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# THE NEUROCHEMISTRY OF THE SPINAL CORD AND ITS PERIPHERAL PROJECTIONS

Sarah Jane Gibson

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## ACKNOWLEDGEMENTS

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I should like to thank all my friends and colleagues with whom I have had the pleasure to work and who have helped through the years. In particular, I am grateful to Pat Wall and Maria Fitzgerald for instilling me with an awe-inspiring interest in the spinal cord. I thank my friends Anne Bishop and Vic Aber for their patience and help, Sandra Lock for her typing expertise and especially for coping with my horrendous tables. I also wish to thank Mr. S. Kar for making recent collaborative ventures an enjoyable experience.

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# NEUROCHEMISTRY OF THE SPINAL CORD AND ITS PERIPHERAL PROJECTIONS

## ABSTRACT

The distributions of 22 peptide immunoreactivities were studied in spinal cords of several species, including man, using immunocytochemistry at the light microscopical level. Distributions of amine, enzyme and structural protein immunoreactivities were included to provide a comprehensive study.

Peptides were found in areas concerned with sensory (dorsal horn), autonomic (intermediolateral cell columns) and motor (ventral horn) regulation. The majority of peptides found in the dorsal horn, were also localised to subsets of cells in the dorsal root ganglia, and thus were thought to have an extrinsic afferent origin. Pharmacological and surgical manipulations confirmed either the extrinsic or intrinsic origins of the peptides in experimental animals. The relationship between regions of the spinal cord which showed a striking peptidergic innervation and peripheral tissues was determined by combining retrograde tracing with immunocytochemistry. VIP/PHI (vasoactive intestinal polypeptide/peptide histidine isoleucine) immunoreactivity was identified as a marker of pelvic visceral afferents and the anatomical and chemical composition of cremaster and pudendal motor nuclei was defined.

The response of peptide-immunoreactive nerves in the dorsal horn to manipulations of the sciatic nerve was undertaken to elucidate

the rôle of peptides in sensory neurotransmission. Most sensory neuropeptides e.g. substance P and calcitonin gene-related peptide, were depleted after sciatic nerve axotomy but VIP/PHI immunoreactivity was increased. This led to the postulate that VIP/PHI is involved in regenerative processes.

During development, motor regions of the spinal cord matured anatomically and expressed the adult, peptide phenotype before sensory areas. This correlated with the physiological maturation of motor and sensory systems. Furthermore, the selective reduction of peptides from sensory or motor regions of spinal cords from rats with a hereditary, sensory neuropathy and humans with motor neurone disease, respectively, suggested that an intact peptidergic innervation is linked to the maintenance of function and possibly to neuronal integrity.

These investigations demonstrate complexities of neurochemical organisation in the spinal cord which were previously unsuspected and emphasise the possible importance of peptides in the normal physiological function of the spinal cord.

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## ABBREVIATIONS

### PEPTIDES, AMINES, ENZYMES AND STRUCTURAL PROTEINS

| | |
|---|---|
| AChE, | acetylcholinesterase |
| ACTH, | adrenocorticotrophin |
| BN, | bombesin/gastrin-releasing peptide |
| CALC, | calcitonin |
| CGRP, | calcitonin gene-related peptide |
| CCK, | cholecystokinin/gastrin-like peptide |
| Chat, | choline acetyltransferase |
| CPON, | C-flanking peptide of neuropeptide Y |
| ENK, | enkephalin |
| DBH, | dopamine β-hydroxylase |
| DYN, | dynorphin |
| FRAP, | fluoride resistant acid phosphatase |
| FMRF, | Phe-Met-Arg-Phe |
| GABA, | gamma amino butyric acid |
| GAD, | glutamic acid decarboxylase |
| GAL, | galanin |
| GFAP, | glial fibrillary acidic protein |
| GRF, | growth hormone-releasing factor |
| GRP, | gastrin-releasing peptide |
| 5-HT, | 5-hydroxytryptamine (serotonin) |
| MCH, | melanin-concentrating hormone |
| NF, | neurofilament triplet proteins |
| NKA, | neurokinin A |
| NmB, | neuromedin B |
| NmC, | neuromedin C |
| NmN, | neuromedin N |
| NmU, | neuromedin U |
| NP, | neurophysin |
| NPY, | neuropeptide Y |
| NT, | neurotensin |
| OXY, | oxytocin |
| PANC, | pancreastatin |
| PHI, | peptide histidine isoleucine |
| PHM, | peptide histidine methionine |
| PYY, | peptide tyrosine tyrosine |
| SOM, | somatostatin |
| SP, | substance P |
| TH, | tyrosine hydroxylase |
| TRH, | thyrotrophin-releasing hormone |
| VIP, | vasoactive intestinal polypeptide |
| VP, | vasopressin |

### MISCELLANEOUS

| | |
|---|---|
| ATP, | adenosine triphosphate |
| DAB, | 3,3' diaminobenzidine |
| DFP, | diisopropyl fluorophosphate |
| DNA, | deoxyribonucleic acid |
| E, | embryonic day of gestation |
| FITC, | fluorescein isothiocyanate |
| mf, | mutilated foot |
| MND, | motor neurone disease |
| 6-OHDA, | 6-hydroxydopamine |
| P, | postnatal day |
| PAP, | peroxidase anti-peroxidase |
| RNA, | ribonucleic acid |
| WGA, | wheat germ agglutinin |

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# CHAPTER 1 INTRODUCTION


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# CHAPTER 1

## 1.1 BACKGROUND: THE CENTRAL AND PERIPHERAL NERVOUS SYSTEM

To provide an anatomical basis for the work presented in this thesis, it is pertinent to give a short description of the spinal cord and its relationship with peripheral nerves. Regions commonly referred to in the spinal cord are shown in Fig.1.1A. The spinal cord is composed of a central core of grey matter, consisting of many thousands of neurones, surrounded by the white matter (Fig.1.1B). The grey matter is generally subdivided into ten layers or laminae (Fig. 1.1C) each characterised by cells of different sizes. The original laminar scheme was proposed by Rexed (1952) based on his cytoarchitectonic studies of Nissl-stained tissue sections of cat spinal cord. Subsequent studies have adopted similar schemes for other species including rat, monkey and man (Molander et al., 1984; Rexed, 1964; Schoenen, 1973).

The dorsal horn (laminae I-VI) and in particular lamina II, the substantia gelatinosa, is involved with the processing of sensory information. This information is transported from the peripheral axons of pseudounipolar, dorsal root ganglion cells (primary sensory neurones) to their central terminals in the dorsal horn. It is generally considered that large ganglion cells give rise to large diameter myelinated fibres, whilst smaller cells give rise to small diameter, finely myelinated A$\delta$ and unmyelinated C fibres (Lawson and Harper, 1985; Lieberman, 1976) (Fig.1.2A). On entering the spinal cord, afferent fibres form collaterals either

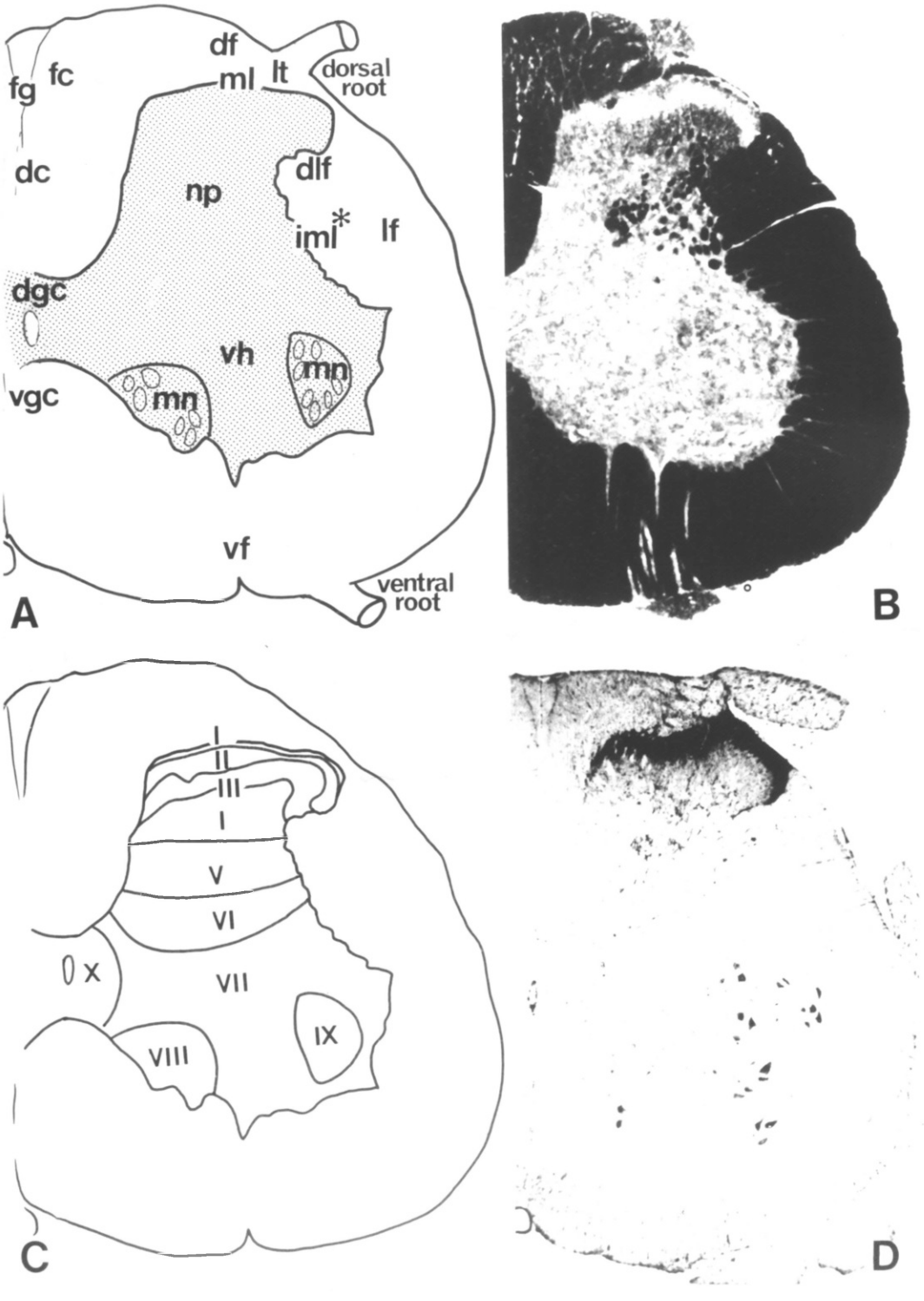



**Fig.1.1** Summary diagram of specific named areas in the spinal cord. df., dorsal funiculus of Lissauer; fg., fasciculus gracilis; fc., fasciculus cuneatus; ml., marginal layer (lamina I); lt., Lissauer's tract; np., nucleus proprius (laminae III-V); dlf., dorsolateral funiculus; iml., intermediolateral cell column; lf., lateral funiculus; dgc., dorsal grey commissure; vh., ventral horn; vgc., ventral grey commissure; mn., motor nuclei; vf., ventral funiculus. B) Transverse, unstained frozen section (50 µm thick) through one half of the $L_4$ segment of the rat spinal cord. Formalin fixation. Lamina II (substantia gelatinosa) is clearly visible as a bright band in the uppermost part of the dorsal horn. C) Diagrammatic representation of the laminar arrangement of the $L_4$ segment of rat spinal cord. D) CGRP immunoreactivity in the $L_4$ segment of rat spinal cord. Immunoreactive fibres are concentrated in lamina II and many of the motoneurones are immunoreactive (B,D x 20). *not present in all segmental levels.

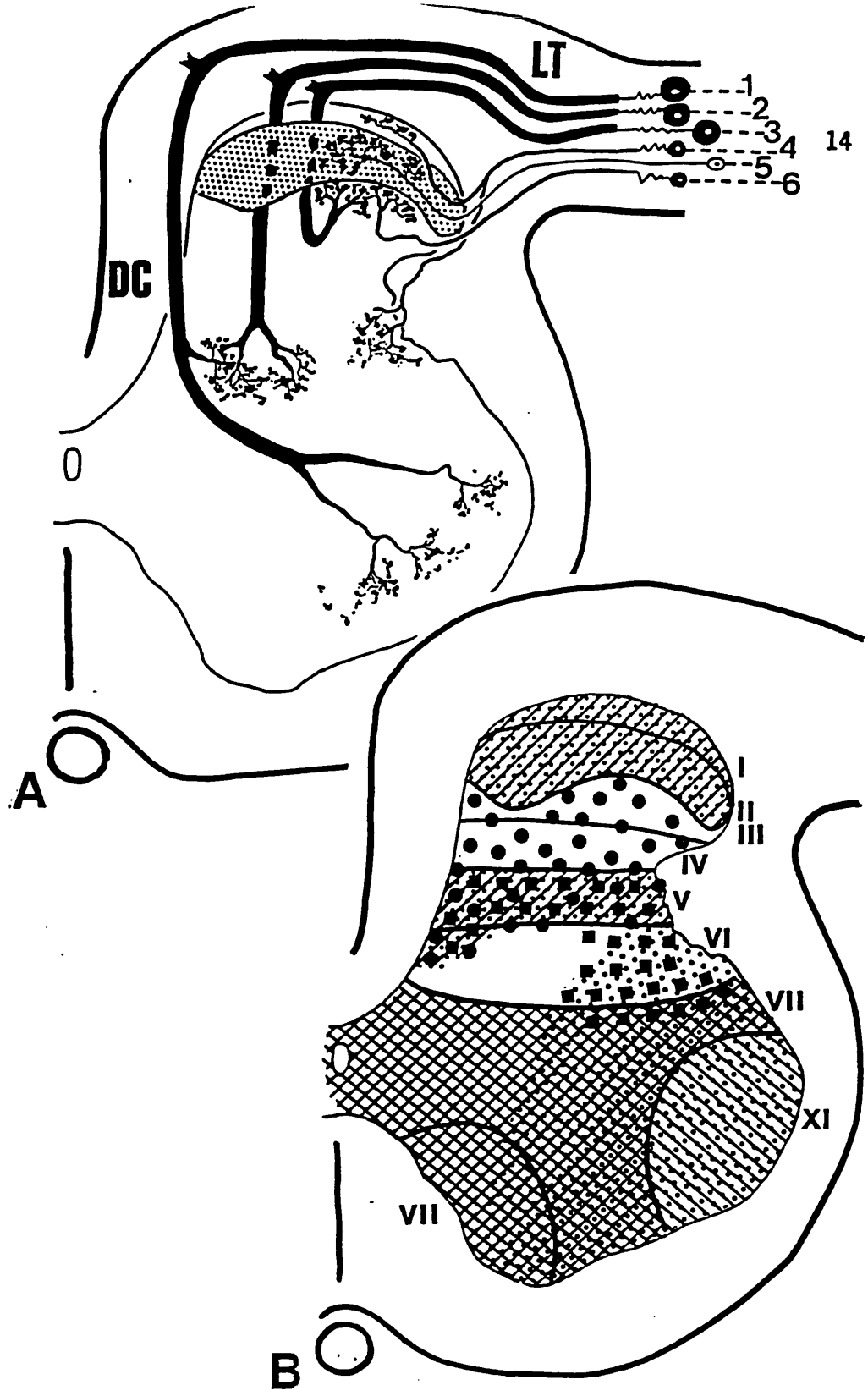



Fig.1.2 A) Afferent input and termination pattern (after Fyffe, 1984). Large dorsal root ganglion cells give rise to large diameter fibres and small cells to small calibre fibres. 1,2,large myelinated, 1a muscle afferents. 3, medium sized myelinated, Aβ cutaneous mechanoreceptors. 4,6, finely myelinated Aδ nociceptors and thermoreceptors. 5, unmyelinated C fibres. Most C fibres terminate in lamina II (stippled). DC., dorsal column; LT., Lissauer's tract. B) Diagrammatic representation of regions in the cat lumbar spinal cord which receive terminals from various descending tracts (see Brown, 1982). Supraspinal tracts in the white matter have been omitted since relatively small amounts of peptide are found here. Corticospinal tract (sensory cortex); corticospinal tract (motor cortex); raphe-spinal tract; reticulospinal tract (magnocellularis nucleus); reticulospinal tract (gigantocellularis nucleus).

in Lissauer's tract or the dorsal columns and eventually terminate (Heimer and Wall, 1968) in the dorsal horn in a stereotyped pattern according to the functional properties of the fibres. The majority of nociceptive afferents terminate in the upper dorsal horn, whereas nerves that convey innocuous events are found in deeper laminae (Cervero and Iggo, 1980; Willis, 1985).

Motoneurones are found in the ventral horn (laminae VII-X) and autonomic neurones in intermediolateral and intermediomedial cell columns in the thoracolumbar and sacral spinal cord. Preganglionic sympathetic neurones are located in the spinal cord at the thoracolumbar level, whereas preganglionic parasympathetic nerves have either a cranial (in the mid- and hind-brain) or sacral origin. Efferent axons of both motoneurones and preganglionic autonomic neurones leave the spinal cord via the ventral roots.

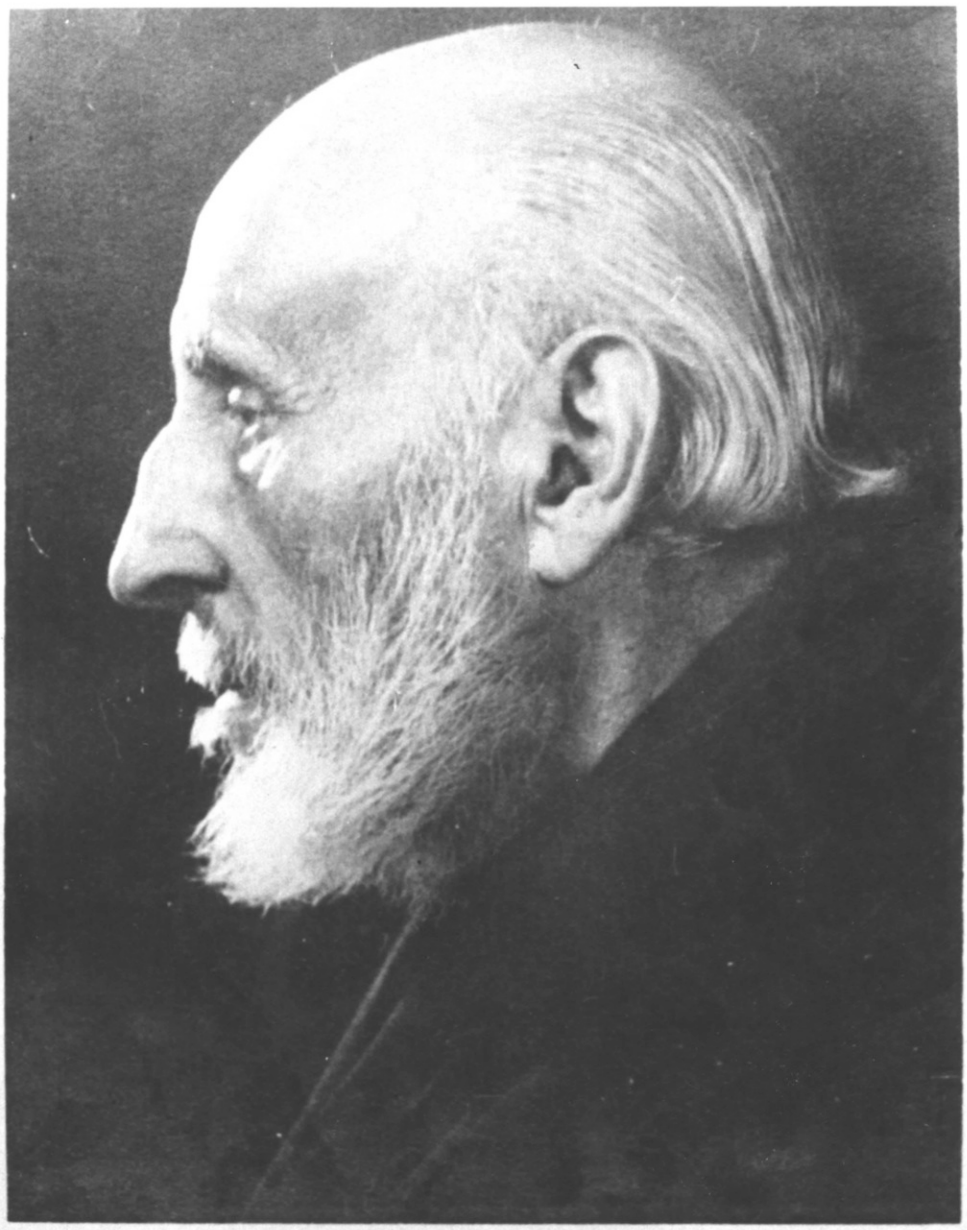
The circuitry of the spinal cord is thus complex, with possible sources of fibres in any area being from i) neurones in the dorsal root ganglia ii) local cell bodies/interneurones or iii) collaterals from descending supraspinal tracts which project to the spinal cord (Basbaum et al., 1978; Brown, 1982; Fields and Basbaum, 1978; Kuypers and Maisky, 1975; Willis and Coggeshall, 1978) (Fig.1.2B).

## 1.2 CHEMICAL NEUROTRANSMISSION - NEUROTRANSMITTERS

In the 17th century, Descartes (see Foster, 1901) suggested that cutaneous impulses were transmitted to the brain by means of the

skin pulling upon delicate threads. The neurone theory, formulated by Waldeyer and predominantly supported by the work of Ramón y Cajal (1954) (Fig.1.3), was finally accepted. However, there remained the question of the mode of communication between neurones. The term "synapse" was proposed to describe the junction between neurones where impulse transmission occurred from one to another. Signals can be transmitted between cells either by direct conduction of action potentials at a specialised gap junction (electrical synapse), or more commonly by molecules of a transmitter substance. Conclusive evidence that chemical substances are released from nerve endings to mediate such transmission was provided by Loewi in 1921.

Classically, the only known transmitters in the peripheral nervous system were acetylcholine (for preganglionic and postganglionic parasympathetic fibres) and noradrenaline (for sympathetic preganglionic fibres). In the central nervous system, acetylcholine was one of the first transmitters to be discovered, when Eccles et al., (1954) demonstrated its release from synapses between motoneurones and Renshaw cells. Today, more than 40 substances are either known or suspected to be, transmitters, each having characteristic inhibitory and/or excitatory effects on neurones. However, many substances modulate synaptic transmission without being transmitters and various workers have proposed a number of criteria which should be fulfilled to characterise a substance as a transmitter (Table 1.1). Established transmitters are the cathecholamines, dopamine, adrenaline and noradrenaline; the amino acids $\gamma$-amino butyric acid (GABA) and glycine, and the



Se ha dicho harlas veces que el problema de España es un problema de cultura. Urge, en efecto, si queremos incorporarnos a los pueblos cultivados cultivar intensamente las germas de nuestra tierra y de nuestro cerebro, salvando para la prosperidad y engrandecimiento patrios todos los ríos que se pierden en el mar y todos los talentos que se pierden en la ignorancia.

S. Ramón Cajal

Madrid 1º de Marzo de 1922

**Fig.1.3** The late Ramón y Cajal

TABLE 1.1 CLASSICAL TRANSMITTER CRITERIA (Orrego, 1979; Werman, 1966)

1. The substance (and its metabolic precursors) must be contained in the cell body and be present in higher concentration in the nerve endings.

2. Enzymes for the synthesis and a mechanism for the release from nerve endings must be present in the neurone.

3. Release by stimulation of the neurone and its presence in the extracellular space.

4. Inactivation mechanisms must exist, either an enzyme or an uptake mechanism.

5. Specific receptors should be present postsynaptically and in increased concentrations subsynaptically.

6. Exogenously applied substances must show a similar reaction to stimulation; in particular, action of drugs e.g. specific antagonists must be the same whether the transmitter is externally applied or arises from the nerve.

Simplification of classical criteria (Furness et al., 1982)

1. Presence in nerve terminal.

2. Stimulation-induced release.

3. Effect on postsynaptic structure.

indoleamines, 5-hydroxytryptamine (5-HT) and histamine (McLennan, 1963; Salt and Hill, 1983 for review). Glutamic and aspartic acids, proline, taurine and purine nucleotides (e.g. ATP [Burnstock et al., 1972]) are also strongly suspected as transmitters.

## 1.3 NEUROPEPTIDES

### 1.3.1 Discovery

In the 1960's, electron microscopic examination of peripheral autonomic nerves showed that the greater majority contained large, dense-core vesicles (p-type vesicles) which are distinct from the small vesicles typical of adrenergic and cholinergic nerves (Baumgarten et al., 1970; Taxi, 1965). The discovery of a third type of nerve, the most abundant in the peripheral nervous system, was consistent with reports of non-adrenergic/non-cholinergic neural mechanisms in peripheral tissues (Ambache and Zar, 1971; Burnstock et al., 1979; Gabella, 1979). Using immunocytochemistry, p-type vesicles were shown to contain a variety of peptides e.g. vasoactive intestinal polypeptide (VIP) (Larsson, 1977), substance P or enkephalin (ENK) (Pickel et al., 1979).

Around the same time as the discovery of p-type vesicles the concept that nerves of the central nervous system also produced, stored and released peptides arose from studies on the neurohypophyseal hormones, oxytocin (OXY) and vasopressin (VP). These peptides were the first to be isolated from the mammalian central nervous system (Du Vigneaud, 1956) and for many years, were consi-

sidered to be unique to the hypothalamus. Subsequent immunocytochemical studies showed OXY and VP were present in many extra-hypothalamic neurones (Buijs, 1978; Dogertrôm et al., 1978). Thus, immunocytochemical localisation became instrumental in the belief that these peptides had a rôle other than that of neurohypophyseal hormones. Henceforth, an ever increasing number of peptides has been localised to the central nervous system.

The impetus in neuropeptide research was stimulated further by the discovery of ENK as the endogenous factor in brain extracts which could mimic effects of morphine and whose actions could be blocked by the opiate antagonist, naloxone (Hughes, 1975). Subsequent localisation of ENK to brain regions including the hypothalamus, amygdala and the dorsal horn of the spinal cord (Hökfelt et al., 1977a) indicated for the first time, a means by which an endogenous opiate could cause euphoria (by actions on the limbic system) and might alleviate pain (at the spinal level).

Fundamental to the study of neurochemistry of the spinal cord and the sensory nervous system was the discovery of substance P in 1931 by Von Euler and Gaddum. A rôle for this peptide in sensory neurotransmission was first proposed by Lembeck (1953) who found the dorsal roots of the spinal cord contained greater concentrations of this peptide than the ventral roots. Early evidence that substance P might be involved in nociceptive afferent transmission, was provided by the discovery of a depletion of substance P from dorsal roots in rats desensitised to noxious stimuli by

capsaicin (see chapter 2) (Gasparovic et al., 1964). In addition, immunocytochemical localisation studies, pioneered by Thomas Hökfelt and colleagues (1975a,b), showed substance P immunoreactivity was concentrated in the dorsal horn and present in small diameter sensory neurones. Whilst a wealth of interest and evidence grew around the idea that substance P was the elusive sensory neurotransmitter (Henry, 1977; Salt and Hill, 1983 for reviews), the discovery of many other peptides in the dorsal horn of the spinal cord and in primary sensory neurones provided alternative candidates (Gibson and Polak, 1986; Hunt, 1983; Ruda et al., 1986 for reviews) (chapters 3 and 4).

### 1.3.2 Isolation

Novel peptides are nowadays so rapidly isolated that potentially bioactive peptides are discovered and localised before any physiological significance can be attributed to them. This contrasts with earlier approaches in which substances mostly, had well defined bioactivity and their anatomical localisation was presented later. Two, relatively new techniques, have been instrumental in setting this trend. One of these, evolved from recombinant DNA technology has allowed the determination of novel, complementary DNA structures e.g. calcitonin gene-related (CGRP) (Rosenfeld et al., 1983). The other has provided a means of chemically identifying new peptides via characteristic amides at the carboxyl terminus e.g. neuropeptide Y (NPY) and galanin (GAL) (Tatemoto and Mutt, 1980).

### 1.3.3 Peptide families

All regulatory peptides share the common property of being initially synthesised as large precursors which are subsequently processed enzymatically to form biologically active products (Andrews et al., 1987 for review). The majority of peptides can therefore be grouped into families, which share close sequence homologies such that they may have all arisen from a common precursor, by gene duplication, or point mutation (Blundell and Humbel, 1980) (Fig. 1.4). The use of recombinant DNA technology and the analysis of genetic codes of a number of regulatory peptides has provided interesting information. Thus, while more than one gene can encode the same or a closely similar peptide, (e.g. $\alpha$- and $\beta$-CGRP) a single gene may encode more than one regulatory peptide (e.g. CGRP and calcitonin).

### 1.3.4 Peptides - transmitter status?

At present there is little evidence that any peptide fulfils all of the criteria for classification as a neurotransmitter. The extent to which some peptides satisfy these criteria are indicated in Table 1.2. In particular, the release of a peptide from nerves after natural, rather than artificial electrical stimulation remains difficult to demonstrate. Inactivation mechanisms for many peptides have not been established, although endopeptidases for some, including ENK, substance P and neurotensin (NT) have been disclosed (Görenstein and Synder, 1979; Griffiths et al., 1982; Probert and Hanley, 1987). Above all, a lack of specific

## Tachykinins

| | 5 | | | | | | | | 10 | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Substance P | R | P | K | P | Q | Q | F | F | G | L | M-$NH_2$ |
| Neurokinin A | | H | K | T | D | S | F | V | G | L | M-$NH_2$ |
| Neurokinin B | | D | M | H | D | F | F | V | G | L | M-$NH_2$ |
| Kassinin | D | V | P | K | S | D | Q | F | V | G | L M-$NH_2$ |
| Physalaemin | pE | A | D | P | N | K | F | Y | G | L | M-$NH_2$ |
| Eledoisin | pE | P | S | K | D | A | F | Y | G | L | M-$NH_2$ |
| Eledoisin-related peptide | | | | | | K | F | I | G | L | M-$NH_2$ |

## Rat CGRP

| | 5 | | | | | 10 | | | | | 15 | | | | | 20 | | | | | 25 | | | | | 30 | | | | | 35 | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| α | S | C | N | T | A | T | C | V | T | H | R | L | A | G | L | L | S | R | S | G | G | V | V | K | D | N | F | V | P | T | N | V | G | S | E | A | F | -$NH_2$ |
| β | S | C | N | T | A | T | C | V | T | H | R | L | A | G | L | L | S | R | S | G | G | V | V | K | D | N | F | V | P | T | N | V | G | S | K | A | F | -$NH_2$ |

**Fig.1.4** Peptide families - amino acid sequences. For one letter notations see IUPAC-IUB Commission on Biochemical Nomenclature (1968).

## VIP and related peptides

| | 5 | | | | | 10 | | | | | 15 | | | | | 20 | | | | | 25 | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| VIP | H | S | D | A | V | F | T | D | N | Y | T | R | L | R | K | Q | M | A | V | K | K | Y | L | N | S | I | L | N | -$NH_2$ | |
| PHI | H | A | D | G | V | F | T | S | D | F | S | R | L | L | G | Q | L | S | A | K | K | Y | L | E | S | L | I | -$NH_2$ | | |
| Helodermin | H | S | D | A | I | F | T | Q | Q | Y | S | K | L | L | A | K | L | A | L | Q | K | Y | L | A | S | I | L | G | S | R | T | S | P | P | P | -$NH_2$ |
| Secretin | H | S | D | G | T | F | T | S | E | L | S | R | L | R | D | S | A | R | L | Q | R | L | L | Q | G | L | V | -$NH_2$ | | |
| Glucagon | H | S | Q | G | T | F | T | S | D | Y | S | K | Y | L | D | S | R | R | A | Q | D | F | V | Q | W | L | M | N | T | -$NH_2$ | |
| GRF | Y | A | D | A | I | F | T | N | S | Y | R | K | V | L | G | Q | L | S | A | R | K | L | L | Q | D | I | M | S | R | Q | Q | G | E | S | N | Q | E | R |
| | | | | | | | | | | | | | | | | | | | | | | | | | | | | | G | A | R | A | R | L | -$NH_2$ | |

## NPY and related peptides

| | 5 | | | | | 10 | | | | | 15 | | | | | 20 | | | | | 25 | | | | | 30 | | | | | 35 | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| NPY | Y | P | S | K | P | D | N | P | G | E | D | A | P | A | E | D | L | A | R | Y | Y | S | A | L | R | H | Y | I | N | L | I | T | R | Q | R | Y | -$NH_2$ |
| CPON | S | S | P | E | T | L | I | S | D | L | L | M | R | E | T | E | N | V | P | R | T | R | L | E | D | P | A | M | W | -$NH_2$ | | | | | | | |
| APP | G | P | S | Q | P | T | Y | P | G | D | D | A | P | V | E | D | L | I | R | F | Y | D | N | L | Q | Q | Y | L | N | V | V | T | R | H | R | Y | -$NH_2$ |
| BPP | A | P | L | E | P | E | Y | P | G | D | N | A | T | P | E | Q | M | A | Q | Y | A | A | E | L | R | R | Y | I | N | M | L | T | R | P | R | Y | -$NH_2$ |
| HPP | A | P | L | E | P | V | Y | P | G | D | N | A | T | P | E | Q | M | A | Q | T | A | A | D | L | R | R | Y | I | N | M | L | T | R | P | R | Y | -$NH_2$ |
| PYY | Y | P | A | K | P | E | A | P | G | E | D | A | S | P | E | E | L | S | R | Y | Y | A | S | L | R | H | Y | L | N | L | V | T | R | Q | R | Y | -$NH_2$ |

KEY: A,H,B avian, human, bovine; PP pancreatic polypeptide
For other abbreviations see page 6
For one letter notation for amino acids see IUPAC-IUB Commission European J. Biochem. 5 (1968)

TABLE 1.2 TRANSMITTER STATUS OF SOME MAJOR PEPTIDES IN THE CENTRAL NERVOUS SYSTEM

| Peptide | Mimickry natural stimulus | Induced release | Vesicular localisation | Receptor sites | Antagonist | Synthesis | Inactivation | Differential distribution |
|---|---|---|---|---|---|---|---|---|
| CGRP | - | Mason et al., 1984 | Merighi et al., 1988b; Harmann et al., 1988 | Tschopp et al., 1985 | - | Amara et al., 1982 | - | Rosenfeld et al., 1983 |
| ENK | - | Iversen et al., 1978b | Pickel et al., 1979 | Pert and Synder, 1975 | Akil et al., 1976 | Clouet and Ratner, 1979 | Görenstein and Snyder, 1979 | Hökfelt et al., 1977a,c |
| GAL | - | - | Köhler et al., 1986 | Skofitsch et al., 1986 | - | Rökaeus and Brownstein, 1986 | - | Rökaeus et al., 1984 |
| NPY | - | - | Pelletier et al., 1984 | Unden et al., 1984 | - | Minth et al., 1984 | - | Allen et al., 1983 |
| SOM | - | Iversen et al., 1978a | Pelletier et al., 1974 | Epelbaum et al., 1982 | - | Schally et al., 1980 | - | Finley et al., 1981 |
| Substance P | - | Otsuka and Konishi, 1976 | Pickel et al., 1977 | Nakata et al., 1978 | Mathison et al., 1984 | Nawa et al., 1983 | Nakata et al., 1981 | Ljundahl et al., 1978 |
| VIP | - | Emson et al., 1978 | Pelletier et al., 1981 | Taylor and Pert, 1979 | - | Itoh et al., 1983 | Fakrenkrug, 1979 | Lorén et al., 1979a |

antagonists has seriously affected their consideration as neurotransmitters (Jessell, 1983; Mathison et al., 1984). Peptides also differ from classical neurotransmitters in a number of characteristics, but if the simplified criteria of Furness et al., (1982) (Table 1.1) are applied, many may be considered putative transmitters.

### 1.3.5 Coexistence of neuroactive substances

With the discovery of an increasing number of peptides having widespread distributions in the central and peripheral nervous systems, it has become evident that most neurones contain more than one messenger molecule (Chan-Palay and Palay, 1984; Cuello, 1982; Hökfelt, 1986; Hökfelt et al., 1980a,b, 1987 for reviews). For each classical neurotransmitter there is at least an example of coexistence with one peptide (Table 1.3). This is in apparent contradiction to the concept, often referred to as Dale's principle, (Eccles, 1957) that neurones secrete a single transmitter at all of their terminals, and thus has raised doubts as to the neurotransmitter status of peptides. In general, these findings can be interpreted to mean that neurones contain more than one transmitter or alternatively that peptides may be responsible for other functions. They may act as neuromodulators, modulating the release of classical transmitters at synapses. Such a rôle has been hypothesised for substance P and thyrotrophin-releasing hormone (TRH) which are co-stored with 5-HT in brainstem neurones and their projections to spinal motoneurones. Evidence suggests that these peptides may enhance transmission at

TABLE 1.3 EXAMPLES OF CLASSICAL NEUROTRANSMITTER AND PEPTIDE COEXISTENCE*

| Classical transmitter | Peptide | Region | Reference(s) |
|---|---|---|---|
| Acetylcholine | CGRP | Medullary motor nuclei | Takami et al., 1985a |
| | ENK | Superior olive, spinal parasympathetic nucleus | Altschuler et al., 1983; Kondo et al., 1985 |
| | GAL | Basal forebrain | Melander et al., 1985 |
| | SOM | Spinal motoneurones | Schrøder et al., 1984 |
| | Substance P | Pons | Vincent et al., 1983 |
| | VIP | Cortex, [sympathetic ganglia] | Eckenstein and Baughman, 1984; [Lundberg, 1979] |
| Dopamine | CCK | Mesencephalon | Hökfelt et al., 1980c |
| | NT | Mesencephalon, arcuate nucleus | Hökfelt et al., 1984 |
| | | Hypothalamus | Hökfelt et al., 1984 |
| Epinephrine | NPY | Medulla oblongata | Everitt et al., 1984 |
| | NT | Medulla oblongata, solitary tract nucleus | Hökfelt et al., 1984 |
| | Substance P | Medulla oblongata | Lorenz et al., 1985 |
| GABA | CCK | Cortex, hippocampus | Hendry et al., 1984; Kosaka et al., 1985 |
| | CGRP | Cerebellum | Kawai et al., 1987 |
| | ENK | Retina, ventral pallidum, hypothalamus | Oertel and Mugaini, 1984; Watt et al., 1984, |
| | GAL | Hypothalamus | Melander et al., 1987 |
| | NPY | Cortex | Hendry et al., 1984 |
| | SOM | Thalamus, cortex, hippocampus | Oertel et al., 1985; Hendry et al., 1984 |
| | Substance P | Hypothalamus | Köhler et al., 1985 |
| | VIP | Hippocampus | Kosaka et al., 1985 |
| Glycine | NT | Retina | Weiler and Ball, 1984 |

TABLE 1.3 (CONT'D)

| Classical transmitter | Peptide | Region | Reference(s) |
|---|---|---|---|
| 5-HT | CCK | Medulla oblongata | Mantyh and Hunt, 1984 |
| | ENK | Medulla oblongata, area postrema | Glazer et al., 1981; Armstrong et al., 1984 |
| | Substance P | Medulla oblongata | Johansson et al., 1981 |
| | Substance P + TRH | Medulla oblongata | Johansson et al., 1981 |
| | TRH | Medulla oblongata | Johansson et al., 1981 |
| Norepinephrine | ENK | Locus coeruleus | Charnay et al., 1982 |
| | NPY | [Sympathetic ganglia] medulla oblongata Locus coeruleus | [Lundberg et al., 1982b]; Everitt et al., 1984 |
| | SOM | [Sympathetic ganglia] | [Hökfelt et al., 1977b] |
| | VP | Locus coeruleus | Caffé and Van Leeuwen, 1983 |

*Coexistence defined mainly by immunocytochemistry using antisera to the classical neurotransmitters or their synthetic enzymes.
[] = peripheral nervous system.

5-HT synapses which may be of importance when 5-HT synapses are in competition with many other inputs to motoneurones (Hökfelt, 1986). Alternatively, peptides may be responsible for long-term trophic effects (Anand et al., 1987; Burnstock, 1982; Wall and Fitzgerald, 1982). For example, substance P exerts growth stimulatory effects on smooth muscle cells (Nilsson et al., 1985) and CGRP regulates expression of acetylcholine receptors at the neuromuscular junction (Fontaine et al., 1986; New and Mudge, 1986).

In some cases, coexistent peptides may have a common precursor or they may arise from different precursors (de Groat, 1987; Gibson and Polak, 1986). Coexistence of substance P and CGRP in sensory neurones is now well documented (see chapter 3) and it seems likely that both are co-released (Diez Guerra et al., 1988; Saria et al., 1986). It has been suggested that CGRP prolongs transmission at synapses by inhibition of a substance P endopeptidase (Le Grevés et al., 1985). Thus, a further type of interaction, independent of membrane bound receptors, could occur in the extracellular space. In view of the gap between the knowledge of the physiological rôle of coexisting peptides in neurones, the significance of coexistence is likely to be difficult to uncover.

## 1.4 MAIN PEPTIDES INVESTIGATED

### 1.4.1 Substance P

Substance P belongs to a family of peptides which share, at their

carboxyl termini, a common sequence of 6 amino acids (Fig. 1.4). Until recently, substance P was thought to be the only mammalian tachykinin, the other peptides (eledoisin, kassinin, physalaemin) of this family having been found only in lower organisms. However, two new members of the tachykinin family, neurokinins A and B, have been isolated from porcine spinal cord, (Kimura et al., 1983). Subsequently, two precursor messenger (m) RNA's have been isolated, one containing the substance P sequence ($\alpha$-preprotachykinin), the other containing substance P and neurokinin A sequences ($\beta$-preprotachykinin) (Nawa et al., 1983) (Fig. 1.5A). Further studies have shown that there appears to be only one gene encoding for both preprotachykinins, with alternative RNA splicing resulting in two messenger mRNAs (Nawa et al., 1984). Even more recently, a third tachykinin, neuropeptide K, was extracted from porcine brain (Tatemoto et al., 1985) and there are now thought to be three types of tachykinin receptor ($NK_1$, $NK_2$, $NK_3$) each having a preferential affinity for the respective tachykinin, substance P, neurokinin A and neuropeptide K (Regoli et al., 1987).

Substance P is an 11 amino acid peptide and is the best known member of the tachykinin family. It has an extensive distribution throughout the body and is found in most peripheral tissues including visceral organs (Alm et al., 1978; Pearse and Polak, 1975) and the cardiovascular system (Reniecke et al., 1980; Wharton et al., 1981). It has a widespread distribution in the central nervous system (Cuello and Kanazawa, 1978; Ljundahl et al., 1978). However, localisation of substance P to primary sensory neurones (Hökfelt et al., 1975b; Katz and Karten,

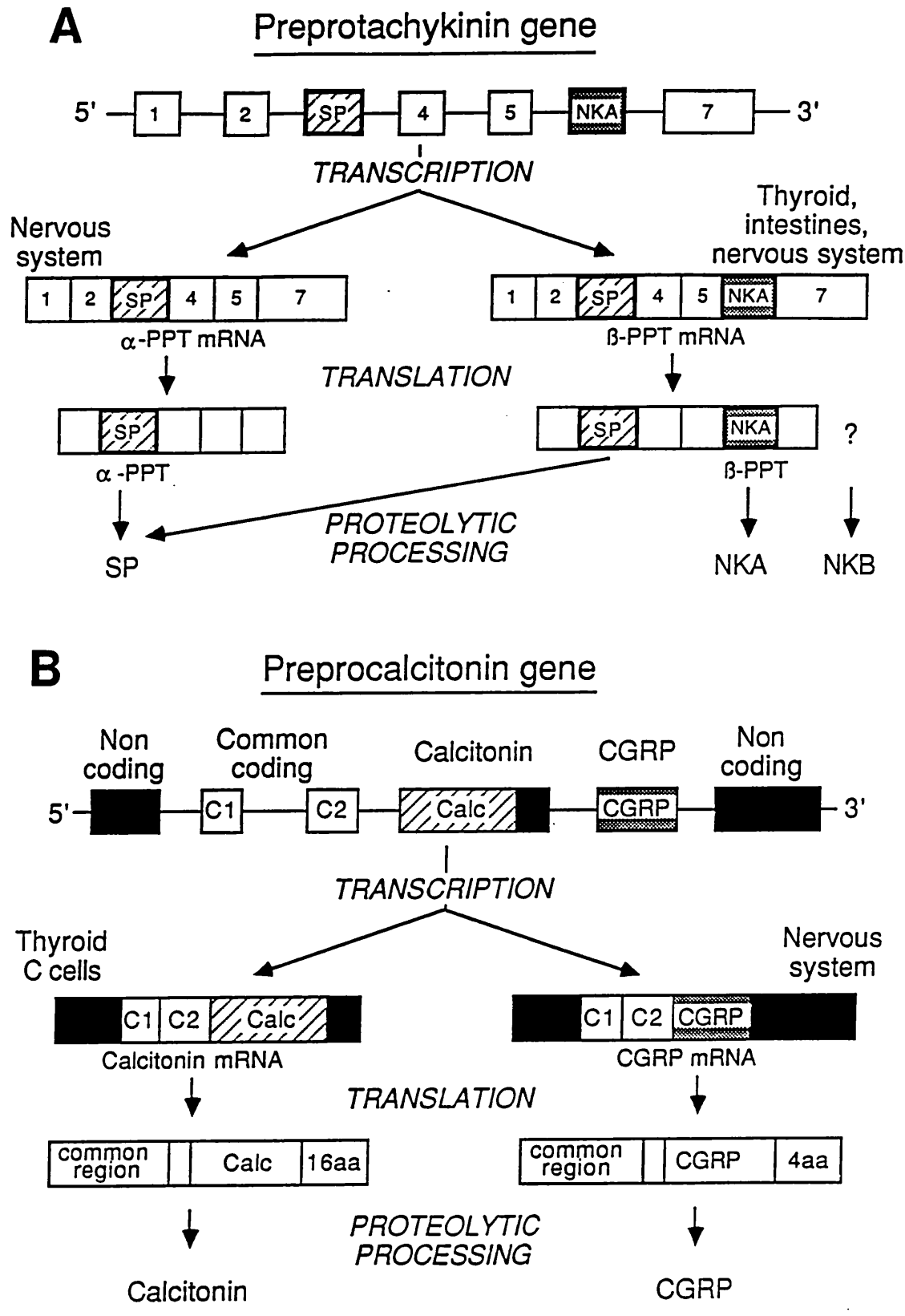



**Fig.1.5** Schematic representation of alternative RNA splicing pathways in the expression of A) the preprotachykinin (PPT) gene and B) the preprocalcitonin gene. The PPT gene encodes both substance P (SP) and neurokinin A (NKA). Although ϒ-PPT mRNA is also generated from the PPT gene through different RNA splicing events, the pathway for generation of this mRNA is not included. The biosynthetic pathway for neurokinin B (NKB) is not known. The preprocalcitonin gene shown encodes calcitonin (CALC) and ∝ CGRP.

1980; Lundberg et al., 1980) and their projections to the dorsal spinal cord (Barber et al., 1979; Chan-Palay and Palay, 1977; Hökfelt et al., 1975a; Ljundahl et al., 1978) and trigeminal nucleus (Cuello et al., 1978; Del Fiacco and Cuello, 1980; Hökfelt et al., 1975b) have added support to evidence that substance P might be a sensory neurotransmitter (Table 1.2).

Lately, the following evidence has implicated substance P as the neurotransmitter of pain. Substance P is present in a subset of dorsal root ganglion cells which correspond to cell bodies of C fibres, some of which conduct nociceptive information (see Hunt, 1983) and it is present in fibres and terminals in high concentrations in the dorsal horn, in the region of C fibre termination. Neonatal capsaicin treatment (chapter 2) produces analgesia (Jancsó et al., 1977) and induces a loss of substance P immunoreactivity from primary sensory neurones and from areas of the spinal cord which receive nociceptive input (Jessell et al., 1978). Substance P is released from the spinal cord following activation of nociceptive afferents (Yaksh et al., 1980). Finally, iontophoresis of substance P excites dorsal horn neurones that are, themselves, excited by noxious stimuli (Henry 1976; Piercey et al., 1980).

Further studies however, suggest that the actions of substance P in the spinal cord may not be as circumscribed as originally suggested. Firstly, capsaicin, which was thought to produce a selective lesion of substance P-immunoreactive dorsal root

ganglion cells, actually produces a non-selective lesion of all small, dorsal root ganglion cells (Nagy et al., 1981, 1983). Secondly, substance P can activate both noxious and non-noxious neurones in the dorsal horn (see Salt and Hill, 1983). Furthermore, the actions of substance P are not restricted to the dorsal horn. Studies with putative antagonists (Åkerman et al., 1982; Loewy and Sawyer, 1982) showed effects, not only on transmission of noxious stimuli but also effects on motor and autonomic function, thus supporting observations that substance P depolarises motoneurones (Otsuka and Yanagisawa, 1980) and preganglionic sympathetic neurones (Backman and Henry, 1983). Finally, there appears to be a functional set of substance P receptors in all of these regions of the spinal cord (Charlton and Helke, 1985). Thus, in the spinal cord, substance P is probably involved not only in regulation of nociception but also in motor and autonomic function.

In accord with evidence which suggests substance P might be involved in nociception (see Henry, 1982), the release of substance P from sensory nerves induces antidromic vasodilatation, plasma extravasation, itching and results in reduced pain sensitivities (Lembeck and Gamse 1982 for review). It has been suggested recently that the neurogenic response may be mediated by release of multiple tachykinins from sensory neurones (Hua et al., 1986). Substance P has also been shown to exert a number of diverse actions including smooth muscle contraction and hypotension (Jordan and Oehme, 1985).

### 1.4.2 Calcitonin gene-related peptide (CGRP)

CGRP is a 37 amino acid peptide produced by alternative processing of the primary transcript of the calcitonin gene (Amara et al., 1982; Rosenfeld et al., 1983) (Fig. 1.5B). Two forms of CGRP have been identified in both rat (Amara et al., 1985) and man (Morris et al., 1984) and are derived from related $\alpha$ and $\beta$ genes. $\beta$-CGRP differs from the previously identified $\alpha$ form by one amino acid in rat and three amino acids in man (Fig. 1.4). RNA mapping studies have shown $\alpha$-CGRP is the predominant form in the central and sensory nervous systems (Amara et al., 1985; Gibson et al., 1988).

CGRP immunoreactivity has been localised to various neuronal groups and tracts in the brain as well as to neurones of the spinal cord (Gibson et al., 1984d; Kawai et al., 1985; Rosenfeld et al., 1983; Skofitsch and Jacobwitz, 1985; Weisenfeld-Hallin et al., 1984) (Fig.1.1D). Notably, CGRP immunoreactivity is abundant in the sensory nervous system, in particular in the dorsal horn of the spinal cord and in cell bodies of spinal and cranial sensory ganglia. Several immunohistochemical studies have shown a substantial CGRP innervation of peripheral tissues including the respiratory (Cadieux et al., 1986) and urinary (Su et al., 1986) tracts, the cardiovascular system (Wharton et al., 1986), oesophagus, tongue and palate (Terenghi et al., 1986). Since these studies also demonstrated a marked reduction of immunoreactivity following capsaicin treatment, it is generally agreed that in these tissues CGRP is mostly of sensory origin.

Several lines of evidence suggest CGRP may play a part in nocicep-

tion. In vitro studies showed CGRP immunoreactivity is released from trigeminal ganglion cells (Mason et al., 1984) and from spinal cord slices following capsaicin administration (Saria et al., 1986). Behavioural studies have shown that co-administration of CGRP and substance P potentiates responses to noxious stimuli over and above that of substance P alone (Wiesenfeld-Hallin et al., 1984; Woolf and Wiesenfeld-Hallin, 1986) (chapter 3). Likewise, CGRP markedly potentiates the substance P-induced plasma extravasation in an inflammatory reaction (Brain and Williams, 1985; Gamse and Saria, 1985).

The demonstration of CGRP in mammalian motor end plates (Matteoli et al., 1988; Rodrigo et al., 1985; Takami et al., 1985b) and in cranial and spinal motoneurones (Gibson et al., 1984d, 1988; Rosenfeld et al., 1983; Takami et al., 1985a) suggested a further rôle for this peptide in motor function. The presence of CGRP in motor nerves is consistent with a number of recently described physiological actions. CGRP will elicit the contraction of striated muscle (Takami et al., 1985b), and is thought to play a part in the synthesis and assembly of acetylcholine receptors at the neuromuscular junction and has been proposed as a muscle trophic factor (Fontaine et al., 1986; New and Mudge, 1986).

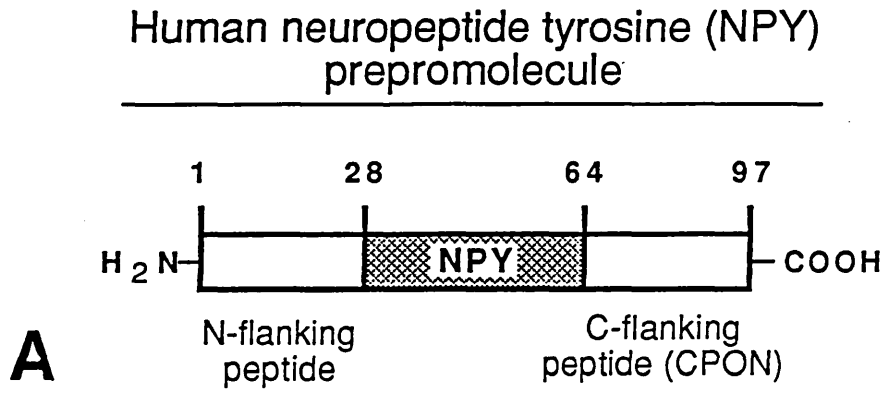
A number of other physiological actions have been reported for CGRP with a major importance as a potent vasodilatory substance which is not inhibited by autonomic blockade (Uddman et al., 1986).

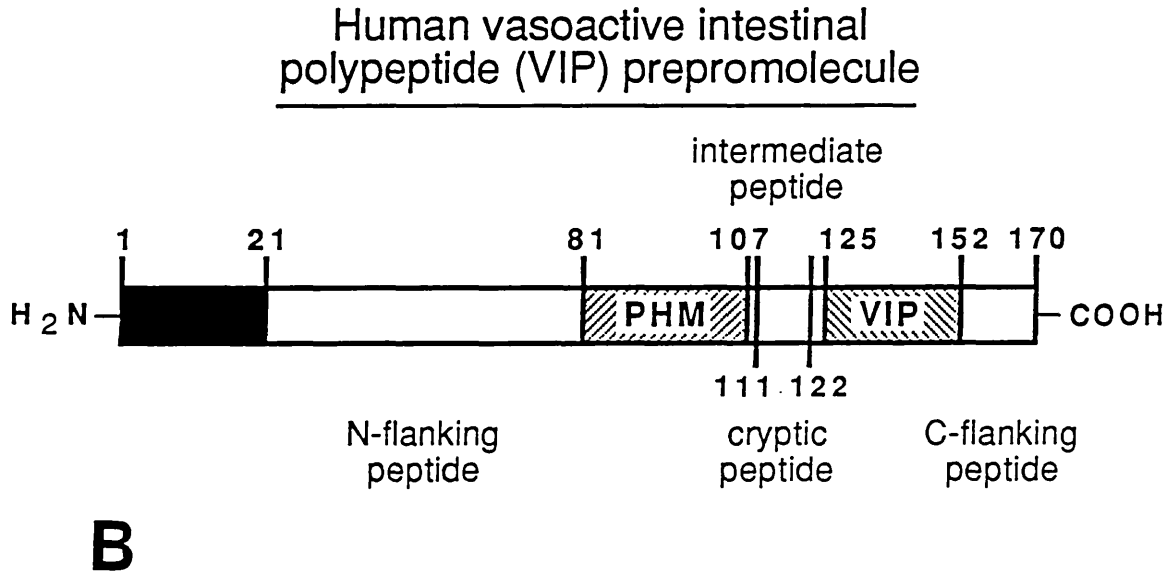
### 1.4.3 Neuropeptide Y (NPY)

This 36 amino acid residue peptide, was first isolated and sequenced from porcine brain (Tatemoto, 1982). It has major sequence homologies with the pancreatic polypeptides and peptide tyrosine tyrosine (PYY) (Tatemoto et al., 1982) (Fig. 1.4). Using antibodies to porcine brain NPY, many immunoreactive structures have been localised to the nervous system and coincide with previous reports of pancreatic polypeptide immunoreactivities (Card et al., 1983; Hökfelt et al., 1981; Hunt et al., 1981a; Lorén et al., 1979b). Since pancreatic polypeptides have been neither extracted nor identified chromatographically in the nervous system, NPY immunoreactivity is probably responsible. Similarly, the molluscan cardioexcitatory peptide, FMRF-amide (Price and Greenberg, 1977) has some sequence similarities with the C-terminus of the pancreatic polypeptides and NPY.

Recent isolation and sequencing of the DNA encoding NPY has predicted the presence of a 97 amino acid precursor peptide (Minth et al., 1984). Proteolytic processing of this precursor could yield three separate products, one of them a 30 amino acid carboxy-terminal flanking peptide (CPON) (Fig. 1.6A). Antibodies to CPON reveal similar distributions and tissue concentrations when compared with NPY (Allen et al., 1985; Gulbenkian et al., 1985), and in most cases is localised to the same neurones.

NPY has been localised to extensive neuronal networks in the brain and several peripheral tissues (Adrian et al., 1983; Allen et al.,




PHM, peptide histidine methionine

**Fig.1.6** A) Neuropeptide Y and B) vasoactive intestinal polypeptide prepromolecules.

1983; Polak and Bloom, 1984). It has also been associated with catecholaminergic nerves because of its colocalisation with tyrosine hydroxylase (TH) and dopamine β-hydroxylase (DBH) in peripheral sympathetic ganglion cells and because of its susceptibility to 6-hydroxydopamine treatment (Lundberg et al., 1982b, 1983). In the central nervous system the colocalisation is not a generalised feature (Everitt et al., 1984), for example, the majority of NPY immunoreactivity in the brainstem is independent of catecholamines although there is some coexistence in the locus coeruleus (Hunt et al., 1981a).

In the central nervous system, NPY induces hypotension (Fuxe et al., 1983b) and influences feeding behaviour (Stanley and Leibowitz, 1984) and secretion of anterior pituitary hormones (McDonald et al., 1985). In peripheral tissues, potent vasoconstrictor effects on vasculature, inhibition of electrically induced contraction of vas deferens, and presynaptic inhibition of transmitters released from noradrenergic, cholinergic and non-adrenergic, non-cholinergic nerves are amongst reported actions of NPY (Allen and Bloom, 1986 for review).

### 1.4.4 Vasoactive intestinal polypeptide (VIP)

VIP was isolated from porcine intestine by Said and Mutt (1970). This 28 amino acid peptide is closely related to secretin and growth hormone-releasing factor (Mutt and Said, 1974) (Fig. 1.4). More recently, another peptide, having close structural similarities to VIP, was isolated from porcine duodenum (Tatemoto and

Mutt, 1981). This peptide consisted of 27 amino acids with an amino-terminal histidine and a carboxy-terminal isoleucine and was called peptide histidine isoleucine (PHI) (Fig. 1.6B). An identical substance was subsequently extracted from brain tissue (Tatemoto et al., 1983a). The finding that equimolar concentrations of VIP and PHI immunoreactivities occurred in many tissues, led to the suggestion that the two peptides might be co-synthesised in the same prohormone (Christofides et al., 1982). Disclosure of the amino acid sequence of the prohormone showed that the human form of PHI (peptide histidine methionine, PHM) was derived from the same prepromolecule as VIP (Itoh et al., 1983). The immunocytochemical localisation of PHI/PHM closely parallels that of VIP and in most tissues they are considered to be co-stored in the same neurone (Anand et al., 1984; McGregor et al., 1984; Polak and Bloom, 1984).

The presence of VIP-immunoreactive neurones in the gastrointestinal tract and hypothalamus was first reported in 1976 (Larsson et al., 1976). Since this time VIP immunoreactivity has been found in most tissues of the body (Polak and Bloom, 1982). In the central nervous system it has a selective distribution in the cerebral cortex and is localised to a number of other regions including the amygdala, periaqueductal grey and the spinal cord (Anand et al., 1983; Fuxe et al., 1977; Roberts et al., 1980; Palkovits, 1984). In peripheral tissues, most VIP-immunoreactive nerves originate from postganglionic sympathetic and parasympathetic neurones or enteric neurones (Larsson et al., 1976; Lundberg et al., 1979). However, VIP immunoreactivity has also

been localised to a subset of sensory ganglion cells (Lundberg et al., 1979) (chapter 3). The presence of VIP in cholinergic neurones supplying exocrine glands (Lundberg, 1981) and in cholinergic interneurones in the cerebral cortex (Eckenstein and Baugham, 1984) represents one of the best examples of the coexistence of peptide and classical neurotransmitter. In the brain, the significance of the coexistence is unknown, but in the salivary glands VIP potentiates acetylcholine-induced secretion and blood flow (Lundberg et al., 1982a).

VIP has a number of diverse biological actions (see Said, 1982; 1984) including dilation of blood vessels, potentiation of oedema, stimulation of intestinal water and electrolyte secretion and neuronal excitation. It also relaxes smooth muscle and much evidence favours VIP as a transmitter candidate of non-adrenergic, non-cholinergic inhibitory nerves in peripheral tissues. VIP is also a potent stimulant of adenylate cyclase activity in many tissues, an action which may underlie many of its biological effects.

## 1.5 OTHER SUBSTANCES

A summary of the main features of all peptides studied is given in Table 1.4. Monoamine pathways are shown in appendix I. Details of other substances are given in relevant chapters.

## 1.6 AIMS OF THE STUDY

The major aims of the study were as follows:-

TABLE 1.4 SUMMARY OF PEPTIDE CHARACTERISTICS

| Peptide | Precursor | Primary source | Main localisation | Main biological actions | First localisation in spinal cord | Action on spinal cord |
|---|---|---|---|---|---|---|
| Adrenocorticotrophin (ACTH) [39] | Preproopiomelanocortin (Nakanishi et al., 1979) | Pituitary | Anterior, intermediate pituitary lobes, CNS | ↑Adrenal corticosteroids | Watson et al., 1978 | ? |
| Enkephalin-met (ENK) [5] | Preproopiomelanocortin (Nakanishi et al., (1979; Preproenkephalin (Gubler et al., 1982) | Porcine brain | Endocrine cells, adrenal medulla CNS and PNS | Opioid | Hökfelt et al., 1977a | Inhibits dorsal horn neurones (Randic and Miletic, 1978) |
| Bombesin/Gastrin-releasing peptide (BN) [14/27] | Preprobombesin/ gastrin-releasing peptide (Spindel et al., 1984) | Frog skin/ porcine antrum | Lung endocrine cells, and CNS PNS | Trophic, ↑release of regulatory peptides | Roth et al., 1982 | Exitatory (Phillis and Kirkpatrick, 1979) |
| Neuromedin B (NmB) [10] | ? | Porcine spinal cord (Minamino et al., 1983) | Anterior pituitary, CNS and PNS | ↑Contracts uterine smooth muscle,↑ release gut & pancreatic peptides (Minamino et al., 1983) | Namba et al., 1985 | ? |
| $CCK_8$/gastrin-like peptide (CCK) [8] | PreproCCK (Deschenes et al., 1985) | Porcine intestine | Endocrine cells CNS and PNS | ↑Gall bladder contraction, pancreatic enzyme secretion | Vanderhaegen et al., 1980 | Excites dorsal horn neurones (Jeftinija et al., 1981) |
| Calcitonin gene-related peptide (CGRP) [37] | Preprocalcitonin Amara et al., 1982 | Medullary carcinoma of thyroid | Endocrine cells (thyroid) CNS and PNS | ↑Vasodilatation, acetylcholine receptor synthesis at motor end plates, striated muscle contraction | Rosenfeld et al., 1983 | Potentiates substance P-induced excitation in dorsal horn (Woolf and Wiesenfeld-Hallin, 1986) |

TABLE 1.4 (CONT'D)

| | | | | | | |
|---|---|---|---|---|---|---|
| Galanin (GAL) [29] | Preprogalanin (Rökaeus and Brownstein, 1986) | Porcine intestine (Tatemoto et al., 1983b) | Endocrine cells, adrenal medulla CNS and PNS | ↑Blood sugar, smooth muscle contraction, plasma growth hormone, food intake | Rökaeus et al., 1984 | ? |
| Melanin-concentrating hormone (MCH) [17] | ? | Salmon pituitary (Kawauchi et al., 1983) | CNS | Melanophore retraction in amphibians (Kawauchi et al., 1983) | Skofitsch et al., 1985a | ? |
| Neuropeptide Y (NPY) [36] | Preproneuropeptide Y (Minth et al., 1984) | Porcine brain (Tatemoto et al., 1982) | CNS and PNS | ↑Vasoconstriction, hypotensive | Hunt et al., 1981a (APP); Gibson et al., 1984b; Emson and de Quidt, 1984 | ? |
| C-flanking peptide of neuropeptide Y (CPON) [37] | Preproneuropeptide Y (Minth et al., 1984) | Human phaeochromocytoma | CNS and PNS | ? | Marti et al., 1987; Gibson and Polak, 1986 | ? |
| FMRF-amide [4] | ? | Mollusc CNS | CNS | Cardioexcitatory in molluscs | Weber et al., 1981 | ? |
| Neurotensin (NT) [13] | Preproneurotensin /neuromedin N (Dobner et al., 1987) | Bovine hypothalamus | Endocrine cells adrenal medulla, CNS and PNS | ↑Vasodilatation, ↓gastric acid secretion | Uhl et al., 1977 | Excites dorsal horn neurones (Miletic and Randic, 1979) |
| Neuromedin N (NmM) [6] | Preproneurotensin/ neuromedin N (Dobner et al., 1987) | Porcine spinal cord (Minamino et al., 1984) | CNS | ↑Smooth muscle contraction (Minamino et al., 1984) | Reported here | ? |

TABLE 1.4 (CONT'D)

| | | | | | | |
|---|---|---|---|---|---|---|
| Neurophysin I (NP) [93] | Preprooxytocin-neurophysin I (Land et al., 1983) | Bovine hypothalamus | CNS | Carrier protein for oxytocin | Sofroniew and Weindl, 1978 | ? |
| Oxytocin (OXY) [9] | Preprooxytocin-neurophysin I (Land et al., 1983) | Bovine hypothalamus | CNS, PNS | ↑ Contraction of uterus, milk let down | Buijs, 1978 | Excites sympathetic preganglionic cells (Backman and Henry, 1984) |
| Vasopressin (arginine) (VP) [8] | Preprovasopressin-neurophysin II (Schmale and Richter, 1981) | Bovine hypothalamus | CNS, PNS | Antiduiretic | Buijs, 1978 | Excites sympathetic preganglionic cells (Backman and Henry 1984) |
| Neuromedin U NmU [25,8] | ? | Porcine spinal cord (Minamino et al., 1985) | Anterior and intermediate pituitary, CNS and PNS | ↑ Contracts uterine smooth muscle (Minamino et al., 1985) | Ballesta et al., 1987 | ? |
| Pancreastatin (PANC) [49] | Chromogranin A (Konecki et al., 1987) | Porcine pancreas (Tatemoto et al., 1986) | Endocrine cells, adrenal medulla pancreas, CNS and PNS | ↓ Insulin release (Tatemoto et al., 1986), meal stimulated exocrine pancreatic secretion (Ishizuka et al., 1987) | Kar et al., 1988a | ? |
| Somatostatin (SOM) [28] | Preprosomatostatin (Goodman et al., 1980) | Bovine hypothalamus | Endocrine cells, gut, thyroid, pancreas, CNS and PNS | ↓ Release + action of many peptides. Antitrophic? | Hökfelt et al., 1976 | Inhibits dorsal horn neurones (Randic and Miletic 1978 cf. excitatory Wiesenfeld-Hallin, 1985) |

TABLE 1.4 (CONT'D)

| | | | | | | |
|---|---|---|---|---|---|---|
| Substance P [11] | $\alpha$- and $\beta$-preprotackykinin (Nawa et al., 1983) | Equine hypothalamus | Endocrine cells of gut, CNS and PNS | ↑Vasodilation muscle contraction | Hökfelt et al., 1975a,b | Excites dorsal horn neurones and motoneurones (Henry, 1976; Konishi and Otsuka, 1974) |
| Neurokinin A (NKA) [10] | $\beta$-preprotachykinin (Nawa et al., 1983) | Porcine spinal cord | CNS and PNS | ↑Vasodilation, muscle contraction more potent than substance P (Mukekata, 1984) | Dalsgaard et al., 1985 | Excites dorsal horn neurones (Matsuto et al., 1984) |
| Thyrotrophin-releasing hormone (TRH) [3] | PreproTRH (Richter et al., 1984) | Hypothalamus | CNS | ↑Release TSH | Hökfelt et al., 1975c | Potentiates glutamate excitation of motoneurones (White 1983) |
| Vasoactive intestinal polypeptide (VIP) [28] | Prepro VIP/PHM (Itoh et al., 1983) | Porcine intestine | CNS and PNS | ↑Smooth muscle relaxation, vasodilatation, secretion | Larsson et al., 1976 | Excites dorsal horn neurones (Jeftinja et al., 1982) |
| Peptide histidine isoleucine (PHI) [27] | Prepro VIP/PHM (Itoh et al., 1983) | Porcine intestine | CNS and PNS | ↑Smooth muscle relaxation, secretion | Anand et al., 1984; McGregor et al., 1984 | ? |

[ ] refers to number of amino acids. For further references see Van Noorden and Polak, 1985; CNS, central nervous system; PNS, peripheral nervous system ↑ increases ↓ decreases. Peptides have been grouped in families where possible.

- To provide a comprehensive map of a number of peptides and other neural substances in the spinal cord and dorsal root ganglia.
- To establish the origin(s) of selected peptides in the spinal cord and to elucidate peptide-containing projection pathways to peripheral tissues.
- To assess the effects of peripheral nerve manipulations on peptide distribution in the dorsal spinal cord.
- To investigate the development of the spinal cord and dorsal root ganglia and the temporal appearance of peptide- and monoaminergic-immunoreactive neurones.
- To determine the involvement of peptides in neuropathological conditions.

# CHAPTER 2: MATERIALS AND METHODS


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# CHAPTER 2

## 2.1 GENERAL

The introduction of immunocytochemistry as a tool in neuro-anatomical research has transformed our concept of classical neuroanatomy from a merely descriptive to a more functional anatomy (Sternberger, 1982). Immunocytochemistry was mostly performed using modifications of the indirect immunofluorescence (Coons et al., 1955) and the the peroxidase anti-peroxidase (PAP) methods (Sternberger et al., 1970) (appendix V,VI). The possible origin(s) and nature of peptide-immunoreactive nerves in the spinal cord were investigated by means of several techniques performed on experimental animals. These included use of surgical and pharmacological manipulations of central and peripheral nervous systems and in some cases projection pathways were confirmed by combining axonal transport tracing techniques with immunocytochemistry. In most experiments, the localisation studies were complemented by biochemical analyses, including radioimmunoassay for determination of peptide content and chromatography for chemical characterisation of the immunoreactive material.

## 2.2 FIXATION

The majority of peptides and enzymes are water soluble and must therefore be fixed before being amenable to immunocytochemical or enzymological studies. Fixation has two main purposes, namely to

preserve tissue structure, and in particular for immunocytochemical studies, to retain the antigen of interest *in situ* without loss of antigenicity. Fixatives commonly used introduce intra- and intermolecular cross-links and may therefore modify antigenicity and create an antibody permeability barrier. Inadequate or uneven fixation results in loss of antigen and tissue damage, whereas over-fixation leads to masking of antigenic sites. Since the antigens investigated vary widely in chemical structure from very small peptides to large proteins/enzymes then optimal fixation conditions will also vary. Thus, a variety of different fixatives were examined to establish which provide the most suitable compromise according to the requirements of the study.

In practise, fixative solutions which contain the bi-functional cross-linking agent formaldehyde are suitable for most purposes, e.g. buffered formaldehyde (Pease, 1962), paraformaldehyde or Bouin's solution (see appendix II). However, p-benzoquinone (Bishop et al., 1978; Pearse and Polak, 1975) (appendix II) has been used as an acceptable alternative and in many cases provides a clearer visualisation of peptide-immunoreactive nerves in the spinal cord (Marti et al., 1987).

The central nervous system has a high metabolic rate and is sensitive to anoxic and autolytic changes and the spinal cord, is very soft and highly susceptible to mechanical damage. The most satisfactory means of preservation is achieved by rapid perfusion of fixative *in situ* via the vascular system (Palay et

al., 1962) (appendix III). However, this method is not always possible. Tissues from large mammals, including man, rat embryos and animals in which radioimmunoassay and immunocytochemical analyses were performed in parallel were fixed by immersion in fixative after dissection. Since a decrease in specific immunostaining for peptide antigens occurs with increased fixation delays, all tissues were fixed as soon as possible whichever route was employed (Gu et al., 1985; Helme and White, 1983).

## 2.3 TISSUE PREPARATION

Wax, cryostat or vibratome tissue sections were employed. Although wax embedding allows the preparation of thin (3-5 µm) sections and has the advantage of providing an indefinitely stable tissue supply, a significant loss of antigenicity can occur during the embedding process. For this reason cryostat and vibratome sections were preferred. Tissues for cryostat sectioning were prepared by rapid freezing in melting Arcton (-158°C) (appendix IV) and prior to cutting they were allowed to equilibrate to the temperature of the cryostat (-20°C). Sections of varying thicknesses (5-40 µm) were mounted on pre-washed slides coated with poly-L-lysine (Huang et al., 1983) as an adhesive. When thicker (40-100 µm) sections were required a vibratome was used. In this case, fixed-tissue blocks were attached to a chuck, with "super glue", and slices cut by means of a vibrating razor blade advancing horizontally through the block. Vibratome sections have better morphological preservaton than frozen sections and so are preferred when high resolution and magnification at light

microscopy is required (Pickel et al., 1979).

## 2.4 IMMUNOCYTOCHEMISTRY

Immunocytochemistry is the localisation of a tissue constituent in situ by means of a specific antigen-antibody reaction using a labelled or unlabelled antibody which can be detected microscopically. The first method pioneered by Coons et al., in 1941 was performed in one step using a primary antibody conjugated directly to fluorescein isothiocyanate (FITC). Since then a number of modifications have been used to improve the sensitivity and specificity of the method. The direct method has thus been largely superseded by indirect methods employing a FITC-conjugated immunoglobulin raised against the animal species in which the primary antiserum was obtained (Weller and Coons, 1954). Such indirect methods have several advantages over the original technique. They prevent loss of valuable primary antisera during labelling, allow use of lower concentrations of primary antisera and since the secondary labelled antibodies may attach to multiple sites of the primary antigen (Sternberger, 1979) they amplify the signal. A further indirect method rendering the antigen-antibody site visible by ordinary light employed enzymes like horseradish peroxidase, which could be conjugated to secondary antibodies without great losses of enzyme or antibody activity (Nakane, 1968, 1971 for reviews). However, it was felt that immunogenic rather than covalent attachment of enzyme to antibodies would result in less reduction of enzyme/antibody activities and this gave rise to unlabelled immunoenzyme techniques (Petrusz et al., 1976;

Sternberger et al., 1970). The best known of these modifications is the PAP technique (Sternberger, 1979; Sternberger et al., 1970). This method involves use of three reagents applied to the tissue stepwise. After the primary antiserum has been applied a second unlabelled antibody, raised against the immunoglobulins of the first antiserum is applied in excess so that one of its two identical binding sites is free. The PAP complex, which is a stable cyclic complex resulting from combination of peroxidase and anti-peroxidase (raised in the donor species of the first antiserum) is then applied and attaches to the free binding site.Thus there is a high ratio of peroxidase label to primary antigen. This method was therefore much more efficient than its predecessors and allowed a greater dilution of primary antibody and concomittant reduction in non-specific, background signal.

In the present study the PAP method was often chosen in preference to the indirect immunofluorescence method. Whichever method was used, several steps were added to improve the quality of immunostain including treatment of tissues with detergents (Hartman, 1973; Hartman et al., 1972) and alcohols and xylene (Costa et al., 1980) to aid antibody penetration or remove residual fat from the tissues respectively. The incubation time and concentrations of primary antisera were varied. In general, prolonged incubation times yielded better results and further improvement of a weak signal could be achieved by repeated applications of primary antisera (Gu et al., 1983) and/or repetition of second or third layer antisera (Vacca, 1982).

In addition, the developed peroxidase reaction (Graham and Karnovsky, 1966) lends itself to chemical modifications which provide further enhancement of the visualised end-product. Cobalt chloride intensification of diaminobenzidene (DAB) (Bowker et al., 1982) and the glucose oxidase-DAB-nickel methods (Shu et al., 1988) were most frequently employed. In extreme cases where concentrations of antigen are very low a modification (Springall et al., 1984) of the immunogold silver staining method (Holgate et al., 1983) was used.

### 2.4.1 Detection of multiple antigens

It is often necessary to localise more than one antigen in a neurone. Several methods can be employed to accomplish this. A 'cocktail' of antisera raised in different species can be applied directly to the section as a primary antiserum. Providing that the secondary detecting, immunoglobulins are specific to the species in which the primary antisera were raised and free of inter-species cross-reactivity they can be used to distinguish the different antigenic sites using complementary fluorophores or chromogens (Mason et al., 1983; Van Noorden, 1986 for reviews). The usefulness of this method is often hampered since most primary antisera are raised in rabbits. Elution of "reacted" primary antiserum with subsequent restaining with the second antiserum of interest provides an alternative method. In chapter 3, the method of Nakane (1968) in which primary antisera were eluted in a glycine/acid solution rather than in potassium permanganate (Tramu et al., 1978) was employed as it provided a less harsh treatment.

The success of elution techniques, relies on lack of cross-reactivity between the two primary as well as secondary antisera, completeness of elution and preservation of tissue antigenicity. To confirm the data from elution experiments a second alternative, serial sections were used. Coexistence studies were restricted to cells of the dorsal root ganglia, and since these are relatively large cells serial sections (5 µm) were in many cases adequate. However, to optimise conditions for detection of two antigens in a cell, pairs of serial sections (10-20 µm) were mounted on slides in such a way as to produce mirror-images of each other (Gibson et al., 1984d). Each section could then be immunostained with different antisera.

## 2.5 ANTISERA

Most primary antisera employed (Table 2.1) were polyclonal and raised in rabbits in conjunction with Professor Bloom's department at Hammersmith Hospital using a protocol similiar to the following:

Prior to immunisation the peptide antigens were frequently, but not always (Table 2.1) chemically coupled to a larger carrier protein to evoke a significant immune response since small peptides are not generally immunogenic (Sofroniew et al., 1978; Szelke, 1983). The choice of coupling agent can to some extent dictate the region specificity of the resulting antiserum. For example, glutaraldehyde preferentially couples with amino groups, bis-diazobenzidine with tyrosine, and carbodiimide with amino and

TABLE 2.1 ANTISERA CHARACTERISTICS

| Antisera to | Source | Carrier | Coupler | Dilution IMF : PAP | Absorption with homologous antigen | [1]cross-reacting antigens 20 nmols/mls dil. antiserum) | Antigens with no effect (20 nmols/ml dil. antiserum) |
|---|---|---|---|---|---|---|---|
| ACTH (1-24) (synth.) | Vale, U.S.A. | BSA | CDI | 1/100:1/1000 | 0.1 | n.s. | n.d. |
| BN (synth. amphibian) | H/H | BSA | GLUT | 1/400:1/2000 | 1.0 | GRP (-) porcine SP (+) | CCK8; NP; NT; PHI; $SOM_{14,28}$ |
| CGRP (synth. rat) | H/H | Human $\alpha$ globulin | BDB | 1/500:1/4000 | 0.1 | n.s. | $SP_{1-9}$; SP; $SOM_{14}$; CALC; BN; NKA; GAL; VIP |
| CCK 8 sulphated (synth.) | H/H | BSA | BDB | 1/500:1/5000 | 1.00 | CCK33 (-); CCK39 (-); CGRP (+) | GAL; VIP; SP; ENK; SOM; PHI |
| ChAT[2] (nat. rat) rat monoclonal | Wainer, U.S.A. | - | - | 1/100:1/2000 | | n.s. | CGRP; SP |
| CPON (synth. human) | H/H | KLH | GLUT | 1/300:1/3000 | 1.00 | n.s. | CGRP; SP; NPY |
| DBH[3] (nat. bovine) | Rush, Australia | - | - | 1/200:1/1000 | | n.s. | n.d. |
| ENK-methionine (synth.) | H/H | BSA | GLUT | 1/400:1/1000 | 1.0 | L-ENK (-) | CGRP; GAL; SP; NKA |
| FMRF-$NH_2$ (synth.) | H/H | BSA | GLUT | 1/100:1/1000 | 1.0 | NPY (+) | CGRP; SP; L-ENK; M-ENK |
| GABA[4] (synth.) | Kingan, U.S.A | KLH | GLUT | 1/200:1/1000 | | n.s. | CGRP; SP |
| GAD[5] (nat. mouse) | Wu, U.S.A. | - | - | 1/50:1/100 | | n.s. | CGRP; SP |
| GAL (nat. porcine) | H/H | - | - | 1/1000:1/5000 | 1.0 | n.s. | $CCK_{8,33,39}$; CGRP; SP; NKA; VIP; PHI |

TABLE 2.1 (CONT'D)

| Antisera to | Source | Carrier | Coupler | Dilution IMF : PAP | Absorption with homologous antigen | [1]cross-reacting antigens 20 nmols/mls dil. antiserum) | Antigens with no effect (20 nmols/ml dil. antiserum) |
|---|---|---|---|---|---|---|---|
| GFAP[6] (nat. rat) | Anderton, U.K. | - | - | 1/500:1/2000 | | n.s. | n.d. |
| 5-HT (synth.) | De Mey, Belgium | BSA | Formaldehyde | 1/1000:1/5000 | 0.1 | n.s. | n.d. |
| MCH (nat. salmon) | Zamir, U.S.A. | BSA | CDI | 1/500:1/1000 | 1.0 | n.s. | ACTH; NmU |
| NF[7] (nat. avian) | Dahl, U.S.A. | - | - | 1/500:1/2000 | | n.s. | n.d. |
| NKA (synth.) | H/H | BSA | GLUT | 1/200:1/5000 | 1.0 | SP (+); NKB (+) | CGRP; $CCK_8$; M-ENK; GAL; NPY |
| NmB (synth. bovine) | H/H | BSA | GLUT | 1/1000/1/3000 | 1.0 | BN (+); Ranatensin (+); GRP (+); Litorin (+); NmC (+) | CGRP; SP; TSH (rat) |
| NmN (synth. porcine) | H/H | GLUT | BSA | 1/200:1/500 | 0.1 | NmN 9-13 (-); NT (-) | n.d. |
| NmU 8 (synth. porcine) | H/H | BSA | GLUT | 1/400:1/3000 | 1.0 | NmN 9-13 (-) | ACTH; CLIP; VIP; POMC (1-76); PP |
| NPI (nat. porcine) | Watkins, Australia | - | - | 1/200:1/2000 | 1.0 | APP (+) PYY (-) | CGRP; $CCK_8$; VIP; PHI; SP; $SOM_{14}$ |
| NT (synth. bovine) | H/H | BSA | BDB | 1/800:1/1400 | 0.1 | n.s. | SP; CGRP; GAL; M-ENK |
| NPY (synth. porcine) | H/H | - | - | 1/800:1/3000 | 1.0 | n.s. | CGRP; GAL; CPON; M-ENK; $SOM_{28}$; SP |
| OXY (synth.) | Pelletier, Canada | BSA | BDB | 1/500:1/1000 | 0.1 | n.s. | CGRP; $CCK_8$; M-ENK; SP; VIP; VP |

TABLE 2.1 (CONT'D)

| Antisera to | Source | Carrier | Coupler | Dilution IMF : PAP | Absorption with homologous antigen | [1]cross-reacting antigens 20 nmols/mls dil. antiserum) | Antigens with no effect (20 nmols/ml dil. antiserum) |
|---|---|---|---|---|---|---|---|
| $PANC_{1-49}$ (synth.) | H/H | BSA | GLUT | 1/100:1/2000 | 1.0 | PANC 33-49 (+); Human CgA (-) | CGRP; GAL; VP |
| PHI (nat. porcine) | H/H | BSA | GLUT | 1/400:1/1000 | 0.01 | n.s. | CGRP; $CCK_{8,33}$; ENK; $SOM_{14,28}$; SP; VIP |
| $SOM_{14}$ (synth.) | H/H | BSA | CDI | 1/400:1/2000 | 1.0 | $SOM_{28}$ (-) | CGRP; M-ENK; GAL; NPY; $CCK_8$ |
| $SOM_{14-28}$ (synth.) | H/H | BSA | CDI | 1/200:1/1000 | 0.1 | $SOM_{14}$ (-) | CGRP; M-ENK; GAL; NPY; SOM; $CCK_8$; NT; |
| Substance P (synth.) | H/H | BSA | GLUT | 1/400:1/5000 | 1.0 | Bn (+) | CGRP; $CCK_8$; GAL; NKA; NKB; NP; NT; PHI; $SOM_{28}$; VIP |
| TH[8] (nat. rat.) | Thibault, France | - | - | 1/100:1/1000 | - | n.s. | CGRP; GAL; SP |
| TRH (synth.) | Jackson, U.S.A Kikuyama, Japan | BSA | GLUT | 1/100:1/500 | 1.0 | n.s. | CGRP; NmU; ACTH |
| VIP (nat. porcine) | H/H | KLH | BDB | 1/200:1/2000 | 0.1 | n.s. | CGRP; GAL; M-ENK; NT; SP; $SOM_{28}$; PHI |
| VP-arginine (synth.) | Coghlan, Australia | BSA | GLUT | 1/200:1/2000 | 0.1 | n.d. | CGRP; GAL; SP; OXY |

KEY: H/H, Hammersmith Hospital; synth., synthetic; nat., natural; n.k., not known; n.d., not done; n.s.; no sequence homology suspected; BSA, bovine serum albumin; BDB, bis-diazobenzidene; CDI, carbodiiamide; CgA, chromogranin A; GLUT, glutaraldehyde; THYR, thyroglobulin; KLH, keyhole limpet haemocyanin; IMF, immunofluorescence; L, leucine; M, methionine; TSH, thyroid stimulating hormone. For other abbreviations see pp. 6. [1]cross-reacting antigens, ( ) = effect the antigen has on immunostaining, (-) completely quenched, (+) reduces immunostaining significantly. 2-8 original publications. 2, Wainer et al., 1984; 3, Lees et al., 1981; 4, Hoskins et al., 1986; 5, McLaughlin et al., 1975; 6, Woodhams et al., 1980; 7, Dahl and Bignami, 1977; 8, Thibault et al.,

carboxyl groups thus exposing specific regions of hapten to the immune system (O'Shaugnessey, 1982). The immunogen was subsequently emulsified and injected subcutaneously at multiple sites in the flanks of New Zealand white laboratory rabbits (approximately 1 mg antigen, 50-200 µl/injection).

At four and eight weeks, and at monthly intervals thereafter booster injections were given. Useful antibody titres were rarely obtained before the third boost and did not generally improve after 7-8 boosts. Peak antibody production occurs 7-10 days subsequent to booster injections. Blood is therefore taken at or near to this time interval. To ensure that a good response was not overlooked sera were diluted to cover a wide concentration range and screened as soon as possible on known positive tissues employing the normal immunostaining controls.

## 2.6 SPECIFICITY IN IMMUNOCYTOCHEMISTRY

### 2.6.1 Serum specificity

Since all immunocytochemical specificity tests on primary antibodies provide only indirect evidence of specificity, immunostaining should always be referred to as antigen-like immunoreactivity. A cautious approach is especially appropriate for the immunolocalisation of peptides since the antigenic determinant may be as few as four or less amino acids and not necessarily at one site in a molecule (Larsson, 1983). Families of structurally related peptides having common amino acid sequences increase the

the chances of cross-reaction and a number of reports have described the localisation of peptides which have subsequently proven to be quite different molecules (see Ju et al., 1986; Williams et al., 1987). When possible, immunocytochemical studies should be complemented by the biochemical isolation of the relevant antigen. The subject of specificity in immunocytochemistry has been extensively reviewed (Larsson, 1981, 1983; Petrusz et al., 1976, 1977; Pool et al., 1983; Sternberger, 1979, 1982; Swaab et al., 1977). The most practical, convenient and generally accepted means to test primary antiserum specificity is liquid phase absorption/inhibition of the antiserum prior to immunostaining. To do this the antiserum is initially incubated with the antigen which was used as immunogen in preparation of the antibodies (see appendix VII). Further characterisation of the primary antiserum can be achieved by similar incubation with related or unrelated antigens known to be present in the tissue. A comparison of the concentrations required to inhibit the reaction can give an indication of the degree of cross-reaction between closely related antigens. The availability of synthetic fragments of peptides may, by the same means, be used to determine the region specificity of the antisera.

The specificity of antisera employed is summarised in Table 2.1. Antisera raised to substance P showed significant cross-reactivity with other tachykinins (neurokinins A and B) and vice versa. Thus although the term substance P is used to describe immunoreactivity in this thesis, the nomenclature tachykinin-like immunoreactivity is more correct. Although, no cross-reactivity between VIP and

PHI or NPY and CPON was noted, since each pair of peptides share a common precursor and coexist in a number of tissues (see chapter 1) the terms VIP/PHI and NPY/CPON immunoreactivity have been adopted.

### 2.6.2 Method specificity

All interference, background or false positive immunostaining due to factors other than the primary antibody-antigen interaction are referred to as method specific. Many remedies have been offered to remove or reduce such interference. Several of those used routinely in the present study are as follows:

i) Use of primary antiserum (from hyperimmune donors) at the highest dilution giving optimum reproducible immunostaining so than any interfering serum proteins are removed by dilution.

ii) Pre-incubation of tissue sections with hydrogen peroxide to saturate endogenous peroxidase sites.

iii) Pre-incubation of tissue sections with normal serum from the donor species of the second bridging antibody. This will saturate tissue sites which may possibly bind the part of the immunoglobulins of the second antibodies.

iv) The carrier protein used to induce the primary anti-hapten antibodies can be added to the primary antiserum to remove high concentrations of unwanted antibodies.

v) Individual primary or subsequent immunoreagents can be omitted to serve as negative controls.

## 2.7 CONVENTIONAL HISTOLOGY AND ENZYME STAINS

Conventional stains: A combination of immunostaining and conventional staining on consecutive sections enabled a more precise localisation of immunostained structures. To assess tissue preservation and morphology sections were stained with either toluidine blue, haematoxylin and eosin, or cresyl fast violet and luxol blue. Haematoxylin and cresyl fast violet were also used as counterstains for tissues immunostained using the PAP method.

Enzyme stains: The presence of FRAP activity was demonstrated in the spinal cord using a modification (Ainsworth et al., 1981) of the Gömori technique (Gömori, 1950). The diisopropyl fluorophosphate (DFP) pharmacohistochemical method was employed for demonstration of acetylcholinesterase (AChE) (Satoh et al., 1983). Rats were injected with DFP (1.5 mg/ml) in arachis oil, perfused with 10% formalin containing 1% cobalt chloride and sections processed for AChE according to the method of Karnovsky and Roots (1964).

## 2.8 MICROSCOPY AND PHOTOGRAPHY

Transmitted light preparations were photographed on a Polyvar microscope, equipped with Nomarski differential interference contrast optics (Reichert-Jung, U.K.). Fluorescent preparations were viewed on an Olympus AH-2 Vanox microscope with a HBO 200 W2 lamp (Olympus Optical Co. Ltd., London, U.K.). Characteristics of the filters used are summarised in Table 2.2.

TABLE 2.2 CHARACTERISTICS OF FILTER SYSTEMS AND FLUOROPHORES

| Excitation | Excitation $\lambda$ nm | Observation light $\lambda$ nm | Fluorophore | Colour |
|---|---|---|---|---|
| Ultra-violet | 330-380 | 420 | True Blue<br>Diamidino Yellow | Blue<br>Yellow |
| Blue | 380-490 | 515 | Fluorescein<br>isothiocyanate | Green/<br>Yellow |
| Green | 465-550 | 590 | Rhodamine | Red |

Black and white fluorescent or ordinary light photographs were obtained by exposure onto Ilford FP4 (ASA 125) or Kodak Technical Pan 2415 (ASA 150-175) films respectively. Negatives were developed in 20% Ilford Contrast FF developer for 2.5 minutes (20°C) and printed on Ilfospeed Multigrade II paper or Kodabrome Grade 5 in 20% Suprol Paper Developer (May and Baker, U.K.).

## 2.9 ASSESSMENT OF IMMUNOSTAIN

A marking system was used to indicate relative numbers of immunoreactive fibres or cell bodies/section. Generally a graded system ranging from 1+ to 4+ was used, with 1+ indicating sparse immunoreactivity and 4+ abundant immunoreactivity. Intensity of immunoreaction was also taken into account. In any assessment at least 5 consecutive sections were studied and in a comparative analysis between experimental groups at least 4 animals were studied.

## 2.10 QUANTIFICATION

Immunocytochemical results are not easily quantifiable because of variations in the limits of detection inherent in the technique. Once optimal conditions for both tissue and antisera had been established changes in distribution could often be assessed using a graded marking system (see 2.9). However, for example, in the dorsal root ganglia cell counts were often necessary. Total cell numbers and numbers of immunostained cells were estimated from cell counts taken from x numbers of sections through the ganglion

(chapters 3 and 4). Final figures were expressed either as mean total cell numbers or as a percentage of the total cell number and significant differences calculated using various statistical analyses. For estimation of immunoreactive cell populations and comparison of retrogradely labelled and immunoreactive cell populations this was often sufficient. However, for more precise studies (chapter 7) where not only cell populations but also cell diameters were required, image analysis systems were employed. To do this a Kontron MOP-AM-03 image analyser linked to a RML 380Z computer and an IBAS 2000 linked to a Z80A based computer were used. The computer takes a television image directly from either a microscope, a photographic print or negative. PAP preparations were analysed directly and fluorescent preparations from photographic negatives. In the latter case, values were calibrated using a known scale length to determine the magnification of the negative used. By altering the parameters of interest, the computer could be programmed to provide measurements of the area occupied by immunoreactive fibres in the dorsal horn of the spinal cord.

## 2.11 RADIOIMMUNOASSAY AND CHROMATOGRAPHY

Concentrations of peptides were determined in normal and experimental tissues. Chromatographic analyses were confined to the spinal cord. The biochemical studies were performed by co-workers in the Department of Medicine, Hammersmith Hospital, London. For further details of technologies see Bloom and Long (1982) and publications derived from the present study.

## 2.12 EXPERIMENTAL PROCEDURES

### 2.12.1 Pharmacological manipulations

Colchicine: To enhance visualisation of peptides within cell bodies axonal transport-blocking agents were employed. Among these substances, the most frequently used include colchicine or the catharanthus alkaloid vinblastine (Dahlström, 1971; Dasheiff and Ramirez, 1985). Both have similar properties in that they bind tubulin and disrupt microtubules (Fitzgerald et al., 1984; Knyihár-Csillik and Csillik, 1981). As a consequence, there is an accumulation of substances produced by the cell body, including peptides. Colchicine and vinblastine have a certain degree of selective neurotoxicity in the central nervous system, some neurones being more susceptible than others (Steward et al., 1984).

In the studies presented colchicine (Sigma Chemicals) (10 mg/ml) diluted in sterile saline was adminstered to deeply anaesthetised rats in various locations (see below). A survival time of 24-48 hours was in most cases adequate.

Spinal cord cell bodies:
5-10 µl of colchicine was injected into the intrathecal space via the magnum cisterna (after Yaksh and Rudy, 1976), or a laminectomy was performed and 5-10 injections of 1-2 µls were made directly into the spinal cord. The latter proved the most reliable method.

Dorsal root ganglion cell bodies:

i) A laminectomy was performed to expose several spinal ganglia and 1-2 µl of colchicine was injected directly into each ganglion.

ii) One sciatic nerve was exposed and a perineural cuff, soaked in colchicine was applied for 15 minutes. To determine the effect on the central terminals of peptide-immunoreactive ganglion cells rats were killed at intervals between 3-42 days.

6-hydroxydopamine (6-OHDA): 6-OHDA was used in order to confirm or exclude the presence of peptides in sympathetic nerves. 6-OHDA acts as a 'false transmitter', being incorporated non-specifically into amine-containing nerves (Thoenen and Tranzer, 1971). The toxin induces a selective degeneration of aminergic neurones (Thoenen and Tranzer, 1968). Rats were injected with 6-OHDA HCl (Sigma Chemicals) (0.5 ml, 50 mg/ml body weight in saline) at days 7,6 and 1 before sacrifice.

Capsaicin: Since the onset of the present study the use of capsaicin (8-methyl-N-vanillyl-6-noneamide), the active ingredient of chilli powder, first recognised for its analgesic properties (Jancsó, 1968), has considerably aided analysis of the sensory nervous system (Fitzgerald, 1983; Holzer, 1988; Nagy, 1981 for reviews). Topical application to the skin or mucous membranes induces an acute burning sensation which is followed by a period of desensitisation. This biphasic response was originally thought to be caused by release and subsequent depletion of a "nociceptive substance" from the terminals of sensory neurones. Experiments in animals show that capsaicin acts selectively on small diameter

afferent fibres. In neonatal rats and mice, systemic administration of capsaicin results in a degeneration of the majority of C fibres (90-95%), which is dose-dependent and a variable loss of Aδ fibres at high doses (50-100 mg/kg body weight) (Jancsó et al., 1977; Lawson and Nickels, 1980; Nagy et al., 1983; Scadding, 1980). While in neonates (< 10 days after birth) systemically administered capsaicin induces a permanent degeneration of small dorsal root ganglion cells, in adults, the effect is reversible. Furthermore, perineural application, e.g. to the sciatic nerve of adult rats, apparently causes no degeneration of the central terminals but induces a long term block of axonal transport of peptides in sensory fibres (Ainsworth et al., 1981; Gamse et al., 1982; Gibson et al., 1982). Thus a capsaicin-induced depletion of peptide not only enables one to establish that the peptide is sensory in origin, but also confirms presence of the peptide in small primary sensory neurones.

i) Preparation of capsaicin solution

Since capsaicin is an relatively insoluble compound in water, a number of vehicle solutions were investigated. The most successful was Tween 80 [poly oxyethylene (20) sorbitan mono-oleate] (Sigma Chemicals), ethyl alcohol and sterile saline in the following proportions 1:1:8 vols.

ii) Neonatal administration to rat pups

Rat pups were anaesthetised by hypothermia and injected

subcutaneously with capsaicin (50 mg/kg) (Sigma Chemicals or Fluka AG) dissolved in a vehicle solution. Animals were injected initially on postnatal day 2, but subsequent experiments showed injection at days 2 and 3 provided a more consistent result. Rats were killed 2-3 months after treatment. Effectiveness of treatment was judged before sacrifice by examination of bladder size. Control litters were treated in parallel with the vehicle solution.

iii) Administration to adult guinea pigs

Guinea pigs were anaesthetised with valium (Diazepam) (Roche) and Hynorm (Fentanyl base, Fluanisone) (Janssen Life Sciences) followed by an intraperitoneal injection of theophylline (5 mg/kg) and an aerosol spray inhalation of isoprenaline sulphate to reduce bronchospasm induced by the capsaicin. Five consecutive daily doses of capsaicin were given (25 mg/kg, day 1; 50 mg/kg day 2; 100 mg/kg day 3; 200 mg/kg day 4; 400 mg/kg day 5). Following the initial injection the animals were desensitised and subsequent injections were given under light ether anaesthesia. Control animals received vehicle solution alone. Tissues were collected up to 2 months after the last injection.

iv) Perineural capsaicin administration

Rats were deeply anaesthetised. One sciatic nerve was exposed and capsaicin was applied perineurally for 15 minutes.

a) Time course: Rats were treated with capsaicin (49 mM) or

vehicle solution alone and killed at intervals from 2 to 200 days later.

b) Dose response: Rats were treated with graded doses of capsaicin solution (0.1 - 49 mM) and killed 14 days later.

c) Controls: Capsaicin was applied intramuscularly (to a cut in biceps femoris) to one leg to assess whether changes induced were due to leakage onto the skin or to uptake by the vasculature. Animals were killed 14 days later.

d) Noxious thermal response: The withdrawal reaction time of rats treated in groups b)- c) was assessed using the hind-paw water immersion test (T = 49°C ± 2°C). Each foot was placed in turn into a water bath and withdrawal times noted in triplicate. An upper limit of 15 sec was set.

### 2.12.2 Surgical manipulations

All manipulations were performed on rats and/or cats under general anaesthesia and in aseptic conditions. Interruptions of nerve pathways were performed on animals of weight and age comparable to normal (unoperated) control and sham-operated animals. When manipulations were performed unilaterally, the contralateral side was used for comparison as an internal control. However, it was first necessary to establish that no significant changes were induced in the contralateral side by comparison with normal animals.

Spinal cord

Deafferentation: Multiple, unilateral dorsal rhizotomies were

performed on cats and rats. A laminectomy was performed, the dura was split and dorsal roots ($L_2$-$S_4$ in cats, $C_5$-$C_8$ or $L_2$-$L_6$ in rats) were cut. Ganglionectomies were performed only in cats by unilateral removal of ganglia from segmental levels $S_1$-$S_4$. Animals were killed 2 (rats) or 4 (cats) weeks later.

Descending and ascending pathways: A thoracic laminectomy was performed in rats and cats. Hemisections and transections (in rats only) were made taking care to minimise vascular damage by employing cautery. Lesions of the dorsolateral funiculus were performed separately in a small group of rats (chapter 4.5.2). Animals were killed 2 weeks later.

Short intrinsic projections: In rats an attempt to isolate the region of the central canal by lesioning either side (1 segment) rostral and caudal to the central canal was made using a sharp needle. Animals were killed 2 weeks later.

Peripheral nerves

i) Unilateral sciatic nerve section and ligation, unilateral sciatic nerve crush (30 sec with a plastic coated haemostat). Rats were killed at intervals depending on experimental design from day 1 up to 12 months later.

ii) Unilateral pelvic nerve section proximal to the pelvic plexus. Cats and rats were killed 4 and 1 week later respectively.

iii) Unilateral genitofemoral nerve section. Rats were killed 7 days later.

Further details of numbers and types of manipulation are outlined

in each chapter. Cat surgery was performed by Dr. J.F.B. Morrison, Department of Physiology, Leeds University. Some spinal cord operations were undertaken by Professors P.D. Wall at University College London, and J.S. Kelly at St. Georges Medical School, London.

### 2.12.3 Tracing techniques

Anterograde and retrograde tracing methods have demonstrated that nerves from many brain and brainstem regions project to the spinal cord and have also helped elucidate many afferent and efferent projections between the spinal cord and peripheral nervous system (Heimer and Robards, 1983). Combination of immunocytochemistry with tracing techniques has proved an extremely powerful and precise tool for demonstrating the origin of peptides/transmitters in the nervous system. At present many tracers are available. Horseradish peroxidase (HRP) and its conjugates including wheat-germ agglutinin (WGA), phaseolus vulgaris leucoagglutinin (PHA-L) and the fluorescent dyes are the most frequently used. WGA-HRP is used for retrograde, anterograde and transganglionic tracing, PHA-L for anterograde tracing and the fluorescent dyes are retrogradely transported. The choice of tracer predetermines the immunocytochemical procedure. Originally HRP was used to map pathways; this necessitated demonstration of monoamine or peptide as a fluorescent product, followed by photography and histochemical processing for HRP. Detection of monoamine or peptide by an immunoperoxidase method using one chromogen and of HRP with another permitted simultaneous visualisation of both reaction

products (Ljungdahl et al., 1975). All methods involving peroxidase chemistry for both tracer and cell product require rigorous control procedures to avoid false positives.

The use of fluorescent dyes combined with aldehyde-induced fluorescence, AChE staining, and, in particular immunocytochemistry has revolutionised neurochemical tract-tracing (Hökfelt et al., 1983; Skirboll et al., 1984; Su and Polak, 1987 for reviews) and provides a precise means for determination of the origin and projections of peptide-immuoreactive neurones (Bowker et al., 1982; Kawatani et al., 1983; Lorenz et al., 1985; Sharkey et al., 1983). A large number of fluorescent dyes are available. The dyes can be visualised in tissue sections with a fluorescence microscope and can be used singly or multiply for tract-tracing and can also be applied in combination with other methods, including immunocytochemistry (Hökfelt et al., 1983). The dyes differ from one another in terms of factors such as excitation and emission characteristics, cellular localisation, solubility, rate of transport and degree of transneuronal labelling (Table 2.2). In the present study the fluorescent dye, True Blue has been used extensively.

Preparation of retrograde fluorescent tracers

**True Blue** (1,2-bis[5-Amidino-2-benzofuranyl]-ethylene-diaceturate) (Sigma Chemicals)-5% aqueous solution.
**Diamidino yellow** (2-[4-Aminophenyl]-idol-6-carboxyamidinodihydro-drochloride) (Sigma Chemicals)-2% aqueous solution.

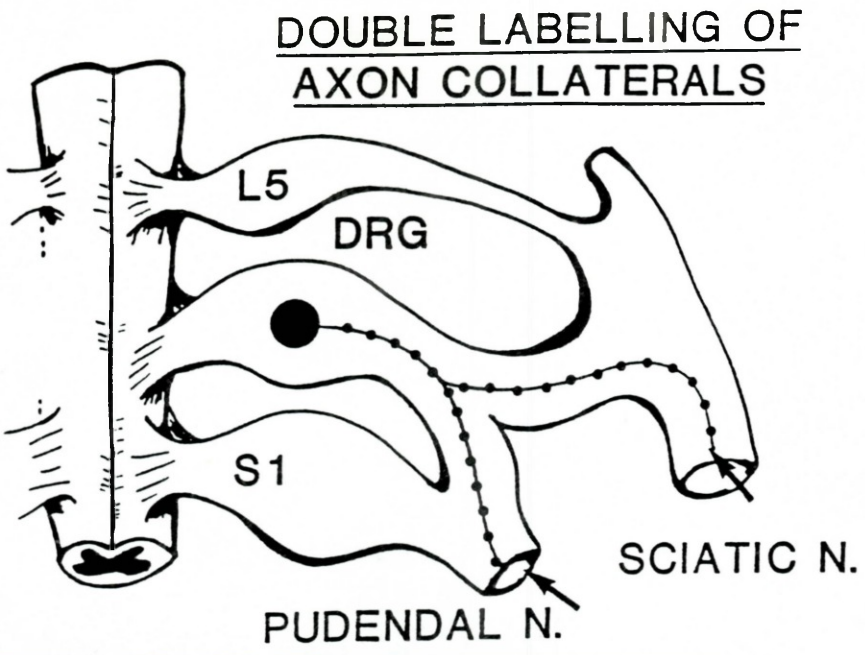
**Rhodamine-labelled latex microspheres** (0.02 µm - 0.2 µm diameter) - concentrated aqueous solution (gift from Dr. Boyse Burge, Santa Clara, U.S.A.)

Double retrograde labelling

Differing characteristics of tracers were used to determine the possibility of divergent axon collaterals of sensory neurones contributing to the innervation of different tissues (Fig.2.1) (chapter 4.5.3). In deeply anaesthetised rats True Blue was injected into pudendal nerves and diamidino yellow or rhodamine-labelled microspheres were injected into the sciatic nerves. Since diamidino yellow is labile and has a short retention time, True Blue was injected first and the animals reanaesthised and injected with diamidino yellow 2 days later. True Blue and rhodamine-labelled microspheres were injected at the same time, since both have long survival times. True Blue and rhodamine-labelled microspheres are both cytoplasmic markers and labelled cells can be identified using appropriate filter systems (Table 2.2). Diamidino yellow marks nuclei a gold yellow colour which is visible at the same excitation wave length as True Blue.

Retrograde tracing and immunocytochemistry

True Blue (2-5 µl) was injected in multiple sites into organs/ tissues or directly into nerve trunks. In the latter case the needle of the Hamilton syringe was gauged to fit the width of the nerve, ensuring dye uptake by the maximal numbers of fibres.



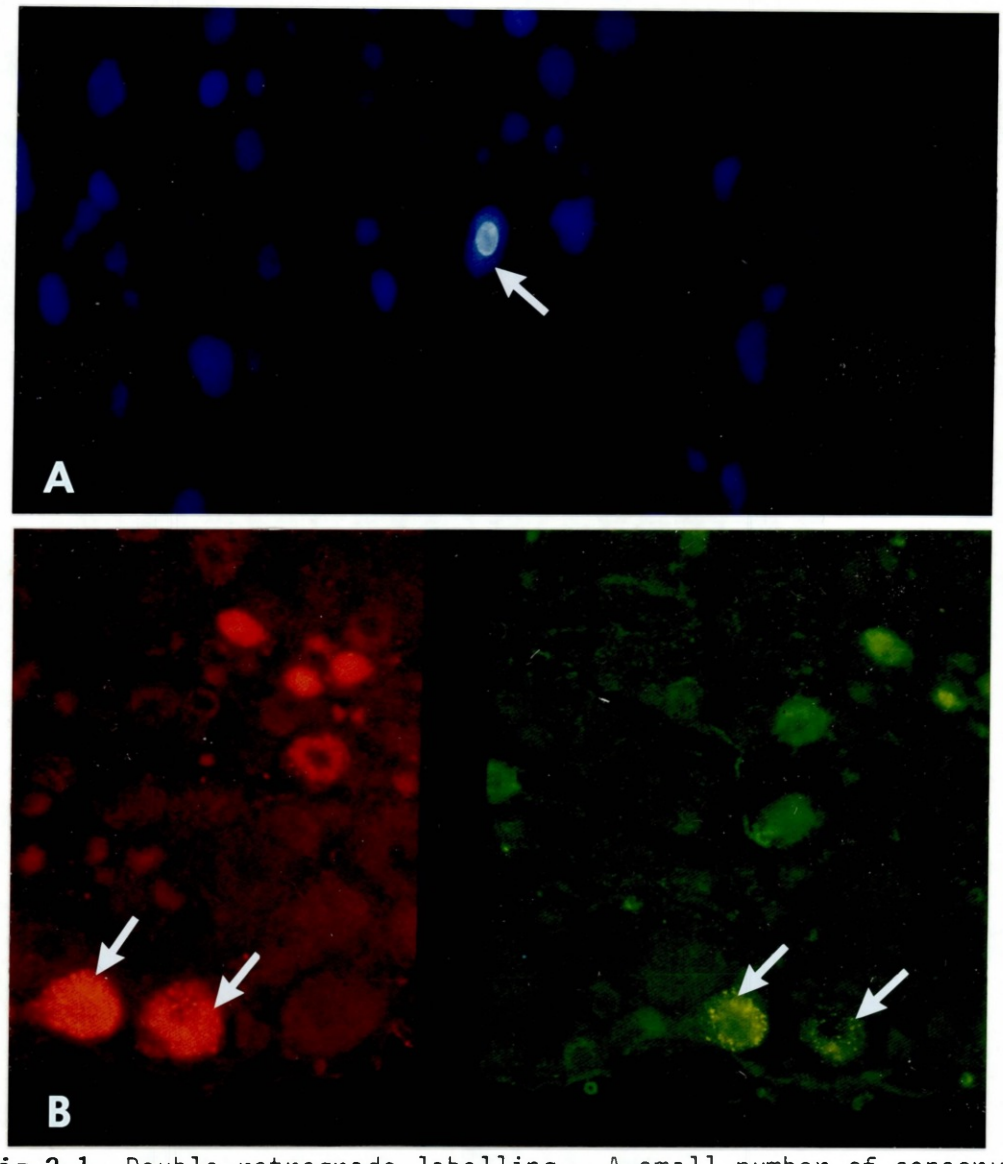




**Fig.2.1** Double retrograde labelling. A small number of sensory neurones in the rat $L_6$ dorsal root ganglia have dichotomising peripheral projections to sciatic and pudendal nerves (N.). A) Diamidino yellow injection to the sciatic and True Blue injection to the pudendal nerve. One double labelled cell body is present (arrow). B) Rhodamine-labelled latex microspheres injected to the sciatic nerve and a section from the $L_4$ dorsal root ganglion subsequently immunostained for substance P. Double labelling is clearly seen (arrows) by using appropriate filter systems to visualise the same section (A,B x 225).

After injection the needle was left in position for 1-2 minutes to avoid leakage due to build up of back pressure. Survival times were derived empirically for each system studied. Animals were perfused with 4% paraformaldehyde, which was found to be the only fixative suitable for combined tracing and immunostaining. True Blue labelled cells and peptide-immunoreactive structures were assessed using appropriate filter systems (Table 2.2). A slight fading of True Blue was seen subsequent to immunostaining, but the numbers of cells were unaltered as established by comparison with control sections. Similar comparative analyses revealed no differences in numbers or in intensity of immunostained structures as a result of retrograde tracing with this dye. Further details of injection sites, and numbers of animals are given in chapter 4.5 and appendix VIII.

# CHAPTER 3 DISTRIBUTION OF PEPTIDES IN THE DORSAL ROOT GANGLIA


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# CHAPTER 3

## 3.1 INTRODUCTION

Mechanisms by which sensory information is conveyed to the central nervous system are of particular importance and the dorsal root ganglion has been a favoured subject for research since late last century. As early as 1851, (Stannius, 1851) it was shown that cell bodies in the dorsal root ganglia were not uniform with respect to size or histochemical staining properties. Electron microscopic analysis has since confirmed the initial observation of two major cell types based on size, density of cytoplasm, organisation of Nissl substance, diameter of cell processes and the presence of a myelin sheath (Andres, 1961). It is now generally agreed that the large cells give rise to large diameter myelinated axons which are generally non-nociceptive and the smaller cells to finely myelinated A $\delta$, or unmyelinated C fibres which may be nociceptive or respond to innocuous stimuli (see chapter 1). This basic morphological classification of two cell types has gained wide acceptance, (Anderton et al., 1982; Duce and Keen, 1977; Lawson and Harper, 1985) although, several other classifications have been proposed (Lieberman, 1976 for review; Rambourg et al., 1983). However, the functional significance of the latter classifications is unknown and methods, other than morphological criteria, maybe needed to characterise modality-specific sensory neurones. Electrophysiological characterisation (Dodd et al., 1983), metabolite identification (Riley et al., 1984; Wong et al., 1983) or transmitter/cytochemical content (Dodd

et al., 1983, 1984; Hökfelt et al., 1976; Ju et al., 1987; Price, 1985) or combined studies of these aspects (Cameron et al., 1986; Dodd et al., 1983; Lawson and Harper, 1985; Sommer et al., 1985) may provide some clue.

The aim of the present study was to establish which peptides are present in neuronal cells of the dorsal root ganglia and whether there is a preferential distribution to a particular cell size. Identification of peptides in sensory neurones was of importance for further studies relating to i) origins of peptide-immunoreactive fibres in the spinal cord and peripheral tissues and ii) possible changes which occur in abnormal conditions affecting the sensory nervous system (chapters 4, 5 and 7).

## 3.2 EXPERIMENTAL APPROACH

### 3.2.1 Distribution of peptide immunoreactivity

Dorsal root ganglia from all (cervical to sacral) levels of the spinal cord were investigated in rat, guinea pig, cat, marmoset, baboon, pig, horse and man. Tissues were fixed, by either immersion or intracardial perfusion of fixative (appendix III). Frozen tissue sections (10-20 µm) or sections (8-12 µm) of paraffin wax embedded tissues were immunostained using the PAP, indirect immunofluorescence (appendix V, VI) or immunogold silver staining methods (Springall et al., 1984). The ratio of CGRP- to substance P-immunoreactive cells was estimated in 6-8 pairs of frozen (20 µm) sections in cat (n = 6) lumbar (L7) dorsal root ganglia. In

pig (n = 3), 4-5 sets of four sections were immunostained for CGRP, substance P, galanin (GAL) or somatostatin (SOM) and the total number of ganglion cells, in addition to those displaying peptide immunoreactivity, was counted in five visual fields (x10 objective) per section.

To ensure maximal visualisation of peptide-immunoreactive cells separate groups of animals were subjected to i) injection of lumbar dorsal root ganglia with colchicine (15 rats, 2 cats) or ii) unilateral sciatic nerve ligation (15 rats) prior to processing for immunocytochemistry.

### 3.2.2 Colocalisation studies (rat, cat and pig lumbar ganglia)

**Serial sections:** Reverse face, frozen (20 µm) or wax (12 µm) sections were arranged on slides so that adjacent faces (mirror images) were uppermost before immunostaining (Gibson et al., 1984d).

**Antibody elution (after Nakane, 1968):** Sections were immunostained for the first peptide according to the PAP method, developed in 4-chloro-1-napthol (blue chromogen) and photographed. Immunoreagents were then removed by elution with glycine-HCL buffer (pH 2.2) and immunostained for the second peptide followed by development in diaminobenzidine (brown chromogen). In a series of controls the elution time for substance P, GAL and SOM immunoreactivities was 3-4.5 hrs, but for CGRP exceeded 24 hr and was never complete. CGRP antiserum was therefore always used as the second immunostain.

## 3.3 RESULTS

### 3.3.1 Distribution of peptide immunoreactivity

The distribution of peptides studied in the rat dorsal root ganglia is summarised in Table 3.1. All peptide immunoreactivities were localised to neuronal cell bodies and fibres (Figs. 3.1-3.2) with the exception of NPY/CPON immunoreactivity which was seen only in fibres (Fig. 3.1F). The distribution of peptide immunoreactivity was similar in all species. In some species cell bodies immunoreactive for a particular peptide, appeared more frequently than in others species (e.g. GAL in pig, substance P in guinea pig and cat, VIP/PHI in cat and horse). In addition, peptide immunoreactivity was found predominantly within smaller cells (Figs. 3.1-3.2) (Table 3.1). On the basis of CGRP immunostaining three cell sizes could be distinguished. The most frequent of these were of small ($< 20$ µm) and intermediate (20-50 µm) diameters whilst the largest ($> 50$ µm) were rarely immunostained (Fig. 3.1A-D).

CGRP-immunoreactive cells were the most abundant and were 2-3 times more numerous than substance P-immunoreactive cells (Table 3.2). In pig, the relative abundance of peptide-immunoreactive cells estimated as a percentage of the total population is shown in Table 3.3.

Increased numbers of cells immunoreactive for the majority of peptides were observed after colchicine treatment or 1-3 days

TABLE 3.1 DISTRIBUTION OF PEPTIDE IMMUNOREACTIVITY IN RAT LUMBAR DORSAL ROOT GANGLIA

| Peptide | Cell body staining characteristics | | | Frequency | | |
|---|---|---|---|---|---|---|
| | Small (10-20 μm) | Intermediate (20-50 μm) | Large (>50 μm) | Fibres | Cells | Cells after colchicine |
| CCK | weak homogeneous **weak perinuclear** | weak granular | weak homogeneous | + | + | ++ |
| CGRP | intense homogeneous **intense perinuclear** | **weak granular** | weak homogeneous | +++ | ++++ | ++++ |
| ENK | **intense perinuclear** | n.s. | weak homogeneous | + | ± | + |
| GAL | **weak perinuclear** | weak homogeneous | n.s. | ++ | ++ | +++ |
| NPY/CPON | n.s. | n.s. | n.s. | ++ | n.s. | n.s. |
| SP | weak homogeneous weak perinuclear | weak granular | n.s | ++ | ++ | +++ |

Key: Homogeneous; cytoplasm evenly stained. Perinuclear; immunoreactive granules densest around nucleus. Granular; immunoreactive granules dispersed throughout cytoplasm. n.s., not seen; n.d., not done. Cells: ±, 1 in 20 sections; +, 1-5/section; ++ 5-10/section; +++ 10-100/section; +++ 100/section; Fibres: +, sparse; ++,moderate; +++, dense. PANC studies were performed in porcine tissues. **Bold type** indicates the predominant cell types. All cell body characteristics, with the exception of VIP/PHI-immunoreactive cells which are only seen after colchicine treatment, refer to normal distributions.

TABLE 3.2 PROPORTION OF SUBSTANCE P- TO CGRP- IMMUNOREACTIVE CELLS IN CAT DORSAL ROOT GANGLIA

| | Cat 1 SP : CGRP | Cat 2 SP : CGRP | Cat 3 SP : CGRP | Cat 4 SP : CGRP | Cat 5 SP : CGRP | Cat 6 SP : CGRP |
|---|---|---|---|---|---|---|
| No. sections | 8 | 7 | 6 | 7 | 6 | 6 |
| Mean | 62.9 : 150.0 | 64.9 : 203.3 | 61.9 : 167.7 | 55.0 : 115.1 | 110.8 : 241.0 | 38.8 : 124.7 |
| S.E.M. | 15.8 : 17.0 | 5.1 : 20.8 | 5.7 : 14.7 | 6.7 : 12.7 | 18.1 : 20.8 | 4.8 : 20.0 |
| Geometric mean SP/CGRP | 2.8 | 3.1 | 2.7 | 2.1 | 2.3 | 3.2 |

($P < 0.001$ for testing whether quotient is greater than 1)

TABLE 3.3 PERCENTAGE OF PEPTIDE-CONTAINING CELLS IN PIG LUMBAR DORSAL ROOT GANGLIA

| Peptide | Section | No. cells* | No. peptide-containing cells | %Peptide containing cells | Mean % |
|---|---|---|---|---|---|
| CGRP | 1 | 359 | 128 | 36 | |
| | 2 | 418 | 160 | 38 | |
| | 3 | 452 | 165 | 36 | 35 |
| | 4 | 520 | 156 | 30 | |
| Substance P | 1 | 424 | 88 | 21 | |
| | 2 | 277 | 26 | 9 | |
| | 3 | 402 | 95 | 24 | 18 |
| | 4 | 375 | 92 | 18 | |
| | 5 | 250 | 31 | 25 | |
| GAL | 1 | 703 | 98 | 14 | |
| | 2 | 481 | 34 | 7 | 12 |
| | 3 | 561 | 90 | 16 | |
| | 4 | 532 | 84 | 12 | |
| SOM | 1 | 392 | 24 | 6 | |
| | 2 | 347 | 19 | 5 | |
| | 3 | 449 | 38 | 8 | 8 |
| | 4 | 275 | 10 | 4 | |
| | 5 | 326 | 31 | 9 | |

* = Mean of number of cells in 5 visual fields (x 10 objective).

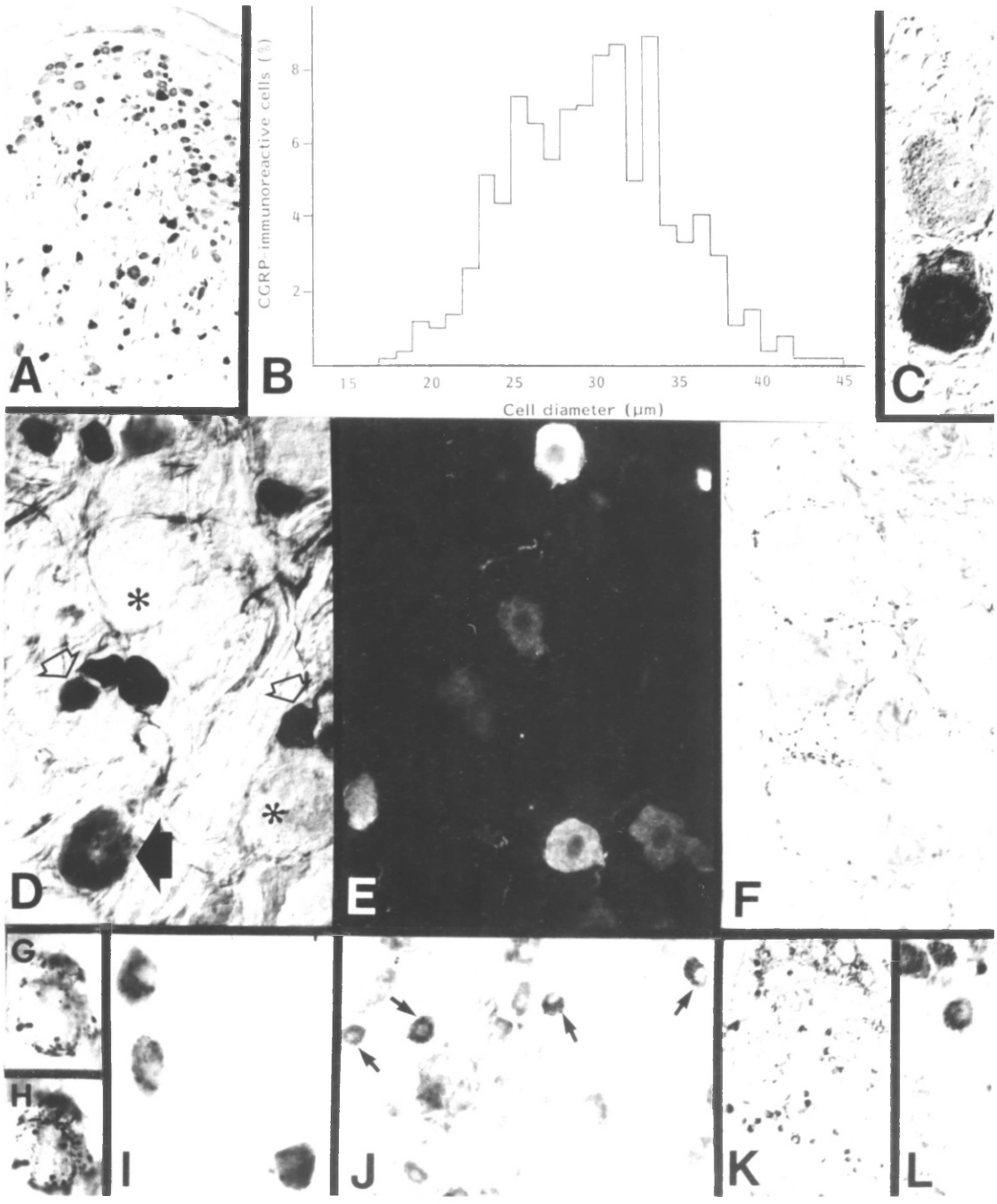



**Fig.3.1** CGRP-immunoreactive cells and fibres in A) guinea pig x 85 C) man x 335 and D) rat lumbar dorsal root ganglia x 270. D) Small (open arrows) and intermediate-sized (blocked arrows) cells are immunoreactive, but no large (asterisks) cells were immunostained. B) CGRP immunoreactivity was localised predominantly to cells with diameters between 20-40 µm. Abscissa, mean diameter, ordinate, number of cells of a particular diameter expressed as a percentage of the total number of cells. A total of 479 cells in the $L_6$ ganglia of rat were analysed using an image analysis system (IBAS 2000, Kontron). E) Substance P-immunoreactive cells in rat dorsal root ganglion x 280. F) NPY-immunoreactive fibres around non-immunoreactive cells in horse lumbar dorsal root ganglia x 230. (G,H) PHI-immunoreactive fibres forming a pericellular network around a non-immunoreactive cell body in cat lumbar dorsal root ganglion x 340. I) Small VIP-immunoreactive cells in rat lumbar dorsal root ganglion after ligation of the sciatic nerve x 240 and J) after colchicine injection (some arrowed) x 150. Numerous GAL-immunoreactive cells in rat lumbar dorsal root ganglion after ligation of the sciatic nerve. K x 69, L x 160. A, C- F Normal, untreated animals. E) Indirect immunofluorescence method, the remainder PAP method.

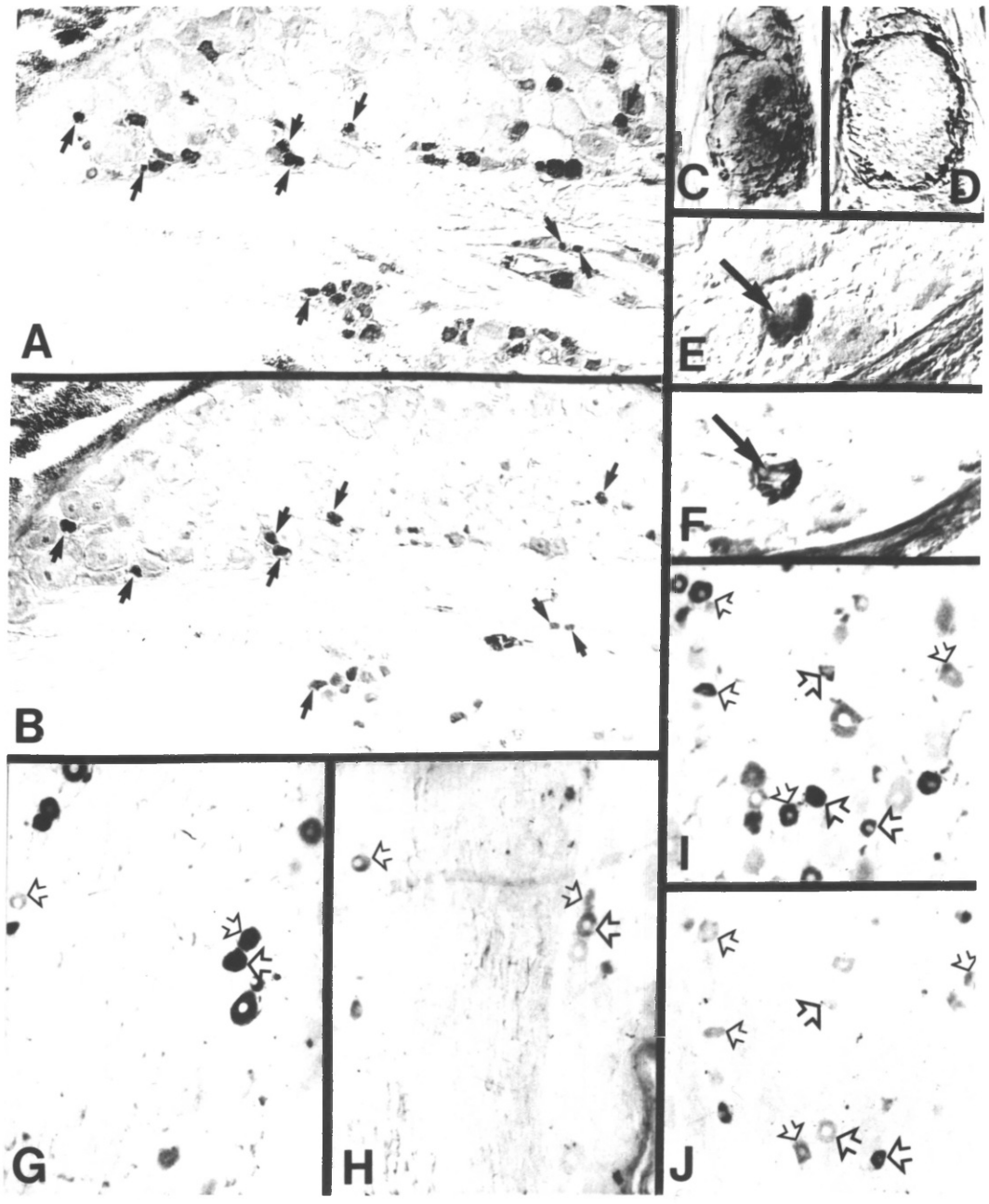



Fig.3.2 Reverse face serial sections of cat thoracic dorsal root ganglia immunostained for A) CGRP and B) substance P showing colocalisation of peptides (arrows) x 80. Reverse face serial sections from the same tissue immunostained for C) CGRP and D) substance P. Fibres immunoreactive for either peptide surround the same non-immunoreactive cell x 345. E) Substance P-immunoreactive cell (arrowed) in rat lumbar dorsal root ganglion. F) Same section, after elution of substance P immunoreactivity and restained with CGRP antiserum x 230. (G,H) and (I,J) pairs of reverse face serial sections of pig lumbar dorsal root ganglion, immunostained for CGRP (G, I), SOM H) and GAL J). GAL- and SOM-immunoreactive cells are colocalised with a subset of CGRP-immunoreactive cells (some arrowed) x 170.

after nerve ligation with the exception of CGRP, which showed only an increased staining intensity (Fig.3.1I-L). VIP/PHI-immunoreactive cells were not seen in control, untreated or non-ligated rats.

### 3.3.2 Colocalisation studies

Most substance P-immunoreactive cells were identified with a subpopulation of small- and intermediate-sized CGRP-immunoreactive cells in rat, cat and pig (Fig.3.2A-F). These peptides were colocalised in all or 90-95% of cells in cat and pig or rat respectively. Serial sections of pig ganglia showed that all GAL- and SOM-immunoreactive cells were colocalised with subsets of CGRP-immunoreactive cells (Fig.3.2G-J). In pig, all GAL-immunoreactive cells contained substance P immunoreactivity, and a few substance P-immunoreactive cells were also immunoreactive for SOM.

## 3.4 DISCUSSION

### 3.4.1 General distribution

The data demonstrate that a number of peptides are found in primary sensory neurones in several mammalian species. CGRP-immunoreactivity was found in a large proportion of these sensory neurones and, with the exception of FRAP and acetylcholinesterase, no other peptide or immunological marker has been shown to be present in such a high percentage of cells[1] (Table 3.4). The rank order in terms of different peptide-immunoreactive cells

[1] in normal non-colchicine treated animals.

TABLE 3.4 SOME CYTOLOGICAL MARKERS OF DORSAL ROOT GANGLION CELLS

| Peptides | Size | % Total cells | Reference(s) |
|---|---|---|---|
| Angiotensin | ? | ? | Fuxe et al., 1976; Hökfelt et al., 1980a |
| CCK | S,I | 15-20 | Lundberg et al., 1980 |
| CGRP | S,I | 30-50 | Rosenfeld et al., 1983 |
| Corticotrophin-releasing factor[1] | S | 5 | Skofitsch et al., 1985b |
| DYN | ? | ? | Botticelli et al., 1981 |
| β Endorphin | ? | ? | Itoga et al., 1980 |
| ENK | S | 1 | Roppolo et al., 1984 |
| GAL | S,I | 10-20 | Ch'ng et al., 1985 |
| Growth hormone-releasing factor | S,I | 1 | Józsa et al., 1987 |
| GRP/BN | S | 5 | Panula et al., 1983 Fuxe et al., 1983a |
| α Melanin-stimulating hormone | L | 25 | Dodd et al., 1983[3] |
| NKA | S,I | 10-20 | Dalsgaard et al., 1985 |
| NPY[2] | ? | 1 | Xue et al., 1987 |
| OXY[1] | S,I | 38 | Kai-Kai et al., 1985 |
| PANC[2] | S,I | 15-20 | Kar et al., 1988a |
| PHI[2] | S,I | 1-5 | Gibson et al., 1986 |
| SOM | S | 5-15 | Hökfelt et al., 1976 |
| Substance P | S | 10-30 | Hökfelt et al., 1975b |
| VIP[1] | S,I | 1-40 | Lundberg et al., 1979 |
| VP[1] | S,I | 50 | Kai-Kai et al., 1985 |
| **Neurofilament triplet proteins** | | | |
| 145 and 200 K dalton (RT97) | L | 25 | Lawson et al., 1984 |
| 68 K dalton | S,I (L) | ? | Price, 1985 |
| **Amines, amino acids and enzymes** | | | |
| AChE | S,I | 50 | Dodd et al., 1983[3] |
| Adenosine deaminase (purine) | S | 10-20 | Nagy and Daddona, 1985 |
| Aspartate amino transferase | S,I, L | 30 | Cangro et al., 1987 |
| Carbonic anhydrase | L | 30 | Dodd et al., 1983[3] |
| FRAP | S,I | 50 | Knyihár-Csillik et al., 1986 |
| Glutamate | S,I, L | 20 | Unpublished data |
| Glutaminase | S,I, L. | 30 | Cangro et al., 1985 |
| Glycogen phosphorylase | S,I, L | 20-100 | Woolf et al., 1985 |
| 5-HT | S | 14 | Kai-Kai and Keen, 1985 |
| Histamine [$H_1$ receptors] | S | 10 | Ninkovic et al., 1982 |
| TH (DBH) | S | 1 | Price and Mudge, 1983 |
| Thiamine monophosphatase | S,I | 50 | Knyihár-Csillik et al., 1986 |
| **Carbohydrates** | | | |
| Globoseries: | | | |
| SSEA 3 | S,I, L | 9 | Dodd et al., 1984 |
| SSEA 4 | S,I, L | 11 | Dodd et al., 1984 |
| Lactoseries (GalβI-3GlcNAc): Lewis $a^+$ | S,I, L | 20 | Dodd and Jessell, 1986 |
| Lactoseries (GalpI-4GlcNAC): A5, LD2, KH10, 2C5 | S | 40-45 | Dodd and Jessell, 1986 |
| LA4, 1B2/1B7 | S,I, L | 45-50 | Dodd and Jessell, 1986 |
| Lectin $UEA_1$ (L-fucosyl glycolipid) (residue) | S | ? | Mori, 1986 |

Methods of detection include use of polyclonal or monoclonal antibodies, enzyme histochemistry, autoradiographic receptor localisation or immunocytochemistry of transported lectins. For most peptides, the first described reference is quoted. In other cases some references are referred to in a review [3]Dodd et al., (1983). Size and estimation of percentage neuronal population expressing the antigens/enzyme/or radiolabel is taken from numerous papers and Dodd et al., (1983). [1]Indicates peptide-immunoreactive cells which are visible only after colchicine treatment. [2]Seen only in chick cell culture. S, small; I, intermediate; L, large cell sizes.

in the normal rat dorsal root ganglia was CGRP > substance P > GAL CCK > SOM > ENK > VIP and is in good agreement with a later study (Ju et al., 1987).

While cells of the dorsal root ganglia are generally classified as either small or large, there is considerable overlap of their distributions (Lawson and Harper, 1985). Using mainly ultrastructural criteria, several investigators (Andres, 1961; Duce and Keen, 1977; Rambourg et al., 1983) found cell types which are of intermediate size. These data therefore support the evidence in the present study for a classification of three cell types according to size and immunostain (Table 3.1) (see also Anderton et al., 1982; Tuchscherer and Seybold, 1985). However, none of the antigens studied were found in all of the cells in any given size category.

Colchicine treatment or nerve ligation enhanced visualisation of peptide-immunoreactive cell bodies. In accord with other studies the most striking change was of VIP/PHI immunoreactivity which was not seen in control animals (Gibson et al., 1984c, 1986; Ju et al., 1987; Kawatani et al., 1983; Shehab and Atkinson, 1986). A number of authors routinely use colchicine, either injected directly into the ganglia, intrathecally, systemically or by *in vitro* incubation. It is of interest to note that peptides which are normally associated only with the hypothalamo-pituitary axis, have now been identified in sensory ganglion cells of colchicine treated animals (Table 3.4).

### 3.4.2 Peptide coexistence

Since a large percentage of ganglion cells are immunoreactive for different peptides some degree of coexistence in afferent neurones is inevitable. Numerous studies have confirmed the first reports (Gibson et al., 1984d; Wiesenfeld-Hallin et al., 1984) of the coexistence of CGRP and substance P immunoreactivity in cells of dorsal root ganglia (Franco-Cereceda et al., 1987; Gibbins et al., 1985, 1987a; Ju et al., 1987; Lee et al., 1985; Lundberg et al., 1985; Molander et al., 1987; Yokakawa et al., 1986). Extensive coexistence of CGRP- and substance P-immunoreactivities has also been reported in peripheral nerve fibres innervating cutaneous, vascular and visceral tissues (Franco-Cereceda et al., 1987; Gibbins et al., 1985, 1987a,b; Gulbenkian et al., 1986; Lundberg et al., 1985) as well as in the central terminals of primary afferent fibres (Franco-Cereceda et al., 1987; Morishima et al., 1986). Furthermore, ultrastructural studies show immunoreactivity for both peptides localised to the same secretory vesicle in the cell body and in the peripheral and central terminals (Gulbenkian et al., 1986; Merighi et al., 1988a,b; Morishima et al., 1986).

Coexistence data obtained on pig tissue was confirmed in a later study of rat dorsal root ganglia (Ju et al., 1987). As for substance P, all GAL- and SOM-immunoreactive cells were colocalised with a subset of CGRP-immunoreactive cells. Furthermore, all GAL-immunoreactive cells were also immunoreactive for substance P. It is therefore probable that some cells contain CGRP,

substance P and GAL immunoreactivities. However, as no GAL/SOM-immunoreactive cells were found, a combination of the four peptides is unlikely. Since only a small number of substance P-immunoreactive cells also contained SOM immunoreactivity both CGRP/SP/SOM and CGRP/SOM combinations could occur. Substance P and SOM coexistence is of interest in several respects. Firstly, in human skin a segregation of fibres containing CGRP/substance P or CGRP/SOM has been reported (Gibbins et al., 1987b). No axons contained both substance P and SOM immunoreactivities. This infers that our proposed CGRP/SP/SOM cells are not cutaneous sensory neurones, and at the same time supports previous studies which suggest substance P- and SOM-immunoreactive cells exist as separate subpopulations in the dorsal root ganglion (Hökfelt et al., 1976; Hunt, 1983; Tuchscherer and Seybold, 1985). However, several reports indicate that some dorsal root ganglion cells contain both substance P and SOM immunoreactivities (Gibbins et al., 1987a; Ju et al., 1987; Leah et al., 1985a, 1985b; Merighi et al., 1988a; reported here).

### 3.4.3 Functional implications

The demonstration of a synthetic apparatus for a peptide is an important step towards establishing it as a neurotransmitter. The ability of ganglion cells to incorporate radiolabelled amino acids has been used to demonstrate *in vitro* synthesis of substance P or SOM (Harmar et al., 1981, 1982). Recently, localisation of peptide messenger RNA by *in situ* hybridisation has provided a more direct method. Precursors of CGRP, substance P, SOM and CCK have

so far been demonstrated in primary sensory neurones using this method (Gibson et al., 1988; Goedert and Hunt, 1986). Thus, the general assumption can be made that peptides localised to dorsal root ganglion cells using immunocytochemical techniques are produced there de novo.

The presence of many peptide-immunoreactive cells in the dorsal root ganglia, suggests that a proportion of the peptide-immunoreactive fibres in the spinal cord originate from these cell bodies. Most peptide-immunoreactive cells were in the small-intermediate range. These cells give rise to different functional classes of A and C fibres which have differential topographic termination patterns in the dorsal horn. While many peptides have specific distributions in the spinal cord (chapter 4; Dodd and Jessell, 1986) the precise contribution from afferent fibres is difficult to assess due to interference from other sources intrinsic to the central nervous system (chapter 4). In addition, there is little information regarding coexistence in the central terminals of primary sensory neurones. Thus, at present there are few correlations between type of termination and peptide content in the spinal cord (chapter 4.5.1).

The significance of peptide colocalisation is unclear. Assuming that all collaterals (see Taylor et al., 1982) of any one neurone contain the same peptide(s), their simultaneous secretion might be expected. Demonstration of CGRP and substance P in the same secretory vesicles together with the study of their release (Diez Guerra et al., 1988; Saria et al., 1986) adds support to a co-

release postulate. Furthermore, several behavioral and physiological studies have shown an interaction between these two peptides. CGRP potentiates a number of substance P-induced effects including scratch/biting behaviour in rats (Wiesenfeld-Hallin et al., 1984), facilitation of flexor withdrawal response (Woolf and Wiesenfeld-Hallin, 1986) and neurogenic plasma extravasation in peripheral tissues (Brain and Williams, 1985; Gamse and Saria, 1985; Lundberg et al., 1985; Unger et al., 1985). Interaction of CGRP and a substance P-degrading enzyme (Le Grevés et al., 1985) or a presynaptic action on substance P-containing synapses (Oku et al., 1987) have been postulated as possible mechanisms whereby CGRP potentiates substance P-mediated nociceptive responses.

Although SOM-immunoreactive neurones also contain CGRP, the latter peptide does not apparently potentiate excitatory effects of SOM when applied intrathecally to the dorsal horn (Wiesenfeld-Hallin, 1985). Further investigations (Kuraishi et al., 1985; Wiesenfeld-Hallin, 1986) implied that substance P and SOM were released by activation of separate populations of physiologically identified C fibre afferents. The observation that most substance P-immunoreactive neurones do not contain SOM immunoreactivity therefore provides a morphological basis for these data. In view of this, it may be proposed that neurochemically distinct populations of sensory neurones may be markers of different groups of physiologically identifiable primary afferent fibres. However, Leah et al., (1985b) were unable to detect a clear relationship

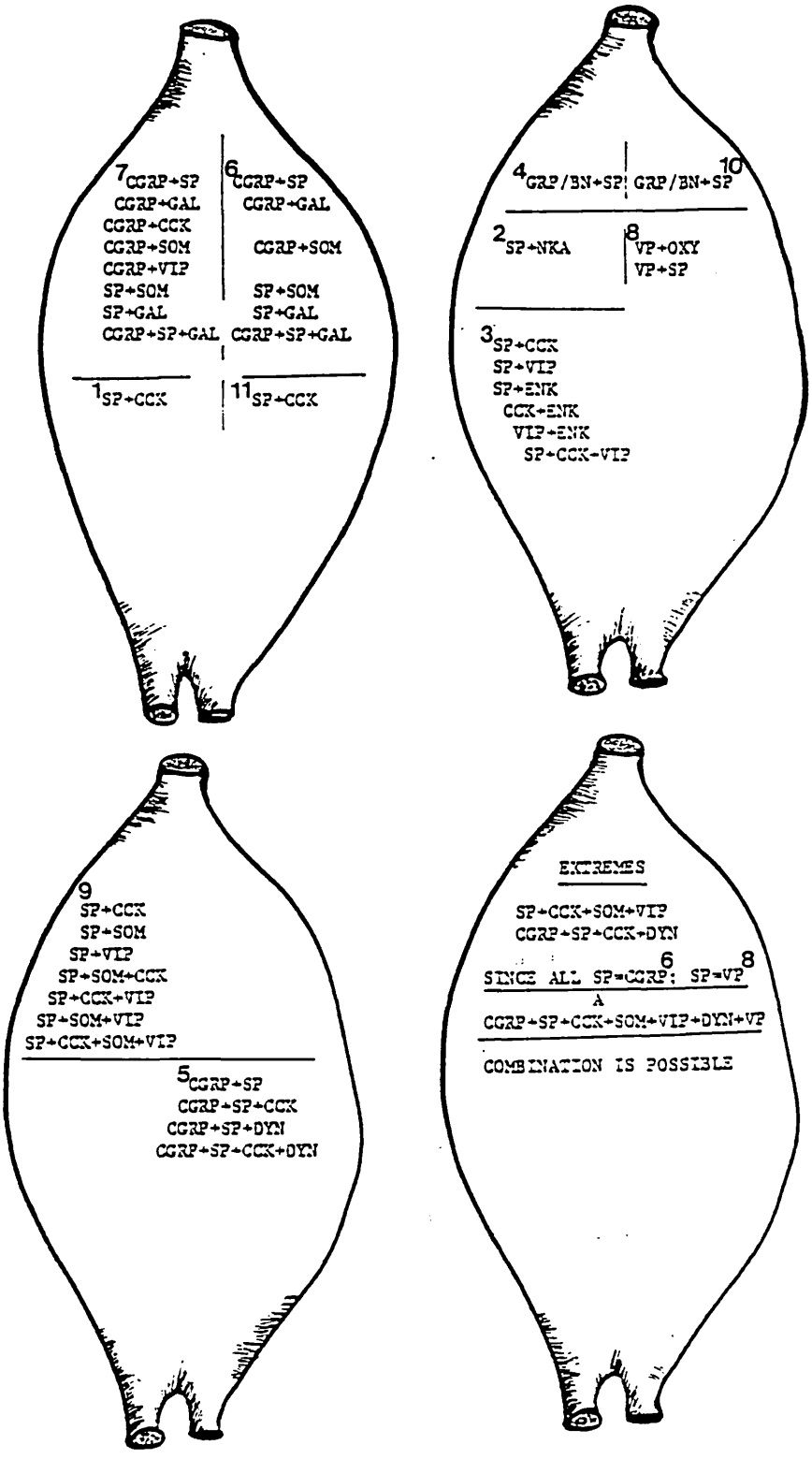



Fig.3.3 Major studies on peptide coexistence in dorsal root ganglion cells. To avoid complication no indication of degree of coexistence is shown. [1]Dalsgaard et al., 1982; [2]Dalsgaard et al., 1985; [3]de Groat, 1987; [4]Fuxe et al., 1983a; [5]Gibbins et al., 1987a; [6]Gibson et al., 1985 and reported here; [7]Ju et al., 1987; [8]Kai-Kai et al., 1986; [9]Leah et al., 1985a; [10] Panula et al., 1983; [11]Tuchscherer and Seybold, 1985.

between the sensory function of electrophysiologically defined dorsal root ganglion cells and the presence of different combinations of peptide immunoreactivities. The situation, therefore, is either more complex, with other peptide combinations not investigated by these authors (Fig.3.3), being implicated in the transmission of noxious stimuli or, alternatively, peptides alone may not provide a classification of modality-specific neurones.

# CHAPTER 4 NEUROCHEMISTRY OF THE SPINAL CORD: ORIGINS AND PROJECTIONS OF PEPTIDE-IMMUNOREACTIVE NERVES


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## 4.1 INTRODUCTION

At the onset of this study (1980), several peptides had been described in the spinal cord but because of varying interests there was little detailed comparison of their localisation. Improvements in the production and quality of antisera within the last decade, has allowed a more precise delineation of specific neurochemical pathways providing insight into the possible functional rôles of their constituents. Antisera are now available, not only to a number of previously undiscovered peptides and peptide precursors, but also to monoamines (e.g. classical neurotransmitters) and structural proteins, including neurofilament triplet proteins and glial fibrillary acidic protein (GFAP).

This chapter summarises the anatomical distribution of various neural antigens and structural proteins in the spinal cord. Having thus established a base-line, further studies dealing with the origin, nature, projection pathways, ontogeny (chapter 6) and possible change in expression under abnormal conditions (chapters 5,7) were undertaken for selected antigens.

## 4.2 EXPERIMENTAL APPROACH

### 4.2.1 Distribution

Spinal cords were collected from a variety of mammalian and non-mammalian species (Table 4.1). Man, cat and in particular rat

TABLE 4.1 SUMMARY OF SUBSTANCES INVESTIGATED IN VARIOUS MAMMALIAN AND NON-MAMMALIAN SPECIES

| Substances studied | AChE[1] | ACTH | BN | CCK | CGRP | ChAT | CPON | DBH | ENK | FRAP | FRMF[1] | GABA | GAD | GAL | GFAP | 5-HT | MCH | NmB | NmN | NmU | NFP | NP | NPY | NT | OXY | PANC | PHI | SOM | SP | TH | TRH | VIP | VP |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Species investigated** | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| Axolotl (n = 8) | | | | * | * | | | | | | | | | | | | | | | | | | | | | | * | | * | | | * | |
| Frog (n = 4) | | | | * | * | | | | | | | | | | | | | | | | | | | | | | * | | * | | | * | |
| Mouse (n = 6) | | | * | * | * | | * | * | * | | | | | | | | | | | | | | * | | * | | * | * | * | * | | * | * |
| Gerbil (n = 2) | | | * | * | * | | | * | * | | | | | | | | | | | | | * | | | | | * | | * | | | | |
| Rat (n = 40+) | * | * | * | * | *[3] | * | *[3] | * | *[3] | * | * | * | *[3] | * | * | *[3] | * | *[3] | * | * | * | *[3] | *[3] | *[3] | * | | *[3] | * | *[3] | *[3] | *[3] | *[3] | * |
| Guinea Pig(n = 40+) | | * | * | * | * | | * | | * | | | | | * | | * | | | | | | * | * | | * | | * | * | * | * | * | * | * |
| Pig[2] (n = 8) (Piglet n = 4) | | | * | * | * | | * | | * | | | | * | | | | * | | | | * | * | | | * | * | * | * | * | | * | * | * |
| Horse[2] (n = 4) | | * | * | * | * | | | | * | | | | * | | * | | | | | | | * | * | | * | | * | * | * | | * | * | * |
| Impala[2] (n = 1) | | | | | * | | | | | | | | | | | | | | | | | | | | | | | | * | | | | |
| Elephant[2] (n = 1) | | | | | * | | | | | | | | | | | | | | | | | | | | | | | | * | | | | |
| Cat (n = 10) | | * | * | * | * | | * | * | * | | | | | * | | * | | * | | | | * | * | | * | | * | * | * | * | * | * | * |
| Dog (n = 3) | | | | | | | | | | | | | | | | | | | | | | | | | | | * | | | | | * | |
| Marmoset (n = 5) | | * | * | * | * | | * | | * | | | | | * | | * | | | | | * | * | * | | * | * | * | * | * | * | * | * | |
| Macaque (n = 2) | | * | * | * | * | | * | | * | | | | | * | | * | | | | | * | * | * | | * | | * | * | * | | | * | * |
| Baboon (n = 1) | | | | * | * | | | | * | | | | | * | | | | | | | * | * | | | * | * | * | * | * | | * | * | |
| Man[2] (n = 20+) | | * | * | * | * | | * | | * | | | | | * | * | | | * | | | * | * | * | | * | | * | * | * | | * | * | * |

*, indicates substances which were studied in detail. □, indicates either the substances were not investigated or that no positive results were obtained. The major species and antigens investigated are in bold type face.

[1] AChE and FRAP which are products of enzymic reactions have been included in the list.

[2] Indicates animals which were fixed by immersion. The remainder were perfusion fixed.

[3] Refers to the antigens studied following intraspinal colchicine treatment. (n =) refers to the number of normal adult animals studied. (n = +) many more animals were studied than shown.

were analysed in greatest detail. Where possible, tissues were fixed by intracardial perfusion with a range of fixatives (appendix II, III). Tissue sections were cut from frozen, paraffin wax embedded or vibratome prepared tissues. The tissues were oriented in transverse or parasagittal planes and taken from cervical, thoracic, lumbar or sacral segments. Sections were immunostained for a range of antigens or processed for demonstration of acetylcholinesterase (AChE) or fluoride resistant acid phosphatase (FRAP) (chapter 2.7). To enhance visualisation of peptide-containing cell bodies, rats (n = 20) were injected intraspinally with colchicine (chapter 2.12.1).

### 4.2.2 Origin(s) of peptide-immunoreactive nerves

To determine the origin(s) of immunoreactive nerves the following experimental groups were studied (see also chapter 2.12).
Deafferentations: Unilateral dorsal rhizotomy or ganglionectomy (rats and cats). Capsaicin administration to a) neonatal rat pups b) adult guinea pigs c) dorsal root ganglia by injection (rats).
Spinal transections: Mid-thoracic transection (rats) and hemisection (cats).

## 4.3 RESULTS

### 4.3.1 Distribution

Since a large number of peptides, amines, enzymes and structural proteins were studied, a full description of each is not within the scope of this thesis. Their distributions are summarised in

TABLE 4.2 LOCALISATION OF PEPTIDES AND FRAP IN THE GREY MATTER OF THE RAT SPINAL CORD[1]

| | | LAMINA | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | DORSAL | | | | | | VENTRAL | | | | T | S |
| Peptide | | I | II | III | IV | V | VI | VII | VIII | IX | X | IML | IML |
| CGRP | F | ++++ | ++++ | ++ | + | +++ | + | + | + | + | ++ | ++ | ++ |
| | C | / | / | / | / | / | / | / | 0 | 0 | / | / | 0 |
| SP | F | ++++ | ++++ | ++ | ++ | +++ | ++ | ++ | ++ | ++ | +++ | +++ | +++ |
| | C | 0 | 0 | 0 | / | 0 | / | / | / | / | / | / | / |
| NKA | F | ++++ | ++++ | ++ | ++ | +++ | ++ | ++ | ++ | ++ | +++ | +++ | +++ |
| | C | / | / | / | / | / | / | / | / | / | / | / | / |
| ENK | F | ++ | +++ | + | + | ++ | + | ++ | ++ | ++ | +++ | +++ | +++ |
| | C | 0 | 0 | 0 | 0 | 0 | / | / | / | / | / | / | 0 |
| SOM | F | + | +++ | + | + | + | + | ++ | + | ++ | +++ | ++ | ++ |
| | C | / | 00 | 0 | 0 | 0 | / | / | 0 | / | / | / | 0 |
| GAL | F | ++ | ++ | + | + | + | + | ++ | + | + | ++ | +++ | ++ |
| | C | / | 00 | / | / | / | / | 00 | 0 | 0 | 00 | 0 | / |
| CCK | F | +++ | +++ | + | ++ | ++ | + | ++ | + | + | +++ | +++ | +++ |
| | C | / | 00 | 00 | 0 | 0 | / | 0 | 0 | 0 | 00 | / | / |
| NPY | F | ++ | ++ | + | + | + | + | + | + | ++ | ++ | ++++ | +++ |
| | C | / | 0 | 00 | / | 0 | / | 0 | 0 | / | 0 | 0 | 0 |
| CPON | F | ++ | ++ | + | + | + | + | + | + | ++ | ++ | ++++ | +++ |
| | C | / | 00 | 00 | / | 0 | / | / | 00 | / | 0 | 0 | 00 |
| FMRF | F | + | ++ | + | + | + | + | + | + | + | + | ++ | + |
| | C | / | / | / | / | / | / | / | / | / | / | / | / |
| NT | F | + | +++ | + | + | ++ | / | / | / | + | + | + | + |
| | C | | 000 | 000 | / | / | / | / | / | / | / | / | / |
| NmN | F | + | +++ | + | + | ++ | / | / | / | + | + | + | + |
| | C | / | 000 | 000 | / | / | / | / | / | / | / | / | / |
| BN | F | + | + | / | + | / | / | + | / | + | + | / | / |
| | C | / | / | / | / | / | / | / | / | / | / | / | / |
| NmB | F | ++ | ++ | ++ | + | / | / | / | / | + | + | + | + |
| | C | / | / | / | / | / | / | / | / | / | / | / | / |
| PANC[3] | F | + | ++ | ++ | + | ++ | + | ++ | ++ | ++ | ++ | ++++ | +++ |
| | C | / | / | 0 | 00 | 00 | 00 | 00 | 000 | 0000 | 0000 | / | / |
| NP | F | ++ | + | + | + | + | + | ++ | + | + | ++ | +++ | + |
| | C | / | / | / | / | / | / | / | / | / | / | / | / |
| OXY | F | ++ | + | + | + | + | + | ++ | + | + | ++ | +++ | + |
| | C | / | / | / | / | / | / | / | / | / | / | / | / |
| VP | F | + | / | / | + | + | / | / | / | / | + | ++ | + |
| | C | / | / | / | / | / | / | / | / | / | / | / | / |
| VIP[2] | F | + | + | + | / | / | / | / | / | + | + | + | +++ |
| | C | / | 00 | 0 | 0 | / | / | 0 | / | / | 0 | 0 | 0 |
| PHI[2] | F | + | + | + | / | / | / | / | / | + | + | + | +++ |
| | C | / | 00 | 0 | 0 | / | / | 0 | / | / | 0 | 0 | 0 |
| TRH[2] | F | + | + | / | + | + | + | ++ | + | +++ | ++ | +++ | ++ |
| | C | / | 0 | / | / | / | / | / | / | / | / | / | / |
| ACTH | F | + | + | + | / | / | / | / | / | / | + | + | + |
| | C | / | / | / | / | / | / | / | / | / | / | / | / |
| MCH | F | + | + | + | / | + | + | / | / | + | + | / | / |
| | C | / | / | / | / | / | / | / | / | / | / | / | / |
| NmU | F | / | / | / | / | / | / | / | / | / | / | / | / |
| | C | / | / | / | / | / | / | / | / | / | / | / | / |
| FRAP | F | + | ++++ | / | / | / | / | / | / | / | / | + | +++ |
| | C | / | / | / | / | / | / | / | / | / | / | / | / |

TABLE 4.3 LOCALISATION OF AMINES AND RELATED ENZYMES IN THE GREY MATTER OF THE RAT SPINAL CORD

| | | LAMINA | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | DORSAL | | | | | VENTRAL | | | | | T | S |
| Substance | | I | II | III | IV | V | VI | VII | VIII | IX | X | IML | IML |
| AChE | F | + | ++ | + | / | + | / | + | ++ | ++ | ++ | ++ | ++ |
| | C | / | / | / | / | / | / | / | 000 | 000 | / | 00 | 00 |
| ChAT | F | + | + | ++ | / | + | / | + | ++ | +++ | +++ | ++ | + |
| | C | / | / | / | / | / | / | / | 0 | 0 | / | 0 | 0 |
| GAD | F | + | ++ | + | + | + | + | + | ++ | ++ | ++ | + | + |
| | C | / | / | / | / | / | / | / | / | / | / | / | / |
| GABA | F | ++ | ++ | + | + | + | + | ++ | ++ | ++ | ++ | + | + |
| | C | 0 | / | 0 | / | / | / | 0 | / | / | 0 | / | / |
| DBH | F | + | + | ++ | + | + | + | + | +++ | +++ | ++ | ++++ | ++ |
| | C | / | / | / | / | / | / | / | / | / | / | / | / |
| TH | F | ++ | + | ++ | + | + | + | + | + | +++ | ++ | ++++ | ++ |
| | C | / | / | / | / | / | / | / | / | / | / | / | / |
| 5-HT | F | ++ | + | + | + | + | + | + | ++ | +++ | ++ | +++ | + |
| | C | / | / | / | / | / | / | / | / | / | / | / | / |

The tables show the relative abundance of a particular substance in the spinal cord. Therefore the density of staining for one substance should not be compared with the density for another. Four pluses for CGRP does not mean it is present in the same concentration as indicated by four pluses for substance P. Peptides are listed in order of descending abundance.

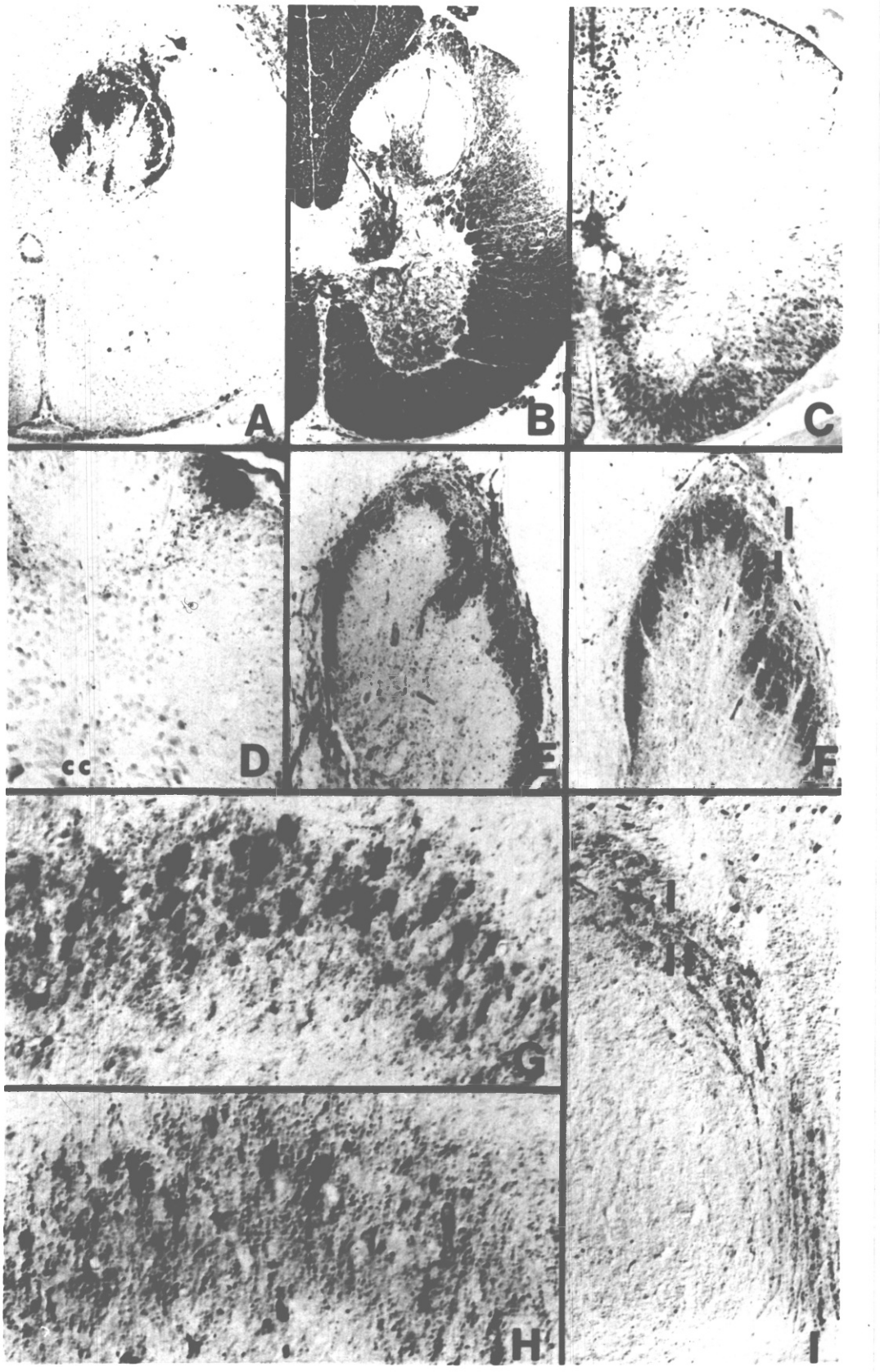
Key: Number of fibres: ++++ very dense; +++ dense; ++ moderate; + sparse; - rarely visible; / not seen; cell bodies: 000, numerous (10-20/5 sections); 00 (moderate 5-10/5 sections); 0 sparse (1-5/5 sections); /, none visible. [1]The laminar distribution in a mid-lumbar spinal cord segment is described. C, cell bodies; F, fibres; T, thoracic; S, sacral; IML, intermediolateral cell columns. [2]Cell bodies visible only after colchicine treatment. [3]Pig spinal cord.

Tables 4.2, 4.3 and in appendix XII. A concise overview is given below, outlining some of the most salient features. For more details, publications accompanying the thesis should be consulted. Overall distributions showed similar trends in most species. The following description refers to rat, unless stated otherwise since this was the species most intensively investigated.

Peptides and FRAP

**Dorsal spinal cord (laminae I-IV):** Each peptide studied was concentrated in the dorsal horn and most were localised to Lissauer's tract and/or laminae I-III (Fig.4.1A,D-I). TRH, was an exception, and showed greater numbers of immunoreactive fibres deeper in the dorsal horn. CGRP, substance P, ENK and CCK immunoreactivities were among the most abundant with distributions ranging from moderate to very dense in the upper dorsal horn. Although there was a generalised concentration of immunoreactivity in the upper three laminae there were notable differences. For example CGRP-, substance P- and ENK-immunoreactive fibres were evenly distributed throughout laminae I-III, whilst fibres immunoreactive for SOM, FRAP and neuromedin B (in pig) were concentrated in lamina II. NT-immunoreactive fibres appeared as a double band in lamina I and lower lamina II. VIP/PHI-immunoreactive fibres were characteristically localised to lamina I and the laminae I/II border. Further differences were noted in the deeper laminae with some peptide-immunoreactive fibres, e.g. substance P and ENK, present in all of them.

Fig.4.1 Peptides and structural proteins in the dorsal horn. (A-C) Thoracic spinal cord of man (6 week neonate). A) GAL immunoreactivity is most concentrated in fibres and terminals in laminae I-II. Some weakly stained motoneurones are visible in the ventral horn. B) Antiserum to neurofilament triplet proteins marks many myelinated axons and the majority of motoneurones. In contrast to A) little immunoreactivity is present in laminae I-II. C) The non-neuronal supporting cells, astrocytes are demonstrated using antiserum to GFAP (A-C x 15). (D,E) Substance P immunoreactivity concentrated in the upper laminae of the dorsal horn. D) Axolotl lumbar spinal cord, cc, central canal. E) Horse thoracic dorsal horn. F) NPY-immunoreactive fibres in horse thoracic dorsal horn (D x 100, E,F x 85). (G,H) Numerous peptide-immunoreactive cell bodies in laminae I-II following colchicine treatment. G) NT- H) VIP-immunoreactive cell bodies in rat lumbar dorsal horn (G, H x 300). I) VIP immunoreactivity in the sacral ($S_2$) dorsal horn of man. Immunoreactive fibres, seen as discrete bundles are present in Lissauer's tract. Fibres are most numerous in laminae I-II with the majority in the lateral dorsal horn (x 105).


A
B
C
cc
D
E
F
G
H
I

The distribution of each peptide was similar in all segmental levels of the spinal cord but there was a general trend for many peptide - (e.g. substance P, CCK, SOM, GAL) immunoreactive fibres to be more abundant in lumbosacral segments. This trend was most exaggerated in the case of VIP/PHI, with sparse immunoreactive fibres seen in rostral segments. In comparison, many VIP/PHI-immunoreactive fibres were present in lumbosacral segments. These were in Lissauer's tract and mainly in the lateral region of lamina I, with scattered fibres also in laminae II-III (Fig. 4.1I). This feature was common to all species studied, but most pronounced in cat and man.

Peptide immunoreactivity was, for the most part, localised to fibres and terminals. However, a few cell bodies immuoreactive for SOM (man), NPY/CPON, CCK, GAL, NT and neuromedin N were seen in the upper dorsal horn of normal animals. In pig, large pancreastatin-immunoreactive cells were numerous in laminae III-V and the dorsolateral funiculus. Intraspinal colchicine treatment markedly increased the number of peptide-immunoreactive cell bodies particularly, in laminae I-III, with VIP/PHI- and TRH-immunoreactive cells visualised only after this treatment (Figs. 4.1G,H, 4.4 B).

**Central canal (laminae X, medial VII):** Most peptide immunoreactivities, concentrated in the upper three laminae of the dorsal horn were also found in a substantial number of fibres around the central canal (Fig. 4.2). A feature peculiar to rat, was a rostrocaudally directed small tract of fibres located directly

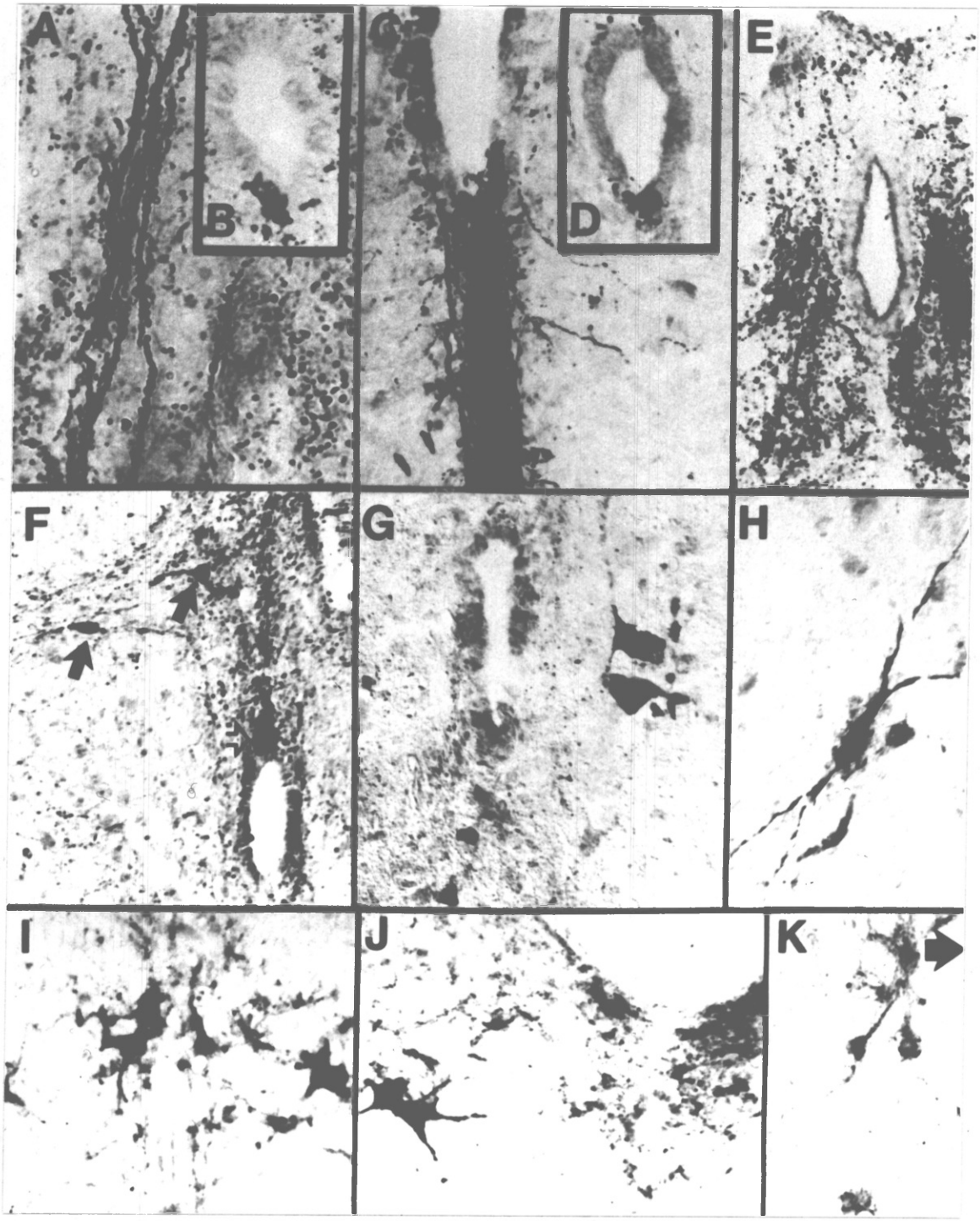



**Fig.4.2** Peptides in the central canal region. (A,B) Substance P- and (C,D) CGRP-immunoreactive fibres ventral to the central canal in the rat spinal cord. (B,D) Transverse sections showing the close proximity of the ventrally positioned bundle of fibres with the ependymal layer (x 225). (A,C) A view of the fibre tracts in longitudinally orientated sections (x 400). E) CPON-immunoreactive fibres around the central canal at the lumbar level in baboon spinal cord (x 185). F) NPY-immunoreactive fibres and cell bodies (blocked arrows) in the dorsal grey commissure in the sacral spinal cord of the rat, note fibre bundle (open arrow) directly dorsal to the central canal (x 200). (G,H) VIP-immunoreactive cell bodies in the lumbar spinal cord of the rat following colchicine treatment. G) Two large cells near the central canal. H) A spindle shaped cell in laminae X/XII (G x 230, H x 210). GAL-immunoreactive fibres and clusters of cell bodies in the lumbar $L_4$ I) and $L_3$ segments J) of the rat spinal cord (I,J x 110). K) Parasagittal section through the $L_4$ segment of the the rat spinal cord stained with antiserum to CCK. A column of cell bodies in laminae X/VII is visible. Arrow points dorsally (x 85).

ventral to the central canal (Fig. 4.2A-D). This tract was intensely immunoreactive for CGRP, substance P, SOM, GAL, CCK, BN, neuromedin B and VIP/PHI. CGRP immunoreactivity was also found in a bundle of fibres directly above the central canal, and NPY/CPON-immunoreactive fibres were found in a bundle above the central canal at the lumbosacral level (Fig. 4.2F). Even without colchicine pretreatment, substance P- (cat), ENK-, SOM- (man) and NPY/CPON-immunoreactive cell bodies were present, mostly in lumbosacral segments. In segments $L_3$-$L_4$, a column of large, multipolar cells in laminae X-VII were immunoreactive for GAL or CCK (Fig. 4.2I-K).

**Ventral spinal cord (laminae VII-IX):** Relative to the dorsal horn and central canal region, the abundance of peptide immunoreactivity in the ventral cord was low. Fibres immunoreactive for substance P, ENK, CCK, SOM, NPY/CPON, pancreastatin (pig) and TRH were most prevalent and present in all laminae. In particular, fibres immunoreactive for these peptides were seen in close apposition to motoneurones in laminae VIII and IX (Fig. 4.3A). However, some of these fibres were present at high densities around specific motor nuclei. Substance P- and/or CCK-immunoreactive fibres densely innervated a motor nucleus in a mediolateral position in segments $L_1$-$L_2$, and the motor nuclei corresponding to the pudendal motor nucleus in segments ($L_6$-$S_1$ rat, $S_2$-$S_3$ man, $S_1$-$S_3$ cat) were richly innervated with fibres immunoreactive for ENK, SOM and NPY/CPON (Fig. 4.3C,D).

Peptide-immunoreactive fibres were also frequently seen in the

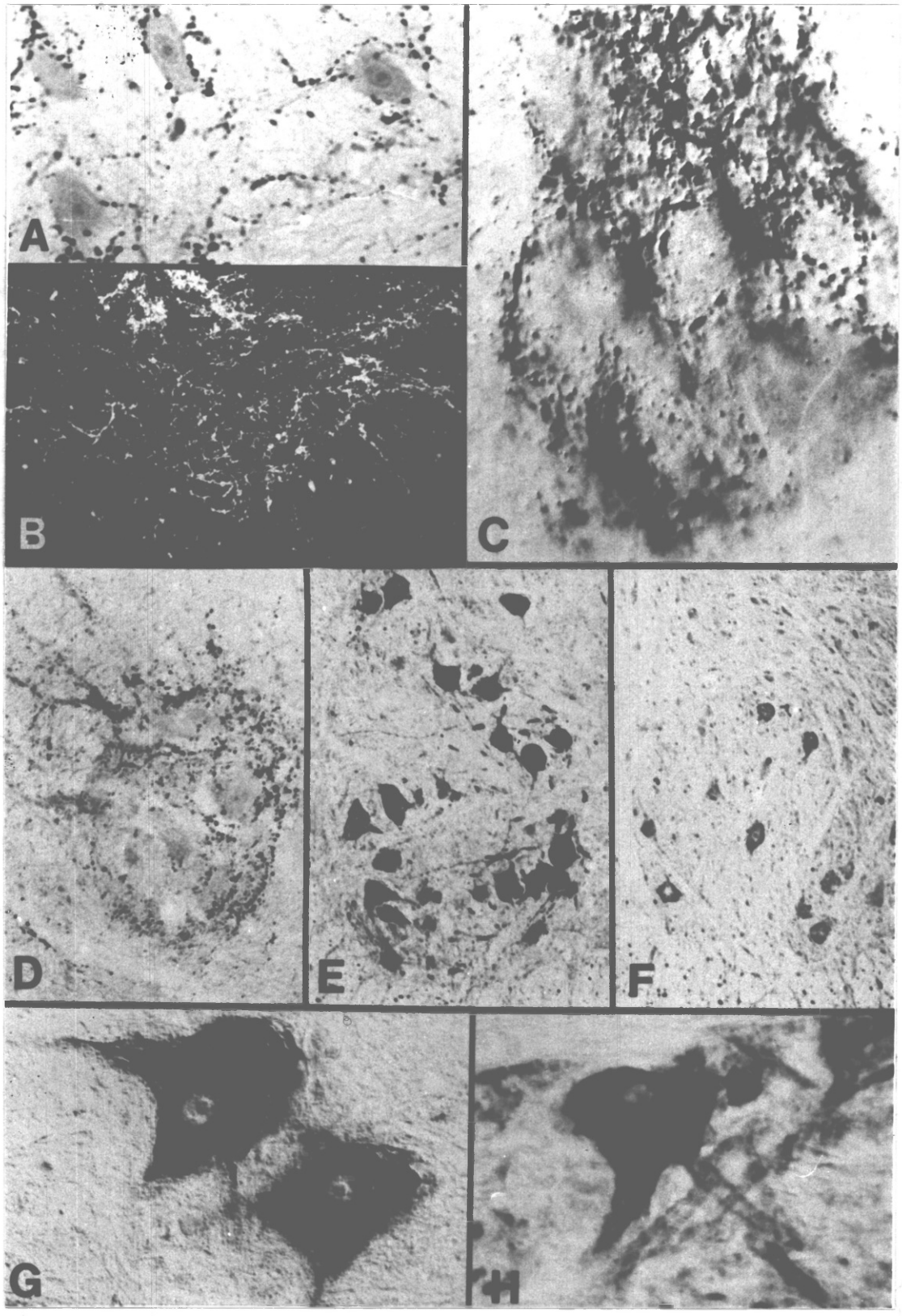



Fig.4.3 Peptides and 5-HT in the ventral horn. A) Substance P-immunoreactive fibres in close apposition to motoneurones and B) dark field photomicrograph of 5-HT immunoreactive fibres in the rat lumbar spinal cord (A x 200, B x 130). C) Substance P-immunoreactive fibres concentrated around a group of motoneurones in the $L_2$ segment of the rat spinal cord (x 330). D) NPY-immunoreactive fibres in and around the dorsolateral motor nucleus in the $L_6$ segment of the rat spinal cord (x 92). CGRP-immunoreactive motoneurones in the lumbar spinal cord of a normal, untreated rat F) and in the same region E) after colchicine treatment (x 95). (G,H) High power photomicrographs of immunoreactive motoneurones in the rat sacral spinal cord G) CGRP and H) GAL following colchicine treatment (x 350).

autonomic nuclei, and in particular, the intermediolateral cell columns (Fig. 4.4 B-H). These fibres often connected the intermediolateral cell columns with the dorsal grey commissures via the intermediomedial cell columns. In general, peptide-immunoreactive fibres were more abundant in thoracolumbar (Table 4.2) than in the lumbosacral autonomic nuclei.

Small, peptide-immunoreactive cell bodies were occassionally seen in laminae VII-IX. In pig, most of the large cells were pancreastatin-immunoreactive. The majority of motoneurones were CGRP-immunoreactive, with numbers increasing following colchicine treatment (Fig. 4.3 E-G). In man, these were less easily demonstrable and seen only in tissues obtained less that 36 hours post-mortem. GAL- and SOM- immunoreactive motoneurones were infrequent, with numbers increasing after colchicine (Fig. 4.3H). In the intermediolateral cell columns, immunoreactive cell bodies, presumably preganglionic, were observed in colchicine-treated rats (e.g. substance P, ENK, SOM, VIP/PHI) whilst CGRP-, NPY/CPON- and SOM-immunoreactive cells were often present in untreated animals and in man (Fig. 4.4 B,D). As in the dorsal spinal cord, immunoreactivity was greater, but less dramatic in caudal segments.

Amines and related enzymes

There was a sparse to moderate distribution of fibres immunoreactive for amines and/or related enzymes throughout the dorsal horn (Table 4.3). A few GABA-immunoreactive cell bodies were seen.

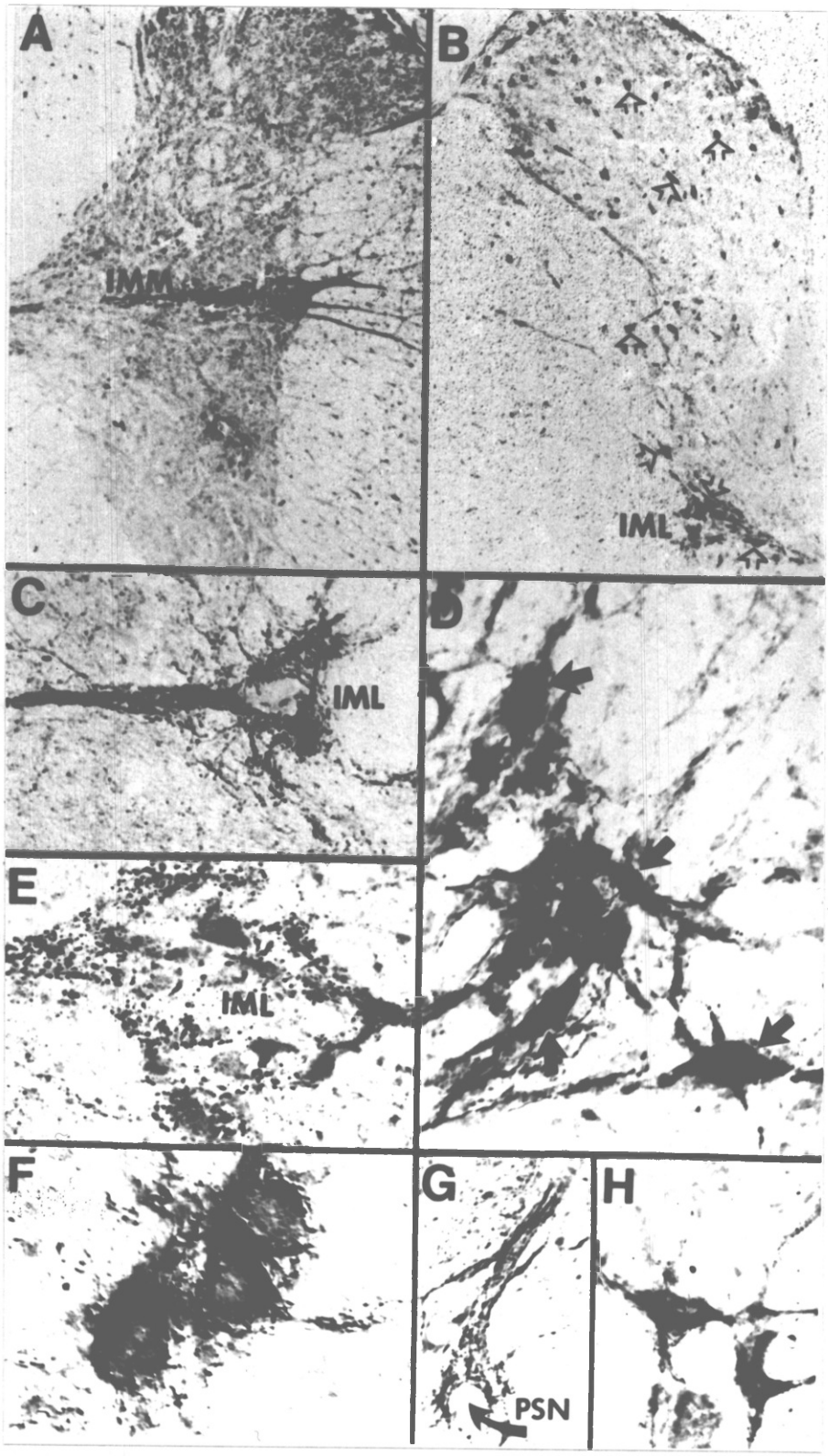



**Fig.4.4** Peptides and TH in autonomic nuclei. A) TH-immunoreactive fibres concentrated in the intermediolateral cell columns, and connecting with the central canal via the intermediomedial cell column in the thoracic spinal cord of rat (x 45). B) VIP-immunoreactive cell bodies (some arrowed) in the rat thoracic spinal cord after colchicine treatment. Numerous cells and fibres are present in the intermediolateral cell column (IML) (x 65). (C-F) Intermediolateral cell columns (IML) in the rat thoracic spinal cord. C) CCK- E), CPON- F), GAL-immunoreactive fibres (C x 195, E,F, x 220). (D,G,H) Parasympathetic nuclei (PSN) in the sacral spinal of D) rat, G) guinea pig and H) marmoset. D) CGRP-immunoreactive fibres and cell bodies (some arrowed) (x 320). G) PHI-immunoreactive fibres and H) SOM-immunoreactive fibres (G x 50; H x 205).

Immunoreactive fibres were more abundant in the ventral spinal spinal cord (laminae VII-X), notably in laminae VIII and IX in association with motor nuclei (Fig. 4.3B). As for substance P and CCK, fibres immunoreactive for DBH, TH and 5-HT were concentrated around a motor column in $L_1$-$L_2$ segments and were also numerous around the pudendal motor nuclei in $L_6$-$S_1$ segments. A number of motoneurones were GABA, ChAT or AChE positive. In the intermediolateral cell columns ChAT and AChE staining showed a moderate distribution of fibres and cell bodies. In contrast, DBH-, TH- and 5-HT-immunoreactive fibres were very numerous in the thoracic intermediolateral cell columns but relatively sparse in the lumbosacral autonomic nuclei (Fig. 4.4A). With the exception of the latter case, little variation in distribution was seen at different segmental levels.

Structural proteins

Neurofilament triplet proteins-immunoreactive fibres were of large diameter, most numerous in the white matter and ventral grey matter and present throughout the spinal cord (Fig. 4.1B). Many large cell bodies were positively stained, notably the cells of Clarke's column and motoneurones. GFAP-immunoreactive astrocytes and their processes were scattered throughout the white matter, some forming the glial limiting membrane. Immunoreactive processes were present in the grey matter and were concentrated dorsal to the central canal around the ependymal cells (Fig. 4.1C).

TABLE 4.4 SUMMARY OF THE EFFECTS OF VARIOUS INVESTIGATIVE PROCEDURES ON THE DISTRIBUTIONS OF PEPTIDES AND FRAP IN THE SPINAL CORD

| | Dorsal rhizotomy/ganglionectomy | Capsaicin | Caudal to transection | Inferred origin(s) |
|---|---|---|---|---|
| CGRP | ↓ F laminae I-III, X | ↓ F laminae I-III, $X^1$ | ⇄ | Afferent, intrinsic cells |
| SP | ↓ F laminae I-III, X | ↓ F laminae I-III, $X^1$ | ↓ F laminae VIII-IX | Afferent, intrinsic cells, descending tracts |
| ENK | ⇄ | ⇄ | ↓ F laminae VIII-IX | Intrinsic cells, descending tracts |
| SOM | ↓ F lamina II | ↓ F lamina II, $X^1$ | ⇄ | Afferent, intrinsic cells |
| GAL | ↓ F laminae I-III, ↑ C lamina II | ↓ F laminae I-III, $X^1$ | ↑ F lamina X | Afferent, intrinsic cells ascending tracts |
| CCK | ↓ F laminae I-III | ↓ F laminae I-III, $X^1$ | ↑ F lamina X | Afferent, intrinsic cells ascending tracts |
| NPY/CPON FMRF | ⇄ | ⇄ | ↓ F thoracic IML | Intrinsic cells, descending tracts |
| NT/NmN | ⇄ | ⇄ | ⇄ | Intrinsic cells |
| Bn/NmB | n.d | ↓ F lamina II, $X^1$ | n.d. | Afferent |
| PANC | n.d. | n.d. | n.d | ? |
| NP/OXY/VP | ⇄ | ⇄ | ↓ F lamina I, thoracic IML | Descending tracts |
| VIP/PHI | ↓ F laminae I-II, X, ventral root exit, sacral IML | ↓ F laminae I-II, $X^1$, sacral IML | ↑ C lamina X, DLF | Afferent, intrinsic cells ascending tracts |
| TRH | ⇄ | ⇄ | ↓ F laminae VIII-IX, thoracic IML | Intrinsic cells, descending tracts |
| ACTH/MCH/ NmU | ⇄ | ⇄ | ↓ F laminae I-III | Descending tracts |
| FRAP | ↓ F lamina II | ↓ lamina II | ⇄ | Afferent |

Inferred origin(s) as indicated from manipulations and presence of cell bodies in the spinal cord. F, fibres; C, cell bodies; IML, intermediolateral cell column; ↓ decrease, ↑ increase; ⇄ no change; n.d, not done; FRAP studied only in rats. $X^1$, refers to fibre bundle below the central canal in rats.

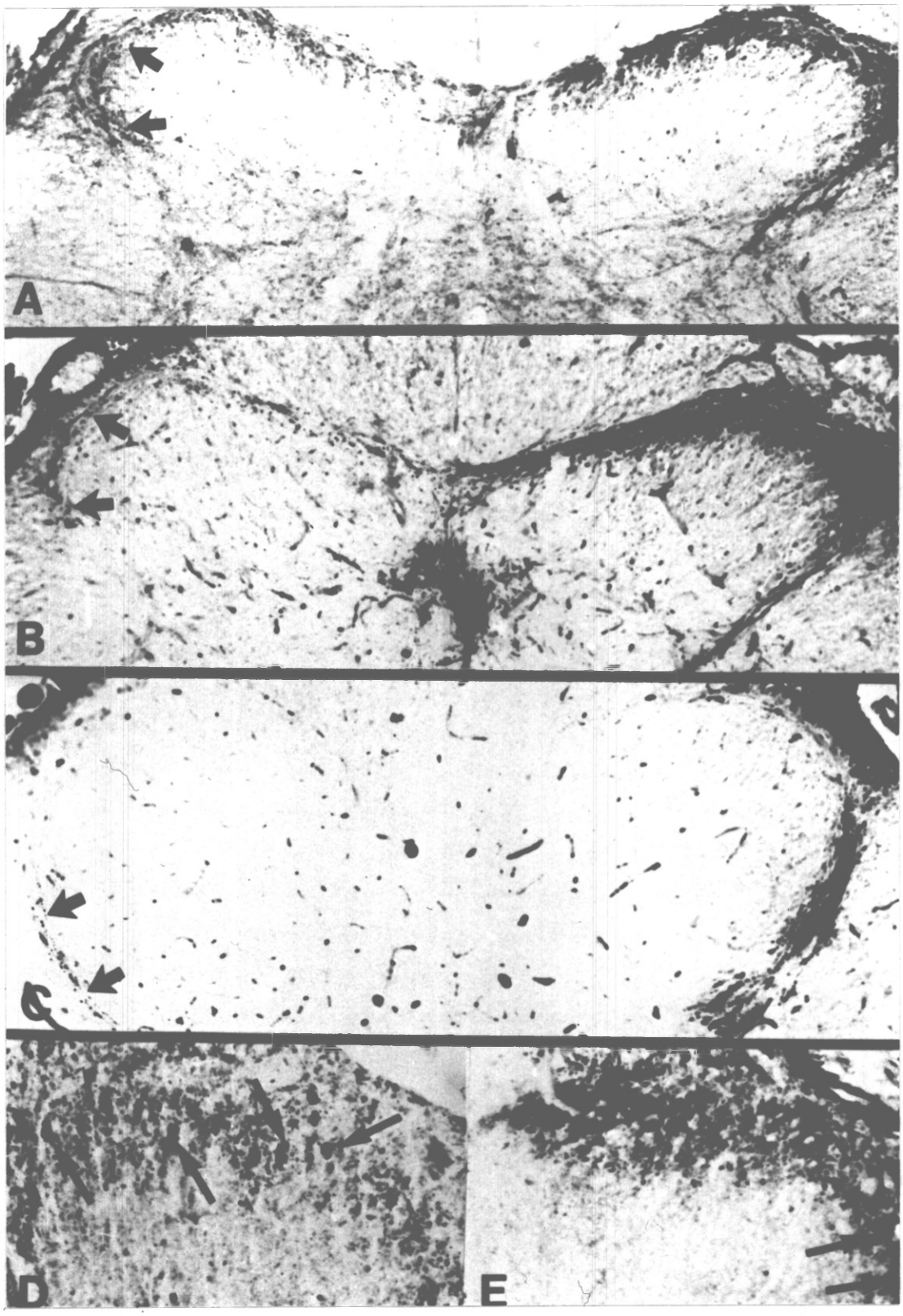



Fig.4.5 (A-C) Cat dorsal spinal cord following unilateral lumbosacral rhizotomy (left side). (A,B) Serial sections at the $S_3$ level immunostained for A) substance P and B) CGRP. C) VIP immunoreactivity in the $S_2$ segment. There is a marked loss of immunoreactivity ipsilateral (arrows) to the lesion (A,B x 55, C x 60). (D,E) GAL immunoreactivity in the rat sacral dorsal spinal cord following unilateral lumbosacral rhizotomy. There is a decrease of GAL-immunoreactive fibres from laminae I-II of the ipsilateral side D) but staining of cell bodies (some arrowed) is more obvious (D,E x 220).

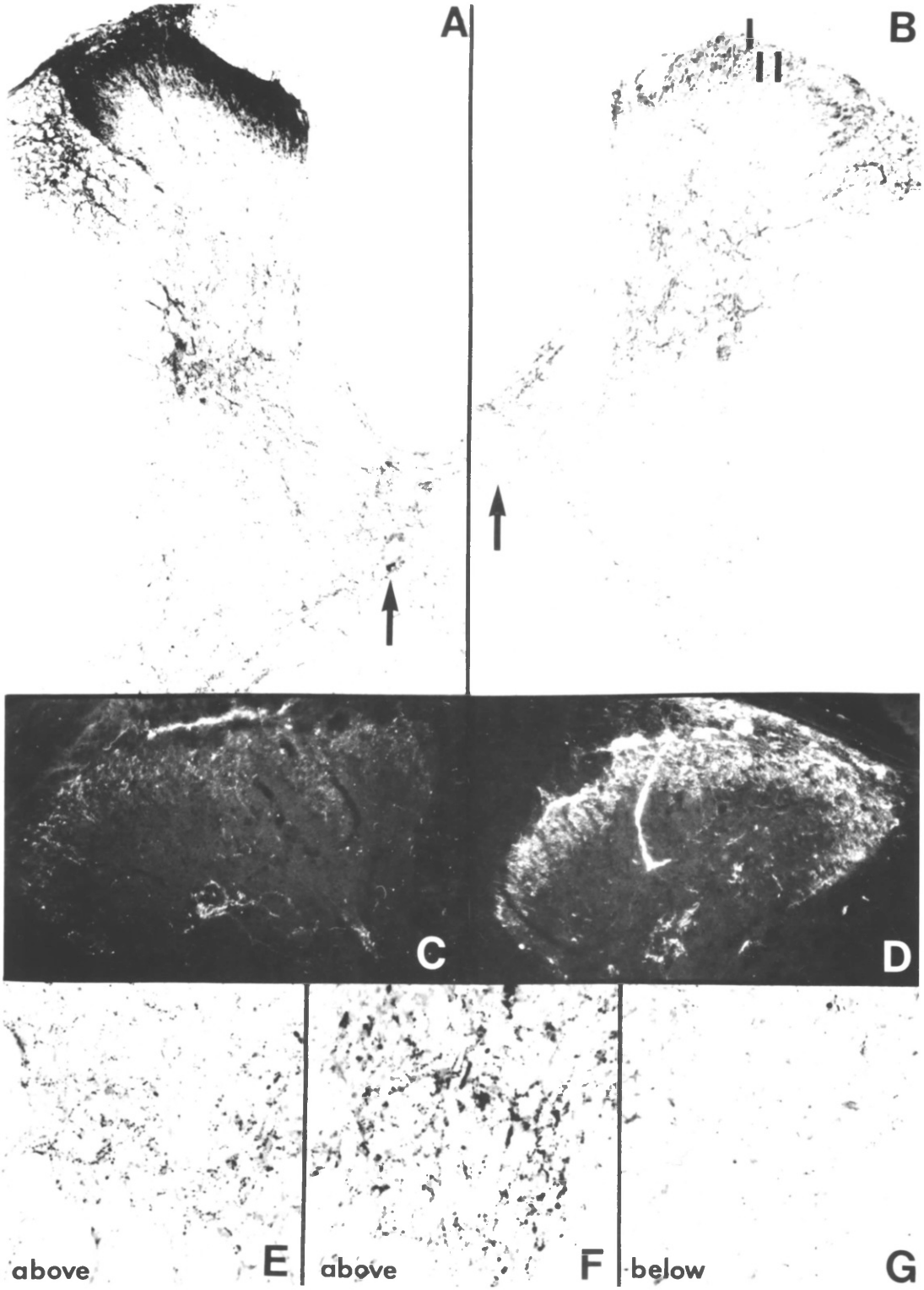



Fig.4.6 Substance P immunoreactivity is reduced dramatically from laminae I-II and from the region ventral to the central canal (arrows) in adult rat spinal cord following neonatal administration of capsaicin B) compared to the control A) (x 75). There is a marked loss of CGRP immunoreactivity from laminae I-II of the guinea pig dorsal horn after systemic administration of capsaicin C) compared to the control D). Thoracic spinal cord, indirect immunofluorescence method (x 90). (E-G) Substance P-immunoreactive fibres in the ventral horn of the rat spinal cord rostral E) immediately rostral F) and G) caudal to spinal transection at the mid-thoracic level. Note build up of immunoreactivity above F) and loss below G) the lesion (x 110).

### 4.3.2 Origin(s) of peptide-immunoreactive nerves

The results of various manipulations used to investigate the origin(s) of peptide immunoreactivity are summarised in Table 4.4. Rhizotomies and ganglionectomies induced a marked loss of CGRP, substance P, SOM, GAL, CCK, BN, neuromedin B and VIP/PHI from the ipsilateral dorsal horn, at the level of the lesion (Fig. 4.5). In cat, sacral rhizotomies induced a further loss of CGRP- and substance P-immunoreactive fibres from lamina X (Fig. 4.5A,B). Capsaicin treatment produced similar results to surgical deafferentation and in neonatally treated rats, no immunostaining was associated with the bundle of fibres located directly ventral to the central canal. No changes were apparent in other areas of the spinal cord (Fig. 4.6A-D).

Spinal transections indicated that at least a proportion of immunoreactive fibres in the ventral (substance P, ENK, TRH) and dorsal horns (NP, OXP, VP, ACTH, neuromedin U, MCH) are from descending systems (Fig. 4.6 E-G). In contrast, increased immunostaining caudal to the transection as noted for GAL, CCK and VIP/PHI suggests an ascending projection.

## 4.4 DISCUSSION

The localisation of peptides, amines, enzymes and structural proteins and investigations of the origin of peptide-immunoreactive fibres in the spinal cord, is in good agreement with other reports. Many of these have been published since completion

of this work. An extensive bibliography now surrounds this subject and is too large to incorporate in this thesis. Some major references are cited in Table 4.5 and for reviews see (Gibson and Polak, 1986; Hökfelt et al., 1980a, 1982; Hunt, 1983; Palkovits, 1984; Ruda et al., 1986). Although peptides were present throughout the spinal cord, laminae I-III was the most densely innervated region. Here, many immunoreactive fibres were derived from primary sensory neurones, as shown by deafferentions, whereas others such as ENK and NPY/CPON were intrinsic to the spinal cord, presumably derived from local interneurones (see also Hunt et al., 1981b).

Since the substantia gelatinosa (lamina II) appears to modulate incoming information about pain from primary sensory neurones (Cervero and Iggo, 1980; Wall, 1978, 1980), a large part of the interest in the dorsal spinal cord has been in mechanisms of pain transmission and analgesia. In 1965, Melzack and Wall, proposed that incoming nociceptive information was modified (or gated) by lamina II interneurones before going to higher control centres. A neurochemical basis was provided for this "gate control theory", when it was shown that ENK (an analgesic substance) could modulate release of substance P (nociceptive substance) from the central terminals of primary sensory neurones (Fields et al., 1980; Jessell and Iversen, 1977). However, although recent ultrastructural studies have shown a number of peptide immunoreactivities in synapses in the spinal cord, little evidence to support a presynaptic opioid-modulation of primary afferent axons has been forthcoming (Aronin et al., 1981; Barber et al., 1979; Harmann et

TABLE 4.5 MAJOR STUDIES WHICH FURTHER DOCUMENT THE IMMUNOCYTOCHEMICAL DISTRIBUTION OF PEPTIDES, AMINES, ENZYMES AND STRUCTURAL PROTEINS IN THE SPINAL CORD

| SUBSTANCE | REFERENCE(S) |
|---|---|
| AChE | Silver and Wolstencroft, 1971[c] |
| ACTH | Gibson et al., 1981[r]; Tsou et al., 1986[r] |
| BN | Panula et al., 1983[r] |
| CCK | Schrøder, 1983[r] |
| CGRP | Franco-Cereceda et al., 1987[g,h]; Gibson et al., 1984d,[f,m,r,g,c,p,ho,mk,h]; Rosenfeld et al., 1983[r], Marti et al., 1987[r,h]; Harmann et al.,1988[h] |
| ChAT | Barber et al., 1984[r] |
| CPON | Marti et al., 1987[r,h] |
| DBH | Aramount et al., 1986[r]; Hartman et al., 1972[r] |
| ENK | Glazer and Basbaum, 1981a,[c]; Schoenen et al., 1985a,[h] |
| FRAP | Knyihár-Csillik and Csillik, 1981[r] |
| FMRF | Dockray and Williams, 1983[r] |
| GABA | Magoul et al., 1987[r] |
| GAD | Barber et al., 1982[r] |
| GAL | Ch'ng et al., 1985[r,p]; Ju et al., 1987[r] |
| GFAP | Bullon et al., 1984[r] |
| 5-HT | Ruda et al., 1982[c]; Steinbusch, 1981[r] |
| MCH | Skofitsch et al., 1985a,[r] |
| NKA | Dalsgaard et al., 1985[r] |
| NmB | Namba et al., 1985[r,c,p] |
| NmN | reported here[r] |
| NmU | Ballesta et al., 1987[r] |
| NFP | Marti et al., 1987[r,h]; Trojanowski et al., 1986[r,h] |
| NP | Swanson and McKellar, 1979[r,m] |
| NPY | Allen et al., 1984a,[h]; Gibson et al., 1984b,[r] |
| NT | Gibson et al., 1981[r]; Seybold and Elde, 1982[r] |
| OXY | Schoenen et al., 1985a,[h]; Swanson and McKellar, 1979[r,mk] |
| PANC | Kar et al., 1988a,[p] |
| PHI | Anand et al., 1984[h]; Gibson et al., 1986[r,c]; McGregor et al., 1984[r] |
| SOM | Schoenen et al., 1985a,[h]; Schrøder, 1984[r] |
| SP | Barber et al., 1979[r]; Schoenen et al., 1985a,[h] |
| TH | Foster et al., 1985[r] |
| TRH | Harkness and Brownfield, 1986[r] |
| VIP | Anand et al., 1983[h]; Basbaum and Glazer, 1983[c]; Gibson et al., 1984c,[m,r,g,c,ho,mk]; Fuji et al., 1983 |
| VP | Gibson et al., 1981[r]; Schoenen et al., 1985a,[h] |

Superscripts refer to [f], frog, [r], rat; [c], cat; [m], mouse; [g], guinea pig; [p], pig; [ho], horse, [mk], monkey and [h], human.

al., 1988; Honda et al., 1983; Hunt, 1983; Hunt et al., 1980; Merighi et al., 1988b; Ruda, 1982, Ruda et al., 1986; Sumal et al., 1982). To date, GABA is the only neurochemical to be identified as presynaptic to primary afferent fibres (Barber et al., 1978).

A number of catecholaminergic fibres were also found in the dorsal horn and are known to be involved in descending modulation of analgesic mechanisms (Dubner and Bennett, 1983 for review; Yaksh et al., 1981). It is of interest, that while in many regions of the nervous system there is a close anatomical and functional interaction between NPY and noradrenergic transmission (Allen and Bloom, 1986 for review), in the dorsal horn NPY/CPON immunoreactivity would appear to derive from intrinsic cell bodies (Table 4.4) (Van Dongen et al., 1985). However, cathecholaminergic fibres are almost exclusively of supraspinal origin (Glazer and Ross, 1980; but see Dietl et al., 1985). At present the action of NPY/CPON in the dorsal horn is unknown. Clearly, a single dorsal horn neurone may receive more than one neurochemical input. Further ultrastructual, electrophysiological and pharmacological investigations are therefore required to understand the rôle of peptides and amines in pain neurotransmission.

Little is known about the region around the central canal, but, like the dorsal horn, it receives nociceptive information (Light and Perl, 1979; Nahin et al., 1983) and is a peptide rich area. The significance of the peptide-immunoreactive bundle of fibres directly ventral to the central canal in the rat is unknown, but

an afferent origin has been proposed on the strength of capsaicin experiments (Table 4.4) (Jancsó et al., 1981) and transganglionic tracing studies (Nadelhaft and Booth, 1984).

Autonomic cell columns were also innervated by peptides and monoamines although there were differences in relative densities of immunoreactive fibres in the sympathetic (thoracic) and parasympathetic (sacral) centres. As expected, NPY/CPON and noradrenergic fibres were more prevalent and of supraspinal origin in thoracic segments (Table 4.4) (Hökfelt et al., 1981; Westlund et al., 1983). Other examples included TRH-, 5-HT-, NP-, OXY-, and VP-immunoreactive fibres, presumably descending projections of brainstem and/or hypothalamic neurones (Bowker et al., 1982; Johansson et al., 1981; Millan et al., 1984; Swanson, 1977). Identification of peptide-immunoreactive cells as preganglionic neurones however, requires further verification by the combined use of retrograde tracing and immunocytochemistry, as shown for CCK-, SOM- and ENK-immunoreactive cells (de Groat et al., 1983; Schrøder., 1983, 1984).

Brainstem raphe neurones have a major projection to the ventral horn and 5-HT and peptides (substance P, TRH or ENK) are known to coexist in these cells and contribute afferents to the ventral horn. The majority of these terminate in the vicinity of motoneurones (Hunt, 1983; Ruda et al., 1986; Tashiro and Ruda, 1985). The loss of substance P-, TRH- and 5-HT-immunoreactive fibres in the ventral horn following transection was therefore an expected finding. A rôle in motor function for both peptides and

5-HT is likely since all three substances depolarise motoneurones (Konishi and Otsuka, 1974; McCall and Aghajanian, 1979; Nicoll, 1978; ). However, the significance of other peptide-immunoreactive fibres innervating motoneurones is unknown. Finally, identification of peptides, namely CGRP and GAL to motoneurones (Ch'ng et al., 1985; Gibson et al., 1984d; Rosenfeld et al., 1983; Sawchenko et al., 1987), opens an exciting new avenue suggesting a direct involvement in the regulation of motor function and a possible interaction with the classical neurotransmitters, acetylcholine and GABA.

The rapidly growing number of peptides which have been localised to the spinal cord has considerably extended our knowledge, but at the same time added to the complexity of understanding neurotransmission/modulation in the spinal cord. Detailed analysis of the distribution and origin(s) of peptides has revealed a number with unique characteristics which in turn suggest specialised functional rôles. Several such examples are described below.

## 4.5 STUDIES OF AREAS DENSELY INNERVATED WITH PEPTIDES AND THEIR RELATIONSHIP WITH THE PERIPHERAL NERVOUS SYSTEM

Investigations were focused on three areas of interest. For simplification, data are presented with experimental approach in a concise format and a short commentary accompanies each subject.

### 4.5.1 VIP/PHI in the lumbosacral spinal cord

Most VIP/PHI immunoreactivity is in the lumbosacral spinal cord

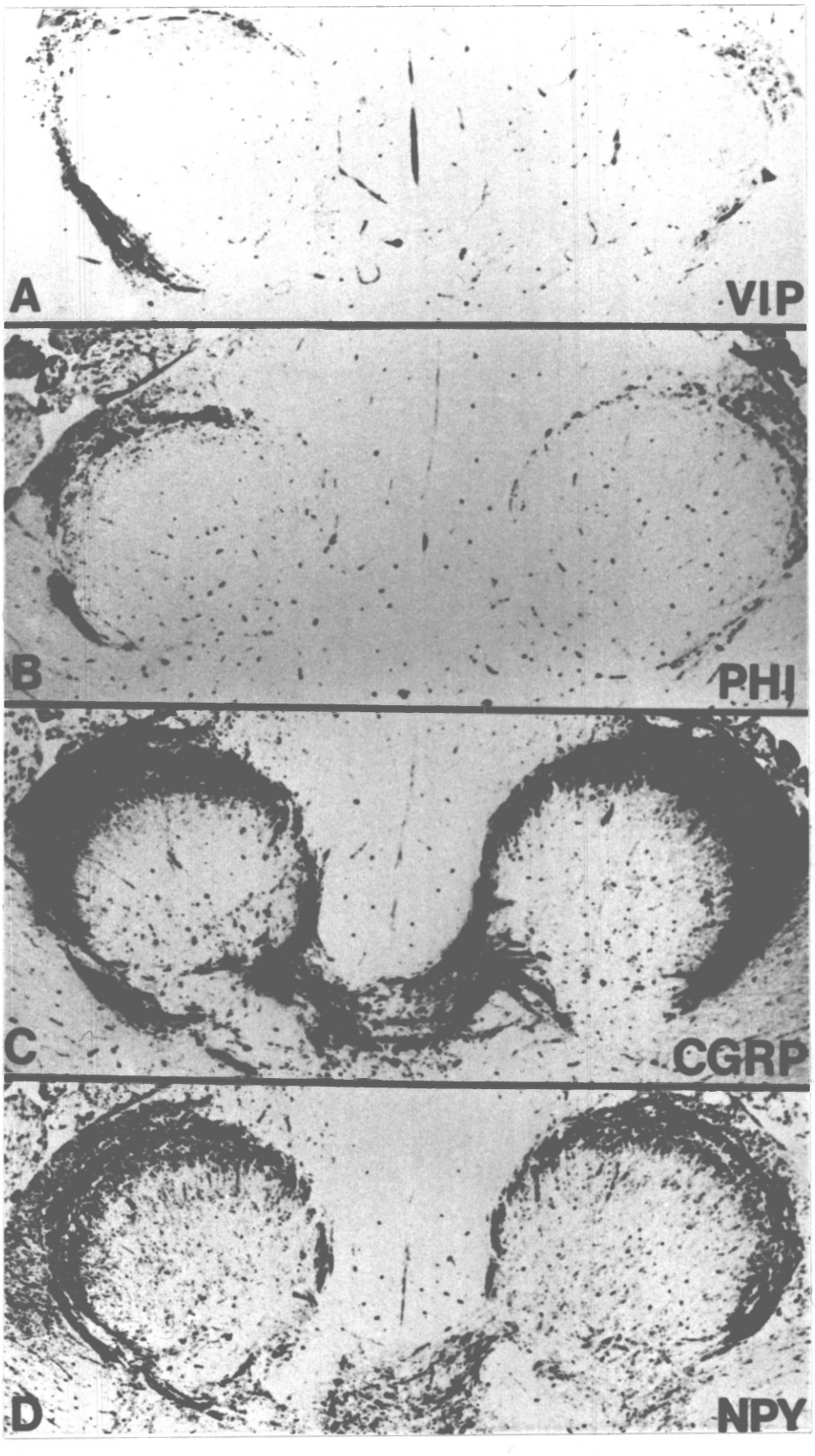



**Fig.4.7** A) VIP, B) PHI, C) CGRP and D) NPY immunoreactivities in the cat dorsal sacral ($S_2$) spinal cord following unilateral section of the pelvic nerve. There is a marked loss of VIP/PHI immunoreactivity from Lissauer's tract and laminae I-II of the ipsilateral side (right) but no apparent change in the distribution of CGRP and NPY (x 50).

TABLE 4.6 RADIOIMMUNOASSAY: SPINAL CORD AND BLADDER AFTER UNILATERAL PELVIC NERVE SECTION (pmol/g WET WEIGHT ± S.E.M)

| Region | VIP concentration | | | |
|---|---|---|---|---|
| | RD | RV | LD | LV |
| Spinal cord | | | | |
| $S_2$ cat | 70.9 ± 5.0 | 2.9 ± 0.4 | *22.4 ± 6.3 | 2.8 ± 0.6 |
| $S_3$ cat | 170.0 ± 30.0 | 11.0 ± 0.7 | **62.0 ± 38.0 | 5.6 ± 1.6 |
| | R | | L | |
| $L_6$ rat | 7.8 ± 2.3 | | 3.4 ± 0.9 | |
| Bladder | | | | |
| dome cat | 3.6 ± 0.9 | | 3.5 ± 1.1 | |
| dome rat | 2.9 ± 1.2 | | 3.1 ± 0.2 | |
| base cat | 10.1 ± 2.8 | | 9.4 ± 1.1 | |
| base rat | 5.9 ± 0.5 | | 11.9 ± 6.3 | |

L, left (operated side); R, right; V, ventral; D, dorsal.
* $P < 10.01$; ** $P < 0.05$ Student's paried 't' test. All other values show non-significant changes.

TABLE 4.7 *MEAN NEURONAL CELL COUNTS $L_6$ SEGMENT

| Injection site | True Blue cells | | | | Total cells | | | |
|---|---|---|---|---|---|---|---|---|
| | DM | V | DL | RDL | DM | V | DL | RDL |
| Pelvic muscle | 122 | 46.7 | 76.7 | - | 213 | 141 | 217 | - |
| [mean % total] | 58 | 33 | 35 | | | | | |
| Pudendal nerve | 193.3 | 137.3 | 171.3 | 232.3 | 193.3 | 137.3 | 171.3 | 409 |
| [mean % total] | 100 | 100 | 100 | 56 | | | | |

TABLE 4.8 DOUBLE LABELLING EXPERIMENT *MEAN NEURONAL CELL COUNTS OF THE RETRODORSOLATERAL NUCLEUS $L_6$ SEGMENT

| | True Blue cells | Diamidino yellow/ rhodamine cells |
|---|---|---|
| Mean number | 200 | 211 |
| Mean % total | 51 | 49 |

DM, dorsomedial; V, ventral; DL, dorsolateral; RDL, retrodorsolateral; *, Mean cell counts = average cell number from 3 rats in 20 µm serial sections through the $L_6$ segment.

and is of afferent origin. There is a striking similarity between the distribution of VIP/PHI-immunoreactive fibres and the central projections of the pelvic nerve (Morgan et al., 1981). Since VIP/PHI-immunoreactive fibres innervate pelvic organs, the following experiments were designed to identify whether VIP/PHI was a marker of pelvic visceral afferents.

Unilateral pelvic nerve section 6 rats killed 4-7 days later; 9 cats killed 4 weeks later.

In the spinal cord VIP/PHI immunoreactivity was markedly reduced in the ipsilateral lumbosacral dorsal horn (segments $S_2$, $S_3$ cat and $L_6$ rat) (Fig.4.7A,B) (Table 4.6). There was no apparent change in the distribution of CGRP, substance P, ENK or NPY/CPON (Fig.4.7C,D). In the bladder, there was a small ipsilateral increase of VIP/PHI-immunoreactive fibres, in contrast there were reduced numbers of fibres immunoreactive for CGRP and substance P and no change in ENK (Fig. 4.8C,F) (Table 4.6).

Neonatal capsaicin 10 treated, 10 vehicle control rats killed 2 months later.

VIP/PHI immunoreactivity was reduced in the dorsal horn and lamina X, but there was no apparent change in the bladder (Fig.4.8A,B). CGRP and substance P immunoreactivities were decreased in the dorsal horn and bladder whereas ENK immunoreactivity was unaffected.

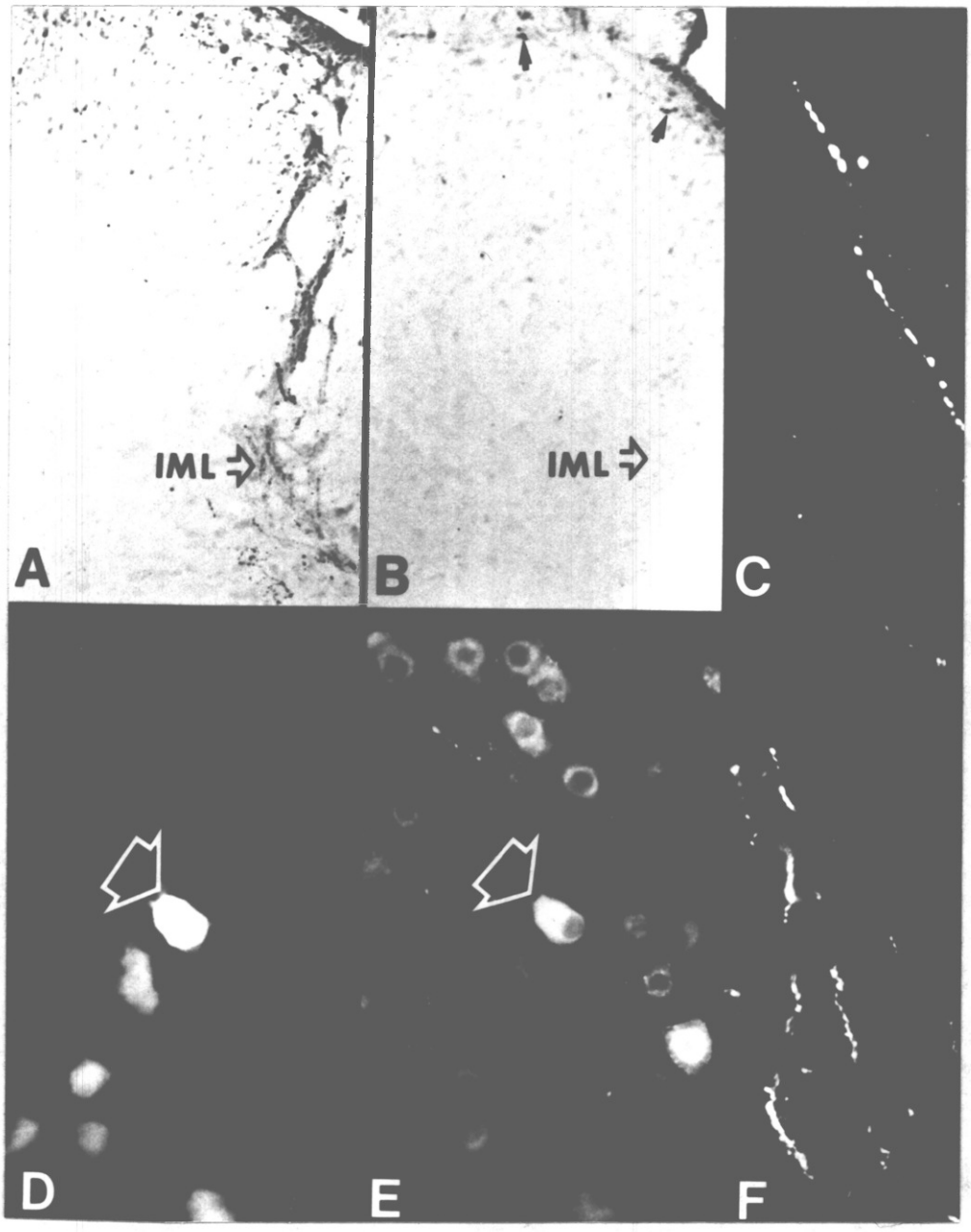



Fig.4.8 A) PHI-immunoreactive fibres in the lateral dorsal horn and in the intermediolateral cell column (IML) in the $L_6$ segment of a control, vehicle treated rat. B) Few immunoreactive fibres (some arrowed) are seen in animals treated as neonates with capsaicin (A,B x 95). C) VIP-immunoreactive fibres in the contralateral side of the rat bladder after unilateral nerve section. F) A small increase in numbers of immunoreactive fibres was noted in the lamina propria of the ipsilateral side (C,F x 300). Following injection of True Blue into the rat bladder some VIP-immunoreactive cells in the pelvic ganglion E) were retrogradely labelled with the tracer D) (see arrow). (D,E x 160).

Retrograde tracing True Blue injection to bladder (19 rats) and pelvic nerve (8 rats). Killed 7 days later.

Retrogradely labelled cell bodies were seen in $L_6$-$S_1$ dorsal root ganglia (approx. 30% of the total), the sacral intermediolateral cell column and in the pelvic ganglion only after injection of the bladder.

Retrograde tracing and immunocytochemistry True Blue injection to bladder + colchicine injection of $L_6$-$S_1$ ganglia 2 days prior to sacrifice (20 rats).

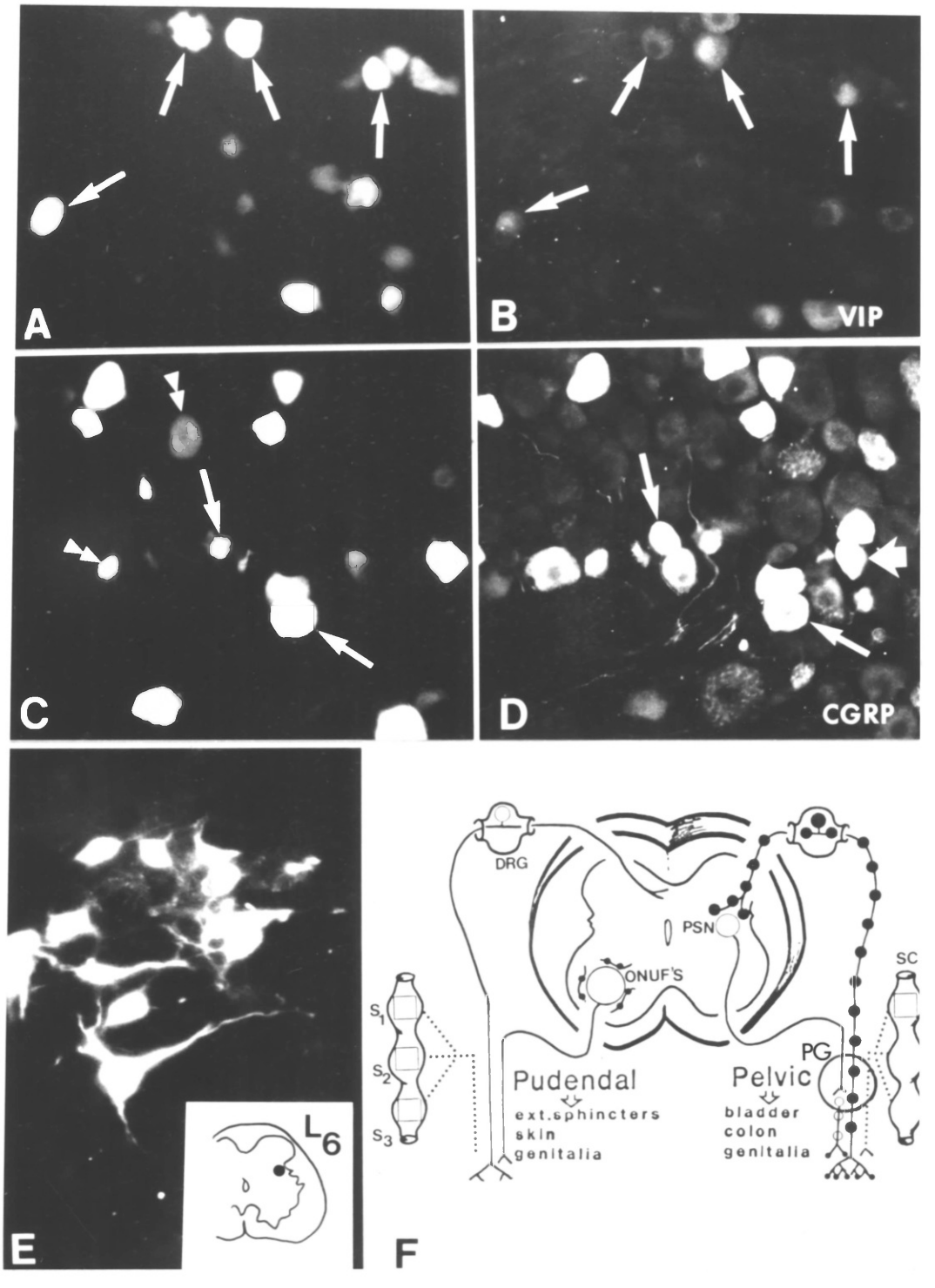
Retrogradely labelled dorsal root ganglion cells were colocalised with cells immunoreactive for VIP/PHI, CGRP and substance P (Fig.4.9 A-D). In the intermediolateral cell columns, no labelled cells were immunoreactive (Fig.4.9E). In the pelvic ganglion, many labelled cells were VIP/PHI-immunoreactive (Fig.4.8D,E).

Retrograde tracing and CGRP immunocytochemistry True Blue injection to urogenital tract, stomach, pancreas (10 rats/group).

CGRP immunoreactivity was identified in many retrogradely labelled cells at several segmental levels (Fig.4.10).

**Commentary:** Loss of VIP/PHI immunoreactivities in the spinal cord following nerve section was comparable to, although not as complete as, that observed after rhizotomy or ganglionectomy. The residual VIP/PHI may thus be derived from local cell bodies or

**Fig.4.9** (A-D) $L_6$ dorsal root ganglia of rat. Following injection of True Blue into the bladder wall many cells are retrogradely labelled (A,C). A) Some True Blue labelled cells contain VIP immunoreactivity B) (arrows) which can be demonstrated after colchicine treatment. D) Same section as C) processed for demonstration of CGRP immunoreactivity. Many immunoreactive cells are present without colchicine treatment. Some cells are retrogradely labelled ( ↑ ). Not all immunoreactive cells are labelled with True Blue (e.g. ▲) and vice versa (e.g. ▶▶) (C,D x 220). E) True Blue labelled preganglionic neurones in the intermediolateral cell column of the $L_6$ rat spinal cord (see diagram) following injection of the tracer into the pelvic ganglion (x 185). F) Summary diagram of the VIP/PHI-containing sensory pathway between the bladder and the lumbosacral spinal cord. VIP/PHI immunoreactivity in visceral afferents (●●●) derives from cells in the dorsal root ganglia (DRG). The central terminals of these cells end in the dorsal horn, some in close proximity to the parasympathetic sacral nucleus (PSN). The peripheral terminals end in the bladder. Not all VIP/PHI-immunoreactive fibres in the bladder are afferent in origin. Postganglionic (∘∘∘) fibres from the pelvic ganglion (PG), intramural cells and sympathetic efferents ( ·····) constitute a major source of peptide. SC, sympathetic chain.


A
B
VIP
C
D
CGRP
E
L6
F
DRG
PSN
ONUF'S
SC
PG
S1
S2
S3
Pudendal
ext.sphincters
skin
genitalia
Pelvic
bladder
colon
genitalia

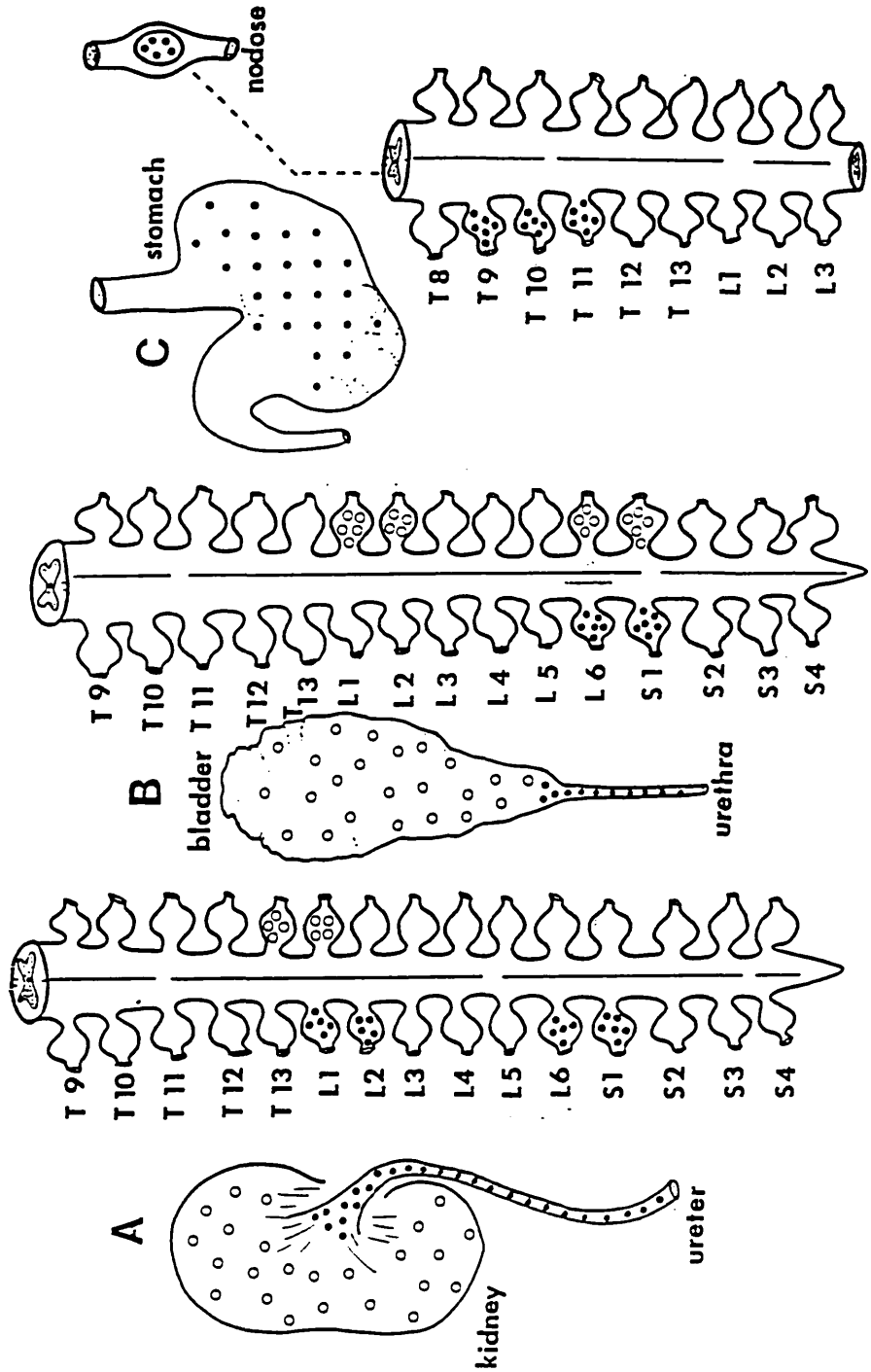



Fig.4.10 Following injection of True Blue into the urinary tract A), B) or stomach C) of the rat, retrogradely labelled sensory neurones were found in segmental levels specific for each of the injection sites. Many True Blue labelled neurones in the dorsal root and nodose ganglia contained CGRP immunoreactivity. Peak distributions of True Blue/CGRP positive cells are shown diagrammatically. Open and closed circles distinguish injection sites.

other afferents e.g. from pudendal or sciatic nerves, which have overlapping distributions with the pelvic nerve in the lumbosacral cord (see chapter 4.5.3) (de Groat, 1987; Katagiri et al., 1986; Morrison, 1987). Failure to cut all vesical branches of the nerve is another possibility. The loss of VIP/PHI, further reinforces the speculation that VIP/PHI occurs in visceral nerves. However, it was surprising that nerve section resulted in no marked change in the distributions of CGRP or substance P in the spinal cord as these peptides are known to be present in dorsal root ganglion cells which also project to pelvic viscera (Sharkey et al., 1983; Su et al., 1986 and shown here). Since the density of CGRP- and substance P-immunoreactive fibres markedly exceeds that of VIP/PHI and both peptides are also common to somatic, vascular and cutaneous afferents (Gibbins et al., 1985; this report), small changes induced by pelvic nerve section could easily be masked.

In the bladder, a major source of VIP/PHI is from postganglionic parasympathetic fibres and intramural ganglia (Alm et al., 1977; Gu et al., 1984). Changes in the relatively minor population of VIP/PHI-immunoreactive afferents could be obscured by the response of local cell bodies to nerve injury and may thus account for the small increase seen after section, as also noted by others (Gu et al., 1984).

Combined retrograde tracing and immunocytochemical studies confirmed that VIP/PHI immunoreactivity exists in sensory neurones projecting to the pelvic viscera, in accord with a study in cat (Kawatani et al., 1983). Further, conclusive evidence of a

selective innervation was subsequently provided by the demonstration that only a minor proportion of pudendal afferents contain VIP, in comparison to pelvic afferents (Kawatani et al., 1986).

Although other peptides show an overlap with pelvic afferents their distribution in the spinal cord is not so selective in caudal segments. For example, CGRP is present in many pelvic visceral afferents which project to the urogenital tract and also in thoracic afferents innervating tissues including the stomach and pancreas (Su et al., 1987). There is now a body of evidence which suggests VIP/PHI immunoreactivity is especially common in a subpopulation of visceral afferents which innervate pelvic organs (Anand et al., 1983, 1984; Basbaum and Glazer, 1983; Gibson et al., 1986; Honda et al., 1983; Kawatani et al., 1983). However, the presence of small numbers of VIP/PHI-immunoreactive fibres, i) which are capsaicin-sensitive and unaffected by spinal transection, in the dorsal horn of all spinal segments (Gibson et al., 1984c; Kawatani et al., 1983; LaMotte and de Lanerolle, 1983) and ii) in the vagus nerve (Lundberg et al., 1980) together with iii) the demonstration of a few positively stained dorsal root ganglion cells at the thoracic level (Kuo et al., 1985), suggests that VIP/PHI may also be present in other visceral afferents.

Electrophysiological studies indicate VIP may be a neurotransmitter in the spinal cord (Jeftinija et al., 1982; Phillis, 1982). A rôle in sacral afferent pathways is further supported by release experiments which have shown that greater levels of VIP

are released into spinal cord superfusate following high intensity stimulation of the pelvic nerve (Blank et al., 1984) than after stimulation of the sciatic nerve (Yaksh et al., 1982a).

In conclusion, evidence points to VIP/PHI as a marker of pelvic afferent fibres in the lumbosacral spinal cord which originate from cell bodies located in lumbosacral dorsal root ganglia.

### 4.5.2 Motoneurone group in $L_1$-$L_2$ segments of the rat spinal cord

The dense innervation of the mediolateral motor nucleus in upper lumbar segments, notably by substance P-immunoreactive fibres was a striking feature and in a separate study the origin of these immunoreactive fibres was further investigated (Gibson et al., 1984a) (Fig. 4.3C, 4.11A,B). The $L_1$-$L_2$ level of the spinal cord gives rise to the genitofemoral nerve (Hodson, 1970). The neurochemical composition and projection pathways of this nerve was investigated using retrograde tracing combined with immunocytochemistry, and a number of pharmacological and surgical procedures were used to establish that the motor nucleus under investigation innervates the cremaster muscle and a part of the scrotal skin.

Origin of substance P-immunoreactive fibres 3-5 rats/group killed 2 weeks later.

The following manipulations were performed to identify a possible i) supraspinal ii) afferent and/or iii) intrinsic (from lamina X)

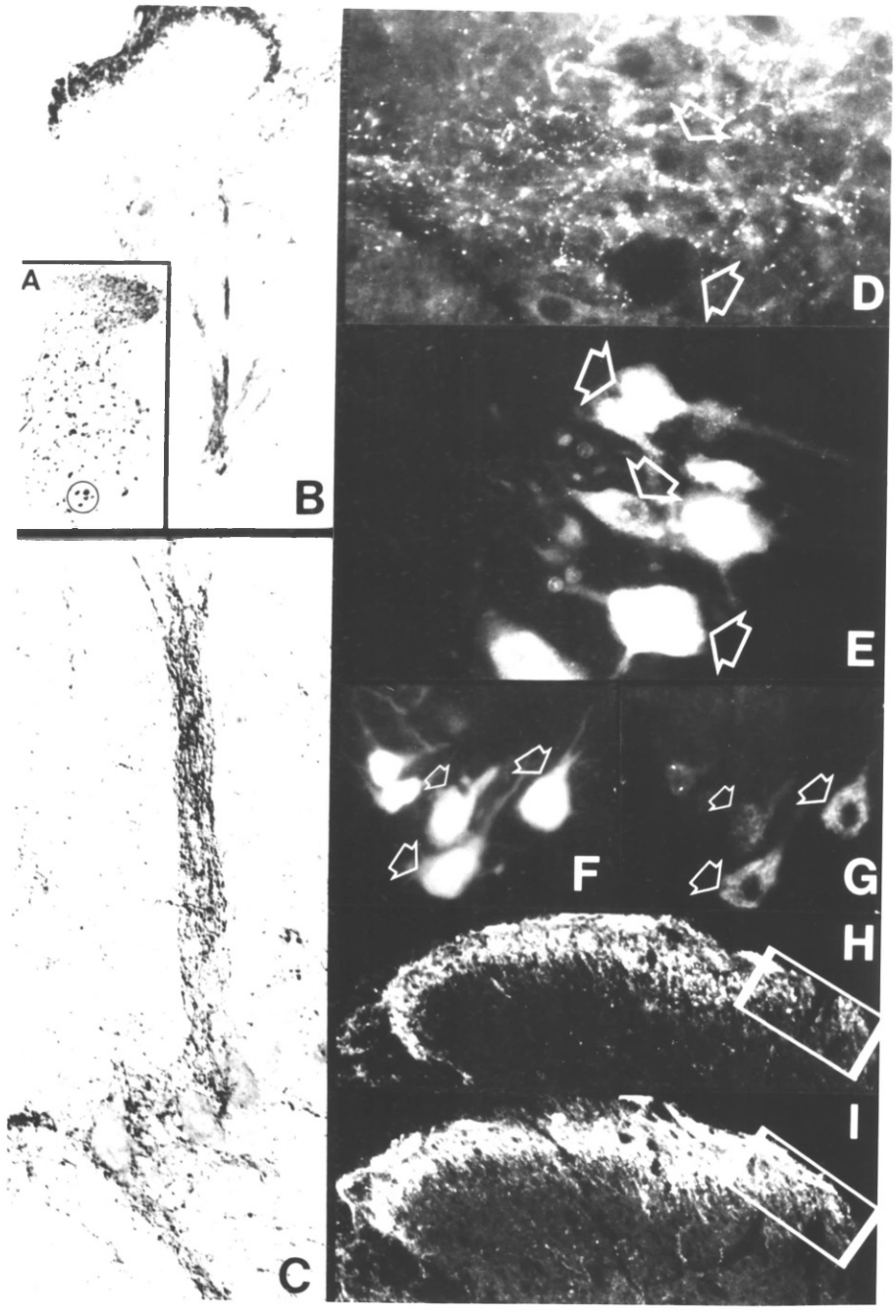



Fig.4.11 A) Toluidine blue stained section through the $L_2$ segment of the rat spinal cord to show the position of the cremaster motoneurones (circle) (x 20). B) Low power photomicrograph of substance P immunoreactivity in the $L_2$ spinal cord. Note aggregation of fibres around the cremaster motor nucleus (x 45). C) Following dorsal rhizotomy, no change in the distribution of substance P-immunoreactive fibres was seen in the vicinity of the cremaster motor nucleus (x 140). (D,E) The same section showing D) substance P-immunoreactive fibres surrounding E) True Blue labelled motoneurones (arrows) in the $L_2$ ventral horn after injection of True Blue into the genitofemoral nerve (x 275). F) True Blue labelled motoneurones in the $L_2$ ventral horn after injection of the tracer into the cremaster muscle. G) The same section as F) immunostained for CGRP demonstrating most cremaster motoneurones are CGRP positive (arrows) (x 185). H) Following unilateral genitofemoral nerve section there is a discrete ipsilateral loss of CGRP immunoreactivity from the medial dorsal horn of the $L_2$ segment compared to the contralateral side I) (see boxed areas for comparison) (x 75).

origin.

i) Hemisection, transection, unilateral and bilateral dorsolateral funiculus lesions (segmental level $T_6$-$T_8$).

ii) Neonatal capsaicin (killed at 2 months), bilateral dorsal rhizotomy ($T_{12}$-$L_3$).

iii) Double lesions of the area around the central canal (cuts at $T_{12}$ and $L_3$.

In all cases no change was seen in the distribution of substance P-immunoreactive fibres around the motor nucleus (Fig.4.11C).

Retrograde tracing and immunocytochemistry True Blue injection of the genitofemoral nerve or cremaster muscle. 20 rats, killed 5 days later.

Retrogradely labelled cells were seen in the mediolateral motor nucleus. These were CGRP-immunoreactive and were surrounded by substance P-immunoreactive fibres (Fig.4.11D-G). True Blue labelled dorsal root ganglion cells were seen in segmental levels $L_1$ and $L_2$ and of these 10% were CGRP- and 5% were substance P-immunoreactive. Fewer, retrogradely labelled cells were seen after injection of cremaster muscle.

Unilateral genitofemoral nerve section 5 rats, killed 7 days later.

There was a decrease CGRP- and substance P-immunoreactive fibres

in the medial 1/5th of laminae I-II in the ipsilateral dorsal horn in segments $L_1$ and $L_2$ (Fig.4.11H,I). There was an almost complete loss of CGRP- and substance P-immunoreactive fibres in scrotal skin and cremaster muscle.

Neonatal capsaicin 4 treated, 4 vehicle control rats killed at 2 months of age.

In capsiacin treated rats CGRP- or substance P-immunoreactive fibres were not visible in scrotal skin. In cremaster muscle, substance P-immunoreactive fibres were lost but there was only a partial reduction of CGRP-immunoreactive fibres.

**Commentary:** It was thought likely that the substance P-immunoreactive fibres were of supraspinal or extrinsic origin for the following reasons i) a substantial proportion of substance P in the ventral horn is from descending fibre projections (Hunt, 1983; Ruda et al., 1986 for reviews) and ii) degeneration studies have shown that some dorsal root afferents terminate on motoneurones (Sprague, 1958). Our data, subsequently confirmed by Nagy and Senba (1985), ruled out such possibilities. A third likely source was from local cells in lamina X, but lesions of this area were without effect. Therefore, it is most probable that the substance P-immunoreactive fibres are collateral branches from interneurones in other laminae.

Using a similar experimental approach it has been shown that the substance P-innervated group of motoneurones projects to the

cremaster muscle via the genitofemoral nerve (Nagy and Senba, 1985). Our findings confirm and extend this work. CGRP immunoreactivity was identified in both cremaster motoneurones and a proportion of sensory neurones which also project to genitofemoral nerve. Thus, although genitofemoral nerve section produced a complete loss of CGRP from the muscle, capsaicin treatment induced a partial loss of immunoreactive fibres which could be attributed to the dual origins of CGRP from motor and sensory axons.

The significance of the close relationship between substance P-immunoreactive fibres and CGRP-immunoreactive motoneurones is unclear. There is considerable evidence to suggest substance P may play a part in motor function (Doi and Jurna, 1982; Piercey et al., 1981). Iontophoresis to motoneuronal membranes increases their excitability, (Konishi and Otsuka, 1974), and high doses of the substance P antagonist, Spantide, induces hind-limb paralysis (Åkerman et al., 1982; Piercey et al., 1981). The effect of peptides on motoneurones is likely to be complex. Thus, although hind-limb responses elicited by intrathecal application of substance P to the lumbar spinal cord are potentiated by CGRP (Wiesenfeld-Hallin et al., 1984; Woolf and Wiesenfeld-Hallin, 1986), a possible mechanism for their release and action on motoneurones remains to be elucidated.

Both CGRP and substance P sensory neurones contribute to the innervation of the genitofemoral nerve, with their central axons terminating in a restricted region of the dorsal horn (shown by axotomy) and their peripheral axons in both cremaster muscle and scrotal skin (axotomy and capsaicin experiments). Cremaster

muscle and scrotal skin play a part in testicular thermoregulation and hence in spermatogenesis (Bishop and Walton, 1960) and it is likely that CGRP and substance P have a contributory rôle since both peptides are potent vasodilators (Brain and Williams, 1985; Gamse and Saria; 1985). In scrotal skin, release of CGRP and substance P from sensory perivascular nerves, would result in increased blood flow and surface heat loss. CGRP will also contract striated muscle (Takami et al., 1985b). If CGRP exerts a similar effect on cremaster muscle, the scrotal sac would be drawn closer to the body wall and heat would be conserved. Thus, male infertility could result as a consequence of imbalance of peptide production or interruption of nerve supply.

### 4.5.3 Pudendal motor nuclei in the rat spinal cord

The pudendal motor nuclei were richly innervated by peptide-immunoreactive fibres. In man, motor nerves innervating the pelvic muscles are thought to originate from one nucleus, the pudendal or Onuf's nucleus (Onuf, 1900). With the advent of retrograde tracing techniques, however, more detailed studies of pelvic muscle projections indicated in monkey (Nakagawa, 1980; Roppolo et al., 1985; Ueyama et al., 1985) and dog (Kuzuhara et al., 1980; Nakano, 1969) that the pudendal nucleus consists of one group of neurones but in cat (de Groat et al., 1983) and particularly in rat, this nucleus probably comprises two subnuclei (Sato et al., 1978; Schrøder, 1980). Thus, to clarify and provide further information on the central projections and neurochemistry of the pudendal nerve, retrograde tracing techniques were combined

with immunocytochemistry.

Retrograde tracing: True Blue injection to pelvic muscles, pudendal or sciatic nerve. 3-10 rats/group killed 7 days later.

Retrogradely labelled cells were seen in segmental levels $L_4$-$S_1$, in one or more of the following locations in the spinal cord; dorsolateral, dorsomedial, ventral, retrodorsolateral motor nuclei, intermediolateral cell columns and in the dorsal root ganglia. Results are summarised in Fig.4.12 and neuronal cell counts after pelvic muscle and pudendal nerve injections are shown in Tables 4.7,4.8 (see pp. 117).

Double retrograde labelling 10 rats, killed 7 days later.

Labelling of sciatic and pudendal nerves showed overlapping distributions in the retrodorsolateral nucleus and dorsal root ganglia. Thus, different tracers (see chapter 2.12.3) were injected into both nerves to determine possible pre-spinal convergence. True Blue was injected into the pudendal nerve and either diamidino yellow or rhodamine-labelled microspheres were injected into the sciatic nerve.

In the retrodorsolateral nucleus each dye labelled a separate cell population (Table 4.8). In the dorsal root ganglia 1-3 sensory neurones were double labelled (Fig.2.1).

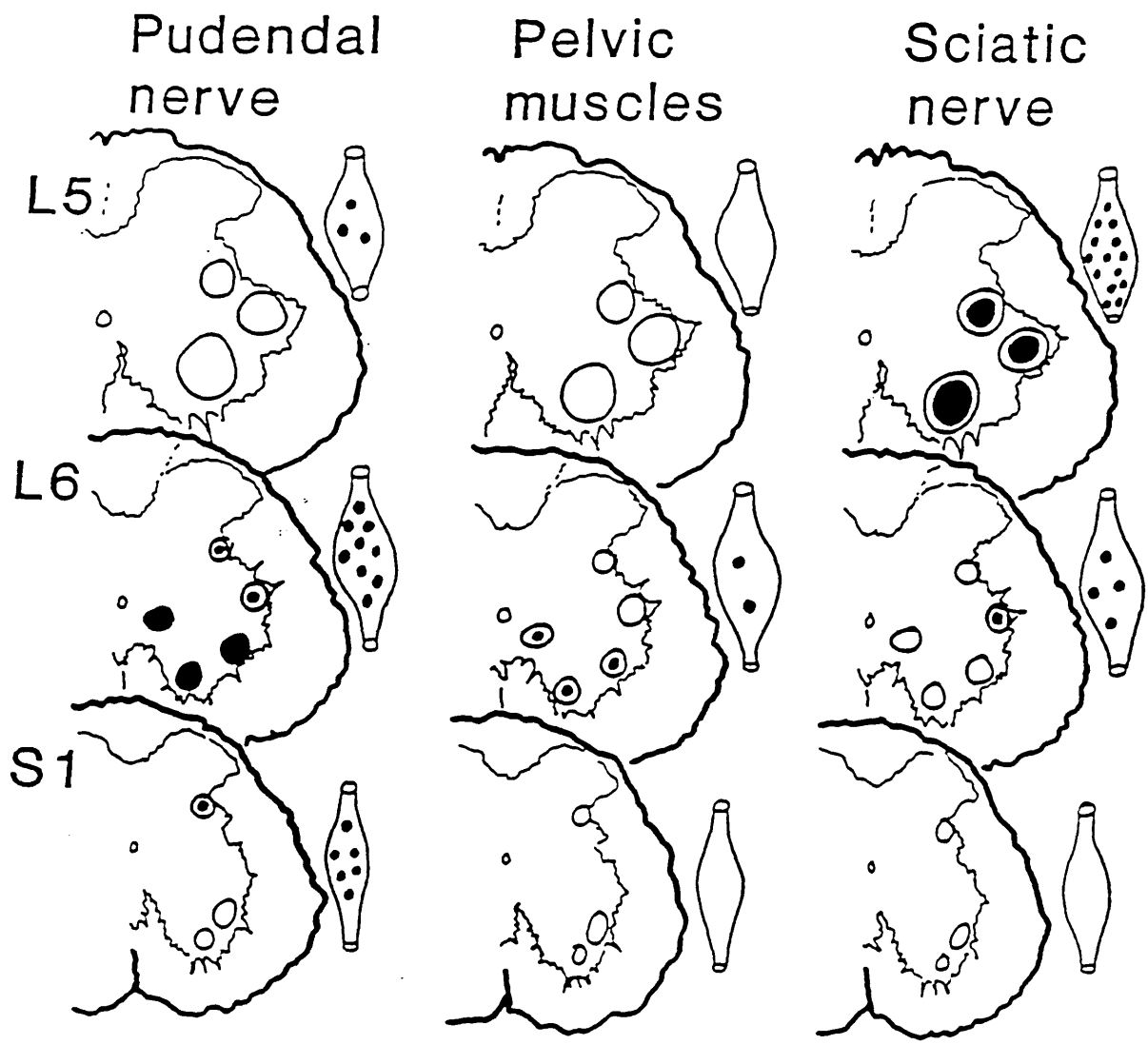



Fig.4.12 Location of retrogradely labelled cells following injection of tracer into pudendal or sciatic nerves or pelvic muscles. Filled or partially filled circles indicate relative proportions of True Blue labelled cells in the spinal cord, and in the dorsal root ganglia dots denote density.

Retrograde tracing and immunocytochemistry True Blue injection to pelvic muscles or pudendal nerves. 4-6 rats/group killed 7 days later.

In the spinal cord SOM-, ENK- and NPY/CPON-immunoreactive fibres were present around all retrogradely labelled neurones. The dorsolateral, followed by the dorsomedial motor nuclei were the most densely innervated and often a tract of immunoreactive fibres connected the two nuclei (Fig.4.13). Most, labelled motoneurones were CGRP positive (Fig.4.13D,E). In $L_6$-$S_1$ dorsal root ganglia approximately 10% of the labelled cells were CGRP- and 2% substance P-immunoreactive.

**Commentary:** The pudendal nerve plays a significant part in regulation of external sphincters and muscles of the urogenital tract. These findings show that three motoneurone groups (dorsomedial, ventral and dorsolateral) project to striated pelvic muscles involved in micturition, erection, ejaculation and defaecation.

In rat, the dorsolateral nucleus is generally considered homologous with Onuf's nucleus in man, monkey and dog on the basis of a number of common cytological features including a circumscript nucleus in a ventrolateral location, a dense longitudinally orientated dendritic bundle and the small size of the motoneurones (Kuzuhara et al., 1980; Nakagawa, 1980; Onuf, 1900; Roppolo et al., 1985). However, there are certain cytoarchitectural and histochemical similarities between dorsolateral and

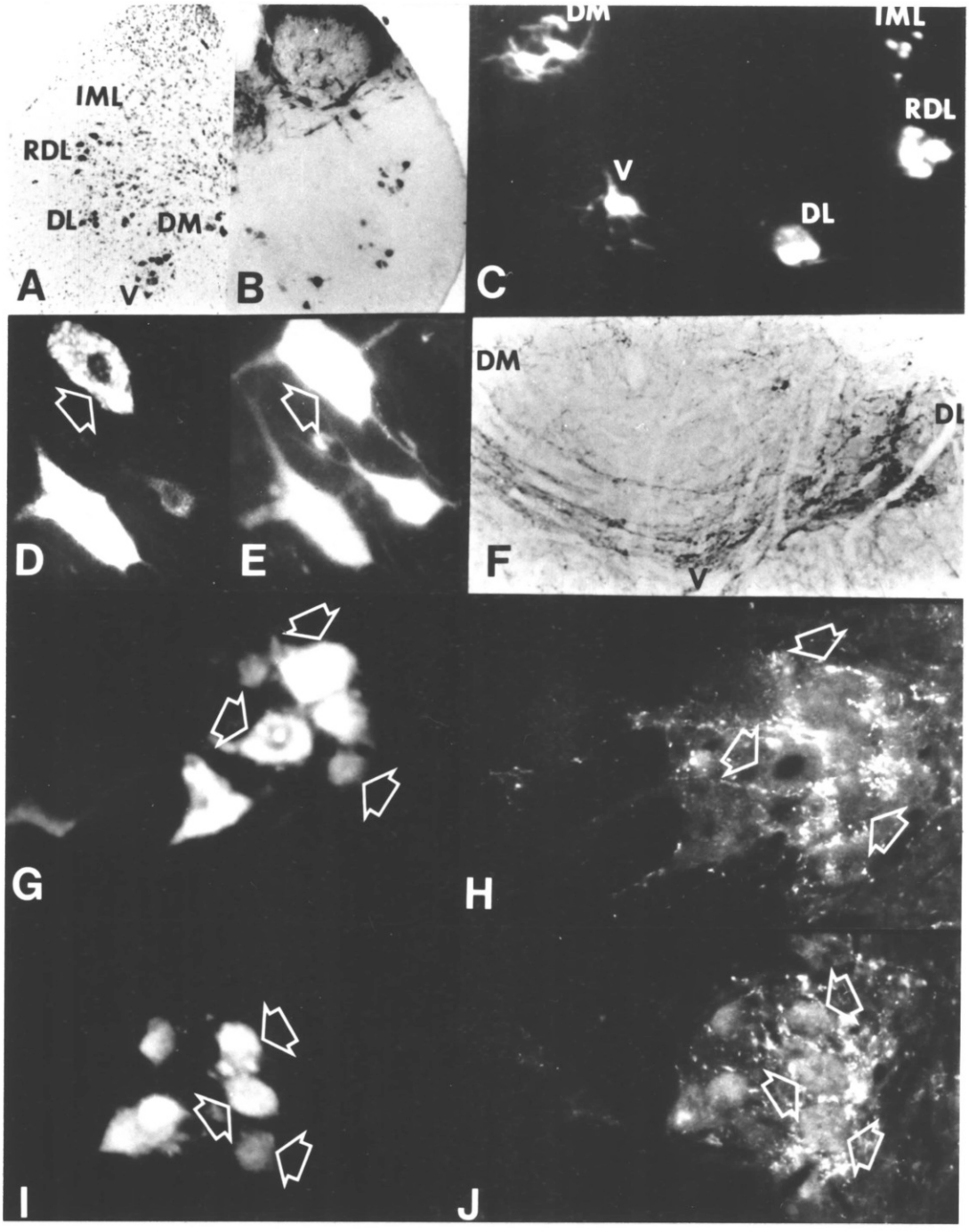



Fig.4.13 A) Toluidine blue stained section through the $L_6$ segment of the rat spinal cord showing various cell columns. B) CGRP immunoreactivity in a similar section, note positive motoneurones are present in each of the motor nuclei (A,B x 35). C) True Blue labelled neuronal groups in the $L_6$ segment following injection of the tracer into the pudendal nerve (x 125). Most of the True Blue labelled neurones D) were CGRP-immunoreactive E). Section showing neurones from the ventral nucleus (D,E x 220). F) ENK immunoreactivity is particularly dense in the dorsolateral (DL) and dorsomedial (not shown) nuclei. Fibres are often present connecting these two nuclei via the ventral (V) nucleus (x 115). (G,I) True Blue labelled neurones in the dorsolateral nucleus. H) ENK-immunoreactive fibres in the same section as G) and J) NPY-immunoreactive fibres in the same section as I). The peptide-immunoreactive fibres are in close proximity to the True Blue labelled neurones (arrows). Immunoreactivity is more pronounced in the medial portion of the nucleus (G-J, x 175). IML, intermediolateral cell column; RDL, retrodorsolateral; DL, dorsolateral; V, ventral; DM, dorsomedial nuclei.

dorsomedial nuclei, including the particularly dense innervation by SOM-, ENK- and NPY/CPON-immunoreactive fibres and presence of peptide-immunoreactive fibre tracts which link the two (reported here, Katagiri et al., 1986; Schrøder, 1984). Thus, on the strength of the immunocytochemical data it would seem that in rat, as suggested by previous studies (Schrøder, 1980; 1984), the pudendal motor nucleus comprises two widely separated subnuclei (see also chapter 7.2).

Injection of pudendal and sciatic nerves with different tracers in the same rat, showed that motor axons from each nerve originate from separate cells in the retrodorsolateral nucleus. In the dorsal root ganglia, in accord with others (Taylor and Pierau, 1983; Taylor et al., 1982) a small percentage of cells were double labelled, indicating some pre-spinal convergence of axon collaterals. As expected, motor and sensory neurones provided the major input to the pudendal nerve, which is generally considered somatosensory. However, as shown here and noted by others, (Hulsebosch and Coggeshall, 1982) there is also a parasympathetic component, since a small number of cells in the intermediolateral cell column were retrogradely labelled following injection of the pudendal nerve.

In conclusion, the dense innervation of both dorsomedial and dorsolateral nuclei by fibres immunoreactive for SOM, ENK and NPY/CPON supports the speculation that, in male rats both nuclei constitute the pudendal motor nucleus and its homologue, Onuf's nucleus, in man.

# CHAPTER 5: SCIATIC NERVE MANIPULATIONS AND THEIR EFFECTS ON PEPTIDES IN THE RAT DORSAL HORN


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# CHAPTER 5

## 5.1 INTRODUCTION

Peripheral axotomy leads to a number of morphological and histochemical changes proximal to the site of the lesion, in the parent cell body and trans-synaptically (Aldskogius et al., 1985; Aldskogius and Risling, 1981; Csillik and Knyihár, 1975; Lieberman, 1971; Sunderland, 1978). There are also profound changes in the neurochemistry of primary afferents in laminae I-II of the dorsal horn with losses of FRAP (Devor and Claman, 1980; Knyihár-Csillik and Csillik, 1981; Schoenen et al., 1968) and substance P (Barbut et al., 1981; Jessell et al., 1979; Wall et al., 1981) being amongst the best documented. These biochemical changes are accompanied by electrophysiological rearrangements which are concerned with the terminals of unmyelinated C fibre afferents in laminae I-II of the dorsal horn (Devor and Wall, 1981; Wall, 1982; Wall and Devor, 1981) (Table 5.1). It is likely that an imbalance in neurotransmitter-like substances, such as peptides, is linked to the disturbed physiological function. The aim of the first part of this chapter therefore was to extend the observations on substance P and to examine possible changes in the distributions of other peptides in the dorsal horn following sciatic nerve lesions.

In the second part, distributions of peptides in the dorsal horn were investigated following local application of capsaicin (chapter 2.12.1) to one sciatic nerve. Although capsaicin can be

TABLE 5.1 SUMMARY OF PHYSIOLOGICAL, ANATOMICAL, CHEMICAL AND BEHAVIOURAL CONSEQUENCES OF DIFFERENT MANIPULATIONS OF THE RAT SCIATIC NERVE (14 DAYS POST-OPERATIVELY)

| Parameters | Section and ligation | Crush | Local capsaicin (49 mM) |
|---|---|---|---|
| Loss of neurogenic oedema | present[13] | present | present[8,12] |
| Axonal transport blockade | present | present | present[1,9] |
| Distal degeneration | present | present | absent[1] |
| Primary afferent depolarisation | present[16] | no change[16] | no change[17] |
| C fibre transmission to dorsal horn cells | present[1] | ? | absent[17] |
| Reactive cells | present[10] | present[10] | n.r |
| FRAP | decreased[3] | decreased[3] | decreased[1] |
| BN | no change[1] | no change[1] | no change[1] |
| CCK | decreased[1,15] | no change[1] | decreased[1] |
| CGRP | decreased[1] | no change[1] | decreased[1] |
| ENK no change[1], increased[11], | decreased[5] | no change[1] | no change[1] |
| NT | no change[1] | no change[1] | no change[1] |
| NP | no change[1] | no change[1] | no change[1] |
| SOM | decreased[1,15] | no change[1] | decreased[1] |
| Substance P decreased[1,2,11,,14,15] | | no change[1,2] | decreased[1] |
| VIP/PHI | increased[1,15] | increased[1] | increased[1] |
| Opiate receptors | decreased[4,6] | n.r | n.r |
| Chemical analgesia | present | present | present[12] |
| Thermal analgesia | present | present | present[1,7] |
| Mechanical analgesia | present | present | absent[7] |

FRAP and peptide data refer to staining in the ipsilateral dorsal horn of the $L_4$ spinal cord segment. [1]Refers to work reported here and published from resultant studies see Ainsworth et al., 1981; Gibson et al., 1982; McGregor et al., 1984; Wall et al., 1981; [2]Barbut et al., 1981; [3] Knyihár-Csillik and Csillik, 1981; [4]Csillik et al., 1982a; [5]Di Giullo et al., 1985; [6]Fields et al., 1980; [7]Fitzgerald and Woolf, 1982; [8]Fitzgerald, 1983; [9]Gamse et al., 1982; [10]Gilmore and Skinner, 1979; [11]Hunt et al., 1982; [12]Jancsó et al., 1980; [13]Jancsó, 1968; [14]Jessell et al., 1979; [15]Shehab et al., 1986; [16]Wall and Devor, 1981; [17]Wall and Fitzgerald, 1981; n.r = not reported.

administered by subcutaneous injection, such a route affects all peripheral nerves and produces a permanent degeneration of unmyelinated fibres. Local application however, allows a comparison of single nerves in the same animal and there is no degeneration (Ainsworth et al., 1981). There are however, behavioural changes which relate directly or indirectly to C fibres (Fitzgerald, 1983) (Table 5.1). It was thus of interest to study the effects of perineural capsaicin on peptide immunoreactivities in the dorsal horn, not only to provide further information on the action of the neurotoxin, but also to provide a comparison with data obtained following surgical interruption of the sciatic nerve.

## 5.2 EXPERIMENTAL APPROACH

### 5.2.1 Animal groups (see also chapter 2.12)

Unilateral nerve section or crush: Rats (3 groups; n = 20/group) were anaesthetised using ether inhalation and the sciatic nerve on one side was exposed. In the first group, the nerve was sectioned and ligated, in the second, the nerve was crushed, and the third group was sham-operated. Animals were killed 14 days later. Peptide levels were assessed quantitatively by radioimmunoassay of spinal cord extracts of six animals from each group.

Local capsaicin application: For immunocytochemistry three groups of at least 4 experimental and 4 control (vehicle treated) rats were assessed at every point in each group.

**Time course:** One sciatic nerve treated with 49 mM capsaicin or vehicle solution and killed at post-operative intervals of 2-3 days from day 2 to day 200.

**Dose response:** One sciatic nerve treated with graded doses of capsaicin (0.01, 0.1, 1.5, 3.0, 5.0, 6.0, 12, 24 and 49 mM). Rats killed 14 days later.

**Unilateral intramuscular capsaicin (49 mM) and unoperated controls:** Rats killed 14 days later.

For radioimmunoassay, 10 rats from each of the following groups: 1.5 mM and 49 mM capsaicin, vehicle treated, intramuscular capsaicin and unoperated were killed 14 days post-operatively, subsequent to analysis of noxious thermal responses using the hind-paw water immersion test (chapter 2.12.1).

### 5.2.2 Tissue processing

For immunocytochemistry rats were perfused with 4% p-benzoquinone (appendix II,III) and frozen tissue sections (20 µm) through $L_3$-$L_5$ segments were immunostained using the the indirect immunofluorescence or the PAP method (appendix V,VI). For radioimmunoassay, the $L_4$ segment was hemisected by a dorsal-ventral cut through the midline and peptides extracted in either, boiling water or acetic acid (0.5 M) according to the peptide of interest. From capsaicin experiments (1.5 mM and 49 mM capsaicin, vehicle treated and unoperated controls), samples of hind-paw skin were also taken for immunocytochemistry and radioimmunoassay.

### 5.2.3 Analysis of results

A subjective comparison of the patterns of immunostaining in the ipsilateral and contralateral sides of the spinal cord (and skin where applicable) of operated and unoperated controls was made. Corresponding, mean peptide concentrations were compared and values obtained for noxious heat responses. The significance of the results were analysed using Students' paired t-tests.

## 5.3 RESULTS

Sciatic nerve section or crush

Immunocytochemical analysis of the spinal cord following nerve section, revealed a marked loss of substance P-immunoreactive fibres from the ipsilateral side of the dorsal horn which was most striking in the $L_4$ segment. This loss was most apparent in the medial two-thirds of laminae I-II of the dorsal horn. No changes were seen in other regions of the spinal cord. Similarly, CCK- and SOM-immunoreactivities were reduced. In marked contrast, however, VIP/PHI-immunoreactive fibres showed an increase in the medial region of laminae I-II of the ipsilateral dorsal horn. No change in the distributions of BN, ENK, NT or NP immunoreactivities were noted (Fig.5.1). In accord with these data, significantly decreased levels of substance P and SOM and increased levels of VIP/PHI were detected by radioimmunoassay of the ipsilateral $L_4$ hemisegment relative to the contralateral side. The concentrations of CCK, BN and NT by radioimmunoassay showed no

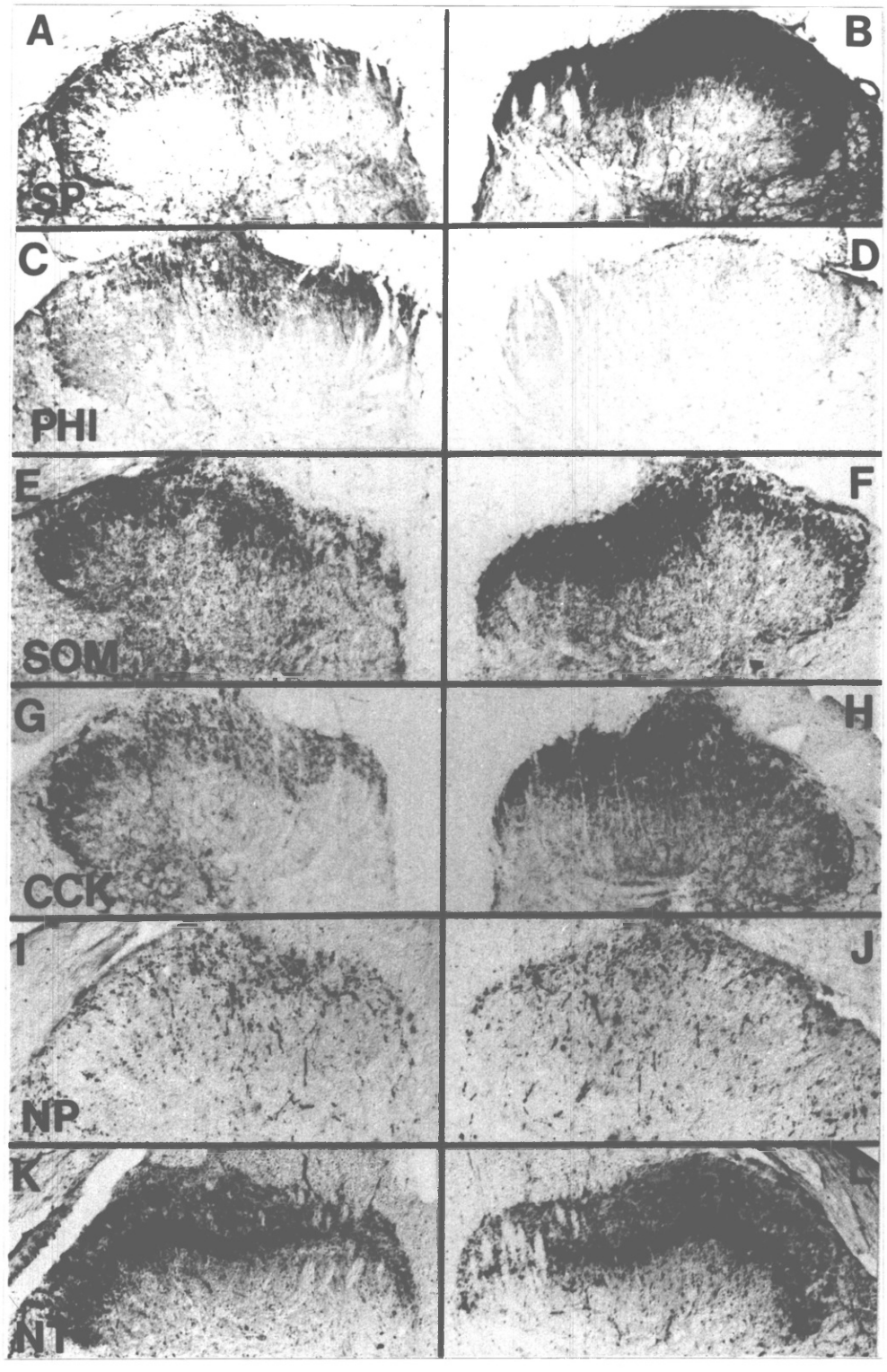



Fig.5.1 (A-L) Sections through the $L_4$ segment of the rat spinal cord 14 days following unilateral section and ligation of the sciatic nerve. Ipsilateral side is shown on the left. There is a marked reduction of substance P (SP) A), SOM E) and CCK G) immunoreactivities from laminae I-II compared to the intact contralateral side B), F) and H) respectively. By contrast, PHI immunoreactivity C) is increased compared to the control side D). The distribution of NP I) and NT K) immunoreactivities in the ipsilateral dorsal horn were unchanged compared to the contralateral sides J) and L) (A-L x 55).

TABLE 5.2 MEAN PEPTIDE CONCENTRATIONS BY RADIOIMMUNOASSAY IN $L_4$ HEMISEGMENTS FOLLOWING UNILATERAL SCIATIC NERVE SECTION, CRUSH OR SHAM OPERATION

| | Nerve section (n = 6) | | Nerve crush (n = 6) | | Sham-operated (n = 6) | |
|---|---|---|---|---|---|---|
| Peptide | Ipsi | Contra | Ipsi | Contra | Ipsi | Contra |
| Substance P | 150 ± 17* | 228 ± 23 | 173 ± 13 | 182 ± 13 | 216 ± 22 | 205 ± 19 |
| SOM | 227 ± 16* | 306 ± 30 | 274 ± 24 | 300 ± 18 | 296 ± 16 | 310 ± 24 |
| CCK | 21 ± 2 | 21 ± 1 | 23 ± 2 | 27 ± 1 | 25 ± 3 | 23 ± 3 |
| VIP | 7.7 ± 1.8* | 2.8 ± 1.1 | 7.2 ± 0.6 | 5.2 ± 0.9 | 5 ± 0.7 | 3.5 ± 1.1 |
| PHI | 11.0 ± 1.2* | 4.7 ± 0.4 | 8.5 ± 1.0* | 5.4 ± 0.6 | 6 ± 0.7 | 4.4 ± 0.6 |
| NT | 45 ± 5 | 45 ± 5 | 48 ± 5 | 46 ± 6 | 40 ± 1.0 | 37 ± 6 |
| BN | 5.6 ± 1.0 | 5.1 ± 0.7 | 4.2 ± 0.6 | 4.2 ± 0.7 | 3.6 ± 0.8 | 4.5 ± 0.8 |

Results are expressed in pmol/g wet weight ± S.E.M. *$P<0.05$ paired t-test. n = number of determinations. ipsi., ipsilateral; contra., contralateral.

change (Table 5.2).

Following nerve crush both radioimmunoassay and immunocytochemistry showed an increase of VIP/PHI immunoreactivity in the ipsilateral spinal cord, but this was not as pronounced as after nerve section. No change was detected in the other peptides studied (Table 5.2).

Local capsaicin application

**Spinal cord:** Fourteen days after perineural application of capsaicin, similar data to the nerve section studies were obtained (Fig.5.2 A-H) (Table 5.3). No changes were found in the distributions or concentrations of any of the peptides studied in hemisegments of $L_4$ spinal cords from control animals treated with vehicle solution or intramuscular capsaicin (49 mM) when compared to unoperated animals.

**Time course:** One week after capsaicin (49 mM) treatment a depletion of substance P-and CCK-immunoreactive fibres was apparent and became maximal 11-14 days post-operatively. This depletion persisted up to 60 days after treatment (Fig.5.3).

**Dose response:** At 0.1 mM, capsaicin produced minor decreases in substance P- and CCK-immunoreactive fibres which became more pronounced in the concentration range 3.0-49 mM (Fig.5.4). Radioimmunoassay, however, showed no significant decrease of substance P or SOM at the lower dose range investigated (1.5 mM) (Table 5.3).

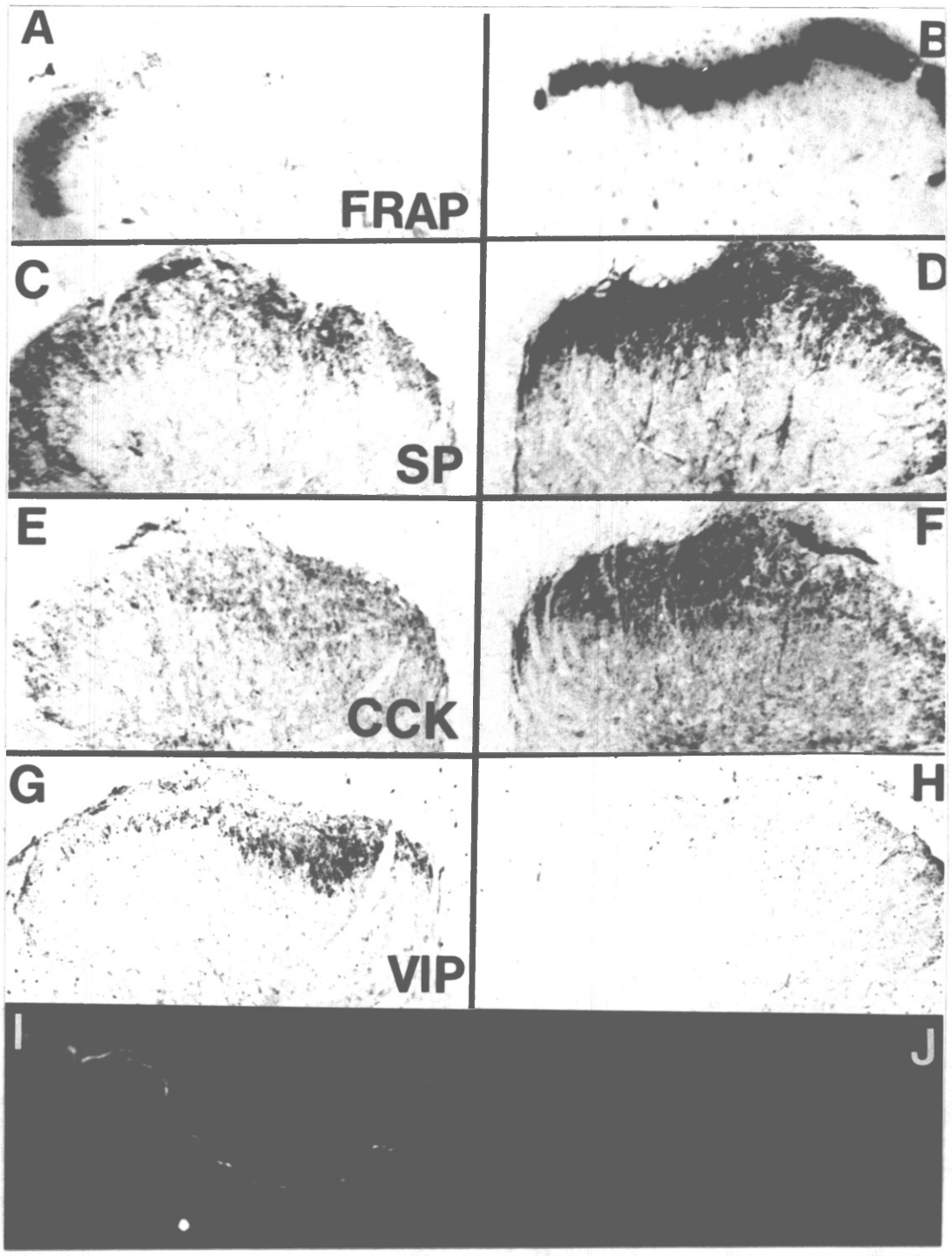


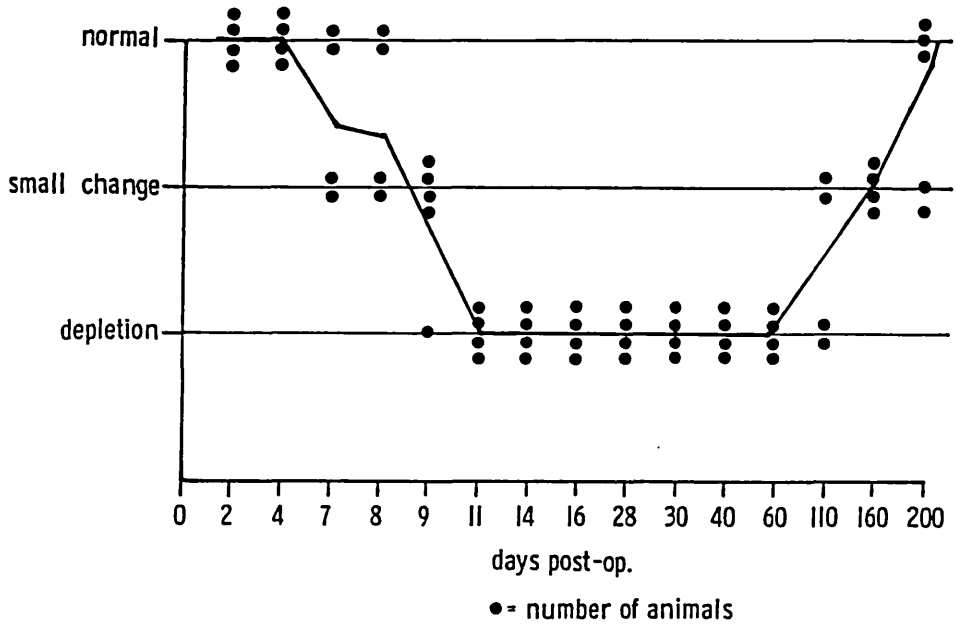

**Fig.5.2** (A-H) Sections through the $L_4$ segment of the rat spinal cord 14 days following local application of capsaicin (49 mM) to one sciatic nerve. A) FRAP activity is almost abolished in the medial two thirds of the ipsilateral dorsal horn compared to the control side B). Substance P C) and CCK E) immunoreactivities are likewise greatly reduced in comparison to the control sides D) and F). G) VIP immunoreactivity shows an increase after capsaicin treatment, whereas few fibres are seen in the contralateral dorsal horn H). (A-H, x 75). (I,J) Ipsilateral hind paw skin taken from I) a vehicle treated and J) capsaicin (49 mM) treated rat 14 days post-operatively immunostained for substance P. No immunoreactive fibres were seen in the skin from the capsaicin treated side J). (I,J x 310).

TABLE 5.3 MEAN PEPTIDE CONCENTRATIONS BY RADIOIMMUNOASSAY IN $L_4$ HEMISEGMENTS FOLLOWING UNILATERAL SCIATIC NERVE CAPSAICIN, VEHICLE SOLUTION OR INTRAMUSCULAR CAPSAICIN

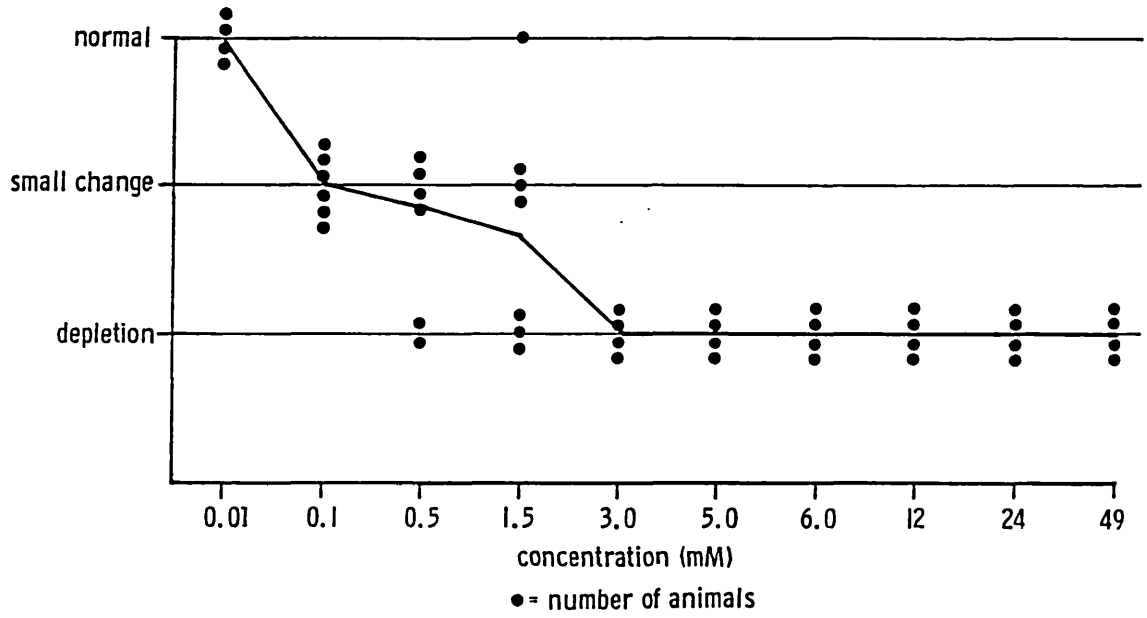
| Peptide | 49 mM capsaicin | 1.5 mM capsaicin | Vehicle | Intrasmuscular 49 mM capsaicin | Unoperated |
|---|---|---|---|---|---|
| | Ipsi : Contra | Ipsi : Contra | Ipsi : Contra | Ipsi : Contra | Ipsi : Contra |
| Substance P | 141 ± 16*:193 ± 16 | 127 ± 18:160 ± 20 | 116 ± 19:93 ± 16 (n = 9) | 92 ± 9:99 ± 14 (n = 9) | 102 ± 12:71 ± 10 |
| SOM | 63 ± 6*:36 ± 5.1 | 44 ± 9:43 ± 7 (n = 6) | 107 ± 11:81 ± 9 | 92 ± 11:88 ± 13 ( n = 9) | 94 ± 12:56 ± 6 |
| CCK | 12.3 ± 1.7:11.2 ± 1.4 | 14.1 ± 2.3:12.1 ± 2.1 | 8.7 ± 1.2:8.1 ± 1.5 | 7.8 ± 0.9:9 ± 0.7 | 7.6 ± 2.0:8.9 ± 1.7 |
| NT | 11.2 ± 2.6:13.5 ± 0.6 | 12.8 ± 2.0:15.0 ± 2.4 (n = 9) | 9.2 ± 1.4:10.5 ± 1.1 (n = 6) | 7.7 ± 0.9:8.6 ± 0.5 | 14.4 ± 0.7:14.0 ± 0.7 |
| BN | 6.5 ± 0.4:7.6 ± 0.8 (n = 8) | 5.4 ± 0.4:7.1 ± 1.0 | 3.6 ± 0.5:2.8 ± 0.6 | 2.3 ± 0.3:3.1 ± 0.4 | 7.2 ± 1.0:6.5 ± 0.5 |

Results are expressed in p mol/g wet weight ± S.E.M. *$P < 0.05$ paired t-test. n = 10 except where shown. ipsi., ipsilateral; contra., contralateral.

5.3 Time course : Substance P changes in the ipsilateral L4 segment following treatment with capsaicin (49mM).




5.4 Dose response : Substance P changes in the ipsilateral L4 segment following treatment with capsaicin.




Figs.5.3 & 5.4 Changes in substance P immunoreactivity induced in the ipsilateral $L_4$ dorsal spinal cord following various capsaicin treatments. Fig.5.3, Time course. Fig.5.4, Dose response (all animals killed 14 days post-operatively). Change in immunoreactivity was assessed subjectively. Results using CCK antiserum were similar.

**Skin:** Peptide-immunoreactive fibres were too rare to allow a useful comparison between animals except for those reacting with antibodies to substance P. Substance P-immunoreactive fibres were present below the epidermis and around blood vessels in the skin of contralateral hind-paws. No substance P-immunoreactive fibres were seen in the skin from ipsilateral limbs of rats treated with capsaicin (49 mM) 14 days previously (Fig.5.2I,J). Radioimmunoassay also showed a significant decrease of substance P (70%, 3.0 ± 0.8 pmol/g contralateral skin; 0.9 ± 0.3 pmol/g ipsilateral skin, $P<0.01$). The effect was less apparent with capsaicin at 1.5 mM. Substance P concentrations in the ipsilateral hind-paw skin of animals treated with vehicle solution and intramuscular capsaicin were not affected.

Noxious thermal response

A significant increase in withdrawal time was observed in animals treated with capsaicin (49 mM) (Fig.5.5). At lower doses, the increase was less significant. No increase in thresholds was observed in any of the control groups.

## 5.4 DISCUSSION

### 5.4.1 The effects of sciatic nerve section, crush or capsaicin on peptides in the dorsal horn

The present study has shown that a number of biochemical changes occur in the lumbar dorsal horn as a result of sciatic nerve

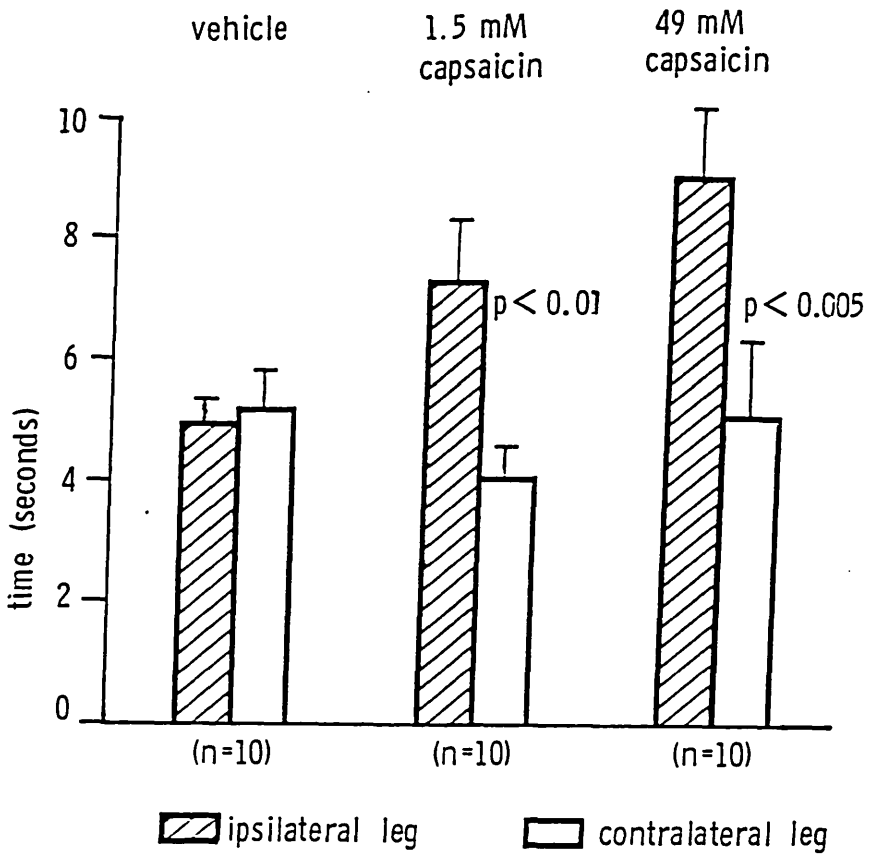



**Fig.5.5** Noxious thermal response assessed in three groups of rats 14 days after manipulation of one sciatic nerve.

TABLE 5.4 EFFECT OF COMBINED MANIPULATIONS ON VIP/PHI- AND SUBSTANCE P-IMMUNOREACTIVE FIBRES IN THE IPSILATERAL $L_4$ SEGMENT OF THE RAT DORSAL HORN

| MANIPULATIONS | IMMUNOSTAINING OF FIBRES | |
|---|---|---|
| | VIP/PHI | SUBSTANCE P |
| Dorsal rhizotomy ($L_3$-$L_6$) with sciatic nerve section 10 days later. | ↓↓↓ | ↓↓↓ |
| Neonatal capsaicin + sciatic nerve section when adult. | ↑ | No change |
| Mutilated foot rats[1] + sciatic nerve section. | ↑ | ↓ |
| Mid-thoracic spinal transection + sciatic nerve section. | ↑↑↑ | ↓↓↓ |
| Neonatal 6-OHDA + sciatic nerve section when adult. | ↑↑↑ | ↓↓↓ |

↑ small increase; ↑↑↑ equivalent to maximal increase in normal rats;
↓ small decrease; ↓↓↓ equivalent to maximal decrease in normal rats.
[1] see chapter 7.3

section. These included a loss of substance P immunoreactivity, as noted by others (Barbut et al., 1981; Di Giulio et al., 1985; Jessell et al., 1979) as well as a decrease in fibres immunoreactive for CCK and SOM and an unexpected increase of VIP/PHI-immunoreactive fibres in the ipsilateral dorsal horn. These changes were limited to the target area of the sciatic nerve in the spinal cord, mainly to the medial two-thirds of laminae I-II in the $L_4$ segment. This region coincides exactly with the area in which FRAP disappears following nerve section (Devor and Claman, 1980). Since the changes in immunoreactivity were confined to this restricted region of the spinal cord it would appear that we are concerned with a phenomenon associated with unmyelinated afferent fibres since these fibres rarely penetrate beyond lamina II and have limited rostro-caudal trajectories (Wall and Werman, 1976). Furthermore, FRAP and the peptides substance P, CCK and SOM, are known to be present in small cells of sensory ganglia and, together, represent markers of the majority of fine axons, including C fibres. The observed changes could thus result from a reorganisation of metabolism in reacting ganglion cells in favour of structural proteins, required for repair (Grafstein and McQuarrie, 1978). However, not all ganglion cells respond identically to peripheral nerve section. FRAP activity (Devor and Claman, 1980) is reduced 3-4 days before substance P, CCK and SOM immunoreactivities and in contrast, VIP/PHI immunoreactivity is increased. Taken together, these observations suggest that individual ganglion cells may respond differently and that the response is more complex than a simple, overall, depletion of biochemicals.

Whether C fibres in the dorsal horn degenerate after peripheral nerve injury, or whether they remain intact, with only some chemical stores being depleted, remains an open question. Several studies have shown degeneration argyrophylia in the dorsal horn (transganglionic degeneration atrophy) following nerve injury (Arvidsson et al., 1986; Csillik et al., 1982b) and a number of sensory neurones are known to die (Alskogius et al., 1985; Arvidsson et al., 1986). Although it would seem that some sensory neurones disappear after nerve section, it is unlikely that this would account for all of the neurochemical changes seen in the spinal cord since some of them are reversible, with FRAP and substance P levels showing eventual recovery (Jessell et al., 1979; Knyihár-Csillik and Csillik, 1981; Tessler et al., 1985). Furthermore, physiological studies show that, after nerve section the number of conducting C fibres remains normal and they are still able to excite dorsal horn neurones (Wall et al., 1981) (Table 5.1).

Nerve section and crush, share a number of effects which include the degeneration of distal fibres, the loss of input of nerve impulses from the original target site, and similar changes in FRAP- and VIP/PHI-containing fibres in the dorsal horn. However, there are marked differences, in particular, the failure of sciatic nerve crush to induce a change in any other peptide, in particular substance P immunoreactivity in the spinal cord (see also Barbut et al., 1981). Since altered chemical synthesis and expression presumably occurs in sensory neurones because of a changed signal from the periphery, it is possible that FRAP- (Nagy

and Hunt, 1982) and VIP/PHI-containing neurones represent particularly sensitive groups of cells.

Data obtained in the present study showed that the local application of capsaicin to the sciatic nerve, induces similar effects on peptide immunoreactivities in the dorsal horn to that of nerve section. At high doses of capsaicin (49 mM), immunostaining for substance P showed a decrease by day 9 and a full recovery after 60 days (reported here; Gamse et al., 1982). This closely approximates with the time course followed by substance P after nerve section (Barbut et al., 1981; Jessell et al., 1979; Wall et al., 1981).

Substance P has been proposed as the mediator of the neurogenic inflammatory response (Lembeck and Gamse, 1982). Its reduction in skin and sensory neurones after systemic (Gamse et al., 1980) or local administration of capsaicin (reported here; Gamse et al., 1982) would support such a rôle, since neurogenic oedema is known to be impaired in these animals (Fitzgerald, 1983; Gamse et al., 1980). Substance P is also thought to be involved in the processing of nociceptive heat stimuli (Henry, 1976; Randic and Miletic, 1977). It is tempting to suggest that the decreased response to noxious heat seen in the capsaicin treated animals, is directly related to loss of substance P from the terminals of sensory neurones in the skin and spinal cord. However, reduced sensitivity to heat is reported to occur as early as 1-2 days after local application of capsaicin to the sciatic nerve, well before any peptide change is seen (Fitzgerald and Woolf, 1982).

In contrast, in adult rats, treated systemically with capsaicin, no changes in responses to noxious heat were reported, although substance P levels were reduced in the spinal cord and skin (Hayes and Tyers, 1980). It could be argued however, that, the method of measurement for detection of heat thresholds is more sensitive than that used for detection of peptide or vice versa. The weight of evidence presented in this chapter would, however, argue strongly in favour of a possible rôle for peptides in the overall response.

The effect of peripheral nerve section or capsaicin on CCK immunoreactivity in the dorsal horn is problematical. A number of immunocytochemical studies have reported a loss of CCK immunoreactivity after nerve section (reported here, McGregor et al., 1984), capsaicin treatment (reported here, Gibson et al., 1982; Jancsó et al., 1981; Marley et al., 1982; Schultzberg et al., 1982) or rhizotomy (Marley et al., 1982). However, these reports have been challenged by radioimmunoassays which showed no change in CCK content (Gibson et al., 1982; Marley et al., 1982; McGregor et al., 1984; Schultzberg et al., 1982). There are several possible explanations. The low levels of $CCK_8$ detected by radioimmunoassays would suggest that the immunostaining may have included another molecular form of CCK (Dockray, 1980) or may reflect depletion of another cross-reacting peptide. Indeed, recent immunochemical studies have shown by specificity-inhibition tests that there is significant cross-reaction between CGRP and CCK in the spinal cord and dorsal root ganglia (Ju et al., 1986; Williams et al., 1987), with CGRP responsible for most

immunostaining. Indeed, in later investigations, CGRP (which was not discovered at the outset of this study) was found to mimic exactly the staining pattern and response of CCK-immunoreactive neurones.

### 5.4.2 Origin and significance of VIP/PHI increase

The increase of VIP/PHI immunoreactivity in the dorsal horn after mechanical or pharmacological manipulations of the sciatic nerve was of considerable interest. In addition, the whereabouts of the cells responsible for the increase was of importance to our understanding of the changes which occur after peripheral nerve injury. A number of possibilities could account for a structural reorganisation of sensory input. Collateral sprouting may occur at the interneuronal level (Hámori, 1983) and, although not seen in untreated rats, those treated with colchicine showed a number of VIP/PHI cell bodies in laminae I-II which could account for the increase (chapter 4, Gibson et al., 1984c). Other mechanisms point to sensory neurones as the possible source of the increase. Presynaptic contacts, previously made by sensory neurones which have died, may be occupied by collateral sprouts of surviving ganglion cells, or by restitution of axon terminals in which nerve injury is followed by a restricted effect on the terminal. As dorsal rhizotomy and neonatal capsaicin treatment both reduce VIP/PHI in the spinal cord (Gibson et al., 1984c, 1986; Jancsó et al., 1981; Yaksh et al., 1982a) it was concluded that a peripheral neuronal system was the most likely source of the VIP/PHI increases. Further experiments (see Table 5.4), and the observa-

tion that sciatic nerve section leads not only to increased VIP/PHI immunoreactivity in the dorsal horn but also to a marked increase in the number of VIP/PHI-immunoreactive sensory neurones (chapter 3), reinforced a sensory neuronal origin. At the same time, similar studies were reported (Shehab and Atkinson, 1986) and in later work conclusive evidence was provided that sensory cell bodies of axotomised nerves were responsible for the increased VIP/PHI immunoreactivity seen after sciatic nerve section (Shehab et al., 1986).

The functional significance of the increased VIP/PHI immunoreactivity is unknown. In the periphery, VIP may regulate blood flow and vessel permeability and may be of relevance in perineural permeability following nerve section (Williams, 1982). An alternative suggestion is that VIP may stimulate glycogenolysis (Magistretti et al., 1981) in the injured nerves thus ensuring an energy source to the cell body for regeneration of the peripheral axon. Whatever the functional rôle, a number of fundamental questions remain unanswered. Firstly, did the cells showing increased VIP/PHI immunoreactivity previously produce substance P which subsequently decreased? Secondly, why is there no increase of VIP/PHI after pelvic nerve section (chapter 4.5.1)? Since the region of the increase of VIP/PHI immunoreactivity and decrease of substance P coincided almost exactly in the dorsal horn, then the answer to the first question is probably affirmative with increased production of VIP/PHI via an aberrant metabolic pathway. The absence of increased VIP/PHI immunoreactivity after pelvic nerve axotomy implies the neurochemical response of visceral and somatosensory afferents to injury differ.

In conclusion, the data presented provide evidence that peripheral nerve lesions induce a complex series of changes in the dorsal horn. Further studies on structural, biochemical and functional responses of primary sensory neurones to peripheral nerve injury are needed, to understand the basic aspects of lesion-induced changes, and why, in man, sensory function frequently does not recover after nerve lesions.

# CHAPTER 6 DISTRIBUTION OF PEPTIDES, AMINES AND STRUCTURAL PROTEINS IN DEVELOPING SPINAL CORD, DORSAL ROOT GANGLIA AND SKIN


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# CHAPTER 6

## 6.1 INTRODUCTION

The spinal cord was chosen as a favourable organ for the study of development of the nervous system by Bidder and Kupffer as early as 1857. Since then much has been learned about the events and mechanisms of spinal cord development (see Altman and Bayer, 1984; Jacobson, 1978; Purves and Lichtman, 1983 for reviews). The spinal cord is formed subsequent to the appearance of a rudimentary brain. An identation appears in the neural tube which divides into alar and basal plates (Fig.6.1). These give rise to dorsal and ventral horns respectively. There is a rostro-caudal progression of maturation in the development of the neural tube, as well as a ventral-to-dorsal cell proliferation (Purves and Lichtman, 1983). Motoneurones are generated first, followed by interneurones and finally, the small cells of the dorsal horn (Bicknell and Beal, 1984). This supports the suggestion by many investigators (including Okado et al., 1979; Sims and Vaughn, 1979; Vaughn and Grieshaber, 1973; Windle, 1944) that neurones in the spinal cord mature in a retrograde sequence with respect to impulse conduction in the reflex pathway. As a result, the motor system develops before the sensory system.

Physiological development follows a similar rostro-caudal and ventral-to-dorsal gradient. Classical studies of behavioural embryology (see Windle, 1944; Windle and Baxter, 1936; Hamburger, 1968) show that motor, reflex responses occur early in prenatal

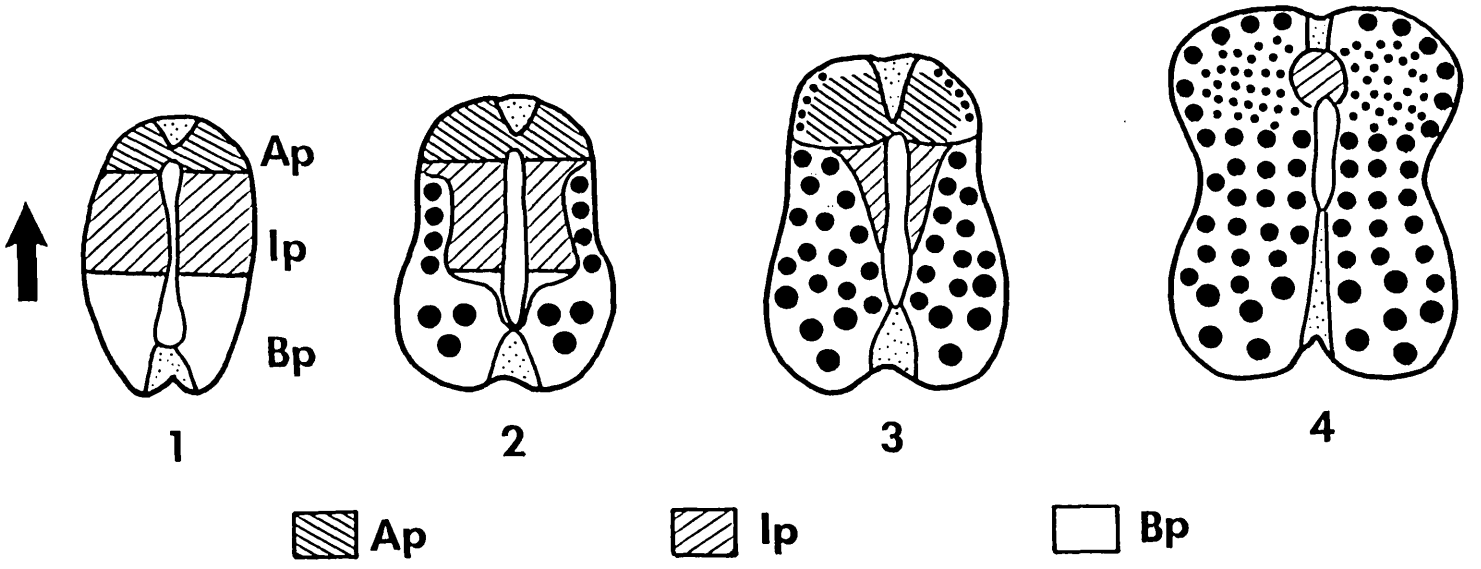



Fig.6.1 Hypothesis of four stages in the production of neurones of the spinal cord. Although, the neural tube comprises five epithelial zones, only three components of the lateral plates produce neurones. The basal plate (Bp) is the source of motoneurones, the intermediate plate (Ip) the relay neurones of the intermediate grey matter and the alar plate (Ap) the small cells of the dorsal horn (from Altman and Bayer, 1984). Arrows points dorsally.

life with spontaneous motility and reflex-elicited movements first emerging in the head and fore-limbs. The appearance of these spinal reflexes is consistent with the early connections made by dorsal root ganglion cells to both the periphery and spinal cord (Fitzgerald and Swett, 1983; Smith, 1983) and with motor axon growth into the muscle (Smith and Hollyday, 1983). However, maturation of central connections in the spinal cord is slow (Ekholm, 1967; Stelzner, 1971) and in rat C fibre responses are not fully developed until the second postnatal week (Fitzgerald, 1985a). In addition, descending, brainstem projections to the spinal cord, which exert strong inhibitory influences on spinal cord neurones, are present from early stages of development in the rat but are not functionally mature until the second postnatal week (Fitzgerald and Koltzenburg, 1986; Gilbert and Stelzner, 1979; Leong et al., 1984). At present, it is not known whether the delay in physiological maturation of the dorsal horn is due to incomplete functional connections (i.e. synaptogenesis) or to insufficient synthesis and/or release of neurochemicals during embryonic life.

Lately, reports have emerged, documenting the ontogenesis of various substances in mammalian brain and, less extensively, in spinal cord and dorsal root ganglia (Table 6.1). Many of these reports emphasise the possibility that these substances regulate developmental processes. Recent evidence suggests that specific peptides and neurotransmitters have trophic rôles in the developing nervous system (Brenner et al., 1983; Burnstock, 1982; Fontaine et al., 1986; Molliver, 1982; New and Mudge, 1986).

TABLE 6.1 Key:

>., immunoreactivity found in the earliest ages studied. Numerical superscripts refer to the references cited below. IMC, immunocytochemistry; RIA, radioimmunoassay; IC, histofluorescence. 1 - Allen et al., 1984a (RIA); 2 - Antanitus et al., 1976 (IMC); 3 Ayer-Le Lievre and Seiger, 1984 (IMC); 4 - Ayer-Le Lievre et al., 1985 (IMC); 5 - Beinfeld et al., 1983 (RIA); 6 - Bignami and Dahl, 1974 (IMC); 7 - Bullon et al., 1984 (IMC); 8 - Bugnon et al., 1978 (IMC); 9 - Cavaghn and Parnavales, 1986 (IMC); 10 - Charnay et al, 1983 (IMC); 11 - Charnay et al., 1984 (IMC); 12 - Charnay et al., 1985 (IMC); 13 - Chayvialle et al., 1980 (RIA/IMC); 14 - Choi, 1981 (IMC); 15 - Daikoku et al., 1986 (IMC); 16 - Emson et al., 1979 (RIA); 17 - Fitzgerald and Gibson, 1984 (IMC); 18 - Foster et al., 1985 (IMC); 19 - Fuji et al., 1985 (IMC); 20 - Inagaki et al., 1982 (IMC); 21 - Khachaturian et al., 1985 (IMC); 22 - Launder and Bloom, 1974 (IC); 23 - Maletti et al., 1980 (RIA); 24 - Mehra et al., 1985 (IMC); 25 - McGregor et al., 1982; 26 - Nemeskéri et al., 1985 (RIA); 27 - Nobou et al., 1985 (RIA); 28 - Palmer et al., 1982 (IMC); 29 - Paulin et al., 1986 (RIA); 30 - Pickel et al., 1980a (IMC); 31 - Pickel et al., 1980b (IMC); 32 - Pickel et al., 1982 (IMC); 33 - Raju et al., 1981 (IMC); 34 - Senba et al., 1982 (IMC); 35 - Senba et al., 1985 (IMC); 36 - Specht et al., 1981 (IMC); 37 - Woodhams et al., 1985 (IMC). * not reported, E = embryonic day rat, P = postnatal day rat, W = foetal week human.

Table 6.1 TEMPORAL APPEARANCE OF PEPTIDES AND AMINES IN RAT AND HUMAN BRAIN, DORSAL ROOT GANGLIA AND SKIN

| Antigen | Brain | | Spinal cord | | Dorsal root ganglia | | Skin |
|---|---|---|---|---|---|---|---|
| | rat | man | rat | man | rat | man | rat |
| Neurofilament triplet proteins | E10-12[4,33] | * | E11[4] E12-13[33] | * | E11-12[4] | * | * |
| GFAP | >P1[6,7] | W14[2] | * | W7.5[14] | * | * | * |
| CGRP | * | * | * | * | * | * | * |
| GAL | * | * | * | * | * | * | * |
| Substance P | >E14[20,25] E15[32] E19[35] | >W8[29] W17[30] | E15-18[32] E16[34] | W8[29] W12[10] W15[24] | E17[3,34] E18[32] | >W12[10] | >P1[17] |
| SOM | E14[25] E15[9] E19[27,35] | W12[8] W16[13] | E15[34] | * | E15[34] | * | * |
| ENK | E12-15[21] E15[28] E18[32] E19[35] | >W17[30] | E15[28] E18[32,34] | W10[11] | P1[34] | * | * |
| NPY | E13[37] E14[1] E19[35] | * | E13[37] | * | * | * | * |
| CPON | * | * | * | * | * | * | * |
| VIP | E17[23] P0[16] P1[25,27] P1-10[5] | W16[12] | E17-18[19] P0[16] | W16[12] | P1[19] | W16[12] | * |
| TRH | >E12[26] | * | * | * | * | * | * |
| TH | E12,5[36] E13-16[15,18] E19[35] | W12[31] | E16[18] | * | * | * | * |
| 5-HT | E11-15[22] E19[35] | * | * | >W15[24] | * | * | * |

The present study was undertaken in order to describe the anatomical maturation of neurones and supporting cells as shown by the expression of neurofilament triplet proteins (Gershon et al., 1983) and GFAP respectively. In addition, the phenotypic expression of eight peptides (CGRP, GAL, NPY/CPON, SOM, substance P, TRH, VIP) and two aminergic markers (TH and 5-HT) in the spinal cord and dorsal root ganglia was studied during foetal and postnatal development in regions associated with motor, sensory and autonomic functions. Skin from rat foot-pads was also studied since cutaneous (sensory) nerves are representative of peripheral terminations of lumbar dorsal root ganglion cells whose central branches show a highly ordered pattern of termination in the lumbar dorsal horn (Fitzgerald and Swett, 1983; Molander and Grant, 1985; Woolf and Swett, 1984). Thus a simultaneous comparison of the neurochemical ontogeny of central and peripheral sensory and postganglionic autonomic nerves could be monitored (Björklund et al., 1986; Lundberg et al., 1979).

## 6.2 EXPERIMENTAL APPROACH

**Tissues and processing:** Embryos of specific gestation were obtained from time-mated rats (Wistar). 10-20 embryos were studied at each day from embryonic day (E)12through to E21. Birth usually occurred at E22 which was noted as postnatal day P1. 6-8 animals were killed at P1, P2, P4, P7, P14, P21 and P36 (adult). Human foetuses (152) from 6 to 26 weeks of gestation were obtained from legal terminations of pregnancy and a further 13 foetuses or neonates at autopsy between the 30th foetal and 6th postnatal week

(appendix IX). Rat embryos from E17 or earlier were too small to dissect successfully and therefore were completely immersed in fixative whilst from E17 until birth, the vertebral column and hind feet could be dissected. Neonatal rats were fixed by transcardiac perfusion with fixative after appropriate anaesthesia. The fixatives employed were, either Bouin's fluid, 0.4% benzoquinone or 4% paraformaldehyde solutions. Human specimens were fixed by immersion in Bouin's, p-benzoquinone solutions (appendix II, III), or formalin containing 7% saturated mercuric chloride.

In rat it was possible to divide the spinal cord into cervical, thoracic, lumbar and sacral levels after E16. In human foetuses, spinal cord levels were identified where possible. Dorsal root ganglia were taken from representative levels of the rat spinal cord and all human dorsal root ganglia found were processed. Bouin's- and mercuric formalin-fixed tissues were embedded in paraffin wax whilst frozen tissues were prepared from paraformaldehyde- and p-benzoquinone fixed tissues. Representative tissue sections from various levels of the spinal cord were stained with haematoxylin and eosin to monitor tissue preservation.

## 6.3 RESULTS

### 6.3.1 Spinal cord

#### Anatomical and cellular differentiation

By E13 the rat spinal cord was already divisible into an outer

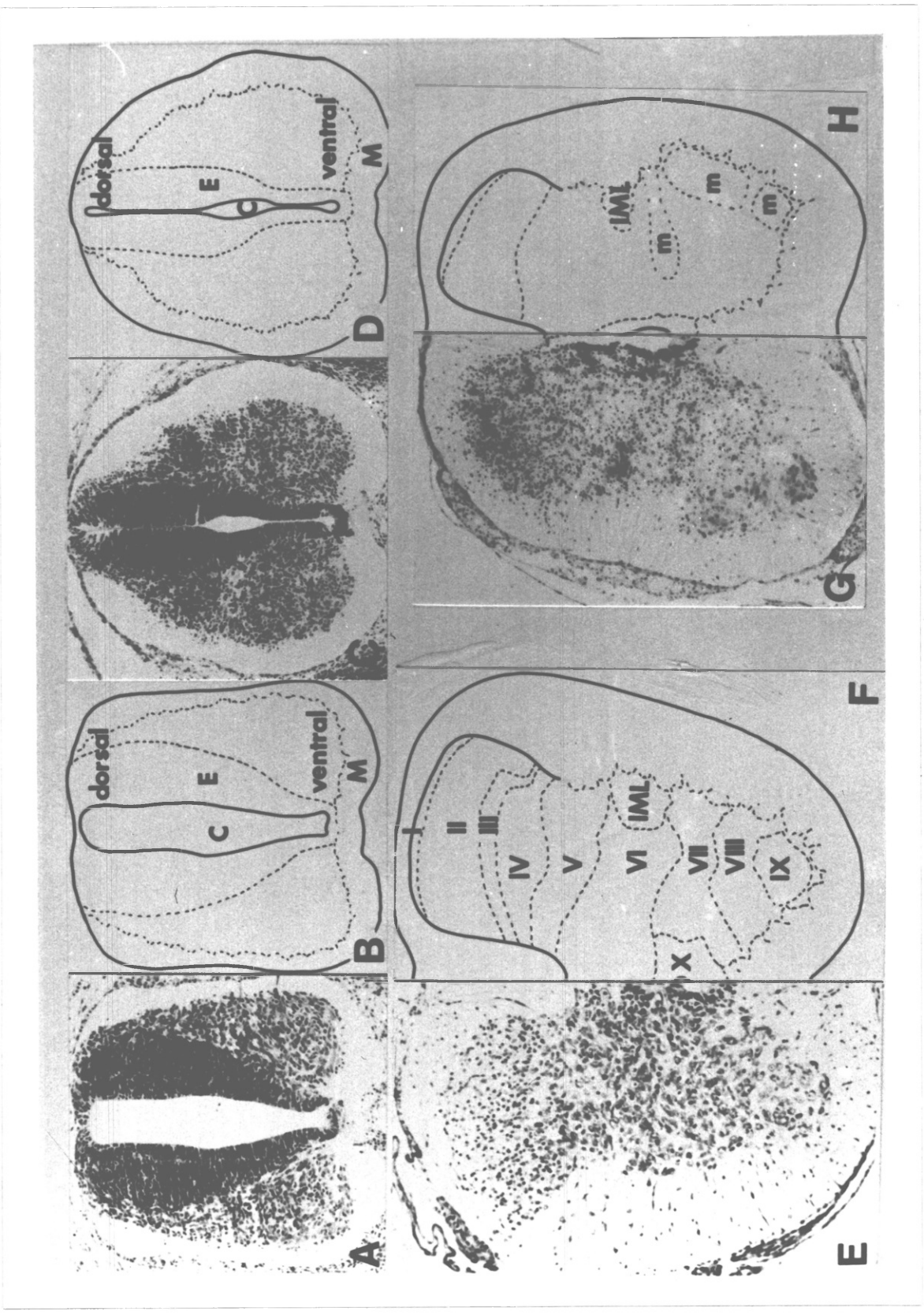


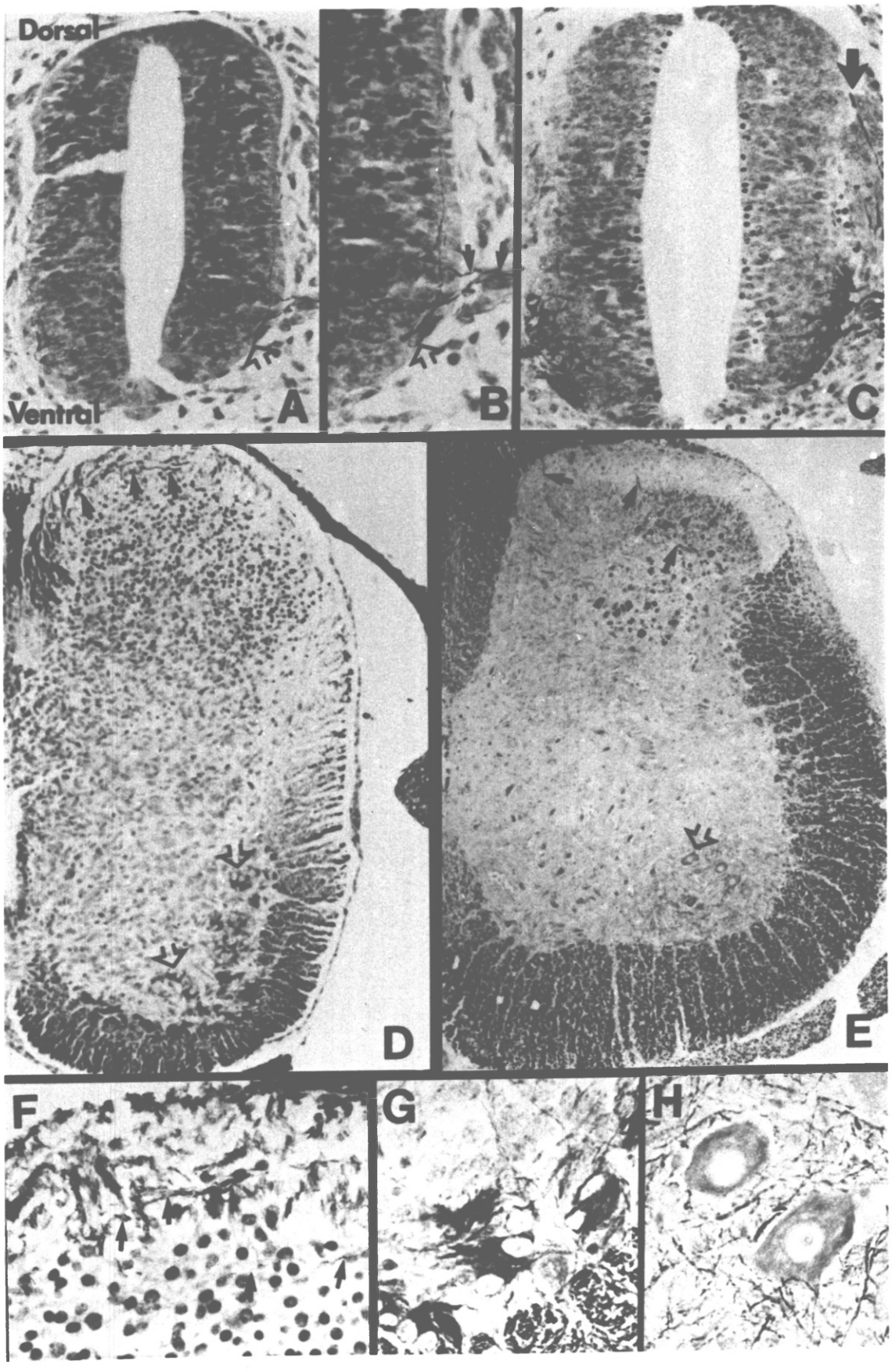

**Fig.6.2** Histological appearance and diagrammatic counterpart of rat and human spinal cord. (A,B) E15 rat cervical spinal cord. (C,D) 6 week human foetal lumbar spinal cord. (E,F) E21 rat mid-thoracic spinal cord. (G,H) 8 week human foetal sacral spinal cord. C, central canal; E, ependymal layer; M, marginal layer; IML, intermediolateral cell column; m, motor nuclei. A,C, E, G Haematoxylin and eosin stained sections. A x 69; C, E x 123; G x 80.

marginal layer, the prospective white matter, and the mantle layer the presumptive grey matter. By E15 its appearance was similar to that of the youngest human foetus studied (6 weeks). Motor nuclei were easily identified and there was a clear distinction between white and grey matter. Also a few small cells were present in the dorsal horns. A laminar arrangement of cells in the grey matter was starting to become evident after E17 in rat and the 8th week of gestation in man (Fig.6.2).

Neuronal differentiation, monitored by the expression of neurofilament triplet proteins immunoreactivity, progressed in a rostro-caudal and ventral-to-dorsal manner. Immunoreactivity for these proteins was first seen at E13 in the rat, in cell bodies located ventrolaterally in the cervical spinal cord (Fig.6.3A,B). One day later immunoreactive motoneurones were present throughout the length of the spinal cord and immunoreactive fibres were seen for the first time in the cervical dorsal horn (Fig.6.3C). By E17 the distribution of neurofilament triplet proteins immunoreactivity was similar to that seen in the adult rat (Fig.6.3 D). In the youngest human foetuses, immunoreactivity was already present throughout the spinal cord with a similar pattern to the rat at E15-17 (Fig. 6.3 D,F,G). In both species, immunoreactivity increased relatively more in the white than in the grey matter until P1 in rat and the 8th-10th foetal week in man. The density of immunostaining at these stages was similar to that observed in adults (Fig.6.3 E,H, 6.4 A-C).

In rat, GFAP-immunoreactive fibres were first noted in the

**Fig.6.3** Neurofilament triplet proteins immunoreactivity in the rat spinal cord. (A,B) E13 cervical spinal cord. Ventrolaterally located immunoreactive cells and fibres (open arrows) are present, with processes passing into the ventral root (blocked arrows). C) E14 cervical spinal cord. Some immunoreactive fibres (arrow) are present in the dorsal roots. D) E17. Some fibres were present in the dorsal horn (solid arrows) shown at higher magnification F) and the motoneurones (open arrows) are clearly defined G). E) P21. Many large calibre fibres are immunoreactive (solid arrows). The white matter is strongly immunoreactive. The motoneurones (open arrow) and H) are less intensely stained than in the foetus. A, C,D x 140; B,F,G x 220; E x 55; H x 300.


Dorsal
Ventral
A
B
C
D
E
F
G
H

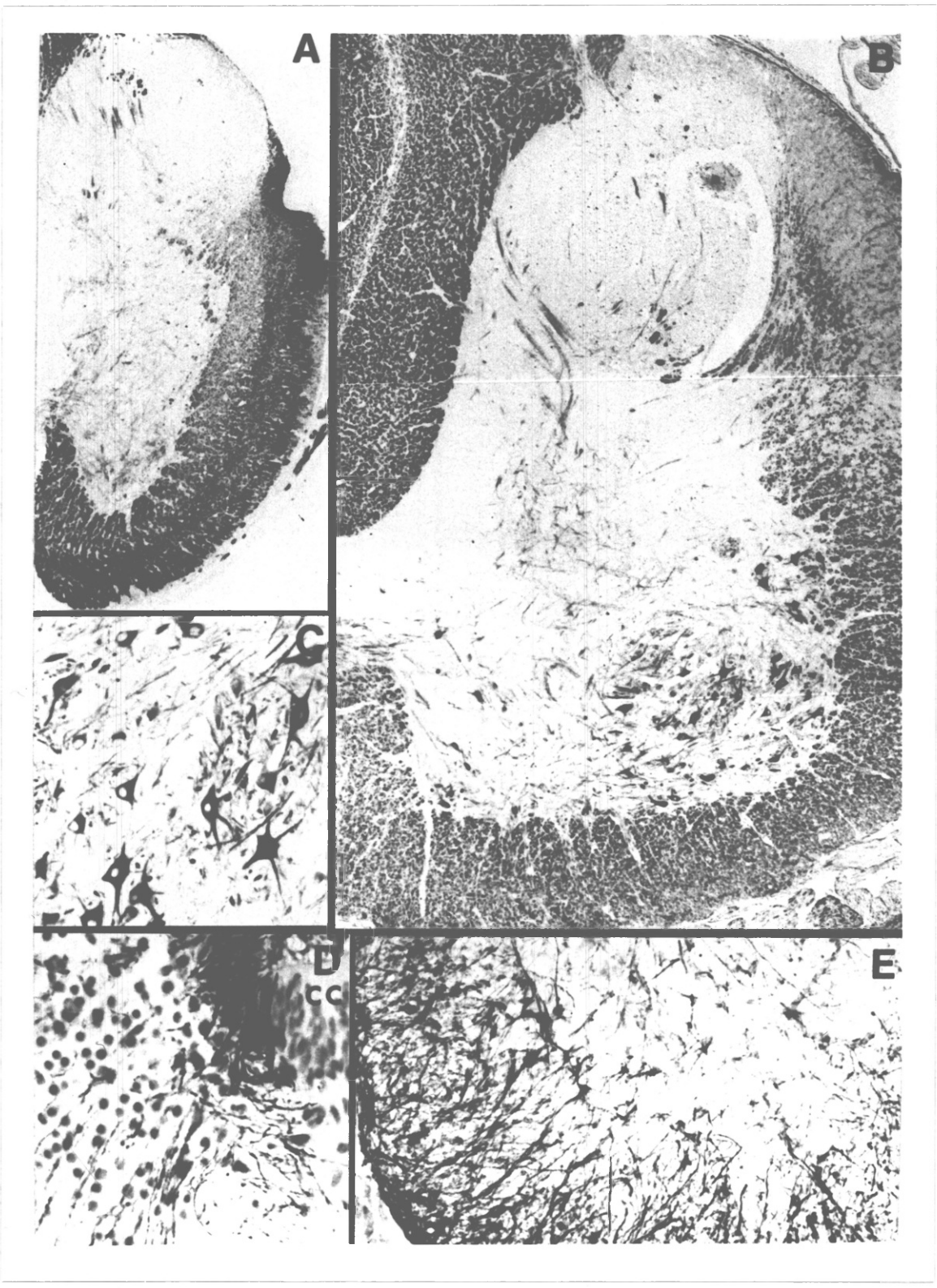



Fig.6.4 (A-C) Neurofilament triplet proteins immunoreactivity in man. A) Thoracic spinal cord 12th foetal week. B) Lumbar spinal cord 26th foetal week. C) High power photomicrograph of motoneurones shown in B). (D,E) GFAP immunoreactivity in a 10 week human foetus. D) Fibres around the ependymal layer of the central canal (cc). E) Immunoreactive fibres and astrocytes in the ventral grey matter. A x 17; B x 70; C x 210; D x 380; E x 245.

TABLE 6.2 TEMPORAL APPEARANCE OF NEUROFILAMENT TRIPLET PROTEINS, PEPTIDES AND AMINES IN DIFFERENT FUNCTIONAL REGIONS OF SPINAL CORD

| | MOTOR | | SENSORY | | | | | AUTONOMIC | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | VENTRAL GREY | | DORSAL GREY | | DRG | | EPIDERMIS | IML | | DERMIS |
| Antigen | C | F | C | F | C | F | F | C | F | F |
| NEUROFILAMENT TRIPLET PROTEINS | E13 [>6W] | E13 [>6W] | * [*] | E14 [>6W] | E14 [>6W] | E14 [>6W] | E16 | E20 [ 35W] | E17-20 [ 35 W] | E16 |
| CGRP | E15 [6W] | E21 [6W] | * [*] | E17 [10W] | E16 [14W] | E16 [24W] | E18 | * [*] | E21 [24W] | E18 |
| GAL | E15 [6W] | E21 [6W] | P1 [*] | E17 [20W] | E16 [24W] | E16 [14W] | * | * [*] | E21 [24W] | * |
| SOM | E15 [8W] | E15 [10W] | E15 [*] | E16 [10W] | * [10W] | E17 [10W] | P1 | * [20W] | E21 [20W] | * |
| SUBSTANCE P | * [*] | E18 [6W] | E17 [*] | E18 [11W] | E21 [24W] | E17 [24W] | P1 | * [*] | E20 [24W] | * |
| NPY | * [*] | E17 [10W] | E17 [*] | E21 [10W] | * [*] | E18 [*] | * | E18 [*] | E21 [24W] | P1 |
| CPON | E17 [*] | E15 [8W] | E15 [8W] | E17 [8W] | * [*] | E17 [*] | * | * [*] | E21 [20W] | P1 |
| ENK | * [*] | E21 [10W] | * [*] | P1 [14W] | * [14W] | * [*] | * | * [*] | P1 [20W] | * |
| TRH | * [*] | E21 [*] | P10 [*] | P7 [*] | * [*] | * [*] | * | * [*] | E20 [*] | * |
| TH | * [*] | E20 [*] | * [*] | E20 [*] | * [*] | * [*] | * | * [*] | P1 [*] | P1 |
| VIP | * [*] | P1 [20W] | * [*] | P1 [20W] | * [*] | P1 [*] | P1 | * [*] | P1 [20W] | P1 |
| 5HT | * [*] | E19 [*] | * [*] | E19 [*] | * [*] | * [*] | * | * [*] | E20 [*] | * |

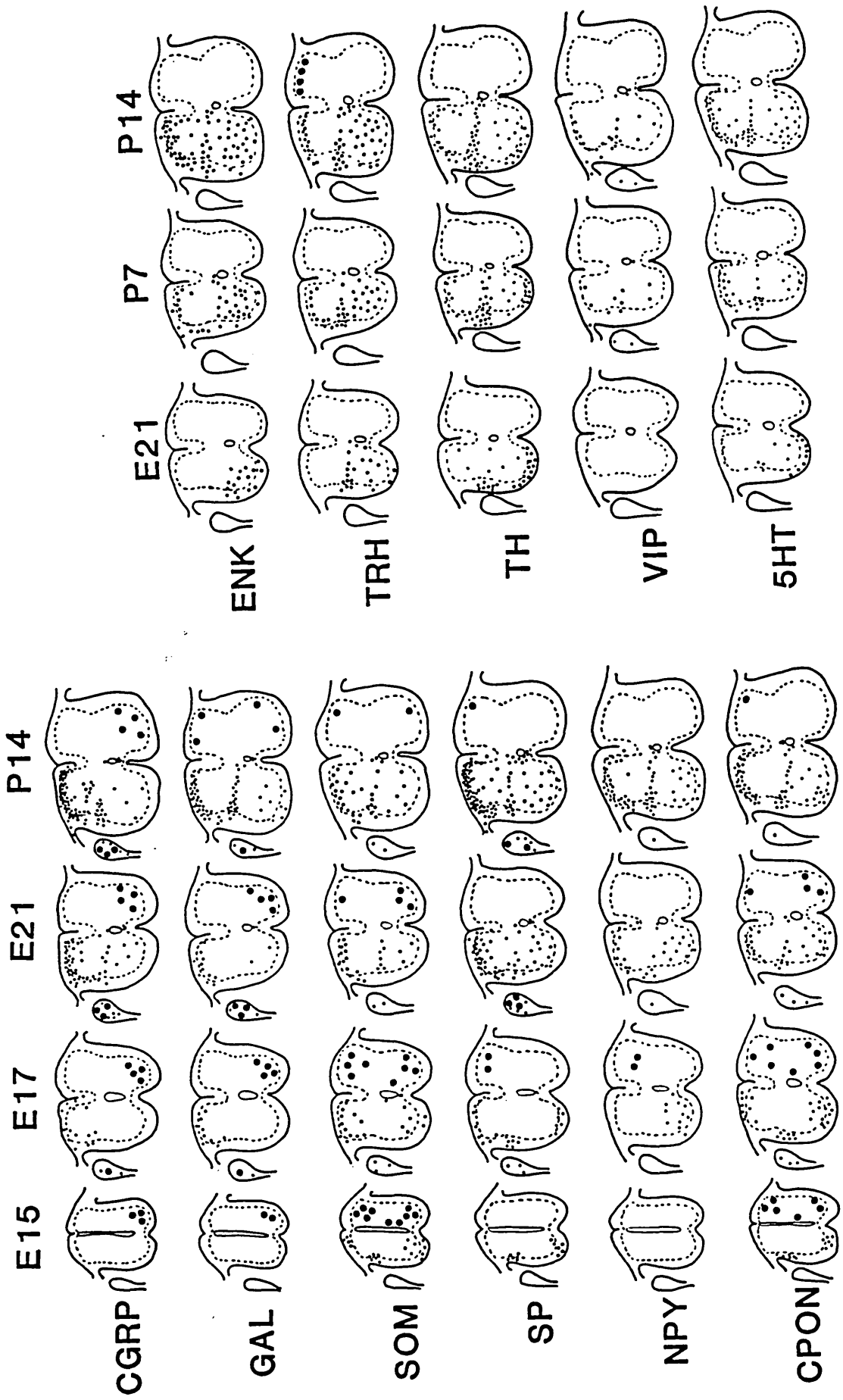



**Fig.6.5** Diagrammatic representation of the distribution of peptide- and amine-immunoreactive structures at specific stages of development in the rat spinal cord. Distribution of immunoreactive fibres is shown on the left side (small dots) and cell bodies (large dots) on the right side of the spinal cord.

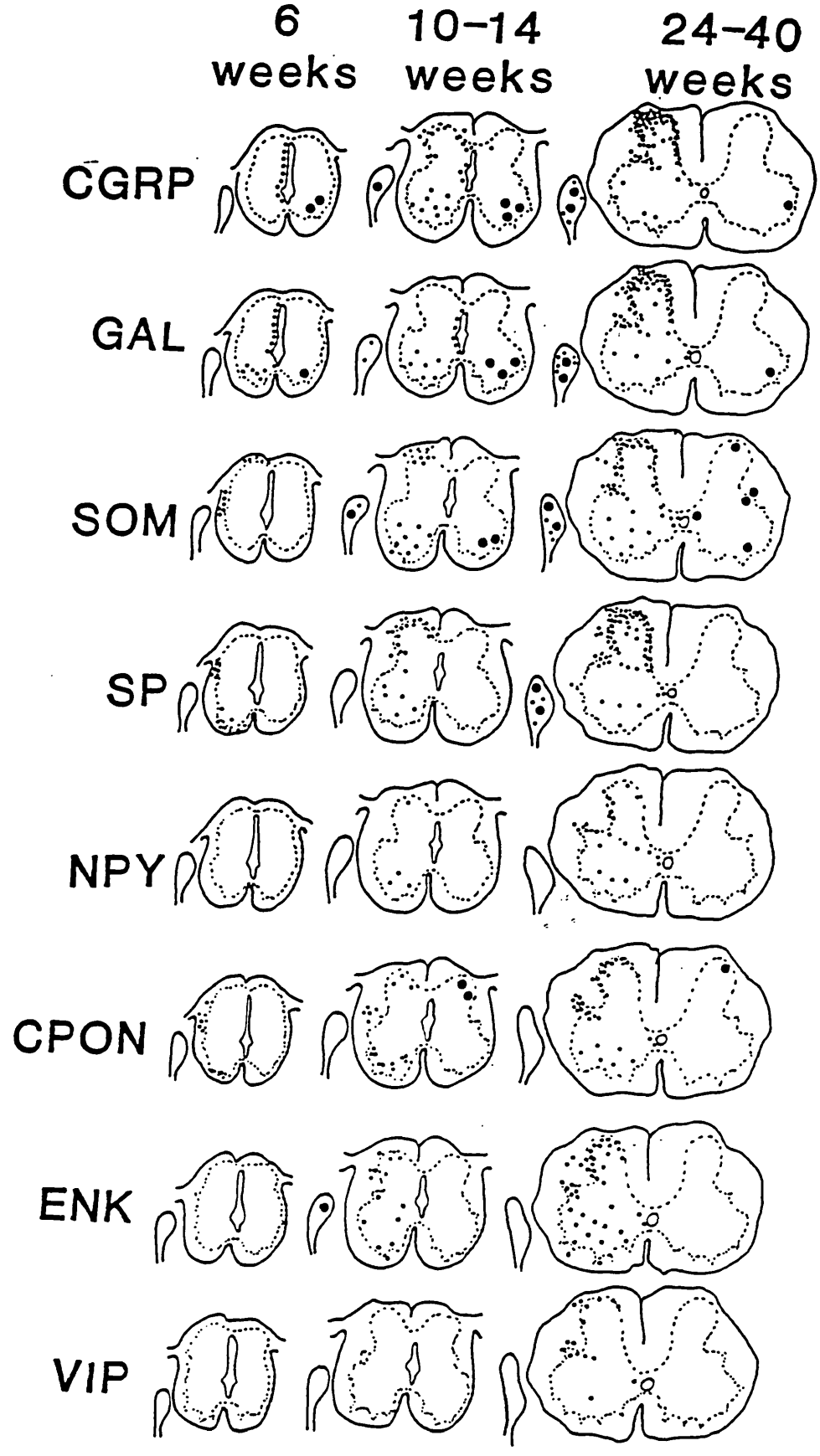



Fig.6.6 Diagrammatic representation of the distribution of peptide-immunoreactive structures in man during early (6-14 weeks) foetal life and in late foetal and early neonatal (24-40 weeks) life (birth occuring at 36 weeks). Immunoreactive fibres (small dots) shown on left side and cell bodies (large dots) shown on the right side of the spinal cord.

commissures at E16 and by E17, immunoreactive fibres were also seen in dorsal and lateral funiculi. From the 10th foetal week in man, radial glia were observed in the ependymal layer. However, these decreased after the appearance of GFAP-immunoreactive astrocytes (Fig.6.4 D,E). GFAP-immunoreactive astrocytes increased with age in rats until the second postnatal week when there was a proliferation of astrocytes. After this they appeared to become fewer until the third postnatal week when no further changes were observed.

## Neurochemical expression

Data are summarised in Table 6.2 and Figs.6.5-6.6 and a brief description of events is given below. Since neurochemical expression, with the exception of VIP, did not follow a rostrocaudal progression, the appearance of peptides[1] and amines at various ages, is described irrespective of segmental level.

**Ventral grey matter:** CGRP, GAL and SOM immunoreactivities were expressed in motoneurones during gestation (Figs. 6.7 B,C, 6.8 A,C, 6.9 A,E) and were first seen in the presumptive motoneurone pools at E15 (rat) and the 6th foetal week (man). A few CPON-immunoreactive motoneurones were present at E15 in rat but were not detected after E17 (Fig.6.10 A,C,D). Motoneurones, displaying CGRP, GAL and SOM immunoreactivities, increased in number until P2 in rat and the 14th-20th foetal week man. With the exception of CGRP, motoneurones immunoreactive for GAL and SOM became progressively less easy to visualise after P10 in rat (Figs.6.7 D,

[1]As in the rest of this thesis, the term NPY/CPON has been used except where obvious differences were noted using antisera to the two antigens.

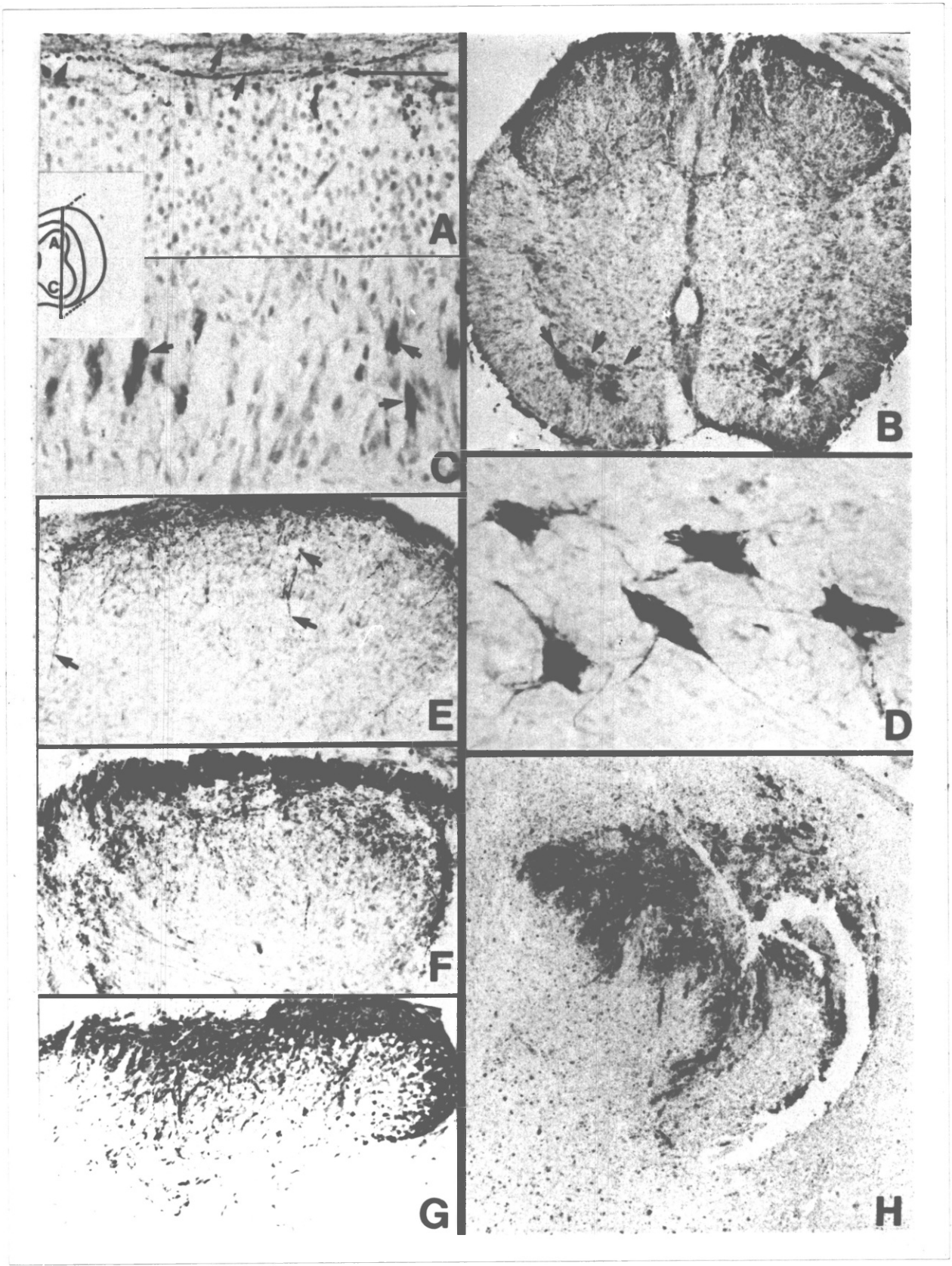



Fig.6.7 CGRP immunoreactivity in rat (A-G) and human H) spinal cord. (A,C) Parasagittal sections through the lumbosacral spinal cord at E17. A) Some fibres (arrows) are present in the dorsal horn, which can be seen more clearly in transverse section E). C) Immunoreactive motoneurones (some arrowed). B) By E21 the dorsal horn is more densely innervated with CGRP-immunoreactive fibres (see also F), and the motoneurones (some arrowed) remain strongly immunoreactive. D) Immunoreactive motoneurones at P14. G) Lumbar dorsal horn at P25. H) Sacral dorsal horn 25th foetal week. A, C, E x 225; B x 75; D x 300; F x 175; G x 108; H x 90.

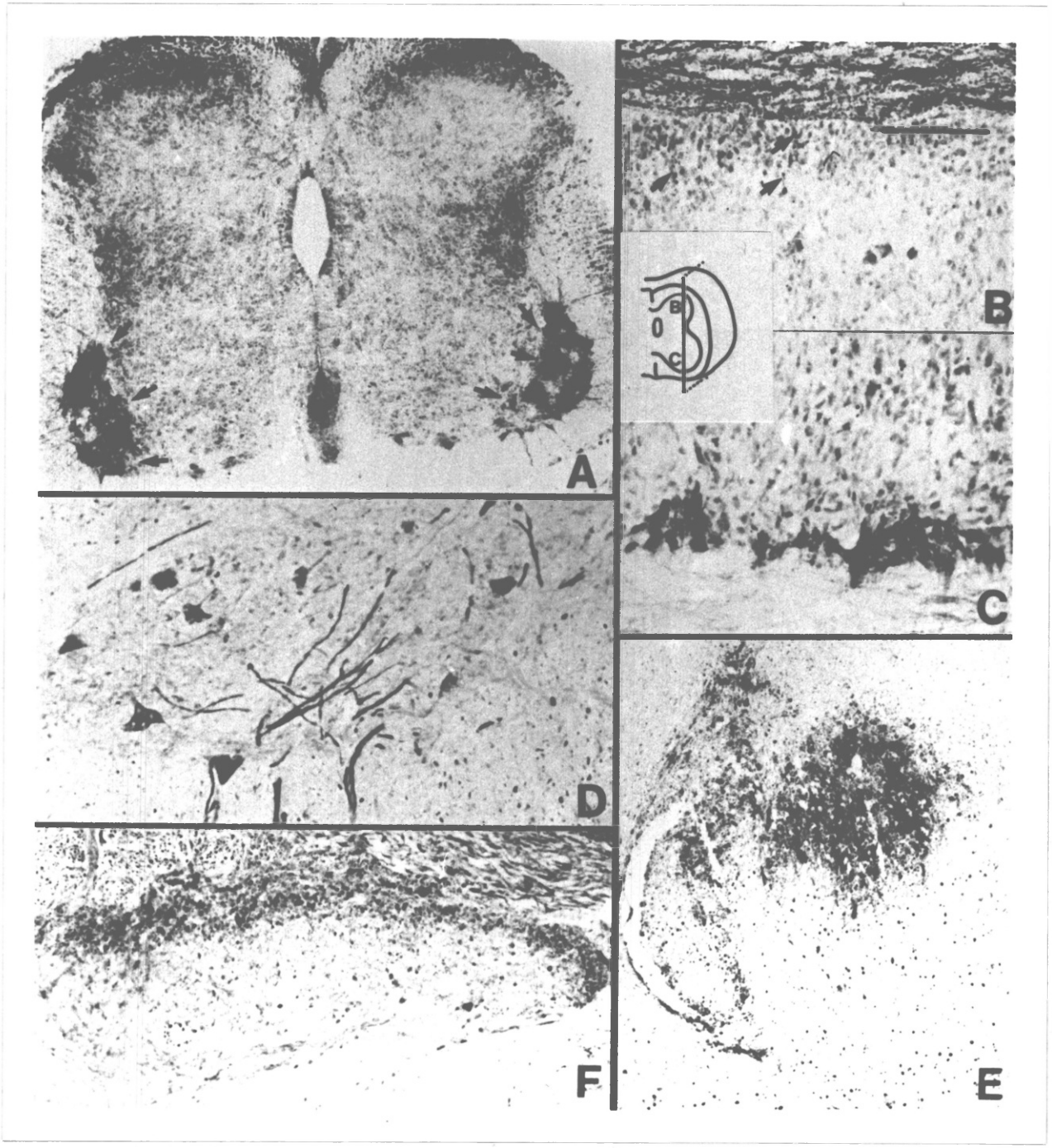
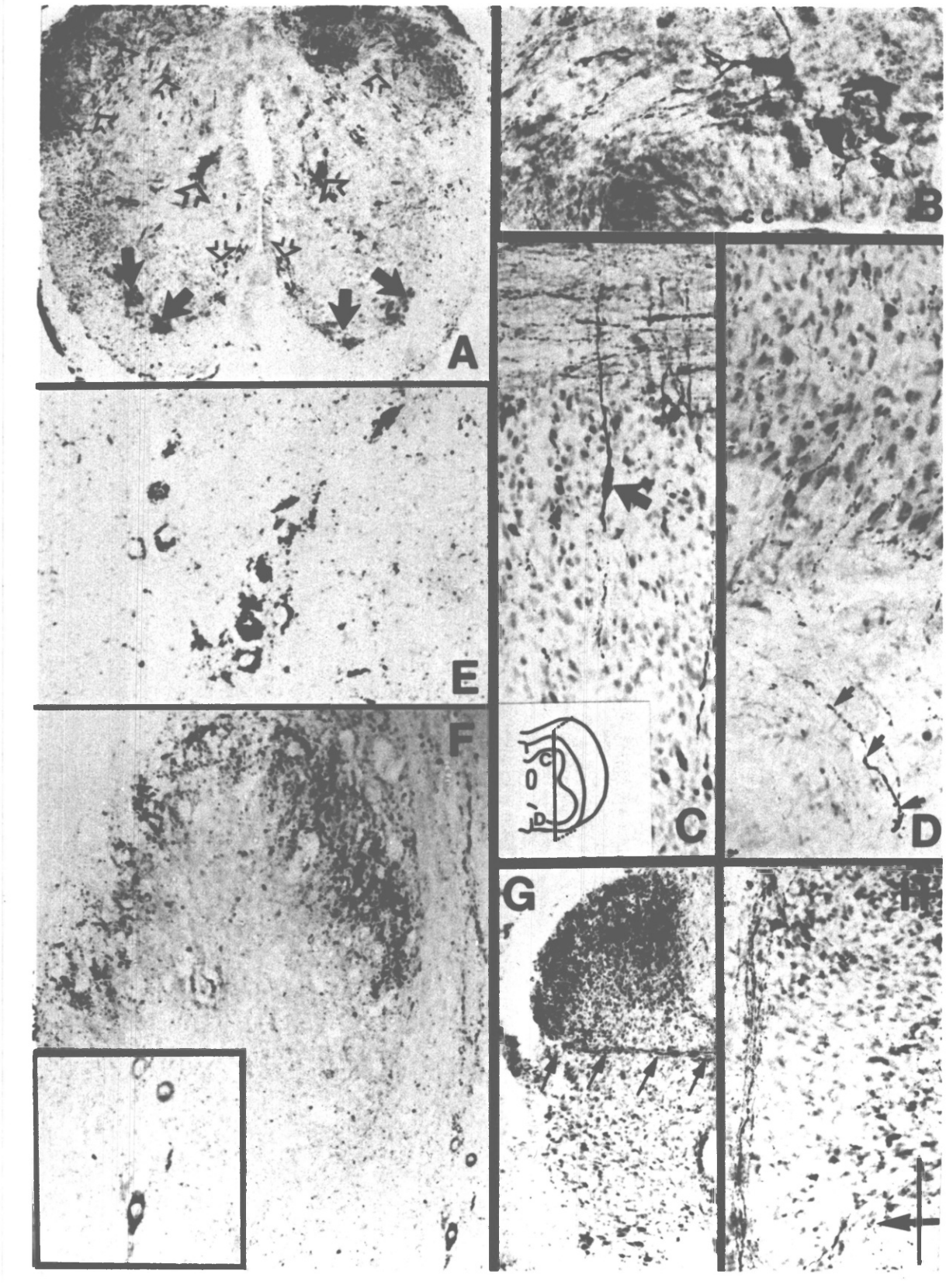



**Fig.6.8** GAL immunoreactivity in rat (A-D,F) and man E). A) Mid-lumbar spinal cord at E16, motoneurone pools are strongly immunoreactive (arrows). (B,C) E17, parasagittal sections through the spinal cord. B) Most fibres run in a rostro-caudal direction (long arrow) in the dorsal horn, a few enter the underlying grey matter (small arrows). C) A strongly immunoreactive motor column. D) Immunoreactive motoneurones and processes in the ventral horn of the cervical spinal cord at P5. E) Immunoreactive fibres in the thoracic dorsal horn in a 25 week human foetus. F) GAL immunoreactivity in the rat cervical dorsal horn at P25. A x 230; B,C x 225; D x 115; E x 83; F x 100.

**Fig.6.9** SOM immunoreactivity in rat (A-D), G), H) and man E), F). A) Lumbar spinal cord E16, note numerous immunoreactive cell bodies (open arrows) and strongly immunoreactive motor nuclei (blocked arrows). B) Immunoreactive cell bodies around the central canal (cc) at E15. (C,D) Parasagittal sections through the lumbar spinal cord at E17. C) An immunoreactive cell body (arrow) is present in the substantia gelatinosa and D) numerous fibres are present in the ventral funiculus (arrows) as well as in the grey matter. E) Immunoreactive motoneurones in the ventral horn of the thoracic spinal cord of a 20 week human foetus. F) Immunoreactive fibres in the thoracic dorsal horn and cell bodies in the intermediolateral cell column (see also insert) in a 20 week human foetus. G) Thoracic spinal cord E21. Note tract of immunoreactive fibres (arrows) connecting the two sides of the spinal cord via the dorsal grey commissure. H) High power view of fibre tract shown in G). Long arrow points to the central canal, large thick arrow points dorsally. A x 155; B x 325; C, D x 245; E,F x 90; G x 65; H x 145.


A
B
C
D
E
F
G
H

6.8 D). In man, motoneurones immunoreactive for all three peptides were difficult to find after the 14th foetal week. CGRP-, GAL-, NPY/CPON-, SOM- and substance P-immunoreactive fibres were among the first to be identified in the ventral horn between E15-17 (rat) and 6th-10th foetal week (man) (Figs.6.9 D, 6.10 E, 6.11C). Most of these were associated with motoneurones expressing immunoreactivity for the respective peptides. The loss of peptide-immunoreactive motoneurones, however, was not accompanied by a loss of immunoreactive fibres. On the contrary, CGRP- and GAL-immunoreactive fibres were not seen in rat until E21. The remaining substances studied, were localised to fibres later (perinatally) and even postnatally in the case of VIP in rat. The density of fibres, in particular those immunoreactive for ENK, NPY/CPON, SOM, substance P, TRH, TH and 5-HT, increased until birth in man and the second postnatal week in rat. Subsequently their distributions were similar to that of the adult (Figs. 6.5-6.6) (Table 6.2) (chapter 4).

**Autonomic nuclei:** SOM-immunoreactive cells were the only immunoreactive neurones found in the autonomic nuclei (Fig.6.9 F). These were apparent from the 20th foetal week in man, in thoracic intermediolateral and intermediomedial cell columns. SOM-immunoreactive cells in similar locations persisted into neonatal life in man.

In both species fibres, immunoreactive for all antigens studied, were present in thoracic and sacral intermediolateral cell columns (except TRH, TH and 5-HT in man). Immunoreactive fibres appeared

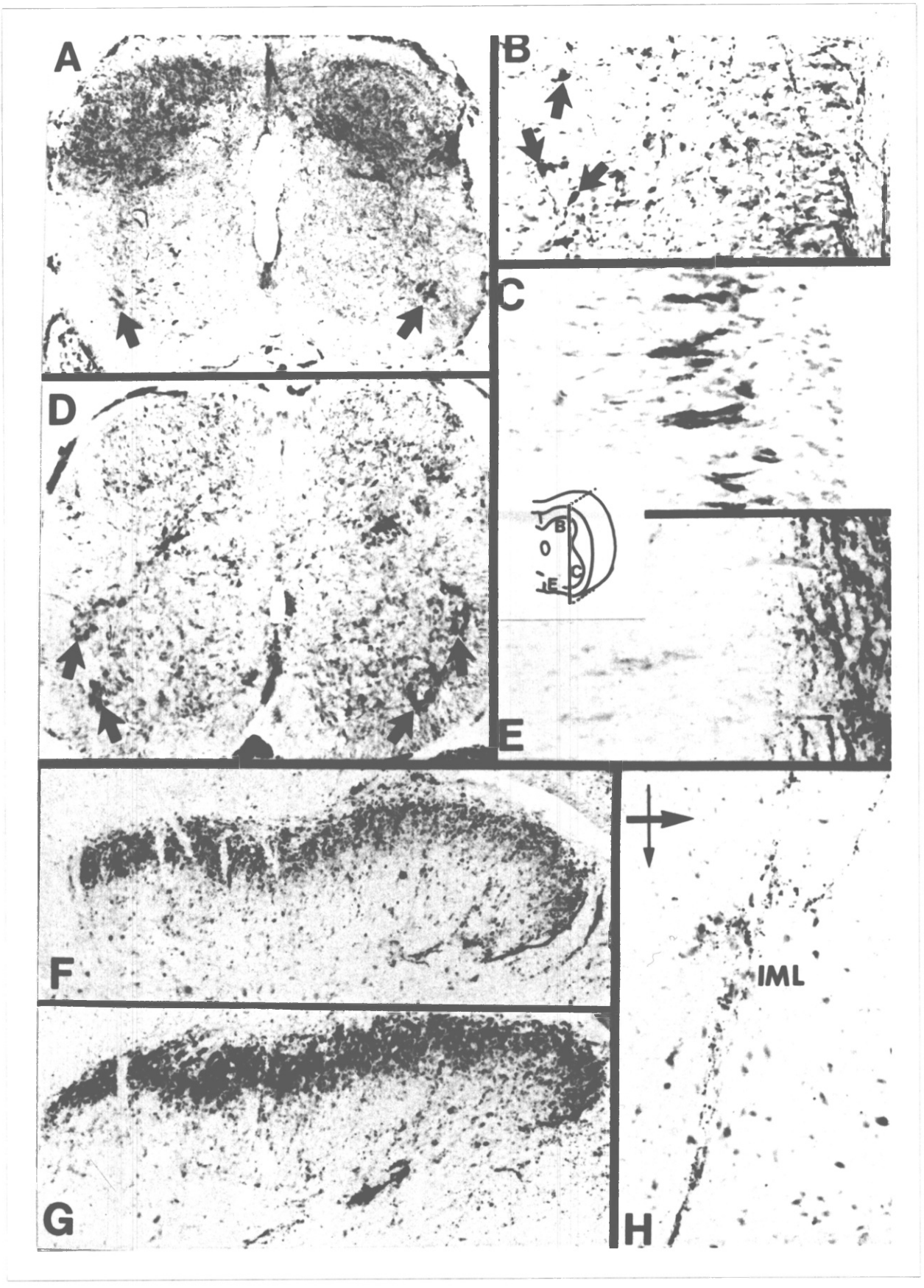



**Fig.6.10** Rat spinal cord immunostained with antisera to CPON (A-E,G) and NPY (F,H). A) E16 and D) E21. Many immunoreactive cells particularly in the ventral horn (some arrowed) are present. (B,C,E) Parasagittal sections through the lumbar spinal cord at E17. B) Some immunoreactive cells (some arrowed) are present in the dorsal grey matter. C) Immunoreactive motoneurones and E) fibres in the ventral funiculus. F) NPY- and G) CPON-immunoreactive fibres in the lumbar dorsal horn P14. H) NPY-immunoreactive fibres in the thoracic intermediolateral cell column at P14. Long arrows points to the central canal, large thick arrow to the dorsal horn. A x 140; B,C,E x 225; D x 110; F G x 100; H x 125.

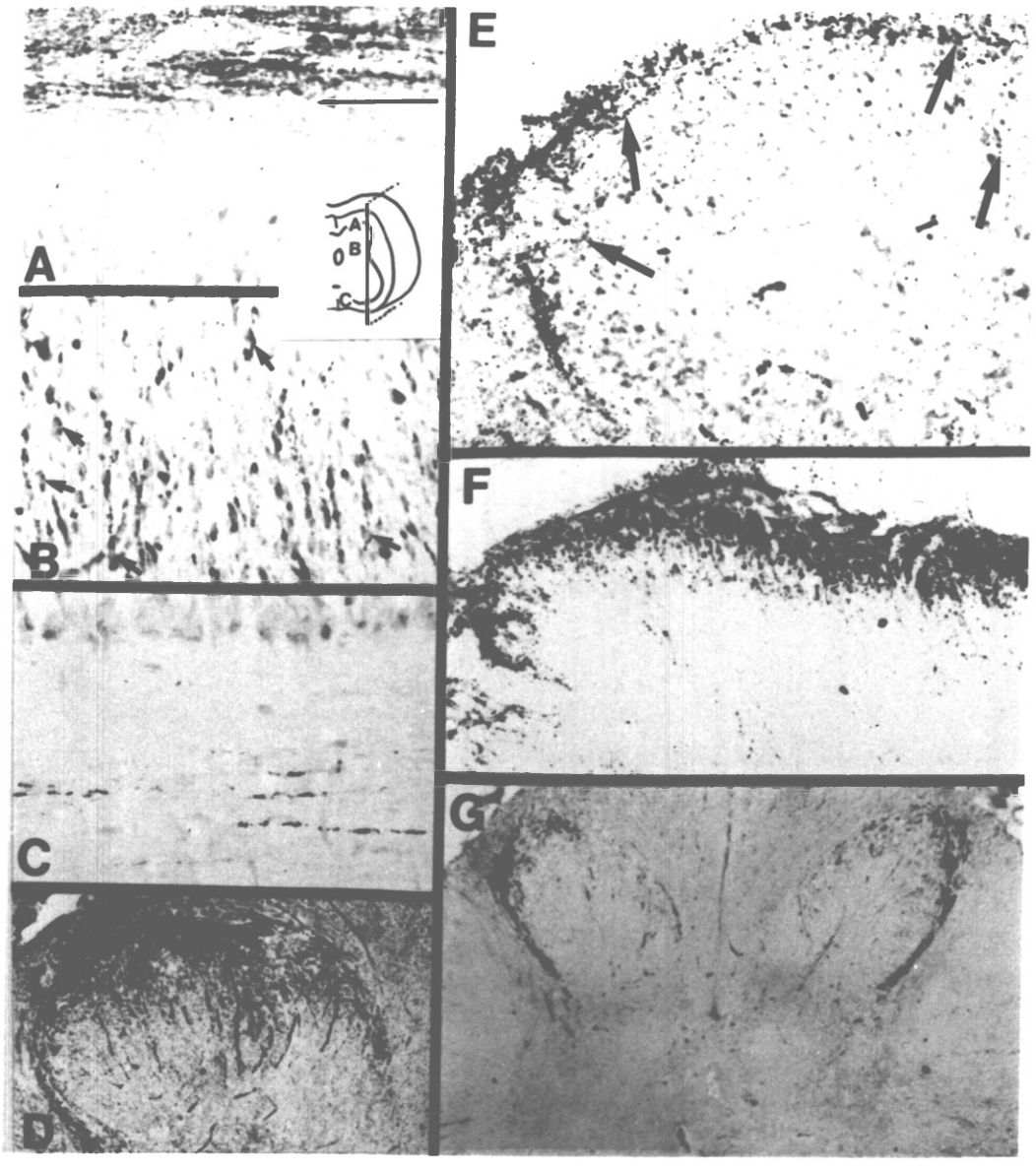



**Fig.6.11** Substance P immunoreactivity in rat (A-C, E, F) and man (D, G). (A-C) Parasagittal sections through the lumbar spinal cord at E17. A) Fibres are present in the white matter, with a few in the underlying grey matter (arrow indicates rostro-caudal axis). B) A few immunoreactive cells (some arrowed) are present in the dorsal horn. C) Fibres in the ventral funiculus. E) Immunoreactive fibres in the cervical dorsal horn at E21. F) Immunoreactive fibres in the lumbar dorsal horn at P25. Photomicrographs of D) cervical and G) thoracic spinal cord of a 20 week human foetus showing immunoreactive fibres in the dorsal horn. A-C x 190; D x 80; E x 150; F x 90; G x 23.

relatively late during foetal life. In rat, immunoreactive fibres were first seen at E20, with most peptides expressed by E21. The remaining substances (ENK, TH and VIP) were not seen in the autonomic nuclei until P1. In man, all peptides appeared between 20-24th foetal week in the intermediolateral cell columns. In both species immunoreactive fibres, in particular those immunoreactive for ENK, GAL, NPY/CPON, SOM and in rat TRH, TH and 5-HT, increased markedly around birth. Their distributions were similar to those seen in the adult spinal cord (Figs.6.9 G,H, 6.10 H, 6.13 D,E).

**Dorsal grey matter:** There was a striking appearance of numerous SOM-immunoreactive cells at E15 in the rat. These cells were scattered throughout the grey matter but were mainly found around the central canal with a few present in the 'presumptive' laminae I-II (Fig.6.9 A-C). Although less abundant, CPON-immunoreactive cells were seen in similar locations and were also present in laminae I-II in man (foetal weeks 10-14) (Fig. 6.10 A,B). Some SOM- and CPON-immunoreactive cells became restricted to the upper dorsal horn in rat by E17, the remaining cells in the intermediate grey matter became less apparent. Also at E17, a few substance P- and NPY-immunoreactive cells appeared in laminae I-II, but were rarely seen after E18 (Fig.6.11B). As parturition approached it became increasingly more difficult to detect immunoreactive cells. GAL- and TRH-immunoreactive cells appeared postnatally in the rat. The former were noted at P2 in the dorsal horn and could be found at all postnatal ages studied. TRH-immunoreactive cells and fibres were expressed transiently in laminae I-II from P5-10 (Fig.6.12 A,B).

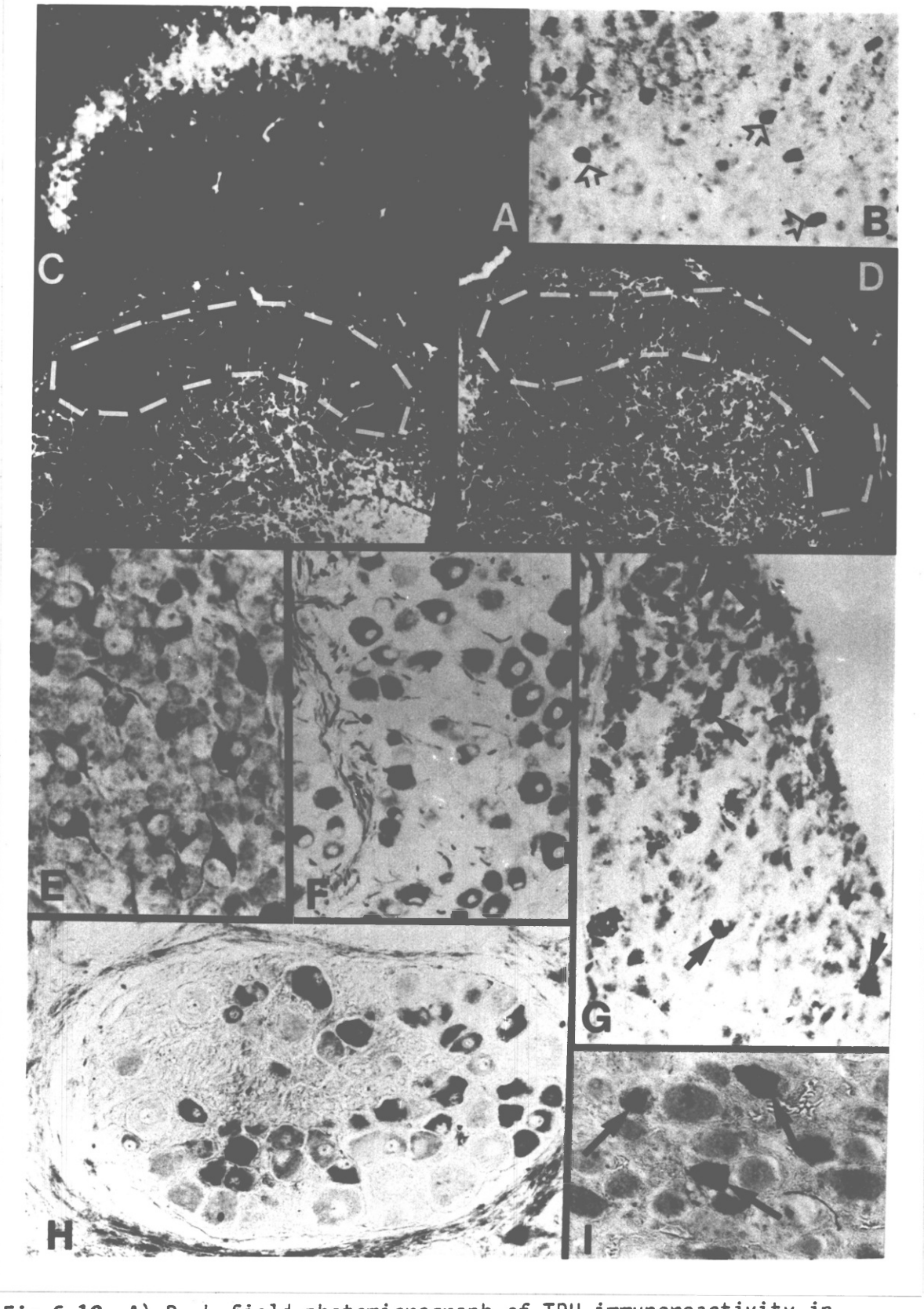



**Fig.6.12** A) Dark field photomicrograph of TRH immunoreactivity in the rat dorsal horn at P10. B) TRH-immunoreactive fibres and cell bodies (some arrowed) in the rat dorsal horn at P10. Dark field photomicrographs of 5-HT-immunoreactive fibres in the dorsal horn at P1 C) and P7 D). Laminae I-II is indicated by the broken lines. Neurofilament triplet proteins-immunoreactive cells and fibres in cervical E) and lumbar F) dorsal root ganglia of man at 6 and 20 weeks of gestation respectively. G) GAL-immunoreactive cells (some arrowed) in rat lumbar dorsal root ganglion at E18. H) GAL-immunoreactive cells in a lumbar dorsal root ganglion from a 30 week human foetus. I) SOM-immunoreactive cells (arrowed) in a lumbar dorsal root ganglion from a 32 week human foetus. A x 85; B x 275; C,D x 100; E x 430; F x 350; G x 310; H x 230; I x 300.

From early foetal ages (E16-18 rat, 8-20th foetal week man) a few CGRP-, GAL-, NPY/CPON-, SOM-, substance P-, (and in man ENK) immunoreactive fibres were present (Figs.6.7 A,E, 6.8B, 6.9 A,C, 6.10A,B, 6.11 A). The loss of many immunoreactive cells from the dorsal horn apparently coincided with greater numbers of fibres immunoreactive for the same antigens being seen as well as with fibres displaying immunoreactivities which were not localised to cell bodies (Fig.6.7 B,E,F,H, 6.8 E, 6.9 F,G, 6.11 D,E,G). Increased fibre densities were most apparent perinatally when TRH-, TH-, VIP- and 5-HT-immunoreactivities were also seen for the first time (Fig.6.12 A-D). The number of immunoreactive fibres gradually increased in rat until postnatal day 10 when an adult pattern was established (Fig.6.7G, 6.8 F, 6.10 F,G, 6.11 F, 6.12 D).

### 6.3.3 Dorsal root ganglia

#### Anatomical and cellular differentiation

Small groups of cells were observed at each side of the spinal cord in the youngest rat embryos. The size of these cells increased until E17, when large and small cells could be distinguished. Ganglia containing large and small cells were also apparent by the 6th foetal week in man.

Cells and fibres were immunoreactive for neurofilament triplet proteins from E14 in rat and immunoreactive cells were more numerous at cervical than at sacral levels. By E15, no segmental

variation was noted. The number of immunoreactive cells increased with age until E21, when nearly all were immunostained. In the youngest human specimens (6th foetal week), numerous immunoreactive cells and fibres were already present at all levels (Fig.6.12 E,F). As in the rat, the majority of cells in the ganglia were immunoreactive throughout the gestation period studied

### Neurochemical expression

The results are summarised in Table 6.2. Cells immunoreactive for CGRP, GAL and substance P and fibres immunoreactive for SOM were first seen between E16-18 in rat (Fig.6.12 G). The number of peptide-immunoreactive cells and fibres increased until birth. Although CGRP-immunoreactive cells appeared to remain constant through to adult life, those displaying GAL and substance P decreased after birth and by P7-10 only a few immunoreactive cells were seen. CGRP-, GAL-, SOM- and substance P-immunoreactive cells were present in man from foetal week 26-33 but became progressively more difficult to detect (Fig.6.12 H,I).

## 6.3.4 Rat skin

### Anatomical and cellular differentiation

At E13-14 the skin is comprised of a single layer of cells, the epidermis, and a fibrous dermis. The epidermis became progressively more stratified and resembled that of adult by E18.

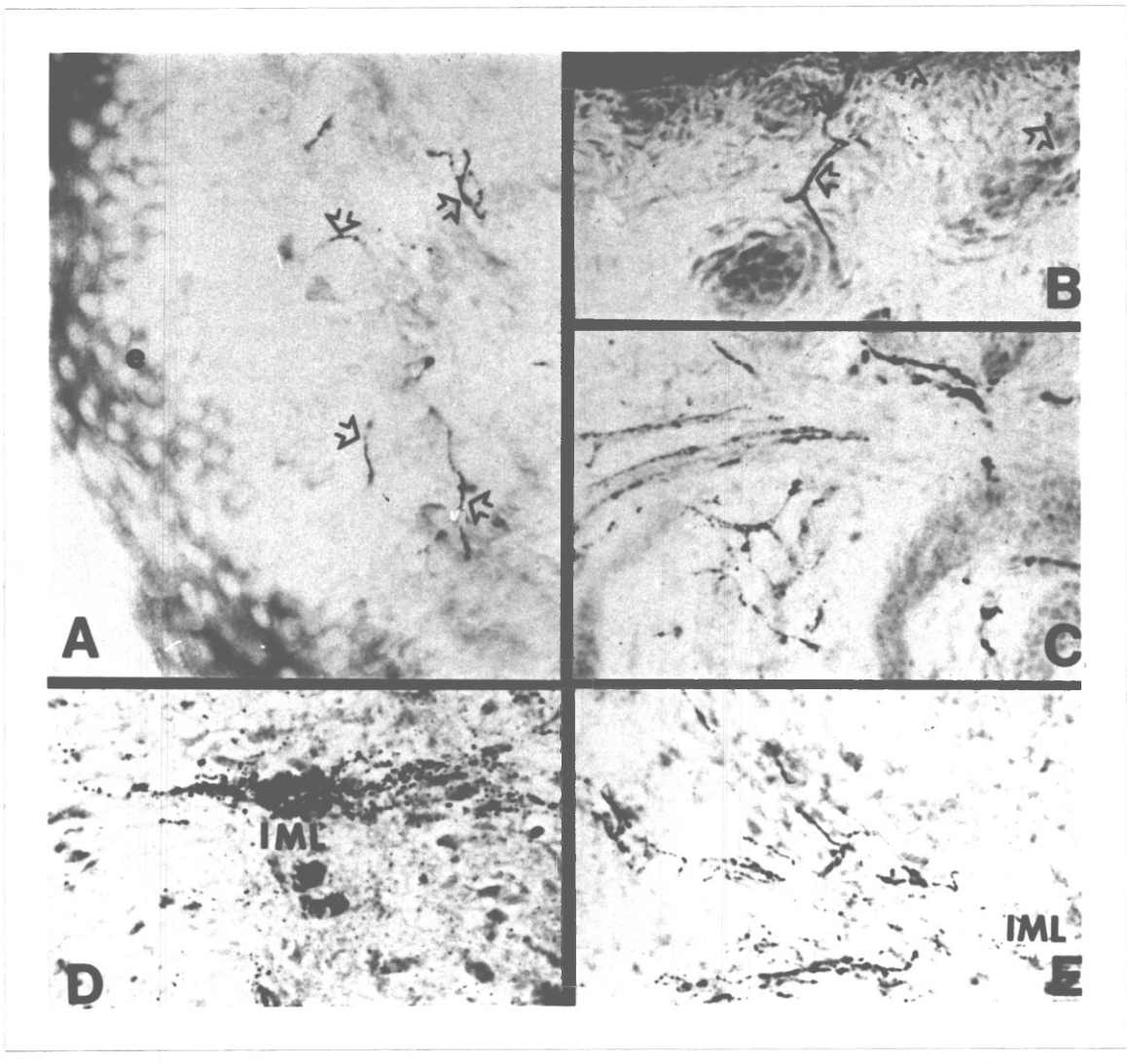



Fig.6.13 (A-C) Immunoreactive fibres (some arrowed) in rat hind foot skin. A) Substance P at P1. B) CGRP at E21. C) TH at P7. D) TRH-immunoreactive fibres in the rat thoracic intermediolateral cell column at P7. E) VIP-immunoreactive fibres in the sacral intermediolateral cell column of a 20 week human foetus. e., epidermis. A x 330; B,C x 225; D x 240; E x 250.

Fibres expressing neurofilament triplet proteins immunoreactivity were detected at E16. These were located in the dermis as thick, nerve bundles, around sweat glands and blood vessels with a few fine nerve fibres penetrating the epidermis. Although immunoreactive nerves were rarely numerous in the epidermis they were widely distributed in the dermis after birth.

### Neurochemical expression

CGRP immunoreactivity was the only substance found in fibres innervating the glaborous skin of the foot during foetal life in rat (E18). NPY/CPON-, substance P-, SOM-, VIP-, and TH-immunoreactive fibres were apparent from P1-2 onwards (Table 6.2) in both glaborous and hairy skin. Fibres immunoreactive for CGRP, substance P and SOM were located mainly near the epidermis (Fig.6.13 A,B). Those fibres immunoreactive for VIP, NPY/CPON and TH were found mostly around blood vessels and sweat glands and increased rapidly during the first postnatal week (Fig.6.13 C).

## 6.4 DISCUSSION

### 6.4.1 Transient expression of peptides and amines during development

In the present study, particularly in the spinal cord, many peptide-immunoreactive cells were present in either greater numbers or in locations where they are few or even entirely absent in the mature animal. This transient expression was most apparent

during early foetal life. Since all cells are potentially able to synthesise the complete enzymatic machinery for all transmitter substances, it may be postulated that external factors, possibly trophic, released by other neurones, will trigger the appropriate genetic mechanism for phenotypic expression (Cohen, 1972; Le Douarin et al., 1975; Norr, 1973). It is possible that the expression of peptide during foetal life may, in fact, be a prerequisite for growth and differentiation, whereas, in the adult, the phenotype may have a different rôle, such as neurotransmission or modulation. Since neuronal phenotypic expression may be plastic it remains to be determined whether transient synthesis of peptides is of functional significance.

The present study adds to the number of reports of transient expression or over-expression of classical transmitter- and peptide-containing cells during development of the central nervous system (Senba et al., 1985; Palmer et al., 1982, Woodhams et al., 1985 for brain) (Fuji et al., 1985; Senba et al., 1982 for spinal cord). Many cells immunoreactive for SOM, NPY/CPON and substance P were present in the dorsal horn and the intermediate grey matter of young foetal rats. In addition, motoneurones displaying CGRP, GAL, NPY/CPON and SOM immunoreactivities were seen in rat and man. It was revealed that all of these immunoreactive cells apparently decreased in numbers towards birth when there was a concomitant increase of immunoreactive fibres, notably in the dorsal horn. The decrease of immunoreactive cells may be attributed in part to natural cell death (Kelly and Cowan, 1972; Oppenheim, 1986) a view supported by a substantial decline in the

number of motoneurones in the ventral horn during the foetal period (Bennett et al., 1986; Oppenheim, 1986; but see Nurcombe et al., 1981; Tada et al., 1979). In the dorsal root ganglia, however, there is no evidence of postnatal ganglion cell death (Hulsebosch et al., 1986) but here also, greater numbers of cells immunoreactive for CGRP, GAL, SOM and substance P were seen perinatally in rat and man and all, with the exception of CGRP, became less abundant with increase in age. It is therefore tempting to speculate that the decreased detection of peptide-immunoreactive cell bodies along with increased detection of immunoreactive fibres reflects an initiation of centrifugal axonal transport of chemicals synthesised in the cell body prior to neural connectivity and acquisition of functional maturation. In support of this speculation, it has been shown that in conditions, (i.e. cultures or grafts) where peptide-immunoreactive neurones are effectively removed from their natural environment and from afferent stimuli, there is a marked increase in perikaryal peptide content (Fontaine-Perus et al., 1981; Jessen et al., 1980) and, as demonstrated for substance P-immunoreactive neurones, a corresponding increase of messenger RNA (Black et al., 1984).

Although the majority of changes in phenotypic expression were seen during foetal life, changes also occurred during the early postnatal period in rat. The brief appearance of TRH-immunoreactive cells in the dorsal horn during the first and second postnatal week was of particular interest. TRH-immunoreactive cells resembled islet cells, the predominant cell type in lamina II and one of the last cell types to mature in the dorsal horn

(Bicknell and Beal, 1984). It is possible that TRH production is linked to maturation of islet cells which occurs during the time TRH-immunoreactive cells were apparent. Many studies of peptides/amines in the nervous system have documented a dramatic increase during the first month of postnatal life. In most cases, these increases exceed adult levels in rats, rabbits and guinea pigs as assessed by radioimmunoassay (Christofides et al., 1983; Gingras-Leatherman et al., 1985; Kessler and Black, 1981; McGregor et al., 1982; Monnet-Tschudi et al., 1986; Nemeskéri et al., 1985; Nobu et al., 1985) and immunocytochemistry (Aramount et al., 1986; Cajal-Agüeras et al., 1986; Dhall et al., 1986; Jaeger and Joh, 1983). While the number of immunoreactive structures (namely nerve fibres) in the rat spinal cord, dorsal root ganglia and skin increased markedly perinatally no further changes were seen after the second postnatal week.

### 6.4.2 Neurochemical expression in motor, autonomic and sensory regions

As mentioned in the introduction, (6.1) there is a temporal gradient of ventral-to-dorsal and rostro-caudal neuronal differentiation in the spinal cord. The somatic motoneurones represent the oldest cell type, having birth dates between E11-13 in rat (Nornes and Das, 1974; Smith and Hollyday, 1983). Whether autonomic neurones differentiate around this time is still a point of contention (see Altman and Bayer, 1984) but studies in chick indicate that preganglionic neurones migrate and settle later than motoneurones (Nornes and Das, 1974; Hamburger and Levi-Montalcini,

1949). The first cells, born in the dorsal horn, do not arise until the latter stages of neurogenesis by which time the cells of the dorsal root ganglia undergo their final division (embryonic day 14) (Lawson et al., 1974; Nornes and Das, 1974; Sims and Vaughn, 1979). Thus, as expected, and as seen in rat, the expression of neurofilament triplet proteins immunoreactivity (indicator of first neuronal differentiation Gershon et al., 1983) followed a rostro-caudal, ventral-to-dorsal sequence.

Although, the expression of peptides and amines did not follow a rostro-caudal progression they generally appeared in the ventral before the dorsal spinal cord. The first peptides expressed were CGRP, GAL, NPY/CPON, SOM and substance P (E15-17 rat, 6-10th foetal week man). At these early ages, all of the above peptides were localised to presumptive motoneurones. A small number of fibres, including those immunoreactive for SOM and substance P were present in the dorsolateral white matter as noted by other authors (Pickel et al., 1982; Senba et al., 1982). From their location, it is not clear whether these fibres are markers of primitive autonomic centres, or if they derive from descending bulbospinal pathways as in the adult rat (Basbaum and Fields, 1979; Hökfelt et al., 1979). Although some immunoreactive structures were observed in the dorsal horn at these stages, fibres, in particular in laminae I-II, were not numerous until later stages of gestation. It was thus, of interest that an increase of CGRP-, GAL-, SOM- and substance P-immunoreactive fibres occurred in laminae I-II around the time of the first detection of the same peptides in the dorsal root ganglia.

However, these early fibres may be intrinsic and, in fact, an even greater increase in immunoreactivity was noted in the dorsal horn perinatally in rat corresponding to the time when C fibres enter the spinal cord (Fitzgerald and Swett, 1983; Scheibel and Scheibel, 1968; Smith, 1983).

Sensory peptides in the skin appeared later than in the dorsal horn. This may be due to incomplete peripheral axonal projections since physically greater distances are involved before peripheral targets are reached. However, peptides were not present in the skin until well after the appearance of neurofilament triplet proteins-immunoreactive fibres at E16. Visualisation of neurofilament triplet proteins around this time is consistent with the studies of English et al., (1980) who described the presence of unmyelinated axons in trunk dermis and is further in keeping with the description of silver staining reported by Fitzgerald (1966). Although it is known that small unmyelinated, C fibres are difficult to demonstrate using antisera to neurofilament triplet proteins (Friede and Samorajski, 1970; Trojanowski et al., 1986), it is possible they were not yet present at E16 in the skin and the future A fibres, whose cell bodies are born before those of C fibres (Hamburger and Levi-Montalcini, 1949; Lawson et al., 1974) were immunostained. The arrival of C fibres in the skin, possibly heralded by the appearance of the sensory peptides, CGRP, SOM and substance P, may therefore represent a second wave of innervation similar to that noted in the dorsal horn. The autonomic nerves of the dermis containing NPY/CPON, TH and VIP, first appeared in rat at P1, and rapidly increased. In addition,

these same antigens did not appear in the sympathetic intermediolateral cell columns of the spinal cord until near birth. These data may indicate a late neurochemical phenotypic expression in all autonomic neurones.

### 6.4.3 Possible functional significance in the spinal cord

The earliest foetal movements are simple, non-purposeful, segmental motor responses (Angulo y Gonzalez, 1932; Ekholm, 1967; Fitzgerald and Windle, 1942; Shiota, 1983). It is notable that motoneurones were the first cells to acquire an adult-like neurochemistry. However, fibres containing 5-HT, TRH and TH (all classically, present in descending systems terminating in the ventral horn) were only seen perinatally. One suggestion therefore, is that they may influence the organisation of reflex circuits, which eventually control rhythmic coordinated, limb activity in later life (Weber and Stelzner, 1977).

In sensory systems, particularly in the dorsal horn, the number of peptide-immunoreactive fibres was not comparable to adult levels until P1 in rat and during late gestation in man. It may be speculated that sensory transmitters/modulators are only required by C fibres after birth since the embryo is not subjected to nociceptive stimuli *in utero*. It has been reported that human neonates experience little pain during the first few days after birth (Craig, 1980). In accord with this, C fibres are not functionally mature in the neonatal rat until P10-11 (Fitzgerald and Gibson, 1984; Fitzgerald, 1985a). The slow physiological

maturation of C fibres may be related to insufficient quantities of peptides in the terminals of sensory neurones or even to a lack of specific neurochemicals in afferent pathways. In support of the latter speculation, 5-HT-immunoreactive fibres, which are known to be involved in descending inhibitory control of incoming primary afferent stimuli in the dorsal horn (Fields and Basbaum, 1984; Rivot et al., 1984), proliferate substantially in rat in the first postnatal week. This is consistent with the postnatal development of this descending control (Fitzgerald and Koltzenburg, 1986).

# CHAPTER 7 DISEASES AFFECTING THE SPINAL CORD IN MAN AND EXPERIMENTAL ANIMALS


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## 7.1 GENERAL INTRODUCTION

Localisation studies of classical neurotransmitters and peptides has contributed to the understanding of how certain regions of the brain function. This in turn has led to the realisation that some neurological disorders are caused by an imbalance of these substances. Loss of dopaminergic neurones in Parkinson's disease, cholinergic cortical neurones in Alzheimer's disease and the selective loss of striatal neurones which contain NPY and SOM immunoreactivities in Hungtingdon's Chorea, are among the most well documented examples. An alternative hypothesis is that neurodegenerative conditions may result from the neurotoxic action (over production) of an endogenous compound rather than from the inability of a cell to produce specific substance(s).
The vulnerability of particular neuronal systems in different syndromes has posed considerable problems for the neuroscientist and the cause of most sensory or motor neuropathies remain unknown. In this chapter, possible peptide changes were studied in examples of motor (motor neurone disease) and sensory (mutilated foot rat) neuropathies to determine whether peptides in specific systems are selectively affected.

## 7.2 MOTOR NEURONE DISEASE (MND)[1]

### 7.2.1 INTRODUCTION

Although the name of the condition is singular, motor neurone

[1]In man it is conventional to refer to anterior and posterior spinal cord. However, for consistency the terms ventral and dorsal have been used throughout.

disease (MND), its etiology, is almost certainly heterogeneous. In nearly all cases of MND, the lower (spinal) motoneurones are affected, either alone or together with the upper motoneurones, and in particular the lower cranial nerve nuclei. (Brownell et al., 1970; Charcot and Joffray, 1869; Iwata and Hirano, 1979; Konno et al., 1986; Mannen et al., 1977; Oyanagi et al., 1983; Sung, 1982). Interestingly, however, not all motoneurones are affected, cells in the oculomotor nuclei are spared, as are the spinal neurones of Onuf's nucleus (Onuf, 1900) which supply the sphincter muscles of the bladder and anus (see chapter 4.5.3). The precise nature of the neurones in this nucleus, has been the subject of debate for a number of years. Several retrograde tracing studies in experimental animals indicate the pudendal motor nucleus, the homologue of Onuf's nucleus, is somatic in nature (Kuzuhara et al., 1980; Sato et al., 1978). This is supported by clinicopathological studies of patients with MND where Onuf's nucleus, and according vesicorectal and genitourinary function, is preserved even to the latest stages of the disease (Mannen et al., 1977; Sung, 1982). In contrast, Shy-Drager syndrome and Fabry's disease, both of which affect autonomic centres, show extensive cell loss from Onuf's nucleus correlating well with disturbed vesicorectal function (Konno et al., 1986; Mannen et al., 1977; Sung, 1982; Sung et al., 1979). Finally, a mixed somatic and autonomic neuronal population has been proposed by Yamamoto et al., (1978) from retrograde tracing experiments in animals.

To date there are relatively few studies on peptides in MND. The aims of the present study were therefore to analyse changes in peptide distribution throughout the spinal cord in order to determine whether changes are restricted to the motor region and to provide more information on the nature (somatic, autonomic or mixed) of Onuf's nucleus in man.

### 7.2.2 EXPERIMENTAL APPROACH

Tissues and fixation: Spinal cords were collected at autopsy from 52 patients having no known neurological disease and from 36 patients with clinically typical sporadic MND. Details of the tissues examined are shown in appendix X,XI. Retrospective cases obtained from hospital files (cases 1-17 controls, cases 1-27 MND see appendix X, XI) were all fixed by immersion in 10% buffered formalin for periods ranging from 1-6 years. Samples were taken from 4 or more segmental levels including rostral and caudal, cervical, thoracic and lumbar levels. Sacral segments ($S_1$-$S_3$) were obtained from four normal (cases 4,5,7,12) and 7 diseased spinal cords (cases 4,6,14,18,19,22,23). Tissues were washed in running tap water (1-2 days) to ensure removal of excess formalin and stored in phosphate-buffered saline containing sucrose (15% w/v) and sodium azide (0.01% w/v) for 1-3 months. Tissues collected at autopsy were dissected on ice. Samples from each of of the four major segmental levels were fixed as follows for 8-12 hours i) 10% buffered formalin ii) Bouin's solution iii) 0.4% p-benzoquinone (appendix II, III). Following fixation the samples were washed in buffer and stored as above.

Since there is often a variable delay before fixation of human spinal cord, the effect of fixation delay on peptide immunoreactivity was assessed on one normal spinal cord obtained 24 hours (earliest time available) post-mortem. This specimen was kept at at 4°C and duplicate samples were taken and fixed in 10% buffered formalin and 0.4% p-benzoquinone every 10 hours up to 94 hours after death.

Histological and immunocytochemical staining methods: To assess neuronal loss and extent of corticospinal tract degeneration representative frozen (20 μm) or vibratome (100 μm) sections from diseased and control tissues were compared after staining with either cresyl fast violet and luxol blue, toluidine blue, haematoxylin and eosin or immunostaining with antisera to neurofilament triplet proteins. Diseased and control tissues were matched according to age, sex and fixation conditions whenever possible and sections from the same segment or approximately same level were processed simultaneously. A range of antisera to peptides known present in sensory, autonomic and motor areas of the spinal cord were employed (Tables 2.1, 7.1).

### 7.2.3 RESULTS

Fixation: Analysis of the normal control spinal cord fixed in formalin after delays of 24-64 hours post-mortem showed little variation in distribution of peptide immunoreactivity. Similarly, little variation in distribution was detected in tissues fixed for either long (several years) or short (8-12 hours) periods in

formalin. However, there was considerable variation of intensity of immunostaining between individual cases even in tissues fixed for the same length of time. In addition, fixation in p-benzoquinone after a 54 hour post-mortem interval, gave weak and diffuse immunostaining along with poor morphological preservation. Nevertheless, p-benzoquinone fixed tissues proved best for the preservation of substance P and CGRP immunoreactivities and was essential for the localisation of TRH. For all other antigens, Bouin's solution gave the best results and optimum preservation.

Morphology of MND spinal cord: Conventional staining showed a loss of motoneurones from all levels of the spinal cord with lumbosacral segments often the most severely affected regions. However, a few large cells, presumed to be motoneurones, always remained. In sacral segments, in contrast to the dorsolateral motoneurone groups where numbers of cells were dramatically reduced, the cells of Onuf's nucleus were spared (Fig.7.1A,B). In the remaining grey matter no other striking abnormalities were observed. In the white matter, a lack of myelin (demonstrated by loss of luxol blue stain) notably from the lateral corticospinal tracts, was a common feature. The extent of this loss varied between cases and often between levels studied.

Abnormalities of the cytoskeleton were also detected using antisera to neurofilament triplet proteins. Immunoreactive motoneurones and large diameter fibres (presumably the efferent axons of motoneurones) were lacking in the ventral horn. In the

TABLE 7.1 COMPARATIVE DISTRIBUTIONS OF PEPTIDE-IMMUNOREACTIVE FIBRES AND CELL BODIES IN SENSORY, AUTONOMIC AND MOTOR AREAS OF NORMAL AND MND SPINAL CORD

| Antiserum to: | Dorsal horn laminae I-III | | Autonomic nuclei | | | | Ventral horn | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | IML | | PSN | | Lamina IX | | Onuf's nucleus | |
| | C | MND | C | MND | C | MND | C | MND | C | MND |
| CGRP | ++++ | ++++ | + | + | ++ | ++ | ± 0 | ± | ± | ± |
| ENK | ++ 0 | ++ 0 | +++ | +++ | +++ | +++ | ++ | + | ++ | ++ |
| GAL | + | + | ++ | ++ | ++ | ++ | ± | ± | n.s | n.s |
| NPY/CPON | + | + | +++ | +++ | ++ | ++ | + | + | ++ | ++ |
| SOM | ++ 00 | ++ 00 | ++ 0 | ++ 0 | ++ | ++ | ++ | + | +++ | +++ |
| Subtance P | +++ | +++ | + | + | ++ | ++ | +++ | + | + | + |
| TRH | ± | ± | +++ | +++ | + | + | ++ | + | n.s | n.s |
| VIP[1] | ++ | ++ | ± | ± | ++ | ++ | ± | ± | + | + |

Key: C, control; MND, motor neurone disease; IML, intermediolateral cell column; PSN, sacral parasympathetic nucleus. Immunoreactive fibres ++++ very dense; +++ dense; ++ moderate; + sparse; ± infrequently seen; n.s not seen. Immunoreactive cell bodies: 0 present and sparse, 00 more than 3/section. [1]Staining intensity in the sacral spinal cord.

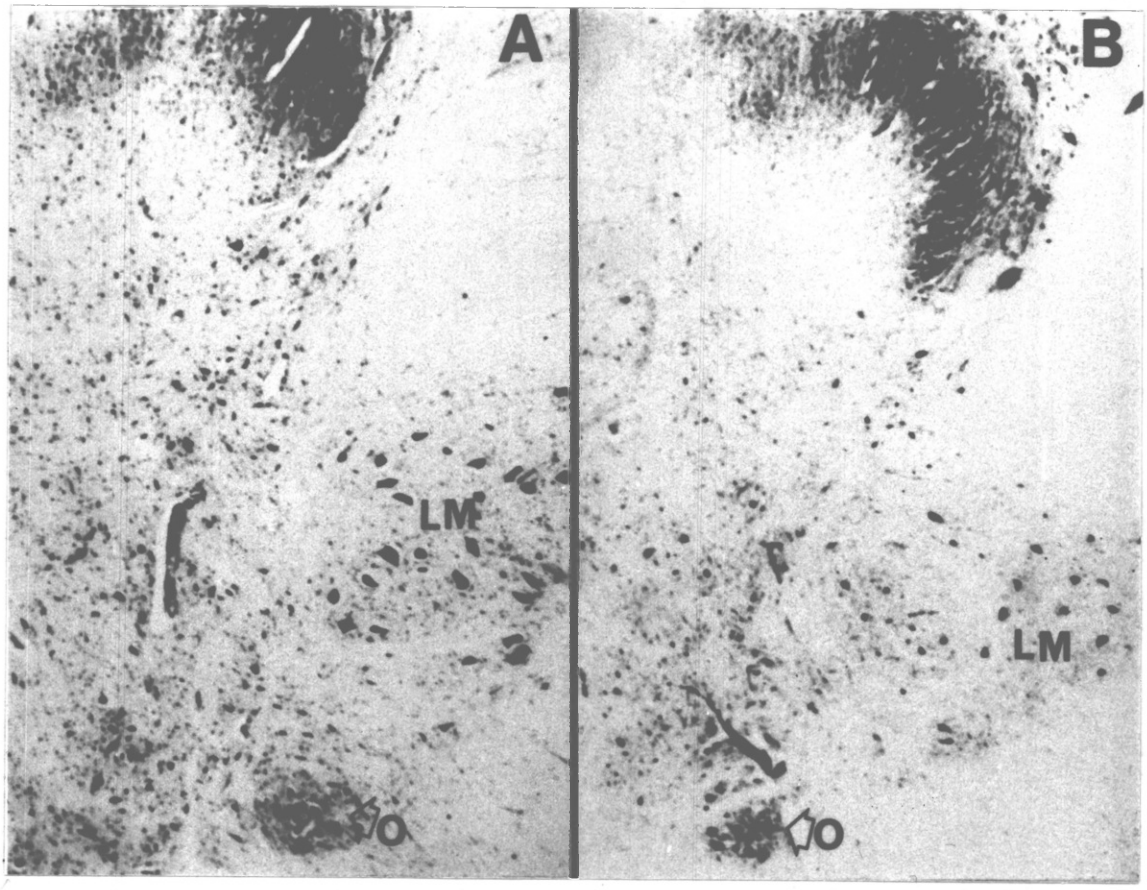



Fig.7.1A-B Sacral ($S_2$) segment of A) normal and B) MND spinal cord stained with toluidine blue. The number and size of motoneurones is reduced in the lateral motoneurone group (LM). Neurones in Onuf's nucleus (O) are well preserved (x 35).

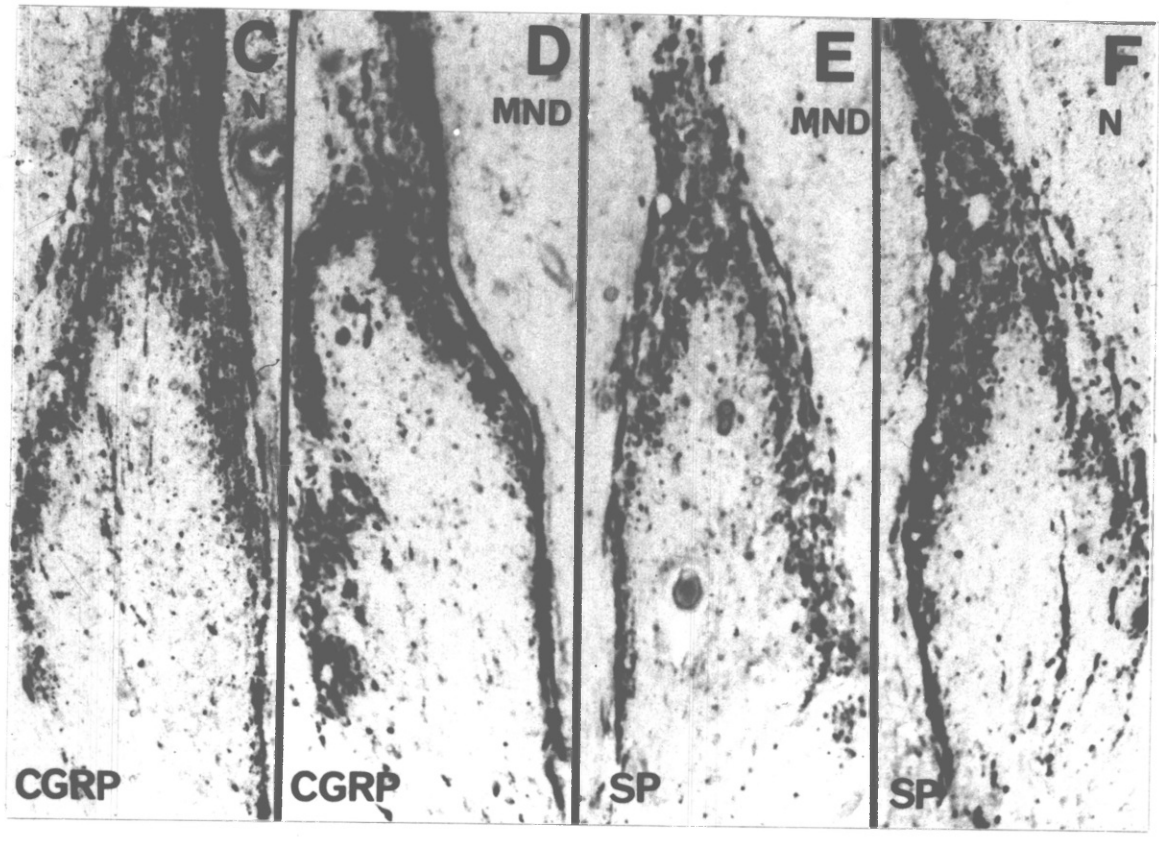



Fig.7.1C-F (C,D) CGRP and (E,F) substance P (SP) immunoreactivities in the thoracic dorsal horn of normal (C,F) and MND (D,E) spinal cord (C-D x 95). N, normal; MND, motor neurone disease.

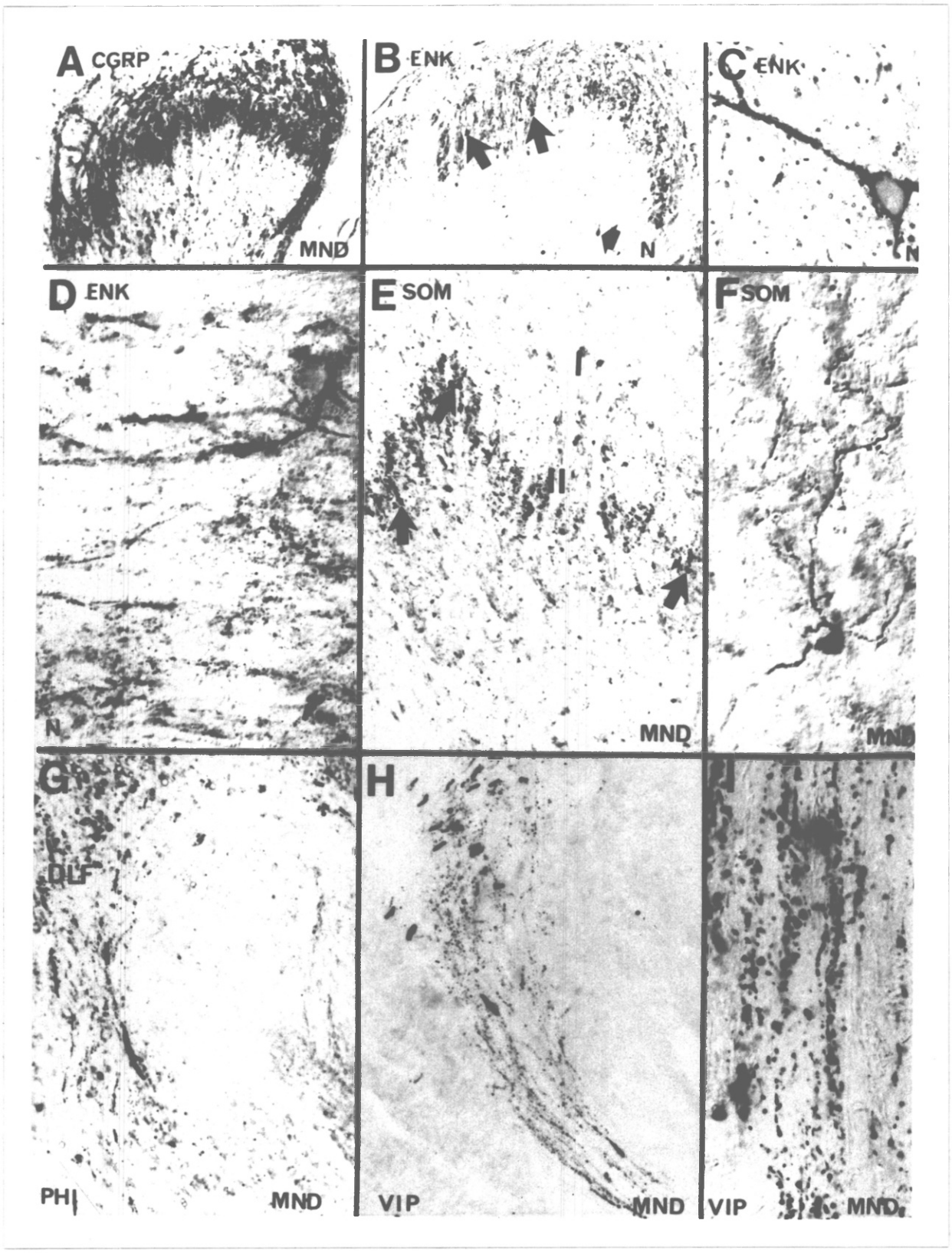



**Fig.7.2** Peptide immunoreactivity in the dorsal horn of MND (A,E-I) and normal (B-D) spinal cord was similar. A) CGRP immunoreactivity in the lumbar dorsal horn (x 30). B) ENK immunoreactivity in the lumbar dorsal horn, some immunoreactive cells are arrowed (x 50). (C,D) ENK-immunoreactive fibres outlining non-immunoreactive cells in C) the dorsolateral funiculus D) lamina II of the lumbar spinal cord (C,D x 175). E) SOM immunoreactivity in the lumbar dorsal horn, some cell bodies are arrowed (x 70). F) SOM-immunoreactive neurone in lamina II of the sacral dorsal horn (x 315). G) PHI- and H) VIP-immunoreactive fibres in the sacral ($S_2$) dorsal horn (G,H x 65). I) High power photomicrograph of VIP-immunoreactive fibres in the lateral dorsal horn of the $S_2$ segment (x 360). N, normal; MND, motor neurone disease; DLF, dorsolateral funiculus.

white matter of both diseased and control tissues from patients aged 40 years or older, spheroids were intensely stained.

Peptide distribution in normal and MND spinal cords: The distribution of ENK, NPY, SOM, substance P and VIP have been previously described in adult human spinal cord. Our findings were in general agreement with previous reports (De Lanerolle and LaMotte, 1982; Schoenen et al., 1985a for ENK; Allen et al., 1984a for NPY; Anand et al., 1983; Cuello et al., 1976; De Lanerolle and LaMotte, 1982; Hunt et al., 1982; Pearson et al., 1982; Schoenen et al., 1985a; Vacca-Galloway, 1985 for substance P; Anand et al., 1983; LaMotte and de Lanerolle, 1983 for VIP). However, only scanty reports of CGRP (Franco-Cereceda et al., 1987; Gibson et al., 1984d; Harmann et al., 1988; Tschopp et al., 1985) the C-flanking peptide of NPY (CPON), GAL (Gibson and Polak, 1986) and TRH (Jackson et al., 1986) exist. These preliminary reports were therefore expanded and together with the remaining peptides studied, their distributions were mapped at cervical, thoracic, lumbar and sacral levels of the spinal cord to form a basis for comparison with neurologically abnormal spinal cords. The distribution of each peptide studied is summarised in Table 7.1 see also chapter 4.

In comparison to neurologically normal spinal cords there was no marked change in the distributions or densities of the peptides studied in the dorsal horn (Figs.7.1C-F, 7.2) or in the autonomic centres of the thoracic and sacral spinal cord (Fig.7.3A-F).

In the ventral horn of MND patients, none of the remaining moto-

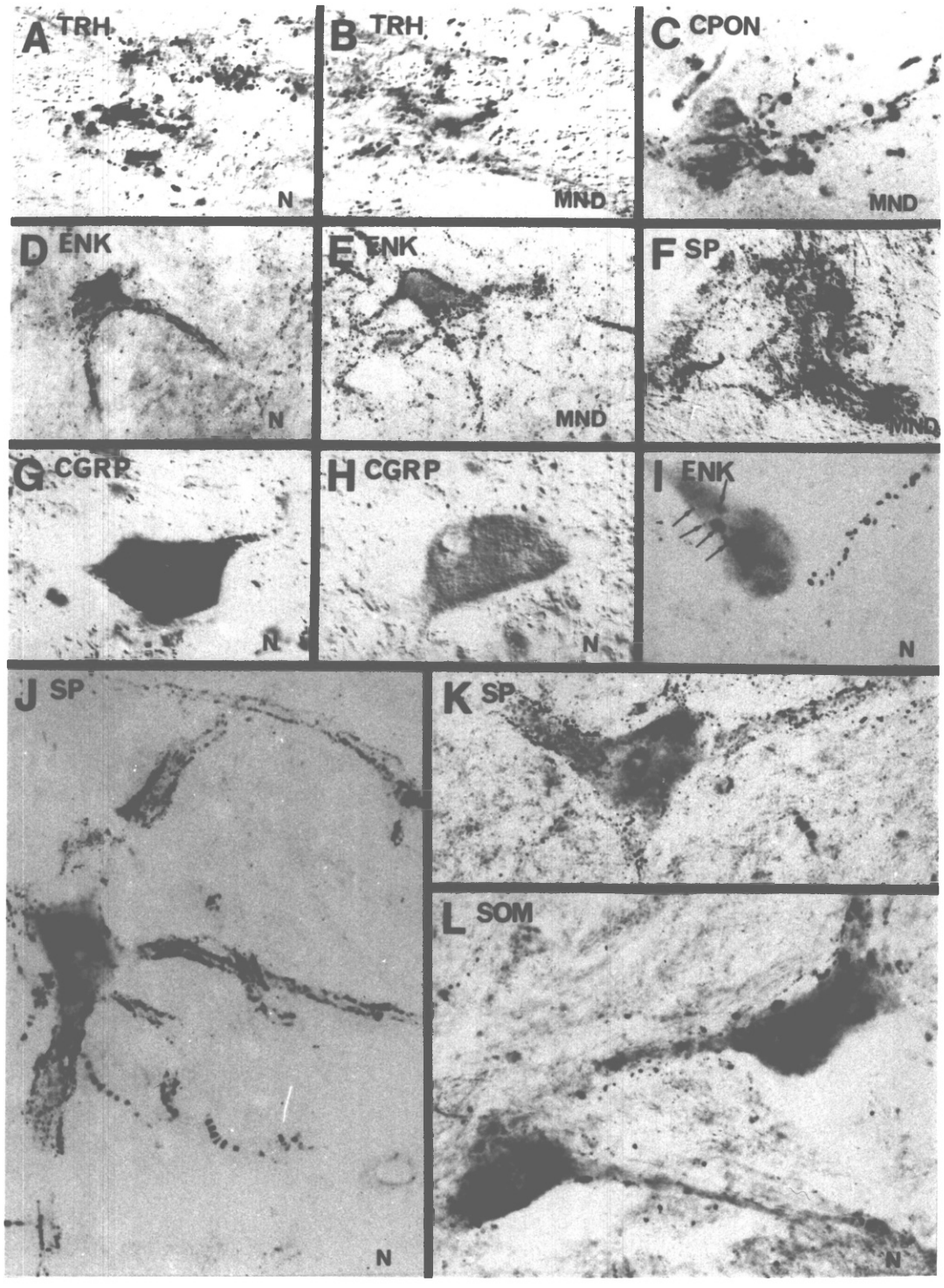



**Fig.7.3** Peptide immunoreactivity in the intermediolateral cell columns was similar in normal and MND spinal cord. (A,B) TRH-immunoreactive fibres in the thoracic intermediolateral cell columns (x 110). C) CPON-immunoreactive fibres in the thoracic intermediolateral cell column (x 100). (D,E) ENK-immunoreactive fibres in the thoracic intermediolateral cell columns outlining non-immunoreactive cells (x 215). F) Substance P-immunoreactive fibres in the sacral intermediolateral cell column. Asterisks, non-immunoreactive, presumptive preganglionic cell (x 130). (G-L) Ventral horn of normal spinal cord. G) CGRP-immunoreactive motoneurone in lamina IX in the lumbar spinal cord. H) A motoneurone from the same case which is not CGRP immunoreactive (G,D, x 375). I) ENK-, (J,K) substance P- and L) SOM-immunoreactive fibres in close apposition to motoneurones in lamina IX of the lumbar spinal cord. N, normal; MND, motor neurone disease (I x 315; J x 330; K,L x 520).

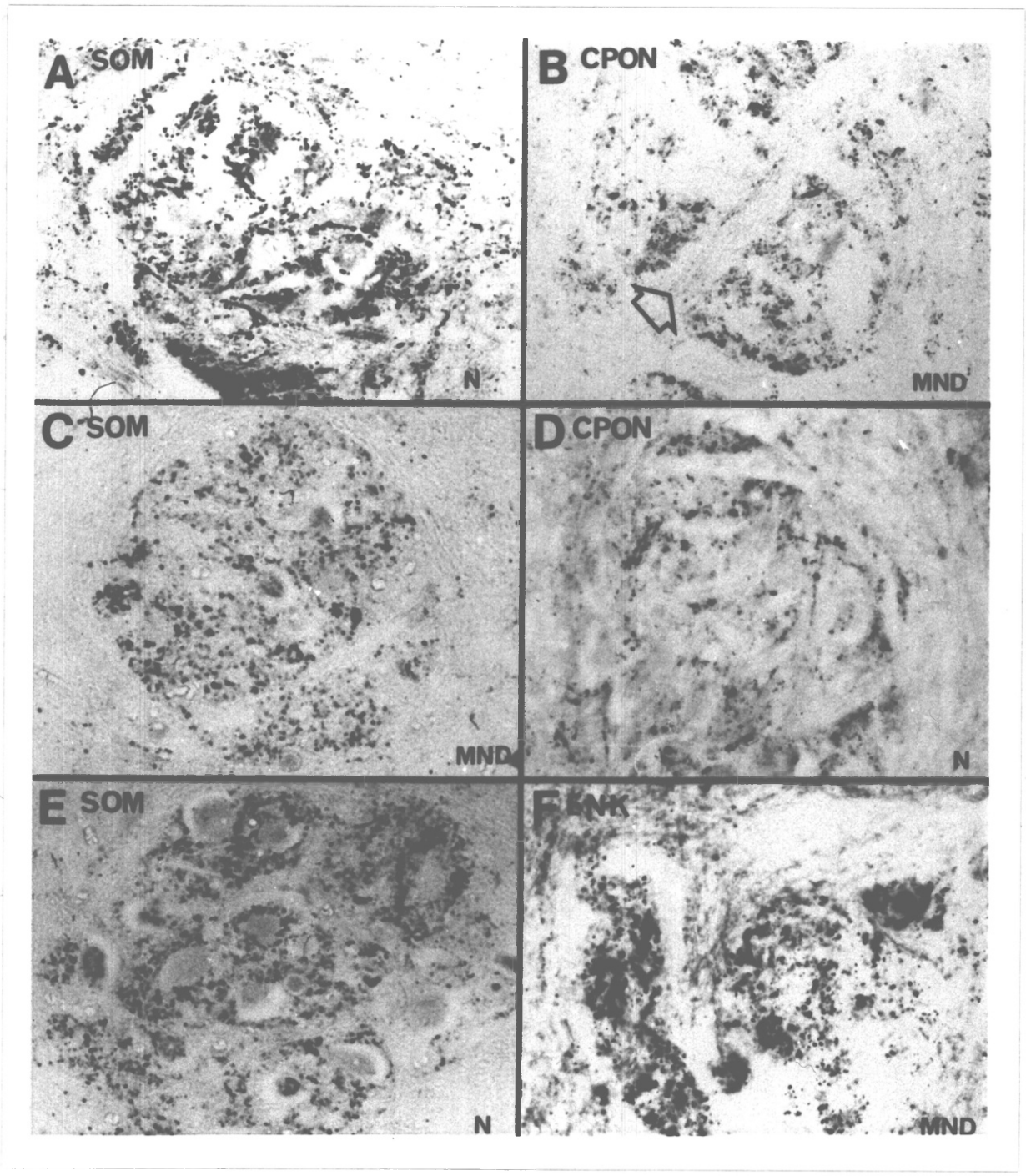



Fig.7.4 Peptide-immunoreactive fibres surrounding the neurones of Onuf's nucleus in the $S_2$ segment of diseased (MND) and normal (N) spinal cord. (A,C,E) SOM-immunoreactive fibres. A,C) Cryostat sections, E) wax section. (B,D) Fibres immunoreactive to CPON antisera. Note the prominent dendritic bundle (arrow) which is characteristic of Onuf's nucleus. F) ENK-immunoreactive fibres (A-F x 180).

neurones displayed CGRP immunoreactivity. In contrast, control spinal cords obtained prior to 36 hours post-mortem, a sub-population of cells, presumably motoneurones according to their size and location, immunoreactive for CGRP were present in lumbar and cervical segments (Fig.7.3 G,H).

In control spinal cords, the close relationship between motoneurones and fibres immunoreactive for ENK, SOM, substance P and TRH was a striking feature (Fig.7.3I-L). In the diseased cords many motoneurones were lost along with their rich peptidergic innervation, whilst the distribution of peptide immunoreactive fibres in the remaining grey matter was apparently unchanged. (Table 7.1). In contrast, while many of the motoneurone-associated peptide-immunoreactive fibres were lost in the diseased cords, Onuf's nucleus was preserved and innervated by fibres immunoreactive for substance P, VIP and in particular, NPY/CPON, ENK and SOM (Fig.7.4). The distribution of peptide immunoreactivity in and around this nucleus showed no significant changes in control tissues.

### 7.2.4 DISCUSSION

#### Are the peptide changes consistent with a motor neuropathy?

Since the motor system is the only part of the nervous system which is affected in MND, it was of interest to study possible change in peptide distribution in the ventral horn to identify which, if any, peptides are implicated in the maintenance of

motoneurone integrity.

No change in peptide distribution was apparent in the dorsal horn which is in agreement with previous studies of ENK, SOM and substance P (Schoenen et al., 1985b). In the ventral horn of diseased spinal cords, there was an extensive loss of motoneurones and the immunoreactive fibres, normally associated with them. The densities of the remaining peptide-immunoreactive fibres although, were similar in normal and diseased spinal cords. Previous immunocytochemical studies have however, reported a similar reduction of substance P-immunoreactive fibres (Patten and Croft, 1984; Schoenen et al., 1985b) but no change in the distribution of ENK, SOM (Schoenen et al., 1985b) or TRH (Jackson et al., 1986) immunoreactivities. Conversely, radioimmunoassay of substance P by two independent groups has failed to demonstrate any significant changes in the ventral horn (Gilberg et al., 1982; Otsuka et al., 1978). The apparent contradiction between these studies remains unexplained. However, since the motoneurones degenerate and the immunoreactive fibres which are normally associated with them disappear, the overall concentration of peptide-immunoreactive fibres should be less than in the control tissues. Thus in normal and MND spinal cord, TRH concentrations per milligram of protein as measured by radioimmunoassay gave similar values, but TRH measured per wet weight of tissue, showed a decrease in MND (Jackson et al., 1986).

The present investigation also provides evidence to support a selective loss of a proportion of ENK-, SOM-, substance P- and

TRH-immunoreactive fibres from the ventral horn. It is important to consider changes which occur in the cell bodies giving rise to these fibres in order to establish whether these changes herald motoneurone degeneration or whether degeneration ensues as a consequence of other events. Many of the immunoreactive fibres which terminate in the ventral horn are thought to originate from the raphe nuclei in the brainstem where there is a notable degree of coexistence of ENK, substance P and TRH immunoreactivities (Hunt, 1983; Ruda et al., 1986 for reviews) (see chapter 2). It is possible therefore, that a chemically coded cell type in the raphe nucleus is selectively vulnerable in MND. Future investigations of changes which occur in the biosynthesis of these peptides in the brainstem nuclei may provide further insights into the pathogenesis of MND.

CGRP, is fast gaining importance as a long-term anterograde muscle trophic factor (Fontaine et al., 1986; New and Mudge, 1986). In the present study a loss of CGRP-immunoreactive motoneurones in MND was noted. A number of motor actions have been described for CGRP, including, contraction of striated muscle (Takami et al., 1985b), increase in cAMP levels in myocytes (Laufer and Changeux, 1987) and CGRP has recently been shown to play a part in the synthesis and assembly of acetylcholine receptors at the neuromuscular junction (Fontaine et al., 1986; Laufer and Changeux, 1987). The inability of motoneurones to synthesise and transport this peptide may thus have a direct bearing on the maintenance of muscle fibre function and the integrity of the motoneurone.

Significance and nature of the peptidergic innervation of Onuf's nucleus

In man, Onuf's nucleus is richly innervated with a number of peptide-immunoreactive fibres including NPY/CPON, ENK and SOM in common with other mammals (see chapter 4.5.3). In order to assess whether the presence or absence of peptide-immunoreactive fibres could be linked to selective sparing of the neurones in this nucleus it would be of importance to study the fate of these fibres in Shy-Drager or Fabry's disease in which Onuf's nucleus is extensively affected (Konno et al., 1986; Mannen et al., 1977; Sung et al., 1979). It is of interest to note, however, that the rich peptidergic innervation of Onuf's nucleus is also common to autonomic nuclei, in particular parasympathetic neurones. Whether the innervation thus provides protection to these neurones or (more probably) reflects their autonomic nature (see below) remains to be established.

Several lines of evidence support the view that the Onuf's nucleus is autonomic. Neuropathological studies indicate that its component neurones show a common, selective vulnerability along with autonomic neurones of the spinal cord in several neurological diseases (Iwata and Hirano, 1979; Konno et al., 1986; Mannen et al., 1977; Sung, 1982; Sung et al., 1979). In addition, the early work of Jacobsohn-Lask (Jacobsohn, 1908; Jacobosohn-Lask, 1931) and Laruelle (1937) suggested that sacral autonomic neurones were not only localised to the parasympathetic nucleus but also to the ventral horn. Furthermore, the neurones of Onuf's nucleus are

atypical of motoneurones being of considerably smaller size (Schrøder, 1981), and a bridge connecting the parasympathetic and Onuf's nuclei, (Rexed, 1954) has been noted in man (Konno et al., 1986). The present study also provides neurochemical evidence for the classification of Onuf's nucleus as autonomic. Peptides have been proposed as selective markers of autonomic neurones (Anand and Bloom 1984) and it was clear that the peptidergic innervation of Onuf's and the sacral autonomic nuclei showed a close correspondence. Whilst a large body of evidence suggests that the cells of Onuf's nucleus are autonomic, the overriding fact remains that their function is at least partly that of classical motoneurones (Breedlove and Arnold, 1981; Katagiri et al., 1986; McKenna and Nadelhaft, 1986; Sato et al., 1978; Schrøder, 1980). Due to the lack of a specific marker for somatic neurones, further evidence to support a somatic nature is not forthcoming.

Finally, there is indirect evidence from retrograde tracing experiments which suggests Onuf's nucleus innervates both striated and smooth muscles and is thus a mixed autonomic and somatic nucleus (Yamamoto et al., 1978). Since no attempt has been made to quantify the number of neurones in Onuf's nucleus in man, it is possible that it comprises two neuronal types, an autonomic and a smaller somatic component, the latter being lost in MND. However, this explanation does not correlate with the unimpaired vesicorectal and genitourinary function characteristic of the disease. Since high densities of peptide-immunoreactive fibres were found in close apposition to the cells of Onuf's nucleus it may be postulated that these peptides (notably

ENK, NPY/CPON, SOM) play a part in the maintenance of bladder and rectal function.

The origin of the peptide-immunoreactive fibres in and around Onuf's nucleus is unknown. Data from experimental animal studies, suggests a possible source is the sacral parasympathetic nuclei, since ENK-, NPY- and SOM-immunoreactive cells have all been identified in this region of the spinal cord (Glazer and Basbaum, 1981b; Sasek et al., 1984; Schrøder, 1984). Other possible sources include the area around the central canal and at least a proportion of ENK-immunoreactive fibres innervating Onuf's nucleus have been shown to derive from cells in this region (Konishi et al., 1985). Other fibres, projecting to Onuf's nucleus, could originate from the dorsal grey commissures or primary sensory neurones. Although a hypothalamic projection to Onuf's nucleus has been identified (Holstege et al., 1986) it is unlikely that peptide immunoreactivity in Onuf's nucleus is of supraspinal origin since no change in peptide distribution is seen in animals (Erdman et al., 1984; Schrøder, 1984) or man (own observations) following transection of the spinal cord.

In conclusion, the data presented suggests ENK-, SOM-, substance P-, and TRH-immunoreactive fibres and CGRP-immunoreactive motoneurones are lost from the ventral horn in MND. In contrast, neuronal cell bodies of Onuf's nucleus are densely innervated with NPY/CPON-, ENK- and SOM-immunoreactive fibres in both normal and diseased states. A similar distribution of cell bodies and fibres

was noted in the sacral autonomic nuclei suggesting that Onuf's nucleus is either autonomic or of a mixed (somatic and autonomic) nature. Loss of peptides which are normally associated with motoneurones and preservation of other cells and their normal peptide constituents provides evidence for a neuronotrophic rôle for peptides.

## 7.3 MUTILATED FOOT (mf) RATS

### 7.3.1 INTRODUCTION

The study of human central nervous diseases is often hampered by the poor availability of tissues. However, similar diseases, inherited in experimental animals can be of help in understanding their pathogenesis (Dyck, 1984). The mutilated foot (mf) rat is one such example and has been suggested as a useful animal model for the study of hereditary sensory neuropathy in man.

The mf rat, is an autosomal recessive mutant (Jacobs et al., 1981) and affected animals become ataxic and display autotomy with their hind-limbs often being mutilated. These animals have markedly reduced numbers of cervical and lumbar dorsal root ganglion cells, diminished pain thresholds and reduced levels of substance P in the cervical and lumbar spinal cord (Scaravilli, 1983). However, no information concerning possible changes in the distribution of substance P in other regions of the nervous system involved with sensory perception or of other peptides has been documented.

The present study has therefore addressed several points. Firstly, which peptides other than substance P show changed distributions and were the changes consistent throughout each spinal segmental level? Secondly, was the peptidergic innervation of the skin altered only in tissues from mutilated appendages and, if so, did this reflect change in peptide distribution in the spinal cord? Finally, did the expected loss of substance P and CGRP immunoreactivities from 'sensory' nerves in the spinal cord and skin, correlate with loss of their cells of origin in the dorsal root ganglia?

### 7.3.2 EXPERIMENTAL APPROACH

Tissues: Spinal cord and dorsal root ganglia from segmental levels, $C_{6-8}$, $T_{6-8}$ and $L_{4-6}$, and skin from fore- and hind-limbs, snout and whisker areas were collected from 10 (Sprague-Dawley) mf rats (2-12 months old) and from an equal number of normal litter-mates as controls which were matched for sex, age and body weight. All tissues were fixed by intracardial perfusion with either Bouin's fluid or 0.4% p-benzoquinone solution (see appendix II, III) followed by immersion in the same fixatives for 1-2 hours.

Histological and immunostaining method: Frozen sections of spinal cord (20 µm), dorsal root ganglia (10-15 µm) or skin were stained for conventional histology with either toluidine blue or haematoxylin and eosin or immunostained using a modified PAP method (appendix VI). The immunofluorescence method (appendix V)

provided optimum visualisation of immunoreactive structures in skin. Antisera to substance P or CGRP were employed in the study of all tissues. ENK antiserum was used to monitor post-synaptic changes in the dorsal horn whilst antisera to NPY or CPON were used to determine whether changes in the mf rat involved autonomic neurones.

Quantitative assessment of peptide change: The area occupied by immunoreactive fibres in laminae I-III in both dorsal horns of mf rats and controls was measured using a computerised image analysis system (IBAS 2000, Kontron) for 4 pairs of animals (chapter 2.10). Five sections from the $L_4$ segment were immunostained for substance P or CGRP. An average of three readings were obtained per section, and an analysis of variance comparing the mean areas was made. Relative changes in ENK immunoreactivity were not included, since normal litter-mate controls showed sparse ENK-immunoreactive fibres and could not be used to obtain a consistent value (see Kar et al., 1988b for further details).

Serial (10 μm) sections were stained sequentially with toluidine blue or with antisera to substance P and CGRP. The total neuronal population and number of cells expressing either substance P or CGRP immunoreactivity was estimated in cervical ($C_6$), thoracic ($T_6$) and lumbar ($L_4$ and $L_5$) dorsal root ganglia from 5 pairs of mf rats and normal litter-mate controls according to Chimelli and Scaravilli (1986) and Tuchscherer and Seybold (1985). The following analyses were performed comparing mutants with litter-matched controls- i) total numbers of neurones, number of sub-

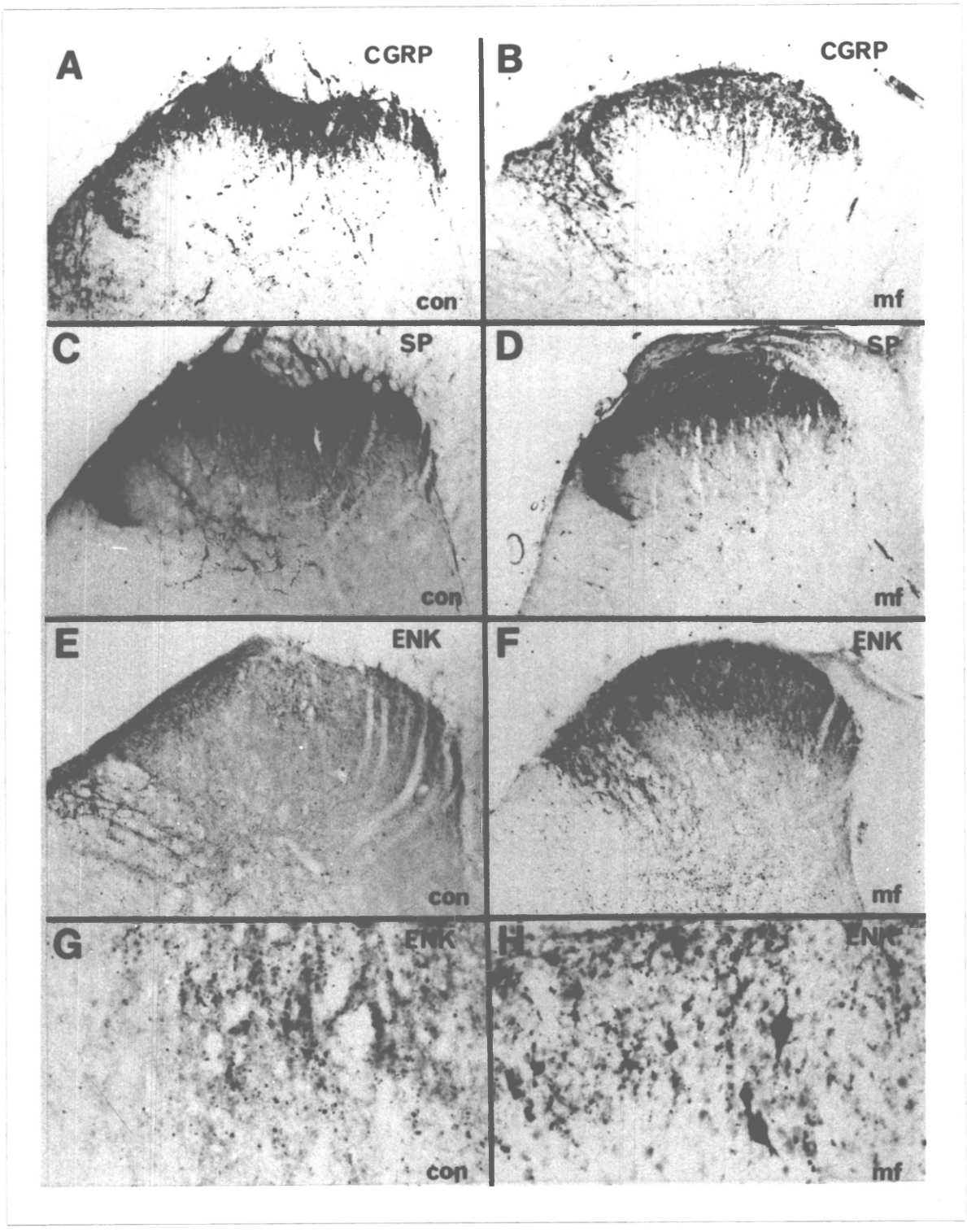



Fig.7.5 Sections through the $L_4$ dorsal horn of normal litter-mate control (A,C,E,G) and mutant (B,D,F,H) rat spinal cords immunostained for CGRP (A,B), substance P (C,D) and ENK (E-H). In mf rats CGRP B) and substance P D) immunoreactivities are markedly reduced. In contrast, ENK F) immunoreactivity is increased with the appearance of immunoreactive cell bodies (some arrowed) (A-F x 55). (G,H) High power photomicrograph of ENK immunoreactivity in lamina II of the lumbar spinal cord of a normal G) and mutant H) rat (G,H x 205).

TABLE 7.2 QUANTITATIVE ASSESSMENT OF CGRP- AND SUBSTANCE P-IMMUNOREACTIVE AREAS IN LAMINAE I-III OF THE $L_4$ SEGMENT OF *mf* AND MATCHED LITTER-MATE CONTROLS

| | CGRP-immunoreactive area ($mm^2$) | | | Substance P-immunoreactive area ($mm^2$) | | |
|---|---|---|---|---|---|---|
| | Control | *mf* | Mean difference | Control | *mf* | Mean difference |
| Animal Pair | | | | | | |
| 1. | 0.114 | 0.067 | 0.047 | 0.103 | 0.066 | 0.037 |
| 2. | 0.104 | 0.071 | 0.033 | 0.131 | 0.085 | 0.046 |
| 3. | 0.111 | 0.062 | 0.049 | 0.104 | 0.068 | 0.036 |
| 4. | 0.112 | 0.067 | 0.045 | 0.115 | 0.076 | 0.039 |
| Mean | 0.110 | 0.067 | *0.043 *$P < 0.001$ | 0.113 | 0.074 | *0.039 *$P < 0.001$ |

Key: Animal pairs = age, sex and litter-mate matched animals; Mean difference = difference between control and *mf* rat values. *$P < 0.001$ for *mf* versus control rats comparison.

stance P- or CGRP-immunoreactive cells and ii) the difference between the total neuronal population and numbers of immunoreactive cells. Analysis of variance was made for each variable using a BMDP package (Dixon, 1983). In addition to analysis of neuronal loss in the mf rat, cell diameters were measured using the same sections on an image analyser (MOP-AM-03) linked to an RML 380Z computer. The computer was programmed to calculate cell diameters assuming circularity of the cross sectional profiles and to group diameters at 5 μm intervals.

### 7.3.2 RESULTS

Spinal Cord: Comparison of the size of the spinal cords of mf and normal animals showed a dramatic reduction in the mutant rats (Fig.7.5). The dorsal horn was considerably smaller and more rounded than in litter-mate controls and these differences, and the differences in peptide distribution, were more pronounced in cervical and lumbar enlargements.

As the distributions of peptides have been extensively described in chapter 4, the following section briefly summarises the data. In the dorsal horn of mf rats the area occupied by fibres immunoeactive for substance P and CGRP, particularly in laminae I-II and Lissauer's tract was greatly reduced in comparison with litter matched controls (Fig.7.5 A-D) (Table 7.2). Fibres in the dorsolateral funiculus, which are strongly immunoreactive for substance P in control animals (but not for CGRP) were also depleted. In the ventral horn, the bundle of fibres located directly ventral to

TABLE 7.3 SUMMARY OF NEURONAL CELL COUNTS IN DORSAL ROOT GANGLIA FROM CONTROL AND *mf* RATS

| Ganglia | Total toluidine blue cells Geometric mean Control | *mf*$^{+}$ | %* | Total CGRP cells Geometric mean Control | *mf*$^{+}$ | %* |
|---|---|---|---|---|---|---|
| $C_6$ | 8734 | 4753 | 54 | 3609 | 1754 | 49 |
| $T_6$ | 4291 | 3685 | 86 | 1420 | 1140 | 80 |
| $L_4$ | 8557 | 3820 | 45 | 3525 | 1328 | 37 |
| $L_5$ | 9436 | 3812 | 40 | 3691 | 1281 | 35 |

| Ganglia | Total Substance P cells Geometric mean Control | *mf*$^{+}$ | %* | Percentage of total cells** CGRP Control | *mf*$^{+}$ | Substance P Control | *mf*$^{+++}$ |
|---|---|---|---|---|---|---|---|
| $C_6$ | 1167 | 480 | 41 | 41 | 37 | 13 | 10 |
| $T_6$ | 491 | 369 | 75 | 33 | 31 | 11 | 10 |
| $L_4$ | 1285 | 425 | 33 | 41 | 34 | 15 | 11 |
| $L_5$ | 1316 | 397 | 30 | 39 | 34 | 14 | 10 |

KEY: *Number of cells in mutants as percentage of those in control animals. **Percentage of CGRP- or substance P-immunoreactive cells in respective groups. $^{+}P < 0.001$, $^{++}P = 0.011$, $^{+++}P = 0.004$ for *mf* versus control rat comparison in all areas. C, cervical; T, thoracic; L, lumbar. Data obtained from pairs of *mf* and litter-mate control rats.

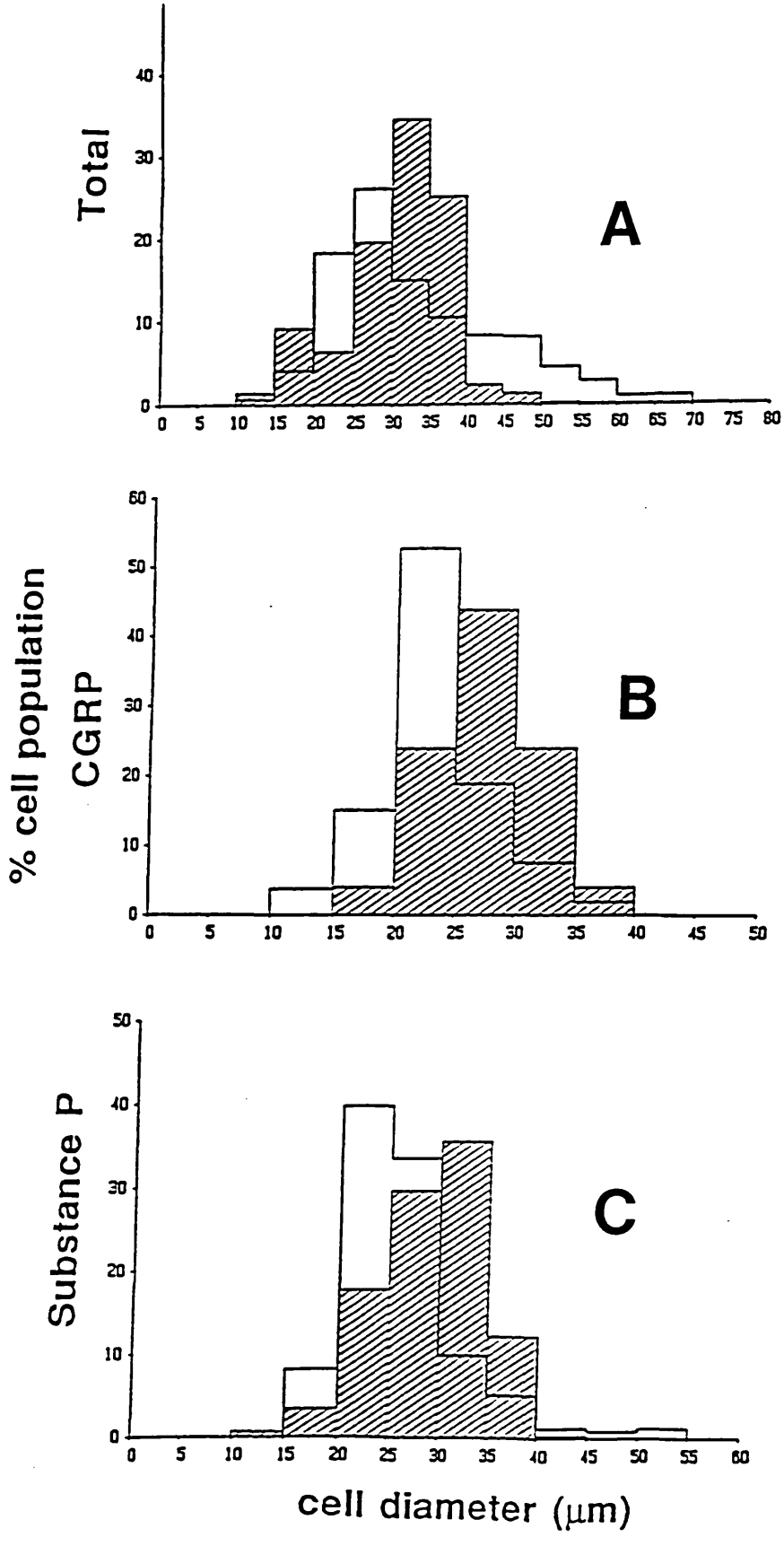



Fig.7.6 Histograms showing the frequency distributions of the diameter range of A) the total neuronal population (toluidine blue cells), B) CGRP- and C) substance P-immunoreactive cells in a lumbar $L_5$ dorsal root ganglion of a litter-mate control (open) and mf (hatched) rat.

the central canal, which stained intensely for substance P and for CGRP in normal animals, was either reduced in size or absent. No marked changes were observed in the remaining grey or white matter.

In contrast to substance P and CGRP immunoreactivities, there was a marked increase in ENK immunoreactivity in mutant rats (Fig.7.5 E-H). In litter-mate controls, a few, scattered ENK-immunoreactive fibres were present in laminae I-II, whilst in mutant rats many intensely stained fibres were seen. In addition, several immunoreactive cell bodies (not seen in controls) were apparent in laminae I-III (Fig.7.5 F,H). No change in the distribution of ENK immunoreactivity was noted in other regions. NPY/CPON immunoreactivity had a similar distribution in both mutant and control animals.

Dorsal root ganglia: Ganglia from mutant rats were considerably smaller than those of controls and neuronal counts were significantly reduced ($P > 0.001$) at all segmental levels studied (Table 7.3). All differences associated with ganglia from mf rats were most noticeable at cervical and lumbar levels. Analysis of cell diameters in ranges 20-30 μm and 30-40 μm showed a relative decrease and increase respectively. Cervical and lumbar ganglia also showed a decrease in the population of large cells ($>45$ μm). A histogram illustrating these points from lumbar ganglia from a pair of animals is shown in Fig.7.6A (for further details see Kar et al., 1986).

Immunostaining of substance P- and CGRP-immunoreactive cells in

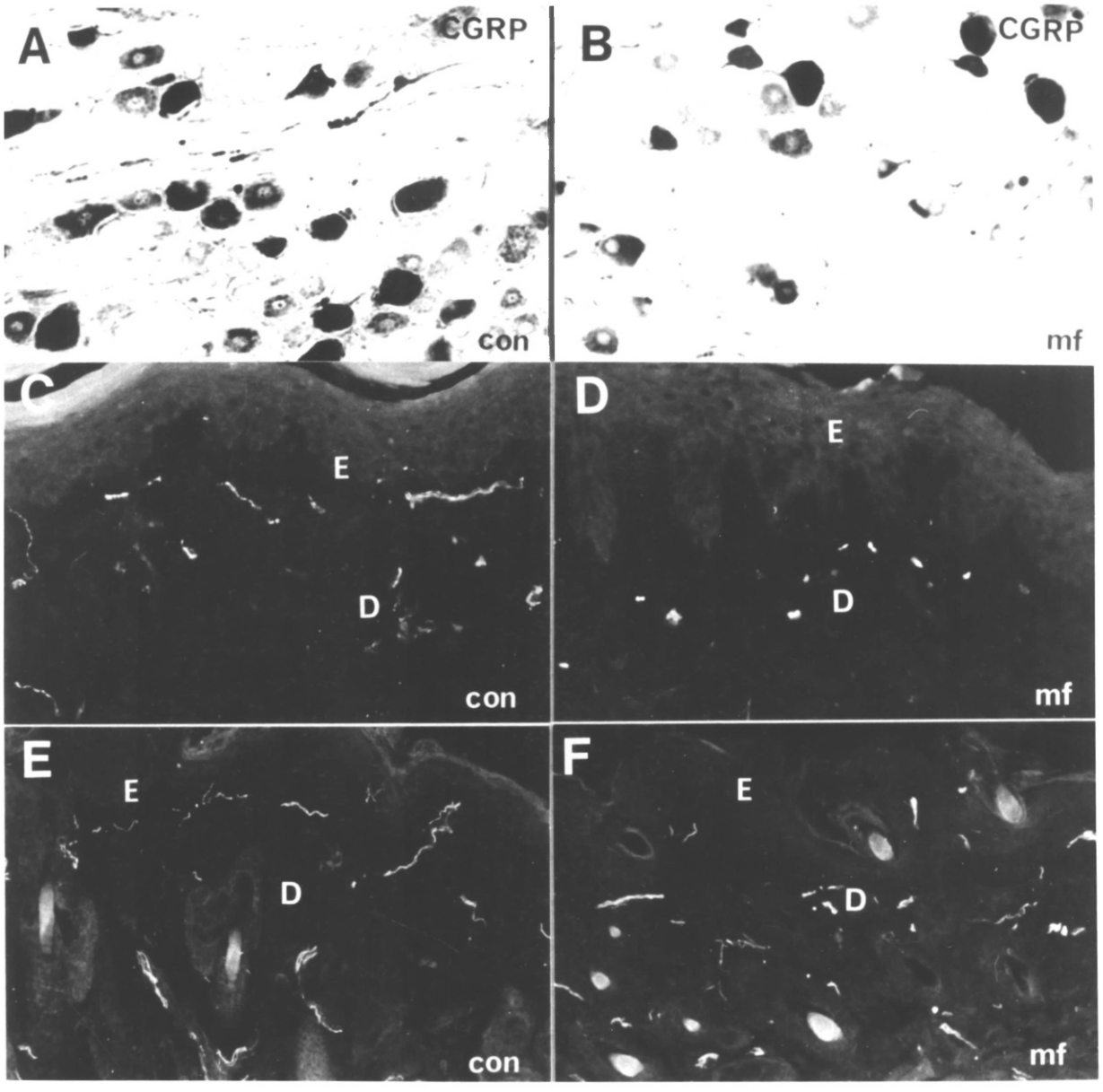



Fig.7.7 CGRP-immunoreactive fibres and cell bodies in the $L_4$ dorsal root ganglia of a litter-mate control A) and mf B) rat (x 185). CGRP-immunoreactive fibres in skin from hind-foot (C,D) and snout (E,F) of control (C,E) and mf (D,F) rats. Note reduced numbers of fibres in hind-foot skin of the mf rat. E, epidermis; D, dermis; con, control. (C,D x 250; E,F x 125).

the dorsal root ganglia appeared to be less intense in mf rats than in controls (Fig.7.7 A,B). The results are summarised in Table 7.3 and show significantly less cells in mf than control rats. Frequency distribution histograms revealed a relative shift in sizes of immunoreactive cells from small (15-25 μm) to the medium (25-35 μm) range (Fig.7.6 B,C).

Skin

In some mutant rats, there was an extensive loss of metacarpals with little evidence of skin lesions, whilst in others there was ulceration, bony destruction and sometimes secondary infection.

Substance P- and CGRP-immunoreactive fibres in skin from hind- and fore-limbs of mf rats were dramatically reduced in both the epidermis and dermis in comparison to controls. No change in the distribution of these antigens was detected in skin from other regions (e.g. whiskers and snout areas) (Fig.7.7 C-F). In contrast to substance P and CGRP no change in NPY/CPON immunoreactivity was seen. Immunoreactive fibres were present either singly or as bundles around blood vessels and sweat glands in equal numbers in both mutant and control rats.

## 7.3.4 DISCUSSION

### Do changes in the spinal cord of mf rats correspond to a loss of sensory input?

The small size of the spinal cord, particularly of the dorsal horn

in mf rats, is consistent with a reduced sensory input. A close parallel can be drawn with the effects of peripheral nerve section in neonatal animals (Fitzgerald, 1985b; Fitzgerald and Vrbová, 1985). The precise mechanism for the lack of growth or shrinkage of the dorsal horn is not known but a number of factors such as dorsal horn cell death could be underlying causes. Sensory stimulation is required for the maturation of several neuronal circuits, (Jacobson, 1978; Loewy, 1972). In mf rats, the loss of sensory neurones is known to occur early in foetal life (Chimelli and Scaravilli, 1986) with necrosis of ganglion cells during develoment of central connections. Thus, failure to establish these connections may act as an inhibitor of growth.

The reduction of substance P-immunoreactive fibres in the dorsal horn of the mf rats is in accord with previously reported results (Scaravilli, 1983). Similar findings have also been noted in conditions characterised by diminished pain sensitivities including other types of hereditary sensory neuropathy in dogs (Cummings et al., 1984) and man (Pearson et al., 1978). The concurrent reduction in CGRP immunoreactivity from regions where primary afferent fibres are known to terminate (laminae I-III, X) was an expected finding in view of the loss of peptide-immunoreactive sensory neurones in the mutants. The loss of both CGRP and substance P immunoreactivity was also expected since these peptides are known to coexist in primary sensory neurones (Gibson et al., 1984d; Wiesenfeld-Hallin et al., 1984; and see chapter 3). The data therefore approximate closely with results obtained following neonatal capsaicin treatment (Jancsó et al., 1981;

Jessell et al., 1978; Nagy et al., 1981; Su et al., 1986) and nerve lesion/dorsal rhizotomy experiments (Gibson et al., 1984b,d; Hökfelt et al., 1975a; Jessell et al., 1979). The fact that the loss of sensory peptides was more pronounced in the cervical and lumbar dorsal horns reflects a more severe reduction of ganglion cells at these levels. Consequently there is a greater reduction of sensitivity to noxious stimuli in the corresponding dermatomes (Chimelli and Scaravilli; 1986).

An intriguing aspect of this work was the marked rise in ENK immunoreactivity in the dorsal horn of the mutants. It has been suggested that enhanced activity of spinal cord, pro-enkephalin derived peptides may play rôles in modulation of nociception (see Millan, 1986 for review). Both opioids and tachykinins (substance P-like peptides) are therefore involved in pain modulation, with ENK related to production of analgesia at the level of the spinal cord and the tachykinins in pain transmission in primary afferents (see chapter 1). It is therefore not surprising that interactions between these two peptide families have been the focus of many studies in central pathways related to pain. In the present study, the decrease in substance P immunoreactivity and elevation in ENK immunoreactivity noted in the mutants showed similarities to a study of human spinal cord post-amputation (Hunt et al., 1982). However, in rats treated neonatally with capsaicin, no increase in ENK has been reported although levels of sensory peptides are reduced (Gibson and Polak, 1986; Jancsó et al., 1981). It is possible that the observed increase in ENK may be related to the development of pain syndromes and to the insurgence

of phantom limb pain which often occurs following peripheral nerve injury and results in autotomy in the denervated tissues (Brown et al., 1976; Culp, 1984; Wall et al., 1979).

The mechanism by which ENK modulates the release of substance P from the central terminals of afferent fibres in the dorsal horn has not been fully established. The cascade of events which lead to alterations of intraspinal ENK-ergic structures are most probably mediated by trans-synaptic mechanisms (see chapter 4.4). The observed increase in ENK immunoreactivity tells little about the biosynthetic activity of the ENK-ergic systems in the mf rat. In vivo release studies in these animals are not yet available however, an increase in messenger(m) RNA encoding a particular product has been associated with increased secretory activity (Höllt et al., 1987; Millan, 1986) and an analysis of mRNA levels may therefore suggest more precisely the functional rôle of spinal ENK-ergic interneurones.

Whilst degeneration of sensory neurones is the major feature of sensory neuropathies, there is often an involvement of other systems including autonomic neurones. A number of factors emerging from the present data support the view that there is predominantly a sensory deficit in the mf rat. The changes of peptide immunoreactivities in the dorsal horn were consistent with disturbed sensory input. In addition, NPY/CPON immunoreactivities which show no response to deafferentation procedures (Gibson et al., 1984b) and which are intrinsic to the central nervous system, were unchanged in the dorsal horns of the

mutants. Furthermore, all peptides studied in regions of the spinal cord associated with autonomic as well as motor function showed no differences in distribution in affected animals in comparison to the controls.

Morphology and biochemistry of sensory neurones and their peripheral projections

Significantly reduced numbers of cells were noted at all segmental levels with the loss being more pronounced in cervical and lumbar enlargements. The underlying reason for the apparent selective vulnerability at these levels is not clear. However, it is evident that the neuronal drop-out was not only restricted to the cells which displayed immunoreactivities for CGRP and substance P(only 25% of the reduction). Thus, chemical markers for the large majority of cells in the dorsal root ganglia of the mf rats remain to be identified in order to determine whether loss of cells occurs in a specific, chemically defined subpopulation.

It was of interest to note a reduction in the population of small-sized neurones as well as the larger diameter neurones. These data are in accord with other conditions in man, such as in familial dysautonomia (Pearson et al., 1978) and Tangier's disease (Schmalbruch et al., 1987). Since small sensory neurones give rise to C and A $\delta$ fibres, the present observations are also consistent with the reported loss of nociception.

The reduced numbers of peptide-containing primary sensory neurones was reflected in reduced substance P- and CGRP-immunoreactive fibres in sensory regions of the skin of the mutant rats. Since the substance P and CGRP, apart from being involved in sensory transmission, are also known to mediate neurogenic oedema, the severe loss of peptide-immunoreactive sensory fibres suggests that mutilation of the limbs does not result in a neurogenic inflammatory response and would therefore explain the apparently 'normal' histology of the skin from affected areas.

In conclusion, these data reinforce the observation that sensory peptides as well as intrinsic spinal ENK-ergic systems show altered distributions in cases where there is loss of pain sensitivity.

## CHAPTER 8: CONCLUDING REMARKS

The significance and interpretation of new information obtained during the course of this thesis has been discussed in relevant chapters. The main focus of these studies was directed towards establishing the occurrence, distributions and origins of peptides in the spinal cord and to correlate alterations in peptide distributions with functional changes associated with nerve injury, during development and pathological states which affect this structure. To acheive this immunocytochemistry has been used in combination with other investigative techniques including surgical and pharmacological manipulations and retrograde tracing studies. Several general conclusions can be drawn from these investigations.

Peptides are found throughout the spinal cord, in regions associated with sensory, autonomic and motor function. They are particularly abundant in laminae I-III of the dorsal horn, the major site of early integration of pain information. At this site most peptide immunoreactivity is derived from afferent axons and/or spinal interneurones (chapter 4). This is in accord with the classical concept that this region of the dorsal horn represents a closed system (i.e. without long projection systems). Many of the small calibre afferent fibres carry nociceptive information and since, in addition to substance P, a number of other peptides were localised to afferent neurones (e.g. CGRP, GAL, SOM etc.) (chapter 3) the list of putative pain neurotransmitters has been expanded considerably. However, it is

taken into consideration that localisation of a substance to a nerve terminal warrants only tentative speculation for a rôle as an intercellular messenger, mediating any particular type of pharmacological or behavioural effect (chapter 1).

Several intrinsic peptide-immunoreactive neurones are also found in laminae I-III. These include neurones which contain ENK and NT (chapter 4) both of which have powerful analgesic actions (Fields and Basbaum, 1984; Yaksh et al., 1982b). Local interactions were suggested as possible means of mediating analgesia caused by stimulation of large myelinated fibres and, although the gate-control system has been expanded, modified and partially supported over the past 20 years (Melzack and Wall, 1965; Wall, 1978, 1980, 1983), the nature of the peptide/peptide integration and interaction with classical transmitters remains complex and not well understood.

Peptide-immunoreactive fibres of primary afferent origin, some of which have characteristic termination patterns in the spinal cord (e.g. SOM, VIP) derive from specific subsets of dorsal root ganglion cells (chapter 3). Factors which control peptide expression in sensory neurones are not known but there is evidence to suggest that at least a proportion of peptide-immunoreactive primary sensory neurones have selective projections to peripheral tissues. For example, VIP/PHI is plentiful in visceral afferents in the pelvic nerve (chapter 4), but not in skin or muscle; substance P is abundant in cutaneous but rarely seen in muscle afferents (McMahon and Gibson, 1987) whilst several other patterns have been suggested in the literature (Gibbins et al., 1987a).

Coexistence of different peptides is a common finding amongst primary sensory neurones (chapter 3) and future studies are needed to expand those presented here. This would enable a precise definition of the combination of peptides in neurones which project to cutaneous, somatic or visceral targets and a determination of whether there is an ordered, tissue-specific chemical coding of these neurones.

There is an impressive number of different peptide-immunoreactive fibres and terminals around autonomic neurones in the spinal cord. These nerves may come from a number of sources including supraspinal descending systems as seen for NPY/CPON and TRH (chapter 4), local interneurones, or afferent fibres as in the case of VIP/PHI (chapter 4). In addition, several peptides were localised to presumptive preganglionic neurones in sympathetic and parasympathetic autonomic nuclei.

In general, peptide immunoreactivity is less abundant in the ventral horn (somatic motor region) than in sensory or autonomic centres, but the intimate relationship between peptide-immunoreactive fibres and motoneuronal soma and processes is in accord with the modulatory effects on motoneurone excitability which has been described for a number of peptides (chapter 4). However, certain specialisations of the neuropil, show biochemical distinctions. In particular, motoneurone groups which innervate the genitourinary system are in turn innervated by a greater number of peptide-immunoreactive fibres than other motor nuclei

(chapter 4). This would imply that peptides play an integral part in the modulation of micturition, defaecation and sexual activity. Motor nerves have received little attention in peptide studies, possibly due to the relatively low concentration of peptides in the ventral horn. However, the localisation of peptides, notably CGRP, to motoneurones has opened a new avenue of research (chapter 4).

Classical studies of neurogenesis show that the central nervous system matures before the peripheral nervous system and that motoneurones develop before sensory or autonomic neurones. The hypothesis that neural antigens are expressed at different times in selected functional regions during development was tested and proven (chapter 6). Motoneurones are the first cells to mature anatomically and express peptides, whilst peptide expression in peripheral terminals of cutaneous sensory nerves is the latest developmental event. An adult-like pattern of peptide and classical neurotransmitter expression emerges by late foetal (man) and early postnatal (rat) life in the spinal cord and is coincident with physiological maturation of afferent and efferent fibres and the development of intrinsic neuronal pathways (i.e. descending systems). In the developing spinal cord and dorsal root ganglia, some peptide-immunoreactive cells are more abundant than in the adult, whilst a number of peptide-immunoreactive cells are expressed transiently during development so that immunoreactive cells cannot be detected after birth. The phenomenon of natural cell death may in part account for loss of supernumary cells which show a peptide phenotype.

The relationship between peptides and primary sensory transmission is of considerable interest. Since peptides occur in nerve fibres which carry altered sensory information from areas of damage, the effects of peripheral nerve manipulations were studied to elucidate the possible involvement of peptides in pain-producing events (chapter 5). A biochemical plasticity exists in the dorsal horn, with substance P and a number of other sensory peptides depleted following peripheral nerve axotomy. In contrast, increase of primary afferent VIP/PHI immunoreactivity in the dorsal horn, which was seen after all manipulations of the sciatic nerve, may indicate a different rôle in regenerative and reorganisation processes in this region. These studies reveal the complexity of biochemical changes which ensue after nerve injury. How these events relate to electrophysiological changes requires further investigations. However, on the basis of these and similar studies made in conjunction with electrophysiological observations, it has been proposed that substance P is not the specific neurotransmitter of pain in the spinal cord and that C fibres have two distinct rôles (Wall and Fitzgerald, 1982). One of these rôles is to exert influence on the dorsal horn in the area of termination via impulses and the other is to signal the presence of peripheral damage via axoplasmic transport of chemical signals. Peptides may thus have an additional or different rôle from that of neurotransmitter to that of a slow, long-term trophic action.

Whatever the exact mode of action of peptides, studies on sensory and motor neuropathies (chapter 7) reinforce the supposition that

the presence of peptide is linked to functional status. In rats with a sensory neuropathy (mf rats), reduced levels of sensory peptides and increased ENK immunoreactivity in the dorsal horn is consistent with diminished sensitivity to noxious stimuli which characterises these animals. In motor neurone disease, the selective loss of motoneurones and peptide immunoreactivity from the ventral spinal cord is consistent with loss of motor function and muscle atrophy. However, the apparent sparing of the neurones of Onuf's nucleus, which are involved in vesicorectal function (which is also spared in motor neurone disease) and the intact peptidergic innervation of this nucleus in the diseased state, implies that peptides contribute to both maintenance of function and neuronal integrity.

The present investigations provide further insight into the significance of peptide-immunoreactive systems in the spinal cord and some peripheral tissues. Future studies employing latest morphological methods including in situ hybridisation for localisation of peptide mRNA's and hence the dynamics of peptide production and in vitro receptor binding studies for identification of site of action together with neurophysiological and pharmacological studies should cast further light on an intriguing subject.

# APPENDICES

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## Appendix I - Classical neurotransmitter metabolism

*Catecholamine*

Tyrosine

↓ Tyrosine hydroxylase (TH)

Dihydroxy phenylalanine (DOPA)

↓ Dopa-decarboxylase (DDC)

Dopamine

↓ Dopamine ß-hydroxylase (DßH)

Noradrenaline

↓ Phenylethanolamine-N-methyl transferase (PNMT)

Adrenaline

*5-HT*

Tryptophan

↓ Tryptophan hydroxylase

5-Hydroxytryptophan

→ $CO_2$

↓ Aromatic L-amino acid decarboxylase

5-Hydroxytryptamine (Serotonin)

*Acetylcholine*

Acetyl-CoA

+

Choline

↓ choline acetyltransferase

Acetylcholine

+

CoA

↓ *Acetylcholinesterase

Choline + $CH_3COOH$

*GABA*

Glutamate

$CO_2$ ← Glutamate decarboxylase (GAD)

GABA

α keto glutarate ← GABA transaminase

glutamate

Kreb's cycle

Boxed areas refer to antisera or *enzyme reaction used to analyse the distribution of aminergic neurones in the spinal cord

## APPENDIX II

## PREPARATION OF FIXATIVES

A. 4% Paraformaldehyde (1 litre)

i) 40 g paraformaldehyde (BDH Chemicals) is partially dissolved in 0.01M phosphate-buffered saline (PBS), pH 7.2 and made to 1 litre with the same buffer.
ii) Heat to 60°C and clear by adding sodium hydroxide (1N).
iii) Cool and filter before use.
Recommended shelf life: 6-8 hr.

B. Bouin's solution (1 litre)

i) 750 ml saturated Picric acid.
250 ml 40% formaldehyde/formalin stock solution.
ii) Add 10 ml glacial acetic acid before use.
Recommended shelf life: Indefinite (- acetic acid)
12-24 hr (+ acetic acid)

C. 0.4% p-benzoquinone solution (1 litre)

i) Dissolve 4 g p-benzoquinone (Köch Light Labs Ltd.) in 1 litre of 0.01 M PBS (pH 7.2) to give a clear, bright yellow solution.
Recommended shelf life: The solution must be freshly prepared (maximum time, 10 min before use). The solution (or solid) should not be exposed to light or heated, both procedures will oxidise the quinone and render it unsuitable for fixation.

## PREPARATION OF BUFFERS

A. Phosphate-buffered saline (PBS) (0.01M) (10 litres)

NaCl 87.9 g; $KH_2PO_4$ 2.72 g; $Na_2HPO_4$ anhydrous 11.35 g; Adjust to pH 7.1-7.4. It is convenient to make this solution x 10 concentrated, and dilute before use.

B. PBS sucrose (1 litre)

Add 150 g sucrose to 1 litre of PBS and include 0.001 g sodium azide (anti-fungal/bacteriostat).

## APPENDIX III

## TISSUE FIXATION

A. Perfusion

i) Prepare a suitable perfusion needle (a syringe needle with a rounded end) and attach to perfusion pump tubing.

ii) Anaesthetise animal with either an overdose of sodium pentobarbitone (Euthatal, May and Baker Ltd., 200 mg/ml) intraperitoneally for large animals (piglets, cats, guinea pigs etc.) or by inhalation of ether (rats, mice etc.).

iii) Fix animal ventral side uppermost to a dissection board. Expose the heart and make a small incision into the left ventricle. Insert the perfusion needle into the cut ventricle and guide into the aorta. (Do not advance too far, otherwise the carotid arteries will be blocked and lead to uneven perfusion).

iv) Fix needle in place by clamping the ventricle.

v) Cut open the right atrium.

vi) Flush out blood slowly (perfusion pump at 10-20 ml/min) with 0.01M PBS (approx. 50 ml/100 g body weight) until liver and kidneys become pale.

vii) Replace the buffer with fixative and perfuse for an appropriate time. (For adult rats perfusion rate is approximately 20 ml per min. Different rates are required depending upon the size of the animal).

viii) Dissect relevant tissues and post-fix as necessary.

B. Immersion Fixation

Place small blocks of tissue, (not exceeding 2 x 2 $cm^2$ or 0.5 cm thick) in a quantity of fixing fluid about 20 times their volume.

C. Fixation times

| Tissue | Post-perfusion (hr) immersion | | | Immersion (hr) | | |
|---|---|---|---|---|---|---|
| | P | B | BQ | P | B | BQ |
| bladder | 0 | 0 | 0 | 2 | 2 | 1½ |
| ganglia | 0 | 0 | 0 | 1 | 1 | 1 |
| gut | 0 | 0 | 0 | 1 | 1 | 1 |
| muscle | 0 | 0 | 0 | 1 | 1 | 1½ |
| peripheral nerves | 0 | 0 | 0 | 1 | 1 | ½ |
| skin (footpad) | ½ | 1 | 1 | 4 | 3 | 3-4 |
| spinal cord (rat) | 3 | 2 | 2 | 4 | 1 | ½ |
| spinal cord (man) | - | - | - | 6-8 | 12+ | 8-10 |
| whole rat embryos | - | - | - | 8-10 | 8-10 | 10-12 |

P, paraformaldehyde; B, Bouin's fluid; BQ, p-benzoquinone solution; hr, hours.

D. Post-fixation treatment

Rinse tissues with several changes of PBS and store in PBS sucrose at 4°C (maximum storage approx. 6 months).

## APPENDIX IV

## FROZEN TISSUE PREPARATION

i) Blot tissue with absorbent paper to remove excess rinsing medium.

ii) Mount tissue block at optimum orientation in embedding medium (O.C.T. compound, Miles Scientific) onto cork support. Very small or delicate tissue can be held in place with larger tissues (e.g. unfixed liver).

iii) Cover entire tissue with embedding medium and plunge into melting Arcton (Dichlorodifluoromethane -158°C) pre-cooled in liquid nitrogen.

## APPENDIX V

## INDIRECT IMMUNOFLUORESCENCE FOR FROZEN TISSUE SECTIONS

i) Mount sections onto poly-L-lysine (1 mg/ml Sigma Chemicals) coated slides and air-dry for 1-2 hr at room temperature.

ii) Block non-specific binding sites by applying normal goat serum (diluted 1/10 in PBS) for 30 min in humid chamber at room temperature.

iii) Remove normal goat serum by blotting with filter paper and apply primary antiserum at optimum dilution for 12-16 hr at 4°C.

iv) Rinse in 3 changes of PBS, 15 min each change.

v) Wipe dry around each section and apply fluorescein-labelled goat anti-rabbit IgG serum (Miles Scientific) diluted to optimum in PBS and incubate for 40-60 min at room temperature.

vi) Rinse in a further 3 changes of PBS, 15 min each change and mount sections in PBS/glycerine (1:1).

## APPENDIX VI

## PEROXIDASE ANTI-PEROXIDASE (PAP) IMMUNOSTAINING FOR FROZEN TISSUE SECTIONS

i) See (i) Appendix V.

ii) Dehydrate through graded alcohols to xylene (or suitable non-toxic alternative).

iii) Block endogenous peroxidase by immersion in 0.3% hydrogen peroxide in methanol for 30 min at room temperature.

iv) Rehydrate to distilled water and partially air-dry.

v) Apply primary antisera diluted to optimum value in PBS containing approximately 30% normal goat serum. Incubate over two nights in a humidified chamber at 4°C.

vi) Rinse in 3 changes of PBS, 15 min for each.

vii) Wipe dry around each section and apply goat anti-rabbit IgG (Miles Scientific) diluted to optimum value in PBS-containing 0.25% Triton X-100, and incubate for 40-60 min at room temperature.

viii) Rinse in 3 changes of PBS, 15 min for each.

ix) Wipe dry around each section and apply PAP (rabbit) complex (Miles Scientific) diluted to optimum value in PBS-containing 0.25% Triton X-100 and incubate for 60 mins at room temperature.

x) Rinse in 3 changes of PBS, 15 min for each.

xi) Prepare a developer solution containing 0.025% 3,3'-diaminobenzidine (DAB; Sigma Chemicals), in PBS and 0.015% hydrogen peroxide. Filter before use.

xii) Immerse sections in developer for 5 min then inspect microscopically.

xiii) Wash sections in running tap water, dehydrate and mount in DPX mountant (Raymond A. Lamb Ltd.), or counterstain with either haematoxylin (25%) and eosin (0.5%), cresyl violet (0.1%) or toluidine blue (1%) prior to dehydration.

## APPENDIX VII

## ANTISERUM SPECIFICITY: ADSORPTION PROCEDURE

i) Determine optimum dilution of antiserum by titration on tissue of interest.

ii) Mix antiserum at optimum dilution with antigen (for peptides this is usually in solid form) to give an antigen concentration of 10 nmol per ml of diluted antiserum.

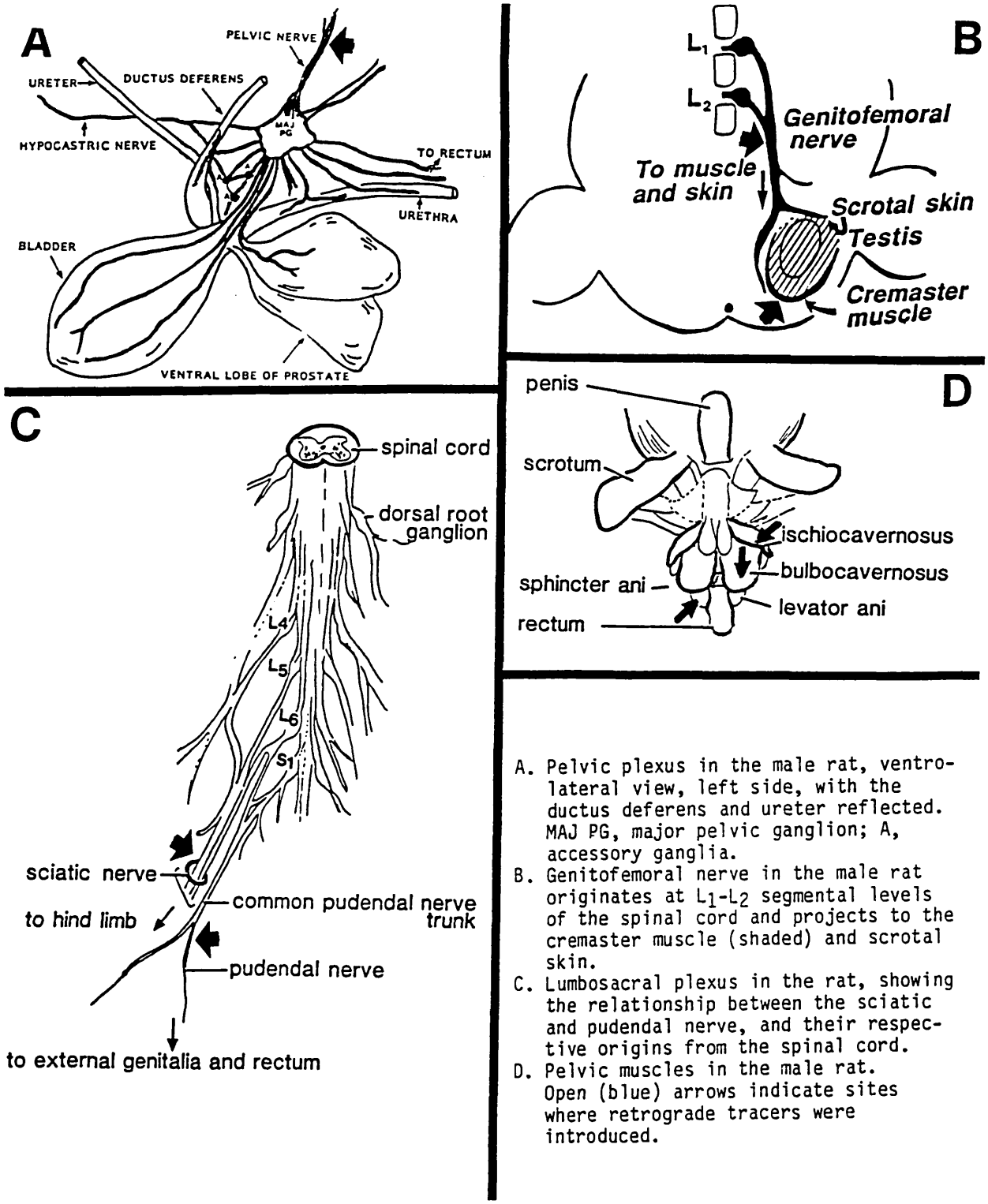
iii) Apply mixture to tissue section in place of primary antiserum.

iv) Follow immunostaining sequence as described above.

N.B. A range of decreasing concentrations of antigen can be used to determine the value at which immunostaining returns and allows a comparison of the degree of cross-reactivity of several antigens.

# APPENDIX VIII

## ILLUSTRATION OF NERVE TRUNKS AND MUSCLES




A. Pelvic plexus in the male rat, ventrolateral view, left side, with the ductus deferens and ureter reflected. MAJ PG, major pelvic ganglion; A, accessory ganglia.

B. Genitofemoral nerve in the male rat originates at $L_1$-$L_2$ segmental levels of the spinal cord and projects to the cremaster muscle (shaded) and scrotal skin.

C. Lumbosacral plexus in the rat, showing the relationship between the sciatic and pudendal nerve, and their respective origins from the spinal cord.

D. Pelvic muscles in the male rat. Open (blue) arrows indicate sites where retrograde tracers were introduced.

## APPENDIX IX

## DEVELOPMENTAL INVESTIGATION: AGES STUDIED

| SIZE VARIATION OF PRENATAL RATS WITH GESTATIONAL AGE | | | FOETAL COLLECTION (MAN) | | PREMATURE AND NEONATAL DEATHS (MAN) | | |
|---|---|---|---|---|---|---|---|
| AGE (days) | CROWN RUMP LENGTH (mm) | FOOT LENGTH (mm) | FOETAL AGE[1] (WEEKS) | No. CASES[2] | AGE (WEEKS) | No.CASES | PM DELAY (hrs) |
| | | | | | foetal | | |
| E12 | 3-7 | 2.5 | 6 | (5) 1 | 30 | (2) 1 | 74 |
| E13 | 8-10 | 3 | 7 | (9) 3 | 32 | (3) 1 | 48 |
| E14 | 9-11 | 4 | 8 | (20) 7 | 33 | (1) 1 | 70 |
| E15 | 12-15 | 4.5 | 8.5 | (22) 7 | 35 | (1) 1 | 29 |
| E16 | 14-18 | 5 | 9 | (15) 4 | postnatal | | |
| E17 | 17-22 | 6 | 9.5 | (11) 7 | 3 | (1) 1 | 48 |
| E18 | 22-29 | 7 | 10 | (12) 4 | 4 | (3) 3 | 24-48 |
| E19 | 26-32 | 8 | 10.5 | (11) 4 | 6 | (2) 1 | 44 |
| E20 | 30-38 | 9.5 | 11.5 | (16) 3 | | | |
| E21 | 34-44 | 11 | 12 | (15) 2 | | | |
| | | 15 | 14 | (5) 1 | | | |
| | | 26 | 18 | (3) 1 | | | |
| | | 29 | 19 | (1) 1 | | | |
| | | 34 | 20 | (2) 2 | | | |
| | | 43 | 24 | (1) 1 | | | |
| | | 45 | 25 | (1) 1 | | | |
| | | 48 | 26 | (3) 1 | | | |

[1] Foetal age estimated by measuring foot length (Trolle, 1948)
[2] Numbers in parentheses represent the total number of cases collected at each age. Those without parentheses, indicate the number of samples which were sufficiently well preserved for immunocytochemical analysis.

## APPENDIX X

## DETAILS OF NEUROLOGICALLY NORMAL CONTROL SPINAL CORDS

| Case Number | Age at death (yrs) | Sex | Cause of death | Post-mortem delay (hrs) | Tissue Handling |
|---|---|---|---|---|---|
| 1 | 70 | M | CVA | n.k | F 9 yr |
| 2 | 65 | M | P | 36 | F 7 yr |
| 3 | 64 | M | Ca | 78 | F 6 yr |
| 4 | 79 | F | Lymphoma | 48 | F 5 yr |
| 5 | 73 | F | Ca | 36 | F 5 yr |
| 6 | 87 | M | CML | 78 | F 5 yr |
| 7 | 66 | M | AML | 78 | F 5 yr |
| 8 | 40 | F | P | 24 | F 4 yr |
| 9 | 52 | F | AML | 72 | F 4 yr |
| 10 | 77 | M | Ca | 35 | F 3 yr |
| 11 | 58 | M | P | 24-40 | F 3 yr |
| 12 | 60 | M | CVD | n.k | F 2 yr |
| 13 | 72 | M | CVD | 32 | F 2 yr |
| 14 | 81 | F | CDA | 27 | F 2 yr |
| 15 | 14 | F | AML | 60 | F 1 yr |
| 16 | 88 | F | CVA | 12-36 | F 1 yr |
| 17 | 75 | F | CVAn | 44 | F 1 yr |
| 18 | 59 | M | P | 36 | FR |
| 19 | 75 | M | MDP | 220 | FR |
| 20 | 91 | M | Ca | 48 | FR |
| 21 | 69 | F | Multiple myeloma | 72 | FR |
| 22 | 16 | M | Lymphoma | 24 | FR |
| 23 | 42 | F | Diabetes | 48 | FR |
| 24 | 82 | F | Scleroderma | 24 | FR |
| 25 | 75 | F | CA | 40 | FR |
| 26 | 74 | M | P | 24 | FR |
| 27 | 85 | M | Ca | 48 | FR |
| 28 | 86 | M | CA | 14 | FR |
| 29 | 85 | M | Ca | 14 | FR |
| 30 | 56 | M | CVAn | 48 | FR |
| 31 | 81 | M | Mesothelioma | 48 | FR |
| 32 | 66 | M | PE | 24 | FR |
| 33 | 81 | F | CVA | 28 | FR |
| 34 | 73 | F | Ca | 20 | FR |
| 35 | 56 | M | Ca | 17 | FR |
| 36 | 81 | M | P | 17 | FR |
| 37 | 75 | F | Ca | 24 | FR |
| 38 | 65 | M | Cardiae arrhythmia | 24 | FR |
| 39 | 71 | M | Ca | 48 | FR |
| 40 | 80 | F | Ca | 30 | FR |
| 41 | 66 | M | Ca | 35 | FR |
| 42 | 74 | F | CA | 32 | FR |
| 43 | 71 | M | Ca | 48 | FR |
| 44 | 77 | F | Ca | 48 | FR |
| 45 | 85 | M | Airways disease | 24 | FR |
| 46 | 75 | M | Ca | 24 | FR |
| 47 | 58 | M | CA | 24 | FR |
| 48 | 42 | M | Suicide | 40 | FR |
| 49 | n.k. | F | P | 20 | FR |
| 50 | 71 | F | P | 10 | FR |
| 51 | 73 | F | Ca | 3 | FR |
| 52 | 83 | M | Ca | 30 | FR |

For key see appendix XI

## APPENDIX XI

## DETAILS OF CASES OF MOTOR NEURONE DISEASE

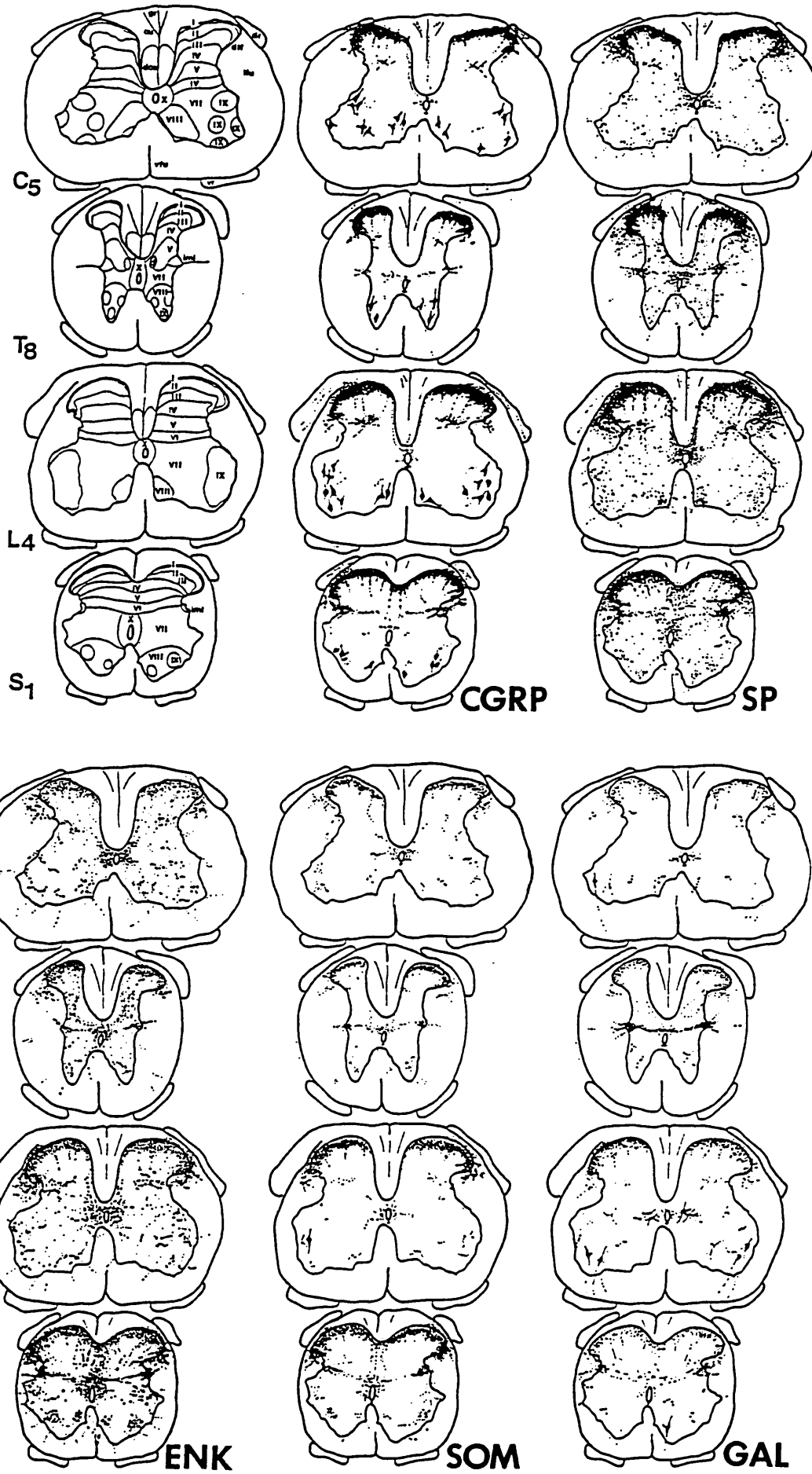
| Case Number | Age at death (yrs) | Sex | Duration | Post-mortem delay (hrs) | Tissue Handling |
|---|---|---|---|---|---|
| 1 | 75 | M | 1 mt | 48 | F 6 yr |
| 2 | 68 | M | n.k | 53 | F 6 yr |
| 3 | 69 | F | n.k | 36 | F 6 yr |
| 4 | 70 | M | 1 yr | 24 | F 6 yr |
| 5 | 53 | M | 2.5 yrs | 25 | F 6 yr |
| 6 | 69 | M | 1.5 yrs | 26 | F 5 yr |
| 7 | 67 | M | 1 yr | 24 | F 5 yr |
| 8 | 76 | F | 1 yr | 36 | F 5 yr |
| 9 | 45 | M | 2 yrs | 37 | F 4 yr |
| 10 | 71 | F | 1 yr | 33 | F 4 yr |
| 11 | 62 | F | 2 yrs | 24 | F 4 yr |
| 12 | 69 | M | 1 yr | n.k | F 4 yr |
| 13 | 69 | M | 1 mt | n.k | F 3 yr |
| 14 | 65 | F | 2 yrs | 60 | F 3 yr |
| 15 | 68 | M | 1 yr | n.k | F 3 yr |
| 16 | 57 | F | 2.5 yrs | 85 | F 3 yr |
| 17 | 62 | M | 3 mts | 30 | F 3 yr |
| 18 | 60 | M | 4 yrs | 24 | F 2 yr |
| 19 | 66 | F | 1.5 yrs | 88 | F 2 yr |
| 20 | 69 | M | 1.3 yrs | 78 | F 2 yr |
| 21 | 58 | F | 2 yrs | 30 | F 2 yr |
| 22 | 75 | M | 3 yrs | 12-48 | F 2 yr |
| 23 | 60 | M | 2.5 yrs | 39 | F 2 yr |
| 24 | 41 | M | 1 mt | 72 | F 1 yr |
| 25 | 77 | M | 3 yrs | 48 | F 3 yr : FW |
| 26 | 63 | F | 2 yrs | 36 | F 2 yr : FW |
| 27 | 70 | M | 2.5 yrs | 36 | F 5 yr : FW |
| 28 | 56 | F | 1.5 yrs | 12 | FR |
| 29 | 64 | F | 2.5 yrs | 11.5 | FR |
| 30 | 54 | F | 4.0 yrs | 30 | FR |
| 31 | 79 | F | 3.0 yrs | 48 | FR |
| 32 | 70 | F | n.k. | 15 | FR |
| 33 | n.k. | M | 5 mts | 17 | FR |
| 34 | 84 | F | n.k. | 36 | FR |
| 35 | n.k. | F | n.k. | 20 | FR |
| 36 | 48 | M | 40 yrs | 15 | FR |

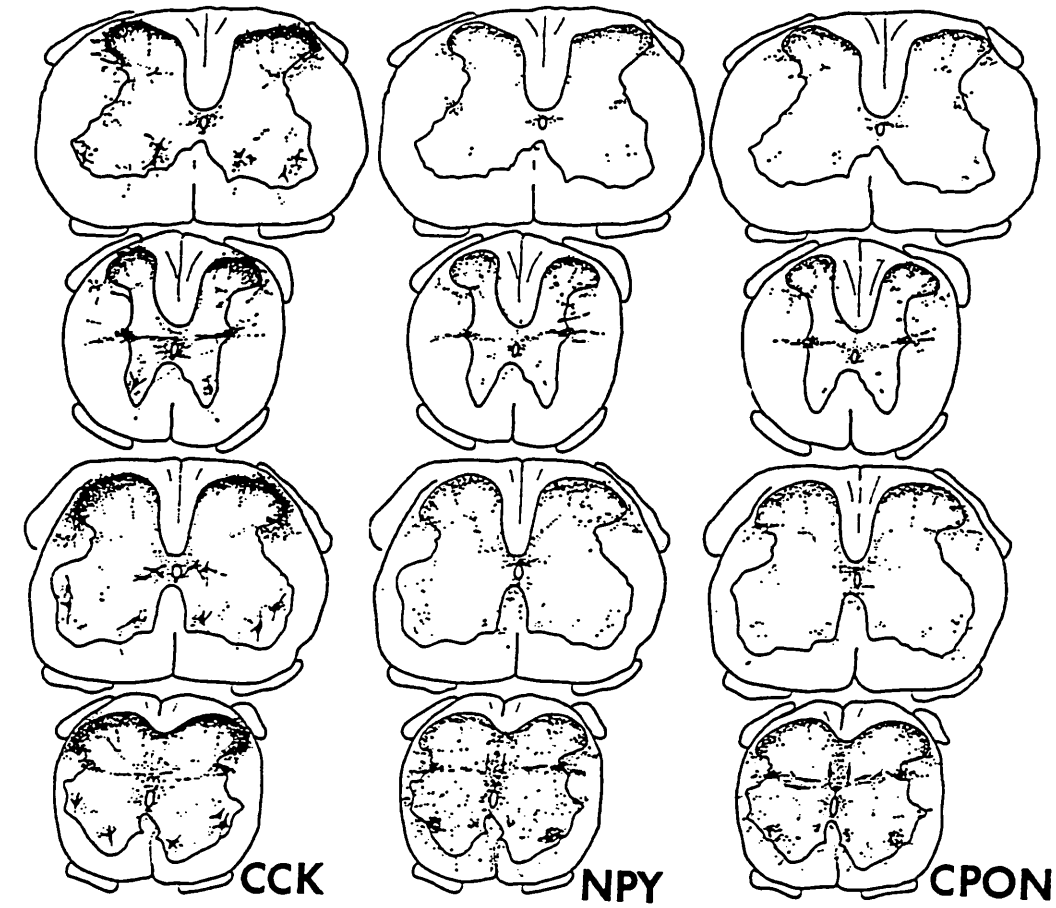
M, male; F, female; yrs, years; mts, months; F x yr, denotes tissues had been fixed in formalin for x years; FW, tissue transferred and processed to wax; FR, denotes tissues were fixed under ideal conditions for immunocytochemistry; n.k, not known; CA, cerebrovascular accident (stroke); CVAn, cardiovascular aneurysm; P, pneumonia; Ca, carcinoma of stomach, colon, bronchus or metastatic; CML and AML, chronic or acute myelogenic leukemia; CVD, cerebrovascular disease; MDP, myelodysplasia; CA, cardiac arrest.

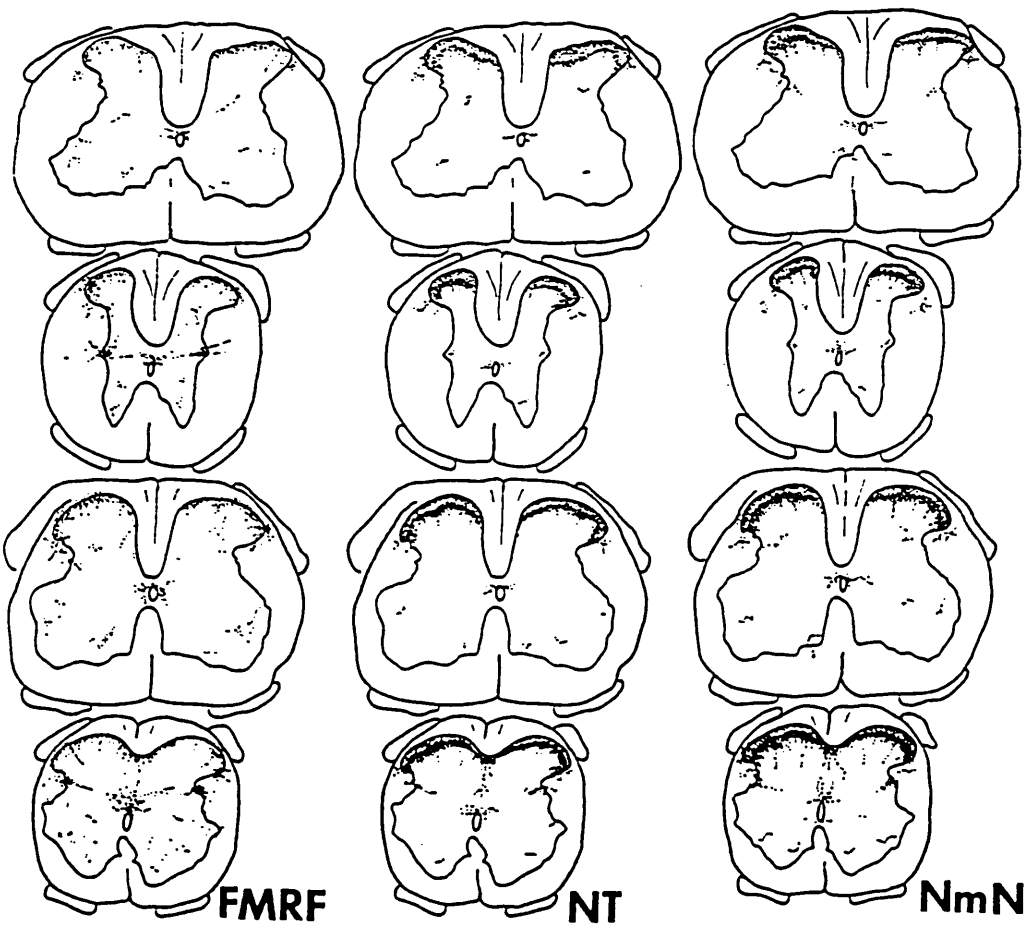
# APPENDIX XII

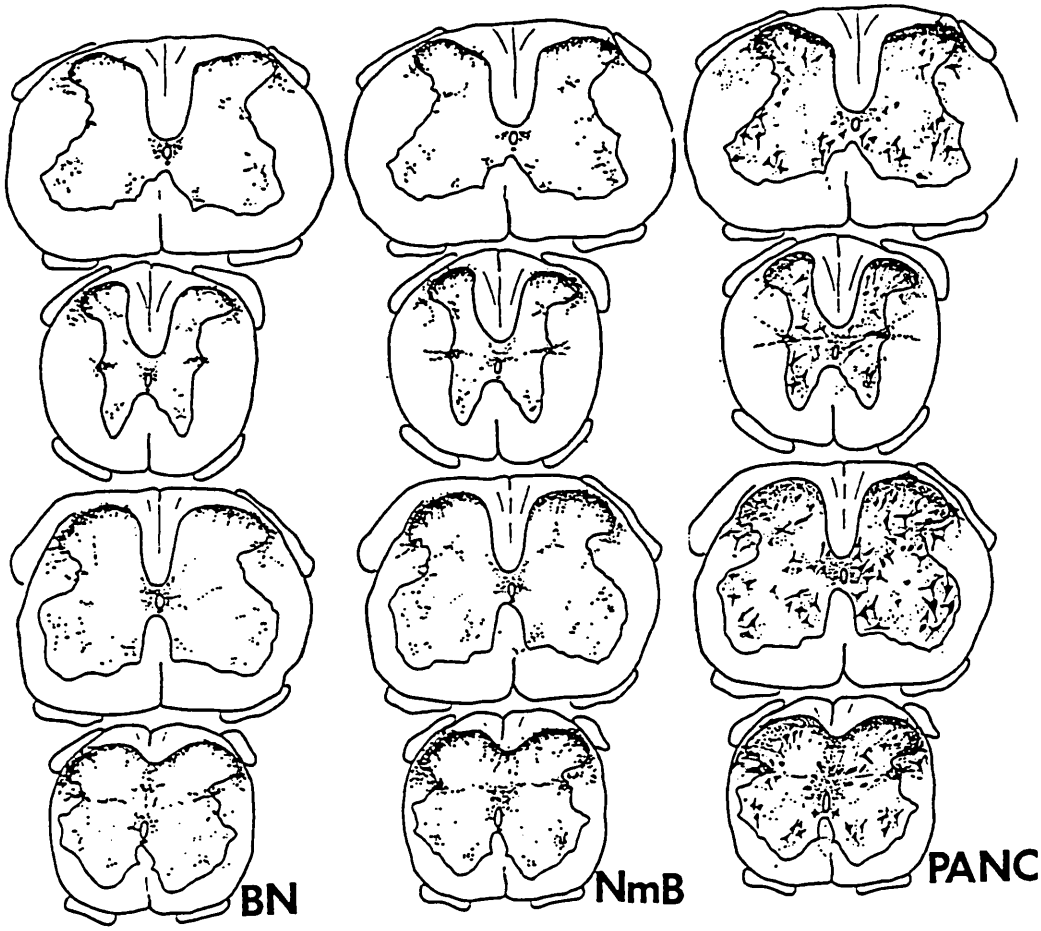
## DISTRIBUTION* OF PEPTIDES, AMINES AND ENZYMES IN THE RAT SPINAL CORD (* see footnote)

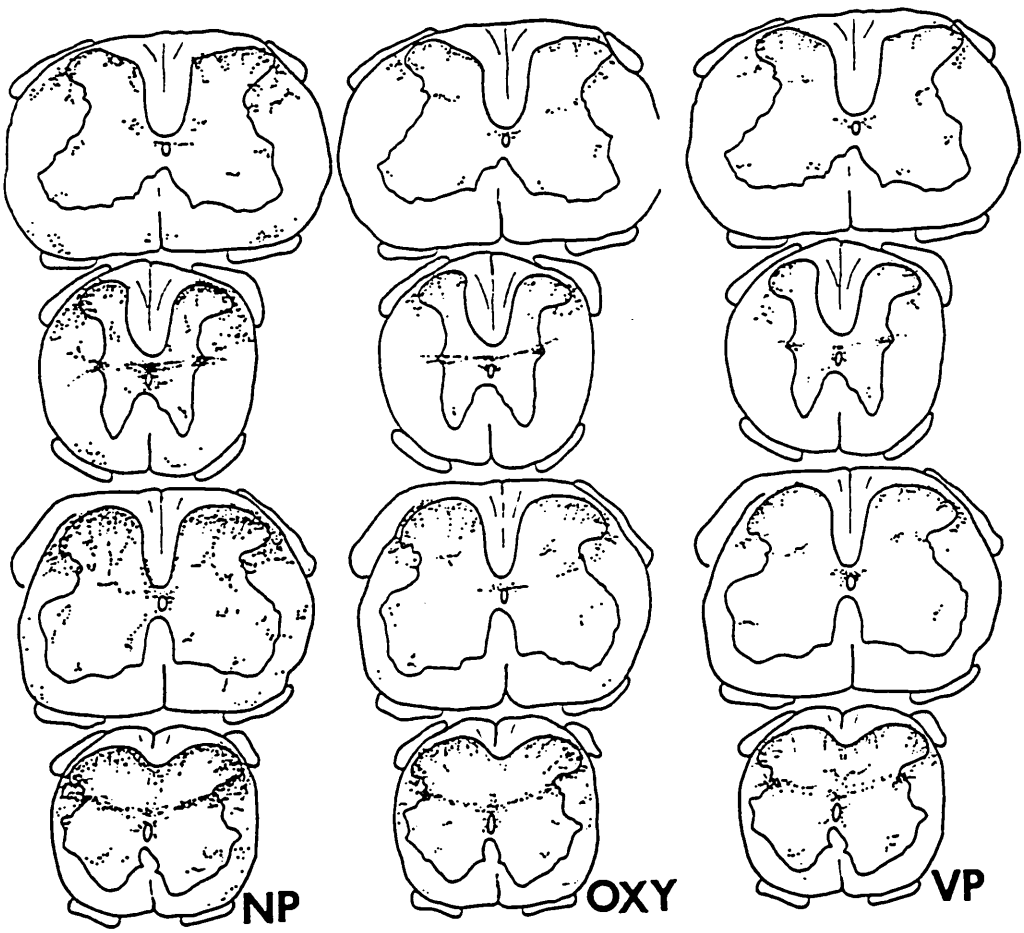
*Immunoreactive cell bodies in laminae I-III have not been included in the above. For the distribution of cell bodies in this region of the spinal cord refer to Tables 4.2.3 and 7.1.

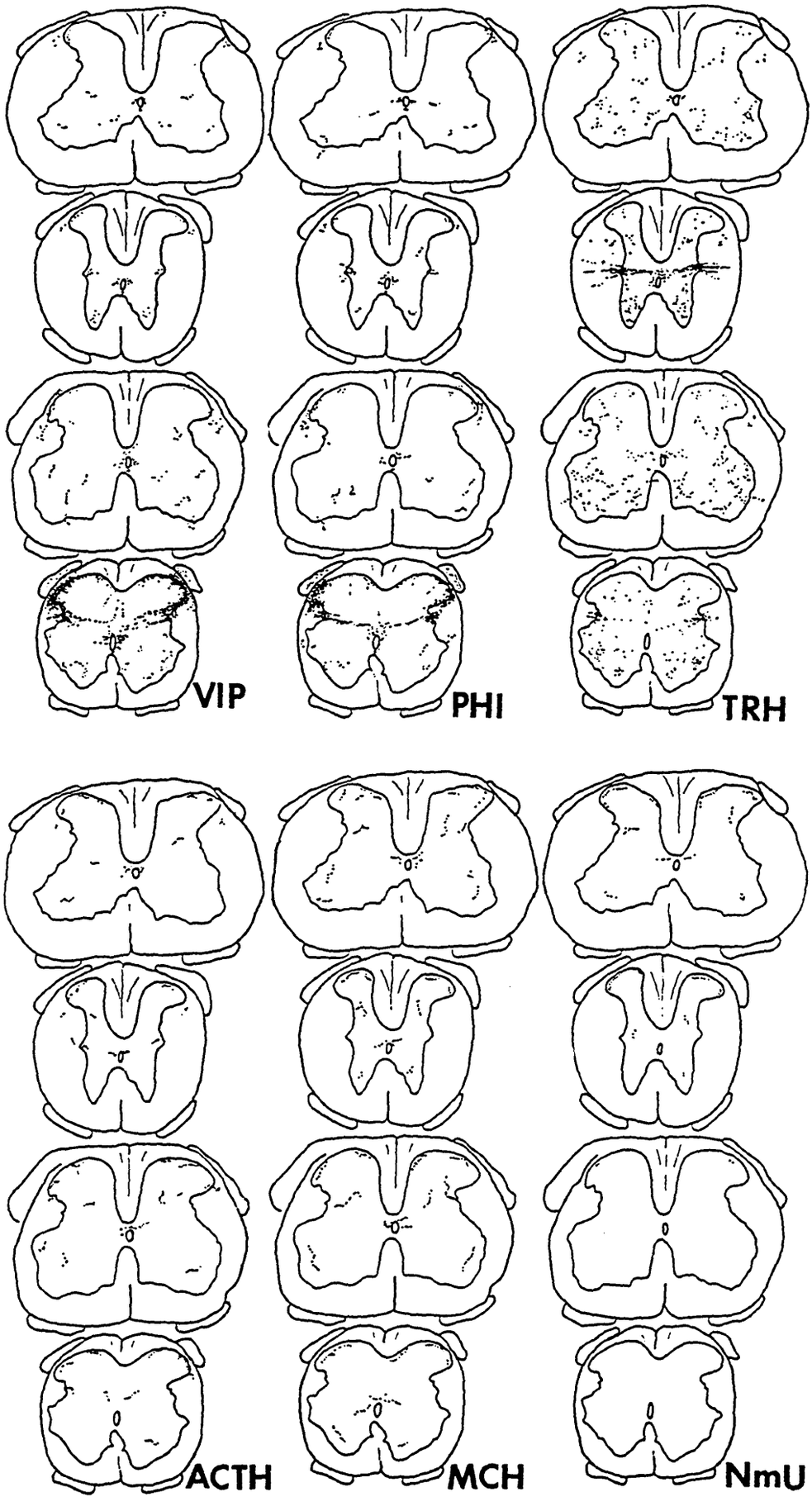

C5
T8
L4
S1
CGRP
SP
ENK
SOM
GAL

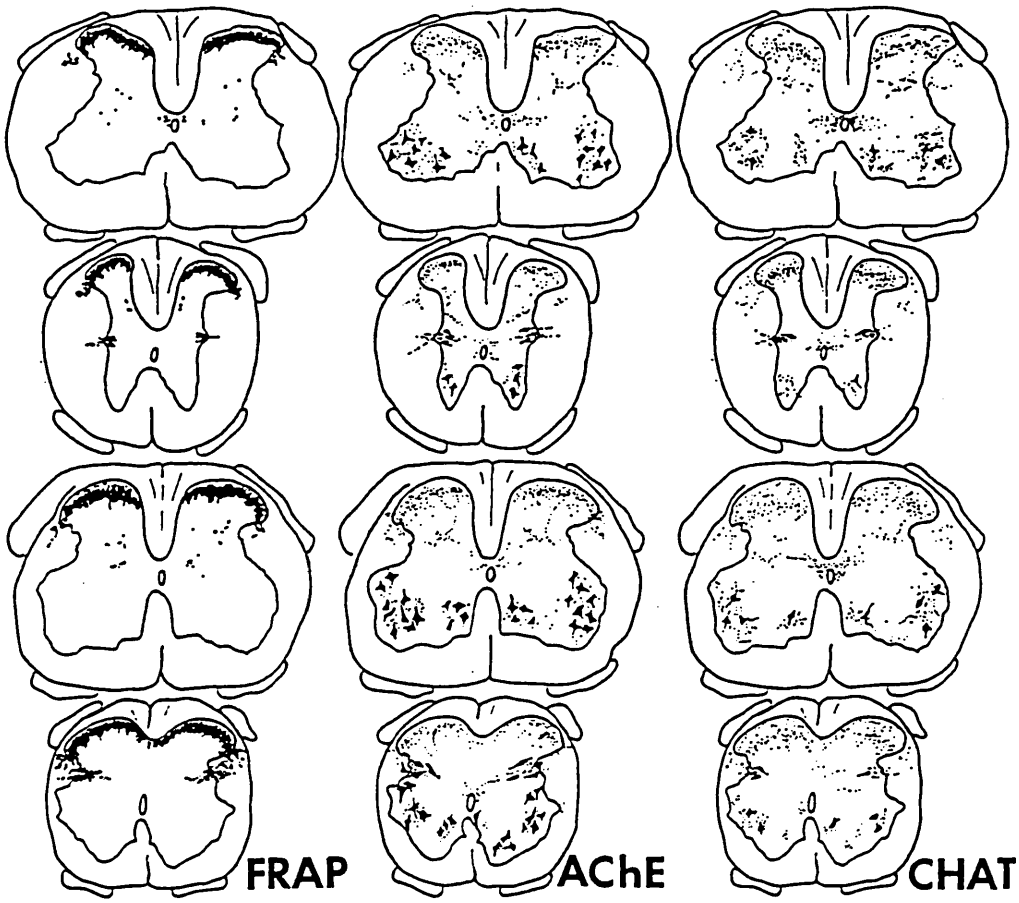

CCK
NPY
CPON

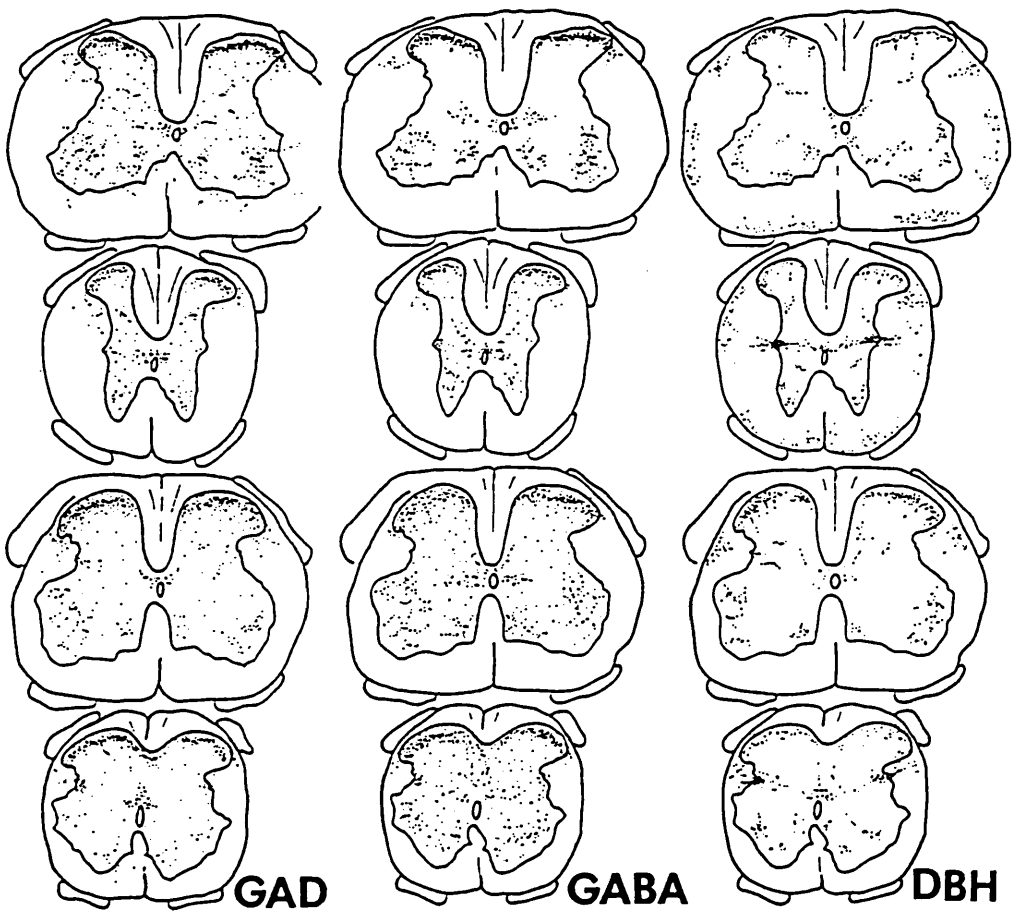

FMRF
NT
NmN

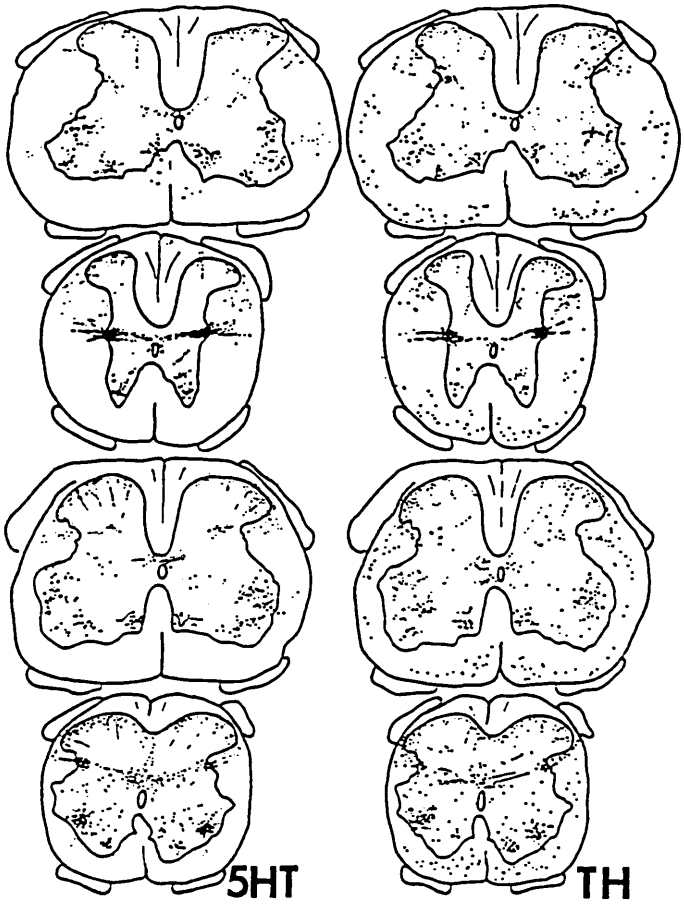

BN
NmB
PANC


NP
OXY
VP


VIP
PHI
TRH
ACTH
MCH
NmU


FRAP
AChE
CHAT


GAD
GABA
DBH


5HT
TH

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