Phenotypic differences among ~~adult rat~~ primary ~~dorsal root ganglion~~ sensory neurons *in vivo* and *in vitro*

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Abstract

An influence of the peripheral target tissue on primary afferent neuron phenotype in the adult dorsal root ganglion has been studied using *in vivo* and *in vitro* techniques.

Firstly the chemical phenotype of dorsal root ganglion neurons innervating skin, muscle or joint of the rat hindlimb, was studied by retrogradely labelling afferent neurons from the different target tissues with fluorescent dye. The target-identified afferents were then counterstained, in sections of dorsal root ganglia, for four putative transmitters or transmitter-related markers of subpopulations of primary afferent neurons: the enzyme thiamine monophosphatase, and the neuropeptides substance P, calcitonin gene-related peptide and somatostatin. Retrogradely-identified afferents were also immunostained with RT97, which labels large light neurons.

The distribution of the four markers among primary sensory neurons was found to be related to the peripheral target they innervated, as was the proportion of large light neurons labelled from each tissue.

The distribution of low-affinity receptors for nerve growth factor was studied among retrogradely-labelled skin and muscle afferents. A higher proportion of muscle afferents than skin afferents were immunoreactive for the NGF receptor.

The possibility that the target-phenotype relationship was modifiable in response to a change in the peripheral environment, was studied by producing a chronic sterile inflammation in an area of skin from which skin afferents were retrogradely labelled. The results suggest that an increased proportion of RT97-positive skin afferents contained substance P immunoreactivity ipsilateral to the inflammation.

An alternative system was used to investigate dynamic target influences on chemical expression by retrogradely-labelled sensory neurons: maintenance of dissociated dorsal root ganglia

(incorporating the target-identified neurons) *in vitro*, where diffusible factors representing target-derived influences could be added to, or removed from, the culture medium. The first experiment indicated that nerve growth factor may regulate thiamine monophosphatase enzyme activity *in vitro* in the total neuron population (as suggested previously *in vivo*), and in muscle afferents. In the second experiment, medium conditioned by myotubes was found to increase neuronal survival, with a selective effect on RT97-positive neurons, and any effects on peptide expression by skin afferents were secondary to this.

A relationship between phenotype and peripheral target has therefore been demonstrated in adult rat primary afferent neurons, along with the possibility that this relationship is plastic.

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"all that is human must retrograde if it does not advance"

Edward Gibbon

The Rise and Fall of the Roman Empire

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List of abbreviations

α-	anti-
AP	action potential
Ara C	cytosine arabinoside
ATP	adenosine triphosphate
BDNF	brain-derived neurotrophic factor
C	cervical
CGRP	calcitonin gene-related peptide
cm	centimetre
CNS	central nervous system
CV	conduction velocity
d	days
DRG	dorsal root ganglion
DY	diamidino yellow
E	embryonic day
F14FCS	F14 + 10% FCS
FB	Fast Blue
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
FRAP	fluoride-resistant acid phosphatase
GAP-43	growth-associated protein 43
h	high-intensity
hr	hours
HRP	horseradish peroxidase
HS	horse serum
IgG	immunoglobulin G
jA	ankle joint
jK	knee joint
l	low intensity
L	lumbar
LHS	left hand side
m	medium intensity
MCM	myotube-conditioned medium
mGA	gastrocnemius muscle
μg	microgram
μl	microlitre
μm	micrometre
mm	millimetre
mRNA	messenger RNA
mTA	tibialis anterior muscle
NGF	nerve growth factor
NGFr	NGF receptor
P	postnatal day
PBS	phosphate-buffered saline
PPTA	preprotachykinin A
RHS	right hand side
S	sacral
Sa	saphenous area skin
s. c.	sub cutaneous
scra	scraped skin
Su	sural area skin
T	thoracic

TMP	thiamine monophosphatase
Ti	tibial area skin
TRG	trigeminal ganglion
TRITC	tetramethylrhodamine isothiocyanate
TX	Triton-X-100
VIP	vasoactive intestinal peptide

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CHAPTER ONE: Introduction

1.1 The problem under investigation

Chemical phenotype in neurons, although broadly defined by genotype, is under environmental control. This conclusion has been reached as a consequence of experiments involving neurons from both the central and peripheral nervous systems.

Most of the experiments examining this topic have been performed on developing systems, including migrating neural crest cells (Le Douarin & Teillet, 1974) and embryonic rat mesencephalic raphé neurons (Foster *et al.*, 1988) *in vivo*; and neonatal rat sympathetic ganglion neurons (Patterson & Chun, 1974) and embryonic chick sensory neurons (Mudge, 1981) *in vitro*. There is, however, evidence that mature neurons, both *in vitro* (Kessler & Black, 1982) and *in vivo* (McMahon & Gibson, 1987; McMahon & Moore, 1988; McMahon *et al.*, 1989), can, when presented with a novel environment, alter their phenotypic expression.

The environment of a neuron is complex and dynamic, including such influences as: synaptic activity, neurotransmitters and modulators from local neurons and terminals; neurohumoral signals from distant neurons; trophic and other factors produced by non-neuronal (glial) cells, macrophages and other unidentified components of tissues; local gradients of metabolites; electrical fields; and exogenous stimuli.

The particular problem addressed in this thesis is whether, in the case of adult rat primary sensory neurons, the peripheral target tissue - incorporating the influences in the environment of the afferent terminals which are peculiar to the tissue type - contributes to determining neuronal chemical phenotype. This has been investigated both in the 'control' *in vivo* situation, and after manipulation of environments both *in vivo* and *in vitro* to assess the plasticity of the relationship.

1.2 Why the problem is interesting

The primary sensory neuron is specialized to provide a passage for information about the external and internal environments, to the central nervous system (CNS). It is important for the correct functioning of the somatosensory system that primary sensory neurons are specialized to perform different tasks and make appropriate connections to particular peripheral receptors or target tissues and to central, second-order, neurons.

The heterogeneity of primary afferent neurons has been examined extensively at different levels of the neuron, for instance: the type of sensory receptor and axon, the presence of putative neurotransmitters and modulators in the cell body in the dorsal root ganglion (DRG) and in peripheral and central terminals, the peripheral target, electrophysiological properties, and branching patterns of central terminals. By any of these characteristics, the population of primary sensory neurons may be classified into a range of subgroups which, in some cases, correspond to subgroups defined by a different characteristic. For example, various inter-relationships between characteristics such as functional class, peripheral receptor, central terminals and peripheral target of the primary sensory neuron have previously been investigated in some detail (described in Chapter 2).

Meanwhile, the relationship between the presence of certain chemical markers found in primary afferents, and the neuron's function within the somatosensory system, is less well understood. 'Chemical markers' refers to a wide range of intracellular peptides, enzymes, hormones and structural components, and surface antigens and receptors, which have been found in subgroups of the primary afferent population (also described in Chapter 2). An examination of marker subgroups in relation to the peripheral target tissue innervated by those neurons may provide insight into the specialization of different primary afferents with particular functions.

The markers chosen for study here were the neuropeptides calcitonin gene-related peptide (CGRP), substance P and somatostatin

which are putative neurotransmitters/modulators, and the enzyme thiamine monophosphatase (TMP) which may be involved in the synthetic or degradation pathway of a putative nucleotide transmitter, adenosine triphosphate (ATP). If such markers were found to be restricted to (i.e. label specifically) different target-classes of primary sensory neurons, the knowledge of which afferents contain which markers might help unravel the function both of the afferents and of the transmitter-related markers.

Such knowledge might be used to develop pharmacological agents that could modulate, selectively, sensory information from one type of peripheral tissue. Furthermore, if a relationship between markers and peripheral target was capable of plasticity in the mature animal, such that the spectrum of neuronal markers changed if the target environment of the neuron was changed, this would enable the investigation of the signals responsible for neuronal specification and response to injury, with respect to function and neurotransmitter type. Some appreciation of the relative contribution of intrinsic and epigenetic determinants would further the understanding of development, of the extent of adaptation and compensatory mechanisms operating in injury, and of the scope and results of neuronal transplant experiments.

1.3 The approach to the problem

The experimental approach employed in this work was that of retrograde labelling of primary afferent neurons from different target tissues such as skin, muscle and joint, in combination with histochemistry to demonstrate TMP activity, and immunocytochemical localization of neuropeptides, in neuronal cell bodies. An important, additional method of characterizing the target-identified neurons, was the use of RT97 immunostaining (Lawson *et al.*, 1984; Lawson & Waddell, 1985), to indicate whether neurons were large light (myelinated fibres; RT97 positive) or small dark (unmyelinated fibres; RT97 negative). In this way, the proportions of fibre types retrogradely labelled from the different tissues could be examined, along with the distribution

of the chemical markers between large light and small dark neurons, and thus more information was provided about the functions of the different target classes of primary sensory neurons.

The development of the technique, along with accompanying basic methods, is described in Chapter 3. The technique was extended, as described in Chapter 7, to include maintenance of retrogradely-identified neurons *in vitro*. Here the neuronal environment was manipulated using different culturing media, with subsequent phenotypic characterization of neurons.

1.4 Why this approach was used

Retrograde identification of afferents from peripheral tissues was necessary because a morphological examination of primary sensory neurons at the level of the dorsal root ganglion, where chemical phenotype was to be examined, yields no information about the target to which each cell body sends a peripheral axon. Reasonable numbers of neurons can be identified with which to generate a chemical profile of afferents supplying different targets. Molander *et al.* (1987) performed a similar study of the distribution of the same four markers among target classes of DRG neurons which had been retrogradely identified after application of tracer to the cut ends of relatively pure peripheral nerves. In the present work the complications introduced by axotomizing the afferent axons were avoided, because tracer was injected into peripheral target fields. Molander *et al.* (1987) did not use RT97 immunostaining to characterize the target-identified neurons, and did not perform detailed counts but instead made estimates of the distribution of markers. The present work is therefore an improvement upon theirs in the above three respects.

While intracellular recording and exploration of peripheral receptive fields [as in the work of Lawson *et al.*, 1987; McCarthy & Lawson, 1989] is the definitive method for identifying the specific peripheral target area, and, in addition, the receptor type, of single neurons in the ganglion for subsequent chemical

characterization, such a technique cannot easily generate data on chemical phenotype for a large sample of neurons.

Maintaining target-identified neurons *in vitro* provides a system for investigating a certain type of environmental influence on neuronal chemical expression: that of diffusible factors. Neurotrophic substances and conditioned medium, chosen to represent the sorts of target-derived instructive factors which might be encountered by neurons *in vivo*, can be added to, or removed from, the culture medium, thus altering the neurons' environment. To perform similar experiments on neurons *in vivo* would be very much more difficult, and the results would not be easy to interpret because of the possibility of unknown interactions with other cells and systems in the whole organism. This last point, however, also indicates one drawback of working with simplified systems *in vitro*: a relationship seen to operate in the culture dish may not represent the true *in vivo* situation precisely because other influences, which would operate *in vivo*, have been removed.

1.5 Specific questions addressed by this work

The questions addressed in each chapter were as follows:

To what extent is the primary sensory neuron's expression of chemical markers (Chapter 4) and neurotrophic factor receptor (Chapter 5), related to peripheral target in the adult rat?

Can the target-phenotype relationship be changed *in vivo* by altering the environment at the peripheral terminals? (Chapter 6).

Is transmitter-related enzyme (Chapter Eight) or neuropeptide (Chapter Nine) expression plastic in target-identified neurons under novel conditions *in vitro*?

Conclusions are presented in Chapter 10.

CHAPTER TWO: The primary afferent neuron

This chapter is divided into three sections: (2.1) Development, (2.2) Physiology and (2.3) Chemistry of primary afferent neurons.

2.1 Development

2.1.1 Genesis of sensory neurons and formation of dorsal root ganglia

Dorsal root ganglia develop from the neural crest, which arises from the neural tube in the neurula (at about E2 in the chick). While spinal cord and brain develop from the neural tube, neural crest cells migrate to colonize variously distant sites, which will become spinal and autonomic ganglia, glial cells of the PNS and some non-neuronal tissues. One theory suggests that somites are also the origin of part of the DRG cell population (Altman & Bayer, 1984). A third source of neural tissue in the embryo is the sensory placodes in the head region, which contribute along with the neural crest cells to formation of cranial sensory (in Barnes & Lindsay, 1985) and autonomic ganglia. Trigeminal ganglia are analogous to spinal sensory ganglia, but contain both crest and placode-derived neurons.

Migration of neural crest cells (after E2 in the chick) is under environmental influence (Noden, 1975), controlled by the transient availability of pathways lined with extracellular matrix proteins, such as fibronectin, laminin and collagen (reviewed in Sanes, 1983; also see Jessell 1988; Lander 1989). When crest cells have aggregated at appropriate sites, differentiation begins. The experiments of Le Douarin in particular demonstrate that neural crest cells possess a range of developmental potentialities (discussed in Le Douarin, 1984a,b), but they differentiate according to the environment at which they arrive after migration, into sensory or autonomic cells (Le Douarin & Teillet, 1974; Le Douarin, 1980).

Neural crest precursors are still undergoing division during migration and the early stages of colonization. This can be

demonstrated by labelling dividing cells with ^{3}H-Thymidine, which when injected into a pregnant rat is incorporated into newly-synthesized DNA in cells in the developing fetus. The 'birthdays' of DRG cells (i.e. the last cycle of mitosis) can be determined by labelling fetuses at successive ages.

Most DRG neurons are generated over the period E11 to E14 in the rat (Lawson *et al.*, 1974; Altman & Bayer, 1984, say E12 to E15 because they called the first detected day of gestation E1, instead of E0), with a slight rostral-to-caudal gradient in the proportion formed on successive days. At each level, the large diameter neurons stop accumulating label before the small diameter neurons, i.e. large diameter neurons are born earlier. In the avian embryo, two populations of DRG cells are segregated in the ventrolateral (large diameter, proprioceptive neurons) and dorsomedial (small diameter neurons) halves of the DRG, and birthdate studies (Carr & Simpson, 1978) indicate that the dorsomedial neurons are not all born until 1 day after the ventrolateral neurons have become post-mitotic.

Neural crest precursors also give rise to the satellite (glial) cells of the DRG, which by ^{3}H-Thymidine labelling are still seen to be dividing at E18 (Altman & Bayer, 1984).

2.1.2 Axonal growth and target projection

After birth of the neuron, morphology alters as the central and peripheral projection axons appear from opposite poles of the cell, which begins at E11 in the rat as indicated by GAP-43 (growth-associated protein) labelling of growing axons (Reynolds *et al.*, 1989), i.e. almost immediately the first neurons are born. The initial portions of each axon eventually fuse to form one stem axon, an event which was shown by Mudge (1984) to occur in embryonic chick DRG cultures under the influence of embryonic rat Schwann cells.

Growth of individual axons depends on locomotive activity of the specialized structure at the neurite tip, known as the growth cone. The growth cone probably interacts with extrinsic factors (such as adhesion, mechanical routes, chemotaxis and electric fields, all of

which are known to be important *in vitro*) to produce and stabilize local membrane extensions (reviewed by Letourneau, 1985).

It is also unclear how, on a larger scale, developing axons reach their appropriate targets. Again, it seems that generation of the stereotyped pattern of nerves in the limb is influenced by the environment which the growing axons traverse, as on the smaller scale of neurite growth: mechanical guidance, differential adhesiveness, electrical activity and tropic gradients emanating from target tissues probably all play a part. Some of the facts we do know are illustrated by the following work:

In the rat, spinal nerves (containing motor and sensory axons) form at E12 (Reynolds *et al.* 1989; Altman & Bayer 1984) and are found waiting at the base of the limb bud at E13. Here they apparently intermix and reorganize into peripheral nerves, and at E14 innervate the limb bud as it is beginning to differentiate. The first nerve branches are clearly directed out towards the peripheral epidermis rather than into the centre of the limb, which contains undifferentiated muscle masses. Axons penetrating the epidermis down about half the length of the limb are first seen at E15. At E15 the first muscle nerves are seen to grow towards proximal muscle tissue, forming fine branches at E15-16 and clusters of terminals at E17 (Reynolds *et al.*, 1989).

The finding by Reynolds *et al.* (1989) that innervation of the epidermis in the rat occurs 1-2 days ahead of that of muscle at the same proximodistal level is interesting in the light of evidence from the chick that sensory fibres follow the pathways in the developing limb bud formed by motor axons (Scott, 1986; Landmesser & Honig, 1986; but see Scott, 1988).

However unlikely it may seem that axons can find their targets in an environment of semi-differentiated tissue, the adult projection pattern of peripheral nerves is established in the embryo under these conditions, as suggested by recording from embryonic nerves (Scott, 1982), and retrograde labelling of motor and sensory neurons achieved by injection of HRP into muscle and skin of the embryonic mammal

(Smith & Hollyday, 1983) and chick (Scott 1982; Honig 1982). Reynolds *et al.* (1989) suggest that factors such as epidermal growth factor may be released by developing skin in a timed proximodistal fashion, encouraging proximal innervation to occur before distal. Other unknown factors may similarly be released by muscle as part of the differentiation process.

For sensory neurons with their bifurcating axons, two types of target projection and connection must be made - central and peripheral, and there must be some correlation between the two so that in the adult the appropriate central pathways or effector cells receive the peripheral sensory information they are best equipped to use. There are two classes of relationship between central and peripheral terminals - first that fibres innervating different receptor types have characteristic branching patterns in the spinal cord with respect to the three-dimensional shape and space that the branching occupies, and second, the correlation between peripheral receptor or target and the specific laminar location of the central terminal arborization and especially of synaptic boutons. Examples of the different sorts of terminations are provided by the hair follicle afferent in the rat (myelinated; Shortland *et al.*, 1989) and the polymodal nociceptor in the guinea pig (unmyelinated; Sugiura *et al.*, 1986).

The hair follicle afferent exhibits a flame-shaped arbor with a restricted mediolateral spread of up to 75µm, with the dorsoventral extent of the 'flame' being long at about 150µm. Rostrocaudally, the arbours of adjacent collaterals are continuous for 300µm. The specific site occupied by the flame of terminals in the spinal cord extends from lamina IV to inner II (Woolf, 1987).

The polymodal nociceptor, in contrast, has central terminals with a mediolateral spread of 200µm which is greater than the spread in the dorsoventral axis (150µm). Rostrocaudally, only a few collaterals exhibited arbours as extensive as the area just described, with distant collaterals having minimal, discontinuous branching patterns. Most of the arborizations and boutons are located in laminae I and II. These two types of receptors are

therefore provided with distinct patterns and sites of central terminations, so that different second order neurons will be involved in receiving their information.

Fitzgerald *et al.* (1989) found that afferent collaterals first penetrated the dorsal horn grey matter at E15, at the same time that peripheral skin innervation begins. Flame-shaped arbors of hair-follicle afferents are first observed at E19 (Beal, 1982).

2.1.3 Neuron-target interactions

The next event in the development of sensory innervation is a phase of neuronal death, which in the chick embryo DRG and ciliary ganglion, for example, occurs over the same period that growing axons reach their target - E5 to E12. Even before the phenomenon of natural cell death was discovered (Hamburger & Levi-Montalcini, 1949; Hamburger & Oppenheim, 1982), the idea had already been suggested by various other groups that a positive correlation existed between the number of cells in nerve centres, and the size of their projection targets. The changes in neuron number resulting from removal or addition of limb buds in embryos suggested that neuron survival involved the acquisition of a target-derived agent that was in short supply. Rather than being due to failure to reach their targets, it seems that competition between neurons on reaching targets is the cause of neuronal 'pruning'.

The target-derived neurotrophic factor hypothesis has developed in tandem with work on Nerve Growth Factor (NGF), the most studied of neurotrophic factors. NGF promotes the survival of embryonic sensory and sympathetic neurons *in vitro* (see Levi-Montalcini & Angeletti, 1968, for review of early work), and prevents loss of these neurons *in vivo* if administered during the period of natural neuronal death (Hamburger *et al.*, 1981; Oppenheim *et al.*, 1982). The target fields of sensory and sympathetic neurons contain trace quantities of NGF protein, and messenger (m)RNA for NGF, in proportion to their sympathetic innervation density (Heumann *et al.*, 1984; Shelton & Reichardt, 1984). A current theory (Davies, 1988) suggests that sensory axons are

subject to pruning due to restricted availability of NGF in their target fields. Neurons which compete successfully for NGF will survive.

The innervating neurons possess specific cell surface receptors which mediate the uptake of NGF in the target tissue (reviewed in Thoenen & Barde, 1980). The internalized receptor-ligand complex is conveyed by fast axonal transport to the cell body (Johnson *et al.*, 1987), where the mechanism of action on cell metabolism is unknown. One suggestion (Martin *et al.*, 1988) is that NGF inhibits endogenous destruction mechanisms, because sympathetic neurons deprived of NGF *in vitro* survive longer in the presence of protein synthesis inhibitors. The specific action of NGF on sensory neurons appears to be restricted to those neurons which are of neural crest origin, not of placodal origin (Davies & Lindsay, 1985).

In addition to target-derived support of neurons, a relationship also exists in the reverse orientation, whereby the presence of innervation is necessary to maintain some aspects of the target - for instance the contractile properties of skeletal muscle fibres can switch from slow to fast and vice versa if the appropriate type of motoneuron is allowed to innervate them (Buller *et al.*, 1960). Similarly, the presence of taste buds depends upon the integrity of an afferent nerve supply (Zalewski 1969, 1974), and human skin shows remarkable changes after denervation (Sunderland, 1978).

2.2 Physiology

In this section, four ways in which the heterogeneous population of DRG neurons has been examined and classified by physiology and function are discussed, along with the relationships between such classifications.

2.2.1 Fibre types and cell sizes

Two classifications are currently used for peripheral nerve fibres, which depend on fibre diameter and conduction velocity. The first, by Erlanger & Gasser (1937), deals with all the fibre types found in a peripheral nerve (sensory, motor and autonomic) and is still used for cutaneous fibres (Aβ, Aδ and C fibre categories). The second, by Lloyd (1953), classifies only afferents, and is used for muscle sensory fibres. Deep tissue and visceral afferents may be grouped according to either system.

There is a positive correlation between fibre diameter and maximum conduction velocity (CV). The smallest diameter fibres - C or Group IV, lack an insulating myelin sheath, while Aδ or Group III fibres are thinly myelinated, and the remaining fibres are thickly myelinated.

It has long been thought that size of the afferent neuron cell body is proportional to the axon diameter, and thereby related to presence of myelin (Ranson, 1912). A section of a DRG viewed by light or electron microscopy exhibits two morphological classes of DRG neuron, the large light and the small dark (reviewed by Lieberman, 1976). Each type has its characteristic size distribution, but the two distributions partly overlap, both in mouse (Lawson, 1979), and rat (Lawson *et al.*, 1984) where cross-sectional areas of small dark neurons range from 80 to 460 μm^2 and the large light neurons from 220 to 1440 μm^2. Harper & Lawson (1985) found a positive correlation between CV and cross-sectional area of soma in rat DRG, suggesting that the myelinated fibres, having higher CVs, also had larger cell bodies. Lee *et al.* (1986) examined single lumbosacral DRG neurons in cat, and found a positive correlation

between soma size and peripheral CV only for A cells, and not for C cells of 35-50 μm in diameter. (NB. cell diameters vary with species, age and fixation procedure).

A further aspect of the relationship is the staining pattern obtained with a monoclonal antibody, RT97 (Wood & Anderton, 1981), on DRG neurons. There are three species of neurofilament in nerve cells: 200, 158 and 60 kD MW, and RT97 reacts with phosphorylated epitopes of the two higher molecular weight forms. Neurofilaments are a subset of intermediate filaments found in all neurons, but RT97 was found to label the somata of a specific subpopulation of dorsal root ganglion neurons - the large light population (Lawson *et al.*, 1984). Friede & Samorajski (1970) also showed, by counting neurofilaments in rat and mouse sciatic nerves, that axon diameter correlated with number of neurofilaments in an axonal section.

RT97 immunoreactivity has since been directly correlated with neurons that have myelinated axons (with a small number of exceptions)* by Lawson & Waddell (1985), who recorded intracellularly from DRG neurons to classify cells according to conduction velocity, and then filled the cells with dye, enabling each one to be visualized in subsequent immunohistochemical processing. RT97 labelling of DRG sections can identify myelinated neurons by an immunocytochemical reaction, a characterisation which otherwise requires single-cell electrophysiological studies.

Various studies have examined possible correlations between soma electrophysiological properties, and afferent fibre type. Cameron *et al.* (1986) found action potential (AP) duration in the soma of cat L7 and S1 DRG neurons was inversely correlated with axon CV. Rose *et al.* (1986) looking at the same system, found the same negative correlation, but that for a given CV, neurons supplying low-threshold cutaneous receptors had shorter APs than those supplying high-threshold receptors. Harper & Lawson (1985) however, report that in rat DRG neurons, most Aδ fibres with slower CVs had fast soma action potentials.

Fulton (1987) examined the same relationship in rats during the first two weeks of postnatal development. Because myelination of

* Lawson (personal communication) now suggests that up to 10% of small dark cells may be RT97 positive.

afferent fibres does not begin until near the time of birth (Friede & Samorajski, 1968), the conducting properties of A fibres change markedly during the early postnatal period: it is not until postnatal day (P)9 that myelin sheathing is sufficient to result in separation of the A and C waves in the afferent volley received by the spinal cord after peripheral nerve stimulation (Fitzgerald, 1988). Before that time, only one wave of activity is seen. Similarly, Fulton (1987) found that at P1, CVs of single neurons fell into one tri-modal but continuous range entirely below 1 metre/second. At P14, however, the range of CVs was spread much wider, up to 12 m/s. Soma action potentials recorded at P1 were of three types, duration being inversely correlated with CV, and most spikes had at least a slight inflection on the repolarization phase. By P14, while a subpopulation of neurons still possessed a broad spike with an obvious inflection, the remainder displayed a variety of sharper, faster spikes: these were the neurons with CVs now well above 1 m/s. For neurons with CVs of less than 8 m/s, there was a negative correlation between CV and the action potential duration.

The different membrane properties among primary afferent neurons are due to the different ionic conductances operating, and developmental changes probably reflect the incorporation of a new set of channel types into the membrane, a process which may even be directed by influences from the peripheral target.

2.2.2 Peripheral receptors

Peripheral sensory receptors take many anatomical forms: the receptor may exist as a specialized organ innervated by the end of the sensory axon - such as the Merkel cell touch receptor in skin (Burgess & Perl, 1973); the sensory axon may be specialized in its terminal organization, by, for example, wrapping around the root of a hair in the follicle in characteristic patterns (Millard & Woolf, 1988); or the axon may exhibit a 'free nerve ending' with no structural specialization, but with a distinct function as in the

case of unmyelinated nociceptive axons in skin (Kruger *et al.*, 1985), and cornea (Rosza & Beuerman 1982).

Each type of peripheral tissue boasts a unique mixture of receptor types, innervated by a matching range of fibres. Classical studies of nociceptive (Aδ and C-fibre) afferents were performed by Beck & Handwerker (1974) and Beck *et al.* (1974) in cat, Handwerker *et al.* (1987) and Lynn & Carpenter (1982) in rat, for cutaneous receptors, Mense & Meyer (1985) on cat muscle and tendon receptors, and Schaible & Schmidt (1983) for cat joint receptors. The major classes of receptors have been summarized by Lynn (1989):

In skin, Aβ fibres supply low-threshold mechanoreceptors including most hair-follicle endings (tylotrich and guard hairs); Aδ fibres innervate d-hair follicles (vellus hairs), and cold thermoreceptors and nociceptors (mostly high-threshold mechanoreceptors); finally C fibres innervate polymodal nociceptors and warmth receptors.

In muscle, Group I and II fibres innervate muscle spindles and Golgi tendon organs; Group III and IV fibres innervate a mixture of nociceptors, low-threshold pressure receptors, contraction-sensitive units and thermosensitive units, in decreasing proportion. Up to 75% of skeletal muscle sensory innervation may be in the form of free nerve endings (Stacey, 1969), between muscle fibres, in connective tissue, in blood vessel walls and in tendons.

In joints, Group I and II fibres innervate proprioceptors. Some Group III fibres are also proprioceptive, and Group III and IV fibres are nociceptive, supplying a mixture of mechanical and chemical receptors. Nerve fibre counts in cat articular nerve revealed that Group III and IV fibres predominate over Group I and II fibres (Langford & Schmidt, 1982)

2.2.3 Central termination patterns

The patterns of terminations of primary afferent fibres in the CNS are an important part of their function, because the site of the

terminal synapses determines which second-order neurons can receive fast monosynaptic inputs.

Many different techniques have been used to map central terminations, from classical Golgi staining to intra-axonal tracer injection. The following account summarizes the results from studies in which single electrophysiologically-characterized afferents were filled with the enzyme horseradish peroxidase (HRP), which was then reacted to form a visible deposit.

After entering the spinal cord, many dorsal root fibres bifurcate, sending one branch rostrally and one caudally. These give off collaterals, most densely in the segment of root entry. Collaterals penetrate the dorsal grey/white matter border and course down through the laminae.

Sugiura *et al.* (1986) traced C fibres (using transport of a plant lectin) in the guinea pig and showed most terminals were in lamina II with some spread to lamina I (but one of two polymodal nociceptors extended to laminae III and IV). HRP-filled Aδ high-threshold mechanoreceptors from skin and deep tissues in cats were shown to have terminals in lamina I and outer II only, or in two sites: lamina I and more ventrally in laminae IV and V (Light & Perl, 1979; Mense & Prabhakar 1986). Single HRP-filled low-threshold cutaneous myelinated fibres have been shown by Brown (cat; 1981a, b) and Woolf (rat; 1987) to have terminals in laminae III to VI for slow- and rapidly-adapting mechanoreceptors, and inner II to IV for hair follicle afferents. The more dense arborizations, and probably synapses, are in III to V.

If, instead of single fibres, whole nerves or tissues are labelled to give a mass labelling of tissue-specific terminals, the following patterns emerge (reviewed in Fitzgerald, 1989a). Cutaneous afferents show a dense projection to lamina II (small diameter fibres, corresponding to C fibres) and also to III and IV (like hair follicle afferents). Fine muscle afferents (not proprioceptive inputs, which go to laminae VI and VII in the ventral horn) terminate in laminae I and V (Mense & Craig, 1988) corresponding to Aδ fibre

terminations. Single muscle C fibres can, however, be antidromically activated from inner lamina II, or III (McMahon & Wall, 1985) suggesting that the labelling may be incomplete. Cat knee articular nerve projects to lamina I and laminae V and VI (Craig *et al.*, 1988), very similar to the small diameter muscle afferent projection. Visceral afferents run medially and laterally around the dorsal horn to terminate in laminae V, VII, X and contralaterally (Cervero & Connell, 1984).

2.2.4 Peripheral terminals with an efferent role

Antidromic stimulation of the peripheral stump of transected dorsal roots or sensory nerves produces vasodilatation and plasma extravasation in the skin area supplied by those axons (first shown by Stricker, 1876, and Gaertner, 1889). Exactly the same inflammatory response can be produced by cutaneous application of irritant chemicals such as capsaicin, the pungent ingredient in capsicum species (Jancso *et al.*, 1967, 1968), and this response was named 'neurogenic' inflammation because it is prevented by sensory denervation (Bayliss, 1901). These findings suggest that some primary afferent neurons have an effector role, participating in the generation of inflammation in response to tissue injury at the peripheral terminals.

Irritant-induced inflammation is not inhibited by local anaesthetic or tetrodotoxin (Jancso *et al.*, 1968; Szolcsanyi, 1984), suggesting that generation of action potentials in the sensory axon is not necessary for its onset. If a noxious stimulus excites sensory nerve endings, mediators (such as the neuropeptide substance P, present in afferent terminals: see part 2.3.2) are released onto effector tissues such as blood vessels, or have an indirect effect by causing degranulation of mast cells (Holzer, 1988). Mast cells release inflammatory mediators such as histamine and prostaglandins, and factors which cause serotonin release from platelets (reviewed in Campbell *et al.*, 1989)

Excitation can also spread, by a mechanism which is sensitive to local anaesthetic action (Jancso *et al.*, 1968; Foreman & Jordan, 1984; Foreman *et al.*, 1983), to nerve endings which have not been directly stimulated - this is called 'flare'. It was suggested by Lewis & Marvin (1927) that an axon reflex occurs within terminal branches of a single neuron, such that if the branch with a sensory ending receives a stimulus, impulses travel centrally, and from the branch point peripherally. Where the second branch supplies a blood vessel, vasodilatation results (Lewis & Marvin, 1927).

The afferent fibres responsible for peripheral vasodilatation have been characterized electrophysiologically as C fibres (Hinsey & Gasser, 1930) and as nociceptive fibres (Celander & Folkow, 1953) with polymodal receptors (Kenins, 1981). The use of capsaicin as a pharmacological tool has provided further evidence for the type of sensory neuron with an effector role. Capsaicin, applied to adult rat peripheral nerve, or administered subcutaneously, blocks irritant-induced and antidromically-evoked neurogenic oedema, in parallel with a block of "chemical pain" sensitivity (reviewed in Fitzgerald, 1983). Non-painful inflammatory compounds still have effect. Thus activation of chemosensitive sensory neurons is required for the initiation of the neurogenic response.

If capsaicin is administered to a neonate, 70% of small dark, 'B' type DRG neurons are destroyed, while the majority of the large cells remain. Counting also dorsal root fibres, Lawson & Nickels (1980) showed a 90-95% drop in the number of C fibres and a 30% drop in Aδ fibres, in neonatally-treated rats. In such rats, the neurogenic inflammatory response is permanently absent, (Jancso *et al.*, 1977), again indicating that (at least some of) the small diameter fibres, most of which are polymodal nociceptors in rat skin (Lynn & Carpenter 1982) are responsible for neurogenic inflammation.

The neurochemical effects of capsaicin are discussed in section 2.3.

2.2.5 Conclusion

Examined by a variety of physiological criteria, subpopulations of DRG neurons have specialized functions, and their anatomical characteristics also differ. This heterogeneity of primary sensory neurons is related to their functional specificity and the different targets they innervate.

2.3 Chemistry

In this section, further subgroupings according to chemical markers in DRG neurons are discussed, beginning with transmitter candidates and ending with a look at correlations between the various classifications that have been mentioned in this and the previous section.

2.3.1 The primary afferent transmitter

Sensory inputs to the dorsal horn may be coded by different transmitters as well as by the site and nature of central terminals. Exactly what the transmitter molecules in primary sensory neurons are, is still not clear (see Salt & Hill, 1983; Iversen & Goodman, 1986). however there is evidence to suggest two main types of transmitter action: fast and slow. Further, the two types of transmitter corresponding to these two types of signals may be stored in, and released by, the same afferents, therefore the potential exists for interactions between transmitter effects on the post-synaptic neuron.

Small clear and large granular vesicles coexist in the same terminals in lamina I and outer lamina II, and in identified C and Aδ fibre terminals. Small clear vesicles alone in terminals are seen in laminae I-IV and in identified Aβ, Aδ and C fibres (Maxwell & Rethelyi, 1987; Jessell & Dodd, 1989).

Low-intensity stimulation of spinal cord dorsal roots *in vitro* elicits a fast excitatory post-synaptic potential in dorsal horn neurons, while higher-intensity stimulation evokes a slow-onset, slow excitatory potential (Urban & Randic, 1984; Yoshimura & Jessell, 1989). The "fast" transmitter(s) is believed to be glutamate or a similar amino acid. There are three types of receptor for glutamate, one of which, the N-methyl d-aspartate receptor, may mediate long-lasting changes in post-synaptic efficacy. This receptor is distributed in many brain areas associated with sensory processing, and in the substantia gelatinosa (Cotman et al, 1987).

The "slow" transmitter candidates are the neuropeptides, which produce slow depolarizations when iontophoresed onto spinal neurons (Iversen & Goodman 1986). Presumably because their breakdown/reuptake is similarly slow, they

can diffuse from the synapse into which they are released, and have the potential to modify the responses of second-order neurons to other input over a long range (many μm) and time scale (minutes). For this and other reasons the neuropeptides have been termed 'neuromodulators'.

In addition to central effects, neuropeptides may have a peripheral role, being released from peripheral terminals of the same neurons which contain them in cell bodies and central terminals (first suggested by Dale, 1935) In fact their peripheral role may be more important than the central function. Receptors for peripherally-released peptides are found on blood vessels, sweat glands, hair follicles, macrophages, lymph nodes, etc. (discussed further in Chapter 4).

2.3.2 Slow transmitter candidates: the neuropeptides

More information exists about peptide location and coexistence within the DRG rather than in central or peripheral terminals. Peptides are easier to visualize in higher concentrations in the cell body, especially if colchicine is administered systemically or locally to block axon transport mechanisms. Recently, however, colchicine has been shown to affect protein synthesis (Rethelyi et al., 1988) and any results obtained with its use must be interpreted with caution.

There are more than thirty neuropeptides which have been identified within DRG neurons, and some are also found in the areas of termination in the spinal cord of C fibres, ie. laminae I and II of the dorsal horn, and in the periphery. A selection will be discussed here in order to illustrate the main features of these transmitter candidates.

<u>Substance P</u>

The undecapeptide substance P was the earliest neuropeptide discovered in primary sensory neurons (von Euler & Gaddum, 1931). It is first seen in fetal rat DRGs at E17 (Senba *et al.*, 1982; Marti *et*

al., 1987) and is present in about 20% of adult lumbar DRG neurons, of small and medium diameter (Hokfelt *et al.*, 1975), some of which are RT97 positive (McCarthy & Lawson, 1989). In the central terminals of sensory neurons in laminae I and II of the dorsal horn, substance P immunoreactivity is associated with large granular vesicles, alongside small clear vesicles of unknown content (reviewed in Jessell & Dodd, 1989). While substance P fulfils many of the transmitter criteria (Salt & Hill, 1983), the evidence is not conclusive due to the lack of a specific antagonist. However, various studies suggest that substance P-containing afferents are involved in nociceptive transmission (reviewed in Salt & Hill 1983; Jessell & Jahr, 1986; Hokfelt *et al.*, 1986; Otsuka & Yanagisawa, 1987; Jessell & Dodd, 1989). Substance P is depleted from dorsal roots (85%), spinal cord (50-60%) and DRG neurons (50-90%) by neonatal capsaicin treatment (reviewed in Fitzgerald, 1983; Lawson, 1987). Gamse *et al.* (1980) showed how such treatment also impaired neurogenic plasma extravasation by 80%, and depleted substance P from hind paw skin, leading them to propose that peripheral substance P release is responsible for neurogenic inflammation. Intradermal injection of substance P causes vasodilatation and protein extravasation (Hagermark *et al.*, 1978). Local application of capsaicin to the sciatic nerve also depletes dorsal horn substance P (Ainsworth *et al.*, 1981), as does peripheral nerve section (Barbut *et al.*, 1981). However, since capsaicin depletes other substances in addition to substance P, such work cannot prove that substance P is the nociception transmitter.

CGRP

By analyzing the primary RNA transcript of the calcitonin gene of the rat, Amara *et al.* (1982) discovered a new 37 amino acid peptide, which they named calcitonin gene-related peptide (CGRP). The mRNA for CGRP as opposed to calcitonin predominates in the peripheral nervous system, and by immunocytochemistry, CGRP has been found in a large proportion of dorsal root and trigeminal ganglion cell bodies, often entirely overlapping with the smaller proportion of sensory neurons containing substance P (Lee *et al.*, 1985; and see Dalsgaard, 1988, for a review). Some CGRP-positive neurons are RT97-positive (Lawson *et al.*, 1987). CGRP is first seen in DRGs at E17 (Marti *et*

al., 1987). CGRP and substance P are colocalized in the same vesicles in DRG somas and their axons and terminals in the guinea pig (Gulbenkian *et al.*, 1986), and the two peptides act synergistically in behavioural studies on the spinal cord (Wiesenfeld-Hallin *et al.*, 1984; Woolf & Wiesenfeld-Hallin, 1986). CGRP in the superficial dorsal horn (Gibson *et al.*, 1984) is depleted by local application of capsaicin to a peripheral nerve (Wall, 1987), and by dorsal rhizotomy (Traub *et al.*, 1989). CGRP is released peripherally where again it potentiates the actions of substance P (Gamse & Saria, 1985), and independently causes vasodilatation (Brain *et al.*, 1985).

Somatostatin

Somatostatin was described first in the bovine hypothalamus as a 14 amino acid peptide, and has since been detected in various other parts of the CNS in this form and in higher molecular weight forms, and also in a subpopulation of small sensory neurons which are distinct, in the rat, from those containing substance P (Hokfelt *et al*, 1976; Nagy & Hunt, 1982), although Ju *et al.*, (1987), saw an overlap using colchicine-treated rats. The peptide appears in fetal rat DRG at E15, two days earlier than the appearance of substance P (Senba *et al.*, 1982; but Marti *et al.*, 1987 did not see it until E17). That substance P and somatostatin function in different physiological pathways onto spinal cord neurons was shown by Wiesenfeld-Hallin (1986). Somatostatin immunoreactivity is especially dense in lamina II of the spinal cord with labelling also in lamina I; only about 20% is thought to be of primary afferent origin (for review see Dalsgaard, 1988). By radioimmunoassay, the responses of somatostatin and substance P levels in DRGs and dorsal spinal cord to neonatal capsaicin treatment were shown to be similar (down regulated in both tissues). Along with a parallel upregulation of these peptides by NGF treatment (Kessler & Black, 1981), this suggests that although they might be in separate populations of neurons, the two are under the same regulation.

VIP

Vasoactive intestinal peptide (VIP), originally isolated from the gastrointestinal tract (Said & Mutt, 1970), has a widespread distribution in both the CNS and PNS (Hokfelt *et al.*, 1982), and was first described in sacral primary sensory neurons by Lundberg *et al.*, (1978). There is some overlap between the substance P, CGRP or somatostatin neurons and the VIP population (Ju *et al.*, 1987). VIP was not seen in DRGS until P7 (Marti *et al.*, 1987). In the sacral spinal cord, VIP-immunoreactive fibres exhibit a similar distribution to retrogradely labelled visceral afferents (Kawatani *et al.*, 1983). VIP exhibits an interesting reaction to axotomy of afferent axons - VIP expression is increased in the axotomized DRG neurons (Shehab *et al.*, 1986) and in the central terminals in the spinal cord (McGregor *et al.*, 1984). Similarly, local application of capsaicin to the sciatic nerve increased VIP in the ipsilateral dorsal horn (Wall 1987).

Dynorphin

The opioid peptide, dynorphin, is expressed in various forms in cultured murine spinal and DRG cells, most frequently in sacral ganglion cells (Sweetnam *et al.* 1982). In the cat, dynorphin immunoreactivity was seen in axons and terminals in laminae I and V of sacral cord, and in dorsal roots. Only by radioimmunoassay could dynorphin be detected in DRG cells, again predominantly those of sacral levels (Basbaum *et al.* 1986). The central terminal staining pattern is similar to that of VIP. Enkephalin has a similar spinal and ganglion distribution (Jessell & Dodd, 1989).

Vasopressin and oxytocin

A further class of peptides found in DRG cells are the hypophysial hormones vasopressin and oxytocin (Kai-Kai *et al.* 1986). Levels of immunoreactivity were low, although colchicine was used for detection, and indeed other laboratories have had difficulty repeating the finding. The two peptides were found to coexist entirely within 50% of DRG neurons, and this staining was entirely complementary to RT97 immunolabelling. Immunoreactivity for

arginine-vasopressin was present in laminae I and II of all lumbosacral cord levels, and was demonstrated to be of sensory origin.

2.3.3 Fast transmitter candidates and related enzymes

The candidates for mediating the fast dorsal horn cell excitatory post-synaptic potential are the excitatory amino acids and the nucleotides.

L-glutamate

While it is difficult to collect evidence for a proposed transmitter substance which is ubiquitous in body tissues, the excitatory amino acid L-glutamate seems to fulfil many of the criteria, especially in the case of large diameter primary afferents carrying non-nociceptive information (reviewed in Salt & Hill, 1983; Jessell & Dodd, 1989). There is an excess of L-glutamate, but not of other amino acids, in dorsal roots compared to ventral roots (Roberts *et al.*, 1973). There is a high density of glutamate receptors/binding sites in the superficial dorsal horn (Greenamyre *et al.*, 1984). Glutamate detected by an antiserum to a glutamate-haemocyanin conjugate, is present in 65-70% of rat cervical DRG neurons; most of the smaller positive neurons also contain substance P (Battaglia *et al.*, 1987). The two substances are colocalized in a class of afferent terminals in the superficial dorsal horn, which contains both small clear and large granular vesicles (De Biasi & Rustioni, 1988), therefore they may be co-released. Cangro *et al.* (1985) find immunoreactivity to glutaminase - the enzyme which synthesizes glutamate from glutamine - to be elevated in 30-40% of DRG cells, these always being small cells. Duce and Keen (1983) showed DRGs *in vitro* to take up ^{3}H-glutamate into satellite cells, and ^{3}H-glutamine into small neurons, and the ganglia can convert glutamine to glutamate.

Adenosine triphosphate

Concerning nucleotides, there is evidence that adenosine triphosphate (ATP) is released from the peripheral endings of sensory nerves upon antidromic stimulation (Holton, 1959). Jahr & Jessell (1983) have examined electrophysiological effects of ATP on dorsal horn neurons *in vitro* and *in vivo*, and find it depolarizes a subset of these neurons. Fyffe & Perl (1984) further characterized the sensitive dorsal horn neurons in cat as A and C fibre mechanoreceptor input. It is not known what neuronal elements are responsible for ATP release.

Fluoride-resistant acid phosphatase

There are some enzymes thought to be related to metabolism of nucleotides fulfilling a tramsmitter function, because the activity associated with these enzymes is localized to dorsal root ganglia and laminae I and II of the spinal cord. The enzyme marker most widely studied is fluoride-resistant acid phosphatase (FRAP). FRAP activity, using the substrate β-glycerophosphate, is demonstrable at all levels of mammalian spinal cord as a dense 'eyebrow' of staining in inner lamina II with occasional fibres in Lissauer's tract and lamina I, and additional reaction in ventral horn cell bodies (Knyihar-Csillik & Csillik, 1981). Using the alternative substrate thiamine monophosphate, a more selective staining in the spinal cord - only the eyebrow - is achieved (Knyihar-Csillik *et al.* 1986). The endogenous substrate for the enzyme is unknown.

FRAP and thiamine monophosphatase (TMP) activities can be seen in small DRG cells (Knyihar-Csillik & Csillik, 1981; Knyihar-Csillik *et al.*, 1986). The FRAP population is contained within the small dark cell population but is distinct from cells immunoreactive for substance P or somatostatin (Nagy & Hunt, 1982; Price, 1985). Dalsgaard *et al.* (1984) saw a slight overlap between these peptides and FRAP, but had used colchicine. FRAP appears in the DRG at E15 (Schoenen, 1978).

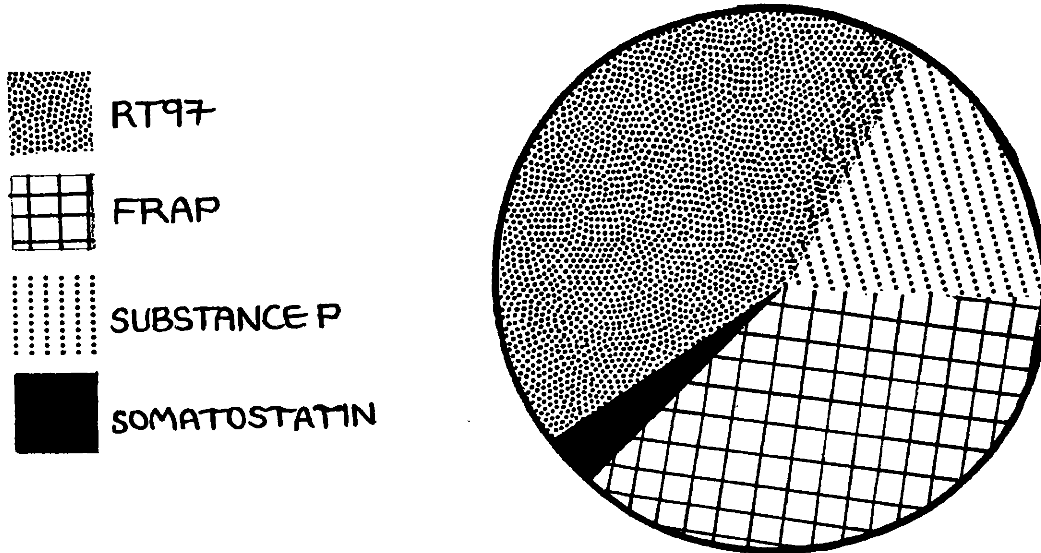
Adenosine deaminase

Adenosine deaminase converts adenosine, another postulated transmitter, into inosine. In rat spinal cord, adenosine deaminase immunoreactive fibres and neurons are confined to lamina I and outer lamina II. In L4 and L5 rat DRG, small cells immunoreactive for adenosine deaminase also contained somatostatin but not substance P or FRAP (Nagy and Daddona 1985).

Figs. 2.1 and 2.2 illustrate that some of these various transmitter candidates and markers of DRG neurons are present in overlapping subpopulations. Data on coexistence of markers has been taken from Lawson *et al.* (1987), McCarthy & Lawson (1989), Ju *et al.* (1987), Nagy & Hunt (1982), Price (1985), Knyihar-Csillik & Csillik (1981).

Fig. 2.1: *Pie chart representing the total population of primary afferent neurons, showing the overlaps between subpopulations expressing immunoreactivity for RT97, substance P, or CGRP*

Fig. 2.2: *Pie chart representing the total population of primary afferent neurons, showing the overlaps between subpopulations containing immunoreactivity for RT97, substance P or somatostatin, or FRAP enzyme activity*



2.3.4 Other markers of dorsal root ganglion neuron subpopulations

Price & Mudge (1983) showed that from P9 a subpopulation of rat DRG neurons is catecholaminergic (probably producing dopamine). The segmental distribution of tyrosine hydroxylase immunoreactive DRG neurons was very variable, being highest in L5, at 1% of total neurons. Price (1985) went on to show that the tyrosine hydroxylase positive neurons constituted a separate population from substance P, somatostatin or FRAP neurons, but were still part of the small dark (RT97 negative) population.

An enzyme which is ubiquitous among body tissues, carbonic anhydrase, displays a selective localization of activity in a subpopulation of large and medium diameter rat DRG neurons, and large diameter dorsal roots (Wong *et al.*, 1983; Riley *et al.*, 1984). Satellite cells wrapping all neurons in the ganglia were also reactive. Robertson & Grant (1989) showed that carbonic anhydrase positive neurons are a subpopulation of DRG neurons expressing the GM1 ganglioside, which in turn are all RT97 positive. Staining of

sensory endings in muscle spindles suggests that this enzyme may be a marker for large muscle afferents (Riley *et al.*, 1984). In the spinal cord, glial cells were stained but the only neuronal components of the grey matter which were positive were a few collateral fibres coming from the heavily-reactive posterolateral region of the dorsal columns.

Calbindin and calretinin, calcium binding proteins, are present in overlapping subpopulations of chick dorsal root ganglia, staining a part of both the large and small neuron populations (Rogers, 1989). Calbindin immunoreactivity is detectable from E10, and is stable until after hatching, in about 20% of DRG neurons (Philippe & Droz, 1988).

Two classes of surface antigens, the globoseries (Dodd *et al.*, 1984) and lactoseries (Dodd & Jessell, 1985) carbohydrates, have been identified in separate populations of DRG neurons. Different lactoseries carbohydrate antigens overlap to varying extents with substance P, somatostatin and FRAP, and immunoreactivity is seen on small diameter neurons and in dorsal horn laminae I and II, suggesting these are nociceptive afferents. Globoseries carbohydrate immunoreactivity is seen in laminae I, III and IV, where low-threshold myelinated afferents terminate, and does not overlap with peptides or FRAP in DRG neurons. Such cell surface determinants may be involved in interactions with spinal cord neurons and in target finding in development.

Various studies have been performed on the detailed ultrastructure of DRG neurons, and have classified six types of neurons according to cellular distribution of cytoplasmic organelles (Duce & Keen, 1977, Rambourg *et al.*, 1983 in rat; Sommer *et al.*, 1985, in mouse).

2.3.5 Conclusions: correlations between chemical and functional characteristics

How can the physiological and chemical studies on DRG neuron subpopulations be drawn together? Some laboratories have attempted

directly to combine electrophysiological characterization of single neurons with analysis of peptide immunoreactivity. Leah *et al.*, (1985) identified the receptor properties of cat DRG neurons, intracellularly labelled them and then stained them for substance P, somatostatin and VIP. A complex pattern emerged with no clear correlation between receptor type and peptides: in particular, some nociceptive C fibres had substance P-negative cell bodies.

Lawson *et al.* (1988) have developed an *in vitro* DRG - peripheral nerve - skin preparation which will allow a similar investigation in rat DRG neurons.

Lynn & Hunt (1984) summarized C fibre chemistry and physiology, concluding that there were only partial correlations: VIP was present in visceral C fibres, substance P in some polymodal nociceptors, some C mechanoreceptors were FRAP positive. Developments since then include the finding of substance P and CGRP in A fibre neurons (Lawson *et al.*, 1987; McCarthy & Lawson, 1989) and Molander *et al.*s (1987) study on nerve-specific chemical distribution (discussed further in Chapter 4).

Schlichter *et al.* (1989) examined sensory neurons in the quail trigeminal ganglion for substance P expression or exhibition of a Ca^{2+}-activated chloride current. Neurons containing substance P were found to have the current three times less frequently than those without substance P.

Sugiura *et al.* (1988) looked at the ultrastructural features of functionally identified neurons with C fibres in the guinea pig and found that all the neurons were of the small dark 'B' type. In addition, specific sensory modalites could be correlated with the three subtypes of B neurons described by Duce & Keen (1977) and Rambourg *et al.* (1983). High-threshold mechanoreceptors and mechanical-cold nociceptors were type B1, polymodal nociceptors type B2, and cooling receptors type B3. This was the first demonstration of a correlation between detailed structural and functional characteristics.

CHAPTER THREE: Retrograde labelling and histological methods

3.1 Aim

The aim was to develop a technique for retrogradely labelling primary afferent neurons so as to provide a means of identifying, in a section of a dorsal root ganglion, those cells whose peripheral axons projected to a distinct target tissue, such as skin or muscle.

Therefore a neuroanatomical tracing technique was employed, whereby dyes deposited at sites of axon termination are endocytosed and transported retrogradely by primary afferents to the DRG. Such dyes accumulate in the cell body, and can be visualized clearly above background, without the need for processing other than fixing and sectioning.

Once target-related cells can be identified in this way, other procedures can be performed on the tissue to demonstrate the localization of cellular constituents which may be particular to certain cell types or functions.

3.2 Background

The idea of retrogradely tracing the paths of neurons from their terminals to their cell bodies was first proposed by Kristensson & Olsson (1971) and La Vail & La Vail (1972), as a way of identifying axon pathways in the CNS.

A commonly used retrograde tracer is horseradish peroxidase, a protein which is picked up by axon terminals and transported by active transport mechanisms. For visualization, the enzyme must be reacted by incubation with its substrate, hydrogen peroxide, and a chromogenic electron donor which results in a highly insoluble coloured deposit. The requirement to process the tissue in order to visualize the tracer, before any further procedures to demonstrate cellular constituents, is a disadvantage because it introduces the

possibility of tissue loss, and destruction of antigenicity and enzyme activity.

Fluorescent dyes were first introduced as neuroanatomical tracers by Kristensson & Olsson (1971), and Kuypers *et al.* (1977) and several such dyes give similar results to HRP (Aschoff & Hollander 1982). The advantage of fluorescent tracers is that they are visible without further processing, and thus combining retrograde tracing with other histochemistry - for transmitters, peptides, etc. is easier. Such techniques are reviewed by Sawchenko & Swanson (1981) and Skirboll & Hokfelt (1983).

In the peripheral nervous system, whole nerves can be labelled with dyes by cutting the nerve and dipping the cut end into dye, or fixing a chamber containing the dye onto the cut end. In this way all the axons in the nerve retrogradely transport the dye, so if nerves which are relatively pure in composition (i.e. all the axons project to one type of tissue) are labelled, then large numbers of retrogradely traced neurons can be identified in the DRG. Dalsgaard *et al.* (1984) labelled the cut ends of the greater splanchnic nerve or the T9 intercostal nerve in rats, and later processed fixed DRG sections for FRAP activity. There was no difference between the visceral or muscle afferents in terms of the percentage which were FRAP positive.

Molander *et al.* (1987) similarly labelled the cut ends of the greater splanchnic, gastrocnemius or saphenous nerves with Fast Blue, and processed the DRGs for FRAP activity or CGRP, substance P or somatostatin immunoreactivity. Then they estimated percentages of retrogradely labelled neurons with counterstains, and found that chemical markers were distributed differently according to tissue type.

The major disadvantage of using cut nerves in order to label neurons retrogradely, is that axotomy is known to cause changes in chemical expression in the cell bodies and central terminals of those neurons. Therefore the results obtained for the presence of chemical

markers in such retrogradely labelled neurons may be an inaccurate representation of the normal situation.

A way of avoiding the problems associated with axotomizing neurons is to label the target tissue itself, so that axon terminals at the site of dye deposition pick up the dye for retrograde transport. Examples of the use of this technique are provided by Kuwayama *et al.* (1987) and Ositelu *et al.* (1987). The former group used cholera toxin β-subunit to label ocular sensory neurons in the trigeminal ganglion from the anterior chamber of the eye, while Ositelu *et al.* (1987) retrogradely labelled skin, tongue and masticatory muscle afferents with injections of the fluorescent dye True Blue into those tissues in rats. The labelled cell bodies in the trigeminal ganglia were processed to demonstrate somatostatin immunoreactivity.

If dorsal root ganglion neurons had peripheral processes which branched, and which innervated two different tissues, then specific retrograde identification of neurons would be a misleading concept, and indeed the study of target-specific influences would not be feasible. Electrophysiological experiments suggest branching may occur in some neurons such that in the peripheral nerve, there are pairs of axons which appear to be linked to one DRG cell body. It is only very rarely (Devor *et al.*, 1984) that a neuron sends branches to two distinct peripheral targets, but there may be frequent occurrences of one neuron projecting two branches to the same target area (Taylor & Pierau, 1982, by fluorescent tracing of axons; McMahon & Wall, 1987, by electrophysiology, who suggested that one branch of each dichotomizing fibre formed a non-functional sensory ending). The frequency of occurrence of branched peripheral axons supplying two distinct targets is therefore low enough to be ignored in the present work.

For this project, the method of retrogradely labelling DRG neurons from peripheral tissues has been chosen as the most useful way of identifying subpopulations of primary sensory neurons which innervate different peripheral targets.

3.3 Methods

This section consists of the following parts:

3.3.1 Fluorescent dye injection method

3.3.2 Tissue fixation

3.3.3 Cutting and dealing with tissue sections

3.3.4 Histochemistry

3.3.5 Immunocytochemistry

3.3.6 A note on the use of RT97

3.3.7 How neurons were counted

3.3.1 Fluorescent dye injection method

The crystalline Fast Blue (FB) or Diamidino Yellow dihydrochloride (DY) were dissolved in ethylene glycol at a concentration of 5% (w/v). Dry powder and solutions were stored at -20°C.

Injections of FB and DY were made using a 30g dental needle attached via polypropylene tubing to a 10μl syringe. Having the needle extended in this way made it more flexible, and when an injection was made, the needle could be left in place for up to a minute to prevent dye leaking out of the tissue along the needle track.

In the case of dye injections into skin, hairy skin was first shaved, and both hairy and glabrous skin injection sites were treated with hair-removing cream (Nair), which as well as removing all hair remaining after shaving, made the skin softer. The needle was inserted tangentially to ensure that dye was deposited only within the skin layers. The needle and dye could both be visualized through the top surface of the skin. The volume injected was about 1 or 2 μl.* If dye leaked back out it was cleaned away with a swab.

From one case, a piece of skin incorporating the dye injection site was dissected after perfusion, at 5d, post fixed, frozen and sectioned.

* subsequently in animals used for experimental studies, several injections per animal of 1-2 μl were made, about 3mm apart, in either skin or muscle.

An area of necrosis, coloured by FB and which therefore appeared likely to be the site of deposition of dye, was present as a restricted zone of about 2mm diameter, well into the epidermis and also abutting on the connective tissue underneath.

For dye injections into muscle, the skin and connective tissue layers were opened to expose the muscle belly. The needle was inserted tangentially, dye injected, and the needle left in place for a while. Sometimes there would be some back leakage of dye, and this was removed with a saline swab.

The FB injection site in a section of fixed and frozen muscle was also examined at 7d. There was an area of necrosis where the dye was present, similar to a skin injection site.

3.3.2 Tissue fixation

For the *in vivo* studies and when the labelling technique was being developed, rats which had received injections of dye solutions into various tissues were sacrificed, after the appropriate survival period, by transcardiac perfusion with 500mls of fixative, under deep anaesthesia with pentobarbitone (Sagatal, 50 mg/kg). A fixative was chosen that was compatible with the retrograde tracers and with preservation of various markers which we intended to visualize concurrently with them. The low solubility of the fluorescent tracers in water allowed the use of an aqueous fixative. Glutaraldehyde is not compatible with fluorescent tracers or immunofluorescent labels because it induces autofluorescence. Paraformaldehyde, although not as good as glutaraldehyde with regard to structural preservation, fixes the tracers and also adequately preserves enzymes and peptides of interest. When tissue requires fixing for subsequent reactions to demonstrate enzyme activity or peptide immunolocalization, there are two opposing constraints - firstly that the protein should be sufficiently fixed to prevent leakage into other cells or out of the tissue altogether, and secondly that the degree of cross-linking of the protein structure

does not obstruct either the catalytic activity of the enzyme or the antigenic epitope of the protein.

4% paraformaldehyde in phosphate buffer (pH 7.4, ice cold) was chosen as the perfusion fixative. After dissecting the required tissue it was post-fixed for two hours in the same solution, and then transferred to 20% sucrose in phosphate buffer overnight, all at 4°C. This latter procedure cryoprotects the tissue by preventing formation of ice crystals in the cells when the tissue is frozen for sectioning.

3.3.3 Cutting and dealing with tissue sections

Fixed tissue was mounted in Tissue Tek/OCT compound on metal chucks in a liquid nitrogen bath (-80°C). Sections were cut using a Bright cryostat, chamber temperature -30°C. Sections of 10μm were used, because at this thickness antibodies employed in immunostaining can penetrate entirely through the section. Sections were collected onto chrome-gelatin subbed slides.

During the development of the labelling technique, serial sections were made of each identified ganglion. In later studies, ganglia of interest were usually pooled and sectioned together. Then, when tissue was to be used for more than one histochemical study, several series of alternate sections - rather than one set of serial sections - were generated.

Migration and fading of the FB upon exposure to warmth and light was a potential problem. It was found that dye in a recently-cut cryostat section of a DRG, dry-mounted on a glass slide, would begin to migrate to satellite cell nuclei initially around the labelled neurons and eventually all over the section if left at room temperature for more than an hour (see Fig 3.1). As can be seen in the photograph, however, such dye leakage did not obstruct identification of labelled neurons.* When sections were being cut and collected, slides were kept inside the cryostat cabinet (at -30°C) and given just 30 mins - 1 hour maximum drying time at room

* when neurons were labelled by tissue FB injections, FB-labelled neurons were almost always present as isolated cells, rather than in groups of two or more, therefore 'sideways' leakage of FB within the DRG was not suspected.

temperature before continuing with processing. Sometimes processing was not to begin until the next day, in which case the air-dried slides were stored in airtight boxes at -80°C.

Alternatively if sections were to be examined without further processing, they were coverslipped with the anti-fade mountant Citifluor and the glass sealed with nail varnish. Such slides, and those which had been further processed and similarly coverslipped, were stored at 4°C.

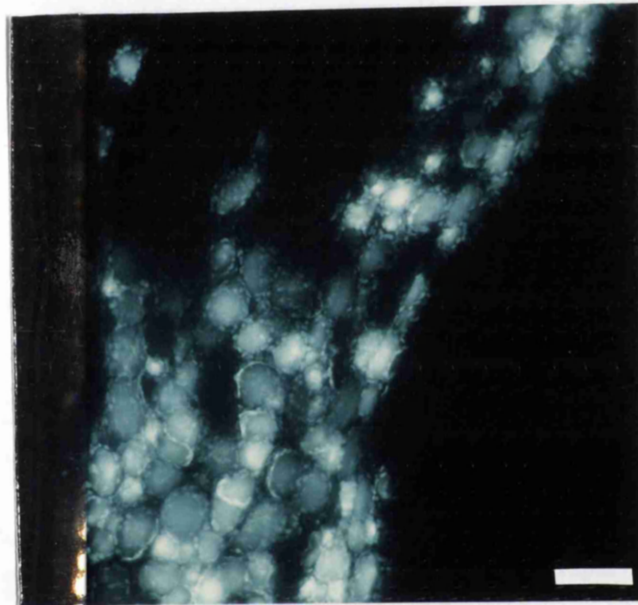
3.3.4 Histochemistry

Due to the observed effect of the histochemical procedure causing a washing-out of the FB, freshly-sectioned and mounted but not coverslipped DRGs were photographed using Ektachrome Daylight film, under ultraviolet (UV) epifluorescence to record the locations of FB-labelled neurons. While not required for photography, sections were kept frozen rather than at room temperature. Then all sections were left at room temperature for 20 mins to dry before the TMP procedure.

Pre-photographed (and some not photographed) series of DRG sections on slides were incubated to demonstrate TMP activity according to the method of Knyihar-Csillik *et al.* (1986). The incubation medium (containing 0.05M Tris buffer pH 5.6 rather than pH 5.0 as in the standard method) was placed in Coplin jars into which slides were fitted, and the jars immersed in a water bath at 37°C for 60 mins. Slides were rinsed gently in Tris buffer and dipped into 1% ammonium polysulphide for 2 mins for the visualization reaction. Then slides were rinsed in distilled water, and either kept moist until immunocytochemical processing for RT97 was begun, or coverslipped immediately.

Fig. 3.1: *Section of an L4 DRG containing FB-labelled neurons after injection of FB into the ipsilateral sciatic nerve*

Under UV epifluorescence, the majority of neurons in the ganglion are seen to contain FB. The rings of tiny fluorescent 'dots' around the neurons are satellite cells, which sometimes take up migrating FB in this way. Scale bar = 100 μm.



3.3.5 Immunocytochemistry

Slides with tissue sections containing FB-labelled cell bodies were coated, around the edge of the sections, with a line of rubber-solution glue (Cow gum) applied with a syringe needle. The gum was allowed to set, resulting in the creation of shallow well, in volume about 150 µl, in which antibody solutions could be applied to the sections. Then sections were preincubated with phosphate-buffered saline (PBS) containing 0.1% Triton-X-100 (PBS/TX, also used for subsequent rinsing) for 10 mins, and then PBS/TX/ 10% horse serum (PBS/TX/HS, also used as antibody diluent) for 10 mins.

Antibody solutions were applied overnight at 4°C in a humid atmosphere, and after 3 x 5 min rinses the second layer antibody was applied for 1 hr at room temperature. The third layer was applied, after similar rinsing, for a further 1 hr, and after a final rinse the slides were coverslipped with Citifluor and the edges of the coverglass sealed with nail varnish.

The primary antibodies used in these studies were: rabbit α-CGRP (CRB, 1:1000; or gift from Dr.P.K. Mulderry, 1:16,000), rabbit α-substance P (gift from Prof.P. Keen, 1:1000), rabbit α-somatostatin (gift from Dr.J. Winter, 1:4000), RT97 (mouse monoclonal, ascites fluid, gift from Dr.J. Wood, 1:2000) and α-NGF receptor (Chandler *et al.*, 1984; ascites fluid obtained from Balb/C mice inoculated with the 192-IgG-secreting hybridoma cell line, the latter kindly supplied by Prof.E.M. Shooter, 1:1000).

For the TMP study of Chapter 4, RT97 was applied alone, with a second layer of α-mouse immunoglobulin G (IgG) conjugated with biotin (Amersham; 1:200) and a final layer of fluorescein isothiocyanate (FITC)- or tetramethylrhodamine isothiocyanate (TRITC)-conjugated streptavidin (Amersham; 1:100). For the neuropeptide studies of Chapters 4 and 6, each α-peptide antiserum was applied concurrently with RT97, the second layer being biotin-conjugated α-rabbit IgG (Amersham; 1:200), and the third layer FITC-conjugated streptavidin, thus labelling the peptide staining green, with TRITC- or Texas Red-

conjugated α-mouse IgG (Amersham, 1:100), thus labelling RT97 stain red.

The α-NGF receptor antibody used in Chapter 5, being also raised in mouse, could not be used in conjunction with RT97. After the primary incubation, the second layer applied was α-mouse IgG conjugated with biotin, and the third layer was Texas Red-streptavidin.

For each of the primary antisera, and the second and third layer antibodies, a control was performed whereby that antibody was omitted from the antibody diluent, while all other layers were applied as normal. In all cases, no positive labelling of sections resulted; if the control was such that one of the first or second layers was missing but the third layer was applied, there would often be a background green or red colour on sections, as appropriate to the type of label, but which was not of a strong, actually fluorescent nature. This tendency for the labels to cause a non-specific colouring can also be seen on sections reacted experimentally - while positive cells stand out as brightly fluorescent, the remaining tissue exhibits a 'background' colouring, and is not completely black (see, for example, Figs. 4.4-4.7 in Chapter 4).

For neuropeptide antisera, further controls were performed by preadsorbing different aliquots of the diluted antisera with 10-50 μg/ml of each of the peptides for 24 hrs, 4°C, prior to application to the tissue. Each antiserum was completely prevented from positively staining DRG sections only in the cases where it was pre-adsorbed with its specific antigen; in other cases the staining pattern was unaffected by the preadsorption.

3.3.6 A note on the use of RT97

The basis of the correlation between large light DRG neurons and RT97 labelled cells (Lawson *et al.*, 1984) is that the size distributions of both populations can be fitted by almost identical normal curves, while the same is true for the small dark, and RT97 negative, DRG neuron population. These size measurements and curve fitting were done with an acoustic computer-linked drawing board with plotting program, and I was kindly allowed by Dr. Lawson to use the

same system to assess whether RT97 staining was behaving the same way in my project.

Serial sections of 7μm were cut at intervals of 200μm from one L5 DRG from a control, perfusion-fixed rat. After reacting with the RT97 antibody and an indirect immunofluorescent label, every cell sectioned at the level of the nucleus was measured in two sections by tracing its drawing-board projection with an acoustic pen. Presence or absence of RT97 label was also recorded. An observation of Lawson *et al.* (1984), who used a peroxidase conjugated antibody, was that a distinct population of very faintly-positive neurons, all of small diameter, should be classified as negative in order to generate a normal curve of RT97-positive cells which most closely approximated that for large light neurons. The same was found to be true on the DRG sections prepared with fluorescent-labelled RT97 immunostain, therefore in subsequent studies the same protocol for classifying RT97 positive and negative cells, where very faintly stained cells were considered negative, was applied.

Presence or absence of neurofilaments as revealed by RT97 immunoreactivity is therefore not an absolute marker for large light and small dark neurons respectively, but serves to represent a relative difference between the two neuronal classes.

3.3.7 How neurons were counted

Examination of sections under epi-fluorescence with the UV filter appropriate for FB (excitation wavelength 390-420 nm) revealed that FB intensity varied between cells, and therefore labelled neurons were classified by inspection into three categories: high, medium and low intensity. High-intensity neurons were characterized by a consistent, brilliant fluorescence which looked almost white rather than blue. Low-intensity neurons were blue-coloured just enough to be distinguishable from the green background characteristic of the tissue under these optical conditions, and those of medium intensity were of intermediate coloration.

In order to make counts of labelled cells without introducing the error of repeatedly counting a cell split between more than one section, only those cell profiles containing a nucleus were counted (easily identified because the nucleus is unlabelled by FB, and the cytoplasm is unlabelled by DY). Where a series of adjacent sections were being examined, each section was compared with its predecessor and its successor, to ensure that cells with nuclei split between more than one section were scored once only.

For microscopy of immunofluorescent labels, once a cell had been identified under the UV filter as containing FB, the filters were switched to those appropriate for FITC (450-490 nm) and TRITC/Texas Red (510-550 nm), and the presence of positive staining for other markers recorded for each FB-labelled neuron.

In the case of sections pre-photographed for the TMP study, the colour transparency record was compared with the reacted sections under light microscopy (and if the UV light was also used to examine FB remaining in the tissue, identification of FB-labelled neurons was relatively easy) and presence of TMP staining recorded, followed by RT97 staining viewed under the FITC filter.

Positive and negative neuronal profiles after immunolabelling with the neuropeptide antibodies were usually unambiguous, but if the staining was unclear on any DRG section, e.g. background colour was high, then data from that section would not be included in the study. Information lost in this way accounted for less than 5% of FB-labelled afferents. However, RT97 immunolabelling was more capricious, and results - particularly in the substance P and somatostatin studies in Chapters 4 and 5 - were compromised because data could not be used.

The TMP histochemical procedure appeared not to affect subsequent RT97 immunolabelling - the labelling was not diminished in intensity, and the results of Table 4.6 show that across the four studies with the different counterstains, none of the procedures drastically affected the RT97 results because the proportions of RT97 negative afferents were fairly constant for a given target tissue.

3.4 Development of retrograde labelling technique

The different types of tissues labelled, and control experiments to determine reliability and specificity of the method.

3.4.1 Initial details

Because DY labels only the nucleus, it is not as easy to visualize as FB, and we decided to use FB rather than DY for the major part of our studies.

Initially FB (about 2μl) was injected into the exposed sciatic nerve trunk, forceps being used to crush the nerve lightly at the point of injection. After 5 days, the rat was perfused and sciatic nerve projection DRGs sectioned. Hundreds of cells were cytoplasmically-labelled with FB in DRG L3, 4, and 5 on the ipsilateral side (see Fig 3.1). The dye was therefore transported to, and visible in, DRG cell bodies after their axons had been exposed. Looking at the spinal cord of this animal revealed some motoneuron cell bodies which were FB-labelled in the ipsilateral ventral horn, although there was no evidence that the FB had been transported transganglionically in primary afferents to label terminals in the dorsal or ventral horn.

The next step was to inject FB into different target areas such as skin, muscle or joint, and investigate the specificity of DRG cell labelling achieved by axonal uptake of FB deposited in these tissues.

The raw data for all the experimental cases listed in this chapter are given in Appendix I, each table numbered by case.

3.4.2 Fast Blue injected into skin

Injections were made into two areas of skin, in different rats or in different limbs in the same rat: the inner thigh, supplied by the saphenous nerve, and the sole of the foot which is mainly supplied by the tibial nerve. Animals were perfused between 5 and 15 days later

in order to establish the optimal time for transport of dye to the dorsal root ganglia.

Table 3.1 presents a summary of the experiments performed (n =1 in each case).

TABLE 3.1: *Summary of experiments where FB was injected into different skin areas*

Case no.	Time(days)	LHS	RHS
1	5d	Ti FB	Sa FB
2	9d	Ti FB	Sa FB
3	15d	Ti FB	Sa FB
4	6d	-	Sa FB
5	6d	-	Sa FB
6	6d	-	Sa FB
7	8d	Ti FB +scra	Ti FB
8	6d	Sa FB +scra	Sa FB

Ti = tibial area skin (sole of foot)
Sa = saphenous area skin (inner thigh)
scra = scraped skin at injection site

The time course of labelling from a saphenous projection area of skin (Sa; inner thigh) and a tibial projection area of skin (Ti; sole of foot) was investigated in cases 1, 2 and 3. Optimum labelling in terms of total numbers of DRG cells labelled, was achieved with a 5d survival time for Sa and 9d for Ti, which was the expected relationship between the two sites, tibial axons having further to transport the FB than the saphenous axons. By inspection, there were no major differences in the proportions of large and small diameter cells labelled at the different time points, nor in the range of intensities of FB labelling (see Fig 3.2). Molander & Grant (1987)

found when labelling DRG cells by applying HRP to whole cut nerves that over the first 12 hours, small diameter labelled cells were more abundant than large or medium - the proportions of these increasing at longer survival times. Here we are looking at a relatively long survival time so that differences in speed of transport along axons of different diameter are no longer apparent.

Cases 4, 5 and 6 were further Sa labelling, establishing that the majority of labelled cells were in L2 and L3, with a few more in L4 and maybe sometimes in L5. Molander & Grant (1987) observed the majority of DRG cells retrogradely labelled from the cut saphenous nerve to be at levels L3 and L4, with a few more in L2. Ti labelling resulted in FB cells in the L3, 4 and 5 DRGs, while they sometimes also found labelled cells in L6. If an effort was made to keep the injection volumes consistent, similar injections could yield similar total numbers of labelled cells, as in cases 4, 5 and 6, where the total number of high intensity labelled cells in ipsilateral ganglia L2-L4 resulting from a Sa injection were 127, 140 and 142 respectively. However interanimal variation could account for the frequent inconsistencies seen in other cases.*

The glabrous skin on the foot was very much thicker and tougher than hairy skin and this made injections more difficult. Abrading the skin surface, by scraping with a scalpel blade after the skin was softened with hair removing cream, was tried in cases 7 (Ti) and 8 (Sa), so that FB could be applied to the scraped surface. This protocol, however, resulted in no better labelling in terms of numbers of FB cells, and in fact it was decided to avoid causing additional damage to axon endings by inflaming the skin in this way, in case this led to an 'injury response'.

* Similarly, the variable distribution of FB-labelled neurons among the three ganglia (L3-L5) in cases 4, 5 and 6 could be due to differences in the contribution of each ganglion to the area of skin which received the FB. The variation may also indicate that slightly different areas of skin were labelled in each case.

Fig. 3.2: *Sections of DRGs from rats which received FB injections into different ipsilateral skin areas, after different survival times*

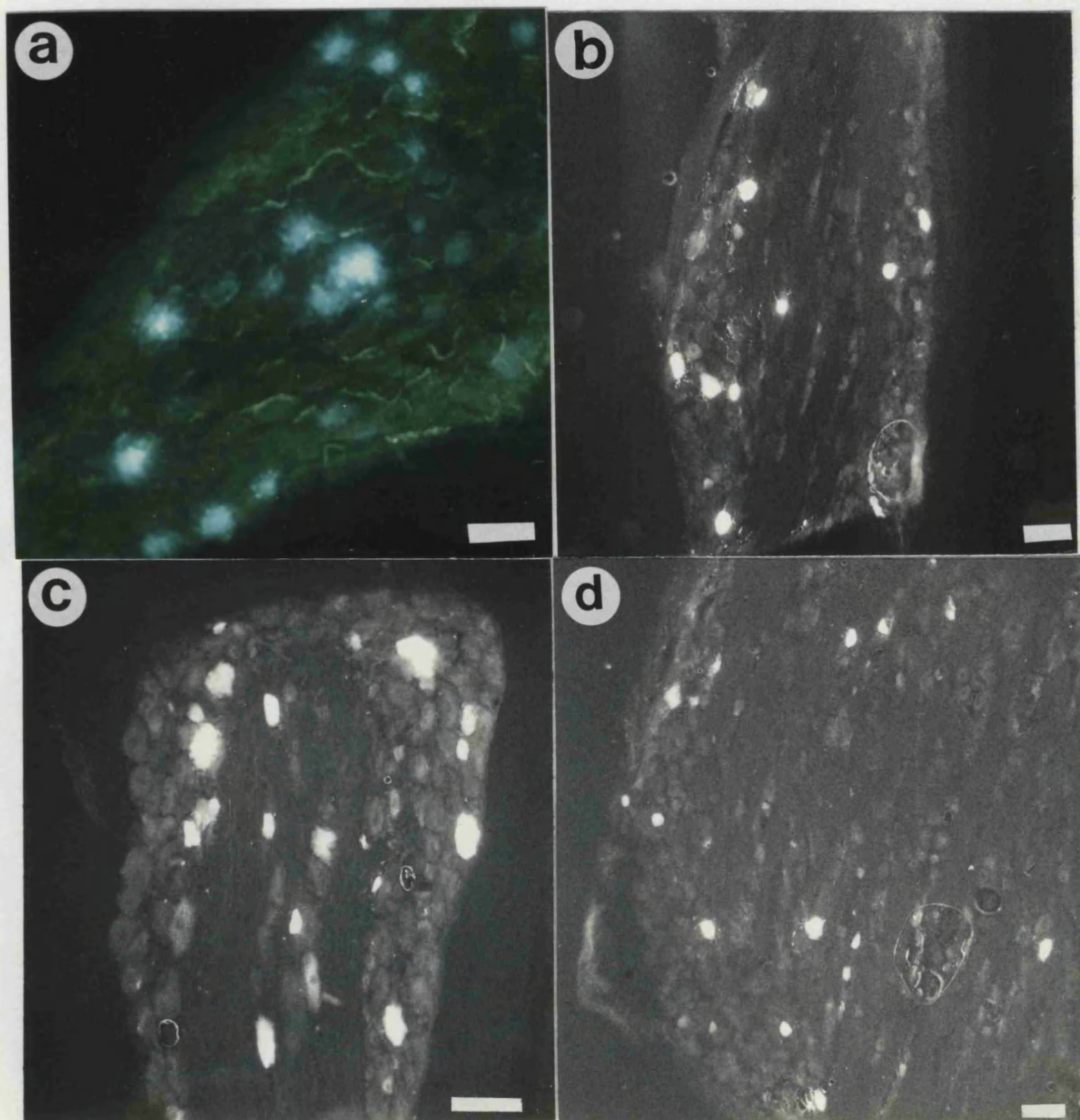
Under UV epifluorescence, there are no major differences in the proportions of large- and small-diameter neurons, or in the range of FB intensities, resulting from the different transport times allowed for the FB to reach the DRGs. Scale bars = 100 μm.

(a) Case 1; saphenous skin FB, 5 d survival, L3 DRG.

(b) Case 2; saphenous skin FB, 9 d survival, L3 DRG.

(c) Case 2; tibial skin FB, 9 d survival, L4 DRG.

(d) Case 3; tibial skin FB, 15 d survival, L5 DRG.



3.4.3 Specificity of skin injections

At this point the specificity of the labelling was investigated, in terms of which afferents, and by what alternative routes, dye was being transported to the DRG cell bodies. Some rats were injected bilaterally in the same skin area, and the nerves known to supply afferent axons to those skin areas were cut and ligated on one side, immediately after the dye injection. Thus tracer transported specifically by direct-projecting afferents would be prevented from reaching the DRG neuron cell bodies. The projection DRGs and others at 'inappropriate' levels of the spinal cord were examined bilaterally.

To investigate the effect of any dye gaining access to the bloodstream, some FB was injected directly into a vein.

Another test of specificity, to investigate leakage from one tissue to another closely apposed, such as skin to underlying muscle, was performed by using the two tracers, FB and DY, each in a different tissue. After opening the skin slightly to the side of the injection site, DY was injected into the top surface of the muscle, just below a skin area which subsequently received a FB injection as normal, a few days later (because DY transports more slowly than FB). The projection DRGs were examined for examples of double-labelled cells, the presence of which could indicate leakage of dye from one tissue to another, to be uptaken by the same terminal portion of an afferent axon as had picked up the other dye. Alternatively, a double-labelled cell might truly have two peripheral branches, each one in one of the tissues. although as examined electrophysiologically the occurrence of such branching is rare (McMahon & Wall, 1987).

Table 3.2 presents a summary of the experiments (n = 1 in each case)

TABLE 3.2: *Summary of experiments examining specificity of skin FB injections*

Case no.	Time(days)	LHS	RHS
9	6d	Sa FB + cut	Sa FB
10	9d	Su FB	Su FB + cut
11	9d	Su FB	Su FB + cut
12	9d	Su FB + all others cut	Su FB
13	7d	-	i.v. FB near Su nerve
14	8d 6d	muscle DY Sa FB	- Sa FB
15	12d 8d	- Ti FB	muscle DY Ti FB

Su = sural area skin (lateral ankle)
Sa = saphenous area skin (inner thigh)
Ti = tibial area skin (sole of foot)

In case 9, cutting and ligating the LHS saphenous nerve at the same time as FB was injected into both left and RHS Sa skin, resulted in a massive reduction in the number of high intensity FB cells in the LHS DRG, halving of the medium intensity cells and a similar drop in low intensity labelled cells. This type of control experiment was also performed with injections of FB into the skin of the calf, supplied by the sural nerve. The sural is a cutaneous branch of the sciatic nerve, which also has a purely muscle branch going to the gastrocnemius muscle in the calf, close to the Su skin injection area. In cases 10 and 11, Su skin injections were made bilaterally, with the Su nerve branch cut and ligated on the RHS. Figure 3.3 compares the L4 DRGs from each side of case 10. In case 12 FB injections were made bilaterally into Su skin, and on the LHS the sural neurve was kept intact while other nerves nearby were sectioned

and ligated (other branches of sciatic and the saphenous and 6th nerves). The majority of labelled cells in all cases were found in L4 and L5, with some in L3. Molander & Grant (1987) find labelling mostly in L5 and L6, with some in L4, and may be due to their different strain of rat having a sciatic nerve which projects some axons to L6, because other of their projection results (tibial and common peroneal) also extend more caudally than in this study.

In case 11, as in case 9, the number of high intensity FB cells in the DRGs on the cut nerve side was substantially reduced, while medium intensity cells were reduced in number by two thirds. Thus specific FB transport along the Su nerve had been obstructed. In case 12, when all nearby nerves were cut but the sural was intact, surgery did appear to reduce the number of labelled cells in the DRG, but by an amount that could instead be accounted for by natural variation in innervation density, and injection size. Since case 11 had shown many fewer cells to be labelled without sural nerve transport, such variation seemed a satisfactory explanation of the 'reduction' in labelled cells in case 12. It was decided that high intensity cells, almost all of which disappeared when the appropriate nerve was sectioned, represented specifically retrogradely labelled afferents, while medium intensity cells, up to half of which remained after nerve section, represented a mixed population of specific and non-specifically labelled neurons. Data for both intensities was collected in subsequent experiments to see if any trends in the high intensity cell population as regards distribution of chemical markers, etc., were followed by medium intensity cells. Emphasis for the purpose of drawing conclusions from results is, however, placed on only high-intensity FB neurons. In subsequent studies, results from medium-intensity FB neurons are not presented in each chapter, but are included as an appendix.

In order to look at one cause of the FB label of different intensities, since we suspected that low intensity labelled cells might be the result of systemic spread of FB (taken up into the bloodstream), an injection of about 1μl FB was made directly into a vein next to the sural nerve (case 13). Checking DRGs of lumbar and

thoracic levels for labelling, we found many low intensity-cells in inappropriate DRGs, but a very small number of high and medium intensity cells in L4 on the injection side (Fig 3.4). It was reasoned that this strong labelling was the result of FB getting straight onto the sural nerve at the vein injection site, since there had been some exudation of dye which had been cleaned up straight away at the time of injection. The low intensity-labelling, however, which appeared in other DRGs was obviously the result of systemic spread of dye, which was then picked up by axons terminating on blood vessels in all manner of other tissues.

Low-intensity labelled cells were from then on regarded as non-specific labelling and data from these was not used in counterstaining experiments. The tiny numbers of high intensity cells remaining in DRGs where the projecting nerve had been cut may be the result of spread of dye to the cut nerves, or dye uptake by fibres in tissue exposed by the surgery. The number of such cells, however, was sufficiently small that they are unlikely to introduce errors.

Fig. 3.3 (next page): *Sections of L4 DRG showing the effect on the number of FB-labelled neurons of cutting the sural nerve unilaterally, after FB was injected bilaterally into skin supplied by the sural nerve*

Under UV epifluorescence, the number of FB-labelled neurons is greatly reduced in the ganglion ipsilateral to the cut nerve. Scale bar = 100 μm. Case 10; RHS and LHS sural skin FB, RHS sural nerve cut, 9 d survival. (a) LHS L4 DRG; (b) RHS L4 DRG.

Fig. 3.4 (next page): *Sections of ipsilateral lumbar and thoracic DRGs showing neurons containing different intensities of FB label after FB was injected into a vein next to the sural nerve*

Under UV epifluorescence, (a) the L4 DRG contained only 4 high-intensity FB neurons believed to result from FB leaking onto the sural nerve, while (b) thoracic DRG contained many low-intensity FB neurons. Case 13. Scale bar = 100 μm.

Fig. 3.3

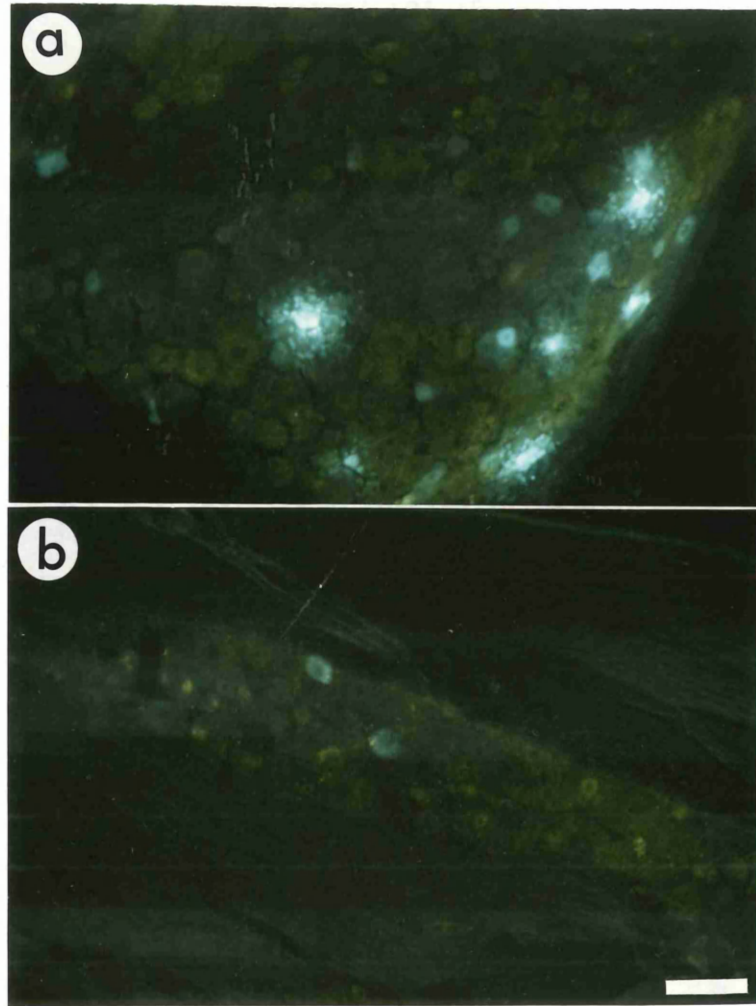
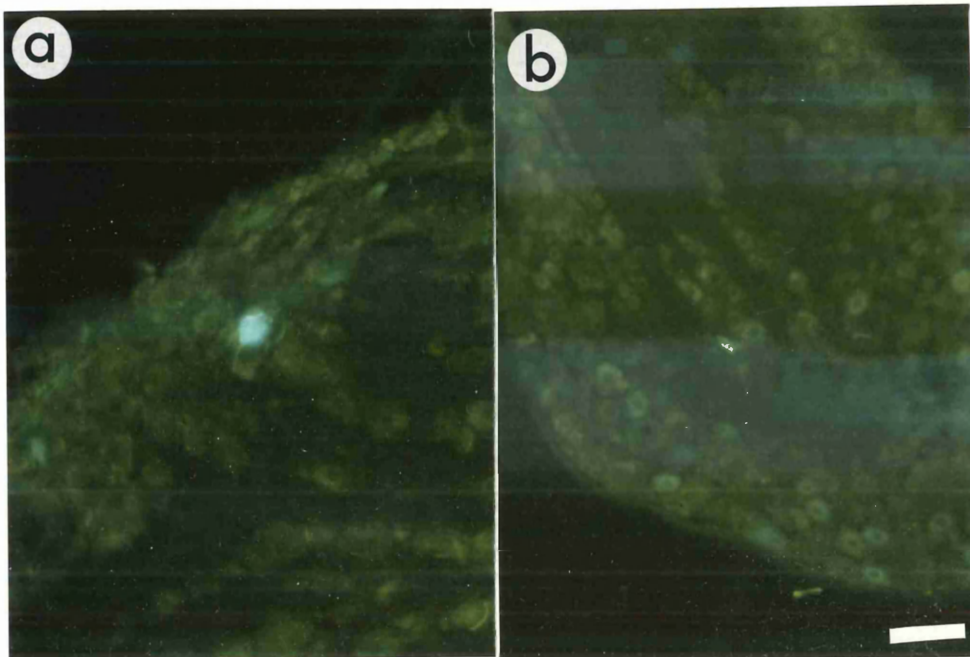


Fig. 3.4



The next type of control experiment was the injection of FB into one tissue type, and concurrent labelling of another tissue with an injection of DY. In case 14, Sa skin injections were made bilaterally, and two days previously DY had been injected into the muscle under the same skin area on the LHS. While FB labelling was found mainly in DRGs L2 and L3 on both sides, DY labelling was found in neuronal nuclei in LHS L3, 4, 5 and 6, i.e. somatotopy was clearly different. There were no double labelled cells, so FB was not leaking from skin to underlying muscle.

In case 15, the same experiment was made for the Ti skin area, with DY injected into the small foot muscles below the sole. While FB labelling was mainly in L2, 3, 4 and 5, DY was virtually restricted to L4 and L5. In this case, however, double labelled cells were found (Fig 3.5) in L4 and L5, suggesting that FB had spread to the underlying muscle. Knowing that it was difficult with Ti skin injections to insert the needle accurately when force was required, thus making subcutaneous deposition of dye more likely, FB labelling of skin from this area was not continued. NB. Double-labelled cells could also indicate the presence of two diverging peripheral branches of the axon, although for reasons already mentioned, this was unlikely. *

3.4.4 Labelling and specificity in other tissues

Having established a general technique for skin injections of FB, we continued by labelling other peripheral tissues - muscle, joint and bladder, and performing similar control experiments.

Table 3.3 presents a summary of the muscle injections and control experiments where the nerve supplying that peripheral target was cut.

* Although the experiments to check for specificity of retrograde transport indicated that not all high intensity FB neurons were the result of transport along the nerve, because some neurons remained where the nerve was cut (cases 9-11), it was decided that the error (up to 10%) was small compared to the magnitude of differences in chemical expression that we were looking for between tissues, and that retrograde labelling should be examined in other tissues anyway as a further indication of whether different neurons were being identified.

TABLE 3.3: *Summary of experiments on FB injection into muscles, and corresponding controls*

Case no.	Time(days)	LHS	RHS
16	7d	mTA FB	-
17	7d	-	mTA FB
18	7d	-	mTA FB
19	7d	mTA FB	mTA FB + cut
20	7d	mGa FB + cut	mGa FB
21	7d	mGa FB + all others cut	mGa FB

mTA = tibialis anterior muscle
mGa = gastrocnemius muscle

For identification and accessibility, the tibialis anterior and gastrocnemius muscles were chosen for trying muscle FB injections. In cases 16, 17 and 18, FB was injected into the tibialis anterior muscle.* The first feature to notice about the results is the much smaller number of labelled cells that were seen, compared to the skin injections of a similar volume of dye, indicating that muscle is probably innervated less densely than skin. Baron *et al.* (1988) retrogradely labelled the saphenous and gastrocnemius soleus nerves with HRP to study the numbers of sensory neurons projecting in these nerves. They estimated that 3750 afferents projected into the saphenous, compared with only 530 projecting into the gastrocnemius nerve. Some of the difference may be accounted for by the relative sizes of the fields of innervation, but part of the difference must be attributable to innervation densities of the two tissues.

Secondly, as in case 16, both projection and inappropriate DRGs (L4 on an uninjected side, and thoracic ganglia on the injected side) contained many low intensity labelled cells, and this was more

* The timing was established by judging the distance from calf muscle to DRG to be longer than the distance from upper-leg skin to DRG, therefore increasing the FB transport time.

frequently a consequence of muscle FB injections than of skin injections. Thus in a DRG sections from a muscle FB case, the ratio of low to high intensity cells was much greater than in a skin FB case (see Fig. 3.6, compared to Figs. 3.2 and 3.3). Densest labelling was seen in L3 and L4, with a few more cells in L2, 5 and 6. When Molander & Grant (1987) labelled the whole common peroneal nerve with HRP, labelled cells were virtually restricted to L4 and L5. Similarly, Baron *et al.* (1988) found most neuronal labelling in L3-4 after saphenous nerve HRP application, and in L4-5 for the gastrocnemius afferents.

With the control experiment where the common peroneal nerve was cut and ligated unilaterally (case 19), the number of labelled cells did not fall as much as in the skin experiments, but labelling on both sides was less than in cases 16, 17 and 18.

Labelling the gastrocnemius muscle, and performing similar controls by cutting the gastrocnemius nerve (case 20) or cutting all nerves in the area except the gastrocnemius (case 21) indicated that muscle labelling (which was predominantly in L3, 4 and 5 in these cases) could be specific as long as injections were made carefully, keeping the needle tangential and near the muscle surface, so the dye could be visualized as it was deposited, away from the centre of the limb where there are large blood vessels. Any fluid coming out at the injection site was immediately mopped up with a cotton bud. Additionally a technique was used where silicone oil was drawn into the syringe system before picking up the FB, and injecting a sufficient volume such that some silicone oil would go into the muscle after the FB, so that if there was leakage out of contracting muscles after the wound layers had been closed then the silicone oil would leak first.

While the results of cases 19 and 20 indicated that some FB reached the DRGs by a route other than transport up the specific nerve, the subsequent adoption of a more careful injection technique was considered a sufficient starting point to begin the phenotypic investigation of afferents identified by FB injections into muscle.

As with skin injections (e.g. cases 4-6), there was interanimal variability in the distribution of FB cells between DRGs after a muscle FB injection (compare cases 20 and 21); on rare occasions the sciatic nerve (of which the gastrocnemius is a branch) was seen to send axons to the L6 DRG, in which case that ganglion was also collected for processing.

Fig. 3.5: *Section of L4 DRG containing neurons double-labelled with FB and DY, after FB was injected into skin on the sole of the foot and DY was injected into the underlying muscle*

Under UV epifluorescence, both FB- and DY-labelled neurons can be seen. Double-labelled neurons are arrowed. Case 15; 12 d survival. Scale bar = 100 μm.

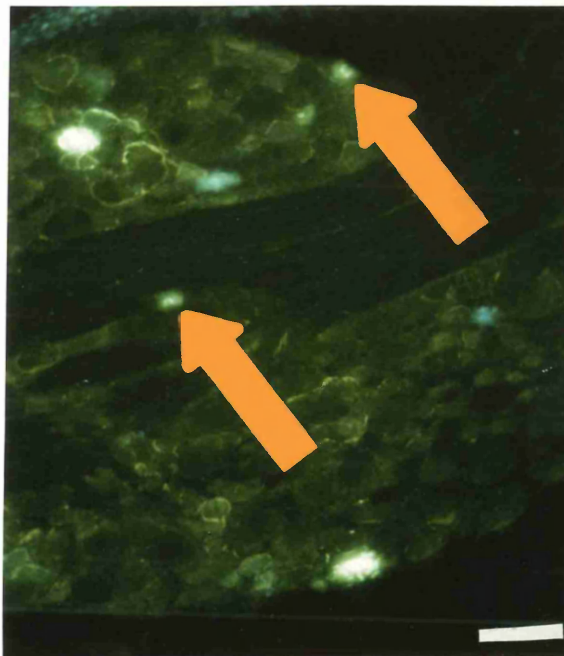


Fig. 3.6: *Section of L4 DRG showing neuronal FB labelling resulting from injection of FB into the ipsilateral tibialis anterior muscle*

Under UV epifluorescence there were many fewer high-intensity FB-labelled neurons, but more low-intensity neurons, than following a skin FB injection. Case 18; 7 d survival. Scale bar = 100 μm.

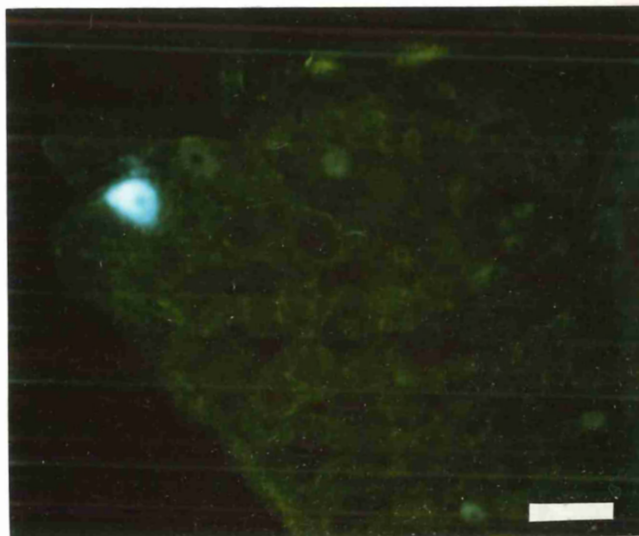


Table 3.4 summarizes the experiments involving injections of FB into joint and bladder, and controls.

TABLE 3.4: *Summary of experiments where FB was injected into joints or bladder, plus controls*

Case no.	Time (days)	LHS	RHS
22	6d	jK FB	s.c. FB in K
23	9d	jA FB + cut	jA FB
24	8d	s.c. DY in K	-
	6d	jK FB	-
25	8d	jK DY	jK FB
26	5d	- bladder wall FB -	
27	5d	- bladder wall FB + ligate	

jK = knee joint
jA = ankle joint
s.c. = sub cutaneous

Injections into joint were first attempted in the LHS knee joint capsule space (by C.J. Woolf) by inserting the needle between the fibula and femur (case 22). On the RHS, FB was injected subcutaneously, and this actually labelled as many DRG cells. Next (case 23), P.D. Wall made injections bilaterally into the ankle joint which would be easier to denervate in the fashion of control experiments. On the LHS, all the many nerves in the lower leg which send branches to the ankle joint were cut. This completely abolished high intensity labelling of DRG neurons, and greatly reduced medium-intensity label. However since there is no specific 'joint' nerve which can be cut in isolation, because all nerves in the area supply some axons to the joint and therefore have to be cut, this control

experiment can only demonstrate how much of the labelling is due to distant leaking rather than leakage to nearby non-specific nerves.

Knee joint injections into the capsule wall and space were performed subsequently by C. Molander, who had previously noticed that uptake of tracers by the trigeminal ganglion (TRG) provided a reliable indication of leakage into the circulation.

Firstly (case 24) another double-labelling experiment was performed in which DY was injected subcutaneously at the knee, and FB injected into the knee joint. Leakage occurred, and so double-labelled DRG cells were found in lumbar ganglia, and in the TRG many medium and faintly labelled cells were also seen. In case 25 we halved the concentration of dyes, injecting DY in one side, FB in the other. With this regime, while only very few DY labelled DRG cells were seen, a reasonable number of neurons were FB labelled, with only a few faintly-labelled cells seen in the TRG. This result established this method as suitable for labelling joint afferents.*

In case 26, several FB injections were made by CJW into the exposed bladder wall, and resulted in labelling of DRG cells at levels L1, 2, 5 and 6, and S1 on both the left and RHS. If a similar experiment was performed, and the pelvic nerve concurrently ligated on the RHS (case 27), the number of bright FB cells on the RHS were almost eliminated, while medium intensity cells were reduced in number by about three fifths.

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* and subsequently in all animals used for labelling joint afferents, the TRGs were removed and sectioned along with the DRGs, and the DRGs were used for counterstaining only if the TRGs were free of FB labelling.

CHAPTER FOUR: The extent to which chemical expression in adult sensory neurons is related to peripheral target.

4.1 Introduction

Experiments were performed to examine the distribution among skin, muscle and joint afferents of four putative transmitter (or transmitter-related) markers known to be present in subpopulations of DRG neurons. These were the enzyme TMP, and the neuropeptides CGRP, substance P and somatostatin. These have previously been shown to exhibit a different distribution among afferents retrogradely labelled from whole nerves with different targets (Molander *et al.* 1987). Here we have avoided the problem associated with sectioning of whole nerves (i.e. the cellular response to axotomy) by labelling afferents from their target tissues.

Substance P and FRAP accumulation at a ligature in a muscle nerve is minimal compared to accumulation in a ligated cutaneous nerve, (McMahon *et al.*, 1984), paralleling the capacity for neurogenic extravasation in these tissues. Such a technique for demonstrating presence of markers provides no information about individual neurons, therefore it was appropriate here to examine the distributions in the afferent cell bodies. Ositelu *et al.* (1987) showed that somatostatin in trigeminal ganglion neurons was present in facial skin afferents, but not masticatory muscle- or tongue-afferents, therefore it was interesting to see if a similar specificity was true for somatostatin in hindlimb sensory neurons.

In the present work, the study of each marker was combined with immunostaining by RT97, to label large light DRG neurons. This enabled a determination of the proportions of large light and small dark neurons retrogradely labelled from each target, and identification of the distribution of each marker among these small dark and large light cells.

Although originally thought to be present only in small dark cells, substance P immunoreactivity was shown by McCarthy & Lawson

(1989) to be present in 30% of small dark neurons and in 10% of large light neurons (as distinguished by RT97 immunoreactivity) in rat L4 DRGs. On the basis of conduction velocities, 10% of A-fibre neurons sampled, all of which had Aδ velocities, contained substance P, as did 50% of C-fibre neurons.

Lawson *et al.* (1987) found CGRP immunoreactivity in neurons with conduction velocities in the C-, Aδ- and Aβ-fibre ranges, and that a proportion of CGRP-positive neurons were consequently also RT97 positive.

4.2 Methods

Twenty-six male Wistar rats were given two or three injections of 1-2μl Fast Blue into either skin (n = 7), gastrocnemius muscle (n = 10) or knee joint (n = 9) of the hindlimb. After 6 (skin), 7 (muscle) or 4 (joint) days survival, the cell bodies of primary afferent neurons innervating these different target tissues were identified in fixed serial sections of L2-L6 DRGs by fluorescence microscopy. The sections were shared between four studies, each examining the presence, in the three types of target-identified neurons, of one of the markers. Thus sections were processed to demonstrate activity of TMP, or immunoreactivity to CGRP, substance P or somatostatin, and immunoreactivity to RT97.

In the TMP study, most of the sections were photographed immediately after being cut, to record the positions of FB-labelled cells, in case the enzyme-histochemical procedure caused loss of FB. RT97 immunostaining was performed directly after the TMP reaction.

In the neuropeptide studies, pre-photographing was not necessary; RT97 and neuropeptide immunostaining were usually performed concurrently.

Counts were made of FB-labelled nucleated cells with and without additional labels. The results presented here are for only high-intensity FB cells; medium-intensity FB cells were also counted, and those results are included in Appendix II.

4.3 Results

This section has the following parts:

4.3.1 Observations on the staining patterns of the markers;

4.3.2 Markers in target-identified neurons

4.3.3 Patterns of RT97 labelling;

4.3.4 Markers in small dark neurons; and

4.3.5 Markers in large light neurons.

4.3.1 Observations on the staining patterns of the markers

All markers stained cells distributed 'randomly' across sections and throughout the DRGs.

TMP study (see figure 4.1 and 4.4): Comparison of the pre-TMP photographs with the reacted sections indicated that in every high-intensity FB cell, enough of the fluorescent dye remained in the cells after the histochemical procedure to allow them to be identified, but the procedure had dimmed the FB intensity. No cells were positive for both TMP and RT97. About three quarters of the small dark cells were TMP positive.

CGRP study (see figures 4.2 and 4.5): Positive staining took several forms: large granules dotted across the cytoplasm, a bright, even staining of all the cytoplasm, or a perinuclear ring of granules with faint, even stain across all the cytoplasm. These patterns were independent of the presence of FB in a cell, but the cells with sparse large granules of CGRP immunoreactivity were frequently also RT97 positive. Both RT97 negative and positive cells were seen to contain CGRP. About half of all DRG cells were CGRP positive, and positive-staining axons were often visible.

Substance P study (see figures 4.3 and 4.6): the intensity of immunoreactive stain for substance P varied from very bright to faint, independently of other markers. Frequently substance P-positive axons were clearly visible. Substance P immunoreactivity coexisted in some cells with RT97 immunoreactivity, but to a lesser

extent than the overlap of CGRP and RT97. By inspection, 15-20% of all DRG cells were substance P positive.

Somatostatin study (see figures 4.2 and 4.7): Positive cells were characterized by a perinuclear ring of very bright immunoreactive granules. None were RT97 immunoreactive. About 5% of all cells were somatostatin positive.

4.3.2 Markers in target-identified neurons

Figures 4.1-4.3 show examples of retrogradely-labelled skin, muscle and joint afferents, counterstained for TMP, CGRP, substance P, somatostatin and RT97, in black and white; and there are examples in colour in Figs. 4.4-4.7).

Figs. 4.1 – 4.3 (over page): *Retrogradely-labelled skin, muscle and joint afferents in DRG sections counterstained for TMP, CGRP, substance P or somatostatin*

Each figure comprises three series of views of one DRG section containing FB-labelled neurons counterstained for TMP enzyme activity, or CGRP, substance P or somatostatin immunoreactivity, and in all cases for RT97 immunoreactivity.

In each row of three views of one DRG section, the first picture is of FB-labelled neurons under UV epifluorescence, the second is of the marker under study (i.e. TMP viewed by transmitted light, or neuropeptide viewed under epifluorescence filter appropriate for FITC), and the third is of RT97 immunostaining viewed under epifluorescence filter appropriate for FITC (in TMP study) or TRITC (in neuropeptide studies).

Fig. 4.1 <u>Top</u>: *Skin FB, TMP study* (a) high-intensity FB cell, (b) is positive for TMP, (c) and is RT97 negative. <u>Middle</u>: *Skin FB, TMP study* (d) medium-intensity FB cell, (e) is TMP negative and (f) RT97 positive. <u>Bottom</u>: *Joint FB, CGRP study* (g) three medium-intensity FB cells, (h) two are CGRP positive (closed arrows), one is negative (open arrow), (j) all three are RT97 positive. Scale bar = 40 μm.

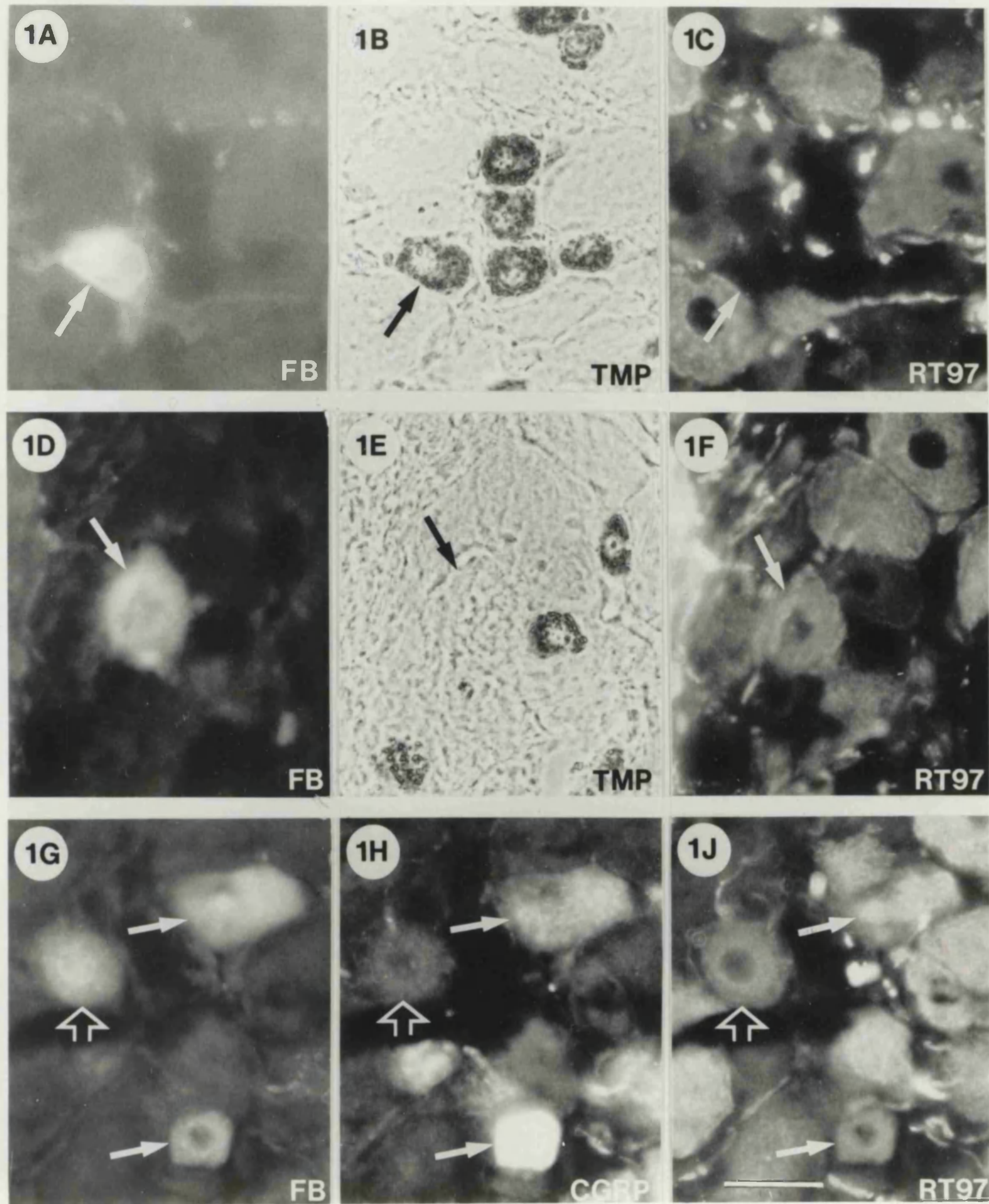


Fig. 4.2 <u>Top</u>: *Muscle FB, CGRP study* (a) high-intensity FB cell, (b) is positive for CGRP, and (c) for RT97. <u>Middle</u>: *Muscle FB, somatostatin study* (d) medium-intensity FB cell, (e) is somatostatin negative and (f) RT97 negative <u>Bottom</u>: *Skin FB, somatostatin study* (g) two high-intensity and one medium-intensity FB cells, (h) only one high-intensity FB cell is somatostatin positive (open arrow), (j) all three are RT97 negative. Scale bar = 40 μm.

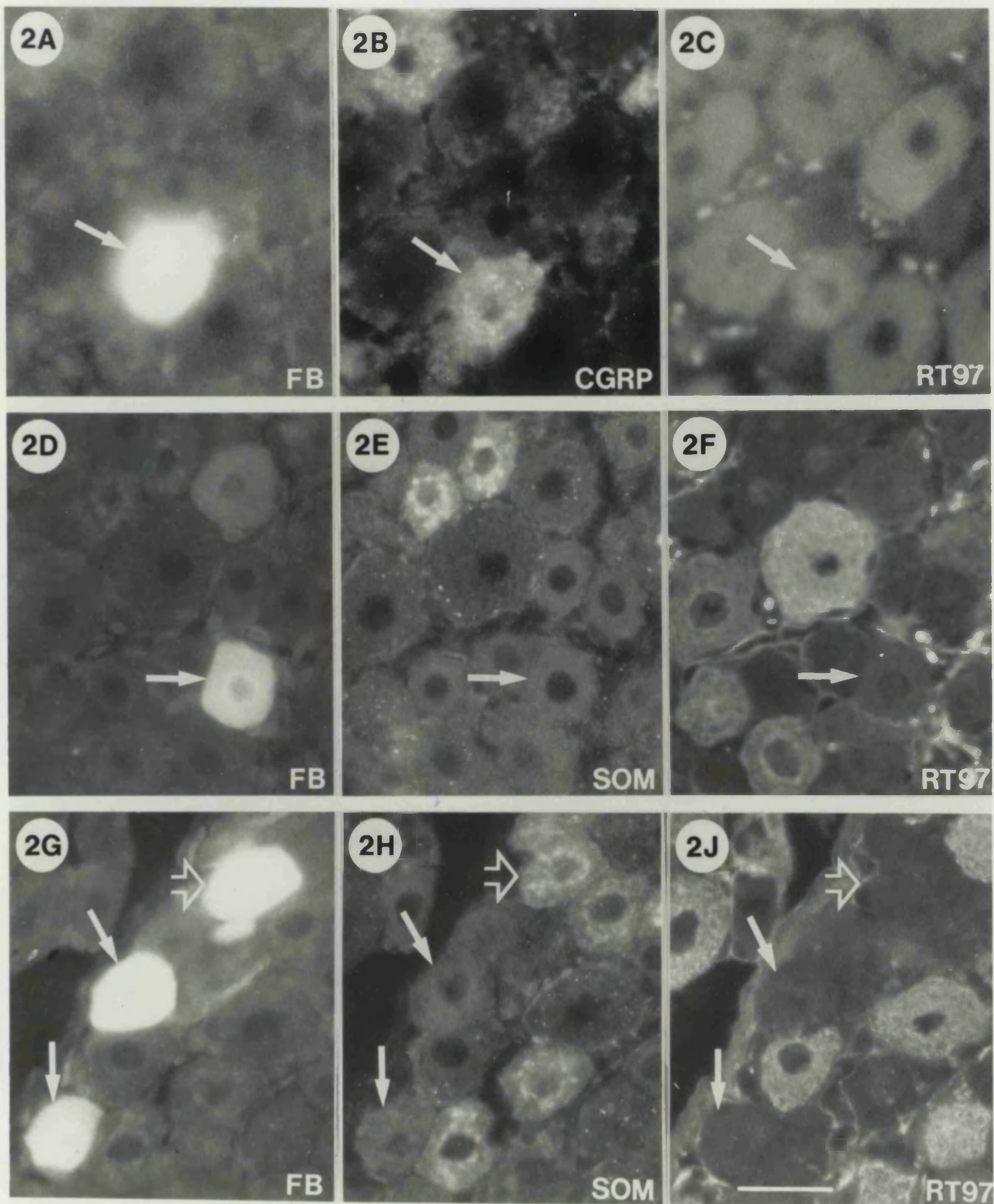


Fig. 4.3 Top: *Skin FB, substance P study* (a) three medium intensity FB cells, (b) only one of which is substance P positive (open arrow), (c) one substance P-negative cell is RT97 positive (curved arrow) and the others are RT97 negative. Middle: *Muscle FB, substance P study* (d) one high-intensity and one medium-intensity FB cell, (e) only the high-intensity FB cell is substance P positive (open arrow), (f) both are RT97 positive. Bottom: *Joint FB, substance P study* (g) one high-intensity FB cell, is (h) substance P positive, and (j) RT97 negative. Scale bar = 40 μm.

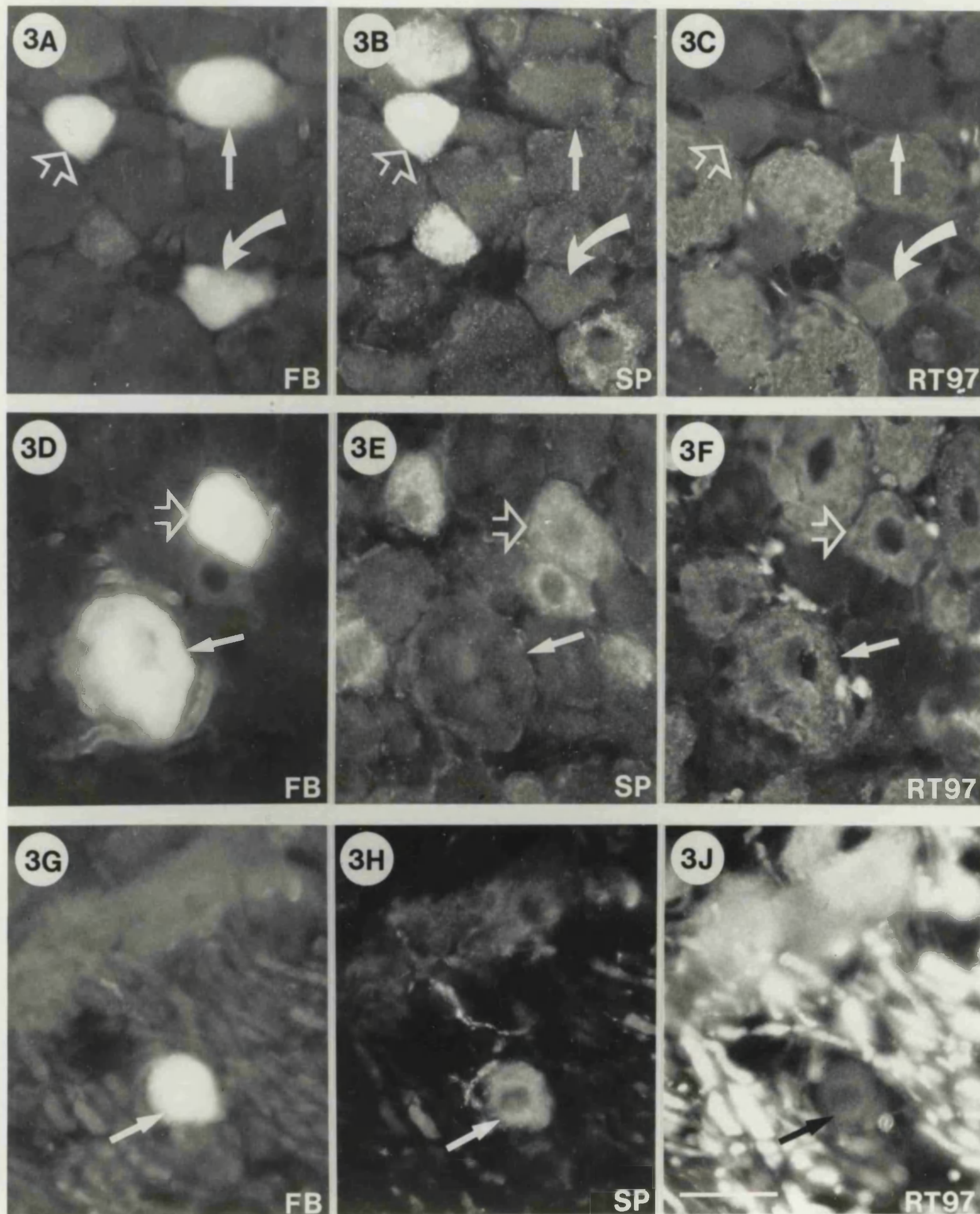


Fig. 4.4 (over page): *One DRG section viewed with filters appropriate for FB labelling, TMP enzyme reaction product, and RT97 immunostaining, to show counterstaining in neurons labelled by FB injection into skin* (a) two FB-labelled neurons (arrows), (b) only one is TMP positive (red arrow), (c) the same neuron is RT97 negative (red arrow) while the other cell is RT97 positive (orange arrow). Scale bar = 50 μm.

Fig. 4.5 (over page): *One DRG section viewed with filters appropriate for FB labelling, immunoreactivity for CGRP, and RT97 immunostaining, to show counterstaining in neurons labelled by FB injection into joint* (a) one FB-labelled neuron (arrow), is (b) positive for CGRP (green arrow) as is a large-diameter neuron (blue arrow), (c) FB-labelled neuron is RT97 negative (green arrow) while the blue-arrowed neuron is RT97 positive. Scale = 50 μm.

Fig. 4.6 (over page): *One DRG section viewed with filters appropriate for FB labelling, immunoreactivity for substance P, and RT97 immunostaining, to show counterstaining in neurons labelled by FB injection into muscle* (a) FB-labelled neuron (orange arrow) is (b) negative for substance P (an example of a substance P-positive neuron is shown by the blue arrow), (c) the FB-labelled neuron is RT97 positive (orange arrow) as is the blue-arrowed neuron. Scale bar = 50 μm.

Fig. 4.7 (over page): *One DRG section viewed with filters appropriate for FB labelling, immunoreactivity for somatostatin, and RT97 immunostaining, to show counterstaining in neurons labelled by FB injection into skin* (a) FB-labelled neuron (white arrow), is (b) negative for somatostatin (a positive neuron is shown by the green arrow), (c) the FB-labelled neuron (white arrow) is RT97 positive, while the green-arrowed neuron is RT97 negative. Scale bar = 50 μm.

Fig. 4.4

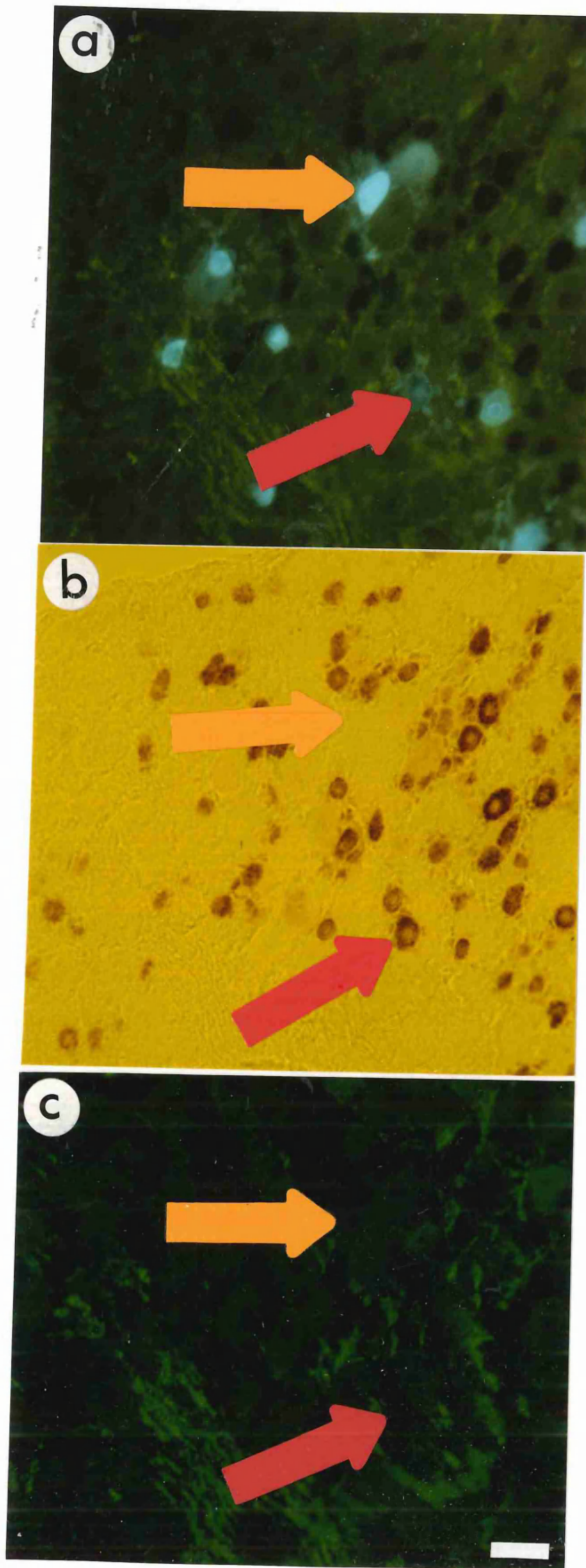


Fig. 4.5

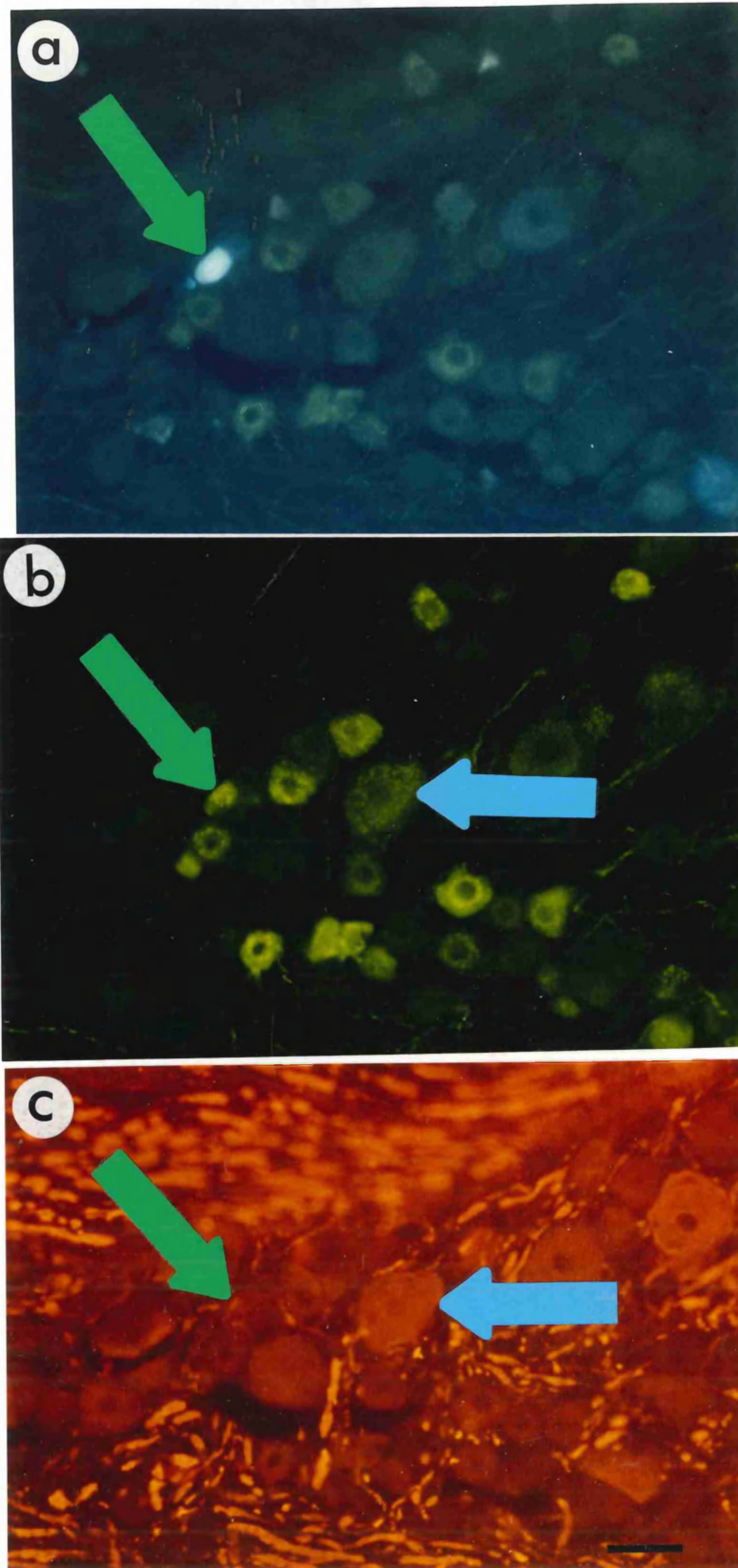


Fig. 4.6

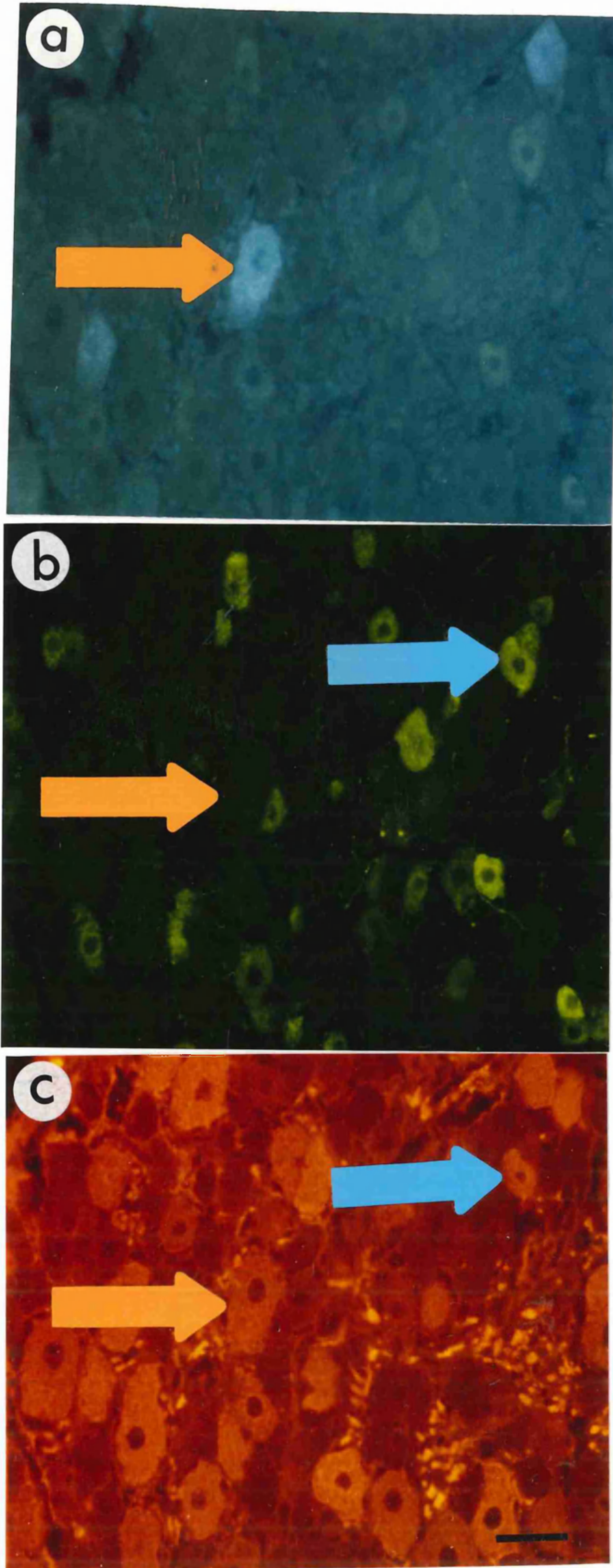
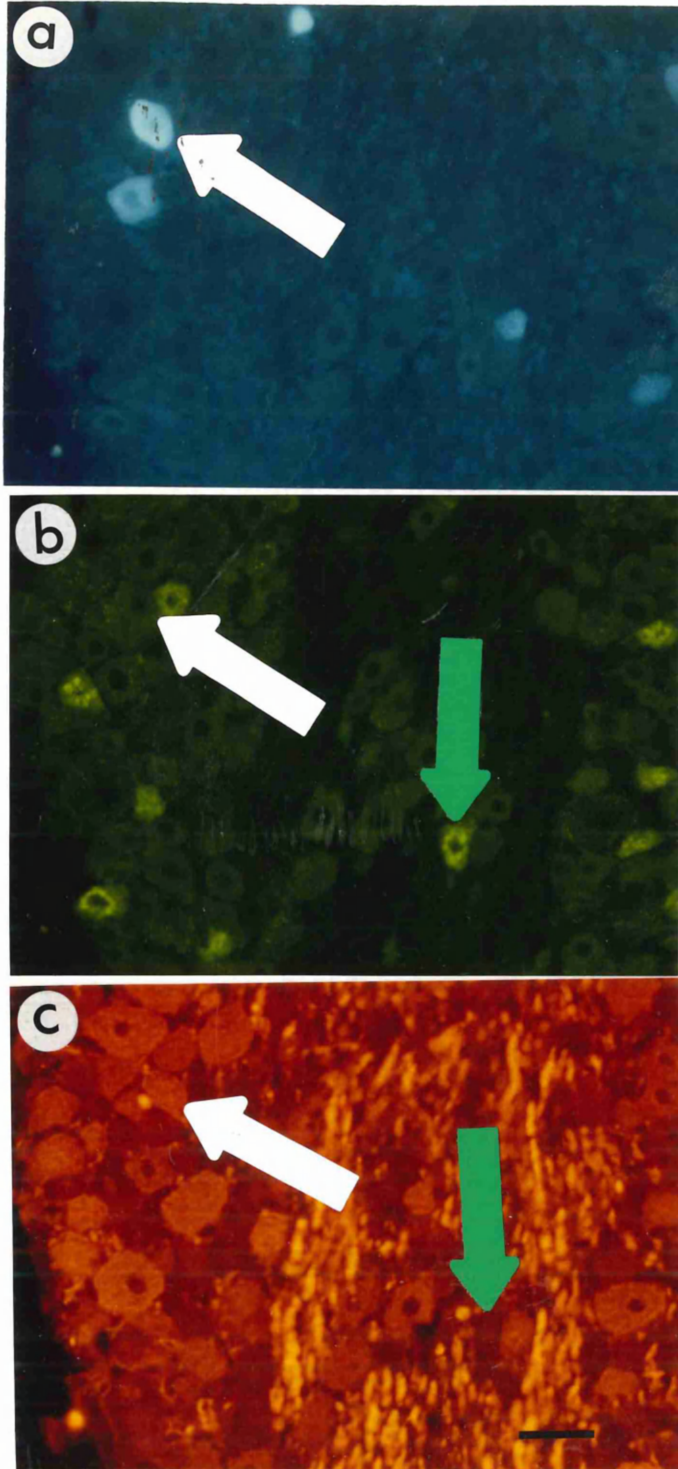


Fig. 4.7



Tables 4.1-4.4 present the raw data for each study i.e. the numbers of afferents found in the ganglia to contain high-intensity FB after injection of the tracer into skin, muscle or joint, how many were negative or positive for RT97, and whether they contained TMP, CGRP, SP or somatostatin, respectively. A total of 26 rats were used. The numbers of rats (n) from which DRG sections were used for each part of the study are indicated in each table. Where DRGs from separate rats were processed and the results counted separately, the same trends of incidence of markers were seen from one animal to the next, even though total numbers of cells were small.

The same trends displayed by the high-intensity FB retrogradely-labelled neurons, were followed by the medium-intensity cells (raw data in Appendix II), although with less pronounced differences between tissues.

In order to make the tables more easily comparable, data for each target tissue is presented in one row of each table regardless of whether data had originally been collected from each animal separately, i.e. results from all animals with the same site of FB injection are given together. At the start of the TMP and CGRP studies, the data was collected for each animal separately because the DRGs had been sectioned and collected on separate sets of slides. Later, however, the time spent cutting sections, and the number of slides generated for staining, was minimized by pooling the relevant DRGs from several animals which had received FB injections in the same tissue. All the data in the substance P and somatostatin studies was generated in this way.

Another change of protocol was made after the TMP and CGRP studies had begun, which was to generate two or three series of sections, on separate slides, from one batch of DRGs. Then the series were used in different studies (i.e. counterstained with different antibodies) so that one FB-labelled neuron split between sections could not be counted twice in the same study, and indeed where neurons were split, more data would be obtained if they were recounted in a different study. However, this method does mean that the total number of FB-labelled afferents counted and added across the studies does not represent the total number of afferents present in the DRGs.

TABLE 4.1: *Thiamine monophosphatase study*

Numbers of: high-intensity retrogradely-labelled DRG cells, those that were RT97 negative (small dark) and those which also contained TMP

TARGET	n	Total FB	RT97 negative	RT97 negative TMP positive
SKIN	2	184	130	96
MUSCLE	4	143	62	8
JOINT	4*	182	-	0
	2	68	-	0

* = not photographed before TMP reaction
- = not determined

TABLE 4.2: *Calcitonin gene-related peptide study*

Numbers of: high-intensity retrogradely-labelled DRG cells, those that were RT97 negative (small dark), and the occurrence of CGRP in RT97 positive and RT97 negative FB cells

TARGET	n	Total FB	Total RT97 negative	RT97 negative CGRP positive	RT97 positive CGRP positive
SKIN	3	116	71	35	22
MUSCLE	4	63	18	18	24
JOINT	3	62	9	7	37

TABLE 4.3: *Substance P study*

Numbers of: high-intensity retrogradely-labelled DRG cells, those that were RT97 negative, and the occurrence of substance P (SP) in both RT97 positive and RT97 negative FB cells

TARGET	n	Total FB	Total RT97 negative	RT97 negative SP positive	RT97 positive SP positive
SKIN	2	226	150	55	9
MUSCLE	2	174	64	50	11
JOINT	3	77	18	12	10
	3	35	-	(	13)

- = not determined

TABLE 4.4: *Somatostatin study*

Numbers of: high-intensity retrogradely-labelled DRG cells, those that were RT97 negative (small dark), and those that also contained somatostatin

TARGET	n	Total FB	RT97 negative	RT97 negative Somatostatin positive
SKIN	2	267	173	34
MUSCLE	2	211	94	1
JOINT	3	48	9	0
	5	127	-	0

- = not determined

TABLE 4.5: *Total target-identified cells with markers*

Distribution of markers as a percentage of total high-intensity Fast Blue retrogradely-labelled neurons

TARGET	TMP POSITIVE	CGRP POSITIVE	SUBSTANCE P POSITIVE	SOM POSITIVE
SKIN	96/184 = 52%	57/116 = 49%	64/226 = 28%	34/267 = 13%
MUSCLE	8/143 = 6%	42/63 = 67%	61/174 = 35%	1/211 = 0.5%
JOINT	0 = 0%	44/62 = 71%	35/112 = 31%	0/48 = 0%

SOM = somatostatin

4.3.3 Patterns of RT97 labelling

Comparing the results from the four studies (Table 4.6 summarizes the relevant numbers), the proportions of FB-labelled cells which were RT97 negative (small dark) were relatively consistent for one target tissue type, but different between targets. The proportions of RT97 negative afferents in the populations labelled from each tissue were: skin, 61-71%; muscle, 29-43%; joint, 15-23%.

4.3.4 Markers in small dark neurons

Table 4.7 summarizes the data on the presence of TMP, CGRP, substance P and somatostatin in small dark DRG cells (RT97 negative) retrogradely labelled from skin, muscle and joint.

TMP is not present in any joint afferents, and although we do not have results for RT97 labelling on these sections, we know that the proportion of small dark afferents in the population labelled from joint is low (15-23%). With a total of 250 high intensity FB joint afferents, however, we would have expected at least 37 (i.e. 15%) to be small dark - and yet none were TMP positive.

While TMP was present in three quarters of small dark skin afferents, only about an eighth of small dark muscle afferents were TMP positive, six times less than the proportion of small dark TMP-positive skin afferents.

CGRP was found in all small dark muscle afferents, three quarters of small dark joint afferents and half of the retrogradely-labelled small dark cutaneous afferents.

Substance P was present in three quarters of small dark muscle afferents, two thirds of small dark joint afferents, and just over one third of skin afferents.

Somatostatin was virtually restricted to small dark skin afferents where it was present in one fifth. No joint afferents, and only 1% of small dark muscle afferents contained this peptide.

4.3.5 Markers in large light neurons

Table 4.8 presents the data for the occurrence of markers in large light DRG cells (RT97 positive), which contain only CGRP ~~or~~ substance P, but not TMP or somatostatin.

TMP and somatostatin were not present in large light neurons. CGRP and substance P were present in large light afferents to all three tissues, the proportion displaying CGRP always higher than that with substance P. CGRP was found in 70% of large light joint afferents, and in about half of both muscle and skin large light afferents. Substance P was present in only one tenth of skin and muscle large light afferents, and in 17% of large light joint afferents.

TABLE 4.6: *Proportions of small dark cells in the populations labelled from each target*

Percentage of high-intensity retrogradely-labelled DRG cells which were RT97 negative (small dark) in each study

TARGET	TMP STUDY	CGRP STUDY	SUBSTANCE P STUDY	SOM STUDY
SKIN	130/184 = 71%	71/116 = 61%	150/226 = 66%	173/267 = 65%
MUSCLE	62/143 = 43%	18/63 = 29%	64/174 = 37%	94/211 = 45%
JOINT	- -	9/62 = 15%	18/77 = 23%	9/48 = 19%

SOM = somatostatin

TABLE 4.7: *Target-identified small dark cells (RT97 negative)*

Distribution of markers, as a percentage of the RT97 negative, high-intensity Fast Blue retrogradely-labelled cells

TARGET	TMP POSITIVE	CGRP POSITIVE	SUBSTANCE P POSITIVE	SOM POSITIVE
SKIN	96/130 = 74%	35/71 = 50%	55/150 = 37%	34/173 = 20%
MUSCLE	8/62 = 13%	18/18 = 100%	50/64 = 78%	1/94 = 1%
JOINT	0 = 0%	7/9 = 78%	12/18 = 66%	0/9 = 0%

SOM = somatostatin

TABLE 4. 8: *Target-identified large light cells (RT97 positive)*

Distribution of CGRP and substance P, as a percentage of the RT97 positive, high-intensity Fast Blue retrogradely-labelled cells. No TMP or somatostatin is found in RT97 positive cells

TARGET	CGRP POSITIVE	SUBSTANCE P POSITIVE
SKIN	22/45 = 49%	9/76 = 12%
MUSCLE	24/45 = 53%	11/110 = 10%
JOINT	37/53 = 70%	10/59 = 17%

4.4 Discussion

This section is presented in the following parts:

4.4.1 Purpose of the experiment;

4.4.2 Previous work on the distribution of markers in DRG neurons;

Comparison of present results with previous work on:

4.4.3 Distribution of markers in all target-identified neurons

4.4.4 Proportions of large light and small dark cells from targets

4.4.5 Distribution of markers in large light and small dark cells

4.4.6 Presence and role of markers at central terminals

4.4.7 Presence and role of markers at peripheral terminals.

4.4.1 Purpose of the experiment

The aim of this experiment was to examine whether, in the adult rat, there was a relationship between the peripheral target tissue of an afferent neuron, and the presence of four putative transmitter (or related) markers in the DRG cell body.

4.4.2 Previous work on the distribution of the markers in DRG neurons

Substance P was originally thought to be found only in small-diameter cells (Hokfelt *et al.*, 1975) which were all RT97 negative (Price 1985; Kai-Kai *et al.*, 1986). Indeed there was a general assumption that peptides were restricted to C-fibre neurons, which send dense terminations into laminae I and II of the spinal cord, the same area to which peptide staining is more or less restricted. The dark appearance of small dark cells was postulated to be due to their extra content of peptide-synthesizing 'machinery', required because the peptides as putative neurotransmitters would need constant replenishment, while large light neurons did not have this extra capacity because they used non-peptide transmitters, which could be taken up from the synapse or resynthesized by enzymes.

Somatostatin and substance P were shown to exist in separate populations of small-diameter DRG neurons (Hokfelt *et al.*, 1976), both of which were different from the FRAP population (Nagy & Hunt, 1982). Price (1985) found that FRAP- and somatostatin-positive neurons were all RT97 negative. CGRP, however, was seen by Ju *et al.* (1987) in large-diameter DRG neurons, and both CGRP and substance P have recently been shown to double-label some RT97 positive neurons (Lawson *et al.*, 1987; McCarthy & Lawson, 1989). Thus it was important in the present experiment to double-stain DRG sections for markers and RT97 to allow specific localization of peptides and TMP in small dark (unmyelinated) and large light (myelinated) neurons.

Comparison of the present results with work on:

4.4.3 Distribution of markers in all target-identified neurons

Molander *et al.* (1987) performed a study with aims similar to this experiment, using a slightly different technique for retrogradely identifying neurons. Whole nerves of (relatively) pure afferent composition were labelled with Fast Blue applied to the cut proximal stump. Thus large numbers of FB-labelled neurons could be identified in the DRGs as projecting to a cutaneous, muscle or visceral target. The distributions of the same four markers were studied as in the present work, except that FRAP staining was examined instead of TMP. Rather than performing detailed counts, however, estimates were made of FB-labelled neurons counterstained for each marker from an unspecified number of neurons, and no significance was attributed to different intensities of FB label.

Considering the present results for distribution of markers in the total target-identified population, the figures for CGRP and substance P in total skin afferents are 50% and 28% respectively, and 66% and 35% in muscle afferents (Table 4.5). These proportions are more than double the percentages found in each case by Molander *et al.* (1987).

The figures for TMP in total skin and muscle afferents are 52% and 6% respectively. Comparing this TMP result with the FRAP result of Molander *et al.* (1987): they found 20% of cutaneous, and 15% of muscle, afferents were FRAP positive, but here there is a much greater difference between the two targets. Similarly in the case of somatostatin, while Molander *et al.* (1987) found only one cutaneous and one muscle afferent containing this peptide, the present results showed that 13% of skin afferents and 0.5% of muscle afferents were somatostatin positive.

Differences in the counting methods used in these two experiments may account for some disparities between results, and another important difference lies in the method of retrogradely labelling the neurons. The technique used by Molander *et al.* (1987) of cutting nerves, thereby producing an axotomy, may be a complicating factor,

* If the present results for medium-intensity FB cells were included with the high-intensity results, then the differences in chemical expression between tissues would be less marked, and therefore these results would be qualitatively more similar to those of Molander *et al.* (1987), although quantitatively the present work would still show higher proportions of counterstained afferents. While it is true that some data is lost in the present work by the exclusion of results from medium-intensity FB cells, this was the only way to maximise the accuracy of the results. Because medium-intensity FB cells showed the same trends, but 'diluted', compared to high-intensity cells, this suggested that a population of medium-intensity FB cells were specifically labelled and exhibited very similar patterns of chemical expression to the high-intensity cells.

because axotomy is known to result in changes in gene expression and possibly transport, and thus the presence of these four markers may be affected (by up- or down-regulation) and the results obtained for counterstaining no longer represent the true control situation. Although such retrograde consequences of nerve injury are shown to become obvious - in terms of greatly increased or decreased numbers of neurons containing immunoreactive peptide - 14 to 28 days post lesion (for example, Himes & Tessler, 1989; Shehab *et al.*, 1986), the possibility still remains that expression of markers had begun to change when the tissue was sampled three days after FB application.

The characteristics of dye uptake may be different at an axon terminal, from that at the site of axotomy. A proportion of afferents which may have resisted uptake of FB at the terminals by the present method, are more likely to have been labelled by Molander *et al.* (1987), with the result that total labelled afferents here may not represent the full spectrum which innervate each target. If the resistant axons happened to be those supplying cell bodies which did not contain the four markers under investigation, then the percentages of retrogradely-labelled neurons with counterstains would be artificially increased compared to the results of Molander *et al.* (1987). Indeed most of the present results do indicate a higher degree of counterstaining, but as mentioned above the results of Molander *et al.* (1987) may, at the same time, be lower than the true value because of the axotomy.*

Ositelu *et al.* (1987) used retrograde tracing from target tissues in the face: the tongue, masseter muscles and facial skin. Only skin afferents contained somatostatin, the proportion (3.5%) being much smaller than the 12% result of the present work. The difference may be due to the use of a different (less sensitive) antibody, or could reflect a real difference between sensory innervation of facial skin and leg skin (related to characteristics such as hairiness, thickness, vascularity; but see below on the presence of somatostatin in skin itself).

Other groups have examined chemical specificity within whole nerves rather than at the level of DRG cell bodies. McMahon *et al.*

(1984) examined substance P and FRAP in 24 hr-ligated sural (cutaneous) and gastrocnemius (muscle) nerves. Sections of the fixed sural nerve showed dense FRAP staining and many substance P-positive fibres on the ganglionic side of the ligature, but in the sectioned gastrocnemius nerve many fewer marker-positive fibres were present.

Ligation of a peripheral nerve allows visualization of accumulated, transported markers, and results from such experiments cannot be expected to match the apparent distribution of markers in cell bodies for the following reasons:

Rates of transport of different markers in different types of afferent axon may not be comparable (in the same way that various tracer substances are transported at different rates or not at all by different fibre types; Borges & Sidman, 1982; Sawchenko & Gerfen, 1985; Plenderleith *et al.*, 1989). Endogenous substances which are transported need not necessarily represent the entire spectrum of markers produced in the DRG cell body, or amounts may be so small as to go undetected after 24 hrs ligation. Alternatively, a substance may be transported and released at such a rate that it is more easily detected by accumulation at a ligation, than in the cell body where absolute levels are always low. This was the case for Gibbins *et al.* (1987), who found somatostatin immunoreactivity on the ganglionic side of lumbosacral dorsal roots *in vivo*, when the peptide was very rarely detected in neuronal perikarya.

Despite the above reasons for mis-matching results, the distribution of TMP shown in the present work, whereby about nine times as many skin as muscle afferents contain TMP, agrees with the results of McMahon *et al.* (1984) in ligated nerves. In the case of substance P however, the present results suggest that the proportion of total muscle afferents containing this peptide is higher than among skin afferents. The disparity between this result and those of McMahon *et al.* (1984) may be due to different characteristics of transport of substance P in the two target-classes of afferents. In addition, the relative numbers of axons in the gastrocnemius and saphenous nerves (Baron *et al.*, 1988) indicate that the total axon populations from which the present percentage results are derived are

widely different, and in a nerve-ligation experiment such percentage differences could be masked or reversed due to this large disparity in the total population sizes. Finally, the fact that substance P-containing neurons form a separate subpopulation from the FRAP positive neurons (Nagy & Hunt 1982) may explain why the results for TMP can be compatible between the present work and that of McMahon *et al.* (1984), at the same time that results for substance P are not.

4.4.4 Proportions of large light and small dark cells from targets

The different proportions of large light and small dark neurons labelled from each peripheral target (Table 4.6) suggest that the spectrum of afferents required to supply each tissue is different, depending on the function of sensory receptors and nerve endings in the targets. Of the three tissues, skin had the highest proportion of RT97 negative retrogradely-labelled afferents (61-71%), indicating that these would be unmyelinated axons, probably mostly polymodal nociceptors (Lynn & Carpenter, 1982) and possibly having also an efferent inflammatory role. Joint afferents, on the other hand, had the lowest proportion of RT97 negative retrogradely-labelled neurons, and therefore of unmyelinated afferents carrying high threshold information, suggesting that more of the sensory receptors are low-threshold with myelinated axons, subserving proprioceptive functions, and Group III nociceptors with thinly-myelinated axons. Langford & Schmidt (1982) found that cat articular nerve Group III and IV fibres outnumbered those of Group II by at least two to one, therefore in order to explain the present result (aside from species differences) that so few joint afferents were RT97 negative, (unmyelinated, therefore Group IV) it can be proposed that most of the RT97-positive afferents fall into the Group III class. This is not incompatible with the results of Schaible & Schmidt (1983) on cat joint receptors. Joint afferents have been demonstrated to participate in inflammatory reactions (reviewed in Fitzgerald, 1989b), and the 15-23% of retrogradely-identified neurons which were RT97 negative are most likely nociceptors which have been attributed with this inflammatory role. Muscle afferents showed a distribution intermediate to skin and joint afferents, with 29-45% of retrogradely-labelled neurons being RT97 negative. Thus myelinated,

fast-conducting axons with stretch receptors, thinly-myelinated high-threshold mechanoreceptors, and unmyelinated axons to nociceptors, may be represented (Mense & Mayer, 1985).

It must be remembered, however, that the proportions of RT97-negative and -positive neurons found by this retrograde identification method may not represent the true distribution, but may be a function of the different capacities of neuronal types to pick up the FB in different tissue environments. (This caveat also applies to the observed distribution of chemical markers in target classes of neurons). In addition, each of the targets investigated contain similar structures such as blood vessels and connective tissue, so part of the results arise from afferents with the same function or projection in each target, i.e. a component of the results is probably the same for each target, partly masking the differences which are due to the structures peculiar to each target.

4.4.5 Distribution of markers in small dark and large light cells

The distribution of markers found here within small dark and large light neurons (as defined by absence or presence, respectively, of RT97 immunostaining) is in agreement with that found by Price (1985) who found that somatostatin and FRAP were restricted to small dark cells; and with McCarthy & Lawson (1989) and Lawson *et al.* (1987) who found overlaps between RT97 and CGRP or substance P immunoreactivity in electrophysiologically-characterized DRG neurons, showing that a proportion of each of these types of peptide-containing afferents are large light neurons. Terenghi *et al.* (1985) have also observed CGRP staining in thinly-myelinated sensory fibres innervating the eye. The presence of myelin suggests that these fibres would have RT97 positive cell bodies (Lawson & Waddell 1985).

4.4.6 Presence and role of markers at central terminals

The four chemical markers (putative transmitters) investigated here have previously been localized in the spinal dorsal horn, mainly

in lamina II. Substance P and CGRP extend into lamina I, and CGRP fibres descend laterally into the deeper dorsal horn. Somatostatin and TMP may be restricted to outer lamina II (Jessell & Dodd, 1989).

The cutaneous unmyelinated afferents containing all four markers are therefore likely to terminate in lamina II. The myelinated afferents from skin containing substance P, and in particular CGRP, may project (eg Brown, 1981a,b; Woolf, 1987) to laminae III and IV - which do not stain so prominently for the peptides as lamina II. Alternatively these afferents may consist of a high proportion of Aδ high-threshold mechanoreceptors terminating in lamina I and outer lamina II, and lamina V. As for the small proportion of muscle unmyelinated afferents containing TMP, it is not clear whether these have central terminals in inner lamina II where FRAP and TMP staining is found (although projections from muscle have not consistently shown terminations in lamina II), or whether they arborize less extensively in other laminae.

The substantial proportion of muscle afferents we have shown to contain CGRP and substance P presumably terminate in lamina I rather than lamina II, as Mense & Craig (1988) showed cat muscle afferent terminals in laminae I and V in the dorsal horn, with no projection in lamina II. These afferents might therefore be nociceptors and thermoreceptors, which have such projection sites (Mense & Mayer, 1985). A similar spinal projection was seen for cat joint afferents (Craig *et al.*, 1988) so again both the RT97 positive and negative joint neurons shown here to contain substance P and CGRP probably terminate in lamina I, and therefore represent Group III and IV mechanical and chemical nociceptors (Schaible & Schmidt, 1983). Single afferent labelling of rat muscle and joint Group IV terminals is required to indicate definitively whether there is any projection to lamina II.

Markers shown to be present in cell bodies may not be so easily detectable in central terminals, therefore in some cases our chemical distribution may not tally with the known spinal distributions of markers.* Also, the axons at either end of a DRG neuron exhibit different patterns of transport: three times as much substance P is

* e.g. The substance P-containing RT97 positive afferents, which are probably myelinated and therefore terminate ventral to lamina II, or else in lamina I. Substance P immunoreactivity is seen only in laminae I and II.

transported down peripheral sensory axons as down central (dorsal root) axons (Harmar & Keen, 1982).

Two sorts of *in vivo* measurements have been made to determine what types of stimuli cause the release of neuropeptides in the dorsal horn: perfusion with a push-pull cannula, and insertion of antibody-coated microprobes. Kuraishi *et al.* (1985), in rabbit hindlimb, found that noxious (pinch) stimulation of skin increased the release of substance P in the perfused ipsilateral dorsal horn, while noxious thermal (radiant heat) stimulation increased somatostatin release. Kuraishi *et al.* (1989) showed that subcutaneous injection of formalin into the hindlimb also caused increased substance P release in the dorsal horn, but other types of stimuli did not.

Duggan & Hendry (1986) developed a substance P-antibody-coated microprobe which could be used to detect more precisely the site of substance P release anywhere in the dorsal horn, upon peripheral stimulation of afferents. Experiments were performed on cats, in which peptide coexistence in primary afferents is more complicated than for rat, and in particular substance P and somatostatin are found in the same DRG neurons (Leah *et al.*, 1985). Duggan & Hendry (1986) showed that there was a basal release of substance P which was increased by electrical stimulation only when it was above C-fibre threshold, and the increased release was confined to the region of the substantia gelatinosa and laminae V-VI. Duggan *et al.* (1987) went on to investigate the specificity of the stimulus which caused this release. Noxious heating of the hindpaw skin (by immersion in water at 50°C and 52°C) for two or three periods of ten minutes, consistently caused substance P release in the region of the substantia gelatinosa.

Thus stimulation of nociceptors in the skin of rabbits and cats cause afferents or spinal cord neurons to release substance P or somatostatin selectively after certain types of peripheral stimuli. The present results show that significant proportions of skin afferents in the rat contain substance P or somatostatin, and

therefore these afferents may subserve similar nociceptive roles in the rat.

4.4.7 Presence and role of markers at peripheral terminals

For each of the three tissues investigated here, the information which exists about presence of chemical markers is in agreement with the results on their distribution in target-identified afferents.

Skin

Gibbins *et al.*, (1987), having demonstrated the presence of substance P and CGRP immunoreactivity in DRGs of the guinea pig, went on to examine axonal structures in peripheral target tissues. Substance P and CGRP colocalized in axons around blood vessels in the dermis of hairy skin. In the plantar skin of the foot, substance P and CGRP axons penetrated into the epidermis. Dalsgaard *et al.* (1983) found substance P-immunoreactive free nerve endings within dermal papillae in human skin.

By radioimmunoassay, CGRP was found in the rat epidermis in decreasing concentrations in the nose, footpad, face, tail and back (Mulderry *et al.* 1984).

Tainio *et al.* (1987) investigated the same two peptides in nerves in human skin, and saw only CGRP in fibres around eccrine sweat glands. Sensory nerves beneath the epidermis and around hair follicles contained both CGRP and substance P.

Evidence that substance P released from peripheral terminals can interact with local 'effector systems' is provided by the distribution of binding sites for ^{125}I-substance P in human and rat skin (Deguchi *et al.*, 1989). Binding sites were localized to dermal papillae, sweat glands and hair follicles, and the highest-density binding in rat was in the paw pad skin as opposed to back and abdominal skin. Thus in dermal papillae and hair follicles, the presence of substance P in nerve endings is matched by the distribution of substance P binding sites, providing a basis for an interaction between substance P released from peripheral terminals,

and local systems such as blood vessels, sweat production, sebaceous glands, mast cells, etc.

Johansson & Vaalasti (1987) examined somatostatin immunoreactivity in human skin, finding it in fibres running in the dermis and forming free nerve endings in the epidermis. Compared to skin from the arm, back and chest, relatively few somatostatin fibres were seen in specialized areas such as fingertips. Very few fibres were found close to blood vessels and sweat glands.

Silverman & Kruger (1988) developed a method for visualizing FRAP-positive fibres in the periphery, using the binding characteristics of a plant lectin. It is technically difficult to stain peripheral tissues directly with the FRAP histochemical method (although Szonyi *et al.*, 1979, did use the method successfully on rat cornea). Working from the knowledge that monoclonal antibodies to specific carbohydrate moieties differentially immunostained classes of DRG neurons (Dodd & Jessell 1985, Lawson *et al.*, 1985), Silverman & Kruger examined plant lectins with binding characteristics (i.e. carbohydrate specificities) similar to the antibodies. The lectin *Griffonia simplicifolia* I-B_4 was discovered to have essentially the same specificity for a class of central terminals and DRG cell bodies as FRAP staining, so Silverman & Kruger went on to stain whole-mounts of the rat testicular *tunica vasculosa* and cornea with lectin, visualized using indirect biotin-streptavidin-peroxidase. The corneal epithelium and the *tunica vasculosa* were richly innervated by lectin-positive fibres. This work is difficult to extend to skin because whole mounts would not be so amenable to staining, but the *tunica vasculosa* might be considered as a model of polymodal receptors (Kumazawa *et al.* 1987), innervated by thin axons (Peterson & Brown, 1973), as found in skin (Lynn & Carpenter, 1982). The present finding that 52% of skin afferents contain TMP would then be in agreement with these findings.

Muscle

Gibbins *et al.* (1987) saw varicose axons containing substance P and CGRP in skeletal muscle of the limbs. The axons surrounded small

and large arterioles, either between muscle fibres or in the connective tissue surrounding bundles of muscle fibres.

Joint

Axons containing substance P were seen in joints in rats by Levine *et al.* (1984).

The role of neuropeptides in inflammatory processes is examined in Chapter 6.

CHAPTER FIVE: Is there a relationship between Nerve Growth Factor (NGF) receptors on afferent neurons, and the peripheral target?

5.1 Introduction

The most studied neurotrophic factor, NGF, is present in sensory neuron target tissues in differing amounts. The protein is selectively internalized by some primary sensory neurons in adult mammals and influences their survival and function (Goedert *et al.* 1981; Richardson & Riopelle, 1984; Johnson *et al.*, 1986). In addition, a variety of *in vivo* and *in vitro* approaches suggest that at least 80% of DRG neurons require NGF for survival at a certain stage in development (reviewed in Lindsay, 1987). This critical period has been examined for chick DRG neurons (Eichler & Rich, 1989): when removed at different ages (E15 and E19) but kept in culture for the same time period (6 days), the neurons showed dramatically different responses to acute withdrawal of NGF. Thus while most E19 neurons survive (absolute age 26 days), E15 neurons (absolute age 21 days) are more likely to die. This suggests that between 21 and 26 days the requirement for NGF is intrinsically "switched off".

High-affinity receptors, half-maximally saturated by picomolar concentrations of NGF, are thought to internalize NGF and mediate its biological actions (Sutter *et al.*, 1979). These receptors can be localized by binding of ^{125}I-NGF. More abundant low-affinity sites of uncertain biological importance can be localized using an α-NGF receptor monoclonal antibody. Low-affinity receptors are also found on many neurons with high-affinity receptors, but the exact overlap is not known (however the two affinities probably represent different states in the same molecule, because there is only one species of mRNA; Green & Greene 1986). Some non-neuronal cells (e.g. Schwann cells) possess only low-affinity receptors.

A postnatal reduction in the levels of mRNA for the receptor in rat DRG has been shown by Buck *et al.* (1987), providing a basis for the decrease in the proportions of NGF-responsive neurons in the mature animal (see Lindsay 1987, 1988). It is not known whether there are developmental changes in the neuronal expression of low- and high-affinity receptors, and indeed the relationship between expression of receptors by mature neurons, and their requirement for NGF, is unclear.

As we had access to the monoclonal antibody to low-affinity NGF receptors, experiments studying the distribution of low-affinity receptors for NGF among skin and muscle afferents have been performed to investigate whether the presence of these receptors on mature DRG neurons matches the availability of NGF in the target tissues: levels of NGF and its mRNA are higher in skin than in muscle, which also correlates with the density of sympathetic innervation (Heumann *et al.* 1984; Shelton & Reichardt 1984).

Because the biological role of low-affinity NGF receptors is unclear, we are unable to answer the question of which retrogradely-identified neurons are potentially sensitive to recognized actions of NGF.

5.2 Methods

Eight male Wistar rats (200-350g) were injected with Fast Blue either in the skin (n=4) or muscle (n=4) of both hindlimbs. Fixed DRGs were removed after six (skin L2-L4) or seven (muscle L3-L6) days survival, and cut into a separate set of sections for each rat. All sections containing FB-labelled neurons were immunostained with NGF receptor antibody, and visualized with a Texas Red immunofluorescent layer.

Sections were examined under epifluorescence, and the presence or absence of NGF receptor immunostaining was scored for high- and medium-intensity Fast Blue neurons. A control-stained section (incubated without primary antibody) displayed no fluorescence.

5.3 Results

5.3.1 Observations on the staining pattern of α-NGF receptor antibody

The immunolabelling by the α-NGF receptor antibody was seen in neuronal cytoplasm of a great proportion of all DRG cells. In addition, there was heavy staining on neuron surface membranes and in the extracellular spaces - probably on satellite cells. The receptor functions *in situ* on the cell membrane, therefore it is expected that dense staining will be seen on this structure, but as receptor molecules are manufactured, recycled or endocytosed, they will also be located at intracellular sites.

Among the neurons, some had a 'woolly' fluorescence in the cytoplasm, while others displayed discrete strongly-labelled structures. Although the woolly fluorescence was weaker than the staining in other cells, it was still above the background colour levels seen in control sections, and was counted as positive.

Figures 5.1 and 5.2 show examples of FB labelling and NGF receptor immunoreactivity, in skin and muscle afferents respectively.

5.3.2 Distribution of NGF receptor immunoreactivity among skin and muscle afferents

Table 5.1 presents the counts of NGF receptor immunoreactive high-intensity FB-labelled skin and muscle afferents. The equivalent data for medium intensity neurons is included in Appendix III.

Among the four rats with FB-labelled cutaneous afferents, the proportion of high-intensity retrogradely-labelled neurons which were NGF receptor-immunoreactive fell into the range 57-68%, with a mean of 63%. For high-intensity FB-labelled muscle afferents, the percentages of NGF receptor positive neurons were in the range 82-95%, with a mean of 91%. These results are significantly different.

TABLE 5.1: *NGF receptor (NGFr) in high-intensity retrogradely-labelled FB neurons (separate counts for four rats each labelled with skin FB or muscle FB)*

RAT	Total FB	NGFr positive	% of total
SKIN FB			
S1	136	93	68
S2	216	126	59
S3	124	71	57
S4	152	100	66
MEAN ± S.E.M.			63 ±2.7*
MUSCLE FB			
M1	80	75	94
M2	64	58	91
M3	74	70	95
M4	74	61	82
MEAN ± S.E.M.			91 ±3.0*

* Significantly different ($p<0.005$)
Student's t-test (two-tailed)

Fig. 5.1: *Section of L3 DRG containing one FB-labelled neuron after injection of FB into skin, counterstained with an antibody to the NGF receptor* Viewed under epifluorescence.

(a) under UV, blue arrow shows FB-labelled neuron, (b) under filter for TRITC, the FB-labelled neuron is faintly immunoreactive for NGF receptor. The green arrow shows two neurons which are negative for NGF receptor immunoreactivity. Scale bar = 50μm.

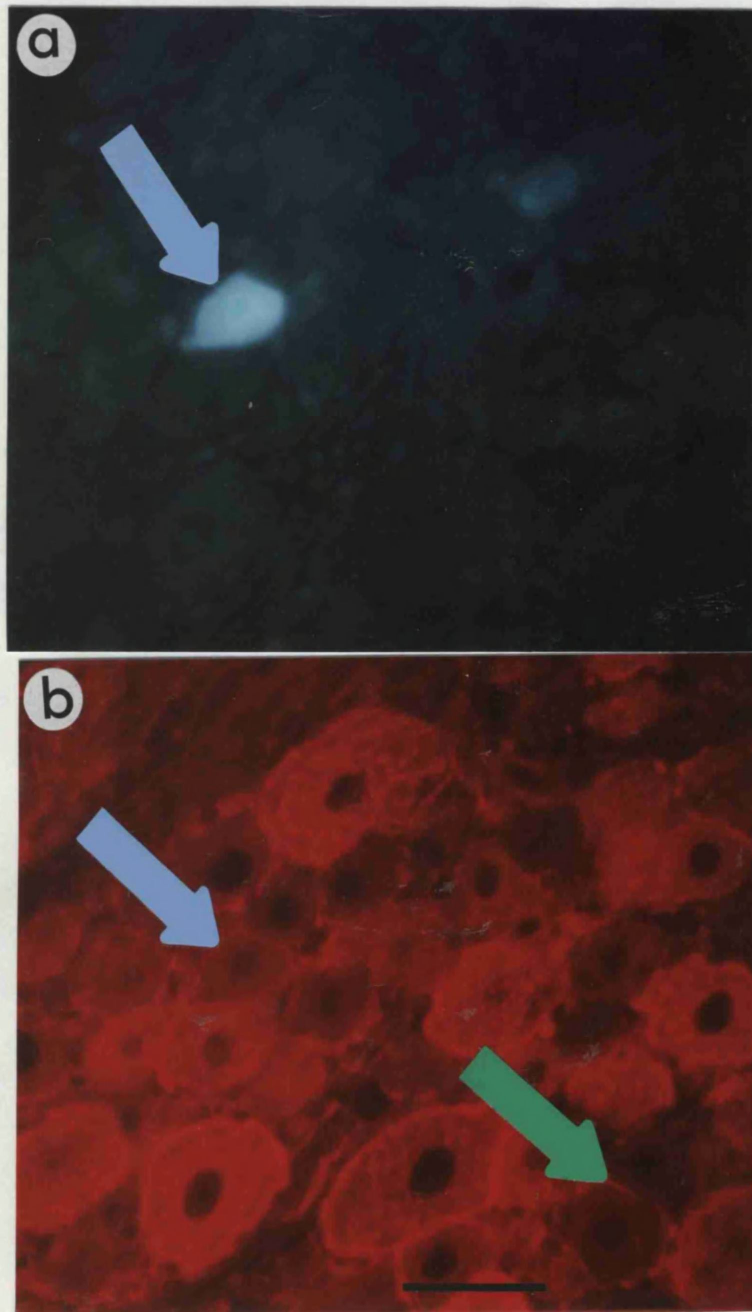
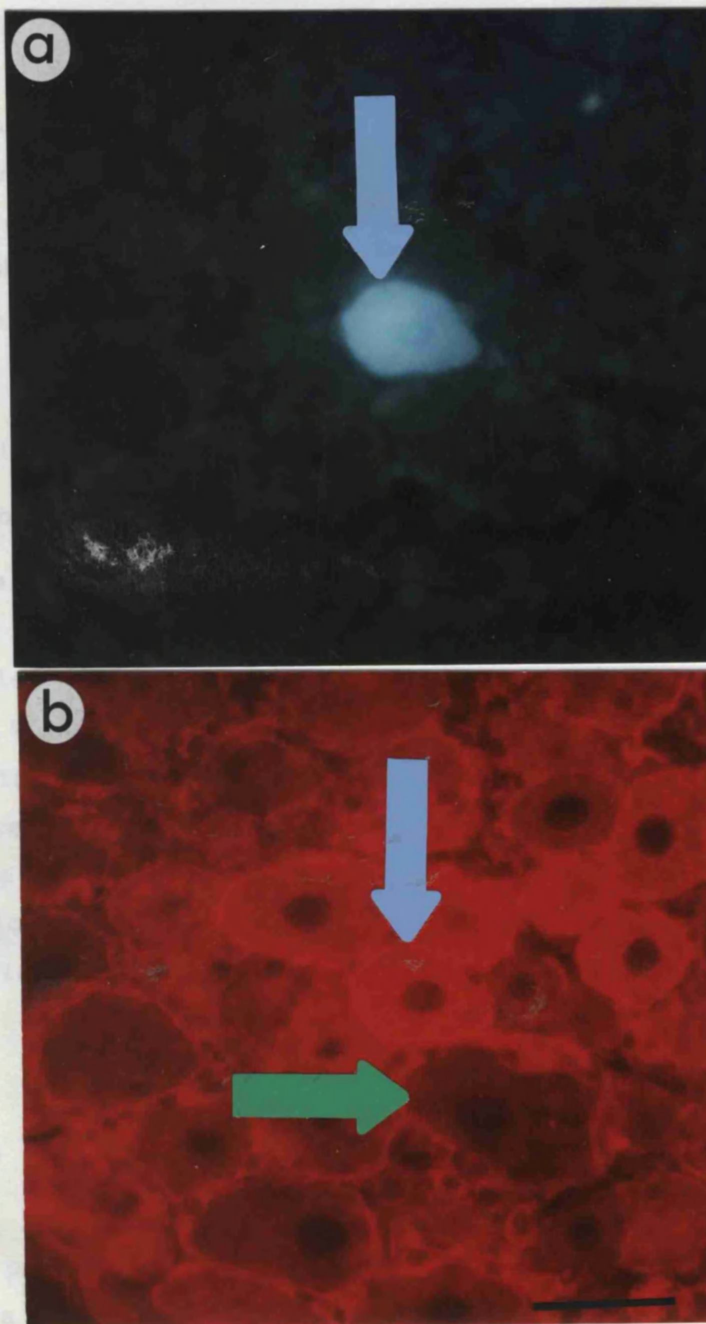


Fig. 5.2: *Section of L5 DRG containing one FB-labelled neuron after injection of FB into muscle, counterstained with an antibody to the NGF receptor* Viewed under epifluorescence.

(a) under UV, blue arrow shows FB-labelled neuron, (b) under filter for TRITC, the FB-labelled neuron is strongly immunoreactive for NGF receptor. The green arrow shows a large-diameter neuron which is negative for NGF receptor immunoreactivity. Scale bar = 50 µm.



5.4 Discussion

5.4.1 Purpose of the experiment

This experiment examined the distribution of low-affinity receptors for NGF on retrogradely-identified skin and muscle afferents from the rat hindlimb. While the biological importance of the low-affinity receptors is uncertain, there is evidence that they and the high-affinity receptors represent two kinetic states of the same molecule (Green & Greene, 1986). If the states were interconvertible, the present results could indicate what proportion of skin and muscle afferents are potentially responsive to the actions of NGF in the mature animal. However the signals or mechanisms for interconversion of kinetic states are unknown. It was also of interest to see whether receptor distribution matched the distribution of NGF and its mRNA previously demonstrated in the target tissues of skin and muscle afferents.

Previous work on this topic and comparison with the present results:

5.4.2 Sizes and proportions of immunoreactive neurons

Richardson *et al.* (1986) used ^{125}I-NGF to bind to high-affinity binding sites (i.e. receptors) on rat L5 DRGs, and found about 45% were heavily labelled. The mean diameter of labelled neurons was only slightly less than that for unlabelled neurons, but relatively few neurons with diameter >50 µm were labelled. Although detailed counts of total receptor immunoreactivity were not performed in the present work, it was estimated that probably 75% of total DRG neurons were labelled for low-affinity receptors. In addition, some of the most strongly labelled neurons were among the largest in the DRG (up to 70 µm).

5.4.3 Correlation between receptor on afferents, and ligand in target fields

Studies of NGF protein and its mRNA in target fields have shown that muscle contains sparse amounts of the protein or the ability to

synthesize it, in contrast to skin which contains much higher concentrations (Heumann *et al.* 1984; Shelton & Reichardt 1984). (However, a high-molecular-weight form of NGF is synthesized and secreted by fused and unfused rat skeletal muscle cells *in vitro*; Murphy *et al.*, 1977). The high localization of low-affinity NGF receptor on muscle afferents as opposed to skin afferents shown here clearly does not match up with the *in vivo* NGF distribution. The reason for this mis-match is not known. If low- and high-affinity receptors represent the same molecule, then high-affinity receptors might also show a distribution among target-identified neurons which does not match the availability of the ligand; conversely, our result with the low-affinity receptor may be misleading due to an incomplete overlap between the two receptor types.

5.4.4 Overlaps between presence of peptides and receptor

The study by Verge *et al.* (1989a) is relevant with respect to the present results. The distribution of ^{125}I-NGF binding (i.e. high-affinity receptors) on neurons containing TMP, CGRP, substance P and somatostatin was examined in rat lumbar DRG sections. Virtually all neurons with CGRP immunoreactivity had NGF binding sites, although 10% of large neurons with dense NGF labelling were not positive for CGRP. Smaller neurons which were substance P positive were consistently heavily labelled by NGF, whereas other small neurons containing somatostatin immunoreactivity or thiamine monophosphatase activity were not specifically labelled by the ligand.

The present results show that around 90% of muscle afferents and 60% of skin afferents were immunoreactive for low-affinity NGF receptor. Referring to the distribution of markers in skin and muscle afferents shown in Chapter 4, some comparisons can be made between this data for NGF receptor, and the work of Verge *et al.* (1989a) on NGF binding sites. In Chapter 4, substance P and CGRP were present in both skin and muscle afferents, but greater proportions of muscle afferents than skin afferents contained each peptide. Verge *et al.* (1989a) find NGF receptor to colocalize with

most CGRP and all substance P neurons, thus any target-specific class of neurons containing those peptides should exhibit NGF receptor, which the present results do indeed show - with most muscle afferents immunoreactive for the low-affinity receptor. In the case of TMP, which was poorly represented in muscle afferents but present in half of skin afferents, that proportion of each target-specific class which does not contain the marker (i.e. >90% of muscle afferents, and 50% of skin afferents) may be represented by those neurons which do have NGF receptor. Similarly with somatostatin, which does not colocalize with NGF receptor (Verge *et al.*, 1989a), but which is present in 12% of skin afferents; these skin afferents presumably belong to a separate subgroup from the 60% of receptor-positive skin afferents. Thus despite the use of techniques which demonstrate two different types of NGF binding site, the present results and those of Verge *et al.* (1989a) are highly comparable, and suggest a co-localization of high- and low-affinity receptors.

5.4.5 Role of NGF in the mature animal

By quantitative autoradiography, the density of high-affinity receptors for NGF on L5 DRG neurons was shown by Verge *et al.* (1989b) to decrease dramatically after sciatic nerve section, due to both decreased receptor density and cell atrophy. Neurons with high-affinity receptors are more likely to atrophy than those without, suggesting that those neurons which are responsive to NGF in the adult require a supply from the target, which is terminated by nerve section. Administration of NGF restored receptor density to normal.

Other neuronal responses to injury have been suggested to be due to NGF deprivation, such as the loss of peptides from the DRG cell bodies and their terminals in the dorsal horn after peripheral nerve section. For example, Fitzgerald *et al.* (1985) showed that administration of NGF via an osmotic pump on the proximal end of the cut sciatic nerve partially reversed the depletion of substance P and FRAP in the superficial dorsal horn. That NGF treatment should partially reverse a FRAP depletion, is interesting with respect to

the results quoted above of Verge *et al.* (1989a) who found minimal localization of high-affinity NGF receptors on TMP positive neurons. *In vitro* experiments (Lindsay & Harmar, 1989) demonstrate that NGF regulates substance P and CGRP, at the level of their mRNA, in DRG neurons which are essentially axotomized in the process of being cultured.

Despite the presence of NGF and its mRNA being greater in skin than muscle, suggesting that cutaneous afferents are sensitive to NGF, or require NGF for maintenance, the present results and those in Chapter 4 suggest that peptide-containing muscle afferents may also have the capacity to respond to NGF because the majority of muscle afferents possess low-affinity receptors. Alternatively the low-affinity receptors seen here may have operated in the high-affinity state during development, when the majority of neurons (probably including muscle afferents) require NGF for survival. Thus in addition to the developmental changes in NGF receptor synthesis (Buck *et al.*, 1987), a change in the neuronal population requiring NGF could be due to a switching between kinetic states of the receptor molecule, for example by loss of an essential component of the receptor.

To resolve some of these questions, it would be useful to identify skin and muscle afferents and perform autoradiography for the high-affinity NGF binding sites, and to combine detection of the low and high-affinity sites on the same tissue to examine the exact overlap between these two receptor types; in addition one could combine the retrograde identification technique with studies on DRG cell atrophy after cutaneous or muscle nerve section, and examine the response to NGF administration in target-identified neurons.

CHAPTER SIX: To investigate whether the target - phenotype relationship can be changed by altering the environment at the peripheral terminals.

6.1 Introduction

Having established that primary afferent phenotype is related to the peripheral target tissue (Chapter 4), this experiment was designed to study whether skin afferents would react to a changed environment at their peripheral terminals, by changing their expression of neuropeptide. The underlying question being asked is this: is the relationship between target and chemical expression fixed in the adult once established during development, or is the relationship plastic, so that when different signals are detected at the target, the neuron is influenced to begin expressing a new neuropeptide?

The method chosen for changing the environment at the afferent terminals in skin was the induction of inflammation, achieved by subcutaneous injection of turpentine oil, which has been shown (Woolf, 1984) to produce decreased thresholds to mechanical stimuli at the inflamed site lasting for two weeks, in addition to a transient oedema. The presence of substance P in skin afferents supplying the inflamed area was examined.

The involvement of chemosensitive afferents, and the peripheral release of substance P, in neurogenic inflammation was discussed in Chapter Two. Thus as well as signalling to the CNS that a noxious stimulus has been received by the tissue, substance P in afferent neurons may have a peripheral function in the response to injury, and indeed may contribute to the resulting pain by itself stimulating nociceptors. Experimentally-induced arthritis in rats is associated with increased substance P levels in afferent nerves (Lembeck *et al.*, 1981) and with an increased density of substance P-containing nerve fibres in the joint most severely affected (Levine *et al.*, 1984).

Associated with tissue damage and inflammation is the development of hyperalgesia, partly due to peripheral sensitization and to increased central excitability in second order neurons in the spinal cord (Cook *et al.*, 1987). Central changes, lasting long after the initial stimulus is over, might also be mediated by changes in transmitter released by the neurons involved in signalling damage. While an increase in levels of signal could be an adequate mechanism, release of signals at a novel site in the spinal cord, from afferents which did not previously contain that transmitter, would be a more striking change.

There is already evidence that inflammation does result in more DRG neurons expressing substance P: The mRNA for substance P has been shown to be present in increased numbers of neurons in lumbar DRGs ipsilateral to a formalin injection into the hindpaw (Noguchi *et al.* 1988). In the following experiments, cutaneous inflammation was induced by injection of turpentine oil in the skin area from which afferents were retrogradely labelled, and then immunoreactivity for substance P examined in these afferents.

6.2 Methods

Wistar rats (n=3) of 200-350g were given injections of FB into the skin of the inner thigh bilaterally, with four injections marking the corners of a square of about 1 cm^2 on each leg. The following day, 25 µl of turpentine oil was injected into the centre of the FB-labelled area unilaterally. Different sizes of turpentine oil injections had been tested on control rats to establish the extent of the inflamed area so produced. A 25 µl volume could be injected without causing an overt protrusion of skin, and within 24 hours had produced an inflamed area of about 1.5 cm diameter, as judged by the redness of the skin.

Five days after the turpentine injection (six days post FB labelling), rats were perfused and the L2-L4 DRGs processed as normal. Experimental DRGs (ipsilateral to the turpentine injection) were collected separately from control DRGs (contralateral), and the

contribution from each animal pooled. Two series of sections were serially cut from each set of ganglia. Only one series was processed for substance P and RT97 immunostaining, to reduce the possibility of counting nucleated profiles more than once when they were split between sections (each section was, however, still compared with those next to it in the series, to ensure no profile was counted twice). The occurrence of substance P and RT97 in FB-labelled neurons was recorded for all sections in both experimental and control DRGs. Some sections could not be counted because the RT97 immunostaining was poor — this resulted in a smaller number of FB-labelled neurons from which data could be used in contralateral DRGs.

6.3 Results

The numbers and calculated percentages, respectively, of high-intensity retrogradely-labelled neurons from both ipsilateral and contralateral DRGs expressing substance P, are presented in Tables 6.1 and 6.2. The equivalent raw data for medium-intensity FB neurons is given in Appendix IV. There are some data points which are incomplete because RT97 staining was not determined, so the results for substance P in total FB neurons (first column, Table 6.2) add up to more than the results for substance P in RT97 positive and negative neurons (second and third columns, Table 6.2). Also included in Table 6.2 are the results of the control study from Chapter 4 on the distribution of substance P in skin afferents from rats without inflammation,

As can be seen in Table 6.2, there are not great differences between ipsilateral afferents and the contralateral and control results, with respect to the total proportion of FB cells expressing substance P, and the proportion of RT97 negative (small dark) cells with substance P. However, there is an increase in RT97 positive (large light) cells which are substance P positive in the ipsilateral population to 24% from a contralateral value of 11% . The proportion of RT97 negative cells within the FB-labelled population was lower in afferents both ipsilateral (38%) and contralateral (45%) to the inflammation, compared with the control result from Chapter 4 (66%).

TABLE 6.1: *Substance P and inflammation study*

For DRG neurons both ipsilateral and contralateral to the inflamed skin, this table shows the **numbers** of: high-intensity retrogradely-labelled DRG cells, those that were RT97 negative, and the occurrence of SP in both RT97 positive and RT97 negative FB cells

SKIN	Total FB	Total RT97 negative	RT97 negative SP positive	RT97 positive SP positive
CONTRALATERAL FB	57	15	6	2
IPSILATERAL FB	241	55	19	21

TABLE 6.2: *Substance P and inflammation study*

For DRG neurons ipsilateral and contralateral to the inflamed skin, and for control DRGs from Chapter 4, this table shows the **percentages** of: SP in total high-intensity FB, and in RT97+ and RT97- separately, and RT97 negative as % of FB neurons

SKIN	SP in total FB	SP in RT97+	SPin RT97-	RT97 neg in FB
CONTRALATERAL	12/57 = 21%	2/18 = 11%	6/15 = 40%	15/33 = 45%
IPSILATERAL	65/241 = 27%	21/89 = 24%	19/55 = 35%	55/144 = 38%
CONTROL	64/226 = 28%	9/76 = 12%	55/150 = 37%	150/226 = 66%

NB. The numbers in the last column indicate that data on RT97 immunolabelling was not obtained for all FB neurons. eg top row, only 33 out of a total of 57 FB neurons could be classified with the RT97 antibody. On remaining sections the RT97 labelling was too poor.

6.4 Discussion

6.4.1 Purpose of the experiment

The aim was to investigate whether mature neurons can respond to a change in their environmnent by *de novo* expression of a neuropeptide. Skin afferents innervating an area of skin in which inflammation had been induced by turpentine oil injection, were examined for immunoreactivity to substance P. Contralateral DRG neurons provided a control.

6.4.2 Can this technique provide a definitive answer

At the crux of the question of whether a neuron can change its neuropeptide expression, is whether we are looking at new expression or increased amounts of peptide. There is a problem with using immunocytochemistry to demonstrate *de novo* expression of peptide, and that is the sensitivity of the technique, and the limits of peptide detection. Rather than new synthesis of peptide, we may be looking at increased levels in neurons which had very low, undetectable, levels previously, due to changes in transport, release and breakdown characteristics of the peptide. While Northern analysis of total mRNA in tissues can show the underlying changes in mRNA for the neuropeptide of interest, there is no information as to the proportion of neurons which have contributed to the result. *In situ* hybridisation histochemistry would seem to offer the necessary method for identifying exactly which cells are expressing the mRNA, but again the question of levels of detection is apposite where radiolabelled probes have been used: as there is always a background level of radioactivity, an arbitrary level of signal (at a certain factor times background radiation) must be chosen to indicate the presence of mRNA. Therefore in this case too, increased levels of signal may bring some cells above the critical signal:noise ratio.

The present results cannot provide a definitive answer about neurons expressing substance P *de novo* in response to an inflammation stimulus, but are sufficiently suggestive to warrant further experiments.

How the present results compare with previous work:

6.4.3 What happens to peptide expression in DRGs

Noguchi *et al.* (1988) used *in situ* hybridisation histochemistry to examine expression of the mRNA which codes for substance P, preprotachykinin A (PPTA). PPTA also codes for a related peptide, substance K, which is produced in a ratio of 1:3 with substance P (Ogawa *et al.* 1985). Looking at DRGs from rats which had received unilateral injections of formalin subcutaneously in the plantar skin,

Noguchi et al (1988) found that the proportion of PPTA-positive neurons was increased from 11% in control ganglia to 17% in ganglia from the formalin-treated side. There were more neurons exhibiting a strong hybridisation signal in experimental ganglia than in control. A significant difference in the number of positive neurons was seen 3 hr after formalin injection, and lasted for at least 24 hr. By 48 hr there was no difference between control and experimental ganglia. Immunostaining for substance P peptide 24 hr after injection showed no increase in peptide-positive neurons on the experimental side, but a time lag for peptide synthesis and post-translational modification must be expected.

Minami *et al.* (1989) analysed PPTA by Northern blots to show that the total levels were signficantly increased in DRGs L4-6 and lumbar spinal cord ipsilateral to adjuvant-induced inflammation of the rat hind paw. The increase persisted for 15 days, which is longer than suggested by Noguchi *et al.* (1988), whose results showed an increased number of PPTA-containing cells only for 24 hrs post induction of inflammation. There may be a transient and reversible increase in the number of cells which express PPTA, but a lasting increase in the level of expression in cells which were always PPTA-positive.

6.4.4 What type of afferents are involved

The present results could suggest that inflammation at the afferent terminals in the skin caused an increase in the proportion of neurons with detectable levels of substance P. The increase was entirely within the large light population of skin afferents, which normally account for only one sixth of substance P-containing skin afferents (Chapter 4) or 30% of total substance P-containing neurons in L4 DRGs (McCarthy & Lawson 1989). This suggests that the afferents in which substance P immunoreactivity becomes detectable under the influence of inflammation have myelinated fibres. Whether the presence of substance P in myelinated fibres is correlated with changes in peripheral sensitivity (i.e. sensitization), neuroeffector function or central function remains to be established.

6.4.5 Effects of inflammation at central terminals

Substance P has been attributed with a role in transmitting nociceptive information to the dorsal horn, and therefore a prolonged noxious stimulus at the peripheral terminals, such as turpentine inflammation, might require increased expression of substance P in order that it does not become depleted at the central terminals. In the chronic pain model of adjuvant-induced arthritis, substance P release in the spinal dorsal horn is increased (Oku *et al.* 1987). Minami *et al.* (1989) have shown that inflammation causes an increase of PPTA in the spinal cord, therefore both DRG cells and intrinsic spinal cord cells increase their substance P and substance K expression, and may be more excitable because of this.

6.4.6 Effects of inflammation in peripheral tissues

Levine *et al.* (1984) showed that joints with a higher risk of becoming affected by injury (e.g. ankles) have a higher concentration of substance P, and further, infusion of substance P itself into the knee joint exacerbates arthritis and joint destruction. Thus the role of the nervous system in arthritic-type inflammation goes beyond that of signalling from the affected joints: the nerves themselves (and their substance P content) are part of the cause. Inflammation could then cause peripheral release of inflammatory mediators (via substance P release) which stimulate neurogenic inflammation. The effect of gold, an antirheumatic therapy, on substance P levels in rat peripheral nerve was investigated by Levine *et al.* (1988), who showed that gold salts, which reduce severity of experimentally-induced arthritis, produce a depletion of substance P from the sciatic nerve as well as selectively decreasing numbers of unmyelinated afferents in peripheral nerve. Thus a neurotoxic action of gold salts on these peptidergic afferents appears to produce the anti-inflammatory effects.

The intestine has a sensory nerve supply which contains both substance P and CGRP (Molander *et al.*, 1987; Gibbins *et al.*, 1985).

Axons containing these peptides follow blood vessels, and are believed to be involved in inflammatory processes.

Mantyh *et al.* (1988) have studied the distribution of binding sites for ^{125}I-substance P and two other members of the tachykinin family in the normal and inflamed human colon. They found that receptor binding sites for substance P only were expressed in the inflamed colon in concentrations up to 2000 times control levels. The sites of binding were arterioles and venules throughout the mucosal and muscle layers, and also within lymph nodules. Popper *et al.* (1988) found CGRP and substance P immunoreactive nerve fibres and receptor binding sites (but not somatostatin binding sites) in canine mesenteric lymph nodes specifically associated with arterioles and venules. These findings have been taken to show a means by which the sensory system can influence intestinal inflammatory and immune responses, with substance P and CGRP being released peripherally from sensory endings in these tissues.

Therefore there is much evidence to suggest that neuropeptides, especially substance P, are upregulated in inflammatory conditions, and that they are part of the cause, as well as a result, of inflammation. Whether the upregulation is due to a change of neuropeptide phenotype of some afferent neurons as a response to the tissue damage remains to be demonstrated conclusively.

CHAPTER SEVEN: *In vitro* culture of target-identified adult rat dorsal root ganglion neurons.

7.1 Aim

Having studied the relationship between the peripheral target tissue innervated by DRG neurons *in vivo*, and their phenotype, the aim of the following work was to investigate whether such a relationship can be altered by a change in the chemical environment of the neurons. Retrograde labelling of DRG neurons *in vivo* was combined with subsequent *in vitro* maintenance of the target-identified cells, where the growth environment could be precisely controlled. By varying the culture conditions, for instance to include neurotrophic factors, or growth medium conditioned by target tissue, the neurons would be exposed to influences characteristic of peripheral target tissues other than those they were originally subject to *in vivo*.

First it was necessary to show that the techniques could be successfully combined: (a) that the retrogradely-transported fluorescent tracer was retained specifically, and (b) whether such labelled neurons would settle and develop normally *in vitro*; and (c) to check that the histochemical and immunocytochemical procedures were compatible with the culture system

7.2 Background

Tissue culture in neurobiology can be considered a reductionist tool, helping to simplify the heterogeneity of neuronal and glial elements which constitute the nervous system. If subclasses of cells can be separated, the study of the biochemistry and physiology of each cell type is made much easier.

In practice this requires methods for separating neurons from glial cells, and enriching procedures for the particular cell of interest. The dorsal root ganglion is already a relatively simple

system containing only one neuronal type, plus associated non-neuronal cells - Schwann and satellite cells and fibroblasts.

The *in vitro* system for culture of adult rat DRG neurons used here was developed by Lindsay (1988) from a procedure first used by Scott (1977). An important modification introduced by Lindsay (1988) is the technique for enrichment of neurons from the dissociated cell suspension, along with greater attention to maximising the yield.

Another aspect of sensory neuron culture is whether these cells depend on neurotrophic factors for survival. Embryonic DRG neurons have been shown to require NGF both *in vivo* and *in vitro* (for review see Lindsay, 1987), and while the situation in the adult appears to be that NGF is not continuously required as a survival factor, this aspect had not been addressed in detail *in vitro*, prior to Lindsay (1988). His finding was that neither NGF, Brain-derived neurotrophic factor (BDNF), nor any other source of neurotrophic activity (serum, non-neuronal cells, other neurons) was required for long term (3-4 week) survival of 80% of adult sensory neurons. Therefore the adult neuron cultures, as opposed to embryonic or neonatal sensory neurons *in vitro*, provide a useful system in which to examine the effects of neurotrophic and other activities.

7.3 Methods

7.3.1 Neuron culture method

DRG neurons were labelled with FB as usual by injections into the Sa area skin or the Ti and Ga muscles. After a seven day survival time, rats were asphyxiated and the DRGs dissected aseptically. Experimental DRGs, i.e. those containing neurons with axons projecting to the dye-injected target, were collected separately from other, non-projecting ganglia which would serve as control populations. For all culture experiments, rats were 200-350g in weight.

The collection medium was F-14 (Imperial Labs., UK) supplemented with 10% heat-inactivated fetal calf serum (Gibco) (F14FCS). F14 is an enriched (double amino acids, extra vitamins and co-factors)

formulation of Ham's F12 medium. Ganglia were trimmed of roots before being enzymatically treated (37°C) and dissociated by a modification of the procedure of Lindsay (1988), as follows:

Ganglia were incubated twice for 1.5hr in F14FCS with 0.125 % collagenase (Boehringer-Mannheim; clostridiopeptidase), washed twice in F14, treated with 0.25 % trypsin (Worthington) in F14 for 30 mins, washed twice with F14FCS, and finally suspended in 2 ml of F14FCS. A single-cell suspension was then generated by trituration of the softened ganglia by 8-10 passages through the constricted tip of a flame-polished Pasteur pipette.

Cells were either plated immediately on 35 mm culture dishes, previously coated with polyornithine (500 µg/ml) and laminin (5 µg/ml; BRL-Gibco), or plated the following day after enrichment of neurons. If small numbers of ganglia (ie <10) were being processed in separate batches, the preplating step was omitted to minimise neuronal loss. Enrichment of larger collections of ganglia was achieved by plating the dissociated cells on a 60 mm culture dish previously coated with polyornithine. After 15-20 hr, the non-neuronal cells were firmly attached to the dish while most of the neurons were only weakly adherent to the dish or to flattened non-neuronal cells. By carefully removing the culture medium, most dead cells and axonal and myelin debris were discarded prior to selectively dislodging the attached neurons with a gentle stream of culture medium delivered from a constricted Pasteur pipette. Further neuronal enrichment (also used for non-preplated suspensions) was also achieved by centrifugation of the cell suspension at 500 rpm for 5 min, whereupon viable neurons were lightly pelleted, leaving myelin debris, dead cells and small non-neuronal cells in suspension, to be discarded before neurons were resuspended in fresh medium.

Cells were maintained at 36.5°C, 97% air, 3% CO_2, in 1.5 ml of medium supplemented with NGF (prepared by Dr. J. Winter by a method modified from Suda *et al.* (1978); 200 ng/ml, which is saturation concentration for the effect of NGF in promoting neurite outgrowth from neonatal rat DRG); or α-NGF antibodies (gift from Dr Y. Barde; sheep anti-NGF (mouse), 1:200, which should neutralize 200 ng/ml NGF)

or myotube-conditioned medium (preparation method below). Whatever medium was used, the final mixture was filtered through a size 0.2μm millipore filter just before addition to dishes. Where cultures would eventually be used for immunostaining, sometimes the cell suspension was plated by adding it as a 50μl drop to the centre of the coated 35 mm dish, and when after several hours the neurons had adhered in a spot of about 1cm diameter, the dish was flooded with 1.5 mls of medium.

During at least the first three days post plating, media also contained the mitotic inhibitor cytosine arabinoside (Ara C; 10^{-5}M) to prevent proliferation of non-neuronal cells. Medium was replaced every 3 or 4 days, and if non-neuronal contamination was high, Ara C was included again for a further few days.

7.3.2 Preparation of myotube-conditioned medium

Thigh muscles were dissected aseptically from 1 day old rat pups, minced with fine scissors in F14FCS, and incubated twice with 0.5% trypsin in F14 for 20 mins, 37°C. After rinsing in F14FCS, the tissue was triturated through a 5 ml plastic pipette and then filtered through 90 μm gauze. The cells were plated at a density of about 1 x 10^{5} per 35 mm collagen-coated petri dish. (Collagen was prepared by stirring tendons from one rat tail in 200 mls of sterile 0.1% acetic acid overnight at 4°C, spinning down undissoved tendons and using supernatant to coat culture dishes by air-drying).

After 24-48 hours, the myocytes began to line up and then fused into long, thin, multinucleated myotubes, a process which was complete by about 6 days post plating, resulting in a network of criss-crossed and parallel myotubes covering the dish. In healthy-looking cultures the myotubes would rhythmically contract at several foci across the dish.

Myotube-conditioned medium (MCM) was collected from such cultures at intervals of two days and replaced with fresh medium. Usually only two or three such collections were possible before the cells (due to their intrinsic contractile activity) were dislodged, as a sheet,

from the dish surface. Collected medium was immediately frozen at minus 20°C. When required for use in neuronal cultures, MCM was diluted 1:1 with fresh F14FCS, then Ara C and NGF were added, and the whole filtered through 5 μm, then 0.2 μm millipore filters. This medium was used on neurons from the plating step onwards, and replaced every 3 days.

7.3.3 Fixing and mounting cultures

(All the following procedures are at room temperature unless otherwise stated). Cultures were fixed as required for microscopy, or enzyme or peptide counterstaining, by first rinsing off culture medium with PBS, then gently flooding with 4% paraformaldehyde in PBS for 30 mins. Fixative was rinsed off with several changes of PBS. Sometimes neurons were kept at this stage in PBS at 4°C for up to 24 hours before use. If cultures were to be examined without further processing, dishes were prepared for microscopy by draining off PBS and coverslipping with anti-fade mountant (Citifluor; City University). The edges of the dish were cut off, and the coverslip sealed with nail varnish. These discs were taped to glass microscope slides.

7.3.4 Histochemistry

To demonstrate TMP activity in cultured DRG neurons, the incubation medium of Knyihar-Csillik *et al.* (1986) (except that the buffer was pH 5.6) was prepared in a wide shallow glass dish placed in a water bath. Fixed culture dishes were submerged in the incubation medium at 37°C for 90 mins, rinsed by submerging in Tris buffer (0.05M, pH 5.6) and reacted with 1% ammonium polysulphide for 2 mins. After several rinses in distilled water, and then PBS, cultures were coverslipped.

7.3.5 Immunocytochemistry

Fixed cultures were prepared for immunostaining by flooding with PBS/0.1% Triton-X-100 (PBS/TX, also used for subsequent rinsing) for 10 mins, and similarly with PBS/TX/10% horse serum (also used as antibody diluent).

Some cultures had been prepared as 'spots' in order to minimise the required volume of diluted primary antibody. A cotton bud was used on these to wipe the dish dry around the area of cells, so that a 50μl drop of antibody solution stayed on the cells without spreading. Otherwise the antibody solution was applied to the whole dish.

Dishes were incubated overnight at 4°C in a humid atmosphere. After several rinses, the second layer was applied for 1 hour, followed by further rinsing, and similarly the third layer. After a final rinse, dishes were coverslipped as above.

The primary antibodies employed here were rabbit α-CGRP (CRB; 1:1000), and, concurrently or alone, RT97 (gift from Dr. J. Wood; 1:2000). Thus for concurrent staining, the second layer of the antibody reaction was biotinylated anti-rabbit IgG (Amersham; 1:200), and the third layer comprised FITC-conjugated streptavidin (Amersham; 1:100) and TRITC- or Texas Red-conjugated anti-mouse IgG (Amersham, 1:100). CGRP immunoreactivity was thus labelled by fluorescein, RT97 immunoreactivity by TRITC or Texas Red. When RT97 was applied alone, sometimes the labelled anti-mouse IgG was applied as a final second layer, and in other cases biotinylated anti-mouse IgG (Amersham, 1:100) was the second layer, followed by FITC- or Texas Red-conjugated streptavidin.

Specificity of antisera: No staining was seen when primary antibodies were omitted. Adsorption of the diluted α-CGRP antiserum with 50 μg/ml of pure CGRP for 24 hours, 4°C, prior to application to the dishes, resulted in no positive staining of cultures.

7.3.6 Microscopy

Cultures were examined on a Nikon Fluophot epifluorescence microscope with filters appropriate for FB (excitation wavelength 390-420 nm), FITC (450-490 nm) and TRITC/Texas Red (510-550 nm), also with transmitted light and phase-contrast optics. The area containing cells was scanned systematically from side to side moving over the dish, and counts made of: (1) FB-labelled neurons with and without additional counterstains by tracking on the FB filter and then switching to the other filters, (2) all neurons, in low density cultures, with and without counterstains, by tracking on phase contrast, and (3) samples of total neurons in high density cultures, with and without counterstains.

7.3.7 Specificity of retrograde labelling

From the *in vivo* project examining retrogradely-labelled and counterstained DRG cells, it was apparent that: (a) FB intensity was not consistent between cells, and that only the high-intensity cells were entirely the result of specific retrograde labelling, and (b) the histochemical procedure for demonstration of TMP activity tended to 'wash out' some of the FB, so that fainter FB labelling could disappear altogether, while the high-intensity cells were always still easily visible (as checked by photographing FB before TMP processing).

A similar range of FB intensities was apparent in neurons *in vitro*, so only the high intensity neurons were counted in the results. Also, a similar FB washing-out effect of the TMP procedure was observed *in vitro*, by comparing an unreacted dish with a reacted one. The intensities of FB labelled cells were therefore lower in TMP-reacted cultures than in unreacted cultures, but it was still possible to pick out the brightest neurons for scoring results. Photographing cultures for a record of the FB-containing cells before reacting them would result in accelerated FB fading, and would have been almost impossible in practice, because of the number of systematic photographs required to record all FB-labelled neurons.

7.4 Progression of technique

7.4.1 FB characteristics *in vitro*

In order to ascertain the stability of FB in dissociated DRG neurons, fixed and mounted cultures containing labelled cells were examined at various intervals after plating (up to 14 days). Under the FB filter, labelled neurons could be seen scattered randomly in the population. The intensity of FB fluorescence varied among neurons in any one culture dish (see Fig. 7.1), and there appeared to be some weakening of fluorescence intensity in neurons after seven days *in vitro*, with the faint blue colour of the background becoming stronger. Despite this, labelled neurons were still visible in the same proportions over two weeks *in vitro*, and were still present as isolated 'positives' even if the labelled cell was closely apposed to unlabelled cells in a cluster (see Fig. 7.2). This suggested that FB did not pass from retrogradely-labelled cells into initially-unlabelled cells in detectable amounts, and that the labelling therefore remained specific over fourteen days *in vitro*.

Fig. 7.1: *DRG neurons in dissociated culture, showing different intensities of FB label in neurons which had been labelled* in vivo *by injection of FB into skin*

Under UV epifluorescence, of the three FB-labelled neurons (arrows), only the high-intensity neuron (left) would be counted as a specifically-labelled skin afferent. 1 d *in vitro* (this culture has also been reacted for TMP therefore some neuronal profiles are black). Scale bar = 50 μm.

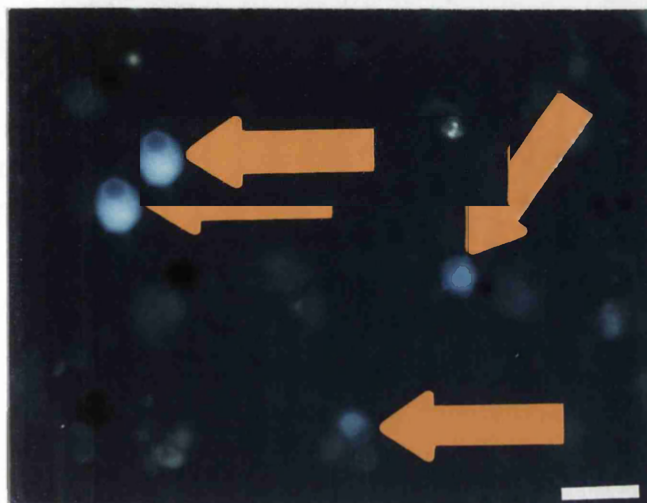
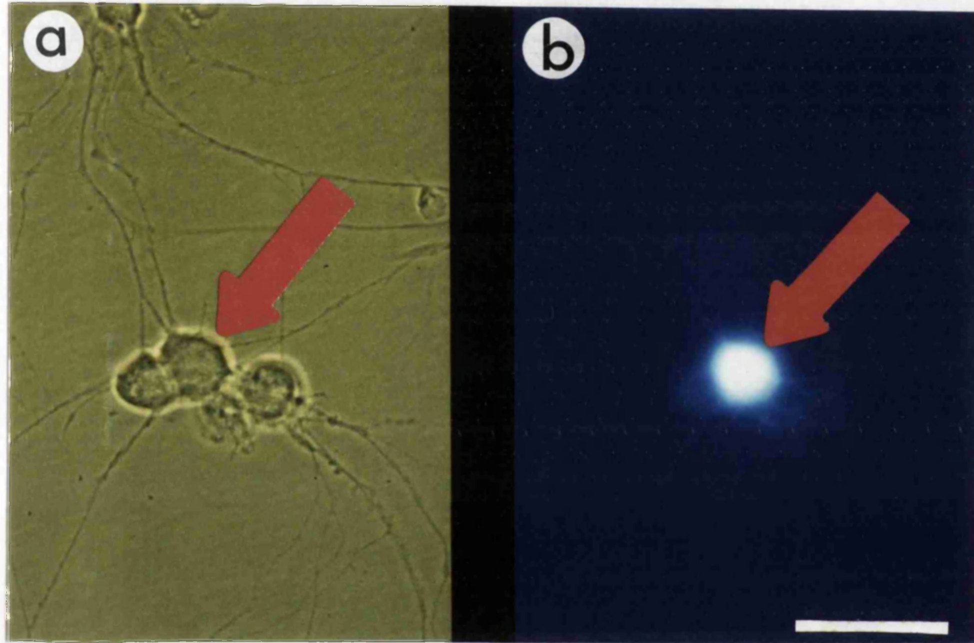


Fig. 7.2: *A cluster of DRG neurons* in vitro, *one of which is FB-labelled, showing that FB is retained specifically by the neuron which received the FB* in vivo *after injection of FB into skin*

(a) phase contrast shows a group of three phase-bright neurons, only one of which (b) is labelled by FB (red arrow) under UV epifluorescence. Scale bar = 50 µm.

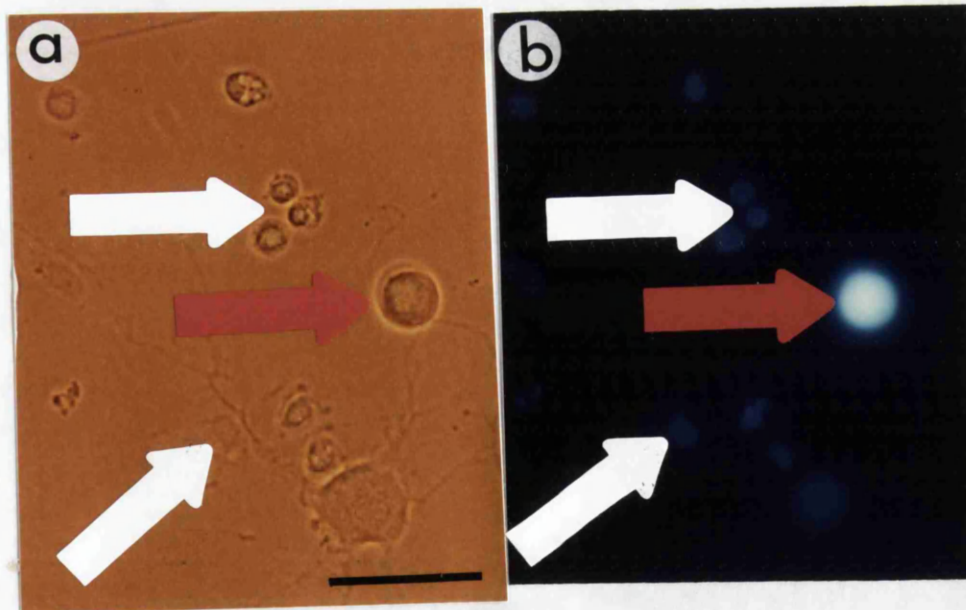


Non-neuronal cells, however, did sometimes accumulate the FB into their nuclei. This was most obvious after several days *in vitro* around the most brightly-labelled neurons (Fig. 7.3), but the much smaller size, and fainter stain of this non-neuronal labelling could not confuse the observation of labelled neurons (which were also easily distinguished under phase-contrast optics).

Fig. 7.3: *DRG neurons* in vitro, *one of which is FB-labelled, and the spread of a faint fluorescence to non-neuronal cells in the culture, which is distinct from specific neuronal label*

(a) under phase contrast, non-neuronal cell (bottom white arrow) and what are probably dead neurons (top white arrow) are clearly distinguishable from larger, round, phase-bright neuron (red arrow),

(b) under UV epifluorescence, only the neuron (red arrow) is FB-labelled although the other elements have picked up a faint fluorescence. Scale bar = 50 μm.

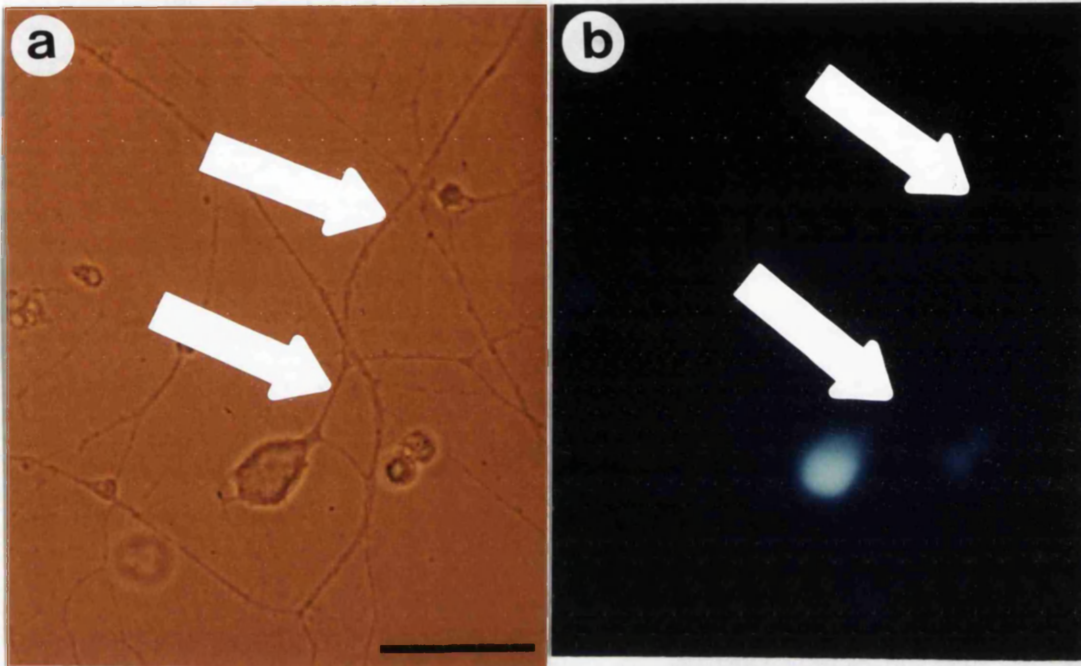


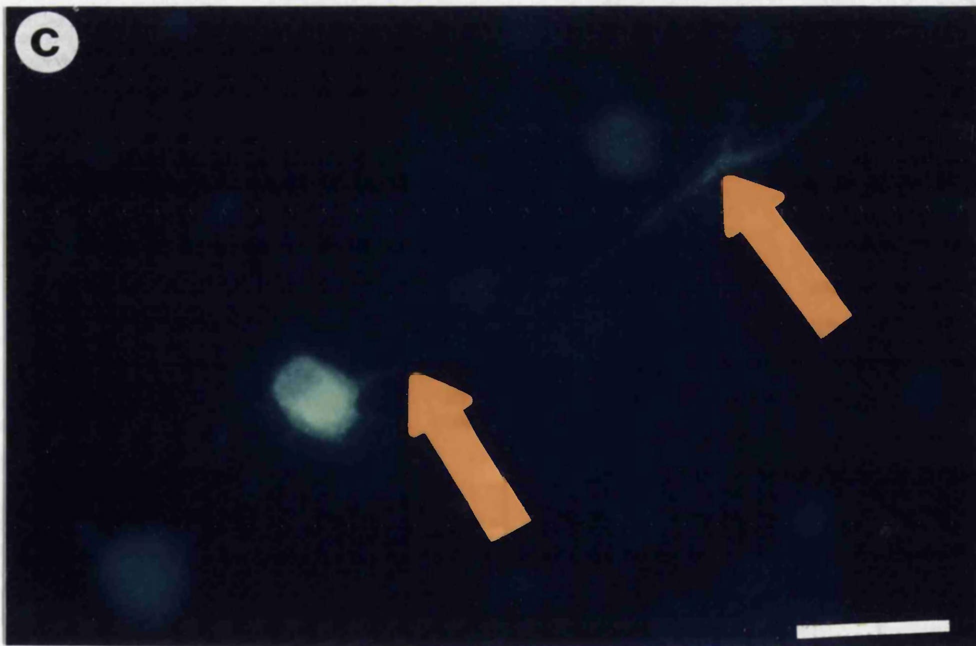
Most neurons successfully adhering to the dishes began extending neurites by 24 hours *in vitro*, or earlier if NGF was present. In FB-labelled neurons, dye could be seen extending into at least the thicker proximal parts of the neurites. This labelling was very susceptible to fading under the microscope epi-fluorescent beam, and indeed fading of dye in the cell bodies, and of the background colour, was discernible in the field of the culture left exposed to excitation wavelengths for even just twenty seconds. In the cell bodies, however, where the FB was heavily concentrated, fading was not sufficient - in the time taken to examine the cells for counterstains, etc., or to photograph them - to completely bleach out all dye. Fig 7.4 shows an example of one neuron which has extensive neurites under phase contrast, but only the soma contains FB, and another neuron which has a long process filled with FB fluorescence. Fixed mounted cultures stored at 4°C still contained clearly-labelled FB cells after twelve months.

Fig. 7.4 (over page): *Examples of FB-labelled neurons* in vitro *which did, and did not, exhibit FB label spreading along the length of the neurites*

(a) phase contrast, and (b) UV epifluorescence, views of a DRG neuron with long neurite (arrows), which is labelled by FB in the cell body but not in the neurite, (c) another neuron under UV epifluorescence exhibits FB labelling right along one neurite (arrows). Scale bar = 50 μm.

Fig. 7.4





Excitation of the FB such that it fluoresces is likely to cause the intracellular release of free radicals ([illegible] 1982) a situation potentially harmful to the cell. The fact that FB-containing cells adhere to the dish and extend neurites in the same way as unlabelled cells, and remain viable for at least two weeks *in vitro*, suggests that any exposure to light received in the dissection, dissociation and culture procedure is not intense enough at the relevant wavelength to cause a damaging excitation. One drawback resulting from the possible deleterious, and the observed fading, effects of exposure to light - and also from the need to mount and coverslip the cultures for any microscopy other than phase-contrast - is that cells cannot be examined during an experiment, only after fixing, etc. Thus control values must be taken from parallel sister cultures rather than the same cultures before and after manipulation.

7.4.2 Thiamine monophosphatase histochemistry

Viewing TMP stain with transmitted light, brown positive small- and medium-sized neurons were seen abundantly in cultures. The stain was generally confined to neuronal cell bodies rather than neurites, and was not present in the non-neuronal cells.

Examination of TMP-positive neurons suggested that the stain did not leak into neighbouring cells because there were closely-apposed negative cells which clearly had no degree of staining. There were two easily-distinguished intensities of brown reaction product, dark and light, as *in vivo* (Fig 7.5). Reaction product tended to form a central mass rather than appearing more peripherally in the cytoplasm, as in DRG sections. This is probably due to having the whole depth of the neuron in view at once.

7.4.3 RT97 and α-CGRP immunocytochemistry

Under the FITC filter, neuropeptide labelling showed as a bright green fluorescence, while all cells picked up a faint green colouring. The background of the dish was black (see Fig 7.6).

With the appropriate filter (depending on which fluorescent label had been used), RT97 labelling was very bright green or red, especially in neurites, but also in the soma cytoplasm of, on the

whole, larger-diameter neurons (see Figs 9.1 and 9.2 in Chapter 9). Negative neuronal cell bodies had a pale colour, with unlabelled neurites. Non-neuronal cells, and the background, were unstained.

There was no 'leakage' of fluorescence from the green to the red filter or vice versa.

7.4.4 Combined FB and counterstains

Presence of FB apparently did not diminish cellular labelling by antibodies, and FB was not visible under FITC or TRITC filters. In some cases where FITC labelling was very bright, a yellow colour showed through under the blue filter when viewing FB, but this was easily distinguishable from the purple-blue fluorescence of FB.

FB-labelled neurons were easily visible in cultures reacted for TMP - in double-stained neurons examined umder UV, the purple glow of FB was seen through and around the the more darkly-brown stained TMP positives, while in faint-TMP-stained neurons, FB showed as normal under UV and on switching to transmitted light, the faint brown TMP stain became visible.

Fig. 7.5 (over page): *Examples of DRG neurons* in vitro *which were reacted to demonstrate TMP enzyme activity; both dark and light reaction product is seen, as* in vivo

Examples of dark (blue arrows) and light (green arrows) TMP reaction product under (a) phase contrast, and (b) transmitted light. Scale bar = 50 μm.

Fig. 7.6 (over page): *Examples of DRG neurons* in vitro *immunostained with an antibody to CGRP*

Under epifluorescence filter appropriate for FITC, three CGRP-positive neurons (white arrow), also with positive-staining neurites, are next to three CGRP-negative neurons (red arrow) which have a very faint green colour. Scale bar = 50 μm.

Fig. 7.5

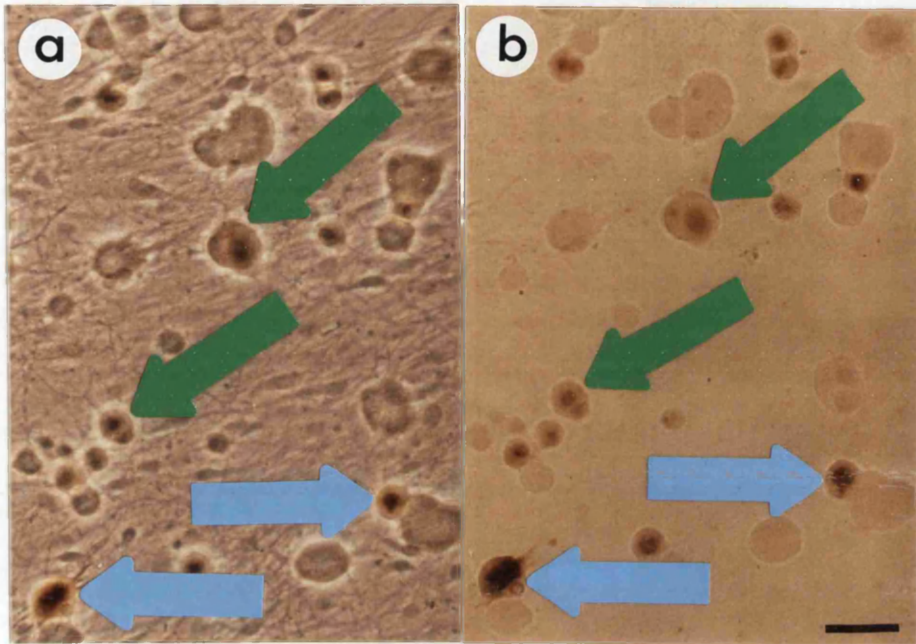
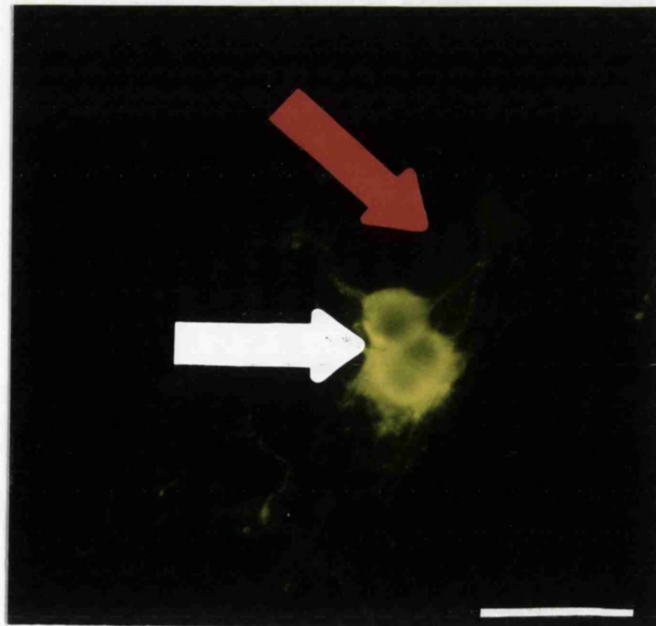


Fig. 7.6



CHAPTER EIGHT: Effects of nerve growth factor (NGF) on thiamine monophosphatase (TMP) expression *in vitro*.

8.1 Introduction

Axotomy of dorsal root ganglion neurons *in vivo*, produced by sectioning a peripheral nerve, results in changes in peptide and enzyme expression in the injured neuronal cell bodies and their central terminals in the dorsal horn; for example substance P (Barbut *et al.*, 1981) and FRAP (Schoenen *et al.*, 1968) are depleted while VIP is increased (Anand *et al.*, 1983). The dissection and dissociation procedure for culturing dorsal root ganglion neurons involves subjecting the neurons to a close axotomy, and therefore cultured neurons cannot be considered to represent, in every respect, neurons *in vivo*. For example, we have found that one early-onset response of DRG neurons to the culture procedure is the increased expression of a growth-associated protein, GAP-43 (Woolf *et al.*, 1989), an indication that these neurons enter a growth (neurite regeneration) mode (see Fig. 8.1). The exact nature of the signal for these changes *in vivo* and *in vitro* is not clear, but the interruption of the retrograde supply of a target-derived neurotrophic activity is likely to be the cause of the axotomy response.

Apart from the stimulation of GAP-43 expression, other changes might be expected to occur in DRG neurons in dissociated culture. Schoenen *et al.* (1989) recently found that just a few hours after dissociation of adult rat DRGs, neurons began to express immunoreactivity for several markers which are not found in the DRGs *in vivo*, such as choline acetyltransferase, γ-amino butyric acid, neurotensin, and β-endorphin. Many of these and other transmitter candidates were expressed in significantly higher proportions of neurons after 3 days *in vitro* compared to freshly-dissociated neurons, for example VIP, substance P, CGRP, and glutamate. Although the low survival (20-30%) rate of these cultures may contribute to the increased proportions of neurons with such markers, the finding

of 'new' markers, which are not present *in vivo*, cannot be explained by an artificial selection of neuron subpopulations.

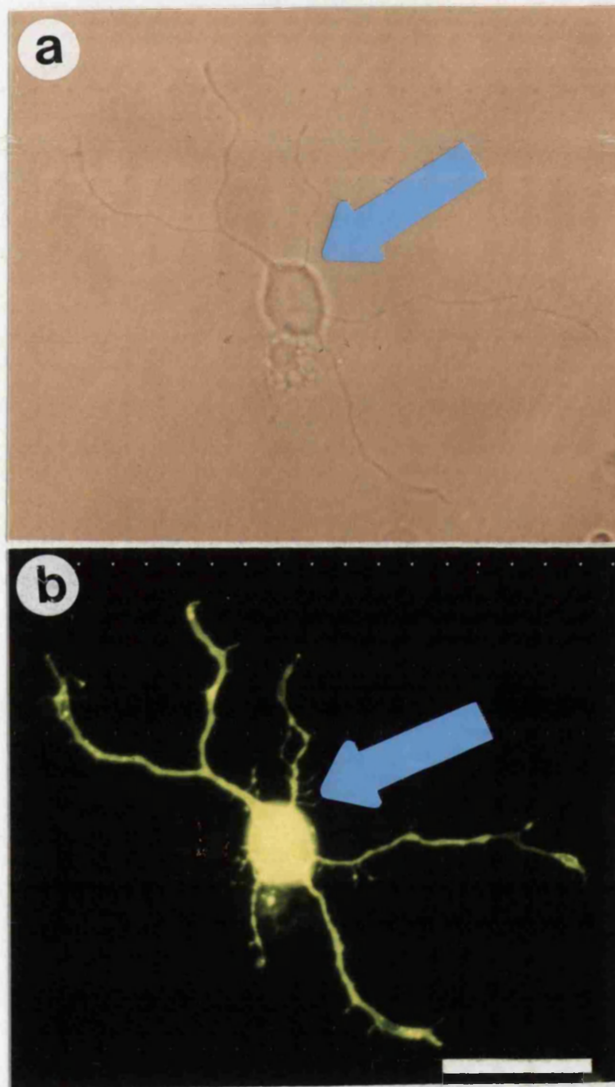
The results of Schoenen *et al.* (1989) are, however, in contrast to the work of Lindsay *et al.* (1989), who performed both counts of immunocytochemically-stained cultures, and radioimmunoassay of peptide levels. When adult DRG neurons are maintained for 2-28 days *in vitro*, the total levels of substance P and CGRP, measured by radioimmunoassay, fall unless NGF is supplied in the medium (Lindsay *et al.*, 1989). This effect is due to regulation of the neuropeptide mRNAs by NGF (Lindsay & Harmar, 1989), and not to selective survival of a neuropeptide-containing subpopulation. Levels of peptide were proportional to the length of time of exposure to NGF.

Results from the immunocytochemical staining also showed that the same proportions of substance P and CGRP positive neurons were found after 1 or 2 days *in vitro* as *in vivo*; and omission of NGF from the culture medium resulted in a drop in the proportions of immunoreactive neurons because levels of staining were so low, which could be restored by continuous or late addition of NGF. These results suggest that axotomized neurons *in vitro* require NGF for maintenance of the normal pattern of substance P and CGRP expression, if not for survival. A separate study has shown that using his culture procedure, 70-80% of adult DRG neurons *in vitro* do not require NGF, BDNF, serum or the presence of non-neuronal cells, for survival (Lindsay, 1988).

Such work, however, might miss any subpopulation-specific effects (e.g. skin afferents reacting differently from muscle afferents) which could include reciprocal changes in marker expression that result in apparently constant proportions when the total neuron population is examined. Counting individual target-identified neurons is required in order to assess whether the proportions of such neurons with and without markers are changed.

Fig. 8.1: *DRG neuron* in vitro *immunolabelled with an antibody to GAP-43*

(a) under phase-contrast, a DRG neuron in low-density culture exhibits several long neurites, (b) under epifluorescence filter for FITC, GAP-43 immunolabelling shows details of the growth cones at the neurite ends, and of small "microspikes" at the proximal end of a neurite (arrow), features which are not visible under phase-contrast. Scale bar = 50 μm.



Whether DRG neuron subpopulations other than those containing neuropeptides are influenced by NGF is unclear TMP-containing neurons for example, which *in vivo* appear to constitute a separate population from substance P-containing neurons (Nagy & Hunt, 1982). The results of the experiments in Chapter 4 indicate different trends in the distribution of TMP and substance P among target-identified afferents; and as discussed in Chapter 5, TMP positive neurons do not possess considerable numbers of high-affinity NGF binding sites (Verge *et al.*, 1989). However, NGF applied to a cut nerve partially reduces the "FRAP gap" which appears after nerve section due to depletion of FRAP from central terminals of axotomized neurons in the dorsal horn (Fitzgerald *et al.*, 1985). This suggests that some FRAP/TMP-containing neurons are capable of responding to NGF. McMahon & Moore (1988) have shown that when a muscle nerve is re-routed to innervate skin, the accumulation of FRAP at a ligature is increased to represent a similar situation to that of a ligated cutaneous nerve. This could be due to a retrograde influence from skin, by NGF or a different factor, altering the chemical phenotype of the newly-innervating muscle afferents. Such an experiment, however, only provides information about total FRAP levels, and not about the proportion of neurons expressing the enzyme.

This experiment was therefore designed to investigate whether NGF can modify the expression of TMP in adult DRG neurons *in vitro*.

8.2 Methods

Lumbar DRGs from eight female Sprague-Dawley rats (200-250g) were dissected and dissociated, and processed as separate pools for each rat. The cell suspensions were spun to light pellet neurons, and the supernatant was discarded. Cells were resuspended in F14 + FCS + Ara C, and each suspension divided between four 35mm polyornithine-laminin coated Petri dishes to give a plating density of about 15,000 neurons. Half the cultures were flooded with F14/FCS/Ara C + NGF, and the other cultures with F14/FCS/Ara C + α-NGF antibodies. NGF antibodies were employed in the minus-NGF cultures to counteract

possible endogenous NGF production by contaminating non-neuronal cells. Neurons had been divided among the two sets of dishes to give pairs of cultures from each rat, each pair consisting of one dish in each medium. Five pairs of dishes were fixed the following day, and processed for TMP activity. Remaining neurons were given changes of medium every three days, with Ara C being omitted after 6 days. A further five pairs of dishes were fixed at 10 days post plating, and similarly reacted for TMP activity. Three further rats were used to prepare three more dishes of neurons in +NGF and α-NGF which were maintained in a similar fashion for 15 days, then fixed and processed for TMP activity. Counts were made, among samples of total neurons, of the numbers of TMP positive and negative cells.

8.3 Results

The results are presented in Table 8.1, and illustrated by Figure 8.2. The raw data is in Appendix V.

The proportion of neurons positive for TMP is relatively stable over the first 10 days *in vitro* in the presence of NGF, and then drops from 56% to 39% at 15 days. In α-NGF cultures, however, the percentage of neurons which are TMP positive drops from 54% to 39% by 10 days post plating, and then declines further to 13% by 15 days Thus at both 10 and 15 days post plating, the proportion of total neurons expressing TMP in +NGF or α-NGF cultures is significantly different.

Neurons which were TMP positive in α-NGF were frequently faintly stained, while those in +NGF were usually very darkly stained.

Figure 8.3 shows examples of TMP staining in cultured neurons.

TABLE 8.1: *The percentage of TMP positive neurons in the total neuron population after 1, 10 and 15 d* in vitro, *in the presence or absence of NGF*

Percentages are calculated from five pairs of dishes at the 1 day and 10 day time points, and from three pairs at 15 days. In all but two dishes, the sample size was over three hundred neurons.

Days post plating	% TMP positive (mean ± S.E.M.) +NGF	α-NGF
1	52.6 ± 3.4	54.0 ± 4.2
10	56.0 ± 1.0	39.2 ± 3.1*
15	39 ± 0.6	13.3 ± 1.8*

* Significant difference between +NGF and α-NGF ($p < 0.01$)
Student's one-tailed t-test.

Fig. 8.2: *Graph illustrating the proportion of DRG neurons* in vitro *which are TMP positive when cultured for 1, 10 and 15 d, in the presence or absence of NGF* (Data from Table 8.1).

Open circles = +NGF; filled circles = α-NGF (i.e. -NGF)

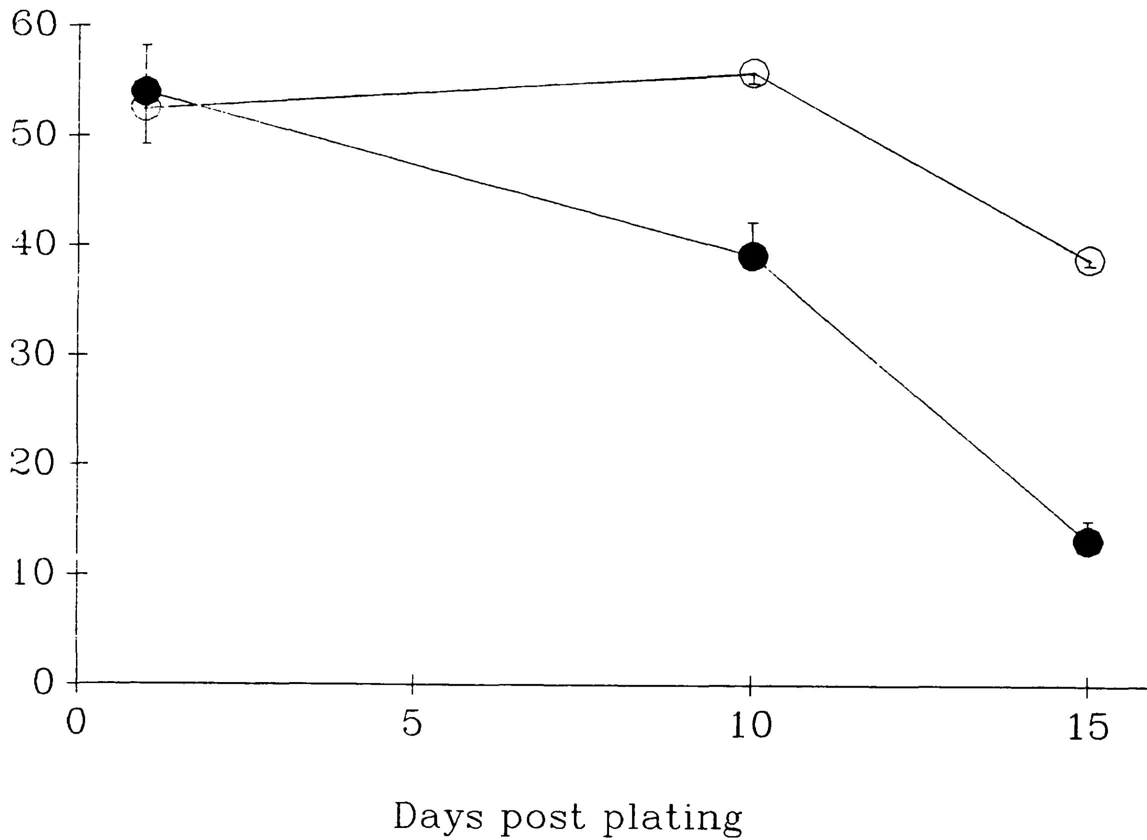
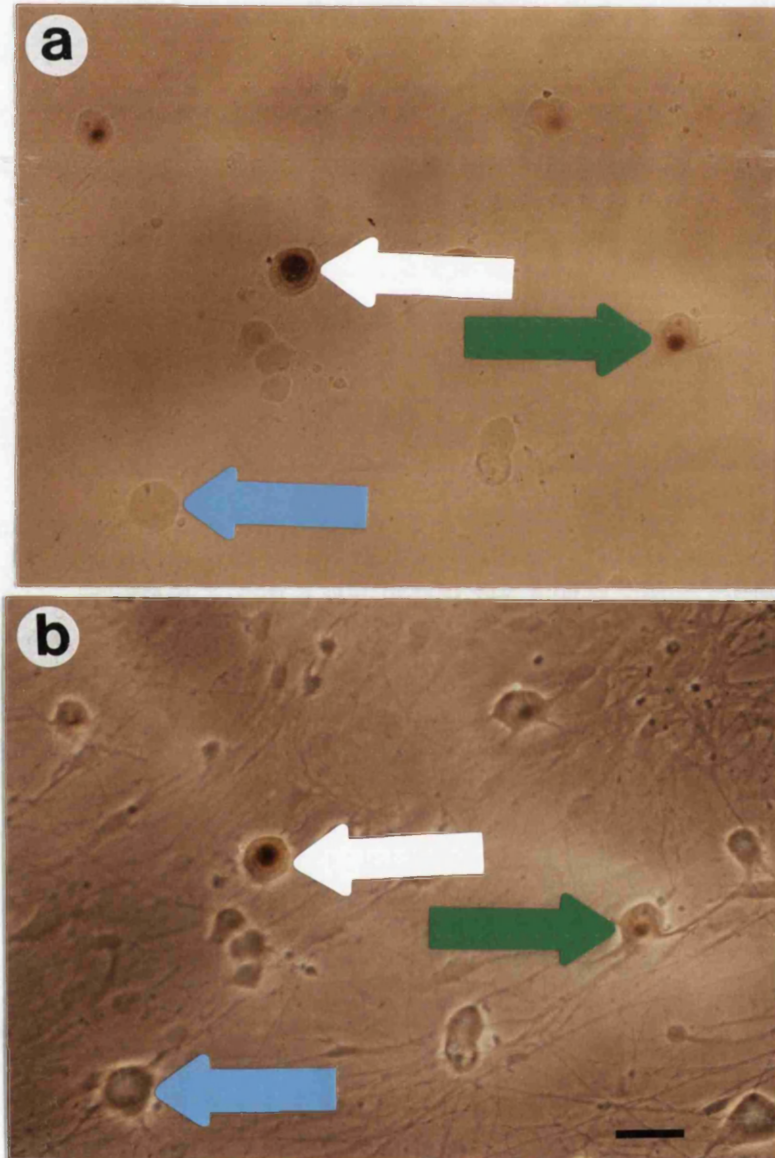


Fig. 8.3: *DRG neurons* in vitro *which were grown in the presence of NGF, and therefore often exhibit a dark TMP enzyme reaction product*

Under (a) transmitted light, and (b) phase-contrast, neurons are visible which are darkly-stained (white arrow), faintly-stained (green arrow) and unstained (bue arrow). Scale bar = 50 µm.



Preliminary results were also obtained for the expression of TMP by muscle afferents *in vitro*, data for which is presented in Table 8.2.

Figure 8.4 shows an example of an FB-labelled muscle afferent in culture, stained for TMP.

TABLE 8.2: *The percentage of retrogradely-identified muscle afferents which were TMP positive after 10 d* in vitro, *in the presence or absence of NGF.*

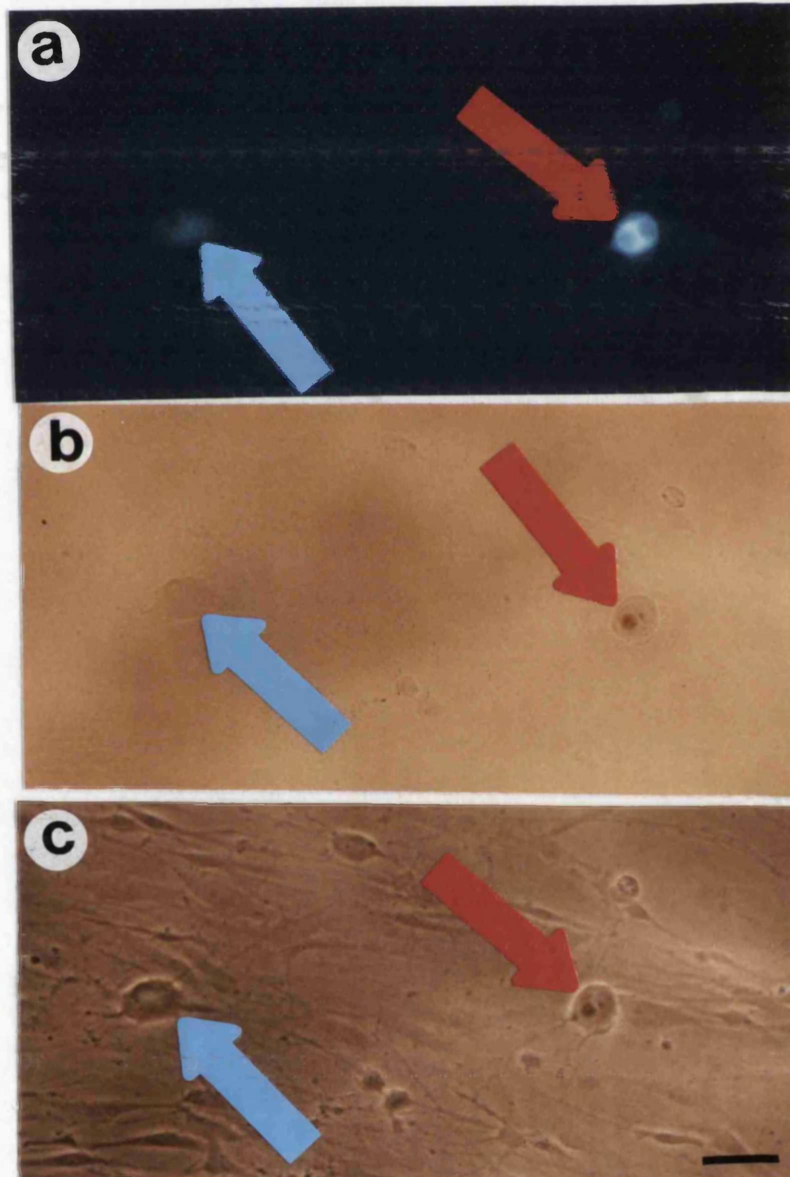
Percentages are calculated from a total of over 600 muscle afferents.

Days post plating	% TMP positive (mean ± S.E.M.) + NGF	α-NGF
10	39.3 ± 5.4	16.9 ± 2.8*

* Significant difference between +NGF and α-NGF ($p < 0.01$) Student's one-tailed t-test.

Fig. 8.4: *DRG neurons* in vitro *including FB-labelled neurons resulting from injection of FB into muscle* in vivo, *reacted for TMP enzyme activity after growing in the presence of α-NGF antibody*

(a) under UV epifluorescence a high-intensity FB-labelled neuron is shown (red arrow), which is also (b) faintly-positive for TMP. The blue-arrowed neuron is neither FB-labelled nor TMP positive. (c) phase-contrast. Scale bar = 50 μm.



8.4 Discussion

8.4.1 TMP in total neurons

When the time course of the effect of NGF on TMP expression in total neurons *in vitro* was investigated, a fall in the proportion of TMP positive neurons was seen in the absence of NGF (in the presence of α-NGF) to about 25% of initial expression over 15 days. In the presence of NGF, however, the levels were maintained at about 75% of the initial value. Lindsay *et al.* (1989) have seen a similar response of adult DRG neurons *in vitro* to NGF, by peptide (substance P and CGRP) levels measured by radioimmunoassay, and by immunoreactive neuron counts.

TMP *in vivo* is present in 50% of DRG neurons (Jessell & Dodd, 1986), and the proportions of total TMP positive neurons *in vitro* are initially very similar to this *in vivo* figure in both +NGF and α-NGF, but the proportion is maintained over 10 days only in the presence of NGF. Therefore with respect to the proportion of total neurons expressing TMP, DRG neurons *in vivo* are comparable to DRG neurons *in vitro* in the presence of NGF. Since lumbar DRGs (from which these neurons were prepared) probably contain a high proportion of skin afferents as opposed to muscle or joint afferents and since TMP is found far more frequently in skin afferents than in muscle or joint afferents (Chapter 4), the major target contribution to the total TMP population is likely to be from skin afferents. NGF is present in higher levels in skin than in other peripheral targets (Heumann *et al.*, 1984), and it seems likely that skin afferents in particular would be responsive to NGF. Therefore the result that NGF maintains TMP activity in total neurons *in vitro* probably reflects the response predominantly of skin afferents to being deprived of NGF by the culture process and when grown in α-NGF, but having the required neurotrophic activity restored in +NGF cultures.

From the difference between +NGF and α-NGF neurons with regard to intensity of TMP stain, it was clear that in α-NGF cultures levels of enzyme activity were decreasing at 10 and 15 days post plating (and thus the proportion of TMP positive neurons would probably have

continued to drop in cultures maintained beyond 15 days). These observations emphasise that the results may indicate only that levels of TMP change, so that they become more or less detectable, and we would need to study mRNA expression to show definitively whether enzyme expression is induced *de novo* or switched off in any particular subpopulation.

8.4.2 TMP in muscle afferents

The results for the muscle afferents are particularly interesting if compared with the *in vivo* frequency of TMP in total muscle afferents from Chapter 4. In both α-NGF and +NGF cultures, the proportion of TMP positive neurons was higher than in total muscle afferents *in vivo* (6%). A differential loss of neurons (i.e. of TMP negatives) might be invoked to explain the apparent increase in TMP positive neurons in α-NGF, and data for a 1 day time point is needed to show what level of TMP expression the muscle afferents had to begin with. However, Lindsay (1988) has shown that NGF is not a requirement for survival in these cultures. Therefore, the process of culturing muscle afferents could increase the proportion of TMP-positive neurons by selection or a survival effect (see Chapter 9), or by increasing expression. Further experiments are needed to establish which explanation is correct.

8.4.3 Which neurons / NGF receptors are involved?

The results imply that NGF can affect TMP expression *in vitro*, which agrees with the *in vivo* data of Fitzgerald *et al.* (1985), but disagrees with Verge *et al.* (1989a) who have shown that there is minimal overlap *in vivo* between TMP-positive DRG neurons and those possessing high-affinity binding sites for NGF. It could be that low-affinity receptors are involved in this effect, because it was shown in Chapter 5 that a large proportion of skin afferents and the majority of muscle afferents had such receptors. Otherwise, NGF may

induce the expression of its receptor on neurons previously devoid of high-affinity sites (Lindsay, personal communication).

Alternatively, if neurons (such as muscle afferents) were being recruited to express TMP *de novo*, these could be neurons which already possess NGF receptors, which according to Verge *et al.* (1989a) would be substance P- and probably CGRP-positive neurons. While substance P and TMP do not normally occur in the same DRG neuron (Nagy & Hunt, 1982), some neurons must contain both TMP and CGRP *in vivo*, because the individual occurrences of each marker quoted in the literature (Jessell & Dodd, 1989) add up to more than 100% of neurons; and as shown in Chapter 4, all small dark muscle afferents contain CGRP and 6% contain TMP, therefore the two markers must overlap in 6% of small dark muscle afferents *in vivo*. If NGF receptor-bearing substance P and CGRP neurons began expressing TMP under the influence of NGF, then overlaps between these peptides and TMP might be seen in more neurons *in vitro*.

NGF has also been shown to regulate sensory neurons' sensitivity to capsaicin *in vitro* (Winter *et al.*, 1988). The capsaicin-sensitive population consists of peptide-containing, mainly C-fibre neurons, (Lawson, 1987) again suggesting that it is the peptidergic afferents which we would expect to be responsive to other actions of NGF, rather than the separate population of TMP-containing afferents.

CHAPTER NINE: The effect of myotube-conditioned medium on peptide expression and neuronal survival among skin afferents *in vitro*.

9.1 Introduction

If phenotypic expression in neurons is related dynamically to influences from target tissues (as the experiments in Chapter 8 may indicate), then culturing neurons which normally innervate one target, with medium conditioned by another target, might induce a change in chemical expression. For example, skin afferents could be cultured in the presence of myotube-conditioned medium (MCM), in which case they would be receiving environmental signals to suggest they were now connected to a muscle target.

It was shown in Chapter 4 of the present work that CGRP is expressed by all small dark sensory neurons retrogradely labelled from muscle, but by only 50% of skin small dark afferents. Under the influence of MCM, small dark skin afferents might all start to produce CGRP, i.e. behave as though they were muscle afferents. Therefore this experiment tested CGRP and RT97 expression in retrogradely-labelled skin afferents, in the presence or absence of MCM. NGF was present at saturating levels in both media, to exclude the possibility that the same trophic activity was present in the MCM (Murphy *et al.*, 1977).

Conditioned medium used in this way represents a model of target-derived, diffusible instructive factors, and does not encompass contact effects between neuron and target, which would be present in cocultures of such cells, and which probably operate *in vivo*.

9.2 Methods

Three Wistar rats (250-300g) were given injections of Fast Blue bilaterally into the skin of the inner thigh in order to label cutaneous afferent neurons. Six days later, DRGs L2-4 (containing

labelled cell bodies) were dissected, pooled and dissociated. In order to minimise loss of retrogradely-labelled neurons, an overnight pre-plating step was not used, but the suspension was spun down to lightly pellet neurons, enabling much of the debris to be discarded with the supernatant. Cells were plated in discrete drops at a density of 3000 per polyornithine-laminin coated 35mm dish, left to settle overnight and flooded with appropriate medium next morning. For the control dishes, medium was F14 supplemented with 10% FCS, plus NGF and Ara C. For the experimental dishes, medium was 50:50 of F14 + 10% FCS and MCM, with NGF and Ara C added to the whole before filtering. Medium was replaced every three days, but Ara C was present only for the first six days. At 14 days post plating, dishes were fixed and reacted with anti-CGRP and RT97 antibodies. A fluorescein label was used for CGRP staining and a rhodamine label for RT97. High- and medium-intensity FB-labelled neurons were identified, and the presence of CGRP and RT97 immunoreactivity scored for each such neuron.

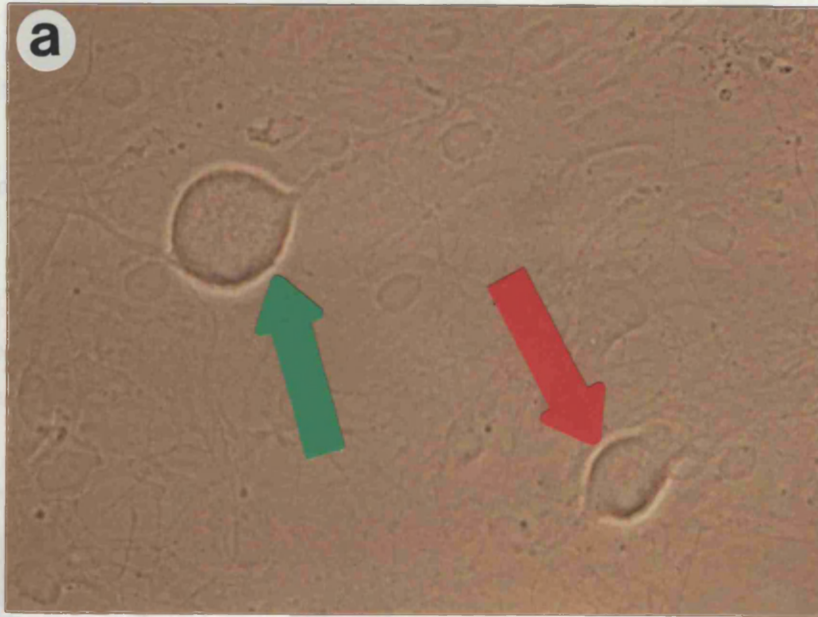
9.3 Results

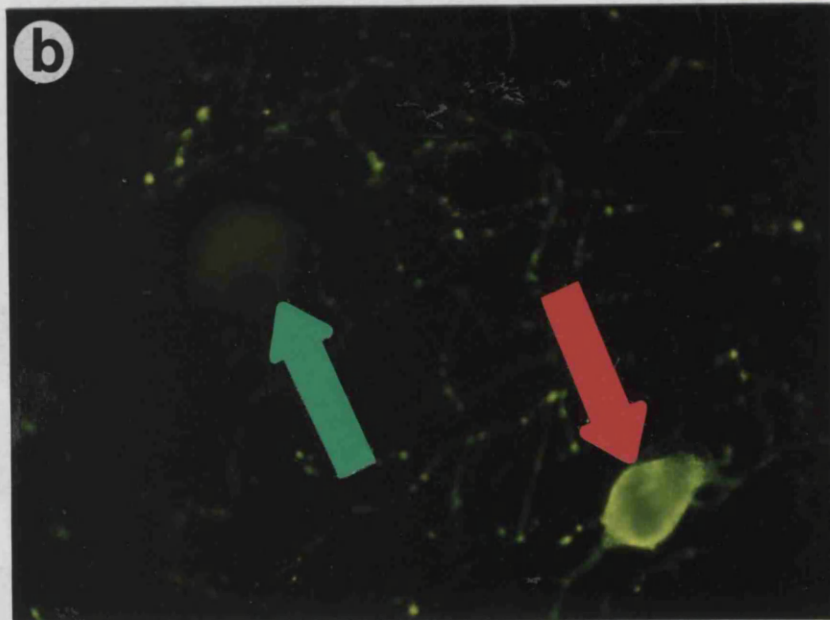
Figures 9.1 and 9.2 show FB-labelled and unlabelled afferents counterstained for CGRP and RT97 immunoreactivity. The data on retrogradely-labelled skin afferents immunoreactive for CGRP and RT97 (for high-intensity FB neurons) are presented in Table 9.1. The equivalent data for medium-intensity FB are included in Appendix VI.

Fig. 9.1 (over page): *Examples of DRG neurons* in vitro *which are counterstained for CGRP and RT97 immunoreactivity*

(a) under phase-contrast two phase-bright neurons are arrowed, (b) under epifluorescence filter appropriate for FITC, only one neuron is immunoreactive for CGRP (red arrow), (c) under filter for TRITC, the other neuron is the only one immunostained by RT97 (green arrow). Scale bar = 50 μm.

Fig. 9.1





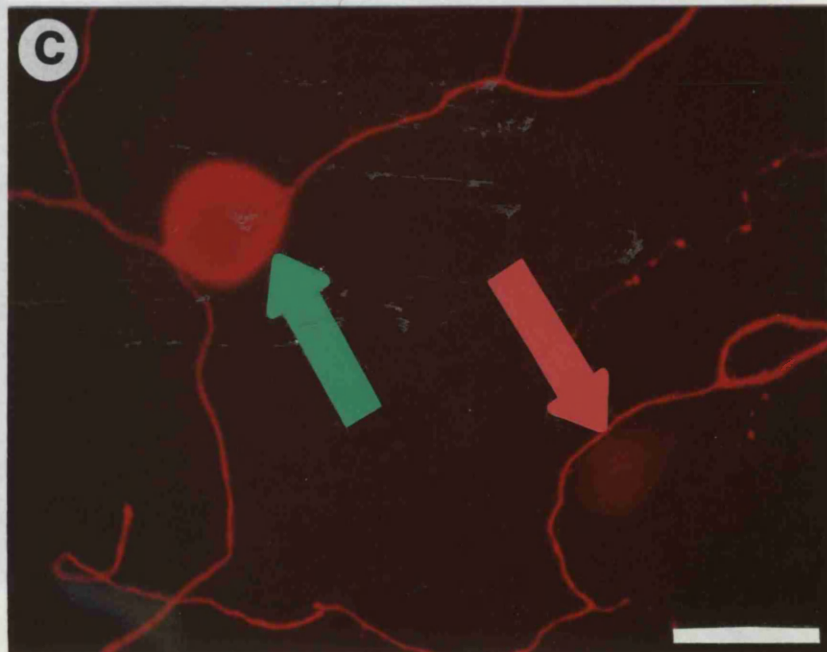


Fig. 9.2: *DRG neurons* in vitro *including one which is FB-labelled as a result of FB injection into the skin* in vivo, *counterstained for CGRP and RT97 immunoreactivity*

(a) under UV epifluorescence, one of three neurons (arrow) is FB-labelled, (b) under filter for FITC, the same neuron is also the only one immunoreactive for CGRP, and (c) all three neurons are RT97 positive under filter for TRITC. (d) phase-contrast (printed as mirror-image of (c)). Fluorescence is weaker than in Fig. 9.1 because of fading over time. Scale bar = 50 μm.

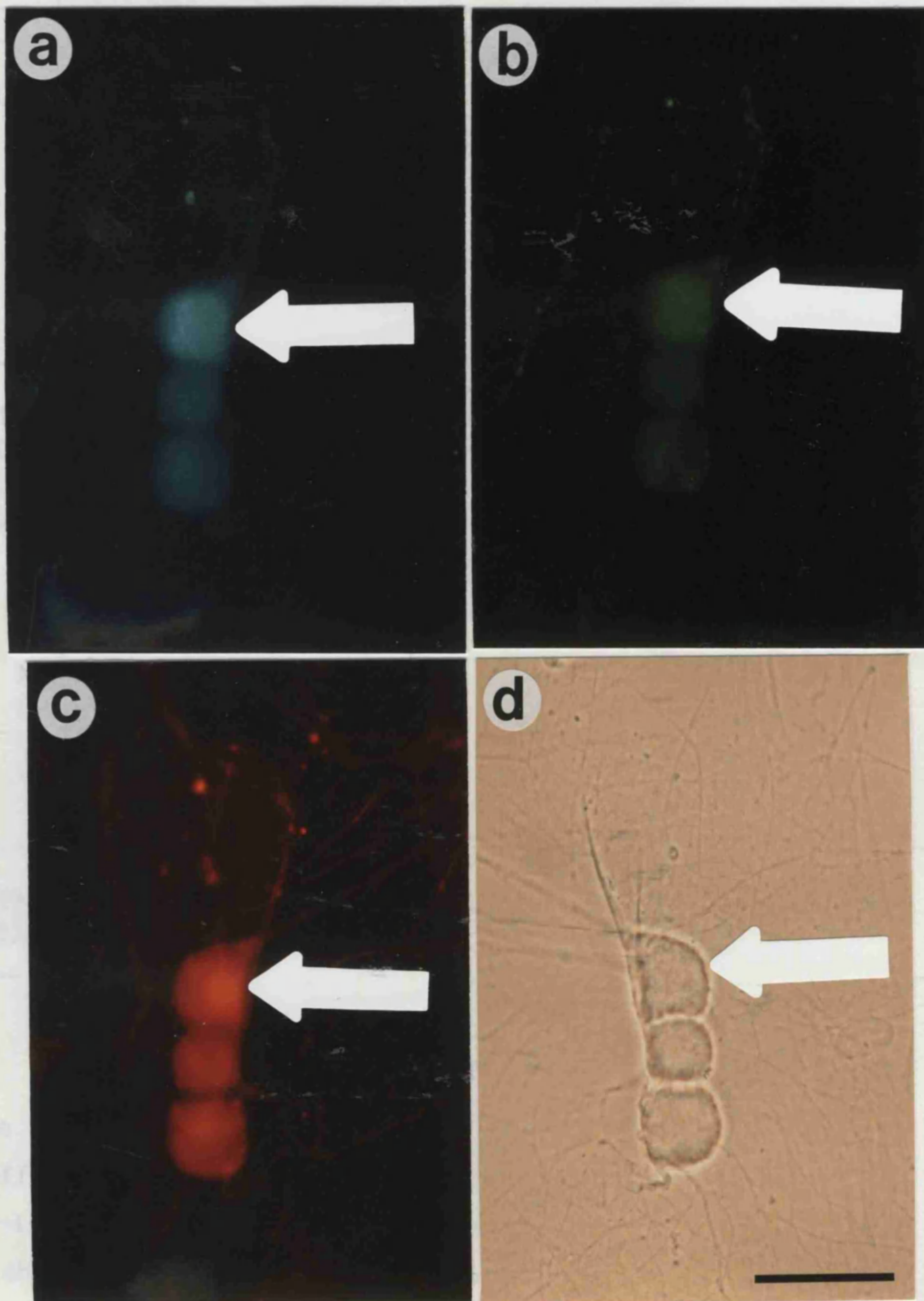


TABLE 9.1: *CGRP immunoreactivity in skin afferents cultured in the presence or absence of myotube-conditioned medium*

Number of RT97 positive and negative retrogradely-labelled skin afferents, and the number of each which contained CGRP, after 14 d *in vitro*, in the absence or presence of myotube-conditioned medium (MCM). NGF was present in all cases.

MEDIUM (& dish)	RT97 positive	RT97 negative	CGRP in RT97+	CGRP in RT97-	Total CGRP +
CONTROL					
1	7	10	5	3	8
2	5	10	4	4	8
3	9	9	6	3	9
4	7	7	5	5	10
5	4	11	2	5	7
6	7	11	3	3	6
7	1	6	1	4	5
TOTAL (n=104)	40/104 = 38%	64/104 = 62%	26/40 = 65%	27/64 = 42%	53/104 = 51%
+ MCM					
8	17	5	9	3	12
9	17	3	8	2	10
10	21	13	11	8	19
11	17	15	9	11	20
12	15	13	11	4	15
13	13	11	6	4	10
14	12	10	5	6	11
TOTAL (n=182)	112/182 = 62%	70/182 = 38%	59/112 = 53%	38/70 = 54%	97/182 = 53%

The presence of MCM resulted in a larger number of FB-labelled skin afferents being encountered in cultures - 182, compared to 104 target-identified neurons in cultures in control medium. Since all the dishes were prepared from one suspension containing FB-labelled

neurons, and initial tests showed a relatively consistent distribution of FB-labelled neurons among dishes, this represented a large discrepancy, indicating that neurons may have been lost from -MCM dishes.

The proportion of RT97 positive (large light) skin afferents was much higher in the presence of MCM - 62% compared to 38% in control medium. Within the large light populations, however, there was a decrease in the proportion expressing CGRP from 65%, to 53% in the presence of MCM. At the same time there was an increase from 42%, to 54% in MCM, of small dark skin afferents expressing CGRP. This resulted in the proportion of total skin afferents expressing CGRP being around 50% in both control and +MCM media.

To investigate the effect of MCM on RT97 expression, a further count was made of RT97 immunoreactivity in a sample of the total neuronal population from three dishes in each medium. The results are presented in Table 9.2. The counts are made by selecting several fields of view randomly, but distributed all over the dish, until about 150 neurons have been counted in each dish.

TABLE 9.2: *RT97 immunoreactivity in total neurons cultured in the presence or absence of myotube-conditioned medium*

Numbers of RT97 immunoreactive neurons among the total population after 14 d *in vitro* in the absence or presence of myotube-conditioned medium (MCM). (NGF was present in all cases; same culture dishes as in Table 9.1).

MEDIUM (+dish no.)	Total neurons	RT97 positive	% RT97 positive
CONTROL			
1	190	59	31
2	182	98	54
3	155	48	31
		MEAN	38.6 ± 7.7%*
+ MCM			
1	160	92	58
2	134	78	58
3	162	105	65
		MEAN	60.3 ± 2.3%*

* significantly different ($p < 0.05$) Student's one-tailed t-test.

Table 9.2 shows that among the total neuron population, MCM significantly increased the proportion of neurons which were RT97 positive by an amount very similar to the effect on retrogradely-labelled skin afferents (Table 9.1).

Observations on the nature of the cultures were:

1) there were more neurons on dishes with MCM, judging roughly by the density of neurons seen as dishes were counted;

2) the network of neurites, especially visible with CGRP immunostaining, was denser in MCM; and

3) the number of non-neuronal cells was much greater on MCM dishes.

9.4 Discussion: purpose of the experiment and how fulfilled

In vivo, all small dark muscle afferents contain CGRP whereas only 50% of skin small dark afferents are CGRP positive; among large light neurons, about 50% of both target classes contain CGRP (shown in Chapter 4). Myotube-conditioned medium (MCM) was used here to expose skin afferents in culture to an element of the environment that may be experienced normally by muscle afferents. This experiment tested whether the proportion of skin afferents which normally do not contain CGRP were induced to do so by the novel influence of MCM.

The result was that MCM did not change the percentage of total retrogradely-labelled skin afferents which expressed CGRP. Within RT97 positive (large light) neurons, there was a slight decrease in CGRP positives in MCM, and within RT97 negative (small dark) neurons there was a corresponding small increase in those immunoreactive for CGRP (Table 9.1). The RT97 negative skin afferents were of particular interest, because *in vivo* only 50% of these are CGRP positive, while RT97 negative muscle afferents all contain CGRP. The effect of MCM was to increase the proportion of RT97 negative skin afferents which contained CGRP from 42 to 54%, both figures being very similar to the *in vivo* percentage. Thus there was no obvious induction of CGRP in a new proportion of skin afferents by the novel target influence of MCM.

The most obvious effect of MCM was the increase in RT97 positive skin afferent neurons from 38% to 62%. The range of RT97 positive skin afferents found *in vivo* (see Table 4.5 in Chapter 4) was 29-39%, corresponding well with the *in vitro* result in standard medium here.

Counts of RT97 expression in the total population of neurons (Table 9.2) indicated that the increase occurred among the whole neuron population as well as among skin afferents. Again, the result in

control medium (39% RT97 positive) corresponds to the *in vivo* situation for total lumbar neurons (Lawson *et al.* 1984).

Therefore the effect of MCM appeared to be to increase the survival of neurons on the dishes, with a selective effect on RT97 positive (large light) neurons. Any possible effects on CGRP distribution in retrogradely-identified skin afferents appear to be secondary to these changes; and in the present results no significant effect of MCM on CGRP expression could be established. Further experiments were designed to examine the survival effect of MCM in more detail. Total neuron counts were made on both live and fixed low-density neuron cultures at various times post plating. For the greatest counting accuracy, RT97 immunostaining was not performed on these cultures so that washing off neurons with all the rinsing steps was avoided.

9.5 Methods

Lumbar DRGs were prepared for culture from a control rat, using exactly the same protocol as described in the previous experiment except that the plating density was carefully regulated to be consistent, supposedly 500 per dish, plated in a spot. Three dishes each in control and +MCM media were repeatedly counted 'live' at 3, 6, 10 and 14 days post plating, taken out of the incubator for the minimum time required to perform the count of total neurons on the dish. Three further dishes in control and +MCM media were fixed at each of the time points: 3, 6, 10 and 14 days. Dishes were not coverslipped, but kept with PBS in to keep neurons hydrated, and in this way the treatment received by live and fixed dishes when counted was more comparable. Counting the total neurons on fixed and live dishes was done under phase contrast microscopy, with the dish on an etched grid for accurate location of consecutive fields of view, so that the whole area occupied by neurons was examined.

9.6 Results

The results for neuronal survival over 14 days in control or +MCM media, in live and fixed cultures, are illustrated in Figures 9.3 and 9.4 respectively.

Both graphs show that there is a greater neuronal survival over the 14 day culture period in +MCM, although the difference between the two culture conditions is more obvious in Fig. 9.3, where consecutive counts came from the same dish. Thus when neurons are followed live, the effect of MCM on increasing neuronal survival is obvious from 6 days post plating, onwards.

Neuron counts are generally lower at all time points on fixed dishes (Fig. 9.4) than on live dishes (Fig. 9.3) especially for -MCM cultures, which may be due to extra dislodging of slightly loose neurons by the solution-changing steps involved in the fixation procedure.

Fig. 9.3: *Graph illustrating the effect of MCM on DRG neuron survival in three cultures each, grown in the presence or absence of MCM, followed* **live** *over 14 d* in vitro. Initial plating density = 500 per dish.

Open circles = -MCM; closed circles = +MCM

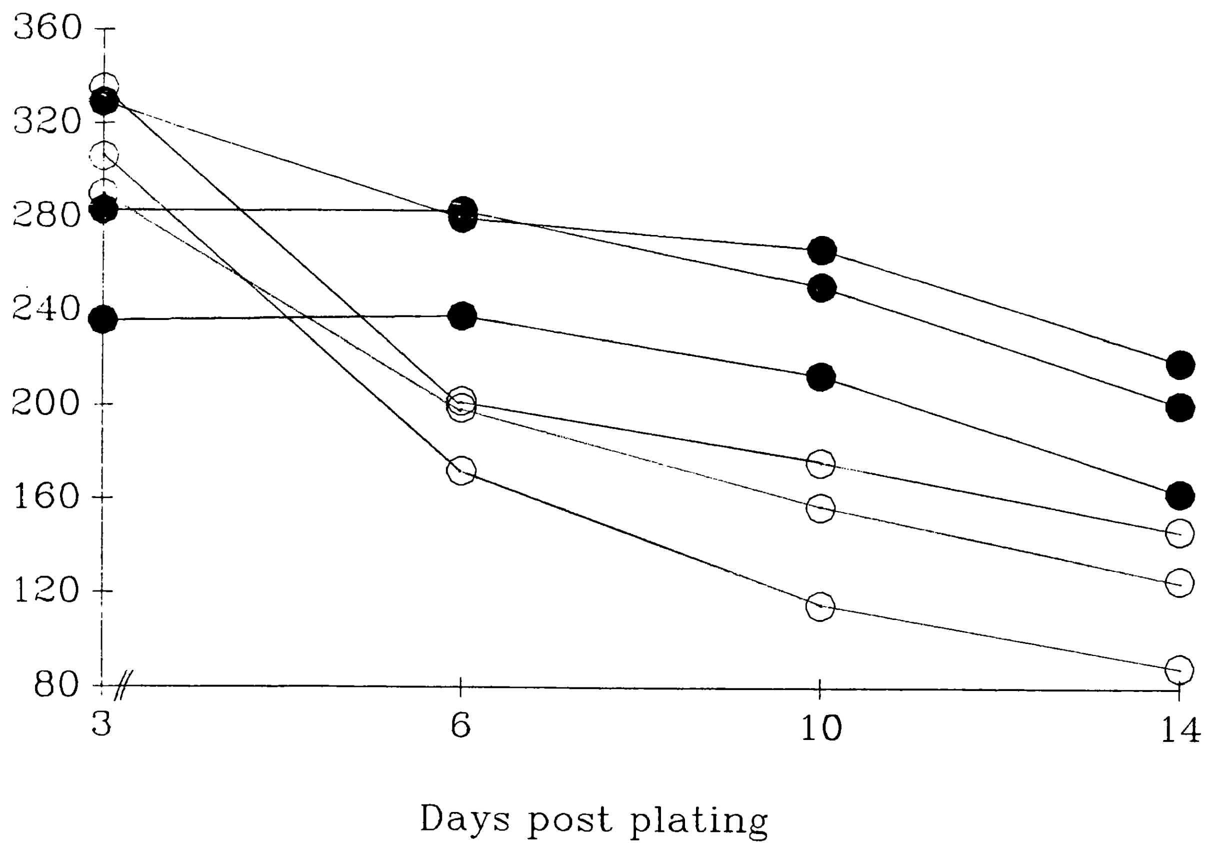
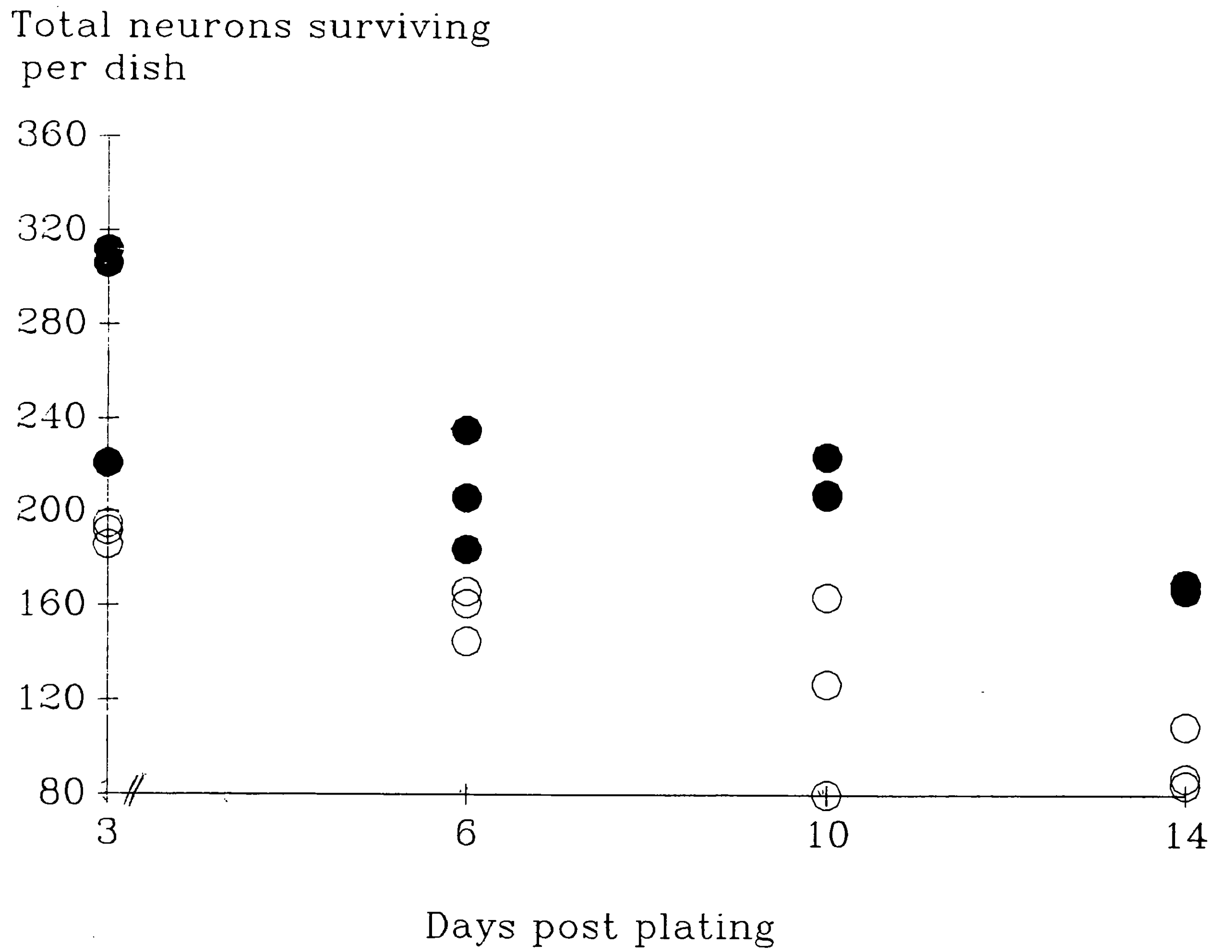


Fig. 9.4: *Graph illustrating the effect of MCM on DRG neuron survival in three cultures* ***fixed*** *at each time point shown, after growing in the presence or absence of MCM* in vitro .
Initial plating density = 500 per dish.
Open circles = -MCM; closed circles = +MCM



9.7 Discussion

Coupled with the observations of increased density of non-neuronal cells, and greater neuritic networks in MCM cultures, a survival effect of MCM had been suspected from the results on CGRP and RT97 expression, and the purpose of this experiment was to examine that directly. The results for neurons examined both live and fixed over 14 d indicate that MCM does increase the numbers of neurons which survive. Therefore under the culture conditions used here, neurons grown in control medium lack a 'survival factor', which is supplied by the MCM.

9.8 Overall discussion

9.8.1 Neuronal survival using Lindsay's system

The culture system described and used here was developed (Lindsay, 1988) for the maintenance of adult sensory neurons without the need for added trophic factors.

Lindsay's (1988) investigations of neuronal survival were performed in cultures both enriched and non-enriched for neurons, and in single-neuron cultures in microwells where there could be no possibility of influences from non-neuronal cells. In non-enriched cultures, >60% of plated neurons survived and extended processes, whereas in enriched cultures 70-80% of neurons survived longer than 3 weeks *in vitro*; 80% of single neurons in microwells were process-bearing after 7d *in vitro*.

The conclusion by Lindsay that "the vast majority" of adult rat primary sensory neurons do not require trophic factors for survival is in opposition to the work of Grothe & Unsicker (1987), who found that non-neuronal cells and pig brain extract provided essential survival factor(s). On a polyornithine substrate, neuron-enriched cultures which exhibited the same frequency of substance P- and somatostatin-positive neurons as *in vivo*, lost 60-70% of the neurons initially present after 7 days *in vitro*. There was no change in the proportions of substance P- and somatostatin-immunoreactive cells

suggesting that the loss was of all neuronal classes equally. Their enrichment procedure entailed centrifugation on Percoll, followed by a preplating step where the debris-free cell suspension was plated on uncoated plastic for 2 hr. The medium from these dishes was then plated out, and neuronal counts indicated losses of about 30% of estimated total neurons *in vivo*. Lindsay (1988) reports losses of less than 20%, but his estimates of total neurons *in vivo* are substantially less to start with than those of Grothe & Unsicker. The question of total DRG neuron number *in vivo* has been difficult to answer. The confocal microscope may eliminate the need for correction factors, required when using the current counting techniques, enabling a definitive value to be obtained.

The differences in neuronal preparation between the two methods may in part explain the different yields; for instance Percoll centrifugation may damage some neurons, which are not removed by the preplating step of Grothe & Unsicker and which die later, contributing to the loss figure. The overnight preplating step used by Lindsay, however, allows for removal of any dead, non-adherent neurons in the medium, so that only live, lightly adherent neurons are harvested for culture.

Another important difference was the culture substrate: only Lindsay (1988) used laminin, which may be very important for adhesion, neurite extension and survival.

9.8.2 How the present culture conditions differed from those of Lindsay

In the present experiments the overnight preplating step was not used, in order to avoid losses from the small population of retrogradely-labelled neurons. Therefore neurons damaged in the dissociation procedure (likely to be the larger diameter neurons) may die over the following few days *in vitro*, contributing to the apparent rapid cell loss between plating, at which time there should have been 500 neurons per dish, and 3 days post plating. (However, accuracy in calculating the total neurons plated at such low density

may be low, and the slopes of the graphs in Fig. 9.3 suggest that actual initial density could have been nearer to 350 neurons). The omission of the preplating step means that the cultures were non-enriched, and Lindsay found that neurons from such cultures (where non-neuronal cells proliferated) exhibited a lower percentage survival than neurons grown in the relative or absolute absence of non-neuronal cells. This could be explained by a detachment of neurons from the underlying non-neuronal cells as the latter divide *in vitro.* Therefore the system as used here is not allowing the optimal survival of neurons.

Fetal calf serum was used in these experiments so that myotube culture medium which uses FCS, was of the same basic composition as neuronal medium, and the survival effects of neurons in this serum have not been tested. However, Lindsay (1988) found that high neuronal survival could be obtained in serum-free medium, therefore FCS does not supply an essential factor.

The low plating density of the cultures used for the survival counts may allow more neurons to be washed off with medium changes than would be seen on higher density cultures (1-3000 or more neurons/dish).

9.8.3 The survival effect seen in the present work

While Lindsay (1988) maintains that neurotrophic factors are not required for survival of adult sensory neurons, his figure for maximum survival is 80%, for neurons grown singly in microwells for 7 days. In non-enriched cultures, survival is " > 60%" of neurons plated. What we may be seeing in the present results is a survival-enhancing effect of MCM on the 30-40% lost in Lindsay's cultures. Calculating percentage survival in the present work by averaging the three counts in each medium at 14 days post plating, and if the original plating density in Figs. 9.3 and 9.4 is taken as 350 neurons (see part 9.8.2), then after 14 d the average survival is only increased from 34% to 56% in live cultures, and from 27% to 48% in fixed cultures, by the addition of MCM.* Therefore the maximium possible survival demonstrated here is never higher than Lindsay's

* If percentage survival is calculated using 500 as the initial density, then after 14d the average survival is increased from 24% to 39% in live cultures, and from 19% to 34% in fixed cultures, by the addition of MCM.

results for non-enriched cultures, and MCM may only be restoring neurons lost by differences in culturing expertise. The present results also suggest that the neurons which are lost in control medium, but which are saved by MCM, are RT97 positive, large light neurons.

9.8.4 Ways in which MCM might affect survival: (i) adherence

Instead of a survival effect due to the supply of an essential component in the medium, one explanation for the effect of MCM may have been to enhance the adhering of neurons onto the dishes, vital for them to remain in the cultures and extend neurites. MCM is likely to contain laminin (Calof & Reichardt, 1984) and fibronectin. An observation on some control cultures was that neurons had tended to clump together in small groups by the end of the culture period. Such behaviour results from insufficient adhesion to the culture dish itself, and the amount of laminin coating on these dishes may have been suboptimal, such that additional adhesion molecules in the MCM actually promoted sticking-down of neurons. A control experiment to test for such an effect would be to add laminin solution to cultures instead of adding MCM.

An alternative, but less likely, explanation is that collagen, on which the myotubes were plated, was present in MCM. To test this, the control medium could be prepared by incubation in collagen-coated dishes without the myotubes. Collagen polymerises as it dries onto the dishes, and it could be shown whether the collected control medium was able to promote adhesion in the same way as MCM.

(ii) via non-neuronal cells

Alternatively, the effect of MCM on the neurons may have been indirect, and mediated by non-neuronal cells, such as satellite cells and fibroblasts, which were seen to proliferate extensively in MCM cultures. Grothe & Unsicker (1987) found that non-neuronal cells provided essential survival factors for their adult DRG neuron cultures.

In cultures of embryonic chick sensory neurons, ganglionic non-neuronal cells and sciatic nerve Schwann cells can influence neuronal transmitter expression and morphology, respectively (Mudge, 1984; 1981). Barakat & Droz (1985) found that the proportions of chick sensory neurons expressing acetylcholinesterase, α-bungarotoxin binding sites or a high uptake capacity for glutamine were enhanced by ganglionic non-neuronal cells. Transmitter expression by neonatal rat sympathetic neurons was increased by non-neuronal ganglionic cells (Patterson & Chun, 1974). Schwann cells in mature peripheral nerve react to axotomy by synthesizing and releasing NGF (Heumann *et al.*, 1987), but whether the equivalent cell type in the ganglion (the satellite cells), or the fibroblasts elaborate a trophic activity *in vitro* in cultures from the adult rat DRG is unclear.

However, since Lindsay (1988) found that neuronal survival was lower in non-enriched cultures than in neuron-enriched cultures, a survival-enhancing effect of the non-neuronal cells was not indicated in this culture system (but this does not rule out an indirect effect of MCM on neurons, via non-neuronal cells).

9.8.5 Other ways in which the target-phenotype relationship has been studied

Other work on the effect of target tissue on marker expression *in vitro* has been performed on avian sensory neurons. Davis & Epstein (1987) cocultured embryonic quail trigeminal ganglion with embryonic aneural chick rectum on the chorioallantoic membrane of chick hosts for 7-8 days. Small trigeminal neurons (10-13μm), different from larger native neurons, were immunoreactive for the peptide VIP, which is not expressed by trigeminal ganglion neurons *in vivo*. VIP was not present in ganglia explanted with embryonic heart, or in ganglia explanted alone, therefore some influence from the transplanted gut tissue was responsible for the change in transmitter expression by trigeminal neurons.

A different way of looking at target interactions was provided by Marusich *et al.* (1986a, b). They produced a monoclonal antibody (SN1) which surface-labels a subpopulation of quail DRG neurons. The

proportion of SN1-positive neurons (first visible at E10 *in vivo* and E7 *in vitro*) varied from 30-40% at brachial and lumbosacral spinal levels, to 80-90% in lower thoracic DRGs. SN1 immunoreactive fibres project to laminae I and II of the spinal cord dorsal horn, and are seen in the skin, but not in deeper tissues such as muscle, of E10-16 embryos and adults, suggesting that SN1 labels a class of cutaneous sensory neurons. The developmental time of appearance of SN1-positive neurons suggests that they normally express the epitope only after they have made contact with their normal peripheral targets, and Marusich *et al.* (1986b) went on to examine this *in vitro*, where the neurons were removed from the possibility of contacting skin. DRGs from all spinal levels were dissected from E6, 8 and 10 quail embryos, dissociated and cultured separately. The percentage of neurons which were SN1 positive was counted 18 hr post plating, and after longer times *in vitro*, with total ages of the neurons (embryonic age + days *in vitro*) being 13-17 days, which is longer than the time required for the major increase of SN1 reactivity *in vivo*, at E11-13.

E6 and E8 neurons initially show no SN1 immunoreactivity, but after 5-9 days *in vitro* up to 17% are SN1 positive. Thus even though they are not in contact with their normal target, the neurons can acquire SN1 immunoreactivity *in vitro*. E10 neurons start with a low proportion of SN1 positives which only increases to 23% after 7 days. The figures for SN1 expression are well below the proportion found in E12-16 DRGs *in vivo* (average 50-60%) and thus target contact is likely to be necessary to increase the proportion. This was backed up by experiments *in vivo* where unilateral wing bud extirpations performed at E3 (prior to wing innervation) resulted in a dramatic selective decrease in the number of SN1-positive neurons within DRGs that normally project to the wing. Therefore the appearance of the SN1 antigen appears to be intrinsically regulated, but target interaction will increase and stabilize the expression.

Another marker of a subpopulation of chick sensory ganglion neurons is a calcium-binding protein, calbindin, the expression of which *in vivo* depends on the formation of connections with developing muscle cells (Philippe *et al.* 1988). Barakat & Droz (1989) showed that DRG neurons cultured from E6 chick embryos, prior to formation

of specific connection with muscles, are devoid of calbindin immunoreactivity for up to 10 days *in vitro*, and then demonstrated that muscle extracts from older animals (E18 or post-hatching) induced the expression of calbindin immunoreactivity in 7% of neurons. Skin extract induced calbindin immunoreactivity in 2.5% of neurons, whereas brain and liver extracts failed to have an effect. The inducing effect was reproduced by cocultures of DRG neurons with myotube-forming myoblasts and, to a lesser extent, by conditioned medium prepared from myoblasts (Bossart *et al.* 1988).

Muscle extract, as opposed to conditioned medium, may have exerted a more obvious phenotype-inducing effect in the present work, but apart from the overriding survival/adherence influence, the numbers of target-identified neurons in the cultures were too low to be confident of picking up any changes of small magnitude.

CHAPTER TEN: Conclusions

10.1 The problem investigated, and its relevance

10.1.1 The question

This thesis has addressed the question of whether the chemical phenotype of a mature primary sensory neuron is both statically and dynamically related to the type of peripheral target tissue to which it projects an afferent axon.

The target tissue, which represents the environment at the peripheral axon terminals, may be important at two stages in the life of a sensory neuron. Firstly during development, when target-derived factors are essential for guidance, innervation and survival, and may also influence subsequent differentiation of the neurons; and secondly in the mature animal, where the neuron might have the capacity to respond to perturbation of its environment due, for instance, to tissue damage, by modifying its phenotype and therefore its function.

10.1.2 Intrinsic versus environmental influences in development

The sequence of events in neuronal development can help to indicate which interactions (e.g. target influences) could operate to direct later events (e.g. chemical differentiation). However we do not have sufficient knowledge of intrinsically-determined events, thus although putative neurotransmitters such as peptides are not detectable in DRG neurons until after the time that peripheral target connections are made (see below), neurotransmitter specificity has not (yet) been shown to be determined by the target. It may be that intrinsic mechanisms specify both the neurotransmitter type and the capacity of the growth cone to respond to environmental cues, (e.g. by expressing certain membrane antigens), to direct the growing axon to a particular target.

Aside from transmitter type or chemical content, 'decisions' about specification must be made about all the other characteristics described in Chapter 2, e.g. receptor type, membrane properties, myelination, effector function. All of the possibilities may be partially or fully decided intrinsically, or may be subject to environmental (peripheral and central) influence. Rat DRG neurons may even communicate with each other at early ages (E13-15), at which time Fulton (1989) has shown that groups of up to 8 neurons are connected by gap junctions, probably at the cell body, so that soluble signals or factors could diffuse among a group of cells, influencing them all in a similar fashion. (It is not known whether each such group of cells all develop to innervate the same target or express the same neurotransmitter, etc).

10.1.3 Is phenotype fixed or plastic

Following the question of intrinsic or environmental determination of neuronal phenotype, is the question of whether the system is capable of change. Neuronal phenotype might be decided and set during development and maintained throughout maturity, or there might be the possibility for plasticity or of lasting changes in the structure and function of the system, in response to particular signals in the environment. There are many documented situations in which the primary afferent neuron, when exposed to altered environmental influences, exhibits a capacity for plasticity of neuronal phenotype in the mature animal.

When the status quo of the mature peripheral nervous system is disrupted or perturbed, for instance by injury directly to the axon or in the peripheral or central target fields, it is not clear whether the neuron's reaction represents a complete de-differentiation followed by re-differentiation as occurs during development, or the acquisition of a novel state in which new responses arise to compensate for the disruption (e.g. regeneration). In fact it is likely that both reactions occur to some extent and

therefore studies on injured neurons (for example, axotomized neurons *in vitro*) should be interpreted with care.

10.2 Summary of results

10.2.1 Chapter 4

The chemical phenotype of neurons projecting to the hindlimb was shown to be related to the target tissue they innervated. Skin, muscle and joint afferents were identified by retrograde labelling from the tissue. Enzyme histochemistry was used to detect TMP, and neuropeptide immunocytochemistry was used to localize CGRP, substance P and somatostatin. TMP and somatostatin were found only in RT97 negative (unmyelinated) afferents. Somatostatin was virtually restricted to skin afferents, TMP was in a much greater proportion of skin afferents than muscle afferents, and was not found in joint afferents. Substance P and CGRP were present in RT97 positive (myelinated) neurons as well as RT97 negative neurons, and found in skin, joint and muscle afferents with increasing frequency.

10.2.2 Chapter 5

A monoclonal antibody to the low-affinity NGF receptor was used to demonstrate the distribution of receptor among retrogradely-labelled skin and muscle afferents. Perhaps surprisingly, a much larger proportion of muscle afferents than skin afferents bore low-affinity NGF receptors. (It was previously thought that NGF would act on small-diameter cutaneous sensory neurons. However, presence of the receptor does not mean that neurons are sensitive to the ligand's action).

10.2.3 Chapter 6

The effect of tissue injury (inflammation) as a model of dynamic change in the target environment, was examined with respect to the proportion of neurons innervating an inflamed area that expressed substance P. The preliminary results suggest that chemical phenotype is dynamically related to the tissue environment, because there was

an increase in the proportion of RT97 positive skin afferents expressing substance P in ipsilateral DRGs.

10.2.4 Chapter 8

As an alternative approach to the question of a dynamic relationship between chemical phenotype and target-derived factors, adult DRG neurons were grown in dissociated culture in the presence or absence of NGF. While NGF was required to maintain the proportion of TMP-positive neurons in the total population, a preliminary result indicates that among retrogradely-labelled muscle afferents, the proportion of TMP-positive neurons was dramatically increased by culturing in the presence of NGF.

10.2.5 Chapter 9

The effect of a novel target influence, i.e. medium conditioned by developing myotubes, was investigated in retrogradely-labelled skin afferents *in vitro*, with respect to expression of CGRP. MCM was found to have a positive effect on neuronal survival, which was biased towards RT97-positive neurons. Survival was also enhanced in the total neuron population; and any novel target influence of myotube-conditioned medium on skin afferents was secondary to these other effects.

10.3 Relating the results to each other and setting them in context

10.3.1 Retrograde labelling studies

Other retrograde labelling and immunocytochemical studies have shown that the expression of neuronal markers is related to peripheral targets such as the intestine (Green & Dockray, 1987), facial skin (Ositelu *et al.*, 1987) and cerebral vasculature (O'Connor & van der Kooy, 1988). Therefore an influence of target tissue upon

primary afferent chemical expression is probably important throughout the neuraxis.

10.3.2 Expression of markers independently of target contact

It is not yet clear whether peripheral target contact helps to direct chemical differentiation as the neuron develops. There are several examples of markers being switched on intrinsically:

An autonomous expression of substance P and VIP was noted by Fontaine-Perus *et al.* (1982) when quail neuroblasts were implanted into chick hosts. The quail neuroblasts differentiated according to the same chronological pattern as those in normal quail embryos, i.e. VIP- and substance P-immunoreactivity appeared earlier in development in the grafted quail neurons than in normal chick gut. The chronology in this respect was not influenced by the host environment.

Davies *et al.* (1987) studied the expression of NGF receptors in developing chick trigeminal axons by ^{125}I-NGF binding to neurons of different ages in culture . At E9, initial fibres (not yet emerged from the ganglion) were devoid of NGF binding sites. Then the proportion of labelled fibres and neurons increased from <30% at E10.5 to almost 100% in E14 cultures. The increase in labelling is closely related to the period of development during which trigeminal nerve fibres arrive in the peripheral target-field. The E10.5 neurons *in vitro* (which are actually 24 hrs older when they are tested for NGF receptor) would not have contacted their targets *in vivo* before removal to culture, yet the expression of receptor occurred at the relevant time.

It would be interesting to know how the timing of expression of NGF receptors correlated with the appearance of, for example, substance P and CGRP, in the trigeminal neurons, as an indication of whether NGF might be required to reach the cell body to induce peptide expression. In the experiments of Fontaine-Perus *et al.* (1982), the quail neurons may have autonomously begun expressing

receptor for a neurotrophic factor, allowing an available factor to induce peptide expression before chick neurons were ready to respond.

The work of Weston's group (reviewed in Weston *et al.*, 1988) also indicates that some subpopulations of neurons arise autonomously - such as the proportion of sensory neurons in chick which become SN1-positive *in vitro* without having contacted their peripheral targets. Target interactions later in development are thought to stabilize and modulate these subpopulation-specific phenotypes (Marusich & Weston, 1988).

10.3.3 Timing of target innervation and neuropeptide expression

Investigations of the timing of appearance of various neuropeptides in rat DRGs (Senba *et al.*, 1982; Marti *et al.*, 1987), suggest that somatostatin appears at E15, CGRP and E16 and substance P at E17. The work of Reynolds *et al.* (1989), using GAP-43 immunoreactivity to follow the development of peripheral innervation in the rat embryo, suggests that innervation of the epidermis in the hindlimb begins on E15, and of the muscle on E17. Part of the same work (Fitzgerald *et al.*, 1989) showed how dorsal root collaterals, after waiting at the grey-white matter boundary for three days, begin to grow into the dorsal horn at E15, exactly coincident with the innervation of the peripheral target, skin. Central connections may thus be directed by whichever peripheral target has been contacted (also the conclusion of a study in the frog by Smith & Frank, 1987). The fact that neuropeptides are first seen in DRGs at or after the time of peripheral and central innervation is consistent with the idea that their expression is related to target (central and/or peripheral) interaction. Somatostatin, shown in Chapter 4 to be a marker for skin afferents, first appears in DRGs at the same time that skin innervation begins - E15, which is before muscle innervation begins.

10.3.4 Different neurotrophic activities

Apart from NGF, evidence for the requirement for, and specific actions of, neurotrophic factors is sparse. Brain-derived neurotrophic factor (BDNF) is specific for a subpopulation of sensory neurons mostly different from those responsive to NGF - neurons derived from the neural placodes (reviewed by Lindsay *et al.*, 1985). The source of BDNF may be the central targets of these neurons (Davies *et al.* 1986); the neural tube itself may elaborate BDNF to promote survival of newly-forming DRGs (Kalcheim *et al.* 1987) at a time when the neurons are unresponsive to NGF (Davies *et al.*, 1987).

It is not known what component of the myotube-conditioned medium was responsible for the promotion of neuronal survival found in Chapter 9, although an adhesion molecule is likely; however the possible selectivity of the medium for RT97-positive neurons is interesting because in Chapter 4 it was shown that a higher proportion of retrogradely-labelled muscle and joint afferents, than skin afferents, are RT97 positive, suggesting that the neurons which were sensitive to the survival effect included muscle and joint afferents.

Further studies would be needed to establish that a neurotrophic activity was responsible, and indeed the search by many groups for a muscle-derived neurotrophic factor for sensory neurons has proved fruitless. Gurney *et al.* (1986) thought they had isolated a neurotrophic factor from mouse salivary gland, naming it neuroleukin. Neuroleukin supported the survival of cultured embryonic sensory neurons which were insensitive to NGF. The protein was also released by denervated muscle grown in organ culture, and antibodies to it partially inhibited terminal sprouting caused by muscle paralysis - and so it was thought to be a myogenic trophic factor. Chaput *et al.* (1988) and simultaneously Faik *et al.* (1988) discovered that the DNA sequence of neuroleukin is 90% homologous with an ubiquitous enzyme, glucose-6-phosphate isomerase, and thus neuroleukin is not a true neurotrophic factor at all. True status for neurotrophic factors

must be achieved by *in vivo* studies, which avoid the problems of the artificial environment of tissue culture.

There is evidence that muscle may elaborate a trophic factor for motoneurons if not for sensory neurons (for instance, Nurcombe *et al.*, 1984; Thompson & Thompson, 1988; Oppenheim *et al.* 1988).

10.3.5 Plasticity of chemical expression in the adult; (i) *in vivo*

Recent work by McMahon's group has employed the technique of cross-anastomosing adult rat peripheral nerves of different specificity to test whether connection to a different target induces sensory neurons to change their chemical expression, i.e. whether a target influence on phenotype is maintained in the adult and is plastic. The (cutaneous) sural nerve and (muscular) gastrocnemius nerve were cross-anastomosed unilaterally. After regeneration the nerves were ligated distally to the anastomosis for 24 hr and stained for FRAP (McMahon & Moore, 1988), and immunoreactivity to substance P (McMahon & Gibson, 1987). The ability of cross-anastomosed nerves *in vivo* to evoke neurogenic extravasation was also correlated with substance P and CGRP accumulation as measured by radioimmunoassay (McMahon *et al.*, 1989).

Control sural nerves but not gastrocnemius nerves show a dense accumulation of FRAP proximal to the ligature. In cross-anastomosed nerves, gastrocnemius nerves now re-routed to skin frequently displayed considerable amounts of FRAP stain with the same appearance as control sural nerve staining. Cross-anastomosed sural nerves, now innervating muscle, showed variable amounts of staining: 50% of cases were stained as densely as controls, but 50% showed decreased levels of FRAP stain. Thus both types of cross-anastomosed afferents showed changes, but in opposite directions, adopting a FRAP staining pattern appropriate to the new tissue they innervated.

Using the same system to examine changes in substance P immunoreactivity, which is present in control sural, but not gastrocnemius, nerve, again it was found that cross-anastomosis caused the substance P staining to change. Muscle nerve re-routed to

skin showed increased substance P, while skin nerve re-routed to muscle showed a decrease.

When accumulated peptide levels were measured by radioimmunoassay, control cutaneous nerves showed higher levels of both peptides than control muscle nerves, and when nerves were cross-anastomosed the levels of peptide fell in skin nerves re-routed to muscle, and rose in muscle nerves re-routed to skin. The capacity of nerves to produce extravasation in their new targets also changed according to the tissue innervated (normally neurogenic extravasation in muscle is about 10% of that in skin; McMahon *et al.*, 1984). These results are consistent with the suggestion that substance P and CGRP are responsible for initiating neurogenic extravasation (Lembeck & Holzer, 1979), there being a high correlation between the levels of these peptide in nerves and their ability to induce extravasation in skin.

The experiments of McMahon's group indicate that in adult rats, influences from peripheral targets, or perhaps in distal nerve sheaths, affect chemical expression in axons as measured by accumulation or levels in nerves, and that there is plasticity in this adult system.

10.3.5 (ii) *in vitro*

McMahon's work cannot address events at the single neuron level, while, in contrast, the system of maintaining dissociated neurons *in vitro* does allow investigation of target effects on specific neuronal subpopulations. There are, undoubtedly, problems associated with *in vitro* experiments, not least in the scope for differences between different scientists' work (even using the same procedures). Neuronal yield, contamination by other cell types, and neuronal requirement for adhesion or survival factors can all be expected to influence the results obtained. Thus while Lindsay (1988) finds that adult DRG neurons do <u>not</u> require neurotrophic factors, serum or non-neuronal cells for survival, Grothe & Unsicker (1987) find that pig-brain extract and non-neuronal cells <u>are</u> requried for survival. Schoenen *et al.* (1989) suggest that the culture process switches on

expression of markers not detectable in DRG neurons *in vivo*, and increases the expression of others. In contrast, Lindsay *et al.* (1989) have shown that while substance P and CGRP levels can be regulated by NGF *in vitro*, the expression of these neuropeptides is most unlikely to be switched on in a new subpopulation of neurons. TMP may normally be subject to regulation other than by NGF, but when exposed to NGF *in vitro* TMP expression may be switched on in certain subpopulations (Chapter 8). The problems associated with the use of novel media, such as myotube-conditioned medium, were illustrated in Chapter 9. While experiments *in vitro* can provide a means of simplifying the system under study, the results require careful interpretation.

10.3.6 The response to different types of injury

The preliminary result of Chapter 6 also indicates that neurons can change their chemical expression when the target tissue is altered by inflammation. The mRNA species for substance P is found in a greater proportion of DRG neurons ipsilateral to an inflamed paw (Noguchi *et al.*, 1988), so a similar trend in expression of neuropeptide would be expected, and indeed was indicated by the results. Within RT97 positive skin afferents, the proportion which were also substance P positive was increased. The mechanism of this chemical response may involve an increase in the target-derived supply of neurotrophic factor as a result of increased expression of trophic factor receptors, since the response is the opposite to that seen after axotomy.

When axons are subjected to axotomy, Schwann cells around the degenerating axons begin to produce NGF (Heumann *et al.*, 1987) and sequester it on low-affinity receptors (Johnson *et al.*, 1988), possibly in order to encourage the regeneration of neurites. Paradoxically, the expression of high-affinity NGF receptors on DRG neuron cell bodies is decreased as the result of axotomy (Verge *et al.*, 1989b), but this might be due to more of the receptors

collecting at the regenerating axon tips. If NGF is applied to the nerve, receptor density is restored.

Afferents themselves contribute to inflammation, especially chemosensitive, peptide-containing neurons which receive the noxious stimulus and then release neuropeptides peripherally, to mediate other reactions. At the same time, a signal may travel to the cell body to increase peptide production, or the released neuropeptides may stimulate another cell type (e.g. macrophages, which have substance P receptors) to generate the signal for the neurons to produce more peptide. The fact that RT97 positive skin afferents with substance P were increased by peripheral inflammation suggests that neurons different from those which receive and initially react to the noxious stimulus (small dark, unmyelinated axons which would be RT97 negative) are stimulated to express substance P. (As discussed in 2.2.4., polymodal nociceptive C-afferents probably account for a large proportion of the afferents reacting to inflammatory stimuli, but Aδ fibre neurons could also be involved, and these would have RT97 positive somata).

10.4 Overview

Many studies in different systems, developing and mature, indicate an important relationship and possibly direct influence between peripheral targets and phenotype of primary afferent neurons. The work in this thesis has similarly shown that primary sensory neurons projecting to three target tissues in the hindlimb, namely skin, muscle and joint, show different distributions of three putative transmitters and a transmitter-related enzyme, and that the sensory neuronal reaction to changes in the environment - such as tissue inflammation and presence of neurotrophic factors - includes a modification of expression of these substances. In this way the target may be able to direct the information carried by, and passed on from, the primary sensory neuron, vital to the correct functioning of the somatosensory system.

10.5 Direction of future work

This work could be extended in several ways using both *in vivo* and *in vitro* systems: retrograde labelling could be performed with

tracer substances that have a specificity for certain neuronal classes, e.g. lectins, or that can travel transneuronally, e.g. viruses; the distribution of other cytoplasmic and surface markers could be studied; other aspects of sensory neuron heterogeneity such as membrane properties or receptor type could probably be related to target influences; the contribution of centrally-derived influences could be assessed; and the processes in development which lead to the establishment of target-phenotype relationships need further investigation. The use of molecular biological techniques such as *in situ* hybridisation, Northern analysis, and polymerase chain reaction (especially useful for small tissue samples), would allow the determination of the underlying changes in gene expression which are responsible for the dynamic relationship between neuronal phenotype and target influence.

References

Ainsworth A., Hall P., Wall P.D., Allt G., MacKenzie M.L., Gibson S. & Polak J.M. (1981) Effects of capsaicin applied locally to adult peripheral nerve. II. Anatomy and enzyme and peptide chemistry of peripheral nerve and spinal cord. *Pain **11**, 379-388*

Altman J. & Bayer S.A. (1984). The Development of the Rat Spinal Cord. *Advances in Anatomy Embryology and Cell Biology* **85**

Amara S.G., Jonas V., Rosenfeld M.G., Ong E.S. & Evans R.M. (1982) Alternative processing in calcitonin gene expression generates mRNAs encoding different polypeptide products. *Nature (London).* **198**, 240-244

Anand P., Gibson S.J., McGregor G.P., Blank M.A., Iangou Y., Bacarese-Hamilton A.J., Polak J.M. & Bloom S.R. (1983) Peripheral neuronal systems show marked increases in VIP/PHI and CCK content following injury. *Regul. Peptides* **7**, 273

Aschoff A. & Hollander H. (1982) Fluorescent compounds as retrograde tracers compared with horseradish peroxidase (HRP). I. A parametric study in the central visual system of the albino rat. *J. Neurosci. Meth.* **6**, 179-197

Barakat I. & Droz B. (1985) Influence of environmental factors on the expression of phenotypic characters by chicken dorsal root ganglion cells in culture. *Dev. Neurosci.* **7**, 351-363

Barakat I. & Droz B. (1989) Inducing effect of skeletal muscle extracts on the appearance of calbindin-immunoreactive dorsal root ganglion cells in culture. *Neuroscience.* **28**, 39-47

Barbut D., Polak J.M. & Wall P.D. (1981) Substance P in the spinal cord dorsal horn decreases following peripheral nerve injury. *Brain Res.* **205**, 289-298

Baron R., Janig W. & Kollmann W. (1988) Sympathetic and afferent somata projecting in hindlimb nerves and the anatomical organization of the lumbar sympathetic nervous system of the rat. *J. Comp. Neurol.* **275**, 460-468

Basbaum A.I., Cruz L. & Weber E. (1986) Immunoreactive dynorphin B in primary afferent fibers of the cat. *J. Neurosci.* **6**, 127-133

Battaglia G., Rustioni A., Altschuler R.A. & Petrusz P. (1987) In:

Schmidt R.F., Schaible H.G. & Vahle-Hinz C. (eds.) *Fine Afferent Nerve Fibres and Pain.* pp 78-84. VCH, Weinheim, F.R.G.

Bayliss W.M. (1901) On the origin from the spinal cord of the vaso-dilator fibres of the hind-limb, and on the nature of these fibres. *J. Physiol. Lond.* **26**, *173-209*

Beal J.A. (1982) The pre- and postnatal development of flame-shaped hair follicle primary afferents in the rat dorsal horn: a Golgi study. Soc. Neurosci. Abs. **3**, 92

Beck P.W. & Handwerker H.O. (1974) Bradykinin and serotonin effects on various types of cutaneous nerve fibres. *Pflugers Archives.* **347**, 209-222

Beck P.W., Handwerker H.O. & Zimmerman M. (1974) Nervous outflow from the cat's foot during noxious radiant heat stimulation. *Brain Res.* **67** 373-386

Borges L.F. & Sidman R.L. (1982) Axonal transport of lectins in the peripheral nervous system. *J. Neurosci.* **5**, 647-653

Bossart E., Barakat I. & Droz B. (1988) Expression of calbindin-immunoreactivity by subpopulations of primary sensory neurons in the chick embryo dorsal root ganglion growth in coculture or conditioned medium. *Dev. Neurosci.* **10**, 81-90

Brain S.D., Williams T.J., Tippins J.R., Morris H.R. & MacIntyre I. (1985) Calcitonin gene-related peptide is a potent vasodilator. *Nature.* **313**, 54-56

Brown A.G. (1981a) *Organisation in the spinal cord.* Springer Verlag, New York.

Brown A.G. (1981b) The terminations of cutaneous nerve fibres in the spinal cord. *Trends Neurosci.* **4**, . 64-67

Buck C.R., Martinez H.J., Black I.B. & Chao M.V. (1987) Developmentally regulated expression of the nerve growth factor receptor gene in the periphery and brain. *Proc. Natl. Acad. Sci. USA.* **84**, 3060-3063

Buller A.J., Eccles J.C., & Eccles R.M. (1960) Interactions between motoneurones and muscles in respect of the characteristic speeds of their responses. *J. Physiol. (Lond.).* **150**, 417-439

Burgess P.R. & Perl E.R. (1973) Cutaneous mechanoreceptors and nociceptors. In: *Handbook of sensory physiology* Iggo A. (ed.)

Vol II, pp 29-78. Springer, Berlin Heidelberg New York

Calof A.L. & Reichardt L.F. (1984) Motoneurons purified by cell sorting respond to two distinct activities in myotube-conditioned medium. *Dev. Biol.* **106**, 194-210.

Cameron A.A., Leah J.D. & Snow P.J. (1986) The electrophysiological and morphological characteristics of feline dorsal root ganglion cells. *Brain Res.* **362**, 1-6

Campbell J.N., Raja S.N., Cohen R.H., Manning D.C., Khan A.A. & Meyer R.A. (1989) Peripheral neural mechanisms of nociception. In: *Textbook of Pain*, Wall P.D. & Melzack R. (eds.) 2nd edition pp 22-45, Churchill Livingstone. Edinburgh.

Cangro C.B., Sweetnam P.M., Wrathall J.R., Haser W.B., Curthoys N.P. & Neale J.H. (1985) Localization of elevated glutaminase immunoreactivity in small DRG neurons. *Brain Res.* **239**, 283-288

Carr V. McM. & Simpson S.D. (1978). Proliferative and degenerative events in the early development of chick dorsal root ganglia. I: Normal development. *J. Comp. Neurol.* **182**, 727-740

Celander O. & Folkow B. (1953) The nature and the distribution of afferent fibres provided with the axon reflex arrangement. *Acta Physiol. Scand. 29, 359-370*

Cervero F. & Connell L.A. (1984) Distribution of somatic and visceral primary afferent fibres within the thoracic spinal cord of the cat. *J. Comp. Neurol.* **230**, 88-98

Chandler C.E., Parsons L.M., Hosang M. & Shooter E.M. (1984) A monoclonal antibody modulates the interaction of nerve growth factor with PC12 cells. *J. Biol. Chem.* **259**, 6882-6889.

Chaput M., Claes V., Portetelle D., Cludts I., Cravador A., Burny A., Gras H. & Tartar A. (1988) The neurotrophic factor neuroleukin is 90% homologous with phosphohexose isomerase. *Nature.* **332**, 454-455

Cook A.J., Woolf C.J., Wall P.D. & McMahon S.B. (1987) Dynamic receptive field plasticity in rat spinal cord dorsal horn following C-primary afferent input. *Nature.* **325**, 151-153

Cotman et al (1987) see page 210.

Craig A.D., Heppelmann B. & Schaible H.-G. (1988) The projection of the medial and posterior articular nerves of the cat's knee to the spinal cord. *J. Comp. Neurol.* **276**, 279-288

Dale (1935) see page 210.

Dalsgaard C.-J. (1988) The sensory system. In: Bjorklund A., Hokfelt T. & Owman C. (eds.) *Handbook of Chemical Neuroanatomy, Vol. 6. The Peripheral Nervous System.* Elsevier, Amsterdam.

Dalsgaard C.-J., Jonsson C.-E., Hokfelt T. & Cuello A.C. (1983) Localization of substance P-immunoreactive nerve fibres in the human digital skin. *Experientia.* **39**, 1018-1020

Dalsgaard C.-J., Ygge J., Vincent S.R., Ohrling M., Dockray G.J. & Elde R. (1984) Peripheral projections and neuropeptide coexistence in a subpopulation of fluoride-resistant acid phosphatase reactive spinal primary sensory neurons. *Neurosci. Letts.* **51**, 139-144

Davies (1988) see page 210

Davies A.M., Bandtlow C., Heumann R., Korsching S., Rohrer H. & Thoenen H. (1987) Timing and site of nerve growth factor synthesis in developing skin in relation to innervation and expression of the receptor. *Nature.* **326**, 353-358

Davies A.M. & Lindsay R.M. (1985) The cranial sensory ganglia in culture; differences in the response of placode-derived and neural crest-derived neurons to nerve growth factor. *Dev. Biol.* **111**, 62-72

Davies A.M., Thoenen H. & Barde Y.-A. (1986) Different factors from the central nervous system and periphery regulate the survival of sensory neurones. *Nature.* **319**, 497-499

Davis J.P. & Epstein M.L. (1987) Trigeminal ganglion cells cocultured with gut express vasoactive intestinal peptide. *Devl. Biol.* **124**, 512-522

De Biasi S. & Rustioni A. (1988) Glutamate and substance P coexist in primary afferent terminals in the superficial laminae of spinal cord. *Proc. Natl. Acad. Sci. USA.* **85**, 7820-7824

Deguchi M., Niwa M., Shigematsu K., Fujii T., Namba K. & Ozaki M. (1989) Specific (^{125}I)Bolton-Hunter substance P binding sites in human and rat skin. *Neurosci. Letts.* **99**, 287-292

Devor M., Wall P.D. & McMahon S.B. (1984) Dichotomizing somatic nerve fibers exist in rats but they are rare. *Neurosci. Letts.* **49**, 187-192

Dodd J. & Jessell T.M. (1985) Lactoseries carbohydrates specify subsets of dorsal root ganglion neurons projecting to the

superficial dorsal horn of rat spinal cord. *J. Neurosci.* **5**, 3278-3294

Dodd J., Solter D. & Jessell T.M. (1984) Monoclonal antibodies against carbohydrate differentiation antigens identify subsets of primary sensory neurons. *Nature* **311**, 469-472

Duce I.R. & Keen P. (1977) An ultrastructural classification of the neuronal cell bodies of the rat dorsal root ganglion using zinc iodide-osmium impregnation. *Cell Tissue Res.* **185**, 263-277

Duce I.R. & Keen P. (1983) Selective uptake of [^{3}H] glutamate into neurons and satellite cells of dorsal root ganglia in vitro. *Neuroscience* **8**, 861-866

Duggan A.W. & Hendry I.A. (1986) Laminar localization of the sites of release of immunoreactive substance P in the dorsal horn with antibody-coated microelectrodes. *Neurosci. Letts.* **68**, 134-140

Duggan A.W., Morton C.R., Zhao Z.Q. & Hendry I.A. (1987) Noxious heating of the skin releases immunoreactive substance P in the substantia gelatinosa of the cat: a study with antibody microprobes. *Brain Res.* **403**, 345-349

Eichler M.E. & Rich K.M. (1989) Death of sensory ganglion neurons after acute withdrawal of nerve growth factor in dissociated cell cultures. *Brain Res.* **482**, 340-346

Erlanger J. & Gasser H.S. (1937) *Electrical Signs of Nervous Activity.* Philadelphia: University of Pennsylvania Press.

Faik P., Walker J.I.H., Redmill A.A.M. & Morgan M.J. (1988) Mouse glucose-6-phosphate isomerase and neuroleukin have identical 3' sequences. *Nature.* **332**, 455-457

Fitzgerald M. (1983) Capsaicin and sensory neurones - a review. *Pain* **15**, 109-130

Fitzgerald M. (1988) The development of activity evoked by fine diameter cutaneous fibres in the spinal cord of the newborn rat. *Neurosci. Letts.* **86**, 161-166

Fitzgerald M. (1989a) The course and termination of primary afferent fibres. In: Wall P.D. & Melzack R. (eds.) *Textbook of Pain,* 2nd edition, pp 46-62. Churchill Livingstone, Edinburgh.

Fitzgerald M. (1989b) Arthritis and the nervous system. *Trends*

Neurosci. **12**, 86-87

Fitzgerald M., Reynolds M.L. & Benowitz L.I. (1989) The development of the rat lumbar spinal cord: a GAP-43 immunocytochemical study. (Submitted)

Fitzgerald M., Wall P.D., Goedert M. & Emson P.C. (1985) Nerve growth factor counteracts the neurophysiological and neurochemical effects of chronic sciatic nerve section. *Brain Res.* **332**, 131-141

Fontaine-Perus J.C., Chanconie M. & Le Douarin N.M. (1982) Differentiation of peptidergic neurones in quail-chick chimaeric embryos. *Cell Differentiation.* **11**, 183-193

Foreman J.C. & Jordan C.C. (1984) Neurogenic inflammation. *Trends Pharmac. Sci.* **5**, 116-119

Foreman J.C., Jordan C.C., Oehme P. & Renner H. (1983) Structure-activity relationships for some substance P-related peptides that cause wheal and flare reactions in human skin. *J. Physiol. (Lond.)* **335**, 449-465

Foster G.A., Schultzberg M., Gage F.H., Bjorklund A., Hokfelt T., Cuello A.C., Verhofstadt A.A.J. & Visser T.J. (1988) Transmitter expression and morphological development of embryonic medullary and mesencephalic raphé neurones after transplantation to the adult rat central nervous system. *Exp. Brain Res.* **70**, 225-241

Friede R.L. & Samorajski T. (1968) Myelin formation in the sciatic nerve of the rat. *J. Neuropath. Exp. Neurol.* **27**, 546-571

Friede R.L. & Samorajski T. (1970) Axon caliber related to neurofilaments and microtubules in sciatic nerve fibers of rats and mice. *Anat. Rec.* **167**, 379-388

Fulton B.P. (1987) Postnatal changes in conduction velocity and soma action potential parameters of rat dorsal root ganglion neurones. *Neurosci. Letts.* **73**, 125-130

Fulton B.P. (1989) Intercellular communication between rat dorsal root ganglion neurones during embryonic development. *Proc. Intl. U. Phys. Sci.* **XVII**, S4100

Fyffe R.E.W. & Perl E.R. (1984) Is ATP a central synaptic mediator for certain primary afferent fibers from mammalian brain? *Proc.*

Natl. Acad. Sci. USA. **81**, 6890-6893

Gaertner G. (1889) Ueber den Verlauf der Vasodilatatoren. *Klin. Wschr.* **51**, 980-981

Gamse R., Holzer P. & Lembeck F. (1980) Decrease of substance P in primary afferent neurones and impairment of neurogenic plasma extravasation by capsaicin. *Br. J. Pharm.* **68**, 207-213

Gamse R. & Saria A. (1985) Potentiation of tachykinin-induced plasma protein extravasation by calcitonin gene-related peptide. *Eur. J. Pharm.* **114**, 61-66

Gibbins I.L., Furness J.B. & Costa M. (1987) Pathway-specific patterns of the co-existence of substance P, calcitonin gene-related peptide, cholecystokinin and dynorphin in neurons of the dorsal root ganglia of the guinea-pig. *Cell Tissue Res.* **248**, 417-437

Gibbins I.L., Furness J.B., Costa M., MacIntyre I., Hillyard C.J. & Girgis S. (1985) Co-localization of calcitonin gene-related peptide-like immunoreactivity with substance P in cutaneous, vascular and visceral sensory neurons of guinea pigs. *Neurosci. Letts.* **57**, 125-130

Gibson S.J., Polak J.M., Bloom S.R., Sabate I.M., Mulderry P.M., Ghatei M.A., McGregor G.P., Morrison J.F.B., Kelly J.S., Evans R.M. & Rosenfeld M.G. (1984) Calcitonin gene-related peptide immunocytochemistry in the spinal cord of man and eight other species. *J. Neurosci.* **4**, 3101-3111

Goedert M., Stoeckel K. & Otten U. (1981) Biological importance of the retrograde axonal transport of nerve growth factor in sensory neurons. *Proc. Natl. Acad. Sci. USA.* **78**, 5895-5898

Green S.H. & Greene L.A. (1986) A single Mr = 103,000 ^{125}I-b-nerve growth factor-affinity-labeled species represents both the low and high affinity forms of the nerve growth factor receptor. *J. Biol. Chem.* **261**, 15316-15326

Green T. & Dockray G.J. (1987) Calcitonin gene-related peptide and substance P in afferents to the upper gastrointestinal tract in the rat. *Neurosci. Letts.* **76**, 151-156

Greenamyre J.T., Young A.B. & Penny J.B. (1984) Quantitative autoradiographic distribution of L(3H) glutamate-binding sites

in rat central nervous system. *J. Neurosci.* **4**, 2133-2144

Grothe C. & Unsicker K. (1987) Neuron-enriched cultures of adult rat dorsal root ganglia: establishment, characterization, survival, and neuropeptide expression in response to trophic factors. *J. Neurosci. Res.* **18**, 539-550

Gulbenkian S., Merighi A., Wharton J., Varndell I.M. & Polak J.M. (1986) Ultrastructural evidence for the coexistence of calcitonin gene-related peptide and substance P in secretory vesicles of peripheral nerves in the guinea pig. *J. Neurocytol.* **15**, 535-542

Gurney M.E., Heinrich S.P., Lee M.R. & Yin H. (1986) Molecular cloning and expression of neuroleukin, a neurotrophic factor for spinal and sensory neurons. *Science* **234**, 566-581

Hagermark O., Hokfelt T. & Pernow B. (1978) Flare and itch induced by substance P in human skin. *J. Invest. Derm.* **71**, 233-235

Hamburger V., Brunso-Bechtold J.K & Yip J.W. (1981) Neuronal death in the spinal ganglia of the chick embryo and its reduction by nerve growth factor. *J. Neurosci.* **1**, 60-70

Hamburger V. & Levi-Montalcini R.(1949) Proliferation, differentiation and degeneration in the spinal ganglia of the chick embryo under normal and experimental conditions. *J. Exp. Zool.* **111**, 457-502

Hamburger V. & Oppenheim R.W (1982) Naturally occurring neuronal death in vertebrates. *Neurosci. Comment.* **1** 39-55

Harper A.A. & Lawson S.N. (1985) Conduction velocity is related to morphological cell type in rat dorsal root ganglion neurons. *J. Physiol. (Lond.)* **359**, 31-46

Handwerker H.O., Anton F. & Reeh P.W. (1987) Discharge patterns of afferent cutaneous nerve fibers from the rat's tail during prolonged noxious mechanical stimulation. *Exp. Brain Res.* **65**, 493-504

Harmar A. & Keen P. (1982) Synthesis, and central and peripheral axonal transport of substance P in a dorsal root ganglion-nerve preparation in vitro. *Brain Res.* **231**, 379-386

Harper A.A. & Lawson S.N. (1985) Conduction velocity is related to morphological cell type in rat dorsal root ganglion neurones.

J. Physiol. **359**, 47-63

Heumann R., Korsching S., Bandtlow C. & Thoenen H. (1987) Changes of nerve growth factor synthesis in nonneuronal cells in response to sciatic nerve transection. *J. Cell Biol.* **104**, 1623-1631

Heumann R., Korsching S., Scott J. & Thoenen H. (1984) Relationship between levels of nerve growth factor (NGF) and its messenger RNA in sympathetic ganglia and peripheral target tissues. *EMBO J.* **3**, 3183-3189

Himes B.T. & Tessler A. (1989) Death of some dorsal root ganglion neurons and plasticity of others following sciatic nerve section in adult and neonatal rats. *J. Comp. Neurol.* **284**, 215-230.

Hinsey J.C. & Gasser H.S. (1930) The component of the dorsal root mediating vasodilatation and the Sherrington contraction. *Am. J. Physiol.* **92**, 679-689

Hokfelt T., Elde R., Johansson O., Luft R., Nilsson G. & Arimura A. (1976) Immunohistochemical evidence for separate populations of somatostatin-containing and substance P-containing primary afferent neurons in the rat. *Neuroscience* **1**, 131-136

Hokfelt T., Everitt B., Holets V.R., Meister B., Melander T., Schalling M., Staines W. & Lundberg J.M. (1986) Coexistence of peptides and other active molecules in neurons: diversity of chemical signalling potential. In: Iversen L.L. & Goodman E.C. (eds.) *Fast and Slow Chemical Signalling in the Nervous System.* pp 205-231. Oxford University Press

Hokfelt T., Kellerth J.-O., Nilsson G. & Pernow B. (1975) Substance P: localization in central nervous system and in some primary sensory neurons. *Science* **190**, 889-890

Hokfelt T., Schultzberg M., Lundberg J.M., Fuxe K., Mutt V., Fahrenkrug J. & Said S.I. (1982) Distribution of vasoactive intestinal polypeptide in the central and peripheral nervous systems as revealed by immunohistochemistry. In: Said S.I. (ed.) *Vasoactive Intestinal Peptide.* pp 65-90. Raven Press, New York.

Holton P. (1959) The liberation of adenosine triphosphate on antidromic stimulation of sensory nerves. *J. Physiol. (Lond.)*

145, 494-504

Holzer P. (1988) Local effector functions of capsaicin-sensitive sensory nerve endings: involvement of tachykinins, calcitonin gene-related peptide and other neuropeptides. *Neuroscience* 24, 739-768

Honig M.G. (1982) The development of sensory projection patterns in embryonic chick hind limb. *J. Physiol.* **330**, 175-202

Iversen L.L. & Goodman E. (1986) *Fast and slow chemical signalling in the nervous system.* Oxford University Press

Jahr C.E. & Jessell T.M. (1983) ATP excites a subpopulation of rat dorsal horn neurons. *Nature* **304**, 730-733

Jancso N., Jancso-Gabor A. & Szolcsanyi J. (1967) Direct evidence for neurogenic inflammation and its prevention by denervation and by pretreatment with capsaicin. *Br. J. Pharm.* **31**, 138-151

Jancso N., Jancso-Gabor A & Szolcsanyi J. (1968) The role of sensory nerve endings in neurogenic inflammation induced in human skin and in the eye and paw of the rat. *Br. J. Pharm.* **32**, 32-41

Jancso G., Kiraly E & Jancso-Gabor A. (1977) Pharmacologically induced selective degeneration of chemosensitive primary sensory neurones. *Nature* **270**, 741-743

Jessell T.M. (1988) Adhesion molecules and the hierarchy of neural development. *Neuron* **1**, 3-13

Jessell T.M. & Dodd J. (1986) Neurotransmitters and differentiation antigens in subsets of sensory neurons projecting to the spinal dorsal horn. In: Martin J.B. & Barchas J.D. (eds.) *Neuropeptides in Neurologic and Psychiatric Disease* pp 111-133. Raven Press, New York,

Jessell T.M. & Dodd J. (1989) Functional chemistry of primary afferent neurons. In: Wall P.D. & Melzack R. (eds.), *Textbook of Pain*, 2nd edition, pp 82-99. Churchill Livingstone. Edinburgh.

Jessell T.M. & Jahr C.E. (1986) Synaptic interactions between dorsal root ganglion and dorsal horn neurons in cell culture: amino acids, nucleotides, and peptides as possible fast and slow excitatory transmitters. In: Iversen L.L. & Goodman E.C. (eds.) *Fast and Slow Chemical Signalling in the Nervous System*, pp

164-184. Oxford University Press

Johansson O. & Vaalasti A. (1987) Immunohistochemical evidence for the presence of somatostatin-containing sensory nerve fibres in the human skin. *Neurosci. Letts.* **73**, 225-230

Johnson E.M., Jr., Rich K.M. & Yip H.K. (1986) The role of NGF in sensory neurons *in vivo*. *Trends Neurosci.* **9**, 33-37

Johnson E.M., Jr., Taniuchi M., Clark H.B., Springer J.E., Koh S., Tayrien M.W. & Loy R. (1987) Demonstration of the retrograde transport of nerve growth factor receptor in the peripheral and central nervous system. *J. Neurosci.* **7**, 923-929

Johnson E.M. Jr., Taniuchi M. & DiStefano P.S. (1988) Expression and possible function of nerve growth factor receptors on Schwann cells. *Trends Neurosci.* **11**, 299-304

Ju G., Hokfelt T., Brodin E., Fahrenkrug J., Fischer J.A., Frey P., Elde R.P. & Brown J.C. (1987) Primary sensory neurons of the rat showing calcitonin gene-related peptide immunoreactivity and their relation to substance P-, somatostatin-, galanin-, vasoactive intestinal polypeptide- and cholecystokinin-immunoreactive ganglion cells. *Cell Tissue Res.* **247**, 417-431

Kai-Kai M.A., Anderton B.H. & Keen P. (1986) A quantitative analysis of the interrelationships between subpopulations of rat sensory neurons containing arginine vasopressin or oxytocin and those containing substance P, fluoride-resistant acid phosphatase or neurofilament protein. *Neuroscience.* **18**, 475-486

Kalcheim, C., Barde Y.-A., Thoenen H. & Le Douarin N.M. (1987) *In vivo* effect of brain-derived neurotrophic factor on the survival of developing dorsal root ganglion cells. *EMBO J.* **6**, 2871-2873

Kawatani M., Lowe I.P., Nadelhaft I., Morgan C. & de Groat W. (1983) Vasoactive intestinal peptides in visceral afferent pathways to the sacral spinal cord of the cat. *Neurosci. Letts.* **42**, 311-316

Kenins P. (1981) Identification of the unmyelinated sensory nerves which evoke plasma extravasation in response to antidromic stimulation. *Neurosci. Lett.* **25**, 137-141

Kessler J.A. & Black I.B. (1981) Similarities in development of substance P and somatostatin in peripheral sensory neurons:

effects of capsaicin and nerve growth factor. Proc. Natl. Acad. Sci. USA. **78**, 4644-4647

Kessler J.A. & Black I.B. (1982) Regulation of substance P in adult rat sympathetic ganglia. *Brain Res.* **234**, 182-187

Knyihar E. & Csillik B. (1976) Effect of peripheral axotomy on the fine structure and histochemistry of the Rolando substance. *Exp. Brain Res.* **26**, 73-87

Knyihar-Csillik E., Bezzegh A., Boti S. and Csillik B. (1986) Thiamine monophosphatase: a genuine marker for transganglionic regulation of primary sensory neurons. *J. Histochem. Cytochem.* **34**, 363-371

Kniyhar-Csillik E. & Csillik B. (1981) FRAP: Histochemistry of the primary nociceptive neuron. *Prog. Histochem. Cytochem.* **14**, 1-137

Kristensson K. & Olsson Y. (1971) Retrograde axonal transport of protein. *Brain Res.* **29**, 363-365

Kruger L., Sampogna S.L., Rodin B.E., Clague J., Brecha N. & Yeh, Y. (1985) Thin-fiber cutaneous innervation and its intraepidermal contribution studied by labeling methods and neurotoxin treatment in rats. *Somatosens. Res.* **2**, 335-356

Kumazawa T., Mizumura K. & Sato J. (1987) Response properties of polymodal receptors studied using *in vitro* testi superior spermatic nerve preparations of dogs. *J. Neurophysiol.* **57**, 702-711

Kuraishi Y., Hirota N., Sato Y., Hanashima N., Takagi H. & Satoh M.(1989). Stimulus specificity of peripherally evoked substance P release from the rabbit dorsal horn in situ. *Neuroscience.* **30**, 241-250

Kuraishi Y., Hirota N., Sato Y., Hino Y., Satoh M. & Takagi H. (1985) Evidence that substance P and somatostatin transmit separate information related to pain in the spinal dorsal horn. *Brain Res.* **325**, 294-298

Kuwayama Y., Terenghi G., Polak J.M., Trojanowski J.Q. & Stone R.A. (1987) A quantitative correlation of substance P-, calcitonin gene-related peptide- and cholecystokinin-like immunoreactivity with retrogradely labeled trigeminal ganglion cells innervating

the eye. *Brain Res.* **405**, 220-226

Kuypers H.G.J.M., Catsman-Berrevoets C.E. & Padt R.E. (1977) Retrograde axonal transport of fluorescent substances in the rat's forebrain. *Neurosci. Letts.* **6**, 127-135

Lander A.D. (1989) Understanding the molecules of neural cell contacts: emerging patterns of structure and function. *Trends Neurosci.* **12**, 189-195

Landmesser L. & Honig M.G. (1986) Altered sensory projections in the chick hind limb following the early removal of motoneurons. *Dev. Biol.* **118**, 511-531

Langford L.A. & Schmidt R.F. (1982) The medial articular nerve: an electron-microscopic examination. *Pfluegers Arch.* **394**, R57

LaVail J.H. & LaVail M. (1972) Retrograde axonal transport in the central nervous system. *Science* **176**, 1416-1417

Lawson S.N. (1979) The postnatal development of large light and small dark neurons in mouse dorsal root ganglia: a statistical analysis of cell numbers and size. *J. Neurocytol.* **8**, 275-294

Lawson S.N. (1987) The morphological consequences of neonatal treatment with capsaicin on primary afferent neurones in adult rats. *Acta Physiol. Hungarica* **69**, 315-321

Lawson S.N., Caddy K.W.T. & Biscoe T.J. (1974) Development of rat dorsal root ganglion neurones. Studies of cell birthdays and changes in mean cell diameter. *Cell Tissue Res.* **153**, 399-413

Lawson S.N., Harper A.A., Harper E.I., Garson J.A. & Anderton B.H. (1984) A monoclonal antibody against neurofilament protein specifically labels a subpopulation of rat sensory neurones. J. Comp. Neurol. **228**, 263-272

Lawson S.N., Harper E.I., Harper A.A., Garson J.A., Coakham H.B. & Randle B.J. (1985) Monoclonal antibody 2C5: a marker for a subpopulation of small neurones in rat dorsal root ganglia. *Neuroscience* **16**, 365-374

Lawson S.N., McCarthy P.W., Reeh P. & Waddell P.J. (1988) An *in vitro* rat sensory ganglion-nerve and skin preparation which allows a direct comparison of cell body and receptor properties. *J. Physiol.* **401**, 11P

Lawson S.N. & Nickels S.M. (1980) The use of morphometric techniques

to analyse the effect of neonatal capsaicin treatment of rat dorsal root ganglia and dorsal roots. *J. Physiol.* **303**, 12P

Lawson S.N. & Waddell P.J (1985) The antibody RT97 distinguishes between cell bodies with myelinated and unmyelinated peripheral processes in the rat. *J. Physiol.* **371**, 59P

Lawson S.N., Waddell P.J. & McCarthy P.W. (1987) A comparison of the electrophysiological and immunocytochemical properties of rat dorsal root ganglion neurones with A and C fibres. In: Schmidt R.F., Schaible H.-G. & Vahle-Hinz C. (eds.) *The Role of Fine Afferent Fibres in Somatovisceral Sensation.* pp 195-203. UCH, West Germany.

Leah J.D., Cameron A.A. & Snow P.J. (1985) Neuropeptides in physiologically identified mammalian sensory neurons. *Neurosci. Letts.* **56**, 257-263

Le Douarin N.M (1980) The ontogeny of the neural crest in avian embryo chimaeras. *Nature* **286**, 663-669

Le Douarin N.M. (1984a) A model for cell line divergence in the ontogeny of the peripheral nervous system. In: I. Black (ed.) *Cellular and Molecular Biology of Neuronal Development*, pp 3-28. Plenum, New York.

Le Douarin N.M. (1984b) The neural crest and the development of the peripheral nervous system. In: W. M Cowan (ed.) *Handbook of Physiology.* American Physiological Society, Bethesda, MD.

Le Douarin N.M. & Teillet M.A. (1974) Experimental analysis of the migration and differentiation of neuroblasts of the autonomic nervous system and of neurectodermal mesenchymal derivatives, using a biological cell marking technique. *Dev. Biol.* **41**, 162-184

Lee K.H., Chung K., Chung J.M & Coggeshall R.E. (1986) Correlation of cell body size, axon size, and signal conduction velocity for individually labelled dorsal root ganglion cells in the cat. *J. Comp. Neurol.* **243**, 335-356

Lee Y., Takami K., Kawai Y., Girgis S., Hillyard C.J., MacIntyre I., Emson P.C. & Tohyama M. (1985) Distribution of calcitonin gene-related peptide in the rat peripheral nervous system with

reference to its coexistence with substance P. *Neuroscience* **15**, 1227-1237

Lembeck F., Donnerer J. & Colpaert F.C. (1981) Increase of substance P in primary afferent nerves during chronic pain. *Neuropeptides* **1**, 175-180

Lembeck F. & Holzer P. (1979) Substance P as neurogenic mediator of antidromic vasodilatation and neurogenic plasma extravasation. *Naunyn-Schmiedeberg's Arch. Pharm.* **310**, 175-183

Letourneau P.C. (1985) Axonal Growth and Guidance. In: Edelman G.M., Einar Gall W. & Maxwell Cowan W. (eds.) *Molecular Bases of Neural Development*, pp 269-293. John Wiley & Sons, New York.

Levi-Montalcini R. & Angeletti P.U. (1968) Nerve growth factor. *Physiol. Rev.* **48**, 534-569

Levine J.D., Clark R., Devor M., Helms C., Moskowitz M.A. & Basbaum A.I. (1984) Intraneuronal substance P contributes to the severity of experimental arthritis. *Science* **226**, 547-549

Levine J.D., Moskowitz M.A. & Basbaum A.I. (1988) The effect of gold, an antirheumatic therapy, on substance P levels in rat peripheral nerve. *Neurosci. Letts.* **87**, 200-202

Lewis T. & Marvin H.M. (1927) Observations relating to vasodilatation arising from antidromic impulses, to herpes zoster and trophic effects. *Heart* **14**, 27-46

Lieberman A.R. (1976) Sensory ganglia. In: D.N. Landon (ed.) *The Peripheral Nerve*, pp 188-278. Chapman & Hall, London.

Light A.R. & Perl E.R. (1979) Spinal termination of functionally identified primary afferent neurons with slowly conducting myelinated fibres. *J. Comp. Neurol.* **186**, 133-150

Lindsay R.M. (1987) The role of neurotrophic factors in development, maintenance and regeneration of sensory neurons. In: Parnevelas J., Stern C. & Stirling V. (eds.) *The Making of The Nervous System*. Oxford University Press.

Lindsay R.M. (1988) Nerve growth factors (NGF, BDNF) enhance axonal regeneration but are not required for survival of adult sensory neurons. *J. Neurosci. 8, 2394-2405*

Lindsay R.M., Barde Y.-A., Davies A.M. & Rohrer H. (1985) Differences and similarities in the neurotrophic growth factor

requirements of sensory neurons derived from neural crest and neural placode. *J. Cell. Sci.* **Suppl. 3**, 115-129

Lindsay R.M. & Harmar A.J. (1989) Nerve growth factor regulates expression of neuropeptide genes in adult sensory neurons. *Nature* **337**, 362-364

Lindsay R.M., Lockett C., Sternberg J. & Winter J. (1989) Neuropeptide expression in cultures of adult sensory neurons: modulation of substance P and CGRP levels by nerve growth factor. *Neuroscience.* (In press)

Lloyd D.P.C. (1953) Neuron patterns controlling transmission of ipsilateral hind limb reflexes in cat. *J. Neurophysiol.* **6**, 293-315

Lundberg J.M., Hokfelt T., Nilsson G., Terenius L., Rehfeld J., Elde R. & Said S. (1978) Peptide neurons in the vagus, splanchnic and sciatic nerves. *Acta Physiol. Scand.* **104**, 499-501

Lynn B. (1989) The peripheral modulation of nociceptive input. In: Paterson & Burn (eds.) *Back Pain: An International Review (Proc. 1989 FIMM Congress, London.)* Kluwer, Lancaster,

Lynn B. & Carpenter S.E. (1982) Primary afferent units from the hairy skin of the rat hindlimb. *Brain Res.* **238**, 29-43

Lynn B. & Hunt S.P. (1984) Afferent C-fibres: physiological and biochemical correlations. *Trends Neurosci.* **7**, 186-188

Mantyh C.R., Gates T.S., Zimmerman R.P., Welton M.L., Passaro E.P. Jr., Vigna S.R., Maggio J.E., Kruger L. & Mantyh P.W. (1988) Receptor binding sites for substance P, but not substance K or neuromedin K, are expressed in high concentrations by arterioles, venules, and lymph nodules in surgical specimens obtained from patients with ulcerative colitis and Crohn's disease. *Proc. Natl. Acad. Sci. USA.* **85**, 3235-3239

Marano (1982) see page 210

Marti E., Gibson S.J., Polak J.M., Facer P., Springall D.R., Van Aswegen G., Aitchison M. & Koltzenburg M. (1987) Ontogeny of peptide- and amine-containing neurones in motor, sensory, and autonomic regions of rat and human spinal cord, dorsal root ganglia, and rat skin. *J. Comp. Neurol.* **266**, 332-359

Martin D.P., Schmidt R.E., Distefand P.S., Lowry O.H., Carter J.G. & Johnson E.M. (1988) Inhibitors of protein synthesis and RNA

synthesis prevent neuronal death caused by NGF deprivation. *J. Cell Biol.* **106**, 829-844

Marusich M.F., Pourmehr K. & Weston J.A. (1986a) A monoclonal antibody (SN1) identifies a subpopulation of avian sensory neurons whose distribution is correlated with axial level. *Dev. Biol.* **118**, 494-504

Marusich M.F., Pourmehr K. & Weston J.A. (1986b) The development of an identified subpopulation of avian sensory neurons is regulated by interaction with the periphery. *Dev. Biol.* **118**, 505-510

Marusich M.F. & Weston J.A. (1988) Role of neuron-target interactions in the development of a subpopulation of avian sensory neurons. *J. Neurosci. Res.* **21**, 480-486

Maxwell D.J. & Rethelyi M. (1987) Ultrastructure and synaptic connections of cutaneous afferent fibres in the spinal cord. *Trends Neurosci.* **10**, 117-123

McCarthy P.W. & Lawson S.N. (1989) Cell type and conduction velocity of rat primary sensory neurons with substance P-like immunoreactivity. *Neuroscience* **28**, 745-753

McGregor G.P., Gibson S.J., Sabate I.M., Blank M.A., Christofides N.D., Wall P.D., Polak J.M. & Bloom S.R. (1984) Effect of peripheral nerve section and nerve crush on spinal cord neuropeptides in the rat; increased VIP and PHI in the dorsal horn. *Neuroscience* **13**, 207-216

McMahon S.B. & Gibson S.J. (1987) Peptide expression is altered when afferent nerves reinnervate inappropriate tissue. *Neurosci. Letts.* **73**, 9-15

McMahon S.B., Lewin G.R., Anand P., Ghatei M.A. & Bloom S.R. (1989) Quantitative analysis of peptide levels and neurogenic extravasation following regeneration of afferents to appropriate and inappropriate targets. *Neuroscience* (In press)

McMahon S.B. & Moore C.E.G. (1988) Plasticity of primary afferent acid phosphatase expression following rerouting of afferents from muscle to skin in the adult rat. *J. Comp. Neurol.* **274**, 1-8

McMahon S.B., Sykova E., Wall P.D., Woolf C.J. & Gibson S.J. (1984) Neurogenic extravasation and substance P levels are low in

muscle as compared to skin in the rat hindlimb. *Neurosci. Letts.* **52**, 235-240

McMahon S.B. & Wall P.D. (1985) The distribution and central termination of single cutaneous and muscle unmyelinated fibres in rat spinal cord. *Brain Res.* **359**, 39-48

McMahon S.B. & Wall P.D. (1987) Physiological evidence for branching of peripheral unmyelinated sensory afferent fibers in the rat. *J. Comp. Neurol.* **261**, 130-136

Mense S. & Craig A.D. Jr. (1988) Spinal and supraspinal terminations of primary afferent fibers from the gastrocnemius soleus muscle in the cat. *Neuroscience* **26**, 1023-1035

Mense S. & Meyer H. (1985) Different types of slowly conducting afferent units in cat skeletal muscle and tendon. *J. Physiol.* **363**, 403-417

Mense S. & Prabhakar N.R. (1986) Spinal termination of nociceptive afferent fibres from deep tissues in the cat. *Neurosci. Letts.* **66**, 169-174

Millard C.L. & Woolf C.J. (1988) Sensory innervation of the hairs of the rat hindlimb: a light microscopic analysis. *J. Comp. Neurol.* **277**, 183-194

Minami M., Kuraishi Y., Kawamura M., Yamaguchi T., Masu Y., Nakanishi S. & Satoh M. (1989) Enhancement of preprotachykinin A gene expression by adjuvant-induced inflammation in the rat spinal cord: possible involvement of substance P-containing spinal neurons in nociception. *Neurosci. Letts.* **98**, 105-110

Molander C. & Grant G. (1987) Spinal cord projections from hindlimb muscle nerves in the rat studied by transganglionic transport using HRP, WGA-HRP, or HRP with DMSO. *J. Comp. Neurol.* **260**, 246-255

Molander C., Ygge J. & Dalsgaard C.-J. (1987) Substance P-, somatostatin- and calcitonin gene-related peptide-like immunoreactivity and fluoride resistant acid phosphatase-activity in relation to retrogradely labelled cutaneous, muscular and visceral primary sensory neurones in the rat. *Neurosci. Letts.* **74**, 37-42

Mudge A.W. (1981) Effect of chemical environment on levels of

substance P and somatostatin in cultured sensory neurones. *Nature* **292**, 764-767

Mudge A.W. (1984) Schwann cells induce morphological transformation of sensory neurones *in vitro*. *Nature* **309**, 367-369

Mulderry P.K., Nicholl C.G., Ghatei M.A., Springall D.R., Polak J.M. & Bloom S.R. (1984) Distribution and possible dual role of CGRP-containing nerves in the skin of the rat. *Reg. Pept.* **9**, 341

Murphy R.A., Singer R.H., Saide J.D., Pantazis N.J., Blanchard, M.H., Byron K.S., Arnason B.G.W. & Young M. (1977) Synthesis and secretion of a high molecular weight form of nerve growth factor by skeletal muscle cells in culture. *Proc. Natl. Acad. Sci. USA.* **74**, 4496-4500

Nagy J.I. & Daddona P.E. (1985) Anatomical and cytochemical relationships of adenosine deaminase-containing primary afferent neurons in the rat. *Neuroscience* **15**, 799-813

Nagy J.I. & Hunt S.P. (1982) Fluoride-resistant acid phosphatase-containing neurones in dorsal root ganglia are separate from those containing substance P or somatostatin. *Neuroscience* **7**, 215-224

Noden D.M. (1975) Analysis of migratory behaviour of avian cephalic neural crest cells. *Dev. Biol.* **42**, 106-30

Noguchi K., Morita Y., Kiyama H., Ono K. & Tohyama M. (1988) A noxious stimulus induces the preprotachykinin-A gene expression in the rat dorsal root ganglion: a quantitative study using *in situ* hybridization histochemistry. *Molec. Brain Res.* **4**, 31-35

Nurcombe V., Hill M.A., Eagleson K.L. & Bennett M.R. (1984) Motor neuron survival and neuritic extension from spinal cord explants induced by factors released from denervated muscle. *Brain Res.* **291**, 19-28

O'Connor T.P. & van der Kooy D. (1988) Enrichment of a vasoactive neuropeptide (calcitonin gene-related peptide) in the trigeminal sensory projection to the intracranial arteries. *J. Neurosci.* **8**, 2468-2476

Ogawa I., Kanazawa I. & Kimura S. (1985) Regional distribution of substance P, neurokinin α and neurokinin β in the rat spinal

cord, nerve roots and dorsal root ganglia, and the effects of dorsal root section or spinal transection. *Brain Res.* **359**, 152-157

Oku R., Satoh M. & Takagi H. (1987) Release of substance P from the spinal dorsal horn is enhanced in polyarthritic rats. *Neurosci. Letts.* **74**, 315-319

Oppenheim R.W., Haverkamp L.J., Prevette D., McManaman J.L. & Appel S.H. (1988) Reduction of naturally occurring motoneuron death in vivo by a target-derived neurotrophic factor. *Science* **240**, 919-922

Oppenheim R.W., Maderdrut J.L. & Wells D.J. (1982). Cell death of motoneurons in the chick embryo spinal cord. VI. Reduction of naturally occurring cell death in the thoracolumbar column of Terni by nerve growth factor. *J. Comp. Neurol.* **210**, 174-189

Ositelu D.O., Morris R. & Vaillant C. (1987) Innervation of facial skin but not masticatory muscles or the tongue by trigeminal primary afferents containing somatostatin in the rat. *Neurosci. Letts.* **78**, 271-276

Otsuka M. & Yanagisawa M. (1987) Does substance P act as a pain transmitter? *Trends Pharmac. Sci.* **8**, 506-510

Patterson P.H. & Chun L.L.Y. (1974) The influence of non-neuronal cells on catecholamine and acetylcholine synthesis and accumulation in cultures of dissociated sympathetic neurons. *Proc. Nat. Acad. Sci. USA.* **71**,, 3607-3610

Peterson D.F. & Brown A.M. (1973) Functional afferent innervation of testis. *Neurophysiology* **36**, 425-433

Philippe E. & Droz B. (1988) Calbindin D-28k-immunoreactive neurons in chick dorsal root ganglion: ontogenesis and cytological characteristics of the immunoreactive sensory neurons. *Neuroscience* **26**, 215-224

Philippe E., Garosi M. & Droz B. (1988) Influence of peripheral and central targets on subpopulations of sensory neurons expressing calbindin immunoreactivity in the dorsal root ganglion of the chick embryo. *Neuroscience* **26**, 225-232

Plenderleith M.B., Cameron A.A., Key B. & Snow P.J. (1989) The plant lectin soybean agglutinin binds to the soma, axon and central

terminals of a subpopulation of small-diameter primary sensory neurons in the rat and cat. *Neuroscience* **31**, 683-695

Popper P., Mantyh C.R., Vigna S.R., Maggio J.E. & Mantyh P.W. (1988) The localization of sensory nerve fibers and receptor binding sites for sensory neuropeptides in canine mesenteric lymph nodes. *Peptides* **9**, 257-267

Price J. (1985) An immunohistochemical and quantitative examination of dorsal root ganglion neuronal subpopulations. *J. Neurosci.* **5**, 2051-2059

Price J. & Mudge A.W. (1983) A subpopulation of rat dorsal root ganglion neurones is catecholaminergic. *Nature* **301**, 241-3

Rambourg A., Clermont Y. & Beaudet A. (1983) Ultrastructural features of six types of neurons in rat dorsal root ganglia. *J. Neurocytol.* **12**, 47-66

Ranson, S.W. (1912) The structure of the spinal ganglia and of the spinal nerves. J. Comp. Neurol. **22**, 159-175

Rethelyi et al (1988) see page 210

Reynolds M.L., Fitzgerald M. & Benowitz L.I. (1989) The development of cutaneous and muscle innervation in the rat hindlimb: a study of peripheral axon growth using GAP-43 immunocytochemistry. (submitted)

Richardson P.M., Issa V.M.K.V. & Riopelle R.J. (1986) Distribution of neuronal receptors for nerve growth factor in the rat. *J. Neurosci.* **6**, 2312-2321

Richardson P.M. & Riopelle R.J. (1984) Uptake of nerve growth factor along peripheral and spinal axons of primary sensory neurons. *J. Neurosci.* **4**, 1683-1689

Riley D.A., Ellis S. & Bain J.L.W. (1984) Ultrastructural cytochemical localization of carbonic anhydrase activity in rat peripheral sensory and motor nerves, dorsal root ganglia and dorsal column nuclei. *Neuroscience* **13**, 189-206

Roberts P.J., Keen P. & Mitchell J.F. (1973) The distribution and axonal transport of free amino acids and related compounds in the dorsal sensory neuron of the rat, as determined by the dansyl reaction. *J. Neurochem.* **21**, 199-209

Robertson B. & Grant G. (1989) Immunocytochemical evidence for the localization of the GM1 ganglioside in carbonic anhydrase-

containing and RT 97-containing rat primary sensory neurones. *J. Neurocytol.* **18**, 77-86

Rogers J.H. (1989) Two calcium-binding proteins mark many chick sensory neurons. *Neuroscience* **31**, 697-709

Rose R.D., Koerber H.R., Sedivec M.J. & Mendell L.M. (1986) Somal action potential duration differs in identified primary afferents. *Neurosci. Letts.* **63**, 259-264

Rozsa A.J. & Beuerman R.W. (1982) Density and organization of free nerve endings in the corneal epithelium of the rabbit. *Pain* **14**, 105-120

Said S. & Mutt W. (1970) Polypeptide with broad biological activity. Isolation from small intestine. *Science* **169**, 1217-1218

Salt T.E. & Hill R.G. (1983) Neurotransmitter candidates of somatosensory primary afferent fibres. *Neuroscience* **10**, 1083-1103

Sanes J.R. (1983) Roles of extracellular matrix in neural development. *Ann. Rev. Physiol.* **45**, 581-600

Sawchenko P.E. & Gerfen C.R. (1985) Plant lectins and bacterial toxins as tools for tracing neuronal connections. *Trends Neurosci.* **8**, 378-384

Sawchenko P.E. & Swanson L.W. (1981) A method for tracing biochemically defined pathways in the central nervous system using combined fluorescence retrograde transport and immunohistochemical techniques. *Brain Res.* **210**, 31-51

Schaible H.-G. & Schmidt R.F. (1983) Activation of groups III and IV sensory units in medial articular nerve by local mechanical stimulation of knee joint. *J. Neurophysiol.* **49** 35-44

Schlichter R., Bader C.R., Bertrand D., Dubois-Dauphin M. & Bernheim L. (1989) Expression of substance P and of a Ca^{2+}-activated Cl^- current in quail sensory trigeminal neurons. *Neuroscience* **30**, 585-594

Schoenen J. (1978) Histochemistry of developing rat spinal cord. *Neuropath. App. Neurobiol.* **4**, 37-46

Schoenen J., Budo C. & Poncelet G. (1968) Effet de la section du sciatique sur l'activité de l'isoenzyme fluorésistant de la phosphatase acide dans la moelle épinière du rat. *C.R. Soc*

Biol. (Paris). **162**, 2035-2937

Schoenen J., Delree P., Leprince P. & Moonen G. (1989) Neurotransmitter phenotype plasticity in cultured dissociated adult rat dorsal root ganglia: an immunocytochemical study. *J. Neurosci. Res.* **22**, 473-487

Scott B.S. (1977) Adult mouse dorsal root ganglia neurons in cell culture. *J. Neurobiol.* **8**, 417-427

Scott S.A. (1982) The development of the segmental pattern of skin sensory innervation in embryonic chick hind limb. *J. Physiol.* **330**, 203-220

Scott S.A. (1986) Skin sensory innervation patterns in embryonic chick hindlimb following dorsal root ganglion reversals. *J. Neurobiol.* **17**, 649-668

Scott S.A. (1988) Skin sensory innervation patterns in embryonic chick hindlimbs deprived of motoneurons. *Dev. Biol.* **126**, 362-374

Senba E., Shiosaka S., Hara Y., Inagaki S., Sakanaka M., Takatsuki K., Kawai Y. & Tohyama M. (1982) Ontogeny of the peptidergic system in the rat spinal cord: immunohistochemical analysis. *J. Comp. Neurol.* **208**, 54-66

Shehab S.A.S., Atkinson M.E. & Payne J.N. (1986) The origins of the sciatic nerve and changes in neuropeptides after axotomy: a double labelling study using retrograde transport of true blue and vasoactive intestinal polypeptide immunohistochemistry. *Brain Res.* **376**, 180-185

Shelton D.L. & Reichardt L.F. (1984) Expression of the β-nerve growth factor gene correlates with the density of sympathetic innervation in effector organs. *Proc. Natl. Acad. Sci. USA.* **81**, 7951-7955

Shortland P., Woolf C.J. & Fitzgerald M. (1989) Morphology and somatotopic organization of the central terminals of hindlimb hair follicle afferents in the rat lumbar spinal cord. *J. Comp. Neurol.* **289**, 416-433.

Silverman J.D. & Kruger L. (1988) Lectin and neuropeptide labeling of separate populations of dorsal root ganglion neurons and associated "nociceptor" thin axons in rat testis and cornea

whole-mount preparations. Somatosens. Res. **5**, 259-267

Skirboll L. & Hokfelt T. (1983) Transmitter specific mapping of neuronal pathways by immunohistochemistry combined with fluorescent dyes. In: Cuello A.C. (ed.) *Immunohistochemistry*, Ch. 18. IBRO Publications, Oxford

Smith C.L. & Frank E. (1987) Peripheral specification of sensory neurons transplanted to novel locations along the neuraxis. *J. Neurosci.* **7**, 1537-1549

Smith C.L. & Hollyday M. (1983) The development and postnatal organization of motor nuclei in the rat thoracic spinal cord. *J. Comp. Neurol.* **220**, 16-28

Sommer E.W., Kazimierczak J. & Droz B. (1985) Neuronal subpopulations in the dorsal root ganglion of the mouse as characterized by combination of ultrastructural and cytochemical features. *Brain Res.* **346**, 310-326

Stacey J.J. (1969) Free nerve endings in skeletal muscle of the cat. *Am. J. Anat.* **105**, 231-254

Stricker S. (1876) Untersuchungen ueber die Gefasswurzeln des Ischiadicus. *Sber. Akad. Wiss. Wien.* **3**, 173-185

Suda K., Barde Y.-A. & Thoenen H. (1978) Nerve growth factor in mouse and rat serum: correlation between bioassay and radioimmunoassay determinations. *Proc. Natl. Acad. Sci. USA.* **75**, 4042-4046.

Sugiura Y., Hosoya Y., Ito R. & Kohno K. (1988) Ultrastructural features of functionally identified primary afferent neurons with C (unmyelinated) fibers of the guinea pig: classification of dorsal root ganglion cell type with reference to sensory modality. *J. Comp. Neurol.* **276**, 265-278

Sugiura Y., Lee C.L. & Perl E.R. (1986) Central projections of identified, unmyelinated (C) afferent fibers innervating mammalian skin. *Science* **234**, 358-361

Sunderland S. (1978) *Nerves and Nerve Injuries. **2nd Ed.**, pp 474-475. Churchill-Livingstone, London.*

Sutter A., Riopelle R.J., Harris-Warrick R.M. & Shooter E.M. (1979) Nerve growth factor receptors. *J. Biol. Chem.* **254**, 5972-5982

Sweetnam P.M., Neale J.H. Barker J.L. & Goldstein A (1982)

Localization of immunoreactive dynorphin in neurons cultured from spinal cord and dorsal root ganglia. *Proc. Natl. Acad. Sci. USA.* **79**, 6742-6746

Szolcsanyi J. (1984) Capsaicin and neurogenic inflammation: history and early findings. In: Chahl L.A., Szolcsanyi J. & Lembeck F. (eds.) *Antidromic Vasodilatation and Neurogenic Inflammation.* pp 7-25. Akademiai Kiado, Budapest

Szonyi G., Knyihar E. & Csillik B. (1979) Extra-lysosomal fluoride-resistant acid phosphatase active neuronal system subserving nociception in the rat cornea. *A. Mikrosk.-Anat. Forsch.* **93**, 974-971

Taylor D.C.M. & Pierau F.K. (1982) Double fluorescence labelling supports electrophysiological evidence for dichotomizing peripheral sensory nerve fibres in rats. *Neurosci. Letts.* **33**, 1-6

Tainio H., Vaalasti A. & Rechardt L. (1987) The distribution of substance P-, CGRP-, galanin- and ANP-like immunoreactive nerves in human sweat glands. *Histochem. J.* **19**, 375-380

Terenghi G., Polak J.M., Ghatei M.A., Mulderry P.K., Butler J.M., Unger W.G. & Bloom S.R. (1985) Distribution and origin of calcitonin gene-related peptide (CGRP) immunoreactivity in the sensory innervation of the mammalian eye. *J. Comp. Neurol.* **233**, 506-516

Thoenen H & Barde Y.-A. (1980) Physiology of nerve growth factor. *Physiol. Rev.* **60**, 1284-1335

Thompson J.M. & Thompson E.G. (1988) Developmental changes in spinal cord neurite-promoting activity from chick muscle extracts. *Dev. Brain Res.* **40**, 158-160

Traub R.J., Solodkin A. & Ruda M.A. (1989) Calcitonin gene-related peptide immunoreactivity in the cat lumbosacral spinal cord and the effects of multiple dorsal rhizotomies. *J. Comp. Neurol.* **287**, 225-237.

Tuchscherer M.M. & Seybold V.S. (1985) Immunohistochemical studies of substance P, cholecystokinin-octapeptide and somatostatin in dorsal root ganglia of the rat. *Neuroscience* **14**, 593-605

Urban L. & Randic M. (1984) Slow excitatory transmission in rat

dorsal horn. Possible mediation by peptides. *Brain Res.* **290**, 336-341

Verge V.M.K., Richardson P.M., Benoit R. & Riopelle R.J. (1989a) Histochemical characterization of sensory neurons with high-affinity receptors for nerve growth factor. *J. Neurocytol.* **18**, 583-591

Verge V.M.K., Riopelle R.J. & Richardson P.M. (1989b) Nerve growth factor receptors on normal and injured sensory neurons. *J. Neurosci.* **9**, 914-922

von Euler U.S. & Gaddum J.H. (1931) An unidentified depressor substance in certain tissue extracts. *J. Physiol. (Lond.)* **72**. 74-87

Wall P.D. (1987) The central consequences of the application of capsaicin to one peripheral nerve in adult rat. *Acta Physiol. Hungarica* **69**, 275-286

Weston J.A., Vogel K.S. & Marusich M.F. (1988) Identification and fate of neural crest cell subpopulations in early embryonic development. In: S. Easter et al. (eds.) *From Message to Mind.* pp 224-237. Sinauer Associates, Sunderland, MA

Wiesenfeld-Hallin Z. (1986) Substance P and somatostatin modulate spinal cord excitability via physiologically different sensory pathways. *Brain Res.* **372**, 172-175

Wiesenfeld-Hallin Z., Hokfelt T, Lundberg J.M., Forssman W.G., Reinecke M., Tschopp F.A. & Fischer J.A. (1984). Immunoreactive calcitonin gene-related peptide and substance P coexist in sensory neurons to the spinal cord and interact in spinal behavioural responses of the rat. *Neurosci. Letts.* **52**, 199-204

Winter J., Forbes C.A., Sternberg J. & Lindsay R.M. (1988) Nerve growth factor (NGF) regulates adult rat cultured dorsal root ganglion neuron responses to the excitotoxin capsaicin. *Neuron* **1**, 973-981

Wong V., Barrett C.P., Donati E.J., Eng L.F. & Guth L. (1983) Carbonic anhydrase activity in first-order sensory neurons of the rat. *J. Histochem. Cytochem.* **31**, 293-300

Wood J.N. & Anderton B.H. (1981) Monoclonal antibodies to mammalian neurofilaments. *Biosci. Rep.* **1**, 263-268

Woolf C.J. (1984) Long term alterations in the excitability of the flexion reflex produced by peripheral tissue injury in the chronic decerebrate rat. *Pain.* **18**, 325-343.

Woolf C.J. (1987) Central terminations of cutaneous mechanoreceptor afferents in rat lumbar cord. *J. Comp. Neurol.* **261**, 105-119

Woolf C.J., Reynolds M.L., Molander C., O'Brien C., Lindsay R.M. & Benowitz L.I. (1989) GAP-43, a growth associated protein, appears in dorsal root ganglion cells and in the dorsal horn of the rat spinal cord following peripheral nerve injury. *Neuroscience* (In press)

Woolf C. & Wiesenfeld-Hallin Z. (1986) Substance P and calcitonin gene-related peptide synergistically modulate the gain of the nociceptive flexor withdrawal reflex in the rat. *Neurosci. Letts.* **66**, 226-230

Yoshimura M. & Jessell T.M. (1989) Primary afferent-evoked synaptic responses and slow potential generation in rat substantia gelatinosa neurons *in vitro*. *J. Neurophysiol.* **62**, 96-108

Zalewski A.A. (1969) Regeneration of taste buds after reinnervation by peripheral or central sensory fibres of vagal ganglia. *Exp. Neurol.* **25**, 429-437

Zalewski A.A. (1974) Neuronal and tissue specifications involved in taste bud formation. *Ann. N.Y. Acad. Sci.* ***228**, 344-349*

ADDENDUM

Cotman C.W., Monaghan D.T., Ottersen O.P. & Storm-Mathiesen J. (1987) Anatomical organisation of excitatory amino acid receptors and their pathways. *Trends Neurosci.* **10**, 273-280.

Dale H.H. (1935) Pharmacology and nerve endings. *Proc. R. Soc. Lond.* **28**, 318-332.

Davies A.M. (1988) Role of neurotrophic factors in development. *Trends Genetics* **4**, 139-143.

Maranto A.R. (1982) Neuronal mapping: a photo-oxidation reaction that makes Lucifer Yellow useful for electron microscopy. *Science* 217, 953-955.

Rethelyi M., Metz C.B. & Lund P.K. (1989) Distribution of neurons expressing calcitonin gene-related peptide mRNAs in the brain stem, spinal cord and dorsal root ganglia of rat and guinea pig Neuroscience 29, 225-239

Appendix I: Raw data from Chapter Three

Tables are numbered according to animal case number as in text

KEY: h = high intensity
m = medium intensity
l = low intensity

L = lumbar
S = sacral
T = thoracic

TRG = trigeminal ganglion
– = not determined.

CASE 1 5d

	LHS Ti FB			RHS Sa FB		
	h	m	l	h	m	l
L2	–	–	–	122	203	347
L3	} only odd one or two			56	138	266
L4	}			0	0	9
L5	}			–	–	–
TOTAL				178	341	622

CASE 2 9d

	LHS Ti FB			RHS Sa FB		
	h	m	l	h	m	l
L2	1	0	–	–	–	–
L3	57	142	–	23	24	–
L4	98	266	–	3	7	–
L5	81	154	–	2	4	–
L6	–	–	–	2	0	–
TOTAL	236	562		30	35	

CASE 3 15d

	LHS Ti FB			RHS Sa FB		
	h	m	l	h	m	l
L2	0	0	-	0	6	-
L3	0	0	-	44	54	-
L4	0	0	-	1	3	-
L5	41	119	-	2	9	-
L6	0	4	-	0	0	-
TOTAL	41	123		47	72	

CASE 4 6d

	LHS -	RHS Sa FB		
		h	m	l
L2		50	-	-
L3		70	-	-
L4		7	-	-
TOTAL		127		

CASE 5 6d

	LHS -	RHS Sa FB		
		h	m	l
L2		57	-	-
L3		67	-	-
L4		16	-	-
TOTAL		140		

CASE 6 6d

	LHS -			RHS Sa FB		
				h	m	l
L2				36	-	-
L3				104	-	-
L4				2	-	-
TOTAL				142		

CASE 7 8d

	LHS Ti FB + scra			RHS Ti FB		
	h	m	l	h	m	l
L2	0	0	-	0	0	-
L3	0	0	-	0	0	-
L4	0	21	-	0	18	-
L5	2	130	-	5	104	-
TOTAL	2	151		5	122	

CASE 8 6d

	LHS Sa FB + scra			RHS Sa FB		
	h	m	l	h	m	l
L1	0	14	-	0	8	-
L2	10	34		7	14	-
L3	4	47	-	34	70	-
L4	0	24	-	4	8	-
TOTAL	14	119		45	100	

CASE 9 6d

	LHS Sa FB + cut			RHS Sa FB		
	h	m	l	h	m	l
L1	0	7	3	3	7	3
L2	3	43	31	49	124	67
L3	2	24	27	10	50	34
L4	1	19	11	1	8	21
L5	0	2	0	1	10	1
TOTAL	6	95	72	64	199	126

CASE 10 9d

	LHS Su FB			RHS Su FB + cut		
	h	m	l	h	m	l
L3	1	0	27	0	0	7
L4	18	39	263	0	4	22
L5	55	70	181	2	5	9
TOTAL	74	109	471	2	9	38
Cx	0	0	0	0	0	0
Tx	0	0	0	0	0	0

CASE 11 9d

	LHS Su FB			RHS Su FB + cut		
	h	m	l	h	m	l
L3	0	2	-	0	0	-
L4	51	77	-	2	42	-
L5	46	70	-	0	11	-
TOTAL	97	149	-	2	53	

CASE 12 9d

	LHS Su FB + others cut			RHS Su FB		
	h	m	l	h	m	l
L3	0	1	66	18	50	133
L4	21	27	92	31	40	112
L5	6	14	44	9	9	40
TOTAL	27	41	202	58	99	285

CASE 13 7d

	LHS -			RHS i.v FB near sural nerve		
	h	m	l	h	m	l
L4	0	0	many	4	18	36
Tmid	0	0	some	0	0	some
Tupper	0	0	some	0	0	some

CASE 14

	LHS 8d muscle DY 6d Sa FB				RHS - Sa FB	
	h FB	m FB	DY	FB+DY	h FB	m FB
L1	4	32	0	0	0	6
L2	52	68	0	0	4	28
L3	56	88	47	0	8	99
L4	10	20	27	0	1	1
L5	0	9	61	0	8	35
L6	0	0	2	0	0	0
TOTAL	122	217	137	0	21	169
Tx	0	0	-	-	-	

CASE 15

	LHS 12d - 8d Ti FB		RHS muscle DY Ti FB			
	h FB	m FB	h FB	m FB	DY	DY + FB
L2	3	14	0	25	1	0
L3	8	26	0	12	0	0
L4	13	26	0	17	38	5
L5	45	80	6	50	60	22
L6	1	4	0	3	0	0
TOTAL	70	150	6	107	99	27

CASE 16 7d

	LHS m TA FB			RHS –		
	h	m	l	h	m	l
L3	25	71	–	–	–	–
L4	29	142	–	0	0	many
L5	0	49	–	–	–	–
L6	0	97	–	–	–	–
Tx	0	0	many	–	–	–
TOTAL	54	359				

CASE 17 7d

	LHS –	RHS mTA FB		
		h	m	l
L2		0	–	–
L3		17	–	–
L4		17	–	–
TOTAL		34		

CASE 18 7d

	LHS –	RHS mTA FB		
		h	m	l
L2		1	–	–
L3		8	–	–
L4		13	–	–
TOTAL		22		

CASE 19 7d

	LHS mTA FB			RHS mTA FB + cut		
	h	m	l	h	m	l
L2	2	47	-	0	74	-
L3	12	116	-	4	91	-
L4	3	118	-	7	51	-
L5	0	4	many	0	60	-
TOTAL	17	285		11	276	

CASE 20 7d

	LHS mGa FB + cut			RHS mGa FB		
	h	m	l	h	m	l
L2	0	0	-	0	0	-
L3	0	0	-	0	0	-
L4	0	0	-	0	0	-
L5	0	7	-	0	52	-
L6	0	0	-	2	19	-
TOTAL	0	7		2	71	

CASE 21 7d

	LHS mGa FB + all others cut			RHS mGa FB		
	h	m	l	h	m	l
L3	0	15	-	4	38	-
L4	0	1	-	5	30	-
L5	9	38	-	13	88	-
L6	0	1	-	0	12	-
Tx	-	-	-	0	5	-
TOTAL	9	55		22	173	

CASE 22 6d

	LHS jK FB			RHS s. c. FB in K		
	h	m	l	h	m	l
L2	16	131	-	21	137	-
L3	39	207	-	8	131	-
L4	66	190	-	0	56	-
L5	19	80	-	0	107	-
Tx	0	19	-	0	30	-
TOTAL	140	627		31	461	

CASE 23 9d

	LHS jA FB + cut			RHS jA FB		
	h	m	l	h	m	l
L2	0	16	-	3	28	-
L3	0	0	-	16	53	-
L4	0	21	-	31	192	-
L5	0	20	-	41	262	-
L6	0	0	-	1	0	-
Tx			many	-	-	-
TOTAL	0	57		92	535	

CASE 24

	LHS 8d s.c. DY in K; 6d jK FB				RHS - -
	h FB	mFB	l FB	FB+DY	
L3 } L4 } L5	some		lots	some	
TRG		lots	lots	0	

CASE 25 8d

	LHS jK DY (2.5%)			RHS jK FB (2.5%)		
	h	m	l	h	m	l
L4} } L5}	few	-	-	40	118	56
TRG	-	-	-	0	0	few

CASE 26 5d

	LHS			RHS		
	bladder wall			FB		
	h	m	l	h	m	l
L1	26	184	-	16	155	-
L2	14	103	-	9	88	-
L5	17	14	-	0	0	-
L6	36	152	-	51	223	-
S1	17	117	-	10	101	-
S2	0	0	-	0	0	-
TOTAL	110	570		86	567	

CASE 27 5d

	LHS			RHS		
	bladder wall			FB	+ ligate	
	h	m	l	h	m	l
L1	1	71	-	1	55	-
L2	8	33	-	0	26	-
L5	0	0	-	0	0	-
L6	20	95	-	0	1	-
S1 (lost)	-	-	-	-	-	-
S2	0	0	-	0	0	-
TOTAL	(29)	(199)		(1)	(82)	

Appendix II: Extra data from Chapter Four

Equivalent data to **Tables 4.1-4.4**, for medium intensity rather than high-intensity FB-labelled neurons

(TABLE 4.1): *Thiamine monophosphatase study*

Numbers of: medium-intensity retrogradely-labelled DRG cells, those that were RT97 negative (small dark) and those which also contained TMP

TARGET	n	Total FB	RT97 negative	TMP positive
SKIN	2	181	124	75
MUSCLE	4	188	89	29
JOINT	4*	246	-	1
	2	39	-	0

* = not photographed before TMP reaction
- = not determined

(TABLE 4.2): *Calcitonin gene-related peptide study*

Numbers of: medium-intensity retrogradely-labelled DRG cells, those that were RT97 negative (small dark), and the occurrence of CGRP in RT97 positive and RT97 negative FB cells

TARGET	n	Total FB	Total RT97 negative	RT97 negative CGRP positive	RT97 positive CGRP positive
SKIN	3	183	93	43	34
MUSCLE	4	192	80	66	56
JOINT	3	72	15	11	47

(TABLE 4.3): *Substance P study*

Numbers of: medium-intensity retrogradely-labelled DRG cells, those that were RT97 negative, and the occurrence of SP in both RT97 positive and RT97 negative FB cells

TARGET	n	Total FB	Total RT97 negative	RT97 negative SP positive	RT97 positive SP positive
SKIN	2	256	144	33	6
MUSCLE	2	337	88	66	13
JOINT	3	58	10	8	7
	3	21	-	(	8)

- = not determined

(TABLE 4.4): *Somatostatin study*

Numbers of: medium-intensity retrogradely-labelled DRG cells, those that were RT97 negative (small dark), and those that also contained somatostatin

TARGET	n	Total FB	RT97 negative	Somatostatin positive
SKIN	2	276	150	18
MUSCLE	2	483	109	2
JOINT	3	51	4	0
	5	29	-	0

- = not determined

Appendix III: Extra data from Chapter Five

The equivalent data to **Table 5.1**, for medium intensity rather than high-intensity FB neurons

(TABLE 5.1): *NGF receptor in medium-intensity retrogradely-labelled FB neurons (separate counts for four rats each with skin FB or muscle FB)*

RAT	Total FB	NGFr positive	% of total
SKIN FB			
S1	106	69	59
S2	297	174	59
S3	120	68	57
S4	132	89	67
		MEAN	61
MUSCLE FB			
M1	69	66	96
M2	143	136	95
M3	138	124	90
M4	97	95	98
		MEAN	95

Appendix IV: Extra data from Chapter Six

Equivalent data to **Table 6.1**, for medium intensity rather than high intensity FB labelled cells

(Table 6.1): Numbers of medium-intensity retrogradely labelled DRG cells, those that were RT97 negative, and the occurrence of SP in both RT97 positive and RT97 negative FB cells

SKIN	Total FB	Total RT97 negative	RT97 negative SP positive	RT97 positive SP positive
CONTRALATERAL	49	12	8	1
IPSILATERAL	295	71	19	6

Appendix V: Raw data from Chapter Eight

Raw data used to compile **Table 8.1** and **Fig. 8.2**

Counts of neurons among the total population which contain TMP in cultures 1, 10 and 15 days post plating, in the presence and absence of NGF

1 DAY POST PLATING

DISH (pair no.)	Total sample	TMP positive	% of total
+ NGF 1	344	173	50
2	481	306	64
3	333	173	52
4	347	189	54
5	394	171	43
- NGF 1	334	147	44
2	372	215	58
3	355	185	52
4	372	252	68
5	437	210	48

10 DAYS POST PLATING

DISH (pair no.)	Total sample	TMP positive	% of total
+ NGF 1	333	184	55
2	318	179	56
3	365	217	59
4	420	238	57
5	386	205	53
- NGF 1	255	111	44
2	177	63	36
3	333	101	30
4	395	189	48
5	330	126	38

15 DAYS POST PLATING

DISH (pair no.)	Total sample	TMP positive	% of total
+ NGF 1	362	137	38
2	316	123	39
3	356	144	40
- NGF 1	356	36	10
2	337	54	16
3	380	52	14

Appendix VI: Extra data and raw data from Chapter Nine

Equivalent data to **Table 9.1**, but for medium-intensity, rather than high-intensity, FB-labelled skin afferents.

CGRP in skin afferents in myotube-conditioned medium

Number of RT97 positive and negative retrogradely-labelled skin afferents, and the number of each which contained CGRP, after 14 d *in vitro*, in the absence or presence of myotube-conditioned medium (MCM). (NGF present in all cases).

MEDIUM (& dish)	RT97 positive	RT97 negative	CGRP in RT97+	CGRP in RT97-	Total CGRP +
CONTROL					
1	11	8	6	4	10
2	6	9	5	5	10
3	14	10	13	6	19
4	18	14	13	3	16
5	7	8	5	3	8
6	7	5	4	2	6
7	1	1	1	0	1
TOTAL (n=119)	64/119 = 54%	55/119 = 46%	47/64 = 73%	23/55 = 42%	70/119 = 59%
+ MCM					
8	23	6	16	2	18
9	19	5	13	3	19
10	28	6	18	4	22
11	21	11	12	7	19
12	12	1	12	1	13
13	14	10	7	7	14
14	6	7	1	2	3
TOTAL (n=170)	123/170 = 72%	47/170 = 28%	79/123 = 64%	26/47 = 55%	105/170 = 62%

Neuron survival raw data used for **Figs. 9.3 and 9.4**

(Fig. 9.3) Counts of total neurons on six **live** dishes followed over 14 days *in vitro*: neurons grown in the absence and presence of myotube-conditioned medium (MCM). (NGF present in all cases; initial plating density = 500 neurons/dish).

DAYS post plating	CONTROL DISH A	CONTROL DISH B	CONTROL DISH C	+ MCM DISH D	+ MCM DISH E	+ MCM DISH F
3	335	306	290	329	283	236
6	202	172	199	280	283	238
10	176	115	157	267	251	213
14	147	89	126	219	201	163

(Fig. 9.4) Counts of total neurons in dishes **fixed** over a period of 14 days *in vitro*: neurons grown in the absence or presence of myotube-conditioned medium (MCM). (NGF present in all cases; initial plating density = 500 neurons/dish).

DAYS post plating	CONTROL replicates			+ MCM replicates		
3	192	186	195	312	221	306
6	145	161	166	235	206	184
10	80	164	127	207	224	208
14	109	84	87	170	167	-

ABSTRACT from the seventh national meeting of the Brain Research Association, 1989. *Neurosci. Letts.*, Suppl. 36, S32.

IMMUNOCYTOCHEMICAL ANALYSIS OF NGF RECEPTOR DISTRIBUTION AMONG SUBPOPULATIONS OF ADULT RAT DORSAL ROOT GANGLION NEURONS.

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Nerve growth factor (NGF) is essential for the survival of a large percentage of rat dorsal root ganglion (DRG) neurons during development and appears to regulate neuropeptide expression in a subpopulation(s) of these neurons in the adult. While it has been shown that around 50% of mature DRG neurons possess high affinity NGF receptors, little is known about the distribution of NGF receptors among functionally or anatomically distinct subpopulations of sensory neurons. To examine possible differences in the distribution of NGF receptor-bearing afferents among peripheral target tissues, we have combined retrograde labelling techniques with immunocytochemical detection of the NGF receptor. Fast Blue was injected into the skin or muscle of the hind limb of adult rats in order to label skin- or muscle-specific afferents in lumbar ganglia L2 - L6. One week after labelling the lumbar ganglia were removed, sectioned and stained with a monoclonal antibody (192-IgG) to the NGF receptor. Surprisingly there was no great difference in the percentage of identified muscle and skin afferents which were immunoreactive for the NGF receptor: 85% and 63% respectively. The extent to which this anatomical distribution of NGF receptors is reflected in function remains to be determined.

ABSTRACT from the satellite symposium of the XXXI International Congress of Physiological Sciences, 1989: Regulators of Peripheral Nerve Regeneration, P11.

GAP-43 IMMUNOREACTIVITY IN ADULT RAT DISSOCIATED DORSAL ROOT GANGLION CELLS IN VITRO.

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The response to axotomy of primary sensory neurons includes an increase in the production of GAP-43. We have examined adult rat dorsal root ganglion cells in vitro, where the cells can be considered to be regenerating after the axotomy involved in their removal, to test the effect of culture environment on GAP-43 immunoreactivity.

Using an accelerated version of R.M. Lindsay's dissociation method (J. Neurosci 8:2394, 1988), cultures could be immunostained as early as 3.5 hours post axotomy, at which time all neurons showed some degree of immunoreactivity. This preceded the initiation of neurite outgrowth, after which enhanced staining was seen in 'blebs' and then in growth cones at the tips of neurites. Presence or absence of serum or NGF in culture media did not influence GAP-43 staining other than by inducing a faster and more exuberant neurite outgrowth - in which case more GAP-43 was apparent.

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DIFFERENCES IN THE CHEMICAL EXPRESSION OF RAT PRIMARY AFFERENT NEURONS WHICH INNERVATE SKIN, MUSCLE OR JOINT

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Abstract—The fluorescent dye Fast Blue was injected in anaesthetized rats into either skin, muscle or knee joint of the hindlimb. Following retrograde transport of the dye to lumbar dorsal root ganglia, the cell bodies of primary afferent neurons innervating these different target tissues were identified in ganglion sections by fluorescence microscopy. The sections were processed to demonstrate activity of the enzyme thiamine monophosphatase, or immunoreactivity to calcitonin gene-related peptide, substance P, or somatostatin, in Fast Blue labelled neurons. In all cases immunoreactivity to the antineurofilament antibody RT97 was used to classify dorsal root ganglion cells as being either small dark (RT97 negative, unmyelinated axons) or large light (RT97 positive, myelinated axons).

The proportion of small dark cells labelled from each target decreased in the order: skin, muscle, joint. Thiamine monophosphatase and somatostatin were present only in small dark cells, while calcitonin gene-related peptide and substance P were found in both small dark and large light cells. In large light cells of all three targets, more contained calcitonin gene-related peptide than substance P. Among small dark cells, thiamine monophosphatase and somatostatin were found predominantly in skin afferents, while calcitonin gene-related peptide and substance P were more common in muscle and joint afferents.

The chemical expression of primary afferents is therefore characteristic of the peripheral target they innervate. This could reflect either a maintained influence of the target on the afferents, or the factors which operate only during development.

Primary afferent neurons are chemically heterogeneous: different subpopulations of these cells, examined typically at the level of the dorsal root ganglion (DRG), contain varied combinations of neuropeptides, enzymes, amino acids, nucleotides, and other antigens (for review see Ref. 15). Some of these chemicals are putative transmitters for the fast and slow central effects of impulses carried by primary afferents,[14,36] and some participate in the efferent axon reflex of unmyelinated afferents.[22]

The chemical heterogeneity could reflect the sensory function of the cell, since some primary afferent neurons relay information from peripheral receptors with a high degree of functional specificity[2] to mechanical, thermal, and/or chemical stimuli.[24] Alternatively, or additionally, the nature of the peripheral target tissue or the tissue along the course of a nerve might determine the cell's chemical specificity. Such influences might operate only during development,[26] but could be maintained in the adult.[28,29]

The aim of this study has been to investigate whether distinct peripheral targets are innervated by primary afferent neurons with different patterns of chemical expression, in the adult rat. The approach we have used is the identification of afferents by retrograde labelling from different peripheral tissues, combined with histochemistry and immunocytochemistry of DRG sections containing labelled cell bodies. The distribution of the enzyme thiamine monophosphatase (TMP), and of the neuropeptides calcitonin gene-related peptide (CGRP), substance P (SP), and somatostatin, have been studied among retrogradely labelled skin, muscle or joint afferent neurons. These four markers are present in subpopulations of DRG cells,[13,16,35,39] and previous studies have begun to correlate afferent target type with presence of these markers.[4,10,11,30,31,33,37] As a refinement of the labelling technique we have, in addition, used the mouse monoclonal antibody RT97 as a marker to distinguish 'large light' and 'small dark' DRG cells.[19]

Abbreviations: CGRP, calcitonin gene-related peptide; DRG, dorsal root ganglion; FB, Fast Blue; FITC, fluorescein isothiocyanate; FRAP, fluoride-resistant acid phosphatase; HS, horse serum; PB, phosphate buffer; PBS, phosphate-buffered saline; RL, Rexed Lamina; SP, substance P; TMP, thiamine monophosphatase; TRG, trigeminal ganglion; TRITC, tetramethyl rhodamine isothiocyanate; TX, Triton X-100.

EXPERIMENTAL PROCEDURES

Animal preparation

Twenty-six male Wistar rats (230–280 g) anaesthetized with sodium pentobarbitone (Sagatal, May & Baker; 50 mg/kg i.p.) were given unilateral or bilateral injections of Fast Blue (FB) (Dr Illing GmbH, West Germany) (5 or 2.5% w/v in ethylene glycol) into either the skin of the inner thigh, the gastrocnemius or tibialis anterior muscles, or the knee joints, using a 30 gauge dental needle. For skin injections the needle was inserted tangentially just below the skin surface, ensuring that the dye was not deposited subcutaneously. In the case of muscle injections, skin and

connective tissue were first opened to fully expose the muscle belly. Silicon oil was drawn into the syringe system before the FB so that any liquid subsequently leaking from the muscle injection site consisted of oil rather than dye. In both skin and muscle, several injections of 1 μl were made within an area of up to 1 cm^2. Injections into joint were made after opening the skin at the knee and inserting the needle such that both the joint capsule wall and cavity received dye. After six (skin), seven (muscle) or four (joint) days survival (at which times an optimum number of DRG cells were found to be retrogradely labelled), animals were re-anaesthetized and perfused via the left ventricle with 0.9% saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4 (PB).

To test for systemic spread of dye, a further six animals were prepared as above with bilateral injections of dye. On one side the nerves supplying the peripherally labelled tissue were ligated and sectioned, immediately after dye injection, while on the other side the nerves were left intact.

Tissue processing

Relevant DRGs (L2-4, skin; L4-6, gastrocnemius or L2-4 tibialis anterior muscle; L4-5, joint), and trigeminal ganglia (to check for systemic spread in joint-FB animals), were dissected, post-fixed for 2 h at 4°C, then transferred to 20% sucrose in PB, overnight at 4°C. Tissue sections (10 μm, cut on a Bright cryostat) were collected either serially or in two or three alternating series on gelatinized slides. Series of sections were then processed differently according to which marker was being studied, as below.

Thiamine monophosphate study

Because the incubation procedure for demonstration of TMP tends to diminish the FB intensity, sections containing FB-labelled nucleated cell profiles were photographed, before being reacted according to the method of Knyihar-Csillik *et al.*[18] to demonstrate TMP activity. Following incubation and visualization of reaction product, sections were rinsed in distilled water and kept moist until the pre-incubation for RT97 immunohistochemistry. Sections were washed in phosphate-buffered saline pH 7.4/0.1% Triton X-100 (PBS/TX), then in PBS/TX/10% horse serum (PBS/TX/HS). All antibodies used in these studies were diluted in PBS/TX/HS.

The monoclonal anti-neurofilament antibody RT97 (gift from Dr J. N. Wood; ascites fluid, 1:2000) was applied overnight, in a humid atmosphere at 4°C. After rinsing in PBS/TX sections were incubated for 1 h at 22°C with biotinylated goat anti-mouse IgG (Amersham, diluted 1:200), rinsed again and similarly incubated with fluorescein isothiocyanate (FITC)-conjugated streptavidin, (Amersham, 1:100). Finally sections were rinsed in PBS and coverslipped with anti-fade mountant (Citifluor; City University).

Neuropeptide studies

Sections were incubated overnight at 4°C with one of the following antisera generated in rabbits: anti-CGRP (Cambridge Research Biochemicals, 1:1000; or gift from Dr P. K. Mulderry, 1:16,000), anti-SP (gift from Prof. P. Keen, 1:2000) or anti-somatostatin (gift from Dr J. Winter, 1:4000). After rinsing sections in PBS/TX, biotinylated donkey anti-rabbit IgG (Amersham, 1:200) was applied for 1 h, 22°C, sections were rinsed again and then incubated with FITC-streptavidin (1:100) for 1 h, 22°C. In some cases, RT97 (1:2000) was applied concurrently with the primary antibody, and then tetramethyl rhodamine isothiocyanate (TRITC)-conjugated anti-mouse IgG applied concurrently with the FITC-streptavidin. In the remaining cases, the overnight incubation for RT97 was performed subsequent to the neuropeptide reactions, and the TRITC anti-mouse IgG applied after that. After a final rinse, sections were coverslipped with anti-fade mountant.

Specificity of antisera

No staining was seen when primary antibodies were omitted. Adsorption of the diluted anti-neuropeptide antisera with 20–50 μg/ml of the respective peptide for 24 h, 4°C, prior to application to the tissue, resulted in no positive staining of sections. Without biochemical characterization of the peptides recognized by these antisera, cross reaction of the anti-neuropeptide antibodies with different, related, peptides cannot, however, be discounted. Therefore the terms 'CGRP-like immunoreactivity', 'SP-like immunoreactivity' and 'somatostatin-like immunoreactivity' are more appropriate to describe positive staining, although 'CGRP', 'SP' and 'somatostatin' will be used for brevity.

Microscopy

Sections were examined on a Zeiss epi-fluorescence or Nikon Fluophot microscope with filters appropriate for FB (excitation wavelength 390–420 nm), FITC (450–490 nm) and TRITC/Texas Red (510–550 nm), and with transmitted light. Counts were made of FB-labelled nucleated cells with and without additional labels, ensuring that where a series of adjacent sections had been used for one study, cells with split nuclei were scored only once.

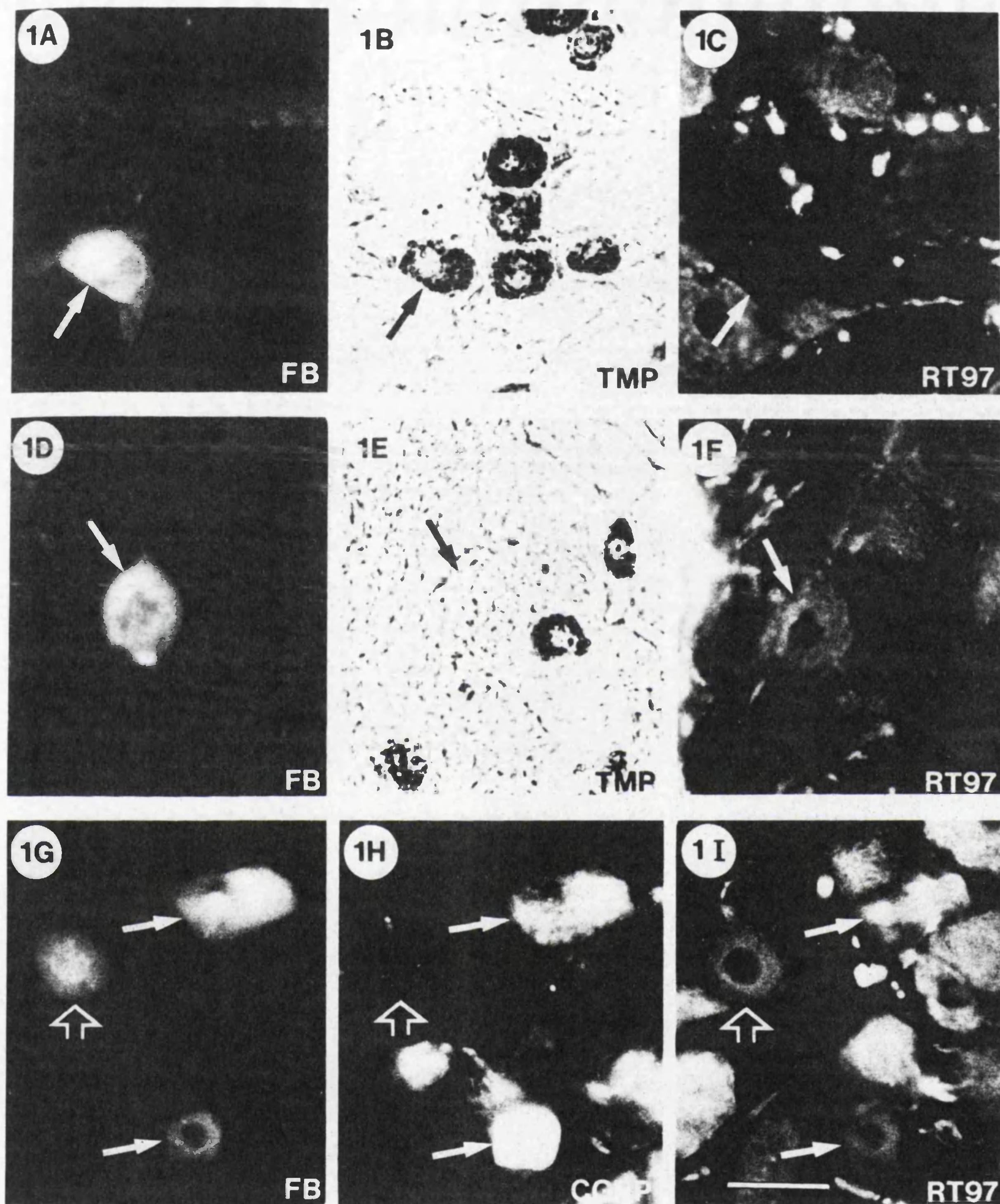
RESULTS

Retrograde identification of afferents

In DRG sections, FB labelling was found in cell bodies of all sizes, distributed throughout the ganglion with no obvious pattern. Label intensity varied independently of cell diameter. Labelled neurons were divided by inspection into three categories according to dye intensity—high, medium and low. High intensity cells had a consistent, brilliant fluorescence, low intensity were coloured just enough to be distinguishable from background, and medium included everything in between. Where the peripheral nerve had been cut, high intensity FB-labelled cells were almost eliminated; at most two such cells remained in a total of three ganglia, compared to 50 or more on the uncut side. These labelled cells may be the result of spread of the dye to the cut nerves, or its uptake by nerve fibres in tissue exposed by the surgery. The number of such cells, however, was sufficiently low—usually none—that they are unlikely to introduce errors.

Medium intensity cells were reduced in number by 50% or more (e.g. from 199 down to 95 FB cells across three ganglia) by peripheral nerve section, while low intensity labelling was apparent in the same numbers of cells as from an uncut side (up to 1000 per ganglion). Low intensity labelling was not always present in tissue from skin- or joint-injected animals, but was almost always seen in muscle-labelled animals. It seems likely that low intensity labelling is the result of systemic spread of FB, and counts of these cells were omitted from the analysis. Medium intensity cells were considered to represent some specific and some non-specifically labelled cells and so data for these is not presented. (See Figs 1, 2 and 3A, D and G for examples of FB intensities).

As an additional test for specificity of labelling in joint-FB animals, the anatomically remote trigeminal ganglia (TRGs) from these rats were examined for



Figs 1–3. Retrogradely-identified DRG cells counterstained for enzyme activity or neuropeptide immunofluorescence, and for neurofilament immunofluorescence. Each figure is divided into rows of three photomicrographs, each row representing the same field of a DRG section from one study, viewed in three ways: (i) with filter appropriate for FB fluorescence to show cell bodies retrogradely labelled after an injection of FB into a peripheral tissue; (ii) with transmitted light (in TMP study) or with filter appropriate for FITC fluorescence (in peptide studies) to show counterstaining by TMP enzyme activity or peptide immunoreactivity; (iii) with filter for FITC fluorescence (in TMP study) or for TRITC/Texas Red fluorescence (in peptide studies) to show labelling by RT97 antibody. Note that due to differences in contrast, it may be difficult to compare intensities of the same stain between some photomicrographs.

Fig. 1. Top row. Skin FB, TMP study. (A) One high intensity FB cell; (B) cell is positive for TMP; (C) cell is RT97-negative. Middle row. Skin FB, TMP study. (D) Medium intensity FB cell; (E) cell is negative for TMP; (F) cell is RT97-positive. Bottom row. Joint FB, CGRP study. (G) Three medium intensity FB cells; (H) two are CGRP-positive (closed arrows), one is CGRP-negative (open arrow); (I) all three are RT97-positive. Scale bar = 40 μm.

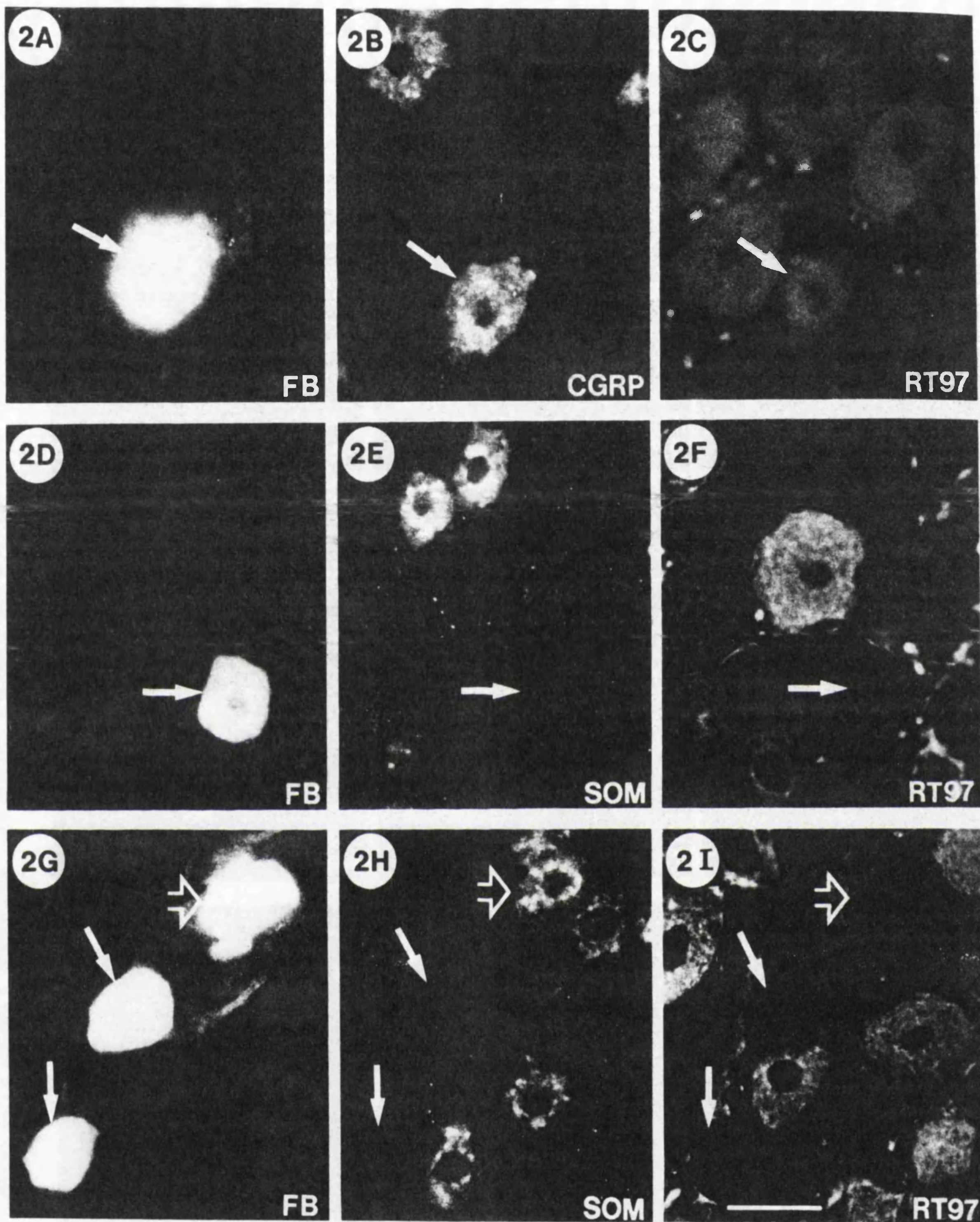


Fig. 2. Top row. Muscle FB, CGRP study. (A) One high intensity FB cell; (B) cell is positive for CGRP; (C) cell is positive for RT97. Middle row. Muscle FB, somatostatin (SOM) study. (D) One medium intensity FB cell; (E) cell is negative for somatostatin; (F) cell is negative for RT97. Bottom row. Skin FB, somatostatin (SOM) study. (G) Two high intensity and one medium intensity FB cells; (H) one high intensity cell is somatostatin-positive (open arrow), the remaining cells are somatostatin-negative (closed arrows); (I) all three are RT97-negative. Scale bar = 40 μm.

presence of the fluorescent tracer, as an indicator of tracer spread to the bloodstream from this particular peripheral tissue. DRGs were used for the study only if there was no FB, or only very few faintly labelled FB cells, in cryostat sections of the TRGs of the same animal, when examined at the stage of perfusion and dissection.

In one skin-FB and one muscle-FB rat the tissue at the injection site was dissected and sectioned for examination. In each case there was a restricted area of about 1 mm of very bright FB fluorescence associated with each lesion caused by the injection needle.

Injection of FB into the leg skin almost always resulted in many more retrogradely labelled DRG

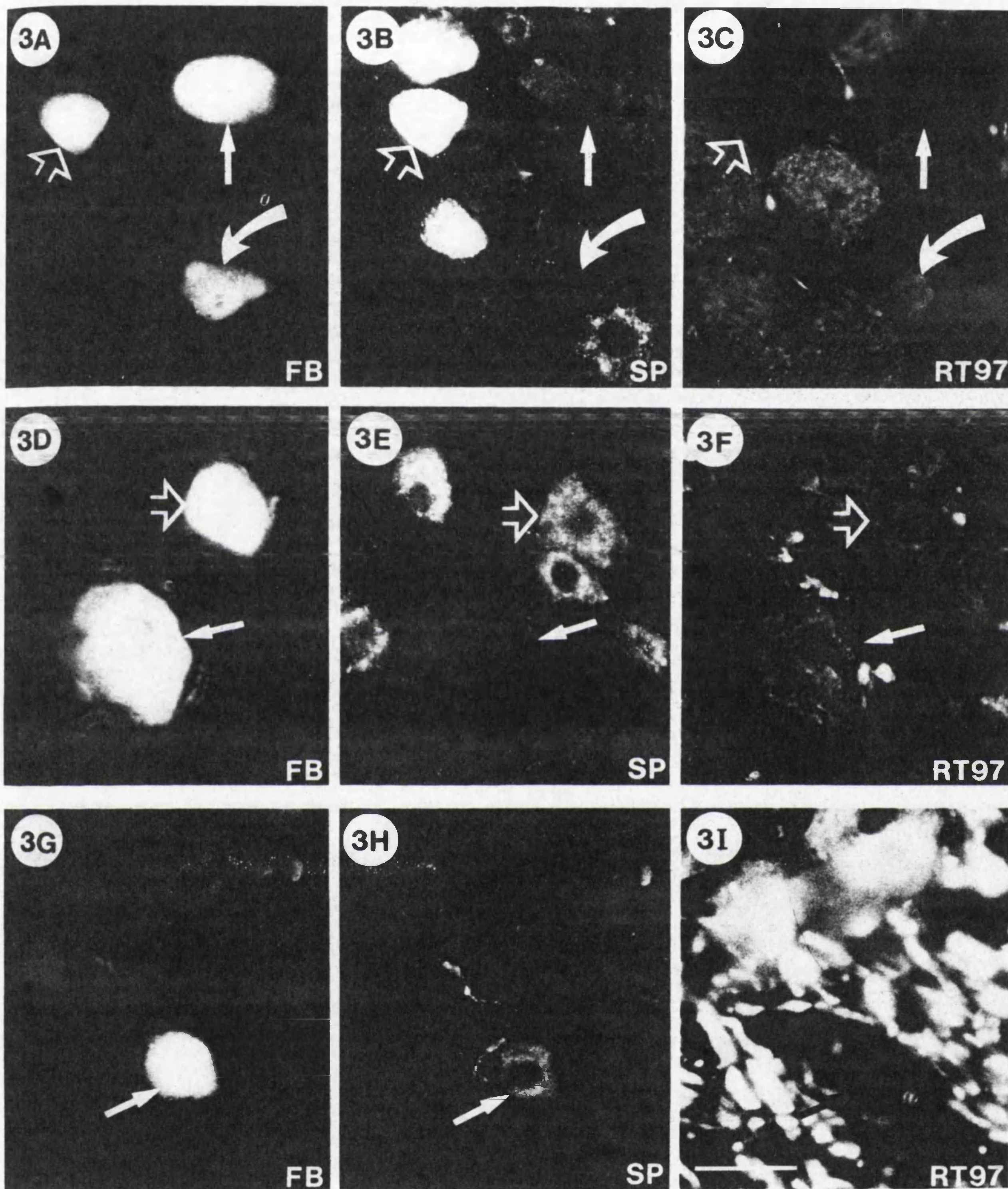


Fig. 3. Top row. Skin FB, SP study. (A) Three medium intensity FB cells; (B) one is SP-positive (open arrow), two are SP-negative (closed arrows); (C) one SP-negative cell is RT97-positive (curved arrow), the remaining cells are RT97-negative. Middle row. Muscle FB, SP study. (D) One high intensity and one medium intensity FB cell; (E) The high intensity cell is SP-positive (open arrow), the medium intensity cell is SP-negative (closed arrow); (F) both are RT97-positive. Bottom row. Joint FB, SP study. (G) One high intensity FB cell; (H) cell is SP-positive; (I) cell is RT97-negative. Scale bar = 40 μm.

cells than a similar injection into muscle or joint, and the absolute number of retrogradely labelled cells from a given target was different for each animal, reflecting different densities of afferent terminations, and differences in size of the injection site. In spite of this, where results from each rat were counted separately, as in the TMP and CGRP studies, the same trends with respect to RT97, TMP and CGRP counterstaining were apparent within the group of labelled afferents from each different animal, so that skin afferents were consistently different from muscle afferents (see Tables 1 and 2).

Table 1. Thiamine monophosphatase study

Target	n	Total FB	RT97 negative	TMP positive
Skin	1	110	77	54
	1	74	53	42
	Total	184	130	96
Muscle	1	32	15	0
	1	38	9	2
	1	23	9	3
	1	50	29	3
	Total	143	62	8
Joint	1*	13	—	0
	1*	38	—	0
	2*	131	—	0
	2	68	—	0
	Total	250	—	0

Numbers of: retrogradely labelled DRG cells, those that were RT97 negative (small dark) and those which also contained TMP.
*Not photographed before TMP reaction.
—Not determined.

Patterns of RT97 staining

Across the four studies, the proportions of FB-labelled cells which were RT97 negative (small dark) were relatively consistent within one target tissue type, but different between targets. The proportions of RT97 negative cells for each tissue were: skin, 61–71%; muscle, 29–43%; joint, 15–23%.

Markers in target-identified cells

The data on the presence of TMP, CGRP, SP and somatostatin in small dark DRG cells (RT97 negative; unmyelinated axons) retrogradely labelled from skin, muscle and joint are summarized in Table 3. The equivalent data for large light DRG cells (RT97 positive; myelinated axons), which contain only CGRP and SP, not TMP or somatostatin are presented in Table 4.

Among small dark cells, TMP and somatostatin are largely markers for skin afferents, being absent from the limited sample of joint afferents and present in only a few muscle afferents. Calcitonin GRP and SP are found predominantly in muscle and joint afferents, but are by no means absent from skin DRG cells. In particular, CGRP is present in all muscle small dark DRG cells.

SP, as well as CGRP, is present in large light DRG cells from all three target tissues. Among large light neurons, CGRP was found in a greater proportion of cells than SP in all cases, and joint afferents contained both peptides in higher proportion than skin and muscle afferents.

The raw data for each study, i.e. the numbers of afferents found in the ganglia to contain high intensity FB after injection of the tracer into skin, muscle or joint, how many were negative or positive for RT97, and whether they contained TMP, CGRP, SP or somatostatin, respectively are presented in Tables 1, 2, 5 and 6. A total of 26 rats were used. Where DRGs were processed separately for each rat, numbers of cells counted each case are given in the tables (i.e. $n = 1$), but otherwise the totals for a group of rats whose DRGs were pooled for processing are presented (i.e. $n > 1$). Examples of retrogradely labelled skin, muscle and joint afferents, counter-stained for TMP, CGRP, SP, somatostatin and RT97 are shown in Figs 1–3.

Observations on the staining patterns of the markers

All markers stained cells distributed 'randomly' across sections and throughout the DRGs.

TMP study (see Fig. 1B and E): Two grades of TMP reaction product could be seen in the DRG

Table 2. Calcitonin gene-related peptide study

Target	n	Total FB	Total RT97 negative	RT97 negative CGRP positive	RT97 positive CGRP positive
Skin	1	46	21	11	14
	1	15	11	5	1
	1	55	39	19	7
	Total	116	71	35	22
Muscle	1	23	9	9	5
	1	12	2	2	9
	1	13	1	1	6
	1	15	6	6	4
	Total	63	18	18	24
Joint	3	62	9	7	37

Numbers of: retrogradely labelled DRG cells, those that were RT97 negative (small dark), and the occurrence of CGRP in RT97 positive and RT97 negative FB cells.

Table 3. Target-identified small dark cells (RT97 negative)

Target	TMP positive	CGRP positive	SP positive	Somatostatin positive
Skin	96/130 = 74%	35/71 = 50%	55/150 = 37%	34/173 = 20%
Muscle	8/62 = 13%	18/18 = 100%	50/64 = 78%	1/94 = 1%
Joint	0 = 0%	7/9 = 78%	12/18 = 66%	0/9 = 0%

Distribution of markers, as a percentage of the RT97 negative, high intensity FB retrogradely labelled cells.

Table 4. Target-identified large light cells (RT97 positive)

Target	CGRP positive	SP positive
Skin	22/45 = 49%	9/76 = 12%
Muscle	24/45 = 53%	11/110 = 10%
Joint	37/53 = 70%	10/59 = 17%

Distribution of CGRP and SP, as a percentage of the RT97 positive, high intensity FB retrogradely labelled cells. No TMP or somatostatin is found in RT97 positive cells.

Table 5. Substance P study

Target	n	Total FB	Total RT97 negative	RT97 negative SP positive	RT97 positive SP positive
Skin	2	226	150	55	9
Muscle	2	174	64	50	11
Joint	3	77	18	12	10
	3	35	—		(13)

Numbers of: retrogradely labelled DRG cells, those that were RT97 negative, and the occurrence of SP in both RT97 positive and RT97 negative FB cells.
—Not determined.

cells, strong in the majority of cases and weak in a few cells. This pattern has been noted before by other groups.[6] Comparing the pre-TMP photographs with the reacted sections indicated that in every high intensity FB cell, enough of the fluorescent dye remained in the cells to allow them to be quickly identified, but FB intensity was diminished. There were no cells positive for both TMP and RT97. About half of the small dark cells were TMP positive.

CGRP study (see Figs 1H and 2B): positive staining took several forms: large granules dotted across the cytoplasm, a bright, even staining of all the cytoplasm, or a perinuclear ring of granules with faint, even stain across all the cytoplasm. These patterns were independent of the presence of FB in a cell, but the cells with sparse large granules of CGRP immunoreactivity were frequently also RT97 positive. Both RT97 negative and positive cells were seen to contain CGRP. About half of all DRG cells were CGRP positive, and positive-staining axons were often visible.

SP study (see Fig. 3B, E and H): the intensity of immunoreactive stain for SP varied from very bright to faint, independently of other markers. Frequently SP-positive axons were clearly visible. SP immunoreactivity coexisted in some cells with RT97 immunoreactivity, but to a lesser extent than the overlap of CGRP and RT97. By inspection, 15–20% of all DRG cells were SP positive.

Somatostatin study (see Fig. 2E and H): positive cells were characterized by a perinuclear ring of very bright immunoreactive granules. None were RT97 immunoreactive. About 5% of all cells were somatostatin positive.

DISCUSSION

We have used retrograde labelling from specific target tissues, to identify afferents which are related in their site of innervation. Afferents are not subjected to axotomy, reasonable numbers of cells can be identified, and their chemical nature explored. This offers advantages over other techniques, such as application of tracer to the cut end of individual cutaneous and muscle nerves[4,31] where axotomy may alter the chemical expression of DRG cells, or direct dye injection into single electrophysiologically identified DRG cells[3,20,27] which yields only a few cells per animal. There are, however, two main disadvantages to the technique used here. Firstly, uptake of tracer may differ between tissues, with different axons transporting the dye to varied extents. Secondly, tissues cannot be considered homogeneous, and afferents innervating blood vessels and connective tissue are likely to be present in all the peripheral tissues investigated, contributing an unspecified component to the results.

Target patterns in RT97 labelling

Distinguished on the basis of their cytoplasmic appearance under light and electron microscopy, DRG cells appear either 'small dark', with an evenly stained dark cytoplasm, or 'large light', with a pale, uneven stain and clear areas of neurofilaments.[7] RT97 reacts with the phosphorylated 155,000 and 200,000 mol. wt subunits of neurofilament protein, and has been shown to label specifically the large light cell population.[19] Despite their name, large light cells display a wide size range which overlaps with the small dark range, and therefore such a classification cannot be made directly on the basis of size measurement. Classification of our retrogradely labelled cells as large light or small dark allows us to make further deductions about the distribution of chemical phenotypes among target-identified DRG cells, in terms of their function. This is because, in the rat, almost all RT97 positive DRG cells have myelinated axons, while all RT97 negative DRG cells have unmyelinated axons.[12] The different proportions of large light and small dark DRG cells we have labelled from the different targets could indicate different receptor populations in the peripheral tissues,[24] or, as mentioned above, they could reflect a variability in uptake by afferents in the different tissues.

Target patterns of markers

TMP, CGRP, SP and somatostatin all display a distribution among subpopulations of primary afferents which relates to the peripheral target of the neurons. Because none of these markers is unique to any target, however, their presence in a DRG cell

Table 6. Somatostatin study

Target	n	Total FB	RT97 negative	Somatostatin positive
Skin	2	267	173	34
Muscle	2	211	94	1
Joint	3	48	9	0
	3	11	—	0
	2	116	—	0
	Total	175	—	0

Numbers of: retrogradely labelled DRG cells, those that were RT97 negative (small dark), and those that also contained somatostatin.
—Not determined.

cannot be used to predict the peripheral specification of that cell. Somatostatin comes very near to being a 'skin marker', and we never found TMP or somatostatin in joint afferents. Joint afferents display a spectrum of markers which resembles muscle afferents more closely than skin afferents. In fact we may have labelled a number of the same types of afferents with the injections into either muscle or joint, e.g. in tendons, connective tissue, and blood vessels.

Medium intensity FB cells showed the same, but less marked, trends in distribution of markers as the high intensity FB cells, therefore it seems likely that they comprise a population of specifically labelled afferents diluted by a population of non-specifically labelled cells.

The results of this study are similar to the findings of Molander *et al.*,[31] who showed that FRAP (fluoride-resistant acid phosphatase, an isoenzyme of TMP), CGRP, SP and somatostatin are present in primary afferents identified by retrograde labelling from whole transected cutaneous and muscle nerves. The proportions of cells containing each marker are generally higher in this study than in that of Molander *et al.*—this could be due to differences in the labelling technique and the method of sampling labelled cells. In the present study the tracer has been injected directly into the target tissue rather than applied to a cut nerve. This should have avoided injury to the axons, which is known to alter the expression of peptides[1] and TMP.[17]

Thiamine monophosphatase study

TMP, which is demonstrated using a more selective substrate in the same method as that used for FRAP,[15,18] is seen in 50% of DRG neurons, all of which are also small dark.[6,18] We find that TMP is preferentially located in skin afferents rather than muscle or joint afferents, and this result agrees with what is known about the central termination pattern of skin and muscle small-diameter afferent fibres in the spinal cord, i.e. skin C-afferent terminals are found in Rexed Lamina (RL) II,[38] which is also the region of dense TMP reaction product in the dorsal horn,[18] whereas no muscle afferents have been demonstrated to terminate in this region (see Ref. 31 for discussion). Our results are also consistent with those of McMahon *et al.*,[30] who showed sparse accumulation of FRAP in a ligated muscle nerve, and much greater amounts of the enzyme in a cutaneous nerve (although their result may reflect differences between the two nerves in terms of transport from the cell body—see below in SP study).

Calcitonin gene-related peptide study

CGRP is present in up to 50% of rat DRG neurons,[6,16,31] which makes it the most frequently occurring sensory neuropeptide. Other studies have demonstrated CGRP in afferents innervating the viscera,[11,37] and in trigeminal ganglion (TRG) cells projecting to the cerebral vasculature.[32] We have confirmed that CGRP is present in large light sensory neurons, as identified by immunoreactivity to RT97, as suggested by Lawson *et al.*[20] Half of the large light afferents from both muscle and skin contained CGRP, as did more than two-thirds of joint afferents. In the small dark DRG cells, while half of those labelled from skin contained CGRP, three quarters of joint small dark cells and all muscle small dark afferents contained this peptide.

Substance P study

SP is known to coexist with CGRP in up to 20% of small and medium sized DRG neurons.[21] We have confirmed that SP is another neuropeptide found in large light DRG cells, in agreement with the work of McCarthy and Lawson[27] on electrophysiologically characterized DRG cells. In all three target tissues, however, SP was not as frequently encountered in large light cells as CGRP. Among small dark cells, we were surprised that three-quarters of muscle afferents, but only one-third of skin afferents, contained SP, in view of the widely-held belief that SP is involved in the C-fibre-mediated phenomena of neurogenic inflammation and plasma extravasation,[22] the latter being a reaction which is readily demonstrable in skin but not so easily in muscle.[30] This apparent anomaly may be another function of differences between afferents with respect to peripheral transport from the cell body, because the work of McMahon and Gibson[28] suggests that very little SP is normally transported in peripheral axons of a muscle nerve, while plenty of the peptide can be visualized after ligation of a cutaneous nerve. The inflammatory reactions in joints may be related to the SP content of the joint afferents.[23]

Somatostatin study

Somatostatin has been shown to be present in 3.5% of TRG afferents from facial skin but not in those from facial muscles.[33] In dorsal root ganglia, somatostatin is found in $<10\%$ of cells, of small and intermediate diameter.[13] In the present study, somatostatin was encountered most frequently in skin afferents, in agreement with the finding by Ositelu *et al.*,[33] although we find a rather higher proportion of skin sensory cells containing the neuropeptide than they did.

The differences in the distribution of somatostatin and SP are in agreement with work by Hokfelt *et al.*,[13] who found the two peptides in separate populations of DRG cells, and also with studies of immunoreactive terminals in the dorsal horn of the spinal cord, where SP is seen in RLI and outer RLII, while somatostatin is more predominant in RLII (for instance, see Ref. 25).

Why is chemical expression related to the target tissue?

The requirements for afferent function vary between different peripheral tissues, due to the range of different stimuli to which these tissues must respond.

By maintaining a range of transmitter types, alone or in combination in afferent cells, the peripheral tissue has the potential to send to the CNS different information signals about the 'external milieu', some of which will be more relevant to one tissue type than another. The four 'markers' of DRG cells whose distribution we have investigated here, are putative neurotransmitters, neuromodulators, or other types of signal-related molecules,[40] and in the case of SP, especially, their main role may be in the periphery,[22] and for such reasons might be expected to show a tissue specificity.

Another mechanism by which target-related chemical expression is probably regulated, depends on the trophic influence exerted by the target tissue on the neurons. Such an influence may operate as the neurons are developing; for instance expression of SP and another neuropeptide, vasoactive intestinal polypeptide, in transplanted neural crest cells in quail-chick chimaeras, depends on the environment into which the neural crest cells migrate, and is not predetermined by their origin in the neural axis or their fate in normal development.[9] Recently, Phillippe *et al.*[34] demonstrated that in the chick embryo, sensory neurons are induced to express calbindin (a calcium binding protein) only when they are able to interact with their peripheral target tissue, skeletal muscle. A similar dependency can be shown to occur *in vitro*.[5] The trophic theory may also be relevant beyond development and the initiation of expression of chemical phenotype: an interaction between neuron and target may be required continously throughout life.[8,28,29]

CONCLUSION

We have been able to show that there is a target-related difference in chemical expression by intact primary afferent neurons *in vivo*.

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REFERENCES

1. Barbut D., Polak J. M. and Wall P. D. (1981) Substance P in the spinal cord dorsal horn decreases following peripheral nerve injury. *Brain Res.* **205,** 289–298.
2. Burgess P. R. and Perl E. R. (1973) Cutaneous mechanoreceptors and nociceptors. In *Handbook of Sensory Physiology* (ed. Iggo A.), Vol. II, pp. 29–78. Springer, Berlin.
3. Cameron A. A., Leah J. D. and Snow P. J. (1986) The electrophysiological and morphological characteristics of feline dorsal root ganglion cells. *Brain Res.* **362,** 1–6.
4. Dalsgaard C.-J., Ygge J., Vincent S. R., Ohrling M., Dockray G. J. and Elde R. (1984) Peripheral projections and neuropeptide coexistence in a subpopulation of fluoride-resistance acid phosphatase reactive spinal primary sensory neurons. *Neurosci. Lett.* **51,** 139–144.
5. Davis J. P. and Epstein M. L. (1987) Trigeminal ganglion cells cocultured with gut express vasoactive intestinal peptide. *Dev. Biol.* **124,** 512–522.
6. Dodd J., Jahr E. and Jessell T. M. (1984) Neurotransmitters and neuronal markers at sensory synapses in the dorsal horn. In *Advances in Pain Research and Therapy* (eds Kruger L. and Liebeskind J. C.), Vol. 6, pp. 105–121. Raven Press, New York.
7. Duce I. R. and Keen P. (1977) An ultrastructural classification of the neuronal cell bodies of the rat dorsal root ganglion using zinc iodide-osmium impregnation. *Cell Tiss. Res.* **185,** 263–277.
8. Fitzgerald M., Wall P. D., Goedert M. and Emson P. C. (1985) Nerve growth factor counteracts the neurophysiological and neurochemical effects of chronic sciatic nerve section. *Brain Res.* **332,** 131–141.
9. Fontaine-Perus J. C., Chanconie M. and Le Douarin N. M. (1982) Differentiation of peptidergic neurones in quail-chick chimaeric embryos. *Cell Different.* **11,** 183–193.
10. Gibbins I. L., Furness J. B. and Costa M. (1987) Pathway-specific patterns of the co-existence of substance P, calcitonin gene-related peptide, cholecystokinin and dynorphin in neurons of the dorsal root ganglia of the guinea-pig. *Cell Tiss. Res.* **248,** 417–437.
11. Green T. and Dockray G. J. (1987) Calcitonin gene-related peptide and substance P in afferents to the upper gastrointestinal tract in the rat. *Neurosci. Lett.* **76,** 151–156.
12. Harper A. A. and Lawson S. N. (1985) Conduction velocity is related to morphological cell type in rat dorsal root ganglion neurons. *J. Physiol. Lond.* **359,** 31–46.
13. Hokfelt T., Elde R., Johansson O., Luft R., Nilsson G. and Arimura A. (1976) Immunohistochemical evidence for separate populations of somatostatin and substance P-containing primary afferent neurons in the rat. *Neuroscience* **1,** 131–136.
14. Iversen L. L. and Goodman E. (1986) *Fast and Slow Chemical Signalling in the Nervous System.* Oxford University Press, Oxford.
15. Jessell T. M. and Dodd J. (1986) Neurotransmitters and differentiation antigens in subsets of sensory neurons projecting to the spinal dorsal horn. In *Neuropeptides in Neurologic and Psychiatric Disease* (eds Martin J. B. and Barchas J. D.), pp. 111–133. Raven Press, New York.
16. Ju G., Hokfelt T., Brodin E., Fahrenkrug J., Fischer J. A., Frey P., Elde R. P. and Brown J. C. (1987) Primary sensory neurons of the rat showing calcitonin gene-related peptide immunoreactivity and their relation to substance P-, somatostatin-, galanin-, vasoactive intestinal polypeptide- and cholecystokinin-immunoreactive ganglion cells. *Cell Tiss. Res.* **247,** 417–431.
17. Knyihar E. and Csillik B. (1976) Effect of peripheral axotomy on the fine structure and histochemistry of the Rolando substance. *Exp. Brain Res.* **26,** 73–87.
18. Knyihar-Csillik E., Bezzegh A., Boti S. and Csillik B. (1986) Thiamine monophosphatase: a genuine marker for transganglionic regulation of primary sensory neurons. *J. Histochem. Cytochem.* **34,** 363–371.

19. Lawson S. N., Harper A. A., Harper E. I., Garson J. A. and Anderton B. H. (1984) A monoclonal antibody against neurofilament protein specifically labels a subpopulation of rat sensory neurones. *J. comp. Neurol.* **228,** 263–272.
20. Lawson S. N., Waddell P. J. and McCarthy P. W. (1987) A comparison of the electrophysiological and immunocytochemical properties of rat dorsal root ganglion neurones with A and C fibres. In *The Role of Fine Afferent Fibres in Somatovisceral Sensation* (eds Schmidt R. F., Schiable H.-G. and Vahle-Hinz C.), pp. 195–203. VCH, West Germany.
21. Lee Y., Takami K., Kawai Y., Girgis S., Hillyard C. J., MacIntyre I., Emson P. C. and Tohyama M. (1985) Distribution of calcitonin gene-related peptide in the rat peripheral nervous system with reference to its coexistence with substance P. *Neuroscience* **15,** 1227–1237.
22. Lembeck F. and Gamse R. (1982) Substance P in peripheral sensory processes. In *Substance P in the Nervous System,* Ciba Foundation Symposium 91, pp. 35–54. Pitman, London.
23. Levine J. D., Clark R., Devor M., Helms C., Moskowitz M. A. and Basbaum A. I. (1984) Intraneuronal substance P contributes to the severity of experimental arthritis. *Science* **226,** 547–549.
24. Lynn B. and Carpenter S. E. (1982) Primary afferent units from the hairy skin of the rat hindlimb. *Brain Res.* **238,** 29–43.
25. Lynn B. and Hunt S. P. (1984) Afferent C-fibres: physiological and biochemical correlations. *Trends Neurosci.* **7,** 186–188.
26. Marusich M. F., Pourmehr K. and Weston J. A. (1986) The development of an identified subpopulation of avian sensory neurons is regulated by interaction with the periphery. *Dev. Biol.* **118,** 505–510.
27. McCarthy P. W. and Lawson S. N. (1989) Cell type and conduction velocity of rat primary sensory neurons with substance P-like immunoreactivity. *Neuroscience* **28,** 745–753.
28. McMahon S. B. and Gibson S. J. (1987) Peptide expression is altered when afferent nerves reinnervate inappropriate tissue. *Neurosci. Lett.* **73,** 9–15.
29. McMahon S. B. and Moore C. E. G. (1988) Plasticity of primary afferent acid phosphatase expression following rerouting of afferents from muscles to skin in the adult rat. *J. comp. Neurol.* **274,** 1–8.
30. McMahon S. B., Sykova E., Wall P. D., Woolf C. J. and Gibson S. J. (1984) Neurogenic extravasation and substance P levels are low in muscle as compared to skin in the rat hindlimb. *Neurosci. Lett.* **52,** 235–240.
31. Molander C., Ygge J. and Dalsgaard C.-J. (1987) Substance P-, somatostatin- and calcitonin gene-related peptide-like immunoreactivity and fluoride resistant acid phosphatase-activity in relation to retrogradely labelled cutaneous, muscular and visceral primary sensory neurons. *Neurosci. Lett.* **74,** 37–42.
32. O'Connor T. P. and van der Kooy D. (1988) Enrichment of a vasoactive neuropeptide (calcitonin gene-related peptide) in the trigeminal sensory projection to the intracranial arteries. *J. Neurosci.* **8,** 2468–2476.
33. Ositelu D. O., Morris R. and Vaillant C. (1987) Innervation of facial skin but not masticatory muscles or the tongue by trigeminal primary afferents containing somatostatin in the rat. *Neurosci. Lett.* **78,** 271–276.
34. Philippe E., Garosi M. and Droz B. (1988) Influence of peripheral and central targets on subpopulations of sensory neurons expressing calbindin immunoreactivity in the dorsal root ganglion of the chick embryo. *Neuroscience* **26,** 225–232.
35. Price J. (1985) An immunohistochemical and quantitative examination of dorsal root ganglion neuronal subpopulations. *J. Neurosci.* **5,** 2051–2059.
36. Salt T. E. and Hill R. G. (1983) Neurotransmitter candidates of somatosensory primary afferent fibres. *Neuroscience* **10,** 1083–1103.
37. Su H. C., Bishop A. E., Power R. F., Hamada Y. and Polak J. M. (1987) Dual intrinsic and extrinsic origins of CGRP- and NPY-immunoreactive nerves of rat gut and pancreas. *J. Neurosci.* **7,** 2674–2687.
38. Sugiura Y., Lee C. L. and Perl E. R. (1986) Central projections of identified unmyelinated (C) afferent fibers innervating mammalian skin. *Science* **234,** 358–361.
39. Tuchscherer M. M. and Seybold V. S. (1985) Immunohistochemical studies of substance P, cholecystokinin-octapeptide and somatostatin in dorsal root ganglia of the rat. *Neuroscience* **14,** 593–605.
40. Wall P. D. and Fitzgerald M. (1982) If substance P fails to fulfill the criteria as a neurotransmitter in somatosensory afferents, what might be its function? In *Substance P in the Nervous System,* Ciba Foundation Symposium 91, pp. 249–266, Pitman, London.

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