THE INVOLVEMENT OF NEUROPEPTIDES IN
NERVE REGENERATION

by

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ABSTRACT

The studies presented in this thesis are concerned with the role of peptides in regeneration of the peripheral nervous system of the adult rat, and the presence of macrophages expressing different antigenic markers in the peripheral nervous system.

The major section examines the time course of accumulation of two neuropeptides, calcitonin gene-related peptide (CGRP) and vasoactive intestinal peptide (VIP), as well as the lymphokine, gamma interferon (IFNγ), following lesion to the sciatic nerve. Using radioimmunoassay, elevated levels of CGRP and VIP-like immunoreactivity expressed in the sciatic nerve after injury are quantified. The time course for IFNγ accumulation is documented by immunohistochemistry.

Data are also presented demonstrating that addition of synthetic human CGRP causes an accumulation of cyclic adenosine monophosphate (cyclic AMP) within macrophages isolated from the sciatic nerve, peritoneum and spleen. However, additional experiments demonstrate that the ability of CGRP to alter such cyclic AMP levels is influenced by the state of macrophage activation. This raises the possibility that CGRP is important for regulating the response of macrophages which infiltrate injured sciatic nerve.
In the second section of the thesis, the distribution of macrophage subpopulations/phenotypes in normal versus lesioned sciatic nerve are described. A series of monoclonal antibodies to macrophage markers including, ED1,2,3, Leukocyte Common Antigen (LCA) and OX42 demonstrate how numbers and distribution of macrophage phenotypes alters in response to peripheral nerve injury.
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LISTS OF ABBREVIATIONS

ACh  acetylcholine
APC  antigen presenting cell
bFGF basic fibroblast growth factor
BrdU bromodeoxyuridine
cAMP 3',5'-cyclic adenosine monophosphate
CGRP calcitonin gene-related peptide
CNS central nervous system
DAB 3,3'-diaminobenzidine tetrahydrochloride
DRG dorsal-root ganglion
FCS foetal calf serum
GFAP glial fibrillary acidic protein
GGF glial growth factor
HCl hydrochloric acid
IBMX isobutyl-methylxanthine
I-CAM intercellular adhesion molecule
IFNγ gamma interferon
IHC immunohistochemistry
IL-1 interleukin-1
IL-4 interleukin-4
KCl potassium chloride
LCA leucocyte common antigen
LFA-1 leucocyte function-associated antigen-1
LPS lipopolysaccharide
MAC-1 monocyte/myeloid adhesion complex
MBP myelin basic protein
mcAb monoclonal antibody
MHC major histocompatibility antigens
NaCl sodium chloride
N-CAM neural cell adhesion molecule
Ng-CAM neural-glia cell adhesion molecule
NF-H high molecular weight neurofilament
NF-L low molecular weight neurofilament
NF-M middle size molecular weight neurofilament
NGF nerve growth factor
NKA neurokinin A
NKB neurokinin B
NP-Y neuropeptide Y
PALS periarteriolar lymphatic sheath
PBS phosphate buffered saline
PDGF platelet-derived growth factor
PNS peripheral nervous system
RIA radioimmunoassay
RNA messenger ribonucleic acid
SCa slow component a
SCb slow component b
s.e.m. standard error of the mean
SK substance K
SP substance P
TNF tumour necrosis factor
VIP vasoactive intestinal peptide
CHAPTER 1

INTRODUCTION
INTRODUCTION

The ability of peripheral nervous system (PNS) neurons in mammals to regenerate following injury, while those of the central nervous system in mammals do not, is a fundamental difference between the two systems which has an important bearing on clinical medicine. Although, the proximal stumps of axons transected within the central nervous system begin to regenerate, this growth ceases after approximately 2 weeks (Cajal, 1928). By contrast, peripheral nerves are able to continue to regenerate beyond 2 weeks and may successfully reinnervate their targets.

Early studies by Augustus Volney Waller and Santiago Ramón Y Cajal pioneered research in peripheral nerve degeneration and regeneration. Waller, using the frog glossopharyngeal nerve, showed that damaged nerve fibres degenerate from the site of transection all the way down to the nerve's target (reviewed by Cajal, 1928). Such degeneration, distal to the site of injury is termed Wallerian degeneration. Waller also observed that the proximal part of the damaged nerve fibre, connected to the neuronal cell body retains its normal form except for a small portion of nerve adjacent to the injury site which undergoes retrograde degeneration. Further to Waller's description of peripheral nerve degeneration, Cajal demonstrated that nerve regeneration occurs by outgrowth from the proximal stump and not by autoregeneration of the
degenerated distal nerve (Cajal, 1928).

With the development of immunohistochemistry and the powerful tools of molecular genetics and molecular biology, more recent studies have re-explored in detail, the events of peripheral nerve regeneration. In this thesis, immunochemical techniques were used to investigate further the cellular and molecular changes that take place during peripheral nerve injury and repair.

The peripheral nerve.

The peripheral nervous system (PNS) consists of all nervous tissue outside the bony structures of the vertebral column and skull. There are two major divisions; the voluntary and the involuntary (or autonomic) nervous systems. The autonomic nervous system, composed of both sympathetic and parasympathetic nerves, is involved in activities such as blood pressure and heartbeat regulation, gastrointestinal motility and exocrine gland secretion. The major functions of the voluntary nervous system are to carry sensory information into the central nervous system (CNS), by sensory axons and to carry motor commands out to muscle or autonomic ganglia, via motor axons. The sensory and motor axons can be found together in mixed peripheral nerves or they can be found in separate sensory or motor nerves respectively; autonomic axons are also found together with sensory and/or motor axons. The organisation
of all but the smallest peripheral nerves consist of axon bundles or fasciculi, involving three connective tissue sheaths. The entire nerve is surrounded by the epineurium. Within the epineurium each bundle of fibres is enclosed by a perineurium sheath, and individual axons with their Schwann cells are embedded in connective tissue termed the endoneurium (figure 1.1).

The neuron.

The functional unit of the nervous system is the neuron, consisting of a cell body, with or without dendrites, and the axon. In the PNS the cell body (perikaryon) is found within the dorsal root ganglion (DRG) for sensory neurons, in the ventral horn for motoneurons innervating muscle. Short branching dendrites associated with the cell body form a major part of the receptive area of the cells. The axon varies greatly in length from one type of neuron to another and conducts impulses away from the cell body.

Glial cells of the peripheral nervous system.

Surrounding the axons are the supporting glial cells. These neural crest-derived glial cells are known as Schwann cells, of which there are two kinds: myelinating and nonmyelinating Schwann cells. In nonmyelinated nerves, one Schwann cell envelopes several (up to 15) thin axons (Terzis and Smith, 1990). In contrast, myelinating Schwann cells envelope only one axon, laying down many layers of specialised cell membrane by spiralling around the axon.
**Figure 1.1:** General structure of a peripheral nerve trunk. Endoneurial connective tissue surrounds each axon, and bundles of axons or fascicles surrounded by multinucleated perineurium are embedded in a loose connective tissue, the epineurium. The inserted box shows a schematic diagram of a neuron, showing the cell body, dendrites, and myelinated axon with basal lamina.
perimeter. This myelin sheath is a proteophospholipid multilayered spiral of compacted apposed cell membranes. The myelin sheath has an important physiological function, in that it insulates the electrical signal passing down the axon. The myelin sheath formed by individual Schwann cells along the length of the axon has gaps at regular intervals, known as nodes of Ranvier, with one Schwann cell ensheathing the distance between each node (internode). The node allows extracellular ions to reach the axon, which is essential for the propagation of action potentials. These regularly spaced gaps in the myelin sheath result in saltatory propagation of impulses from node to node.

**Fibroblasts and macrophages.**

A peripheral nerve also consists of other cell types: fibroblasts, important for connective tissue formation such as the perineurium, and a third cell type, the macrophage. The role of the resident macrophage population in a normal peripheral nerve remains unclear. By contrast, the population of macrophages recruited into the peripheral nerve after injury plays a major part in the events following injury and is discussed in detail later.

Significant advances have been made over the last few years in understanding how each component of a peripheral nerve contributes to the regenerative response of the PNS following injury. The following discussion of peripheral nerve response to injury will review recent data which has
provided a more detailed picture of cellular events following injury.

**The damaged axon.**

**Inflammatory changes.**

Traumatic nerve injury, such as crush lesion or transection produces, as in other well vascularised tissues, an inflammatory reaction. The integrity of the specialised blood-nerve barrier maintained by the perineurium and endoneurial vessels is lost, with a resulting increase in vascular permeability and leakage of proteins from the blood into the interstitium. Vascular permeability is also altered by mast cell vasoactive amines released in response to the injury (reviewed by Terzis and Smith, 1990). These changes to a peripheral nerve move in a distal direction from the site of injury, with the entire nerve distal to an injury showing increased vascular permeability by four days after the injury. There is also a small spread in the proximal direction.

**The cell body response.**

Lesioning of the axon also produces characteristic structural and functional changes in the nerve cell body (for review see Lieberman, 1971). The classical chromatolysis response, first described by Nissl in 1892 is now known to be associated with morphological and biochemical alterations within the cell body. Nissl
described an increase in cell body volume, displacement of the nucleus to the periphery, and a disappearance of basophilic material from the cytoplasm (see Cajal, 1928). The reaction reflects an alteration in the arrangement and concentration of RNA-containing material (Nissl substance) in the cell, leading to changes in protein synthesis. Ultrastructurally, chromatolysis represents a disorganisation of the structure of the rough endoplasmic reticulum and release of associated ribonucleoprotein particles (ribosomes, the site of protein synthesis in the cell). Changes in the cell’s metabolic activity, such as increases in glycogen phosphorylase activity and iron uptake in axotomised motoneurons (Woolf et al., 1984; Graeber et al., 1989), are some of the other lesion-induced biochemical alterations. Peripheral nerve injury also causes a general increase in RNA and protein synthesis which may be dependent upon the type of lesion (reviewed by Leiberman, 1971).

The degenerating distal stump.

Several structural changes take place during Wallerian degeneration of the distal segment of a severed peripheral nerve. (i) Within the first few minutes of injuring the nerve, myelin retracts from the nodes of Ranvier close to the site of injury and the retraction spreads distally over the next six hours (Cajal, 1928). By twenty eight hours after the lesion nearly all the nodes, along the entire length are unrecognisable. (ii) Axonal disintegration
usually commences with a varicose appearance of the axon approximately two to four hours after the lesion. Within twenty four hours, there is a general disintegration of the neurofilaments, extending along the entire distal portion of the nerve by four days (Lunn et al., 1990). (iii) Fragmentation of myelin leading to the formation of ellipsoid bodies, occurs four to five days post-injury, and by fourteen days the myelin has almost completely disappeared from fine and medium size nerve fibres (Cajal, 1928).

The general pattern and rate of axonal degeneration in the distal stump is known to be affected by different types of lesions; a cut nerve degenerates faster than a crushed nerve (Lunn et al., 1990). Even where the outer sheath, the epineurium, is cut and the blood supply damaged at the site of injury, degeneration after a severe crush still proceeds at a similar rate to that of a simple crush. However, when a suture remains tightly tied after crushing, the nerve behaves as though it had been severed (Lunn et al, 1990), suggesting that interruption of axonal flow, either by transection or suture, influences the speed at which degeneration occurs.

The dual role of the macrophage.

(i) Macrophages in degeneration

Research in the last five years has uncovered some of
the most convincing evidence for the importance of macrophages in both Wallerian degeneration and regeneration of the PNS following injury. Macrophages are "professional" phagocytic cells in addition to being a major component of the immune system. They are found in most tissues, especially in haematopoietic and lymphoid organs (bone marrow, liver, spleen, lymph nodes) and in connective tissues. Cajal noted that a large number of macrophages were present within the PNS following axotomy (Cajal, 1928). Although Cajal held the view that macrophages were important for the phagocytosis and removal of myelin debris, it was difficult to provide clear evidence at that time for their ability to phagocytose and remove such debris. The opposing view of Bungner, Zalla and Stroebe, (see Cajal, 1928) amongst others, was that the peripheral nerve phagocytic cells were derived from the Schwann cells. There is now considerable data to support the hypothesis that the removal of debris formed by nerve degeneration is dependent upon the recruitment of macrophages from the vasculature (Beuche and Friede, 1984; Bonnekoh et al., 1989; Lunn et al., 1989). A reduction in myelin debris removal and decreased removal of myelin proteins are some of the consequences of either preventing the entry of macrophages into a severed sciatic nerve or blocking phagocytosis (Beuche and Freide, 1984; Scheidt et al, 1986).

New light was shed upon the importance of macrophages in
Peripheral nerve degeneration following the discovery of an unusual mouse strain, C57Bl/6/Olac (Lunn et al., 1989). Whilst examining nerve degeneration, Lunn et al. (1989) discovered that sciatic nerves in C57Bl/6/Olac mice degenerated at a slower rate when compared to a number of other strains; the rate of degeneration measured by neurofilament breakdown, endplate degeneration, and the disappearance of myelinated axons was dramatically reduced in C57Bl/6/Olac mice (Lunn et al., 1989). Furthermore, action potentials recorded from the distal segment of the sciatic nerve of the C57Bl/6/Olac mice were maintained for a significantly longer period after injury, compared to other strains. When the injured nerves were stained for macrophages, the normal recruitment of macrophages into the distal nerve segment following injury was not observed in C57Bl/6/Olac mice. (Perry et al., 1987; Lunn et al., 1989). Subsequent experiments on this mouse strain have also provided evidence for the existence of a factor, unique to the nervous system, which attracts macrophages into the lesioned nerve. Perry et al. (1990a) showed that macrophage recruitment in the C57Bl/6/Olac still occurs in inflammatory sites other than the nerve. Furthermore, it was shown, using bone marrow chimaeric experiments, that the haemopoietic cells of the C57Bl/6/Olac still invade the sciatic nerve of a different mouse strain (Perry et al., 1990a). This suggests that the failure to recruit these cells into the nerve results from a defect within the C57Bl/6/Olac nerve. Lack of degeneration is also observed
in the central nervous system and is controlled by a single autosomal dominant gene (Perry et al., 1990b; Perry et al., 1991a).

The expression of adhesion molecules, such as macrophage antigen-1 (MAC-1) or lymphocyte function-associated antigen-1 (LFA-1), on the macrophage cell surface, in addition to the expression of similar structures such as intercellular adhesion molecule (I-CAM) on the endothelial cells, is important for migration of macrophages into inflamed tissue (Wawryk et al., 1989). Therefore, the defect in the C57Bl/6/Olac mouse strain may be due to either an absence of appropriate adhesion molecules on the endothelial cells, or lack of a neurally derived chemotactic factor.

(ii) Macrophages and regeneration.

The absence of macrophages in the nerves of C57Bl/6/Olac mice during Wallerian degeneration does not seem to hinder regeneration of motoneurons (Brown et al., 1991), whereas peripheral sensory nerve regeneration is dependent upon their presence (Brown et al., 1991). The ability of macrophages to modify the normally nonpermissive state of the optic nerve to one which aids neurite outgrowth from embryonic DRGs also supports the importance of macrophages in regeneration (David et al., 1990).

Although macrophages were originally noted for their
powerful phagocytic properties, it has now been well established that they also secrete a large number of bioactive products (Nathan, 1987; Rappolee and Werb, 1988). Secretion of the enzyme collagenase, along with a number of other proteases, suggests a mechanism by which macrophages may contribute to the degenerative events following nerve injury (Welgus et al., 1985). However, the ability of macrophages to produce collagenase inhibitor, fibronectin and apolipoprotein E, may also be an important role of these cells in nerve regeneration (Welgus et al., 1985; Alitalo et al., 1980; Stoll and Muller, 1986). Recent studies on the macrophage cytokine, interleukin-1 (IL-1), have provided data in support of their role in nerve regeneration. IL-1 regulates the production of nerve growth factor (NGF) (Lindholm et al., 1987) and activity blocking antibodies raised against IL-1 inhibit cytokine-induced Schwann cell proliferation (Lisak and Bealmear, 1991). Furthermore, IL-1 is a mitogen for Schwann cells in vitro (M.Khan, pers. comm.). Since macrophages express properties important for both degeneration and regeneration of peripheral nerves, it is possible that different macrophage subpopulation, previously described by Dijkstra et al. (1985) perform different functions in response to peripheral nerve injury. Alternatively, the changing environment of the nerve following axotomy may influence macrophage function, regulating their phagocytic properties in addition to its secretory properties.
Rebuilding of the axon.

Regeneration of a peripheral nerve usually commences after a latent period of one to two days, at which point formation of a growth cone occurs; the growth cone is a swelling at the tip of the proximal disrupted axon. Reconstruction of lesioned axons requires the presence of essential building materials such as extracellular matrix and cytoskeletal elements. The cell body responds to injury by increasing the manufacture of a number of cytoskeletal elements for example actin, perpherin and tubulin are known to be up regulated in DRGs in response to peripheral nerve injury (Tetzlaff et al., 1988; Troy et al., 1990; Wong and Oblinger, 1990a and b). The cytoskeletal elements are then transported from the cell body down to the growth cone.

Transportation of proteins from the nerve cell body, along the axon to the newly formed growth cone occurs at either fast or slow rates (reviewed by Alberts et al, 1989). Neuronal cytoskeletal elements (actin, spectrin, tubulin, and the three neurofilament (NF) subunits, NF-H, NF-M, NF-L) are all transported via the slow rate transport which is subdivided into two separate components. The slow component 'a' (SCa), is mainly composed of microtubules and neurofilaments and does not appear to be affected by axotomy (Jacob and McQuarrie, 1991), whereas the slow component 'b' (SCb), comprised of actin and spectrin in addition to clathrin and calmodulin, has recently been
shown to accelerate after axotomy (Jacob and McQuarrie, 1991). The importance of SCb transport during regeneration is also reflected by the fact that the rate of SCb transport is further increased, along with the regeneration rate, following a second nerve injury inflicted 14 days after the first "conditioning" lesion (McQuarrie and Grafstein, 1973; Bisby and Pollok, 1983; McQuarrie and Jacob, 1991).

Structural support for peripheral nerve regeneration is also provided by a number of extracellular matrix substances. Fibronectin, tenascin, collagen (produced by endoneurial fibroblasts), and laminin (produced by Schwann cells), provide the substratum for neurite outgrowth during regeneration (Longo et al., 1984; Kuecherer-Ehret et al., 1990; Wehrle and Chiquet, 1990). Further support for axon regeneration is gained by the cell adhesion molecules (CAMs). Two CAMs which have been widely studied in peripheral nerve regeneration are neural cell-adhesion molecule (N-CAM) and the L1 glycoprotein (also known as the neuron-glial cell-adhesion molecule or Ng-CAM). Recent studies in rats showed that mRNA and protein levels for N-CAM and L1 increase as a result of sciatic nerve transection (Tacke et al., 1990; Martini and Schachner, 1988; Daniloff et al., 1986). Also, when antibodies directed against N-CAM are applied to a transected sciatic nerve, muscle reinnervation is delayed for up to 30 days (Remsen et al., 1990) and abnormal regeneration and
reinnervation processes occur (Reiger et al., 1988). In the presence of anti-N-CAM antibodies, ectopic synapses occur and the axon terminals lack a terminal Schwann cell capping the nerve-basal lamina contact area (Reiger et al., 1988).

It appears that, if regeneration of a peripheral nerve is to be successful then the expression of many different protein structures within the PNS microenviroment are required.

The distal stump and regeneration.

Cajal recognised and documented in 1928 the importance of the distal stump for effective regeneration. Subsequently, with the aid of modern techniques, more recent studies have uncovered some of the properties of the distal stump, including Schwann cells with its basal lamina and a number of diffusible trophic and tropic factors, which promote axonal regeneration (Politis et al., 1982; Ide et al., 1983;).

Proliferation of Schwann cells.

Schwann cell proliferation is a major event in response to peripheral nerve injury. Early studies on the Schwann cell proliferative response describe mitosis commencing some 4 days after transection of the peripheral nerve and peaking between 6 to 9 days (Cajal, 1928). The numbers of total cell nuclei increase 3 days after injury and reach a
maximum of 8 times the initial population after 25 days (Joseph, 1950; Abercrombie and Johnson, 1946). More advanced techniques, for example, the incorporation of tritiated thymidine into cells, have facilitated more accurate measurement of mitosis. There are known to be two periods of Schwann cell proliferation following axotomy in vivo. The first period commences almost immediately following injury within the immediate vicinity of the crush (Pellegrino et al., 1986; Clemence et al., 1989). It is independent of blood monocyte/macrophage recruitment, with the peak of proliferation occurring three to four days after injury (Clemence et al., 1989; Brown et al., 1991). In contrast the second period of Schwann cell proliferation which occurs during the regenerative phase also coincides with the recruitment of macrophages (Brown et al., 1991).

The importance of Schwann cell proliferation during nerve regeneration has lead to an extensive search to identify Schwann cell mitogens, produced as a result of peripheral nerve injury. This has proved to be a complex task. Potential Schwann cell mitogens include axon derived factors (Bunge, 1987; Salzer et al., 1980), products of activated lymphocytes and macrophages (Lisak et al., 1985), in addition to a myelin membrane fraction (Bigbee et al., 1987). A number of growth factors including platelet-derived growth factor (PDGF), fibroblast growth factor (FGF) and transforming growth factor β (TGF) are mitogenic for Schwann cells in vitro (Ridley et al., 1989; Davis and
Stroobant, 1990). However, none of these agents are mitogenic when applied alone, in serum free culture medium (Weimaster and Lemke, 1990). Work by Weimaster and Lemke (1990) suggests that there is an absolute requirement for elevation of the second messenger, cyclic adenosine monophosphate (cAMP), by a second factor, in order for Schwann cells to respond to the above growth factors (Weimaster and Lemke, 1990). Additional data has revealed that voltage-gated ion channels, such as potassium channels, expressed on the membrane of Schwann cells are also important for their proliferative response (Chiu and Wilson, 1989). Chui and Wilson (1989) showed that when potassium currents in Schwann cells are blocked by using potassium channel blockers, Schwann cell proliferation was inhibited in a dose dependent manner. It would seem that one factor alone is not an adequate signal for proliferation but that the combination of factors present in an injured nerve contributes to the control of Schwann cell proliferation.

Schwann cells and axon guidance.

A role for Schwann cells in the guidance of axon regrowth to their target, and as trophic support providing nutrients, was suggested early in the 20th century, (Cajal, 1928). However, recent studies have cast doubt over whether Schwann cells are an essential requirement for such growth. If, for example, acellular nerve explants are sutured to the proximal stump of a transected nerve, then they will
support the growth of regenerating axons (Anderson and Turmaine, 1986; Tohyama et al, 1990). Moreover, if segments of sciatic nerve distal to a crush are repeatedly frozen and thawed, killing the Schwann cells, only a 30% reduction in the rate of elongation of sensory and motor axons is observed (Skeljaji et al., 1989). As the nerve was crushed rather than transected, the extracellular matrix and basal lamina remains intact. However, if the Schwann cell basal laminae is denatured, by scalding the segments with moist heat, then there is a greater reduction in the rate of axon elongation (Skeljaji et al., 1989). These findings question the importance of Schwann cells themselves in the distal stump for regeneration, although the products of Schwann cells, such as laminin, are clearly important.

However, there are several studies which provide conflicting data, supporting the necessity of Schwann cells in regeneration. When Schwann cell mitosis is inhibited in the distal stump of a severed nerve, using mitomycin C, neurite outgrowth is virtually inhibited (Hall, 1986). It is also the case that Schwann cell surfaces and conditioned medium provide the best substrate to be identified which promotes neurite outgrowth in culture and peripheral motoneuron outgrowth in vitro (Kleitman et al., 1988; Assouline et al., 1987; Bixby et al., 1988). A role for Schwann cells in regeneration is also reflected by the ability of sciatic nerve grafts to support regeneration of a severed optic nerve which normally fails to regenerate
Although axonal regeneration has been described in the absence of Schwann cells, it would appear that optimum regeneration does require their presence.

The interstump gap.

The extent to which peripheral nerve regeneration takes place following transection is dependent upon the distance separating the distal stump from the regenerating proximal stump. A gap of 10mm is thought to be the maximum distance that sciatic nerves are able to grow across unaided (Lundborg et al., 1989). However, transected nerves can traverse a distance greater than 10mm providing a guidance channel or implanted chamber is employed (Aebischer et al., 1989). The use of such chambers has also provided a means of testing different factors which enhance the regenerative response. For example, the release of basic fibroblast growth factor (bFGF) from synthetic guidance channels provides sufficient support for the reconstruction of a transected rat sciatic nerve across a 15mm gap (Aebischer et al., 1989). Furthermore, basic FGF can also accelerate the growth of new fibres through similar chambers (Danielsen et al., 1988). Although several studies have shown the distal stump to be essential for successful nerve regeneration (Scaravilli et al., 1984; Williams et al., 1984; Jenq and Coggeshall, 1986), especially where nonmyelinated axons are involved (Jenq and Coggeshall, 1986), its absence does not prevent regeneration when a
A semipermeable guidance channel is used (Aebischer et al., 1988). Structural support from the channel and trophic factors secreted from the surrounding environment, which includes the distal stump, is sufficient for regeneration to occur.

**Nerve Growth Factor (NGF): A role in regeneration?**

Nerve growth factor (NGF), the best characterised target-derived trophic factor in the nervous system, was discovered in the early fifties by Levi-Montalcini and Hamburger (Levi-Montalcini and Hamburger, 1951). It was found to enhance the outgrowth of neurites selectively from neurons of sympathetic and embryonic dorsal root ganglia both *in vivo* during foetal development and *in vitro* (Levi-Montalcini and Hamburger, 1951; Levi-Montalcini and Angeletti, 1963; Levi-Montalcini et al., 1954). NGF is produced by the targets of NGF-sensitive fibres, taken up by the presynaptic terminals and retrogradely transported to the perikaryon (Hendry et al., 1974; Stoeckel et al., 1975; Stoeckel et al., 1976). The importance of NGF for neuronal survival during development has been demonstrated conclusively by immunosympathectomy. Administration of NGF antiserum to newborn rats results in a significant reduction of neuronal number in the lumbar DRG (Yip et al., 1984). Moreover, Gorin et al. (1979) observes that peripheral sympathetic neurons in offspring of NGF-immunized female rats are destroyed. A similar result is
observed for rat and guinea pig DRG after exposure in utero to maternal antibody to NGF (Johnson et al., 1980; Johnson et al., 1983). The importance of NGF for neuronal survival during development is strengthened by studies which show that the addition of exogenous NGF during development reduces the amount of naturally occurring cell death (Hamburger et al., 1981). This ability to reduce naturally occurring cell death is thought to be via the ability of NGF to suppress an endogenous, active cell death program (Martin et al., 1988).

A considerable amount of neuronal cell death also occurs in sympathetic and dorsal root ganglia following peripheral axotomy in both newborn and adult rats (Hendry, 1975; Yip and Johnson, 1984; Tessler et al., 1985; Arvidsson et al., 1986). The importance and function of NGF in peripheral nerve regeneration has not been defined to the same extent as its role in the development of the nervous system. Deprivation of NGF does not increase the extent of cell death in the lumbar DRG or the extent of regeneration after sciatic nerve transection (Rich et al., 1984). However, NGF partially reverses the central changes in the cell body which occur after nerve transection, if it is applied locally to the lesioned nerve (Fitzgerald et al., 1985; Rich et al., 1987). Furthermore, NGF will also prevent the decrease in neuronal number which normally follows combined central and peripheral lesions (Yip and Johnson, 1984). The importance of NGF after axotomy is also supported by the
recent discovery of its involvement in collateral sprouting of sensory axons in the skin (Diamond et al., 1992) and the observation that NGF mRNA increases distal to the injury site following axotomy (Heumann et al., 1987a). This lesion-induced increase in NGF mRNA is biphasic, with an initial increase 6 hours after transection, followed by a second peak occurring 3 days after transection (Heumann et al., 1987a; Heumann et al., 1987b). When the nerve is prevented from regenerating, i.e. cut rather than crushed, the increase in NGF mRNA remains elevated (Heumann et al. 1987b).

In culture, only a single rapid and transient increase in NGF mRNA is observed following axon injury (Lindholm et al, 1987). However, failure to observe the second, more persistent increase is rectified by the addition of either macrophage conditioned medium or the macrophage cytokine, interleukin-1 (IL-1) (Lindholm et al., 1987), indicating a role for macrophages in regulation of NGF expression.

Although the role of NGF in regeneration is unclear, its increased expression following nerve injury may enable the distal stump to act as a surrogate target, providing support to regenerating sympathetic and sensory neurons. If NGF is important for such regeneration, then its receptor should also be expressed after injury. The re-expression and redistribution of the NGF receptor was shown to occur after nerve transection (Raivich and Kreutzberg, 1987). NGF
receptors accumulate transiently on both sides of crushed or transected sciatic and brachial nerves, due to retrograde and anterograde axonal transport of the receptor (Johnson et al., 1987; Raivich and Kreutzberg, 1987). However, a rapid decrease in uptake and both retrograde and anterograde axonal transport of NGF receptors starts one day after injury (Raivich and Kreutzberg, 1987; Raivich et al., 1991), thus calling into question the relevance of NGF for axon regeneration.

NGF receptors expressed on Schwann cells throughout the distal part of axotomised nerves demonstrate a different time course. Receptors are not detected until 4 days after injury, becoming maximal 6 days after injury (Raivich and Kreutzberg, 1987). Subsequently, as the axons regenerate, Schwann cell NGF receptor expression decreases (Taniuchi et al., 1988). In contrast to NGF, expression of the receptor does not appear to be regulated by the recruited macrophages (Heumann et al., 1987b; Brown et al., 1991) but it may be controlled by axonal contact.

The precise role of the Schwann cell NGF receptor in nerve regeneration is unclear, since NGF has no discernable effect on these cells. It was thought that, together with the release of NGF from the Schwann cells and fibroblasts (Heumann et al., 1987a; Matsuoka et al., 1991) in the distal stump, NGF receptors may support the regeneration of sensory neurons via cell adhesion-like activity, similar to
the role of N-CAM (Taniuchi et al., 1988). NGF may act as 'bridge' between the axon and the surrounding Schwann cells via its ability to bind to receptors on both the Schwann cell and the axon. (Taniuchi et al., 1988). However, with the disappearance of axonal expression and retrograde transport of the NGF receptor following axotomy (Raivich et al., 1991), the regenerating axons may not themselves be the targets for the increased levels of NGF present in the distal stump. The NGF produced by the Schwann cells and fibroblasts after axotomy may interact locally with Schwann cells distal to the injury site which are known to expressing NGF receptors (Raivich et al., 1991). Clearly the role of NGF in the damaged nerve is unresolved, however, its up-regulation in the distal stump indicates a role in nerve regeneration.

Neuropeptides: A trophic role following nerve injury?

The identification and distribution of biologically-active peptides throughout the nervous system, so called neuropeptides, has rapidly expanded over the last twenty years with the aid of immunohistochemistry. This technique has produced some interesting findings concerning the effect of peripheral nerve injury on neuropeptide expression.

For a number of years, neuropeptides had been regarded as simple chemical transmitters, manufactured in the cell
body and transported to the presynaptic membrane, where they are released as signalling molecules. Most effects of neuropeptides on their targets seem to be relatively short-term, acting over a range of seconds or at most minutes. Neuropeptides can be divided into two groups according to whether they have a direct effect on a postsynaptic membrane (neurotransmitter) or modulates the effects of other molecules (neuromodulator). The distinction between neurotransmitters and neuromodulators is frequently unclear, since the same neuropeptide acts at some synapses as a transmitter and at others as a modulator. One such peptide is substance P (SP), which has been proposed to be a sensory neurotransmitter. It is present in small-diameter, primary afferent neurons that project to the superficial laminae of the dorsal horn (Hökfelt et al., 1976). It induces slow excitation in the dorsal horn cells that respond to noxious stimuli (Henry, 1976). However it also depresses the nicotinic acetylcholine (ACh)-induced excitation of various cell types, including adrenal chromaffin cells (Mizobe et al., 1979).

Although many peptides have relatively short-term effects on their target cells, there is now considerable evidence to suggest a trophic role for neuropeptides acting over a time scale of many minutes or even longer. The regulation by calcitonin gene-related peptide (CGRP), of muscle ACh receptor expression and suppression of disuse-induced terminal sprouting after chronic block of nerve-
muscle activity are examples of neuropeptide involvement in a trophic response (New and Mudge, 1986; Tsujimoto and Kuno, 1988).

The characterisation of the neuropeptide receptors, localisation and regulation has been made possible by the use of gene cloning, one of the many molecular biology techniques developed in the last ten to fifteen years. All of the neuropeptide receptors discovered, including the receptors for SP and neurotensin, belong to the family of G protein-coupled receptors with seven membrane-spanning segments (reviewed by Hökfelt, 1991).

Interest in a possible trophic role for neuropeptides during peripheral nerve regeneration has developed from the observations that vasoactive intestinal (VIP) and neuropeptide Y (NPY) increase in the DRG neurons, and CGRP expression increases in the motoneurons, after peripheral nerve injury (Shehab and Atkinson, 1986; Wakisaka et al., 1991; Jessell et al., 1979; Streit et al., 1989; Arvidsson et al., 1990; Noguchi et al., 1990). In contrast to the expression of CGRP in motoneurons, its expression in DRGs decreases following injury, suggesting that the regulation of CGRP in the PNS following injury is complex. However, since peptides with transmitter-like functions would be expected to be redundant after injury, because they could not be released at the terminal synapse, these increases in certain peptides were intriguing. They suggested a role for
neuropeptides in nerve regeneration, since only those peptides and proteins involved in rebuilding the axon and supporting the cells survival are thought to increase after nerve injury. The hypothesis that certain neuropeptides expressed in the PNS are involved in peripheral nerve regeneration is strengthened by the finding that CGRP acts in vitro as a permissive signal for Schwann proliferation via its ability to stimulate Schwann cell cyclic adenosine monophosphate (cAMP) production (M.Khan, personal communication); Schwann cell proliferation is a major event following peripheral nerve injury.

Neuropeptides and the inflammatory response.

A lesion within a peripheral nerve, as in other tissue, causes inflammation. This inflammatory response encompasses a vast array of events, including vascular changes, the release of histamine and the migration of leucocytes, neutrophils and macrophages into the inflammatory site. The ability of the nervous system to influence certain aspects of inflammation is recognised by the capacity of neuropeptides, such as SP, to induce histamine release and vascular changes following skin lesions (Foreman and Jordan, 1983). This is supported by the demonstration that afferent nerve stimulation produces vasodilation and plasma extravasation into the skin, a process termed neurogenic inflammation (reviewed by Foreman and Jordan, 1984). The absence of these events after blockage of nerve
transmission either by mechanical injury, or application of capsaicin, producing a loss of small-diameter primary afferent neurons, supports the hypothesis that peripheral nerves modulate the inflammatory response (Helme and Andrews, 1985). In addition to the effects of SP in neurogenic inflammation, additional neuropeptides such as neurokinin A (NKA) and B (NKB), induce plasma protein extravasation, and CGRP augments extravasation induced by histamine. (Gamse et al., 1987). The possibility arises that certain neuropeptides expressed following peripheral nerve injury have a role in the ensuing inflammatory response. The observation that SP, substance K (SK) and the carboxyl-terminal peptide SP(4-11) increase the release of the inflammatory cytokines IL-1, tumour necrosis factor-α (TNF-α) and interleukin-6 from monocytes (Lotz et al., 1988), supports the hypothesis that neuropeptides regulate certain events of the inflammatory response following nerve injury, in particular the macrophage response. Further evidence for such regulation is provided by studies demonstrating that VIP up regulates cAMP levels in blood monocytes, and CGRP inhibits peritoneal macrophage activation (Wiik, 1989; Nong et al., 1989).

If certain neuropeptides, present following peripheral nerve injury, regulate the macrophage response then they may indirectly influence regeneration of the peripheral nervous system. Therefore, it is important to determine what changes occur, if any, in levels of expression of
certain neuropeptides around the injury site as well as distal to the crush during regeneration.
**Thesis objectives.**

This thesis aims to clarify the importance of neuropeptides to PNS regeneration by examining in detail the expression of CGRP and VIP as well as the lymphokine, gamma interferon in the damaged rat sciatic nerve. It also investigates whether CGRP has a role to play in regulating the function of macrophages recruited into an injured sciatic nerve.

The second section investigates whether the multiple functions of macrophages, which may be important in peripheral nerve degeneration and regeneration, are related to the presence of different macrophage phenotypes in the sciatic nerve, before and after injury. It aims to document the time course and spatial distribution of these macrophages.
CHAPTER 2

CGRP AND VIP IN THE PERIPHERAL NERVOUS SYSTEM: A ROLE FOR CGRP IN PERIPHERAL NERVE INJURY.
Introduction.

Calcitonin gene-related peptide (CGRP) is a 37 amino acid neuropeptide which exists in two forms, $\alpha$-CGRP and $\beta$-CGRP. $\alpha$-CGRP is generated by alternative splicing of the primary transcript of the calcitonin gene (Amara et al., 1982). However, $\beta$-CGRP, which was discovered in brain and thyroid (Amara et al., 1985), is generated from a different gene. When the peptide sequence of rat $\alpha$-CGRP is compared with rat $\beta$-CGRP, there is only a single substitution, a lysine in $\beta$-CGRP for a glutamate in $\alpha$-CGRP at position 35 (Amara et al., 1985). A similar comparison of $\alpha$ and $\beta$ human CGRP shows that they differ by 3 amino acids (Steenbergh et al., 1985).

CGRP is widely distributed within the central and peripheral nervous system (Skofitsch and Jacobowitz, 1985; reviewed by Yamamoto and Tohyama, 1989). It is found in a large population of primary sensory neurons, and it was the first peptide to be localised in motoneurons (Gibson et al., 1984; New and Mudge, 1986; Fontaine et al., 1986). Furthermore, when the distribution of the mRNA for $\alpha$ and $\beta$-CGRP is analysed, it is $\alpha$-CGRP which is more abundant in sensory ganglia and motoneurons (Gibson et al., 1988). However, only $\beta$-CGRP is present in enteric autonomic neurons (Mulderry et al., 1988).

The localisation of CGRP within chick spinal motoneurons
during development and enhanced biosynthesis of the AChR in primary cultures of chick muscle by CGRP (New and Mudge, 1986; Fontaine et al., 1986) raises the possibility that CGRP may be important at the neuromuscular junction. This is supported by the observation of CGRP-like immunoreactivity within synaptosomal vesicles and that CGRP-like immunoreactivity is also found at motor end plates (Takami et al., 1985; Gulbenkian et al., 1986; Freid et al., 1989). Furthermore, either electrical or high K+ stimulation of the phrenic nerve, in vitro, causes release of a CGRP-like immunoreactive substance (Uchida et al., 1990).

The ability of CGRP to regulate AChR expression appears to be via stimulation of adenylate cyclase and cyclic adenosine monophosphate (cAMP) production (Laufer and Changeux, 1987). The addition of CGRP to skeletal muscle in vitro causes the activation of adenylate cyclase and elevates intracellular cAMP of skeletal muscle (Laufer and Changeux, 1987; Roa and Changeux, 1991). Consistent with this observation is that electrical stimulation of the phrenic nerve, in addition to causing the release of CGRP-like immunoreactivity, also causes an increase in cAMP content in the diaphragm (Uchida et al., 1990).

Although CGRP is expressed at a high level in spinal motoneurons during development, CGRP expression in the adult stage is at a low level (Gibson et al., 1988).
Interestingly, recent studies have shown that peripheral axotomy induces an increased immunostaining for CGRP in injured motoneurons, as well as up-regulation of mRNA encoding α-CGRP but not β-CGRP (Streit et al., 1989; Arvidsson et al., 1990; Noguchi et al., 1990). This contrasts with decreased α and β-CGRP mRNA in dorsal root ganglia following peripheral nerve injury (Noguchi et al., 1990). Since levels of transmitter-related enzymes usually decrease in response to axotomy, it was surprising that CGRP in motoneurons increases after injury. This raises the question as to whether CGRP has role in peripheral nerve regeneration.

This thesis examines whether the initial increase of CGRP at the injury site remained elevated as the axons regenerate. The technique of radioimmunoassay was employed to measure levels of CGRP as far distal as fifteen millimetres and up to fourteen days following crush injury.

Two of the main events following peripheral nerve injury are macrophage recruitment and Schwann cell proliferation, as discussed in chapter one. Substantial evidence has recently been provided to support the ability of CGRP to interact with Schwann cells in vitro (M.Khan, pers. comm.). As with a number of other cell types, including astrocytes, endothelial cells and skeletal muscle cells (Lazar et al., 1991; Hægerstrand et al., 1990; Laufer and Changeux, 1989), CGRP activates adenylate cyclase, thus elevating cAMP.
within Schwann cells as well as fibroblasts (M.Khan, pers.comm.). This thesis will examine whether CGRP interacts with the third major cell type of peripheral nerve regeneration, the macrophages, by regulating their cAMP production.

This thesis will also investigate the expression of another neuropeptide, VIP, following peripheral nerve injury. VIP, first isolated from porcine duodenum (Said and Mutt, 1970), is a 28-amino acid peptide and a member of the glucagon-secretin family of gastrointestinal peptide hormones. It has been identified in neurons of the CNS, including neurons in the cerebral cortex and hypothalamic regions (see Hökfelt et al., 1980), in addition to its localisation within primary sensory neurons, intrinsic spinal neurons and autonomic neurons innervating various exocrine glands (Fuji et al., 1983; see Hökfelt et al., 1980). A large number of VIP expressing nerves have also been observed within the gastrointestinal tract (Dimaline and Dockray, 1982; Hökfelt et al 1980).

Interestingly, although VIP is not normally detectable in sensory neurons in lumbar 4/5 DRG, both mRNA and peptide for VIP appear in these sensory ganglia following sciatic nerve injury (Nielsch and Keen, 1988). VIP mRNA appears 3 days following injury with increasing levels with time, measured up to 9 days following injury. Similar studies have shown that VIP peptide also increases in the dorsal
horn following sciatic nerve injury (McGregor et al., 1984; Shehab and Atkinson, 1986). In order to postulate a role for VIP in peripheral nerve regeneration, one needs to show, like CGRP, that VIP is present and maintained at elevated levels in the sciatic nerve during regeneration. Therefore, this thesis also investigated levels and determined the time course for VIP in the sciatic nerve following injury.
MATERIALS AND METHODS.

Animal Surgery.

Male adult Sprague-Dawley rats were anaesthesized with halothane and the sciatic nerve exposed at mid-thigh level. The nerves were subjected to two types of injury. The first (i) allowing regeneration and the second (ii) where regeneration was prevented.

(i) crushing the nerves with either watchmaker forceps (No.5) left in position for approximately 1 minute or with a silk suture (2/0 Mersilk) for approximately 1 minute. (ii) transecting the nerve or leaving a silk suture attached to the nerves until the nerves were removed from the animals.

In sham operated animals the nerve was exposed and manipulated but not damaged.

The animals were killed between 2 hours to 18 days following nerve lesioning. Nerve segments measuring 5mm were removed proximal and distal to the lesion site (see figure 2.1). CGRP or VIP levels in these 5mm nerve segments were determined by radioimmunoassay (RIA). Nerve segments, 12mm in length, were removed 6 days post-suture crush; these segments included regions proximal and distal to the crush, and were used for immunohistochemical detection of CGRP. Similar lengths of nerve were removed 2 hours to 18 days post-crush for IFNY immunohistochemistry.
Figure 2.1: Diagram of lesioned sciatic nerve with an area around the lesion site divided into a number of 5mm segments. Levels of VIP and CGRP in the segments were quantified by RIA.
Tissue preparation for immunohistochemistry.

After dissection, the nerves were immediately immersed in 4% (w/v) paraformaldehyde in phosphate buffered saline, pH 7.4 (PBS) and fixed overnight at 4°C. The following day they were placed in 30% (w/v) sucrose in PBS for up to 2 hours to prevent crystal formation within the tissue. The tissue was mounted in OCT embedding medium (Tissue-tek, Miles) and stored in liquid nitrogen until sectioned on a freezing microtome (Bright).

CGRP immunohistochemistry.

Longitudinal sections of rat sciatic nerves, 10-15 μm thick, removed 2 hours and 6 days post-suture crush, were labelled with a rabbit anti-human CGRP polyclonal antibody (Peninsula Labs). This antibody was applied at a 1/700 dilution overnight at 4°C in 4% (v/v) horse serum (Gibco), 1% (v/v) Triton-X100 (Sigma), 0.01% (v/v) sodium azide and 20mM L-lysine in PBS ("immunohistochemistry buffer"). The next day, sections were washed 3 times for 5 minutes each in the same buffer. The second antibody, a goat anti-rabbit fluorescein-conjugated antibody (Amersham) was diluted 1/100 in immunohistochemistry (IHC) buffer and preadsorbed with 15-20% (v/v) rat serum for 30 minutes. It was then centrifuged in a micro-centrifuge for 10 minutes to remove precipitate. The sections were incubated with the second antibody for 45 minutes at room temperature. The sections were washed a further 3 times in IHC buffer and then mounted in Citifluor (City University, U.K). Sections were
viewed with a fluorescence microscope (Zeiss Universal).

**IFNγ immunohistochemistry.**

Longitudinal sections, 12μm thick, of rat sciatic nerves removed 2 hours, 2, 6 and 18 days post-crush were labelled with the monoclonal antibody, DB-1 (1/1000), which recognises IFNγ (a gift from P van der Meide). The staining protocol was the same as that for detection of CGRP except the second antibody was a goat anti-mouse biotinylated antibody (1/100) (Amersham). After a 45 minutes incubation at room temperature and 3 washes for 5 minutes each in the ICH buffer, a third layer of fluorescein-conjugated streptavidin (1/100) (Amersham) was added for a further 45 minutes to visualise the IFNγ staining. All sections were washed a further 3 times in ICH buffer, mounted in Citiflour (City University) and viewed with a fluorescence microscope (Zeiss Universal).

**Tissue Extraction for Radioimmunoassay.**

Each 5mm segment of sciatic nerve was placed in a 2ml Eppendorf microtube, and then frozen immediately in liquid nitrogen. Frozen tissue was stored for up to one month before assay. To extract CGRP and VIP, each 5mm segment of nerve was placed in 1ml of ice cold 2M acetic acid and homogenised using an electric homogeniser (Ultra-Turrax with UT-dispersing tool 10G; Janke and Kunkel). The homogenates were placed in a boiling water bath for 5 minutes to inactivate proteases and were then lyophilised
overnight.

Radioimmunoassays.

Amount of CGRP or VIP in each nerve segment were measured on the basis that CGRP or VIP in the tissue extract would compete with 

\[ ^{125}\text{I-CGRP (Amersham)} \] or 

\[ ^{125}\text{I-VIP (Amersham)} \] for binding to the CGRP or VIP antibodies respectively. The buffer used for diluting all RIA components was 10mM ethylene diamine tetra acetic acid (EDTA), 0.01% (w/v) sodium azide, 0.05% (w/v) Polybrene (Sigma), 0.1% bovine serum albumen (BSA) (w/v) (Sigma; RIA grade) and 0.1% (w/v) gelatin in PBS. The RIAs were carried out in borosilicate glass tubes (Corning) using the CGRP antibody (Peninsula Labs) at 1/1000 and VIP antiserum L25, supplied by R.Dimaline (Liverpool University) (pers.comm. A.Mudge), at 1/22,000 final dilution respectively. Radiolabelled peptides 

\[ ^{125}\text{I-CGRP} \] or 

\[ ^{125}\text{I-VIP} \] were used at ca.5,000 counts/min (cpm) per tube (Amersham; specific activity 2,000 Ci/mmol).

The lyophilised samples were resuspended in 1ml of RIA buffer and both 100µl and 200µl aliquots were assayed in duplicate. The final incubation volume was 400µl which includes 100µl of antibody and 100µl of 

\[ ^{125}\text{I-CGRP} \] or 

\[ ^{125}\text{I-VIP} \] (the trace); controls for separation were included and contained only trace without antibody or sample. The tubes were incubated at 4°C for 24-48 hours. Trace that was bound to antibody was separated from free trace by adsorbing free
trace with activated charcoal. At the end of the initial incubation period, 400μl of charcoal mixture, 3.2% (w/v) activated charcoal (Sigma), 0.32% (w/v) Dextran T70 (Pharmacia) and 0.01% (w/v) sodium azide in PBS, was added to each tube and the tubes vortexed. The tubes were centrifuged at 2,500g for 15 minutes and the supernatants were decanted and counted for 1 minute in a gamma counter (Nuclear Enterprises, NE1600). The counts obtained represent the trace bound to antibody (the bound fraction).

CGRP radioimmunoassay.

In each RIA a standard curve for synthetic human CGRP (Bachem) was obtained in the range of 8-100 fmol CGRP/tube and two dilutions of nerve extract, both in duplicate, were assayed to allow displacement slope comparisons to be made between sample and standard. This provided a means to compare the nature of the immunoreactive samples compared to standard CGRP. Standard and sample curves were calculated using the logit-log linearisation method (Rodbard et al., 1969). The following terminology is used for each RIA: 'D' represents the counts/minute in the bound fraction in the absence of antibody used at 1/400,000 dilution, 'B,' is the maximum binding (counts/minute in the bound fraction in the absence of peptide). 'B' is the binding with sample (counts/minute in the bound fraction in the presence of antibody and either CGRP, VIP or nerve extract). The percentage binding was calculated from the equation (B-D)/(B,-D) x 100. The logit of the percentage
binding was plotted against the log of the sample dose so that a curve closely approximating linearity was obtained, over the percentage binding range of 12-88%.

**VIP radioimmunoassay.**

The VIP RIA was performed as described for the CGRP assay except that the standard curve for synthetic human VIP (Bachem) was obtained in the range of 3-50 fmol VIP/tube. The trace was \(^{125}\text{I-}\text{VIP}\) (Amersham) and the anti-VIP antibody (R.Dimaline, Liverpool University) used at 1/22,000. The percentage binding was calculated as above and the logit of the percentage binding was plotted against the log of the sample dose.

**Interassay variability.**

Each RIA incorporated an aliquot of the same spinal cord sample which contained a quantity of CGRP. This provided a means of measuring the variation between each RIA. Also sciatic nerve segments from control unoperated animals were assayed in most RIAs.

**Gel permeation chromatography.**

**Materials and equipment.**

A Superose-12 column (Pharmacia) was used together with a 2111 Multirac fraction collector, 2238 Uvicord SII UV recorder, and 2210 2-channel chart recorder, all from LKB. Fractions were collected into borosilicate glass tubes.
(Corning). Bovine thyroglobulin, Cytochrome C (Type VI) and BSA (RIA grade) were from Sigma.

Gel permeation methods.

CGRP-immunoreactive material obtained from nerve segments was characterised by gel filtration chromatography. The tissue extract was obtained as described above and 500μl was applied to a Superose-12 column (Pharmacia). The peptide was eluted with PBS at a flow rate of 1ml/minute. Aliquots of each fraction were assayed for CGRP content using the RIA. The elution profile was compared with that obtained by gel filtration of synthetic 125I-CGRP.
MACROPHAGE TISSUE CULTURE.

Peritoneal macrophage isolation (resident or activated).

Sprague-Dawley rats were killed and injected intraperitoneally with 20ml of Hams F12 medium (Flow). The peritoneum was gently massaged to obtain the maximum possible number of macrophages. The medium was drawn out of the cavity and placed into a conical tube (Falcon) and centrifuged at 1600rpm for ten minutes. The cell pellet was resuspended in 1-2 mls of F12 medium and the cell concentration determined using a haemocytometer. Exclusion of Trypan blue stain was used as a measure of cell viability. The cells were plated into 96 well plates (Falcon).

Activation of peritoneal macrophages in vivo.

Lipopolysaccharide (LPS) (Sigma), 50μg, in Hams F12 medium (Flow) was injected into the peritoneal cavity of Sprague-Dawley rats under halothane anaesthetic. The rats were left for 4 days after which the animals were killed and the peritoneal cells removed as described above.

Spleen macrophage isolation.

Sprague-Dawley rats were killed and their spleens removed and placed into Hams F12. Up to eight spleens were crushed through a sheet of gauze and the resulting suspension containing rat spleen macrophages was incubated for 2 hours in F12 medium containing 10% (v/v) foetal calf serum (FCS) (Gibco) in tissue culture grade 100mm dishes (Falcon) and
placed into a 37°C, 5% CO₂ incubator (LEEC). The macrophages were observed to adhere to the plastic dishes after 2 hours. Nonadherent cells were removed by extensive washing with a defined salt solution, and the remaining adherent cells were cultured overnight in the above incubator. Cells which detached from the dish overnight were collected and used without further purification as a source of macrophages. The cells were centrifuged at 1600rpm for 10 minutes, counted as above and plated into 96 well plates (Primaria grade, Falcon).

Preparation of panning dishes.

Petri dishes (100mm) (Falcon) were coated with a nonspecific rabbit anti-mouse immunoglobulin (Dakopatts) at 10μg/ml in 15mM Trisma base buffered to pH9.7 with hydrochloric acid (HCl). After an overnight incubation at 4°C the buffer was aspirated and the dishes washed 2-3 times with a balanced salt solution to remove excess antibody. Hams F12 medium with 0.2% (w/v) BSA (Sigma) was added for 45 minutes to block nonspecific binding. Panning dishes used for the isolation of macrophages from unlesioned sciatic nerves were then incubated a further 45 minutes with a second antibody. The second antibody used was either anti-leucocyte common antigen (Seralab) or OX42 (Seralab), both of which are known to label macrophage surface antigens. They were added at a 1/400 dilution. The dishes were then washed as before.
Macrophage isolation from injured sciatic nerve.

A total of 4 sciatic nerves, 3 days after crushing, were removed from 4 Sprague-Dawley adult rats. Two sets of two nerves were washed in dissociation buffer (136mM NaCl, 5mM KCl, 5mM Na phosphate, 33mM glucose, pH 7.4) and chopped to small pieces in 1ml of 0.6% (w/v) collagenase (Sigma) in dissociation buffer. The chopped nerves were placed into a 15ml conical tube (Falcon) and 1ml of 0.0175% (w/v) trypsin (Sigma), in dissociation buffer, added. After a dissociation period of 40-50 minutes in a water bath (37°C), growth medium with 10% (v/v) FCS was added to the solution in order to stop further trypsin activity. The resulting suspension was centrifuged at 1600rpm for 10 minutes and the pellet resuspended in 2-3ml of medium containing 10% FCS and passed through gauze to remove excess debri. The cells were distributed evenly into two 100mm Petri dishes which had been coated with a nonspecific rabbit anti-mouse immunoglobulin (Dakopatts). The dishes were shaken briefly every 5 minutes for 15 minutes (37°C). The nonadherent cells were then aspirated, and the remaining cells were removed from the dishes by adding 0.125% trypsin for ten minutes at 37°C. The enzyme solution, with the cell population was placed into a 15ml conical tube (Falcon) containing Hams F12 medium with 10% FCS. After the cells had been centrifuged for 10 minutes they were resuspended in 1ml of Hams F12 medium, counted and plated out into 96 well plates (Falcon, Primaria).
Preparation of macrophages from uninjured sciatic nerve.

Sciatic nerves were removed from 3 or 4 day old Sprague-Dawley rat pups (P3/4) immediately following decapitation. The nerves were digested with 0.025% (w/v) trypsin (Sigma) and 0.15% (w/v) collagenase (Sigma) in dissociation buffer for 30 minutes. Hams F12 medium containing 10% (v/v) FCS was added to the enzyme mixture preventing further trypsin activity. The dissociate was centrifuged at 1600rpm for 10 minutes and the supernatant discarded. The pellet was resuspended in 2ml of Hams F12 medium and the cell suspension was passed through gauze removing excess tissue debris. The cells were added to Petri dishes (Falcon) coated with either leucocyte common antigen (LCA) or OX42 antibody, and incubated at 37°C, 5% CO₂ for 20 minutes following which nonadherent cells were removed by washing the Petri dishes extensively with dissociation buffer. The cells were removed from the Petri dish by adding 0.125% trypsin for 10 minutes. The trypsination was terminated as before and the suspension was centrifuged at 1600rpm for 10 minutes. The pellet of cells was resuspended in Hams F12 medium and plated into 96 well tissue culture plates (Primaria grade, Falcon) at a density of 5000 cells/well.

Cell culture techniques.

The macrophages plated into 96 well plates were cultured in Hams F12 medium. If the cells were incubated overnight or longer, then 2mM glutamine, 50µg/ml streptomycin (Gibco), 50U/ml penicillin (Gibco) and 10% FCS (v/v) were
added to the medium. The cells were cultured at 37°C in the presence of 5% CO₂.

**Macrophage activation in vitro**

Peritoneal and spleen macrophage cultures are treated with gamma interferon (IFNγ) at 300 Units/ml (a gift from P. van der Meide) for 2 hours before the addition of CGRP.

**Cyclic adenosine monophosphate (cAMP) assay.**

Culture medium was replaced by fresh medium without FCS 2 hours before adding the phosphodiesterase inhibitor, isobutyl-methylanthine (IBMX) (Sigma). After a period of 30 minutes, CGRP (Bachem) was added to the cells. Cyclic AMP was extracted from the cells by removing the medium and adding ice cold 70% (v/v) ethanol in water. The cells were left overnight at -20°C. Ethanol from each well was transferred to an Eppendorf tube and dried down using a Speed Vac SC100 (Savant). The cAMP in each sample was measured in duplicate using the more sensitive acetylation method of a dual range cAMP kit (Amersham). The radioactive counts present in each pellet, containing cAMP bound to antibody, was measured using a gamma counter (Nuclear enterprises NE1600) gamma counter.
RESULTS.

**Standard curves for the CGRP and VIP RIA.**

An example of an RIA standard slope for human synthetic CGRP RIA is shown in figure 2.2. The mean of twelve standard slopes for the human synthetic CGRP was calculated and a value of $-3.0325 \pm 0.16$ was obtained. Similarly, a mean of twelve slopes was obtained for CGRP extracted from lesioned sciatic nerves. Values of $-2.90 \pm 0.28$, $-3.05 \pm 0.36$ and $-2.64 \pm 0.33$ were calculated for proximal, distal and contralateral segments of sciatic nerve, respectively (mean $\pm$ s.e.m. $n=12$ for all segments). The small differences between the slopes including the standard slope were not statistically significant, measured by students t-test ($p=0.1$).

A similar example of an RIA standard curve for synthetic human VIP is shown in figure 2.3. The mean of four standard slopes for VIP extracted from lesioned sciatic nerve was calculated and a value of $-3.12 \pm 0.09$ was obtained. Values of $-3.12 \pm 0.24$, $-3.05 \pm 0.36$ and $-2.81 \pm 0.44$ were calculated for proximal, distal and contralateral segments of sciatic nerve, respectively (mean $\pm$ s.e.m. $n=4$ for all the segments). The small differences between the slopes including the standard slope were not statistically significant, measured by students t-test ($p=0.1$).
Figure 2.2: A standard displacement slope of log dose against logit percentage binding is plotted for human synthetic CGRP (○). The slope shown represents an example of the slopes obtained for each RIA. Examples of slopes obtained for CGRP extracted from segments of sciatic nerve are also plotted and regression lines fitted.
Figure 2.3: A standard displacement curve of log dose against logit percentage binding is plotted for human synthetic VIP (○). The slope shown is an example of the slopes obtained for each RIA. Examples of slopes obtained for VIP-like immunoreactivity extracted from segments of sciatic nerve are also plotted and regression lines fitted.
Interassay standard for RIA.

The mean ± s.e.m. for the interasssay spinal cord sample was 25 ± 1.5 pmol, n=12.

Elution profile for nerve extract and synthetic \(^{125}\)I-CGRP.

The elution profile of CGRP immunoreactive material derived from either pooled distal segments or pooled proximal segments from injured sciatic nerve (figure 2.4a) was virtually identical to that of synthetic \(^{125}\)I-CGRP (figure 2.4b).

CGRP immunohistochemistry in lesioned sciatic nerve.

CGRP immunoreactivity was clearly observed within a subset of axons found approximately 6mm distal to the crush site (figure 2.5).

CGRP expression after sham lesion versus control unlesioned sciatic nerve.

There was no significant variation (p=0.1) in the amount of CGRP expressed between any of the 5mm segments of sciatic nerve analysed following a sham operation, measured by RIA (figure 2.6). However, there was a significant elevation (p=0.001) in CGRP content in the nerves both ipsilateral and contralateral after sham operation compared to the amount of CGRP in the sciatic nerve of an unoperated animal (figure 2.6).
Figure 2.4: Elution profile of CGRP–immunoreactivity (a) extracted from proximal and distal segments of crushed sciatic nerve and (b) synthetic $^{125}$I–CGRP. Fractions 8–30 (1ml/tube) of nerve extract were measured for CGRP content by RIA. The elution profile of $^{125}$I–CGRP was determined by measuring the level of radioactivity in each fraction eluted and converted to fmol.

$V_o$, void volume; $V_T$, total volume.
Figure 2.5: Staining of CGRP-like immunoreactivity in the axons 6mm distal to the crush site of a 6 days post-crush rat sciatic nerve. Scale bar = 45µm
Figure 2.6: Amount of CGRP in 5mm nerve segments sham operated (P₂P₁D₁D₂), contralateral (C.*) and from unoperated (C) rats determined by RIA. In this and following histograms (figures 2.6 to 2.12) each bar represents the mean +/- s.e.m for 4 animals.

* The amount of CGRP in segment C is significantly below the amount CGRP measured in any of the nerve segments of sham operated rats, measured by students t-test (p=0.001).

- sensitivity of the assay
CGRP expression after sciatic nerve lesion.

Changes in the level and distribution of CGRP in a defined length of sciatic nerve (see figure 2.1) at several time points following lesion were quantified using the RIA. Results are presented in the following order:

1) Contralateral nerves examined 1 to 14 days after lesioning;
2) Regenerating nerves examined either 1, 2 and 6 days following crush with forceps or 6, 10 and 14 days following crush with suture;
3) Nonregenerating nerves examined either 1, 2 and 6 days following transection or 1, 6 and 14 days following crush with suture left transection.

Amount of CGRP was measured in 5mm segments of the sciatic nerve both proximal and distal to the lesion site (see figure 2.1 for definition of the different segments analysed).

Contralateral nerve.

Lesioning the sciatic nerve produced a significant increase (p=0.001) in CGRP expression in the contralateral nerve at all time points monitored (figures 2.6–2.10). CGRP levels in the contralateral nerve remained above control, unoperated levels, for up to 14 days after crush and 6 days after transection.

CGRP levels in regenerating sciatic nerves.

1 and 2 days post-crush: When the level of CGRP observed in
nerve segment P₁ was compared to that observed in a similar length of nerve from the contralateral sciatic nerve (C₁), there was a 3 fold increase in CGRP content 1 day post-crush (figure 2.7a). A further increase was seen in P₁ 2 days after crushing (figure 2.7b). A smaller accumulation was observed distal to the lesion (D₁) at both time points which became significant (p=0.001) 2 days following lesion when compared to contralateral levels (figures 2.7a and b). Although CGRP was detectable in P₁ 1 day after crush, it was below that found in the contralateral nerve. However, an increase in CGRP significantly above the level in the contralateral nerve segment was seen in P₁ 2 days after crush (figure 2.7b).

6 days post-crush: A shift in the location of the peak of CGRP going from proximal to distal to the lesion site was observed (figure 2.7c) after the nerve had been crushed with forceps. The level of CGRP was significantly higher in D₁ than in P₁ (p=0.001). When a suture was used to crush the nerve, the shift in CGRP from proximal to distal was not as great as in forceps-crushed nerves (figure 2.8a). This result indicated that the suture caused greater damage to the axons and therefore, sutures were used to crush the nerves in subsequent experiments. When the level of CGRP in nerve segments P₁ to D₁ was totalled, a reduction from a peak of 6342 ± 355fmol 2 days after crush to 3544 ± 92fmol 6 days after crush was observed.
CGRP LEVELS IN REGENERATING SCIATIC NERVES CRUSHED WITH FORCEPS.

(a) 1 Day post-crush.

(b) 2 Days post-crush.

(c) 6 Days post-crush

Figure 2.7: Amount of CGRP found in 5mm sciatic nerve segments at (a) 1 day, (b) 2 days and (c) 6 days after crush injury with watchmaker forceps.
Figure 2.8: Amount of CGRP in 5mm nerve segments from regenerating sciatic nerve (a) 6 days, (b) 10 days and (c) 14 days after crush with suture.

* Amount of CGRP in D₃ is significantly above that found in any of the other segments 10 days post-crush, measured by students t-test (p=0.001)
10 and 14 days post-crush: There was an overall reduction in the level of CGRP measured in the segments of sciatic nerve P; to D; 10 and 14 days post-crush (figures 2.8b and c). However, the amount of CGRP present in the segment furthest distal to the crush (D,) was significantly above the amount measured in any of the other segments, 10 days after lesion (p=0.005) (figure 2.8b). However, by day 14, CGRP levels had returned to contralateral levels in all segments examined, with no significant difference (p=0.05) between any of the segments from the crushed nerve (figure 2.8c).

**CGRP levels in nonregenerating sciatic nerves.**

When the suture was used to block nerve regeneration, a significantly lower level (p=0.01) of peptide was found in D,, 2 days post-crush (figure 2.9a) compared to 2 days post-crush with forceps (figure 2.7b). Moreover, CGRP could not be detected in the distal segments at the later time points of 6 and 14 days post-crush. However, a high level of CGRP was still observed in the proximal segment P, at the latter time points (figures 2.9b and c).

Cutting the sciatic nerve produced a 3 fold elevation in CGRP proximal to the lesion site 1 day after transection, which increased to a 4 fold elevation 2 days post-transection (figures 2.10a and b). The amount of CGRP found distally, 1 day after transection, was only slightly below that expressed in the contralateral nerve and not
CGRP LEVELS IN NONREGENERATING SCIATIC NERVES CRUSHED WITH A SUTURE

Figure 2.9 Amount of CGRP in 5mm nerve segments from nonregenerating sciatic nerves (a) 2 days (b) 6 days and 14 days after crush with a suture. CGRP was not detected in D₁ or D₂ at the latter time points as the levels were below the sensitivity of the RIA. (- sensitivity of the assay)
Figure 2.10: Amount of CGRP in 5mm sciatic nerve segments (a) 1 day, (b) 2 days and (c) 6 days after transection. CGRP was not detected in D₁ 6 days post-crush as the level was below the sensitivity of the RIA. (— sensitivity of the assay)
significant (p=0.1). However, the level of CGRP in D, 2 days after transection, had declined to levels significantly below that found in the contralateral nerve (p=0.001) and it became undetectable distal to the cut, 6 days following the injury (figure 2.10c). Although the amount of CGRP in P, 6 days after transection was significantly above that in the contralateral nerve, it was significantly lower (p=0.001) than the amount of CGRP found in P, of nonregenerating sciatic nerves 6 days after crush with suture (figure 2.9b).

**Total CGRP levels in lesioned nerve.**

The total amount of CGRP expressed over the area of P, to D, declined at a much faster rate in transected nerves compared to all other types of lesions (figure 2.11). The total level of CGRP throughout this area of sciatic nerve, 6 days after lesion, was significantly greater (p=0.001) in nerves which had the suture left attached after crushing compared to transected nerves (figure 2.11). When a suture was left attached for 14 days then the levels of CGRP in the sciatic nerve (P, to D,) declined to similar levels found in a 6 day post-transected nerve.

**VIP-like immunoreactivity in lesioned sciatic nerve.**

Sciatic nerve lesion caused an accumulation of VIP-like immunoreactivity proximal and distal to the crush site measured 2 days post-crushed (figure 2.12a). This is compared to levels in the contralateral nerve and
THE EFFECT OF DIFFERENT TYPES OF LESIONS
ON CGRP LEVELS IN THE SCIATIC NERVE.

Figure 2.11: Total amount of CGRP found in sciatic nerve segments $P_2 - D_1$ (see figure 2.1) following sciatic nerve lesioning.

$n = 4$ for each method of lesion.
Figure 2.12: Amount of VIP immunoreactivity in 5mm nerve segments from regenerating sciatic nerves (a) 2 days, (b) 6 days and (c) 14 days after crush with suture.

The amount of VIP in segments C, C', P', D', and D in (a) and P' and C in (b) and C in (c) was below the sensitivity of the RIA. No value is given for D' in (b) since the sample slopes were not parallel with the standard slope. (—sensitivity of assav)
unoperated nerve, which were below the sensitivity of the assay. The level found proximal to the crush ($P_1$) was significantly higher than the level distal to the crush ($D_1$) ($p=0.001$). A shift in the peak of VIP-immunoreactivity from proximal to distal to the crush was observed 6 days after crushing (figure 2.12b). However, this was not significant until 14 days after crushing ($p=0.001$) (figure 2.12c).

**Gamma interferon-like immunoreactivity in the sciatic nerve.**

Gamma interferon (IFNγ)-like immunoreactivity was clearly visible in a subset of axons in the sciatic nerve 2 hours post-crush (figure 2.13a). Furthermore, the staining intensity, distal to the crush site was observed a much higher level 2 days after crush (figure 2.13b). No IFNγ-like immunoreactivity was, however, visible in the axons distal to the crush site, 3-6 days post-crush. However, a low level of IFNγ-immunoreactivity was seen distal to the crush site, 18 days post-crush (figures 2.14a and b).
Figure 2.13: Staining of DB-1 immunoreactive axons in the sciatic nerve (a) 2hrs and (b) 2 days post-crush. The sections are 2mm distal to the crush. Scale bar = 45μm
Figure 2.14: Staining of DB-1 immunoreactive axons in the sciatic nerve (a) 6 days and (b) 18 days post-crush. The section is 2mm distal to the crush. Scale bar = 45μm
CGRP AND cAMP ACCUMULATION IN MACROPHAGES.

The following experiments were performed in order to determine whether CGRP accumulated after sciatic nerve crush has the ability to interact with macrophages recruited into the nerve after injury. CGRP was also added to cultures of spleen and peritoneal macrophages and its affect on intracellular cAMP was measured.

Effect of CGRP on cAMP accumulation in peritoneal macrophages: Resident vs Activated.

The time course of cAMP accumulation by peritoneal macrophages in response to CGRP stimulation is shown in figure 2.15. The time course was not significantly altered by the state of macrophage activation. The peak of cAMP production was reached after approximately 1 minute after CGRP addition and dropped to around 40% of the maximum at approximately 5 minutes. A significant lower level (p=0.005) of cAMP was seen in macrophages activated in vivo with LPS compared to resident macrophages, 15 minutes after the addition of CGRP.

Resident peritoneal macrophage cAMP accumulation was stimulated by CGRP in a dose-response manner, in the presence or absence of IBMX (figure 2.16). A significant increase in cAMP accumulation by peritoneal macrophages, occurred with the lowest dose (1nM) of CGRP. Furthermore, the presence of IBMX enhanced the accumulation of cAMP.
TIME COURSE OF cAMP ACCUMULATION BY
ACTIVATED AND RESIDENT PERITONEAL MACROPHAGES

Figure 2.15: Time course of CGRP stimulated cAMP accumulation by peritoneal macrophages. Macrophages were incubated with IBMX (10^{-5} M, 37°C) for 30min prior to the addition of CGRP (10^{-6} M). Culture medium was removed and 70% ethanol added to the cells at different time points after CGRP addition. cAMP levels were determined by RIA. Each time point represents the mean and S.D. for duplicate wells of macrophages.

* p = 0.005
EFFECT OF CGRP ON cAMP ACCUMULATION BY
ACTIVATED AND RESIDENT PERITONEAL MACROPHAGES

Figure 2.16: Dose–response curves of CGRP stimulated

cAMP accumulation by activated and resident peritoneal

cells, in the presence or absence of IBMX \(10^{-5}\) M. 

Experiments were performed as explained in the legend
to figure 2.15, except the medium was removed and the
cells extracted 2 minutes after CGRP addition. Each point
represents the mean and S.D. for duplicate wells of
macrophages. The experiment was repeated twice, with
similar results (data not shown).
after 2 minutes of stimulation (figure 2.16). Activation of the macrophages in vivo, with LPS (50μg), decreased their ability to accumulate cAMP in response to the addition of CGRP; even in the presence of IBMX the response was significantly reduced (p=0.005) when CGRP at a concentration of 10^-M or greater was added (figure 2.16). In vitro treatment of resident peritoneal macrophages with 300U/ml of IFNγ, 2 hours before addition of CGRP, caused a significant reduction (p=0.005, p=0.001) in accumulation of cAMP in response to the addition of 10^-7 and 10^-4 CGRP respectively (figure 2.17).

**Spleen macrophage production of cAMP in response to CGRP.** The peak production of cAMP in spleen macrophages occurred 5 minutes after addition of CGRP (10^-4), which was slightly later than that of peritoneal macrophages (figure 2.18). The presence of IBMX did not alter the time course of the peak. However, the levels of cAMP produced after the peak were maintained at a higher level in the presence of IBMX. Spleen macrophages demonstrated a more acute dose dependent response to CGRP (figure 2.19) compared to peritoneal macrophages (figure 2.16). However, this response was blocked if these cells were pretreated for 2 hours with CGRP (figure 2.20). Similarly, the spleen macrophage cAMP response to CGRP was slightly depressed if they were pretreated with IFNγ for 2 hours (figure 2.21). However, the level of inhibition was much less compared to that obtained with peritoneal macrophages.
Figure 2.17: Dose–response curves of CGRP stimulated cAMP accumulation by peritoneal macrophages, in the presence of IBMX ($10^{-6}\text{M}$), with or without a 2 hour preincubation with gamma interferon (300U/ml).

Experiments were performed as explained in the legend to figure 2.15, except cells were extracted 2 minutes after CGRP addition. Each point represents the mean and S.D. for duplicate wells of macrophages.

The experiment was repeated twice, with similar results (data not shown).

# $p=0.005$, * $p=0.001$ (measured by students t-test)
TIME COURSE OF cAMP ACCUMULATION BY SPLEEN MACROPHAGES.

Figure 2.18: Time course of CGRP (10^-6 M) stimulated cAMP accumulation by spleen macrophage in the presence or absence of IBMX (10^-5 M). Experiments were performed as explained in the legend to figure 2.15. Each time point represents duplicate wells of macrophages and shows the mean and S.D. The experiment was repeated with similar results (data not shown).
Figure 2.19 Dose–response curves of CGRP stimulated cAMP accumulation by spleen macrophages, in presence or absence of IBMX (10⁻⁵M). Experiments were performed as explained in the legend to figure 2.15, except cells were extracted 5 minutes after CGRP addition. Each point represents the mean and S.D. for duplicate wells of macrophages. The experiment was repeated, with similar results (data not shown).

* p=0.001, # p=0.005
EFFECT OF CGRP PREINCUBATION ON THE cAMP RESPONSE BY SPLEEN MACROPHAGES TO CGRP

Figure 2.20: Dose-response curves of CGRP stimulated cAMP accumulation by spleen macrophages, in the presence of IBMX (10^{-5} M) and with or without a 2 hour preincubation with CGRP (10^{-6} M). Experiments were performed as explained in the legend to figure 2.15, except cells were extracted 2 minutes after CGRP addition. Each point represents the mean and S.D. for duplicate wells of macrophages. The experiment was repeated twice, with similar results (data not shown).

* p=0.001 (measured by students t-test.)
Figure 2.21: Dose–response curves of CGRP stimulated cAMP accumulation by spleen macrophages, in the presence of IBMX (10⁻⁵ M), with or without a 2 hour preincubation with gamma interferon (300U/ml). Experiments were performed as explained in the legend to figure 2.15, except cells were extracted 5 minutes after CGRP addition. Each point represents the mean and S.D. for duplicate wells of macrophages. The experiment was repeated twice, with similar results (data not shown).

* p=0.001
The response of peripheral nerve macrophages.

Incubation of macrophages isolated from lesioned sciatic nerves with CGRP stimulated a accumulation of cAMP, in a dose dependent manner (figure 2.22). However, this was only seen in the presence of IBMX (figure 2.22). Macrophages isolated from unlesioned sciatic nerves also demonstrated a dose-dependent accumulation of cAMP when stimulated with CGRP (figure 2.23). Furthermore, the level of cAMP accumulated by macrophages from unlesioned nerve, after the addition of CGRP (10⁻⁴), was greater than the level accumulated by macrophages from lesioned sciatic nerve (figures 2.22 and 2.23).
EFFECT OF CGRP ON cAMP ACCUMULATION BY MACROPHAGES ISOLATED FROM CRUSHED SCIATIC NERVE

Figure 2.22: Dose–response curves of CGRP stimulated cAMP accumulation by macrophages isolated from crushed sciatic nerve, in the presence or absence of IBMX (10^{-5} M). Experiments were performed as explained in the legend to figure 9, except cells were extracted 5 minutes after CGRP addition. Results are means with s.e.m, n=4 wells of macrophages.
Figure 2.23: Dose–response curves of CGRP stimulated cAMP accumulation by macrophages isolated from unlesioned sciatic nerve, in the presence of IBMX (10^{-5} M). Experiments were performed as explained in the legend to figure 2.15, except the cells were extracted 5 minutes after CGRP addition. Each point represents the mean and S.D. for duplicate wells of macrophages. The experiment was repeated with similar results (data not shown).
DISCUSSION.

This study has provided a detailed time course for the expression of both CGRP and VIP following sciatic nerve injury. It has shown that the rise in CGRP and VIP-like immunoreactivity around the injury site, previously observed immediately following injury (Kashihara et al., 1989; Lundberg et al., 1981;) remains above control levels for up to 14 days. CGRP produced in motoneurons in response to injury (Streit et al., 1989; Arvidsson et al., 1990; Noguchi et al., 1990) is anterogradely transported at about 1mm/hr (Kashihara et al., 1989). The movement of the peak of CGRP, observed by RIA, corresponded with the rate of axon regeneration of 2-4mm/day (reviewed by Terzis and Smith, 1990). Furthermore, the axonal-like distribution of CGRP, shown by the immunohistochemical study, suggests that CGRP is transported down the axons and accumulates at the tips of the regenerating axons. Also, the disappearance of CGRP in the distal stump soon after injury, if regeneration was prevented, suggests that CGRP is not produced by non-neuronal cells in response to injury. The initial accumulation of CGRP seen in the distal stump was probably due to a small amount of retrograde axonal transport.

The accumulation of CGRP observed immediately proximal to the lesion site 1 to 2 days following a crush injury, is consistent with the temporal changes in CGRP-like immunoreactivity and CGRP mRNA in the ventral horn
following injury (Arvidsson et al, 1990; Noguchi et al, 1990). Furthermore, the gradual decline in CGRP peptide levels in the sciatic nerve, back to control levels after it had peaked at 2 days, is also observed for both mRNA and peptide for CGRP in the ventral horn (Dumoulin et al, 1991; Arvidsson et al, 1990).

The level of CGRP observed in a length of 15mm which includes the lesion site, appears to be influenced by the direct contact of the distal stump with the proximal stump. Removal of the distal segment of sciatic nerve by transection led to a more rapid decrease in the amount of CGRP measured over the 15mm length of nerve, compared to when the distal segment remained attached. Since the presence of the distal stump is thought to enhance regeneration (Williams et al., 1984; Scaravilli, 1984), it is possible that this influence of the distal stump upon regeneration is connected to its ability to maintain CGRP expression. Alternatively, it is possible that transmission of action potentials down the sciatic nerve triggers the release of CGRP from the newly formed terminals, and since the distal stump is not attached to the proximal stump after the nerve is severed, CGRP released from the terminals would not be contained within the epineurium. Thus, contact with the distal stump in the case of crush, even with the suture left attached, would prevent such release.
The increase in CGRP in contralateral sciatic nerve compared to unoperated sciatic nerve may reflect increased usage of the contralateral leg and hence increased muscle activity. It is possible that this rise in CGRP is important in modulating the increased muscle activity, since early studies show that CGRP modulates the contraction of striated muscle (Takami et al., 1985). The small increase observed in the ipsilateral nerve after sham operation may reflect the manipulation of muscle whilst exposing the nerve.

Although VIP-like immunoreactivity within the sciatic nerve is also maintained above control levels several days after crush, it is not expressed at such a high level as CGRP. This may reflect a smaller number of VIP expressing neurons in comparison to CGRP expressing neurons. The change in distribution of VIP-like immunoreactivity with time also appears to correlate with the advance of regenerating axons, but at a slower rate than CGRP positive axons. Furthermore, Giachetti and Said (1979) have shown that, if the sciatic nerve is prevented from regenerating, then VIP continues to accumulate just proximal to the lesion site for at least 72 hours. This is slightly later than that found for CGRP which peaked at 2 days, under nonregenerating condition. A further difference between the production of CGRP and VIP following peripheral nerve injury is that mRNA and peptide for VIP increases in the DRG (Neilsch and Keen, 1989), in addition to an increase in
peptide for VIP in the dorsal horn (McGregor et al., 1984; Shehab and Atkinson, 1986). The mRNA and peptide for CGRP does not increase in the DRG, although it does increase in the motoneurons, as discussed above. The different time course for VIP expression in the sciatic nerve compared to the CGRP time course, is consistent with a slower response in VIP mRNA production in the DRG compared to the production of CGRP mRNA in the motoneurons (Nielsch an Keen, 1989; Dumoulin et al., 1991).

Since both CGRP and VIP are normally thought to be released at the nerve terminals, and have neurotransmitter-like properties, it was intriguing to find that both peptides are up-regulated and maintained at elevated levels for a period of time following injury. This raises the possibility that they have a role in peripheral nerve regeneration.

There is evidence to suggest that the initial rise in CGRP expression, shown in this study to have remained elevated in the distal stump for at least 14 days after crush, is an important permissive signal for the wave of Schwann cell proliferation in the distal segment following injury. This is thought to be via CGRP mediated elevation of cAMP within Schwann cells (M.Khan, pers.comm.). CGRP may be released from the regenerating axon tips, triggered by action potentials travelling down the nerve. The CGRP would then be able to interact with the Schwann cells in the
A recent study describing a biphasic time course for CGRP expression following nerve injury supports a role for CGRP in both regeneration and at the reinnervated target (Dumoulin et al., 1991). They describe an early peak in CGRP mRNA within motoneuron cell bodies following facial nerve transection, similar to the results of previous studies (Streit et al., 1989; Noguchi et al., 1990; Arvidsson et al., 1990). However, Dumoulin et al. (1991) also observe a second peak 21 days after injury but only if the nerve is allowed to reinnervate the target (Dumoulin et al., 1991). This second rise in CGRP, which coincides with renewed contact of the regenerating axons at the neuromuscular junction, supports a role for CGRP in peripheral nerve regeneration. Furthermore, the ability of CGRP to prevent disuse-induced sprouting of the motor nerve terminals (Tsujimoto and Kuno, 1988) and to regulate the expression of chick AChR in vitro (Fontaine et al., 1986; New and Mudge, 1986) supports the importance of CGRP for successful reconnection to the target tissue.

This thesis has provided evidence for the ability of CGRP to regulate macrophage function, as well as Schwann cell proliferation (M.Khan, pers.comm.), via activation of adenylate cyclase and production of cAMP. Macrophage recruitment is one of main events which occurs after...
sciatic nerve injury and is important for regeneration, as discussed in chapter one. The data presented in this chapter showed that CGRP produced an accumulation of cAMP within macrophages isolated from both normal and injured sciatic nerve. This effect on macrophage cAMP levels, recently shown in peritoneal macrophages (Vignery et al., 1991), has important implications for the regulation of the macrophage response following peripheral nerve injury; such elevation of cAMP inhibits phagocytosis and IFNγ-induced major histocompatibility (MHC) class II gene product expression (Figueiredo et al, 1990; Newman et al, 1991). Furthermore, since regulation of IL-1 expression by macrophages is influenced by their cAMP levels (Ohmori et al, 1990), it is possible that CGRP may be important during the IL-1 dependent stage of NGF production after peripheral nerve injury (Lindholm et al, 1987).

Although CGRP inhibits macrophage function in vitro (Nong et al, 1989), its ability to do so may depend upon their state of activation. It is clear that CGRP did not elevate cAMP levels within activated peritoneal macrophages to the same extent as in resident peritoneal macrophages. Similarly, macrophages isolated from injured vs normal sciatic nerve are possibly in an activated state and this, therefore, may be the reason why CGRP failed to produce the level of cAMP accumulation observed in resident macrophage populations. Alternatively, macrophages isolated from injured sciatic nerve may not respond so well to exogenous
CGRP since they could have previously encountered CGRP secreted from damaged axons. This is supported by the observation that preincubation of spleen macrophages with CGRP inhibited elevation of cAMP levels when CGRP was added to the cell cultures.

The ability of IFN\(\gamma\) to reduce the level of cAMP accumulated in peritoneal and spleen macrophages in response to CGRP may reflect the in vivo situation of lesioned peripheral nerve. The IFN\(\gamma\) present in the axons of the sciatic nerve may be released from the damaged axons and may influence the state of macrophage activation. Whilst in such an activated state, they phagocytose and break down myelin, removing debris which would otherwise hinder regeneration. During the first two to three days following injury, CGRP present in the peripheral nerve may not be able to influence macrophage function, as the macrophage activating factor, IFN\(\gamma\), is still present, and may maintain the macrophages in an activated state. However, the disappearance of IFN\(\gamma\), as early as 3 days after crush, may allow CGRP released from the regenerating axons to influence macrophage function via elevation of cAMP.

VIP-like immunoreactivity in lesioned peripheral nerve may have a similar role to CGRP since it has been shown to increase cAMP within blood monocytes (Wiik, 1989). The secretion of both CGRP and VIP from the axons, as they
regenerate back to the target, may be important in preventing the inflammatory response progressing into an autoimmune type peripheral neuritis.

Macrophages play a central role in the immune response, acting as antigen presenting cells (APC) during the induction stage of an immune response. They are also an important component of the nonspecific arm of the immune system, due to their powerful phagocytic and secretory activities. Their production of an extraordinary variety of biologically active substances, such as the cytokines IL-1 and tumour necrosis factor (TNF), and acute phase proteins provides a means by which they can contribute and regulate many aspects of the immune system. Therefore, the ability of CGRP in addition to other neuropeptides e.g. VIP, to regulate cAMP levels of macrophages has important implications for the regulation of both an inflammatory and immune response. The observation of CGRP immunoreactive nerve-like profiles within certain lymphoid organs including the spleen (Bellinger et al., 1990), together with the finding of this thesis that CGRP regulated cAMP levels of spleen macrophages, provides further evidence for neuroimmune interactions.

Innervation of both primary and secondary lymphoid tissues and the close association of nerve terminals with cells of the immune system e.g lymphocytes, suggests that released neurotransmitters may interact with surrounding
cells (Felten et al., 1985; Felten et al., 1987; Felten and Olschowska, 1987; Felten and Felten, 1990). The presence of numerous neuropeptides including neurotensin SP, VIP and CGRP, in nerve-like profiles in primary and secondary lymphoid organs may also indicate sensory and sympathetic innervation and neurotransmission by neuropeptides (Felten et al., 1985; Weihe et al, 1989; Lorton et al, 1990; Lorton et al, 1991; Bellinger et al, 1990). Substance P positive fibres have been described to enter the spleen in the hilar region and arborize along the venous sinuses. Such fibres are found in the white pulp, in the marginal zone, and in the outer regions of the periarteriolar lymphatic sheath (PALS) (Lorton et al., 1991). A similar pattern for noradrenergic innervation is also observed in the spleen (Ackerman et al., 1989). Nerve fibres showing positive immunoreactivity for CGRP have been observed along the marginal zone of the white pulp of the spleen, and in the thymus (Bellinger et al., 1990; Weihe et al., 1989). Such fibres are found in the capsule and intralobular septa of the thymus.

Neuropeptide or neurotransmitter regulation of the immune cells may be via direct communication at the cell surface, if the appropriate receptors are expressed. Receptors for both VIP and CGRP have previously been described on T lymphocytes (Ottaway et al., 1984; Umeda and Arisawa, 1989). Furthermore, both neuropeptides inhibit the proliferation of T-lymphocytes in response to concanavalin
A (Boudard and Bastide, 1991). Alternatively, neuropeptides or neurotransmitters may be able to regulate the immune response by influencing other cells in the environment in which the immune cells are situated, thus exerting their effects indirectly.

The data presented here supports a role for CGRP in nerve regeneration, regulating one of the most important cells of the inflammatory and immune response i.e macrophages, which enter following injury. The ability of CGRP to regulate macrophage properties, via elevation of cAMP has important consequences for nerve regeneration. If the macrophage response is not appropriately controlled then any attempt to form new myelin could be continuously disrupted due to its removal via phagocytosis by the recruited macrophages. Moreover, if class II MHC antigen expression, important for presentation of exogenous antigens, is not regulated then myelin phagocytosis could lead to the inappropriate presentation of myelin proteins in association with class II MHC antigens. Such presentation of self antigens could ultimately produce an autoimmune type response, as in experimental allergic neuritis. This would obviously be detrimental to any attempt made by the peripheral nervous system to regenerate.
CHAPTER 3

DISTINCT MACROPHAGE PHENOTYPES
IN LESIONED VERSUS UNLESIONED SCIATIC NERVE.
Introduction.

There is now convincing evidence that the infiltration of macrophages into a peripheral nerve, in response to injury, is essential for the normal degeneration and regeneration of peripheral nerves. The removal of myelin debris by phagocytosis, and the release of cytokines such as IL-1 are two of the known roles that macrophages play in degeneration and regeneration, respectively (Beuche and Freide, 1984; Lindholm et al., 1987). Beuche and Freide (1984) demonstrated that if peritoneal macrophages are prevented from entering a sciatic nerve segment implanted into the peritoneal cavity, then one fails to see the usual breakdown and phagocytosis of myelin. Furthermore, the Schwann cells fail to proliferate in response to the lesion. Subsequently it was shown that, following transection of a peripheral nerve, there is a rapid infiltration of monocytes to the distal nerve segment (Perry et al., 1987).

Some of the most recent data supporting the importance of macrophages in peripheral nerve degeneration has been provided by the discovery of an unusual mouse strain, C57Bl/6/Olac, (Lunn et al., 1989; Perry et al., 1990a and 1990b). It was found that following injury, the sciatic nerve of C57Bl/6/Olac mice degenerate at a slow rate compared to other mouse strains (Lunn et al., 1989). This was subsequently found to correlated with the absence of
the normal recruitment of macrophages into peripheral nerves. Further studies with the C57Bl/6/Olac mouse strain have shown that a property of the nerve is important in macrophage recruitment (Perry et al., 1990a).

It would appear that macrophages are not only important for nerve degeneration but that they also contribute to certain events of peripheral nerve regeneration. In particular, macrophages appear to influence the production of NGF in non-neuronal cells via an IL-1 mediated pathway (Lindholm et al., 1987). More recently, Brown et al (1991) found that mRNA levels for both NGF and its receptor only increased slightly after axotomy of the sciatic nerve in the C57Bl/01ac mouse when compared to normal and this correlated well with an impaired sensory nerve regeneration.

Further support for the importance of macrophages in peripheral nerve regeneration is provided by Baichwal et al. (1988). They showed that conditioned medium from peritoneal macrophages treated with a myelin membrane fraction could produce a mitogenic signal for cultured Schwann cells. Furthermore, Schwann cells in the nerves of C57Bl/6/Olac mice failed to proliferate to the same extent as in normal mice (Lunn et al., 1989).

The majority of the published studies which have analysed macrophage recruitment into peripheral nerves after injury
have used the mouse. Furthermore, macrophages expressing the phenotypic marker myeloperoxidase (myelomonocytic cells) have often been analysed in the mouse. However, there has been, to my knowledge, no documented data describing similar macrophages in the peripheral nerve of the rat following peripheral nerve injury.

The discovery in the rat of different macrophage phenotype markers, the ED protein series, has provided a method of carrying out more detailed studies of the distribution of macrophage phenotypes in many rat tissues including the peripheral nervous system.

The existence of macrophage subpopulations, defined by the monoclonal antibodies ED1, ED2 and ED3, was originally described by Dijkstra et al. (1985). They observed that macrophages recognised by the ED monoclonal antibodies had a preference for definite compartments within the lymphoid organs, for example, the cortex of the thymus stained conspicuously with ED2, whereas the medulla was completely negative for ED2.

Although ED1-positive macrophages have previously been visualised during Wallerian degeneration (Stoll et al., 1989) there is no data, to date, describing the different subpopulations within the peripheral nervous system before and/or after injury. Since macrophage phenotypes have been found to vary in different environments it is important to
characterise macrophage phenotypes which may be present in injured vs uninjured peripheral nerves.

Several monoclonal antibodies which recognise monocyte/macrophage markers and phenotypes such as the ED monoclonal antibody series (ED1-3) were used to characterise macrophage phenotypes in both normal and lesioned peripheral nerves. This study also used the macrophage markers to follow the time course and extent of recruitment into the damaged nerve. Furthermore, incorporation of bromodeoxyuridine (BrdU) into dividing cells, in vivo, was used to study whether macrophages found in lesioned peripheral nerves contribute to the population of cells which proliferate following injury to peripheral nerves. Finally, using a polyclonal antibody to S100, a calcium binding protein found in Schwann cells and frequently used as a phenotypic marker for these cells, the distribution of S100 positive cells was correlated with the distribution of macrophages before and after lesion.
MATERIALS AND METHODS.

Animal Surgery.

Male adult Sprague-Dawley rats were anaesthetized with halothane and the sciatic nerve exposed at mid-thigh level. The nerve was crushed with a silk suture (2/0 Mersilk). At various times ranging from 3-18 days postoperative, the animals were killed and a segment of sciatic nerve, approximately 12-15mm in length, was removed. Sciatic nerves from B10 strain mice were also crushed as described for rats and the nerves removed after 2 or 6 days.

Tissue preparation.

After dissection, the nerves were immediately immersed in 4% paraformaldehyde (w/v) in PBS, pH 7.4 and fixed overnight at 4°C. The following day they were transferred to 30% sucrose (w/v) in PBS for up to 2 hours to prevent crystal formation within the tissue. The tissue was mounted in OCT embedding medium (Tissue-tek, Miles) and stored in liquid nitrogen until sectioned on a freezing microtome (Bright).

BrdU labelling.

Sprague-Dawley rats were injected intraperitoneally with 30mg of bromodeoxyuridine (BrdU) (Sigma) 4 days post-crush. After 1 hour the animals were killed and the sciatic nerve removed and placed in ice-cold 60% (v/v) ethanol in
water, fixing for 20 minutes. The nerve segment was then transferred to 30% sucrose (w/v) in PBS at 4°C and prepared as above.

Immunohistochemistry.

Longitudinal sections of rat sciatic nerve, 10-15μm thick, removed 2 hours, 3, 6 and 18 days post-crush were treated with immunohistochemistry buffer (IHC) for 1 hour. The buffer consisted of 4% horse serum (v/v) (Gibco), 1% Triton-X100 (v/v), 0.01% sodium azide (v/v) and 20mM L-lysine in PBS. The sections labelled with BrdU were treated first with 2M hydrochloric acid (HCl) for 10 minutes and then 0.1M sodium borate (NaB₄O₇), pH8.5, for 5 minutes before adding the IHC buffer. The sections were then labelled with one of a selection of mouse monoclonal antibodies (mcAbs) raised against macrophage antigens and/or BrdU, or rabbit antiserum to S100 protein or a mcAb to the class II MHC antigen (table 1). The primary antibodies (table 1) were applied overnight at 4°C in the IHC buffer. The sections were washed 3 times for 5 minutes each in the same buffer. The second antibody was preadsorbed with 15-20% rat serum (v/v) for 30 minutes and centrifuged to remove precipitate before use. Goat anti-mouse fluorescein-conjugated antibody was used to visualise macrophage or class II labelling. Goat anti-mouse rhodamine conjugated antibody was used to visualise the BrdU label and sheep anti-rabbit rhodamine conjugated antibody to visualise S100. All antibodies were applied for 45 minutes
**TABLE 1: Antibodies used for immunohistochemistry.**

<table>
<thead>
<tr>
<th>ANTIBODY</th>
<th>SOURCE</th>
<th>ANTIGEN</th>
</tr>
</thead>
<tbody>
<tr>
<td>ED1</td>
<td>Dijkstra'</td>
<td>Cytoplasmic/membrane protein</td>
</tr>
<tr>
<td>ED2/ED3/ED7&quot;</td>
<td>Dijkstra'</td>
<td>Membrane protein</td>
</tr>
<tr>
<td>OX42</td>
<td>Seralab</td>
<td>Membrane protein, complement receptor (C3bi)</td>
</tr>
<tr>
<td>OX1</td>
<td>Seralab</td>
<td>Leucocyte common antigen (LCA)</td>
</tr>
<tr>
<td>OX4</td>
<td>Seralab</td>
<td>Membrane, MHC class II antigen</td>
</tr>
<tr>
<td>S100</td>
<td>Sigma</td>
<td>Cytoplasmic protein</td>
</tr>
<tr>
<td>BU20</td>
<td>Magaud et al 1988</td>
<td>Bromodeoxyuridine (BrdU), Nuclear</td>
</tr>
</tbody>
</table>

* Antibodies were a gift from Christine Dijkstra.
** ED7 labels granulocytes
at room temperature. The sections were washed a further 3 times as above and mounted in Citiflour (City University, U.K.). Sections were viewed with a fluorescence microscope (Zeiss Universal).

Histochemistry.

To visualise monocytes which express endogenous peroxidase (myeloperoxidase), frozen sections of rat or mouse sciatic nerve removed 2 or 6 days post-crush were exposed to 3,3'-diaminobenzidine tetrahydrochloride (DAB) and 0.01% hydrogen peroxide (v/v) in either phosphate buffer or Tris-HCl, for 5-6 minutes at room temperature. The sections were then washed in PBS and mounted in glycerol.
RESULTS.

**Myelomonocytic cell distribution in rat and mouse sciatic nerve after lesion.**

A large infiltration of myelomonocytic cells into mouse sciatic nerve, distal to a lesion site, was detected by the DAB/H₂O₂ reaction product catalysed by their endogenous peroxidase (figure 3.1a). In contrast, very few cells expressing endogenous peroxidase were seen in the endoneurium in response to either a cut or crush injury to rat sciatic nerve (figure 3.1b). There was, however, a high density of peroxidase-positive cells in the sheath of rat nerves which was comparable to that seen in mouse nerve sheaths.

**Sciatic nerve lesion and macrophage subtypes.**

*Control unlesioned nerve:* A number of macrophages labelled with either ED3, OX42 or ED2 were observed in the length of nerve endoneurium analysed (12mm) (figure 3.2a,b and 3.3a). Cells expressing the other markers, namely ED1, LCA, and Class II MHC were rarely seen in the endoneurium (figure 3.3b). The nerve sheath contained macrophages which can be labelled with the above markers, in the following approximate proportions: ED2=ED3>OX42>ED1>LCA>ClassII (see also table 2). ED2 and ED3 positive (ED2' and ED3') cells were the predominant macrophage populations in normal nerves and their morphology was either ramified or
Figure 3.1: Staining of myeloperoxidase-positive cells in (a) mouse or (b) rat sciatic nerve, at least 3mm distal to the crush site. The nerves were analysed 6 days post-crush. The diaminobenzidine reaction product appears black. The scale bar = 70μm
Figure 3.2: Staining of (a) ED3' and (b) OX42' macrophages in the endoneurium of an unlesioned rat sciatic nerve. ED3 mAb recognises a membrane antigen and OX42 recognises the C3bi receptor on the cell surface. Scale bar = 45μm
Figure 3.3: Staining of (a) ED2\textsuperscript{*} and (b) ED1\textsuperscript{*} macrophages in the endoneurium of an unlesioned rat sciatic nerve. ED2 mAb recognises a membrane antigen whereas ED1 mAb recognises an antigen located within the cytoplasm, but is also on the cell surface. Scale bar = 45\mu m
TABLE 2: Summary of macrophage staining and distribution within a 12mm length of sciatic nerve.

<table>
<thead>
<tr>
<th></th>
<th>ED1</th>
<th>ED3</th>
<th>ED2</th>
<th>LCA</th>
<th>OX42</th>
<th>ClassII</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Control:</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Endoneurium</td>
<td>occasional</td>
<td>sparse</td>
<td>sparse</td>
<td>occasional</td>
<td>sparse</td>
<td>occasional</td>
</tr>
<tr>
<td>Sheath</td>
<td>v.sparse</td>
<td>sparse</td>
<td>sparse</td>
<td>occasional</td>
<td>sparse</td>
<td>occasional</td>
</tr>
<tr>
<td><strong>2hr post crush:</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Endoneurium</td>
<td>as above</td>
<td>as above</td>
<td>as above</td>
<td>many at crush site</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Sheath</td>
<td></td>
<td></td>
<td></td>
<td>dense</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>3 day post-crush:</strong></td>
<td>Endoneurium</td>
<td>sparse</td>
<td>many</td>
<td>same as ED3 &amp; ED1</td>
<td>same as ED3 &amp; ED1</td>
<td>many</td>
</tr>
<tr>
<td>proximal (3mm)</td>
<td></td>
<td>dense(1.2mm)</td>
<td>many</td>
<td></td>
<td></td>
<td>many</td>
</tr>
<tr>
<td>crush site</td>
<td></td>
<td></td>
<td></td>
<td>ED3 &amp; ED1</td>
<td></td>
<td>many</td>
</tr>
<tr>
<td>distal (6mm)</td>
<td></td>
<td>many</td>
<td></td>
<td></td>
<td></td>
<td>occasional</td>
</tr>
<tr>
<td>Sheath (12mm)</td>
<td>dense</td>
<td>dense</td>
<td></td>
<td></td>
<td></td>
<td>dense</td>
</tr>
<tr>
<td><strong>6 day post-crush:</strong></td>
<td>Endoneurium</td>
<td>as above</td>
<td>as above</td>
<td>as above</td>
<td>as above</td>
<td>as above*</td>
</tr>
<tr>
<td>proximal (3mm)</td>
<td></td>
<td>except, dense at crush site for 2.4mm</td>
<td></td>
<td></td>
<td></td>
<td>as above</td>
</tr>
<tr>
<td>crush site</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>as above</td>
</tr>
<tr>
<td>distal (6mm)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>as above</td>
</tr>
<tr>
<td>Sheath (12mm)</td>
<td>as above*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>as above</td>
</tr>
<tr>
<td><strong>18 day post-crush:</strong></td>
<td>Endoneurium</td>
<td>dense</td>
<td>sparse</td>
<td>sparse</td>
<td>undetected</td>
<td>sparse</td>
</tr>
<tr>
<td>(12mm)</td>
<td></td>
<td>dense*</td>
<td>sparse</td>
<td>sparse</td>
<td></td>
<td>sparse</td>
</tr>
<tr>
<td>Sheath (12mm)</td>
<td>sparse</td>
<td>many</td>
<td>many</td>
<td>sparse</td>
<td>sparse</td>
<td>many</td>
</tr>
</tbody>
</table>

* - less intense.
amoeboid-like, as was the morphology of ED1' cells. The majority of stained cells found in the endoneurium, however, were process-bearing and were close to blood vessels (figure 3.4a and b).

2 hours post-crush: There was no change in the numbers of macrophages at this time point compared with undamaged nerves. There was, however, a large infiltration of granulocytes around the crush site that was visualised with ED7 and also with OX1/LCA monoclonal antibody (mcAb) (figure 3.5a,b and table 2). There were no ED7' cells in the endoneurium, either proximal or distal to the lesion, but there were a surprisingly large number of OX1/LCA' and ED7' cells in the 12mm length of the nerve sheath, either proximal and distal to the lesion (figure 3.6a and b).

3 day post-crush: Large numbers of bloated ED1' macrophages were observed in a 1.2mm area at the crush site (figure 3.7a and table 2), with a gradual decrease in ED1' cells extending 6-8mm distally from the crush site. The ED1, cells further from the crush site were not swollen to the same extent as those at the crush site (figure 3.7b). There were also a number of ED1', process-bearing cells, proximal to the crush site (figure 3.8). The sheath contained a large number of ED1' cells throughout the length of the nerve examined (12mm). The staining with ED3 antibody showed a similar cellular distribution compared to ED1 (figure 3.9a). In contrast to ED1 and ED3, there were fewer ED2'
Figure 3.4: Staining of (a) ED1' macrophages in a blood vessel with (b) the corresponding phase photograph of the endoneurial blood vessel in a rat sciatic nerve. Scale bar = 30μm
Figure 3.5: Staining of (a) ED7* and (b) OX1/LCA* leukocytes found in the endoneurium within 1.2mm of the crush site of a 2 hour post-crush rat sciatic nerve. ED7 mAb recognises a membrane antigen on granulocytes. Scale bar = 45µm
Figure 3.6: Staining of (a) ED7* and (b) OX1/LCA* leukocytes found in the sheath of a 2 hour post-crush rat sciatic nerve. Scale bar = 45μm
Figure 3.7: Staining of ED1' macrophages in the endoneurium (a) within 1.2mm of the crush site and (b) 6mm distal to the crush site of a 3 day post-crush rat sciatic nerve. Scale bar = 45µm
Figure 3.8: Staining of ED1' macrophages found in the endoneurium, 3mm proximal to the crush site of a 3 day post-crush sciatic nerve. Scale bar = 45μm
Figure 3.9: Staining of (a) ED3* and (b) ED2* macrophages in the endoneurium, within 1.2mm of the crush site of a 3 day post-crush rat sciatic nerve. Scale bar = 45μm
macrophages at the crush site or distal to the crush site (figure 3.9b), although large numbers of the ED2' cells were found in the sheath. A number of ED2' process-bearing cells were found proximal to the lesion; these cells were more intensely labelled than those at the crush site (figure 3.10). The OX42 mcAb and OX1/LCA mcAb labelled macrophages with a similar distribution to ED3 and ED1 except that there were very few LCA' cells distal to the crush (figure 3.11a and b). The LCA' macrophages found in the sheath, however, had a different morphology (rounded, lymphocyte-like) than with ED3' or OX42' cells. Class II MHC antigens labelled with OX4 were found on a small number of cells proximal to the lesion with increasing numbers towards the site of lesion, but only a small number of cells expressed class II around the site of crush and distally (figure 3.12); a large number of cells in the sheath expressed class II antigens. Surprisingly, ED7 labelled only a few cells around the crush site at this time.

6 day post-crush: The general distribution of ED1' macrophages had not changed significantly from that of 3 days post-crush (table 2), except the mass accumulation of cells around the crush site extended further distally (2.4mm compared with 1.2mm at 3 days). The distribution of ED3 was similar to that above, but the intensity of the staining had reduced with time. A small number of ED2' macrophages were found at the crush site and distally; there were still a number of ED2' cells in the sheath, but
Figure 3.10: Staining of ED2* macrophages in the endoneurium, 3mm proximal to the crush site of a 3 day post-crush sciatic nerve. Scale bar = 45μm
Figure 3.11: Staining of (a) OX42' and (b) OX1/LCA' macrophages within 1.2mm of the crush site in the endoneurium of a 3 day post-crush rat sciatic nerve. Scale bar = 45μm
less than the number of ED1+ macrophages. OX42 and OX1/LCA labelled cells at the crush site, but like ED1, the staining intensity had decreased. A few OX42 cells were found distant in the site of lesion. Finally, the number of class II positive cells (OX41) was similar to the earlier time point and there were no ED1+ cells found in the nerve sheath (Figure 3.10). There were a small number of small round LCA cells scattered in the endoneurium and in the sheath, but the staining intensity was very low. The OX42 labelling on the macrophages in the endoneurium was virtually undetectable.

Figure 3.12: Staining of OX4/MHC classII+ macrophages in the endoneurium, within 1.2mm of the crush site of a 3 day post-crush rat sciatic nerve. Scale bar = 45μm
less than the number of ED1' macrophages. OX42 and OX1/LCA labelled cells at the crush site, but like ED3, the staining intensity had decreased. A few OX42' cells were found distal to the site of lesion. Finally, the number of class II positive cells (OX4') was similar to the earlier time point and there were no ED7' cells found in the nerve at this time.

18 day post-crush: A large number of ED1' and ED3' macrophages extended throughout the length of the endoneurium examined (12mm) (figure 3.13a,b and table 2). This contrasts with a very low number of ED2' cells in the endoneurium (figure 3.14). The intensity of the ED3' immunoreactivity had decreased further compared with 6 days post-crush. There were, however, brightly labelled ED2' and ED3' macrophages found in the nerve sheath (figure 3.15a,b) but a surprisingly low number of ED1' macrophages in the sheath (figure 3.16). There were a small number of small round LCA' cells scattered in the endoneurium and in the sheath, but the staining intensity was very low. The OX42 labelling on the macrophages in the endoneurium was virtually undetectable.

S100 staining in unlesioned versus lesioned sciatic nerve.

Lesioning the sciatic nerve produced a very disorganised S100 staining pattern after 3 days compared to a normal nerve; this loss of S100 immunoreactivity was observed around the lesion site extending for a short distance
Figure 3.13: Staining of (a) ED1' and (b) ED3' macrophages in the endoneurium of an 18 day post-crush rat sciatic nerve. Scale bar = 45µm
Figure 3.14: Staining of ED2+ macrophages in the endoneurium of an 18 day post-crush rat sciatic nerve. Scale bar = 45\mu m
Figure 3.15: Staining of (A) ED3' and (B) ED2' macrophages in the sheath of an 18 day post-crush rat sciatic nerve. Scale bar = 45μm.
distally after 3 or 6 days post-crush. The lack of S100 staining corresponds with the mass accumulation of EDI' and ED3' macrophages (Figures 3.17a,b and 3.18a,b). S100 at 18 days post-crush is virtually absent in the 12mm of nerve analysed, with only discrete areas of staining observed (Figures 3.17a and b).

BrdU labelling in a crush sciatic nerve.

Figure 3.16: Staining of EDI' macrophages in the sheath of an 18 day post-crush rat sciatic nerve. Scale bar = 45µm
distally after 3 or 6 days post-crush. The lack of S100 staining corresponds with the mass accumulation of ED1' and ED3' macrophages (figures 3.17a,b and 3.18a,b). S100 at 18 days post-crush is virtually absent in the 12mm of nerve analysed, with only discrete areas of staining observed (figure 3.19a and b)

**BrdU labelling in crushed sciatic nerve.**

A large number of BrdU' cells were found around the crush site (figure 3.20a). However, the majority of ED1' macrophages found around the crush site were not BrdU' (figure 3.20b). Only a very small number ED1' macrophages found in the sciatic nerve after injury expressed BrdU. Proliferating Schwann cells were the main cell type expressing BrdU.
Figure 3.17: Staining of (a) ED1' macrophages with (b) the corresponding S100 staining in the endoneurium within 1.2mm of the crush site of a 6 day post-crush rat sciatic nerve. Scale bar = 45μm
Figure 3.18: Staining of (a) ED1' macrophages with (b) the corresponding S100 staining in the endoneurium 6mm distal to the crush site of a 6 day post-crush rat sciatic nerve. Scale bar = 45μm
Figure 3.19: Staining of (a) ED1* macrophages with (b) the corresponding S100 staining in the endoneurium of an 18 day post-crush rat sciatic nerve. Scale bar = 45µm
Figure 3.20: Staining of (a) BrdU* cells and (b) ED1* and Brdu* cells 2mm distal to the crush site of a 4 day post-crush rat sciatic nerve. Scale bar = 45μm
DISCUSSION.

Previous data has suggested that the classical inflammatory response, that of early migration of granulocytes into an inflammatory lesion, is muted in the distal segment of a lesioned peripheral nerve injury (reviewed by Perry and Brown, 1992). My data, however, supports the presence of a classical inflammatory response confined to the crush site. Although a large number of ED7* granulocytes were recruited to the epineurial nerve tissue, both proximal and distal to the crush, they failed to move into the endoneurial tissue. The large number of granulocytes in the epineurium could be caused by increased blood supply to the nerve. Since these cells do not move into the endoneurium, except for an area in and around the crush site (which probably reflects damaged blood vessels) they may not have a direct role in degeneration. Conversely, large numbers of different macrophages phenotypes/subpopulations were found in the epineurium and endoneurium.

The distribution of ED3*, OX42* and LCA* cells in the lesioned sciatic nerve suggests the existence of one population of macrophages expressing the three recognised antigens on the cell surface. This could be further analysed by double labelling. When the expression of the C3bi receptor (OX42) and the leucocyte common antigen (LCA/OX1) was monitored over a period of 18 days after
crush, there was a significant down regulation of both antigens. The expression of the C3bi receptor is related to the adhesive and phagocytic properties of macrophages as well as their activation (Ding et al., 1987; Arnaout et al., 1983; Brück and Friede, 1990). Therefore, it would seem that the observed increase in expression of this membrane protein following axotomy correlates with the requirement to move into the endoneurium after injury and the removal of myelin debris. Moreover, macrophage recruitment is prevented in lesioned mouse sciatic nerve by the antibody '5C6' which, like OX42, recognises the C3bi receptor (Robinson et al., 1986). Down regulation and virtual loss of C3bi receptor expression, demonstrated in this study, may correspond with a decrease in phagocytosis of myelin debris and a change in the activation state of this macrophage subpopulation. Subsequently, the process of regeneration could proceed as myelin phagocytosis is reduced.

The response of macrophages to activation is dependant upon the type of stimulus. For example, macrophages activated by either lipopolysaccharide (LPS) or IFNγ affect gene expression differently (Yu et al., 1990), also thyoglicolate-, peptone-, and con A-elicited macrophages respond differently when IL-4 or IFNγ is added to the cell cultures (Molina and Huber, 1991). It seems that, although the macrophages within an injured nerve are in an activated state as they phagocytose myelin debris, the majority of
macrophages in the endoneurium fail to express class II antigens. This is also observed in the mouse sciatic nerve and the rat optic nerve (Beuche and Friede, 1986; Stoll et al., 1989). In contrast, a large number of class II expressing cells were present in the sheath of lesioned sciatic nerves. The low level of class II expression in the endoneurium may be due to either active inhibition of expression, or alternatively activated macrophages occupied with phagocytosing myelin do not trigger the necessary machinery to cause class II expression. However, the fact that a large number of class II expressing cells are found in the perineurium with only very few in the endoneurium, suggests that they may lose their ability to express class II antigens as they migrate into the endoneurium, or some factor released from the lesioned axons is actively down-regulating class II antigen expression. Such absence of class II expression may be beneficial to the process of nerve regeneration. If the macrophages that are phagocytosing the myelin debris did express class II antigens, then myelin could become processed within the macrophage, with certain myelin proteins eventually presented on the cell surface. This would lead to an immune type inflammatory response and therefore prevent nerve regeneration, since the nerve would be under constant bombardment from the immune system. This may bear some relation to some neurological diseases, such as multiple sclerosis, which are thought to have an immunological aspect to the pathology.
Although a normal sciatic nerve contains more ED3' than ED1' macrophages, lesioning produced a similar accumulation and distribution of both ED1' and ED3' macrophages in the sheath and endoneurium of the sciatic nerve; the mass accumulation of macrophages in endoneurium results from the migration of ED1', ED3', ED2' monocytes from the blood stream. This is supported by the failure to observe significant numbers of dividing macrophages, measured by BrdU labelling. The distribution of ED1' and ED3' macrophages within a lesioned sciatic nerve suggests that the environment of the nerve is influencing macrophage phenotype, causing the expression of ED3 by the cells as they move into the tissue; this supports previous data which suggests that environmental factors influence macrophage phenotype (reviewed by Perry and Brown, 1992). Moreover, since I have shown that the number of ED2' macrophages increases to a lesser extent after lesioning, it is possible that ED3' and ED1' macrophages are more suited to the environment of a degenerating nerve, than is the separate subpopulation of ED2' macrophages. In contrast, no ED3' macrophages are found in the sciatic nerve during the immune mediated model of experimental allergic neuritis (Stevens et al., 1989). However, ED1' and ED2' macrophages are present in increased numbers during experimental allergic neuritis (Stevens et al., 1989). This data brings into question the importance of the ED3' macrophage subpopulation during peripheral nerve injury. It also supports the hypothesis that the environment can influence
macrophage phenotype and that the expression of certain antigens reflect a particular function.

The bloated morphology of all macrophage types around the crush site, compared with a ramified/spindly appearance proximally, probably reflects the phagocytosis of myelin. This is supported by the observation of a reduction in intensity of all ED proteins analysed, on or in the macrophages around the crush site, compared with those found proximally. This may reflect a constant renewal of cell membrane during phagocytosis.

In addition to the ED macrophage phenotypic markers, the presence of myeloperoxidase within monocytes (myelomonocytic cells) is another marker routinely used to detect the presence of macrophages in both mice and rat tissue. However, the data presented in this chapter has shown that the distribution of myelomonocytic cells found in injured sciatic nerves of mice was significantly different compared to rats. Although there was a low number of myelomonocytic cells recruited into the peripheral nervous system in the rat, compared with mouse sciatic nerve, this does not appear to affect phagocytosis and removal of myelin. This brings into question the significance of the large number of myelomonocytic cells found in the mouse sciatic nerve after injury. It is not clear what importance, if any, the presence of myeloperoxidase in these cells has for nerve degeneration.
and regeneration.

The distribution of S100 immunoreactivity observed in this study correlates with the extent of nerve degeneration. S100 protein was originally isolated and purified from bovine and rabbit brains and is used as a marker for cultured Schwann cells (Moore, 1965; Spreca et al., 1989). It is not clear whether the reduction in S100 protein levels observed after injury is related directly to the loss of axonal contact of the Schwann cells or to the action of macrophages. Loss of S100 immunoreactivity occurred within 3 days after nerve lesion and although the loss was restricted to only a small area around the injury site, it coincided with the presence of large numbers of bloated macrophages. As the macrophage staining extended along the nerve with time, so the S100 was observed to decline in the same direction, with only patches of S100 found 18 days after a crush. It is possible that macrophages may cause the decrease in protein either directly, by secretion of a cytokine, or indirectly via disrupting the surrounding environment. Although down regulation of S100 mRNA has been observed after sciatic nerve lesion (De León et al., 1991), there is conflicting data concerning the regulation of S100 expression after injury. Spreca et al. (1989) found that crushing did not affect the intensity or localisation of S100 but a decrease was seen after cutting the nerve. The reason for the discrepancy between the data presented in this chapter as
well as that of Kato and Saroh (1983), with that of Spreca et al. (1989) is not entirely clear. One possibility is that the extent to which the nerve is damaged may be important.

This study has provided evidence for the existence of different macrophage phenotypes/subpopulations within the peripheral nervous system. It has also described how crush injury to the peripheral nervous system alters the distribution of the macrophage phenotypes. Although similar macrophage phenotypes, defined by the ED markers, have also been observed in the central nervous system (Sminia et al., 1987), it still remains unclear as to whether this is a reflection of different macrophage activation states or whether separate macrophage populations perform different functions. Furthermore, the expression of ED3 by recruited macrophages may be important during peripheral nerve injury but appears not be required during immune mediated experimental allergic neuritis (Stevens et al., 1989). Further studies are required to determine the function of the ED3' macrophages following peripheral nerve injury.
Communication between the nervous system and the immune system has been hypothesised for a number of years. The presence of receptors for neuropeptides, along with the identification of nerve fibres within primary and secondary lymphoid tissue support such a hypothesis. This thesis has provided further evidence of communication between elements of the nervous system and the immune system. It has shown how a component of the inflammatory response, produced as a consequence of a peripheral nerve lesion, may be regulated by one of a number of neuropeptides expressed at elevated levels after injury.

Lesioning of a peripheral nerve leads to an inflammatory response that can be split into two major events: 1) the classical inflammatory response at the site of injury with the granulocytes found at the site within hours of lesioning the nerve but with no granulocytes recruited into the endoneurium, distal to the crush; 2) a recruitment of distinct macrophage phenotypes/subpopulations throughout the distal segment of the lesioned nerve, which occurs at a later time point of 3–4 days after injury. This thesis has provided evidence that the neuropeptide, CGRP, observed within a subset of axons in the sciatic nerve, is an important regulatory factor of the macrophage response to peripheral nerve injury. Furthermore, the time course of expression of the lymphokine, IFNγ, provided by this thesis, along with documented evidence that IFNγ influences macrophage recruitment (Mebius et al., 1990; Sethna and
Lampson, 1991), suggests a potential role for IFNγ in macrophage recruitment.

Gamma interferon was thought be expressed only within the immune system. During an immune response it is produced and released by the CD4+ subset of T cells. Its production by certain neurons in the central and peripheral nervous system (Ljungdahl et al., 1989; Olsson et al, 1989; Kiefer and Kreutzberg, 1990) and its transportation along the axons was intriguing. Subsequently it was suggested that IFNγ may be considered as a new member of the neuropeptide family. Moreover, the pattern of IFNγ-like immunoreactive fibres observed within the spleen was found to resemble tyrosine hydroxylase-positive fibres which were previously proposed to be important in neuronal control of immune cell activity. Activation of macrophages by gamma interferon has been known for a considerable period. Increase in both macrophage H2O2 production and MHC class II expression are two of the changes which take place as a consequence of the addition of IFNγ to macrophages. In addition to these direct effects upon macrophages, strong evidence exists to support the above finding that recruitment of both lymphocytes and monocytes/macrophages into injury sites is influenced by IFNγ. Injection of IFNγ into rat popliteal lymph nodes, which has the afferent lymphatic severed, mediated an accumulation of ED1+ macrophages into the operated node (Mebius et al., 1990). A similar study has also shown that intracerebral injection of IFN-γ caused the
recruitment of OX42' macrophages and CD4' T cells into the brain parenchyma (Sethna and Lampson, 1991). The ability of IFNγ to influence lymphocyte traffic (Issekutz et al., 1988) has been shown to involve the regulation of MECA-325, an antigen specific for endothelial cells involved in the above trafficking (Duijvestijn et al., 1986).

Although there is no documented data supporting IFNγ as a chemotactic factor for macrophages, there is data suggesting that IFNγ regulates the expression of adhesion proteins found on macrophage and endothelial cells (Dustin et al., 1986; Blanchard and Djeu, 1991). One such adhesion molecule is intercellular adhesion molecule (I-CAM). During an inflammatory response I-CAM expression on endothelial cells increases. Subsequently it was found that a number of inflammatory cytokines, including IL-1 and IFNγ caused an increase in I-CAM expression on endothelial cells (Dustin et al., 1986). One of the ligands for I-CAM is leukocyte function-associated antigen-1 (LFA-1), also referred to as CD11a/CD18. This antigen appears to be essential for the migration of inflammatory cells into sites of inflammation. The expression of this antigen on macrophages is increased by IFNγ (Blanchard and Djeu, 1991). Therefore, it is possible that IFNγ in the axons is secreted from the damaged axons after nerve injury, thus causing an increase in expression of the above adhesion molecules. This would, consequently, allow the macrophages to migrate into the endoneurium of the sciatic nerve. Since IFNγ-like
immunoreactivity is not restricted to lymphoid organs it is possible that it regulates inflammatory processes in peripheral tissue.

The finding that CGRP can prevent macrophage activation (Nong et al., 1989) supports the hypothesis that it regulates the macrophage response after peripheral nerve injury. Although the simultaneous addition of CGRP and IFNγ to macrophage cultures fails to inhibit macrophage H₂O₂ production, a marker for activation, preincubation with CGRP inhibited the ability of IFNγ to activate the cells (Nong et al., 1989). Since CGRP remained expressed at a high level in the distal stump for a significantly longer period than IFNγ, constant release of CGRP around the macrophages, as the axons regenerate, may lead to the eventual down regulation of the macrophage activation state. Potentially, CGRP could regulate macrophages recruited into the injured nerve via increasing macrophage intracellular cAMP levels. The inhibition of both macrophage phagocytosis and class II MHC expression has been linked with increasing intracellular cAMP (Eppell et al., 1989; Figueiredo et al., 1990; Newman et al., 1991). Moreover, the small number of class II expressing cells in the endoneurium, compared to a large number of such cells in the sheath following trauma, may be related to CGRP expression following peripheral nerve injury. Since IFNγ increases class II MHC expression on macrophages and it was present at elevated levels for a short while after nerve
injury, then it is possible that an inhibitory signal is present in the nerve to prevent over expression of such class II in the endoneurium. This inhibitory signal, like CGRP and VIP, probably acts via an ability to elevate cAMP.

If nerve regeneration is going to be successful then regulation of the inflammatory and immune response could be essential. Regeneration of the peripheral nervous system appears to be one particular situation where regulation of the most influential cell type of the immune system, the macrophage, may be essential for reformation of a peripheral nerve. Furthermore, since macrophages are fundamental to the immune response, then regulation of macrophage function in the nervous system following trauma is probably essential if autoimmunity within the peripheral nervous system is to be prevented. This thesis has provided data supporting the ability of CGRP, present in a lesioned peripheral nerve, to prevent such autoimmunity within the PNS by its capacity to regulate the response of recruited macrophages via elevation of cAMP.

However, CGRP is not only important for regulation of the macrophage response. It also seems to have a role in the Schwann cell proliferative response following injury. Furthermore, with its vasodilatory properties and its ability to regulate the expression of AChR on muscle, CGRP can be classed as a pleiotropic factor. The ability of other neuropeptides, like VIP, to interact with different
cell types including immune cells, expands the regulatory role of neuropeptide secretion from the nervous system. Neuropeptides can no longer be thought of as simple chemical transmitters.
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