BEHAVIOURAL AND MORPHOLOGICAL STUDIES ON EXPERIMENTAL
CHRONIC COMPRESSION OF PERIPHERAL NERVE IN RATS

by
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ABSTRACT

A morphological, behavioural and correlation study was carried out in rats using a method described by Bennett and Xie (1988) to produce neuropathic pain-related behaviour. This involved the placing of multiple loose ligatures around the sciatic nerve.

In this thesis, behavioural tests involving total foot immersion were used to confirm that hyperalgesia, allodynia and possibly spontaneous pain were produced. Section of the saphenous nerve at the time of, or within a week of, tying ligatures resulted in abolition of the early hyperaesthesia, suggesting that this nerve plays an important role in neuropathic pain-related behaviour from 4 to 12 days post-operation.

The morphological study included a qualitative and quantitative investigation of the pathological changes produced within the nerve. It showed a variable amount of nerve fibre damage with a preferential loss of large diameter fibres in most cases and some loss of unmyelinated fibres. The variation is a reflection of the difficulty in tying ligatures with a consistent degree of 'tightness'.

The mechanisms of nerve damage caused by this mild lesion were studied from 8 hours post-operation. Morphological changes indicated that ischaemia played a major role within the first 24-48 hours. The presence of endoneurial oedema results in 'self strangulation' of the nerve, and compression is the overwhelming effect subsequently.

Correlation studies between neuropathic pain-related behaviour and the quantitative assessment of nerve damage were made. Results were consistent with the notion that not only
A-δ and C afferent fibres, but also A-α, A-β afferent and perhaps motor efferent fibres were involved in mediating neuropathic pain-related behaviour.
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I wish to dedicate this work to my wife and daughter and to thank my wife, Teresa, in particular, for her tolerance, encouragement and constant support.
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INTRODUCTION

The use of multiple loose ligatures around the rat sciatic nerve has been proposed as an animal model for the study of causalgia, alldynia and spontaneous pain in man (Bennett and Xie, 1988). Other studies (Attal et al., 1990, Sommer et al., 1993) have confirmed the behavioural changes caused by this procedure.

Pain-related behaviour was examined using a method involving immersion of the whole foot of the rat. This region is innervated not only by the sciatic nerve (the lateral part of the foot), but also by the saphenous nerve (the medial area of the foot).

It is known that after complete interruption of the sciatic nerve, the spread of receptive fields of the saphenous nerve begins within a few days (Devor et al., 1979). The spread of sensitization of polymodal nociceptors from a nearby injury is also found (Fitzgerald, 1979). It was thought likely that the saphenous nerve could play a role in pain-related behaviour since the nearby sciatic nerve is injured by the multiple loose ligatures. Therefore, the role of the saphenous nerve in pain-related behaviour formed a major part of the behavioural study.

When work on this thesis began, little was known of the morphological changes caused by the loose ligatures, the original papers having dealt mainly with the behavioural aspects. Over the last few years a number of morphological studies have been published (Munger and Bennett, 1990; Basbaum et al., 1990; Carlton et al., 1991; Coggeshall et al., 1993). A common observation was the loss of large myelinated fibres. However, there are many other aspects of the pathology of this lesion that have not been addressed in these studies.
This thesis includes an account of the investigation of the initial cause of a severe nerve lesion produced by an apparently mild procedure. The ligatures were tied round the nerve loosely enough not to cause compression of the nerve but sufficiently tightly to restrict blood flow in some epineurial vessels.

A quantitative assessment of nerve damage was made, both to correlate this with the behavioural changes, but also to demonstrate the great variation in amount of damage due to the difficulty in applying ligatures of equal 'tightness'.

The aims of the present study are:

1. To study and quantify the neuropathic painful behaviour caused by placing loose ligatures around the rat sciatic nerve.
2. To study the contribution of the saphenous nerve to pain-related behaviour caused by loose ligatures.
3. To study and quantify the pathology of this lesion.
4. To correlate the quantified pain-related behaviour with quantified morphological changes.
NORMAL STRUCTURE OF PERIPHERAL NERVES

Peripheral nerves of vertebrates consist of one or more fascicles containing bundles of nerve fibres enclosed by a perineurial sheath. The fascicles are embedded in a loose connective tissue called the epineurium. Individual nerve fibres lie within an intrafascicular space, the endoneurium, which is largely composed of longitudinally orientated collagen fibrils and of endoneurial fluid. Other cellular components of the intrafascicular compartment include Schwann cells (about 90% of endoneurial cells), fibroblasts (about 5% or less), endothelial cells, smooth muscle cells and pericytes of endoneurial blood vessels and occasional mast cells and macrophages (Oldfors, 1980).

The perineurium is the sheath enclosing the nerve fibres. It is composed of specialized, flattened, circularly orientated cells forming discrete lamellae, each covered by a basal lamina. The number of lamellae varies directly with the size of the fascicle. In between these lamellae are collagen fibrils which are arranged in circular, longitudinal and oblique orientations. Pinocytotic vesicles are a characteristic feature of perineurial cells, and they suggest the presence of a transport system across the cells.

The perineurium acts as a barrier due to the presence of tight junctions between neighbouring perineurial cells (Thomas, 1963). The presence of a range of phosphorylating enzymes, and high levels of ATPase and creatine kinase also suggests that these cells are able to function as a metabolically active barrier (Shanthaveerappa and Bourne, 1962).
The perineurium is a component of the blood-nerve barrier. Blood vessels in the epineurium are of a non-specialised type, and are freely permeable, whilst those in the endoneurium have tight junctions forming a barrier, similar to but less 'tight', than that in the brain. The perineurial lamellae, also joined by tight junctions, separate these two regions of differing vascular permeability.

**MYELINATED FIBRES**

During development, axons of peripheral nerves grow out from neurons in the central nervous system and ganglia in association with Schwann cells which initially multiply as their associated axons lengthen. Some axons become associated with a single Schwann cell and these are destined to become myelinated fibres. The Schwann cells become spaced out along the axons at intervals of 200-300μm. Myelination begins by elongation of the mesaxon, an extension of the Schwann cell plasma membrane formed by its envelopment of the axon. The elongated mesaxon wraps spirally round the axon, and the membranes compact to form the myelin sheath (Geren, 1954). Mammalian myelinated fibres range in size from about 3-20μm.

Ultrastructurally, the myelin sheath appears as a laminated structure with regular, alternate bands of dense and less dense lines. The major dense lines are formed by apposition of the cytoplasmic surface of the Schwann cell plasma membrane, while the less dense, or intraperiod lines result from joining together of the external surfaces of the membrane. At either end of the territory of a Schwann cell, the myelin lamellae terminate on the axon as a series of opened out loops each containing Schwann cell cytoplasm. The region between the termination of two adjacent myelin sheaths is the node of Ranvier. In larger fibres, the size of the axon
is considerably reduced at the node compared with its cross sectional area in the internodal region.

The myelin sheath is interrupted at intervals along its length by obliquely arranged Schmidt-Lanterman incisures. These are regions at which the myelin lamellae open up at the major dense line to contain small amounts of Schwann cell cytoplasm. Thus a continuous spiral of cytoplasm is formed connecting the cytoplasmic compartments internal and external to the myelin sheath. The number of Schmidt-Lanterman incisures along an internode is directly related to the size of the fibre (Hiscoe, 1947). The incisures are the site of early changes seen in myelinated fibres undergoing Wallerian degeneration when the sheath segments into ovoids.

The internodal length of myelinated fibres is directly related to the size of the fibre; in mammalian nerves the range is from about 200-1500µm, the maximum length being determined by the length of the nerve. For example maximum internodal length in a limb nerve such as the sciatic is much greater than in a short nerve such as the facial.

There is also a relationship between myelin sheath thickness and fibre diameter, large fibres having thicker sheaths than small fibres. The relationship between axon diameter (d) and fibre diameter (D) is expressed as a ratio d/D or 'g' (Schmitt and Bear, 1937). These relationships do not hold true when the internodal length is below the normal range for axon size as is seen at the initial segments of dorsal root ganglion cells (Lieberman, 1976) or in regenerated nerves in adult animals.
THE SCHWANN CELL

The largest concentration of Schwann cell cytoplasm lies in the region of the nucleus; elsewhere along the internode it is present as very attenuated threads of cytoplasm internal and external to the myelin sheath, joined across the sheath through the Schmidt-Lanterman incisures (see above). In the paranodal region a spiral of cytoplasm extends through the opened out loops of myelin as they terminate on the axon, and the Schwann cell itself terminates on the axon at the node of Ranvier in a series of finger-like processes. In large fibres a quantity of cytoplasm occupies the depressions made by the fluting of the myelin sheath in the paranodal region, and these contain large numbers of mitochondria. In fact, in all fibres, the paranodal region can usually be recognised by the increased number of small mitochondria in Schwann cell cytoplasm. The mitochondria are thought to provide a source of energy for the maintenance of the ionic changes that take place at the node of Ranvier in relation to nerve impulse transmission.

UNMYELINATED FIBRES

Unmyelinated fibres consist of a number of axons associated with the processes of a single Schwann cell. The axons lie in depressions of the Schwann cell surface whose processes enclose them, and become apposed near to the surface to form the mesaxon. The axons have a unimodal distribution of sizes with a range from 0.2-3.0μm and a mean of about 1.5μm (Ochoa, 1976). In some nerve preparations Schwann cell processes are clearly distinguishable from axons, but in others they appear rather similar and differentiation is sometimes difficult. Criteria for distinguishing unmyelinated axons from Schwann cell processes include:- their
generally circular contour; their envelopment by the processes of another cell; the absence of rough endoplasmic reticulum or ribosomes; generally, the more conspicuous content of microtubules.

**THE AXON**

The axonal cytoplasm (axoplasm) is a more or less cylindrical extension of the nerve cell body bounded by a cell membrane, the axolemma. The axoplasm contains microfilaments (5-7nm), neurofilaments (8-11nm), neurotubules (hollow cylinders, 23-25nm in diameter), mitochondria, smooth endoplasmic reticulum (SER) and some secondary lysosomes. Dense-core vesicles and coated vesicles may be seen occasionally. Axoplasm lacks ribosomes, rough endoplasmic reticulum and golgi membranes (Landon and Hall, 1976; Thomas and Ochoa, 1984; Thomas, Landon and King, 1992).

The structure of the axoplasm is simple when compared with the cytoplasm of the neuron. The main component is an electron lucent amorphous matrix in which is suspended a population of longitudinally orientated organelles and mitochondria. Microfilaments are thought to be composed of paired helical chains of actin and they possibly have a contractile function. They contribute approximately 10 per cent to the total complement of axonal protein. They are most conspicuous in the growth cones of actively elongating neurites, but are less evident in unmyelinated axons and in the internodal portions of myelinated axons, where they appear to be confined to the cortical zone of axoplasm subjacent to the axolemma. It is suggested that they may have a contractile function and play a part in the mechanisms responsible for intraxon-axonal transport (Schwartz, 1979).
Neurofilaments (NF) are longitudinally orientated and of indefinite length. NF content has been correlated with area of axons in the PNS and CNS (Friede and Samorajski, 1970; Friede, Miyagishi and Hu, 1971; Hoffman, Griffin and Price, 1984; Price, Lasek and Katz, 1990; Gold et al., 1991), in unmyelinated and myelinated fibres and in various regions of the same axon. Under normal conditions NF density (100-300/μm²) remains relatively stable and axonal area changes proportionately to the NF content of an axon (Friede, Miyagishi and Hu, 1971; Berthold, 1978; Price, Lasek and Katz, 1990).

NF's consist of 3 protein subunits which have molecular weights of 68KDa (NF-L), 155 KDa (NF-M) and 200 KDa (NF-H) (Hoffman and Lasek, 1975; Sharp, et al., 1982; Mata et al., 1992). These are synthesized in the cell body and rapidly assembled into polymers (Lasek, Oblinger and Drake, 1983). The 200KDa (NF-H) subunits appear to be associated with the sidearms that can be seen extending from neurofilaments. The sidearms may influence the spacing of filaments, and are also potential sites of phosphorylation. Although NFs are poorly phosphorylated in the neuronal perikaryon, they are highly phosphorylated in axons (Bennett and DiLullo, 1985; Hart, Nuckolls and Wood, 1987; Lee et al., 1987; Pestronk, Watson and Yuan, 1990).

NFs are delivered to the axon in the slow component of axonal transport (Hoffman and Lasek, 1975; Black and Lasek, 1980) at rates of several mm/day. Although neurofilaments appear to be highly stable polymers, it is possible that they are capable of undergoing reversible assembly and disassembly.

Other filamentous structures in the axoplasm are microtubules or neurotubules. They are arranged longitudinally and occur singly or in parallel arrays.
Ultrastructurally they appear to be unbranched 23-25nm hollow cylinders, which according to Bray and Bunge (1981) are 100-800μm in length, although other sources (Thomas, Landon and King, 1992) suggest that they are of indefinite length. They are composed of globular subunits of the protein tubulin. This has been shown to exist in two distinct monomeric forms, α and β, having molecular weights of 57 and 54KDa respectively, each microtubule being composed of helically arranged chains of alternating α and β monomers. The tubulin subunits have a definite orientation, and are arranged so that their (+) ends are away from the cell body. Evidence from studies on the effects of agents causing depolymerisation of microtubules, such as colchicine, led to the idea that these organelles were involved in axonal transport. Later, more direct evidence using special microscopic techniques, showed movement of membrane-bound organelles along microtubules. Theories of fast transport mechanisms involve other force-generating proteins, such as kinesin and dynein, which are discussed later.

The number of microtubules in myelinated fibres varies inversely with axon size, ranging from 30-40/μm² in small axons to 10-15/μm² in large axons. Unmyelinated axons contain a proportionately larger numbers of microtubules, with a density of 50-100/μm² (Friede and Samorajski, 1970). The number of tubules in the terminal branches of some large mammalian axons is many times greater than the number found in the parent axon (Zenker and Hohberg, 1973).

Smooth endoplasmic reticulum (SER) is composed of a mesh of tubules which is thought to extend from the neuron to the distal end of the nerves. The arrangement of SER has been demonstrated by Droz, Rambourg and Koenig (1975) using special high voltage EM and metal impregnation techniques. The axonal endoplasmic
reticulum appears as a continuous system of tubules extending from the perikaryon to the axon terminal. Narrow and wide tubules of SER appear occasionally to be closely apposed to the axolemmal and presynaptic membrane. It is postulated that the exchange of fast axonally transported macromolecules takes place at the contact between the subaxolemmal plate and the SER tubules. At the distal tip of thin tubular branches of SER, synaptic vesicles appear as blebs which may originate either by fission from, or fusion with the SER.

**AXONAL TRANSPORT**

Somatofugal or anterograde transport mechanisms have been the most extensively studied (Schwartz, 1979; Grafstein and Forman, 1980; Ochs, 1982) and have been found to operate at a minimum of two and probably in excess of five different rates which are divided into a slow transport component moving at 0.25-4 mm/day, and a fast transport component which may reach speeds in excess of 400 mm/day (Table I.1). Axonal transport has recently been reviewed by Ochs and Brimijoin (1993).

**Anterograde transport**

*Slow transport*

Slow anterograde transport has two components, component 'a', consisting of tubulin and neurofilament proteins which travel at a speed of 0.25-1 mm/day (Tytell, et al., 1981). Component 'b' consists of actin monomers, soluble enzymes and small oligomers which travel together at a speed of 2-4 mm/day. The neurofilament proteins were thought to be transported in the form of polymerized neurofilaments (Lasek, 1982). Alterations in slow transport produce changes in the axonal cytoskeleton which
may cause alterations in axonal calibre.

*Fast transport*

Fast anterograde transport is energy utilizing and ATP dependent. It transports mainly mitochondria and transmitter storage vesicles (i.e. transmitter-synthesizing enzymes, glycoprotein and membrane components) (Droz et al., 1979; Griffin et al., 1981).

*Retrograde transport*

Labelled proteins carried anterogradely have been shown to undergo a turnaround at their distal end and are then carried back towards the nerve cell body by retrograde transport. Retrograde transport is of considerable interest in studies of nerve regeneration following injury (Forman, 1983). Their return to the cell body also probably provides a part of the necessary, feedback signal which will enable the cell to modulate the balance of its anabolic and catabolic activity in response to the needs of its far distant axon terminals.

*Slow transport.*

A slow component of retrograde transport has been described in mammalian peripheral nerve fibres (Gainer and Fink, 1982). The component moves at 3-6 mm/day, and has been tentatively identified as serum albumin. Heavy metal ions may also use this component at a rate of some 10 mm/day (Baruah et al., 1981).

*Fast transport*

Retrograde or somatopetal axonal transport consists primarily of the fast transport of membrane-bound structures toward the cell body at a speed around 200 mm/day. The structures include secondary lysosomes, multivesicular bodies and smaller vesicles ready for recycling or destruction, together with exogenous materials
absorbed at the nerve terminal (Holtzman, 1971; Bunt and Hasche, 1978). Nerve growth factor also reaches developing sensory dorsal root ganglion cell and sympathetic nerve cell bodies via retrograde fast transport (Hendry et al., 1974; Raivich and Kreutzberg, 1987; Raivich, Hellweg and Kreutzberg, 1991), by active receptor-mediated absorptive pinocytosis (Dumas, Schwab and Thoenen, 1979).

Possible mechanisms of fast transport

One suggestion is that movement is generated by interactions between actin and myosin, both of which are known to be present within neurons (Trifaro, 1978), and that this may result in cytoplasmic streaming capable of transporting particulate components (Isenberg, Schubert and Kreutzberg, 1980). Support for this is provided by the requirement for calcium ions (Ochs, 1983), and by the observations that the substance cytochalasin B is able to inhibit cell movements including neurite growth cones, and axonal fast transport systems.

An alternative explanation is that microtubules are capable of generating movement of adjacent structures, either by interacting with them directly (Schmitt and Samson, 1968) or via an intermediate carrier mechanism (Smith, 1970; Ochs, 1971a, b, 1982). To isolate the essential components, motility assays were developed; kinesin, a protein isolated from mammalian brain and squid neuronal tissue was found to be necessary to support anterograde transport. Kinesin and the related protein, dynein (Sheetz et al., 1987; Schroer and Sheetz, 1991; Toyoshima et al., 1992) have also been shown to be associated with brain microtubules.

Kinesin is composed of two light and two heavy chains. The heavy chains terminate with two globular, folded regions bearing microtubule-binding and ATPase sites which can promote ATP-dependent movement of isolated microtubules in vitro.
(Howard, Hudspeth and Vale, 1989). At the fanned-out tails of heavy chains are folded light chains. A region of bending, the hinge, is found along the main shaft of the kinesin structure. The role of kinesin has been demonstrated in the movement of lysosomes, melanophores and axonal vesicles (Schroer et al., 1988; Urrutia et al., 1991).

Cytoplasmic dynein is a microtubule-based, mechano-chemical ATPase found in virtually all animal cells (Koonce and McIntosh, 1990; Verde et al., 1991). Ultrastructurally and biochemically similar to axonemal dynein, cytoplasmic dynein is a microtubule-activated ATPase that powers movement toward the minus ends of tubulin filaments (Paschal et al., 1987). Dynein may function as a force producer for neurite extension and other microtubule-dependent cytoplasmic rearrangements. How the force-generating proteins actually produce the movement of organelles is as yet speculative. A proposed model is that when the hinge region of the shaft straightens, it rolls the organelle forward to the next kinesin chain which then straightens to hand it on to the next kinesin, and so on (Hirokawa et al., 1989). Another transport model proposes that the carrier is a filament which by bridging cross-arms to microtubules could apply more force to the carrier which would then allow movement of larger structures. Additionally, with several cross-arms, a direction would be given to the movement of the carrier along the microtubules. Cytoplasmic dynein plays a role in axonal transport which is similar to that proposed for kinesin.

How kinesin and cytoplasmic dynein bond to the organelles to direct their anterograde and retrograde transport has been proposed in two models (Sheetz, Steuer and Schroer, 1989). In one, kinesin and cytoplasmic dynein connect to different organelles that are then each moved along different microtubules or along different
aspects of the microtubule. Alternatively, kinesin and cytoplasmic dynein both attach to the same organelle, the direction of transport being determined by some accessory factors activating either kinesin or cytoplasmic dynein.

Table I.1. Different forms of axonal transport (Modified from Tomlinson (1988)).

<table>
<thead>
<tr>
<th>Component</th>
<th>velocity (mm/day)</th>
<th>material transported</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anterograde fast</td>
<td>200-400</td>
<td>membranous organelles, proteins, glycoproteins, glycolipids, phospholipids, vesicle-associated enzymes, neurotransmitters</td>
</tr>
<tr>
<td>slow component &quot;b&quot;</td>
<td>3 - 5</td>
<td>soluble proteins (e.g. actin, calmodulin, CPK, PFK, LDH, enolase, choline, acetyltransferase)</td>
</tr>
<tr>
<td>component &quot;a&quot;</td>
<td>0.5 - 2.5</td>
<td>soluble tubulin, insoluble tubulin, neurofilament triplet neurofilament-associated proteins</td>
</tr>
<tr>
<td>Retrograde fast</td>
<td>200</td>
<td>secondary lysosomes, multivesicular bodies, recycling smaller vesicles, exogenous materials absorbed at nerve terminal</td>
</tr>
<tr>
<td>slow</td>
<td>10</td>
<td>heavy metal ions</td>
</tr>
<tr>
<td></td>
<td>3 - 6</td>
<td>serum albumin</td>
</tr>
</tbody>
</table>

THE BLOOD SUPPLY OF PERIPHERAL NERVES

Peripheral nerves are supplied by two independent vascular systems, the extrinsic and intrinsic systems, which are connected by extensive anastomoses. The extrinsic system is made up of regional nutritive arteries, arterioles and venules together with the epineurial vessels, whilst the intrinsic system consists of
longitudinally orientated microvessels lying in the endoneurial space of the nerve. An abundant network of interconnecting epineurial and perineurial vessels link the two systems. This rich anastomosis results in the high resistance to ischaemia displayed by peripheral nerves.

Because the peripheral nerve is extremely long and far from its parent cell body, it is exquisitely dependent on nerve microenvironment for its blood supply, oxygenation and nutrition and for the removal of toxic metabolic products. McManis, Low and Lagerlund (1993), in a review of the endoneurial micro-environment, point out several characteristic properties of peripheral nerve vasculature. The first, is a lack of autoregulation; this is normally achieved by varying arteriolar tone and is dependent on myogenic rather than neurogenic mechanisms. The vessels in rat nerve are mainly capillaries of large diameter (Bell and Weddell, 1984a); the arterioles have a poorly developed smooth muscle layer (Bell and Weddell, 1984b). The second feature is the greater intercapillary distance in nerve compared to other tissues such as muscle. This would tend to make perfusion of the endoneurium inefficient. A third feature is based on observations by Nukada, Dyck and Karnes (1985) that capillary density is greater in the superineurial region of a fascicle than in the centre. This morphological difference could account for the relative resistance to ischaemia of the subperineurial region because the shorter diffusion distance provides a greater safety margin.

In addition to its characteristic vascular morphology, the metabolism of peripheral nerve is unique. It has about 10% of the oxygen requirement of the brain, although stores of energy are similar (Low et al., 1985). When maximally active, nerve increases its energy demand far less than the brain. The relatively large energy
stores, and the low resting and maximal energy expenditure enable nerve to function well on anaerobically generated high energy phosphate.

Ischaemia in peripheral nerves

Mild to moderate degrees of ischaemia cause no pathological changes in peripheral nerves. Severe reduction in blood flow in human neuropathies may produce infarction of the centre of fascicles with large diameter fibres, or wedge-shaped infarcts (Dyck, Conn and Okazaki, 1972). In sural nerve biopsies there may be degeneration of all myelinated and unmyelinated fibres.

Experimental models of ischaemic neuropathy require extensive ligation of limb arteries, demonstrating the resistance of peripheral nerve to ischaemia. An effective model was developed by Korthals and Wiśniewski (1975) involving tying the abdominal aorta and femoral artery of one limb of the cat. This caused extensive lesions in the distal sciatic nerve and its branches. Sampling of many levels of these nerves showed that between 5 and 12 hours after ligation a zone of abnormal myelinated fibres could be delineated (Korthals, Korthals and Wiśniewski, 1978). The axons of the proximal part of this zone contained large numbers of organelles including mitochondria, dense bodies and vesicles. The distension of axons caused by these organelle accumulations resulted in thinning or loss of the myelin sheaths. More distally was a zone in which fibres showed early changes of Wallerian degeneration; this zone eventually became necrotic, with evidence of degeneration of Schwann cells, endoneurial cells and cellular elements of the blood vessels. In some animals, a zone of fibres with axonal organelle accumulations also formed distal to the necrotic region.

There is no adequate explanation for the increased numbers of organelles, but
it appears to be quite different from that seen near to a crush lesion. Korthals, Korthals and Wiśniewski (1978) suggest that initially lack of energy required for fast axonal transport is the cause of organelle accumulation.

In another study by McManis and Low (1988) individual nutrient branches of the extrinsic system were occluded, causing a regional reduction in nerve blood flow (NBF) with no selectivity for subperineurial or centrifascicular regions. This suggests that longitudinal anastomoses are not as efficient as the regional radial anastomoses. Blunt and Stratton (1956), in a study of the rabbit sciatic nerve, emphasized the importance of regional blood supply at the lower end of the nerve. The abundance of nutrient vessels in the proximal segments of the sciatic nerve provide a head of pressure for the intrinsic system that has decreased by the time it reaches the more distal part of the nerve, which then becomes increasingly dependent upon the regional nutrient arterioles.

**PHYSIOLOGICAL CLASSIFICATION OF NERVE FIBRES**

Mammalian nerve fibres, on the basis of their electrical properties, have been subdivided into three groups, A, B and C. Group A includes the largest fibres with the fastest conduction velocities (i.e. the myelinated somatic afferent and efferent fibres); Group B includes smaller, myelinated preganglionic fibres; and group C is composed of the smallest diameter slowly conducting fibres, the unmyelinated visceral and somatic afferent fibres, and the postganglionic autonomic efferent fibres. Group A fibres have been further subdivided into four efferent groups $\alpha$, $\beta$, $\gamma$ and $\delta$ in order of descending conduction velocity.

$A\alpha$: Exclusively skeletomotor fibres; 9-20μm diameter; conduction velocity
50-100 m/s.

\( \text{A\&} \): Fusimotor and skeletomotor fibres; 9-15\( \mu \)m: 50-85 m/s.

\( \text{A\,\gamma} \): Exclusively fusimotor fibres; 4.5-8.5\( \mu \)m; 20-40 m/s (Boyd and Davey, 1968).

Later, a second classification system was based on the sensory nerves, subdividing them into:

- **Group Ia**: Primary sensory fibres to muscle spindles (12-22\( \mu \)m).
- **Group Ib**: Smaller fibres of Golgi tendon organs.
- **Group II**: Secondary sensory terminals of muscle spindles and cutaneous afferent receptors (6-12\( \mu \)m).
- **Group III**: Free sensory endings in the connective tissue sheaths around and within muscles (1-6\( \mu \)m). These appear to be nociceptive, related to 'pressure-pain' in externally stimulated muscles (Lloyd, 1943).
- **Group IV**: Unmyelinated fibres (0.2-3.9\( \mu \)m); include free endings in muscle, mainly nociceptive. This group also includes C fibres, postganglionic sympathetic and parasympathetic axons.

**TRANSMISSION OF THE NERVE IMPULSE**

The function of the axon is to transmit action potentials along its length. This property depends on the polarization of the axolemma whose internal surface is maintained at a negative potential of around -70 mV by energy-dependent, ATP-utilizing, ionic-pumping mechanisms principally involving movement of the sodium ion through channels in the membrane. The channels can be activated either by ligands such as neurotransmitters, or by a change in membrane potential. When the
channels are activated, an action potential current can depolarize adjacent segments of the axolemma, resulting in a continuously propagated wave of depolarization which spreads along the nerve.

In unmyelinated axons the large capacitance of the axonal membrane attenuates forward axial flow of current within the axoplasm, and limits conduction to a speed of around one metre per second. In myelinated fibres, electrical activity is restricted to the nodes of Ranvier, with ionic activity leaping from one node to the next in series; this is described as saltatory conduction.

This mechanism of conduction can enhance conduction velocity to as much as 100 metres per second, depending on the axon diameter and thickness of the myelin sheath. Loss of one or more myelin segments due to injury or pathology will result acutely in conduction block, but the axon eventually develops the capacity to sustain continuous conduction by a mechanism similar to that occurring in normal unmyelinated axons (Bostock and Sears, 1978; Sears and Bostock, 1981).

**AXONAL DEGENERATION**

After a peripheral nerve injury such as nerve crush or transection, degeneration of the distal segment of the nerve takes place. Waller (1850) studied the sequence of events in the glossopharyngeal nerve of the frog and was the first to describe them, so giving his name to the process. Nerve degeneration was later described in great detail by Cajal (1928), who made use of silver stains to show both degenerative and regenerative changes.

By light microscopy, early changes in myelinated fibres consist of disruption of the myelin sheath, usually beginning at the Schmidt-Lanterman incisures, with later
interruption of the axon and breaking down of the axon into ovoids containing axonal debris. Ovoids were originally thought to be taken up first by a proliferating population of Schwann cells, but more recent studies indicate that the breakdown products are transferred to macrophages which appear in the endoneurium.

The importance of macrophages was demonstrated in elegant studies by Beuche and Friede (1984) who showed that there was no proliferation of Schwann cells and only very slow myelin breakdown if macrophages were prevented from entering an isolated degenerating peripheral nerve. This work also indicated that macrophages are predominantly derived from a blood-borne source.

As the fibres begin to degenerate, macrophages appear and there is Schwann proliferation in the distal part of the nerve. This begins from two to three days after nerve injury and reaches a peak at about three to four weeks (Abercrombie and Johnson, 1946; Bradley and Asbury, 1970). By this time, most of the breakdown products have been removed although small amounts of debris may persist for many weeks. Schwann cell proliferation was observed to occur within the persisting basement membranes, resulting in longitudinal columns of Schwann cells, called the bands of Büngner. The amount of Schwann cell proliferation is a reflection of the size of the degenerating fibres, and a correlation was noted between the degree of proliferation and the extent of myelination (Bradley and Asbury, 1970).

At least a dozen mitogens for Schwann cells have been identified; these include an axolemmal-rich fraction of the axon, myelin-related mitogens and many soluble mitogens such as fibroblast growth factor and platelet-derived growth factor (De Vries, 1993).

Changes in the expression of various markers of Schwann cells associated with
myelinated fibres take place in a nerve soon after nerve injury; and Schwann cells of both myelinated and unmyelinated fibres begin to synthesize nerve growth factor (NGF) and nerve growth factor receptor. These changes have been described briefly by Griffin and Hoffman (1993).

By electron microscopy the earliest axoplasmic changes, seen at about 12 hours, are the accumulation of mitochondria, multivesicular bodies and lamellar osmiophilic bodies at nodes of Ranvier (Webster, 1962; Ballin and Thomas, 1969). By 24 hours, the axoplasmic SER breaks up into rows of vesicles, followed by granular disintegration of the microtubules and neurofilaments and swelling of mitochondria (Schlaepfer and Micko, 1978). These changes are advanced at 48 hours, when the axons become filled with clumps of granular debris. The times given are approximate and are known to vary between species (Cajal, 1928).

Axonal disintegration is probably initiated by an increase in axoplasmic calcium concentration, and mediated by calcium-sensitive proteases (Schlaepfer, 1974).

Degeneration of unmyelinated axons occurs rapidly following nerve fibre damage. Within 24 hours there is loss of microtubules and break-up of the endoplasmic reticulum with gradual disintegration of the neurofilaments, swelling of mitochondria and disappearance of axons within 2-3 days. Although some Schwann cell proliferation occurs it is less extensive than for myelinated fibres (Dyck and Hopkins, 1972; Aguayo and Bray, 1975). When the axons disappear the Schwann cell processes come together to form stacks which are regarded as a sign of loss of unmyelinated axons; there may also be increased numbers of collagen pockets, Schwann cell processes enclosing groups of collagen fibrils.
AXON CHANGES IN THE PROXIMAL STUMP

Immediately proximal to nerve section, reactive axon swellings develop within a few hours, consisting of accumulations of vesicular profiles (Lampert, 1967). A few days later, when axonal sprouting has begun, there may be a reduction in axon size just above the site of injury. Nerve conduction velocity and the size of the largest fibres were decreased in rabbit peroneal nerves proximal to several different types of nerve lesion causing distal fibre degeneration (Cragg and Thomas, 1961).

VASCULAR PERMEABILITY CHANGES

Protein tracers have been used to study permeability changes in nerves undergoing Wallerian degeneration. Mechanical lesions will cause an immediate leakage of vascular tracers from endoneurial blood vessels at the site of injury. At a later stage there is another wave of increased permeability in the distal part of the nerve which appears to follow the front of regenerating axons (Mellick and Cavanagh, 1968; Sparrow and Kiernan, 1981). There do not appear to have been any studies on vascular permeability changes in axonal degeneration produced without direct trauma to the nerve. However, studies during the development of a demyelinating lesion, produced without trauma in a mutant mouse (twitcher) (Jacobs and Scaravilli, 1981a) showed that vascular permeability was increased at the first sign of myelin breakdown and the concurrent entry of macrophages into the nerve. It is likely that the process of axonal degeneration, with the entry of macrophages into the nerve, is also associated with increased vascular permeability.

NERVE REGENERATION

Within a few hours of interruption of an axon, sprouts appear either from the
end of the severed axons, or from nodes of Ranvier proximal to the site of injury (Cajal, 1928). The growing tips of the regenerating axons are known as growth cones which can be recognised ultrastructurally by the large numbers of profiles of endoplasmic reticulum (Bunge, 1973).

When there is a small gap between the severed ends of the nerve, many regenerating axons, accompanied by Schwann cells, grow across the gap into the distal stump and invade the existing basement tubes filled with Schwann cells (bands of Büngner). This appears to be a random process, so that most axons will innervate inappropriate distal bands of Büngner. Regenerating axons will also grow out in all directions, some turning back on themselves and growing in a retrograde direction along the proximal stump (Cajal, 1928). The regeneration process after a nerve crush injury is more effective than after transection of the axons, because the regenerating axons are guided to their correct terminations through the continuous basement membrane lined tubes of Schwann cells in the distal stump.

During regeneration the process of myelination is similar to that during development. However, one proximal axon may give rise to many regenerating axons within the same basement membrane; myelination of more than one of these axons may take place leading to the formation of regeneration clusters.

Schwann cells at regular intervals along the regenerating axons form myelin sheaths in the same manner as during development. More Schwann cells are produced in the bands of Büngner than are needed for myelination and those not required are excluded. In adults, because there is no further increase in length of the part in which the nerve lies, the regenerating internodes will be uniformly short, about 200-400µm, instead of showing the normal linear relationship between fibre
size and internodal length (Vizoso and Young, 1948). Schröder (1972) showed that the thickest regenerated fibres were thinner than the largest control fibres, and that 24 months after a nerve crush in the rat the myelin sheaths were still thinner than normal. A linear relationship has been shown between myelin volume and internodal axonal mantle area (Friede and Bischhausen, 1980; Smith et al., 1982), therefore short internodes would be expected to have thinner myelin sheaths.

In the regeneration of unmyelinated fibres, large groups of unmyelinated axon sprouts are associated with Schwann cell processes within a common basement membrane. In regenerating nerves with a mixed population of myelinated and unmyelinated axons, King and Thomas (1971) suggested that regenerating unmyelinated axons are diverted and come to be preferentially associated with regenerating myelinated axons.

The study of growth factors is now an important part of the study of nerve regeneration. Macrophages, apart from their function in the removal of debris, also induce the secretion of NGF by Schwann cells. There is a dramatic fifty-fold reexpression of low-affinity nerve growth factor (NGF) receptors on Schwann cells within bands of Büngner in the distal segment during the first two weeks after nerve transection. (Taniuchi, Clark and Johnson, 1986, 1988; Johnson, Taniuchi and DiStefano, 1988). In line with the general concept that growth factors induce the synthesis of their own receptors, it has been suggested that 'reactive' Schwann cells produce NGF and subsequently sequester it on the induced receptors, providing a favourable substrate over which NGF-dependent (sensory) axons can grow (Taniuchi, Clark and Johnson, 1986; DiStefano and Johnson, 1988). Evidence from in vivo studies suggests that macrophages may process myelin debris and present a myelin-
derived mitogen for Schwann cells (Baichwal, Bigbee and De Vries, 1988); it is also possible that a product of degraded myelin may induce the secretion of a soluble Schwann cell mitogen by macrophages.

A sequence of events during Wallerian degeneration was suggested by Johnson, Taniuchi and DiStefano (1988) involving firstly the expression of NGF receptors on the Schwann cell surface, and also the release of NGF which is taken up by low affinity receptors and concentrated on the Schwann cell surface (Raivich, Hellweg and Kreutzberg, 1991). As axons of sensory and sympathetic neurons invade bands of Büngner, they are guided over the Schwann cell surface by the binding of NGF. The expression of NGF and NGF receptors is then suppressed by axonal contact, so that the Schwann cell is ahead of the growth cones that possess NGF, giving a directionality to axon elongation.

NERVE CELL REACTION AFTER NERVE FIBRE DAMAGE

Changes take place in the neuronal perikaryon after peripheral nerve injury which were first described by Nissl (1892), and are now known as 'chromatolysis' or the 'axon reaction' (Lieberman, 1976). The following changes occur:- the cell body becomes rounded (particularly in motor neurons) and the nucleus is displaced to the periphery; the Nissl substance or Nissl bodies in the centre of the cell disappear and are replaced by finely granular material which no longer has the staining characteristics of the Nissl bodies, so explaining the word 'chromatolysis'. The nucleus and nucleolus are enlarged. By electron microscopy, there is loss of organisation of the rough endoplasmic reticulum (or Nissl substance) with loss of ribosomes from the reticulum and the appearance of polyribosomes in the cytoplasm;
there is proliferation of smooth endoplasmic reticulum, Golgi bodies and neurofilaments. The rate of response of the cell body to axon damage is related to the distance between the cell and the axon injury, suggesting the passage of a signal from the injured axon (Kristensson, 1984). Chemical changes were described by Brattgård, Edström and Hydén (1957) and these led to the interpretation of the morphological changes as regenerative rather than degenerative phenomena.

COMPRESSIVE NERVE INJURY

ACUTE NERVE INJURY

Acute nerve injury may be caused by sudden compression, transection or stretching. Several types of injury can be identified and these have been classified by two different systems.

Class 1 or Neurapraxia

This lesion is associated with mild or moderate focal compression of nerve and commonly results in a blockade of nerve conduction. Two types of lesions can be recognised. In one, most commonly resulting from transient ischaemia of the nerve, there is a mild and rapidly reversible conduction block. A second type is assumed to be due to paranodal or internodal demyelination causing a more persistent conduction block. This type of lesion has been produced experimentally by inflating a pneumatic cuff around the limbs of cats (Mayer and Denny-Brown, 1964) and baboons (Ochoa, Fowler and Gilliatt, 1972). At the edges of the cuff, myelin sheaths are displaced away from the site of compression, and become invaginated, causing damage to the myelin sheath which results in localized demyelination and in conduction block. Large diameter fibres are more affected than small fibres.
Remyelination occurs rapidly and functional recovery is usually complete by about 6 weeks after the injury. Ochoa, Fowler and Gilliatt (1972) concluded that the lesion is caused by mechanical processes.

**Class 2 or Axonotmesis**

This lesion is associated with percussion and closed crush injuries. The axons are interrupted, but the Schwann cell basement membrane around each fibre remains intact, as does the endoneurial connective tissue (Thomas, 1964). Wallerian degeneration occurs distal to the lesion site, but regeneration proceeds at a rate of about 1-2mm per day. All functions are eventually restored since regenerating axons are guided back to their original terminations through the continuous basement-membrane-contained Schwann cells that develop distally following Wallerian degeneration (Haftek and Thomas, 1968).

**Classes 3, 4 and 5 or Neurotmesis**

These are all injuries involving the severance of nerve fibres but with varying degrees of disruption of the associated tissues. In class 3 injuries, the fascicular orientation is preserved; in class 4, there is disruption of the perineurium and in class 5, there is total loss of continuity of the nerve and its connective tissue components. Nerve fibres distal to the injury undergo Wallerian degeneration, and subsequently regenerating axon sprouts grow from the proximal ends (Cajal, 1928; Morris, Hudson and Weddell, 1972). Many axons may traverse the lesion site and successfully grow along the distal Schwann cell columns, although misrouting occurs and inappropriate end-organ connections are commonly established. Many regenerating fibres grow out randomly at the injury site since they lack guiding basement membranes. Continued growth of these aberrantly regenerating fibres may result in the formation of a
neuroma at the site of injury (Spencer, 1974). Neuroma formation may be prevented by careful surgical anastomosis of the divided ends of the injured nerve, either as individual fascicles or of the whole nerve.

Clinically, injuries associated with partial or complete nerve transection produce clearly defined areas of total motor, sensory and autonomic dysfunction. Denervated muscle may lose 80% of its bulk, but function can be recovered if reinnervation takes place within three years of transection. Shrinkage of the autonomous sensory zone of a nerve, its area of superficial cutaneous sensation, follows within days of complete nerve section. This phenomenon reflects the overlap of normal innervation; it is important to recognise that this does not reflect regeneration.

CHRONIC NERVE COMPRESSION

Three types of injury are commonly associated with chronic trauma to peripheral nerve.

1. Compression in fibro-osseous tunnels (carpal, cubital or tarsal tunnels)
2. Angulation and stretch over arthritic joints, anomalous fibrous bands, under ligaments
3. Recurrent external compression e.g. occupational trauma to hands and feet

As far as the tunnel syndromes (Type 1) are concerned, histopathological changes in human and experimental animals have been well studied but little is known of Type 2 and 3 injuries.

In human carpal tunnel syndrome, the nerve is enlarged above the site of compression and is narrowed in the compressed zone (Neary and Eames, 1975;
Neary, Ochoa and Gilliatt, 1975). The morphological features of demyelination in chronic nerve entrapment differ markedly from those in acute compression. A naturally occurring nerve entrapment is found in the guinea pig, and studies of the compressed nerves show that the myelin sheath becomes deformed, with a bulbous swelling at one end of an internode and tapering of myelin at the opposite end (Ochoa and Marotte, 1973). The bulbous ends are orientated away from the site of compression, indicating the direction of the mechanical force. The myelin sheaths degenerate and bare axons are remyelinated. In older animals, there is eventual axonal degeneration (Fullerton and Gilliatt, 1967). Unmyelinated fibres resist degeneration until late.

Mechanical factors appear to be dominant in the demyelination of chronic compression. The role of ischaemia is less certain, but it may be responsible for the acute attacks of pain suffered in the carpal tunnel syndrome. Short periods of ischaemia can reversibly block conduction in damaged fibres (Fullerton, 1963).

**NEUROPATHIC PAIN AND ITS BACKGROUND**

Neuropathic pain is pain associated with abnormalities of the peripheral nervous system. This particular aspect of pain will be discussed since it is most relevant to the topic of this thesis. A number of clinical features of neuropathic pain can be recognised in man. These include:-

- Pain in the absence of detectable ongoing tissue damage
- Dysesthesiae, often of burning quality
- Delay in onset after precipitating injury
- Pain in region of sensory deficit
Paroxysmal shooting or stabbing component

Mild innocuous stimuli cause pain (allodynia)

Pronounced summation and after-reaction with repetitive stimuli

In man, not all of these features will be present, but any single one should lead to the suspicion that the pain is neuropathic.

In the rat model of neuropathic pain which is the subject of this thesis, both allodynia and pain in the absence of apparent ongoing tissue damage can be recognised.

Wall (1989) has extensively reviewed the topic of pain, and this is the source of part of this literature overview.

THE RELATIONSHIP OF INJURIES OF THE PERIPHERAL NERVOUS SYSTEM WITH PAIN

Peripheral nerves contain nerve fibres subserving many functions, including motor, autonomic and sensory. It is the latter group which is of the greatest importance in the study of pain; these include large and small myelinated fibres and unmyelinated axons.

AFFERENT FIBRES

Afferent fibres of all sizes may contribute to neuropathic pain.

Large (6-22μm) myelinated fibres (Aα and Aβ)

These are mostly excited by mild mechanical stimuli; they do not respond to noxious stimuli and in normal subjects their activity does not produce pain. In fact, afferent barrages in Aα fibres inhibit the response of cord cells to noxious stimuli and decrease pain (Wall and Cronly-Dillon, 1960; Wall, 1964). This was one of the major
observations which led to the gate control theory (Melzack and Wall, 1965). Support for the role of Aα fibres in pain comes from clinical observations that selective electrical stimulation of large diameter primary afferent fibres can give striking relief to the burning pain caused by some forms of peripheral nerve injury (Wall and Sweet, 1967; Meyer and Fields, 1972).

If the electrical stimulus is raised to include the smaller fibres (Aδ) of this group, the inhibition of painful sensation and of the flexor reflex to noxious inputs is replaced by a facilitation (Willer et al., 1980). In tender areas distant from injury, light mechanical stimuli (A-β activated) may evoke pain (Kugelberg and Lindblom, 1959; Raja, Campbell and Meyer, 1984; Campbell et al., 1988).

**Small myelinated (2-5μm) fibres (Aδ)**

Myelinated nociceptors are included in this group; they respond to noxious mechanical stimuli and are called high threshold mechanoreceptors (HTM's). A significant proportion (20-50%) of Aδ fibres also respond to heat stimuli (mechanothermal nociceptors). Some of these respond to skin temperatures below pain threshold, but all respond as temperatures are raised into the noxious range (45-47°C), and some also respond to cooling of their receptive fields. Both HTM's and mechanothermal nociceptors have the property of sensitization, showing increased sensitivity with repeated stimulation (Fitzgerald and Lynn, 1977; Campbell and Meyer, 1983).

**Unmyelinated afferent (0.3-2.0μm) axons (C fibres)**

About 75% of primary afferent axons in a peripheral nerve are unmyelinated C fibres; and in a primate cutaneous limb nerve, over 90% of the C fibres are nociceptive. In studies of human nerves (Torebjörk, 1974) all C fibre afferents were
found to be nociceptive. Most C fibres are polymodal (C-PMN), responding to noxious thermal, mechanical and chemical stimuli applied to the skin. Like the myelinated nociceptors, C-PMN's sensitize with repeated noxious stimuli. Selective stimulation of C fibres produces pain (Collins, Nulsen and Randt, 1960; Sinclair, 1981).

There is now good evidence for other functions specific to C fibres beyond the immediate effect of delivering impulses to the spinal cord; these include:-

(a) The axon reflex, in which activation of C fibres in the periphery leads to leakage of substances (e.g. substance P) from their distal endings, causing vasodilatation, neurogenic oedema and the release of histamine from mast cells.

(b) Long-latency, long-duration, widespread sensitization of spinal cord circuits (See later).

(c) Central consequences of the transport of neuropeptides and other substances. C fibres may respond to the metabolic state and nature of the tissue in which they terminate. They convey information to the central nervous system from their peripheral terminals either by nerve impulses or by the transport of chemicals (Campbell, Meyer and LaMotte, 1979; LaMotte et al., 1982).

The many functions of C fibres could help to explain their vast numbers. Loss of C fibres would reveal an absence of peripheral inflammatory responses and of central connectivity control but would leave the rapid painful reaction to injury largely intact, since most of that information could be mediated by Aδ fibres.

THE EFFECT OF INJURY ON AXONS
Early physiological change

Section of a nerve is followed by an immediate, brief and repetitive discharge in all type of axons. This injury discharge activity dies down within seconds and the cut end of the nerve becomes relatively insensitive for some time (Wall and Gutnick, 1974).

Morphological change

Within a day of the section of an axon, the cut end seals over, growth cones form and sprouts begin to grow (Cajal, 1928). When a nerve is sectioned, there is loss of continuity of the basement membrane surrounding each myelinated fibre, and this has important implications in regeneration. In experimental studies on rat sciatic nerve, even with precise sectioning and immediate careful resuturing, over 25% of axons fail to cross into the distal stump (Gibby, Koeber and Horch, 1983; Pover and Lisney, 1988).

If axons have been damaged in a simple crush injury, the basement membranes remain intact (Haftek and Thomas, 1968). In this situation, almost 100% of the severed fibres succeed in growing sprouts into distal Schwann cell tubes and reinnervating the area which was previously innervated (Horch and Lisney, 1981). In general more than one sprout is produced, and the total number of axons in the nerve distal to a crush lesion may exceed the number in the proximal part of the nerve (Toft, Fugleholm and Schmalbruch, 1988).

Later physiological changes

The physiological properties of the outgrowing sprouts differ from normal nerve in three respects (Wall and Gutnick, 1974).

(a) *Ongoing activity*
As sprouts grow out, all types of sensory fibres begin to generate spontaneous nerve impulses. In the case of crushed nerves, where the sprouts successfully penetrate into distal Schwann cells, this activity rises for about 7 days and then declines to zero. In the cut-ligated nerve where all fibres form a neuroma, the activity reaches a peak at about 2 weeks and declines to a low level over the next 2 weeks. These active sprouts then continue to produce nerve impulses indefinitely (Govrin-Lippmann and Devor, 1978).

(b) **Mechanical sensitivity**

Normal axons are relatively insensitive to mechanical distortion. However, nerve sprouts become spontaneously active, and they become sensitive to slight mechanical distortion.

(c) **Sensitivity to adrenalin**

Outgrowing sprouts become extremely sensitive to the alpha receptor action of adrenalin. Stimulation of the sympathetic system releases sufficient noradrenaline in the area of a neuroma to generate a powerful barrage of nerve impulses in sensory afferents. The beta agent, isoprenaline, has no excitatory effect.

Outgrowing axons in a neuroma may establish close contact with other sprouts, without intervening Schwann cell cytoplasm. This may allow impulses to jump ephaptically from one axon to another. Seltzer and Devor (1979) describe a stable electrical (ephaptic) interaction between pairs of injured sensory and motor axons, when the nerve ends in a neuroma.

**Collateral sprouting**

Intact nerve innervating skin adjacent to a denervated region produce sprouts which occupy at least part of the denervated zone. This phenomenon has been
investigated behaviourally and electrophysiologically by Devor et al. (1979). It explains the rapid phase of filling in of anaesthetic areas and may contribute to the abnormal sensation associated with the edge of such areas. Recent behavioural, electrophysiological and morphological studies on a sensory field isolated within a large area of denervated skin, have shown that collateral sprouting of cutaneous nerves in this situation is dependent upon nerve growth factor (NGF) (Diamond, Holmes and Coughlin, 1992). This contrasts with regeneration of a damaged nerve which appears to be independent of NGF (Diamond et al., 1992).

Collateral sprouting occurs in C-fibres and Aδ fibres, but not from Aα fibres in adult animals.

**INJURY OF CENTRALLY DIRECTED AXONS**

The injury discharge which results from the damage to peripheral axons will be conducted centrally over the afferent fibres.

When a peripheral sensory nerve is cut, the centrally directed axons, which project to the spinal cord through the dorsal roots, are presumed to remain intact. Some ganglion cells degenerate following damage to their peripheral axons; this occurs particularly in immature animals (see Thomas, Scaravilli and Belai, 1993), but has also been described in adult animals Aldskogius and Arvidsson (1978).

One of the factors causing pain following chronic peripheral nerve lesions may be due to the irreversible but scattered degeneration of spinal cord afferents as a result of DRG cell loss.

**Electrical activity in the dorsal root ganglion (DRG)**

Some days after nerve section, generation of nerve impulses begins in the region of
the DRG (Wall and Devor, 1983). After sciatic nerve section in the rat, this rises to a maximum at about 3 weeks and then declines slowly over subsequent months but never ceases as long as a neuroma exists. There are two different sources of abnormal peripheral afferent nerve impulses in chronic pain, one from the region of injury and one from the DRG cell (see Wall, 1989).

**Changes in peptides and other substances**

DRG cells synthesise proteins and peptides which are transported to the peripheral endings and to the spinal cord terminals. Each neuropeptide may play some part in neurotransmission, although this is still a matter of uncertainty. When a peripheral nerve is cut, the presence of these substances (e.g. substance P) in the spinal cord afferent terminals in the substantia gelatinosa drops very substantially (Barbut, Polak and Wall, 1981). Whatever may be their function, this type of change could be a link in the slow modification of postsynaptic function. However, after 6 months some of these substances begin to reappear, particularly fluoride-resistant acid phosphatase and substance P. These very slow changes indicate that the central nervous system alterations may continue for many months after a peripheral nerve lesion.

**THE EFFECT OF PERIPHERAL INJURY ON CELLS IN THE SPINAL CORD**

The grey matter of the spinal cord was recognised by Rexed (1952) to be organised into laminae (Fig. I.1); these were defined in terms of the size, orientation and density of the neurons within them.

**Lamina I.** This marginal layer, contains large flat "marginal" cells and many intermediate sized neurons and is traversed by fibre bundles.

**Lamina II.** Because of its clear "gelatinous" appearance in fresh spinal cord, this
lamina has been given the name 'substantia gelatinosa'. It is a clearly demarcated region containing very small, tightly packed neurons.

Lamina III. The neurons in this lamina are larger and less densely packed than those in lamina II.

Lamina IV. This is the thickest lamina, containing very large neurons with dendrites that spread into the more superficial layers.

Lamina V. The neurons are smaller than those in lamina IV.

These are the laminae most involved in pain mechanisms in the spinal cord. Myelinated nociceptors project to laminae I and V; unmyelinated nociceptors project to lamina II and probably lamina I; the larger myelinated non-nociceptive afferents (Aβ fibres) terminate in laminae III, IV and V.
Fig. 1.1  Rexed's scheme for lamination of the spinal grey matter (From Rexed, B., A cytoarchitectonic atlas of the spinal cord in the cat. *J. Comp. Neurol.*, 96:415-495, 1952).

Fig. 1.2  The main projections of different cutaneous fibre groups (Aβ, Aδ, C-fibres and ventral root, VRA) to the dorsal horn (From McMahon, S.B., Spinal mechanisms in somatic pain. In: *The Neurobiology of Pain*, Eds., Holden, A.V. and Winlow, W., Manchester University Press, 1984, pp.159-178, by permission).
Fig.1.2 (taken from McMahon, 1984) shows diagrammatically, the major components of the upper laminae of the spinal cord.

Peripheral nerve injury may produce selective damage to myelinated primary afferents, with sparing of unmyelinated nociceptors. This may be the case, for example, where nerves are subjected to chronic compression as in entrapment neuropathies which are often painful. As stated above, evidence that large fibre loss contributes to the pain in some types of neuropathy comes from clinical experience that stimulation of large fibres can give relief from pain. Observations such as these led Melzack and Wall (1965) to propose a 'gate control' theory to explain a mechanism of pain.

**Gate control theory**

The gate control theory has four main components.

(a) **Afferents**

Unmyelinated nociceptors which terminate in the two outer laminae; and Aδ fibres which also terminate in this area and some extend to a zone in lamina V. Low-threshold Aβ fibres terminate in the deeper laminae.

The low-threshold afferents may be related to pain through the following findings:

1. Tenderness is mainly explained by a sensitization of nociceptors, such as allodynia, (innocuous stimuli to normal tissue which produce pain). As we know, repeated noxious stimuli will sensitize both myelinated and unmyelinated nociceptors (Meyer and Campbell 1981). Thus, allodynia could be partially explained by sensitization of nociceptors due to threshold decrease.
2. Allodynia in some patients can be abolished by selective blockade of large myelinated afferents (Meyer, Campbell and Raja, 1985) which include few if any nociceptors. This indicates that large myelinated afferents can contribute to pain under pathological conditions.

(b) *Interneurons within the dorsal horn*

The cells which receive incoming afferents cells are not simply collecting information from particular types of afferents and transmitting these to their destination. They select and compute combinations of the signals received. Some combinations summate signals, others are inhibitory. Such excitatory and inhibitory interaction requires the presence of interneurons which are present in laminae I and II.

(c) *Descending controls*

Many regions in the brain project into the dorsal horn and have mainly inhibitory effects on the firing of dorsal horn nociceptive neurons. The inhibition of nociceptive responses in the cord produced by electrical stimulation of some of these regions appears to correlate well with behavioural analgesia. The raphe nuclei and the reticular formation have been emphasized to be the origin of descending control, but other sources (in man) include the periaqueductal grey matter (Fields and Basbaum, 1978).

(d) *Transmission cell*

Synaptic decisions made in the dorsal horn are received by several different types of cell, whose axons terminate in distant structures. The spinothalamic tract is one pathway thought to be involved, since the surgical operation of anterolateral cordotomy as a treatment for pain was assumed to be the equivalent of cutting the
spinothalamic tract. However, other projections systems may be cut during that operation.

Although the term 'gate control' is now restricted to the rapidly acting mechanism, the theory still offers an explanation of central summation and the phenomenon of allodynia.

According to the gate control hypothesis of Melzack and Wall (1965) (Fig.1.3), the interaction between myelinated and unmyelinated inputs to the spinal cord occurs at two sites: inhibitory neurons in the substantia gelatina (SG) (Lamina II) and dorsal horn transmission neurons (Lamina I and V). Both myelinated and unmyelinated primary afferents were proposed to have a direct excitatory action on the pain transmission (T) neurons. The SG neurons (inhibitory interneurons) were proposed to inhibit transmitter release or inputs from both classes of primary afferents, thus presynaptically inhibiting all afferent input to the pain transmission (T) neurons. The myelinated afferents were proposed to excite the inhibitory (SG) interneurons, thereby reducing input to transmission (T) neurons and consequently inhibiting pain. In contrast, activity in unmyelinated nociceptors was proposed to inhibit the inhibitory neurons, resulting in an enhancement of inputs from primary afferents to the transmission (T) neurons and consequently increasing pain intensity. The descending controls from periaqueductal grey matter and reticular formation (and possibly other sites) have inhibitory effects on the transmission neurons of spinal cord and consequently reduce pain intensity.
Fig. I.3 The inhibitory interneuron (I) is excited by the myelinated non-nociceptive afferent (M). The unmyelinated nociceptor (U) inhibits the inhibitory interneuron secondarily exciting the transmission cell (T). The unmyelinated primary afferent thus has both direct and indirect excitatory effects on the T cell, whose activity usually results in the sensation of pain. (Modified from Fields, H.L., *Pain*, Ed., Fields, H.L., McGraw-Hill Book Company, 1987).
Later changes of spinal cord

Nociceptive pain produced by tissue injury, and neuropathic pain due to damage to peripheral nerve function, each have different peripheral mechanisms but both are influenced by changes in the CNS. When the tissue or peripheral nerve is damaged, the nature of the afferent barrage in the peripheral nerve changes with time, and the receiving mechanisms within the brain and spinal cord also change under conditions of ongoing injury detection. Therefore the relationship of the four main components of the gate control theory will not be as simple as originally proposed now that central plasticity is known to occur. The role of central neuroplasticity in pathological pain has recently been extensively reviewed by Coderre et al., (1993).

CENTRAL PLASTICITY IN NOCICEPTIVE PAIN

This will only be briefly mentioned since the main topic of this review is neuropathic pain. Two types of hyperalgesia can be recognised (Hardy, Wolff and Goodell, 1950). Primary analgesia is the increased sensitivity to noxious stimulation at the site of an injury, and is probably mediated by peripheral mechanisms; secondary analgesia is the increased sensitivity extending beyond the site of injury and is thought to be related to central hyperactivity or sensitization.

Evidence for a central mechanism of hyperalgesia comes from a number of different observations of the phenomenon of referred pain and hyperalgesia, including the fact that pain and hyperalgesia can occur in regions far removed from the injured site.

Central plasticity in neuropathic pain

*Phantom pain*
Phantom pain associated with the removal of limbs and other parts of the body provides striking evidence that central mechanisms are involved since the 'peripheral' source of the pain no longer exists.

Denervation hypersensitivity

The development of hypersensitivity in the hind paw of a rat after sciatic nerve section occurs at the time of expansion of the projection of the saphenous nerve in the spinal cord (Markus, Pomeranz and Krushelnycky, 1984). This finding will be discussed in more detail elsewhere, since it is pertinent to the topic of this thesis.

There are many other examples of nerve injury which are associated with increased activity in the central nervous system.

Prolonged central changes triggered by nerve impulses

In the clinical setting, minor injuries may cause prolonged pain and tenderness that spreads far from the site of injury. Central changes are likely to be implicated in these widespread effects. Studies in decerebrate rats following peripheral thermal injury have shown a prolonged increase in the excitability of both ipsilateral and contralateral flexor reflexes (Woolf, 1983). C fibre activity is essential to produce the central changes. Increased excitability in the contralateral flexor efferent nerve continues after impulses from the injured paw are blocked by local anaesthetic, suggesting that central rather than peripheral changes are implicated.

Expansion of the receptive fields of dorsal horn neurons

Neurons in the dorsal horn of the spinal cord whose receptive fields lie adjacent to neurons related to a cutaneous heat injury expand their receptive fields to incorporate the site of injury (McMahon and Wall, 1984). Similar receptive field expansions have been observed in the spinal cord following injury caused by a variety
of insults including nerve damage (Devor and Wall, 1978) and electrical nerve stimulation (Cook et al., 1987).

Noxious stimulus-induced neurochemical mediators in central plasticity

C-fibre neuropeptides

Noxious stimulation or peripheral inflammation causes the release of a number of neuropeptides in spinal cord dorsal horn, suggesting their involvement in nociception. These include substance P (SP) (Oku, Satoh and Takagi, 1987; Duggan et al., 1988), neurokinin A (Hua et al., 1986; Duggan et al., 1990), somatostatin (Kuraishi et al., 1985; Morton, Hutchison and Hendry, 1988), calcitonin gene-related peptide (CGRP) (Saria et al., 1986) and galanin (Morton and Hutchison, 1990). C-fibre neuropeptides may also be implicated in noxious stimulus-induced plasticity as suggested by the fact that repetitive stimulation of dorsal roots elicits a slow depolarization in dorsal horn neurons which is mimicked by SP (Murase and Randic, 1984), neurokinin A (Murase, Ryu and Randic, 1989), CGRP (Ryu et al., 1988), and vasoactive intestinal polypeptide (VIP) (Urban and Randic, 1984). This effect is blocked by SP antagonists or by capsaicin (Urban and Randic, 1984). Intrathecal application of SP, CGRP (Woolf and Wiesenfeld-Hallin, 1986), neurokinin A (Xu, Maggi and Wiesenfeld-Hallin, 1991), somatostatin, VIP and galanin (Wiesenfeld-Hallin, Villar and Hökfelt, 1989) produces prolonged enhancements in the excitability of the flexion reflex.

Excitatory amino acids

Noxious stimulation or peripheral inflammation also causes the release of glutamate and aspartate in spinal cord dorsal horn (Skilling, Smullin and Larson,
1988), again suggesting its role in nociception. Iontophoretic application of excitatory amino acids (EAAs) produces an excitation of dorsal horn neurons (Curtis and Watkins, 1960; Willcoxon et al., 1984a; Schneider and Perl, 1988), while intrathecal treatment produces both behavioural hyperalgesia and spontaneous nociceptive behaviours (Aanonsen and Wilcox, 1986, 1987). A role of EAAs in noxious stimulus-induced plasticity is suggested by the observation that repetitive C-fibre stimulation produces a 'wind-up' of dorsal horn neuron activity which is mimicked by the application of glutamate or NMDA (Gerber and Randic, 1989; King et al., 1988). This effect is blocked by competitive (Dickenson and Sullivan, 1987a; Thompson, King and Woolf, 1990) or non-competitive (Davies and Lodge, 1987; Thompson, King and Woolf, 1990) NMDA antagonists. In a recent study in humans (Gordh and Kristensen, 1992) intrathecal treatment with the competitive NMDA receptor antagonist CPP abolished spreading pain and hyperalgesia (symptoms proposed to be associated with wind-up) in a patient with neuropathic pain.

The mechanism by which neuropeptides and EAAs contribute to central neuroplasticity is not known. Intracellular calcium ($Ca^{\text{2+}}$), second messenger systems and protein kinases may play a part in altering membrane excitability.

(see review in Coderre et al., 1993)

Molecular mechanisms of noxious stimulus-induced CNS plasticity

Expression of c-fos and other proto-oncogenes

Noxious stimulation also leads to the expression of proto-oncogenes and their protein products which act as third messengers thought to be involved in transcriptional control of genes encoding a variety of neuropeptides. The c-fos product Fos is expressed in dorsal horn neurons following a number of noxious procedures.
including neuroma formation after a nerve injury (Chi, Levine and Basbaum, 1990).

Noxious stimulation also leads to the expression of other proto-oncogene products including Fos B, Jun, Jun B, Jun D, NGF1-A, NGF1-B and SRF in the spinal cord (Wisden et al., 1990; Herdegen et al., 1990a,b). Increased expression of Fos has also been demonstrated in other CNS structures involved in pain transmission, including the periaqueductal grey, thalamus, habenula and somatosensory cortex (Bullit, 1989; Iadorola, Sanders and Draisci, 1990; Herdegen et al., 1991b). There is a strong correlation between pain behaviour and the number of cells expressing Fos (Presley et al., 1990). Morphine pretreatment produces a dose-dependent suppression of Fos expression which corresponds with its analgesic effects (Presley et al., 1990; Tölle et al., 1991).

Relationship between c-fos, central neuroplasticity and hyperalgesia

There is growing evidence of a relationship between noxious stimulus-induced Fos expression, central neuroplasticity and behavioural hyperalgesia. Noxious stimuli that produce Fos expression such as heat injury, formalin injection and inflammatory agents, also produce behavioural hyperalgesia which is associated with central neuroplasticity (Coderre and Melzack, 1985, 1987; Kayser and Guilbaud, 1987; Coderre, Vaccarino and Melzack, 1990), and follows the same time course.

SUMMARY

When tissue is damaged in the periphery, the central nervous system receives an afferent barrage in nociceptors as determined by a gate control mechanism. Subsequent to this rapid phase, slow onset, long-duration changes are triggered by impulses in unmyelinated afferents, particularly those originating from deep tissue.
These central changes consist of widespread increases of excitability of nerve cells projecting to the brain and to reflex circuits. The increases of excitability are so great that previously ineffective inputs become effective. The practical effect is that some cells were previously excited only by nociceptors now respond to low-threshold inputs. This might provide a basis for the widespread secondary tenderness or allodynia which characterizes so many types of pain. In such pathological states, the nervous system is reorganised with less specificity, less somatotopy and less precise timing. Furthermore, there are slowly acting brain-to-spinal cord circuits which control the extent of this spinal cord plasticity.
CHAPTER 2: MATERIAL AND METHODS

ANIMALS

A total of 93 young adult male Sprague-Dawley rats, weighing 250-330g, were used. They were housed in small groups in plastic cages with solid floors and soft bedding; food and water were available ad libitum. Animals were divided into seven experimental groups as shown in Table II.1

Table II.1 Experimental groups

<table>
<thead>
<tr>
<th>Procedure</th>
<th>No of animals</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sciatic nerve loosely ligated only</td>
<td>40</td>
</tr>
<tr>
<td>Sciatic nerve loosely ligated + saphenous nerve cut &amp; ligated at the same time</td>
<td>6</td>
</tr>
<tr>
<td>Sciatic nerve loosely ligated + saphenous nerve cut &amp; ligated 1 week later</td>
<td>6</td>
</tr>
<tr>
<td>Sciatic nerve loosely ligated + saphenous nerve cut &amp; ligated 2 weeks later</td>
<td>6</td>
</tr>
<tr>
<td>Sciatic nerve loosely ligated + saphenous nerve cut &amp; ligated 3 weeks later</td>
<td>6</td>
</tr>
<tr>
<td>Sciatic nerve loosely ligated; killed 8.5 hours later, after i.v. HRP</td>
<td>3</td>
</tr>
<tr>
<td>Sciatic nerve loosely ligated; killed 24 hours later, after i.v. HRP</td>
<td>4</td>
</tr>
<tr>
<td>Sciatic nerve loosely ligated; killed 48 hours later, after i.v. HRP</td>
<td>8</td>
</tr>
</tbody>
</table>

A Control group consists of normal rats (n=6) without any manipulation.

In a Sham group (n=8) the procedure for tying ligatures was followed precisely, but without actually tying the ligatures, which were left lying loosely beneath the nerve.
EXPERIMENTAL PROCEDURES

SURGICAL PROCEDURES

Anaesthesia was initially induced with isoflurane, and maintained with i.p. hypnorm (fentanyl citrate and fluanisone) and midazolam using 1 part of each + 2 parts water (0.315 mg/ml) : (10 mg/ml) : (5 mg/ml) at a dose of 2.5 ml/kg.

Placing of ligatures Under a dissecting microscope an incision was made approximately 40mm in length. The common sciatic nerve was exposed at the level of the middle of the thigh by blunt dissection through biceps femoris. The chromic gut was placed in a dish of distilled water so as to keep it moist, since it became brittle and impossible to tie if allowed to dry.

A 10mm length of nerve was freed of adhering tissue and 4 ligatures (4/0 chromic gut) were tied loosely around it with about 1mm spacing. The length of nerve thus affected was about 5mm. Great care was taken to tie the ligatures so as not to cause obvious constriction of the nerve and circulation through the epineurial vasculature was not totally interrupted.

The wound was closed and covered with a Sprilon spray for protection.

Saphenous nerve section The right saphenous nerve was exposed by making an incision on the ventral aspect of the thigh. The saphenous nerve was freed and cut. In order to ensure a complete transection and prevent reinnervation, about 10mm of the nerve was removed at mid-thigh level, and both proximal and distal ends were ligated.

EXAMINATION OF ANIMALS

Some rats, used in the study of the evolution of the lesion, were examined at
8.5 and 24 hours after operation. The majority of animals were examined 2 days after operation, every day during the first two post-operative (p.o.) weeks, three times weekly during the third and fourth weeks and thereafter once every week until 4 months p.o.. The unoperated limb was examined first, and then the operated limb to prevent heterosynaptic facilitation (Woolf, 1983; Woolf and Wall, 1986). Six normal rats, with no surgery, were used as controls. Statistical analysis was performed using the two-tailed Mann-Whitney U test. Values of \( P < 0.01 \) were considered to be significant. For each set of values the mean and standard error of the mean (SEM) was determined.

**Observation of posture and gait: assessment on a semiquantitative scale.**

This was always the first test, and a rating system was devised. The behavioural changes observed may be due to both sensory-related effects and motor deficits, and it is important to recognize this. However, it was impracticable to attempt to differentiate between sensory and motor deficits, and the rating scale therefore includes changes of both sensory and motor function.

The rats were placed on a wire grid to carefully observe the hind limb grasping reflex for a minute or two. Normal rats maintain their grip with all five toes. Particular attention was given to the animal's gait, the posture of the affected hind paw, the condition of the hind paw skin, and whether there were signs of autotomy. The behaviour of the rats could be described either as hypersensitive or allodynia (negative grade) or as hyposensitive or paretic (positive grade) by a semiquantitative scale.
Table II.2  Definition of the assessment of gait and grip on a semiquantitative rating scale

<table>
<thead>
<tr>
<th>Behaviour</th>
<th>Definition</th>
<th>Rating</th>
</tr>
</thead>
<tbody>
<tr>
<td>Allodynia or hypersensitivity</td>
<td>A limping gait in a guarded posture with reluctance to put the foot down</td>
<td>-3</td>
</tr>
<tr>
<td></td>
<td>Very brief (≤ 5 sec) grip of the cage wires followed by licking and vocalization</td>
<td>-2</td>
</tr>
<tr>
<td></td>
<td>Longer period (&gt; 5 sec) of grip followed by licking and vocalization</td>
<td>-1</td>
</tr>
<tr>
<td>Normal</td>
<td>Maintenance of grip with all five toes</td>
<td>0</td>
</tr>
<tr>
<td>Paresis or hyposensitivity</td>
<td>Firm grasp with all claws but not sustained</td>
<td>+1</td>
</tr>
<tr>
<td></td>
<td>Grasp with 2-4 claws but not sustained</td>
<td>+2</td>
</tr>
<tr>
<td></td>
<td>Loss of grip, dragging the limb while walking</td>
<td>+3</td>
</tr>
</tbody>
</table>

Tests using thermal stimulation

*Heat* The animal was carefully held so that the head and limbs were free. The lower limb was placed in a hot water bath at 48°C and the time taken for withdrawal of the foot was measured; as previously mentioned the unoperated side was examined first. Every rat was tested at least twice, with an interval of at least 10 min between each test. If the foot was not withdrawn within 30 secs, it was removed from the bath by the observer, to avoid any possible tissue damage. Values of this magnitude were only obtained within the first 2 days following application of ligatures.

The two withdrawal reflex latencies of the ligated side were averaged and a difference score was computed by subtracting the average latency of the unoperated side from the average latency of the ligated side. Negative difference scores thus indicate a lower threshold on the ligated side. The duration of the time that the hind
paw was withdrawn was measured with a stopwatch.

In normal rats and humans the nociceptive threshold temperature ranges from 44 to 46°C (Bennett and Xie, 1988, Fields, 1987); the 48°C test was therefore presumed to test noxious stimulation.

A pilot study using methods described above at temperatures ranging from 40°C to 54°C at intervals of 2°C showed a marked difference in response from 48°C to 50°C (Fig. II.1). The data from the tests with 48°C stimulus were the most informative and are presented here.

In tests at 48°C, the mean of the difference between right and left sides of control rats was 0.04 sec ± 0.15 (SEM) at day 1, -0.29 sec ± 0.1 (SEM) at day 7, and -0.06 ± 0.02 (SEM) at day 14. The measured values of latencies in the same control rats were 5.75 sec ± 0.57 (SEM) and 5.71 ± 0.61 (SEM) on right and left sides respectively on day 1, 4.88 sec ± 0.49 (SEM) and 5.17 sec ± 0.46 (SEM) on day 7, and 5.73 sec ± 0.64 (SEM) and 5.78 sec ± 0.55 (SEM) on day 14.

The 'difference' method was chosen in preference to giving absolute latencies since it showed less daily variation.

Cold The method used was similar to heat tests, but with the water bath maintained at 6°C. In some animals there was immediate withdrawal of the limb (within 1 sec); this was regarded as a false positive. The limb was dried and the test repeated after 10 min. As with tests at 48°C the 'difference' method was used to record the findings. Similar variations in readings were found to those recorded in the 'heat' tests. The mean of the difference between right and left sides of control rats was -0.042 sec ± 0.16 (SEM) at day 1, -0.277 sec ± 0.14 (SEM) at day 7, and -0.31 ± 0.17 sec (SEM) at day 14. The measured values of latencies in the same control rats...
were 6.79 sec ± 0.56 (SEM) and 6.83 sec ± 0.54 (SEM) on right and left sides respectively on day 1, 6.22 sec ± 0.46 (SEM) and 6.5 sec ± 0.44 (SEM) on day 7, and 5.94 sec ± 0.41 (SEM) and 6.25 sec ± 0.28 (SEM) on day 14.

The temperature of 6°C was non-noxious for the experimenter and was presumed to test non-noxious stimulation (Attal et al., 1990) according to IASP guidelines (Zimmermann, 1983).

Measurement  A negative difference score in either 'heat' or 'cold' tests indicated hyperaesthesia or hyperalgesia, and a positive result, hypoaesthesia or hypoalgesia.

Results were expressed in graphical form, showing the mean withdrawal reflex time in seconds and standard error of the mean (SEM) for each set of readings.

There was considerable variation in the values obtained in these behavioural tests, no doubt reflecting the difficulty in tying ligatures with a consistent degree of 'looseness' (see Discussion).
Fig. II.1  Pilot study showing withdrawal reflex times (secs) for a range of temperatures from 38-54°C. The results of tests on three rats are shown (○) and their means (■) at each temperature. It was decided to use 48°C as the test temperature for the main study (see text).
MORPHOLOGICAL METHODS

FIXATION

Most rats used for morphological studies requiring fixation were killed by intracardiac perfusion of fixative under deep pentobarbitone anaesthesia. The animals were pinned out in the supine position. The rib cage was opened, and the right atrium and left ventricle were incised. The apex of the heart was held with blunt forceps and a cannula was inserted and tied into the aorta. Fixative was delivered mechanically using a peristaltic pump at a pressure of about 100 mm of mercury. The interval between opening the thorax and the beginning of perfusion was kept to a minimum. Successful perfusion was recognisable by rapid stiffening of the body and tail and discolouration of the viscera.

In a few cases i.e. for some vascular tracer studies and for early changes in which the state of the blood vessels was to be examined, immersion fixation was used. The rats were given an overdose of pentobarbitone anaesthetic, and whilst under deep anaesthesia, the sciatic nerve was carefully dissected out, placed on a card and held at either end by watchmakers forceps to prevent its natural tendency to shorten. After adhering to the card (about 10 secs), the nerve was placed in fixative. In later experiments, the nerve was fixed in situ, with the limb held at maximum length to ensure that the nerve was extended during fixation.

FIXATIVES

A modification of Karnovsky’s fixative (1965) was used. This fixative was freshly prepared and used at about 10°C

10% paraformaldehyde 20 ml
5% glutaraldehyde 12 ml
0.2 M sodium cacodylate buffer 40 ml
Distilled water 28 ml
Anhydrous calcium chloride 50 mg

PROCESSING

(1) Fix nerves overnight in Karnovsky at 4°C, then cut with a blade into appropriate pieces

(2) Rinse in 0.1M sodium cacodylate buffer.

(3) Transfer to 1% aqueous osmium tetroxide for 2-4 hrs at 4°C.

(4) Rinse in 0.1M sodium cacodylate buffer.

(5) Dehydrate in grades of ethanol:
   70% ethanol - 10 mins
   90% ethanol - 10 mins
   Absolute ethanol I - 10 mins
   Absolute ethanol II - 10 mins
   Absolute ethanol III - 10 mins

(6) Transfer to propylene oxide (1,2 epoxy propane) - 15 mins at room temperature.

(7) Transfer to fresh propylene oxide - 15 mins at room temperature.

(8) Transfer to a 1:1 mixture of propylene oxide and resin mixture, place on rotator for 1/2 to 1 hour.

(9) Transfer to pure resin mixture and rotate slowly overnight.

(10) Embed tissue in fresh resin mixture and polymerise 15-40 hrs at 60°C.
Resin mixture

<p>| | |</p>
<table>
<thead>
<tr>
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<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Araldite</td>
<td>20 ml</td>
</tr>
<tr>
<td>DDSA</td>
<td>25 ml</td>
</tr>
<tr>
<td>DMP30</td>
<td>0.8 ml</td>
</tr>
</tbody>
</table>

**NERVES FOR TEASING**

The same schedule as above except

i  DMP 30 is omitted from the resin mixture, so that the resin will not polymerise

ii Stage 10 is omitted; instead the nerves are stored in a refrigerator until required for teasing

**STAINING PROCEDURES**

For light microscopy (semi-thin sections)

(1) Place slides with sections on hotplate

(2) Stain with 1% toluidine blue until steam rises from section for 5 - 10 min

(3) Rinse with distilled water

(4) Drain excess water and blot section

(5) Mount in araldite resin

For electron microscopy

(1) Place grids on drops of uranyl acetate for 3 mins (protect solution from light because of photosensitivity)

(2) Wash in two changes of methanol

(3) Dry on filter paper

(4) Transfer grids to drops of lead citrate for 10 mins (stain in the presence of
sodium hydroxide pellets and avoid breathing on solution to prevent lead carbonate precipitation)

(5) Wash in two changes of distilled water

(6) Dry on filter paper

(7) Place in appropriate grid-box

SELECTION OF BLOCKS

After perfusion with modified Karnovsky’s fixative, the sciatic nerves were exposed and the limb immersed in the same fixative at 4°C overnight. Segments of sciatic nerves proximal or distal to the outermost ligatures, and of sural nerves at mid-gastrocnemius levels were taken. These blocks of nerve were processed and cut by methods given above. More detailed explanations of the selection of blocks particularly in the short interval experiments (8.5, 24 and 48 hour animals) are given in the Results section.

TEASED FIBRE PREPARATIONS

In some cases, the whole segment of nerve, including the ligatured region and portions of the proximal and distal ends was used for fibre teasing; in other cases approximately 1 cm lengths of distal ends of nerves were used. These were processed into araldite resin without hardener and stored in a refrigerator. After removing the perineurium, small bundles of nerve fibres were transferred from non-polymerised resin to a slide with a drop of resin with hardener. For the whole nerve, fibres were separated out into bundles to show the change in contour of the fibres over the ligated area.
For the smaller pieces of nerve, fine needles were used to separate fibres and a permanent preparation was obtained after placing a coverslip over the teased fibres and allowing the resin to polymerise.

ULTRATHIN SECTIONS

Ultrathin section from selected blocks were cut in the silver grey area of interference colour with a diamond knife. They were collected on copper grids, stained with saturated uranyl acetate in 50% ethanol and lead citrate (Reynolds, 1963) (see method above) and examined in a Joel 100 CX electron microscope at an accelerating voltage of 80KV.

HRP METHOD

Fixative for horseradish peroxidase (HRP) method

25% Glutaraldehyde 16 ml
0.2M Cacodylate buffer 84 ml

Horseradish peroxidase (Type II, Sigma chemical Co., St. Louis, U.S.A.) was injected into the femoral vein at a dose of 0.3mg/g in a volume of 0.5-0.7 ml saline. Some rats were killed 5-10 min later by perfusion through the aorta with 4% glutaraldehyde in 0.2M cacodylate buffer. In other rats the nerves were fixed by immersion or fixation in situ.

Treatment of tissues for the cytochemical reaction of peroxidase with 3.3'-diaminobenzidine tetrahydrochloride (DAB) and H₂O₂ was carried out according to the method of Karnovsky (1967). DAB is a carcinogenic substance and care was taken during its handling.
Method:

1. Keep tissue for 1 hr in fixative (4% glutaraldehyde in 0.2M cacodylate buffer) after perfusion or immersion fixation.
2. Cut sections at 30μm using a vibroslice.
3. Rinse in cacodylate buffer for 1 hr at 4°C
4. Rinse in distilled water 4x over 10 min
5. Incubate for 1 hr on rotator in DAB solution prepared as follows:
   a. 15mg DAB in 10ml 0.05M tris buffer at pH 7.6
   b. Place 30 min in 60°C oven
   c. Filter in fume cupboard
   d. Add 0.1ml of 1% H₂O₂
6. Rinse in distilled water 4x over 10 min
7. Post-fix in 1% OsO₄ for 1 hr on rotator
8. Rinsed in cacodylate buffer and leave at 4°C for normal EM processing
9. Semi-thin and ultrathin sections were cut

QUANTITATIVE METHODS

Measurement of number and density of myelinated axons

The purpose of this quantitation was to assess the proportion of myelinated fibres that remained following placing of ligatures. This was achieved by counting the number of normal fibres remaining in each nerve and comparing this with results obtained from 4 normal, control nerves.

Method 1

Numbers of normal myelinated axons were counted from 1μm transverse
sections. The counting was done at the microscope using a x 40 oil immersion objective with an eye-piece graticule marked in squares, and a hand counter. The total number of normal fibres in the sural nerve, and where possible, the sciatic nerve were counted. Using this high magnification, normal fibres could be distinguished from those that were degenerating, although it is possible that fibres at a very early stage of degeneration might have been miscounted as normal fibres.

The sections of a few sciatic nerves were incomplete, but it was possible to count the majority of fibres. Fascicular area was measured in a microscope with an extension tube using a cursor with a light-emitting diode (LED) positioned over the bitpad of a Mini-MOP to trace the outline of the fascicle. The inner edge of the perineurial sheath was used to define the fascicular area. The myelinated fibre density/mm² was calculated from the total number of nerve fibres/ fascicular area.

Method 2

Counts and measurements of nerve fibres were made from light micrographs taken with an oil-immersion objective at x40, and printed to give a final magnification of about x 3,000. The magnification was accurately calculated using a ruler marking superimposed on each photograph. Again, at this magnification, degenerating fibres could be distinguished from degenerating fibres with a high degree of certainty.

Nerves from 21 days onward p.o. began to contain regenerating fibres. For reasons explained in the Discussion, these were included in the counts.

The sural nerve could be photographed in its entirety. In the sciatic nerve, there was no difficulty in sampling areas for photographing when the fibre degeneration was evenly distributed. However, a few nerves were unevenly affected, and in these cases, sampling was non-random in order to include both badly affected
and less affected areas.

Myelinated fibre density was calculated from the area of the prints and the number of fibres counted in this area. Both methods were compared in the study of sural nerves and the variation was less than 10%.

Axon and fibre areas were measured by tracing the inner and outer circumferences of the myelinated fibres, using a pen cursor over the bit-pad of a Kontron Minimop. The Minimop was interfaced to an IBM computer and programmes used to convert area measurements to diameters, assuming circularity of the fibres, and to store data. Fibres sectioned at paranodal regions, recognised by characteristic folding of the myelin sheath, were excluded. At Schmidt-Lanterman incisures, compensation was made for any swelling of the incisures due to fixation artefact. When such swelling occurs, it appears to impinge upon the axon, causing some decrease in axon size. Tracing the inner and outer contour of such fibres would result in axon measurements being smaller, and myelin sheaths thicker than is actually the case. Therefore measurements were made estimating the myelin sheath thickness as it would be without the incisural swelling.

For the demonstration of myelinated fibre and axon parameters, histograms showing fibre diameter distribution, and scatterplots showing myelin sheath thickness for diameter, and 'g' ratios (axon diameter/fibre diameter) were prepared from the stored data.

The density of acutely degenerating myelinated fibres was assessed by counting these in the electron micrographs used for unmyelinated axon counts. They were recognised by collapse of the myelin sheath, loss or alteration of axonal organelles, or loss of the axon.
Measurement of number and density of unmyelinated axons

The density of unmyelinated axons was obtained from electron micrographs of representative regions of sciatic and sural nerves. The sampled region was unavoidably very small (about 0.02mm²), and this is a possible source of inaccuracy.

Criteria for recognising unmyelinated axons were:-

1. Generally circular profiles
2. Completely, or almost completely enclosed by Schwann cell processes
3. Absence of ribosomes or rough endoplasmic reticulum
4. Usually more conspicuous microtubules

It is not possible to distinguish 'original' unmyelinated axons from regenerated axons, therefore the counts obtained will include both types of axon.

Electron micrographs were taken at an initial nominal magnification of 3,300 and micrographs were printed to a final magnification of about x9,000. A grating replica, 2160 lines/mm was used for calibration at each EM session. The total area counted was estimated from the area of each print multiplied by the number of prints. The density of unmyelinated axons /mm² was calculated from the total number of axons in this area.

Statistical methods and formulae

(a) Behavioural studies

Group means were calculated, and the standard error of the mean (SEM = SD/√N). The significance level of the difference between means of various groups at different post-operative times was examined with a 2-tailed Mann-Whitney U (M-W U) test. Values of p <0.05 were considered to be significant.

(b) Morphological correlations
Using Graphpad software and an IBM personal computer, the line of best fit was calculated using regression analysis for some of the data. The relationship between the two parameters was then further analysed by calculating the Pearson's correlation coefficient (r) of the data. A t-test was then carried out on both the slope of the regression line and (r) value.
CHAPTER 3: RESULTS

CLINICAL OBSERVATION OF NEUROPATHIC PAINFUL BEHAVIOUR
AFTER TYING LOOSE LIGATURES ROUND THE SCIATIC NERVE

OBSERVATION OF GAIT AND POSTURE

This was assessed using a quantitative scale, using criteria described in Materials and Methods p.65.

Chronic loose ligatures only

Within a few hours of placing ligatures the animals showed foot drop or dragging, and this continued during the first few post-operative days. From 5 days p.o. this group showed significant neuropathic painful behaviour when compared with controls (p<0.01, n=18, M-W U test). From 4 days p.o. some of the rats (4/18) displayed allodynia (tactile-induced nociceptive behaviour) or spontaneous pain, inferred from a reluctance to put the foot down, and holding the limb in a guarded position while walking, and frequent licking of the foot or vocalization. Maximal allodynia or spontaneous pain behaviour was noted to occur from 1-2 weeks p.o., with a gradual return to normal behaviour by 60 days p.o. (Fig. III.1).

Chronic loose ligatures + concurrent saphenous nerve section

No pain related behaviour was noted at 5 days p.o., the animals showing paretic symptoms only. From 7 days p.o. some of the rats (2/6) displayed pain-related behaviour i.e. allodynia and spontaneous pain. After 10 days, the animals developed
significant painful behaviour when compared with controls (p <0.01, n=6, M-W U test), and an even greater degree of pain-related behaviour than the ligature-only group which continued until at least 30 days p.o.. The return to normal behaviour took longer than 60 days.

**Chronic loose ligatures + saphenous nerve section 7 days later**

All animals showed the same tendency as the ligature-only group within the first 7 days p.o., but following saphenous nerve section 7 days after tying ligatures, they displayed a dramatic change from pain-related behaviour to paretic symptoms such as foot drop or dragging, and insensitivity to external stimuli, from the 8th day. By the 12th day, there was a return to 'allodynic' behaviour. As with the previous group (ligature + concurrent saphenectomy) there was a greater degree of pain-related behaviour and this took rather longer than 60 days to return to normal.

**Chronic loose ligatures + saphenous nerve section 14 and 21 days later**

Both groups showed a similar response to the ligature-only group, but the return to normal behaviour was slightly longer than 60 days p.o.

It was usually easy to differentiate between limping due to weakness, and limping because of reluctance to place the foot on the ground due to pain. However, in a few cases this distinction was difficult to make, although it could often be recognised after several tests had been made.
TESTS USING THERMAL STIMULATION

Chronic loose ligature only

*Thermal stimulation at 48°C*

This was presumed to test the nociceptive response. The earliest hyperalgesic reaction was found in 2 out of 18 rats at 4 days p.o. By the 7th day, 16/18 showed hyperalgesia. After 14 days p.o. all rats were hyperalgesic (p = <0.001, n=18, M-W U test). There was return to a normal response by the 8th week (Fig. III.2A).

*Thermal stimulation at 6°C*

This was presumed to test hyperaesthesia or allodynia (increased reaction to non-noxious stimuli). The earliest hyperalgesic reaction occurred at 4 days p.o.; by the 7th day 18/18 rats showed hyperalgesia compared with normal rats (p = <0.001, n=18, M-W U test), becoming maximal from 7-15 days. There was a gradual return to normal by the 8th week (Fig. III.2B).

Chronic loose ligature + concurrent saphenous nerve section

*Thermal stimulation at 48°C*

From 4-10 days p.o. there was hypoesthesia instead of the hyperaesthesia displayed by the ligature-only group (p= <0.001, n=6, M-W U test) (Fig. III.3A). After the 10th day p.o. the behaviour patterns were not significantly different from the ligature-only group. The return to normal took rather longer than 8 weeks.

*Thermal stimulation at 6°C*

These animals also showed hypoesthesia from 4-8 days p.o. (p < 0.001, n=6, M-W U test) (Fig. III.3B). Thereafter the animals became hyperaesthetic, falling into the ligature-only range, although tending to show rather more marked and longer hyperaesthesia.
Chronic loose ligatures + saphenous nerve section 7 days later

*Thermal stimulation at 48°C*

The responses were not different from those of the ligature-only group until the saphenous nerve was cut and ligated at 7 days p.o., after which there was a period of hypoaesthesia from 8-12 days p.o. (p< 0.001, n=6, M-W U test) (Fig. III 4A). From 14 days after tying ligatures the responses were becoming hyperaesthetic, similar to those of the ligature-only group although again tending to be more delayed in the return to normal.

*Thermal stimulation at 6°C*

As in the 48°C responses, there was no difference from the ligature-only group until the saphenous nerve was cut and ligated at 7 days p.o.. There was a shorter but equally significant period of hypoaesthesia (p<0.001, n=6, M-W U test) (Fig. III.4B) followed by a return to the normal range.

Chronic loose ligatures + saphenous nerve section 14 and 21 days later

These animals showed no significant differences in behaviour from the ligatures-only group after stimulation at 48°C or 6°C (Figs. III.5A,B and III.6A,B).

Sham operated rats

An identical procedure was performed to that used for the normal operation, but without actually tying the ligatures, which were left lying loosely beneath the nerve. In animals so treated, stimulation at 48°C and 6°C resulted in the demonstration of a period of hyperaesthesia (p = <0.01, n=8, M-W U test) during the first 4 days following operation (Fig.III. 7). There was a return to normal values of responses at 6 days p.o.
Autotomy

Only one rat of the 78 in which loose ligatures were tied showed evidence of autotomy. This animal (loose ligatures only) had self mutilated the distal ends of the 4th and 5th digits of the operated foot from the 8th day p.o. There had been a change from hyperaesthesia to hypoaesthesia on the operated side on the 7th day p.o.

As for the other rats, there was no evidence of a change in claw length or thickness between the ligatured and normal sides. Increase in claw length and thickening of the claws may be regarded as evidence of a lack of normal grooming behaviour.

SUMMARY

These behavioural studies have shown that following placing of loose ligatures round the sciatic nerve, hyperalgesia and hyperaesthesia was detected from the 4th post-operative day, and had become maximal by the 14th day. Normal behavioural responses had returned by the 8th p.o. week. Using thermal tests involving immersion of the whole foot, it was found that section of the saphenous nerve at the time of, or within a week of, placing ligatures, had the effect of abolishing the hyperaesthetic behaviour and instead causing hypoaesthesia from the 4th to the 10-12th days. This was followed by a change to hyperaesthetic behaviour. The findings are interpreted as indicating that the saphenous nerve plays a role in the early hyperaesthesia produced in this experimental model.

The behavioural study can be summarized in the accompanying diagram (Fig. III.8).
Abnormal (touch-related) behaviour graded as a rank index (see text) in rats with ligature only (□) \( (n=18) \), ligature with saphenous nerve section at the same time (●) \( (n=6) \) and ligature + saphenous nerve section 7 days later (●) \( (n=6) \). All rats show paresis (+2.5 - +3) for the first few days. In ligature only rats there is a sudden change to allodynia or spontaneous pain (see text) from the 5th day po, with a gradual return to normal by the 60th day (not shown on graph). In rats with concurrent ligature and saphenous nerve section, allodynic behaviour does not occur until later and is even more severe. In rats with ligature and saphenous nerve section at 7 days, the behaviour changes abruptly after saphenous nerve section but by 12 days has returned to an allodynic pattern similar to the ligature only group.
Fig. III.2. Withdrawal reflex times (secs) expressed as the difference between left (normal) and right (experimental) sides at different temperatures. Results are shown as means (■) ± SEM (n=18).

A. 48°C. Following initial hypoesthesia there is hyperaesthesia which is maximal from 2-3 weeks post-operatively (po). A return to a normal response has occurred by the 8th week.

B. 6°C. Hyperaesthesia and/or allodynia is maximal at about the 2nd week po, with a return to a normal response by the 8th week.
Fig. III.3. Results comparing the withdrawal reflex times at (A) 48°C and (B) 6°C in ligature + concurrent saphenous nerve section groups (□ mean ± SEM) (n=6) with that of ligatures only groups (■ mean ± SEM) (data taken from figure 2A and B). There is a highly significant (*) hypoesthetic reaction until the 10th day po (A) and the 12th day po (B). There is a tendency towards greater and longer lasting hyperaesthesia in the saphenous nerve section + ligature group than in the ligature only group.

(* = p<0.001, Mann-Witney U Test)

(Arrow indicates timing of saphenous nerve section.)
Fig. III.4. Results comparing the withdrawal reflex times at (A) 48°C and (B) 6°C in groups with ligature + saphenous nerve section 7 days later (□ ± SEM) (n=6) with that of ligatures only groups (■ mean ± SEM) (data taken from figure 2A and B). For the first 7 days the response is not significantly different from the ligature only group. Following saphenous nerve sections there is a highly significant (*) hypoalgesic response from the 8th day po until the 2nd week po (A and B). There is a tendency towards greater and longer lasting hyperaesthesia in the saphenous nerve section + ligature group than in the ligature only group. (* = p<0.001, Mann-Witney U Test)
(Arrow indicates timing of saphenous nerve section.)
Fig. III.5. Results comparing the withdrawal reflex times at (A) 48°C and (B) 6°C in groups with ligature + saphenous nerve section 14 days later (□ ± SEM) (n=6) with that of ligatures only groups (■ mean ± SEM) (data taken from figure 2A and B). There is no difference between the ligature + saphenous nerve section groups and the ligature only groups before the time of saphenous nerve section. The hyperalgesic response tends to be greater and longer lasting in the ligature + saphenous nerve section group than in the ligature only group. (Arrow indicates timing of saphenous nerve section.)
Fig. III.6. Results comparing the withdrawal reflex times at (A) 48°C and (B) 6°C in groups with ligature + saphenous nerve section 21 days later (□ ± SEM) (n=6) with that of ligatures only groups (■ mean ± SEM) (data taken from figure 2A and B). There is no difference between the ligature + saphenous nerve section groups and the ligature only groups before the time of saphenous nerve section. The hyperalgesic response tends to be greater and longer lasting in the ligature + saphenous nerve section + ligature group than in the ligature only group. (Arrow indicates timing of saphenous nerve section.)
Fig. III.7. Withdrawal reflex time (secs) at 48°C (n=8) in sham-operated rats (see text). There is an initial hyperaesthetic response which returns to normal by the 6th day.
Fig. III.8. Diagram showing the hypothetical temporal relationship between pain-related behaviour (thermal and cold response) caused firstly (A) by the contribution from the saphenous nerve, lasting from the 4th to the 14th day and possibly later, and (B), from about the 10-12th day to the 8th week, that is due mainly to the sciatic nerve.
MORPHOLOGICAL EXAMINATION OF SCIATIC NERVES AFTER PLACING LOOSE LIGATURES

CHANGES UP TO 48 HOURS p.o.

Examination of sciatic nerves at short time intervals after tying ligatures was made in order to study the evolution of the lesion. It will be recalled that the ligatures were tied loosely, so as not to compress the nerve, although there was some constriction of superficial epineurial vessels. Despite this apparently mild procedure, the animals became totally or partially paralysed in that limb shortly after operation.

At periods of 8.5, 24 and 48 hours post-operatively, after in situ immersion fixation, the sciatic nerve was dissected out and placed in fixative overnight. After careful removal of the ligatures, the nerve was examined macroscopically. At 8.5 and 24 hours, there was very little evidence of any change in the contour of the nerve. Occasionally a slight indentation could be seen affecting just a part of the nerve surface.

Segments of nerve were cut 5mm proximal to the topmost ligature. Transverse and longitudinal segments were taken from the ligature region, and a segment 5mm distal to the lowest ligature. Diagrams made at the time of taking these blocks indicated their position in relation to the ligatures. The pieces of nerve were processed routinely into araldite for semithin and ultrathin sectioning. Nerves from two control limbs, without surgery, were treated in the same way. Fig. III. 9 shows the appearance of a control nerve after in situ immersion fixation.
8.5 hours p.o.

Total fascicular areas

The fascicular areas of sections taken at various levels showed little change, compared with their control sides (Table III.1). In one animal, sections were cut at the level of a ligature and the block then reversed to cut a level between ligatures. There was no difference in areas of these two levels.

Epineurial changes

At proximal, and at ligature levels, the epineurial vessels immediately surrounding the nerve were often seen by light microscopy to be congested with tightly packed red cells (Fig. III.10). Arterioles in the epineurium only have a narrow layer of smooth muscle cells; when the vessels are distended it is difficult to differentiate them from venules. Vessels not filled with cells often appeared to be distended with plasma. At the distal level, an occasional small vessel was engorged with red cells. In control nerves fixed in the same way (by in situ immersion), epineurial vessels contained some red cells or were filled with plasma but were not distended.

Scattered macrophages were seen particularly in the region where the ligatures had been situated (Fig. III.11). Mast cells were seen, and those near to the perineurium were sometimes degranulated (Fig. III.12).

Changes within the endoneurium

Blood vessels

At proximal levels, a region of subperineurial oedema was seen, often affecting only a part of the fascicle (Fig. III.13). Scattered macrophages were seen but only near to the perineurium, lying in this oedematous region (see Fig. III.11).
Within the endoneurium, the vessels were all open, and sometimes quite distended, but usually only containing a few red cells. Endothelial cells appeared plump with nuclei protruding into the vessel lumen. At a distal level there was marked subperineurial oedema (Fig. III.14).

At the level of the ligatures there occasionally appeared to have been extravasation of red cells, which were seen lying in the endoneurial space (Fig. III.15); this was not necessarily accompanied by clear evidence of endoneurial oedema in the vicinity. Definite separation of endothelial cells was not seen, and tight junctions appeared to be intact (Fig. III.16). Mast cells were always present, but unlike those in the epineurium they had in most cases not become degranulated. Endothelial cells were again prominent, with nuclei protruding into the vessel lumen. Also there was often conspicuous pinocytotic activity of these endothelial cells.

Nerve fibre changes

Nerve fibre changes were most obvious in the general region of the ligatures. Levels proximal and distal to the ligatured region appeared normal except for some subperineurial oedema (Figs. III.10,13), and a very occasional abnormal fibre showing changes to be described below. By electron microscopy, the subperineurial oedema could be seen to have separated the inner layers of the perineurial sheath (Fig. III.17) although these laminae remain intact. The outer perineurial cells appear to have prevented passage of endoneurial oedema through to the epineurium.

In the ligature regions, various types of abnormality were recognised. In some fibres the axons were quite deeply stained and swollen, often with marked thinning of the myelin sheath (see Fig. III.15). In other fibres the axons appeared shrunken and there was a space between the axon and the myelin sheath. These changes
sometimes affected groups of fibres, but were also scattered throughout the nerve. Transverse sections viewed by electron microscopy showed changes in many fibres; some axons were shrunken, leaving a wide periaxonal space (Fig. III.18). In others there was axonal swelling due to an accumulation of organelles, often of a vesicular nature, and subsequent thinning or loss of the myelin sheath (Fig. III. 19a,b). In other fibres the axon appeared to be degenerating and the myelin sheath had a 'moth-eaten' appearance (Fig. III.20). In many fibres the Schwann cell cytoplasm was abnormal; often it was more abundant then normal, and was of increased electron density due to close packing of organelles; mitochondria were often enlarged, or degenerating. In other Schwann cells, there was loss of all recognisable organelles leaving just granular material (Fig. III.21); this was also seen in some myelinated fibres which were not degenerating (Fig. III. 22). In Fig. III.23 the axon contains aggregations of tubulo-vesicular organelles. There is a periaxonal space; the myelin sheath is intact, but the Schwann cell cytoplasm contains no recognisable organelles. There was little change in unmyelinated fibres.

In longitudinal sections of one nerve, a group of fibres showed axon swelling, clearly associated with the accumulation of organelles, and with loss or thinning of the myelin sheath (Fig.III. 24). The fibres in continuity with these, showed some folding of the sheaths and shrinkage of axons.

Electron microscopy showed accumulation of organelles including vesicular bodies, probably part of the SER system, mitochondria in various stages of degeneration and dense lysosomal bodies in association with thinning of the myelin sheath (Fig.III. 25). In other fibres accumulations of neurofilaments and granular material as well as vesicular material caused axonal swelling and thinning and loss
of myelin (Fig. III.26). The aggregations of organelles had often collected at the edges of the axons. The increase in axonal size often appeared to be consistent along the length of the fibre for many microns, with no focal swelling such as might be expected from a mechanically produced lesion. The Schwann cell cytoplasm of some of these fibres was often very abnormal with few recognisable organelles (see Fig. III. 18). Beyond the region of swelling there were shrunken axons with a wide periaxonal space and folded myelin. The unmyelinated axons were sometimes seen to swell focally along their length.

24 hours p.o.

Total fascicular areas

Macroscopic examination still showed no gross changes in contour along the nerve (see Table III.1).

Epineurial changes

Blood vessels in the ligatured region were sometimes packed with red cells, others appeared distended by plasma. By electron microscopy there was evidence of separation of the endothelial cells allowing the free passage of red cells and plasma (Fig. III. 27) and there was also evidence of degeneration of some of these endothelial cells. Scattered macrophages were present throughout the epineurium from proximal to distal levels.

Changes within the endoneurium

Some subperineurial oedema was seen at the proximal and distal levels, but was not conspicuous at ligature levels. Endoneurial blood vessels were generally open but mostly only containing plasma. Very occasionally, there was congestion of an endoneurial vessel with red cells, with probable extravasation of red cells which were
seen lying freely in the endoneurium (Fig. III. 28). Occasionally, the vessels were distended with plasma. As at 8.5 hours there was no obvious accumulation of endoneurial oedema near to the site of red cell extravasation. In one nerve, there was evidence that the perineurium had split and nerve fibres could be seen emerging from the fascicle (Fig. III. 29). The site of rupture of the perineurium was clearly not at a surface facing onto the ligature, but at an inner surface between two fascicles. This was the only example seen, in the whole study, of disruption of the perineurium. In this nerve the different appearances of fascicles is shown (see below).

Nerve fibres at proximal and distal levels were generally normal. Appearances at transverse 'ligature' levels could vary very much, both between and within fascicles (see Fig. III.29) and at different levels. For example, at one level, scattered or small groups of myelinated fibres showed appearances similar to those described at 8.5 hours p.o., with swollen, darker staining axons, sometimes with periaxonal spaces, or with thinning or loss of sheaths (Fig. III.30); at another level, many of the fibres showed markedly shrunken axons, periaxonal spaces and split or disintegrating myelin sheaths (Fig. III. 31).

In longitudinal section there were fibres with thinning of sheaths at the nodes of Ranvier, often affecting only one side of the node (Fig.III. 32), whilst elsewhere there was marked folding of sheaths.

**48 hours p.o.**

**Epineurial changes**

By this time, macroscopic examination showed indications of constriction of the nerve. Measurement of transverse sections in one case (Table III.1) showed a marked increase in the size of the proximal segment with narrowing at the level of a ligature.
The onset of compression appeared to be related to the increase in size of the proximal and distal segments due to endoneurial oedema; another factor could be the increase in fascicular size due to the marked swelling of axons affected by ischaemia.

By light microscopy, epineurial vessels were full, but not packed, with red cells. Endoneurial vessels appeared small, containing a few red cells, and some looked collapsed. Increase in fascicular size of the proximal segment was due to endoneurial oedema. A few fibres with swollen axons and thinned myelin sheaths were scattered throughout the nerve.

In the region of the ligatures epineurial vessels were sometimes packed with red cells; others were empty. In the endoneurium, blood vessels varied from small and shrunken-looking structures, to distended vessels, full of fluid but with few red cells. Appearances of nerve fibres varied considerably from level to level.

Changes within the endoneurium

In longitudinal section, narrowing of the nerve seemed to be associated with reduction in the size of nerve fibres due to thinning of the sheaths (Fig. III. 33a,b). In these fibres no accumulation of axonal organelles was visible by light microscopy, but electron microscopy showed a conspicuous increase in the density of axonal neurofilaments (Fig. III. 34). In some cases, the increased neurofilament density appeared to be related to myelination since there was a noticeable increase in neurofilament density where the axon was demyelinated (Fig. III. 35). This increase did not produce any focal swelling but was distributed evenly along the nerve, at least over the region examined. Changes were also observed in the Schwann cell cytoplasm of these fibres, which was often electron dense due particularly to the large numbers of mitochondria present, but also to the close packing of other organelles.
such as filaments and ribosomes. This is seen in Fig. III.34 and also in Fig. III.36 in association with a demyelinated fibre with increased axonal neurofilaments; the myelin of the original sheath has become widely separated forming a loose mesh of membranes. In some fibres, thinning of the axon was due to withdrawal of the terminal loops of myelin lamellae, which could be seen at situations other than at the paranodal regions producing gradual thinning along the internode. Elsewhere the axons were completely bare of myelin and of Schwann cell cytoplasm (Fig. III.37). Occasionally a demyelinated axon did not show increased neurofilaments but granular material was present (Fig. III.38); in this fibre, terminal loops of the adjacent myelin sheath had been pulled away from their original sites of termination. In this narrowed region unmyelinated axons were swollen, with numerous microtubules and granular material (Fig. III.39).

On either side of this narrowed region with axons rich in neurofilaments, there were fibres with evidence of axonal accumulation of other organelles (Fig. III.40).

The appearances in the narrowed region possibly represent the first evidence of a direct acute compressive effect upon the nerve fibres, the thinning or loss of myelin not being due to obvious swelling of the axons, as in the ischaemic-related changes, and not apparently associated with degenerative changes to the Schwann cell, although these showed other and different changes.

Appearances of fibres varied considerably in transverse section at levels other than those of the narrowed regions. Some myelinated fibres showed accumulation of organelles, mostly of a vesicular nature; unmyelinated axons were also involved (Fig. III. 41). These may represent fibres affected by ischaemia at other levels. Unmyelinated axons sometimes showed massive swelling with a granular change to
the axoplasm while adjacent myelinated fibres were much less affected (Fig. III.42).

In summary, the study of nerves within two days of tying ligatures has suggested possible mechanisms for the drastic clinical effects of an apparently mild lesion. Congestion of many epineurial vessels appears to be associated with early changes to nerve fibres. These take place before any marked alterations in the contour of the nerve, and are therefore not likely to be due to direct compression. The fibre changes are identical to those described in experimental ischaemic studies (Korthals and Wiśniewski, 1975; Korthals, Korthals and Wiśniewski, 1978), (see discussion).

Increase in fascicular area takes place due to endoneurial oedema, particularly proximal and distal to the ligatured region, and this eventually does cause the ligatures to have a compressive effect. Thinning of the myelin sheath or loss of myelin, with increased numbers of axonal neurofilaments, is possibly a change due to compression, and was identified by 48 hours p.o.
Fig. III.9. 1μm resin section (immersion fixation): A control nerve showing normal nerve fibres. There is no subperineurial oedema; the endoneurial blood vessels are open but not distended and contain an occasional red cell. x740
TABLE III.1 Measurement of fascicular areas in mm² from 1µm araldite sections
Areas of control side in brackets. *: indicates significant differences (p< 0.05, student t test).

<table>
<thead>
<tr>
<th>Level of section</th>
<th>Proximal</th>
<th>Ligature</th>
<th>Distal</th>
</tr>
</thead>
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<tr>
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<td>0.58</td>
<td>0.45(0.43)</td>
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<tr>
<td>Ligatured side</td>
<td>0.39(0.38)</td>
<td>0.39</td>
<td>0.45(0.38)</td>
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<tr>
<td>Ligatured side</td>
<td>-</td>
<td>0.48</td>
<td>0.49</td>
</tr>
</tbody>
</table>

8.5 hours p.o.

24 hours p.o.

| Ligatured side   | 0.60 | 0.52 | 0.54 |
| Ligatured side   | 0.53 | 0.51 | 0.57 |

48 hours p.o.

| Ligatured side   | 0.74* | 0.312* | 0.46 |
Fig. III.10. 1μm resin section (immersion fixation): chronic multiple loose ligation, proximal to the ligatures, 8.5 hours p.o. The transverse section shows that the epineurial vessels are congested with tightly packed red cells. Endoneurial blood vessels are open and contain a few red cells. Nerve fibres appear normal. There is no marked subperineurial oedema in this part of the nerve. x460
Fig. III.11. 1μm resin section (immersion fixation): chronic multiple loose ligation, at ligatures level, 8.5 hours p.o. The transverse section shows that epineurial vessels are congested with tightly packed red cells. There is also prominent inflammatory cell infiltration in the epineurium. Macrophages are also seen in the perineurium and some in the subperineurial area (arrows) but none in the endoneurium. Some abnormalities of nerve fibres are seen, consisting of shrunken axons staining more deeply than normal, and folded myelin sheaths. x 460
Fig. III.12. 1μm resin section (immersion fixation): chronic multiple loose ligation, proximal to the ligatures, 8.5 hours p.o. One epineurial vessel is filled with red cells. There are scattered macrophages in the epineurium. Degranulated mast cells (arrows) are seen. There is some subperineurial oedema; the nerve fibres appear normal. x740
Fig. III.13. 1μm resin section (immersion fixation): chronic multiple loose ligation, proximal to the ligatures, 8.5 hours p.o. A region of marked subperineurial oedema is shown. x740
Fig. III. 14. 1μm resin section (immersion fixation): chronic multiple loose ligation, distal to the ligatures, 8.5 hours p.o. The transverse section shows an almost normal appearance, but there is prominent subperineurial oedema extending along a dividing septum. x460
Fig. III.15. 1μm resin section (immersion fixation): chronic multiple loose ligation, at the ligatures level, 8.5 hours p.o. There is extravasation of red cells into the endoneurial space (arrow). Several macrophages are seen in the subperineural space. Some myelinated fibres have darkly staining axons which are either swollen or have a periaxonal space. In one or two fibres the axons have disappeared. x740.
Fig. III.16. Electron micrograph: chronic multiple loose ligation, at the ligatures level, 8.5 hours p.o. Red cells are seen lying between the endothelial cells and the pericytes (arrow), as well as in the endoneurial space. The red cells have almost certainly passed through endothelial cells, although all the junctions were found to be intact. x10,625.
Fig. III.17. Electron micrograph: chronic multiple loose ligation, proximal to the ligatures, 8.5 hours p.o. showing granular proteinaceous oedema in the subperineurial space and separating individual inner perineurial lamellae. The lamellae, however, remain continuous. Part of a macrophage is seen in the subperineurial space. x12,750
Fig. III.18. Electron micrograph: Chronic multiple loose ligation at the ligatures level, 8.5 hours p.o. A myelinated fibre with a very shrunken axon which contains closely packed filaments and microtubules; there is a large periaxonal space. x15,000.
Fig. III.19a. Electron micrograph: chronic multiple loose ligation, at the ligatures level, 8.5 hours p.o. A grossly swollen axon filled with vesicular bodies. The fibre has no myelin sheath and this may have been displaced due to axonal swelling. However, the few mitochondria present in the Schwann cell cytoplasm might suggest that this section is at the paranodal region. x10,000

Fig. III.19b. At a higher magnification, the vesicular nature of the accumulated organelles can be seen. x25,000
Fig. III.20. Electron micrograph: chronic multiple loose ligation, at the ligatures level, 8.5 hours p.o. A myelinated fibre showing degenerating change of axon, myelin sheath and Schwann cell. x 10,000.
Fig. III.21. Electron micrograph: chronic multiple loose ligation, at the ligatures level, 8.5 hours p.o. The presence of Schwann cell processes (arrows) suggests that this section is at the level of a node of Ranvier. The cytoplasm of the two participating Schwann cells is abundant and contains no recognisable organelles. x15,000
Fig. III.22. Electron micrograph: chronic multiple loose ligation, at the ligatures level, 8.5 hours p.o. A myelinated fibre showing some axonal atrophy. However, the most noticeable feature is the abnormal appearance of the abundant Schwann cell cytoplasm. Only an occasional small mitochondrion is still recognisable, otherwise the cytoplasm consists of dense granular material. x12,750
Fig. III.23. Electron micrograph: chronic loose ligation at the ligatures level, 8.5 hours p.o. A myelinated fibre with aggregations of axonal organelles, periaxonal space and intact myelin sheath. The Schwann cell cytoplasm appears degenerate. x10,625
Fig. III.24. 1 µm resin section (immersion fixation): chronic multiple loose ligation, at the ligatures level, 8.5 hours p.o. In the longitudinal section, a group of fibres shows axonal swelling associated with accumulation of organelles and myelin thinning. x180.
Fig.III.25. Electron micrograph: chronic multiple loose ligation, at the ligature level, 8.5 hours p.o. The longitudinal section shows prominent accumulation of organelles, thinning of myelin sheath and increase of periaxonal space toward the right of the figure. Vesicular organelles are collected mainly at the edges of the axons, and the neurofilaments tend to lie at the centre. There is also thinning of the myelin sheath. The Schwann cell cytoplasm consists only of dense granular material. x10,250.
Fig.III.26. Electron micrograph: chronic multiple loose ligation, at the ligatures level, 8.5 hours p.o. The longitudinal section shows accumulation of organelles (mainly neurofilaments and vesicular profiles) and granular material. There is tapering and eventually loss of the myelin sheath. x10,250.
Fig. III.27. Electron micrograph: chronic loose ligation at the ligatures level, 24 hours p.o. The endothelial cells of this epineurial vessel are shrunken and have wide spaces between them. The vessel is packed with red cells. One endothelial cell has a degenerate appearance. x15,000
Fig.III.28. 1μm resin section (immersion fixation): chronic multiple loose ligation, at the ligatures level, 24 hours p.o. There are many myelinated fibres with deeply stained axons, often with thinning of myelin sheaths. Some fibres appear with shrunken or collapsed axons. Some endoneurial blood vessels are congested with red cells. There are some red cells extravasating from blood vessels and lying freely in the endoneurium, but with no marked endoneurial oedema. x470.
Fig.III.29. 1μm resin section (immersion fixation): chronic loose ligation, just distal to the ligatures 24 hours p.o. The perineurium of the larger tibial nerve has ruptured and nerve fibres are emerging into the perineurial space. The smaller peroneal nerve is more severely affected than the tibial nerve. x 180
Fig. III.30. 1μm resin section (immersion fixation): chronic multiple loose ligation, at the proximal part of ligature level, 24 hours p.o. There are some myelinated fibres with swollen, darkly stained axons of which myelin sheaths are thinning or lost. Other fibres have shrunken, darkly stained axons with large periaxonal spaces. x470.
Fig.III.31. 1μm resin section (immersion fixation): chronic multiple loose ligation, at the distal part of ligatures level, 24 hours p.o. Most of the myelinated fibres show markedly shrunken axons, periaxonal spaces and split or disintegrating myelin sheaths. The appearance markedly contrasts with the previous section at more proximal level. x470.
Fig III.32. 1μm resin section (immersion fixation): chronic multiple loose ligation, at the ligatures level, 24 hours p.o. In longitudinal section, there are fibres with thinning of myelin sheaths at the node of Ranvier, often affecting only one side of the node (arrow). x740.
Fig. III.33a. 1μm resin section (immersion fixation): chronic multiple loose ligation, at the ligatures level, 48 hours p.o. In longitudinal section, there is some local reduction in the size of the nerve possibly due to a direct compressive effect. To the right side of ligature, the nerve apparently contains mainly myelin sheaths (see Fig. III.33 in transverse section). The asterisk marks the original position of the ligature. x74

Fig. III.33b. At a higher magnification, the nerve fibres in the narrowed region show only thinning of their myelin sheaths without obvious evidence of organelle accumulation. The appearance is in contrast to the changes at 8.5 and 24 hours p.o. which show clear evidence of organelle accumulation. x740
Fig.III.34. Electron micrograph: chronic loose ligation, at ligature level in the region of fascicular narrowing (see Fig.33) 48 hours p.o. A longitudinal section showing increased axonal neurofilaments. The myelin sheath is thin relative to fibre size. The Schwann cell cytoplasm is electron dense and contains large numbers of mitochondria, (the section is not at a paranodal region where these might be expected). x25,000
Fig. III.35. Electron micrograph: chronic loose ligation, at ligature level in the region of fascicular narrowing, 48 hours p.o. This fibre shows demyelination of part of the internode illustrated. Axonal neurofilament density increases sharply at the demyelinated part of the fibre. The loops of the myelin sheath do not terminate normally (arrows). Part of a larger fibre shown, also has increased axonal neurofilaments. x19,000
Fig. III.36. Electron micrograph: chronic loose ligation, at ligature level in the region of fascicular narrowing, 48 hours p.o. An oblique section through a demyelinated axon with increased neurofilaments. The associated Schwann cell cytoplasm contains large numbers of mitochondria. The myelin of the sheath has separated into a mesh of loose lamellae. x15,000
Fig. III.37. Electron micrograph: chronic loose ligation, at ligature level in the region of fascicular narrowing, 48 hours p.o. A demyelinated axon with a high density of neurofilaments is partially denuded of Schwann cell cytoplasm. x19,000
Fig. III.38. Electron micrograph: chronic loose ligation, at ligature level in the region of fascicular narrowing, 48 hours p.o. A demyelinated axon contains granular material, with few or no neurofilaments. The terminal loops of the adjacent myelin sheath do not terminate normally (arrows). A single Schwann cell is related to both demyelinated and myelinated parts of the fibre, and shows increased electron density and large numbers of mitochondria. x30,000
Fig.III.39. Electron micrograph: chronic loose ligation, at the ligature level in the region of fascicular narrowing, 48 hours p.o. Unmyelinated axons are swollen, with granular material and numerous microtubules. x19,000
Fig. III.40. Electron micrograph: chronic loose ligation, at the ligature level, adjacent to the narrowed region of the fascicle, 48 hours p.o. The axon is swollen with large numbers of vesicular and lamellar inclusions, and the myelin sheath is thin. x15,000
Fig. III.41. Electron micrograph: chronic loose ligation, at the ligatures level, 48 hours p.o. Axons are swollen mainly with vesicular organelles. Some of these are probably unmyelinated axons. Schwann cell cytoplasm often has an abnormal appearance or is difficult to identify, particularly in what appear to be unmyelinated fibres. x10,000
Fig.III.42. Electron micrograph: chronic loose ligation, at the ligatures level, 48 hours p.o. Grossly swollen unmyelinated axons are seen, mostly filled with granular material. The myelinated fibres are less affected. x10,000
CHANGES FROM 5-14 DAYS p.o.

Qualitative changes

By the 5th day, constrictions of the nerve could be seen at ligature sites more definitely than at 48 hours p.o. This was most clearly shown in teased bundles of the nerve (Fig. III.43). Compressed regions which were without myelin were interspersed with swollen segments containing much myelin, mostly in the form of large ovoids. Fibres passing from the compressed to the swollen region showed marked thinning and/or folding of their myelin sheaths (Fig. III.44). Immediately proximal to the ligatures, the nerve fibres were often swollen, gradually becoming normal in appearance at a low power magnification (Fig. III.45). Distal to the ligatures some fibres were breaking up into ovoids (Fig. III.46).

Assessment of the degree of fibre degeneration caused by the ligatures was made from transverse sections of the sciatic nerve about 5mm distal to the ligatures. In some animals the sural nerve was also examined. At this stage degeneration was at an early stage, and was usually recognised by light microscopy as collapse of the myelin sheath or degenerative change or absence of the axon (Fig. III.47). There was variation in the amount and distribution of nerve fibre damage and in the degree of endoneurial oedema. A few nerves showed degeneration of the majority of myelinated fibres (Fig. III.48). Nerve fibre degeneration may not always be recognised by light microscopy, and fibres such as that seen in Fig. III.49, show that electron microscopy may be required for correct identification of the early stages of fibre degeneration.

Unmyelinated axons were infrequently seen in the process of degeneration. A swollen appearance, with loss of axonal organelles was assumed to indicate early
degeneration. Schwann cells rarely showed the banding of their processes which is described as the typical appearance of denervated unmyelinated axons (Fig. III. 50).

A conspicuous feature of one nerve, 7 days after tying ligatures was the presence of many vacuolated fibroblasts (Fig. III. 51). The nerve showed rather little fibre degeneration, but it was oedematous. Both tibial and peroneal branches of the sciatic nerve were similarly affected and contained the fibroblasts. A very occasional vacuolated fibroblast was seen in one or two other nerves.

By 8 and 14 days p.o. fibre degeneration was further advanced. Not all degenerated fibres are recognisable by light microscopy at these later stages, since some will be sectioned at a level which does not include myelin debris. It is necessary to use electron microscopy to identify the bands of Büngner which signify earlier degeneration of myelinated fibres (Fig. III.52).

A change noted at 8 days and more so at rather later stages was the presence of fibres with axons small for the thickness of their myelin sheaths. These are described as atrophic axons (Fig. III. 53). Some of the axons of these fibres contained very few neurofilaments, although microtubules were numerous (Fig. III. 54). There were still some fibres showing acute degeneration at 14 days, and this would have been too late to be caused directly by compression. However, these became less frequent with time (Table III.2).

21-28 days p.o.

As with the previous nerves, there was wide variation in the degree of myelinated fibre damage. Transverse sections showed normal fibres and some myelin debris. A low power figure illustrates patchy distribution of fibre loss (Fig. III.55). At higher power there is still evidence of recent fibre degeneration which again would
be too late to have been caused by the initial compressive lesion (Fig. III.56, 57); in addition there are also atrophic fibres Fig. III.56). From previous studies on the effects of compressive lesions (Baba et al., 1982), it seems likely that a slow and continuing process of axonal atrophy, as a result of the continuing compressive effect of the ligatures, could lead eventually to distal fibre degeneration. This would account for the presence of the recently degenerated fibres.

At this stage, myelinated fibre regeneration was evident although the fibres were still small and thinly myelinated and could generally be distinguished from the 'original' fibres (Fig. III.56).

In some nerves, unmyelinated axons, probably regenerated, were seen in close apposition, without intervening Schwann cell cytoplasm (Fig. III. 58, Fig. III.59). This is a possible source of ephaptic transmission. Some of the axons in these unmyelinated fibre bundles showed accumulation of organelles, and were possibly at an early stage of degeneration.

1-3 months p.o.

Assessment of damage was more difficult as regenerating fibres were less easy to differentiate from 'original' fibres, although they were generally clearly smaller (Fig. III.60). Small numbers of recently degenerated fibres could still be identified at one month p.o. (Fig. III.60) and two months p.o. Fig. III.61) indicating that the process of distal degeneration could continue for many weeks.

In only one of all the nerves examined was there interruption of the perineurium, a common finding in the study by Coggeshall et al., (1993) and Sommer et al., (1993); and in the present study, there was no evidence of minifascicle formation outside the nerve, a feature also reported by Sommer et al.,
(1993) and no doubt due to rupture of the perineurium.

**Sham operation**

Eight rats were examined after sham operations, using the same procedure as for the usual operation but leaving the ligatures untied. In sections taken distal to the level of placing of the ligatures, only one nerve showed evidence of occasional scattered fibre degeneration (Fig. III.62). This shows that the isolation of the nerve from its vasculature for several centimetres, in order to tie the ligatures, had very little effect.

**Tissue response to the ligature**

Sections taken through the ligatures showed, in one animal, a band of macrophages surrounding the material, but not so as to obviously have any compressive effect upon the nerve (Fig. III.63). In another animal, by 7 days p.o. macrophages had begun to erode, as well as surround the ligature material (Fig III.64). The ligature material could still be found up to eight weeks p.o.

**HRP**

The purpose of this part of the study was to attempt to identify the origin of endoneurial oedema which contributes to the lesion. Increased fascicular size is clearly an important factor in causing the ligatures, originally loosely tied, to eventually have a self-strangulating effect. Unfortunately the results were less helpful than had been hoped, largely because of the difficulty of sampling in a lesion which varies from fascicle to fascicle and one level to another. Also, there were technical problems with the method, and the reaction product was often not sufficiently dark to clearly see the route of the tracer either by light or electron microscopy.

At 8.5 hours p.o. successful preparations were only obtained from the ligature
region. Reaction product was present, as expected in the epineurium; it was seen within some endoneurial vessels, mostly at the luminal surface of the endothelial cells, but there was no evidence of leakage from these vessels into the endoneurial space. HRP reaction product was present in the subperineurial region in one small segment of a fascicle in one nerve (Fig. III. 65) but it was not possible to identify the source of this leakage. Electron microscopy failed to show any passage of tracer through the perineurium, although this would seem to be the most likely route. Permeability of the perineurium is also suggested by the presence of macrophages in the subperineurial region (described above). None of the sections examined were at sites of extravasation of red cells, which would have been expected to show some leakage.

At 24 hours p.o., there was evidence of considerable leakage of the tracer into the endoneurium in sections proximal to ligatures, and passage of HRP between endothelial cells was seen by electron microscopy (Fig. III. 66). At ligature level reaction product was seen lying between perineurial lamellae but not in the endoneurial space. By 48 hours p.o., there was leakage of HRP particularly into the subperineurial space, but also more generally in the endoneurium.

The HRP studies have shown passage of the tracer through endoneurial blood vessels in the proximal part of the nerve at an early stage, and the subperineurial distribution of leakage of the tracer elsewhere suggests its passage through the perineurium, although this was not confirmed. The absence of leakage of tracer from endoneurial vessels at ligature level 8.5 hours p.o. would be in keeping with ischaemia in this region.
Quantitative changes

The degree of axon loss was assessed by measuring the density of myelinated axons remaining in the nerves, and calculating the percentage of axons lost compared with densities from control nerves. Control values (mean density/mm² ± S.D.) were obtained from 4 normal sciatic and 4 normal sural nerves (Table III.3)

Myelinated fibres

(a) Estimation of myelinated fibre loss

In this first section only results up to 14 days p.o. are considered because beyond that time quantitative studies are complicated by the presence of regenerating fibres. The very wide variation of loss of fibres is shown (Table III. 4), ranging from 17-97%. From measurements of fibre size, it could be seen that in many cases there was greater loss of larger fibres than of small fibres. A graph from one nerve (Fig. III. 67) shows the myelinated fibre distribution with greater loss of large fibres. Also, data in the same Table shows the % loss of fibres above and below 6μm. In most cases the loss of large fibres was greater than that of small ones, but in some cases this selectivity was not obvious especially when the nerve injury was very severe (Fig. III. 68).

(b) Density of myelinated fibres

Findings are shown in Table III.5. (This Table also includes values for pain related behaviour (PRB) which are later used for correlation studies). In many nerves after 21 days p.o. the numbers of small fibres exceeded the normal range due to inclusion of regenerated fibres. The densities of small fibres is greater in sciatic than in sural nerves, illustrating the fact that the level of section was more distal in the
sural than the sciatic nerve.

(c) Estimation of 'g' ratios

The 'g' ratio (axon/fibre diameter) can be used to identify fibres showing axonal atrophy, since rarely do normal fibres have 'g' ratios of less than 0.5. (Fig. III. 69). Also, high values of 0.8-0.9 probably indicate regenerated fibres with thin myelin sheaths. Figure III.70 shows a number of fibres with 'g' values of less than 0.5. As observed by Baba, Gilliatt and Jacobs, (1983) and O'Neill et al., (1984) in their studies of fibres distal to a tight, but not completely constricting ligature, atrophic fibres were seen up to several weeks after tying the ligature (Figs. III.71,72)

Estimation of the density of unmyelinated axons

The normal range of unmyelinated axon density is quite wide, therefore calculations of the proportions of axons lost are at best only a rough estimate, being based on the mean control values. The loss of unmyelinated axons during the first 14 days is generally less than that of myelinated fibres (Table III. 4). As mentioned in the Methods section, from 21 days it is not possible to differentiate regenerating unmyelinated axons from original axons. In general, the increases were not very marked, despite the fact that in addition to possible regenerated fibres, unmyelinated axon sprouts, associated with the regeneration of myelinated fibres, would unavoidably have been included in these counts. However, the numbers of regeneration clusters was generally not very great.
Fig. III.43. A bundle of teased fibres: chronic loose ligation; 5 days p.o. The nerve showing three constricted regions, at the site of ligatures, which are unstained because of loss of myelin sheaths. x5
Fig. III.44. Teased fibres: chronic loose ligation, 5 days p.o. Fibres from beyond a constricted region showing irregular thinning of the myelin sheath and variation in the contour of the axons. x740
Fig.III.45. Teased fibres: chronic loose ligation, 5 days p.o. Fibres passing from a ligatured region to the level proximal to the ligatures. At the left there is loss of myelin staining; the central region shows fibres with thin myelin and irregular contours. At the right, the proximal fibres are becoming more normal in appearance. x470
Fig. III.46. Teased fibres: chronic loose ligation, 5 days p.o. Fibres just distal to the lowest ligature show thinning of myelin at the right, and the beginning of ovoid formation more distally at the left. x470
Fig. III.47. 1μm section (immersion fixation): chronic loose ligation, distal to ligatures, 5 days p.o. There are many degenerating fibres without visible axons but only collapsed myelin sheaths. The nerve fibre damage has a patchy distribution. x470
Fig. III.48. 1μm section: chronic loose ligation, distal to ligatures, 7 days p.o. The majority of myelinated fibres show early axonal degeneration. x 470
Fig. III.49. Electron micrograph: chronic loose ligation, distal to ligatures, 8 days p.o. A myelinated fibre showing early axonal degeneration. The myelin sheath is still intact. x20,000
Fig. III.50. Electron micrograph: chronic loose ligation, distal to ligatures, 5 days p.o. The Schwann cell processes of a denervated unmyelinated fibre do not show banding. x15,000
Fig. III.51. 1μm section: chronic loose ligation, distal to ligatures, 7 days p.o. A nerve showing little evidence of fibre degeneration but with pronounced endoneurial oedema. Several large vacuolated fibroblasts are seen. x740
Fig. III.52. Electron micrograph: chronic loose ligation, distal to ligatures, 14 days p.o. Schwann cell processes of a denervated myelinated fibre form a band of Büngner. x25,000
Fig. III.53. 1μm section; chronic loose ligation, distal to ligatures, 14 days p.o. Many fibres are at an early stage of degeneration (arrows); other fibres are atrophic with small axons relative to myelin sheath thickness (arrowheads). These are often of irregular shape. x740
Fig. III.54. Electron micrograph: chronic loose ligation, distal to ligatures, 14 days p.o. An atrophic fibre with an axon which is small for the thickness of the myelin sheath. The external contour of the Schwann cell is irregular. There are many axonal microtubules but very few neurofilaments. x20,000
Table III.2  Sciatic nerves: Acute degenerated myelinated fibre density/mm² and PRB (pain-related behaviour)

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<td>No.19 - 100D.PO</td>
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Fig. III.55. 1μm section: chronic loose ligation, distal to ligatures, 21 days p.o. There is a patchy distribution of fibre degeneration seen in both peroneal and tibial branches of the nerve. ×74
Fig. III.56. 1μm section: higher power of Fig. III.55 showing recently degenerated fibres (arrows), atrophic fibres (arrowheads) and scattered regenerating fibres. x470
Fig. III.57. Electron micrograph: chronic loose ligation, distal to ligatures, 21 days p.o. A myelinated fibre is at an early stage of degeneration. x 10,000
Fig. III.58. Electron micrograph: chronic loose ligation, distal to ligatures, 21 days p.o. A group of unmyelinated axons is incompletely surrounded by Schwann cell processes. x25,000
Fig.III.59. Electron micrograph: chronic loose ligation, distal to ligatures, 21 days p.o. Accumulations of organelles in one unmyelinated axon may be an early degenerative change (asterisk). Some unmyelinated axons are closely apposed with no intervening Schwann cell cytoplasm (arrows). x7,800
Fig. III.60. 1μm section: chronic loose ligation, distal to ligatures, 28 days p.o. There is patchy fibre degeneration with some regenerating fibres. Myelin debris is present, and some recent fibre degeneration (arrow). x740
Fig. III.61. 1μm section: chronic loose ligation, distal to ligatures, 8 weeks p.o. There is myelin debris and some recent fibre degeneration. Many thinly remyelinated, regenerating axons are seen. x 740
Fig. III.62. 1μm section: sham operation, distal to placing of untied loose ligatures. A few degenerating myelinated fibres are seen. x740
Fig.III.63. 1μm section (immersion fixation): chronic loose ligation, at level of ligatures, 7 days p.o. A band of inflammatory cells surround the ligature. There is no evidence at this level, that the inflammatory response is causing nerve compression. x470
Fig. III.64. 1μm section (immersion fixation): chronic loose ligation, at the level of the ligatures, 7 days p.o. in another rat. A piece of ligature is surrounded, and being eroded by macrophages. x740
Fig. III.65. HRP 1μm resin section (immersion fixation): chronic loose ligation, at level of ligatures, 8.5 hours p.o. HRP reaction product is present in the epineurium and in the subperineurial space to the left of the picture, with some spread between nearby myelinated fibres. Reaction produce is seen at the luminal surface of endoneurial endothelial cells (arrows) but there is no evidence of leakage. The scattered few red cells seen in the endoneurium are probably accidental 'contaminants'. x180
Fig.III.66. HRP Unstained electron micrograph (immersion fixation): chronic loose ligation, proximal to the ligatures, 24 hours p.o. Reaction product is seen in the lumen, and outside the endoneurial blood vessel, and tracer is present in the spaces between adjacent endothelial cells (arrows). x10,000
Fig. III.67. Graph: chronic loose ligation, 5 days p.o. Controls are shown as mean (□) nerve fibre densities with bars showing the standard deviation (SD). The loss of large diameter fibres is seen to be greater than that of small diameter fibres (■) in chronic loose ligation.
Fig. III.68. Graph: chronic loose ligation, 5 days p.o. There is no selective loss when the nerve injury is severe.
Table III.3 Control sciatic nerves: myelinated and unmyelinated axons. MF (myelinated fibre density/mm²), LMF (large myelinated fibre density/mm²), SMF (small myelinated fibre density/mm²) and UMA (unmyelinated axon density/mm²)

<table>
<thead>
<tr>
<th>Control</th>
<th>MF (mean ± SD)</th>
<th>LMF &gt; 6μm (mean ± SD)</th>
<th>SMF ≤ 6μm (mean ± SD)</th>
<th>UMA (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sciatic N</td>
<td>14,311 ± 1,176</td>
<td>8,065 ± 1,176</td>
<td>6,246 ± 990</td>
<td>24,666 ± 4,635</td>
</tr>
<tr>
<td>Sural N</td>
<td>19,646 ± 2,062</td>
<td>6,293 ± 2,062</td>
<td>13,353 ± 2,654</td>
<td>58,102 ± 13,229</td>
</tr>
</tbody>
</table>

Table III.4 Percentage reduction of myelinated and unmyelinated axons from 5-14 days p.o. compared with control fibre density.

<table>
<thead>
<tr>
<th>Control</th>
<th>MF</th>
<th>LMF &gt; 6μm</th>
<th>SMF ≤ 6μm</th>
<th>UMA</th>
</tr>
</thead>
<tbody>
<tr>
<td>No.1 5D.PO</td>
<td>49.4%</td>
<td>53.6%</td>
<td>44%</td>
<td>19.9%</td>
</tr>
<tr>
<td>No.2 5D.PO</td>
<td>97%</td>
<td>97.1%</td>
<td>97.1%</td>
<td>99%</td>
</tr>
<tr>
<td>No.3 5D.PO</td>
<td>49.3%</td>
<td>70.3%</td>
<td>22.1%</td>
<td>-0.5%</td>
</tr>
<tr>
<td>No.4 8D.PO</td>
<td>86.4%</td>
<td>89.9%</td>
<td>81.9%</td>
<td>31%</td>
</tr>
<tr>
<td>No.5 8D.PO</td>
<td>53.6%</td>
<td>36.2%</td>
<td>22.7%</td>
<td>35.1%</td>
</tr>
<tr>
<td>No.6 14D.PO</td>
<td>47.7%</td>
<td>54.6%</td>
<td>38.9%</td>
<td>3%</td>
</tr>
<tr>
<td>No.7 14D.PO</td>
<td>16.9%</td>
<td>33.7%</td>
<td>-4.7%</td>
<td>5.6%</td>
</tr>
</tbody>
</table>
### TABLE III.5

Myelinated fibre densities/mm$^2$ (MF), large myelinated fibres density/mm$^2$ (LMF), small myelinated fibres density/mm$^2$ (SMF) and unmyelinated axons density/mm$^2$ (UMA); (% of control) = % of control mean values; PRB (pain-related behaviour).

(see Table III.1 for control values)

<table>
<thead>
<tr>
<th>No.</th>
<th>Sciatic</th>
<th>MF (% of control)</th>
<th>LMF &gt; 6μm (% of control)</th>
<th>SMF ≤ 6μm (% of control)</th>
<th>UMA (% of control)</th>
<th>PRB</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sural</td>
<td>7,240 (50.6%)</td>
<td>3,743 (46.4%)</td>
<td>3,497 (56%)</td>
<td>19,764 (80.13%)</td>
<td>48°: + 2.15; 6°C: +1</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>423 (3%)</td>
<td>241 (2.98%)</td>
<td>182 (2.9%)</td>
<td>239 (1%)</td>
<td>48°: -7; 6°: -24</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>7,260 (50.7%)</td>
<td>2,396 (29.7%)</td>
<td>4,864 (77.9%)</td>
<td>24,797 (100.51%)</td>
<td>48°: +2; 6°: -3.5</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>1,944 (13.6%)</td>
<td>816 (10.1%)</td>
<td>1,128 (18.1%)</td>
<td>17,017 (68.99%)</td>
<td>48°: +2.75; 6°: +4.75</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>48°: -4.5; 6°: -23</td>
</tr>
</tbody>
</table>

Sural
<table>
<thead>
<tr>
<th>No.</th>
<th>Day</th>
<th>Sciatic</th>
<th>Sural</th>
<th>MF (% of control)</th>
<th>LMF &gt; 6μm (% of control)</th>
<th>SMF ≤ 6μm (% of control)</th>
<th>UMA (% of control)</th>
<th>PRB</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>8D.P0</td>
<td>Sciatic</td>
<td>Sural</td>
<td>9,972 (67.7%)</td>
<td>5,146 (63.8%)</td>
<td>4,826 (77.3%)</td>
<td>16,003 (64.87%)</td>
<td>48° : +2; 6° : -7</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>13,869 (70.59%)</td>
<td>5,409 (67.1%)</td>
<td>8,460 (63.4%)</td>
<td>64,901 (111.7%)</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>14D.P0</td>
<td>Sciatic</td>
<td>Sural</td>
<td>7,479 (52.3%)</td>
<td>3,665 (45.4%)</td>
<td>3,814 (61.1%)</td>
<td>23,932 (0.9702)</td>
<td>48° : +2.5; 6° : -3.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>11,260 (57.3%)</td>
<td>3,873 (61.5%)</td>
<td>7,387 (55.3%)</td>
<td>41,707 (0.718)</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>14D.P0</td>
<td>Sciatic</td>
<td>Sural</td>
<td>11,887 (83.1%)</td>
<td>5,349 (66.3%)</td>
<td>6,538 (104.7%)</td>
<td>23,295 (94.4%)</td>
<td>48° : +1.5; 6° : +3</td>
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<tr>
<td>9</td>
<td>21D.P0</td>
<td>Sciatic</td>
<td>Sural</td>
<td>12,028 (84%)</td>
<td>7,217 (89.5%)</td>
<td>4,811 (77%)</td>
<td>24,328 (98.6%)</td>
<td>48° : +3.4; 6° : +4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>14,680 (74.7%)</td>
<td>4,962 (78.8%)</td>
<td>9,718 (72.8%)</td>
<td>53,037 (91.3%)</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>21D.P0</td>
<td>Sciatic</td>
<td>Sural</td>
<td>13,814 (96.5%)</td>
<td>1,174 (14.6%)</td>
<td>12,640 (202%)</td>
<td>53,999 (218.9%)</td>
<td>48° : +4.5; 6° : +4.5</td>
</tr>
<tr>
<td></td>
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<td></td>
<td></td>
<td>20,286 (103.3%)</td>
<td>5,011 (79.6%)</td>
<td>15,275 (114.4%)</td>
<td>94,558 (162.7%)</td>
<td></td>
</tr>
<tr>
<td>No.</td>
<td>Sciatic</td>
<td>Sural</td>
<td>MF (% of control)</td>
<td>LMF &gt; 6μm (% of control)</td>
<td>SMF ≤ 6μm (% of control)</td>
<td>UMA (% of control)</td>
<td>PRB</td>
<td></td>
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</tr>
<tr>
<td>11</td>
<td>21D.PO</td>
<td></td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>28D.PO</td>
<td></td>
<td>10,113 (70.7%)</td>
<td>5,997 (95%)</td>
<td>4,116 (51%)</td>
<td>24,915 (101%)</td>
<td>48° : -2; 6° : -15</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>12,397 (63.1%)</td>
<td>8,368 (62.7%)</td>
<td>8,368 (62.7%)</td>
<td>56,208 (96.7%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>30D.PO</td>
<td></td>
<td>13,296 (92.9%)</td>
<td>8,111 (100.5%)</td>
<td>5,185 (83%)</td>
<td>20,293 (82.3%)</td>
<td>48° : 0; 6° : 0</td>
<td></td>
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<td>-</td>
<td></td>
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</tr>
<tr>
<td>14</td>
<td>28D.PO</td>
<td></td>
<td>11,324 (79.1%)</td>
<td>6,625 (82.1%)</td>
<td>4,699 (75.2%)</td>
<td>40,989 (166.2%)</td>
<td>48° : + 1; 6° : +3</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>15,322 (78%)</td>
<td>6,435 (102.2%)</td>
<td>8,887 (66.6%)</td>
<td>58,188 (100.1%)</td>
<td></td>
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</tr>
<tr>
<td>15</td>
<td>56D.PO</td>
<td></td>
<td>26,789 (187.1%)</td>
<td>3,349 (41.5%)</td>
<td>23,440 (375%)</td>
<td>44,063 (164.5%)</td>
<td>48° : 0; 6° : -1</td>
<td></td>
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</tr>
<tr>
<td>No.</td>
<td>Date</td>
<td>Tissue</td>
<td>MF (% of control)</td>
<td>LMF &gt; 6μm (% of control)</td>
<td>SMF ≤ 6μm (% of control)</td>
<td>UMA (% of control)</td>
<td>PRB</td>
<td></td>
</tr>
<tr>
<td>-------</td>
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</tr>
<tr>
<td>15</td>
<td>60D</td>
<td>Sciatic</td>
<td>16,896 (118.1%)</td>
<td>9,377 (116.3%)</td>
<td>7,518 (120.4%)</td>
<td>26,171 (106.1%)</td>
<td>48° : 0; 6° : 0</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Sural</td>
<td>16,108 (81.99%)</td>
<td>9,118 (144.9%)</td>
<td>6,990 (52.4%)</td>
<td>49,180 (84.64%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>84D</td>
<td>Sciatic</td>
<td>10,789 (75.39%)</td>
<td>7,391 (91.6%)</td>
<td>3,398 (54.4%)</td>
<td>18,559 (75.24%)</td>
<td>48° : 0; 6° : 0</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Sural</td>
<td>16,108 (81.99%)</td>
<td>9,118 (144.9%)</td>
<td>6,990 (52.4%)</td>
<td>49,180 (84.64%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>84D</td>
<td>Sciatic</td>
<td>24,461 (170.9%)</td>
<td>5,357 (66.4%)</td>
<td>19,104 (305.8%)</td>
<td>27,416 (111.1%)</td>
<td>48° : 0; 6° : 0</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Sural</td>
<td>21,335 (108.6%)</td>
<td>1,152 (18.3%)</td>
<td>20,183 (151.1%)</td>
<td>78,839 (135.69%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>100D</td>
<td>Sciatic</td>
<td>30,807 (229.2%)</td>
<td>3,348 (41.5%)</td>
<td>29,459 (471.6%)</td>
<td>43,369 (175.8%)</td>
<td>48° : 0; 6° : 0</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Sural</td>
<td>16,998 (86.42%)</td>
<td>7,905 (125.6%)</td>
<td>9,093 (68.1%)</td>
<td>49,590 (85.35%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>100D</td>
<td>Sciatic</td>
<td>16,888 (118.01%)</td>
<td>7,768 (96.32%)</td>
<td>9,120 (146.0%)</td>
<td>32,143 (130.3%)</td>
<td>48° : 0; 6° : 0</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Sural</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Fig. III.69. Scatterplot. The 'g' ratios (axon/fibre diameter) of a normal control are from 0.5 to 0.7, most are around 0.6.
Fig.III.70. Scatterplot: chronic loose ligation, 14 days p.o. The 'g' ratios show a wide distribution from 0.4 to 0.8, and a preferential loss of large diameter points.
Fig. III.71. Scatterplot: chronic loose ligation, 28 days p.o. There is a wide range distribution of 'g' ratios from 0.4 to 0.8.
Fig. III.72. Scatterplot: chronic loose ligation, 12 weeks p.o.
There are still some 'g' ratios that are not within normal range (0.5-0.7), either above 0.7 (regenerated fibres) or below 0.5 (atrophic fibres).
CORRELATION OF NEUROPATHIC PAINFUL BEHAVIOUR WITH SURVIVING NERVE FIBRES

The A-δ range myelinated fibres (≤ 6μm) were chosen as those responsible for cold stimuli, and unmyelinated axons (UMA, mainly C-fibres) as being involved in heat stimuli. It is known that neuropathic painful behaviour from 12 days to 2 months p.o. is mainly due to the sciatic nerve damage (Ro and Jacobs, 1993a). The sciatic nerves (n=8) and sural nerves (n=6) were selected for the correlation study from 2-4 weeks p.o.. For statistical convenience, the number of surviving fibres is expressed as nerve fibre density/mm² (NFD) and is correlated with values of pain-related behaviour (PRB) tests to heat and cold. For this analysis, the values assigned to PRB tests have had their signs reversed so that hyperalgesia, which was given a negative value in the behavioural study, now becomes a positive value; similarly the formerly positive values for hypoalgesia now become negative numbers.

A. The correlation of sural (sensory) nerve fibres with PRB

(1) The number of surviving A-δ fibres (≤6μm) shows a significant positive correlation with PRB to cold (6°C) (r=0.902, P=0.0139 <0.05, two-tailed test) (Table III. 6, Fig. III. 73).

(2) The number of surviving myelinated fibres (MF) including large and small fibres, shows an even stronger positive correlation with PRB to cold (6°C) (r=0.951, p=0.014 < 0.05 two-tailed test)(Table III. 6, Fig. III.74).

(3) The number of surviving large myelinated fibres (LMF) including A-β and A-α fibres shows a significant positive correlation with PRB to cold (6°C) (r=0.932, p=0.007 <0.05, two-tailed test (Table III. 6, Fig. III.75).
(4) The number of surviving unmyelinated axons (UMA) is positively correlated with PRB to heat (48°C) \((r=0.8504, p=0.0319 < 0.05, \text{ two-tailed test})\) (Table III. 6, Fig. III.76).

**B. The correlation of sciatic nerve (mixed nerve) fibres with PRB**

(1) The number of surviving A-δ fibres \((\leq 6\mu \text{m})\) shows a significant correlation with PRB to cold \((6^\circ \text{C})\) \((r=0.7467, p=0.0333 < 0.05, \text{ two-tailed test})\) (Table III. 6, Fig. III.77).

(2) The number of surviving myelinated fibres (MF) including large and small fibres shows a stronger positive correlation with PRB to cold \((6^\circ \text{C})\) \((r=0.950, p=0.0003, \text{<} 0.05, \text{ two-tailed test})\) (Table III. 6, Fig. III.78).

(3) The number of surviving large myelinated fibres (LMF) including A-β and A-α afferents and motor efferents was not significantly correlated with PRB to cold \((6^\circ \text{C})\) \((r=0.571, p=0.139)\) (Table III. 6, Fig III. 79).

(4) The number of surviving unmyelinated axons (UMA) shows a positive correlation with PRB to heat \((48^\circ \text{C})\) \((r=0.7641), p=0.00273, \text{two-tailed test})\) (Table III. 6, Fig80).

**C. Correlation of neuropathic painful behaviour with acute myelinated fibre degeneration**

The number of acutely degenerating myelinated fibres (ADMF) does not show any significant correlation with PRB to cold \((6^\circ \text{C})\) \((r=-0.2445, p=0.5595)\) or heat \((48^\circ \text{C})\) \((r=0.2863, p=0.4918)\).
Table III.6  Correlation between nerve fibre densities/mm² and pain-related behaviour (PRB) of 6°C and 48°C stimuli.
SMF = small myelinated fibres, LMF = large myelinated fibres, MF = myelinated fibres, UMA = unmyelinated axons
*= p < 0.05

<table>
<thead>
<tr>
<th>Correlation coefficient (r)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>PRB (6°C) and sural SMF</td>
<td>0.902</td>
</tr>
<tr>
<td>PRB (6°C) and sural LMF</td>
<td>0.932</td>
</tr>
<tr>
<td>PRB (6°C) and sural MF</td>
<td>0.951</td>
</tr>
<tr>
<td>PRB (48°C) and sural UMA</td>
<td>0.850</td>
</tr>
<tr>
<td>PRB (6°C) and sciatic SMF</td>
<td>0.748</td>
</tr>
<tr>
<td>PRB (6°C) and sciatic LMF</td>
<td>0.571</td>
</tr>
<tr>
<td>PRB (6°C) and sciatic MF</td>
<td>0.950</td>
</tr>
<tr>
<td>PRB (48°C) and sciatic UMA</td>
<td>0.7641</td>
</tr>
</tbody>
</table>
Fig. III.73. The number of surviving sural A-δ range fibres (≤ 6μm) shows a significant positive correlation with the value of pain-related behaviours (PRB) to 6°C cold stimuli, (r=0.902, P=0.0139 < 0.05, two-tailed test).
Fig. III.74. The number of surviving sural myelinated fibres (MF) including large and small afferent fibres (A-α, A-β and A-δ) shows a strong positive correlation with the value of pain-related behaviours (PRB) to 6°C cold stimuli ($r=0.951, p=0.014 < 0.05$, two-tailed test).
Fig. III.75. The number of surviving sural large myelinated fibres (LMF) including A-δ and A-β afferent fibres shows a significant positive correlation with pain-related behaviours (PRB) to 6°C cold stimuli ($r=0.932, P=0.007<0.005$, two-tailed test).
Fig. III.76 The number of surviving sural unmyelinated axons (UMA) is positively correlated with pain-related behaviours to 48°C heat stimuli ($r=0.8504$, $P=0.0319 < 0.05$, two-tailed test).
Fig. III.77. The number of surviving sciatic A-δ range fibres (≤ 6μm) shows a significant positive correlation with pain-related behaviours (PRB) to 6°C cold stimuli ($r=0.7467$, $P=0.0333 < 0.05$, two-tailed test).
Fig. III.78. The number of surviving sciatic myelinated fibres (MF) including large and small fibres (both afferent and efferent fibres) shows a stronger positive correlation with pain-related behaviours (PRB) to 6°C cold stimuli ($r=0.950$, $P=0.0003 <0.05$, two-tailed test).
Fig. III.79. The number of surviving sciatic large myelinated fibres (LMF) including A-α, A-β afferents and motor efferents is not significantly correlated with pain-related behaviours (PRB) to 6°C cold stimuli ($r=0.571$, $P=0.139 > 0.05$).
Fig. III.80. The number of surviving sciatic unmyelinated axons (UMA) shows a significant positive correlation with pain-related behaviours (PRB) to 48°C heat stimuli ($r = 0.7641$, $P = 0.00273 < 0.05$, two-tailed test).
CHAPTER 4: DISCUSSION

Neuropathic Pain-Related Behaviour

The present study provides behavioural evidence that experimental unilateral mononeuropathy caused by multiple loose ligatures around the sciatic nerve produces pain-related disorders in the rat, further confirming the data previously reported by Bennett and Xie (1988) and Attal et al. (1990). In addition, it allows a quantifiable analysis of the animals' abnormal behaviours. The pain-related disorders include hyperalgesia (increased reaction to noxious stimuli), and allodynia (nociceptive reaction to normally non-noxious stimuli) and spontaneous pain-related behaviour.

In our studies, hyperalgesia was indicated by a marked decrease in the mean immersion duration for a noxious temperature of 48°C compared with contralateral limb without surgical operation. Aversive behaviours such as licking the paw or vocalization were frequently detected after the thermal stimuli. These findings may suggest that peripheral nerve injury does produce thermal hyperalgesia. In addition, rats would often exhibit abnormal reactions lasting up to several minutes after the end of stimulation, which may also be related to sensitization or prolonged after discharges (Bennett and Xie, 1988).

Allodynia or hyperaesthesia was indicated by a reduced hind paw immersion duration in a 6°C water bath. Furthermore rats sometimes showed behavioural signs of sensitization or sustained pain after termination of stimulation. These behaviours suggest that a non-painful stimulus can evoke pain in the hind limb after peripheral nerve injury.

Allodynia or spontaneous pain-related behaviour was judged by placing the rat on a wire grid to observe the hind limb grasping reflex. Allodynia was indicated by
touch-induced nociceptive behaviour and spontaneous pain was inferred from the reluctance to put the foot down while walking and frequent licking or vocalisation. Licking of the toes may be a behaviour that is correlated with that of the human neuropathic patient who attempts to relieve pain by wetting the painful extremity with water (Richards, 1967; Tahmoush, 1981).

Neuropathic pain-related behaviour is produced by four ligatures tied loosely around the common sciatic nerve at the mid-thigh level. Hyperalgesia and allodynia were detected from 4 days after tying ligatures, becoming maximal after 14 days; normal thermal reaction returned by 8 weeks post-operation. This time course of pain-related behaviours in the present study was generally similar to that reported by Bennett and Xie (1988) although they described hyperalgesia as early as 2 days p.o. In most animals in the present study, hyperalgesia and allodynia started from the 4th day p.o.; however, in two animals tested at 8.5 hours p.o. there was evidence of hyperalgesia followed by marked hypoaesthesia to all tests, and paresis instead of pain-related behaviour. The findings of Attal et al. (1990) were generally similar to ours although they did not test their animals at the early time of 8.5 hours p.o.

Variation in the demonstration of early neuropathic pain-related behaviour between different studies could be due to the use of different test methods. For example, the noxious heat test used by Bennett and Xie (1988) involved the use of a radiant heat source with an aperture of 5mm x 10mm, place beneath an elevated sheet of glass on which the rat was stood. Only the proximal part of the plantar surface of the paw was irradiated by this small heat source. In the experiments described in this thesis, the whole foot was immersed in a water bath. In both cases, the withdrawal time was noted. Differences in temporal-spatial summation could possibly explain the
early differences in the two experimental situations.

The advantage of using a water bath compared with a hot plate (which is often used in behavioural studies) for thermal stimulation is that it reduces tactile sensation, which also contributes to neuropathic pain, making the results easier to interpret and simpler to carry out. However, a disadvantage is that it tests not only the sciatic nerve territory (lateral part) but also the saphenous nerve territory (medial part), which possibly participates in secondary hyperalgesia (Lewis, 1935) or nociceptor sensitization (Fitzgerald, 1979), and makes the pain-related behaviour more complicated. But this method of testing (Immersion of the whole foot) has also proved to be an advantage in studies on the role of the saphenous nerve (See later in Discussion).

Relevance of the Model

It has been suggested (Bennett and Xie, 1988) that the model of neuropathy studied here has features in common with human causalgia. Obviously one has to be very careful not to infer that the model reproduces human behaviour since we have no means of knowing what the animal really feels. It is difficult to assert that the spontaneous pain-related behaviour in the rats may be related to the spontaneous pain observed in human causalgia, the typical characteristic of 'superficial burning pain' experienced in causalgia being obviously undetectable in an animal model except by altered behavioural responses.

The latency of occurrence of pain-related disorder (by day 4 in the loose ligature model) is more prolonged than in the human causalgia syndrome, which generally has a very rapid onset (Bonica, 1979), although a delayed onset has also been reported for this phenomenon. However, in the loose ligatures model, a short
period of early pain-related behaviour (at 8.5 hours p.o.) does occur and is gradually replaced within one day p.o. by hypoalgesia or paresis.

This early onset of pain-related behaviour can possibly be explained by an ischaemic effect produced by the loose ligatures. Gammel (1927) described a case after an intragluteal injection of potassium bismuth tartrate for syphilis. The patient noted numbness of the ipsilateral foot immediately after injection, which was accompanied by pain, swelling and necrosis of the skin of buttock. Within an hour the patient developed weakness of dorsiflexion and eversion of the foot. Gammel postulated that the inferior gluteal artery and its distal branch were occluded by bismuth crystal emboli leading to an ischaemic lesion of the sciatic nerve. The notion is similar to the findings of rats after chronic loose ligatures. In morphological study it has been confirmed that ischaemia played a major role in the development of the lesion during the first two days after loose ligation (see later discussion).

The trophic changes sometimes seen in patients with causalgia, such as asymmetries in skin temperature, have also been reported by Bennett and Xie (1988) and investigated in their rats using thermography. In the present study, a rather crude measurement of temperature (placing the thermometer on the plantar surface of the foot) showed no significant asymmetry in skin temperature. However, autonomic dysfunction still could not be ruled out.

Lastly, it is clear that the hyperalgesia to thermal stimuli exhibited by these rats, as well as the dramatic sensitization after non-noxious stimulation (i.e. cold stimulation), are similar to human causalgia (Bonica, 1979; Campbell et al., 1988), and it has been reported that allodynia to cooling is a characteristic of certain 'reflex sympathetic dystrophy' (Frost et al., 1988).
Thus, there are some common features between this experimental neuropathy and clinical causalgia in man. The animal model of neuropathy possibly provides a useful tool for pathophysiological investigation of abnormal pain sensation in man.

**Comparison with other models**

Other animal models for the study of pain-related behaviour have been developed in recent years. Seltzer, Dubner and Shir (1990) reported another model that involves a tight ligation of one-half to one-third of the sciatic nerve in the rat. This surgical procedure produces an immediate onset of behavioural signs of spontaneous pain, allodynia and hyperalgesia. Kim and Chung (1992) produced another model by segmental spinal nerve ligation of either L5 or L5 + L6. It has the disadvantage of extensive surgery. However, the surgical procedure is stereotyped and probably less liable to the variations which are unavoidable in the Bennett and Xie (1988) (in which the degree of tightness of the ligatures is very difficult to control) and the Seltzer, Dubner and Shir (1990) model, in which the number and localization of damaged nerve fibres is also difficult to control. A further advantage of the Kim and Chung (1992) model is that the levels of injured and intact spinal segments are completely separated, allowing independent experimental manipulations of the injured and intact spinal segments in future experiments to answer questions regarding underlying mechanisms of causalgia. Furthermore, surgical sympathectomy immediately alleviated the symptoms in this model, which suggested that reflex sympathetic dystrophy could possibly be underlying the mechanisms of neuropathic pain, and provided a possible model to investigate reflex sympathetic pain.

**Autotomy**

Autotomy is a well recognised behavioural activity in animals (including rats)
involving the intentional chewing or damaging of a limb. Animals showing this behaviour include those with inherited congenital loss of sensory fibres, e.g. the mutilated foot rat (Jacobs et al., 1981b), and rats subjected to sciatic nerve injury or to dorsal rhizotomy. The degree of autotomy varies considerably, and its effects range from mild trauma to the ends of one or two digits, to severe damage involving loss of the foot.

Autotomy has been the subject of much discussion. It is now generally accepted that behaviour producing autotomy is generated centrally (Kruger, 1992; Blumenkopf and Lipman, 1992) but peripheral effects (such as spontaneous ectopic discharges) also play a role (Devor, 1992). However, the question as to whether there is underlying abnormal sensation is still controversial (Kruger, 1992).

In the present study, the rate of autotomy was very low with only one rat out of 72 showing a minor degree of damage to the distal lateral parts of two digits. Two other studies of this same model describe widely differing incidences of autotomy. Bennett and Xie (1988) found that 70% of rats showed self mutilation although this was generally limited to gnawing of claw tips. Only 5 rats were affected out of 133 in the study of Attal et al. (1990). In other models produced by partial sciatic nerve injury (Seltzer, Dubner and Shir, 1990) and segmental spinal nerve ligation (Kim and Chung, 1992), none of the animals showed signs of autotomy despite the presence of signs of spontaneous pain. There was no obvious association between the severity of autotomy and the degree of hyperalgesia or allodynia (Attal et al., 1990; Ro and Jacobs, 1993b). It is likely that the variation in autotomy rate is due to the variable degree of fibre damage in the different models.

In a pilot study, using tighter ligatures (Anand, personal communication), a
much higher incidence of autotomy was found, as well as claw changes, which were also reported by Bennett and Xie (1988).

Devor et al. (1979) showed that within the first few weeks following sciatic nerve section in the rat the functional distribution of the remaining saphenous nerve expands across the foot and reinnervates all but the lateral edge of the foot and the lateral two or three toes. There is evidence that this zone that remains anaesthetic is the target for autotomy (Wall et al., 1979; Devor et al., 1979). Using a similar model to study autotomy, Wall et al. (1979) suggested that if the saphenous nerve is severed simultaneously, the protection it afforded the foot is removed. Autotomy is maximal when both sciatic and saphenous nerve are severed.

A suggested explanation of autotomy is that both the distal ends of damaged nerves and the associated dorsal root ganglion cells may contribute spontaneous discharges (Wall and Gutnick, 1974; Govrin-Lippmann and Dover, 1978; Wall and Devor, 1983) which then induce a state of hyperexcitation in the spinal cord dorsal horn cell (Dubner and Ruda, 1992; Coderre et al., 1993), so accounting for the abnormal sensation. Because the whole foot is anaesthetic, the self-mutilation does not cause pain.

This explanation is supported by the findings in the ligation model. In the one rat showing autotomy in the present experiments, there was damage to the distal lateral phalanges of two toes. These lie within the sciatic nerve innervation territory and are supposed to still remain anaesthetic at the time (10 days p.o.) (Ro and Jacobs, 1993a). Also, the thermal test in this rat showed hypoesthesia (or anaesthesia) instead of hyperaesthesia. Attal et al., (1990) also describe rats with autotomized lateral phalanges together with hypoesthesia within the first two weeks p.o.. Jacobs
et al., (1981b) studied and quantified hind limb nerve fibres of mutant rats with autotomy (mutilated foot). They found a severe reduction in the number of sensory ganglion cells and dorsal root axons, including unmyelinated axons in the mutant rats. The severe loss of sensory fibres associated with autotomy may be compatible with the notion that the rats which had autotomized after loose ligations usually displayed hypoaesthesia rather than hyperaesthesia. These findings may explain why the methods of nerve damage used by Seltzer, Dubner and Shir (1990) and Kim and Chung (1992) did not produce autotomy, since their partial nerve injury and segmented spinal ligation would be less likely to produce a total "anaesthetic area" in the distal hind limb. It is well known that axonal rearrangement occurs throughout the length of the nerve trunk (Sunderland, 1968). That is why partial injury in the proximal part may only produce punctate anaesthetic area in the distal hind limb.

**Sham Operation**

Unlike Bennett and Xie (1988) and Attal et al. (1990), a transient behavioural response in the sham-operated rats was found. Morphologically, there was evidence of damage limited to a few myelinated fibres in one of several rats examined. There was no evidence of loss of unmyelinated fibres. It is suspected that cutaneous and muscular nerve damage during the sham operation procedure, involving as it does a relatively long skin incision, possibly produces prolonged increases in the excitability of the flexion reflex in the rat and may account for the transient behavioural response during the first few days (Wall and Woolf, 1984).

An alternative, although unlikely explanation is that in sham operations a few large myelinated fibres were injured, which could lead to transient disturbance of the gate control (Metzack and Wall, 1965). Because no unmyelinated fibres were injured,
long-lasting neuropathic pain-related behaviour would not be sustained (Thomas, 1974; Woolf and Wall, 1986); that is why only a transient neuropathic pain is detected. However, it is likely that the numbers of damaged myelinated fibres would be too small to produce any detectable effects.

The Role of Saphenous Nerve

It has long been known that collateral sprouting of the saphenous nerve following damage to the sciatic nerve contributes to the early return of sensation to the rat foot beginning on the 4th day (Devor et al., 1979). Secondary hyperalgesia following early nerve damage was also described by Lewis (1935). We studied the possible contribution of saphenous innervation in the ligature model. When the saphenous nerve was sectioned at the time of placing ligatures, the tests showed hypoaesthesia, significantly different from the ligature only group, lasting from the 4th to the 10th day. This suggests that the early hyperaesthesia and hyperalgesia caused by loose ligatures might be due to early collateral sprouting of the saphenous nerve or to secondary hyperalgesia from nociceptor sensitization (Fitzgerald, 1979). Section of the saphenous nerve 7 days after placing ligatures also changed the hyperalgesic response to hypoaesthesia from about the 8th to the 12th day. This reinforced the hypothesis that the saphenous nerve plays a role in early hyperaesthesia and hyperalgesia. There was no modification of hyperaesthesia when the saphenous nerve was sectioned on the 14th or 21st days after tying ligatures. These findings suggest that hyperaesthesia and hyperalgesia due mainly, but not exclusively, to sciatic nerve damage began from about the 10th to 12th day after placing ligatures. Devor et al. (1979) found that functional recovery after a crush lesion of the sciatic nerve begins from 21 days post-crush. Our findings of earlier hyperaesthesia and
hyperalgesia originating from the sciatic nerve itself (10 to 12 days p.o. in chronic loose ligation model compared with 21 days p.o. in sciatic nerve crush model) probably reflect the less severe nerve degeneration caused by multiple loose ligatures.

In studies of saphenous nerve collateral innervation, Brenan (1986) found that the increased field extended mainly to the dorsal surface of the foot. However, Markus, Pomeranz and Krushelnycky, (1984) and Kingery and Vallin (1989), using different methods showed substantial plantar collateralization. It seems that the increased plantar field may be variable in extent. In Bennett and Xie's model (1988), the saphenous nerve was not proved to play a part in neuropathic pain behaviours. Our method involving total immersion of the foot, with the possibility of greater spatial summation, differs from the more focal method used by Bennett and Xie (1988). This may possibly explain why Bennett and Xie (1988) obtained different results from ours. Sectioning of the saphenous nerve three months after sciatic nerve ligation had no effect in abolishing neuropathic pain behaviours (Attal et al., 1990). In our study, there was no effect of saphenous nerve section two weeks after tying ligatures (Ro and Jacobs, 1993a).

It is of interest that in a chronic sciatic nerve injury without regeneration (Markus, Pomeranz and Krushelnycky, 1984) there was hyperalgesia, often with vocalisation, indicating a possible nociceptive response in the dorso-medial foot. This was only demonstrated at 21 days p.o., but had probably occurred earlier. This region is normally innervated mainly by the saphenous nerve. In a similar model, histological studies showed an increase in the number of nerve endings in the sensitive region, confirming cutaneous sprouting (Weddell, Gutmman and Gutmann, 1941). Kingery and Vallin (1989) found that saphenous nerve territory (medial toes)
showed marked hyperalgesia within one week after sciatic nerve section but the collateralization territory (middle toe) did not become hyperalgesic until 3 weeks after nerve section. The early hyperalgesic response in our study would be comparable to the early hyperalgesic effect (within one week) shown by Kingery and Vallin (1989) and Vallin and Kingery (1991) which in both studies may be due to nociceptive sensitization, although collateral sprouting still remains a possible explanation.

This study has shown that the saphenous nerve plays a role in the behavioural changes following sciatic nerve damage caused by loose ligatures. Whether this is due to saphenous nerve collateralization has not yet proven, but still remains a possibility. An alternative hypothesis would be spreading nociceptor sensitization due to adjacent nerve fibre injury. The sciatic nerve damage may induce a central excitable state e.g. by NMDA-mediated dorsal horn hypersensitivity (Yaksh, 1989; Seltzer et al., 1991a; Dubner and Ruda, 1992; Coderre et al., 1993) which is further excited by intact saphenous afferents.

Morphological changes

Comparison with other studies of the Bennett and Xie model

When the present study was first begun, little was known of the morphological changes underlying the behavioural changes described by Bennett and Xie in their loose ligature model (1988). Since then a number of morphological studies have been made (Basbaum et al. 1990; Carlton et al. (1991); Coggeshall et al., 1993; Guilbaud et al., 1993, Sommer et al., 1993). In general the numbers of animals examined have been small; Basbaum et al. (1990) examined two rats two weeks p.o. There was a severe loss of large (>5µm) and medium-diameter (3-5µm) myelinated fibres, and a decrease in unmyelinated axons. Quantitation was limited to counts of unmyelinated
axons using the nerve proximal to the ligature as a control to compare the distal nerve. Guilbaud et al. (1993) examined rats at different intervals after operation; using teased fibres, between 91 and 98% of fibres showed ovoid formation up to three weeks p.o. However, histograms of fibre diameters of transverse sections show peaks of at least 2000/mm² myelinated fibres of between 5-9µm at 1 week, and about 5000/mm² at 2 weeks, indicating much less nerve fibre degeneration than was found in the teased fibre quantitation. The widely varying results perhaps illustrate the unsuitability of teased fibres for quantitative studies in this type of neuropathy and/or the extensive differences between animals. Carlton et al., (1991) examined five rats two weeks after loose ligatures were tied. Their quantitation on transverse sections made use of x900 montages, and the myelinated fibres in an area equivalent to half of the nerve were counted. An 84-99% decrease in myelinated fibres and a 62-84% decrease in unmyelinated axons was found, comparing the counts proximal to ligatures with those distally. The greatest loss was of the largest myelinated fibres. The wide disparity of fibre loss from animal to animal was noted, but neuropathic behavioural changes were found in all these animals, as measured by paw withdrawal latency to radiant heat.

Coggeshall et al. (1993) examined rats at intervals of 3,5,14,28 and 56 days p.o., and included some of the data from the Carlton et al. (1991) paper. The percentage decrease was maximum at 14 days, when 94 ± 3% standard error (SE) of myelinated, and 73 ± 5% SE of unmyelinated fibres had degenerated. It is probable that Coggeshall et al. (1993) tied ligatures more tightly than in the experiments described in the present thesis, since their values for the percentage of degenerated fibres are all very high. The morphological findings were correlated
with behavioural studies (Coggeshall et al., 1993) and it was concluded that heat hyperalgesia develops concurrently with the preferential loss of large myelinated fibres, but the hyperalgesia lessens as axon loss progresses.

Sommer et al. (1993) are the first to have examined the lesion at an early stage. An illustration of a nerve at day 1 p.o. shows marked constriction already, and it seems likely that their ligatures were tied more tightly than those in the experiments described in this thesis. Their findings of perineurial splitting and minifascicle formation, particularly outside the nerve fascicles, also confirm that their lesion was very severe. They concluded that ischaemia was a factor in the sequence of pathological events following tying of ligatures, although no specific evidence was shown of ischaemic damage, such as described in the present thesis. Quantitation of unmyelinated axons showed a reduction to 32.5% of control values; myelinated fibre loss was not estimated but was thought to involve the loss of most large fibres.

In summary, all the studies describe loss of myelinated fibres, particularly of the large diameter fibres, and some loss of unmyelinated axons. However, in common with the behavioural studies it is clear that there is considerable variation between animals.

None of these studies have questioned the cause of this often severe axonal neuropathy caused by an apparently very mild procedure. The present study has shown that this could only be identified by examination of the nerves at very short intervals after tying ligatures.

The morphological findings in our studies suggest that ischaemia may play an important role in causing nerve damage in the very early stages following loose nerve ligations. The alternative suggestion would be that it is due to nerve compression, but
this seems very unlikely at this early stage since there was little or no evidence of any nerve compression. Moreover, some of the changes seen would not be consistent with a compressive type lesion. There are several lines of evidence for an ischaemic basis to the nerve damage. The scattered or occasionally focal presence of axons swollen with accumulated organelles, particularly of a tubulo-vesicular nature is very similar to findings described by Korthals et al. (1978) in an ischaemic nerve lesion produced in rabbits (See Literature review). In another ischaemic lesion caused by microspheres, Nukada and Dyck (1984) described similar swollen axons. In their skip serial sections they also showed a sequence of changes along the myelinated fibres, with more distal changes involving a reduction in the size of the axon, or even its disappearance, but with an extensive adaxonal space. Most distally still, the fibres frequently showed a collapsed sheath with no axon, apparently a very early stage of fibre degeneration. Fibres showing these types of change were also seen in the nerves examined in this thesis. It was not possible to easily distinguish a 'proximal' and 'distal' change along the fibres because the multiple ligatures probably cause more than one region of ischaemia. The pattern and distribution of organelle accumulation described differs from that occurring near to a nerve cut, crush or ligature lesion.

Of considerable relevance are the degenerative changes found in Schwann cells associated with fibres showing the axon organelle accumulations. Degenerate Schwann cells were also seen in relation to fibres with altered myelin sheaths, which were probably beginning to break down. Schwann cell changes were also associated with myelinated fibres that appeared otherwise normal. Such Schwann cell changes do not occur as a result of nerve compression. Although Korthals and Wiśniewski (1975) and Korthal, Korthals and Wiśniewski (1978) refer to 'infarction' and 'necrosis' in their
model of nerve ischaemia, their descriptions concentrate on the axonal changes rather than those of the Schwann cell. Degenerative changes of endoneurial endothelial cells were not found in the present study.

Macrophages were seen in the oedematous subperineurial region within 8.5 hours of tying ligatures. This would be far too early for the macrophage recruitment that occurs in Wallerian-type degeneration. The source of these macrophages is not clear. However, since they are only found in the subperineurial region (at this early stage) it may be that changes in perineurial permeability, perhaps due to the effects of degranulation of mast cells, observed to occur in epineurial tissues near to the perineurial sheath, are involved.

Vascular permeability studies with HRP were used to indicate the source of endoneurial oedema. This seems to be an important factor in the increase in size of the nerve leading ultimately to its 'self-strangulation'. The studies have shown leaked HRP in the subperineurial fluid, although there was no evidence that this had leaked into the nerve from the epineurium. However, this could easily be missed since it is sometimes difficult to identify the tight junctions, or the lack of tightness, between perineurial cells. There was, however, evidence of leakage of HRP through open junctions between endoneurial cells, and morphological evidence of open junctions was seen. This does not explain the subperineurial distribution of the leaked tracer in the early stages of the lesion.

The part of the sciatic nerve used for this experiment has been shown to be a 'watershed' zone as far as its vascular supply is concerned (McManis, Low and Lagerlund, 1993). It is therefore all the more likely that it would be vulnerable to the loose-ligature lesion.
Compressive lesions

By 48 hours p.o., there was evidence that compression might be causing some of the observed changes to nerve fibres in the ligature region. Of particular interest were the changes involving thinning or loss of myelin sheaths that did not appear to be related to axonal swelling. Electron microscopy showed that these fibres contained increased densities of axonal neurofilaments, with dramatic changes in neurofilament densities at regions of myelin loss. There has been recent interest in the manner in which local surroundings of the axon can influence the numbers and structure of axonal neurofilaments. Price, Lasek and Katz (1990) have proposed that regional differences in the density of axonal neurofilaments along an internode are related to the circumferential pressures on the axon by different external components e.g. myelin sheath, myelin sheath plus Schwann cell nucleus, Schwann cell cytoplasm at paranodal and nodal regions and at Schmidt-Lanterman incisures.

de Waegh, Lee and Brady (1992) studied the mutant Trembler hypomyelinating mouse as a model to test the importance of the Schwann cell/axon relationship in regulating axonal neurofilament content. In the Trembler, lack or absence of myelin leads to a reduction in axon calibre, decreasing neurofilament phosphorylation and increasing neurofilament density. The studies were made using grafts of Trembler nerves in normal hosts and from them it was proposed that a transmembrane signal localized to the myelin-axon interface (possibly myelin-associated protein) modulates the kinase and phosphatase activities in axons, changing phosphorylation levels for neurofilaments, which in turn influences neurofilament density.

Demyelination is well recognised to occur in regions of chronic compression.
Changes in the axon have not been noted in these situations. In the present study, the earliest stages of a compressive lesion were studied and they happen to have provided a good illustration of the axonal neurofilament changes occurring along a demyelinating fibre, much as that described (although not illustrated) in the Trembler/normal mouse graft experiments. The cause of the general increase in axonal neurofilament density (without focal increase in axon size) along some fibres in the early compression lesion is not known, but exogenous external forces may be operating to affect neurofilament numbers through the kind of phosphorylation changes suggested by de Waegh, Lee and Brady (1992), and from the circumferential pressures described by Price, Lasek and Katz (1990). Axonal transport has not been mentioned in this context, but clearly is another factor to be considered.

In none of the other studies of the Bennett and Xie model has the delayed degeneration of nerve fibres been noted. This was however, a feature described in another model of chronic nerve compression studied by Gilliatt and colleagues in the early 1980’s (Baba et al., 1982; Baba, Gilliatt and Jacobs, 1983, O’Neill et al., 1984). A silk ligature was tied round the tibial nerve of a rabbit, tightly enough to cause degeneration of most of the large myelinated fibres. This produced a lesion rather similar to that caused by the Bennett and Xie model. There is no record from Gilliatt and colleagues’ studies that the lesion caused pain.

Examination of the nerves distal to this chronic tight ligature showed a reduction in external fibre diameter for fibres of similar internodal length due to atrophy of the axons when compared with control nerves, and these fibres were shown to have lower ’g’ ratios than controls (Baba et al., 1982). Fibre teasing showed some examples of distal degeneration of these atrophic fibres - a type of 'dying back'.
In the present study, the Bennett and Xie model was found to cause similar atrophic changes in some nerve fibres, affecting larger more than small fibres, as was also the case in the tight ligature model of Gilliatt's. From the finding of fibre degeneration up to several weeks p.o., far too late for it to be due to the original lesion, it is assumed that atrophic fibres may degenerate at their distal ends, as occurred in the Gilliatt model.

It was of interest in the present study that in some of the most atrophic axons, the numbers of filaments were greatly reduced or even absent, although many microtubules were present. It has been proposed that axon calibre is controlled by neurofilament density (Hoffman and Griffin, 1993); the number of neurofilaments in large myelinated fibres correlates closely with axon cross-sectional area. Presumably, chronic nerve constriction affects transport of neurofilaments. When the compressive effects are released, as occurs eventually in the Bennett and Xie model once the ligature material breaks down, then normal movement of neurofilaments resumes, and fibres are no longer atrophic. A reversal of axonal atrophy was demonstrated by Baba et al., (1983) following removal of the single tight ligature.

Tissue response to the ligatures

Ligatures in the original Bennett and Xie (1988) model and a number of subsequent studies using the model were of 4.0 chromic gut. In the present experiment, the earliest response to the presence of the gut (at 8.5 and 24 hours p.o.) was the appearance of macrophages scattered throughout the epineurium external to the sciatic nerve fascicles. After a week the macrophages had aggregated to form a band several cells deep around the ligature. The ligature material was clearly identified in all nerves examined until about the third month p.o. when it had
disappeared. It is presumed therefore that the chromic gut was maintaining a ligating effect throughout most of the period of this study. Bennett and Xie (1988) remark that 'pieces of partly digested suture were recovered ....as late as the third postoperative week'. Guilbaud et al. (1993) described thinning due to resorption of chromic gut (5-0) by six weeks p.o., with progressive disappearance by 15 weeks. Coggeshall et al. (1993) noted that the ligature resulted in an 'inflammatory swelling around the sutures. The sutures and the associated swelling constricted the nerve....' They describe the sutures as being largely resorbed between 14 and 28 days p.o. Thus in some of the other studies, the ligating effect of the suture may not have been as long-lasting as in the present study.

**Vacuolated fibroblasts**

These curious cells were only seen in quantity in one nerve, a sciatic nerve 7 days p.o., in which there was relatively little fibre degeneration but marked endoneurial oedema. Fibroblasts with much smaller vacuoles, only recognisable by electron microscopy, were seen occasionally in other nerves.

These cells were first described by Schoene et al., (1970) in sural nerves from cases of hereditary sensory neuropathy. The nerves contained virtually no myelinated fibres and there was no evidence of recent fibre degeneration. Illustrations indicate that the cells lay in regions of endoneurial oedema. Schoene et al. describe a range of appearances of these cells, from those with very small and discrete vacuoles to others with huge vacuoles, easily visible by light microscopy. It is suggested that vacuoles form within the cell, and increase in size due to expansion or confluence of smaller vacuoles; alternatively, they could be caused as result of complex infolding of the surface membrane.
Jacobs (personal communication) has found vacuolated fibroblasts in sural nerve biopsies from a number of different neuropathies, the common feature being the presence of endoneurial oedema; again the size of the vacuoles varies considerably. In the loose-ligatured nerves, their presence was also associated with endoneurial oedema.

Possible causes of neuropathic pain

There are several possible explanations for neuropathic pain caused by the loose ligatures. Firstly, the preferential loss of large fibres, according to the gate control theory (Melzack and Wall, 1965) can activate central transmission neurons because of the loss of inhibitory control exerted by these large fibres (discussed in Chapter 1). Secondly, the occasional finding of abnormal contacts between unmyelinated axons suggests a potential cross-talk between fibres. Basbaum et al. (1991) postulated that unmyelinated axons in contact with each other become responsive to local mechanical stimulation and to circulating catecholamines because they are no longer protected by the normally complete envelop of Schwann (Remak) cell processes. Whether it is appropriate to compare nerve fibres of a neuroma with those resulting from the Bennett and Xie ligation model is open to question. However, spontaneous discharges originating in the dorsal root ganglia and in A-\(\beta\) and A-\(\delta\) afferents have been described in the Bennett and Xie model (Kajander and Bennett, 1988, 1992).

Munger, Bennett and Kajander (1992) suggested, in relation to the Bennett and Xie model, that interrupted myelinated fibres may be the source of spontaneous discharges, and this may be the basis of abnormal spontaneous sensation.

Fibres proximal to the loose ligatures are intact, and their intraspinal terminals
are intact and functional. However, some publications (Garry et al., 1989; Kajander et al., 1989a) reported significant changes in the level of peptide neurotransmitters and their binding sites in the superficial laminae of the spinal cord, which are possibly responsible for remodelling of the synaptic circuit in the spinal dorsal horn after peripheral nerve injury. Changes of peptide neurotransmitters have been suggested as playing a role in central plasticity (Coderre et al., 1993).

**Correlation studies**

Four loose ligatures around the sciatic nerve induced "neuropathic pain" from 2-5 days p.o., reaching a peak of severity at 2-3 weeks p.o., and lasting for about 2 months (Bennett and Xie, 1988; Attal et al., 1990; Ro and Jacobs, 1993a). But the pain-related behaviour (PRB) (to heat and cold responses) seems to be caused firstly by the contribution from the saphenous nerve, and this lasts from 4 to about 14 days and a second phase, from about 10-12 days to 2 months p.o. which is due mainly to the sciatic nerve damage (Ro and Jacobs, 1993a).

Possible correlations between PRB and nerve fibre damage was sought. For the correlation study, the neuropathic periods between 2 to 4 weeks p.o. were selected since they exclude effects due to the saphenous nerve as much as possible. The number of remaining $\leq 6\mu m$ (A-δ) fibres was correlated with PRB to cold stimuli, which is proposed to be mediated mainly by A-δ afferents (Darian-Smith, Johnson and Dykes, 1973; Mackenzie et al., 1975; Adriaensen et al., 1983). The surviving number of unmyelinated axons was correlated with PRB to heat stimuli, which is proposed to be mediated by polymodal nociceptive C fibres (Torebjörk 1974; Van Hees and Gybels, 1981).

The correlation of the number of $\leq 6\mu m$ fibres of the sural nerve (sensory
Correlation of the total number of myelinated fibres (including A-β, A-α afferents) of the sural nerve with PRB to cold stimuli shows a more significant result ($r=0.905$, $p=0.003$). The number of large-diameter fibres (mainly A-β and A-α afferents) shows a high correlation with PRB to cold stimuli ($r=0.932$, $p=0.007$). This may support the suggestion that other afferents also play a role in mediating a nociceptive response during the "neuropathic pain" period.

Correlation of the number of $\leq 6\mu m$ (A-δ) fibres of the sciatic nerve (mixed nerve) with PRB to cold stimuli is also significant ($r=0.748$, $p = 0.033$), whilst the number of large-diameter fibres ($>6\mu m$) of sciatic nerves does not show a significant correlation with PRB to cold stimuli. This is possibly partly due to the fact that the sciatic nerve consists not only of sensory afferents but also quite a few motor efferents, and these are not supposed to convey a nociceptive response. However, total myelinated fibres, including A-β, A-α afferents and motor efferents of sciatic nerves show an even better correlation with PRB to cold stimuli ($r=0.95$, $p<0.0001$). This may suggest that fibres other than A-δ range are not signalling nociceptive responses directly but are possibly participating in the peripheral sensitization (Bessou et al., 1969; Campbell, Meyer and LaMotte, 1979; LaMotte et al., 1982; Raja, Campbell and Meyer, 1984) and central sensitization (Woolf, 1983; Woolf, 1989; Woolf and Thompson, 1991) to sustain neuropathic pain. However, these suggestions are not supported by interaction statistical studies.

The number of unmyelinated axons of the sural nerve (sensory cutaneous nerve), which includes 70-80% afferents and about 20-30% postganglionic sympathetic efferents (Chad et al., 1983; Baron, Jänig and Kollman, 1988) shows a
good correlation with PRB to heat stimuli ($r = 0.76$, $p = 0.0273$). The number of unmyelinated axons of the sciatic nerve (mixed nerve), which includes fewer afferents but more postganglionic efferents (Lisney, 1989), also shows a good correlation with PRB to heat stimuli ($r = 0.8504$, $p = 0.0319$). This reinforces the idea that the postganglionic sympathetic efferents may also play a role in mediating neuropathic pain.

The possible underlying mechanisms, could be explained by Livingston's vicious circuit (1943). According to the vicious circuit concept, as primary afferent nociceptors are activated due to axonal damage, they in turn activate the sympathetic preganglionic neurons in the intermediolateral column. The preganglionic neurons activate the noradrenergic postganglionic neurons in the sympathetic ganglion, which sensitize and activate the primary afferent nociceptors that feed back to the spinal cord thus maintaining the pain. The nociceptive input may also set another pathway by activating motorneurons that cause muscle spasm. The prolonged muscle spasm activates muscle nociceptors that feed back to the spinal cord to sustain the spasm. The statistical studies would be in accord with Livingston's vicious circuit theory. The dysautonomic behaviour (skin temperature changes) and muscle spasm (inferred from prolonged muscle activity) which are mentioned in the loose ligature model by Bennett and Xie (1988) may support the explanation.

An alternative explanation for the positive correlation is that regenerated axons, both myelinated and unmyelinated, which may have been included in the axon density measurements, present the possibility of spontaneous ectopic (Kajander, Wakisaka and Bennett, 1989b; Kajander and Bennett, 1992), or ephaptic discharges (Seltzer and Devor, 1979) and would thus more easily transmit or enhance a
nociceptive response to build up the neuropathic behaviour (Wall and Gutnick, 1974: Wall and Devor, 1983).

Dyck (1976) proposed that pain in his patients with peripheral neuropathy correlated well with acute fibre degeneration. This suggested another approach in the correlation studies. The number of acute degenerated myelinated fibres was correlated with PRB to cold or heat stimuli. In the sciatic nerve there was no significant correlation with PRB. But an interesting finding is that acute myelinated fibre degeneration continues well after 3 weeks p.o., (discussed in the section on morphological changes) indicating a continuing injury process which possibly participates in 'winding up' the dorsal horn neuron (Seltzer et al., 1991a,b), then producing peripheral and central sensitization.

Basbaum et al. (1990), in studies on the Bennett and Xie model, suggested that the magnitude and variability of behavioural deficits observed in different rats reflects the number of fascicles that are severely damaged. However, Coggeshall et al. (1993) postulated that a preferential loss of large diameter fibres would be a key morphological event underlying the onset of the hyperalgesia and other alterations in pain sensation caused by the model. When the neuropathic pain symptoms are established or start resolving, there is no obvious relation between symptoms on one hand and numbers or size of fibres on the other hand. This thesis has demonstrated different but interesting positive correlations. A possible explanation for the lack of obvious relationship between symptoms and fibre numbers or sizes in other studies (Basbaum et al., 1991, Coggeshall et al., 1993) is that the contribution of the saphenous nerve in early hyperalgesia (Ro and Jacobs, 1993a) was not excluded in their studies.
One rat in the studies presented here showed a nearly total loss of all nerve fibres. The rat displayed firstly a short period of hyperalgesia which was then replaced by hypoalgesia. This can support the hypothesis that the number of nerve fibres damaged establishes the neuropathic pain behaviour (Coggeshall et al., 1993) but when the A-δ or C fibres continue to decrease until total or almost total loss, the hyperalgesia gradually changes to hypoalgesia. These results also highlight the fact that a partial nerve injury is the usual antecedent of painful neuropathy in man.

Other factors may also contribute to the build up neuropathic pain. This experimental neuropathy produces a preferential loss of large myelinated fibres, which leads to loss of central inhibitory controls exerted by large diameter afferents (Gautron et al., 1990; Basbaum et al., 1990; Munger, Bennett and Kajander, 1992; Coggeshall et al., 1993) as suggested by the gate control mechanism (Melzack and Wall 1965). In addition there is some loss of the smaller myelinated and unmyelinated fibres (including C fibres). Loss of unmyelinated afferents may produce slow onset, long-duration changes, which consist of widespread increases of excitability of neurons projecting to the brain and to reflex circuits (Chung et al., 1979; Woolf, 1983) by way of changes to peptide neurotransmitters (Garry et al., 1989; Kajander et al., 1989a) . Subsequent to the rapid phase, the continuous injury signal, ectopic discharge from damaged primary afferent nociceptors either from regenerated sprouts (Wall and Gutnick, 1974) or from secondary sites near the cell body in DRG (Wall and Devor, 1983; Burchiel, 1984) and ephaptic discharges from regenerated axons in close apposition (Seltzer and Devor, 1979) can all 'wind up' the spinal cord dorsal horn neurons. The increases of excitability (by wind up) are so great that previously ineffective inputs, such as low-threshold mechanoreceptors, can activate the
transmission neuron. In such pathological states, the nervous system is reorganised by either activating normally present but ineffective connections, or by activating indirect pathways (Fields, 1987; Wall 1992).

It has been found that pain-related behaviour (PRB) shows a positive correlation with the number of sensory afferents (C, A-δ, and even A-α afferents). However, there are some drawbacks to the morphological part of these studies. Firstly, the numbers of surviving axons are calculated as nerve fibre densities, and are compared with control nerve fibre densities; for myelinated fibres the variation between controls is reasonably low, but it is very large for unmyelinated axons and this is clearly, but unavoidably a source of inaccuracy. A second source of inaccuracy is the effect of endoneurial oedema on nerve fibre density calculations. Ideally the total fibre population should be counted in order to avoid this source of error, but technical problems did not always allow this, therefore all counts were calculated as densities /mm². Thirdly, because of the inclusion of regenerated fibres, not all of the counted nerve fibres are properly functioning (the actual number and type of nerve fibres can not be decided by fibre diameter only). Some of them are regenerated fibres which do not reach their target tissue during the 2-4 weeks p.o. However, even though some regenerated fibres may not be functional, they may possibly contribute to the ectopic (Wall and Gutnik, 1974; Wall and Devor, 1983; Burchiel, 1984; Kajander and Bennett, 1992) or ephaptic discharge (Seltzer and Devor, 1979) which also plays a role in neuropathic pain. In addition, some of the myelinated fibres are abnormal, with atrophic axons and low 'g' ratios and these may have degenerated at their distal ends and have become non-functional. A fourth drawback is that in reality there is no sharp line between A-δ and A-β fibres in histograms of nerve fibre...
diameters or in electrophysiological studies relating to the response properties of the receptors. Depending on fibre diameters to classify different functional type fibres is not totally reliable. Fifthly, the possibility of coincidence cannot be totally ruled out due to the small samples used (n=6 in sural, n=8 in sciatic nerve).
SUGGESTIONS FOR FURTHER STUDY OF THE BENNETT AND XIE MODEL

C-fos protein and pain-related behaviour

Hunt, Pini and Evans (1987) showed that activation of small-diameter cutaneous afferents by noxious heat or by chemical stimuli resulted in the rapid appearance of c-fos protein within neurons of the superficial laminae of the dorsal horn. Numerous other studies reported the expression of c-fos in spinal cord neurons, following various acute noxious peripheral stimuli (Draisci and Iadarola, 1989; Menétrey et al., 1989; Bullit, 1990; Presley et al., 1990; Tölle et al., 1991; Herdegen et al., 1991b,c; Noguchi et al., 1991). More recently, c-fos expression was also induced by peripheral nerve injury (Basbaum, Chi and Levine, 1991; Williams, Evans and Hunt, 1991). Non-noxious stimuli could also induce c-fos expression but in a different distribution (Hunt, Pini and Evans, 1987). However, there is accumulating evidence that c-fos is preferentially expressed in dorsal horn neurons receiving noxious inputs. More interestingly, it has been shown using peripheral nerve stimulation that Fos-like immunoreactivity was only induced by Aδ and c-fibre activation (Herdegen et al., 1991b). If c-fos protein is really closely linked with noxious stimulation, this is one immunocytochemical study which could be carried out to confirm the suggestion made in this thesis that the saphenous nerve plays an important role in the behavioural changes following sciatic nerve damage caused by loose ligatures.

Williams et al. (1989) showed, after sciatic nerve section, a massive increase in the number of lamina II neurons labelled with c-fos within the saphenous representation of the dorsal horn, particularly in the area adjacent to the sciatic
representation where numbers and intensity of labelled neurons are usually low 28 days following sciatic nerve section. This demonstrated that c-fos protein was higher in dorsal horn saphenous nerve territory than in sciatic nerve territory even at twenty-eight days after sciatic nerve crush injury, and suggested that the nociceptive response was more likely to be due to the saphenous nerve than the sciatic nerve in the early stage of chronic sciatic nerve injury. If this is true in the loose ligatures model, the c-fos protein would be expected to be higher in the saphenous territory represented in the dorsal horn from the 4th-12th days p.o., or even longer; and it would then become greater in the representation of the sciatic nerve territory from 14 days p.o.

Nerve growth factor (NGF) and pain-related substances

NGF is essential for the survival of sympathetic and sensory neurons during development (Barde, 1989). Almost all sensory neurons die if exposed to antisera to NGF before birth, whereas only about 20% of all dorsal root ganglion (DRG) cells appear to be dependent upon NGF postnatally (Yip et al., 1984; Lewin, Ritter and Mendell, 1992). Anti-NGF treatment in pre- or postnatal periods can lead to a reduction in the number of substance P-containing neurons in the DRG (Goedert et al., 1984) and there is evidence that calcitonin gene-related peptide (CGRP) is also regulated by NGF levels (Lindsay et al., 1989). The CGRP and substance-P-containing neurons have been suggested to be closely linked to nociceptive afferents (Bennett et al., 1989). Thus it is clear that NGF has a kind of link with nociceptive afferents.

Diamond et al., (1992) have shown that endogenous NGF can regulate the collateral sprouting of nociceptive sensory axons in the skin of the adult rat after denervation.
NGF-induced hyperalgesia due to sensitization of peripheral intact nociceptors (reduction of stimulus threshold) in the neonatal and adult rat has been demonstrated by Lewin et al. (1993). NGF mRNA expression is low in normal rat skin, but is significantly increased in denervated skin (Diamond et al., 1992). Thus the early hyperalgesia may be explained in the following ways. Firstly, NGF is expected to increase in the partly denervated skin of the sciatic nerve territory; it may then sensitize the intact nociceptors of the sciatic nerve territory and the saphenous nerve territory (due to anatomical overlapping). Secondly, high levels of NGF can regulate collateral sprouting of nociceptive A-δ and c fibres of intact nerves, which are also possibly involved in pain-related behaviour in rats.

Ro and Jacobs (1993a) suggested that the early hyperalgesia (4-12 days p.o.) occurring in the chronic loose ligature model is mainly contributed by the saphenous nerve from either nociceptor sensitization or collateral sprouting. Therefore, the introduction of anti-NGF may help to clarify the cause of early hyperalgesia in the rats with chronic loose ligatures. If anti-NGF were to abolish early hyperalgesia, it would provide evidence that collateral sprouting was playing a role in early hyperalgesia. As we know, anti-NGF can halt the collateral sprouting from intact nerve (Diamond et al., 1992). However, it is still unknown whether or not anti-NGF can abolish the peripheral nociceptor sensitization induced by NGF.
CHAPTER 5: CONCLUSIONS

1. Multiple loose ligatures around the rat sciatic nerve produce neuropathic pain-related behaviour, including hyperalgesia, allodynia and possibly spontaneous pain, lasting from 4 days to 8 weeks post-operation (p.o.).

2. The saphenous nerve plays an important role in early neuropathic pain-related behaviour from 4 to 12 days p.o.

3. Neuropathic pain-related behaviour from about 12 days to 8 weeks can mainly be attributed to the sciatic nerve.

4. Multiple loose ligatures produce a variable degree of sciatic nerve damage. A preferential loss of large diameter myelinated fibres was noted in most cases.

5. The underlying mechanisms of the early nerve pathology include ischaemia, within the first 48 hours, and then compressive effects due to nerve swelling, causing the ligatures to strangulate the nerve.

6. Most nerve fibre degeneration takes place within the first 7 days p.o. However, as a result of the compressive lesion, nerve fibres continue to degenerate over a period of many weeks, although with decreasing frequency.

7. Neuropathic pain-related behaviour may be related to the preferential loss of large diameter fibres, leading to dysfunction of the gate control mechanism and hyperexcitability of superficial dorsal horn cells. Unmyelinated axons in close contact may contribute to ephaptic discharges.

8. Correlation studies suggest that not only A-δ and C afferent fibres, but also A-α, A-β and possibly motor-efferents can influence neuropathic pain-related behaviour.
APPENDICES

APPENDIX A

Hindlimb withdrawal reflex (lesion side minus normal side in seconds) to 6° or 48°C stimuli.

A1  Sham operation; 48°C thermal stimuli

<table>
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<tr>
<th>Day</th>
<th>No. of rats</th>
<th>Mean ± SEM (secs)</th>
</tr>
</thead>
<tbody>
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<td>-2.41 ± 0.89</td>
</tr>
<tr>
<td>4</td>
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<td>-0.58 ± 0.23</td>
</tr>
<tr>
<td>6</td>
<td>6</td>
<td>-0.13 ± 0.19</td>
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<tr>
<td>9</td>
<td>4</td>
<td>-0.38 ± 0.24</td>
</tr>
<tr>
<td>12</td>
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<td>0 ± 0.36</td>
</tr>
<tr>
<td>21</td>
<td>4</td>
<td>0 ± 0</td>
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</table>

A2  Sham operation; 6°C cold stimuli

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<th>Mean ± SEM (secs)</th>
</tr>
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<td>6</td>
<td>0.31 ± 0.51</td>
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<tr>
<td>4</td>
<td>6</td>
<td>-0.33 ± 0.42</td>
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<tr>
<td>6</td>
<td>6</td>
<td>0.42 ± 0.52</td>
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<tr>
<td>9</td>
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A3  Loose-ligatures only group: Hindlimb withdrawal reflex (secs) (lesions side minus normal side); 48°C thermal stimuli

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<td>6</td>
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<td>7</td>
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A4  Loose-ligatures only group: Hindlimb withdrawal reflex (secs) (lesion side minus normal side); 6°C cold stimuli

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</table>
A5  G1 = 4 loose-ligatures + saphenectomy at the same time
G2 = 4 loose-ligatures followed by saphenectomy 1 week later
G3 = 4 loose-ligatures followed by saphenectomy 2 weeks later
G4 = 4 loose-ligatures followed by saphenectomy 3 weeks later

Hindlimb withdrawal reflex (secs); lesion side minus normal side; 48°C

<table>
<thead>
<tr>
<th>Days</th>
<th>G1 (n=6) mean ± SEM</th>
<th>G2 (n=6) mean ± SEM</th>
<th>G3 (n=6) mean ± SEM</th>
<th>G4 (n=6) mean ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>11.33 ± 2.78</td>
<td>10.67 ± 1.48</td>
<td>7.59 ± 1.99</td>
<td>9.72 ± 2.93</td>
</tr>
<tr>
<td>5</td>
<td>6.83 ± 2.06</td>
<td>1.5 ± 1.65</td>
<td>0.22 ± 1.39</td>
<td>2.89 ± 0.59</td>
</tr>
<tr>
<td>7</td>
<td>4.67 ± 2.72</td>
<td>-0.89 ± 0.52</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>3.64 ± 1.63</td>
<td>3.22 ± 0.33</td>
<td>-1.83 ± 0.44</td>
<td>1 ± 2.27</td>
</tr>
<tr>
<td>10</td>
<td>-0.33 ± 0.99</td>
<td>3.33 ± 0.62</td>
<td>-2.99 ± 0.51</td>
<td>-2.17 ± 1.37</td>
</tr>
<tr>
<td>12</td>
<td>-2.41 ± 0.51</td>
<td>0.83 ± 1.21</td>
<td>-2.33 ± 1.02</td>
<td>-1.83 ± 1.17</td>
</tr>
<tr>
<td>14</td>
<td>-2.92 ± 0.26</td>
<td>-1.17 ± 2.02</td>
<td>-3.67 ± 0.34</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>-3.58 ± 0.35</td>
<td>-1.58 ± 1.22</td>
<td>-3.67 ± 0.60</td>
<td>-3.33 ± 0.17</td>
</tr>
<tr>
<td>19</td>
<td>-3.17 ± 0.73</td>
<td>-2.58 ± 1.13</td>
<td>-3 ± 1.00</td>
<td>-3 ± 1.00</td>
</tr>
<tr>
<td>21</td>
<td>-3.5 ± 0.29</td>
<td>-2.33 ± 0.17</td>
<td>-4.17 ± 0.93</td>
<td>-2.33 ± 0.73</td>
</tr>
<tr>
<td>22</td>
<td></td>
<td></td>
<td></td>
<td>-2.83 ± 0.33</td>
</tr>
<tr>
<td>24</td>
<td>-2.75 ± 0.63</td>
<td>-2.17 ± 0.44</td>
<td>-3 ± 0</td>
<td>-2.67 ± 0.34</td>
</tr>
<tr>
<td>32</td>
<td>-3 ± 0.29</td>
<td>-2.33 ± 0.68</td>
<td>-3 ± 0.58</td>
<td>-3.33 ± 0.17</td>
</tr>
</tbody>
</table>
Hindlimb withdrawal reflex (secs): lesion side minus normal side: 6°C

<table>
<thead>
<tr>
<th>Days</th>
<th>G1 (n=6) mean ± SEM</th>
<th>G2 (n=6) mean ± SEM</th>
<th>G3 (n=6) mean ± SEM</th>
<th>G4 (n=6) mean ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>9.83 ± 0.92</td>
<td>8.5 ± 2.15</td>
<td>7.83 ± 1.33</td>
<td>8.83 ± 0.59</td>
</tr>
<tr>
<td>5</td>
<td>4.33 ± 0.73</td>
<td>-0.16 ± 1.52</td>
<td>-1.61 ± 0.57</td>
<td>2.94 ± 1.38</td>
</tr>
<tr>
<td>7</td>
<td>4.3 ± 2.11</td>
<td>0.16 ± 1.00</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>2 ± 1.01</td>
<td>3.19 ± 0.44</td>
<td>-3.39 ± 0.20</td>
<td>-2.67 ± 0.60</td>
</tr>
<tr>
<td>10</td>
<td>-0.78 ± 1.39</td>
<td>-0.25 ± 1.88</td>
<td>-3.22 ± 0.64</td>
<td>-1.5 ± 0.50</td>
</tr>
<tr>
<td>12</td>
<td>-3.67 ± 0.28</td>
<td>-2.20 ± 1.98</td>
<td>-4.39 ± 0.68</td>
<td>-4.5 ± 0.77</td>
</tr>
<tr>
<td>14</td>
<td>-3.83 ± 0.24</td>
<td>-0.33 ± 2.96</td>
<td>-3.33 ± 0.60</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>-4.08 ± 0.08</td>
<td>-1.75 ± 2.07</td>
<td>-3 ± 0.29</td>
<td>-4.17 ± 0.84</td>
</tr>
<tr>
<td>19</td>
<td>-4 ± 1.16</td>
<td>-2.67 ± 0.34</td>
<td>-3 ± 0.29</td>
<td>-3.5 ± 0.50</td>
</tr>
<tr>
<td>21</td>
<td>-4.5 ± 0.35</td>
<td>-2.5 ± 0.58</td>
<td>-2.33 ± 0.17</td>
<td>-3.67 ± 0.44</td>
</tr>
<tr>
<td>22</td>
<td></td>
<td></td>
<td>-3 ± 0.29</td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>-3.5 ± 0.50</td>
<td>-2.33 ± 0.17</td>
<td>-3 ± 0.58</td>
<td>-2.5 ± 0.77</td>
</tr>
<tr>
<td>32</td>
<td>-3.33 ± 0.67</td>
<td>-2.66 ± 0.67</td>
<td>-2.5 ± 0.87</td>
<td>-3.16 ± 0.60</td>
</tr>
</tbody>
</table>
## APPENDIX B

**B1 Control sciatic nerves (n=4):** Myelinated fibre diameter frequency at 1\(\mu\)m intervals; mean, percentage and standard deviation (SD)

<table>
<thead>
<tr>
<th>Fibre diameter ((\mu)m)</th>
<th>Mean (No/mm(^2))</th>
<th>(%)</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1-2</td>
<td>45.8</td>
<td>0.32</td>
<td>41.5</td>
</tr>
<tr>
<td>2-3</td>
<td>855.8</td>
<td>5.98</td>
<td>506.6</td>
</tr>
<tr>
<td>3-4</td>
<td>1,824.6</td>
<td>12.75</td>
<td>246</td>
</tr>
<tr>
<td>4-5</td>
<td>1,784.6</td>
<td>12.47</td>
<td>208.9</td>
</tr>
<tr>
<td>5-6</td>
<td>1,750</td>
<td>12.23</td>
<td>446.5</td>
</tr>
<tr>
<td>6-7</td>
<td>2,143.8</td>
<td>14.98</td>
<td>455</td>
</tr>
<tr>
<td>7-8</td>
<td>1,974.9</td>
<td>13.80</td>
<td>369</td>
</tr>
<tr>
<td>8-9</td>
<td>1,602.8</td>
<td>11.21</td>
<td>233</td>
</tr>
<tr>
<td>9-10</td>
<td>1,512.7</td>
<td>10.57</td>
<td>791.4</td>
</tr>
<tr>
<td>10-11</td>
<td>758.5</td>
<td>5.30</td>
<td>476.5</td>
</tr>
<tr>
<td>11-12</td>
<td>55.8</td>
<td>0.39</td>
<td>50.1</td>
</tr>
<tr>
<td>12-13</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>13-14</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Density of myelinated and unmyelinated axons in control sciatic nerve (n=4); means and standard deviations

<table>
<thead>
<tr>
<th>Myelinated fibre density/mm(^2)</th>
<th>Unmyelinated axon density/mm(^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>14,514</td>
<td>31,407</td>
</tr>
<tr>
<td>14,635</td>
<td>21,025</td>
</tr>
<tr>
<td>12,657</td>
<td>23,794</td>
</tr>
<tr>
<td>15,437</td>
<td>22,439</td>
</tr>
<tr>
<td>mean ± SD</td>
<td>mean ± SD</td>
</tr>
<tr>
<td>14,311 ± 1,176</td>
<td>24,666 ± 4,635</td>
</tr>
</tbody>
</table>
B2  Control sural nerves (n=4) : Myelinated fibre diameter frequency at 1μm intervals; mean, percentage and standard deviation (SD)

<table>
<thead>
<tr>
<th>Fibre diameter (μm)</th>
<th>Mean (No/mm²)</th>
<th>(%)</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1-2</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2-3</td>
<td>2,890.9</td>
<td>5.13</td>
<td>148.1</td>
</tr>
<tr>
<td>3-4</td>
<td>9,579.8</td>
<td>17.0</td>
<td>497.1</td>
</tr>
<tr>
<td>4-5</td>
<td>11,833.9</td>
<td>21.05</td>
<td>253.5</td>
</tr>
<tr>
<td>5-6</td>
<td>13,794.9</td>
<td>24.48</td>
<td>1,127</td>
</tr>
<tr>
<td>6-7</td>
<td>12,172</td>
<td>21.6</td>
<td>1,994.8</td>
</tr>
<tr>
<td>7-8</td>
<td>4,981.5</td>
<td>8.84</td>
<td>337</td>
</tr>
<tr>
<td>8-9</td>
<td>873.4</td>
<td>1.55</td>
<td>121.3</td>
</tr>
<tr>
<td>9-10</td>
<td>135.2</td>
<td>0.24</td>
<td>30.8</td>
</tr>
<tr>
<td>10-11</td>
<td>67.6</td>
<td>0.12</td>
<td>23.9</td>
</tr>
<tr>
<td>11-12</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12-13</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>13-14</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Density of myelinated and unmyelinated axons in control sural nerves (n=4); means and standard deviations

<table>
<thead>
<tr>
<th>Myelinated fibre density/mm²</th>
<th>Unmyelinated axon density/mm²</th>
</tr>
</thead>
<tbody>
<tr>
<td>20,343</td>
<td>51,845</td>
</tr>
<tr>
<td>17,544</td>
<td>70,047</td>
</tr>
<tr>
<td>22,200</td>
<td>56,580</td>
</tr>
<tr>
<td>18,496</td>
<td>46,938</td>
</tr>
<tr>
<td>mean ± SD</td>
<td>mean ± SD</td>
</tr>
<tr>
<td>19,646 ± 2,062</td>
<td>56,352 ± 9,942</td>
</tr>
</tbody>
</table>
APPENDIX C

C1 Correlation between sural nerve fibre densities/mm² and pain-related behaviour (PRB). MF = myelinated fibres, LMF = large myelinated fibres, SMF = small myelinated fibres, UMA = unmyelinated axons.

<table>
<thead>
<tr>
<th></th>
<th>Y₁</th>
<th>Y₂</th>
<th>X₁</th>
<th>X₂</th>
<th>X₃</th>
<th>X₄</th>
</tr>
</thead>
<tbody>
<tr>
<td>PRB (6°C)</td>
<td>PRB (48°C)</td>
<td>MF</td>
<td>LMF</td>
<td>SMF</td>
<td>UMA</td>
<td></td>
</tr>
<tr>
<td>-3.5</td>
<td>2.5</td>
<td>11,260</td>
<td>3,873</td>
<td>7,387</td>
<td>41,707</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>3.4</td>
<td>14,680</td>
<td>4,962</td>
<td>9,718</td>
<td>53,037</td>
<td></td>
</tr>
<tr>
<td>4.5</td>
<td>4.5</td>
<td>20,286</td>
<td>5,011</td>
<td>15,275</td>
<td>94,558</td>
<td></td>
</tr>
<tr>
<td>-15</td>
<td>-2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>120</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>15,322</td>
<td>6,435</td>
<td>8,887</td>
<td>58,188</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>12,397</td>
<td>4,029</td>
<td>8,368</td>
<td>56,208</td>
<td></td>
</tr>
</tbody>
</table>

(1) Correlation between PRB(6°C) and LMF (large myelinated fibre density/mm²)

The regression equation is

\[ Y_1 = -13.8 + 0.202 X_2 \]

<table>
<thead>
<tr>
<th>Predictor</th>
<th>Coef</th>
<th>Stdev</th>
<th>t-ratio</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Constant</td>
<td>-13.826</td>
<td>2.818</td>
<td>-4.19</td>
<td>0.008</td>
</tr>
<tr>
<td>X₂</td>
<td>0.20202</td>
<td>0.03931</td>
<td>5.14</td>
<td>0.007</td>
</tr>
</tbody>
</table>

\[ s = 3.047 \quad \gamma^2 = 0.868 \]

Correlation co-efficient (\( \gamma \)) = 0.932
P value = 0.007
(2) Correlation between PRB (6°C) and SMF (small myelinated fibre density/mm²)

The regression equation is

\[ Y_1 = -12.2 + 0.184 \times X_3 \]

<table>
<thead>
<tr>
<th>Predictor</th>
<th>Coef</th>
<th>Stdev</th>
<th>t-ratio</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Constant</td>
<td>-12.247</td>
<td>3.105</td>
<td>-3.94</td>
<td>0.017</td>
</tr>
<tr>
<td>X_3</td>
<td>0.18419</td>
<td>0.04405</td>
<td>4.18</td>
<td>0.014</td>
</tr>
</tbody>
</table>

\[ S = 3.625 \quad \gamma^2 = 0.814 \]

Correlation coefficient (\(\gamma\)) = 0.902

P value = 0.014

(3) Correlation between PRB (6°C) and MF (myelinated fibre density/mm²)

The regression equation is

\[ Y_1 = -13.8 + 0.207 \times X_1 \]

<table>
<thead>
<tr>
<th>Predictor</th>
<th>Coef</th>
<th>Stdev</th>
<th>t-ratio</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Constant</td>
<td>-13.794</td>
<td>2.348</td>
<td>-5.87</td>
<td>0.004</td>
</tr>
<tr>
<td>X_1</td>
<td>0.20661</td>
<td>0.03343</td>
<td>6.18</td>
<td>0.003</td>
</tr>
</tbody>
</table>

\[ S = 2.587 \quad \gamma^2 = 0.905 \]

Correlation coefficient (\(\gamma\)) = 0.951

P value = 0.003

(4) Correlation between PRB (48°C) and UMA (unmyelinated axon density/mm²)

Least squares linear regression

\[ \text{Shape} = 3.720 \times 10^{-2} \pm 1.151 \times 10^{-2} \]

\[ 1/\text{shape} = 26.8821 \]

Correlation coefficient (\(\gamma\)) = 0.7641

\[ S = 1.351 \quad \gamma^2 = 0.7231 \]

P value = 0.0319 (two tailed)
(5) Correlation between PRB (6°) and SMF - LMF interaction.

The regression equation is

\[ Y_1 = -15.1 + 0.156X_3 + 0.151X_2 - 0.00106X_2 \times X_3 \]

<table>
<thead>
<tr>
<th>Predictor</th>
<th>Coef</th>
<th>Stdev</th>
<th>t-ratio</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Constant</td>
<td>-15.086</td>
<td>2.766</td>
<td>-5.45</td>
<td>0.032</td>
</tr>
<tr>
<td>X_3</td>
<td>0.1558</td>
<td>0.1170</td>
<td>1.33</td>
<td>0.314</td>
</tr>
<tr>
<td>X_2</td>
<td>0.15147</td>
<td>0.07088</td>
<td>2.14</td>
<td>0.166</td>
</tr>
<tr>
<td>X_3 \times X_2</td>
<td>-0.001063</td>
<td>0.001606</td>
<td>-0.66</td>
<td>0.576</td>
</tr>
</tbody>
</table>

\[ S = 2.767 \quad \gamma^2 = 0.946 \]

P value = 0.576
Correlation between sciatic nerve fibre density/mm² and pain-related behaviour (PRB). MF = myelinated fibres, LUM = large myelinated fibres, SMF = small myelinated fibres, UMA = unmyelinated axons

<table>
<thead>
<tr>
<th>Y₁</th>
<th>Y₂</th>
<th>X₁</th>
<th>X₂</th>
<th>X₃</th>
<th>X₄</th>
</tr>
</thead>
<tbody>
<tr>
<td>PRB (6°C)</td>
<td>PRB (48°C)</td>
<td>MF</td>
<td>LMF</td>
<td>SMF</td>
<td>UMA</td>
</tr>
<tr>
<td>-3.5</td>
<td>2.5</td>
<td>7,479</td>
<td>3,665</td>
<td>3,814</td>
<td>23,932</td>
</tr>
<tr>
<td>3</td>
<td>1.5</td>
<td>11,887</td>
<td>5,349</td>
<td>6,538</td>
<td>23,295</td>
</tr>
<tr>
<td>4</td>
<td>3.4</td>
<td>12,028</td>
<td>7,217</td>
<td>4,811</td>
<td>24,328</td>
</tr>
<tr>
<td>4.5</td>
<td>4.5</td>
<td>13,814</td>
<td>1,174</td>
<td>12,640</td>
<td>53,999</td>
</tr>
<tr>
<td>-15</td>
<td>-2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>120</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>11,324</td>
<td>6,625</td>
<td>4,699</td>
<td>40,989</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>13,296</td>
<td>8,111</td>
<td>5,185</td>
<td>20,293</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>10,113</td>
<td>4,116</td>
<td>5,997</td>
<td>24,915</td>
</tr>
</tbody>
</table>

(1) Correlation between PRB (6°C) and SMF (small myelinated fibre density/mm²)

The regression equation is

\[ Y_1 = -7.80 + 0.0864 \times X_3 \]

<table>
<thead>
<tr>
<th>Predictor</th>
<th>Coef</th>
<th>Stdev</th>
<th>t-ratio</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Constant</td>
<td>-7.795</td>
<td>3.193</td>
<td>-2.44</td>
<td>0.050</td>
</tr>
<tr>
<td>X₃</td>
<td>0.08636</td>
<td>0.03131</td>
<td>2.76</td>
<td>0.033</td>
</tr>
</tbody>
</table>

\[ S = 4.6563 \quad \gamma^2 = 0.559 \]

Correlation co-efficient (\(\gamma\)) = 0.748

P value = 0.033
(2) Correlation between PRB (6°C) and LMF (large myelinated fibre density/mm²)

The regression equation is

\[ Y_1 = -6.10 + 0.104 X_2 \]

<table>
<thead>
<tr>
<th>Predictor</th>
<th>Coef</th>
<th>Stdev</th>
<th>t-ratio</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Constant</td>
<td>-6.10</td>
<td>3.989</td>
<td>-1.53</td>
<td>0.177</td>
</tr>
<tr>
<td>( X_2 )</td>
<td>0.104</td>
<td>0.06109</td>
<td>1.70</td>
<td>0.139</td>
</tr>
</tbody>
</table>

\( S = 5.755 \quad \gamma^2 = 0.326 \)

Correlation coefficient (\( \gamma \)) = 0.571
P value = 0.139

(3) Correlation between PRB (6°C) and MF (myelinated fibre density/mm²)

The regression equation is

\[ Y_1 = -1.40 + 0.197 X_1 \]

<table>
<thead>
<tr>
<th>Predictor</th>
<th>Coef</th>
<th>Stdev</th>
<th>t-ratio</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Constant</td>
<td>-13.979</td>
<td>1.992</td>
<td>-7.02</td>
<td>&lt;0.0001</td>
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<tr>
<td>( X_1 )</td>
<td>1.966</td>
<td>0.02630</td>
<td>7.47</td>
<td>&lt;0.0001</td>
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\( S = 2.183 \quad \gamma^2 = 0.903 \)

Correlation coefficient (\( \gamma \)) = 0.950
P value = < 0.0001

(4) Correlation between PRB (48°C) and UMA (unmyelinated axon density/mm²)

Least squares linear regression

\[
\text{Shape} = 2.440 \ E-02 \pm 8.409 \ E-03 \\
1/\text{shape} = 40.99096 \\
S = 1.41832 \quad \gamma^2 = 0.5838 \\
\text{Correlation coefficient (}\gamma\text{)} = 0.7641 \\
P \text{value} = 0.0273
\]
(5) Correlation between PRB (6°) and SMF - LMF interaction.

The regression equation is

\[ Y_1 = -14.1 + 0.0932X_3 + 0.140X_2 - 0.00045X_2 \times X_3 \]

<table>
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<th>Predictor</th>
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<td>X_3</td>
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<td>0.02587</td>
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<tr>
<td>X_2</td>
<td>0.1400</td>
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<tr>
<td>X_3 * X_2</td>
<td>-0.000445</td>
<td>0.001563</td>
<td>-0.28</td>
<td>0.790</td>
</tr>
</tbody>
</table>

\[ S = 2.636 \quad \gamma^2 = 0.906 \]

P value = 0.790
REFERENCES


Gordh, T. and Kristensen, J.D. (1992) The NMDA receptor antagonist CPP abolishes neurogenic 'wind-up pain' after intrathecal administration in humans. *Regional Anesthesia*, 17, s82.


Hua, X.Y., Saria, A., Games, R. Theodorsson-Norheim, E., Brodin, E. and


Kayser, V. and Guilbaud, G. (1987) Local and remote modifications of nociceptive


Munger, B.L. and Bennett, G.J. (1990) The peripheral axonal pathology in the constrictive model of peripheral neuropathy. *Anatomical Record, 226*, 70A.


Neary, D. and Eames, R.A. (1975) The pathology of ulnar nerve compression in


Ochoa, J. and Marotte, L (1973) The nature of the nerve lesion caused by chronic entrapment in the guinea-pig. *Journal of the Neurological Sciences*, 19, 491-495.


mechanisms of primary and secondary hyperalgesia following heat injury to the glabrous skin. *Brain*, 107, 1179-1188.


Vizoso, A.D. and Young, J.Z. (1948) Internode length and fibre diameter in developing and regenerating nerves. *Journal of Anatomy*, 82, 110-134.


Wall, P.D. and Devor, M. (1983) Sensory afferent impulses from dorsal root
ganglia as well as from the periphery in normal and nerve injured rats. *Pain*, 17, 321-339.


The role of the saphenous nerve in experimental sciatic nerve mononeuropathy produced by loose ligatures: a behavioural study

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Summary
Four loose ligatures were tied round the sciatic nerve of rats to produce the model of altered pain sensation first described by Bennett and Xie (1988). Hyperalgesia and hyperaesthesia were detected from 4 days after tying ligatures, becoming maximal after 14 days; normal behaviour returned by 8 weeks post-operation. Using thermal tests involving immersion of the whole foot, it was found that section of the saphenous nerve at the time of, or within a week of, placing ligatures had the effect of abolishing the hyperaesthetic behaviour and instead caused hypoaesthesia from the 4th to 10-12th days. There was then a change to hyperaesthetic behaviour. The findings are interpreted as indicating that the early hyperaesthesia is possibly due to collateral sprouting, spreading nociceptor sensitivity of saphenous nerve fibres or both of these.

Key words: Hyperalgesia; Allodynia; Hypersensitivity; Neuropathy; Autotomy; (Rat)

Introduction
The use of multiple loose ligation of the rat sciatic nerve has been proposed as a model for the study of causalgia, allodynia or spontaneous pain in man (Bennett and Xie 1988). Other behavioural studies of this model have been made (Attal et al. 1990) and severe damage to the nerve has been reported (Munger and Bennett 1990; Basbaum et al. 1991; Carlton et al. 1991).

As part of a study of the mechanisms leading to nerve damage, and of the morphological changes caused by the multiple ligations (to be published), we have also re-examined the behavioural aspects of the model. It is known that a complete interruption of the sciatic nerve induces, within a few days, the spread of receptive fields of the saphenous nerve which innervates the medial aspect of the foot (Devor et al. 1979). Whether partial deafferentation produces an adequate stimulus for sprouting is not known. However, on this supposition, we have examined the behavioural effects of section of the saphenous nerve at the time of application of the loose ligatures and at 1, 2 and 3 weeks after tying the ligatures.

Materials and methods

Animals
A total of 84 young adult male Sprague-Dawley rats, weighing 250-330 g, were used. They were housed in small groups in plastic cages with solid floors and soft bedding; food and water were available ad libitum.

Surgical procedures
Anaesthesia was initially induced with isoflurane and maintained with i.p. hypnorm (fentanyl citrate and fluanisone) and midazolam (1 part of each + 2 parts water) at a dose of 2.5 ml/kg.

Placing of ligatures. The right sciatic nerve was exposed at mid-thigh level and 4 ligatures (4/0 chronic gut) were placed loosely 1-2 mm apart around the nerve. The ligatures were tied so as not to cause constriction of the nerve, and circulation through the epineurial vasculature was not totally interrupted. In sham operations, exactly the same procedure was followed except that the ligatures were not tied but were left lying loosely beneath the nerve.

Saphenous nerve section. After anaesthetisation, the right saphenous nerve was exposed by making an incision on the ventral aspect of the thigh. About 10 mm of the nerve was cut off at mid-thigh level, and both ends were ligated to prevent regeneration.
Forty rats had sciatic nerve ligatures alone and 12 rats were sham-operated. Four groups each of 6 rats had sciatic nerve ligation and saphenectomy, in group I at the time of ligature and in groups II, III and IV at 1, 2 and 3 weeks respectively after ligature.

**Examination of animals**

Each rat was examined 2 days after operation, every day during the first two post-operative (p.o.) weeks, 3 times weekly during the 3rd and 4th weeks and thereafter once every 2 weeks until 4 months p.o. The unoperated limb was examined first, and then the operated limb to prevent heteronymous facilitation (Woolf 1983; Woolf et al. 1986). Six normal rats with no surgery were used as controls. Statistical analysis was performed using the 2-tailed Mann-Whitney U test. Values of P < 0.01 were considered to be significant. For each set of values the mean and standard error of the mean (S.E.M.) was determined.

**Observation of grip and gait: assessment on a semiquantitative scale.** This was always the first test, and a rating test was devised. The behavioural changes observed may be due to both sensory-related effects and motor deficits, and it is important to recognise this. However, it is impracticable to attempt to differentiate between sensory and motor deficits, and our rating system therefore includes changes of both sensory and motor function.

The rats were placed on a wire grid to observe the hind limb grasping reflex. Normal rats maintain their grip with all 5 toes. The behaviour of the rats could be described either as hypersensitive or allodynic (negative grade) or as hyposensitive or paretic (positive grade). Allodynia was graded as a limping gait with reluctance to put the foot down (~3); very brief (~5 sec) grasping of the cage wires followed by licking and vocalisation (~2); or a longer period (>5 sec) of grip followed by licking and vocalisation (~1). Normal behaviour was grade 0. Paretic behaviour was graded as: firm grasp with all claws which could not be sustained (+1); grasp with 2–4 claws but not sustained (+2); or loss of grip, the animal often dragging the limb when walking (+3).

**Heat.** All experiments were performed by the same individual (L.-S.R.). The animal was carefully held so that the head and limbs were free. The lower limb was placed in a hot water bath at 48°C and the time taken for withdrawal of the foot was measured; as previously mentioned the unoperated side was examined first. Every rat was tested at least twice, with an interval of at least 10 min between each test. If the foot was not withdrawn within 30 secs, it was removed from the bath by the observer to avoid any possible tissue damage. Values of this magnitude were only obtained within the first 2 days following application of ligatures; they are not included in the graphical representation of data.

At each time of measurement, the value of the withdrawal reflex on the unoperated side was subtracted from that of the operated side.

A pilot study using methods described above at temperatures ranging from 40°C to 54°C at intervals of 2°C showed a marked difference in response from 48°C to 50°C (Fig. 1). The data from the tests with the 48°C stimulus were the most informative and are presented here.

In tests at 48°C, the mean of the difference between right and left sides of control rats was 0.04 sec ± 0.15 (S.E.M.) at day 1, ~0.29 sec ± 0.10 (S.E.M.) at day 7, and ~0.06 sec ± 0.02 (S.E.M.) at day 14. The measured values of latencies in the same control rats were 5.75 sec ± 1.40 (S.E.M.) and 5.71 sec ± 1.50 (S.E.M.) on right and left sides respectively on day 1, 4.88 sec ± 0.49 (S.E.M.) and 5.17 sec ± 0.46 (S.E.M.) on day 7, and 5.73 sec ± 0.64 (S.E.M.) and 5.78 sec ± 0.55 (S.E.M.) on day 14. The 'difference' method was chosen in preference to giving absolute latencies since it showed less daily variation.

**Cold.** The method used was similar to the heat testing, but with the water bath maintained at 6°C. In some animals there was immediate withdrawal of the limb (within 1 sec); this was regarded as a false positive. The limb was dried and the test repeated after 10 min. As with tests at 48°C we used the 'difference' method to record the findings. Similar variations in readings were found to those recorded in the 'heat' tests. The maximal mean of the difference between right and left sides was ~0.31 sec ± 0.17 (S.E.M.).

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**Figure 1.** Pilot study showing withdrawal reflex times (sec) for a range of temperatures from 38–54°C. The results of tests on 3 rats are shown (○) with their means (□) at each temperature. It was decided to use 48°C as the test temperature for the main study (see text).
A negative difference score in either ‘heat’ or ‘cold’ indicated hyperaesthesia and a positive result, hypoaesthesia.

Results

Rats with ligations only

Thermal stimulation at 48°C. This was presumed to test the nociceptive response. The earliest hyperalgesic reaction was at 4 days p.o. (2 of 18); by the 7th day, 16 of 18 showed hyperalgesia (Fig. 2A). After the 14th day all rats were hyperalgesic ($P < 0.001$, $n = 18$, Mann-Whitney $U$ test). There was a return to normal behaviour by the 8th week.

Thermal stimulation at 6°C. This was used to test hyperaesthesia or hyperalgesia (increased reaction to noxious stimuli).

The earliest hyperalgesic reaction occurred in 3 of 18 rats 4 days p.o.; by the 7th day 18 of 18 rats showed hyperalgesia compared with normal rats ($P < 0.001$, $n = 18$, Mann-Whitney $U$ test), becoming maximal from 7 to 15 days p.o. There was a gradual return to normal until about the 8th week (Fig. 2B).
Ligations + saphenous nerve section at different times

Concurrent ligations and saphenous nerve section: 48°C. From 4 to 10 days p.o. there was hypoaesthesia in contrast to the hyperaesthesia displayed by the ligation only group ($P < 0.001$, $n = 6$, Mann-Whitney $U$ test) (Fig. 3A). After the 10th day p.o. the behaviour pattern was not significantly different from the ligation-only animals.

Concurrent ligations and saphenous nerve section: 6°C. These animals also showed hypoaesthesia from the 4th to 8th days p.o. ($P < 0.001$, $n = 6$, Mann-Whitney $U$ test) (Fig. 3B). Thereafter the animals became hyperaesthetic, falling into the ligation-only range, although tending to show rather more marked hyperaesthesia.

Saphenous nerve section 1 week after ligations: 48°C. The response was no different from that of the ligation-only group until the saphenous nerve was cut on day 7 after which there was a period of hypoaesthesia from 8 to 12 days p.o ($P = < 0.001$, $n = 6$, Mann-Whit-

![Graph A](image1.png)

![Graph B](image2.png)

Fig. 3. Results comparing the withdrawal reflex times at (A) 48°C and (B) 6°C in ligature + concurrent saphenous nerve section groups (□ mean ± S.E.M.) ($n = 6$) with that of ligatures only groups (● mean ± S.E.M.) (data taken from fig. 2A and B). There is a highly significant (*) hypoaesthetic reaction until the 10th day p.o. (A) and the 12th day p.o. (B). There is a tendency towards greater and longer lasting hyperaesthesia in the saphenous nerve section + ligature group than in the ligation only group. * $P < 0.001$, Mann-Whitney $U$ test; arrow indicates timing of saphenous nerve section.
ney U test) (Fig. 4A). From 14 days p.o. the response was similar to that of the ligature-only group although again tending towards hyperaesthesia.

*Saphenous nerve section 1 week after ligations 6°C.* As in the 48°C group, the response was no different from the ligature-only rats until the 7th day. There was a shorter but equally significant period of hypoesthesia ($P < 0.001$, $n = 6$, Mann-Whitney U test) (Fig. 4B) followed by a return to ligature-only behaviour.

*Saphenous nerve section 2 and 3 weeks after ligations.* These groups showed no significant differences from the ligature-only group at 48°C or 6°C (Figs. 5A, B and 6A, B).

**Alldynia or paresis judged from grip and gait**

All rats showed foot drop (paresis) for the first few days following placing of ligatures. From 5 days p.o. the ligature-only group displayed allodynia (tactile-induced nociceptive behaviour) or spontaneous pain (inferred from the reluctance to put the foot down while walking, and frequent licking) (Fig. 7). There was a gradual return to normal behaviour by the 60th day.

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**Fig. 4.** Results comparing the withdrawal reflex times at (A) 48°C and (B) 6°C in groups with ligature + saphenous nerve section 7 days later ($\square$ mean ± S.E.M.) ($n = 6$) with that of ligature only groups (■ mean ± S.E.M.) (data taken from fig. 2A and B). For the first 7 days the response is not significantly different from the ligature only group. Following saphenous nerve section there is a highly significant (*) hypoalgesic response from the 8th day p.o. until the 2nd week p.o. (A and B). There is a tendency towards greater and longer-lasting hyperaesthesia in the saphenous nerve section + ligature group than in the ligature only group. * $P < 0.001$, Mann-Whitney U test; arrow indicates timing of saphenous nerve section.
p.o. In animals with concurrent saphenous nerve section and ligations, no pain-related behaviour was noted at the 5th day p.o., the animals showing paretic symptoms only. After 10 days p.o. the animals developed an even greater degree of allodynia than the ligature-only group until at least the 30th day p.o. In ligature + 7 days saphenous nerve section, there was a dramatic change to 'paretic' type behaviour on the 8th day with subsequent return to 'allodynic' behaviour by the 12th day. Rats with saphenous nerve section at 14 and 21 days showed no major differences in behaviour to the ligature only animals.

It was usually easy to differentiate between limping due to weakness, and limping because of reluctance to place the foot on the ground due to pain. However, in a few cases this distinction was difficult to make. Even so, after several tests the changes in behaviour could be recognised.

**Sham operation**

In both hot and cold tests, there was some hyperaesthesia ($P < 0.01$, $n = 8$, Mann-Whitney $U$ test) during the first few days p.o. (Fig. 8). Behavioural tests returned to control values by the 6th day p.o.

**Autotomy**

Autotomy, affecting the distal end of the 4th and 5th digits, was recognised in only 1 out of a total of 72
rats. There was no difference in claw length or the condition of the claws between the ligatured and the normal sides.

Discussion

Preliminary morphological studies (to be published) suggest that there is considerable variation in the amount of nerve damage following the application of multiple loose ligatures, due to the difficulty in placing ligatures with a consistent degree of 'tightness' or due to variations in the amount of nerve swelling evoked by the ligatures. This variation no doubt accounts for the wide ranges of values obtained in the behavioural studies. A pilot study (Anand, personal communication) indicates that greater (although still relatively slight) compression of the nerve caused by tighter tying of ligatures may result in complete degeneration of all myelinated and unmyelinated fibres. In the present study ligatures were placed so as not to cause any obvious compression of the nerve, with correspondingly less severe nerve fibre damage.

The results of the behavioural tests we used were generally similar to those reported by Bennett and Xie (1988) and by Attal et al. (1990). Using tests to heat and cold involving total immersion of the foot, we found hyperalgesia and hyperaesthesia, respectively,

Fig. 6. Results comparing the withdrawal reflex times at (A) 48°C and (B) 6°C in groups with ligature + saphenous nerve section 21 days later (□ mean + S.E.M.) with that of ligature only groups (data taken from Fig. 2A and B). There is no difference between the ligature + saphenous nerve section and ligature only groups before the time of saphenous nerve section. The hyperalgesic response tends to be greater and longer in the saphenous nerve section + ligature group than the ligature only group. Arrow indicates timing of saphenous nerve section.
Fig. 7. Abnormal (touch-related) behaviour graded as a rank index (see text) in rats with ligature only (□) (n = 18), ligature with saphenous nerve section at the same time as ligature (○) and ligature + saphenous nerve section 7 days later (●). All rats show paresis (+2.5 - 3) for the first few days. In ligature only rats there is a sudden change to allodynia or spontaneous pain (see text) from the 5th day p.o., with a gradual return to normal by the 60th day (not shown on graph). In rats with concurrent ligature and saphenous nerve section, allodynic behaviour does not occur until later and is even more severe. In rats with ligature and saphenous nerve section at 7 days, the behaviour changes abruptly after saphenous nerve section but by 12 days has returned to an allodynic pattern similar to the ligature only group.

from the 4th day p.o. Before this, there was marked hypoaesthesia to all tests. The findings of Attal et al. (1990) were similar to ours, although Bennett and Xie (1988) described hyperalgesia from day 2 following application of ligatures.

It has long been known that collateral sprouting of
the saphenous nerve following damage to the sciatic nerve contributes to the early return of sensation to the rat foot beginning on the 4th day (Devor et al. 1979). Secondary hyperalgesia following early nerve damage was also described by Lewis (1935). We studied the possible contribution of saphenous innervation in the ligature model. When the saphenous nerve was sectioned at the time of placing ligatures, the tests showed hypoaesthesia, significantly different from the ligature only groups, lasting from the 4th to 10th day. This suggests that the early hyperaesthesia and hyperalgesia caused by the loose ligatures might be due to early collateral sprouting of the saphenous nerve or to secondary hyperalgesia from nociceptor sensitization (Fitzgerald 1979) of the nerve. Section of the saphenous nerve 7 days after applying ligatures also changed the hyperalgesic response to hypoaesthesia from about the 8th to 12th day. This reinforces the hypothesis that the saphenous nerve plays a role in early hyperaesthesia and hyperalgesia. There was no modification of hyperaesthesia when the saphenous nerve was sectioned on the 14th or 21st days after tying ligatures. These findings suggest that hyperaesthesia and hyperalgesia due mainly, but not exclusively, to sciatic nerve degeneration caused by the multiple ligatures. Tests of grip and observation of gait revealed signs of paresis during the first few days. Morphological studies (Gautron et al. 1990; Munger and Bennett 1990; Basbaum et al. 1991; Carlton et al. 1991) and our unpublished studies show considerable nerve damage particularly affecting the larger fibres. Therefore it would be expected that motor function will be compromised. It is important that behavioural change is not attributed to sensory-related effects only, and the occasional difficulty in interpreting limping illustrates the problem of distinguishing sensory from motor deficits.

Allodynia and/or hyperaesthesia which in the ligature-only rats was maximal from about the 5th to 12th day p.o. then began to lessen but persisted for 8 weeks. It is possible that mechanisms in the spinal cord involving long-lasting changes due to NMDA receptor activation may be responsible (Seltzer et al. 1991; Dubner and Ruda 1992). After concurrent ligature and saphenous nerve section, the animals showed only paresis from about the 5th day, lasting for several days, but thereafter signs of allodynia and hyperaesthesia were even more marked than those of the ligature-only rats. The role of collateral sprouting in allodynia or hyperalgesia is not known. Wall et al. (1979) have suggested that in studies of nerve section, intact neighbouring nerves (with the possible implication of collateral sprouting or spreading nociceptor sensitization) could have a protective role in reducing autotomy.

In studies of saphenous nerve collateral innervation Brennan (1986) found that the increased field extended mainly to the dorsal surface of the foot. However, Markus et al. (1984) and Kingery and Vallin (1989) using different methods showed substantial plantar collateralization. It seems that the increased plantar field may be variable in extent. Our method involving total immersion of the foot, with the possibility of greater spatial summation, differs from the more focal method used by Bennett and Xie (1988). This may possibly explain why Bennett and Xie (1988) obtained different results from ours.

It is of interest that in a study of chronic sciatic nerve injury (without regeneration) (Markus et al. 1984) there was hyperalgesia, often with vocalisation, indicating a possible nociceptive response in the dorso-medial foot. This was only demonstrated 21 days p.o., but had probably occurred earlier. This region is normally innervated mainly by the saphenous nerve. In a similar model, histological studies showed an increase in the number of nerve endings in the sensitive region, con-

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Fig. 9. Diagram showing the hypothetical temporal relationship between pain-related behaviour (thermal and cold response) caused firstly (A) by the contribution from the saphenous nerve, lasting from the 4th to 14th day and possibly later, and (B) from about the 10-12th day to the 8th week, that due mainly to the sciatic nerve.
firming cutaneous sprouting (Weddell et al. 1941). Kingery and Vallin (1989) found that the saphenous nerve territory (medial toes) showed marked hyperalgesia within 1 week after sciatic nerve section (without regeneration) but the collateralization territory (middle toe) did not become hyperalgesic until 3 weeks after nerve section. The early hyperalgesic response in our study would be comparable to the early effects (within 1 week) shown by Kingery and Vallin (1989) and Vallin and Kingery (1991) which in both studies may be due to nociceptive sensitization, although collateral sprouting still remains a possible explanation.

Sectioning of the saphenous nerve 3 months after sciatic nerve ligation had no effect (Attal et al. 1990). In our study we found no effect of saphenous nerve section 2 weeks after tying ligatures.

Unlike Bennett and Xie and Attal et al. we found a transient behavioural response in the sham-operated rats. Morphologically, there was evidence of damage to a very few myelinated fibres in some rats (unpublished). Although the nerves have not been examined for unmyelinated fibre damage, their involvement is likely to have been negligible. We conclude that cutaneous nerve damage arising from the sham operation procedure, involving as it does a relatively long skin incision, may account for the transient behavioural response (Wall and Woolf 1984).

In the present study, the rate of autotomy was very low, with only 1 rat out of 72 showing a minor degree of damage to the distal parts of 2 digits. There were no claw changes. Two other studies of this same model describe widely differing incidences of autotomy. Bennett and Xie (1988) found that 70% of rats showed self-mutilation although this was generally limited to gnawing of claw tips. Only 5 rats were affected out of 133 in the study of Attal et al. (1990). It is likely that the variation is due to differences in the tightness of the ligatures and, therefore, to the degree of fibre damage. In the pilot study referred to above, using tighter ligatures (Anand, personal communication), a much higher incidence of autotomy was found in addition to claw changes which were also reported by Bennett and Xie (1988). These included thickening and curving elongation of the claw. In their study on peripheral nerve lesions in rats and mice Wall et al. (1979) found that the incidence of autotomy varied with the type of lesion.

Our conclusions from this study can be summarised in the accompanying diagram (Fig. 9).

This study has shown that the saphenous nerve plays a role in the behavioural changes following sciatic nerve damage caused by loose ligatures. Whether this is due to saphenous nerve collateralization has not been proven, but this remains a possibility. An alternative hypothesis would be spreading nociceptive sensitization (Fitzgerald 1979) due to adjacent nerve fibre injury. The sciatic nerve damage may induce a central excitatory state, e.g., by NMDA-mediated dorsal horn hyperalgesia (Dubner and Ruda 1992) which is further excited by saphenous afferents.

Acknowledgements

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References


Fitzgerald, M., The spread of sensitization of polymodal nociceptors in the rabbit from nearby injury and by antinociceptive nerve stimulation, J. Physiol., 297 (1979) 207-216.


Munger, B.L. and Bennett, G.J., The peripheral axonal pathology in the constrictive model of peripheral neuropathy, Anat. Rec., 226 (1990) 70A.


and phagocytosed bacteria were present in the cervical lymph nodes 3 h after injection and from 48 h onwards plasma cell pleocytosis was observed in the medulla of the cervical lymph nodes reflecting the immunological responses in cervical lymph nodes. However, the routes by which inflammatory cells leave the subarachnoid space remain unclear.

Blakemore W. F., Crang A. J.* & Ryder S.* University of Cambridge
Repopulation of areas of demyelination in the rat by mouse cells. A new model of immune mediated demyelination
Transplants containing isogeneic astrocytes and mouse O-2A enriched glial cells reconstitute the glial environment of spinal cord ethidium bromide lesions in immuno-suppressed rats. Glial cells prepared from male mice were identified using a probe to repeat sequences on the mouse Y-chromosome. These mouse glial cells are rejected when immunosuppression is removed. This leads to a second-wave of demyelination followed by prompt host-mediated remyelination. The host response can be abolished by local \( x \)-irradiation. This inhibition of remyelination leaves demyelinated axons lying in an astrocytic environment. Thus by mixed-species glial cell transplantation, not only has it been possible to create a new model of immune-mediated demyelination, but by suppressing remyelination of such lesions, it also has been possible to create a facsimile of a multiple sclerosis plaque.

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A morphological study of chronic ligature-induced neuropathic pain in rats
Bennett and Xie (Pain 33, 87, 1988) have described a new model for producing pain-related behaviour (PRB) by tying four loose ligatures round the rat sciatic nerve: the method is now being widely used. This mild traumatic lesion may produce marked nerve fibre degeneration, but because of difficulties in tying ligatures with a consistent degree of 'looseness' there is great variation in the effects produced. This paper describes quantitative morphological changes in nerves distal to the ligatures. From 30.3–86.4% of myelinated fibres and 0–35.2% of unmyelinated fibres degenerate acutely. There is relatively greater loss of large fibres. The presence of atrophic myelinated fibres, and of acutely degenerating fibres up to at least 2 months after producing the lesion indicates a continuing process of degeneration which includes both large and small fibres. This may in some way contribute to the long-lasting 'neuropathic pain' behaviours.

Oligodendrocyte reactions and cell proliferation in human demyelinating diseases
There is increasing evidence of a capacity of oligodendrocytes to react to demyelination in experimental animals. In this study we have used antibodies to two enzymes involved in myelination—carbonic anhydrase isoenzyme II and 2',3-cyclic nucleotide 3'-phosphodiesterase—to study oligodendroglial reactions in archival material from cases of diverse demyelinating diseases. In this material we have found evidence of oligodendrocyte hyperplasia and increased expression of these two enzymes in oligodendrocytes. In the same material we have found, using an antibody to proliferating cell nuclear antigen, cells that have entered the cell proliferation cycle. Such cells were not found in normal adult central nervous system, and their presence clearly demonstrates that proliferative glial reactions are induced in response to demyelinating disease.

Expression of MHC class II molecules in the degenerating optic nerve of the rat
The expression of MHC class II molecules in central nervous tissue under pathological conditions has been studied extensively in recent years, though the underlying mechanism is not altogether clear. The present study was undertaken to study the issue of whether degenerating myelin product is essential in triggering the expression of MHC II in the optic pathway in the rat using immunocytochemical methods (Smetanka et al. Brain Res. 521, 343, 1990). After unilateral transection of the optic tract, no expression of MHC II was observed in the contralateral retina at any time up to the fourth week. In contrast, expression of MHC II was observed from the lesion site to the proximal end of the optic nerve by 16 days. As intraretinal optic axons are unmyelinated while the large majority of axons in the optic nerve are myelinated, our