EFFECT OF ISCHAEMIA AND REPERFUSION ON DISCHARGE PATTERNS OF NOCICEPTIVE AFFERENT NERVE FIBRES IN THE RAT TAIL

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ABSTRACT

In rats anaesthetised with enflurane, I examined the response of coccygeal primary afferents fibres to noxious thermal and mechanical stimulation and to innocuous brushing, during transient ischaemia and reperfusion of their receptive fields on the tail. Ischaemia was induced by occluding the blood supply to the tail for 30 min using a tourniquet. I discovered four different groups of afferent fibres, distinguished by conduction velocity and modality. Aβ fibres responding to both brush and pinch of their receptive fields showed decreased sensitivity to brush during both ischaemia and reperfusion; Aδ fibres responding to pinch were unaffected by either ischaemia or reperfusion. C fibres responding to noxious heat (49°C) and pinch showed hypersensitivity during reperfusion, especially immediately after release of the tourniquet. Another group of C fibres, presumably chemosensitive, became more active during ischaemia and exhibited a 7-fold increase in firing rate during receptive field reperfusion in the absence of obvious stimuli.

These results indicate that during reperfusion of the rat tail following transient ischaemia, myelinated fibres do not increase their input to the CNS, while C fibres became more active and showed sensitization to noxious stimulation of their receptive fields. The enhanced CNS nociceptive activity which occurs during reperfusion consequently results from both peripheral and central sensitization.

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DECLARATION

I declare that this dissertation is my own work, this dissertation is being submitted for the degree of Master of Science in the Faculty of Science at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination in any other university.

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6 day of March, 1996

I certify that the studies contained in this dissertation have the approval of the Animal Ethics Committee of the Witwatersrand (AEC Number 93/36/2b)

Johannesburg Date 6 March 1996

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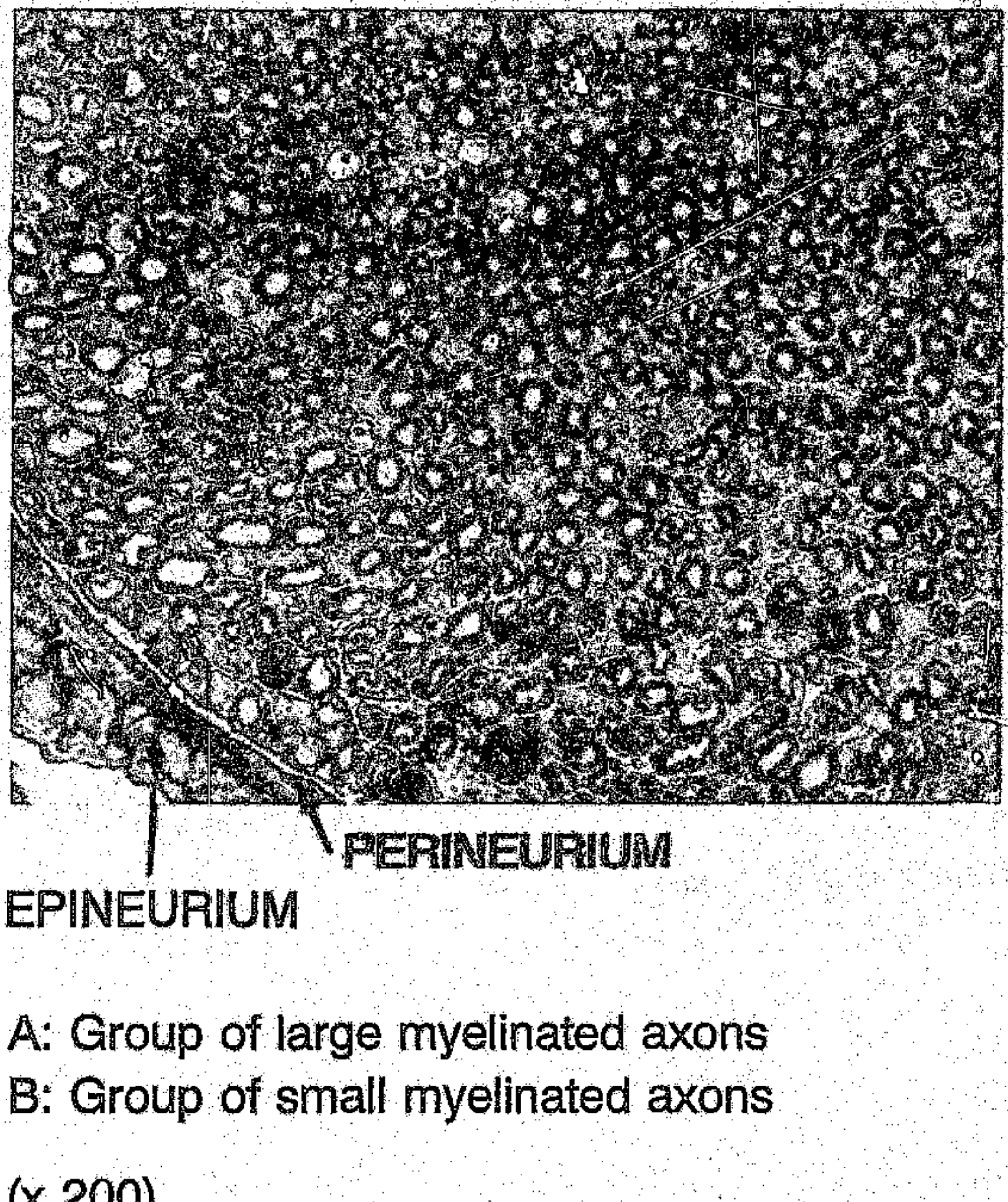
Schematic representation of the model used in this research.



A: Group of large myelinated axons
B: Group of small myelinated axons

(x 200)

PLATE I

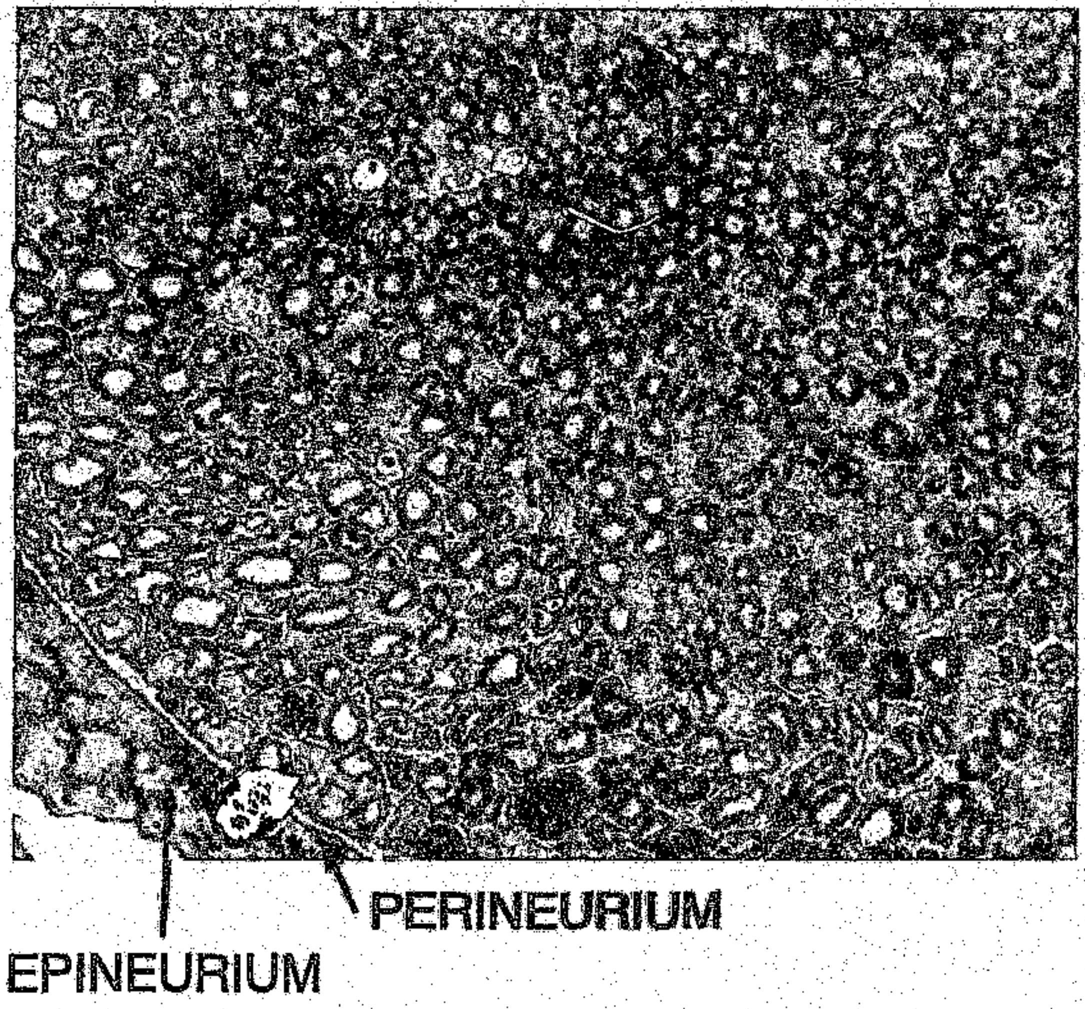


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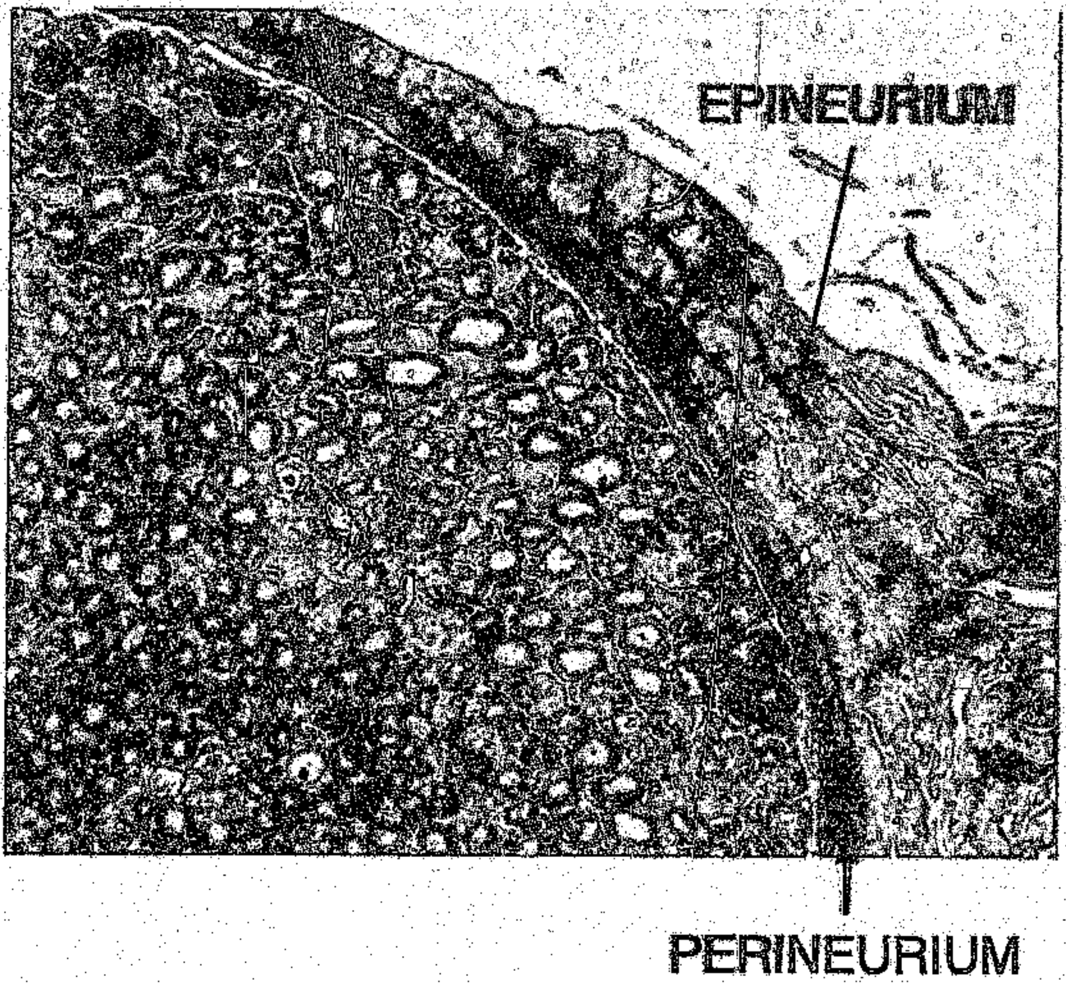
PLATE I



A: Group of large myelinated axons
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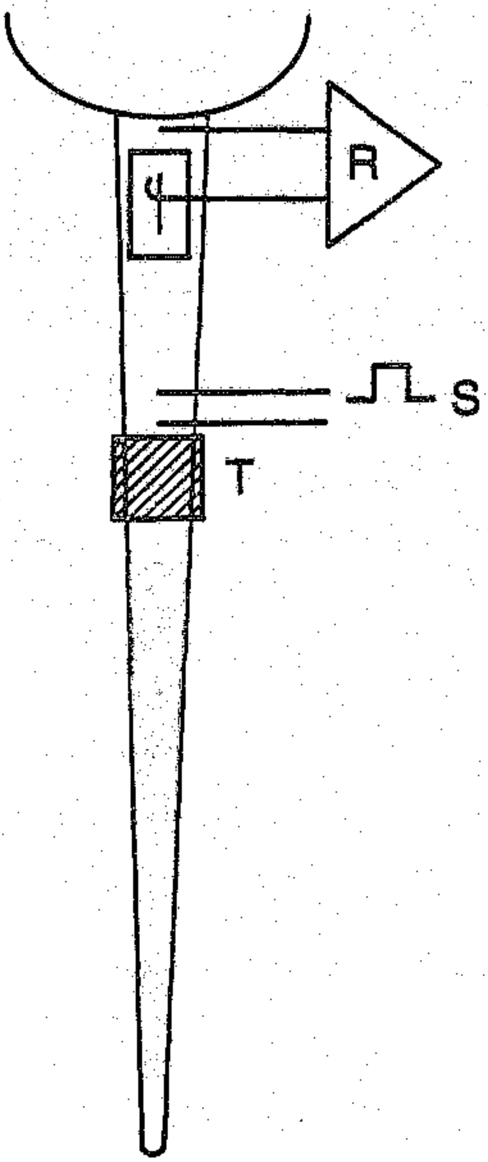

Plate III

Schematic representation of recording, electrical stimulation and application of mechanical and thermal stimulation, T Tourniquet.

The yellow area represents the part of the tail where I looked for receptive fields and applied mechanical and thermal stimulation.

CHAPTER 1

INTRODUCTION: Afferent pathways in nociception

1.1 Somatosensory afferent fibres

Mammalian skin is innervated by terminals of primary nociceptive afferents that belong to thinly myelinated Aδ fibre and unmyelinated C fibre families. These nociceptors have been studied in several species including humans (for review see Raja *et al.* 1988, Campbell *et al.* 1989). The most commonly studied C-fibre nociceptor responds to mechanical as well as heat stimuli. This receptor type is therefore called the C-fibre mechano heat nociceptor (CMH). Responses to cold stimuli or chemical stimuli may also be evident, in which case the term polymodal nociceptor is appropriate. Some C-nociceptors may respond preferentially to mechanical stimuli (Bessou and Perl 1969, Beck *et al.* 1974, Georgopoulos 1976, Kumazawa and Perl 1977), heat stimuli (Beck *et al.* 1974, Georgopoulos, 1976) cold stimuli (LaMotte and Thalhammer 1982, Leem *et al.* 1993), or chemical stimuli (Davis *et al.* 1993).

The size of a CMH receptive field for mechanical stimuli is usually small (1-2 mm diameter) in cat, rabbit and rat (Beck *et al.* 1974, Lynn 1979, Handwerker *et al.* 1987). In contrast CMHs in hairy skin in humans and monkeys often have large (3-10 mm diameter) and complex receptive fields, containing multiple areas of punctate sensitivity to mechanical stimuli (Torebjörk 1974, Croze *et al.* 1976, Kumazawa and Perl 1977).

The majority of A-fibre nociceptors have been classified as high-threshold mechanoreceptors (HTMs). They are not readily activated by heat stimuli, but respond with high threshold to mechanical stimuli. They are also referred to as A-fibre mechano-heat nociceptors, type 1 AMH (Treede *et al.* 1995). These nociceptors have complex receptive fields with multiple sensitive spots (Fitzgerald and Lynn 1977) and exist in both hairy and glabrous skin. When the human hairy skin is briefly touched with a hot object or when skin temperature is rapidly increased with a laser stimulator, a pain sensation is elicited with a short latency of 400 msec (Campbell and LaMotte 1983). A second type of Aδ-nociceptor (type 2 AMH) has been identified in humans and monkeys. Type 2 AMHs respond to the onset of a heat stimulus within a few milliseconds (Adriansen *et al.* 1983, Treede *et al.* 1995). The heat response properties of type 2 AMHs are similar to those of CMHs and they seem to exist only in hairy skin (Treede *et al.* 1995).

Some nociceptors do not respond to mechanical stimuli. Mechanically insensitive afferents (MIAs) have been reported to exist in cats (Schaible and Schmidt 1985, Häbler *et al.* 1990) and monkeys (Meyer *et al.* 1991). MIAs represent a large proportion of the Aδ-fibres (48%) and C-fibre (30%) population and may well contain novel classes of nociceptors. Some of these mechanically insensitive afferents exhibit pronounced chemosensitivity and can be sensitized to mechanical stimuli after injection of inflammatory mediators in their receptive fields (Häbler *et al.* 1990, Meyer *et al.* 1991, Davis *et al.* 1993).

Apart from nociceptors, there are at least six other kinds of afferent in the rat cutaneous nerves. These are: air rapidly adapting (RA) which respond to air puffs applied parallel to the skin, Pacinian corpuscle (PC), slowly adapting type I (SA-I) and type II (SA-II) mechanoreceptor, C-mechanoreceptor and cold receptor (Leem *et al.* 1993). Table 1 shows a summary of responses of rat's cutaneous afferents to different stimulus modalities.

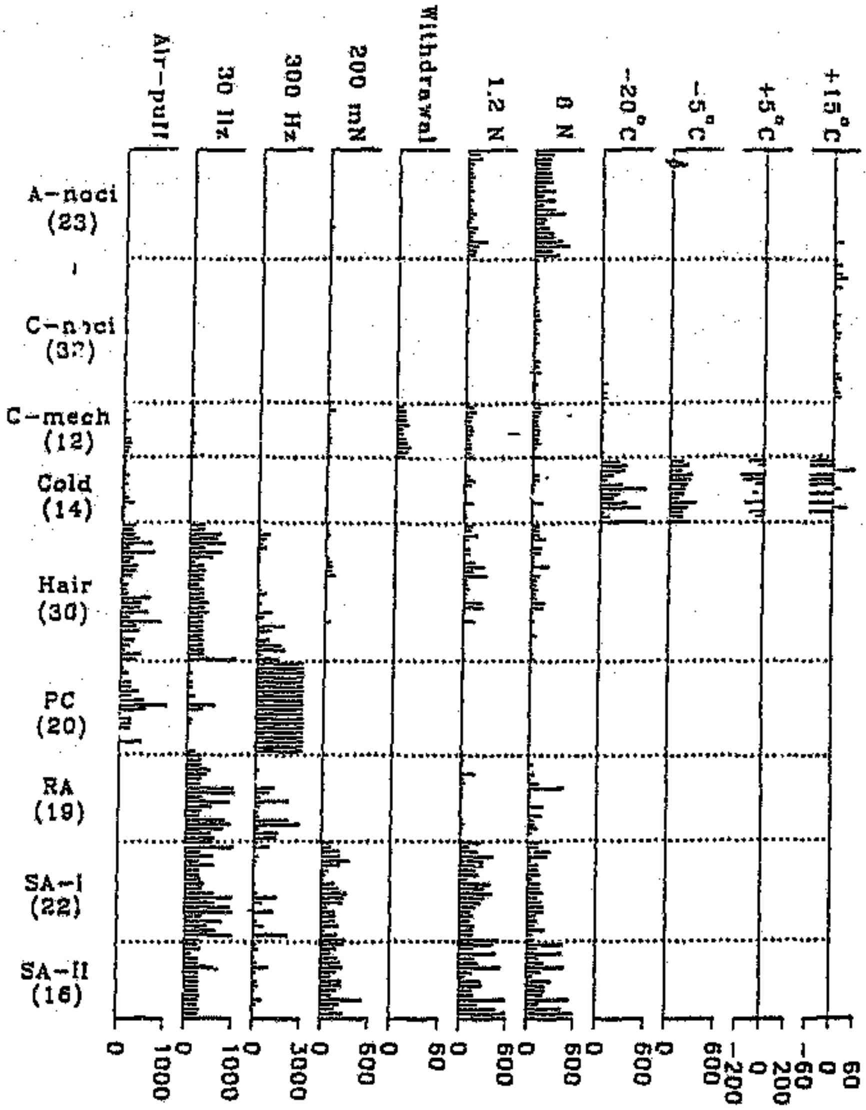

Table 1. No. of impulses/stimulation.

Responses of afferent units of 9 different types described by the 11 stimuli variables. The responses of each unit were measured as the number of impulses evoked by 10 stimuli for 10 s. Additionally, the number of impulses occuring for 5 s after withdrawal of 200-mN skin indentation stimulus (1 of 10 stimuli) was counted (shown as Withdrawal in the *top panel*). Responses were obtained from 188 individual afferent units identified as belonging to receptor types (shown along the ordinate) and represented by horizontal histograms along the abscissa. The response pattern described by any 1 variable across the 11 variables is read horizontally. The response pattern described by any 1 variable across afferent units is read vertically. Numbers in parentheses: number of afferent units sampled for each receptor category. The "supressive" responses of cold units to heat pulse of 47°C (+15°C) were truncated. (From Leem *et al.* J. Neurophysiol. 6, 1993, pg 2417. For abbreviations see page 4.

1.2 Nociceptive neurones in the spinal cord

In the central nervous system, two classes of neurone have been described that may be involved in signalling pain sensation. Nociceptive specific (NS) neurones (sometime referred to as class 3 neurones) have an elevated threshold for cutaneous stimuli such that activity in peripheral nociceptors appears to be necessary to activate them. NS neurones were first found in superficial layers of the dorsal horn (lamina I, Christenser [illegible] Perl 1970), but they also exist in deep layers (lamina V). Another class of neurone also encodes the intensity of noxious stimuli, and may in fact account better for the sensory discriminative aspect of pain (Dubner *et al.* 1989). These neurones are called wide dynamic range (WDR) neurones, multireceptive neurones, or class 2 neurones (Wall 1960). They typically can be excited by mechanical and heat stimuli, and convergence of A-fibre and C-fibre input onto these neurones can be demonstrated (Woolf and King 1987).

WDR neurones are located in lamina V of the dorsal horn, but they exist in lamina I and II, as well. Both NS and WDR neurones in the spinal cord are found among interneurones and spinothalamic projection neurons. The receptive fields of WDR neurones are usually larger than those of primary afferents, due to convergence. A further characteristic of these neurones is their inhibition by Aβ-fibre input from the periphery of their receptive field (Wall 1978).

The electrophysiological effects of C-afferent fibre input on the spinal cord can be studied either by applying natural stimuli to the periphery that exclusively or predominantly activate C-nociceptors (e.g. noxious heat or chemical irritants) or by graded electrical stimulation. Using these approaches, many laboratories have shown with extracellular recording in cats (Gregor and Zimmermann 1972), primates (Chung *et al.* 1979) or rats (Schouenberg and Sjolund 1983) that C fibre inputs excite dorsal horn neurones, evoking, in the case of electrical stimulation, a long-latency, relatively long-lasting burst of spikes followed frequently by an afterdischarge. Mendell and Wall (1965) showed in the cat that repeated single stimuli to a peripheral nerve at C-fibre strength at a frequency > 0.2 Hz resulted in a progressive increase in the number of action potentials evoked in lamina V neurones. The term "windup" was coined to describe this phenomenon and has since been demonstrated also in primates (Wagman and Price 1969) and in the rat (Woolf 1983) dorsal horn neurones. In addition to resulting in an increase in the C-evoked discharge, repeated C-strength stimulation is also associated with an increase in spontaneous activity, afterdischarge (Mendell and Wall 1965) and the response to myelinated afferent input (Schouenberg and Sjolund 1983). Over relatively prolonged poststimulation periods there is also an expansion of the cutaneous receptive field of dorsal horn neurones (Cook *et al.* 1987) and a prolonged facilitation of the flexion withdrawal reflex (Woolf and Wall 1983).

No equivalent changes in dorsal horn neurones have been found by repeated A-fibre inputs. Temporal changes in the response properties of spinal neurones also occur following the activation of C fibres by natural stimuli that cannot be accounted for by changes in the responses of the primary afferents (Beitel and Dubner 1976, Kenshalo *et al.* 1979). C fibre inputs, therefore, produce effects in dorsal horn neurones that outlast the period of stimulation for prolonged periods.

1.3 Hyperalgesia and hypersensitivity

Sensitization is a neurophysiological phenomenon that corresponds to the psychophysical phenomenon of hyperalgesia. Sensitization is characterized by a lowering in threshold, an increase in the suprathreshold response, and spontaneous activity. Hyperalgesia has been therefore defined as an altered state of sensitivity characterized by a decrease in pain threshold and an increase in pain induced by suprathreshold stimuli.

The related term *allodynia* is used by some authors (e.g Simone *et al.* 1989, LaMotte *et al.* 1991) to describe states in which usually nonpainful stimuli, such as light touch, induce pain (Torebjörk *et al.* 1992).

Sensitization of primary afferent nociceptors appears to account for some characteristics of cutaneous hyperalgesia. An increased responsiveness in primary afferents results in increased responses in central neurones as well, without the requirement that central nociceptive neurones develop enhanced sensitivity. There are, however, additional mechanisms that can lead to increased responses in central neurones without primary afferent sensitization. One possibility is increased spatial summation: if the receptive field size of primary afferents increases, any given stimulus will recruit more afferents. True *central sensitization* implies that the same peripheral input leads to an increased response. This could mean an increased responsiveness to nociceptor input or, especially in the case of WDR neurones, increased responsiveness to non-nociceptive afferents. Alternatively, the inhibitory input from mechanoreceptors may be reduced by injury, resulting in increased synaptic efficacy of nociceptive input. It is important to note that a drop in pain threshold below the activation threshold of primary nociceptive afferents can be explained only by nociceptor sensitization or by increased synaptic efficacy from non-nociceptive mechanoreceptors. Small decreases in pain threshold and increases in suprathreshold pain can be due to central summation of nociceptor input.

1.3.1 Primary hyperalgesia

Lewis (1935) introduced the distinction between the "primary" hyperalgesia occurring at the site of an injury and the "secondary" hyperalgesia in the surroundings of the injury.

The capacity to sensitize as a consequence of persisting or repeated noxious events is a prominent feature of most primary nociceptive afferents. When an experimental injury has been induced by prolonged noxious heating of a skin site (LaMotte *et al*, 1984) or by algogenic chemicals (Handwerker *et al*. 1991), hyperalgesia is observed to both heat and mechanical stimuli (Raja *et al*. 1984). The sensitization of nociceptors to heat stimuli has been studied most extensively (Kumazawa and Perl 1977, Beck *et al*. 1974, Lynn 1979, Thalhammer and LaMotte 1982, Fleischer *et al*. 1983) and is thought to be the peripheral neural substrate of primary hyperalgesia to heat.

The relative role of myelinated and unmyelinated nociceptors in primary heat hyperalgesia depends on skin type. In hairy skin, both AHMs and CMHs can be sensitized to heat stimuli. Psychophysical studies showed no changes in the time course of heat hyperalgesia in hairy skin under preferential A-fibre blockade, indicating a dominant role for CMHs (LaMotte *et al*. 1982). In glabrous skin, AMHs sensitize to heat stimuli after a thermal injury (Campbell *et al*. 1979). Many A-fibre nociceptors in glabrous skin are not initially responsive to mild heat stimuli, but develop pronounced heat sensitivity after injury. This recruitment phenomenon is a form of spatial summation. In contrast, the CMHs in glabrous skin show an increased threshold and a decreased response to suprathreshold stimuli following the same type of injury (Meyer and Campbell 1981). These data suggest that AMHs, not CMHs, code for thermal hyperalgesia in the area of primary hyperalgesia in glabrous skin. This suggestion was supported by preferential nerve blocks in humans, which showed that heat hyperalgesia in glabrous skin can be suppressed by an A-fibre block (Meyer and Campbell 1981). In addition, either more distant, or normally unresponsive ("sleeping"), units may be recruited in inflammation and contribute to spatial summation at central synapses. Indeed, it has been found that the receptive fields of some nociceptors become larger and spread into the area of injury (Thalhammer and LaMotte 1982). Furthermore, in rat (Handwerker *et al.* 1991) and monkey skin (Meyer *et al.* 1991), afferent C- and Aδ-units have been demonstrated that are unresponsive under physiological conditions but possibly are recruited in inflammation.

In contrast to changes in heat responsiveness, several authors found it difficult to demonstrate mechanical sensitization after a heat injury either in CMHs (Thalhammer and LaMotte 1982) or AMHs (Campbell *et al.* 1979). Significantly, injuries such as a burn, that clearly caused mechanical hyperalgesia, have failed to yield evidence for nociceptor sensitization to mechanical stimuli. Thus hyperalgesia to mechanical stimuli can not be readily explained by primary afferent nociceptor sensitization. Likewise, after intracutaneous injections of carrageenan or topical application of mustard oil, CMHs in the rat were sensitized to heat, but not to mechanical stimulation (Kocher *et al.* 1987, Reeh *et al.* 1986).

The inability of heat or noxious chemical stimulation to sensitize nociceptors to mechanical stimuli is remarkable since sensitization of deep nociceptors to mechanical stimuli in inflammation has been observed in several animal models; for example, in acute experimental arthritis in the cat, many C- and A-units normally responding only to extreme movements become activated by slight movements (Grigg *et al.* 1986, Schaible *et al.* 1987). The development of mechanical sensitivity in afferents that are initially insensitive to mechanical stimuli may contribute to mechanical hyperalgesia.

Expansion of the receptive field of nociceptors into an adjacent area of injury is an alternate peripheral mechanism that may account for primary hyperalgesia. A stimulus within the area of injury will activate more nociceptors than before injury and lead to an increased sensory response from the injured site (LaMotte *et al* 1991).

Finally, there is some evidence for suppression of Aβ-mechanoreceptors by injury (Beck *et al.* 1974). A decreased response of low threshold mechanoreceptors could lead to a reduction in inhibitory input onto dorsal horn neurones. A similar disinhibition has been reported for cold pain sensation during a block of Aδ fibre cold receptors (Yarnitsky and Ochoa 1990).

1.3.2 Secondary Hyperalgesia

Peripheral and central neuronal mechanisms have been claimed to be the underlying causes of secondary hyperalgesia. Lewis (1935) assumed an axon reflex mechanism by which impulses from stimulated nociceptive nerve endings spread antidromically to other branches of the same primary afferent unit, resulting in the release of sensitizing substances. This axon reflex mechanism has also been thought to account for the flare which surrounds an injury. The idea of hyperalgesia mediated by an axon reflex is supported by the finding that hyperalgesia induced by intradermal application of capsaicin does not spread beyond a narrow strip of skin made insensitive by local anaesthetic (LaMotte *et al.* 1991). However, the direct evidence for a peripheral mechanism of secondary hyperalgesia is not convincing. Sensitization of nociceptors by a nearby injury and by antidromic nociceptor stimulation has been found in one study in the rabbit (Fitzgerald 1979), but this finding could not be replicated in the monkey (Campbell *et al.* 1988, Meyer *et al.* 1988) or the rat (Reeh *et al.* 1986).

Some spreading of the mechanical hyperalgesia from the edge of a trauma may be explained by an extension of receptive fields observed in some HTM Aδ-units (Reeh *et al.* 1987). This does not explain, however, the pure mechanical hyperalgesia observed in an extended area of the skin after heat injury. In this case, the area of secondary hyperalgesia is apparently larger than the flare area, and thus an axon reflex mechanism is unlikely to be the reason (Raja *et al.* 1984).

Because this kind of secondary hyperalgesia involves only mechanical and not heat stimulation as does the primary form (Raja *et al.* 1984), the mechanisms of both forms are likely to be different.

Central mechanisms of secondary hyperalgesia have been postulated four decades ago (Hardy *et al.* 1952). In recent studies, a distinction between mechanical hyperalgesia (to pinprick) and allodynia (to gentle touch) has been made. Because the latter is prominent in the surrounding of the trauma and because no clear proof for nociceptor sensitization to mechanical stimuli was encountered, mechanoreceptor input probably is the source of allodynia (Torebjörk *et al.* 1992). In addition, microstimulation of identified afferent nerve fibres has been used to study the mechanism of secondary hyperalgesia; in unimpaired skin, stimulation of Aβ-units induced tapping or touch sensations. When capsaicin was injected into an adjacent skin area and secondary hyperalgesia developed spreading into the receptive field, a quality of pain was added to the touch sensation induced by the microstimulation (Torebjörk *et al.* 1992). Because microstimulation bypasses the sensory nerve endings, these findings also point to a change in central nervous processing of mechanoreceptor input as a source of the secondary hyperalgesia.

1.4 Ischaemia and reperfusion injury

An ischaemic event is characterized by a lack of oxygen and a conversion of cellular metabolism to anaerobic pathways. Oxygen debt and cellular energy depletion lead to various biochemical alterations including an atypical buildup of cytoplasmic metabolites and malfunction of membrane transport systems. An important result of this latter change is a marked increase in intracellular calcium concentration. This increase is further augmented during the subsequent reperfusion phase as incompetent cellular membranes are exposed to a replenished intravascular supply of calcium ions. Acting as a "second messenger", calcium triggers activation of various enzymes crucial to the production of proinflammatory mediators. These changes are injurious at the cellular level in a manner directly proportional to the duration of ischaemia (Kerrigan and Stotland 1993). Serotonin and bradykinin are also released during ischaemia, respectively by the platelets and plasma kininogen (Sicuteri *et al.* 1974).

The paradox of reperfusion injury is that the reestablishment of normal vascular supply can incite continued, and often intensified, tissue injury. Reactive oxygen intermediates formed within reperfusing post-ischaemic tissue lead to a variety of microvascular alterations including endothelial cell swelling and increased capillary permeability (Kerrigan and Stotland 1993). In addition to direct oxygen radical effects on the endothelial cell, a reperfusion phase increase in intracellular Ca^{2+} concentration results in non-specific protease and phospholipase activation. These enzymes trigger a multitude of cellular processes, many of which lead to proinflammatory mediator synthesis. Early response factors include platelet activating factor (PAF) and a variety of *eicosanoid* compounds such as, leukotriene, thromboxane, and prostaglandin species (Kerrigan and Stotland 1993).

Nociceptors can be sensitized by many substances released during ischaemia and reperfusion or during tissue injury. These substances include arachidonic acid derivatives, substance P, bradykinin, serotonin, noradrenaline, histamine, and Ca^{2+}, all of which are capable of either sensitizing nociceptors or producing pain themselves (Beck and Handwerker 1974, Levine *et al.* 1984, Cohen and Perl 1990, Häbler *et al.* 1990, Kumazawa *et al.* 1991, Kessler *et al.* 1992, Dray *et al.* 1992, Davis *et al.* 1993).

A possible basis for sensitization by serotonin, as well as bradikinin and prostanoids, is a reduction of the slow, inhibitory afterpotential that follows the action potential in some sensory neurones. The inhibition is due to cAMP generation and a reduction of the inhibitory K^+ current. The overall effect is to increase the likelihood that the neurone will respond to a relatively weak stimulus with a train of action potentials rather than with a single spike (Dray *et al.* 1994). Histamine may increase membrane Ca^{2+} permeability of the neurones through the activation of H_1 receptors (Tani *et al.* 1990).

1.5 Nociception in rats during tail ischaemia and reperfusion

The rat tail as an animal preparation has been used by many authors (Necker and Hellon 1978, Fleischer *et al.* 1983, Handwerker *et al.* 1987, Reeh, *et al.* 1987, Cervero *et al.* 1988, Dray *et al.* 1992) since noxious stimulation applied to the tail of anaesthetized rats evokes responses in cutaneous primary afferents that adapt like the responses from human afferent fibres (Handwerker *et al.* 1987). Gelgor and coworkers (1986) developed an ischaemic pain model applying an inflatable tourniquet to the rat's tail.

Occluding the blood supply to the tail of a conscious rat, using a pneumatic tourniquet, induces escape behaviour which is characterised initially by a visible increase in respiratory rate and movement of the head from side to side, followed by either vigorous grooming or by attempts to turn round in the restrainer, or by jumping forward. The mean response latency to tail ischaemia in rats is 12.5 ± 0.2 mins (Gelgor *et al.* 1986.).

Ischaemia also caused significant hyperalgesia to subsequent noxious thermal stimulation. Tail flick latencies during tail immersion in water at 49°C are shorter during reperfusion and this thermal hyperalgesia is greatest immediately after release of the tourniquet (Gelgor *et al.* 1986).

The ventrobasal thalamus of the rat contains somatotopically organized neurones which respond to noxious thermal stimulation of the tail (Mitchell and Hellon 1977). During reperfusion the threshold tail temperature needed to elicit a neuronal response to thermal stimulation is decreased. Most of these thalamic neurones, responding to noxious thermal stimulation of the tail, also increased firing rate during ischaemia. Threshold temperatures for neuronal response and behavioural response are not different (Gelgor *et al.* 1988).

The response of dorsal horn neurones to tail ischaemia and reperfusion was recently investigated by Gelgor and Mitchell (1993). Occluding the blood supply to the rat tail, in the absence of other stimuli to the tail, produced a three-fold increase in spontaneous activity of dorsal horn neurones. Following removal of the tourniquet, the spontaneous firing rate of convergent neurones decreased, but did not return to the pre-ischaemic value within 60 min of recording (Gelgor and Mitchell 1993). Immediately following release of the tourniquet, the mean response to mechanical stimulation did not differ significantly from the response before the tourniquet was applied. However, after 30 and 60 min of reperfusion, there was a significant increase in WDR neurones response to both innocuous and noxious mechanical stimulation of their receptive fields in the tail. A minority (37%) of these convergent neurones responded to thermal stimulation of their receptive fields and only two of these exhibited an increased responsiveness to noxious thermal stimulation during reperfusion of the tail. Receptive field size, during reperfusion, also increased in 80% of the cells studied. The authors concluded that dorsal horn neurones, with receptor fields in the tail, have a modality-specific hypersensitivity (Gelgor and Mitchell 1993).

1.6 Aims of this study

Rats possess two coccygeal nerves which run along the left and right ventro-lateral aspect of the tail. They convey primary afferent information from the tail to central nervous system cells located in the dorsal horn (L1-L5) of the spinal cord.

In order to understand how WDR, with receptive fields in the tail, function and modality-specificity responses they have to noxious and innocuous stimulation during ischaemia and reperfusion my study aimed to:

a) employ the single fibre recording procedure to the animal model currently used in our laboratory and apply it to study coccygeal afferent nerve fibres during ischaemia and reperfusion.

b) discover the modalities represented in afferent fibres in the nerve.

c) identify the fibre types associated with each modality.

d) measure how responses to each modality respond to ischaemia and reperfusion.

CHAPTER 2

Material and methods

2.1 Electrophysiological recording

The recording procedure followed that of Necker and Hellon (1978). Anaesthesia was induced in 56 male Sprague-Dawley rats (420-560 g) with sodium pentobarbitone (50 mg/kg i.p.). Atropine sulphate (0.25 mg/kg i.p.) was administered to reduce bronchial secretions. A tracheal cannula was inserted, through which enflurane (Ethrane, Abbott Laboratories) in 45% oxygen/55% nitrogen was administered at a concentration of 2.5% for surgery and 1.5% for maintenance. The rats breathed spontaneously throughout the experiments. Core body temperature was maintained at 38°C by means of a heating blanket. The right ventral coccygeal nerve was exposed close to the tail base and protected from drying in a pool of mineral oil. To facilitate dissection a black perspex plate was placed beneath the nerve. Under a dissecting microscope, the epi- and perineural sheaths were slit lengthwise, individual fibre bundles were cut proximally and small filaments further divided with micro-dissection scissors (Trident) and insect pins. I used a platinum wire recording electrode (0.2 mm in diameter) with a silver reference electrode in the surrounding tissue. The small filaments were placed on the recording electrode and further dissected until impulses from individual fibres could be identified by electrical stimulation of the coccygeal nerve. Electrical stimulation (Digitimer DS9A) took place via two L-shaped stainless-steel needle electrodes, which were inserted subcutaneously along the path of the nerve, approximately 40 mm distal to the recording site. Typical stimuli used for searching for single fibres were 1 Hz pulses of 0.04-0.06 ms duration at 1-7 V for A fibres and 1 Hz pulses 0.5-0.7 ms duration at 10-40 V for C fibres. Filaments containing fibres that could not be distinguished easily by difference in spike amplitude were rejected, as were filaments containing more than five active fibres. Spikes were amplified (Digitimer NL104), filtered through a bandpass of 200-1500 Hz, and discriminated using a spike processor (Digitimer D130) connected to a laboratory interface (CED 1401, Cambridge Electronic Design), attached to a microcomputer. Raw activity also was stored on magnetic tape for off-line analysis.

Once a single afferent fibre had been isolated with the electrical search stimulus, I located its receptive field and determined its response to innocuous and noxious stimuli. Innocuous stimulation consisted of brushing the tail with a camel hair brush along the entire length of the receptive field, with repeated strokes, for a 10 sec period. Noxious mechanical stimulation was achieved by pinching (1.7 N) the centre of the receptive field with serrated forceps for 10 s (Sher and Mitchell 1990). I took care not to flex the tail during the mechanical stimulation, because of the profusion of flex-sensitive fibres in the nerve (Necker and Hellon 1978). Noxious thermal stimulation was applied by immersing the distal part of the tail for 50 s in water at 49°C; I did not follow up fibres with receptive fields which were not in the distal tail.

Conduction velocities were calculated from the post-stimulus time histogram, generated by the analysis software (Mrate, CED), following suprathreshold electrical stimulation of the fibre. In some preparations several afferent fibres could be characterised within the same filament of nerve, on the basis of their conduction velocities, amplitude and shape of their spikes (Bessou and Perl 1969).

2.2 Ischaemia and reperfusion

A tourniquet in the form of an inflatable cuff (IITC, diameter 15 mm, length 16 mm), positioned 45 mm distal to the recording electrode, was used to induce ischaemia. The cuff was inflated to 280 mm Hg, twice the systolic blood pressure of rats from our stock (Norton *et al.* 1993), for 30 min. The tourniquet was the same as that used previously (Gelgor and Mitchell 1993). Such a tourniquet not only induces ischaemia of the distal tail, but also compression of the nerve immediately underneath the tourniquet (Dahlin *et al.* 1989), and any alteration in activity measured proximal to the tourniquet must be interpreted accordingly.

I measured the response of each fibre to the three stimulus modalities after 15 min of ischaemia, immediately after releasing the tourniquet and after 30 and 60 min of reperfusion. Control experiments followed the same experimental procedure but without inflation of the cuff. I also watched for the appearance of spontaneous activity from additional fibres during the ischaemia and reperfusion.

2.3 Histology

The tails of 5 experimental and 5 control rats were examined histologically. Cross sections of the tail taken from the area beneath the cuff and from the receptive field of an identified afferent fibre were fixed in 10% formalin, embedded in wax, stained with haematoxylin-eosin and observed at 40-100x magnification to check for the presence of oedema and polymorphonuclear leucocyte invasion.

2.4 Ethical considerations

The animals were euthanazed by anaesthetic overdose, without regaining consciousness. All procedures were approved by Animal Ethics Committee of the University of the Witwatersrand (Clearance certificate 93/36/2b).

CHAPTER 3

Results

3.1 Spontaneously active C-fibres

I was able to distinguish four kinds of afferent fibre by virtue of their physiological characteristics. The first of these consisted of fibres which were spontaneously active before the tail was rendered ischaemic, but which were not excited by mechanical stimuli and showed a decrease in firing rate when subjected to noxious thermal stimulation. Recordings were made from 13 of these fibres and for 7 of them the tail was subjected to ischaemia and reperfusion. The conduction velocity of this group of fibres was 0.8 ± 0.2 m/s (mean ± SD, range 0.5-1.1 m/s), identifying them as C fibres. Their spontaneous firing rate was 0.5 ± 0.5 spikes/s (mean ± SD) before ischaemia was induced, and this firing rate increased slowly during the period of ischaemia and rapidly during the first 3 minutes of reperfusion, reaching a maximum within 20-30 min (fig. 1). There was a trend towards recovery after 60 min of reperfusion.

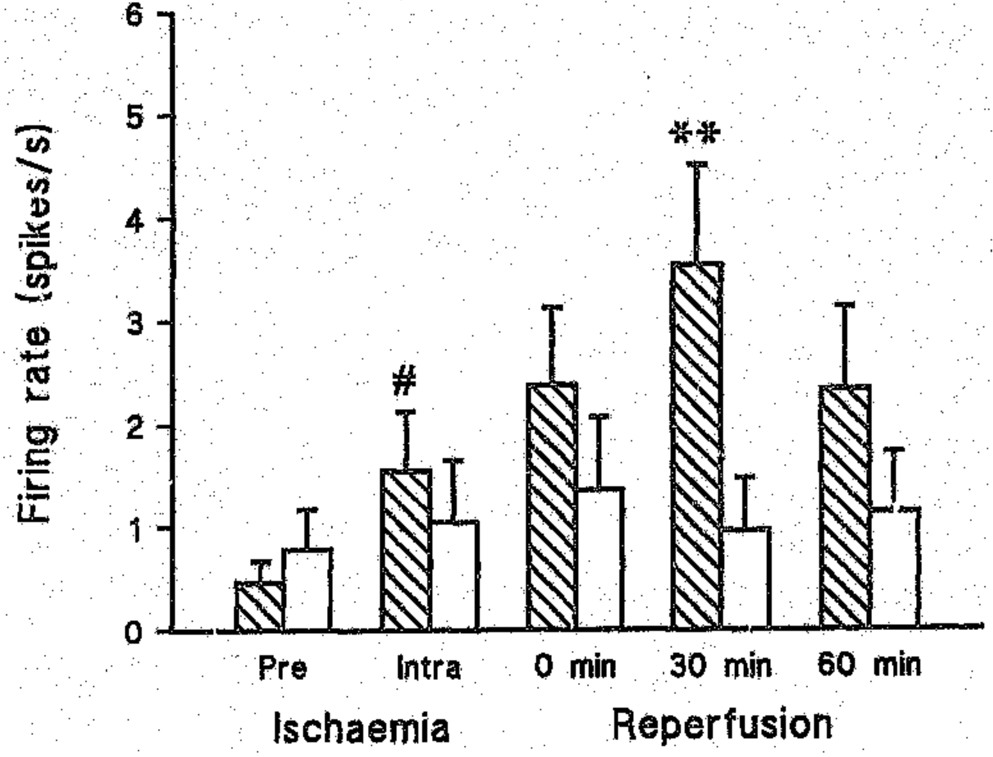

Figure 1.

The firing rate (mean ± SEM, $n = 7$) of spontaneously active C fibres during torniqet-induced ischaemia of the tail, and during reperfusion. These fibres increased their firing rate during ischaemia and reperfusion. They were not sensitive to mechanical stimuli, and decreased their firing rate when the tail was heated to 49°C (# denotes $P<0.05$, Sign test, ** denotes $P<0.01$, repeated measures ANOVA and Dunnett multiple comparisons test, both relative to firing rate before ischaemia). Open bars (mean ± SEM, $n = 6$) show the firing rates of similar fibres when the tourniquet was not applied.

Fig. 2 shows the activity, during reperfusion, of one such spontaneously active C-fibre. None of the 6 spontaneously active fibres which were not subjected to tail ischaemia showed a significant change of firing rate over the 90 min of recording.

3.2 C-fibres

The second group of afferent fibres consisted of fibres which were not spontaneously active but which responded to noxious mechanical and noxious thermal stimuli. I recorded from 17 of these fibres, the receptive fields of 8 of which were subjected to ischaemia and reperfusion. The conduction velocities of these fibres was 0.6 ± 0.2 m/s (mean ± SD, range 0.5 - 1 m/s), so they too were C fibres. For most of these C fibres I investigated only the thermal response during ischaemia and reperfusion because instability of C fibre preparations, and deleterious effects of repeated thermal and mechanical stimulation on the receptive field, confounded attempts to measure the effects of ischaemia and reperfusion on both forms of noxious stimuli. I chose the noxious thermal stimulus rather than the noxious mechanical stimulus because the response to noxious mechanical stimulus always was less intense than the response to the noxious thermal stimulus. Fig.3 shows a typical response of one of the heat-sensitive C fibres to 49°C stimulation of the tail, before, during, and after ischaemia. Before ischaemia, these fibres showed a rapid increase in firing rate when the tail was immersed in 49°C water, followed by a rapid recovery when

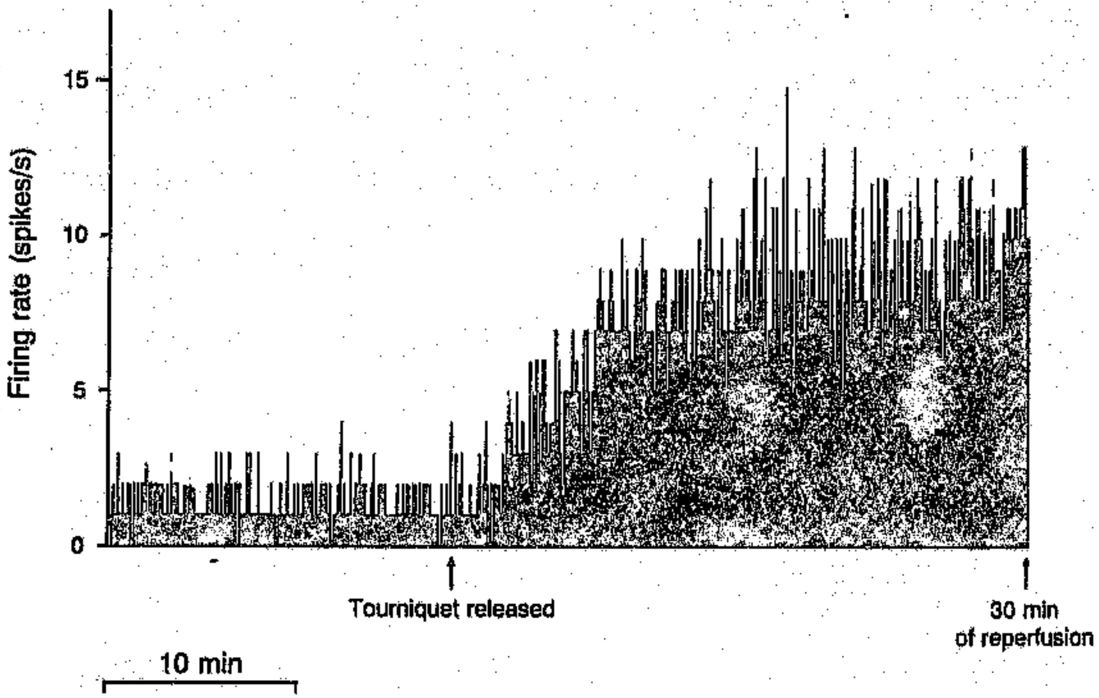

Figure 2.

Firing rate of spontaneously active C fibre during ischaemia and reperfusion of its receptive field. The removal of tourniquet (first arrow) resulted in a sustained increase in firing rate, reaching a maximum between 20 and 30 min of reperfusion. The firing rate of this fibre before application of the tourniquet was 1.6 spikes/s.

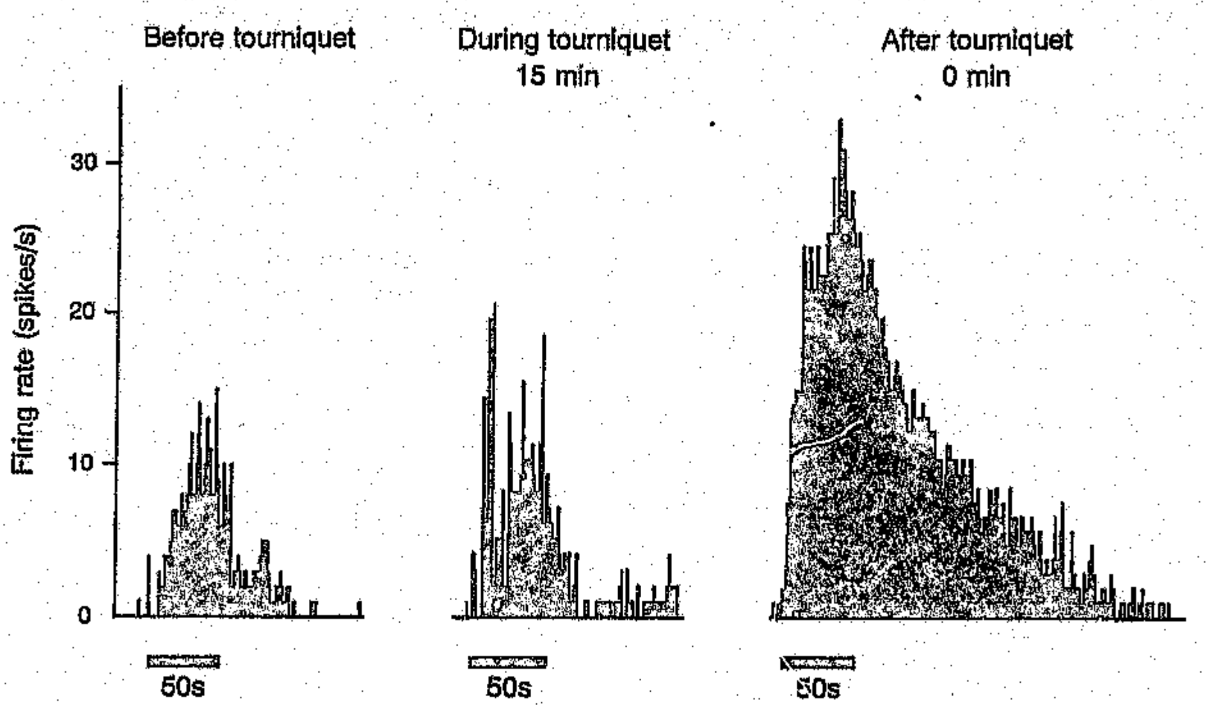

Figure 3.

Response of a thermally-sensitive C-fibre to 49°C stimulation (solid bar) of its receptive field, before, during and after ischaemia. The response of the fibre during ischaemia was similar to that before the application of the tourniquet.

the tail was removed from the water (fig. 3). This response was very similar during the period of ischaemia, but was greatly exaggerated immediately after termination of ischaemia, with an increased recovery time (figs. 3 and 4). The 9 control fibres showed no significant change in their response to 49° stimulation of their receptive fields over the 90 min of recording.

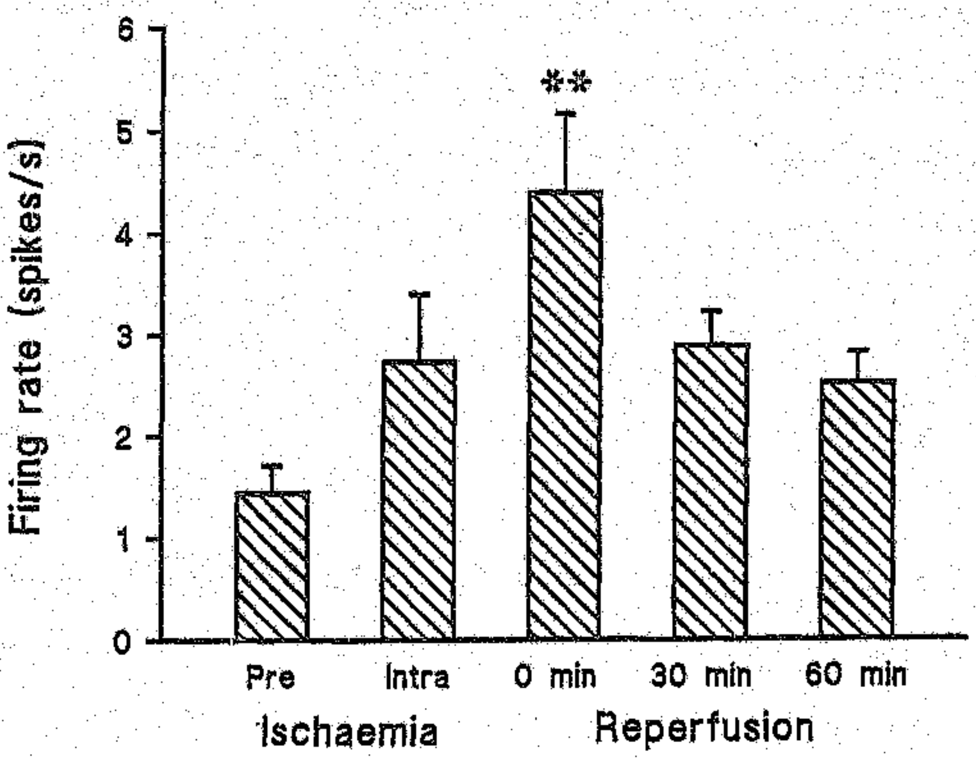

Figure 4.

Response of thermally-sensitive C-fibres (mean ± SEM, n = 8) to 50 s, 49°C stimulus applied to their receptive fields on the tail before, during, and after ischaemia. These fibres were not spontaneously active and also responded to noxious mechanical stimuli. The firing rates are the averages over the 50 s of application of the noxious thermal stimuls, (** denotes $P<0.01$, repeated measures ANOVA and Dunnett multiple comparisons test).

3.3 A-delta fibres

The third group of fibres I encountered responded to noxious pinch but not to brush or thermal stimulation (49°C) of their receptive fields and were not spontaneously active. I recorded from 26 such fibres, and subjected the receptive fields of 11 to ischaemia and reperfusion. Their conduction velocities were 3.0 ± 1.5 m/s (mean ± SD, range 1.5 - 6.4 m/s). This conduction velocity was significantly higher than those of the previous two groups, and in the A-delta range. The response of one of the fibres to pinch before, during, and after ischaemia is shown in fig. 5A. Ischaemia and reperfusion of its receptive field had no effect on the response of this fibre to the noxious mechanical stimulus, nor on that of the responses of the other fibres of this group (Fig. 6). The control group of 15 similar fibres not subjected to receptive field ischaemia and reperfusion also showed no change in their response to noxious pinch stimulation over 90 min of recording.

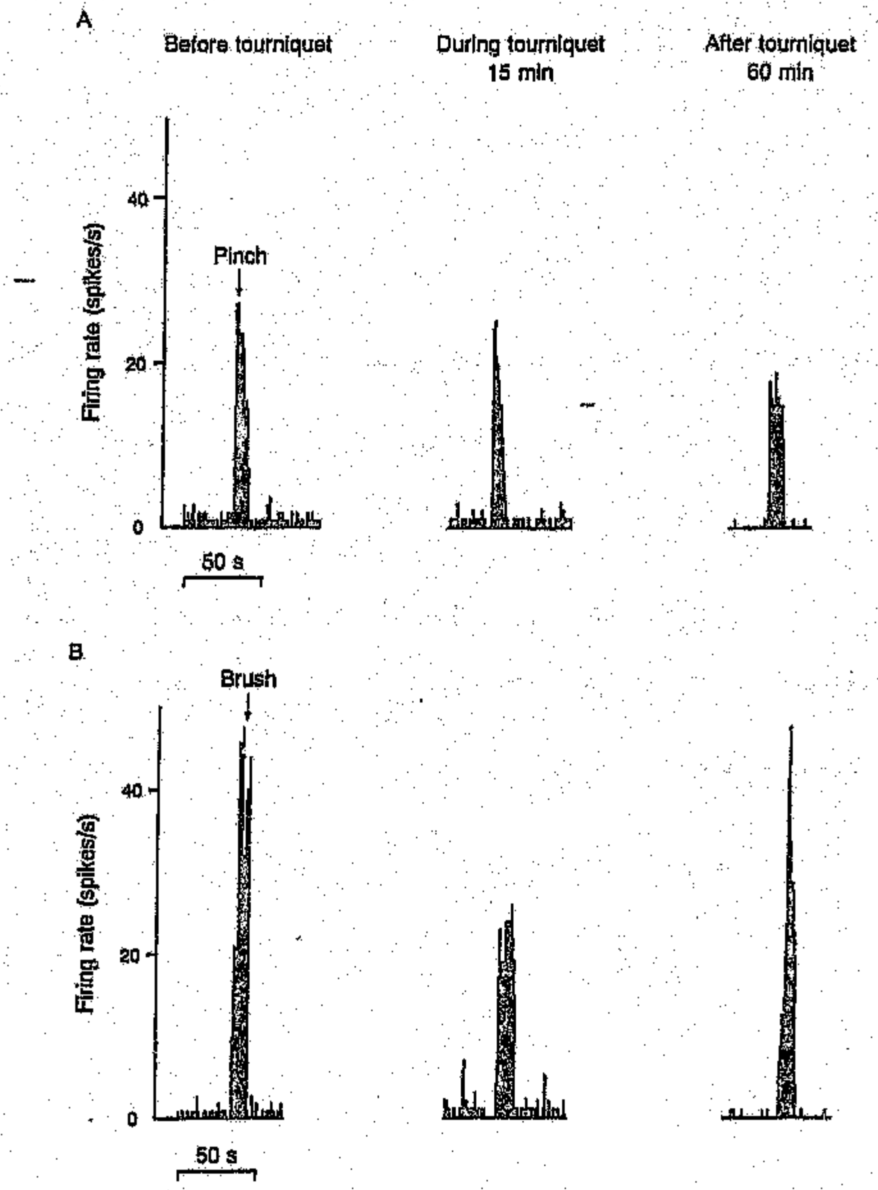

Figure 5. A recording of two mechanoreceptive afferents in the same nerve filament. The afferent in A responded to noxious pinch stimulation, but not brush stimulation, of its receptive field. The response of this fibre was not affected by ischaemia or reperfusion. The fibre in B responded to brush stimulation of its receptive field, in a different region of the tail to A; this response was decreased during ischaemia and reperfusion. Neither of these fibres responded to 49°C heat stimulation of their receptive fields.

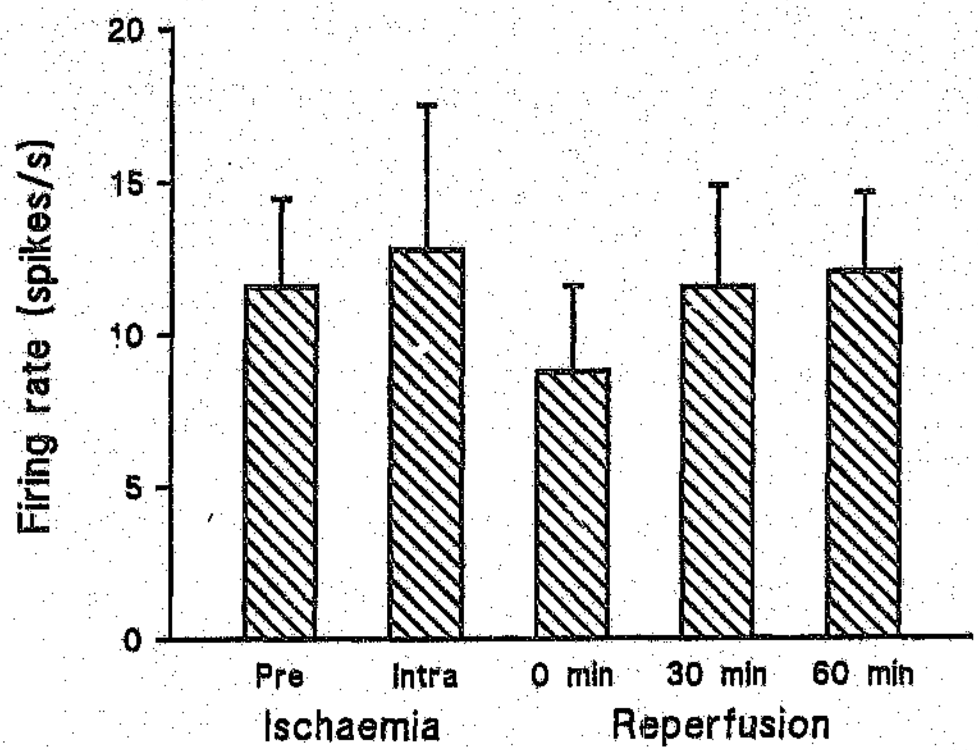

Figure 6. Response of A-delta nociceptors (mean ± SEM, n = 11) to pinch of the centre of their receptive fields, before, during and after ischaemia. The firing rates are the averages over 10 s application of the noxious mechanical stimulus. Neither ischaemia nor reperfusion had any significant effect on the response. These fibres did not respond to brush, nor to 49°C thermal stimulation of their receptive fields.

3.4 A-beta fibres

The final group of fibres from which recordings were made responded to both brush and pinch but not to noxious heat, and were not spontaneously active. Their conduction velocity was 20 ± 7 m/s (mean ± SD, range 15 - 36 m/s). There was no overlap between the conduction velocities of the fibres of this group and those of the fibres of the previous group, and I presume they belonged to the A-beta family. While the response of the fibres to brush stimulation did not show noticeable adaptation, the response to pinch stimulation adapted rapidly. Ten fibres were subjected to ischaemia and reperfusion of their receptive fields, and 12 served as controls. Only the response to brush was investigated during ischaemia and reperfusion, for fear of damaging the low-threshold receptors by repeated noxious stimulation. The response of a typical fibre is shown in Fig. 5B, and the average response of the group in Fig. 7. The response to brush stimulation was decreased during both ischaemia and reperfusion of the receptive field. The response of control fibres to brush stimulation did not change over 90 min of recording.

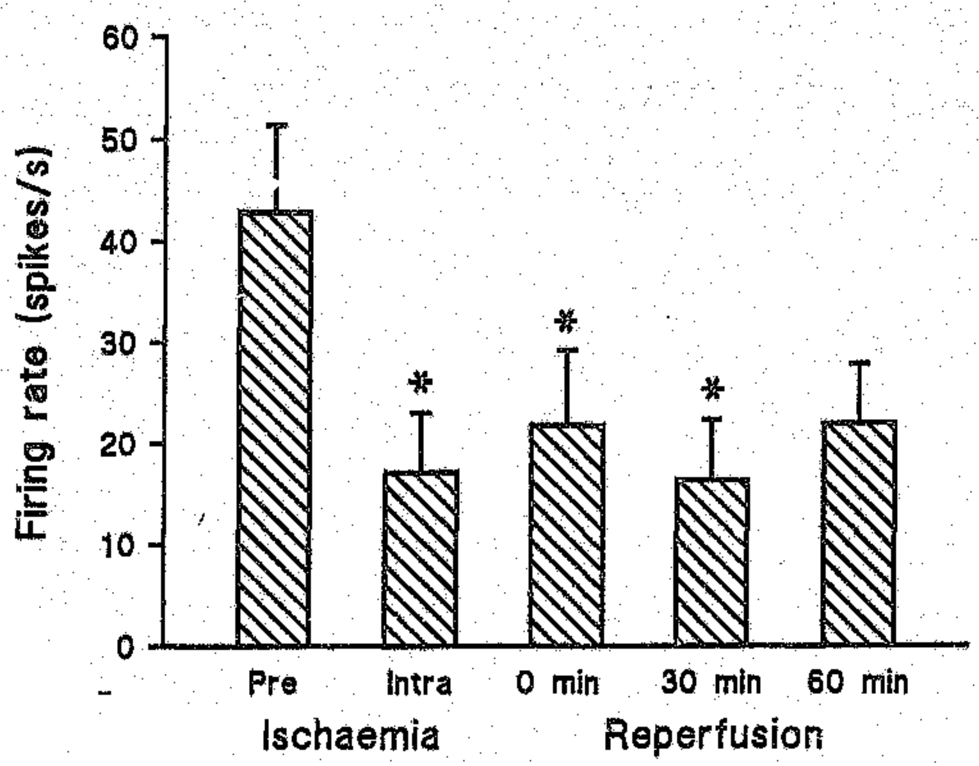

Figure 7. Response of low threshold mechanoreceptors (mean ± SEM, $n = 10$) to brush stimulation of their receptive fields, before, during, and after ischaemia. The firing rates are the averages over the 10 s application of the innocuous mechanical stimulus. These fibres also responded to pinch of their receptive fields, but not to 49°C thermal stimulation. (* denotes $P<0.05$, repeated measurement ANOVA and Dunnett multiple comparisons test).

3.5 Histology

Histological examination of cross sections of the tail revealed no obvious microscopic differences between control tails and those which had been subjected to ischaemia and reperfusion. In particular, there was no oedema nor leucocyte infiltration, therefore this model is different from the formalin model which is of inflammatory type.

CHAPTER 4

Discussion

My investigation has revealed four different groups of primary afferent fibres in the rat coccygeal nerve, with distinct responses to stimulus modality as well as to transient ischaemia and subsequent reperfusion of their receptive fields on the tail (see Table 2). During transient ischaemia, a group of spontaneously active C fibres, for which the adequate stimulus was not obvious, became more excitable, while the sensitivity of a group of large-diameter A fibres to its principal stimulus, brush, decreased. The activity of other groups of fibres was not affected by ischaemia. The subsequent period of reperfusion induced a marked increase of activity of C fibres, but not of A- fibres. Spontaneously active C fibres became even more excitable during reperfusion; over 30 min of reperfusion, the firing rate increased to more than seven times the initial firing rate. Thermally sensitive C fibres showed increased responsiveness to noxious thermal stimuli immediately after release of the tourniquet.

				Response to receptive field stimulation		
Fibre type	Conduction velocity (m/s)		Stimulus	Before ischaemia	During ischaemia	During reperfusion
	Mean	SD				
C	0.80	0.2	Chemical?	+	+	+++
			Heat	–	?	?
C	0.60	0.2	Heat	+	+	+++
			Pinch	+	?	?
Aδ	3.00	1.5	Heat	0	0	0
			Pinch	+++	+++	+++
			Brush	0	0	0
Aβ	20.00	7.4	Heat	0	0	0
			Pinch	+	?	?
			Brush	+++	+	+

Table 2. Summary of the responses of primary afferent fibres in the rat coccygeal nerve before, during and after transient ischaemia of their receptive fields on the tail.

+ reponse to stimulus; - decreased response; 0 no response;

? effect of the stimulus was not investigated.

The attributes of the C-fibre groups, in the absence of ischaemia and reperfusion, are congruent with those of C-fibre afferents which have been observed in rats previously. Necker and Hellon (1978) found afferent fibres suppressed by heating and insensitive to mechanical stimuli originating in the rat tail; Leem *et al.* (1993) found similar fibres originating in the rat hind paw, and showed them to be C fibres. Thermal nociceptors, with feeble responses to mechanical stimuli, have been observed in the rat by Necker and Hellon (1978), Lynn and Carpenter (1982), Fleischer *et al.* (1983), and Leem *et al.* (1993), who again showed them to have C-fibre conduction velocities. High and low threshold mechanoreceptors, innervated by A-fibre groups, and insensitive to noxious heat, also have been described in the rat (Lynn and Carpenter 1982, Handwerker *et al.* 1987, Leem *et al.* 1993). In my study, the conduction velocities of both the presumed A-delta fibres innervating mechanical nociceptors, and the presumed A-beta fibres innervating low-threshold mechanoreceptors, were appreciably lower than those of their counterparts observed by Leem *et al.* (1993) in the rat hind paw. However, relatively slow conduction velocities also were recorded, in similar preparations from rat coccygeal nerve, by Handwerker and coworkers (1987).

My contribution has been to investigate how the properties of the somatosensory afferents arising in the rat tail are affected by transient ischaemia and subsequent reperfusion. A pneumatic tourniquet, such as I used, not only produces ischaemia distal to the site of application, but also compresses the nerve trunks beneath the tourniquet. The activity of afferent fibres proximal to the tourniquet must be tourniquet. The activity of afferent fibres proximal to the tourniquet must be interpreted in the context of the combination of compression and ischaemia. Compression blocks large myelinated fibres before small myelinated fibres (Ochoa *et al.* 1972, Dahlin *et al.* 1989). Even when all myelinated fibres are blocked by nerve compression, C fibres remain active (Clark *et al.* 1936). In addition to being more resistant to compression, unmyelinated fibres are more resistant to ischaemia than are myelinated fibres (Dahlin *et al.* 1989). The compression and ischaemia induced by the tourniquet was sufficient to reduce activity in the larger myelinated afferent fibres in the coccygeal nerve. Small myelinated fibres were not affected by the tourniquet. The robustness of A-delta fibres under compression and ischaemia is consistent with previous observations in nerve conduction studies (MacKenzie *et al.*1975, Rydevik *et al.* 1980).

In contrast to the A-fibre afferents, C-fibre afferents can be activated both by ischaemia and by reperfusion of previously ischaemic tissue (Longhurst *et al.* 1987, Longhurst *et al.* 1991, MacIver and Tanelian 1992, Lagier-Tassonier and Balzamo 1993, Stahl *et al.* 1993). In the rat tail, I found two groups of C-fibre afferents, with the same conduction velocity, which were activated during ischaemia and reperfusion. One of these groups responded to noxious heating and pinch of the receptive fields, and such nociceptors can be sensitized by repeated noxious thermal stimulation (Lynn 1979). That was not the basis of the sensitization I saw, because control fibres, subjected to the same repeated noxious stimulation but not subjected to ischaemia, were not sensitized. The other group of C-fibre afferents activated by ischaemia and reperfusion did not appear to respond to mechanical or to noxious thermal stimulation. I believe them to be chemosensitive, and that their stimulation, as well as the sensitization of thermal nociceptors, was derived from metabolites released during ischaemia and by metabolites released during the reperfusion (Kerrigan and Stotland 1993). Although these metabolites may be the same as, or similar to, those released during inflammation, there was no detectable inflammation in the tail of my rats at the time of C-fibre activation.

The characteristics of the C fibres innervating thermal nociceptors are reminiscent of the behavioural responses of rats to the same noxious thermal stimulus following transient tail ischaemia. Tail flick latencies during tail immersion at 49°C are shorter during reperfusion and this thermal hyperalgesia is greatest immediately after release of the tourniquet (Gelgor *et al.* 1986). Consequently, the thermal hyperalgesia demonstrated by rats following the conditioning stimulus of transient ischaemia applied to the tail may well arise because peripheral thermal nociceptors themselves become sensitized.

Peripheral sensitization of thermal nociceptors, however, is not the only sensitization phenomenon resulting from transient ischaemia and reperfusion. Previous studies in our laboratory have shown that ischaemia and subsequent reperfusion of the rat's tail induce hypersensitivity of wide dynamic range (WDR) neurones, in the dorsal horn of the spinal cord, to both noxious pinch and innocuous brush (Gelgor and Mitchell 1993). This hypersensitivity cannot arise from increased primary afferent traffic, because I have now shown that, following ischaemia and reperfusion of the rat tail, the responses of the A-fibre afferents mediating pinch and brush decrease or remained unchanged. Consequently the hypersensitivity of WDR neurones to mechanical stimuli must arise from a central mechanism, whereby the response of the WDR neurones to afferent input is enhanced (Woolf 1983, Cook *et al.* 1987, Neugebauer and Schaible 1990, Coghill *et al.* 1993).

If, following a conditioning stimulus, afferent fibres conveying a particular modality were sensitized peripherally, and they were to converge on centrally sensitized WDR neurones, then the spinal signal generated by that modality would be amplified disproportionately. As I have shown, afferents conveying the modality of noxious heat from the rat tail are sensitized peripherally during reperfusion. However, the population of WDR neurons investigated by Gelgor and Mitchell (1993) although becoming more sensitive to mechanical stimuli, did not become more sensitive to thermal stimuli during reperfusion. I therefore concur with Gelgor and Mitchell (1993) that those WDR neurones receive little input from heat-sensitive C fibres. The central processing of information arriving from thermal nociceptors must depend, therefore, on another population of dorsal horn neurones

The conditioning stimuli which induce central sensitization have in common that they produce a C-fibre barrage, which generates a sustained hypersensitivity of higher order neurones in nociceptive pathways (Dickenson and Sullivan 1987, Woolf and King 1987, Woolf 1989, Thompson *et al.* 1993). The group of apparently chemosensitive C fibres which became so active during tail ischaemia and reperfusion would be capable of providing such a barrage, and thus may be responsible for the central sensitization of WDR neurones. Indeed, Gelgor and Mitchell (1993) showed that while WDR neurones were hypersensitive to peripheral mechanical stimuli only during tail reperfusion, they were hyperexcitable (that is they demonstrated increased activity in the absence of obvious peripheral stimuli) during both reperfusion and tail ischaemia. I may have found, in this investigation of the coccygeal nerve, the C fibres which could be responsible for a conditioning barrage during ischaemia itself.

CHAPTER 5

Conclusions

By recording directly from primary afferent nerve fibres in the rat coccygeal nerve during tail ischaemia and reperfusion, I have shown that the myelinated fibres in the nerve do not increase their input to the CNS during either ischaemia or reperfusion of their receptive fields, while C fibres in the nerve become more active during both ischaemia and reperfusion and show sensitization to noxious thermal stimulation of their receptive fields during reperfusion. During reperfusion, spinal WDR neurons exhibit hypersensitivity to the mechanical stimulation of their tail receptive fields, even though the mechanosensitive primary afferents are less active at that time (Gelgor and Mitchell 1993). Consequently, the CNS events which follow transient ischaemia and subsequent reperfusion of peripheral receptive fields in the rat's tail result both from plasticity in spinal neuronal circuitry and from changes in the afferent traffic.

The size of the receptive fields of convergent neurones changes during reperfusion (Gelgor and Mitchell 1993). This may depend on an increase in primary afferent receptive field size *per se* or on recruitment of more afferents. Further studies to investigate C fibre receptive field changes during the same period of reperfusion are required.

Spinal administration of non-steroidal anti-inflammatory drugs (NSAIDs) attenuated the hypersensitivity of convergent neurones to both noxious and innocuous mechanical stimulation during reperfusion of their receptive fields on the tail. NSAIDs also attenuated the enlargement in receptive field size as well as the increase in spontaneous firing rate evident during reperfusion of the rat tail. The NSAIDs did not affect the activity of neurones during the transient receptive field ischaemia, nor did they affect responses to stimuli applied without prior ischaemia (Gelgor and Mitchell 1995). Now that I have shown that ischaemia and reperfusion of the rat tail activate nociceptors and consequently afferent pathways, it would be interesting to see if NSAIDs can also prevent C fibre sensitization during the same events. Local anaesthetic drugs could also be injected locally to try to prevent such C-fibre barrage and sensitization to thermal stimulation.

Behavioural and neurophysiological experiments have demonstrated that events occurring during ischaemia are different to those during reperfusion. Indeed ischaemic pain cannot be controlled using NSAIDs. This might be related to a different mechanism which, in my opinion, could also be triggered by the compression effect of the tourniquet and not only by tissue ischaemia. Therefore investigating fibres with receptor fields beneath the tourniquet may contribute to a better understanding of the model we use.

CHAPTER 6

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