

**THE PRIMARY AFFERENT NOCICEPTOR AND  
NEUROPEPTIDE GENE EXPRESSION:  
IMPORTANCE IN EXPERIMENTAL ARTHRITIS**

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## DECLARATION

I declare that this thesis was written entirely by me and represents all my own work except for the procedures listed below and acknowledged in the text.

1. Surgical adrenalectomies were performed by June Noble and Keith Chalmers of the Biomedical Research Facility, Western General Hospital.

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## **Publications arising from this thesis**

The publications (full papers and abstracts) arising from this thesis are listed below;-

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Donaldson, L.F., McQueen, D.S. and Seckl, J.R. Early induction of preprotachykinin (PPT) mRNA expression in sensory ganglia during the development of experimental arthritis (Abstract) *Scottish Medical Journal* 37(1992) 90

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## ABSTRACT

Neuropeptides in dorsal root ganglia (DRG) have been implicated in the pathogenesis of pain and neurogenic inflammation in experimental and clinical arthritis. Sensory neuropeptides are released centrally and peripherally on noxious stimulation and, recently increased levels of substance P (SP) and calcitonin gene-related peptide (CGRP), confined to innervating DRG, were demonstrated in adjuvant-mediated monoarthritis. In these studies, the primary afferent neuronal response to peripheral inflammation with respect to neuropeptide mRNA expression, and the role of the primary afferent and its neuropeptides in the spread of arthritis have been examined.

A mild adjuvant-induced arthritis has been adapted from classical polyarthritis in the rat. Arthritis is induced by adjuvant injection around one tibio-tarsal joint which causes either a discrete monoarthritis or, with a larger dose of adjuvant, a monoarthritis spreading to bilateral arthritis involving both tibio-tarsal joints, which consistently occurs 14 days after adjuvant injection. This model of arthritis thus enables study of the arthritic process and mechanisms underlying the spread of inflammation, while reducing animal discomfort and the confounding effects of systemic illness seen in polyarthritis.

It has been hypothesised that substance P may act upon the peripheral nociceptor terminals to sensitise them to other inflammatory mediators, and that other mediators may act through the release of substance P. Exogenous substance P administered by close arterial injection had no significant effects on C-fibre nociceptor activity in either monoarthritic or normal anaesthetised rats. Substance P may exert an effect upon  $A\delta$  high threshold mechanoreceptors but these fibres were not studied here.

Analysis of preprotachykinin (PPT, encoding SP), CGRP and somatostatin (SS) messenger RNAs showed that expression of all three mRNAs are altered in chronic monoarthritis only in ganglia innervating the affected joint. PPT and CGRP showed especially dramatic increases during the first 8 hours of inflammation. Somatostatin mRNA was the only neuropeptide to show contralateral changes 8 hours after adjuvant injection; this effect being attributable to the injection itself rather than inflammation. All three mRNAs were also increased in chronic arthritis. In bilateral arthritis, ganglionic PPT and CGRP levels were elevated bilaterally whereas SS mRNA increased in ganglia ipsilateral to the adjuvant injection and not in

contralateral DRG. Vasoactive intestinal peptide mRNA was not found in DRG neurons of normal or inflamed animals at any point.

Specific perineural capsaicin lesions of C-fibres innervating the joint demonstrated that capsaicin-sensitive sensory neurons and their neuropeptides are pivotal to both the maintenance and the spread of arthritis in this model. This lesion attenuated adjuvant-induced monoarthritis and totally prevented of spread of arthritis. The inhibition of spread was associated with a reduction in the expression of PPT mRNA in remaining neurons in contralateral DRG. Neonatal capsaicin, a less specific neurotoxic lesion of unmyelinated afferents, had no effect on the severity of arthritis and only delayed the contralateral spread of arthritis, but attenuated increases in neuropeptide mRNA during arthritis. The central nervous system plasticity and alterations in dorsal horn excitability following neonatal capsaicin may be responsible for the lack of effect of this lesion on arthritis.

The regulation of neuropeptide genes in DRG during inflammation is not well understood. Blockage of the increase in primary afferent spontaneous activity known to be observed within hours of the onset of inflammation using local anaesthetic prevented the increase in PPT and CGRP mRNA seen in DRG 8 hours after adjuvant injection. While endogenous glucocorticoids have been shown to regulate neuropeptide levels in DRG, adrenalectomy had no effect on the degree or spread of arthritis and had only subtle effects on the expression of PPT and CGRP mRNA.

Expression of transcription factors in DRG on noxious stimulation has not been demonstrated, and this has been substantiated in these experiments as expression of NGFI-A, NGFI-B and c-jun was not detectable in DRG innervating inflamed or uninflamed joints. AP-2 mRNA, encoding a transcription factor constitutively expressed in tissue of neural crest origin, showed a rapid increase in expression one hour after injection, returning to control levels within 2 hours. The possible involvement of AP-2 in the regulation of these neuropeptides remains to be determined.

In conclusion, these data support the hypothesis that polymodal nociceptors and the neuropeptides they express are closely associated with the maintenance and spread of arthritis. Changes in neuropeptide mRNA expression may be regulated through many mechanisms, including neuronal activity, and transcription factor induction.

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## List of commonly used abbreviations

AA	adjuvant arthritis
ANOVA	analysis of variance
AP-1	activator protein-1
AP-2	activator protein-2
ATP	adenosine triphosphate
°C	degrees Celsius
cAMP	cyclic adenosine monophosphate
CAPL	perineural capsaicin application to the left sciatic nerve
CAPR	capsaicin application to the right sciatic nerve
CBG	corticosterone binding globulin
CGRP	calcitonin gene-related peptide
CRE	cAMP response element
CREB	CRE binding protein
CTP	cytidine triphosphate
d	day
DNA	deoxyribonucleic acid
cDNA	complementary DNA
DRG	dorsal root ganglion
g	gram
GTP	guanosine triphosphate
5-HT	5-hydroxytryptamine
i.p.	intraperitoneal
ISH	in situ hybridisation
kg	kilogram
L4/L5	fourth or fifth lumbar spinal segment
m	metre
m(prefix)	milli ( $10^{-3}$ )
M/mol	mole
min	minute
M. tub.	Mycobacterium tuberculosis
n(prefix)	nano( $10^{-9}$ )
NaCl	sodium chloride
NEP	neutral endopeptidase

NGF	nerve growth factor
NKA	neurokinin A
NKB	neurokinin B
p(prefix)	pico( $10^{-12}$ )
PACR	primary articuloctaneous ramus
PGSN	postganglionic sympathetic neuron
PMN	polymorphonuclear leucocytes
PPE	preproenkephalin
PPT	preprotachykinin
RA	rheumatoid arthritis
RNA	ribonucleic acid
mRNA	messenger RNA
r.p.m.	revolutions per minute
s	second
S.E.M.	standard error of the mean
SP	substance P
SS	somatostatin
UTP	uridine triphosphate
$\mu$ (prefix)	micro ( $10^{-6}$ )
VIP	vasoactive intestinal (poly)peptide

## **1. INTRODUCTION**

### **1.1 Adjuvant disease as a model of arthritis: clinical and histological features**

Adjuvant arthritis (AA) has been widely used as a model of human disease as the pathological and biochemical features closely resemble human rheumatoid disease. AA is thought to be initiated by a delayed hypersensitivity reaction, as overt pathology does not become apparent until 11-16 days post-injection of adjuvant. Some evidence that the disease has an immunological basis was presented by Wakesman et al, (1960), who showed that whole body irradiation prior to adjuvant injection delayed the appearance of joint swelling. However, these studies did not exclude the possibility of the involvement of other systems in the pathogenesis of adjuvant disease. In the classical model, a disease of the whole animal develops following intradermal injection of a mycobacterial suspension in mineral oil, resulting in reduced weight gain (Pearson and Wood, 1959; Sarlis et al., 1992; Colpaert, 1987), reduced mobility (de Castro Costa et al., 1987), increased vocalisation and irritability (Colpaert et al., 1982) and hyperventilation (Colpaert and Van den Hoogen, 1983).

Gross lesions in animals with adjuvant disease are oedematous swellings of multiple joints, particularly the tarsal joints of the hind paws. As the disease progresses, periarticular swellings develop in the hind limbs and tail adjacent to the intervertebral discs (Pearson and Wood, 1959). The disease follows a relapsing and remitting course after the initial two weeks, and may persist for several months, eventually resulting in chronic joint deformation (Pearson, 1963).

Detailed examination of the histological and histochemical features of AA have been made by numerous workers (Pearson and Wood, 1959; Jones and Ward, 1963; Nusbickel and Troyer; 1976; Rainsford, 1982). Microscopic features of arthritis



appear before the gross manifestations, and progress to a suppurative arthritis and peri-arthritis. As AA progresses there are signs of joint destruction, new bone formation, and fibrous and bony ankyloses. Inflammatory changes are seen in synovium and synovial spaces, periarticular tissues and bone marrow (Jones and Ward, 1963). Histochemical changes indicative of chondrocytic hypertrophy, calcification, and loss of glycosaminoglycans from arthritic cartilage are seen (Nusbickel and Troyer, 1976). Similarities between AA and rheumatoid disease in humans have been summarised by Rainsford (1982). In general AA shares many pathological features with RA, with the exception of the presence of circulating rheumatoid factor, but differs in the gross skin, genital and vascular lesions seen in AA but not in RA.

### 1.2 The dorsal root ganglion

Primary sensory neurons are the first stage in the relay of peripheral sensory information to the central nervous system. These neurons have a simple anatomical structure, being pseudounipolar neurons with their somata in dorsal root ganglia, located either just inside (rat) or outside (human) the vertebral column, peripheral axons ending in receptor structures, and central processes synapsing in the dorsal horn of the spinal cord. Primary afferents have been classified by their conduction velocity, the modality of stimulus to which they respond, morphometric, ultrastructural and cytochemical criteria (Carr and Nagy, 1993). The cell bodies of sensory neurons have been divided into two populations on the basis of Nissl staining, and, more recently neurofilament content, into large light, and small dark neurons. In general, large, myelinated fibres have large somata and mediate innocuous stimuli, whereas fine myelinated afferents are either nociceptive or non-nociceptive and unmyelinated afferents mediate noxious information. However, in

terms of both sensory modality and neurochemical content, the dorsal root ganglion represents an extremely heterogeneous population of neurons.

### 1.3. Neuropeptide content of DRG neurons

A large number of neuropeptides, enzymes and other marker substances have been localised in DRG, usually restricted to a subpopulation of the total (Carr and Nagy, 1993). The subpopulations of DRG neurons expressing SP and CGRP are discussed further in Section 5.4.3.3. Due to the large number of different neurochemical markers found in DRG it is perhaps not surprising that there is a considerable degree of colocalisation between peptides, enzymes and others.

#### 1.3.1. Substance P and Neurokinin A

Substance P (SP) neurokinin A (NKA) and neurokinin B (NKB) all belong to the tachykinin family of peptides which share a common C terminus sequence (Figure 1.1).

*Figure 1.1. Amino acid sequences of three members of the tachykinin family.*

*Common amino acid sequences are in bold.*

Substance P	Arg-Pro-Lys-Pro-Gln-Gln- <b>Phe-Phe-Gly-Leu-Met-NH<sub>2</sub></b>
Neurokinin A	His-Lys-Thr-Asp-Ser- <b>Phe-Val-Gly-Leu-Met-NH<sub>2</sub></b>
Neurokinin B	Asp-Met-His-Asp-Phe- <b>Phe-Val-Gly-Leu-Met-NH<sub>2</sub></b>

Substance P and NKA are translated from the same gene, preprotachykinin A, by alternative RNA splicing and neurokinin B from a second gene, PPT-B. The two tachykinin genes exhibit a marked structural similarity and are thought to have

evolved from a common ancestor gene by duplication events (Kotani et al., 1986). Two further members of the tachykinin family, neuropeptide K and neuropeptide  $\gamma$ , are also encoded by the PPT-A gene. The primary structure, alternative mRNA splicing of the PPT-A gene and the neuropeptides produced from each mRNA species was initially determined for the bovine mRNA species and is shown below in Figure 1.2. Similar structures have been demonstrated in the rat (Carter and Krause, 1990; Krause et al., 1987; Harmar et al., 1990) where  $\alpha$ -PPT levels are extremely low and  $\gamma$ - and  $\beta$ -PPT comprise 80, and 20% of the mRNA respectively (Krause et al., 1987)

Thus all the splicing variants of the PPT-A primary transcripts encode substance P,  $\beta$ - and  $\gamma$ -PPT also encode neurokinin A. Additionally neuropeptides K and  $\gamma$  can be produced through post-translational processing of the relevant RNA species. Neurokinin B is thought to be the only tachykinin encoded by the PPT-B gene. It has two transcripts, both very similar and both encoding NKB. It is clear from this that the tachykinin family achieves a large diversity through gene diversity, alternative RNA slicing and post-translational processing.

The tachykinins also exhibit tissue specific expression, even to the extent of expression restricted to subpopulations of DRG neurons (see Section 5); however the promoter sequences responsible for this have not yet been identified. A construct containing 3.3kb of 5' promoter sequence plus the first intron from the rat PPT-A gene directing the expression of the reporter gene beta-galactosidase ( $\beta$ -GAL) microinjected into cultured DRG neurons appears to be selective for neuronal expression of PPT- $\beta$ -GAL. Unfortunately this region of the gene does not seem to direct the expression to a specific subset of DRG neurons as seen in vivo, as the reporter gene marker was observed in neurons which did not show endogenous SP-like immunoreactivity, in addition to neurons which expressed SP endogenously (Mulderry et al., 1993a).

Figure 1.2. Primary structure and alternative splicing of the preprotachykinin-A gene encoding substance P, neurokinin A, neuropeptides K and  $\gamma$ . Open boxes represent exons as numbered.

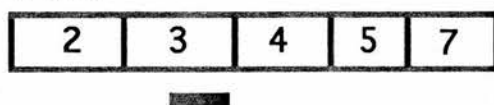
PPT-A gene

Primary structure

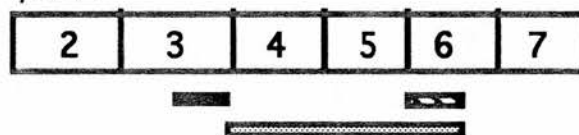


Alternative splicing variants of the PPT-A gene

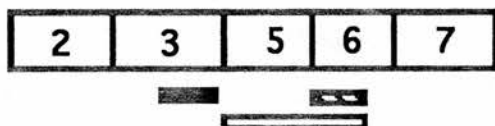
$\alpha$ -PPT



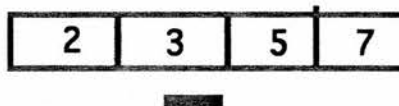
$\beta$ -PPT







$\gamma$ -PPT



$\delta$ -PPT



-  Substance P
-  Neurokinin A
-  Neuropeptide K
-  Neuropeptide  $\gamma$

Substance P is found in many areas of the central nervous system (Cuello et al., 1982), in the dorsal horn of the spinal cord and in a subpopulation of DRG neurons (see Section 5). This consists of 20-25% of the total neuronal number when either immunoreactivity (Hokfelt et al., 1980; McCarthy and Lawson, 1989) or mRNA is measured (Henken et al., 1988; Warden and Young, 1988; Henken et al., 1990); the populations showing PPT mRNA and substance P immunoreactivity being identical (Henken et al., 1988). Using radioimmunoassay, while both substance P and neurokinin A are present in significant amounts in DRG and peripheral nerve, neurokinin B is either present in low amounts or is undetectable (Moussaoui et al., 1992; Ogawa et al., 1985).

### 1.3.2. Calcitonin gene-related peptide

Calcitonin gene-related peptide (CGRP), a 37 amino acid peptide (sequence below), is produced by alternative RNA splicing of the calcitonin gene in neuronal tissues. This was first described by Amara and colleagues (Amara et al., 1982; Rosenfeld et al., 1983) in rat nervous tissue and later in human (Nelkin et al., 1984). Subsequently a second gene encoding a related peptide called  $\beta$ -CGRP, was also identified in rat (Amara et al., 1985) and human (Steenburgh et al., 1985) and the peptide products were found to differ from  $\alpha$ -CGRP by 1 and 3 amino acids respectively. The calcitonin gene and production of CGRP by tissue-specific alternative splicing has been extensively studied. The primary transcript of the calcitonin gene is spliced in different tissues according to the schematic diagram below (Figure 1.3), which is adapted from Emeson et al. (1992). The gene encoding  $\beta$ -CGRP has five exons and only one transcript, (Bennett and Amara, 1992), however this gene does have a "potential" splice site and a segment similar to the calcitonin exon, suggesting a possible gene duplication as in the case of the tachykinin family.

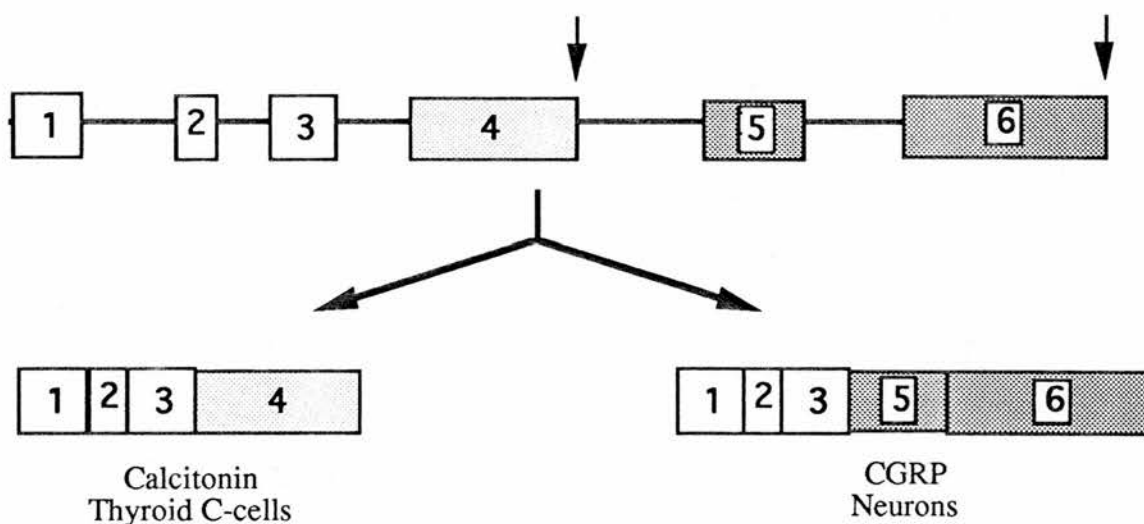
Figure 1.3. Amino acid sequence of rat  $\alpha$ - and  $\beta$ - CGRP (single amino acid difference above sequence in bold) and post-transcriptional splicing of the calcitonin gene. Open and filled boxes represent exons as numbered: (calcitonin specific exon; light stippling; CGRP specific exons; heavy stippling) and arrows show the position of splicing sites.

$\alpha$ -CGRP: Ser-Cys-Asn-Thr-Ala-Thr-Cys-Val-Thr-His-Arg-Leu-Ala-Gly-Leu-Leu-

Ser-Arg-Ser-Gly-Gly-Val-Val-Lys-Asp-Asp-Phe-Val-Pro-Thr-Asn-Val-Gly-Ser-

(Lys)

**Glu**-Ala-Phe-NH<sub>2</sub>



CGRP is also found in many areas of the central nervous system (Ishida-Yamamoto and Toyama, 1989) and the spinal cord and DRG of several species including rat and human where it is found in approximately 50% of neuronal somata by either immunohistochemistry (Skofitsch and Jacobowitz, 1985a; Gibson et al., 1984) or in

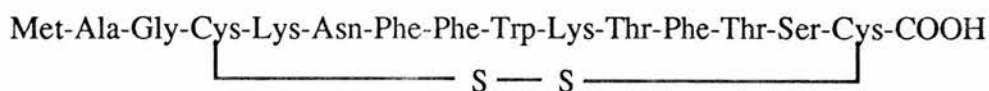
situ hybridisation (Rethelyi et al., 1989) (see also Section 5). CGRP is known to coexist with a large number of other peptides and enzymes particularly substance P (Cameron et al., 1988; Gibbins et al., 1987; Ju et al., 1987; Lee et al., 1985; Skofitsch and Jacobowitz, 1985b), but also somatostatin, dynorphin, cholecystokinin, bombesin, galanin and fluoride resistant acid phosphatase (FRAP: Carr and Nagy, 1993). Colocalised neuropeptides are known to be contained in the same secretory granules (Merighi et al., 1988) and interact in the dorsal horn (Wiesenfeld-Hallin et al., 1984; see also below). Some neuropeptide populations are thought to be discrete e.g. SP and somatostatin, suggesting that these may represent functionally different populations (see below).

### 1.3.3. Somatostatin

Somatostatin exists in two forms in mammalian tissues, somatostatin-14 and an amino-terminally extended form, somatostatin-28 encoded by the same gene (Shen, 1982; Tavianini et al., 1984). The amino acid sequence of somatostatin is depicted below (Figure 1.4). The rat and human genes are very similar and consist of two introns, the second encoding somatostatin-14 and -28 (Shen et al., 1982).

Somatostatin exerts its effects through high affinity G-protein coupled receptors which have been cloned and found in both mouse and human brain (Yamada et al., 1992).

*Figure 1.4 Amino acid sequence of somatostatin-14*



Somatostatin has been detected in 8-10% of DRG neurons (Heppleman et al., 1993; Henken et al., 1988). It is often stated that somatostatin and SP exist in separate

populations of DRG neurons (Carr and Nagy, 1993) even though some workers have found a variable degree of tachykinin and somatostatin colocalisation in neurons (Cameron et al., 1988; Ju et al., 1987) and even in the same secretory granules (Merighi et al., 1988). Somatostatin has been shown to be expressed in 8-10% of DRG neurons when localised by either immunohistochemistry or in situ hybridisation (Henken et al., 1988; Heppleman et al., 1993).

#### 1.3.4. Vasoactive intestinal peptide (VIP)

The VIP gene has been cloned from rat, mouse and human (Gozes, 1988; Lamperti et al., 1991) and has been shown to give rise to a single precursor transcript encoding both vasoactive intestinal peptide and peptide histidine isoleucine (PHI) (Nishizawa et al., 1985; Lamperti et al., 1991). There is no evidence to suggest that RNA splicing produces separate mRNAs encoding these two peptides; therefore differential tissue distributions of VIP and PHI have been attributed to post-translational processing (Lamperti et al., 1991). The gene consists of seven exons, the fourth encoding PHI and the fifth, VIP (Giladi et al., 1990), giving rise to PHI which differs from the human peptide by 4 amino acids, and an identical VIP to that in both human and mouse (Gozes, 1988; Lamperti et al., 1991).

The amino acid sequence of rat VIP is shown in Figure 1.5

*Figure 1.5 Amino acid sequence of rat vasoactive intestinal peptide*

His-Ser-Asp-Ala-Val-Phe-Thr-Asp-Asn-Tyr-Thr-Arg-Leu-Arg-Lys-Gln-Met-Ala-  
Val-Lys-Lys-Tyr-Leu-Asn-Ser-Ile-Leu-Asn-COOH

VIP has also been colocalised with substance P in autonomic nerve fibres in the cat airway (Dey, 1992). VIP is not usually expressed under normal conditions in



sensory ganglia (Noguchi et al., 1989; Nielsch and Keen, 1989; Ju et al., 1987), but is potently induced following nerve injury (Atkinson and Shehab, 1986) by an increase in VIP mRNA content of the DRG (Nielsch and Keen, 1989), whereas tachykinins are decreased. It is not known whether VIP expression occurs in neurons previously expressing other neuropeptides or in a new population, however some colocalisation of CGRP and VIP after axotomy has been reported (Ju et al., 1987).

#### 1.4 Function of primary afferent neuropeptides

##### 1.4.1. Relationship between physiological function and neurochemical content

Recently there have been attempts to find a neurochemical marker for functionally defined groups of afferents, particularly nociceptors. The colocalisation of neurochemical markers has given rise to the suggestion that these populations of neurons may each represent a functional subset of primary afferents. Neuropeptide content and neuronal size studies showed that neuropeptide-containing somata all belonged to the small ganglion cell population (CGRP was not studied: Kummer and Heym, 1986). Combined electrophysiological and immunohistochemical studies have found that while substance P and CGRP are not confined to either C- or A-fibres, approximately 50% of C-fibres contain substance P, 50% contain CGRP, whereas somatostatin-immunoreactivity is only found in neurons with axons conducting in the C-fibre range (Lawson et al., 1993; McCarthy and Lawson, 1989; McCarthy and Lawson, 1990). Substance P is also found in A-fibres, thought to be A $\delta$  afferents as the conduction velocity of substance P-immunoreactive neurons did not exceed 10ms<sup>-1</sup> (McCarthy and Lawson, 1989). Unsurprisingly, due to the range of size of soma in which CGRP immunoreactivity is found, 50% of all CGRP-immunoreactive neurons were found to belong to the A-fibre category (Lawson et

al., 1993). A correlation between neuropeptide expression and functional modality has, however, proved to be elusive (Leah et al., 1985).

Recent work has suggested that nociceptors may be the only functional modality of primary afferents which will respond to exogenous nerve growth factor in the adult animal (Lewin and Mendell, 1993); it remains to be seen whether the expression of the high affinity nerve growth factor receptor is indeed limited to this population of neurons (see below).

#### 1.4.2. Neuropeptide involvement in the transmission of nociceptive information

There is now strong evidence that some neuropeptides, particularly substance P, act as transmitters in the spinal dorsal horn in the transmission of nociceptive information.

##### 1.4.2.1. Substance P and CGRP

The postulated involvement of substance P in the central transmission of nociceptive stimuli is based upon behavioural and electrophysiological data. Intrathecal substance P elicits caudally directed scratching and biting behaviours which have been taken to be indicative of pain (Hylden and Wilcox, 1981). Substance P, neurokinin A and CGRP are known to be released in the dorsal horn on capsaicin application (Zhao et al., 1992; Gamse et al., 1979), in response to inflammatory mediators (Andreeva and Rang, 1993), by noxious stimuli (Yaksh et al., 1980; Klein et al., 1990; Morton and Hutchison, 1989) and in acute and chronic arthritis, (Duggan et al., 1987; Oku et al., 1987b; Duggan et al., 1988; Morton et al., 1989; Duggan et al., 1990; Schaible et al., 1990; Garry and Hargreaves, 1992; Collin et al., 1993). Substance P causes prolonged depolarisation in dorsal horn neurons *in vivo*, and has been shown to excite those neurons which respond to noxious

stimulation (Otsuka and Yoshioka, 1993). Neurokinin A and CGRP also exert direct excitatory effects on dorsal horn neurons and are increased upon noxious stimulation and in arthritis (Otsuka and Yoshioka, 1993). Spinal cord nociceptive transmission in the formalin test is also mediated through release of substance P and can be blocked by a specific NK<sub>1</sub> antagonist (Chapman and Dickenson, 1993).

Increases in expression of tachykinins and CGRP also occur in nociceptive second order projection neurons in the central nervous system during inflammation (Noguchi and Ruda, 1992; Sluka et al., 1992; Minami et al., 1989).

Additionally studies using immunoneutralisation (Louis et al., 1990; Elliot et al., 1989; Louis et al., 1989; Kawamura et al., 1989; Kuraishi et al., 1988), agonists (Mjellem-Joly et al., 1992; Kuraishi et al., 1991; Hylden and Wilcox, 1981) and specific antagonists (Chapman and Dickenson, 1993; Yamamoto and Yaksh, 1992; Murray et al., 1991; Yamamoto and Yaksh, 1991) have revealed an excitatory role for tachykinins and CGRP which interact with many other spinal systems such as excitatory amino acids (Nagy et al., 1993; Murray et al., 1991; Smullin et al., 1990) and prostanoids (Malmberg and Yaksh, 1992) in the spinal processing of nociceptive information.

#### 1.4.2.2. Somatostatin

In the central nervous system, somatostatin is a potent inhibitor of growth hormone release (Berne and Levy, 1990), inhibits the release of substance P from primary afferent terminals (Gazelius et al., 1981) along with other spinal systems such as opioids (Aimone and Yaksh, 1989) and inhibits the responses of dorsal horn neurons (Otsuka and Yoshioka, 1993). Somatostatin has been postulated to mediate the spinal transmission of thermal noxious information as it was found not be released by other noxious or innocuous stimuli (Morton et al., 1989). Intrathecal administration of somatostatin and anti-somatostatin antisera have further supported

the hypothesis that this neuropeptide is involved in thermal and not mechanical nociceptive processes (Ohno et al., 1988; Wiesenfeld-Hallin, 1986). However somatostatin and its stable analogue, sandostatin (Octreotide, Sandoz), have also been shown to inhibit both phases of the formalin test (Chapman and Dickenson, 1992), whereas somatostatin antiserum also inhibits the second phase (Ohkubo et al., 1990). The identical actions of the agonists and the antiserum indicate that the role of somatostatin in nociceptive transmission is not as simple as previously thought. These studies may well be complicated by the known neurotoxic effects of somatostatin, and the problems associated with intrathecal drug administration (see below).

#### 1.4.2.3. Vasoactive intestinal peptide

Vasoactive intestinal peptide is expressed in various nuclei in the central nervous system, particularly the suprachiasmatic nucleus of the hypothalamus where it may be involved in the mediation of the light-dark regulation of the circadian pacemaker (Shinohara et al., 1993). VIP levels are unchanged in the spinal cord of polyarthritic rats (Chery-Croze et al., 1985), and while there are VIP containing systems in the lumbar spinal cord, it is probable that these represent the central terminals of primary afferent nerves from visceral afferents (Anand et al., 1983). As with somatostatin, the role of VIP in nociceptive processing is unclear, as although it is not normally expressed in primary afferents, VIP is known to excite dorsal horn neurons when applied directly to both intact spinal cord and slice preparations (Jeftinija et al., 1982) and causes analgesia in the tail flick test when used intrathecally (Komisaruk et al., 1992).

## 1.5. A note on the intrathecal administration of neuropeptides

A large number of studies have used the behavioural responses following intrathecal neuropeptides as a measure of their involvement in nociceptive transmission. It must always be borne in mind that many intrathecally administered neuropeptides are neurotoxic to motoneurons and therefore behavioural analysis may be confounded by the impairment of motor activity (Gaumann et al., 1990; Mollenholt et al., 1990). In the case of somatostatin, sham operations have also been shown to affect the effectiveness of the drug (Dirkson et al., 1990).

## 1.6. Peptidergic primary afferents and inflammation

### 1.6.1 Neurogenic inflammation

Neurogenic inflammation is the term coined to describe the effects of vasodilation and plasma extravasation seen on antidromic stimulation of sensory nerves, and is thought to result from the release of active substances from peripheral sensory nerve terminals. This has been rigorously reviewed recently (Holzer, 1988). The involvement of the sensory nervous system in inflammatory responses was first proposed by Lewis in his experiments on the axon reflex (1959). Antidromic electrical stimulation or application of substances such as mustard oil to the skin, which directly activates sensory nerves, results in local vasodilatation and extravasation of plasma. That this effect was directly dependent upon an intact sensory nervous system was elegantly shown by Jancso et al. (1967), who also showed that the effect was mediated primarily through capsaicin-sensitive primary afferents, i.e. unmyelinated polymodal C-fibres. Recently A $\delta$  fibres have also been

shown to mediate neurogenic vasodilation (Janig and Lisney, 1989). Neurogenic inflammation is only evident two weeks postnatally, which has been postulated to be due to a delay in development of either the sensory or postganglionic sympathetic nervous systems (Fitzgerald and Gibson, 1984; Gonzales et al., 1991). Lewis again suggested that this effect was due to the release of various substances from the terminals of sensory nerves. This was based on Dale's principle (1935) that the same mediators should be released from both the central and peripheral terminals of sensory neurons, and is supported by the finding that 90% of substance P translated in DRG is transported to the periphery (Harmar and Keen, 1982). There is now strong evidence for the release of many neuropeptides from primary afferent neurons, including substance P, CGRP, somatostatin, neurokinin A, VIP and adenosine triphosphate, by both antidromic stimulation and the action of other inflammatory mediators such as bradykinin, 5-HT and histamine on nerve terminals (Holzer, 1988; Geppetti et al., 1990; Hua and Yaksh, 1993).

#### 1.6.2. Interactions between neuropeptides and the vascular and immune systems

Many neuropeptides, particularly substance P and CGRP, have been shown to interact in the production of vasodilatation and plasma extravasation. Substance P and CGRP are both known to be potent vasodilators in their own right (Gazelius et al., 1981; Brain et al., 1985; Pernow, 1985), and neurokinins A and B also cause transient vasodilatation in the rat knee joint (Lam and Ferrell, 1993b). While CGRP does not cause plasma extravasation independently, unlike substance P (Louis et al., 1989), it can potentiate that caused by substance P in both the skin (Gamse and Saria, 1985; Newbold and Brain, 1993) and the joint (Cambridge and Brain, 1992; Cruwys et al., 1992). This is mediated through a combination of inhibition of SP degradation in both the periphery and the spinal cord (le Greves et al., 1985; Mao et al., 1992) and potentiation of SP release (Oku et al., 1987a) by CGRP. Somatostatin

and galanin can abolish the extravasation produced by capsaicin, possibly acting presynaptically to inhibit release of pro-inflammatory mediators such as substance P and CGRP (Green et al., 1992). In joints, various models of acute inflammation have been shown to have a neurogenic component (Lam and Ferrell, 1991) with only a slight contribution from the sympathetic nervous system; acute inflammation may even attenuate sympathetic and enhance neuropeptide-mediated vascular effects in the joint (Lam and Ferrell, 1993a). Vascular effects in the knee joint induced by electrical antidromic nerve stimulation and carrageenin-induced inflammation can both be blocked by a substance P antagonist, further implicating neuropeptides in inflammation (Lam and Ferrell, 1989; Khoshbaten and Ferrell, 1990). In chronic adjuvant-induced arthritis both myelinated and unmyelinated afferents have been implicated (Levine et al., 1985a;1985b;1985c; Levine et al., 1986) together with postganglionic sympathetic efferents.. The role of the sympathetic nervous system in inflammatory processes is further discussed in Section 6.

Neuropeptides are also known to have potent trophic effects, and may also play a role in wound healing (Holzer, 1988). The effects of the four neuropeptides under study here on various arms of the immune system are summarised in Table 1.1.

*Table 1.1 Neuro-immune interactions*

Neuropeptide	Effect	Reference
Substance P	↑ Lymphocyte proliferation,	1, 2,3
	↑ PMN migration/activation,	4,7
	↑ neutrophil adherence,	5
	↑ histamine release,	6
	↑ phagocytosis	3
	↑ Neutrophil adherence,	5
CGRP	activation	8
Somatostatin	↓ lymphocyte proliferation,	1
	↑/- PMN migration	4,7
	↓ macrophage cAMP accumulation	6
	↓ lymphocyte proliferation/ migration,	1,3
VIP	↓ lymphoblastic transformation	3

References: 1. Tang et al., 1992; 2. Kavelaars et al., 1993; 3. O'Dorisio et al., 1985; 4. Partsch and Marucci-Cerinic, 1992; 5. Zimmerman et al., 1992; 6. Krumins and Broomfield, 1993; 7. Kolasinski et al., 1992; 8. Richter et al., 1992.

### 1.6.3. Primary afferents and arthritis

Following the identification of the interactions between the nervous, vascular and immune systems detailed above, an interest in the possible neuronal involvement in arthritis has developed. The observation that patients with disruption of the innervation of a limb due to poliomyelitis (Glick, 1967) or stroke (Thompson and Bywaters, 1962) did not develop rheumatoid arthritis in the affected limb has



concentrated investigation on the peripheral nervous system. Since the work of Levine and colleagues in the mid 1980s there has been a growing recognition of the involvement of specific primary afferent modalities and some of the neuropeptides they synthesise, in the maintenance and exacerbation of inflammatory arthritis, and numerous hypotheses on the nervous system and arthritis have been suggested (Green et al., 1993a; Garrett et al., 1992; Kidd et al., 1990; Kidd et al., 1989b; Fitzgerald, 1989; Levine et al., 1985b; 1985c). A recent report of a hemiplegic patient with a sparing of the paralysed limb from psoriatic arthritis combined with substance P levels in synovial fluid increased and those in synovium decreased only in the affected knee, further supports a role for primary afferent neuropeptides in clinical inflammatory arthritis (Veale et al., 1993; see also below).

#### 1.6.3.1. Primary afferent neuropeptides in rheumatoid arthritis

A number of the speculations on the involvement of primary afferent neuropeptides have arisen as a result of the discovery of various neuropeptides in the synovial fluid of patients with rheumatoid arthritis. Substance P has been found to be elevated in the majority of studies, but the absolute levels found in both control and rheumatoid patients varies enormously (Table 1.2) which may be due to differing antibody specificities and cross-reaction with other tachykinins. There is a gradient in patients with rheumatoid arthritis between the synovial fluid and synovial tissue, suggesting that there is active release of this neuropeptide into the joint cavity (Menkes et al., 1993).

*Table 1.2. Substance P- and tachykinin-like immunoreactivity in synovial fluid from patients with rheumatoid arthritis. Amounts have been converted to pg/ml where necessary and are shown as means  $\pm$  S.E.M. (n). Controls are all patients with osteoarthritis except for Larsson et al., where traumatic arthritis was used. ND = not detectable.*

Reference	Control	Rheumatoid arthritis
Marshall et al., 1990	1034 $\pm$ 117 (15)	947 $\pm$ 830 (24)
Larsson et al., 1991 (SP)	ND (13)	ND (18)
Larsson et al., 1991 (NKA)	6 $\pm$ 0.8 (13)	4 $\pm$ 0.9 (18)
Marabini et al., 1991	16171 $\pm$ 1751 (12)	57947 $\pm$ 13206 (8)*
Aloe et al., 1992a	70 $\pm$ 10 (6)	135 $\pm$ 23 (8)*
Hernanz et al., 1993	70 $\pm$ 4 (20)	120 $\pm$ 11 (40)*
Matucci-Cerinic et al., 1993	12 $\pm$ 13 (10)	43 $\pm$ 17 (26)*
Menkes et al., 1993	52 $\pm$ 9 (9)	157 $\pm$ 15(19)

CGRP, VIP and somatostatin have been investigated to a lesser extent and also show variation in the levels found by different groups. CGRP is the only peptide apart from substance P to show a consistent rise, however it has only been examined in two groups of patients (Table 1.3). Hernanz et al., (1993) have recently shown a strong positive correlation between the CGRP and substance P content in synovial fluid, as would be expected from their frequent colocalisation. Interestingly, there was also a strong correlation between the ratio of CGRP and substance P and the Mallya index, an index of rheumatoid disease activity analysing morning stiffness, pain, grip strength, articular index (involvement), haemoglobin and erythrocyte sedimentation rate (Mallya et al., 1982). Larsson et al. (1991) also found a correlation, in this instance negative, between CGRP and neurokinin A content of synovial fluid. Recently one of the enzymes involved in the degradation of

substance P, neutral endopeptidase (3.4.24.11) (NEP) has also been found to be elevated in both the plasma and synovial fluid of patients with rheumatoid arthritis (Matucci-Cerinic et al., 1993; Appelboom et al., 1991) compared to patients with osteoarthritis. It has been speculated that the levels of this enzyme may be a marker of the inflammatory process per se, rather than directly linked to the levels of substance P in the synovial fluid as angiotensin converting enzyme, also known to degrade substance P was unaltered in either the plasma or synovial fluid of patients with RA (Matucci-Cerinic et al., 1993). The localisation and activity of NEP is of increasing interest as it is also known to have a potent degrading action on enkephalins (Hughes et al., 1975), consequently the relative proportions of algescic and analgesic neuromodulators may be, in part determined by the activity of NEP. The finding of increased NEP levels in synovial fluid suggests that the high levels of substance P found in the synovial fluid is not a result of impaired enzymatic degradation in RA contributing to the severity of arthritis but rather due to an active secretion (Menkes et al., 1993)

The origin of the substances found in the synovial fluid of patients with RA, shown in Tables 1.2 and 1.3 is as yet unclear. Neuropeptides are thought to be of sensory origin as neuropeptide containing nerves are found in synovium (see below) and bone (Bjurholm et al., 1988) and are known to be released in the periphery (Holzer, 1988). This is a local effect as plasma neuropeptides are, in general, unaltered in these patients (Lygren et al., 1986; Matucci-Cerinic et al., 1993; Hernanz et al., 1993; Menkes et al., 1993; but see also Marshall et al., 1990). NEP is produced by fibroblasts, and in RA its probable source are fibroblast-like synoviocytes or chondrocytes (Matucci-Cerinic et al., 1993) whereas NGF is produced by perivascular cells and lymphocytes in synovitis (Aloe et al., 1992a)

*Table 1.3. Other peptides found in synovial fluid of patients with rheumatoid arthritis. Units are as in Table 1.2 except for neutral endopeptidase (NEP; pmol/ml/min) and expressed as means  $\pm$  S.E.M. except Lygren et al., 1986 which are medians (range). ND = not detectable; NGF = nerve growth factor.*

Reference	Peptide	Control	RA
Lygren et al., 1986	VIP	33 (8-173)	296 (56-565)*
	Somatostatin	66 (0-229)	90 (0-147)
Larsson et al., 1991	VIP	0.7 $\pm$ 0.6	0.8 $\pm$ 0.6
	CGRP	59 $\pm$ 10	144 $\pm$ 7
Marabini et al., 1991	Somatostatin	22275 $\pm$ 7698	37344 $\pm$ 2293*
Aloe et al., 1992b	NGF	ND	4000 $\pm$ 1000
Hernanz et al., 1993	VIP	16 $\pm$ 2	30 $\pm$ 3*
	CGRP	76 $\pm$ 11	152 $\pm$ 19*
Matucci-Cerinic et al., 1993	NEP	15 $\pm$ 11	134 $\pm$ 32*

The significance of the changes in neuropeptide content of synovial fluid in patients with rheumatoid arthritis is as yet unclear; however as outlined above and below these neuropeptides can have potent effects on the immune system and synovium and may therefore interact to exacerbate inflammation or promote repair, depending on the relative proportions of agents present.

#### 1.6.4. Neuropeptidergic nerves and synovium

It has been recently stated (Mapp et al., 1990) that the innervation of human synovium has been poorly demonstrated. However, in a comprehensive review of the joint from Gardner (1950) using serial sectioning of the human foetus, there is a clear description of innervation of various regions of the joint capsule, the infrapatellar fat pad and numerous blood vessels. More particularly, innervation of the synovium and epiphyses is also noted. In the experimental animal

neuropeptidergic nerves have been localised to the synovial lining and sublining tissue, periosteum, marrow and epiphyseal growth plates in the normal rat (Hukkanen et al., 1992) and mouse (Buma et al., 1992). That these nerves, or rather a proportion thereof, contain neuropeptides was first described by Gronblad et al., (1988) and this study also suggested an increased release of these peptides in rheumatoid disease as manifest by decreased staining intensity compared to osteoarthritic and normal controls. Neuropeptide containing nerves appear to be located primarily around synovial blood vessels, perhaps reflecting the potent effects of neuropeptides on the vasculature (vide supra). This has also been seen in the joints of rats with adjuvant arthritis in a study which demonstrated additionally that levels of both substance P and CGRP are raised in arthritic joints when compared to controls (Ahmed et al., 1993). Subsequently, studies involving both animal models and human rheumatoid synovium have demonstrated a loss of neuropeptide containing nerves in inflamed synovium. Initially this was attributed, as above, to an increased release of the peptides in these inflammatory conditions, however using double staining techniques for neural proteins and neuropeptides it was shown that there was an actual loss of fine nerve terminals in the superficial synovium (Mapp et al., 1990; Pereira da Silva and Carmo-Fonseca, 1990; Konttinen et al., 1990). The destruction of these nerve terminals may be attributable to the local production of proteolytic enzymes by inflammatory cells which are known to be in close proximity to neuropeptide immunoreactive nerves in normal synovium (Konttinen et al., 1992; Hukkanen et al., 1991). This has led to the suggestion that rheumatoid synovium has a disturbed neural control (Pereira da Silva and Carmo-Fonseca, 1990) which is supported by the observation that synovial cells from patients with rheumatoid arthritis behave differently in culture than those from controls, spontaneously releasing interleukin 1 $\beta$  and prostaglandin E<sub>2</sub> (Goto et al., 1990). There have, naturally been studies to elucidate the role of neuropeptides in synovium and as usual, the most studied neuropeptide is substance P which has been shown to cause

synoviocyte proliferation (Lotz et al., 1987) and stimulate collagenase production from synoviocytes (Partsch et al., 1991; Lotz et al., 1987) and monocytes (Lotz et al., 1988).

#### 1.6.5. Location of neurokinin receptors

There has recently been a rationalisation of the nomenclature used for the receptors for the tachykinins, which are termed NK<sub>1</sub>, NK<sub>2</sub> and NK<sub>3</sub> on the basis of their affinities for SP, NKA and NKB. These are summarised in Table 1.4.

It can be seen from these values that substance P has a greater affinity for the NK<sub>1</sub> receptor, NKB has a much higher affinity for NK<sub>3</sub> receptor and NKA has equal affinities for the NK<sub>1</sub> and NK<sub>2</sub> receptors., but a greater affinity for the NK<sub>2</sub> than substance P.

There have been numerous studies of the localisation of the tachykinin receptors in the central nervous system and in peripheral tissues using autoradiographic binding studies and mRNA localisation.

*Table 1.4. Half-maximal effective concentrations (EC<sub>50</sub>) of tachykinins on receptor subtypes expressed from recombinant mRNA in Xenopus oocytes (taken from Otsuka and Yoshioka, 1993; refs. 875, 1206, 1442).*

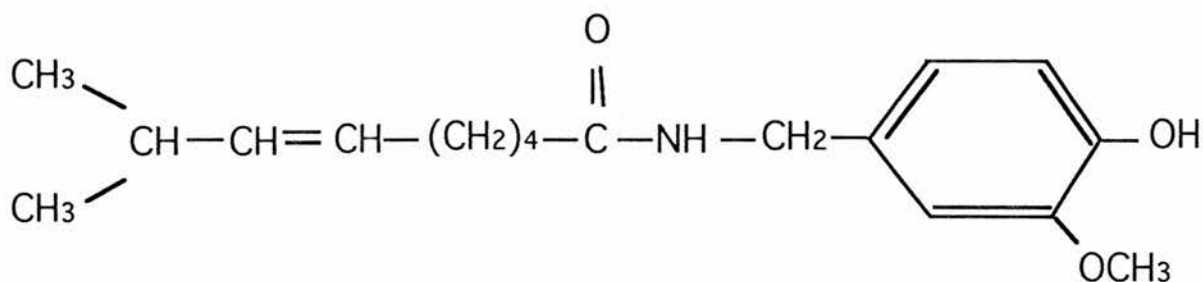
Receptor subtype	EC <sub>50</sub> . M		
	Substance P	Neurokinin A	Neurokinin B
NK <sub>1</sub>	3x10 <sup>-9</sup>	5x10 <sup>-8</sup>	3.6x10 <sup>-7</sup>
NK <sub>2</sub>	8.7x10 <sup>-6</sup>	6x10 <sup>-8</sup>	3.4x10 <sup>-7</sup>
NK <sub>3</sub>	1.9x10 <sup>-7</sup>	2.9x10 <sup>-8</sup>	4.2x10 <sup>-10</sup>

The NK<sub>3</sub> receptor is the only one not found in peripheral tissues. The NK<sub>1</sub> receptor is more widely distributed than the NK<sub>2</sub> (Otsuka and Yoshioka, 1993), and is the only receptor found in skin. Substance P binding sites showing characteristics of the NK<sub>1</sub> receptor have been localised to perivascular sites in human synovium (Walsh et al., 1992) and under inflammatory conditions, the NK<sub>1</sub> receptor is increased in vascular sites and lymph nodes (Mantyh et al., 1989) and in rheumatoid synovium (Garrett et al., 1992).

### 1.7. Capsaicin

Capsaicin (8-methyl-N-vanillyl-6-nonenamide) is a natural extract which is the pungent element of the capsicum family. Its structure is shown in Figure 1.6.

*Figure 1.6. The structure of capsaicin*



It has been known for some time that this substance has neurotoxic effects on peripheral sensory neurons. There have been few reports of an action of capsaicin upon the central nervous system (Buck and Burks, 1986; Fitzgerald, 1983; Sivam and Krause, 1992). Indirect pharmacological evidence has suggested that capsaicin acts through a specific receptor (Holzer, 1988; Szallasi and Blumberg, 1989) to

cause intracellular calcium accumulation through which it is thought to exert its many actions. Both the route of administration of capsaicin, and age of the animal is of importance to the action of capsaicin (Fitzgerald, 1983) and the extent of the lesion caused. Capsaicin has been administered systemically either to the neonate or the adult, intrathecally, or directly onto the peripheral nerve or its target e.g. skin. The action of capsaicin upon the nervous system when injected subcutaneously in the neonate, or applied directly to peripheral nerve are the only methods of administration used in this thesis, and therefore are the two routes which will be considered in detail here.

### 1.7.1. Systemic administration of capsaicin

#### 1.7.1.1. Neuroanatomical effects of systemic capsaicin administration

Systemic administration in the neonatal rat (postnatal day 2; 50mg/kg) results in a loss of up to 95% of C-fibres (Jancso et al., 1977); lower doses cause axon terminal degeneration. Higher doses are thought to cause loss of A $\delta$  fibres also; this effect varies between workers (Wall et al., 1982b; Lawson and Nickels, 1980; Nagy and Hunt, 1983). However all workers report a small percentage (~5%) of C-fibres which are capsaicin-insensitive. Systemic administration in the adult causes no peripheral sensory neuronal degeneration (Joo et al., 1969), however there is a reduction in the numbers of primary afferent neurons expressing these neuropeptides (Lynn, 1987). This effect has also been observed in DRG cell culture following capsaicin (Jeftinija et al., 1992). Administration in the adult has also been reported to result in damage to the hypothalamus (Jancso-Gabor et al, 1970; Szolcsanyi et l. 1971).



### 1.7.1.2. Behavioural effects of systemic capsaicin

One of the reasons why capsaicin has become such a popular pharmacological tool was an initial report of its ability to impart analgesia when neonatal rats were treated systemically with 50mg/kg capsaicin (Holzer et al., 1979). However subsequent investigations of the effect of neonatal and adult capsaicin treatments on nociceptive thresholds have proved inconclusive, with some groups finding capsaicin analgesic in thermal tests (Nagy et al., 1980; Holzer et al., 1979) and others finding effects only on mechanical thresholds (Hayes et al., 1981; Cervero and McRichie, 1981). Subsequent work by Nagy (1983) showed a variable response to different nociceptive stimuli and also to the dose of capsaicin used, with the dose needed to produce thermal analgesia being higher than that required for mechanical analgesia.

### 1.7.1.3. Effect on neurochemical markers

Originally, there were reports that capsaicin had specific effects upon neuronal substance P, as neonatal treatment of rats causes a marked reduction in Substance P immunoreactivity in the dorsal horn and the trigeminal nucleus (Buck et al., 1982; Hayes et al., 1980; Meller et al., 1992), and in many non-neuronal sites such as skin (Holzer et al., 1982) and joint (Levine et al., 1986). The reported dorsal horn reduction is in SP rather variable, but ranges between 30-80% (Buck et al., 1982; Hayes and Tyers, 1980; Gamse et al., 1981). However, it has become more apparent that systemic capsaicin affects other primary afferent neuropeptides as well as substance P. Jancso and colleagues (1981) have shown depletion of cholecystokinin (CCK), somatostatin, fluoride resistant acid phosphatase (FRAP) and vasoactive intestinal peptide (VIP) in dorsal horn and dorsal root ganglia, and this has been confirmed by others (Nagy et al., 1981; Singer et al., 1982). Calcitonin gene-related

peptide (CGRP) has also been shown to be depleted in laminae I, II and V of the dorsal horn (Hammond and Ruda, 1991) and in DRG (Kashiba et al., 1990). This neuropeptide depletion is considered to be permanent as effects on substance P persist for up to 7 months (Cuello et al., 1981); however there is a suggestion that there may be a recovery of primary afferent neuropeptide content in the adult rat treated neonatally with capsaicin, particularly in those afferents expressing CGRP and FRAP (Hammond and Ruda, 1991). Dorsal horn peptides of non-primary afferent origin such as enkephalin, dynorphin (Sivam and Krause, 1992), neurotensin or 5-hydroxytryptamine are not affected by neonatal capsaicin (Jancso et al., 1981; Singer et al., 1982).

Systemic treatment in the adult results in an immediate fall in primary afferent substance P followed by a rise, probably due to increased synthesis in response to the insult. Higher doses of capsaicin cause depletions of SP in dorsal horn which do not recover even after 9 months, although DRG peptide levels recover much more quickly, in 4 months (Gamse et al., 1981).

#### 1.7.1.4. Effect on physiological responses of primary afferent and dorsal horn neurons

Capsaicin is an acute excitant of particular peripheral primary afferents, and will result in excitation of polymodal nociceptors in many mammalian species including the rat (Kenins, 1982; Szolcsanyi et al., 1988). Occasionally specific A $\delta$  modalities may be excited (mechano-heat nociceptors; Szolcsanyi et al., 1988). Capsaicin also causes depolarisation of DRG neurons (Heyman and Rang, 1985).

In neonatally treated animals the majority of the C-fibres have been destroyed; consequently there is a loss of the C-fibre component to the action potential when recorded from peripheral nerve (Wall 1982b). The capsaicin-insensitive C-fibres

remaining show a normal distribution of modality and normal stimulus thresholds (Carpenter and Lynn, 1981)

The afferent denervation caused by neonatal capsaicin administration, while having no direct effect on somatosensory cells in the central nervous system, does have an indirect one on their physiological properties. It is known that neonatal C-fibre denervation leads to aberrant innervation of the superficial dorsal horn with remaining primary afferents (Nagy and Hunt, 1983; Rethelyi et al., 1986; Beal and Knight, 1987; Shortland et al., 1990). Treatment of the neonate with capsaicin reduces the number of dorsal horn neurons, from both deep and superficial layers, responsive to C-fibre stimulation (Cervero et al., 1984). This reduction is somewhat less than the total reduction in C-fibres seen following this lesion, being less than 50% rather than 90%. This study also showed decreased responsivity of dorsal horn neurons showing "wind-up", that is an increase in C-fibre response following repetitive stimulation, and a shift of the neurons which did show "wind-up" to those with smaller receptive fields and those responding to nociceptive stimuli. These neurons do not usually show "wind-up" in the normal animal (Schouenborg and Sjolund, 1982). Dorsal horn neurons are less responsive to noxious thermal stimulation, as are those in the trigeminal system (Fitzgerald, 1983), but show unaltered responses to noxious mechanical stimuli (Cervero et al., 1984).

### 1.7.2. Application of capsaicin to peripheral nerves

#### 1.7.2.1. Neuroanatomical consequences of perineural capsaicin

As with the majority of early work on the actions of capsaicin, Jancso and colleagues first showed that capsaicin could have a direct action upon axons to affect

the sensory function of the nerve (Jancso et al., 1980). The morphological consequences of perineural capsaicin were originally thought to be brief and reversible, resulting in no overall loss of primary afferents (Fitzgerald, 1983). More recent studies, using the capsaicin gap technique (Jancso and Lawson, 1990), however, have shown that perineural capsaicin will cause degeneration of 12-14% of small sized L4 DRG neurons, and that this results in profound transganglionic changes in the dorsal horn (Jancso, 1992). This treatment also causes a reduction of 30-40% in the numbers of C-fibre axons, but not A-fibres, in treated nerves (Pini et al., 1990) and an abolition of neurogenic inflammation (Pini and Lynn, 1990). Discrepancies between cell and axon counts could arise due to branching of C-fibres in the axon (McCarthy et al., 1993).

#### 1.7.2.2. Neurochemical consequences of perineural capsaicin

The degeneration of primary afferents does not seem to be restricted to one particular neurochemical population, as substance P, somatostatin, FRAP and CGRP containing neurons in DRG are reduced by 61%, 46%, 37% and 23% respectively (Holzer, 1991). These peptides are also markedly depleted in the areas of primary afferent termination in the dorsal horn (Ainsworth et al., 1981) and skin (Pini and Lynn, 1990). The changes observed by Gibson and colleagues (1982) in substance P and CCK staining in dorsal horn and skin were found to be maximal after 14 days and to persist for 4-5 months.

#### 1.8.2.3. Physiological consequences of perineural capsaicin

The actions of capsaicin upon the function of the sensory nerve have been divided into acute and chronic effects. Acute application of 1% capsaicin solution to a nerve trunk initially causes depolarisation and action potential discharge (Holzer, 1988).

Following this acute depolarisation capsaicin causes block of the C-fibre conduction which recovers, if not completely, on removal of the solution (Wall and Fitzgerald, 1981), and, in the intermediate term, results in a reduced conduction velocity in these fibres.

After two weeks, there is a large reduction in the numbers of C-fibres responding to strong heat or pressure stimulation (Pini and Lynn, 1990). Electrophysiological experiments have demonstrated that the action of capsaicin applied directly to a nerve is purely restricted to polymodal nociceptors (Pini and Lynn, 1990) and it was observed that the responses of polymodal nociceptors to electrical or heat stimulation, were invariably blocked by the application of capsaicin (Petsche et al., 1983). This effect is associated with a reduction in neurogenic inflammation in areas innervated by the treated nerve and neuropeptide levels in the skin (Pini and Lynn, 1990) and significant increases in thermal nociceptive thresholds without effect on mechanical thresholds (Fitzgerald, 1983; Jancso, 1992).

Dorsal horn neurons responses to noxious heat and C-fibre stimulation are both affected, beginning 24 hours after capsaicin application. There is a marked reduction in the number of deep and superficial dorsal horn neurons which respond to either noxious heat or peripheral C-fibre stimulation (Fitzgerald, 1982; McMahon et al., 1984).

## 1.8. Nerve growth factor

### 1.8.1. Nerve growth factor: role in the prenatal period

Nerve growth factor (NGF) was the first of a family of neurotrophic factors including brain-derived neurotrophic factor (BDNF), neurotrophin-3, -4 and -5 (NT-3, NT-4, NT-5). Nerve growth factor is required for the survival of postganglionic sympathetic neurons as immunoneutralisation of NGF in the neonatal rat results in

the destruction of sympathetic ganglia (Korsching, 1993). NGF is also crucial for the survival of a sub-population of primary sensory neurons during embryonic development; if endogenous NGF is neutralised in embryonic rats by sensitisation of the mother, up to 85% of DRG neurons are lost, in addition to all the sympathetic neurons. Initially, nerve growth factor was thought to be necessary for the development of substance P-containing neurons (Otten et al., 1980) although NGF deprivation has been shown to have effects on the ganglionic content of various primary afferent neurochemical markers including somatostatin, FRAP and VIP (Goedert et al., 1984; Otten, 1984). Augmentation of endogenous NGF levels by administration of exogenous NGF will prevent the naturally occurring cell death normally seen in sensory neurons around birth and increase neurotransmitter levels in DRG (Otten et al., 1980; Vedder et al., 1993) without increasing neuronal numbers. The fact that a large number of neurochemically different primary afferents appear to be dependent upon NGF during embryogenesis suggests that the effects on the neuropeptides described above are related to the direct effects of NGF on the neurons rather than an effect on the expression of the neuropeptide *per se*. It is possible that the population of primary afferent neurons which are dependent on NGF are those which are capsaicin-sensitive, as NGF is known to reverse the effects of neonatal capsaicin administration (Otten et al., 1983).

#### 1.8.2. Role of NGF in the adult animal

In the adult animal, chronic systemic deprivation of NGF does not result in cell death although neuropeptide levels are decreased (Schwartz et al., 1982). Chronic nerve section or crush does however result in the loss of approximately 20% of the axotomised neurons (Johnson and Yip, 1985) and this can be reversed by exogenous nerve growth factor (Fitzgerald et al., 1985). Postnatal deprivation of NGF results in the loss of one sensory modality, the A $\delta$  high-threshold mechanoreceptor, by

conversion to D-hair afferents (Ritter et al., 1991; Lewin et al., 1992a; 1992b) and increased NGF available in the periphery results in increased spinal cord responsiveness to nerve simulation (Lewin et al., 1992c). Using administration of both anti-NGF and NGF (albeit in high doses) Ritter and colleagues have suggested that NGF exerts its effects specifically upon nociceptive afferents and does not affect other sensory modalities (Ritter et al., 1993). Intraperitoneal injections of NGF have also been shown to result in a profound thermal and mechanical hyperalgesia (Lewin and Mendell, 1993); these results however have been criticised for the extremely large doses of NGF used (1mg/kg).

NGF has also been investigated with regard to its role in the regulation of neuropeptide expression. Both *in vitro* and *in vivo*, NGF has been shown to increase substance P, neurokinin A (Vedder et al., 1993; MacLean et al., 1991), CGRP (Delree et al., 1992; Lindsay et al., 1989) and mRNA levels encoding these neuropeptides (Lindsay and Harmar, 1989). NGF has no effect on the expression of somatostatin, whereas BDNF appears to regulate the levels of both somatostatin and its mRNA in cortical neurons (Nawa et al., 1993). Following nerve injury, levels of substance P are reduced whereas VIP is increased (Anand et al., 1991; Noguchi et al., 1989; Atkinson and Shehab, 1986). It was initially suggested that this was a reciprocal regulation through NGF (Nielsch and Keen, 1989); subsequently the increase in VIP expression in culture has been shown to be independent of NGF (Mulderry and Lindsay, 1990). However injured nerve is known to express NGF and other neurotrophins (Andeev et al., 1993), and interleukin -1 (Rotshenker et al., 1992) which may induce NGF synthesis in this situation as in inflammation (see below).

The effect of NGF on substance P expression is thought to be mediated through direct regulation of gene transcription through NGF responsive regions in the PPT promoter region (Gilchrist et al., 1991; Helke et al., 1990). This may also be the case for CGRP as there is a sequence similar to that found to be responsive to NGF

in the PPT gene in the CGRP promoter region (Gilchrist et al., 1991). NGF may also exert regulatory effects through the induction of other transcription factors such as c-fos, c-jun, NGFI-A or NGFI-B, all of which are known to be induced by NGF in rat pheochromocytoma cell lines (see Section 8). The possible role of these immediate early genes and other transcription factors in the regulation of primary afferent neuropeptide expression is discussed in Section 8.

### 1.8.3. The significance of NGF in adjuvant arthritis and the expression of primary afferent neuropeptides.

Nerve growth factor is of interest in inflammatory processes as neuropeptide expression is known to be altered in inflammatory states. Inflammatory mediators such as tumour necrosis factor and interleukin-1 $\beta$  stimulate the production of NGF (Hattori et al., 1993; Lewin and Mendell, 1993), retrograde transport of NGF is increased in the sciatic nerve during the first 2 days of adjuvant inflammation (Donnerer et al., 1992), and NGF levels are increased in joints in both experimental and rheumatoid arthritis (Aloe et al., 1992a; 1992b). The increased content of substance P and CGRP in sensory nerves innervating inflamed tissue has been attributed to increased levels of NGF (Donnerer et al., 1992)

### 1.9. In situ hybridisation: advantages of the technique

In situ hybridisation (ISH) is a potent molecular biological tool for the quantification of DNA and RNA in tissue sections rather than immobilised on solid supports (as in Northern blotting) or in solution, as ISH allows DNA or mRNA to be precisely localised to the cellular site of transcription (Hofler, 1990). This is of particular use in studying heterogeneous cell populations such as exist in the dorsal root ganglion. This technique was first developed approximately 25 years ago and



has been extensively used to localise DNA, and more recently mRNA to individual cells. Use of radioisotopically labelled probes and computer-aided analysis systems in ISH has also provided a reliable method of quantification of mRNA levels in individual cells either compared to RNA standards, or as in the studies described here, to basal expression in normal animals, and has shown an excellent correlation with the estimation of mRNA levels as assessed by Northern analysis with other mRNAs (Segerson et al., 1987).

#### 1.10. Aims of the thesis

Previous work has identified a possible role for the primary afferent neuron and neuropeptides in both the maintenance and spread of clinical and experimental arthritides. However, these studies have been complicated by the use of the polyarthritic rat model which suffers profound systemic immune stimulation and subsequent illness as described in Section 1.1. While a number of studies have shown increased DRG content of neuropeptides in this model, it is difficult to attribute these changes to the arthritic process *per se*. Using a modified adjuvant-induced monoarthritis, increases in SP and CGRP were found only in innervating DRG (Smith et al., 1992). However this study and others examining peptide content have not identified whether the observed changes are attributable to increased synthesis, or decreased axonal transport and/or peripheral release. Additionally the mechanisms through which neuropeptide gene expression is regulated during inflammation are poorly understood.

The aims of this thesis were:-

1. To develop and characterise a mild modified adjuvant-induced arthritis in the rat for use in the study of primary afferent involvement in the arthritic process without the complicating effects of systemic illness. This model has also been used to

determine whether spread of arthritis is an inevitable consequence of monoarthritis and study the possible mechanisms through which spread of inflammation occurs.

2. To determine the effect of exogenous substance P on both the spontaneous and mechanically evoked activity in primary afferent nociceptors with receptors in the ankle joint in normal and monoarthritic rats.

3. To examine the expression of four neuropeptide messenger RNAs, preprotachykinin, calcitonin gene-related peptide, somatostatin and vasoactive intestinal peptide, during the development of monoarthritis and in established bilateral arthritis of the hind limbs in order to determine whether changes in neuropeptide levels are reflected in changes at the level of the mRNA.

4. To identify the contribution of the primary afferent neuron and signals from the periphery in the regulation of any observed changes in neuropeptide mRNA expression during inflammation.

5. To determine the possible role of transcription factors including immediate early genes and glucocorticoid hormone receptors in the possible regulation of these mRNA species during inflammation.

## SECTION 2. MATERIALS AND METHODS

### 2.1 Induction of arthritis

#### 2.1.1. Preparation of adjuvant

Adjuvant used for the induction of symmetrical arthritis was prepared using attenuated *Mycobacterium tuberculosis* (MAFF) suspended in paraffin oil. Suspensions of 5mg/ml or 3mg/ml were prepared and dispersed in a warm ultrasonic water bath for 30 minutes. Suspensions were well mixed before injection into the animals.

#### 2.1.2 Induction of arthritis

Han Wistar rats (male; 7 weeks; 200-250g) were used in all experiments except those involving neurotoxic lesions when younger animals were used (see section 2.4) and for the comparisons between male and females. All groups of animals were age matched. Arthritis was induced under 4% halothane anaesthesia by intradermal injection of adjuvant administered at two sites around the left tibio-tarsal joint. Monoarthritis was induced with either 0.15ml of Freund's Complete Adjuvant (1mg/ml; Sigma) or 0.05ml adjuvant prepared as described above (3mg/ml). Animals showed no difference in reaction to either adjuvant preparation. Symmetrical arthritis was induced using 0.05ml of 5mg/ml adjuvant prepared as above. Control animals were left untreated. Additional controls for vehicle itself were made by injecting 0.15ml sterile paraffin oil.

## 2.2 Assessment of inflammation

### 2.2.1 Overt indices of inflammation

Animals were weighed throughout each experiment as an assessment of general health. The joint circumference was measured using a loop of dressmakers tape three times per week for the duration of each experiment and inflammation was scored according to the overt signs discernible around the joint as follows; 0=normal, 1=mild redness, 2=moderate redness and swelling, 3=severe swelling/lesions over the joint. At the termination of each experiment, when animals were killed, left and right tibio tarsal joints were removed and processed for histological assessment of arthritis.

### 2.2.2 Histological methods

Animals were either anaesthetised with sodium pentobarbitone and killed by cervical dislocation, or were decapitated if tissue was also being collected for in situ hybridisation. For initial studies on joint histology, hind limbs were fixed by perfusion with heparinised saline (10ml; 500 Units/kg) followed by 50-100ml formal saline (0.9g NaCl; 10ml 40% formaldehyde solution; 90ml distilled water) via a cannula inserted anterogradely in the abdominal aorta until the hind limbs were fixed. Hind limbs were removed by section of tibia and fibula, post-fixed in 10% formal saline for 2 weeks. In studies where fresh frozen tissue was required for in situ hybridisation, hind limbs were removed and immersion-fixed for at least 4 weeks in formal saline. Tissues were then decalcified in Gooding and Stewart's solution (15% formic acid; 5% formaldehyde; 80% distilled water) for at least two weeks. After decalcification, tissues were trimmed closely around the tibio tarsal joint and placed in 5% gelatin overnight at 37°C. Blocks for sectioning were made

in 10% gelatin and frozen in Arcton 12 (BOC) cooled to -70°C with liquid nitrogen. 20µm sections were cut from each joint using a cryostat (SLEE), mounted on glass slides, counterstained with haematoxylin and eosin and sealed with glass coverslips. The histological inflammation was assessed microscopically and scored for histological inflammation for both left and right limbs (classified as:- 0=normal, 1=subdermal inflammatory infiltrate, 2=mild synovitis, 3=moderate synovitis/arthritis, 4=severe arthritis; scores  $\geq 3$  were taken as indicative of arthritis).

### 2.2.3. Statistical analysis of data

Body weights and limb circumferences were compared using 1 or 2 factor analysis of variance (ANOVA) followed by Dunnetts post hoc test (Lewis, 1971; Phillips, 1971). Analogue scales such as those used for inflammation or histology were compared using Mann Whitney U test (between groups) and Wilcoxon signed rank test (paired data, within groups; Pipkin, 1984). The Null Hypothesis was rejected at the 5% probability level.

## 2.3 Neuropharmacology of fine articular afferents

### 2.3.1. In vivo rat preparation

#### 2.3.1.1 General preparation

Male Han-Wistar rats (250-300g) were used for all in vivo experiments. Both normal, untreated animals and monoarthritic rats were used in these experiments.

Rats were irreversibly anaesthetised by i.p. injection of 0.6ml/100g body weight urethane (ethyl carbamate, 25% w/v in distilled water). Tracheal and left carotid cannulae (external diameter 0.75mm) were inserted via a midline incision in the

ventral aspect of the neck. The tracheal cannula was inserted immediately below the larynx and secured with a suture. The carotid artery was closed with a ligature just proximal to the bifurcation of the common carotid artery, the cannula inserted retrogradely proximal to the ligature and secured in position with two sutures. The carotid cannula was connected to a pressure transducer (Bell and Howell, 4-442) and arterial blood pressure was monitored following signal amplification and displayed on a chart recorder (Devices M4).

The right femoral artery and vein were exposed through a mid thigh level incision on the ventral surface of the right hind limb. A cannula (external diameter 0.63mm) was inserted retrogradely into the femoral artery following distal ligation of the vessel and advanced approximately 4cm so that the tip lay at the iliac bifurcation. Positioning of the tip of the cannula was routinely checked at the end of each experiment.

Body temperature was monitored continuously via a rectal probe and maintained at  $37\pm 1^{\circ}\text{C}$  by an electronic heating blanket (Harvard Apparatus Ltd).

#### 2.3.1.2 Identification of articular innervation

The primary articulo-cutaneous ramus (PACR; the branch of the medial plantar nerve which innervates the ankle joint in the rat) was identified from the tibial nerve. The tibial nerve was exposed through a longitudinal incision in the medial aspect of the left leg from the knee to a position over the ankle joint. The musculature overlying the tibial nerve, that is semitendinosis, semimembranosis, biceps femoris and medial gastrocnemius muscles, was removed and the PACR identified where it leaves with the medial plantar nerve (Guilbaud et al., 1985). The skin around the incision was dissected from underlying connective tissues; a pool was then created

by suturing the skin flaps to a copper loop and filling the space thus created with paraffin oil at 37°C. This dissection of the surrounding skin also served to rupture any cutaneous fibres around the joint ensuring that recordings made from the PACR were from units innervating the joint capsule, adjacent fascia and skin on the medial aspect of the heel.

### 2.3.2 Recording primary afferent activity

The PACR was dissected free from surrounding tissues, cleaned of attached fat and desheathed. Afferent activity in the PACR was recorded extracellularly either from the intact PACR or from dissected filaments of the ramus. The tibial nerve was routinely cut proximally to abolish efferent activity in the PACR. Activity was recorded via bipolar platinum-iridium wire electrodes mounted on a micromanipulator, in close contact with the undersurface of the PACR. Single afferent unit receptive fields were identified by manual probing with a smooth perspex probe, tip diameter approximately 1mm, over the medial aspect of the joint capsule while recording electrically from the PACR. The electrical signal was amplified (x1000) and displayed on a storage oscilloscope (Tektronix 5113). The signal was also digitised via a digital audio processor (Sony PCM 701-ES) and stored on a videotape recorder (Sony Betamax SL-HF100 UB) for subsequent off-line data analysis. Output from the amplifier was also relayed to a loudspeaker.

### 2.3.3. Unit identification and stimulation of the receptive field

All units were identified as C-fibres by measurement of conduction velocities. The receptive field was electrically stimulated using a mechanical transducer probe consisting of a silver wire core isolated from a metal cylinder casing and action potentials recorded with the bipolar electrodes. The tip of the transducer probe was

smooth, sealed with plastic resin and approximately 1mm in diameter. Conduction velocity was calculated from the conduction distance (d) as measured *in situ* in centimetres, and the conduction time in seconds (t) for the action potential to travel from the stimulating to the recording electrodes, measured from the oscilloscope as below.

$$\text{Conduction velocity (v)} = d/t \text{ (ms}^{-1}\text{)}$$

For all units studied, v ranged from 0.4-2.5 ms<sup>-1</sup>. Only slowly adapting units were used in these experiments. All units were tested for their chemoresponsivity to capsaicin immediately before termination of each experiment.

Receptive fields were stimulated mechanically using the same mechanical transducer probe. The tip of the probe was placed over the receptive field and mechanical stimuli delivered using an electromechanical indentation generator (Somedic, Sweden). A ramp stimulus waveform of maximum displacement 600µm and duration 2 seconds was used routinely. Stimuli were applied at two minute intervals to minimise receptor fatigue. Both mechanically evoked and background activity in the units were analysed.

#### 2.3.4 Drug administration

Drugs were administered in a bolus by close arterial injection via the femoral cannula in a total volume of 0.1ml and washed in with 0.1ml normal saline (0.9% w/v NaCl in distilled water), 15s before a mechanical stimulus. Injections were completed within 1 second. As the tachykinins are known to induce tachyphylaxis, a period of at least 10 minutes was left between injections. Injections of 0.1ml saline



were used as a control of the effect of injection alone on primary afferent activity, were made before the injection of drug and repeated throughout an experiment.

All drugs were dissolved in 0.9% saline except for capsaicin (8-methyl-N-vanillyl-6-nonenamide; Sigma) which was dissolved in 10% Tween 80 (polyoxyethylenesorbitan monooleate; Sigma); 10% ethanol; 80% 0.9% saline, and were equilibrated to room temperature before injection.

### 2.3.5 Off-line analysis of primary afferent activity

The electrical recording stored on videotape was passed through an amplifier and CED 1401 filter module and fed through a WPI window discriminator. This allowed the counting of selected action potentials by appropriate positioning of a window discriminator. Each action potential falling in the window generated a pulse of 1ms duration. The number of impulses occurring in 0.1s intervals was collected and stored using a personal computer and software designed to collect over a period of up to 900 seconds (software written and kindly supplied by Dr M. Dutia, Dept. Physiology, University of Edinburgh). The point of saline or drug injection was indicated with a marker. Collected data were stored on floppy disc for analysis using the same software.

Action potential discharge frequency as a function of time was displayed as a computer-generated plot. Discharge was analysed in the control (pre-injection) period (defined as the 10-60s period immediately prior to injection), and in defined periods after drug injection. Mechanically evoked activity was defined as the number of action potentials produced per stimulus and were analysed for 1 stimulus prior to, and three stimuli after the injection. Background activity was analysed over the first 15 seconds post-injection and in 1 minute periods thereafter for 5 minutes after drug injection. These periods of time were defined using a moving cursor on the plot.

### 2.3.6 Statistical analysis of data

All data was normalised with pre-injection activity expressed as 100%. Each dose of substance P activity was compared to saline injection and also to baseline activity with respect to time using 2 factor ANOVA (Lewis, 1971).

## 2.4 Neurotoxic lesions

### 2.4.1 Perineural capsaicin lesions

A 1.5% capsaicin solution was prepared as in section 2.3.4. Capsaicin was applied perineurally to the exposed sciatic nerve according to the method of Ainsworth et al. (1981) under aseptic conditions. Male rats (Han Wistar, 150g) were anaesthetised with 4% halothane and either right or left sciatic nerves exposed at the mid-thigh. The nerve was carefully separated from the surrounding tissues and approximately 1cm cleaned of attached fascia and fat. Cotton wool soaked in 1.5% capsaicin solution was applied to the nerve trunk for 15 min while anaesthesia was maintained. Exposed tissue was covered in saline-soaked gauze to prevent dehydration. Cotton wool and excess solution were then removed, muscle and skin closed and the animals allowed to recover. Since Tween/ethanol/saline vehicle itself has effects on the physiological characteristics of primary afferents (Petsche et al., 1983), sciatic nerve exposure was used as a control for the surgery; additional controls were untreated. After 2 weeks, animals were re-anaesthetised with 4% halothane in oxygen and symmetrical arthritis induced by injection of 250µg *M.tub.* as outlined in section 2.2.1.

### 2.4.2 Neonatal capsaicin lesions

Capsaicin solution was prepared at a concentration of 10mg/ml in Tween/ethanol/saline vehicle as described above. Vehicle solution alone was also prepared. Neonatal male rats (2-4 days postnatal) were anaesthetised with 4% halothane in oxygen. Capsaicin or vehicle was injected subcutaneously in the dorsal cervical region while anaesthesia was maintained. Parent animals were also treated topically with capsaicin or vehicle to their nose and mouth; this desensitises the parents to the taste of capsaicin and prevents cannibalism of the litter. On recovery from the anaesthesia, the neonatal rats were returned to the mother until weaning. At seven weeks of age, the rats were re-anaesthetised with 4% halothane and either mono- or symmetrical arthritis induced as described previously in section 2.2.1.

### 2.5 Surgical procedures

#### 2.5.1 Adrenalectomy

All adrenalectomies were kindly performed by June Noble and Keith Chalmers.

Adrenalectomy was performed under 4% halothane anaesthesia and aseptic conditions. An incision was made in the flank immediately over each kidney, the adrenals located and removed. Sham operated animals served as controls; adrenals were located but left *in situ*. All adrenalectomised animals received 0.9% saline for drinking. Animals were allowed to recover for five days before being re-anaesthetised for induction of mono- or symmetrical arthritis (Section 2.2.1).

## 2.6 In situ hybridisation

### 2.6.1 Preparation of solutions

#### 2.6.1.1 Solutions for isolation of plasmids and cDNAs

Luria Broth: 10g bacto-tryptone (BDH); 10g NaCl (Sigma); 5g bacto-yeast extract (BDH) per litre distilled water. pH of solution = 7. Sterilise by autoclaving (20 minutes @ 121°C and 15lb/in<sup>2</sup> pressure).

Glucose/Tris/EDTA buffer (GTE): 50mM glucose (BDH); 25mM Trisma base (pH8; Sigma); 10mM ethylenediaminetetraacetic acid (EDTA; pH8; Sigma).

Sterilise by autoclaving.

5M potassium acetate solution: 60ml 5M potassium acetate; 11.5ml acetic acid; 28.5ml distilled water. Sterilise by autoclaving.

Tris/EDTA (TE) buffer: 10mM Tris (pH8); 1mM EDTA (pH8). Sterilise by autoclaving.

#### 2.6.1.2. Solutions for in situ hybridisation

Diethyl pyrocarbonate (DEP) treated water: 0.02% DEP in distilled water. Leave to stand for at least 2 hours and sterilise by autoclaving.

4% paraformaldehyde in 0.1M phosphate buffer:

20mM NaH<sub>2</sub>PO<sub>4</sub> (BDH); 80mM Na<sub>2</sub>HPO<sub>4</sub> (BDH); 40g paraformaldehyde (BDH) in 1 litre DEP water.

20xSSC (sodium-saline citrate): 3M NaCl (Sigma); 0.3M trisodium citrate (Sigma).

2xSSC (for postfixation rinsing): Dilute 50ml sterilised DEP treated 20xSSC or sodium saline citrate solution (Sigma) in 450ml DEP water.

2xHybridisation buffer: 1.2M NaCl; 20 mM Tris-Cl (pH 7.5); 0.04% Ficoll; 0.04% polyvinylpyrrolidone; 0.2% BSA; 2 mM EDTA; 0.2 mg denatured salmon sperm DNA; 0.1 mg/ml total yeast RNA; 20% dextran sulphate (w:v) and 20 mM dithiothreitol in DEP water.

Box buffer (for hybridisation): 50% deionised formamide; 20% 20xSSC; 30% DEP water.

RNase buffer: 0.5M NaCl; 0.01M Tris; 0.01mM EDTA.

### 2.6.1.3. Gel preparation

10xTris/borate/EDTA buffer (TBE) 108g Trisma base; 55g boric acid (Sigma); 40ml 0.5M EDTA (pH8).

Agarose gel: 1-1.5% agarose powder (Gibco); 1xTBE buffer; 1µl/25ml ethidium bromide solution (10mg/ml; Sigma) in distilled water. Heat to melt.

4% polyacrylamide mini-gel: 4.2g urea (Sigma), 1ml 40% acrylamide solution (Sigma), 1ml 10xTBE. Make up to 10ml with distilled water. Polymerise acrylamide with 100µl 10% ammonium persulphate (Sigma) solution and 10µl TEMED (N,N,N',n'-tetramethylethylenediamine; Sigma). Pour immediately.

### 2.6.2 Preparation of tissue

All in situ hybridisation experiments were performed on primary afferent neurons whose cell bodies are located in dorsal root ganglia, which are sited adjacent to the spinal cord. For the time course experiments, animals were decapitated at various times after adjuvant injection, ranging from 30 minutes to 8 hours for the transcription factor studies, and 8 hours to 14 days for studies on neuropeptide mRNA expression. The efficacy of perineural capsaicin lesions was assessed using

tissue taken two weeks after lesioning was performed. In all other experiments, animals were killed by decapitation fourteen or fifteen days after the induction of arthritis. Control tissue was taken from untreated animals.

Following decapitation lumbar L4/L5 dorsal root ganglia were removed by performing a laminectomy, and were rapidly frozen on dry ice. Tissue was then mounted in M1 embedding medium (Bright) on cork squares and stored at -80°C until sectioning.

### 2.6.3 Preparation of slides

Glass microscope slides with frosted ends were treated for 3 minutes in each of 0.2M HCl in DEP treated water, DEP treated water and acetone before thorough drying in an oven at 60°C. Slides were then subbed in 0.15% gelatin, 0.03% sodium azide solution in DEP treated water for five minutes and dried overnight at 60°C. Finally slides were dipped in poly-L-lysine (Sigma) solution (100mg/500ml DEP water) for 2x10 seconds, rinsed in DEP water for 10 minutes and dried at 60°C.

Sections of DRG were cut at 10µm thickness, following equilibration of the tissue to -20°C, in a Cryostat (Bright). Sections were then mounted on poly-L-lysine subbed slides and stored at -80°C until processed for in situ hybridisation. All slides with DRG sections were coded when sectioned. All subsequent analysis was performed blind to experimental group until all analysis of in situ hybridisation data was complete.

## 2.6.4 Preparation of radiolabelled probes

### 2.6.4.1 Preparation of plasmid vectors

cDNAs for the appropriate neuropeptides and immediate early genes under study were obtained from the sources listed below:-

$\beta$ -PPT, Dr A.J. Harmar;  $\alpha$ -CGRP, Dr S. Amara; somatostatin and VIP; Dr R. Goodman; AP-2; Dr P. Mitchell; NGFI-A and -B, Dr J. Milbrandt; c-jun, Dr M. Seckl and jun-D, Dr J. Quinn. The cDNA, vector and appropriate references are detailed in Table 2.1.

*Table 2.1 cDNAs used for in preparation of riboprobes by in vitro transcription*

<b>Neuropeptides</b>	<b>Vector</b>	<b>Reference</b>
$\beta$ -PPT	pGEM3	Harmar et al., 1990
$\alpha$ -CGRP	pSP64	Amara et al., 1985
Somatostatin	pSP65	Shen et al., 1982
VIP	pSP64	Nishizawa et al., 1985
<b>Transcription factors</b>		
c-jun	pGEM3	Hattori et al., 1988
jun D	pGEM3	Ryder et al., 1989
NGFI-A	Bluescript KS	Milbrandt , 1987
NGFI-B	Bluescript KS	Milbrandt, 1988
AP-2	Bluescript SK+	Mitchell et al., 1991

Competent *E. coli*. (HB101) were prepared using the calcium chloride method (Sambrook et al., 1989). A colony of HB101 was transferred to 50ml of Luria Broth (LB) medium and grown for 2-3 hours at 37°C with vigorous shaking. Cells were then cooled rapidly on ice, transferred to a sterile centrifuge tube and spun for 5 minutes @ 4000 r.p.m at 4°C. The medium was drained and the pellet resuspended in 10ml ice cold 0.1M calcium chloride solution. The solution was left to stand on ice for 10 minutes, respun @ 4000 r.p.m. at 4°C, the calcium chloride drained and the pellet resuspended in 2ml of fresh calcium chloride. The competent cells were then stored on ice until transformed with the appropriate plasmid containing the required cDNA and an ampicillin resistance gene.

Transformation was carried out using a heat shock method adapted from Sambrook et al. (1989). 100µl of cells were placed in each eppendorf tube and either the plasmid containing the required cDNA or a control plasmid vector with no cDNA were added. The mixture was stored on ice for 20 minutes, heat shocked in a water bath at 42°C for exactly 90 seconds and stored on ice. The cells were then plated out onto LB agar plates (15g/L LB) containing 100µg/ml ampicillin (Sigma). Plates were grown overnight at 37°C.

A single colony containing the plasmid vector and cDNA was then used to inoculate a 500ml culture of LB medium containing 100µg/ml ampicillin and grown overnight at 37°C with vigorous shaking. Plasmids were then isolated according to Sambrook et al., 1989. Cultures were spun (4000 r.p.m.; 15 mins; 4°C) and the pellet resuspended in 18ml glucose/Tris/EDTA buffer (pH8). Cells were lysed with 2ml lysozyme (10mg/ml in 10mM Tris pH8) and 40ml 1%SDS/ 0.2M sodium hydroxide solution, and then neutralised with 5M potassium acetate solution. Following centrifugation and filtration to remove the precipitate of bacterial proteins/SDS complexes, chromosomal DNA and high molecular weight RNA, plasmid DNA was precipitated using 0.6 volumes of isopropanol (BDH). Following



centrifugation (5000 r.p.m.; 15 min; room temp.), the DNA pellet was rinsed in 70% ethanol and allowed to dry before dissolving in 3ml of Tris. EDTA buffer (pH8).

#### 2.6.4.2 Purification of circular DNA using ethidium bromide-caesium chloride gradients

Plasmid DNA was purified according to the method of Sambrook et al. (1989). 1mg/ml of caesium chloride (Boeringer Mannheim) and 0.8ml/10ml ethidium bromide (Sigma; 10mg/ml) were added to the Tris/DNA solution, sealed in Beckman Quickseal ultracentrifuge tubes and centrifuged overnight at 70 000 r.p.m. at 20°C. Plasmid DNA bands were then recovered from the centrifuge tube, and ethidium bromide extracted using equal volumes of water saturated butanol. Plasmid DNA was then precipitated with 3 volumes of 70% ethanol, spun at 10 000 r.p.m. for 15 mins at 4°C, rinsed in 70% ethanol, air dried and resuspended in Tris/EDTA buffer. Concentration of DNA was determined spectrophotometrically at 260nm.

#### 2.6.4.3 Template DNA

Plasmid dissolved in Tris.EDTA buffer (pH8) was digested with the appropriate restriction enzyme as in Table 2.2. Digests were performed in a total volume of 20µl in DEP treated eppendorf tubes with 2µl 10x restriction buffer, 1 unit of restriction enzyme and 17µl plasmid solution. 1µl of digested plasmid was electrophoresed on a 1% agarose gel to verify linearisation.

#### 2.6.4.4 *In vitro* transcription of complementary riboprobes

Probes were transcribed *in vitro* from linearised plasmid constructs containing cDNAs encoding the neuropeptides and immediate early genes. Linearised plasmids

were incubated in a total volume of 10µl containing [35S]-UTP (800 Ci/mmol, Amersham International, UK ) and "cold" UTP to a total concentration of 20µM. CTP, ATP and GTP were also present in excess. The reaction mixture also contained  $1 \times 10^{-7}$ M dithiothreitol (Sigma), RNase inhibitor (Gibco) and the appropriate polymerase as summarised in Table 2. After incubation at 37°C or 40°C for 60 minutes, transcription was halted by incubation with 1 unit DNase I (RNase free, GIBCO) for 10 minutes at 37°C. Probes were then extracted in phenol: chloroform: isoamyl alcohol and RNA precipitated using glycogen, ammonium acetate and 100% ethanol at -80°C for 10 minutes. Following centrifugation @ 14000 r.p.m., pellets were dried under vacuum and resuspended in DEP water. Neuropeptide probes were labelled to a specific activity of  $3-5 \times 10^8$  Ci/mmol and immediate early genes to a specific activity  $6-10 \times 10^8$  Ci/mmol depending upon probe length. The length, appropriate restriction enzymes and RNA polymerases for each of the riboprobes is detailed in Table 2.2.

#### 2.6.4.5. Verification of probe labelling

1µl of RNA solution was counted in 5ml scintillation fluid in a beta counter to estimate the total activity of the probe. 1µl of RNA was also electrophoresed on a vertical 4% polyacrylamide denaturing gel, and exposed against β-max film (Amersham International) for 3-16 hours to check for incorporation of nucleotide and any degradation of the probe. The remaining RNA was stored at -20°C in hybridisation buffer.



*Table 2.2. Restriction enzyme used for plasmid linearisation, RNA polymerase and riboprobe length for the mRNAs under study*

<b>Neuropeptides</b>	<b>Probe length (nucleotides)</b>	<b>Restriction enzyme</b>	<b>Polymerase</b>
$\beta$ -PPT	441	EcoR1	SP6
$\alpha$ -CGRP	450	EcoR1	SP6
Somatostatin	450	HindIII	SP6
VIP	350	PvuII	SP6
<b>Transcription factors</b>			
c-jun	732	SphI	T7
jun D	404	EcoRI	SP6
NGFI-A	230	EcoRI	T7
NGFI-B	160	SmaI	T7
AP-2	240	EcoR1	T3

### 2.6.5 In situ hybridisation

#### 2.6.5.1 Hybridisation and washing

Sections were removed from the -80°C freezer and immediately postfixed in 4% paraformaldehyde in 0.1M phosphate buffered saline solution for 10 min and rinsed three times in 2xSSC. All solutions were treated with diethylpyrocarbonate (0.02%). [35S]-labelled cRNA probes in hybridisation buffer were denatured by heating at

70°C and added to hybridisation mix (50% deionised formamide, 0.6 M NaCl, 10 mM Tris-Cl (pH 7.5), 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.1% BSA, 1 mM EDTA, 0.1 mg denatured salmon sperm DNA, 0.05 mg/ml total yeast RNA, 0.05 mg/ml yeast tRNA, 10% dextran sulphate and 10 mM dithiothreitol) to give  $10 \times 10^6$  counts/ml. Hybridisation mix (200  $\mu$ l) was added to each slide and hybridisation was carried out overnight in sealed humid chambers at 45 or 50°C, humidified with box buffer (Section 2.6.1.2). After hybridisation slides were rinsed in 2xSSC and treated with RNase A (30  $\mu$ g/ml) for 60 min at 37°C in boxes humidified with buffer (Section 2.6.1.2). Washes consisted of 2xSSC at room temperature and 0.1xSSC at 50-55°C for 60 minutes for all probes. Sections were then dehydrated in 50,70 and 90% ethanols in 0.3 M sodium acetate, air dried overnight and dipped in K5 nuclear emulsion (Ilford, UK) diluted 1:1 with distilled water at 42°C. Sections were exposed at 4°C for 2-4 weeks, developed, counterstained with haematoxylin and eosin and coverslipped with DPX mountant (BDH). Control sections were pretreated with RNase A (100  $\mu$ g/ml) for 60 min at 37°C prior to hybridisation. Positive control brain sections were exposed to  $\beta$ -max film (Amersham) for 3-10 days before dipping in emulsion.

#### 2.6.5.2 Positive controls

As VIP and some of the immediate early genes are not basally expressed in DRG neurons, positive control sections of brain tissue were used in these in situ hybridisations.

Sections through the hypothalamic suprachiasmatic nucleus were used as positive controls for VIP expression and hippocampal and cerebellar sections used for positive control of NGFI-A, NGFI-B, the JUN family and AP2 expression.

### 2.6.6 Quantification of mRNA expression

All neurons studied belonged to the class of small, dark neurons i.e. with neuronal cell bodies of less than 30  $\mu\text{m}$  diameter, which are those thought to belong to the polymodal nociceptor class involved in neurogenic inflammation.

In the initial time course experiments mRNA levels were quantified by direct visual counting of the silver grains overlying individual cells expressing neuropeptide mRNAs. Expression of mRNA was later quantified using computer image analysis (Seescan, Cambridge) of sections. Neuropeptide in situ hybridisation (which gives a high silver grain density) was assessed using software which identifies single grains and clumps independently. Immediate early gene in situ hybridisation was quantified using the software which identifies only single grains (identical to that in the first stage of neuropeptide mRNA assessment). A correlation between analysis by eye and computer was also performed. The relative advantages/disadvantages of the two methods of analysis are discussed in Chapter 5.

A positive cell was classified as one which showed a hybridisation signal to background ratio of at least 4:1. In the time course experiments, four representative cells and four equal areas of background expression in each of three separate sections of each DRG were counted for each animal. In all other experiments a greater number of neurons was analysed due to the increased speed of counting with the computer-aided system. Background values were subtracted from cell counts and the mean number of silver grains per neuron per animal ( $\bar{x}$ ) calculated according to Equation 2.1.

Equation 2.1.

$$X = \frac{\sum_{i=1}^n (y_n - b_n)}{n}$$

where:- n = the total number of cells counted

y = the number of silver grains overlying a cell

b = the number of silver grains overlying an equal area of background

L5 DRG mRNA expression from experimental animals was expressed as a percentage of L5 DRG mRNA expression from untreated controls to eliminate variability in silver grains due to probe specific activity, emulsion layer thickness and time of exposure. The method of calculation of the percentage of control expression is described below and discussed in Chapter 5.

#### 2.6.7. Calculation of in situ hybridisation results as a percentage of untreated control expression

In situ hybridisation results are all expressed as a percentage of the expression of each messenger RNA in L5 DRG sections taken from a group of normal male Han Wistar rats. Control sections are hybridised, exposed to emulsion and developed at the same time as the experimental sections.

The mean for each group is then calculated using Equation 2.2

$$\text{Equation 2.2} \quad \% \text{ control} = 100 * \frac{\sum [(X_e) / (\sum X_c / n_c)]}{n_e}$$

where:-  $\bar{X}$  = mean number of silver grains per cell in an animal

e denotes the experimental group

c denotes the control group

n is the number of animals per group

#### 2.6.8. Statistical analysis of in situ hybridisation data

Data from in situ hybridisation experiments were compared using ANOVA followed by Dunnetts post hoc test. The Null Hypothesis was rejected at the 5% probability level.

#### 2.7 Subcloning of JUN family cDNA fragments

A Sal I- Bgl II human genomic c-jun cDNA fragment cloned into GEM, was kindly given by Dr. M. Seckl. The full length mouse jun-D cDNA cloned into pGEM2 was generously supplied by Dr J. Quinn.

### 2.7.1 Selection of appropriate cDNA restriction fragments

Restriction fragments specific for c-jun and jun-D were identified from 3' untranslated sequences of a c-jun human genomic DNA Sal I- BglII fragment (Hattori et al., 1988) and the mouse jun-D cDNA (Ryder et al., 1989).

The restriction fragments were digested with the appropriate restriction enzymes as detailed in Table 2.3 (Northumbria Biochemicals Ltd) at 37°C for 1 hour and purified by size electrophoresis on 1.5% low melting point agarose gel. Bands were cut from the gel, melted at 70°C and extracted with 1:1 phenol:chloroform/isoamyl alcohol (24:1), ethanol precipitated and centrifuged at 14000 r.p.m. for 10 minutes. The pellet was air dried and resuspended in 10µl sterile distilled water. Recovery of the fragment was checked using horizontal agarose gel electrophoresis on a 1.5% agarose gel.

*Table 2.3. Restriction fragments and vector cloning sites for jun family subclones*

<b>Probe</b>	<b>Restriction fragment</b>	<b>Cloning site</b>	<b>Length (fragment)</b>
c-jun	Hae III	Hinc II	732 (2747-3479)
jun D	Hinc II- Dra I	Hinc II	404 (1154-1558)

### 2.7.2 Subcloning of c-jun and jun-D restriction fragments

The fragments were ligated into the Hinc II site of linearised pGEM3 vector using T4 DNA ligase (NBL), 10mM ATP in ligase buffer, and two different ratios of insert to vector in order to optimise conditions. pGEM vector was religated as a control. Ligations were incubated at room temperature for at least 4 hours. The ligation



mixture was then digested with Sal I (NBL) which shares the restriction site with Hinc II, to linearise any religated vector and the ligation mixtures were then used to transform competent E.coli. (HB101) as described in section 2.6.3.1.

Individual colonies transformed with ligation mixtures were cultured overnight in 3ml Luria Broth and DNA extracted according to the method of Sambrook et al., 1989. Bacterial pellets were centrifuged in eppendorf tubes for 30 seconds, drained of medium and resuspended in glucose/Tris/EDTA buffer (200µl). Bacterial cells were lysed with NaOH/SDS (100µl) solution and neutralised with acetate solution (150µl). Tubes were centrifuged to remove protein/SDS/ high molecular weight RNA and the supernatant removed. DNA was precipitated with 2 volumes of 100% ethanol, centrifuged for 10 minutes at 14000 r.p.m., the pellet air dried and resuspended in 50µl TE buffer and 20µg/ml RNase A. Aliquots of DNA were then double digested with EcoRI and HindIII to cut out the entire polylinker region plus insert if present. Digests were then electrophoresed on 1% agarose gels to determine whether the insert was present.

#### 2.7.4 Specificity of subclones

Specificity of the jun subclones was determined by in situ hybridisation in control rat brain and Northern blot analysis of whole brain mRNA. This is further discussed in Section 8.

### 2.7.4.1 Northern analysis

#### 2.7.4.1A Extraction of whole brain mRNA

Total messenger RNA was extracted from whole brain by the guanidium thiocyanate method (Chomczynski and Sacchi, 1987).

Whole brain was homogenised in 1ml denaturing solution (0.025M sodium citrate, 0.5% sarcosyl, 4M guanidium thiocyanate, 0.1M  $\beta$ -mercaptoethanol in DEP water) in sterile glass/glass homogenisers and 500 $\mu$ l aliquots transferred to DEP treated eppendorf tubes. DNA was precipitated with 50 $\mu$ l 2M sodium acetate solution, and extracted using citrate saturated phenol and chloroform/isoamyl alcohol (49:1). After centrifugation for 20 minutes at 4°C and 14000 r.p.m. the aqueous phase was transferred to a DEP eppendorf and precipitated with isopropanol at -20°C overnight.

The RNA was recovered by and resuspended in 60 $\mu$ l denaturing solution. The RNA was then re-precipitated with 1 volume isopropanol and precipitated for 4 hours at -20°C. RNA was then recovered by centrifugation for 20 minutes at 4 °C and 14000 r.p.m, washed in 75% ethanol, dried *in vacuo* and resuspended in 5-20 $\mu$ l DEP water depending on pellet size.

#### 2.7.4.1B. RNA electrophoresis and capillary transfer

Total brain RNA samples were denatured in 12.5% MOPS buffer, 12.5% formaldehyde and 50% formamide at 65°C for 15 minutes and electrophoresed on a submerged horizontal denaturing agarose gel (1xMOPS buffer, 0.5% formaldehyde, 1% agarose in DEP water). The gel was photographed under ultraviolet trans-illumination to check for RNA degradation and blotted overnight by capillary

transfer onto nylon membranes (Amersham International). The gel was placed on a 3MM paper wick over an upturned gel plate in a reservoir of sterile 20xSSC solution. The nylon membrane, cut to exactly the same size as the gel, was placed in direct contact with the gel. Three layers of 3MM paper were placed on top of the filter, and covered with a 5cm thickness of absorbent paper. A weight was placed on top of the paper and the blot left overnight. RNA was then crosslinked to the nylon membrane by baking at 80°C for 3 hours.

#### 2.7.4.1C. Radiolabelling of cDNAs

The jun cDNA fragment was isolated from the vectors by digestion with EcoRI and HindIII and gel purified on a low melting point agarose gel as described in section 2.7.2. The cDNA fragments were then diluted in 2 volumes TE buffer and melted at 65°C. The cDNAs were labelled using a random priming kit (Boehringer Mannheim). 25ng DNA was incubated at 37°C for 30 minutes with <sup>32</sup>P labelled dCTP, unlabelled deoxynucleotides and Klenow enzyme. The reaction was stopped by the addition of 2µl 0.25M EDTA, pH 8. Labelled probe was then precipitated using 1/20 volumes 5M NaCl and 2 volumes absolute ethanol and incubating for 10 minutes at -80°C. The DNA was recovered by centrifugation, air dried briefly and resuspended in 50µl DEP H<sub>2</sub>O.

#### 2.7.4.1D. RNA hybridisation

Filters were prehybridised in 10ml hybridisation buffer at 42°C for at least 2 hours in a Hybaid hybridisation oven. The buffer was then removed, probe added to the buffer and then replaced in the hybridisation tube, and the filter hybridised overnight at 42°C. Following hybridisation, filters were washed in 2xSSC, 0.1% SDS (sodium lauryl sulphate; Sigma) at 42°C for 30 minutes. Stringent washes consisted of 2

consecutive washes in 0.2xSSC, 0.1% SDS at 50°C for up to 1 hour each. Filters were then covered in Saran wrap and exposed to X-AR film (Amersham International) in cassettes with intensifying filters, for 1-5 days.

Films were developed for 2.5 minutes in D19 developer (Kodak) and fixed for 3 minutes in Amfix high speed fixer (Champion Photochemistry).

#### 2.7.4.2. In situ hybridisation

Messenger RNA distribution for c-jun and jun-D was determined in control rat brain sections through the hippocampus using the same protocol as described in Section 2.6. Distribution of mRNAs was determined by film autoradiography for 10 days (Hyperfilm,  $\beta$ -max; Amersham International).

### 2.8 Neuropeptide radioimmunoassay

All neuropeptide radioimmunoassays were kindly performed by John Bennie and Sheena Carroll (MRC Brain Metabolism Unit, Royal Edinburgh Hospital, Edinburgh).

Sample preparation and radioimmunoassay were performed as described previously (Pierotti and Harmar, 1984; Smith et al., 1991)

#### 2.8.1. Sample preparation

Animals were killed by decapitation and a laminectomy performed to expose the lumbar enlargement. The sciatic nerve was exposed, L4 and L5 DRG identified and dissected free from the dura. DRG were rapidly frozen on dry ice and stored at

-20°C prior to peptide extraction. Left and right L4 and L5 DRG respectively, were pooled to assess peptide levels from left (lesioned) or right (unlesioned) DRG. DRG were homogenised on ice in 100µl 2M acetic acid, homogenates centrifuged at 14 000 r.p.m. to remove insoluble material and supernatants were assayed for SP, CGRP and SS.

### 2.8.2 Radioimmunoassay

Samples were evaporated to dryness in a vacuum oven (45°C, 700 mmHg) and resuspended in assay buffer (0.1M PO<sub>4</sub>, pH 7.6, 1g bovine serum albumin/l).

For SP assay, standards (0-500 pg/ml) and samples were prepared in a volume of 200µl in duplicate. Standards and samples were incubated at 4°C for 16 hours with 200µl of 1:80 000 dilution of rabbit anti-SP antiserum and 5000 cpm in 100µl of <sup>125</sup>I labelled SP. Bound radiolabel was separated from free by precipitation with donkey anti-rabbit gamma globulin antiserum (Scottish Antibody Production Unit, Carlisle, UK, SAPU).

For CGRP assay, standards (human CGRP 0-5000 pg/ml, Peninsula; 200µl/tube) or samples were incubated for 16 hours at 4°C with 100µl of 1:2000 dilution of rabbit anti-human CGRP antiserum (Amersham International) and 5000 cpm 2-[<sup>125</sup>I]iodohistidyl CGRP (human, Amersham International) in 100µl assay buffer. Bound radiolabel was separated from free by precipitation with donkey anti-rabbit gamma globulin antiserum (SAPU).

For SS assay, standards containing SS-14 (0-120 fmol) or samples diluted to 200µl in assay buffer were incubated with 200µl <sup>125</sup>I SS-14 (5000 cpm) and 200µl antiserum (1:250 000 dilution; antiserum directed against SS-14) for 20-24 hours at 4°C. Charcoal suspension (0.2 ml; 25g/l in 0.1M PO<sub>4</sub> buffer pH 7.6 containing 10g/l bovine serum albumin) and 0.8ml phosphate buffer were added and the tubes incubated in ice for 20 minutes before centrifugation. The radioactivity of the

supernatant was counted. The antiserum detected SS-14, SS-28 and higher molecular weight forms of SS.

Cross-reactivity between peptide antibodies, inter- and intra assay coefficients of variance and assay sensitivity are summarised in Table 2.4.

*Table 2.4. Antibody cross-reactivity and coefficients of variance for neuropeptide radioimmunoassay*

Antibody	Sensitivity	Crossreactivity	Interassay CoV	Intra-assay CoV
Substance P	12.5pg/ ganglion	to CGRP & SS; <0.01%	<20%	<10%
CGRP	50pg/ ganglion	to SP & SS; <0.01%	<20%	<10%
Somatostatin	12pg/ ganglion	to SP & CGRP; <0.01%	<15%	<10%

## 2.9 Assessment of chronic stress in arthritis

### 2.9.1 Corticosterone radioimmunoassay

#### 2.9.1.1 Preparation of samples

Whole blood samples (500-1000µl) were taken from adult male Han Wistar rats with both mono- and symmetrical arthritis by tail nicks, morning and evening (2 hours after lights on/off), three times per week during the development of arthritis. Samples were taken in tubes containing anticoagulant, kept on ice until spun and plasma samples collected and kept at -20°C until assayed for corticosterone.

Animals were killed by decapitation in the morning of day 15 after adjuvant injection, trunk blood collected into lithium chloride tubes and stored on ice. Blood samples were spun and plasma samples stored at -20°C until assayed for corticosterone.

#### 2.9.1.2 Corticosterone radioimmunoassay

Rat plasma samples diluted 1:25 in assay buffer were incubated for 30 minutes at 75-80°C. Samples (50µl) or corticosterone standards (0.005-6.4 pmol/50µl) in sodium borate assay buffer were incubated in duplicate with 100µl of 1:5000 specific anti-corticosterone antiserum and 50µl (15 000 c.p.m.) [<sup>3</sup>H] corticosterone overnight at 4°C. Bound and unbound fractions were separated on ice by addition of dextran-coated charcoal suspension (0.5g activated charcoal; 0.05g Dextran T-70; 50mg bovine serum albumin/ 100ml sodium borate buffer) and centrifugation at 4°C for 30 minutes at 3000g. The supernatant was removed into scintillation vials containing 5ml Picofluor 40 scintillant and counted in a β-counter.

Standard curve plots and sample calculations were calculated using computer software (Assayzap).

#### 2.9.2 Corticosterone binding globulin assay

Corticosterone binding globulin (CBG) assays were kindly performed by Isobel Forbes.

Plasma samples from morning trunk bloods were assayed for CBG as previously described (Martin et al., 1977). Endogenous steroids were removed from plasma by running samples through a Sephadex LH-20 column (10 x 1cm). Plasma samples were diluted 1:50 with TEGMD buffer, pH7.4 (30mM Tris, 1mM EDTA, 10mM

sodium molybdate, 10% glycerol, 1mM dithiothreitol) and incubated overnight at 4°C with 200nM solution of [<sup>3</sup>H] corticosterone in TEGMD to give total binding. Non-specific binding was determined by incubation with excess (0.1M) cold corticosterone. Separation of bound from free corticosterone was achieved on Sephadex LH-20 columns as described above. CGB levels were expressed as pmol [<sup>3</sup>H] corticosterone bound/mg protein.

## 2.10 Immunohistochemistry

### 2.10.1. Section preparation and solutions

Parallel fresh frozen DRG tissue sections to those used for in situ hybridisation were mounted on gelatin/poly-L-lysine subbed slides and processed for immunohistochemical localisation of SP-like immunoreactivity.

4% paraformaldehyde in 0.1M PB pH7.4 as described in section 2.4 except the water is not DEP treated.

0.1M phosphate buffer (PB): 20mM NaH<sub>2</sub>PO<sub>4</sub> (BDH); 80mM Na<sub>2</sub>HPO<sub>4</sub> (BDH); in 1 litre distilled water.

0.1M phosphate buffer (PB)+ 0.3% Triton X-100 (Sigma): 3ml TX-100 per litre 0.1M PB

Tris buffered saline (TBS) 0.05M, pH 7.6; 9g NaCl; 6.05g Trizma base; per litre distilled water.

### 2.10.2. Immunohistochemistry method

The anti-SP antiserum used was that also used for SP radioimmunoassay (Section 2.8; a generous gift from Dr Tony Harmar). Slide mounted fresh frozen tissue



sections were postfixed in 4% paraformaldehyde in 0.1M phosphate buffer (0.1M PB), pH 7.4 for 30 minutes, and then washed thoroughly in 0.1M phosphate buffer/0.3% Triton X-100 (TX-100; Sigma). Non-specific binding was blocked by incubation of the tissue sections, in a humid box, with 10% normal sheep serum (NSS; SAPU) in 0.1M PB + 0.3% TX-100 for twenty minutes at room temperature. Slides were then drained and incubated overnight at 4°C in a humid box with primary anti-SP antiserum diluted 1:1000 in 0.1M PB/0.3% TX-100/1% NSS. After primary incubation, slides were thoroughly washed in 0.1M PB/1% NSS/0.3% TX-100 before incubation for 90 minutes with a 1:400 dilution of sheep anti-rabbit IgG-biotin conjugate (Boehringer Mannheim) in 0.1M PB/1% NSS. Tissue was then thoroughly washed in 0.1PB and incubated in avidin-peroxidase complex (Vectorstain Elite, Vector Laboratories) for 1 hour. After rinsing in 0.1PB, antibody binding was localised using diaminobenzidine (0.3ml 5mg/ml diaminobenzidine in TBS; 1.2ml TBS; 0.02% hydrogen peroxide). Following the peroxidase reaction (approx. 5 minutes) tissue sections were counterstained with methyl green for 15 seconds, dehydrated through 70% and absolute ethanol and coverslipped with DPX mountant. Control for the specificity of the signal was omission of the primary antiserum.

## SECTION 3 EVALUATION OF A MODIFIED MODEL OF ADJUVANT MEDIATED ARTHRITIS IN THE RAT

### 3.1 INTRODUCTION

#### 3.1.1 Classical adjuvant-induced polyarthritis in the rat

Arthritis in the rat was originally described in 1959 by Pearson and Wood as a reproducible polyarthritis induced by intradermal injection of heat-attenuated mycobacteria suspended in mineral oil into the tailbase or footpad. The reaction is generally one of systemic illness, with inflammatory involvement of peripheral joints, particularly of the hind limbs. After eleven to sixteen days a severe polyarthritis of the tarsal, carpal, phalangeal and spinal joints develops, accompanied by lesions to the eyes, ears, nose, skin and genitals as well as anorexia and profound weight loss. (Pearson and Wood 1959; Rainsford, 1982) Adjuvant arthritis (AA) shows similar histological features to rheumatoid arthritis in humans and is therefore useful in studies of the chronic pain and inflammation of this condition. AA has been widely accepted as a model of chronic pain as rats show an increased self administration of both non-steroidal anti-inflammatory drugs and opiate analgesics when compared to normal animals (Colpaert et al., 1980; Colpaert et al., 1982). Adjuvant arthritis has been widely exploited in the study of all aspects of arthritic disease including neurophysiology, peptide biochemistry and immunology (Guilbaud 1991; Ohno et al., 1990; Kuraishi et al., 1989). However, the widespread and severe disease induced makes it difficult to attribute changes observed to the arthritic processes *per se*.

It is extremely difficult to study the pathophysiology of inflammation and arthritis in vitro as the interactions between joint, immune system, vasculature and sensory and sympathetic nervous systems are disrupted. Therefore whole animal models

which facilitate the study of inflammation and arthritis are continually being evaluated.

### 3.1.2. Other models of arthritis

In the light of the severity of AA, and the ethical problems involved in the care of such severely affected animals, there have been numerous attempts to modify AA or develop other animal models of chronic pain or inflammation. With such a severe disease process, it is obviously more difficult to attribute observed changes specifically to the arthritic process. There have been several attempts to develop models which show milder disease than the classical AA.

The injection of urate crystals directly into the joint space of either cats (Okuda et al., 1984) or rats (Otsuki et al., 1986; Coderre and Wall, 1987) has been suggested as an alternative to adjuvant arthritis. This intervention gives rise to an inflammatory process peaking at 24 hours and resolving after approximately 1 week, which is milder than adjuvant disease. Intra-articular urate crystals do not result in arthritic changes, but joints show tissue oedema and polymorphonuclear leucocytic infiltration.

Injection of adjuvant into the footpad of the rat has been suggested as a way of limiting AA to one paw (Millan et al., 1988) but this has long been recognised as a very effective site of injection for the induction of polyarthritis (Waksman et al., 1960; Ward and Jones, 1962) and indeed the article proposing this model did describe significant contralateral paw swelling after 21 days (Millan et al., 1988).

A localised adjuvant-induced monoarthritis which shows no behavioural or histological evidence of contralateral effects, where arthritis is induced by periarticular injection of adjuvant, has been extensively used in the study of joint receptors in the intact animal (Grubb et al., 1988; Grubb et al., 1991; McQueen et

al., 1991) and has been adapted to produce a limited adjuvant induced monoarthritis which is stable for six weeks (Butler et al., 1992).

In these studies, the monoarthritis described by Grubb et al. (1988) has been further evaluated in view of recent hypotheses that ipsilateral inflammation necessarily results in contralateral changes indicative of inflammation (Levine et al., 1985b; 1985c; Kidd et al., 1989a). Preliminary studies showed that localised or more distant inflammation may be dependent upon the total injected dose of *Mycobacterium* species. Therefore the effect of increasing amounts of *Mycobacterium tuberculosis* suspended in paraffin oil on the arthritis induced by periarticular injection of adjuvant have been further investigated. The relationship between measures of inflammation and the histological appearance of the joint was also studied to determine whether use of these common indices is valid as such "blind" assessments have been criticised (Rainsford 1982). Rheumatoid arthritis is thought to have a diurnal variation, being more severe in the mornings (Chowdrey and Lightman, 1993); therefore measurements of joint swelling and inflammation were routinely performed at the same time of the day to prevent the possibility of bias due to this effect.

### 3.1.3. The arthritic rat as a model of chronic stress

Chronic inflammatory polyarthritis in the rat was first proposed as a model of chronic stress by the authors of some of the original evaluations of the model (Colpaert, 1987; Colpaert and Van den Hoogen, 1983; Colpaert et al., 1982). However, these studies were primarily concerned with the arthritic rat as a model of chronic pain, and therefore did not seek to establish any changes in the activity of the hypothalamo-pituitary-adrenal axis in these animals. More recent work has shown that there is a chronic hypothalamic-pituitary-adrenal stimulation in these animals,

with a loss of diurnal rhythm of both adrenocorticotropin releasing hormone (ACTH) and corticosterone (Sarlis et al., 1992). These animals also show a disturbance of the normal circadian rhythm of motor activity seen in rats.

Susceptibility to AA in the inbred Lewis rat has been postulated to be due to a defective HPA axis response to inflammatory stimuli (Sternberg et al., 1989).

However, this ineffective HPA axis response may not be not an absolute requirement for susceptibility to AA as strains which show a vigorous HPA response have been shown to be susceptible to AA (Harbuz et al., 1993) and AA has been studied in a variety of different strains of rat (Waksman et al., 1960; Jones and Ward, 1963; Vernon-Roberts et al., 1976; Nusbickel and Troyer, 1976). However it has also been postulated that an underlying defect in hypothalamic responses may contribute to the aetiopathogenesis of human rheumatoid disease (Chikanza et al., 1992). It was, therefore, of interest to examine the corticosteroid status of these arthritic animals to assess whether this model also represents a chronic stressor.

## **3.2 MATERIALS AND METHODS**

### **3.2.1 Dose dependency of adjuvant-induced arthritis**

Male Wistar rats (200-250 g) were allocated to groups (n=5 per group). Animals, under halothane anaesthesia (5% in oxygen), were injected intradermally with either paraffin oil or Freund's Complete adjuvant containing 50 µg, 150 µg or 250 µg attenuated *Mycobacterium tuberculosis* (M. tub.; MAFF, UK) suspended in 0.05 ml paraffin oil, divided between two injection sites around the left tarsal joint. Controls were untreated.

Before induction of arthritis and twice weekly thereafter, the animals were weighed, their left and right tarsal joint circumferences measured, and left tarsal joints scored for inflammation and response to a mild noxious stimulus. Nociceptive threshold

was determined by the pressure required to elicit limb withdrawal was measured either subjectively and scored on an analogue scale 0-3 (0=intense pressure, 3=slight pressure), or objectively using an electrical meter connected to a pressure transducer and scored 0-8 based on the meter reading (0=very slight pressure, 8=intense pressure). All measurements were performed blind to treatment by the same experienced operator.

Preliminary histological studies demonstrated chronic monoarthritis 14 days after adjuvant injection. Therefore, 15 days after injection, animals were deeply anaesthetised with sodium pentobarbitone (60 mg/kg i.p.) and perfused through the descending aorta with heparinised saline (500 U/kg, 10 ml) followed by 10% formal saline (~50 ml) until the hind limbs were fixed. An additional group of animals injected with 150µg M. Tub. was killed after 69 days to determine whether bilateral arthritis would eventually develop and joints were also assessed histologically. Both hind limbs were removed and processed for histological examination as detailed in Section 2. Each section was scored blind for histological evidence for the presence and degree of joint inflammation; assessed on the following histological features; inflammatory cell infiltrate, synovial proliferation, articular cartilage breakdown and new bone formation and classified as:- 0=normal, 1=subdermal inflammation, 2=mild, 3=moderate and 4=severe joint inflammation; scores  $\geq 3$  were taken as indicative of arthritis.

### 3.2.2 Measures of chronic stress

Groups of male Han Wistar rats (200-250g; n=5 per group) were induced for study of either mono- or bilateral arthritis. Tail blood samples were taken three times per week (morning and evening, 2 hours after lights on/off) during the development of chronic arthritis, collected and stored on ice until centrifuged to obtain plasma samples. On day 15 post-injection, animals were killed in the morning by

decapitation, and trunk blood collected. Thymus, spleen and adrenals were also collected and weighed. Control tissue from age-matched untreated animals was also taken. Plasma samples were stored at -20°C until assayed for corticosterone and CBG as outlined in Section 2.9.

### 3.2.3. Statistical procedures

Statistical analysis was by ANOVA (factorial or repeated measures as appropriate) followed by Dunnett's post hoc test. Spearman Rank correlation coefficient was used to determine correlations between the histological arthritis and gross measures of inflammation, joint circumference, nociceptive threshold and inflammation scores. Values are means  $\pm$  S.E.M. The Null Hypothesis was rejected at the 5% probability level.

## **3.3. RESULTS**

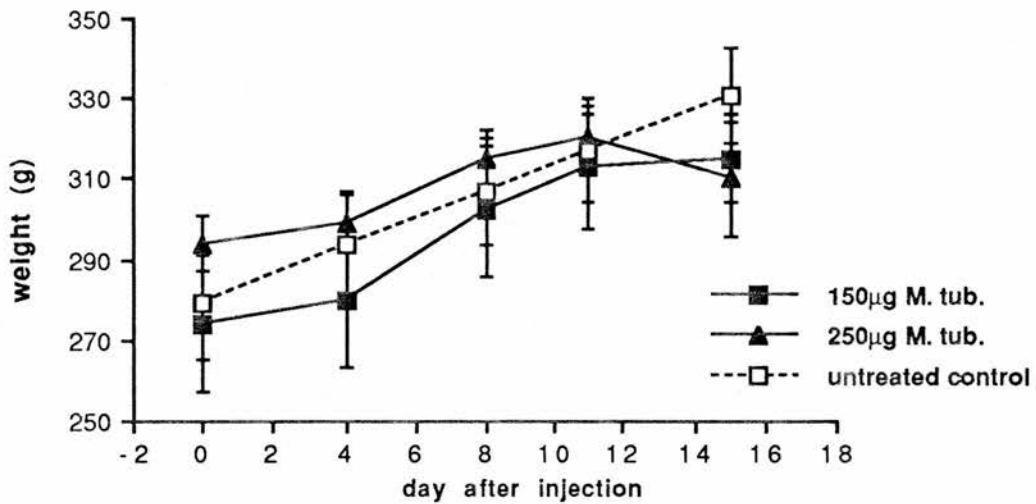
### 3.3.1. Behaviour of animals injected with *Mycobacterium tuberculosis*, or vehicle

During development of inflammation, animals showed little or no apparent discomfort and locomotor activity was near normal, with only occasional guarding of the affected limb. Only in the group receiving the highest dose of *M.tub.* (250  $\mu$ g) was the left hind limb not used for walking, and then only occasionally, fourteen days post-injection.

### 3.3.2. Gross parameters of inflammation

The normal gain in weight was not affected by any treatment although the group receiving the largest amount of *M. Tub.* showed a non-significant drop in weight on day 15 (Figure 3.1).

**Figure 3.1**



*Figure 3.1. Mean body weights of rats injected around left ankle with 150µg, and 250µg M. Tub. and untreated controls during the course of the study. Only the group injected with 250µg M. Tub. show a non-significant alteration in the normal weight gain seen in rats of this age. (Data from paraffin and 50µg groups not shown; not significantly different from untreated controls).*

Animals injected with *M. tub.* showed a dose-related increase in left (injected) joint circumference, redness and swelling, and histological inflammation score. Injection of 50 µg *M. tub.* or paraffin oil alone caused slight, non-significant increases in left



tarsal joint circumference which remained constant for 15 d. By contrast, injection of 150  $\mu\text{g}$  or 250  $\mu\text{g}$  *M.tub.* caused acute inflammation and significant swelling of the left tarsal joint within 24 hours, reaching an initial peak 4 days post-injection ( $p<0.01$ ). This initial swelling partially resolved at days 8-11 (although remaining significantly swollen compared to controls), but increased again 14 days post-injection (Figure 3.2).

Figure 3.2

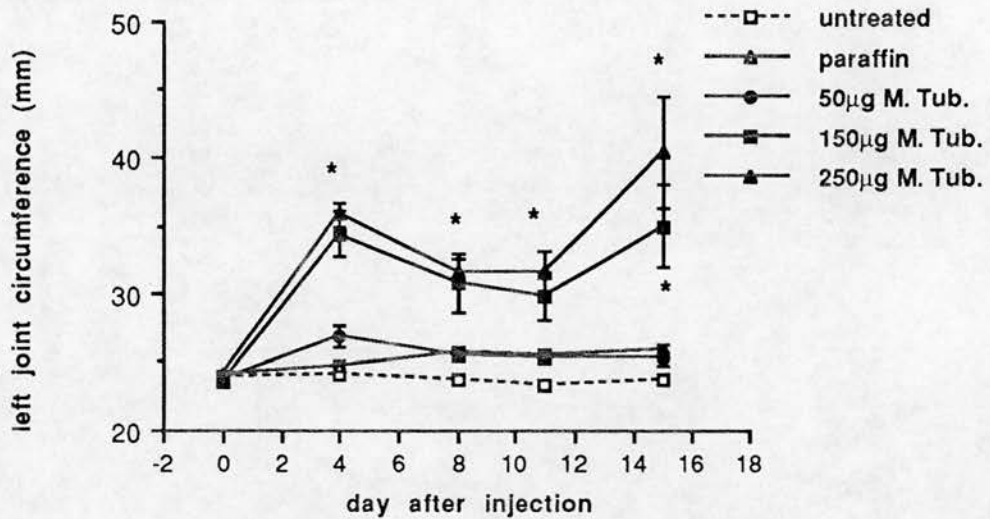


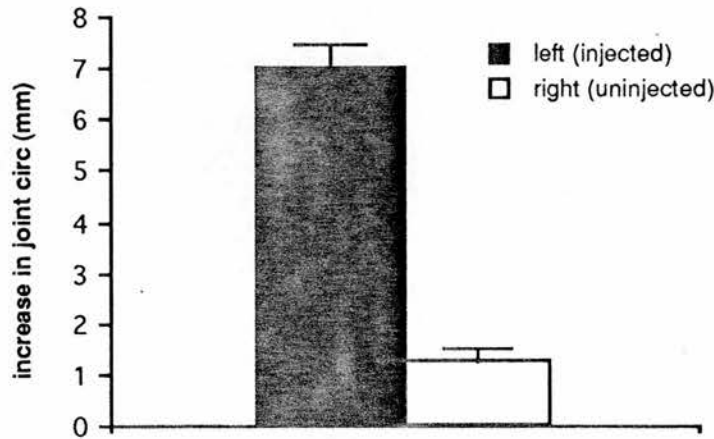
Figure 3.2. Mean left joint circumferences during the development of arthritis in rats injected around the left ankle joint with either *M. Tub.*, paraffin, or untreated.

\* $p<0.05$  compared to untreated control animals.

The group of rats injected with 150 $\mu\text{g}$  *M.tub.* and killed after 69 days showed swelling of the left tibio-tarsal joint with no effect on right joint circumference up to 69 days post adjuvant injection (Figure 3.3). Histological assessment of the right

and left joints verified that inflammation remained confined to the left (injected) limb (see Section 3.3.3, Figure 3.9 and Table 3.1).

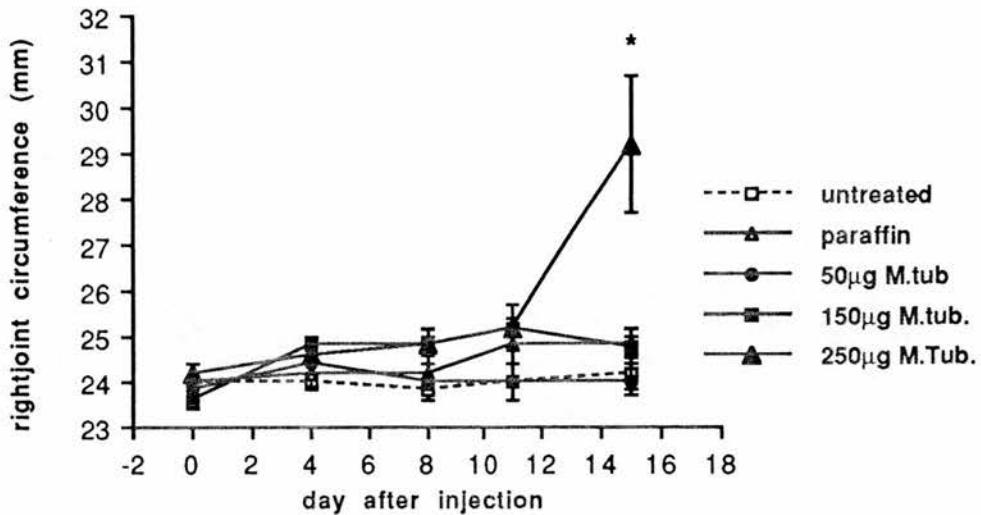
**Figure 3.3**



*Figure 3.3. Mean increase in left and right joint circumferences in rats 69 days after injection of 150µg  $\mu$ . *M. Tub.* around the left ankle joint showing joint swelling affecting the joint only on the injected side.*

Animals treated with the 250 µg dose showed redness and swelling of the contralateral tarsal joint at 14 days post-injection. Those receiving 250 µg only occasionally showed mild forepaw swelling, nose and eye inflammation. Only animals injected with 250 µg of *M. tub.* showed a statistically significant increase in contralateral (right) joint circumference after 14 d (Figure 3.4).

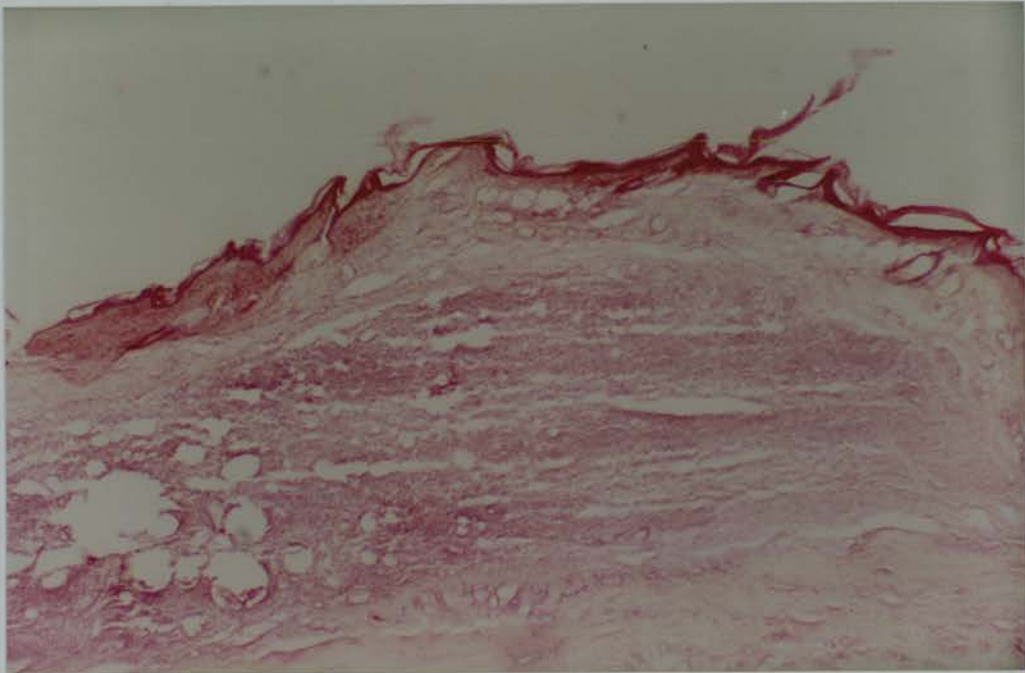
**Figure 3.4**



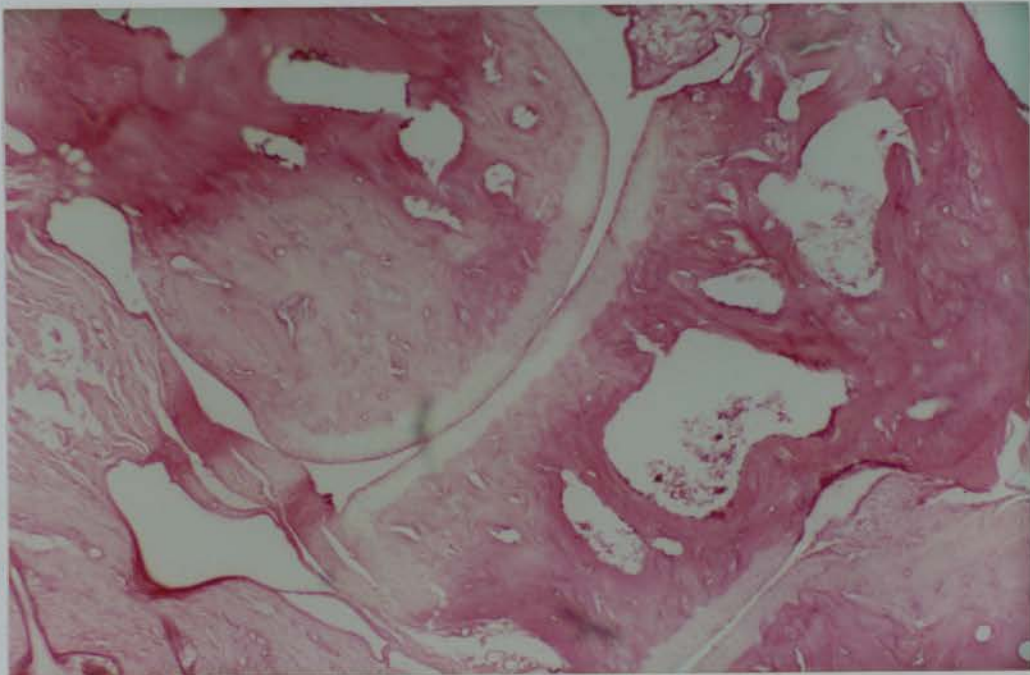
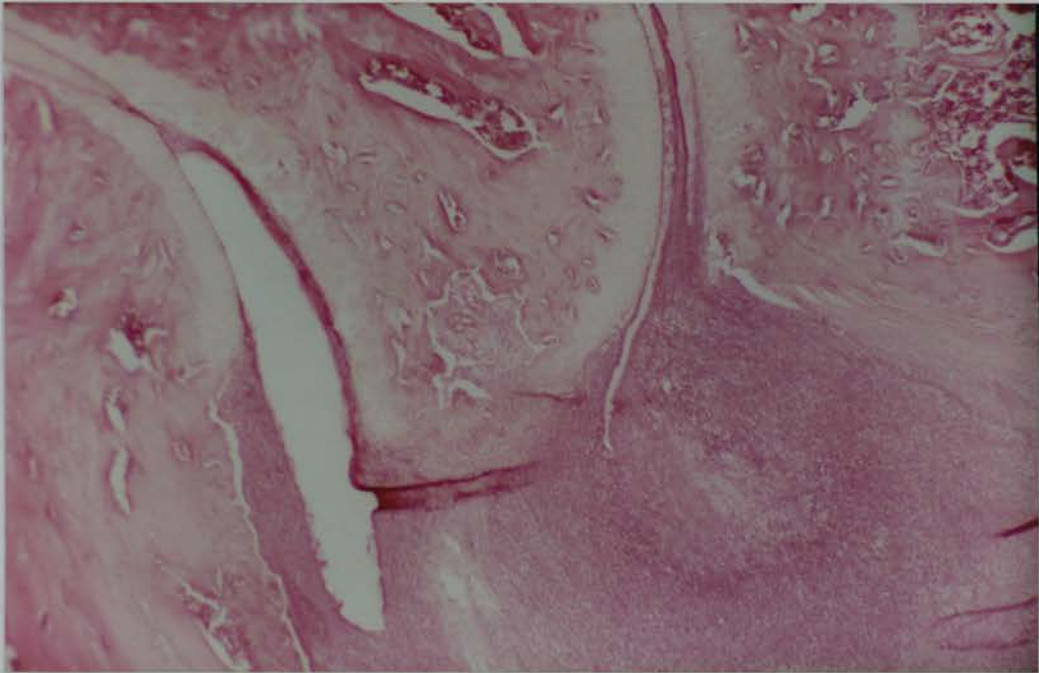
*Figure 3.4 Mean right joint circumferences of rats injected around left ankle joint with either *M. Tub.*, paraffin oil or untreated. \* $p < 0.05$  compared with untreated controls.*

### 3.3.3. Histological features of adjuvant-induced inflammation

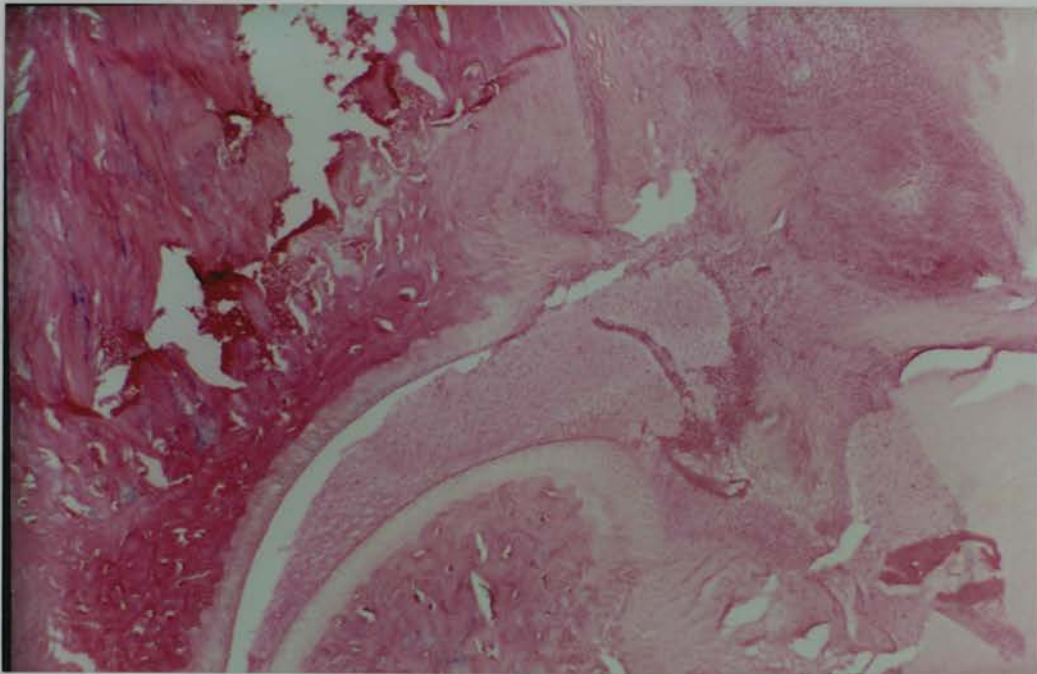
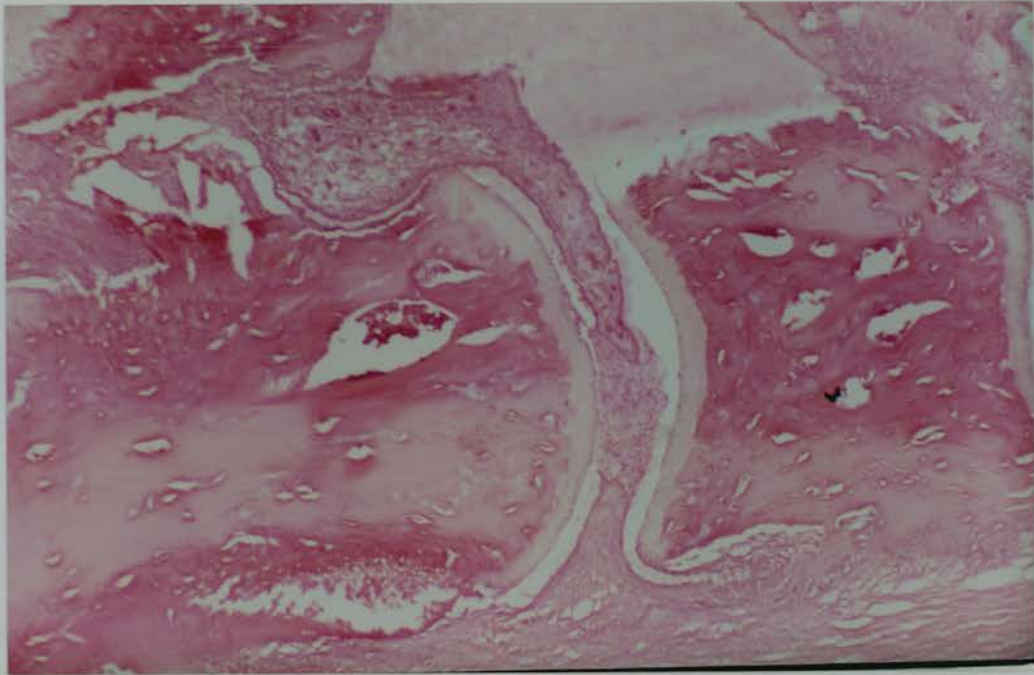
Animals receiving no treatment, paraffin, or 50 µg *M.tub.* showed no histological evidence of arthritis of the left tarsal joint, although an inflammatory infiltrate in overlying subcutaneous tissues was evident in the latter group (Figure 3.5). The histological features in the left (injected) joint from an animal injected with 150µg *M.tub.* are illustrated in Figure 3.6. Similar histopathology was also seen in the right (uninjected) ankle joint of animals receiving 250 µg *M. Tub.* and is shown in Figure 3.7. Histopathological features seen in the joints of these animals were mononuclear cell infiltration, synovial hyperplasia and oedema, pannus formation and subchondral bone resorption and are illustrated in Figure 3.8. Neither gross nor



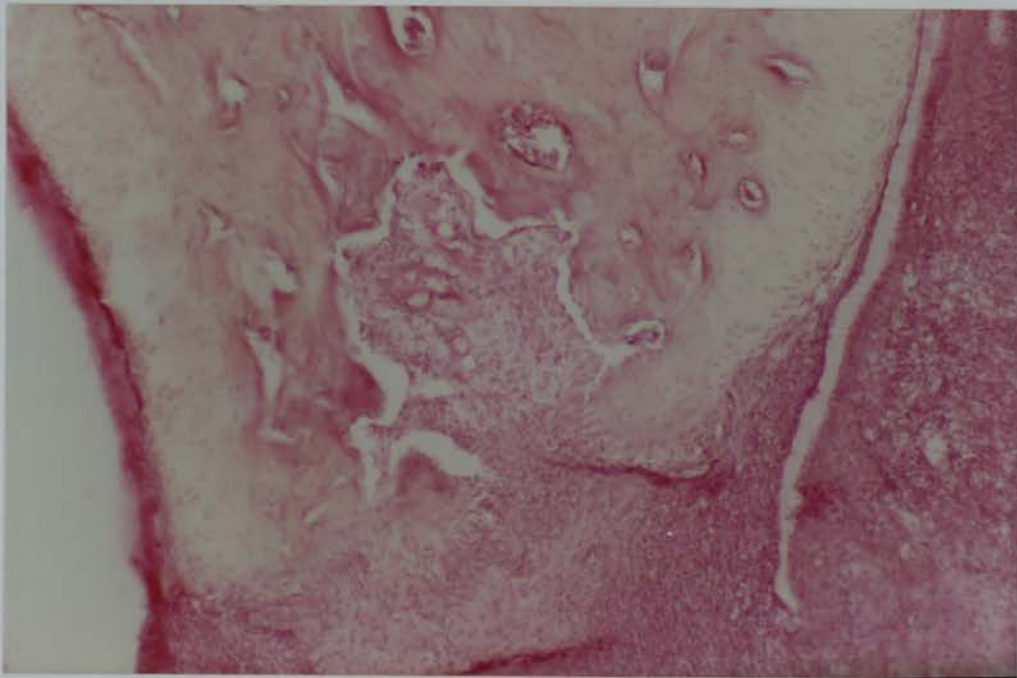
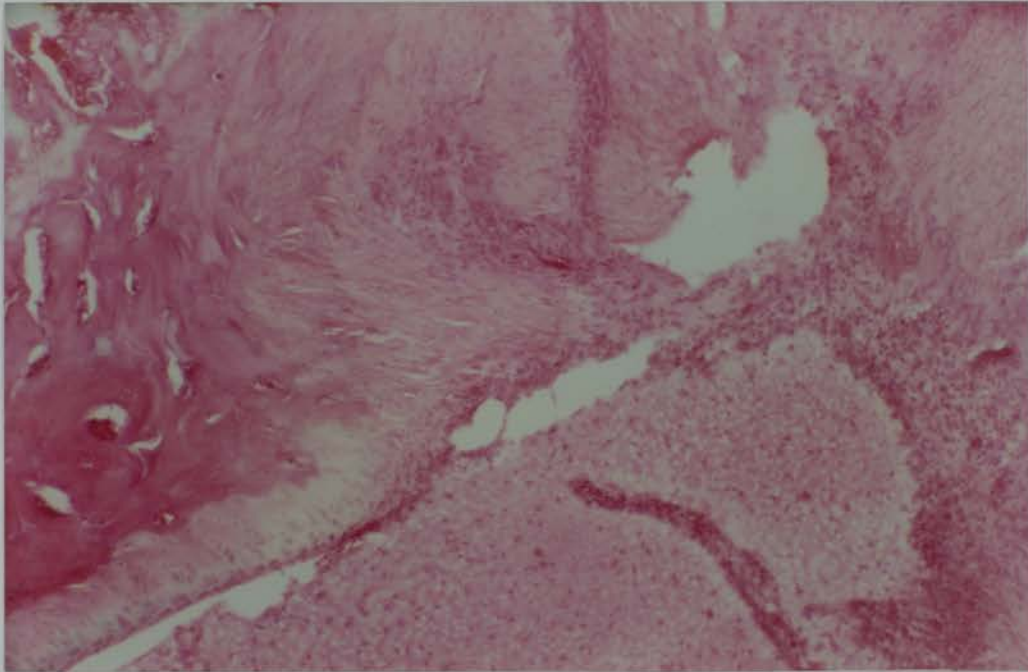
*Figure 3.5. Histological appearance of subdermal infiltrate from an animal injected with 50 $\mu$ g *M.tub.* which showed no other signs of joint inflammation.*



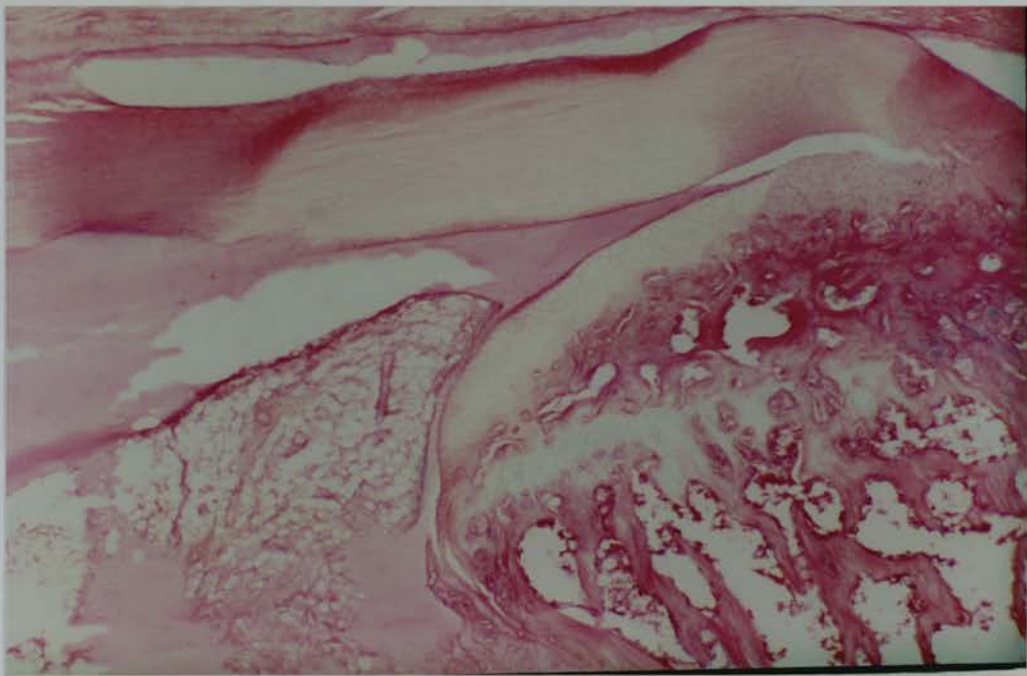
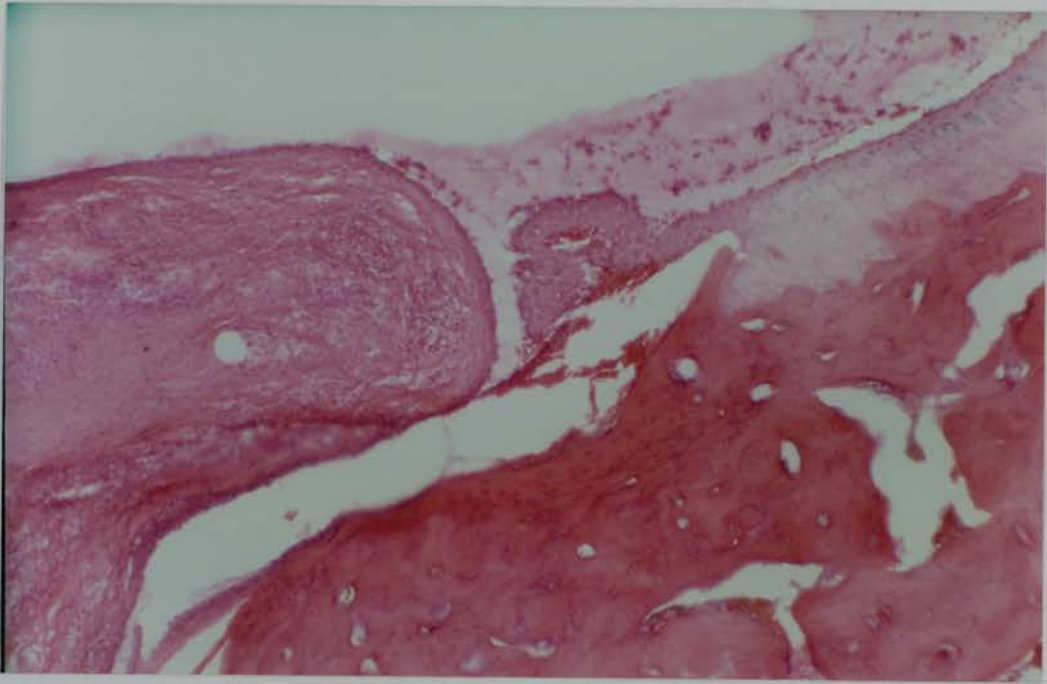
*Figure 3.6. Photomicrographs showing the left (top) and right (bottom) tibio-tarsal joints from a rat injected around the left tibio-tarsal joint with 150 $\mu$ g *M.tub.*, showing arthritis limited to the left (injected) joint.*



*Figure 3.7. Photomicrographs of left (top) and right (bottom) tibio-tarsal joints from a rat injected around the left tibio-tarsal joint with 250 $\mu$ g *M.tub.*, 14 days after adjuvant injection, showing arthritis affecting both joints.*



*Figure 3.8. Photomicrographs showing some features of adjuvant-induced arthritis; inflammatory infiltration of synovium and joint cavity (top) and pannus invasion of subchondral bone (bottom). Original magnification x10.*



*Figure 3.9. Photomicrographs of left (top) and right (bottom) tibio-tarsal joints 70 days after injection of 150µg M.tub. around the left tibio-tarsal joint showing arthritis affecting only the left (injected) joint.*



histopathological changes indicative of arthritis were seen in the right joints of animals injected with 150µg *M. Tub.* at either 15 (Figure 3.6) or 69 days (Figure 3.9) post-adjvant injection, agreeing with and extending the observations of Grubb et al. (1988). Histological scores are summarised in Table 3.1.

*Table 3.1. Histological scores for left and right tibio-tarsal joints in the five treatment groups of rats at day 15 after adjuvant injection of the left limb. Median (range). \*p<0.05, \*\*p<0.01 compared to untreated controls (Mann Whitney U Test).*

	LEFT	RIGHT
Untreated	0	0
Paraffin oil	1 (0-1)	0 (0-1)
50µg <i>M. Tub.</i>	1 (0-2)	0 (0-1)
150µg <i>M. Tub.</i>	3.5 (3-4)**	1 (1-2)
250µg <i>M. Tub.</i>	4 (2-4)**	2 (1-4)**
150µg <i>M. Tub.</i> (70 days)	3 (3)*	0.5 (0-1)

### 3.3.4. Relationship between gross parameters and histological features of inflammation

There was a significant correlation between the histological score and (i) the increase in joint circumference (n=37, r=0.71, p<0.0005) and (ii) the inflammation score (n=25, r=0.86, p<0.0005). The response to a subjectively scored mild noxious stimulus (squeeze) did not correlate significantly with joint histology (n=23, r=0.36, p>0.05), whereas when this parameter was subsequently assessed objectively, a highly significant correlation was seen (n=30, r= -0.699, p<0.0001).

### 3.3.5. Parameters of chronic stress

Thymus, adrenal and splenic weights and final CBG values for each group are summarised in Table 3.2. There were no significant differences in any of these parameters between controls, mono- and bilaterally arthritic rats.

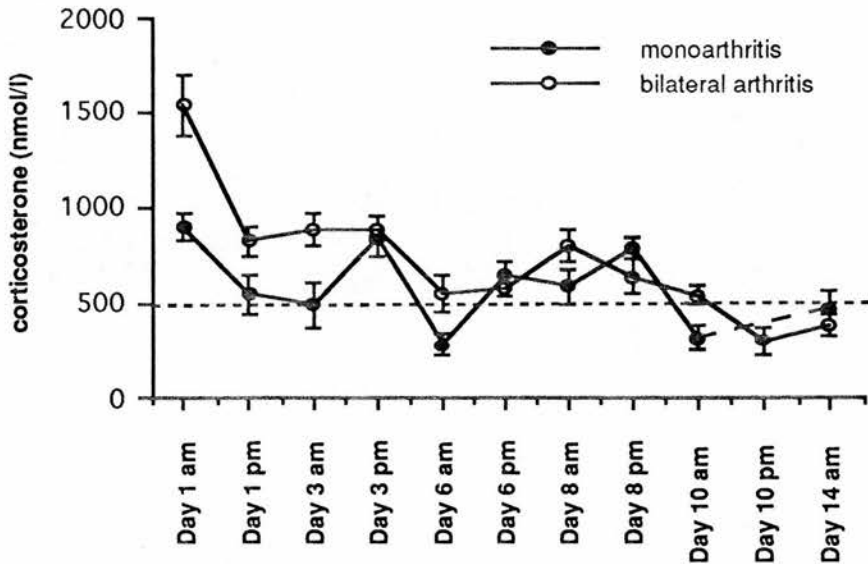
*Table 3.2 Thymic, adrenal and splenic weights and CBG values in control, mono- and bilaterally arthritic male rats. CBG levels are expressed as pmol. <sup>3</sup>H/mg protein. All values are mean ± S.E.M.*

Group	Thymus weight (g)	Adrenal weight (g)	Splenic weight (g)	CBG
Control	0.58±0.057	0.077±0.004	0.903±0.035	13.2±2
Monoarthritic	0.523±0.04	0.065±0.006	0.941±0.074	16.8±2
Bilaterally arthritic	0.539±0.015	0.079±0.008	1.057±0.065	10.7±2

None of these measures, which are known to be sensitive to increased levels of circulating corticosterone were significantly different from control animals.

The morning and evening corticosterone levels in both groups of arthritic rats are shown in Figure 3.10. Control basal corticosterone levels are usually below 50nmol/L and maximal control evening levels are represented by the dashed line at approximately 500 nmol/L. It can be seen from this graph that circulating corticosterone levels are generally raised in both monoarthritic and bilaterally arthritic rats showing levels indicative of stress. Importantly the normal diurnal variation in corticosterone secretion is lost from the onset of inflammation throughout the period of study.

**Figure 3.10**



*Figure 3.10 Morning and evening plasma corticosterone levels in mono- and bilaterally arthritic rats during the development of inflammation. The dashed line shows the maximal evening corticosterone levels in control rats (Dr. M. Holmes, personal communication).*

### **3.4. DISCUSSION**

These results show that adjuvant injected periarticularly can be used to induce either a monoarthritis which remains confined to one joint for up to 69 days, or, with a small increase in the dose of *M. Tub* used, can produce a bilateral arthritis of the hind limbs, assessed by histological features of arthritis. It has also been shown that

the gross assessment of arthritis by measurement of joint swelling shows a high correlation with the histological assessment of arthritis.

#### 3.4.1. Models of inflammatory arthritis

There are other models of arthritic disease commonly used in experimental animals, in addition to adjuvant polyarthritis as described by Pearson and Wood (1959). The subcutaneous injection of sterile air in the rat results in a pouch granuloma, the lining cells of which have many similarities with synovial lining cells (Edwards et al., 1981). The study of cartilage breakdown has been facilitated by an adaptation of the air pouch model (Da Silva et al., 1993).

Many models involve the sensitisation of an animal to an antigen such as collagen and bacterial cell wall components for example, collagen induced arthritis in either rats or mice which is induced by two injections of native Type II collagen (Trentham et al., 1977). Cell mediated immunity to collagen has been reported in patients with rheumatoid arthritis (Stuart et al., 1980). Streptococcal cell wall induced arthritis in rats, first described by Cromartie et al. (1977) induces symmetrical synovitis in all animals treated. Both of these models affect all peripheral joints and are of use in the study of the immune reactions involved in this disease as they are T-cell dependent (Taurog et al., 1983; Trentham et al., 1977; Ridge et al., 1985). Like adjuvant-induced arthritis, collagen and streptococcal cell wall arthritis show many similarities to rheumatoid arthritis clinically, histologically and radiologically. All show involvement of peripheral joints, chronic proliferative synovitis, pannus formation and marginal destruction of joints (Cromartie et al., 1977; Pearson and Wood 1959). Obviously a major difference between all these animal models and human rheumatoid arthritis is the absence of circulating rheumatoid factor in experimental models.

There are also two models of spontaneous arthritis both affecting mice. MRL/MpJ-lpr/lpr mice spontaneously develop polyarthritis by five months of age, particularly involving the hind limbs (Hang et al., 1982). This is associated with an increase in circulating levels of rheumatoid factors and is therefore useful in the study of the aetiology of rheumatoid disease. The second model involving spontaneous development involves a transgenic mouse expressing human tumour necrosis factor. This mouse shows gross and histological evidence of arthritis with associated weight loss, at around 3-4 weeks of age, increasing in severity up to 9-10 weeks when complete loss of hind limb movement occurs (Keffer et al., 1991). This model was not initially developed as a model of arthritis and is proposed as a model suitable for the study of the treatment and pathogenesis of arthritis. While this model would be useful for the study of treatments of arthritic disease, and is strongly indicative of a role for tumour necrosis factor in the pathology of arthritis, its use in the evaluation of the pathogenesis of arthritis is complicated by the overexpression of a human protein in an experimental mouse.

#### 3.4.2. The validity of monoarthritic models

There has been some debate about the validity of models of monoarthritis as many models which are initially confined to one limb eventually become more widespread (Millan et al., 1988), and some workers have documented contralateral effects following a very mild ipsilateral stimulus (Levine et al., 1985a; Denko and Petricevic, 1978; Kidd et al., 1989a). The model described here (monoarthritis) has been utilised for a number of years as a model of monoarthritis; animals have been kept for up to 70 days after the injection of adjuvant and contralateral evidence of inflammation has been extremely rare. Another unilateral model of arthritis induced by adjuvant injected intra-articularly (Butler et al., 1992) also did not show any contralateral effects when the animals were followed for six weeks. It could,

therefore, be hypothesised that adjuvant represents a stimulus which can be graded to produce arthritis which is either localised to the immediate area of adjuvant injection or more widespread.

### 3.4.3. The use of gross measurements of inflammation in the assessment of arthritis.

Hind-limb swelling or diameter has been widely used in the assessment of arthritic inflammation (de Castro Costa et al., 1981; Coderre and Wall, 1987; Butler et al., 1992) but has been criticised (Rainsford, 1982), who states that " hind-paw swelling ... is admittedly a convenient measure ... but tells absolutely nothing about what is happening in the joints that are affected with the disease". The strong positive correlations shown here between the histopathology and the hind paw swelling show that, in this model, this statement is incorrect. The fact that inflammation scores (when performed blind by the same experienced operator) and the response to a noxious stimulus also correlate highly with joint pathology confirms that complicated methods of assessment of joint destruction are not always necessary. Rainsford also maintains that in addition to radiological studies, only complex biochemical investigations can give an accurate picture of the state of joint pathology. The changes seen in classical polyarthritis, and the model adapted by Butler et al. (1992) can be assessed by radiological examination as the bony joint destruction is marked. However, many models, including that described here have been purposefully developed to give a milder arthritis. It has not been established whether the histological features seen in this adapted model are too mild to show obvious changes on radiographic analysis but total joint destruction with joint ankylosis, as seen in more severe models, is never seen in the model described here. In addition, it is not always possible for workers to gain access to the facilities for radiography and complex biochemical analysis regarded as necessary by Rainsford.

Therefore the correlations here should enable use of this model without the need of recourse to complicated assessment of joint pathology.

#### 3.4.4 The arthritic rat as a model of chronic stress.

The circulating corticosterone in both mono- and bilaterally arthritic rats shows both elevated overall levels and a loss of diurnal rhythm throughout the fourteen days of study. However, none of the other measures documented to change under conditions of chronic stress (Sarlis et al., 1992) such as CBG levels and thymus, spleen and adrenal weight, show any significant differences in arthritic rats compared to controls. The thymus is particularly sensitive to increased corticosterone and involution would be expected to occur over this period of time. It is unclear why these parameters are unaltered in the face of raised corticosterone, however as this arthritis is immunologically mediated, the immune stimulus may counteract the effects of raised corticosterone on the thymus gland. In addition this model may represent an intermittent stress, as animal discomfort may only occur when the animal moves, and this is then reflected in raised corticosterone levels with no alteration in any of the other parameters under study.

The particularly striking feature of corticosterone secretion in these animals is the obvious loss of diurnal variation, due to an apparent increase in morning values. Control values used for comparison in the studies presented here were kindly provided by Dr. M. Holmes and were obtained from trunk blood samples in untreated control rats of the same strain (Han-Wistar). Concurrent samples were not taken from control animals during the fifteen days of the development of arthritis due to the problems of obtaining unstressed control blood samples and this obviously limits the interpretation of the data presented. It has proved difficult to obtain control corticosterone levels in the animal facility used due to the conditions of housing. The control data provided by Dr. Holmes were obtained using careful

housing and handling conditions and provide a guide as to the unstressed corticosterone levels in this strain. It is more difficult to interpret this data as a result; however it is unlikely that the method of sampling i.e. tail nicks is complicating the data. Tail nicks are an accepted method of sampling blood from animals without the effects of handling producing a rise in circulating corticosterone in itself, as the sample is taken too rapidly for circulating steroids to be altered in tail blood. Other authors have shown a loss of diurnal rhythm in corticosterone in polyarthritic rats (Sarlis et al., 1992) where morning corticosterone levels were raised to control evening levels and above, whereas evening corticosterone secretion remained unaltered until 14 days after adjuvant injection. These authors postulate that this may be due to increased HPA activation in response to chronic stress. This phenomenon is also apparent in other models of chronic stress (Bhatnagar et al. in press) and certain clinical conditions such as Cushing's syndrome and depression, where the most notable feature is a loss of the evening nadir in cortisol secretion (Wilson and Foster, 1992). However, the significance of the altered secretion is unexplained as these animals show none of the features seen in conditions characterised by a loss in diurnal cortisol secretion in humans. As has already been stated in Section 3.3.1., the ambulatory and exploratory behaviour and weight gain in these rats are all normal. Interleukin 1 (IL-1), known to be produced in inflammatory states (Dinarello et al., 1986), is thought to have stimulatory effects on the HPA axis (Harbuz and Lightman, 1992) and therefore increased circulating IL-1 levels in these animals may underlie the loss of diurnal rhythm in these animals.

In human rheumatoid arthritis (RA), cortisol diurnal variation was found to be normal compared to normal and osteoarthritic controls, and patients with osteomyelitis by Chikanza et al. (1992). However the cortisol levels were at the lower limit of normal and were inappropriately low for the degree of active joint inflammation in the patients. Patients with osteomyelitis had cortisol levels at the high normal level which would be expected in severe inflammation (Chikanza et al.,



1992). However others have reported both abnormally high and low circulating cortisol levels in active RA (Chowdrey and Lightman, 1993). It is therefore unclear how the disturbances in corticosterone secretion in the arthritic rat relate to the situation in the human disease.

### **3.5. SUMMARY**

It can be seen that this model of adjuvant-induced arthritis in the rat has several advantages over the classical model as described by Pearson and Wood. Slight manipulations in the concentration of *Mycobacterium tuberculosis* used in the induction of the disease can result in either a discrete monoarthritis, or a bilateral arthritis, the point at which contralateral spread occurs being easily predicted. The monoarthritis remains confined to a single joint for up to seventy days post-injection. The animals used do not suffer profound systemic disease, unlike other models, so changes observed are more likely to be directly attributable to the inflammatory process *per se*. The inflammation has been shown to be arthritic in nature, with severe synovitis but only mild destruction of hard tissues, thereby reducing the discomfort of the animals as ankylosis and joint deformation do not occur. There is a strong correlation between easily measured parameters of inflammation and the histological arthritis present, which facilitates the assessment of disease progression by non-invasive means.

In general these animals show significantly elevated corticosterone levels and a marked loss of the diurnal corticosterone secretion, however there are no significant changes in other parameters sensitive to corticosterone. This loss of diurnal variation may be attributable to increased endogenous cytokines. This model may therefore represent a mild form of chronic or intermittent stress, with the only evident change in "stress parameters" being a loss of diurnal variation in corticosterone.

## SECTION 4. SUBSTANCE P AND ITS ACTIONS ON CHEMOSENSITIVE PRIMARY SENSORY FIBRES WITH ARTICULAR RECEPTORS IN NORMAL AND ARTHRITIC RATS.

### 4.1. INTRODUCTION

Substance P, synthesised in dorsal root ganglion neurons, has long been postulated to be a neurotransmitter, as outlined in Section 1. The Dale principle suggested that the chemical transmitter released from the peripheral terminal of a neuron might be the same as that released centrally. This appears to be the case with the tachykinins and CGRP, which have been shown to be released from peripheral nerve terminals following antidromic nerve stimulation, capsaicin application or in inflammatory joint diseases (Holzer, 1988), and also in the dorsal horn on noxious stimulation.

#### 4.1.1. Effects of inflammatory mediators on primary afferent nociceptors

High threshold articular afferents innervating an acutely inflamed joint are known to become spontaneously active and to begin to respond to normally innocuous stimuli and movements within the working range of the joint (Schaible and Schmidt, 1985). In rats with chronic adjuvant arthritis, lowered mechanical thresholds have been shown in joint nociceptors (Guilbaud et al., 1985). This activation and sensitisation of nociceptors is mediated through the action of inflammatory mediators on nociceptors and has been thought to be responsible for inflammatory pain and hyperalgesia. Various products of immune cells, the peripheral vasculature and sympathetic nerves have been shown to exert effects on peripheral nociceptors. Among these are 5-hydroxytryptamine (5-HT; Grubb et al., 1988; Herbert and Schmidt, 1992), prostanoids and other arachadonic acid metabolites (Cohen and

Perl, 1990; Grubb et al., 1991; McQueen et al., 1991; Birrell et al., 1991), bradykinin (Koltzenburg et al., 1992), platelet activating factor and low pH. The interaction of these mediators was recently discussed at a symposium on Inflammatory Mediators which was reported on by Dray and Bevan (1993).

It has been hypothesised that substance P may act upon the peripheral nociceptor terminals to sensitise them to other mediators, and that in turn other mediators may act through the release of substance P (Nakamura-Craig and Smith, 1989). CGRP, another neuropeptide which may be involved in the activation or sensitisation of peripheral nociceptors is known to be released from afferent nerves by the action of known inflammatory mediators bradykinin and histamine (Hua and Yaksh, 1993).

#### 4.1.2. Aims of the study

In view of the effects of neuropeptides on trigeminal ganglion and second order sensory neurons, and the fact that neuropeptides are released in the periphery, it was of interest to examine the effects of substance P on primary afferent terminals in normal and arthritic rats. The effect of close arterial injection of synthetic substance P on both noxious mechanical and spontaneous activity in normal and arthritic rats was studied to examine:-

1. whether substance P directly excites, or sensitises articular nociceptors in the normal state, and
2. whether substance P contributes to the increased activity observed in afferents supplying the inflamed joint.

#### 4.2. MATERIALS AND METHODS

The in vivo monoarthritis preparation, neural recording and off-line analysis has been described in detail in Section 2 (Materials and Methods).

Briefly, the trachea, external carotid artery, and right femoral artery and veins of urethane anaesthetised male rats (Han Wistar, 200-250g) were cannulated. Blood pressure was monitored throughout the experiments. Drugs were administered by close arterial injection through the femoral arterial cannula. Extracellular recording was performed using a bipolar platinum electrode from the PACR which supplies the tibio-tarsal joint in the rat. The effects of drugs on single identified units were analysed off-line as described previously.

### 4.3. RESULTS

All units studied responded to capsaicin (1-10 $\mu$ g i.a.), adapted slowly to a noxious mechanical stimulus, and were identified as C-fibres by conduction velocity (<2.5ms<sup>-1</sup> Berne and Levy, 1990). All arthritic animals were used between 14 and 21 days after adjuvant injection

#### 4.3.1. Effect of close arterial injection of synthetic substance P on articular nociceptors in normal rats

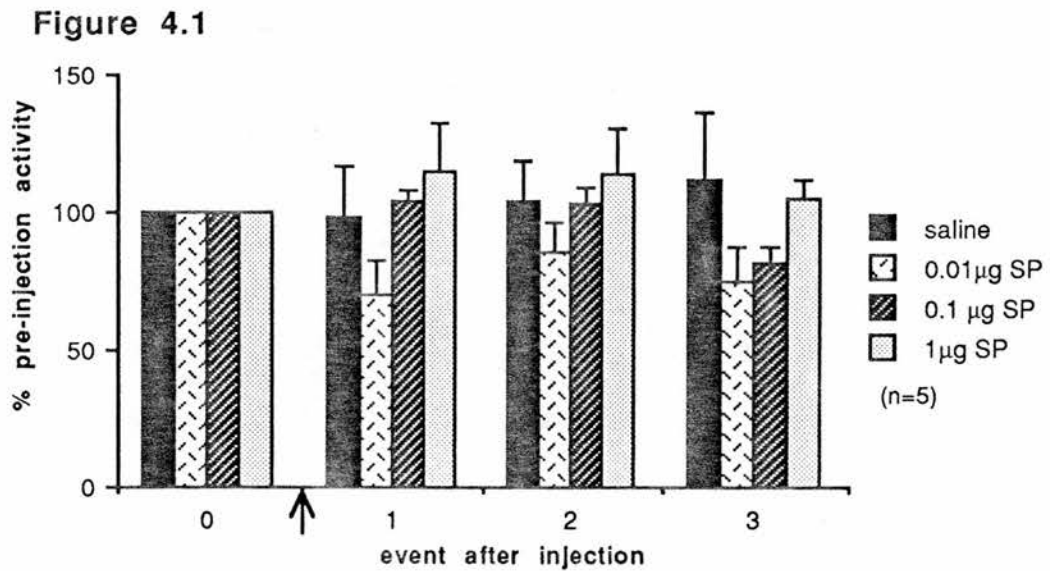
Comparison of pooled data from 5 animals for the three doses of substance P with either; a) the effect of saline injection or b) the preinjection period showed that substance P had no significant effect on the articular nociceptors on either mechanically evoked or spontaneous activity (Figures 4.1 and 4.2).

Data are shown normalised to the pre-injection activity as absolute basal numbers of action potentials in spontaneously active afferents, or those evoked by the mechanical indent varied considerably between experiments (Raw mean basal counts per minute (c.p.m.) for each graph are shown in the figure legends.). Mean activity is calculated for the minute preceding drug injection, the first 15 seconds after injection and in blocks of 1 minute thereafter.

4.3.2. Effect of close arterial injection of substance P on spontaneous and mechanically evoked activity in arthritic rats.

Neither mechanically evoked (Figure 4.3) nor spontaneous discharge (Figure 4.4) recorded from joint nociceptors in arthritic rats was affected by substance P when compared to either preinjection control activity, or saline injection. However, 1 unit showed increased activity 2 minutes post-injection of 1 $\mu$ g substance P (Figure 4.5).

Data are shown normalised to the pre-injection activity for both spontaneous and mechanically evoked activity due to inter-animal and intra-experimental variation.



*Figure 4.1. Mechanically evoked responses of articular nociceptors in normal rats to a 2s indent repeated at intervals of 120s following saline and substance P injection (i.a.). Arrow indicates Substance P injection (15s before stimulus 1). Plots are means  $\pm$  S.E.M. Mean basal evoked activity in c.p.m. ( $\pm$  S.E.M.) was 246 (48).*

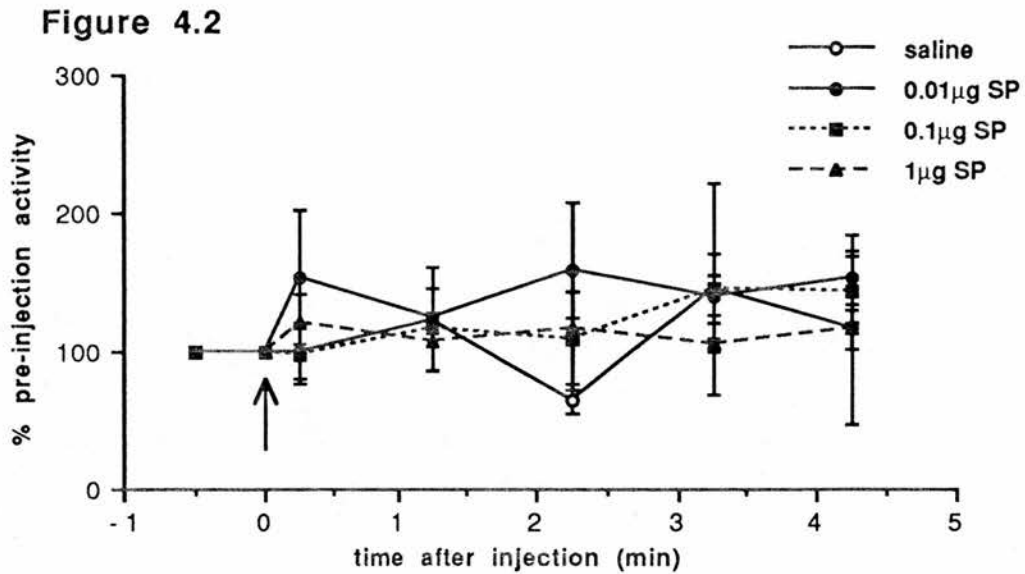


Figure 4.2. Normalised spontaneous activity of articular nociceptors in normal rats following substance P injection (i.a.; arrow at time 0). Values are means  $\pm$  S.E.M. Mean basal spontaneous activity in c.p.m. ( $\pm$  S.E.M.) was 58 (32)

#### 4.3.3. Effects on single articular nociceptors

In the meaned values for spontaneous activity in arthritic rats., 2.25 minutes post-injection, there appears to be a non-significant response to 1µg substance P. This is due to the effect of one nociceptor which responded slightly to 0.1µg SP and dramatically to 1µg SP (Figure 4.5). This unit had a conduction velocity of 1.8ms<sup>-1</sup>. Activity is again expressed as a percentage of the pre-injection control due to variation in nociceptor activity throughout the duration of the experiment (3-21 counts per minute).

**Figure 4.3**

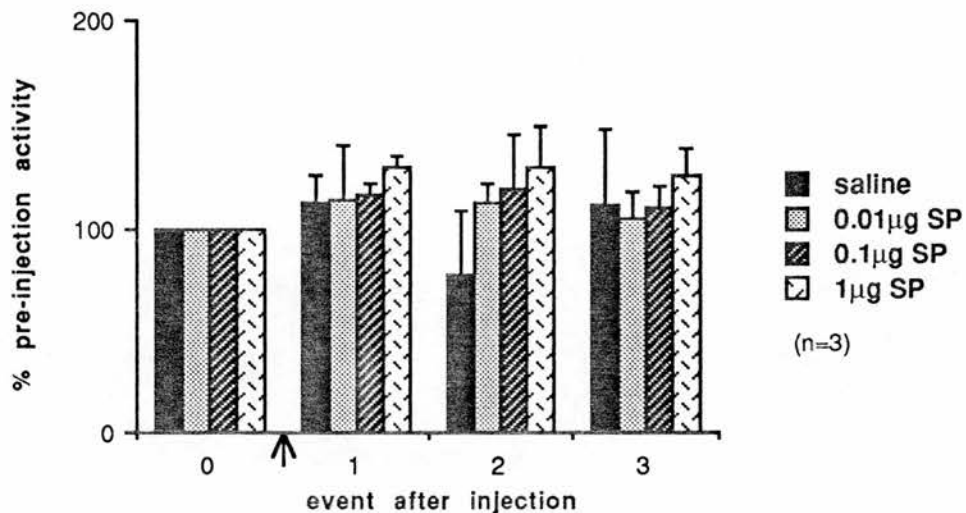


Figure 4.3. Mechanically evoked responses in arthritic rats in response to a 2s mechanical indent before and after injection of substance P (arrow). Values are means  $\pm$  S.E.M. Mean basal evoked activity in c.p.m. ( $\pm$  S.E.M.) was 349 (89).

**Figure 4.4**

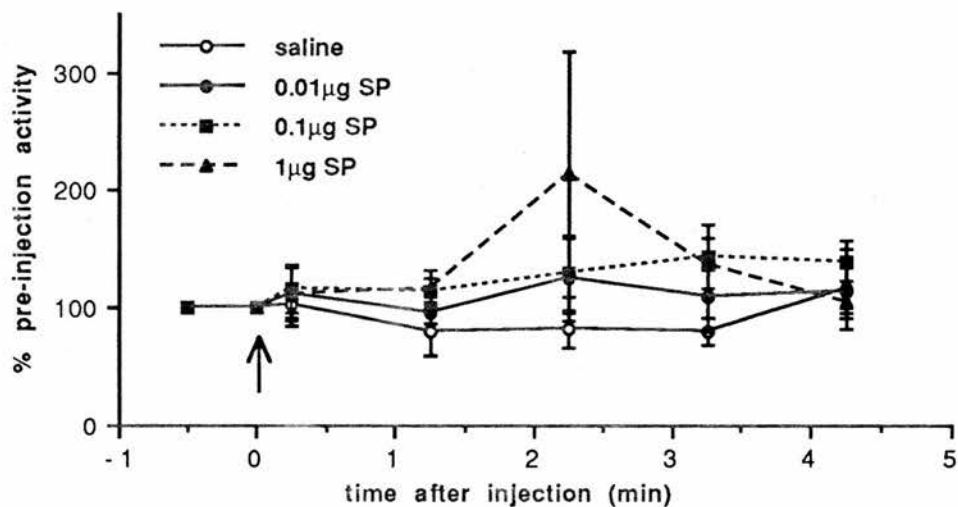
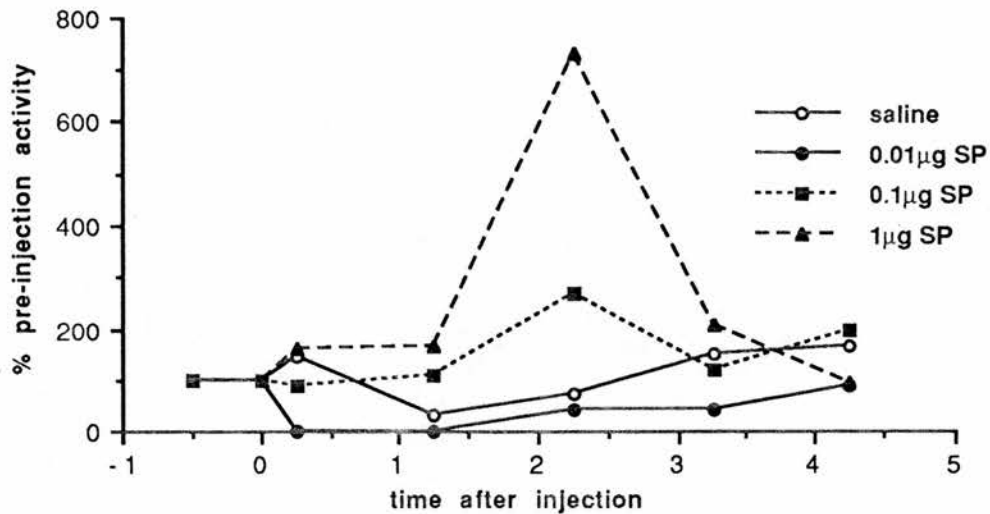


Figure 4.4. Effect of i.a. injection of substance P on spontaneous activity in articular nociceptors in arthritic rats. Activity is shown as normalised means  $\pm$  SEM. Mean basal spontaneous activity in c.p.m. ( $\pm$  S.E.M.) was 76 (25) n=5

**Figure 4.5**



*Figure 4.5. Activity in a single nociceptor from an arthritic rat after injection of saline or substance P (SP), normalised to pre-injection controls. Drug injection was at time 0. Mean basal spontaneous activity in c.p.m. ( $\pm$  S.E.M) for the experiment illustrated in Figure 4.5. was 12 (3).*

#### 4.4. DISCUSSION

In behavioural studies, Nakamura-Craig and colleagues have shown a peripheral hyperalgesia induced by intraplantar injection of substance P or neurokinin A (Nakamura-Craig and Smith, 1989; Nakamura-Craig and Gill, 1991). CGRP also caused hyperalgesia but was significantly less potent than either of the neurokinins. Mechanical hyperalgesia can be induced by repeated saline injections (Levine et al., 1985a) and this hyperalgesic effect may not be specific to tachykinins although this is unlikely as neurokinin antagonists prevented the hyperalgesia, and saline did not have an effect in the studies reported by Nakamura-Craig and Gill (1991). These studies have been supported in humans when substance P and neurokinin A caused



significantly more cutaneous pain than saline placebo, whereas CGRP had no effect (Pedersen-Bjergaard et al., 1991). From these data it was hypothesised that substance P could act on nociceptors to sensitise them to other mediators, or could directly excite them.

#### 4.4.1. Effects on single articular afferents and criteria for inclusion in the analysis

In the course of these studies, one unit responded to the close arterial injection of substance P. From earlier studies group III articular nociceptors were shown to be sensitised to noxious movements by substance P (Herbert et al., 1987). In the experiments presented here, substance P seemed to have a minor effect on spontaneously, not mechanically-evoked activity in an arthritic rat. This effect may be due to the fact that a conduction velocity of  $< 2.5\text{ms}^{-1}$  was used as the cut-off for C-fibre categorisation whereas there is some overlap in conduction velocity of C- and A $\delta$ - fibres (Lawson and Waddell, 1991) resulting in the inclusion of a nociceptor from a class of afferent which may be sensitive to substance P. However the criteria for classification in the analysis were defined before the experiments were performed and as statistical analysis cannot be performed on the responses of a single nociceptor, this unit was included in the data shown here. It seems therefore that the criteria for inclusion in this analysis may not have been appropriate for this animal preparation.

#### 4.4.2. The effects of substance P on nociceptive afferents

The results presented here show that substance P does not have an effect on unsensitised articular nociceptors in the normal rat, or on sensitised nociceptors in inflammatory arthritis. In general, this agrees with reports from other workers who have studied the direct effect of substance P on cutaneous A $\delta$  and C-fibre afferents.

It is possible that substance P is exerting a tonic effect during inflammation, in conjunction with the other mediators of inflammation in the periphery, and therefore exogenous substance P would have no effect in established inflammation, or in the normal animal, without other sensitising agents. Recent work by Asghar and McQueen (unpublished) has not supported this. Administration of an NK1/NK2 antagonist FK224 (Fujisawa) has no effect on either the mechanically evoked or spontaneous activity in articular nociceptors innervating inflamed joints, and substance P does not potentiate the action of bradykinin on nociceptors in normal animals (Asghar and McQueen, unpublished).

The vast majority of previous work has been performed on cutaneous nociceptors, although substance P action on neuroma afferents has also been studied and found to be non-existent on either A- or C-fibres (Devor et al., 1992). In a more physiological situation, Fitzgerald and Lynn (1979) found that synthetic substance P injected in greater amounts than in these studies (2-20 $\mu$ g), induced only weak excitation of a small number of polymodal nociceptors in rat skin and did not potentiate responses to noxious stimuli. More recent studies on cutaneous C-fibre nociceptors in ex vivo conditions have shown no direct effect of substance P or of substance P antagonists (Cohen and Perl, 1990), but a potentiation of the response to an "inflammatory soup" containing bradykinin, 5-HT, histamine and prostaglandin E<sub>2</sub> (Kessler et al., 1992). In vivo, substance P did not excite testicular nociceptors in the dog (Kumazawa and Mizumura, 1979), and in a study on the cat knee joint, it sensitised only group III, but not group IV afferents to specifically noxious movements of either normal or acutely inflamed joints (Herbert et al., 1987). In this classification by conduction velocity used by Schmidt and colleagues, the group IV units probably belong to the same population as those under study here, which were also not responsive to substance P either in the normal or arthritic state.

An excitatory action for substance P on primary sensory neuronal perikarya in the trigeminal ganglion has, however, been demonstrated (Spigelman and Pail, 1991). This excitation, along with the behavioural effects of substance P on peripheral hyperalgesia have suggested that substance P may excite primary afferents through an autoreceptor. The published literature on the action of substance P on peripheral terminals along with the data shown here, earlier *in vitro* work which showed no effect of substance P on dialysed trigeminal neurons or DRG neurons in culture (Krishtal and Pidoplichko, 1981; Nowak and MacDonald, 1982) and recent evidence that primary afferent neurons do not express the NK1 receptor (Brown et al., 1993) does not support this hypothesis.

#### 4.5. SUMMARY

Neurokinins have strong excitatory actions on motoneurons, second order sensory neurons in both the dorsal horn and trigeminal nucleus (Otsuka and Yoshioka, 1993), and postganglionic sympathetic neurons (Dun and Karczmar, 1979). However it would appear from these data and the literature that the predominant peripheral function of neuropeptides in general, and substance P in particular is on the vascular and immune systems (Section 1) rather than on autoreceptors directly on primary afferent nociceptors. Some workers have shown weak facilitatory actions for substance P on primary afferents; it may be that substance P only has effects on C-fibre nociceptors in conjunction with a number of other inflammatory mediators. The behavioural effects of substance P and neurokinin A in peripheral hyperalgesia may not be direct effects of substance P on nociceptors, particularly as primary sensory neurons do not express NK1 receptors. Alternatively, this hyperalgesia may be mediated via A $\delta$  afferents, some of which are also nociceptive and may be sensitised to noxious stimuli by substance P (Herbert et al., 1987).

#### 4.6. CONCLUSION

Substance P does not have an excitatory action on C-fibre nociceptors in either the normal or the arthritic rat (present data) but an effect on spontaneous activity in A $\delta$  mechanoreceptors in this adjuvant mediated monoarthritis in the rat cannot be excluded.

## **SECTION 5. NEUROPEPTIDE MESSENGER RNA EXPRESSION IN MALE AND FEMALE RATS WITH MONO- AND BILATERAL ARTHRITIS**

### 5.1 INTRODUCTION

#### 5.1.1. Primary afferent neuropeptides in experimental arthritis.

Neuropeptides in primary afferents and projection neurons in the spinal cord have been extensively studied in inflammatory and arthritic states, principally as they have been implicated in both mediation of neurogenic inflammation and the spinal processing of nociceptive stimuli. Numerous studies have shown that neuropeptides in primary afferent neurons increase in established inflammatory conditions particularly substance P (Smith et al.; 1992; Colpaert et al., 1983; Lembeck et al., 1981), CGRP (Hanesch et al., 1993; Smith et al. 1992; Kuraishi et al., 1989) and somatostatin (Ohno et al., 1990)-although the latter has also been reported to remain unchanged in arthritic rats (Smith et al., 1992). Messenger RNA levels of species encoding substance P and neurokinin A also increase in primary afferents following peripheral noxious (Noguchi et al., 1988), and inflammatory stimuli (Iadarola and Draisci, 1988).

VIP shows very low or absent expression in DRG under basal conditions but its expression is dramatically increased following nerve injury such as axotomy or crush in the rat (Atkinson and Shehab, 1986; Nielsch and Keen, 1989). Primary afferent VIP is not increased on nerve damage in primates (Zhang et al., 1993) highlighting inter-species variation. VIP expression has not previously been investigated in experimental arthritis.

### 5.1.2. Male/female differences in adjuvant-induced monoarthritis.

Many autoimmune conditions in humans are known to affect proportionally greater numbers of females than males, notably so in systemic lupus erythematosus (Inman et al., 1978). It has been hypothesised that this is due to an effect of oestrogens since both pregnancy and oestrogen containing oral contraceptives exacerbate the condition (Mund et al., 1963; Jungers et al., 1982). The susceptibility to rheumatoid arthritis is also greater in females than males; UK statistics show a 3:1 female to male prevalence ratio and a 2.5:1 incidence ratio (Hazes and Silman, 1990), but the hormonal influences in this condition are much less clear cut. It has been suggested recently that, in humans, androgen levels may be more important in reducing susceptibility to RA, than oestrogens in increasing risk (James, 1993), which would account for the amelioration of disease severity often seen in the third trimester of pregnancy (Hazes, 1991) when androgen levels are increased due to production in the corpus luteum, placenta and foetal gonads and adrenal (James, 1993).

The situation in rodents is also complex. For example, inbred female LEW/N rats are more susceptible to streptococcal cell wall arthritis than are males (Wilder et al., 1982). In adjuvant arthritis, oestradiol has been shown to reduce the incidence of the disease in rats (Mueller and Kappas, 1964) but pregnancy causes no improvement in the disease process, in contrast to the situation in patients with rheumatoid arthritis (Mathers and Russell, 1990; Unger et al., 1983). In gonadectomised animals exogenous androgens were shown to eliminate glycosaminoglycan breakdown whereas this was exacerbated by oestradiol administration (Steward and Bayley, 1992). However in the initial studies on adjuvant arthritis in rats, Pearson and Wood (1959) found no sex differences in the incidence of polyarthritis. Holmdahl et al. (1986) showed that female sex hormones will reduce the severity of arthritis induced by Type II collagen in mice and that female mice were much less susceptible to arthritis than male or oophorectomised female mice. In view of this conflicting data

it was of interest to investigate the male/female differences in reaction to adjuvant in the modified model of adjuvant disease used throughout the studies presented here.

### 5.1.3. Aim of the experiments

The role of the primary afferent in inflammatory conditions has been widely studied in terms of the effector function of C-fibres. As neuropeptides are released both centrally and peripherally, thorough study of the precise role of neuropeptide-containing primary afferents in inflammatory arthritis is highly desirable. Studies of peptide levels, while valuable, still leave the question of whether observed changes are due to increased production, altered transport and/or metabolism, unanswered. Studies involving animal models of chronic inflammation, for example, polyarthritis in rats, are complicated by severe and widespread systemic disease. Different neuropeptides may also have changing roles in the models of acute and chronic inflammation routinely used. Therefore neuropeptide messenger RNA expression has been investigated during the development of monoarthritis in DRG of male and in established arthritis in both male and female rats to determine whether changes in peptide are reflected in changes in the level of mRNA. In addition, the primary afferent neuropeptide response in symmetrical disease in male rats has been determined to elucidate the possible role of peptidergic primary afferents in the contralateral spread of arthritis.

## 5.2. MATERIALS AND METHODS

### 5.2.1. Neuropeptide mRNA expression in DRG

Monoarthritis was induced in female or male Wistar rats (7 weeks of age) and bilateral arthritis in male rats as detailed in Section 2, i.e. by the subdermal injection of either 150µg or 250µg *M.tub.* around the left "ankle" joint. The appearance and circumference of both ankle joints was recorded at intervals to assess inflammation as detailed in section 2.2.

Monoarthritic male animals (n=3-5 per time point) were killed by decapitation at various times after adjuvant injection (as outlined in the Results) in order to study responses in the acute and chronic stages of inflammation. Monoarthritic females and symmetrically arthritic males were monitored throughout development of arthritis and were killed by decapitation fifteen days after adjuvant injection. Both left and right fifth lumbar (L5) DRG (which innervate the tarsal joint via the sensory component of the sciatic nerve) were rapidly dissected from all animals, snap frozen and stored at -80°C. Control L5 DRG from untreated animals (n=10) and animals injected with filter-sterilised paraffin oil (n=3) were similarly taken. Sections were hybridised with riboprobes complementary to all four neuropeptide mRNAs as detailed in Section 2.4. Sections were then exposed to nuclear emulsion for two weeks, developed and counterstained. Expression of mRNA was estimated by counting silver grains overlying neuronal cell bodies of less than 30 µm diameter either by eye or using computerised image analysis.

### 5.2.2. Quantification of in situ hybridisation results

Initial in situ grain counts were determined by eye at x40 magnification under the light microscope. Subsequent analysis was done using computerised image analysis



designed by Seescan (Cambridge UK). These two methods of analysis were subjected to linear regression analysis to determine the relationship between them. Correlations were performed on silver grain counts from either cells expressing neuropeptide mRNA or hippocampal cells expressing steroid receptor mRNA (the latter counts were performed by Dr. J. Seckl). All in situ hybridisation data is expressed as a percentage of the untreated control expression, calculated as described in Section 2, however the mean ( $\pm$  S.E.M.) grain numbers for the control groups are also given in the figure legends.

### 5.2.3. Statistics

Data were assessed by ANOVA followed by Dunnetts post hoc test. Inflammation scores were compared using Wilcoxon's signed ranking test for paired data, and Mann Whitney U test for unpaired data. The Null Hypothesis was rejected at the 5% probability level. Values are shown as means  $\pm$  standard error of the mean (S.E.M).

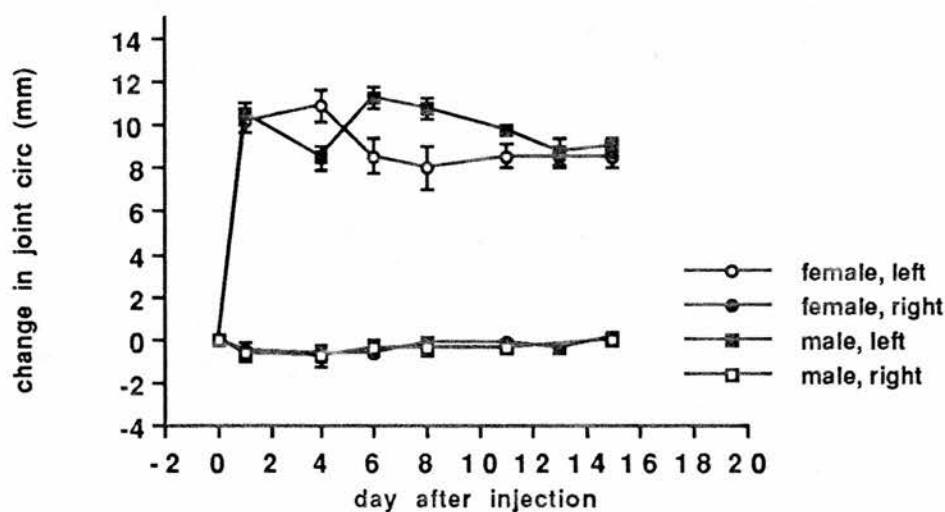
## 5.3 RESULTS

### 5.3.1. Indices of inflammation during development of monoarthritis

Male and female rats show a significant difference in body weight at 7 weeks of age and females tend not to increase in weight as rapidly as males, but development of arthritis did not affect the normal gain in weight seen within each group. Animals behaved normally with respect to grooming and exploratory behaviour throughout development of arthritis, and showed only slight guarding of the injected limb. All animals bore weight on the affected limb throughout the fifteen days of study.

In both male and female groups of rats, all animals developed significant ( $p < 0.01$ ) increases of the tibio-tarsal joint circumference ipsilateral to the side of adjuvant injection (Figure 5.1). In no case was there involvement of the contralateral (uninjected) joint. Although there were slight differences in the pattern of development of joint swelling, overall there were no significant differences in disease between males and females.

**Figure 5.1**



*Figure 5.1 Increase in left(injected) and right joints circumferences in monoarthritic male and female rats during the development of monoarthritis. Values are the difference between pre-injection joint circumference, and circumferences on each day of measurement post-injection, in mm  $\pm$  SEM.*

Overt signs of inflammation (redness, swelling) were also similar in the male and female monoarthritic animals (Table 5.1).

	Left	Right
Untreated controls	0	0
Female; monoarthritic	1.5 (1-3)*	0
Male; monoarthritic	3 (1-3)**	0
Male; bilaterally arthritic	3 (0-4) †	1 (0-4) †

*Table 5.1 Inflammation scores in female and male monoarthritic and bilaterally arthritic rats. Values are median (range) and \*p<0.05, \*\*p<0.01 c.f. contralateral limb (Wilcoxon signed rank test), †p<0.01 c.f. untreated controls (Mann Whitney U test).*

### 5.3.2. Quantification of neuropeptide messenger RNA levels.

Linear regression analysis of the two methods of determining the numbers of silver grains in high density neuropeptide in situ hybridisation showed a strong positive correlation between the two methods (Figure 5.2a). The ratio between eye and machine grain counts was not 1:1, rather 1:4.9 (Equation 1). The linear regression curve has the formula below:-

$$y = 4.9x - 154 \quad \text{Equation 1}$$

When low density grain counts by eye and computer were compared there was also a strong positive correlation, with the linear regression curve represented by the equation below:-

$$y = 0.8x + 4 \quad \text{Equation 2}$$

and illustrated in Figure 5.2b.

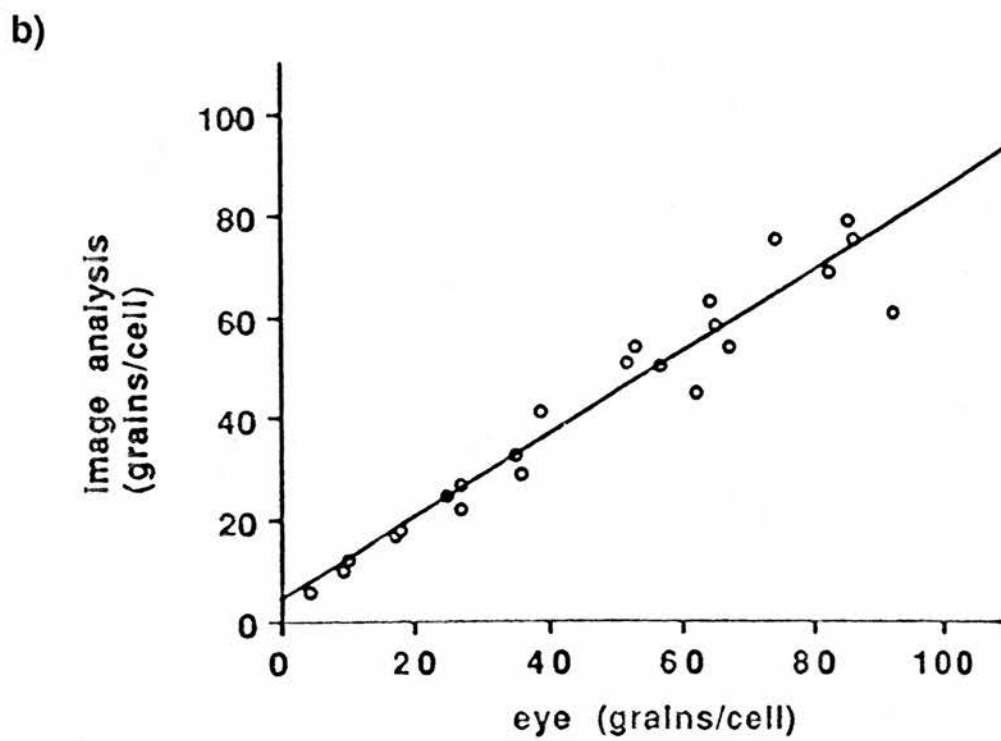
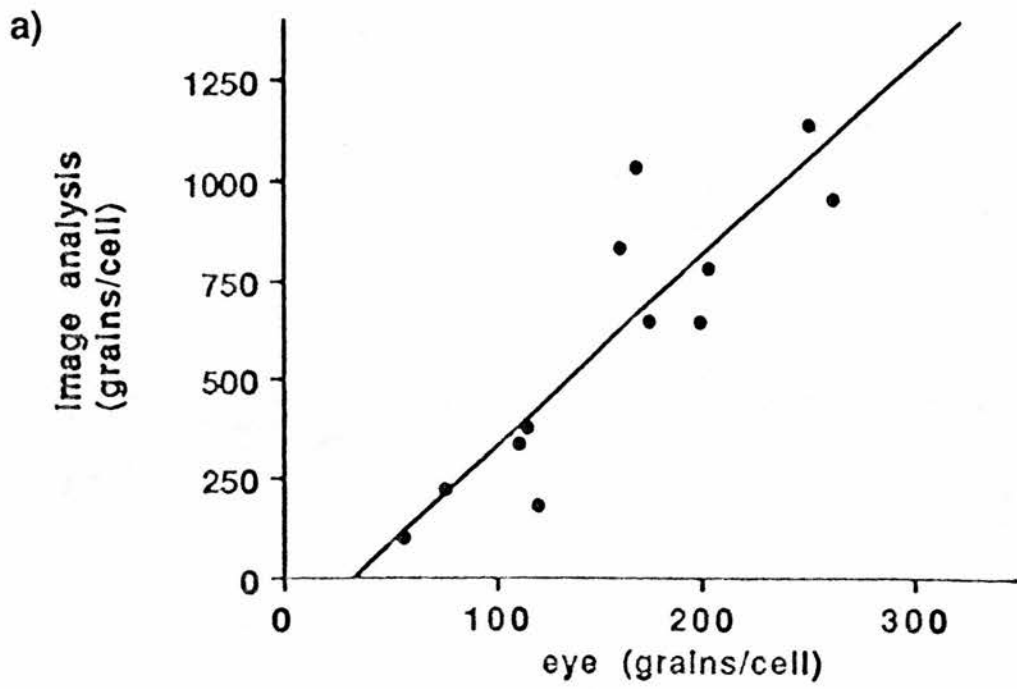


Figure 5.2. Linear regression curves for eye against computerised silver grain counts:-

a) for high density silver grains ( $r=0.90$ ,  $n=24$ ,  $p<0.0001$ )

b) for low density silver grains ( $r=0.97$ ,  $n=23$ ,  $p<0.001$ )

### 5.3.3. Neuropeptide messenger RNA expression during development of monoarthritis

PPT mRNA in control L5 DRG was expressed in  $22\pm 1\%$  of neurons. No change in the proportion of neurons expressing PPT mRNA was seen at either 8 hours or 14 days ( $19\pm 1\%$ ) after FCA injection, in either left or right L5 DRG. Eight hours after injection of adjuvant in male rats there was a significant increase ( $81\pm 24\%$  rise) in PPT mRNA expression per neuron in L5 DRG innervating the injected joint, in comparison with either the contralateral (uninjected) L5 DRG or control DRG. The early induction of PPT mRNA expression in innervating L5 DRG neurons resolved at 24 h, but recurred by 48 h and persisted until arthritis was evident at 14 days ; at no time was there any increased expression in contralateral L5 DRG (Figure 5.3). Expression of PPT mRNA in left and right DRG 8 hours after adjuvant injection is also illustrated in Figure 5.6.

PPT mRNA levels were also significantly increased ( $42\pm 13\%$  rise) in L5 DRG from female rats, fourteen days after adjuvant injection (Figure 5.4). This was not significantly different from the increase in mRNA values in male animals ( $56\pm 7\%$  rise;  $p>0.05$ ). Contralateral L5 DRG PPT mRNA levels in females were not significantly different from controls.

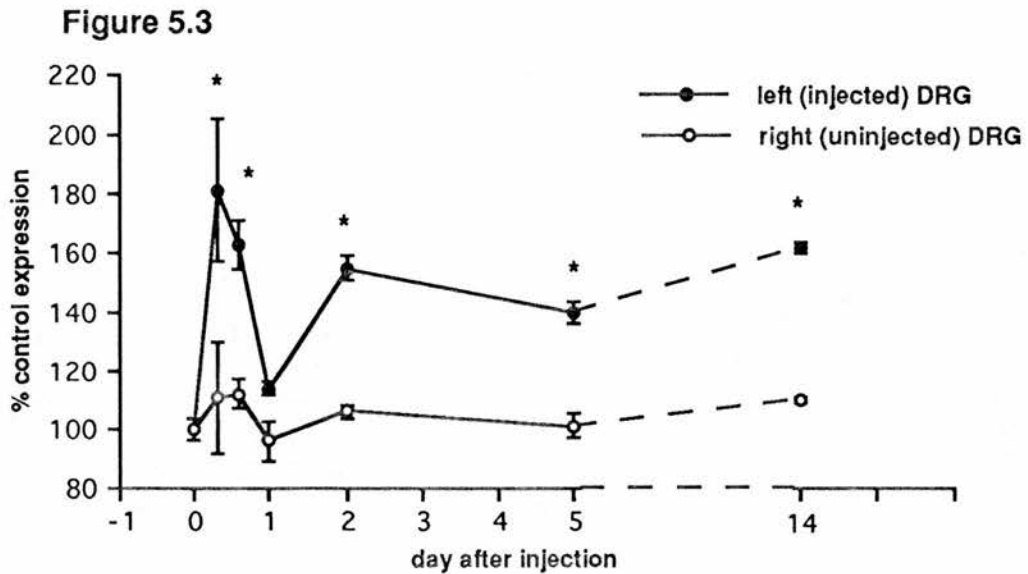


Figure 5.3. PPT mRNA expression during development of monoarthritis. Each point is mean ( $n=3$ )  $\pm$  SEM expressed as a percentage of control expression. \* $p<0.05$  compared with controls. Mean grain counts ( $\pm$  S.E.M..) for the control group were  $168\pm 2$  left;  $175\pm 4$ , right.

CGRP mRNA was expressed in  $37\pm 3\%$  of control DRG neurons, and there was no significant change in the proportion of L5 DRG cells expressing CGRP mRNA at the time of onset of chronic arthritis (day 14;  $36\pm 1\%$ ). As with PPT, the expression of CGRP mRNA per neuron was significantly increased ( $44\pm 6\%$  rise) 8 hours after FCA injection. CGRP mRNA levels remained significantly elevated up to and including the time of development of chronic monoarthritis. These changes were again limited to ipsilateral L5 DRG (Figure 5.5).

Figure 5.4

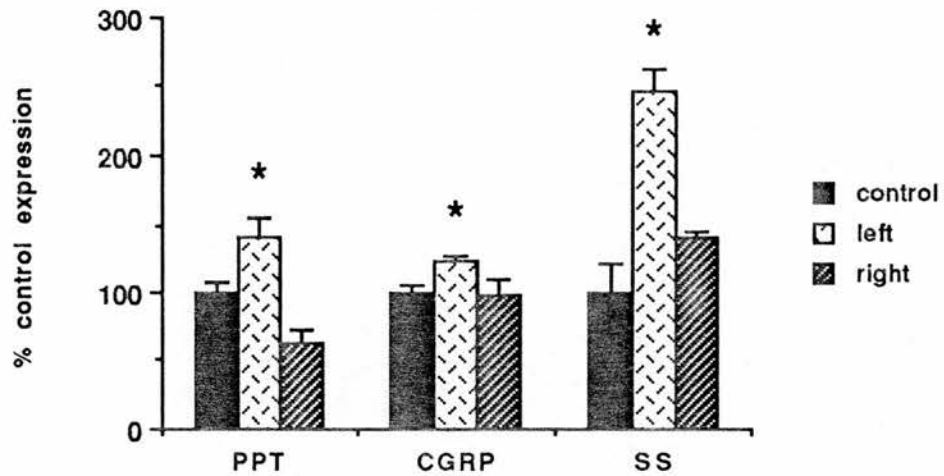


Figure 5.4. Neuropeptide mRNA expression in DRG of monoarthritic female rats. 15 days after adjuvant injection. Values are percentages of untreated controls and are means  $\pm$  SEM. \* $p < 0.05$  cf. control. Mean control grain counts were PPT,  $179 \pm 29$ ; CGRP,  $179 \pm 2$ ; SS,  $355 \pm 46$ .

Levels of CGRP mRNA in female rats also rose fourteen days post-injection ( $24 \pm 3\%$ ), and were significant when compared to contralateral mRNA levels (Figure 5.4). The rise in females was significantly lower than CGRP mRNA values in male rats ( $39 \pm 4\%$ ;  $p = 0.02$ ).

CGRP and PPT mRNA expression 8 hours and 14 days after unilateral adjuvant-inflammation of the left ankle joint are also illustrated in Figures 5.6 and 5.7.

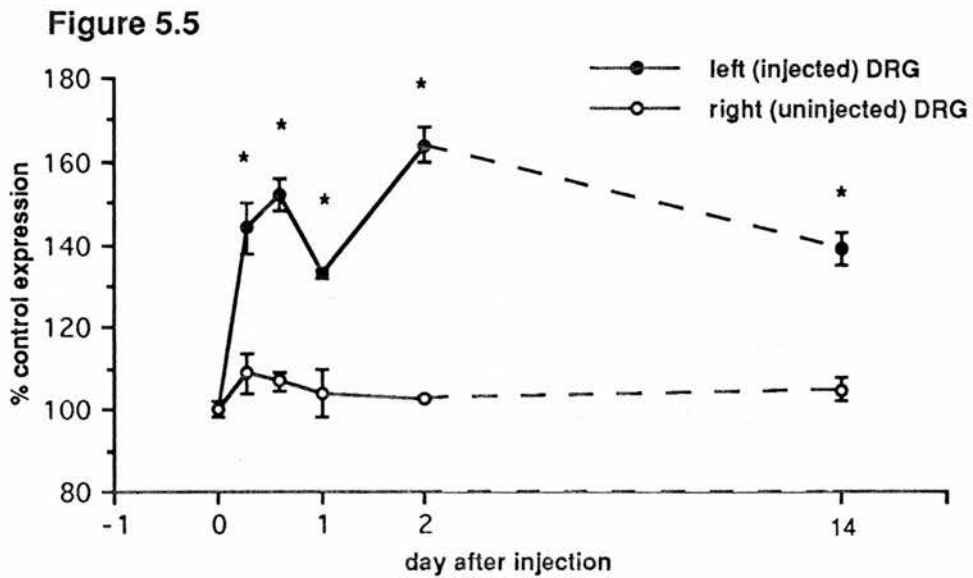


Figure 5.5. CGRP mRNA expression in DRG of monoarthritic rats expressed as a percentage of untreated controls. \* $p < 0.05$  compared to control value. (Means  $\pm$  SEM;  $n = 3$  per point). Mean control grain counts were  $182 \pm 3$ , left;  $193 \pm 5$ , right.

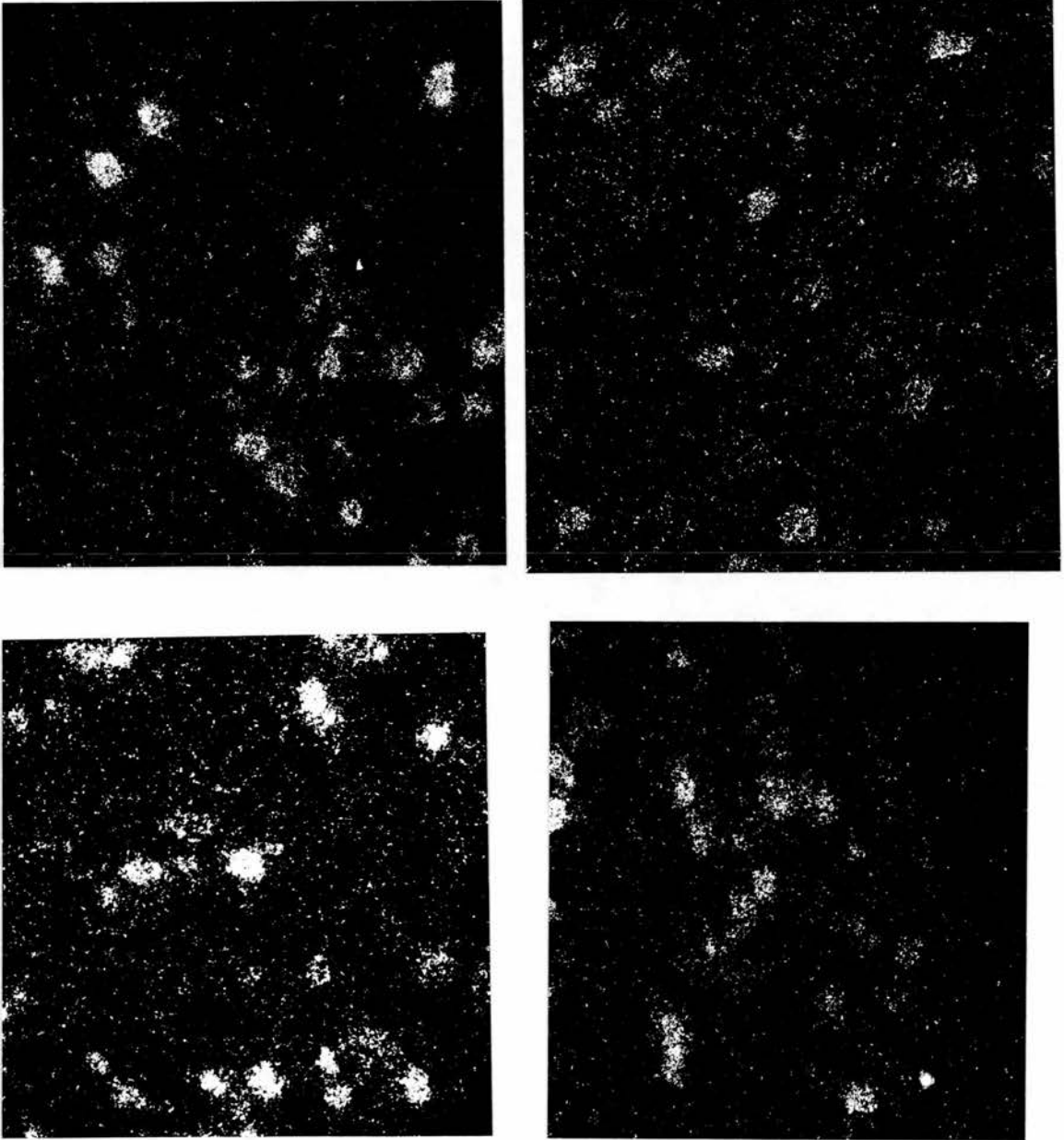
Somatostatin was the only neuropeptide mRNA species to show both ipsi- and contralateral changes during the fourteen day time course of development of arthritis in male rats. Ipsilateral mRNA expression was significantly reduced in the first 16 hours ( $-24 \pm 2\%$ ), returned to control values at 24 hours and was elevated when arthritis is evident at 14 days ( $39 \pm 10\%$ ). Contralateral mRNA expression was initially increased compared to controls at 24 hours ( $41 \pm 11\%$ ) and was reduced by 5 days remaining significantly lower when arthritis was evident at 14 days ( $-20 \pm 3\%$ ). Ipsilateral and contralateral data are illustrated in Figure 5.6.

Somatostatin mRNA expression was also significantly increased in female rats ( $147 \pm 16\%$ ) 14 days after adjuvant injection compared to controls (Figure 5.4) and to the increase seen in males ( $39 \pm 10\%$ ;  $p = 0.001$ ). There were no contralateral changes in SS mRNA expression in female rats in established arthritis.



Left

Right



*Figure 5.6. Dark field photomicrographs showing PPT mRNA (top) and CGRP mRNA (bottom) expressing neurons in left and right L5 DRG taken 8 hours after injection of adjuvant around the left ankle joint. Note the increased density of silver grains overlying expressing neurons in the left DRG compared to the right DRG. Original magnification x20.*



**Figure 5.8**

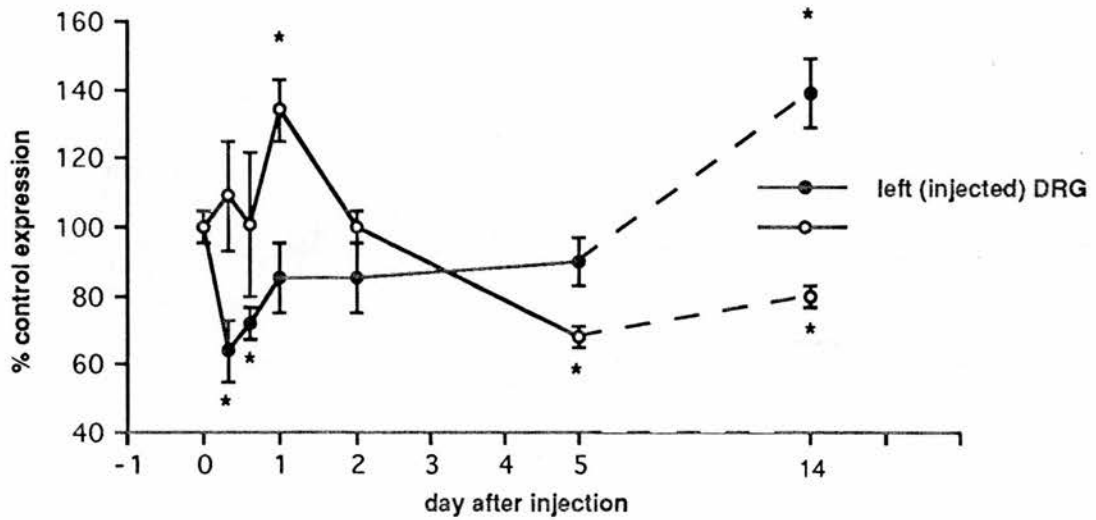


Figure 5.8. Somatostatin mRNA expression in DRG of monoarthritic rats. Values shown are means  $\pm$  S.E.M. \* $p < 0.05$  compared to controls ( $n = 6$  per point). Mean control grain counts were left,  $81 \pm 3$ ; right  $94 \pm 5$ .

#### 5.3.4. Effect of paraffin oil injection on neuropeptide mRNA expression

Injection of sterile paraffin oil (vehicle) did not cause inflammation and there was no change in CGRP mRNA expression eight hours after injection.

Injection of paraffin oil caused a significant decrease in PPT mRNA expression in ipsilateral DRG neurons and did not affect contralateral expression ( $104 \pm 2\%$  control) eight hours after injection. Injection of vehicle did not affect ipsilateral somatostatin mRNA expression but caused a significant increase in contralateral mRNA expression after eight hours. Neuropeptide mRNA levels after vehicle injection are summarised in Table 5.2.

	Preprotachykinin		CGRP		Somatostatin	
	Vehicle	Control	Vehicle	Control	Vehicle	Control
Left	86 ± 2*	100 ± 3	103 ± 7	100 ± 5	98 ± 2	100 ± 5
Right	104 ± 5	99 ± 3	104 ± 2	111 ± 2	121 ± 10*	100 ± 5

*Table 5.2. Neuropeptide messenger RNA expression in response to intradermal injection of 0.15ml sterile paraffin oil. PPT, CGRP and somatostatin mRNA expression in L5 DRG (as a percentage of expression in untreated control rats) in untreated control and paraffin oil injected rats, eight hours after injection.*

*(\*p<0.05; n=3) Mean grain counts for control groups are PPT, 171±8 left, 178±4 right; CGRP, 173±7 left, 197±6 right; SS, 81±5 left, 94±7 right.*

### 5.3.5. Indices of inflammation during development of bilateral arthritis

Male rats injected with the higher dose of M.tub. showed a normal increase in weight over the first fourteen days after adjuvant injection. There was a non-significant reduction in weight on development of symmetrical disease (see Figure 3.1). Rats also showed a rapid unilateral increase in left joint circumference (Figure 5.7) and inflammation score (Table 5.1) immediately after adjuvant injection which was maintained throughout the development of inflammation. Fourteen days after adjuvant injection, contralateral joint involvement was also apparent, evidenced by a significant increase in right joint swelling and inflammation score (Figure 5.9; Table 5.1).

Figure 5.9

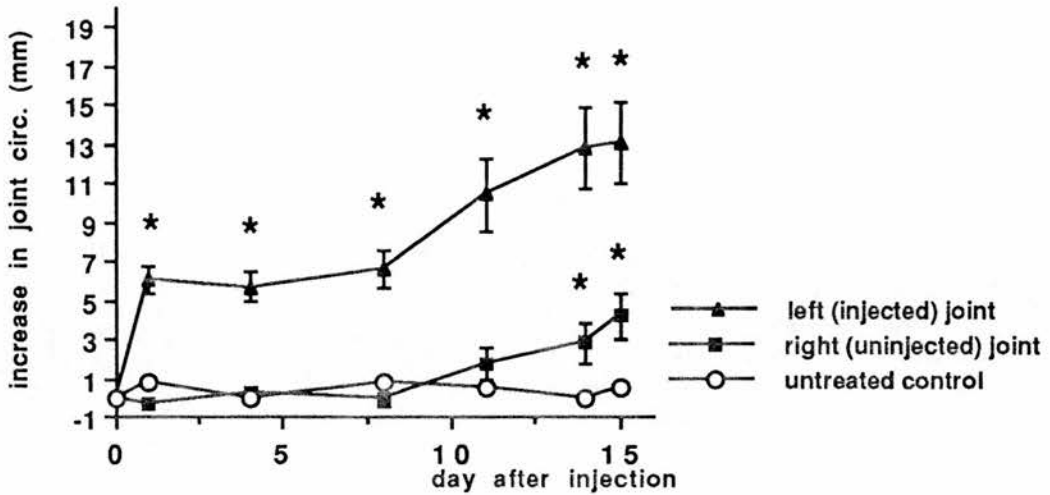
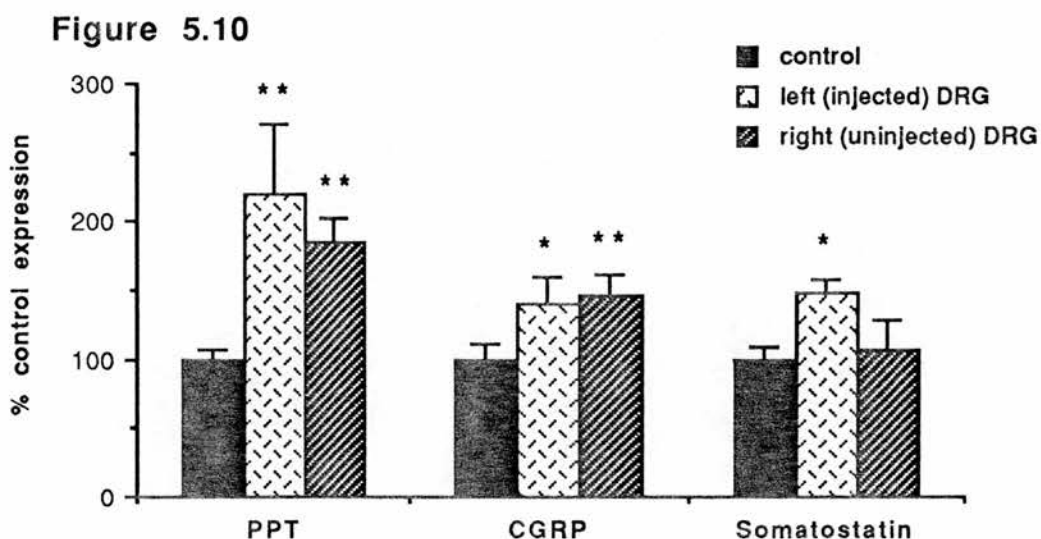


Figure 5.9. Increase in left (injected) and right (uninjected) ankle joint circumferences during development of bilateral arthritis in male rats. (Injection of 250 $\mu$ g *M.tub.* around left ankle joint) Points are calculated as the increase over pre-injection measurement, in mm, and are means  $\pm$  SEM. \* $p < 0.05$  cf. untreated control.

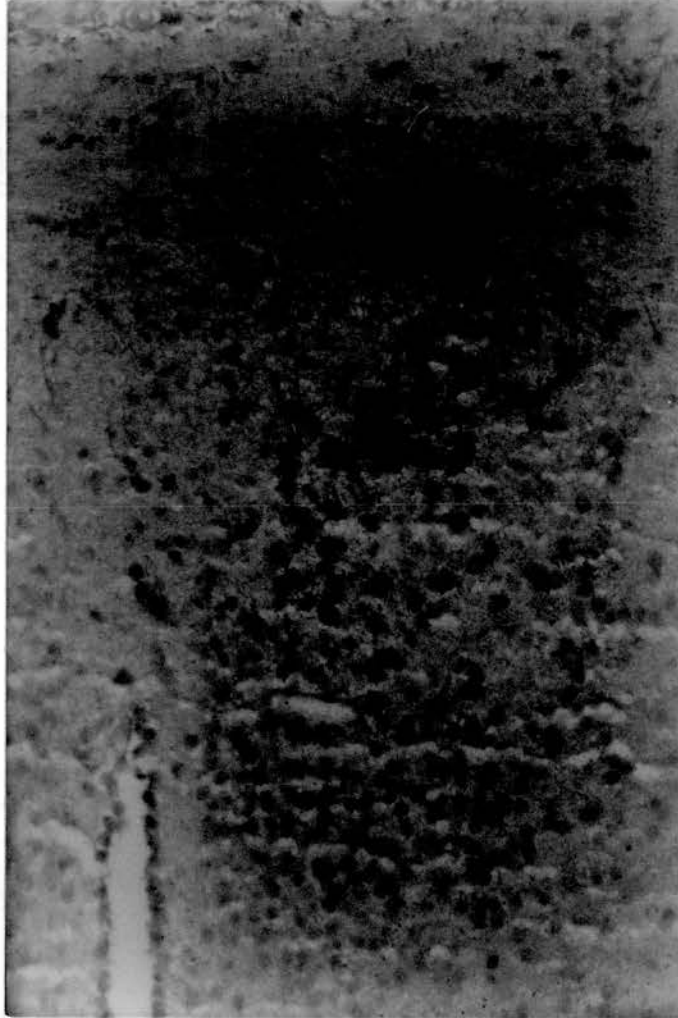
### 5.3.6. Neuropeptide messenger RNA expression in bilateral arthritis

PPT and CGRP mRNA increased bilaterally - PPT (121 $\pm$ 50% rise, left; 85 $\pm$ 17% rise, right) and CGRP (40 $\pm$ 19% rise, left; 47 $\pm$ 15% rise, right). SS mRNA increased in left DRG neurons but was unchanged in right DRG compared to control values (48 $\pm$ 9% rise, left, 8 $\pm$ 20% rise, right). The changes in neuropeptide gene expression in bilaterally arthritic rats are shown in Figure 5.10



*Figure 5.10. Neuropeptide mRNA expression in DRG of bilaterally arthritic rats (15 days after adjuvant injection) expressed as a percentage of untreated control rats. Values are means  $\pm$  SEM. \* $p < 0.05$ , \*\* $p < 0.01$  cf. controls ( $n = 5$  per group). Mean control grain counts were: PPT:  $107 \pm 4$ ; CGRP:  $328 \pm 37$ ; SS  $244 \pm 42$ .*

Vasoactive intestinal peptide mRNA was only detected in the occasional cell in control DRG but was always seen in positive control tissue taken from the hypothalamic suprachiasmatic nucleus (Figure 5.11). No change in VIP mRNA expression was detected during the development of arthritis. Injection of 0.15ml vehicle did not induce expression of VIP.



*Figure 5.11. Light field photomicrograph showing VIP mRNA expression in control section through the suprachiasmatic nucleus*

## 5.4. DISCUSSION

### 5.4.1. Neuropeptide messenger RNA expression in mono- and bilateral arthritis

#### 5.4.1.1. PPT and CGRP mRNA expression in experimental arthritis

PPT and CGRP show similar changes in these experiments and hence will be discussed together.

It has been shown that PPT and CGRP messenger RNA species show extremely rapid and marked increases in individual cells innervating the inflamed joint. This change in PPT and CGRP messenger RNA expression is confined to the DRG ipsilateral to the inflammation, and does not occur in DRG containing neurons innervating regions distant from the area of inflammation (L1), or in contralateral L5 DRG. PPT and CGRP show a biphasic increase in expression, with an initial peak at around 8-16 hours, a trough at 24 hours and rising again as more chronic inflammation appears with expression remaining raised at day 14. This increase in expression of PPT per cell agrees with a previous study by Noguchi et al. (1988), where cellular PPT expression was increased over the first 24 hours and returned to control levels by 48 hours. This initial peak in expression also agrees with previous work where PPT and CGRP mRNA were measured by Northern analysis in rat DRG following bilateral hind limb inflammation induced by carrageenan (Iadarola and Draisci, 1988). The changes they observed were confined to the early stages of the inflammation, peaking at 2 hours, but their data did not go beyond twenty four hours. It is therefore conceivable that the mRNA levels of both these neuropeptides actually attain much higher levels even earlier than 8 hours; this early time period was not examined in the experiments here as it was of interest to examine the changes in both acute and chronic inflammation.



In the present experiments the analysis was confined to neurons with cell bodies of diameter  $<30\mu\text{m}$ . These neurons are thought to be involved in nociception; they give rise to unmyelinated or thinly myelinated axons and are capsaicin-sensitive (Nagy et al., 1992). Most SP-containing neurons are thought to belong to this subset of primary afferents where they coexist with other neuropeptides particularly CGRP (Skofitsch and Jacobowitz, 1985b). Therefore the results of Iadarola and Draisci agree with those presented here on the change in PPT mRNA, as all the mRNA present probably originated in this population of neurons. As regards the CGRP-containing primary afferents, these are known to have both large and small perikarya (Lee et al., 1985) and the inclusion of mRNA levels in large neurons by using Northern analysis may have obscured any changes in mRNA levels in small neurons alone, which was the only population under study here. A more recent study (Minami et al., 1989) examining the expression of PPT, again by Northern analysis showed that bilateral adjuvant-inflammation induces a 1.7 fold increase in PPT expression in lumbar DRG after 4 days, agreeing with the present data at 5 days, which show a  $140\pm 4\%$  increase.

It is clear from mRNA expression following injection of sterile paraffin oil that the injection itself is not causing the initial increase in PPT and CGRP mRNA expression, so it seems reasonable to conclude that it is attributable to the very rapid inflammation which is induced by adjuvant injection. The mechanisms underlying this early increase in mRNA expression are further investigated and discussed in Section 7.

In bilateral arthritis, when contralateral inflammation is apparent, PPT and CGRP mRNA increase in small primary afferent perikarya in both ipsi- and contralateral DRG. This agrees with the results from monoarthritis and from previous studies which demonstrated increased substance P and CGRP in DRG and peripheral nerves

in response to inflammation (Donnerer et al., 1992) and arthritis (Colpaert et al., 1983; Kuraishi et al., 1989). It is unclear whether induction of PPT and CGRP mRNA expression initiates the contralateral spread of inflammation or is a consequence of it, as mRNA expression was examined in animals with overt contralateral disease. Both neuropeptide genes, however, show increases in expression concurrent with the onset of bilateral disease, suggesting a close or causal relationship between the spread of inflammation and the induction of synthesis of these peptides. This assertion is supported by the previous data demonstrating induction of both PPT and CGRP mRNA expression in innervating DRG within 8 hours of adjuvant injection, during the initial inflammatory response, many days before histological arthritis becomes manifest.

The mechanism(s) underlying the contralateral induction of neuropeptide mRNA expression are unclear. It may be that ipsilateral inflammation induces contralateral mRNA changes through contralateral "sensitisation" of dorsal horn neurons. Unilateral noxious or electrical stimulation induces contralateral expression of immediate early gene/transcription factors in dorsal horn neurons, including the AP-1 components c-fos and c-jun (Noguchi et al., 1991; Herdegen et al., 1991a) and the nerve growth factor (NGF)-induced factor NGFI-A (also known as krox24 and zif268; Herdegen et al., 1990). The number of expressing cells and the amount of fos protein produced is increased on a subsequent contralateral stimulus (Leah et al., 1992). Since AP-1 and other transcription factors may regulate PPT and CGRP gene expression this mechanism of "molecular cross-talk" in the spinal cord may underlie the contralateral increases in PPT and CGRP gene expression and provide a possible basis for increased sensitivity of the symmetrical joint to inflammation and arthritis. However, the exact neuronal pathways involved and the underlying molecular mechanisms remain to be determined. The role of the primary afferent and other neuronal systems is further examined and discussed in Section 7.

#### 5.4.1.2. Numbers of cells expressing PPT and CGRP mRNA.

In this study the criterion for a positive cell was a hybridisation signal overlying the neuron of at least four times the background silver grain count. There was no change in the numbers of DRG neurons expressing either PPT or CGRP mRNA between untreated controls ( $22\pm 1\%$ , PPT;  $37\pm 3\%$  CGRP) and in chronic arthritis ( $19\pm 1\%$ , PPT;  $36\pm 1\%$  CGRP). While there is variability in the literature regarding the numbers of cells expressing Substance P/PPT or CGRP, most studies have found that approximately 20% of DRG neurons express Substance P, and 40% express CGRP. In a study examining both immunohistochemistry and in situ hybridisation (Henken et al., 1988) there was no significant difference in the number of PPT/SP positive cells assessed by either method.

Other workers have found increases in the numbers of PPT expressing cells following peripheral noxious stimuli (Noguchi et al., 1988) and adjuvant-induced inflammation (Heppleman et al., 1993), and CGRP immunoreactivity (Hanesch et al., 1993). In all of these studies the numbers of positive cells in control DRG are rather lower than those found by other workers. In fact, Noguchi et al (1988) also found many less cells expressing PPT in controls than they did in a subsequent study one year later (Noguchi et al., 1989). In particular the difference between the numbers of CGRP positive cells in the study by Hanesch et al. (1993) and others locating CGRP by immunohistochemistry or specific in situ hybridisation for either  $\alpha$ - or  $\beta$ -CGRP is striking. It is unclear why these workers have identified such a low number of positive cells in these studies; although differences in fixation technique and tissue processing may account for this. Increases in the numbers of neurons expressing a peptide detected by immunohistochemistry may be attributable to an increase in individual cellular expression of PPT and CGRP mRNAs resulting in increased detection of peptide following the onset of inflammation, in neurons which

were initially below the detection limit of immunohistochemistry. This cannot explain why changes in the number of neurons positive for PPT mRNA have also been observed (Noguchi et al., 1988; Heppleman et al., 1993); it is obvious that this area requires further study.

In the only study to stringently quantify increases in DRG neuropeptide levels in arthritis (Smith et al., 1992), the increases in SP and CGRP were 33% and 200% respectively. In the two studies which found increased numbers of cells expressing PPT (Heppleman et al., 1993) and CGRP immunoreactivity (Hanesch et al., 1993) also in adjuvant-induced monoarthritis, the numbers of cells increased by 16 and 17% respectively. In the case of CGRP particularly, this would not appear to be sufficient to account for the large increase in peptide content. It is not possible to explain why no change in the numbers of PPT and CGRP expressing neurons was found in this study, however the aim of the studies presented here was not to determine the numbers of neurons expressing any of the three neuropeptide mRNAs which are usually expressed in DRG neurons, but to establish the mRNA changes in individual neurons, which is a strength of the in situ hybridisation technique.

#### 5.4.1.3. Somatostatin mRNA changes in mono- and bilateral arthritis.

Somatostatin is the only mRNA species under examination which shows contralateral changes in the early stages of inflammation (Figure 5.8). In the first twenty four hours the contralateral increase in mRNA expression appears to be due to the injection itself, rather than any inflammatory response, as a similar effect is seen on injection of paraffin oil (Table 5.2). In bilaterally arthritic animals somatostatin mRNA does not show any contralateral change (Fig. 5.10) unlike PPT and CGRP mRNA. As mRNA changes were examined immediately after the appearance of contralateral inflammation, this suggests that increased somatostatin biosynthesis is not an early event in the inflammatory arthritic process. Examination

of mRNA later in the disease process may reveal a later change in messenger RNA expression as somatostatin has been shown to increase in DRG of bilaterally arthritic rats (Ohno et al., 1990). However the studies in this thesis have been concerned with the early events in the development of disease and the contralateral effects; therefore the possibility of later changes in somatostatin expression remain to be determined. The contralateral decrease in somatostatin mRNA expression in arthritis (day 14) seen initially (Fig. 5.8) has not been reproducible in either bilaterally arthritic rats, where contralateral somatostatin mRNA expression is not different from control values, or in other monoarthritic male or female animals (see Section 7). Somatostatin mRNA only shows reproducible contralateral changes in the early stages of unilateral inflammation, which are attributable to the initial injection stimulus as demonstrated by the injection of paraffin oil (Table 5.2). The role of somatostatin in arthritis remains an enigma. In general, somatostatin is thought to have inhibitory properties; it is known to inhibit the release of Substance P, for example (Gazelius et al., 1981). However, it is also known to increase in primary afferent neurons in bilateral arthritis (Ohno et al., 1990), although this is disputed (Smith et al., 1992), and has been implicated in the central modulation of thermal nociception (Morton et al., 1989).

The functional significance of the changes in somatostatin, both ipsilateral and contralateral to inflammation are still unclear. The contralateral increase which occurs so rapidly after an ipsilateral stimulus (injection) may be involved in the early priming of the nervous system with regard to the contralateral activation of primary afferents in established inflammation. Contralateral events following an ipsilateral noxious stimulus occur within hours of the initial stimulus (Leah et al., 1992; Herdegen et al., 1990; Herdegen et al., 1991b). Many hyperalgesic states show contralateral effects (Thompson et al., 1993) and the contralateral increase in somatostatin mRNA may be involved in the initiation of these, possibly involved in an inhibitory role. It is unlikely that somatostatin has a direct role in the

contralateral spread of arthritis in this model as it does not show any contralateral change in the first day on which contralateral inflammation is apparent.

#### 5.4.1.4. Vasoactive intestinal peptide in experimental arthritis.

VIP expression was detected in only occasional cells in the experiments described in this Section and showed no change in expression in response to inflammation or arthritis. It has been suggested that VIP expression can be regulated by electrical activity in neurons (Agoston et al., 1991). However, these investigations were performed in dissociated spinal cord cell cultures and VIP expression is known to be dramatically altered unless culture conditions are strictly maintained. In cultures of DRG neurons, depolarisation and cAMP accumulation are thought to regulate the expression of VIP and it has been hypothesised that VIP could be an inducible neurotransmitter in inflammatory conditions (Mulderry, 1993). In arthritic animals, articular primary afferent activity is known to be increased early in the time course of development of inflammation and previously silent afferents develop spontaneous activity (Schaible and Schmidt, 1988). If afferent activity was involved in the regulation of VIP expression in vivo, inflammation could be expected to induce expression of the mRNA. The results presented here suggest that VIP expression is not induced by neuronal depolarisation in vivo and has no role in this model of inflammatory arthritis. It is known that there is no neuronal damage in adjuvant induced arthritis in the rat (Guilbaud et al., 1985); the results on VIP expression support this data, as VIP expression is induced in rat primary afferent neurons by axotomy or crush injury (Atkinson and Shehab, 1986; Nielsch and Keen, 1989).

#### 5.4.2. Male/ female differences in adjuvant-induced monoarthritis

These results show that there are no differences in the susceptibility or disease progression in male or female animals in a mild adjuvant-mediated monoarthritis. Monoarthritis in females follows the same course as that seen in males with a sustained increase in left joint circumference of 9-11mm throughout the fifteen days of study.

These results agree with those of Pearson and Wood (1959) who used both male and female animals in their initial description of adjuvant arthritis and found no sex dependence on either incidence or severity of the disease. Indeed in subsequent studies on the passive transfer of adjuvant disease only female animals were used (Pearson and Wood, 1964). Sex-linked mechanisms are known to affect a variety of immunological conditions in a variable manner and experimental models of autoimmune conditions often show non-uniform influences of sex hormones (Schuurs and Verheul, 1989). Systemic lupus erythematosus shows a profound female dominance, as testosterone suppresses both development of disease and autoantibody production (Steinberg et al., 1979). Some models of inflammatory arthritis have been shown to have some dependence upon sex hormones, but different models show differing male/female susceptibilities. Collagen-induced arthritis in the mouse has been clearly shown to be more severe in male and oophorectomised female mice (Holmdahl et al., 1986), whereas streptococcal cell wall-induced arthritis is more severe in female rats; with the less severe disease in males being dependent upon male testicular hormones (Allen et al., 1983), and androgens inhibit cartilage breakdown in the mouse air pouch model (Da Silva et al., 1993; Steward and Bayley, 1992). In adjuvant disease, oestradiol will reduce the incidence of joint disease if administered to either male or female rats (Mueller et al., 1964). Studies of adjuvant disease related to pregnancy in the rat have also shown

that female hormonal levels can have a beneficial effect on the disease if animals are pregnant prior to induction, but no protective effect if pregnant animals are made arthritic (Mathers and Russell, 1990). In humans, there is a definite female preponderance in both prevalence and incidence of rheumatoid arthritis (RA) (Hazes and Silman, 1990) and pregnancy usually causes a remission in the severity of RA with a 'relapse' after birth. The data in humans also shows that pregnancy has a protective effect against the onset of RA, both during pregnancy and for three months thereafter (Silman et al., 1992).

The reasons for the disparity in the influence of sex hormones in RA and adjuvant arthritis is unclear. It has been hypothesised recently that the female predisposition to RA is due to low androgen levels and not high oestrogen levels (James, 1993), particularly as men with RA also show low levels of androgens (Spector et al., 1989; Spector et al., 1988). However, adjuvant arthritis in the rat and RA do not share the same pathogenesis although they do show similar pathophysiology (Rainsford, 1982), and therefore it may be inappropriate to extend the "androgen theory" to the experimental animal. Adjuvant arthritis in rats is primarily T-cell mediated (Chang, 1984) and this could explain why exogenous oestrogens will suppress adjuvant arthritis (Mueller et al., 1964) in both sexes, since female sex hormones have a suppressive effect on T-cell mediated functions (Kappas et al., 1963; Stimson and Hunter, 1980) so removing this important component of the pathogenesis of the disease. While many autoimmune diseases in humans and animals show varying dependence upon sex hormones adjuvant arthritis has not been shown to be more severe in either sex, merely susceptible to exogenous steroid hormones, consequently, it is not surprising that there is not a male/female difference in the rats in this study.



#### 5.4.3. Expression of in situ hybridisation results

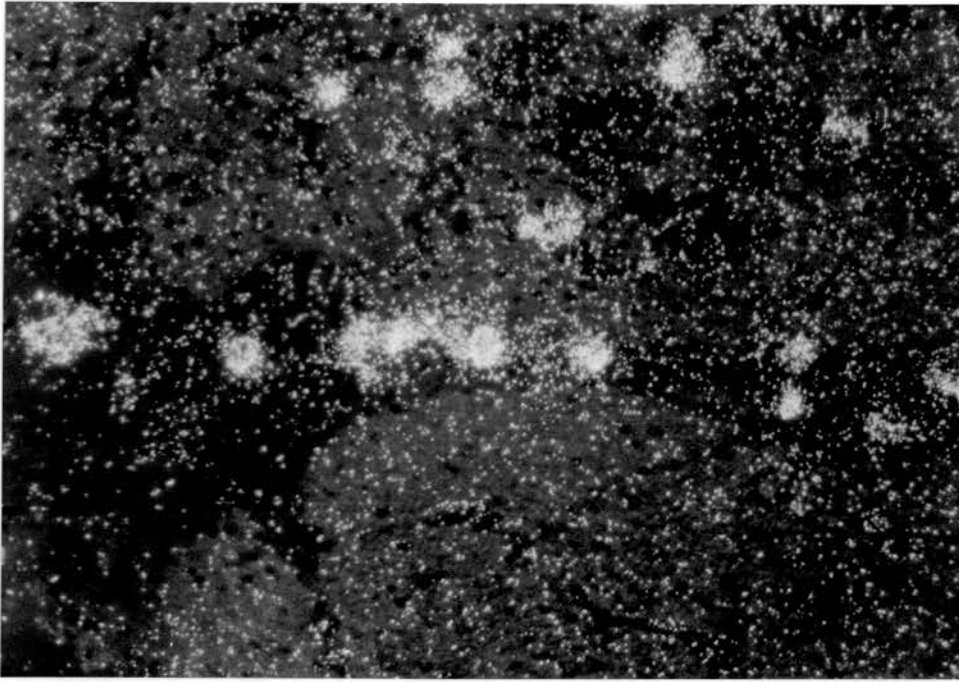
All results shown are expressed as a percentage of the basal expression of messenger RNAs in untreated, normal animals. This is due to the interexperimental variability inherent in the isotopic in situ hybridisation technique. If all parameters such as hybridisation, washing temperatures and times, and exposure times are strictly maintained, there is usually a difference in the specific activity to which the riboprobes are labelled due to template length or activity of the RNA polymerase, which will introduce variation in the numbers of silver grains in the final processed section. Even in cases where all parameters are as closely maintained as possible, there is usually some variation in the absolute grain numbers in control animals. The in situ hybridisation technique as described here, is semi-quantitative in nature because without standards for specific radioisotopic activities the absolute grain numbers overlying any particular cell can not be related to any finite value for the amount of messenger RNA. Therefore the grain count is a representation which must always be compared to a standard, i.e. the expression in untreated control DRG. For these reasons, all data are expressed as a percentage of the untreated controls which are always included in each hybridisation.

#### 5.4.4. Human versus computer image analysis of in situ hybridisation silver grain numbers.

The selection of cells which were analysed in these experiments was perhaps the point at which a degree of subjectivity could have been introduced. In those studies analysed by direct visual counting of silver grains (neuropeptide mRNA expression in the development of monoarthritis, section 5) four cells were counted from each

DRG section, along with four areas of background. This number was primarily determined as that which could give a representation of the level of expression without becoming too arduous in the time required to count the silver grains. In all subsequent experiments, when grain counting was computer-aided, more cells per section were counted, as time limits again would allow. An attempt was made in all the analysis to select cells which showed neither the lowest nor highest hybridisation signal. Obviously this method of the selection of neurons for analysis could introduce some sampling bias; however this was substantially eliminated by coding all slides when sections were cut, and only breaking the code as to experimental group after all in situ silver grain counting was completed. Unfortunately there is no option on the computer software to facilitate the random selection of cells for analysis by the computer itself. This would be a superior method of analysis as sampling bias would be eliminated; however due to the coding of slides it is unlikely that sampling bias introduced a large error in any of the studies presented in this thesis.

The analysis programs used for counting high and low density silver grains are different in the method in which the images are processed and this is reflected in the different correlations between eye and machine counts (Figure 5.2a and b). Low density counts identify only individual silver grains, and this is extremely useful in the quantification of mRNAs which are not highly expressed such as transcription factors (see Section 8). However this method of counting grains cannot be applied to more highly expressed mRNAs such as the neuropeptides under study here. This analysis routine cannot give accurate grain counts in neuropeptide in situ hybridisation due to the clumping of silver grains, which is illustrated in Figures 5.6, 5.7 and below. Therefore the software designed for high density grain counts must be used for quantitation of neuropeptide mRNA expression.



*Figure 5.12. Dark field photomicrograph showing neurons expressing PPT mRNA showing the density of silver grains overlying individual neurons. Original magnification x20.*

A high density grain count is calculated in two stages. Firstly all single grains are counted, then clumps are detected and the number of grains calculated by dividing the total area of clumped grains overlying any cell by a constant representing the average silver grain size in the particular experiment. Totals are then calculated by simple addition. Thus the computer uses defined rules to calculate the number of silver grains in a clump, rather than an arbitrary determination as occurs when counting by eye. This is of value as the computerised technique is consequently more objective in the determination of numbers of grains. Equation 1 shows that in high density silver grain counting, the computerised image analysis system appears to detect nearly five times the number of silver grains detected by eye, indicated by

the x value. This could indicate either that the machine is 5 times more sensitive in detecting silver grains than the human eye, or that a multiplication error has somehow been introduced into the analysis software. On investigation, the latter was found to be true, as the constant representing the average grain size was found to be four times less than the actual grain size. However, this does not introduce a error into the final data analysis as all in situ hybridisation results are always expressed as a ratio of the basal mRNA expression in controls (Section 5.4.3.) which negates any multiplication error introduced by the analysis software. This fault has been eliminated in an updated version of the software.

Therefore this image analysis software represents a more objective and reproducible method of determining silver grain numbers than direct visual counting, particularly in situations where silver grain numbers are high.

## 5.5. CONCLUSIONS

This model of adjuvant arthritis in the rat shows equal disease patterns and severity in both male and female animals, agreeing with other studies on adjuvant-induced polyarthritis.

The numbers of primary afferent neurons expressing neuropeptides under conditions of noxious stimulation and inflammation is still in dispute. Further studies with rigorous and methodical methods of neuronal counting need to be performed.

Neuropeptide mRNAs in primary afferent neurons show distinct, rapid changes in response to developing arthritis and in the bilaterally arthritic animal. It is suggested that PPT and CGRP encoded proteins may play a role in the contralateral spread of arthritis in this model; this is discussed further in Section 6.

## SECTION 6 THE ROLE OF CAPSAICIN-SENSITIVE PRIMARY AFFERENTS AND NEUROPEPTIDE mRNA EXPRESSION IN THE SPREAD OF EXPERIMENTAL ARTHRITIS

### 6.1. INTRODUCTION

The effects of capsaicin systemically administered to the neonate on nociceptive thresholds, inflammatory responses and, in particular, arthritis, is still in dispute. As outlined in Section 1, the effects of either neonatal or perineural capsaicin in adults, on thermal and mechanical nociceptive thresholds are equivocal, with authors reporting both increased and unchanged values in both types of nociceptive test. All workers, however, report decreased sensitivity to noxious chemical stimuli in capsaicin-treated animals (Fitzgerald, 1983).

#### 6.1.1. The peripheral sensory nervous system in articular inflammation

In recent years there have been investigations to determine the extent to which the peripheral, central and sympathetic nervous systems contribute to the development of inflammation and hyperalgesia. Joints are known to be innervated by capsaicin-sensitive afferents (He et al., 1988), and both mechanical and chemical nociceptive stimuli can be inhibited by close arterial injection of capsaicin (He et al., 1990).

Investigations of capsaicin treatment on the development of inflammatory hyperalgesia have shown that polyarthritic rats treated with capsaicin as neonates (which ablates 90% of C-fibre afferents) show normal ambulatory and rearing behaviours when compared with vehicle-treated controls (Hara et al., 1984; Bartho et al., 1990). In a study of the decreased nociceptive thresholds seen in rats with adjuvant arthritis (Hara et al., 1984), neonatal capsaicin was shown to attenuate

decreases in thermal nociceptive thresholds (tested by the hot plate, but not in tail flick tests), chemical nociception tested by the formalin test and also in mechanical nociceptive tests. Development of a neuropathic thermal hyperalgesia is also prevented by neonatal capsaicin administration (Meller et al., 1992).

In rat adjuvant polyarthritis, both Hara and colleagues (1984) and Cervero and Plenderleith (1987) failed to show an amelioration of the joint swelling in animals treated with capsaicin at birth. Only Levine and colleagues (1986) have shown a significant reduction in joint inflammation, assessed by radiography, in animals treated neonatally with capsaicin. Colpaert and colleagues (1983) showed that capsaicin treatment of the adult rat (either before or after adjuvant injection), which is a more restricted lesion than neonatal capsaicin, resulted in a reversal of the decreased weight gain seen in arthritis, and an attenuation of the joint swelling occurred in these animals. In a first attempt using a more confined nerve lesion, Courtright and Kuzell (1965) showed that peripheral axotomy attenuated the severity of adjuvant-induced arthritis in the rat. Levine et al. (1985a) showed that perineural capsaicin will inhibit "reflex neurogenic inflammation", an effect they described of contralateral paw swelling following ipsilateral paw injury and intra-articular capsaicin will also reduce the severity of antigen-induced arthritis (Inman et al., 1989). These latter studies therefore supported the findings of Courtright and Kuzell that disruption of the peripheral sensory system could attenuate joint injury.

#### 6.1.2. The effects of capsaicin on neuropeptides

The effects of both systemic and perineural capsaicin on the neuropeptides in primary afferents has been outlined in Section 1. Capsaicin has acute effects on primary afferents resulting in release of neuropeptides into the spinal cord and in the periphery, and these effects can be blocked using ruthenium red (Amann et al., 1990; Ohkubo et al., 1993), leaving other responses e.g. activation by mechanical or

chemical stimuli unaffected. Ruthenium red will block the capsaicin-induced release of peptides from peripheral terminals but does not affect release stimulated by bradykinin or potassium depolarisation (Holzer, 1991). Systemic administration of capsaicin to neonatal and adult rats results in a depletion of the primary afferent neuropeptides substance P, somatostatin (Gamse et al., 1981), and CGRP (Kashiba et al., 1990) but not neurotensin (a peptide not found in primary afferent neurons) in DRG, peripheral nerve, dorsal roots and dorsal spinal cord. The effect of capsaicin on primary afferent neuropeptides appears to be mediated through nerve growth factor (NGF) as the neurotoxic effects of capsaicin on neuronal death and substance P content can be blocked by NGF (Otten et al., 1983), and perineural capsaicin is known to block retrograde transport of NGF which regulates substance P production (Miller et al., 1982; Lindsay and Harmar, 1989).

### 6.1.3. The sympathetic nervous system in inflammation

Evidence is accumulating that the postganglionic sympathetic nervous system contributes to inflammation, and particularly may influence the severity of joint injury in experimental and clinical arthritis. Reflex sympathetic dystrophy is a disease characterised by increased sympathetic activity which is associated with bilateral joint inflammation (Kozin et al., 1976). It was proposed by Levine and colleagues (1986) and Fitzgerald (1989) that the PGSN contributed to the joint injury seen in experimental arthritis through reflex sensory activation of PGSN via the sensory afferents and preganglionic sympathetic neurons located in the ventral horn of the spinal cord. However, there are conflicting reports on the relative contributions of sensory afferents and sympathetic efferents in, for example, joint inflammation (Lam and Ferrell, 1991; Green et al., 1993a), and on the effects of the inflammatory mediator bradykinin (Green et al., 1993b; Koltzenburg et al., 1992). Unfortunately, systemic sympathectomy using guanethidine, while reducing

peripheral noradrenaline and joint damage (Levine et al., 1986; Green et al., 1993a), also significantly reduces the numbers of both substance P and CGRP immunoreactive neurones in both the trigeminal and dorsal root ganglia (Mione et al., 1992) and cannot be considered as a "clean" sympathetic lesion. A large number of workers is attempting to clarify this situation.

It was also suggested by Levine et al., (1986) that distant hyperalgesic effects were also dependent on the postganglionic sympathetic nervous system. This suggestion was extended by Kidd and colleagues (1989b) in a hypothesis that a contralateral sensitisation of nociceptors occurring through contralateral activation of the preganglionic sympathetic nervous system could underlie the symmetrical spread in experimental and clinical arthritis. Further, it has been proposed that neurogenic plasma extravasation is dependent upon the postganglionic sympathetic neuron (Gonzales et al., 1991). In a recent study of bradykinin-induced plasma extravasation in the rat knee joint, Green and colleagues (1993b) found no evidence for the release of noradrenaline in the contralateral joint, whereas bilateral changes in sensory neuropeptide release have been observed during acute monoarthritis (Bileviciute et al., 1993), further supporting a fundamental role for sensory afferents in mediating contralateral effects of inflammation.

#### 6.1.4. Aims of the studies

The experimental protocols used here, involving the neurotoxin capsaicin were designed to examine the role of the unmyelinated sensory afferents in the mechanism underlying contralateral spread of arthritis. The effect of neonatal and perineural capsaicin on the maintenance and spread of arthritis in a modified model of adjuvant-arthritis has been examined. The role of primary afferent neuropeptides in



this spread has also been determined by assessing neuropeptide mRNA expression in DRG innervating the inflamed joints by in situ hybridisation.

## 6.2. MATERIALS AND METHODS

### 6.2.1. Neonatal capsaicin treatment

Neonatal rats were injected with either 50mg/kg capsaicin solution or vehicle subcutaneously on postnatal day 2, under 4% halothane anaesthesia. Capsaicin solution was obviously exerting an immediate effect as pups showed signs of respiratory disturbance and hypoxia. After recovery animals were left for seven weeks before induction of either mono- or bilateral arthritis by injection of *M. tub.* suspension around the left tarsal joint as described in section 2.

### 6.2.2. Perineural capsaicin treatment

Sciatic nerves were exposed in the mid thigh region and capsaicin solution applied directly to the nerve under halothane anaesthesia (1.5% capsaicin for 15 minutes as described in detail in Section 2.4.1). Sciatic nerve exposure was used as a control for surgical exposure of the nerve, since the vehicle is known to exert direct effects upon axon conduction (Wall and Fitzgerald, 1981; Petsche et al., 1983). Following perineural capsaicin treatment, animals were allowed to recover for two weeks before induction of bilateral arthritis by intradermal injection of 250µg *M. tub.* around the left ankle joint, under halothane anaesthesia. Animals with capsaicin directly applied to either left or right sciatic nerves will be referred to as CAPL and CAPR respectively.

### 6.2.3. Monitoring of progress of arthritis

Weights, joint swelling and inflammation were monitored for fifteen days, when the animals were killed by decapitation and L5 DRG taken and processed for neuropeptide mRNA in situ hybridisation. Parallel sections from neonatally treated animals were also processed for substance P immunohistochemistry (Section 2). Left and right tarsal joints were removed by section of the tibia and fibula, post-fixed in 10% formal saline, decalcified in Gooding and Stewart's solution, and processed for histological examination. Joint sections were scored blind for signs of inflammation/arthritis.

### 6.2.4. Efficacy of the perineural capsaicin treatments

The efficacy of perineural capsaicin treatment was determined by radioimmunoassay of L4/5 DRG neuropeptides. A group of CAPL animals were killed before induction of arthritis, L4/5 DRG taken and substance P, CGRP and somatostatin content assayed as described in Section 2

### 6.2.5. Expression of in situ hybridisation data

All neuropeptide mRNA levels are expressed as a percentage of the basal expression in untreated control animals as described in Section 5.4.3. Data were compared by ANOVA followed by Dunnetts post hoc test

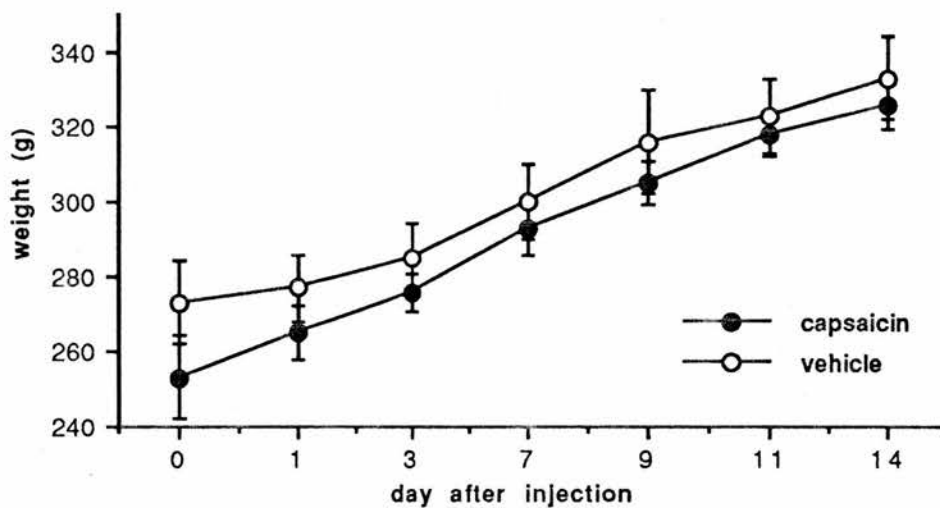
## 6.3. RESULTS

### 6.3.1. Neonatal capsaicin treatment

#### 6.3.1.1. Effect of capsaicin on the development of arthritis

Induction of monoarthritis in either capsaicin or vehicle treated rats did not affect the normal weight gain (Figure 6.1) in rats of this age (see also figure 3.1 for normal weight gain in untreated rats).

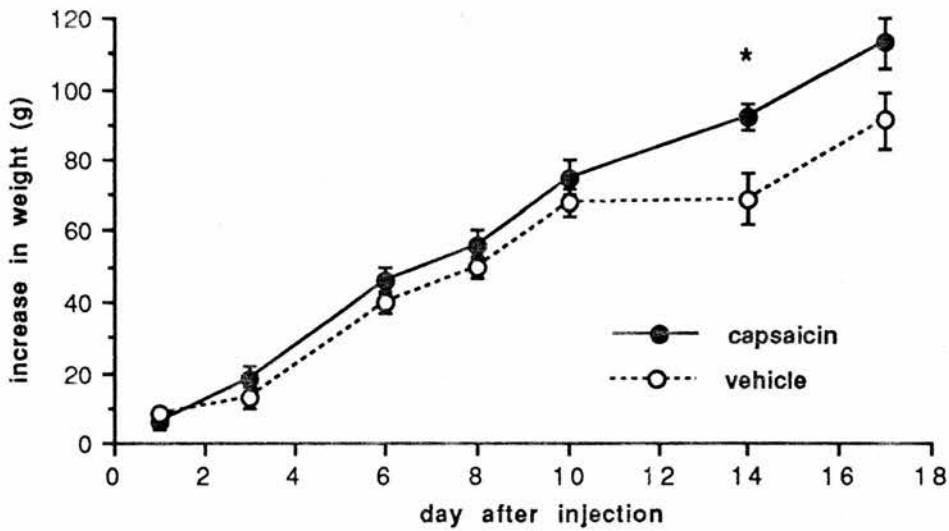
**Figure 6.1**



*Figure 6.1. Mean body weight of monoarthritic rats treated neonatally with either capsaicin or vehicle (n=4 per group)*

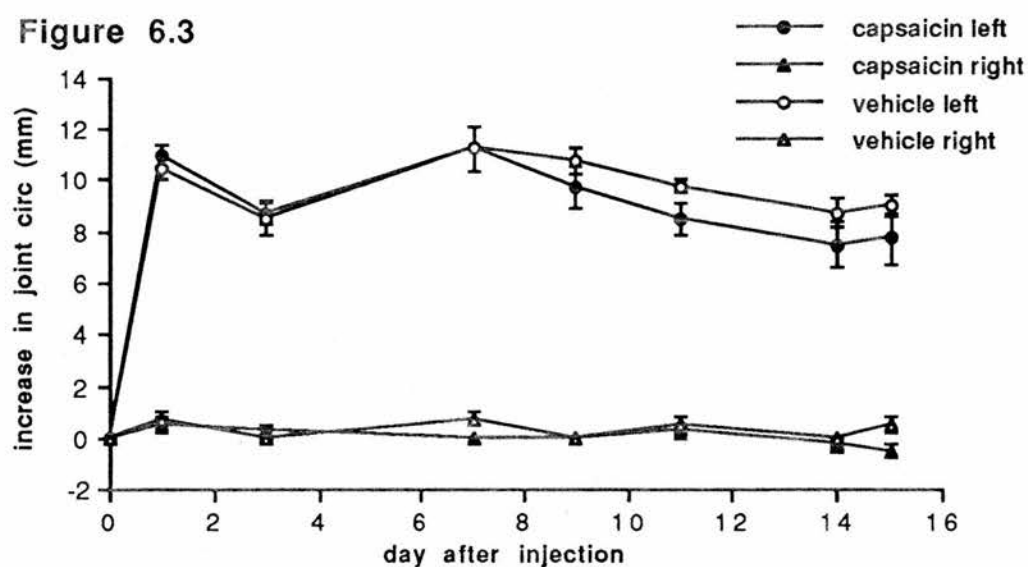
The groups of rats in which arthritis was induced for the study of bilateral disease showed significant differences in weight before arthritis was induced ( $230 \pm 4\text{g}$  capsaicin;  $286 \pm 8\text{g}$  vehicle). If changes in weight are compared, vehicle treated rats showed a significant attenuation of the normal increase in weight with the onset of bilateral arthritis (day 14) compared to capsaicin treated rats (Figure 6.2). Increases in weight plotted in Figure 6.2 are calculated from the difference between the measured value on each day minus the preinjection value for each animal.

**Figure 6.2**



*Figure 6.2. Increase in weight during development of arthritis in capsaicin and vehicle treated bilaterally arthritic rats (n=9 vehicle; 6 capsaicin).*

Neonatal capsaicin slightly attenuated but did not significantly affect left joint swelling in either mono- or bilaterally arthritic rats (Figures 6.3 and 6.4), and slightly delayed, but did not prevent right joint swelling in bilateral arthritis (Figure 6.4). This was obviously not a function of weight gain as capsaicin slightly attenuated joint swelling, but maintained the increase in weight in these animals.



*Figure 6.3. Increase in left (upper) and right (lower) joint circumferences in monoarthritic rats treated on postnatal day 2 with either capsaicin or vehicle. There were no significant differences between capsaicin and vehicle treated groups (n=4).*

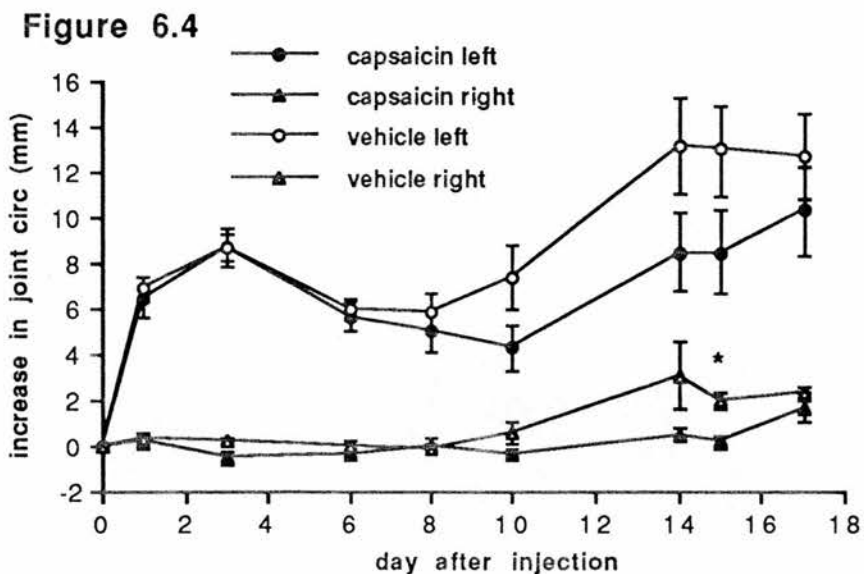


Figure 6.4. Increase in left (upper), and right (lower) joint circumference in bilaterally arthritic rats treated neonatally with either capsaicin or vehicle. (\* $p < 0.05$ ;  $n = 6$  capsaicin;  $9$  vehicle).

Histological assessment of right and left joints from bilaterally arthritic animals showed no difference in the degree of arthritis 17 days after adjuvant injection, between capsaicin or vehicle treated animals (Table 6.1). These animals showed the same features of joint inflammation as those illustrated in Section 3.

Table 6.1. Histological scores for capsaicin and vehicle treated bilaterally arthritic rats (day 17). Values shown are medians (range)

	Left	Right
Capsaicin	3 (2-4)	3 (0-4)
Vehicle	4 (3-4)	4 (2-4)

### 6.3.1.2. Neuropeptide mRNA expression in monoarthritic rats

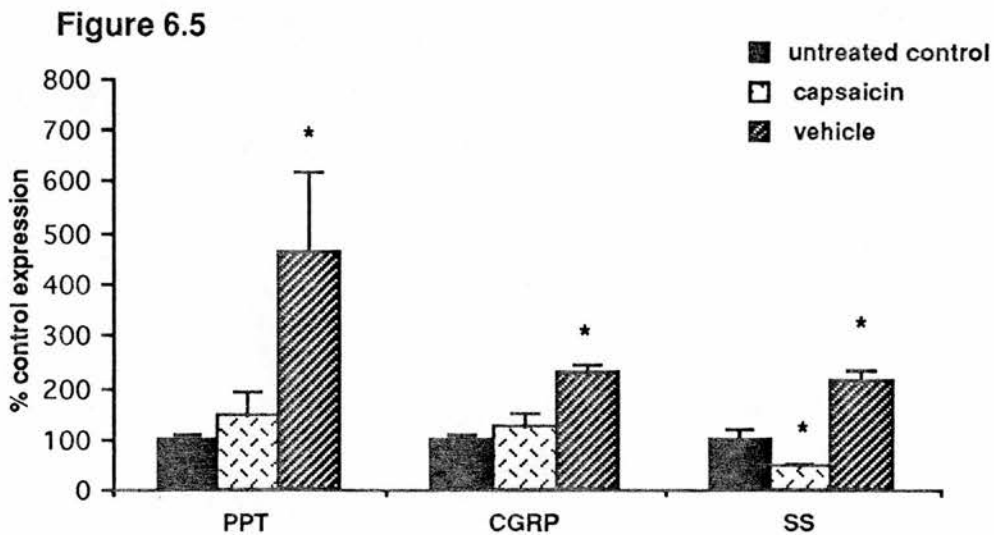
It can be seen from Table 6.2 that in vehicle treated animals the usual rise in all three neuropeptide mRNAs is seen in ipsilateral, but not contralateral L5 DRG. In animals treated on postnatal day 2 with 50mg/kg capsaicin, in the remaining neuropeptide expressing cells, only somatostatin mRNA shows a significant decrease in expression compared to untreated control values. PPT and CGRP mRNA expression in this population of cells is not significantly different from that in untreated control animals, and do not exhibit the usual rise in these neuropeptides seen during monoarthritis (Section 5). The changes in left (injected side) DRG mRNA expression are additionally illustrated in Figure 6.5.

Table 6.2. Expression of neuropeptide mRNAs in monoarthritic rats (day 15 after adjuvant injection) treated at postnatal day 2 with either capsaicin or vehicle.

\*denotes significantly different from control values (untreated animals)  $p < 0.05$ ;  $n = 4$  per group. Mean  $\pm$  S.E.M. control grain counts were; PPT,  $108 \pm 12$ ; CGRP,  $135 \pm 20$ ; SS,  $157 \pm 10$ .

	PPT		CGRP		Somatostatin	
	Left	Right	Left	Right	Left	Right
Untreated Control	100 $\pm$ 9		100 $\pm$ 11		100 $\pm$ 17	
Capsaicin	146 $\pm$ 48	153 $\pm$ 27	124 $\pm$ 29	77 $\pm$ 12	45 $\pm$ 5*	24 $\pm$ 3*
Vehicle	622 $\pm$ 195*	122 $\pm$ 18	226 $\pm$ 18*	110 $\pm$ 26	212 $\pm$ 20*	114 $\pm$ 15

Figure 6.5. Expression of PPT, CGRP and somatostatin in left DRG from untreated control animals, and animals treated neonatally with either capsaicin or vehicle, 15 days after adjuvant injection. Neonatal capsaicin treatment prevented the rise in left DRG usually seen in monoarthritic rats (see also section 5).



### 6.3.1.3. Expression of neuropeptide mRNAs in bilaterally arthritic rats

Treatment of neonatal rats with vehicle, again does not affect the expression of the three neuropeptide mRNAs in bilateral arthritis, as would be predicted from the changes observed in vehicle treated monoarthritic rats, as all show the usual rises in both left and right L5 DRG (PPT and CGRP) or only left L5 DRG (somatostatin). In animals treated neonatally with capsaicin, the usual rise in mRNA seen in both L5 DRG in bilateral arthritis was attenuated and expression of PPT mRNA in the remaining cells did not show any significant differences from untreated controls. CGRP mRNA expression was significantly lower in right DRG from capsaicin-treated animals than in untreated controls. Expression of somatostatin mRNA was

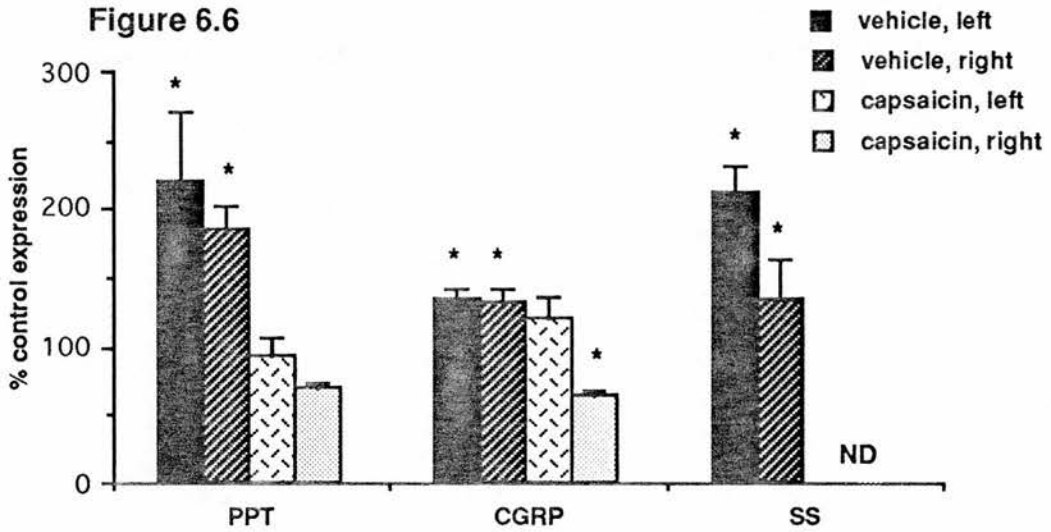


also significantly reduced and indeed was not detectable in animals treated neonatally with capsaicin. The neuropeptide mRNA changes are detailed in Table 6.3 and Figure 6.6.

*Table 6.3. Expression of PPT, CGRP and somatostatin mRNAs in L5 DRG of bilaterally arthritic rats treated on postnatal day 2 with either capsaicin or vehicle, 17 days after adjuvant injection. \*p<0.05 compared to untreated control values; n=6 capsaicin, 9 vehicle; ND no detectable expressing cells Mean  $\pm$  S.E.M. control grain counts were; PPT, 121 $\pm$ 24; CGRP, 254 $\pm$ 71; SS, 244 $\pm$ 84*

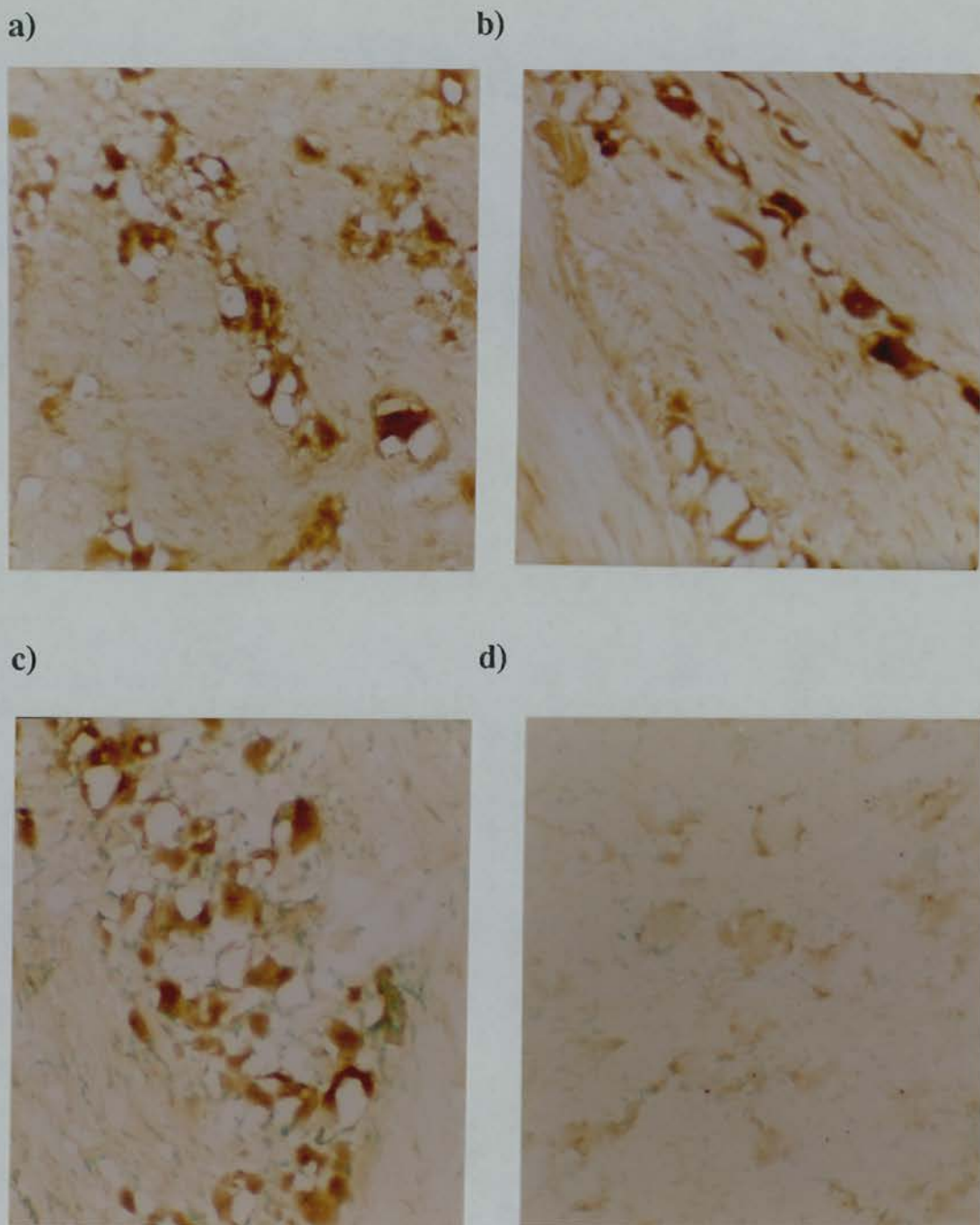
	PPT		CGRP		Somatostatin	
	Left	Right	Left	Right	Left	Right
Untreated Control	100 $\pm$ 7		100 $\pm$ 11		100 $\pm$ 7	
Capsaicin	94 $\pm$ 14	70 $\pm$ 5	121 $\pm$ 16	64 $\pm$ 4*	ND	ND
Vehicle	221 $\pm$ 50*	185 $\pm$ 17*	135 $\pm$ 7*	133 $\pm$ 10*	212 $\pm$ 20*	135 $\pm$ 29

Figure 6.6. Neuropeptide mRNA expression 17 days after adjuvant injection in DRG from rats treated neonatally with either capsaicin or vehicle. ND = not detectable. \* $p < 0.05$  cf. untreated controls (Table 6.3).



#### 6.3.1.4. Substance P immunoreactivity in DRG from capsaicin-treated animals

It can be clearly seen from Figure 6.7 that substance P immunoreactivity is present in both vehicle and capsaicin treated arthritis animals compared to the control without primary antibody. This indicates that the remaining neurons which express PPT mRNA are still capable of substance P-like protein translation.



*Figure 6.7. Light field photomicrographs of DRG neurons expressing substance P-like immunoreactivity from a) control; b) capsaicin and c) vehicle treated rats. A control section was incubated without the primary antibody (d).*

### 6.3.2. Perineural capsaicin treatment

#### 6.3.2.1. Efficacy of perineural capsaicin lesions; effects on DRG neuropeptide content

CAPL caused a significant reduction in L4-5 DRG content of substance P, CGRP and somatostatin, only on the treated side, in otherwise normal animals. Substance P showed the greatest decrease (62% fall compared with untreated controls), CGRP fell by 37% and somatostatin by 35%. Contralateral neuropeptide content was not significantly different from control values. (Table 6.4). Neuropeptide content is expressed as pg/ganglion as small lengths of peripheral nerve or dorsal root are often left attached to DRG on dissection and may bias expression by weight.

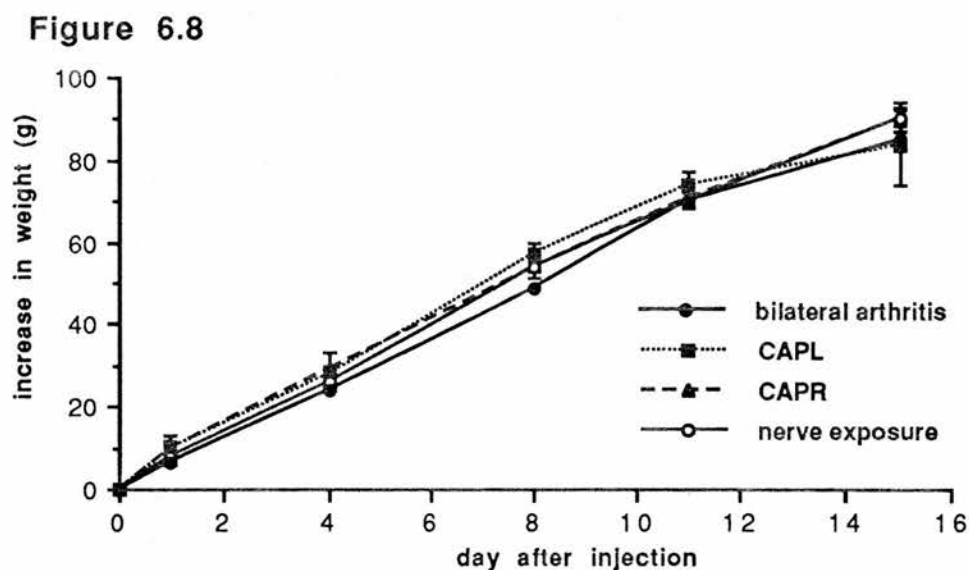
*Table 6.4. Neuropeptide content of L4/5 DRG in normal and CAPL animals.*

*Values are pg/ganglion  $\pm$  SEM (\* $p < 0.05$ , paired and unpaired  $t$  test compared to control or contralateral;  $n = 5$  per group).*

	Substance P		CGRP		Somatostatin	
	Control	CAPL	Control	CAPL	Control	CAPL
Left	186 $\pm$ 26	70 $\pm$ 24*	1178 $\pm$ 110	748 $\pm$ 75*	46 $\pm$ 2	30 $\pm$ 3*
Right	192 $\pm$ 32	164 $\pm$ 9	1171 $\pm$ 116	1217 $\pm$ 39	46 $\pm$ 3	40 $\pm$ 2

### 6.3.2.2. Effect of perineural capsaicin on animal behaviour

No animal showed any abnormal motor effects or infection following capsaicin application to the sciatic nerve. None of the surgical interventions significantly affected the increase in weight of the rats during the fifteen days of the study (Figure 6.8).



*Figure 6.8. Increase in weight of bilaterally arthritic, CAPL, CAPR and arthritic rats following sciatic nerve exposure during development of arthritis. (n=10 bilateral arthritis; 9 CAPL; 4 CAPR; 5 nerve exposure).*

### 6.3.2.3. Effect of perineural capsaicin on left and right joint inflammation

Bilaterally arthritic animals showed a rapid and sustained increase in left ankle joint circumference immediately after adjuvant injection (Figure 6.8) and subsequent involvement of the right ankle joint involvement after 14 days, evidenced by a significant increase in joint circumference (Figure 6.10) as detailed in section 5.

Rats with bilateral arthritis showed no significant differences in either left or right limb circumference in comparison to arthritic animals with sciatic nerve exposure alone (Figure 6.9).

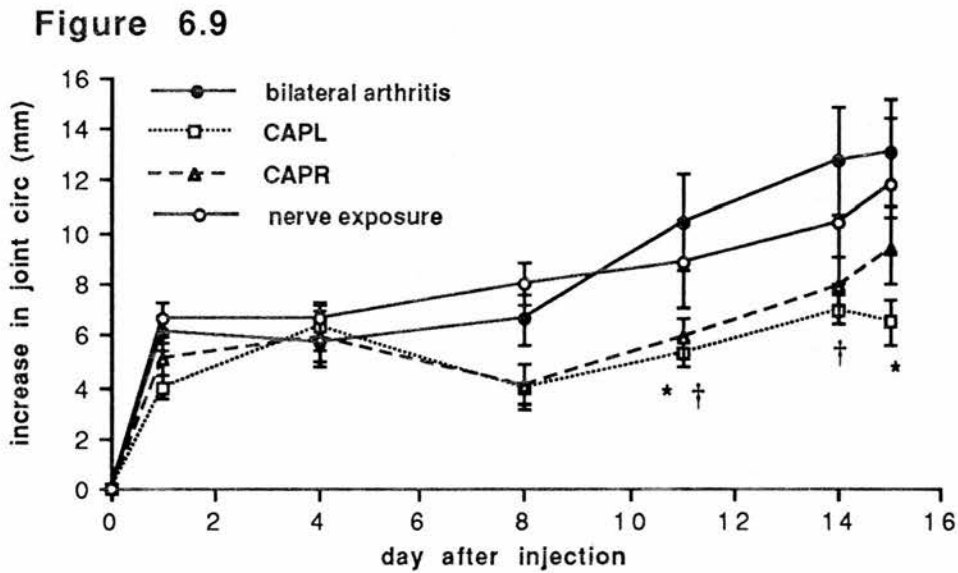


Figure 6.9. Increase in left joint circumferences in bilaterally arthritic and sciatic nerve exposed, CAPL and CAPR rats. (\* $p < 0.05$  CAPL ; † $p < 0.05$  CAPR vs either bilateral arthritis or nerve exposure). *n* values are as in Figure 6.8.

### 6.3.2.3A. Capsaicin application to the left sciatic nerve (CAPL)

CAPL significantly reduced both left (Figure 6.9) and right (Figure 6.10) joint swelling when compared to animals with bilateral arthritis and sciatic nerve exposure.

The reduction in joint swelling for the right ankle was accompanied by a reduction in inflammation score (Table 6.5) and in histological index (Table 6.6).

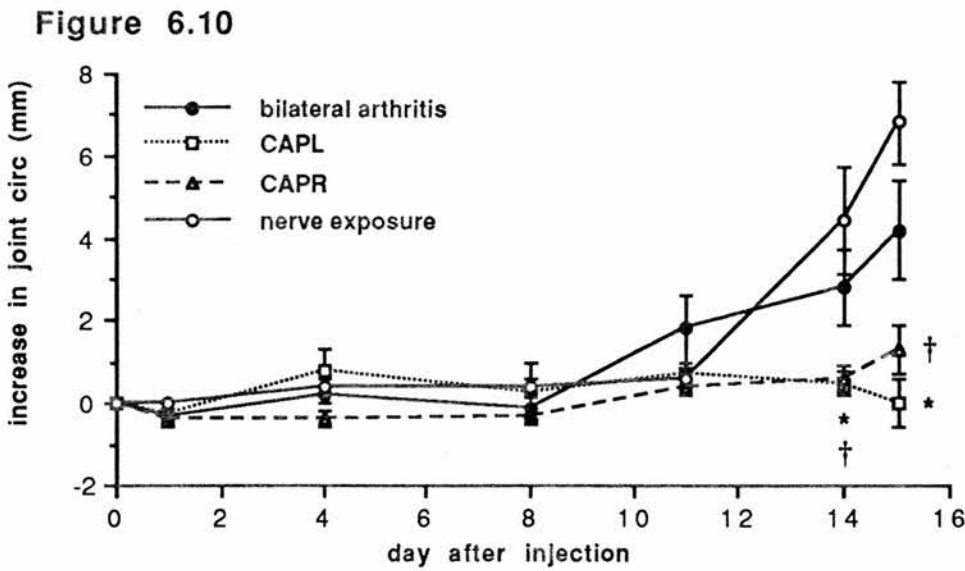


Figure 6.10. Increase in right joint circumferences in bilaterally arthritic and sciatic nerve exposed, CAPL and CAPR rats. (\* $p < 0.05$  CAPL ; † $p < 0.05$  CAPR vs either bilateral arthritis or nerve exposure). *n* values are as in Figure 6.8.

Table 6.5. Inflammation scores for right and left joints from bilaterally arthritic, CAPL and CAPR rats. Inflammation scores for right joints before day 14 are all zero. Values shown are median (range).

Day	Bilaterally arthritic		CAPL		CAPR	
	Left	Right	Left	Right	Left	Right
14	3 (0-4)	0.5 (0-2)	2 (1-3)	0	2 (1-3)	0
15	3 (0-4)	1 (0-3)	1.5 (1-3)	0	2 (1-3)	0 (0-1)

#### 6.3.2.3B. Capsaicin application to the right sciatic nerve (CAPR)

CAPR, surprisingly, also caused a significant reduction in both left joint swelling compared to bilaterally arthritic rats only on days 11 and 14 (Figure 6.9). There is some evidence that unilateral nerve lesions can affect the normal functioning of the contralateral nerve and this is discussed in section 6.4.2.2. There was also a significant attenuation of right joint swelling (Figure 6.10) reflected in lower histological and inflammation scores of the right joint (Tables 6.5 and 6.6).

Table 6.6. Histological scores (as defined in Section 2) of left and right tarsal joints in bilaterally arthritic, CAPL and CAPR animals, 15 days after adjuvant injection. Values shown are median (range). (\* $p < 0.05$  vs bilaterally arthritic animals;  $n = 5$  bilat. 4, CAPL and CAPR)

	Left	Right
Bilaterally arthritic	3 (2-4)	2 (2-4)
CAPL	2.5 (1-4)	1.5 (1-2)
CAPR	2.5 (1-4)	0.5 (0-2)*



#### 6.3.2.4. Effect of perineural capsaicin on neuropeptide mRNA expression

Neuropeptide mRNA expression in animals with bilateral arthritis has been described in Section 5 and is summarised in Table 6.7; briefly mRNA expression of PPT and CGRP, but not somatostatin was increased in both left and right L5 DRG neurons when compared to untreated controls. VIP mRNA was not detected in any section of DRG from either untreated, control arthritic or capsaicin-treated arthritic animals, but was always detected in positive control sections of the hypothalamic suprachiasmatic nucleus.

##### 6.3.2.4A. Animals with CAPL and arthritis

CAPL caused a significant reduction in PPT, CGRP and somatostatin mRNA levels compared to bilaterally arthritic rats, in the remaining left L5 DRG neurons capable of expressing these neuropeptides (also associated with a small, but significant attenuation in joint swelling). CAPL also prevented the rise in neuronal PPT mRNA expression in contralateral (right) L5 DRG, but did not affect the rise in CGRP mRNA seen in L5 DRG of bilaterally arthritic animals. Somatostatin mRNA expression remained at control levels in right DRG neurons (Table 6.7).

##### 6.3.2.4B. Animals with CAPR and arthritis

CAPR prevented the rise in PPT and CGRP mRNA expression seen in right L5 DRG neurons in symmetrically arthritic animals, and did not alter somatostatin mRNA expression. This was associated with a reduction in right joint swelling. CAPR also prevented the rise of CGRP and somatostatin mRNA expression in left L5 DRG. However, the elevation of PPT mRNA expression in the left DRG (the

side injected with adjuvant) was maintained (Table 6.7), as was inflammation and arthritis, although joint swelling was non-significantly reduced. Neuropeptide mRNA expression is additionally illustrated in Figures 6.11 and 6.12.

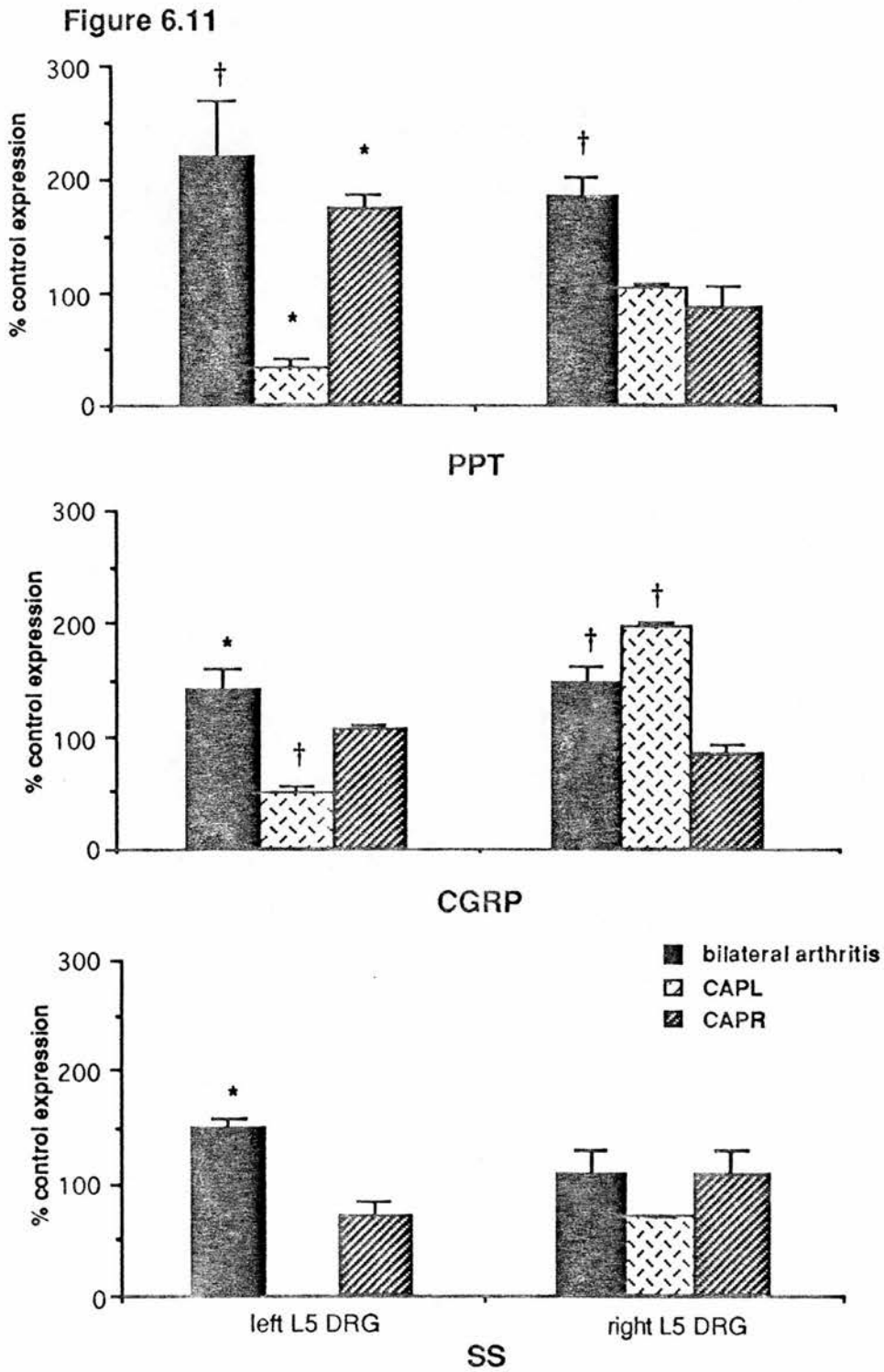
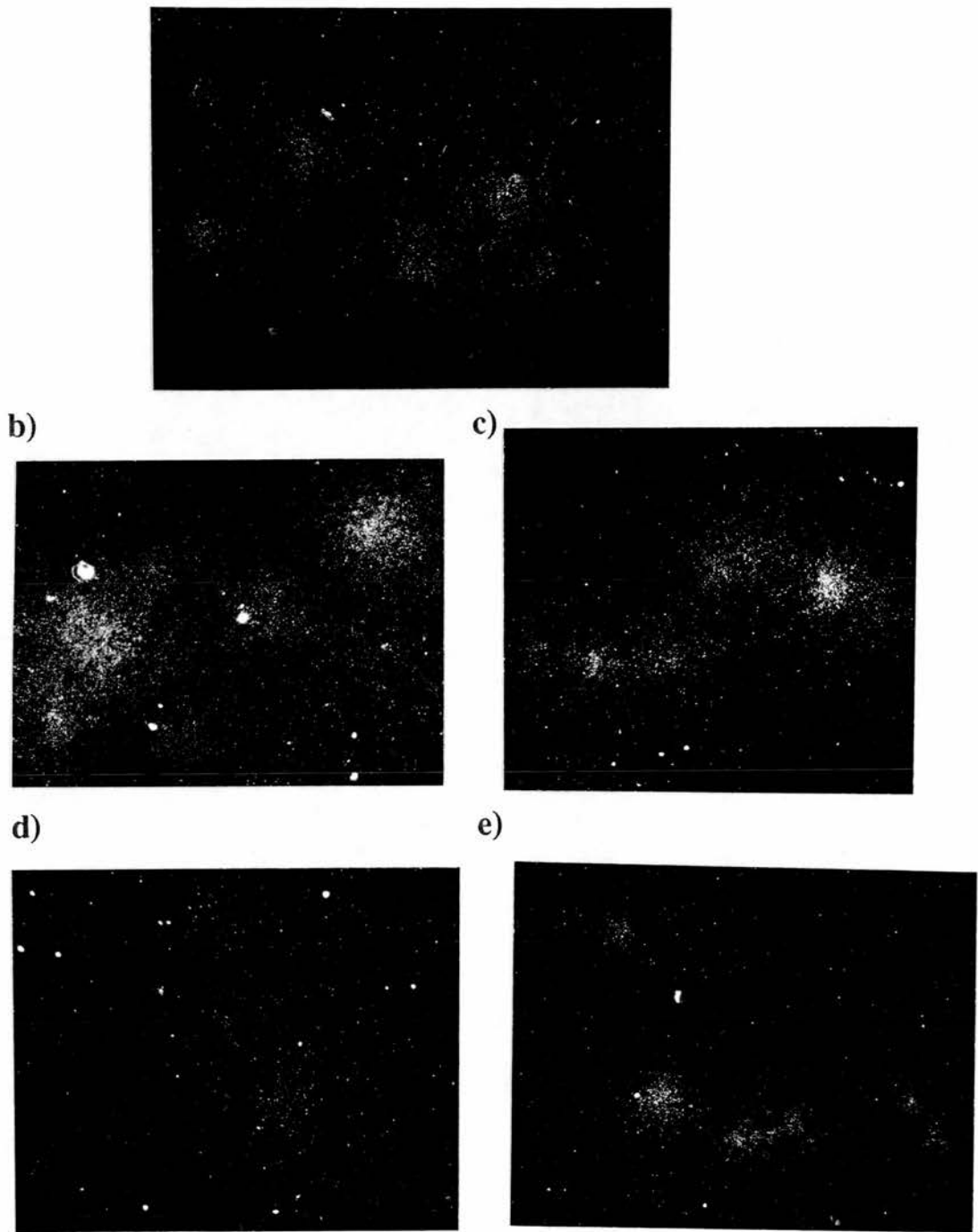


Figure 6.11. Neuropeptide mRNA expression in DRG of bilaterally arthritic, CAPL and CAPR animals 15 days after injection of adjuvant. \* $p < 0.05$ , † $p < 0.01$  cf. untreated controls.  $n = 5$  per group.



*Figure 6.12. Dark field photomicrographs of PPT mRNA in L5 DRG from a) control, b) and c) left and right DRG from a bilaterally arthritic rat, d) and e) left and right DRG from CAPL rat. PPT mRNA represented by silver grain density is increased in both left and right DRG in the bilaterally arthritic rat. Note decrease in left, and no increase in right silver grain density in CAPL animal.*

Table 6.7. Neuropeptide mRNA expression in DRG of bilaterally arthritic, CAPL and CAPR animals 15 days after injection of adjuvant. (\* $p < 0.05$ , † $p < 0.01$  cf. untreated controls). ND = no detectable expressing cells. Mean  $\pm$  S.E.M.. control grain counts were; PPT,  $107 \pm 4$ ; CGRP,  $328 \pm 37$ ; SS,  $244 \pm 42$ .

	PPT		CGRP		Somatostatin	
	Left	Right	Left	Right	Left	Right
Control	100 $\pm$ 8		100 $\pm$ 11		100 $\pm$ 10	
Bilateral arthritis	219 $\pm$ 48 <sup>†</sup>	190 $\pm$ 20 <sup>†</sup>	140 $\pm$ 20*	147 $\pm$ 15 <sup>†</sup>	148 $\pm$ 9*	108 $\pm$ 20
CAPL	34 $\pm$ 6*	105 $\pm$ 4	49 $\pm$ 6 <sup>†</sup>	196 $\pm$ 5 <sup>†</sup>	ND	70 $\pm$ 4
CAPR	174 $\pm$ 13*	88 $\pm$ 19	105 $\pm$ 6	83 $\pm$ 11	71 $\pm$ 13	108 $\pm$ 20

#### 6.4. DISCUSSION

It has been proposed that the sensory and sympathetic nervous systems interact to mediate the spread of inflammation and arthritis (Kidd et al., 1989b). The present results have shown that a specific neurotoxic lesion of polymodal nociceptors will significantly attenuate the spread of arthritis, whereas neonatal destruction of unmyelinated primary afferents will not. The implication, and possible significance of these results is discussed below.

##### 6.4.1. Neonatal capsaicin

The results obtained may appear to be surprising in view of the general acceptance that systemic capsaicin attenuates the severity of adjuvant-induced arthritis (Fitzgerald, 1989; Kidd et al., 1989b; Levine et al., 1986). These results show that

destruction of ~90% of C-fibre afferents has only mild effects on the initial, left inflammatory focus and a slight delaying effect on the contralateral spread. As mentioned in the introduction, only Levine and colleagues (1986) have shown a reduction in disease severity of adjuvant arthritis in rats treated neonatally with capsaicin. At first assessment, this could be due to the differences in the method of determining the disease severity; Levine and colleagues used radiographic analysis of the joints, whereas other workers who have found no reduction in severity assessed the disease by measurement of joint swelling (Hara et al., 1984; Cervero and Plenderleith, 1987). In the current experiments, the severity of arthritis has been assessed both by joint swelling, and histological examination (which correlate strongly with each other; Section 3), and there was no difference between groups of animals treated with capsaicin or vehicle.

#### 6.4.1.1. Effects of neonatal capsaicin on the development of related structures

It is now recognised that the central nervous system is not hard-wired and may show both functional and morphological plasticity in response to nerve injury (Woolf, 1993). It is well documented that neonatal treatment with capsaicin results in disorganised somatotopic representations in both the spinal cord of rats, and the whisker barrel representations in the mouse (Wall et al., 1982c). These animals also show changes in physiological responses such as expanded receptive fields (Wall et al., 1982a) and morphological changes in the innervation of the dorsal horn (Jancso et al. 1977).

Following the destruction of the central terminals of the unmyelinated primary afferents in the substantia gelatinosa of the dorsal horn by capsaicin, there is reinnervation of the substantia gelatinosa by the remaining primary afferents. Nagy and Hunt, (1983), used anterograde labelling techniques to confirm that there was a considerable loss of inputs, but also showed that there was a reorganisation of

primary afferents involving reinnervation of lamina II (Rexed, 1952) in particular. The methodology used in this study did not identify the functional modality of the fibres which were seen to reinnervate structures within the spinal cord following the destruction of the unmyelinated fibres by capsaicin. Subsequent studies attempted to identify the invading afferents in lamina II on the basis of the terminal arborisation patterns as hair follicle afferents (Rethelyi et al., 1986; Beal and Knight, 1987), and this was confirmed by physiological studies (Shortland et al., 1990). Rethelyi et al. (1986) found terminals corresponding to hair follicle afferents in the lateral aspect of the dorsal horn, with functionally unidentified sprouting fibres of different terminal arborisation pattern in the medial aspect of the dorsal horn. These authors suggest that these may be slowly or rapidly adapting mechanoreceptors, or Pacinian corpuscle afferents innervating glabrous skin on the foot. Furthermore, it is apparent that the chronic afferent denervation caused by neonatal capsaicin causes an increase in sympathetic innervation density and transmitter content. It has been suggested that this may be caused by increased availability of growth factors due to lack of competition from the sensory nervous system (Holzer, 1991).

#### 6.4.1.2. Primary afferent neuropeptides following neonatal capsaicin administration: role in adjuvant arthritis

It has been shown that in vehicle-treated animals, the neuropeptide mRNAs under study show the same changes as those described previously in bilaterally arthritic rats, namely bilateral increases in PPT and CGRP mRNA and unilateral increase in somatostatin mRNA expression. Capsaicin-treated rats, however, do not show significant changes in PPT mRNA expression, when compared to normal untreated controls. Individual cellular expression of CGRP was significantly lower than untreated controls only in right DRG. Neonatal capsaicin, while destroying the vast majority of unmyelinated primary afferents, does not remove all neuropeptide

expressing neurons although the numbers of neurons expressing substance P, CGRP and somatostatin are known to be markedly reduced in number (Holzer, 1991) and neurons expressing somatostatin mRNA were undetectable in this study. It is conceivable that the neurons in capsaicin-treated animals, in which neuropeptide mRNA expression was examined are a different population to those in untreated controls, and show lowered basal levels of mRNA expression than those in untreated animals. Capsaicin-sensitive neurons are thought to be those primarily involved in the mediation of neurogenic inflammation but capsaicin-resistant neurons, which also contain neuropeptides, may well be found to possess efferent functions (Holzer, 1988). It must also be borne in mind that neonatal capsaicin does not result in a total lack of capsaicin-sensitive afferents in the adult; whether this is a result of development at a later stage, or escape from the neurotoxin at the time of injection is unknown (Holzer, 1991). The relative proportions of C-fibre modalities in adults treated as neonates is also not significantly different from vehicle treated animals, and these primary afferents show normal function suggesting a degree of recovery in the adult animal (Holzer, 1991). The effects of neonatal capsaicin treatment on primary afferent neuropeptide gene expression is unknown. Decreases in the amount of peptides in DRG following capsaicin have been measured by radioimmunoassay and it is unknown whether these changes are attributable to decreased numbers of neurons or decreased expression in individual cells or a combination of both (Gamse et al., 1981; Wimalawansa, 1992). This complicates the interpretation of the data shown here as it is unknown whether the neuropeptide mRNA levels in arthritic capsaicin-treated rats could have been increased from a lower level, to control values in response to adjuvant injection. This possibility is supported by the finding that CGRP mRNA expression in bilaterally arthritic rats is significantly lower than that in untreated controls. It is a likely explanation for somatostatin mRNA-expressing neurons as while these were undetectable in the group of animals used for study of bilateral arthritis, the levels in monoarthritic animals were significantly lower than

untreated controls (Table 6.2). In order to be able to fully appreciate the implications of the mRNA levels presented here they would be better compared to capsaicin-treated, non-arthritic controls rather than the totally untreated animals used. This control group was not included initially as the experiment was designed to assess the effect of neonatal capsaicin on adult arthritis, rather than the actions of capsaicin *per se*. However the exclusion of this group limits the interpretation which may be placed on the data obtained.

There may be some intra-spinal compensation for the loss of primary afferent-derived substance P as evidenced by the increase in PPT mRNA in spinal cord (Sivam and Krause, 1992). There is also some evidence to indicate that there may be recovery of the CGRP- and FRAP-positive primary afferents, and substance P-positive intraspinal neurons at six weeks of age (Hammond and Ruda, 1991). While the neurons found to be expressing PPT and CGRP do not increase their expression in arthritis in capsaicin treated animals, it is known that dorsal horn neurons in adult rats treated neonatally with capsaicin are supersensitive to tachykinins (Salt and Hill, 1983). This could indicate that while the absolute amount of translated neuropeptide are no different from control levels, the sensitivity of the dorsal horn to release of these peptides is increased, thereby maintaining the stimulatory effect. In addition to this, as somatostatin mRNA is decreased or completely abolished in these animals and somatostatin is known to inhibit the release of substance P from primary afferent terminals (Brodin et al., 1981; Gazelius et al., 1981), the loss of inhibition of release may also potentiate the action of PPT-derived neuropeptides on dorsal horn neurons.

The evidence discussed above indicates that the morphological and functional changes seen after neonatal capsaicin treatment can profoundly alter both the normal physiology of the spinal cord and its reactions in pathophysiological conditions. The effect of this lesion in regulating the spread of inflammation cannot be clearly defined until the reorganisation of the dorsal horn and its primary afferent input are more thoroughly understood. Therefore it is impossible to draw any firm



conclusions on the function of capsaicin-sensitive primary afferents in inflammatory arthritis using this lesion especially as the effects of capsaicin treatment on basal neuropeptide mRNA expression were not studied..

#### 6.4.2. Perineural capsaicin

Perineural capsaicin has time-dependent effects on the afferent nerve. Initially, perineural capsaicin stimulates the afferents, for example resulting in decreased heart rate and mean arterial blood pressure if the vagal nerve is treated (Buck and Burkes, 1986) or release of neuropeptides into the dorsal horn from a cutaneous sensory nerve (Zhao et al., 1992), followed by a reversible conduction block (Wall and Fitzgerald, 1981). The long term effects of perineural capsaicin are a permanent 70-80% reduction in functional polymodal nociceptors after two weeks (Pini and Lynn, 1990) and an abolition of neurogenic inflammation (Jancso et al., 1980). This is similar to the lesion caused by systemic administration in the adult which seems to be restricted to a sub-population of small primary sensory neurons consisting of approximately 15-18% of the total cell population (Lynn, 1990; Jancso, 1992) and therefore these are much more specific lesions than that caused by neonatal administration. Systemic administration in the adult rat has been shown to attenuate AA (Colpaert et al., 1983).

The importance of primary afferents in arthritis has been attributed by various authors to the neuropeptides contained in these primary afferents, which are also known to be transported to (Harmar and Keen, 1982), and released in the periphery (Diez Guerra et al., 1988). The neuropeptide changes and possible functions in inflammatory arthritis has been outlined in Sections 1 and 5

These results show increases in specific neuropeptide mRNA expression in innervating DRG on development of bilateral arthritis, i.e. both ipsilateral to the

initial inflammatory focus and contralaterally. Perineural capsaicin lesions of the sciatic nerve inhibited spread of arthritis and attenuated the arthritis on the side of immune stimulant injection in this model. These lesions also resulted in a reduction in primary afferent neuropeptide mRNA expression, suggesting an association between arthritis, its spread and induction of DRG neuropeptide mRNA expression, particularly that encoding PPT.

The possible mechanisms and significance of the bilateral increases in PPT and CGRP mRNAs have been discussed in Section 5.

#### 6.4.2.1. Effects of capsaicin treatment of the left sciatic nerve (CAPL).

The results presented here show that perineural capsaicin will reduce the ipsilateral (injected side) swelling by only ~50%. As the histological features of arthritis are similar in the left joints of all bilaterally arthritic and CAPL animals in this experiment, this implies that the local, profound immune stimulation caused by adjuvant (Freund and Lipton, 1955) may largely override a smaller, but still significant modulating effect of primary afferent neuropeptides on peripheral oedema and vascular effects. This assumption is supported by the observation that substance P and CGRP can cause oedema formation and vascular effects (Gamse and Saria, 1985; Brain and Williams, 1985). Substance P has, however been implicated in the mediation of arthritic damage; infusion of the peptide into joints will result in a greater degree of joint damage in adjuvant arthritis (Levine et al., 1984). Also, as PPT mRNA was studied, the importance of the second protein translated from this mRNA, neurokinin A (NKA) should not be overlooked. While under cell culture conditions the relative quantities of substance P and NKA translated from the PPT-A gene are approximately equal (MacDonald et al., 1989), it is not known whether treatment with capsaicin will alter the post-transcriptional processing of these neuropeptides. Recent evidence suggests that NKA has an

important spinal function in nociceptive processes (Fleetwood-Walker et al., 1993; Wiesenfeld-Hallin, 1993), and it is also known to be released from peripheral nerve terminals (Diez Guerra et al., 1988).

Importantly CAPL completely blocks the contralateral spread of arthritis, suggesting that distant spread of inflammation is mediated by capsaicin-sensitive components of the peripheral nervous system. PPT mRNA in contralateral DRG was expressed at control (non-arthritis) levels, whereas CGRP mRNA expression was elevated to levels associated with arthritis, although overt spread of inflammation and arthritis were prevented. This could indicate that some other mechanism, other than the capsaicin-sensitive primary afferents (e.g. the sympathetic nervous system which is unaffected by perineural capsaicin; Cervero and McRitchie, 1982), was functioning to activate the contralateral neurons expressing CGRP mRNA. As this effect on CGRP mRNA was associated with a significant attenuation of contralateral joint inflammation, this would support the contention that sympathetic efferent activation of nociceptors is not of major importance in the spread of inflammation. These data further suggest that peptides encoded by the PPT gene - e.g. substance P and neurokinin A (MacDonald et al., 1989) - may be of greater importance than CGRP in mediating the contralateral spread of arthritis, as suggested by the pro-arthritic effects of substance P demonstrated by Levine et al. (1984).

#### 6.4.2.2. Effect of capsaicin treatment of the right sciatic nerve (CAPR)

CAPR had a significant effect on swelling of the left joint at two points in the time of the experiment but this was not reflected in the degree of arthritis assessed histologically. This agrees with the results obtained by Levine and colleagues (1985a) in a study of "reflex neurogenic inflammation". This was essentially a study

of contralateral hyperalgesia and swelling following minor ipsilateral paw injury, rather than a defined inflammatory lesion, where the authors investigated the effect of perineural capsaicin on small increases in ipsi- and contralateral paw thickness (0-3% change). In Levine's study, perineural capsaicin treatment of the nerves in the uninjured limb also produced a decrease in the contralateral injured paw hyperalgesia, agreeing with the results presented here that perineural capsaicin can have effects contralateral to the lesion in itself. This effect is unlikely to be due to a systemic effect of the locally applied neurotoxin. Capsaicin applied perineurally for a longer period (30 minutes) than that used in the studies discussed here is absorbed into the circulation, but in amounts too small to exert a neurotoxic effect on distant tissues (Holzer, 1991).

The effects of CAPR on the spread of arthritis from the initial left focus to involve the right hind limb further emphasises the importance of capsaicin-sensitive primary afferents in the mediation of this spread. CAPR completely prevented the contralateral spread of arthritis and the induction of CGRP and PPT mRNAs in the right L5 DRG. CAPR also markedly attenuated the increase in CGRP mRNA in left DRG in the face of the continuing local inflammation. By contrast, the increase in PPT mRNA ipsilateral to adjuvant injection was maintained, as was arthritis, further indicating the likely importance of this gene and its products in neurogenic inflammation.

#### 6.4.2.3. Neuropeptide mRNA changes in DRG contralateral to the capsaicin application; mechanisms through which perineural capsaicin exerts distant effects

CAPR has distant effects on the neuropeptide mRNAs in left DRG, preventing the rise in PPT and somatostatin mRNAs. The mechanism(s) underpinning the different changes in neuropeptide mRNA expression contralateral to the capsaicin lesion with

CAPL and CAPR are unknown. Contralateral effects may reflect the subsequent state of activation (i.e. presence or absence of adjuvant) of the respective sciatic nerves. Others have shown that nerve injury suppresses neurogenic vasodilation and plasma extravasation in response to antidromic stimulation in nerves contralateral to an injury (Allnatt et al., 1990). If polymodal nociceptors are destroyed using perineural capsaicin before the nerve section, the contralateral impairment of neurogenic inflammation is no longer seen (Stephen Lisney, personal communication) suggesting that the contralateral effects observed in this model of injury and regeneration are also mediated through capsaicin-sensitive polymodal nociceptors.

The neurotoxic effect of perineural capsaicin can be reversed by NGF (Otten et al., 1983) and NGF is known to regulate spinal cord conductivity in the adult rat (Lewin et al., 1992c). Following perineural capsaicin application, transganglionic degeneration of primary afferents is apparent within 14 days (Jancso G, 1992), and this treatment alters afferent inputs to dorsal horn neurons both ipsilateral and contralateral to the injury (Fitzgerald 1982). Thus, there may be plastic changes within the dorsal horn resulting in altered primary afferent reactions to inflammation. It is conceivable that a similar process to that seen after neonatal capsaicin, of sprouting of non-injured primary afferent terminals and synaptic reorganisation in the dorsal horn could occur following perineural capsaicin application, although to a lesser extent, particularly since morphological changes indicative of neuronal plasticity have been noted within hours of damage (Goshgarian et al., 1989). Furthermore, the increase in neuronal activity seen during inflammation will itself induce plastic changes in the spinal cord (Dubner and Ruda, 1992). Clearly the summation of these effects, together with more direct capsaicin-mediated actions on neuropeptide mRNA expression and their regulation by transcription factors, are difficult to predict but might well combine to explain the discordant increases in

primary afferent neuropeptide mRNA expression that were found after CAPL and CAPR.

While the results from the perineural capsaicin experiments do not disprove an involvement of sympathetic efferents in the contralateral spread of arthritis in this model, they are more strongly indicative of a fundamental role for the peripheral sensory system. The problem inherent in using the "end point" of contralateral disease to attribute fundamental importance to either the sympathetic and sensory nervous systems in the spread of inflammation is that, if the activation of nociceptors and PGSN is sequential as proposed by Kidd and associates (1989b), then lesion of either system would, inevitably result in an attenuation of the arthritis. The sequence of events at a spinal level, and whether either dorsal horn sensory neurons or preganglionic sympathetic neurons are involved, needs to be determined so that effect of lesions on neuronal activation can be precisely pinpointed with respect to both time, and the system involved.

Capsaicin-sensitive primary afferents and their central connections are involved in the maintenance of arthritis and are necessary for the distant spread of inflammation in our attenuated model of adjuvant arthritis. Contralateral inflammation is associated with a concurrent increase in PPT and CGRP, but not somatostatin mRNA expression, or induction of VIP expression, suggesting that specific neuropeptide products may be involved in the mediation of contralateral spread of arthritis in this model. Neuropeptides translated from the preprotachykinin gene may be of greater importance in the symmetrical response to inflammation than CGRP.

## 6.5. CONCLUSIONS

Neonatal capsaicin and the destruction of unmyelinated afferents does not affect the contralateral spread of arthritis. This may reflect an increased sensitivity of dorsal horn neurons to primary afferent neuropeptides, decreased inhibition of release of substance P by somatostatin, or some recovery of primary afferents. The lesion may also allow the sympathetic nervous system to assume a greater importance than under normal circumstances.

Perineural capsaicin treatment, a more selective lesion, suggests that capsaicin-sensitive components in the peripheral nervous system are of fundamental importance in the mediation of the spread of arthritis seen in this modified model of adjuvant arthritis in the rat.

Neuropeptides encoded by PPT and CGRP mRNAs expressed in these capsaicin-sensitive neurons are also of importance in the spread of arthritis. PPT encoded peptides may be of more importance than CGRP, although a synergistic relationship is more probable.

## SECTION 7. *IN VIVO* REGULATION OF NEUROPEPTIDE mRNA EXPRESSION

### 7.1. INTRODUCTION

Very little is known about the *in vivo* regulation of sensory neuropeptides particularly in DRG neurons. Much of the work investigating the expression of neuropeptide genes, particularly PPT and CGRP has concentrated on the alternative splicing seen in these genes and the tissue specificity of expression. However, it is still unknown why only limited populations of DRG neurons express particular neuropeptides, although the neuropeptide phenotype of DRG neurons appears to be already determined at birth, at least in the case of VIP (Mulder, 1993). In the chick embryo, the majority of DRG neurons express substance P and it appears that both central and target derived factors act to suppress PPT expression in neurons which are not "destined" to express this gene in the adult (Barakat-Walker et al., 1991). It has not yet been determined what these factors may be.

#### 7.1.1 Regulation of sensory neuropeptides by glucocorticoids

Circulating hormones such as glucocorticoids and sex steroids are known to regulate the expression of various genes through direct interaction with DNA sequences in the 5' flanking portions of target genes. Regulation can also occur at several other levels; it is known that adrenalectomy increases the axonal transport of substance P and, conversely, increased (exogenous) corticosterone levels increase the axonal transport of somatostatin (MacLean, 1987) in the vagus nerve. Glucocorticoids also regulate neuropeptide expression in other areas of the central and peripheral nervous systems such as in brain and sympathetic neurons. It is



probable, however that different regulatory mechanisms are important in different tissues.

The neuropeptides substance P and Leu-enkephalin are both expressed in rat striatum and both preproenkephalin (PPE) and PPT have been shown to be regulated by circulating glucocorticoids. Adrenalectomy results in a decrease in both PPT and PPE mRNA expression which can be reversed by corticosterone administration, although increasing corticosterone levels by restraint stress does not affect PPT mRNA expression. This suggests that maintenance of PPT mRNA expression requires at least basal corticosterone levels. Although PPE shows diurnal variation paralleling that of corticosterone, this is not seen with PPT (Chao and McEwan, 1991). In cultured sympathetic neurons, glucocorticoids inhibit the stimulation of PPT mRNA caused by the inflammatory mediator IL-1 $\beta$  (Kessler and Freidin, 1991). Rat VIP gene expression is also regulated in the intact hypothalamus by exogenous corticosterone and oestrogen (Gozes, 1988). It is difficult, however, to extrapolate data obtained under culture conditions to the *in vivo* situation as DRG ganglion cells lose both central and peripheral targets, and different regulatory mechanisms may act in the CNS and the PNS. Sensory neurons, particularly, are thought to be dependent on centrally and peripherally derived factors for maintenance of phenotype (see Section 1.9); therefore data obtained under culture conditions must be treated with caution. *In vivo*, DRG neuropeptides have recently been shown to be regulated by glucocorticoids. Adrenalectomy caused increases in ganglionic content of substance P and CGRP and a decrease in somatostatin, effects which were abolished by dexamethasone administration (Smith et al., 1991). Rat DRG neurons express both mineralocorticoid and glucocorticoid receptors (J.R. Seckl, personal communication) and it is therefore possible that these changes in peptide content are mediated at the level of transcription. However, assessment of neuropeptide content of whole ganglia may disguise changes in subpopulations of neurons, and changes in peptide

content may reflect regulation at the level of post-translational processing rather than transcription.

Glucocorticoids may affect the inflammatory process in ways other than directly via modulation of gene expression. Synthetic glucocorticoids inhibit the expression of the cyclooxygenase 2 gene, an inducible form of the enzyme thought to be a major mediator of inflammation (O'Banion et al., 1992), and are also known to have potent anti-inflammatory effects on the immune system and neuronal excitability (Joels and de Kloet, 1992).

### 7.1.2. Nerve growth factor

The dependence of adult DRG neurons on NGF for the maintained expression of certain neuropeptides, particularly substance P and CGRP has been outlined in Section 1.8. It has been suggested that NGF may exert its effects on neuropeptide gene expression in the adult either directly (Gilchrist et al., 1991), via the cAMP or Ca<sup>2+</sup>/phosphatidylinositol pathways (Kageyama et al., 1991) or via inducible transcription factors (Section 8). It is possible that glucocorticoids and NGF effects may interact in the regulation of gene expression as dexamethasone is known to repress both c-fos expression (Yin and Howells, 1992), c-jun DNA binding (Yang-Yen et al., 1990) {transcription factors known to be NGF-inducible} and NGF-receptor mRNA induction by NGF (Foreman et al., 1992).

### 7.1.3. Regulation of neuropeptide gene expression by other mechanisms

As mentioned in Section 4, various inflammatory mediators are known to act on peripheral nociceptive afferents to both activate and sensitise them. This results in ongoing spontaneous activity in many nociceptors and increased response to usually innocuous stimuli. Depolarisation with 40mM K<sup>+</sup> has been shown to increase the

expression of VIP and PPT in DRG cultures (Mulderry, 1993b), VIP in spinal cord cultures (Agoston et al., 1991), and intra-hippocampal injection of the excitotoxin kainic acid increases PPT mRNA ipsilateral to the injection site (Brene et al., 1992).

#### 7.1.4. Aims of the study

It was of interest to determine whether adrenalectomy would potentiate the increases in PPT and CGRP mRNA expression seen in arthritic rats, in view of the effects seen on adrenalectomy at the level of peptide expression, and thereby alter the severity of arthritis. Also, as nociceptive afferents are known to have increased spontaneous activity in arthritic rats, the effect of blocking action potential conduction to the DRG on neuropeptide mRNA expression has also been studied.

## 7.2. MATERIALS AND METHODS

### 7.2.1. Adrenalectomy and induction of arthritis

Animals were surgically adrenalectomised under halothane anaesthesia (Section 2.5.1) and allowed to recover for three days from surgery before induction of either mono- or bilateral arthritis as previously described. Monitoring of weight and joint swelling was performed as before. Fifteen days after adjuvant injection animals were killed by decapitation, and trunk blood and DRG collected. In situ hybridisation for PPT, CGRP and somatostatin mRNA expression was performed on sections of DRG as described previously.

### 7.2.2. Blockage of action potential conduction

Action potential conduction in the left sciatic nerve was blocked by direct injection of 2% lidocaine (Sigma) solution in sterile saline without surgical incision or exposure of the nerve. Only animals exhibiting complete motor paralysis of the left hind limb were then injected with 150µg *M.tub.* around the left tibio-tarsal joint. Animals were killed by decapitation 8 hours later and DRG processed for in situ hybridisation for PPT, CGRP and somatostatin mRNA expression as described previously.

## 7.3. RESULTS

### 7.3.1. Assessment of inflammation

#### 7.3.1.1. Effect of adrenalectomy on indices of joint swelling and inflammation

Adrenalectomised animals had no circulating corticosterone as determined by corticosterone radioimmunoassay (< 20 nmol/L). Sham operated rats were found to have circulating corticosterone levels equivalent to those previously found in mono- or bilaterally arthritic rats (section 3) ( $886 \pm 682$  nmol/L morning corticosterone levels for sham operated arthritic rats; n=5). One ADX rat in the monoarthritis group was found to have detectable corticosterone levels and was therefore excluded from the data analysis. Adrenalectomised (ADX) monoarthritic rats showed the same joint swelling and weight gain as sham operated rats (Figures 7.1 and 7.2).

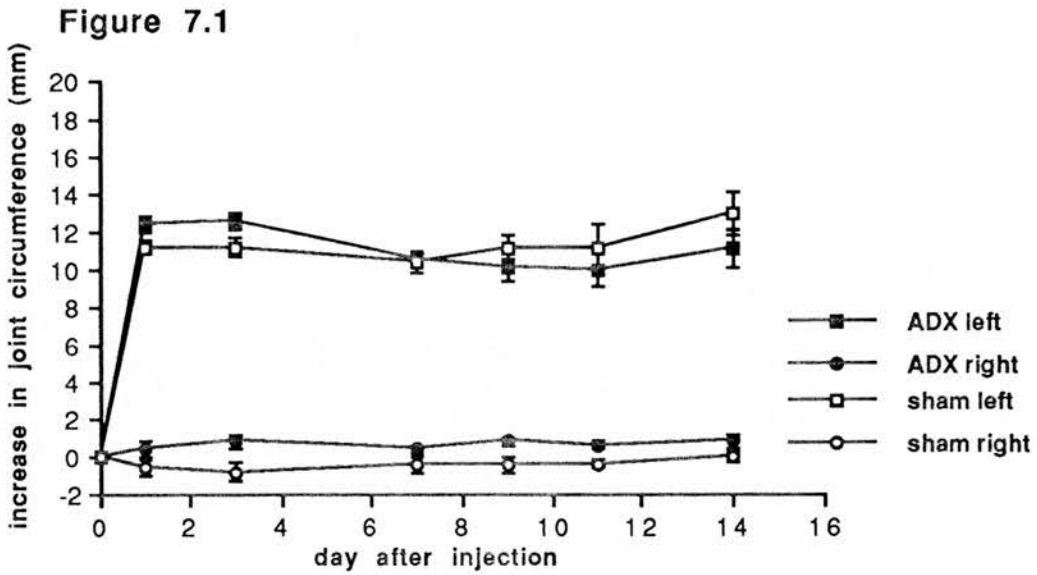


Figure 7.1. Left and right joint circumferences from ADX and sham operated rats during the development of monoarthritis. (Means  $\pm$  S.E.M. n=4 ADX; 5 sham)

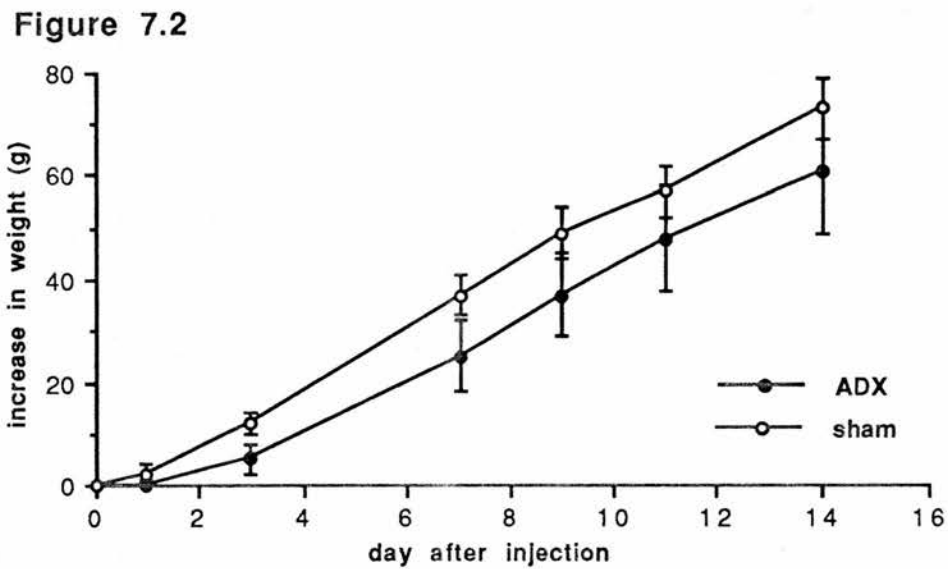


Figure 7.2. Weight gain during development of monoarthritis in ADX and sham operated rats. Groups showed significantly different initial mean weights ( $206 \pm 5$ g, ADX;  $229 \pm 8$ g, sham) but the gain in weight was not significantly different between groups. (Means  $\pm$  S.E.M. n=4 ADX; 5, sham)

Joint swelling in bilaterally arthritic rats also showed no significant differences between ADX and sham operated rats, although there was a tendency for the left swelling to be greater in ADX rats (Figure 7.3) and right swelling to be slightly less. As in the monoarthritic group, the sham and ADX groups had significantly different body weights before induction of arthritis ( $200 \pm 4$  g, ADX;  $238 \pm 8$  g, sham; means  $\pm$  S.E.M.) and when the gain in weight over the 15 day period was compared, ADX rats showed a significantly reduced weight gain at all time points except day 1 (Figure 7.4).

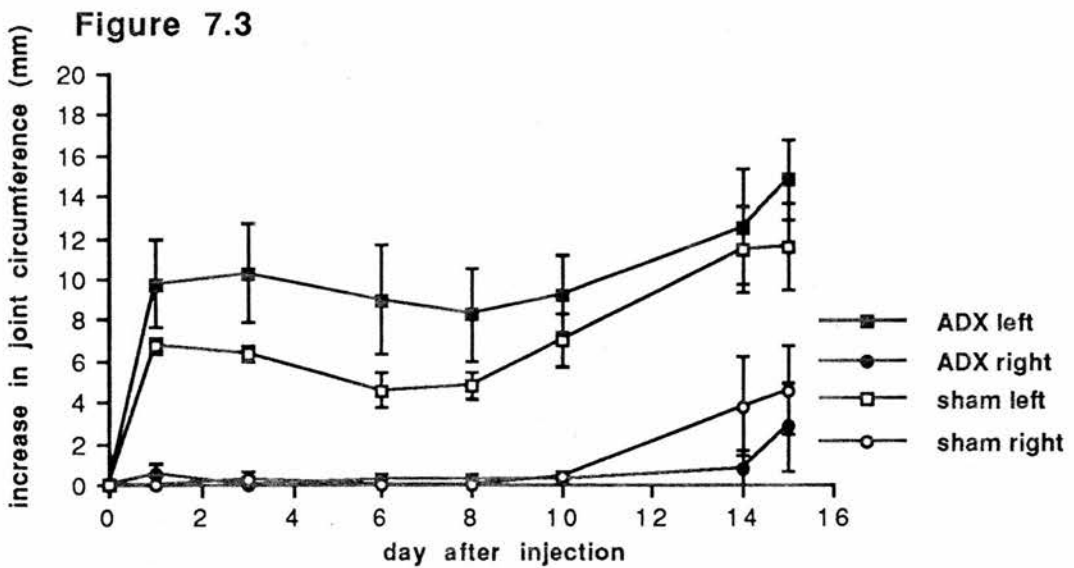
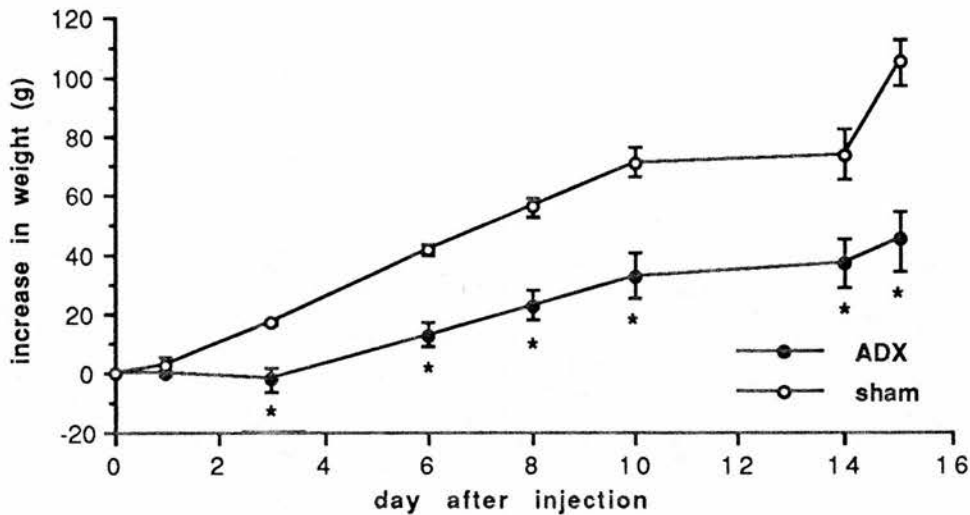


Figure 7.3. Increase in left and right joint circumferences in bilaterally arthritic ADX and sham operated rats. (Means  $\pm$  S.E.M.;  $n=5$  per group)

**Figure 7.4**



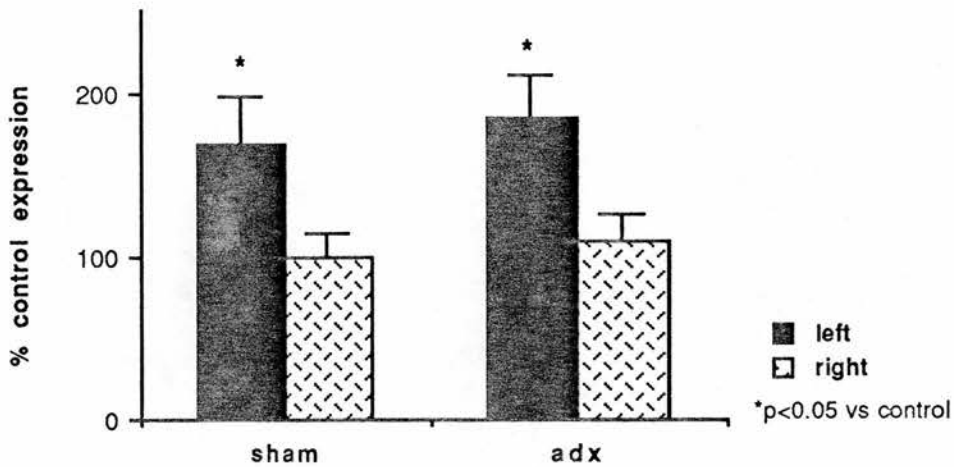
*Figure 7.4. Mean increase in body weight of bilaterally arthritic ADX and sham operated rats during development of arthritis. \* $p < 0.05$  compared to sham operated group (Means  $\pm$  S.E.M.  $n=5$  per group).*

### 7.3.2. Neuropeptide mRNA expression

#### 7.3.2.1. Effect of adrenalectomy on neuropeptide mRNA expression

Although adrenalectomy did not significantly affect the degree of arthritis, it did have subtle effects upon PPT and CGRP mRNA expression. Somatostatin mRNA expression was examined in only bilaterally arthritic animals (as this mRNA shows unilateral increases in expression in both mono- and bilateral arthritis [Section 5]) and was unaffected by adrenalectomy (Figure 7.5). As expression in right DRG from monoarthritic animals is not significantly different from untreated controls, all results were normalised to the expression in right DRG from monoarthritic sham operated animals in this study.

**Figure 7.5**



*Figure 7.5. Somatostatin mRNA expression in DRG from ADX or sham operated bilaterally arthritic rats 14 days after adjuvant injection. Values are means  $\pm$  S.E.M.; n=5 per group. There were no significant differences between ADX and sham animals. Mean  $\pm$  S.E.M. control grain counts were  $184 \pm 24$*

PPT mRNA was increased in left (injected side) DRG neurons in monoarthritic, and both left and right DRG in bilaterally arthritic, sham operated animals as observed previously (Section 5). In ADX monoarthritic rats, PPT mRNA expression was not different from sham operated rats; however bilaterally arthritic ADX rats showed a significant PPT mRNA increase in left (injected) DRG and no increase in right DRG (Figure 7.6A and B).



Figure 7.6A

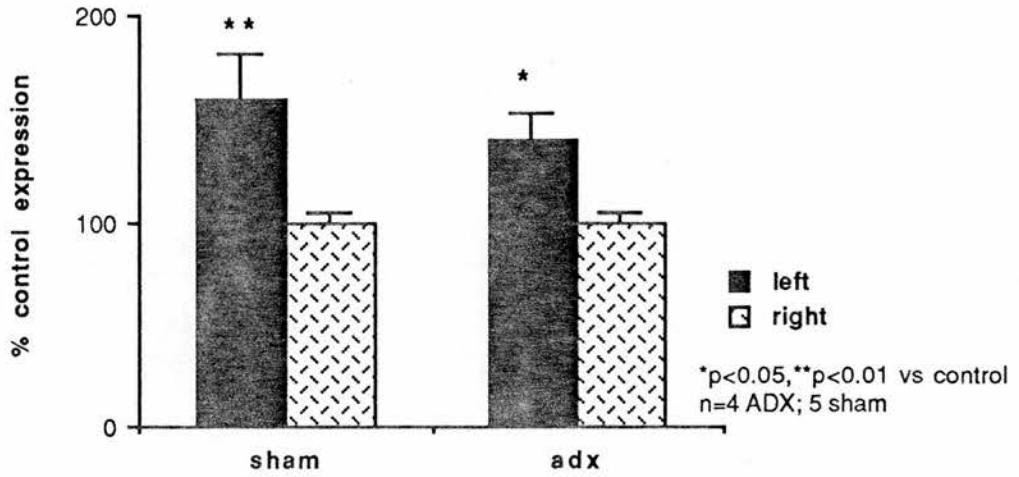


Figure 7.6A PPT mRNA expression in monoarthritic ADX and sham operated rats (day 14). Adrenalectomy does not significantly affect the expression of PPT mRNA. Mean ( $\pm$  S.E.M.) control grain counts were  $331 \pm 17$ .

Figure 7.6B

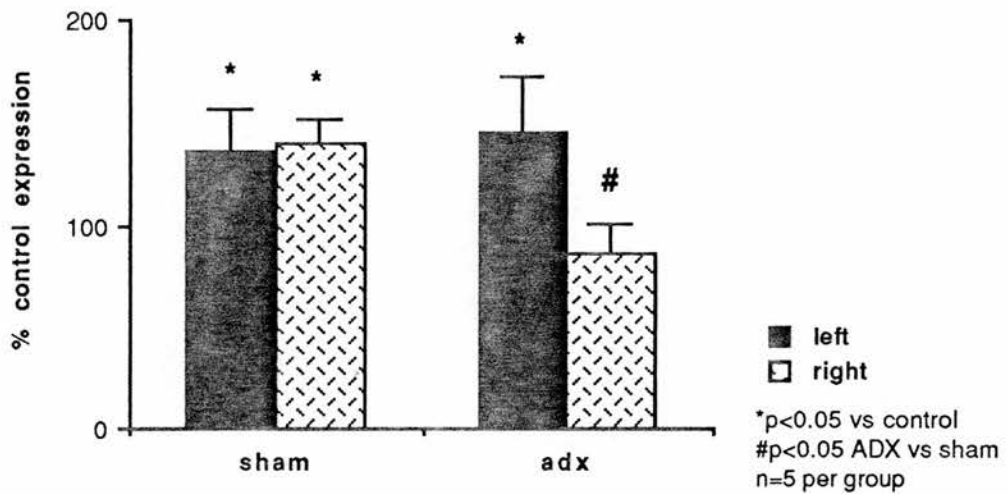
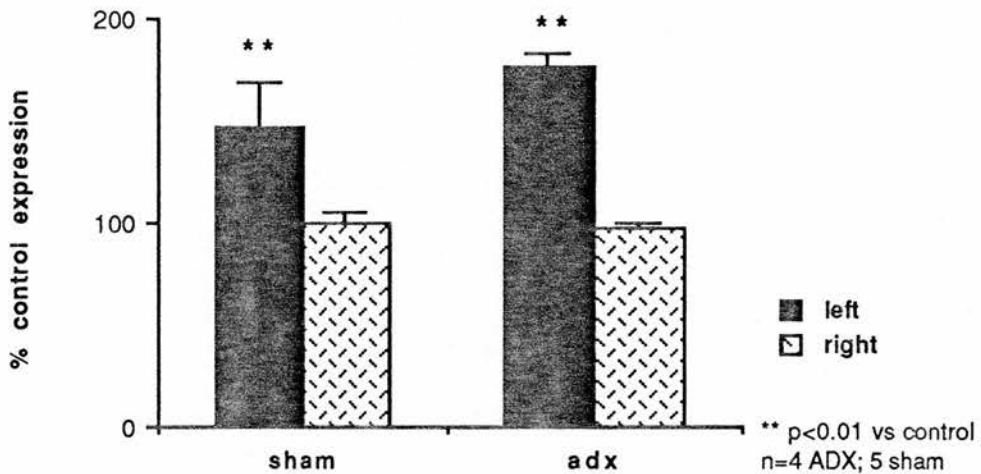


Figure 7.6B PPT mRNA expression in bilaterally arthritic ADX and sham operated rats. (day 15) Adrenalectomy does not affect the left (injected side) PPT mRNA expression but attenuates the increase in right PPT mRNA expression usually seen in bilaterally arthritic rats. Values are means  $\pm$  S.E.M. Mean control grain counts were  $466 \pm 38$

CGRP mRNA expression in sham operated rats was as previously observed in untreated mono- and bilaterally arthritic rats, increased ipsilaterally in monoarthritis and bilaterally in bilateral arthritis. In ADX monoarthritic rats, CGRP mRNA expression was not significantly different from sham operated rats (Figure 7.7A). Bilaterally arthritic ADX rats showed significantly increased expression in left DRG neurons over sham operated rats. Right CGRP mRNA expression was also increased in bilaterally arthritic ADX rats, but this was not significantly different when compared to sham operated controls (Figure 7.7B).

**Figure 7.7A**



*Figure 7.7A. CGRP mRNA expression in DRG from monoarthritic ADX or sham operated rats (day 14). Left (injected side) mRNA expression is significantly raised compared to right. There are no significant differences between the two groups.*

Figure 7.7B

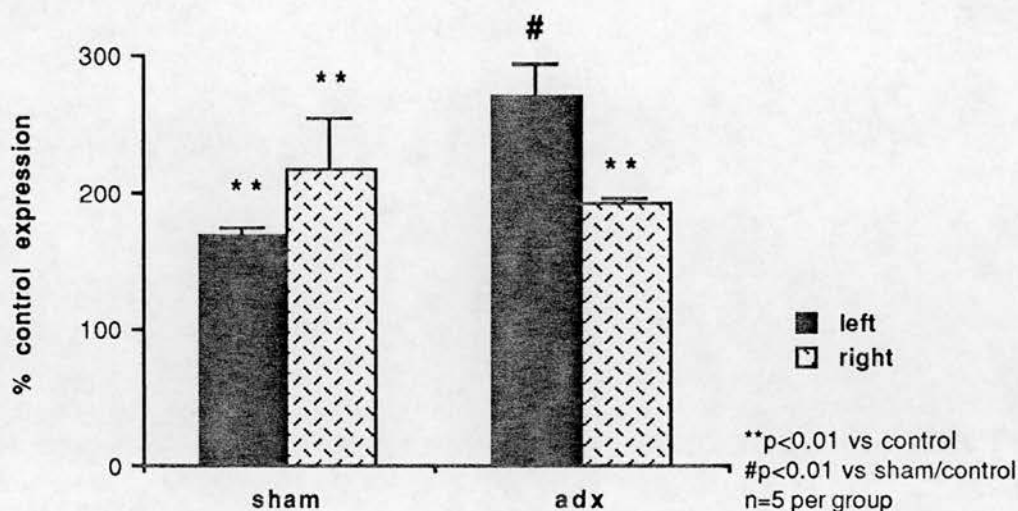


Figure 7.7B. CGRP mRNA expression in DRG from bilaterally arthritic ADX or sham operated rats; (day 15). Sham operated rats show bilateral increases in CGRP mRNA expression. ADX rats show significantly increased left (injected) side CGRP mRNA levels compared to sham animals. Expression in right DRG is significantly raised compared to controls but not sham animals. Values are means  $\pm$  S.E.M.

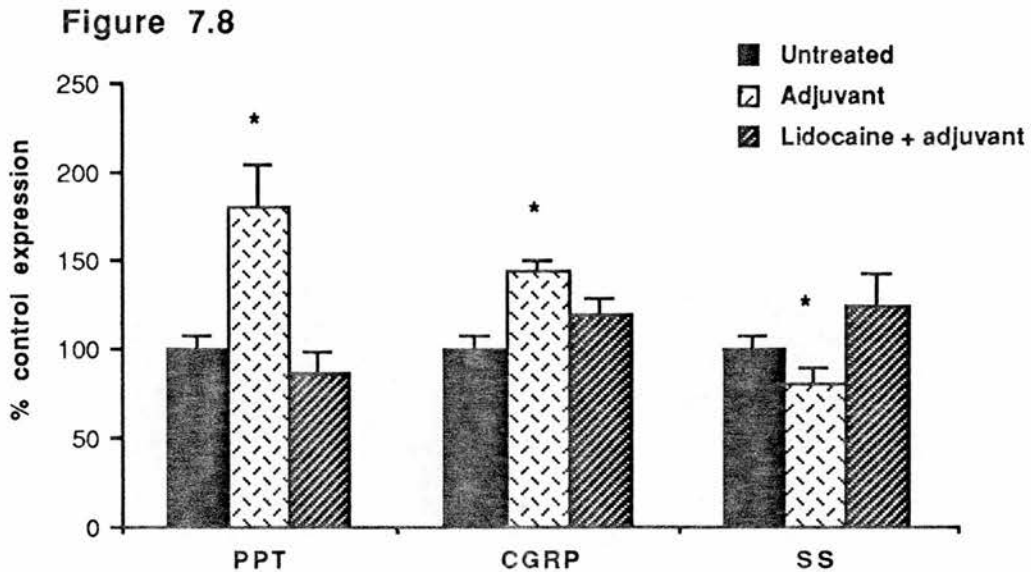
### 7.3.2.2. Effect of local anaesthetic nerve block on neuropeptide mRNA expression

Expression of PPT, CGRP and somatostatin mRNA in left DRG from rats subjected to local anaesthetic block of the left sciatic nerve, and injected with 150 $\mu$ g *M.tub.* was not significantly different from untreated controls (Figure 7.8). Thus lidocaine nerve block prevented the changes in PPT, CGRP and somatostatin mRNA expression seen eight hours after adjuvant injection described in Section 5. This indicates that action potential conduction to the cell body may, in part, regulate the increase in PPT and CGRP mRNA and the decrease in somatostatin mRNA at this

time after adjuvant injection. The increase in PPT mRNA was totally prevented whereas CGRP showed only a partial reduction and somatostatin mRNA expression showed a non-significant rise (Table 7.1). This suggests that the increased neuronal activity seen in primary afferents innervating inflamed joints differentially regulates neuropeptide gene expression in DRG neurons.

*Table 7.1. Neuropeptide mRNA levels in left DRG 8 hours after adjuvant injection with and without nerve block with 2% lidocaine solution. Values shown are means  $\pm$  S.E.M. \* $p < 0.05$  cf. controls.*

	PPT	CGRP	Somatostatin
Control	100 $\pm$ 7	100 $\pm$ 13	100 $\pm$ 21
Adjuvant	181 $\pm$ 24*	144 $\pm$ 6*	81 $\pm$ 8*
Adjuvant+ lidocaine	86 $\pm$ 12	119 $\pm$ 10	125 $\pm$ 17



*Figure 7.8. Effect of lidocaine nerve block on expression of PPT, CGRP and somatostatin mRNAs 8 hours after adjuvant injection. Lidocaine nerve block inhibited the rise in PPT, CGRP mRNA and the fall in somatostatin mRNA. Values are means  $\pm$  S.E.M.  $n=5$  per group. Mean control grain counts were; PPT,  $179\pm13$ ; CGRP,  $445\pm51$ ; SS,  $355\pm65$ .*

## 7.4. DISCUSSION

### 7.4.1. Effect of adrenalectomy on signs of inflammation

Adrenalectomy only significantly affected the weight gain in bilaterally arthritic animals and did not have profound effects upon joint circumferences of either mono- or bilaterally arthritic rats. Although the degree of arthritis, as assessed by measurement of the joint circumference was not significantly affected by adrenalectomy, the general well-being of the animals as reflected in the normal weight gain seen over this period of time, was affected in bilaterally arthritic

animals. As monoarthritic rats continued to gain weight normally this loss of weight may be indicative of the systemic consequences of the lack of an hypothalamo-pituitary-adrenal response to the slightly more severe inflammation, in combination with the effects of adrenalectomy itself on normal weight gain.

These results are in contrast to those obtained by Sarlis et al., (1992) who found that adrenalectomy significantly affected the degree of polyarthritis in rats assessed by paw volume, to the extent of causing increased mortality in the animals. However it must be remembered that the model used in the studies discussed here is not identical to the polyarthritis used by Sarlis, and even bilateral arthritis cannot be directly compared to the polyarthritic model. Animals with polyarthritis are already systemically ill and therefore it is not surprising that adrenalectomy, which is known to attenuate the body's response to severe illness, would exacerbate their condition. The model used in the studies outlined in this thesis generally shows no systemic effects, and this is the only situation in which a significant drop in body weight has been observed using this model.

#### 7.4.2. Effect of adrenalectomy on neuropeptide mRNA expression

In view of the lack of effect of adrenalectomy on the degree of arthritis, it was surprising that the intervention had significant effects on both PPT and CGRP mRNA expression. However, the raised morning corticosterone levels in these arthritic rats might be expected to have some effect in terms of the genes regulated by circulating corticosteroids. The only study examining the in vivo regulation of neuropeptides in DRG showed that adrenalectomy increased substance P and CGRP and decreased somatostatin in DRG (Smith et al., 1991). It has been hypothesised that adrenalectomy would further potentiate the increases in PPT and CGRP mRNA expression seen in inflammation and thereby potentiate the arthritis. This, however, only occurs in the case of CGRP mRNA in left but not right DRG. PPT mRNA

shows the normal increase in left DRG of bilaterally arthritic rats, but does not increase in right DRG despite continued contralateral arthritis. The lack of contralateral increase in PPT mRNA is associated with a slight, non-significant reduction in right joint swelling, which may again support a role for the peptides translated from the PPT gene in the contralateral effects seen in this model of arthritis. However the effects of adrenalectomy on these neuropeptide mRNAs occur in the face of ongoing inflammation, which is unaffected by the surgical intervention. It would therefore appear that while adrenalectomy does have subtle effects on the expression of PPT and CGRP mRNAs, the high circulating corticosteroids in arthritic animals have minor importance in the modulation of the disease in this model.

#### 7.4.3. The influence of neural activity on neuropeptide mRNA expression

Depolarisation is known to increase PPT mRNA expression in cell culture (Mulderry, 1993b). However, VIP is also increased on depolarisation (Mulderry, 1993b) and yet is unaltered in experimental arthritis *in vivo* (Section 5) when primary afferents are known to become spontaneously active within hours of the onset of inflammation (Schaible and Schmidt, 1985). This illustrates the danger of extrapolation of data obtained in culture systems to the *in vivo* situation.

As only animals showing complete motor paralysis were used in this study, unmyelinated afferent conduction was definitely blocked as the action of local anaesthetics is dependent upon fibre diameter, with fibres mediating painful stimuli being affected first and those with large myelinated fibres affected last and recovering most rapidly. Nerve blocks were performed directly, ("blind") to avoid any possible gene induction due to surgical exposure of the nerve. It was possible to determine in which animals a successful block had been achieved as motor paralysis was evident; injection of sterile saline as a control was unsatisfactory as there was no

method of determining that the solution had been delivered around the sciatic nerve, so untreated animals were used as a control in these studies as in all other experiments on neuropeptide mRNA expression

In view of the data presented in this section, it seems that action potential conduction to the soma of unmyelinated primary afferents may be responsible, in part, for the changes in mRNA expression seen during the first 8 hours after adjuvant injection.

### 7.5. SUMMARY

Adrenalectomy had no significant effect on overt parameters of arthritis in either mono- or bilaterally arthritic rats, but did affect the normal weight gain in bilaterally arthritic animals. Alterations in neuropeptide mRNA in arthritic adrenalectomised rats exert only subtle effects on the inflammatory process.

Primary afferent activity developing within hours of the onset of inflammation may mediate the changes in neuropeptide mRNA seen eight hours after adjuvant injection, suggesting that immune stimulation or circulating humoral mediators alone are insufficient for early neuropeptide mRNA induction.



## SECTION 8. THE POSSIBLE ROLE OF TRANSCRIPTION FACTORS IN THE REGULATION OF NEUROPEPTIDE mRNA EXPRESSION DURING INFLAMMATION

### 8.1. INTRODUCTION

As shown in Section 5, during the acute inflammation seen following adjuvant injection,  $\beta$ -PPT,  $\alpha$ -CGRP and somatostatin show very early changes in mRNA expression. The expression of these genes is known to be under the control of a number of different factors, including nerve growth factor (see Section 1.8) electrical activity, glucocorticoids and cAMP as has been discussed in Sections 1 and 7. Analysis of the 5' flanking DNA from both the PPT, somatostatin and VIP genes has shown multiple transcription factor recognition, or consensus, sequences including those for the transcription factors studied here (see below).

#### 8.1.1. Immediate early genes

Immediate early genes are a group of genes, the expression of which is normally at extremely low or undetectable levels, but which can be activated within minutes by the addition of a growth factor in culture or following a physiological or pharmacological stimulus. Their expression is usually transient, peaking within minutes, and as these genes have a short half-life, shortly becoming undetectable once more. Many of these genes were initially characterised in the rat pheochromocytoma cell line (PC12) as being inducible following NGF-stimulated cell differentiation. Members of this family of factors include the fos and jun families which interact to form the AP-1 complex, and an NGF-inducible (NGFI) family. Many of these immediate early genes encode transcription factors, which together with their tightly controlled expression strongly suggests that they are

involved in the regulation of so called late response genes in response to external stimuli (Sheng and Greenberg, 1990).

### 8.1.2. The AP-1 complex; fos and jun

Fos and jun are the two most widely studied members of a family of immediate early genes which are the cellular correlates of viral oncogenes. Transcriptional activation of fos usually occurs within 5 minutes of stimulation and continues for 15-20 minutes causing a peak mRNA level at 30-45 minutes post-stimulation (Morgan and Curran, 1991). Other members of this family are fos-related antigens (fra) 1 and 2 (Cohen and Curran, 1988; Nishina et al., 1990), jun B (Ryder et al., 1988), and jun D (Ryder et al., 1989) among others. These two groups of nuclear proteins dimerise through a "leucine zipper" dimerization domain in each protein. This consists of an  $\alpha$ -helical structure with 4 leucine residues spaced 7 amino acids apart such that all the leucines are aligned along one face of the helix. The leucine zipper domain is also required for required for DNA binding (Mitchell and Tjian, 1989). Fos-Jun, Fra-Jun and Jun-Jun dimers also require a highly conserved basic region for DNA binding; all the dimers forming the AP-1 complex recognise the AP-1 consensus sequence 5'-TGACTCA-3' (Mitchell and Tjian, 1989). Fos-Jun dimers are more stable than Jun-Jun dimers and have a greater DNA binding affinity (Morgan and Curran, 1991). ATF/CREB (activating transcription/cyclic AMP responsive element binding proteins), the transcription factors first identified as leucine zipper proteins, which respond to intracellular cAMP also dimerise with the Fos/Jun family. This cross-family interaction results in an altered DNA binding specificity for the AP-1 and ATF/CRE binding sites (Hai and Curran, 1991). Regulation by cAMP is of importance as PPT, somatostatin and VIP genes all have an active cAMP response element in their 5' flanking DNA sequences (Chapman et al., 1993; Lamperti et al.,

1991; Tsukada et al., 1987), and cAMP is thought to mediate the hyperalgesic actions of eicosanoids (Taiwo et al., 1989).

### 8.1.3. Other nerve growth factor inducible transcription factors

On the discovery that NGF could induce the expression of the fos/jun family in PC12 cells, other workers investigated the induction of further genes by nerve growth factor. Milbrandt and colleagues have isolated two genes, NGFI-A (also known as krox-24, zif268, egr-1 and TIS-8 (see Morgan and Curran 1991 for refs.)), a "zinc-finger" protein (Milbrandt, 1987) recognising the consensus sequence 5' -GCGGGGGCG- 3', and NGFI-B (nurr77, N10, TIS-1), which belongs to the steroid receptor superfamily (Milbrandt, 1988). The 5' flanking region of the NGFI-A gene has been shown to possess an NGF-responsive element (Changelian et al., 1989). Other genes are also known to be induced by NGF in PC12 cells but they will not be discussed here.

### 8.1.4. Activator protein-2 (AP-2)

Transcription factor AP-2 is expressed developmentally in tissues of neural crest origin such as dorsal root ganglia (Mitchell et al., 1991). AP-2, which also exists as a dimer (Williams and Tjian, 1991a) recognises the specific DNA sequence 5'-GCCN<sub>3</sub>GGC-3' and requires an amino-terminal proline-rich sequence in order to stimulate transcription (Williams and Tjian, 1991b). The presence of AP-2 site confers cAMP inducibility on a gene (Mitchell and Tjian, 1989).

### 8.1.5. Transcription factors and neuropeptide genes

Many neuropeptides have been shown to have AP-1 binding sites within their

promoter regions and in PPT, somatostatin and VIP these have been found to be active (Quinn, 1992; Goodman, 1990). AP-2 sites have been identified in the 5' flanking regions of a number of genes, and have been shown to be active in regulation of expression of the human growth hormone and proenkephalin genes (Courtois et al., 1990; Goodman, 1990), the rat TNF- $\beta$ 3 gene (Lafyatis et al., 1990) and human immunodeficiency virus 1 (Muchardt et al., 1992). The PPT promoter has two non-consensus AP-2 sites at positions -78 and -564 of the 5' flanking sequence; it is still unknown whether these are active. There are also several AP-2 sites after the transcription start site of the PPT gene.

#### 8.1.6. Aim of the experiments

These experiments were designed to determine whether any of the transcription factors described above were expressed in small DRG neurons in the early response to peripheral inflammation, and if so, whether their expression could be associated with any of the neuropeptide mRNAs which show changes in this condition.

## 8.2. MATERIALS AND METHODS

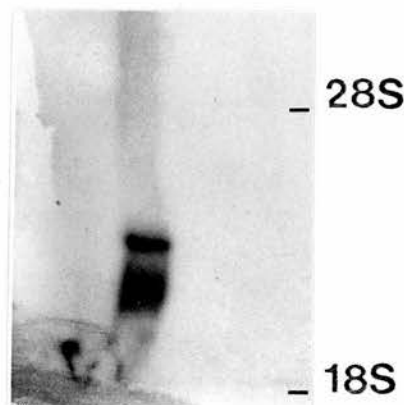
Adjuvant-inflammation was induced as described in Section 2. Animals were killed by decapitation at intervals of 30 minutes, 1, 2, 4 and 8 hours after adjuvant injection, left and right L5 DRG dissected, rapidly frozen and processed for in situ hybridisation. Complementary RNA probes for c-jun, NGFI-A, -B and AP-2 mRNAs were used. Expression of mRNAs was determined using computer-aided image analysis and expressed as a percentage of the basal expression in untreated control animals. Positive control sections of normal rat hippocampus were used to ascertain the efficacy of hybridisation.

For c-jun, a specific probe was subcloned from the full length cDNA as outlined in Section 2. Similar brain sections were also hybridised with a specific jun D cRNA probe and the differential distributions in brain compared to those in the literature. The c-jun cDNA probe was also hybridised to a Northern blot of whole brain total RNA to check specificity.

### 8.3. RESULTS

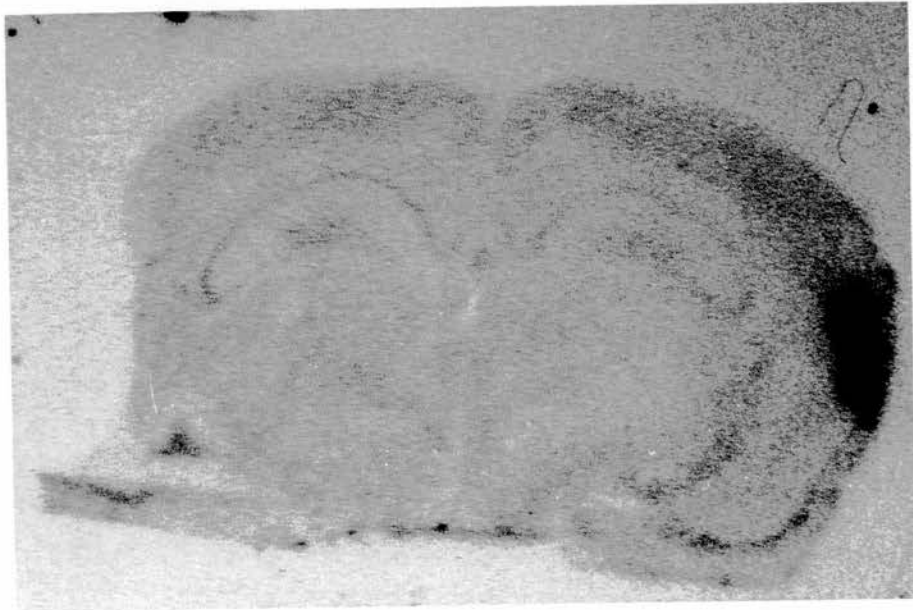
#### 8.3.1. Specificity of the c-jun probe

The c-jun cDNA probe hybridised to two bands of approximately 2.6-2.7 and 3.4kb in length on a Northern blot of total brain mRNA as previously described (Figure 8.1; Ryder et al., 1988). These two transcripts are thought to represent primary unspliced (3.4kb) and mature (2.6-2.7kb) transcripts. A comparison of the distributions of jun-c and jun D in rat brain at the level of the hippocampus by in situ hybridisation revealed a differential distribution of the mRNAs recognised by these two probes which agreed closely with that previously published (Mellstrom et al., 1991), with basal c-jun expression being very low, if not undetectable in all areas of normal brain except hippocampus, and jun D being much more widely expressed in hippocampus, cortex, and many thalamic and hypothalamic nuclei as shown in Figure 8.2.

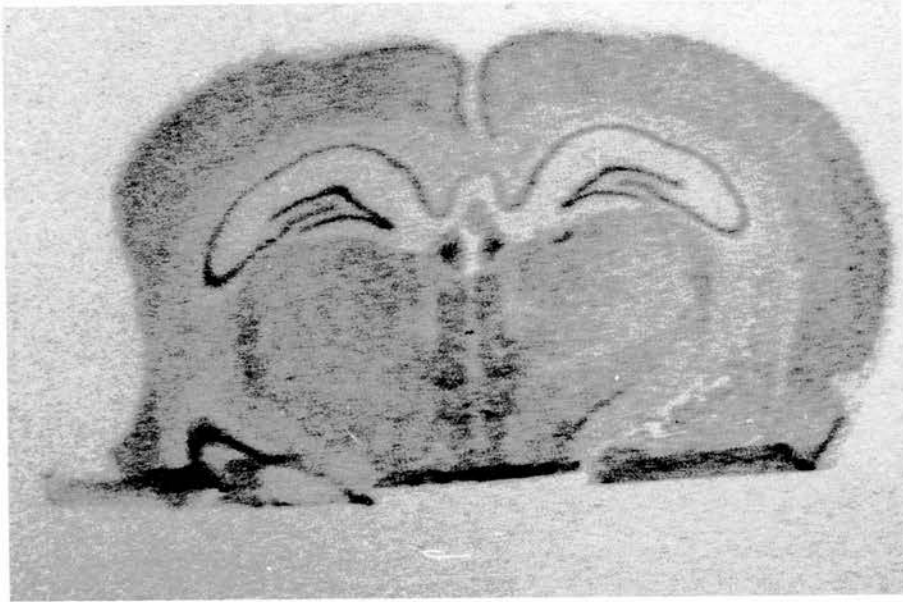


*Figure 8.1. Northern blot of whole rat brain mRNA hybridised with <sup>32</sup>P-l labelled cDNA subclone fragment described in Section 2.*

A)



B)



*Figure 8.2. Autoradiographs of coronal rat brain sections through anterior hippocampus hybridised with A) c-jun cRNA probe and B) jun D cRNA showing the differential distributions of these transcription factors in brain.*

### 8.3.2. Expression of transcription factors in positive control sections

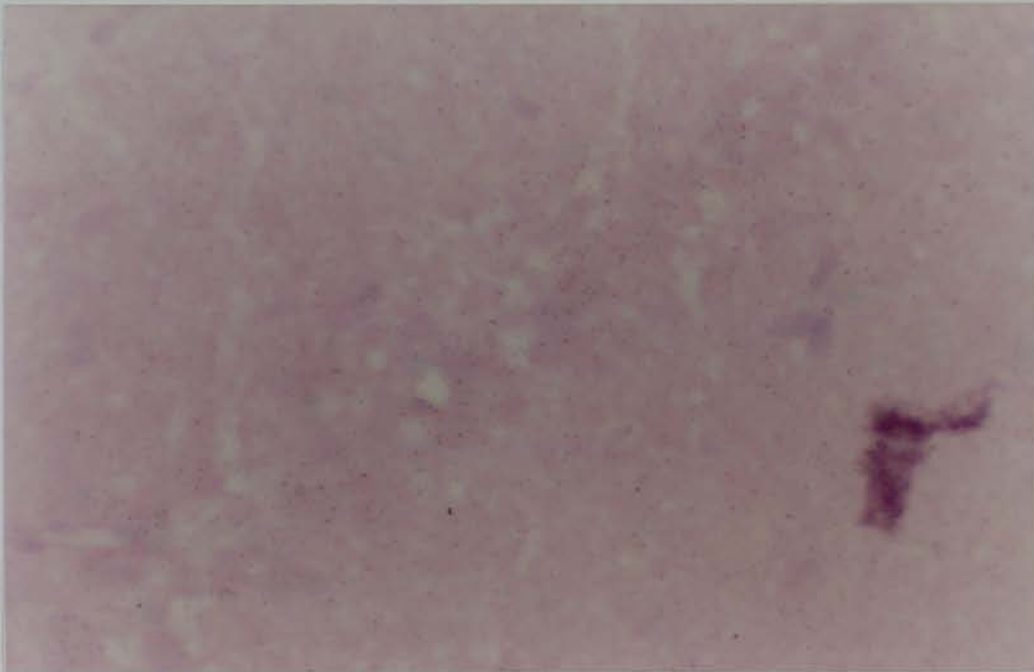
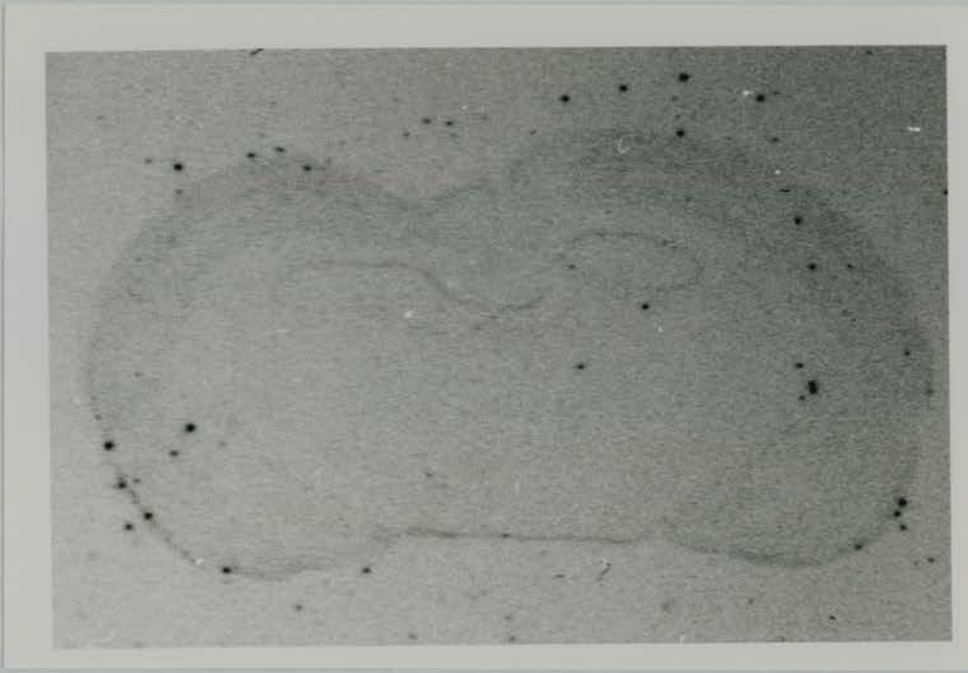
All other transcription factors studied were basally expressed in normal hippocampus agreeing with the previous literature for NGFI-A and -B (Figures 8.3 and 8.4). AP-2 was also found to be basally expressed at low levels in hippocampus and this represents the first description of this expression (Figure 8.5).

### 8.3.3. Expression of NGFI-A, -B and c-jun in DRG neurons

NGFI-A, NGFI-B and c-jun mRNAs were not expressed in either DRG neurons from normal animals, or at any point following adjuvant injection.

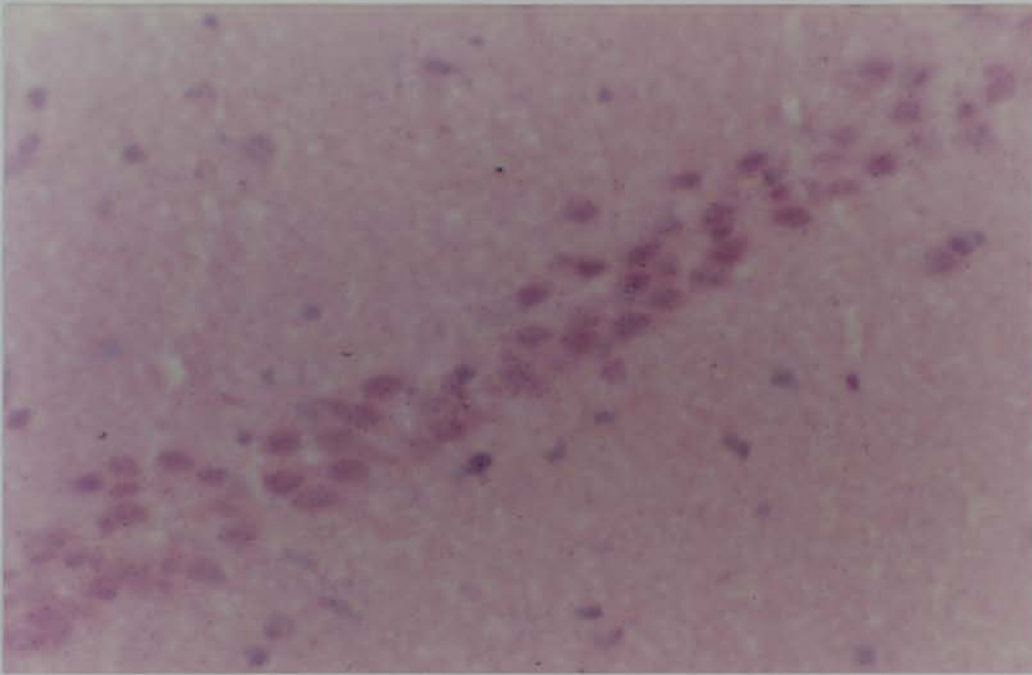
### 8.3.4. Expression of AP-2 in DRG neurons

AP-2 mRNA was constitutively expressed in all DRG neurons irrespective of size. When expression of AP-2 mRNA was assessed in DRG neurons of diameter less than 30 $\mu$ m in normal animals, it was found to be similar to the basal expression of AP-2 mRNA in control hippocampus CA1 neurons. In DRG neurons in animals with adjuvant injection, the expression of AP-2 mRNA was found to be altered in left (injected side) DRG while not significantly different from controls in right (uninjected side) DRG. The changes in expression of AP-2 mRNA following adjuvant injection are shown in Figures 8.6 and 8.7.

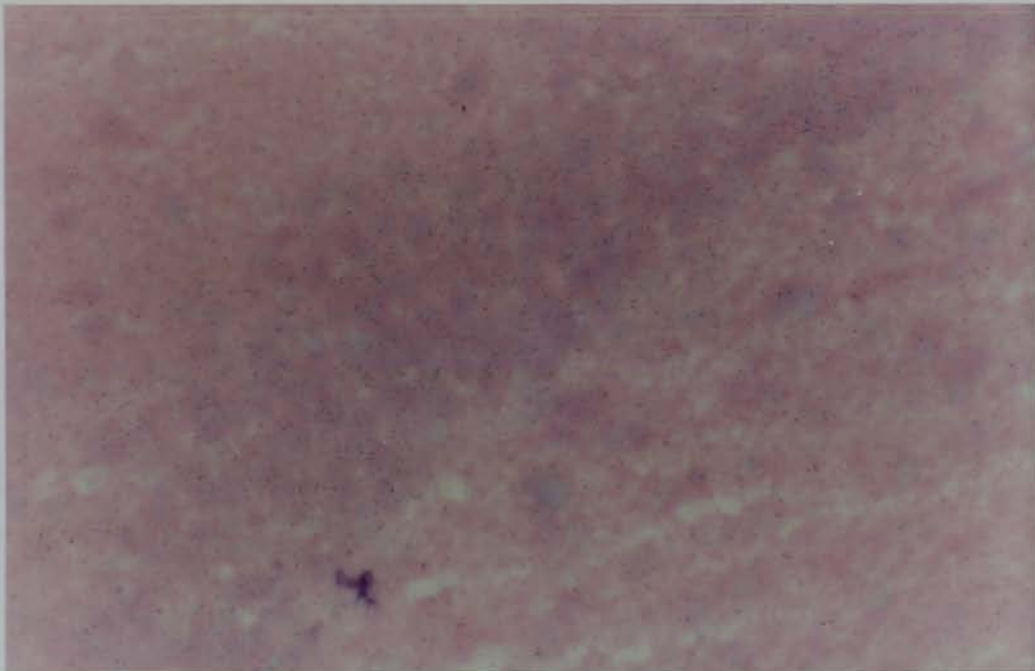


*Figure 8.3. Basal expression of NGFI-A mRNA in rat hippocampus. NGFI-A mRNA is shown in hippocampus by film autoradiography (top) and microautoradiography (bottom) showing higher silver grain density overlying the CA1 region of the hippocampus.*





*Figure 8.4. Basal expression of NGFI-B mRNA in rat hippocampus. NGFI-B mRNA is shown in hippocampus by film autoradiography (top) and microautoradiography (bottom) showing higher silver grain density overlying the CA1 region of the hippocampus.*



*Figure 8.5. Basal expression of AP-2 mRNA in rat hippocampus. AP-2 mRNA is shown in hippocampus by film autoradiography (top) and microautoradiography (bottom) showing higher silver grain density overlying the CA1 region of the hippocampus.*

Figure 8.6

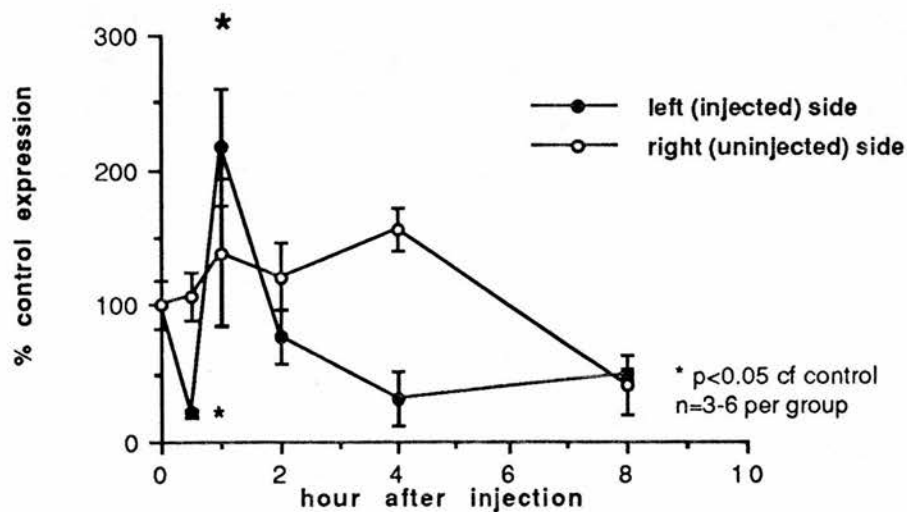
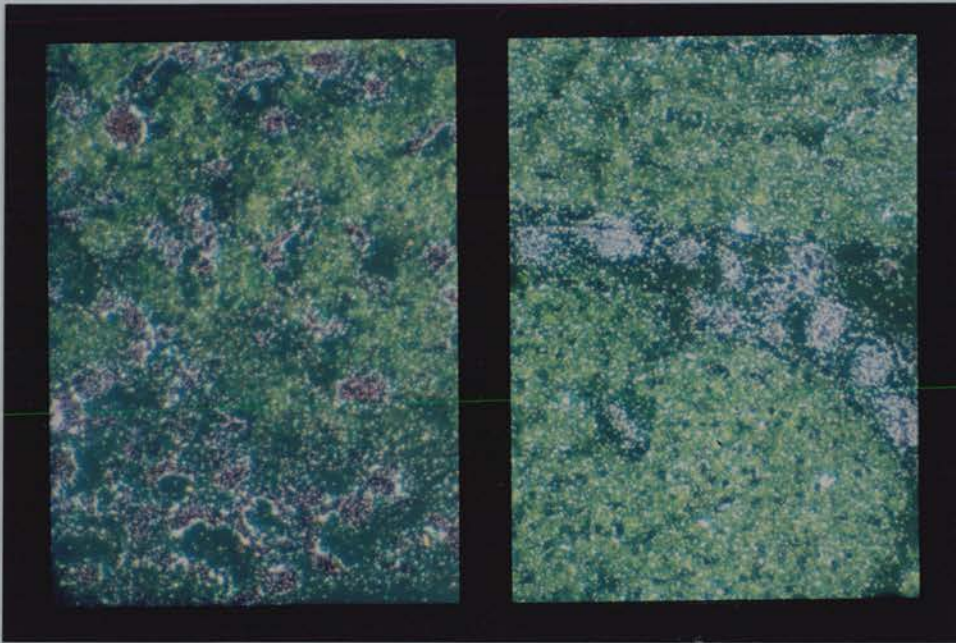
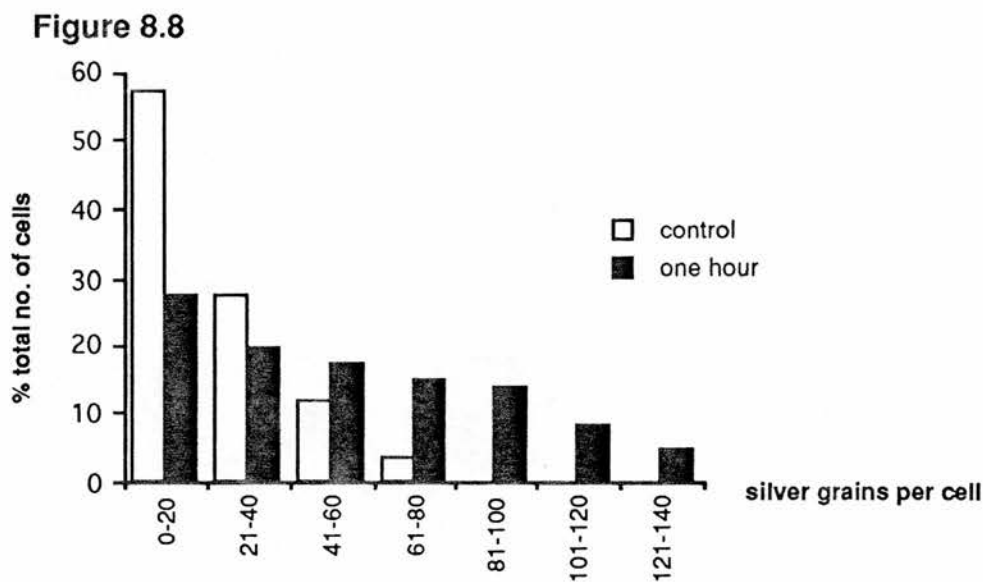


Figure 8.6. Expression of AP-2 mRNA during the first eight hours after adjuvant injection around the left tibio-tarsal joint. AP-2 mRNA shows a significant reduction in expression after 30 minutes and a significant increase in expression after 1 hour, returning to control values at 2 hours. Mean control grain counts were  $30 \pm 5$  per small cell (diameter  $< 30 \mu\text{m}$ ).



*Figure 8.7. Dark field photomicrographs showing expression of AP2 mRNA in DRG neurons from control (left) and adjuvant injected rats (1 hour; right). Original magnification x40.*

The populations of small DRG neurons expressing AP2 mRNA in DRG from control rats and 1 hour after adjuvant injection are illustrated in Figure 8.8. It can be seen that the majority of DRG neurons in control DRG express very low amounts of AP2 mRNA (open bars). One hour after ipsilateral adjuvant injection, this population is altered, showing a reduction in the numbers of cells expressing low amounts of AP2 mRNA and the appearance many more cells expressing much higher amounts of AP2 mRNA.



*Figure 8.8. Populations of small DRG neurons expressing AP2 mRNA in control DRG and 1 hour after ipsilateral adjuvant injection.*

## 8.4. DISCUSSION

### 8.4.1. Expression of c-jun

The subclone for c-jun, taken from the 3' untranslated region of the c-jun cDNA was hybridised to both normal brain sections and total rat brain mRNA Northern blot. On the basis of the differential distributions of c-jun and jun D assessed by in situ hybridisation, this probe appears to be specific for c-jun mRNA; however this still needs to be confirmed by Northern blot analysis of total brain mRNA for jun D and jun B, to confirm that cross-hybridisation is not occurring within members of the jun family. It has been shown here that c-jun mRNA as detected with this probe is not expressed under basal conditions or during the development of inflammation agreeing with previous workers who failed to show induction of c-fos or c-jun mRNA or immunoreactivity following noxious thermal or electrical nerve stimulation (Jenkins and Hunt, 1991). A low basal level of Jun-like immunoreactivity has been seen in DRG (<5% cells), although no c-jun mRNA was evident. c-jun mRNA has been shown to be induced in DRG neurons on axotomy (Jenkins and Hunt, 1991) where it does not act as a typical immediate early gene, but shows long term increases in both mRNA and protein expression over the seven days of this study. The increase in c-jun on axotomy has suggested a role in axonal regeneration as it is not expressed in situations where axonal growth was prevented (Jenkins et al., 1993). Axotomised sensory and motoneurons show increased expression of galanin and CGRP respectively, which are both colocalised with c-Jun protein under these conditions (Herdegen et al., 1993). This is suggestive of a role for c-jun in the regulation of these peptides. However, CGRP in motoneurons is also increased following axonal transport blockade with vinblastine (Kato et al., 1992) suggesting that at least in motoneurons retrograde transport of some factor acts as a

repressor of CGRP expression. This indicates that the regulation of CGRP in motoneurons is distinct from sensory neurons and therefore c-jun may not influence CGRP expression in sensory neurons as indicated by the results presented here. However, PPT and somatostatin are known to have active AP-1 sites in their 5' flanking regions (Quinn, 1991; Goodman, 1990) and therefore may be regulated by AP-1 in DRG. Only one member of the jun family has been examined here, further experiments to examine the expression of jun D and jun B need to be undertaken. Also, while c-fos does not appear to be expressed in DRG either basally or on electrical stimulation of unmyelinated afferent fibres (Hunt et al., 1987), fos-related antigens may be important in PPT gene regulation under culture conditions (J. Quinn, personal communication). Therefore further experiments to study the expression of fra-1 and fra-2 will also be undertaken. Alternatively it is conceivable that nerve growth factor may be exerting a direct effect on neuropeptide genes to increase expression, as peripheral NGF levels are known to be increased in inflammatory conditions (Andreev et al., 1993) and sciatic nerve NGF content increases in adjuvant-induced inflammation.

#### 8.4.2. Expression of NGF-induced transcription factors on noxious stimulation

The majority of studies investigating the expression of immediate early genes, particularly those which constitute the AP-1 complex, have looked at the response of dorsal horn neurons to noxious peripheral stimulation. In primary sensory neurons, NGFI-A expression has been examined following noxious thermal stimulation and mRNA expression was not found in DRG neurons either before or after stimulation (Jenkins and Hunt, 1991) agreeing with the results presented here. NGFI-B expression has not been studied in primary afferents during noxious stimulation or inflammation, but from these results it would appear to be unimportant in primary afferent responses to nociceptive stimulation.

In spinal cord, expression of numerous immediate early genes has been demonstrated by either in situ hybridisation or immunohistochemistry, including c-fos (Hunt et al., 1987), c-jun, NGFI-A (Herdegen et al., 1991a) and NGFI-B (Wisden et al., 1990). Induction of these genes is seen in superficial dorsal horn laminae on noxious thermal (Wisden et al., 1990) electrical (Herdegen et al., 1991a; Herdegen et al., 1990; Hunt et al., 1987) visceral (DeLeo et al., 1991) surgical (Herdegen et al., 1991b) and arthritic stimuli (Abbadie and Besson, 1992). Electrical stimulation of the sciatic nerve also induces NGFI-A-immunoreactivity in various areas of the brain especially in the parabrachial nucleus, the dorsal hypothalamic nucleus, the lateral habenular nucleus and the amygdala (Herdegen et al., 1990). The expression of Jun B, Jun D and Fos B is sequential following a noxious stimulus suggesting differential gene regulation dependent upon the genes expressed either immediately on stimulation or some hours afterwards (Herdegen et al., 1991a). c-fos expression has been implicated in the regulation of spinal expression of dynorphin, as fos has been colocalised with prodynorphin mRNA in dorsal horn neurons during inflammation (Noguchi et al., 1991), and a functional AP-1 site has been demonstrated in the prodynorphin promoter (Naranjo et al., 1991). The transactivation of the prodynorphin gene appears to be under the control of the descending serotonergic raphe fibres (Lucas et al., 1993).

In the majority of cases the changes seen in dorsal horn immediate early gene expression are ipsilateral to the stimulus (Wisden et al., 1990). However, a priming contralateral stimulus will potentiate both ipsi- and contralateral expression of fos-immunoreactivity to a subsequent stimulus (Leah et al., 1992), and late (4-8 hours after stimulation) contralateral expression of c-fos, NGFI-A, c-jun and jun D has been observed after C-fibre stimulation (Herdegen et al., 1991a; 1991b;). Contralateral expression of jun B and c-fos was in a precise mirror image location to the area showing the heaviest expression on the stimulated side (Herdegen et al.,



1991b). The contralateral activation of dorsal horn neurons may reflect, in part, a mechanism through which neurally mediated contralateral spread of inflammation may occur (see Section 6).

#### 8.4.3. AP-2 mRNA expression

This transcription factor was the only one studied here to show a change in small DRG neurons. The expression of AP-2 mRNA was constitutively low (~30 grains/cell), but this showed a 100% increase in expression after one hour, returning to control levels within 2 hours. The PPT promoter contains two putative non-consensus AP-2 binding sites at positions -78 and -564. AP-2, as an enhancer acts in a position-independent manner (Goodman, 1990), however it remains to be determined whether either of these sites do bind AP-2 and are active. It is also of interest to determine whether AP2 mRNA expression increases in neuropeptide-containing neurons or a different population. Colocalisation studies are planned to address this issue. This study also revealed basal expression of AP-2 in rat hippocampus which has not been described previously.

It must be remembered that these results are preliminary and require further work in order to verify the role of transcription factors in the regulation of these neuropeptides. Other members constituting the AP-1 complex need to be studied to determine their possible roles, and the activity (if any) of the AP-2 sites on the PPT promoter require further study. It is also entirely possible that the changes in AP-2 mRNA levels observed are entirely coincidental and may have no bearing on the changes in neuropeptide gene expression seen in this model of adjuvant-induced inflammation. Nevertheless it is intriguing that changes in AP2 and PPT and CGRP mRNAs all occur in small DRG neurons ipsilateral to the stimulus.

## SECTION 9. DISCUSSION

### 9.1. The immune system and adjuvant-induced arthritis

This thesis has dealt exclusively with the possible nervous system involvement in experimental arthritis, as suggested by observations in both the experimental animal and in clinical rheumatoid arthritis. It is a common oversight of the study of inflammatory processes to ignore any possible contribution of any other system, particularly the immune system. While the data presented here are strongly suggestive of a fundamental role for the peripheral sensory nervous system and the neuropeptides expressed therein in this condition, it is not intended to imply that there is not a large immune component in this disease. Rather, this would suggest an extremely close relationship between the immune and sensory nervous systems in inflammatory processes. The arthritogenicity of *Mycobacterium* has been attributed to activation of T-cell subtypes which cross-react with articular tissues, thus causing joint destruction and amplification of a local immune response (Wooley, 1991). This could arise by a failure to clonally delete self-reactive T-cell populations during thymus development, giving rise to susceptible strains of animals, which do indeed exist (see Section 3). It seems clear that in monoarthritis, the contribution of the nervous system to the maintenance of joint swelling and destruction, while evident (Section 6), is minor compared to that of the immune reaction caused by the local injection of adjuvant. The immune system is of obvious importance in the initiation of classical adjuvant disease, as immunosuppressants will prevent the development of arthritis (Wooley, 1991). It would therefore appear that an intimate reaction between the immune and nervous systems is probably of great importance in the spread of disease.

## 9.2. Contralateral spread of arthritis

In passive transfer experiments, labelled T cells sensitised to *Mycobacterium tuberculosis* were injected into naive animals and their tendency to migrate to joints was examined. Labelled cells were found to be more numerous in the joints of rats injected with cells taken from arthritic rats rather than from controls. However it has proved notoriously difficult to initiate adjuvant arthritis by passive transfer, requiring the use of extremely large numbers of activated cells taken from spleen and not thymus, and the use of a highly inbred strain of rat (Pearson and Wood, 1964). Passive transfer was not possible under any other circumstances, particularly in immunologically normal outbred strains such as that used routinely in the studies presented here. Also, an immunological basis cannot fully explain the precise symmetrical nature of the arthritis in the modified model used throughout these studies as it would be expected that if establishment of arthritis was purely dependent on the production of a T-cell subset sensitised to articular tissues, then other joints would all have equal disease susceptibility, which is not the case. Thus it would appear that the initiation (and spread as these are concurrent in the classical model) of arthritis is not wholly dependent upon immunological phenomena, and requires the participation of an additional system. However, it could suggest that, due to an intimate interaction between the nervous and immune systems, the initiation of overt arthritis could be critically dependent upon both systems. The presence of an articular T-cell reaction could induce susceptibility in a joint, and whether overt arthritis would develop would then depend on the state of activation of the peripheral nervous system and whether, for example, neuropeptides were released into the joint thereby stimulating the initiation of arthritis. Evidence supporting this hypothesis exists in both the clinical and experimental situations, as hemiplegic patients with rheumatoid arthritis can show sparing of the paralysed limb when assessed clinically, but concurrently show very mild histological inflammation

of the "unaffected" denervated symmetrical joint (Veale et al., 1993). As has already been mentioned, a "mild" stimulus of intra-articular injection of inert spheres results in contralateral synovial inflammatory infiltration (Kidd et al., 1989a).

The histological studies presented here do not disprove this hypothesis; light microscopic examination of the joints was performed and sections were scored on the presence or absence of a marked inflammatory infiltrate or actual destruction of synovium or bone. It would be difficult to resolve whether an extremely mild inflammatory infiltrate was present without using markers for these cells and accurately quantitating numbers in experimental and control joints.

The results shown here and the evidence from the literature strongly suggest that, even if there is a dependence on the immune system for the initiation of monoarthritis in this model, there is a fundamental requirement for the peripheral nervous system, particularly the capsaicin-sensitive primary afferent, in the maintenance, and more importantly, the spread of arthritis. This would explain the precise topographical spread of arthritis seen in both this experimental model and clinical rheumatoid arthritis.

### 9.3. The role of primary afferent neuropeptides

The aim of the experiments reported in this thesis was to further elucidate the role of the peptidergic primary afferent in experimental arthritis. Studies of neuropeptide levels in DRG suggested that increased central and peripheral release of these neuropeptides in arthritis may be due to increased production of particularly substance P and CGRP within the primary afferent neuron. However, studies of absolute neuropeptide quantity per ganglion are complicated by the fact that this gives no indication of any change in peptide degradation or rate of axonal transport, both of which could affect the steady state levels of ganglionic neuropeptide. The

results presented here, however, support the hypothesis that peptide increases are due to increased transcription of the genes studied, in response to peripheral inflammation, but cannot conclusively prove this due to limitations of the *in situ* hybridisation technique. This method of analysing mRNA levels, while extremely powerful in its ability to precisely localise study to a single population of cells, measures steady state mRNA and therefore also gives no indication of possible changes in mRNA stability, which could also result in apparently increased mRNA levels. While it is unlikely that this is indeed the case, as accumulating evidence from many laboratories supports the data presented here, it is a disadvantage of the *in situ* hybridisation technique which must be remembered. Future use of intron-specific probes to localise and quantitate unspliced RNA transcripts could resolve this.

It has not been ascertained whether the changes in mRNA reported here are a consequence of the inflammation, an initiating event or even merely coincidental. In the case of the changes observed in monoarthritis, it is likely that the observed events (among many others) result from the peripheral excitation and sensitisation of the primary afferents by the inflammatory stimulus of the injection of adjuvant. This is supported by the result obtained on local anaesthetic nerve block, which clearly inhibited the rise in PPT mRNA and partially blocked the rise in CGRP mRNA, both of which are seen within the first eight hours of the onset of inflammation.

While it has been shown here that contralateral spread of arthritis in this modified model of adjuvant arthritis is critically dependent on the capsaicin-sensitive afferents innervating the joint, it is also evident that the neuropeptides, particularly those translated from the PPT-A and CGRP genes are also of importance. The bilateral changes observed in PPT and CGRP mRNAs in bilateral disease are evident when disease has become symmetrical. Further studies on the time course of bilateral

changes need to be undertaken to determine how contralateral changes are related to the onset of contralateral disease.

It is unlikely that neuropeptides released in the periphery have significant effects on the primary afferent terminals themselves and therefore the neuropeptides produced in primary afferents probably have two main functions; modulation of a) the immune and vascular systems in the periphery and b) the activity of spinal cord neurons. If the hypothesis, outlined above, that the peptidergic primary afferent is crucial for the initiation and maintenance of contralateral arthritis is correct, it could be assumed that while contralateral primary afferent activity may not be increased, the neuropeptide transport to the periphery may be. In addition to determining the relationship of onset of inflammation and increased neuropeptide production, it is also of interest to establish whether there is indeed an increased transport of pro-inflammatory neuropeptides in nerves innervating arthritic joints both ipsilateral and contralateral to the adjuvant injection site.

#### 9.4. The central nervous system

All the studies presented in this thesis have concentrated upon the primary afferent in arthritis and have somewhat neglected the central nervous system, particularly the spinal cord. It is obvious from the work of other laboratories that the spinal cord has a major role in the processing of nociceptive information, but it obviously also affects the contralateral spread of arthritis as seen in this model. Studies are planned which should determine whether contralateral neuronal activation occurs through the sensory or sympathetic nervous systems, using neuroanatomical labelling of activated neurons by examining immediate early gene expression as discussed in Section 8.

Furthermore, it is of interest to ascertain whether it is indeed increased release of neuropeptides in the dorsal horn which mediate the contralateral spread of arthritis. Further studies are planned which will utilise intrathecal neutralisation of neuropeptides using either immunological or antagonist approaches, to determine the importance of release of these neuropeptides in this condition.

#### 9.5. The relevance of the observations to arthritis.

The joints from rats with adjuvant-induced arthritis show, in addition to joint involvement, both gross and histological evidence of dermal inflammation. It is impossible to state conclusively that all the neurons showing changes in neuropeptide content (Smith et al., 1992) or mRNAs (present data) innervate articular structures. Indeed some evidence exists that the observations of neuropeptide mRNA levels presented here may be common to all inflammatory states rather than specific to arthritis (Nahin and Byers, 1992). It is likely that the apparent relationship between peptidergic afferents and spread of disease is arthritis-specific; there are no reports of, for instance, dermal inflammation spontaneously involving a symmetrical site. One method of overcoming this problem of the specificity of the changes to the disease state would be to retrogradely label neurons innervating the joint by intra-articular injection of a tracer which was specific for either capsaicin-sensitive neurons (Nagy et al., 1992) or unmyelinated afferents. This would enable mRNA changes only in identified articular afferents to be studied. However, this regime would probably require the development of another model using a different joint, probably the knee, as intra-articular injection into the tibio-tarsal joint is notoriously difficult due to both the small size of the joint and its complex nature which results in there being no large defined joint space into which to inject drugs etc.

## 9.6. The regulation of primary afferent neuropeptide gene expression *in vivo*

As already stated, gene regulation is usually studied under culture conditions as this facilitates dissection of the regulatory mechanisms. The studies presented here on glucocorticoid regulation of the neuropeptides under investigation highlights the problems of addressing this *in vivo*, as this pharmacological manipulation can have profound effects upon the whole animal which may affect the gene under study by some complex mechanism not directly attributable to the manipulation itself. It seems from the literature that cAMP may be of importance in the regulation of these genes in inflammation, as many hyperalgesic states particularly those involving arachadonic acid metabolites seem to be cAMP-dependent.

Possible regulation by transcription factors has, so far proved inconclusive. While AP-2 mRNA was altered in small DRG neurons it remains to be determined whether this effect is a) specific to these peptidergic neurons or generally in DRG neurons during inflammation, b) whether AP-2 expression is correlated in any way with any of the neuropeptides under study here or whether it is involved in the regulation of additional genes and c) whether the putative AP-2 sites in the PPT promoter region are active.

Therefore there are numerous studies which need to be performed in order to reach any definite conclusions on the *in vivo* regulation of these neuropeptide genes.



## SECTION 10. CONCLUSIONS

A novel model of adjuvant-induced inflammatory arthritis which can be easily manipulated to produce either monoarthritis or disease involving both tibio-tarsal joints has been characterised for the study of peptidergic primary afferent involvement in inflammatory arthritis.

Using this model it has been determined that synthetic substance P has no significant role in the activation or sensitisation of unmyelinated articular nociceptors in this condition.

The primary afferent neuropeptide gene responses to inflammation have been examined, and it has been shown that the mRNAs encoding PPT and CGRP in DRG neurons show extremely large, rapid rises in expression in response to adjuvant-induced monoarthritis. Bilateral arthritis is also associated with bilateral rises in expression of these mRNAs in DRG. Somatostatin mRNA is also increased in mono- and bilateral arthritis but only in DRG ipsilateral to the initial adjuvant injection. VIP appears to have no role in inflammation as induction of this mRNA was never seen.

Specific neurotoxic lesions of capsaicin-sensitive primary afferents have defined a fundamental role for this component of the peripheral sensory nervous system and the neuropeptides expressed therein in the contralateral spread of arthritis seen in this model.

Preliminary investigations on the *in vivo* regulation of these neuropeptides during inflammation have indicated that the mechanisms underlying the changes seen are extremely complex, but are in part determined by the interaction of the effects of changes in neuronal activity, circulating corticosteroids, and may be mediated through certain transcription factors.

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## **APPENDIX 1. PUBLICATIONS ARISING FROM THIS THESIS**

BRESM 70511

## Increased expression of preprotachykinin, calcitonin gene-related peptide, but not vasoactive intestinal peptide messenger RNA in dorsal root ganglia during the development of adjuvant monoarthritis in the rat

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**Key words:** Preprotachykinin; Substance P; Calcitonin gene-related peptide; Vasoactive intestinal polypeptide; Adjuvant monoarthritis; Inflammation; Dorsal root ganglia; In situ hybridization; Gene expression

Neuropeptides in dorsal root ganglia (DRG) have been implicated in the pathogenesis of pain and neurogenic inflammation in experimental and clinical arthritis. Recently we demonstrated increased levels of substance P (SP) and calcitonin gene-related peptide (CGRP) confined to innervating DRG in adjuvant-mediated monoarthritis. We have now investigated whether changes in peptide content are reflected in altered neuropeptide gene expression and the time course involved. Using in situ hybridization we found marked increases in expression of  $\beta$ -preprotachykinin (PPT;  $81 \pm 24\%$  rise) and  $\alpha$ -CGRP ( $44 \pm 6\%$  rise) mRNAs in innervating (ipsilateral L<sub>5</sub>) DRG neurones only. These increases occurred at the onset of acute inflammation (8 h) and persisted until chronic arthritis developed after 14 days. There were no changes in the proportion of DRG neurones expressing PPT or CGRP mRNAs. Messenger RNA encoding vasoactive intestinal polypeptide (VIP) was not induced. These data suggest that increased synthesis of PPT and CGRP peptides in DRG may play a role in the pathogenesis both of adjuvant-mediated acute inflammation and chronic arthritis.

### INTRODUCTION

Much evidence suggests a role for the nervous system in the pathogenesis and maintenance of experimental and clinical arthritis. Joints are innervated by primary afferents whose cell bodies are located in dorsal root ganglia (DRG). These neurones synthesize a variety of neuropeptide neurotransmitters, many of which have been implicated in nociception and neurogenic inflammation. Section of primary afferents or neonatal treatment with capsaicin (which destroys 90% of unmyelinated primary afferents) attenuates the severity of adjuvant arthritis<sup>14,5</sup>, reducing joint swelling and raising nociceptive thresholds to normal levels. In both experimental and clinical arthritis the synovial innervation and synovial fluid neuropeptide concentrations are altered<sup>20,27,26,43</sup>. Furthermore denervation (e.g. due to hemiplegia or poliomyelitis) protects joints from rheumatoid arthritis<sup>42,8</sup>.

Of DRG neuropeptides thought important in arthritis, much emphasis has been placed on the role of the tachykinins, particularly substance P (SP) which mediates the flare and wheal reaction in neurogenic inflammation, and calcitonin gene-related peptide (CGRP), with which SP is colocalised in a subpopulation of DRG neurones<sup>15,40</sup>. SP is translated from all transcripts of the preprotachykinin (PPT) A gene, some of which also encode neurokinin A<sup>12</sup>. SP-immunoreactive fibres project from DRG to both the dorsal horn and synovium, and SP causes plasma extravasation on peripheral release in the skin<sup>7</sup>. Following development of arthritis tachykinin levels are increased in primary afferents and DRG neurones innervating affected joints<sup>21,41</sup>. SP is released in the dorsal horn in polyarthritic rats and, when administered intrathecally, elicits a behavioural response suggestive of pain<sup>38,39</sup>. Infusion of SP into knee joints leads to increased inflammation and joint destruction<sup>23</sup>.

CGRP is expressed in DRG neurones and is thought to modulate the actions of SP. CGRP levels also increase in DRG and spinal dorsal horn after adjuvant injection<sup>15,18,30,41</sup>. Intrathecal administration of CGRP lowers nociceptive thresholds to mechanical stimuli and enhances release of SP. Immunoneutralisation of endogenous CGRP, either intrathecally or systemically, attenuates hyperalgesia and inflammation in arthritis<sup>19,24</sup>. CGRP is also a potent vasodilator<sup>4,25</sup> and may contribute to the peripheral changes seen in neurogenic inflammation both directly and by potentiating SP-mediated increases in capillary permeability<sup>6</sup>.

A number of previous investigations have demonstrated increased DRG content of neuropeptides in adjuvant-mediated polyarthritis, but their interpretation is complicated by the severe and widespread systemic disease in these models<sup>35,36</sup>. We have recently developed a rat model of mild adjuvant monoarthritis with inflammation and arthritis confined to one joint and no obvious systemic disturbance<sup>9,41</sup>. Using this model, we found increased SP and CGRP content in innervating DRG, but not in other ipsilateral or any contralateral (unaffected) DRG, suggesting the peptide content changes were specific for the affected joint. However, the radioimmunoassay methods employed in this and other studies are unable to distinguish whether increased synthesis, decreased axonal transport and/or peripheral release underlie the increase in DRG peptide levels. We have therefore examined expression of mRNA encoding sensory neuropeptides in DRG in monoarthritis. In addition we have examined DRG expression of mRNA encoding vasoactive intestinal peptide (VIP). VIP shows very low or absent expression in DRG under basal conditions but its expression is dramatically increased following axotomy, crush injury or even electrical activation<sup>3,15,31,29,1</sup>. As VIP is known to modulate the actions of SP on the vasculature in neurogenic inflammation in skin<sup>16</sup>, we investigated its biosynthesis in experimental monoarthritis.

## MATERIALS AND METHODS

### Induction of arthritis

Monoarthritis was induced in male Wistar rats (200–250 g) by a total intradermal injection of 0.15 ml Freund's Complete Adjuvant (FCA; 1 mg/ml, Sigma) at two sites around the left tarsal joint, under halothane anaesthesia, and the animals allowed to recover<sup>41</sup>. The appearance and circumference of both ankle joints was recorded at intervals to assess inflammation. At various times after FCA injection, animals ( $n = 3-5$  per time point) were killed by decapitation and both left and right L<sub>5</sub> DRG (which innervate the tarsal joint via the sensory component of the sciatic nerve) and the left L<sub>1</sub> DRG (non-sciatic distribution) were rapidly dissected, snap frozen and stored at  $-80^{\circ}\text{C}$ . Sections (10  $\mu\text{m}$ ) were thaw-mounted on gelatin and poly-L-lysine-coated slides and stored at  $-80^{\circ}\text{C}$ . Control L<sub>5</sub> DRG from untreated animals ( $n = 10$ ) and animals treated with filter-sterilised paraffin oil ( $n = 3$ ) were similarly taken (acute and

chronic inflammatory changes are seen after injection of Freund's incomplete adjuvant<sup>41</sup>).

### Synthesis of radiolabelled cRNA probes

Probes were transcribed *in vitro* from plasmid constructs containing cDNAs encoding  $\beta\text{PPT}$  (441 bp full-length cDNA cloned in pGEM3<sup>12</sup>),  $\alpha\text{-CGRP}$  (450 bp fragment encoding the 3' untranslated sequence cloned into pSP64<sup>2</sup>) and VIP (350 bp EcoRI fragment in pSP64). <sup>35</sup>S-labelled cRNA antisense probes were transcribed *in vitro* using SP6 polymerase (Gibco BRL, UK), [<sup>35</sup>S]UTP (800 Ci/mmol, Amersham Int., UK) and unlabelled UTP to a specific activity of  $3-5 \times 10^8$  Ci/mmol.

### *In situ* hybridisation

Sections were postfixed in 4% paraformaldehyde in 0.1 M phosphate buffered saline solution for 10 min and rinsed three times in  $2 \times \text{SSC}$ . All solutions were treated with diethylpyrocarbonate (0.02%). <sup>35</sup>S-labelled cRNA probes were denatured by heating at  $70^{\circ}\text{C}$  and added to hybridisation mix (50% deionised formamide, 0.6 M NaCl, 10 mM Tris-Cl (pH 7.5), 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.1% BSA, 1 mM EDTA, 0.1 mg denatured salmon sperm DNA, 0.05 mg/ml total yeast RNA, 0.05 mg/ml yeast tRNA, 10% dextran sulphate and 10 mM dithiothreitol) to give  $10 \times 10^6$  counts/ml. Hybridisation mix (70  $\mu\text{l}$ ) was added to each slide which was then coverslipped and sealed with DPX. Hybridisation was carried out overnight in sealed humid chambers at  $50^{\circ}\text{C}$ . After hybridisation slides were soaked in  $2 \times \text{SSC}$  to remove coverslips, rinsed in  $2 \times \text{SSC}$  and treated with RNase A (30  $\mu\text{g}/\text{ml}$ ) for 60 min at  $37^{\circ}\text{C}$ . Washes consisted of  $2 \times \text{SSC}$  at room temperature and  $0.1 \times \text{SSC}$  at  $50^{\circ}\text{C}$  for 60 min. Sections were then dehydrated in graded ethanols in 0.3 M sodium acetate, air dried and dipped in K5 nuclear emulsion (Ilford, UK). Sections were exposed at  $4^{\circ}\text{C}$  for 2 weeks, developed and counterstained with haematoxylin and eosin. Control sections were pretreated with RNase A (100  $\mu\text{g}/\text{ml}$ ) for 60 min at  $37^{\circ}\text{C}$  prior to hybridisation. Expression of mRNA was estimated by counting silver grains overlying neuronal cell bodies of less than 30  $\mu\text{m}$  diameter. For each animal, four representative cells in each of three separate sections of each DRG were counted. Left L<sub>5</sub> DRG from animals injected with FCA were compared to L<sub>5</sub> DRG from (i) the contralateral side and (ii) untreated controls. The percentage of neurones expressing each mRNA in the various treatment groups was also estimated.

### Statistics

Data were assessed by ANOVA followed by paired or unpaired Student's *t*-tests, as appropriate. Significance was defined as  $P < 0.05$ . Values are means  $\pm$  S.E.M.

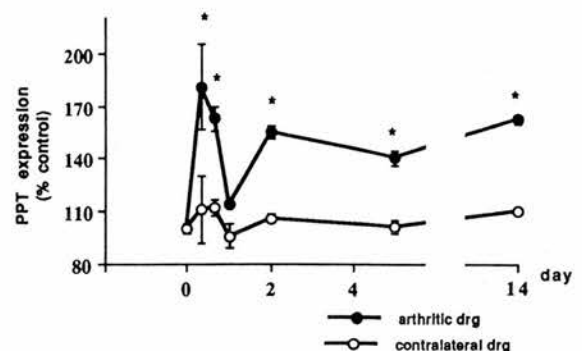


Fig. 1. Expression of  $\beta\text{-PPT}$  mRNA in left (arthritic) and right (control) rat L<sub>5</sub> DRG neurones during development of adjuvant monoarthritis. Values are expressed as % control mean  $\pm$  S.E.M.

\*  $P < 0.05$  compared with control and right DRG.

## RESULTS

Injection of adjuvant resulted in the rapid induction of unilateral inflammation (redness and swelling) within 24 h. This persisted for 4–5 days followed by some resolution, until swelling recurred with signs of chronic monoarthritis at 14 days. No changes in circumference or appearance were observed in the contralateral limb, other joints or in untreated animals. Animals were not severely affected by the arthritis although they tended to favour the affected limb.

In control  $L_5$  DRG PPT mRNA was expressed in

$22 \pm 1\%$  of neurones. No change in the proportion of neurones (diameter  $< 30\mu\text{m}$ ) expressing PPT mRNA was seen at either 8 h or 14 days ( $19 \pm 1\%$ ) after FCA injection, in either left or right  $L_5$  DRG. Eight hours after injection of FCA there was a significant increase ( $81 \pm 24\%$  rise) in PPT mRNA expression per neurone in  $L_5$  DRG innervating the injected joint, compared to either the contralateral (uninjected)  $L_5$  DRG, non-innervating  $L_1$  ( $94 \pm 6\%$  of control  $L_1$ ) DRG or controls (Figs. 1 and 2A,C). The early induction of PPT mRNA expression in innervating  $L_5$  DRG neurones resolved at 24 h, but recurred by 48 h and persisted until

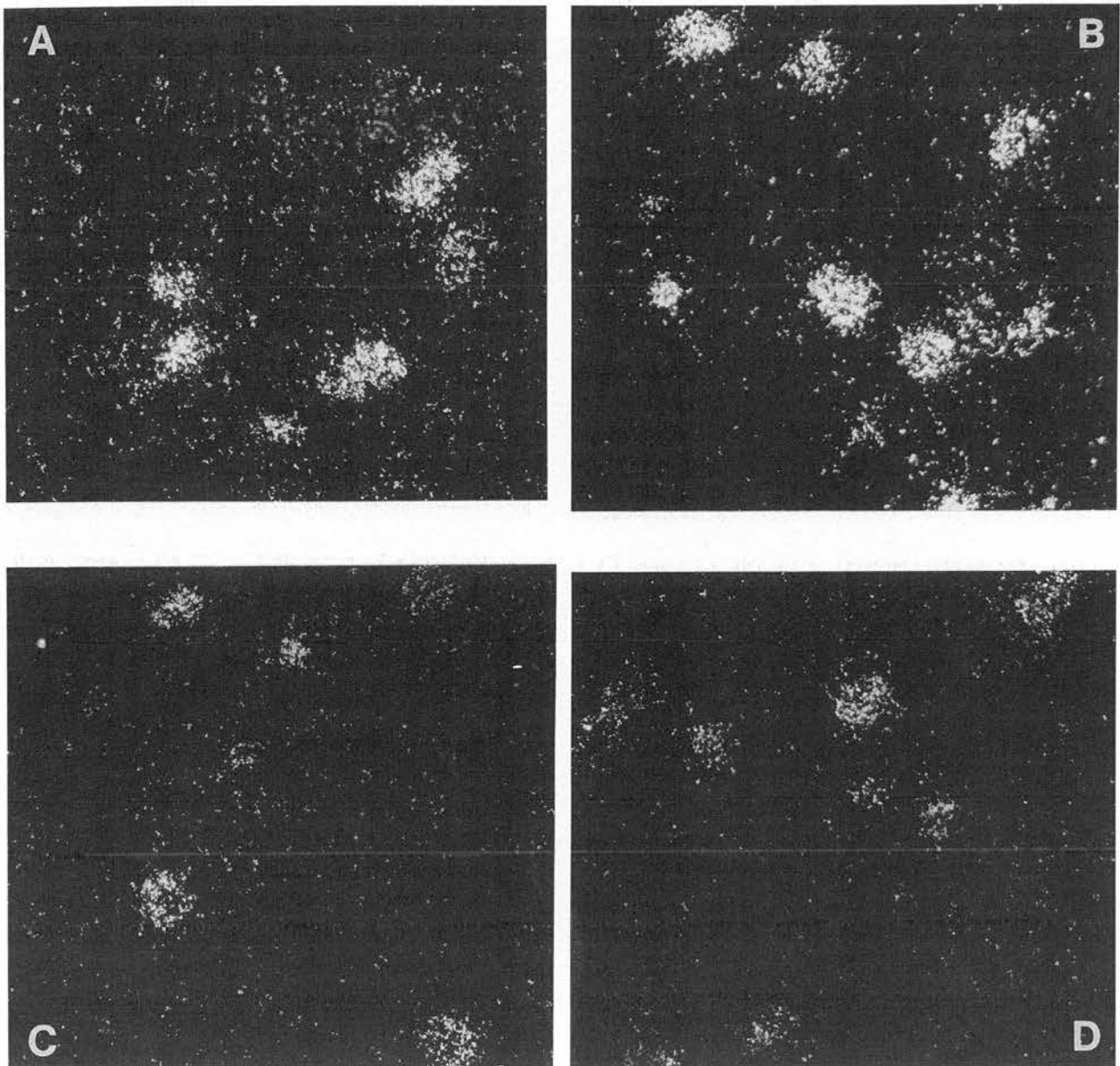


Fig. 2. Dark-field photomicrograph (magnification  $\times 360$ ) showing cells expressing  $\beta$ -PPT mRNA in rat  $L_5$  DRG neurones. A: left (injected side) DRG; 8 h after adjuvant injection. B: left (injected side) DRG; 14 days after adjuvant injection. C: right (contralateral) DRG; 8 h after adjuvant injection. D: right (contralateral) DRG; 14 days after adjuvant injection. Note increased density of silver grains (white) over injected side (left) DRG neurones at both time points.

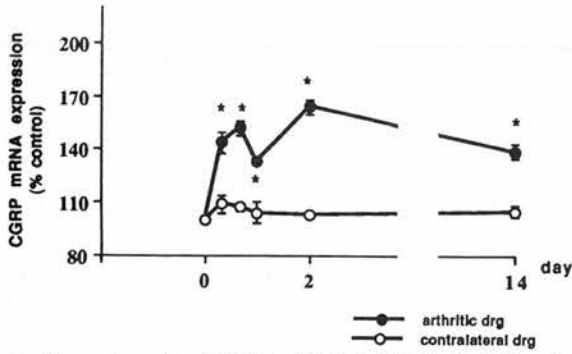


Fig. 3. Expression of  $\alpha$ -CGRP mRNA in left (arthritic) and right (control) rat L<sub>5</sub> DRG neurones against time after adjuvant injection. Values are expressed as % control mean  $\pm$  S.E.M. \*  $P < 0.05$  compared with control and right DRG.

arthritis was evident at 14 days (Figs. 1 and 2B,D); at no time was there any increased expression in the ipsilateral L<sub>1</sub> or contralateral L<sub>5</sub> DRG.

CGRP mRNA was expressed in  $37 \pm 3\%$  of control DRG and there was no significant change in the proportion of L<sub>5</sub> DRG cells expressing CGRP mRNA at the time of onset of chronic arthritis (day 14;  $36 \pm 1\%$ ). As with PPT, the expression of CGRP mRNA per neurone was significantly increased ( $44 \pm 6\%$  rise), 8 h after FCA injection (Figs. 3 and 4A,C). CGRP mRNA levels remained significantly elevated up to and including the time of development of chronic monoarthritis (Figs. 3 and 4B,D). These changes were limited to ipsilateral L<sub>5</sub> DRG.

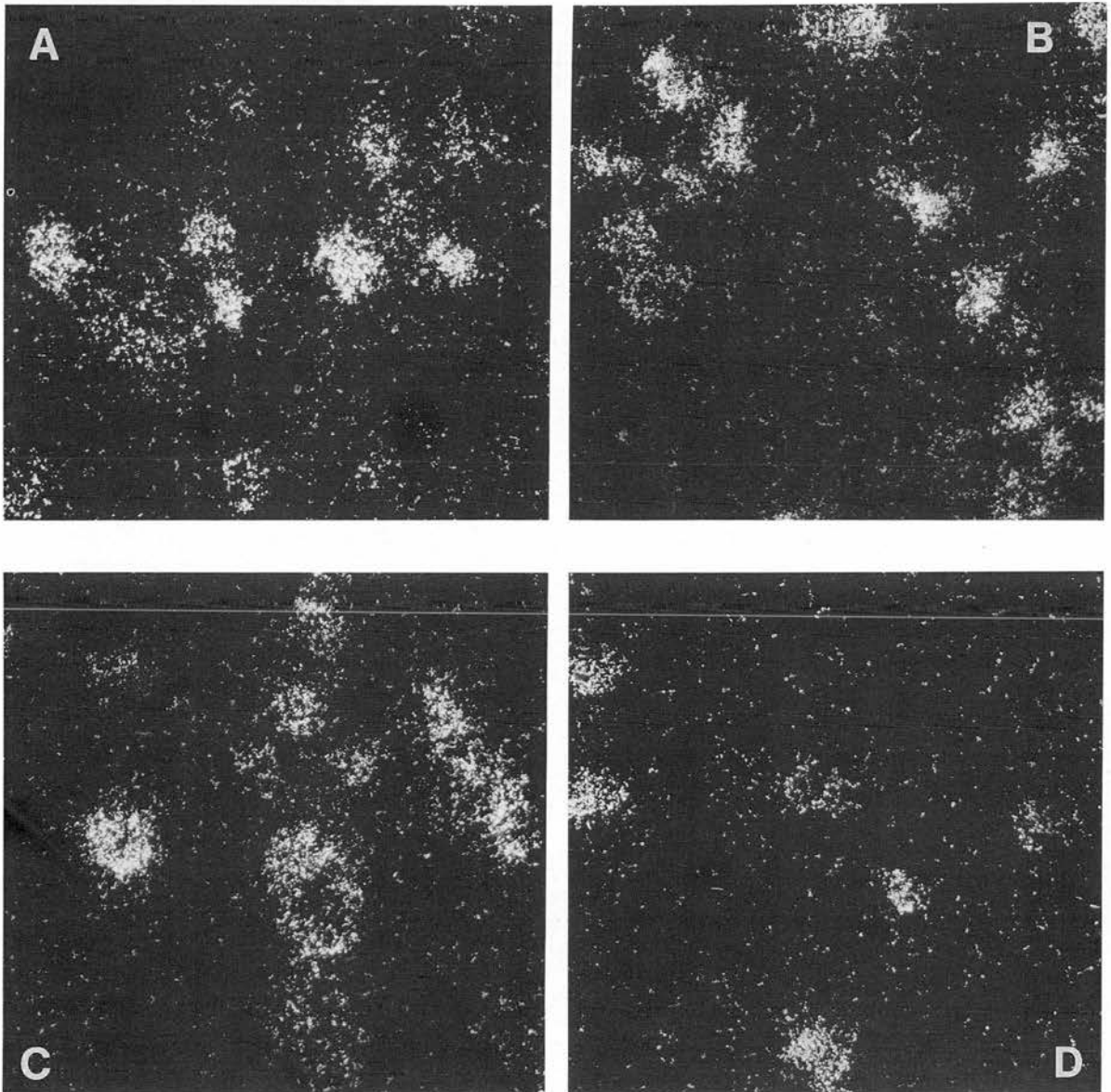


Fig. 4. Dark-field photomicrograph (magnification  $\times 360$ ) showing cells expressing  $\alpha$ -CGRP mRNA in rat L<sub>5</sub> DRG neurones. A: left (injected side) DRG; 8 h after adjuvant injection. B: left (injected side) DRG; 14 days after adjuvant injection. C: right (contralateral) DRG; 8 h after adjuvant injection. D: right (contralateral) DRG; 14 days after adjuvant injection. Note increased density of silver grains (white) over injected side (left) DRG neurones at both time points.

Injection of paraffin oil (vehicle) caused no increase no obvious inflammation and no change in either PPT mRNA or CGRP mRNA expression, 8 h after injection.

At no point during the development of monoarthritis was expression of VIP mRNA detected either in innervating or contralateral L<sub>5</sub> DRG.

## DISCUSSION

These results demonstrate that within 8 h of unilateral injection of a low-dose of adjuvant around the ankle joint there is marked induction of PPT and CGRP mRNA expression in innervating L<sub>5</sub> DRG neurones. This increase in mRNA expression is due directly to the action of FCA as vehicle injection alone produced no such change. The increased gene expression in treated animals persists at least until chronic monoarthritis is established (14 days), but no induction of VIP mRNA expression is found.

Previous studies of neuropeptide gene expression in DRG have shown induction of PPT mRNAs 4 days after bilateral hindpaw injection of adjuvant<sup>28</sup> and within 3 h of unilateral noxious stimulation (formalin injection) of the hindpaw<sup>34</sup>, although the latter largely represented an increased proportion of DRG neurones expressing PPT mRNA rather than any induction in mRNA expression per neurone. Early (~16 h) induction of CGRP mRNA has been shown<sup>11</sup> in motor neurones after axotomy (which causes decreased CGRP mRNA expression in DRG;<sup>32</sup>). We now show rapid (<8 h) induction of PPT mRNA expression localised to innervating DRG, without alteration in the proportion of expressing neurones. The discrepancy between our findings and those of Noguchi et al.<sup>34</sup> may relate to the rather low proportion of neurones expressing PPT mRNA in control DRG (~11%) in the previous study, whereas we and others<sup>13</sup> find ~20% of DRG neurones express PPT mRNA. Alternatively, the more noxious formalin stimulus might recruit an additional population of neurones to express PPT peptides. In parallel with the early induction of PPT mRNA we also found CGRP mRNA induction within 8 h; again this represented an increase in expression per neurone rather than any change in the proportion of neurones expressing the  $\alpha$ -CGRP gene. Our data on the proportion of neurones expressing  $\alpha$ -CGRP agree closely with previous studies<sup>32</sup>.

Increased neuropeptide gene expression was limited to DRG innervating the affected joint, consistent with our previous radioimmunoassay data<sup>41</sup>. These data do not support recent suggestions that unilateral inflammation per se induces both gross and microscopic

contralateral inflammation of neurogenic origin<sup>17,22</sup>. Furthermore, the increased SP- and CGRP-like immunoreactive content of innervating DRG that we have previously demonstrated in monoarthritis<sup>41</sup> is likely to be due, in large part, to induction of biosynthesis of these neuropeptides, although alterations in peptide degradation and/or axonal transport may also occur.

Studies on the time course of articular primary afferent activity during acute arthritis have shown an early induction of resting discharge and response to movement in group III and IV afferents and previously quiescent fibres become responsive within 2–3 h<sup>37</sup>. Thus electrophysiological evidence shows primary afferents become sensitized rapidly following injection of adjuvant. We found PPT and CGRP gene induction in innervating DRG within 8 h of adjuvant administration. Given the substantial pre-existing pools of both CGRP and PPT mRNAs in DRG it is likely that the injection of adjuvant leads directly (i.e. via increased sensory neuronal activity) to induction of expression of the respective genes. It seems unlikely that the changes in neuropeptide gene expression observed are mediated via humoral or other systemic factors secondary to adjuvant injection as (i) the model used here is very mild in comparison to many other polyarthritic models and systemic illness is not a feature at any stage and (ii) gene induction was limited to the innervating DRG. This is suggestive of a causal relationship between the mRNA changes and the inflammatory process. Expression of both peptide mRNAs fell at 24 h, when inflammation is still marked, but thereafter remained elevated until 14 days, at which time histological evidence of monoarthritis becomes manifest (ref. 9 and Donaldson et al., in preparation). Therefore the early changes in PPT and CGRP gene expression cannot be attributable to arthritis, and are most probably associated initially with the inflammation seen around the joint within 24 h of injection, when histological evidence of inflammation is also seen (L. Donaldson et al., unpublished observations).

VIP modulates the actions of SP on the vasculature in cutaneous neurogenic inflammation<sup>16</sup> and is thought to be involved in the reorganisation or regeneration of peripheral nerves as its expression is greatly increased following axotomy<sup>33</sup>. We found little or no VIP mRNA expression in control DRG neurones, confirming previous data<sup>15,33</sup>. Furthermore, we did not find any induction of VIP expression in innervating or contralateral DRG at any stage following adjuvant injection. Thus, although it has been suggested that electrical activity per se induces VIP gene expression in some neurones<sup>1</sup>, since electrical activity increases in sensory afferents,



and previously quiescent afferents are activated, during arthritis<sup>10</sup> it is unlikely that this is regulating VIP gene expression in DRG neurones.

In conclusion, we show for the first time that the response of SP and CGRP mRNAs to low dose adjuvant injection is rapid, marked, specific to the DRG innervating the joint and maintained throughout the development of monoarthritis. There is no evidence to support the involvement of VIP in experimental monoarthritis. It follows from the effects on SP and CGRP mRNA shown here that peptide translation may be increased equally rapidly and maintained throughout the course of the disease, suggesting a role for these peptides in the pathogenesis and maintenance of experimental inflammation and arthritis.

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## **A discrete adjuvant-induced monoarthritis in the rat: effects of adjuvant dose**

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## A discrete adjuvant-induced monoarthritis in the rat: effects of adjuvant dose

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Much evidence suggests an important role for the nervous system in the pathogenesis of peripheral inflammatory conditions such as arthritis. The classical adjuvant-induced arthritis model in the rat is a severe condition in which polyarthritis is accompanied by widespread systemic disease, complicating the interpretation of data. We have developed an adjuvant-induced arthritis of the tibio-tarsal joint in the rat, using a low dose of Freund's adjuvant administered locally. Initial inflammation is followed, after 14 days, by chronic monoarthritis which is maintained without generalised effects or loss of use of the limb until at least 30 days postinjection. A higher dose of adjuvant produces contralateral inflammation and arthritis, but only after 14 days, and without the complicating effects seen in classical adjuvant disease. Indirect measures of arthritis (joint circumference, inflammation score and nociceptive threshold) correlate closely with the histological state of the joint, supporting the use of these indices. This model can be easily exploited by alteration of adjuvant dose, to determine the roles of the nervous system in the pathogenesis, maintenance and symmetrical spread of inflammatory arthritis.

### Introduction

Studies of the cutaneous 'triple response' to injury by Lewis (1942) originally demonstrated the important role of the nervous system in the pathogenesis of peripheral inflammation. Since then much interest has focused on the mechanisms underlying such neurogenic inflammation, generating a plethora of studies at anatomical, biochemical, neurophysiological and, more recently, molecular biological levels.

One of the most frequently exploited models of neurogenic inflammation is adjuvant-induced

arthritis in the rat which involves severe polyarthritis of the tarsal, carpal, phalangeal and spinal joints, accompanied by lesions to the eyes, ears, nose, skin and genitals as well as anorexia and profound weight loss (Pearson and Wood, 1959; Rainsford, 1982). Many studies have demonstrated changes in peripheral and spinal neuronal function and neurotransmitter concentrations in this model (Kuraishi et al., 1989; Ohno et al., 1990; Guilbaud, 1991). However, the widespread and severe disease induced makes it difficult to attribute changes observed to the neurogenic/arthritis processes per se. Moreover, the disease process is erratic with a remitting and relapsing course. Clearly, adaptations of this model which limit disease severity would be of interest not only to reduce animal discomfort but also to demonstrate effects specific to arthritis.

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Unilateral injection of a low dose of adjuvant into rat footpad has been used by other researchers to investigate changes in central (Guilbaud et al., 1985; Hylden et al., 1989) and peripheral sensory neuronal function (Bartho et al., 1990) and spinal cord gene expression (Minami et al., 1989) in response to peripheral inflammation. However, since apparently small alterations in the dose and route of administration of adjuvant appear to produce major differences in the extent of the inflammation produced, we have investigated the effect of increasing doses of heat-killed *Mycobacterium tuberculosis* on inflammation and the histological appearance of the joint in this model. The relationship between indirect measures of inflammation, such as joint swelling and nociceptive threshold, and the histological appearance of the joint has also been investigated to determine whether use of these common indices is valid.

### Materials and methods

Male Wistar rats (200–250 g) were allocated to groups ( $n = 5/\text{group}$ ). Animals, under halothane anaesthesia, were injected subdermally with either paraffin oil vehicle or Freund's complete adjuvant containing 50  $\mu\text{g}$ , 150  $\mu\text{g}$  or 250  $\mu\text{g}$  attenuated *M. tuberculosis* (MAFF, UK) suspended in 0.05 ml of paraffin oil, divided between two injection sites around the left tarsal joint. Controls were untreated. Before induction of arthritis and twice weekly thereafter, the animals were weighed, their left and right tarsal joint circumferences measured, and left tarsal joints scored for inflammation and response to a mild noxious stimulus. Nociceptive threshold was determined by the pressure required to elicit limb withdrawal and was measured either subjectively and scored 0–3 (0 = intense pressure, 3 = slight pressure), or objectively using a pressure gauge and scored 0–8 based on a meter reading (0 = very slight pressure, 8 = intense pressure). Index of inflammation, 0 = normal, 1 = mild redness, 2 = moderate redness and swelling, 3 = severe swelling/lesions over the joint. All measurements were performed blind to treatment by the same experienced operator.

Preliminary histological studies demonstrated chronic monoarthritis 14 days after adjuvant injection. Therefore, 15 days after injection, animals were deeply anaesthetised with sodium pentobarbitone (60 mg/kg i.p.) and perfused through the descending aorta with heparinised saline (500 U/kg, 10 ml) followed by 10% formal saline (~50 ml) until the hind limbs were fixed. Both hind limbs were removed and decalcified in Gooding and Stewart's solution (15% formic acid, 5% formaldehyde in distilled water) for 2 weeks. Tissues were blocked in gelatin and sections (20  $\mu\text{m}$ ) cut by cryostat, mounted and counterstained with haematoxylin and eosin. Each section was scored blind for the presence and degree of joint inflammation, assessed on the following histological features; inflammatory cell infiltrate, synovial proliferation, articular cartilage breakdown and new bone formation and classified as: 0 = normal, 1 = subdermal inflammation, 2 = mild, 3 = moderate and 4 = severe joint inflammation; scores  $\geq 3$  were taken as indicative of arthritis.

Statistical analysis of joint circumferences was by ANOVA followed by post-hoc Dunnett's test. Spearman's rank correlation coefficient was used to determine correlations between the histological arthritis and gross measures of inflammation. Values are means  $\pm$  SEM. Significance was set at  $P < 0.05$ .

### Results

During development of inflammation, animals showed little or no apparent discomfort and locomotor activity was near normal, with only occasional guarding of the affected limb. Only in the group receiving the highest dose of *M. tuberculosis* (250  $\mu\text{g}$ ) was the left hind limb not used for walking, and then only at 14 days postinjection. The normal gain in weight was not affected by any treatment (data not shown). Animals injected with *M. tuberculosis* showed a dose-related increase in left (injected) joint circumference, redness and swelling, and histological inflammation score. Injection of 50  $\mu\text{g}$  *M. tuberculosis* or paraffin oil alone caused slight, non-significant increases in left tarsal joint circumference which

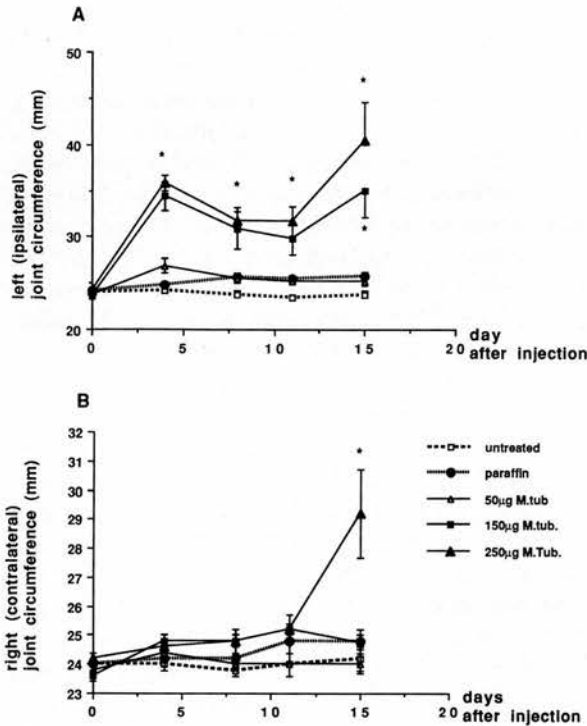


Fig. 1. Effect of dose of adjuvant injected around the left tarsal joint on (A) left (ipsilateral) and (B) right (contralateral) tarsal joint circumference (mm). \*  $P < 0.01$  compared with circumference before injection, untreated and paraffin injected control groups (Dunnett's).

remained constant for 15 days. By contrast, injection of 150  $\mu\text{g}$  or 250  $\mu\text{g}$  *M. tuberculosis* caused acute inflammation and significant swelling of the left tarsal joint within 24 h, reaching an initial peak 4 days postinjection ( $P < 0.01$ ). This initial swelling partially resolved at day 5 (although remaining significantly swollen compared to controls), but increased again 14 days postinjection (Fig. 1A).

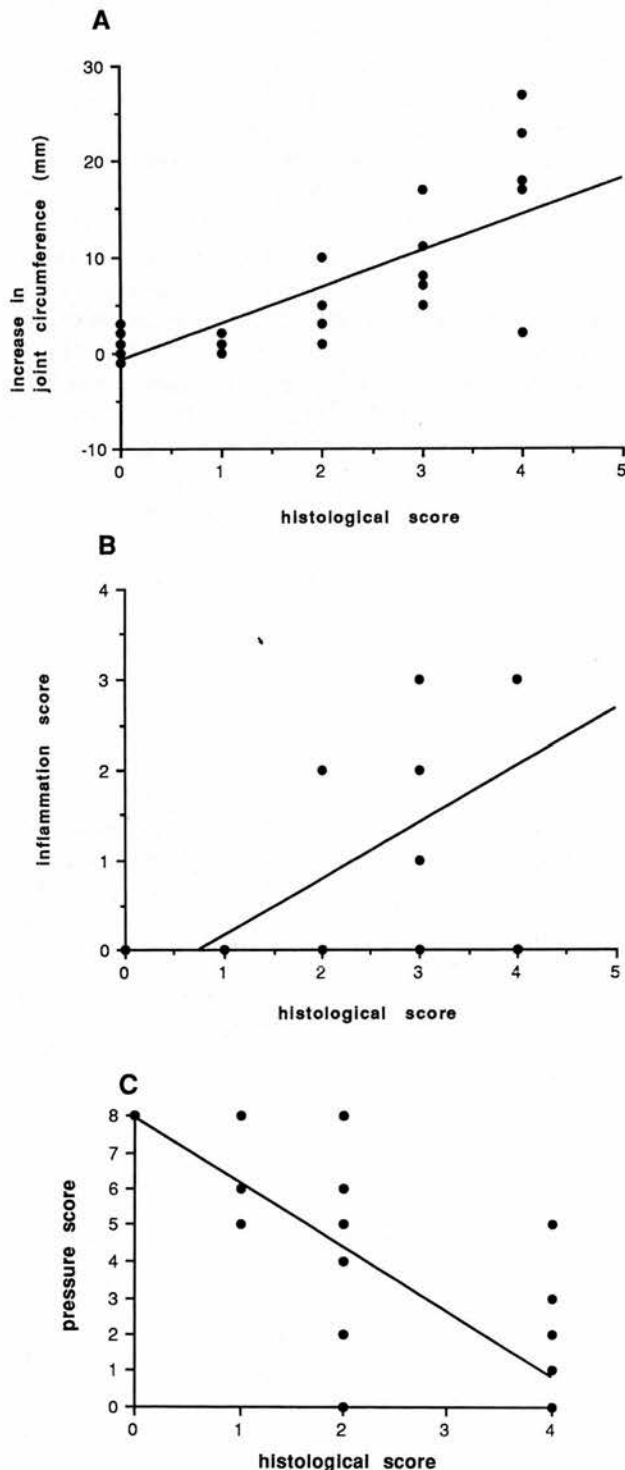
Animals receiving the 250  $\mu\text{g}$  dose showed redness and swelling of the contralateral tarsal joint at 14 days postinjection (Fig. 1B). Those receiving 250  $\mu\text{g}$  also showed mild forepaw swelling, nose and eye inflammation and visceral lesions. Only animals injected with 250  $\mu\text{g}$  of *M. tuberculosis* showed a statistically significant increase in contralateral (right) joint circumference after 14 days ( $P < 0.01$ ) (Fig. 1B).

Animals receiving no treatment, paraffin, or 50  $\mu\text{g}$  *M. tuberculosis* showed no histological evidence of arthritis of the left tarsal joint, although an inflammatory infiltrate in overlying subcutaneous tissues was evident in the latter group. Histological features of arthritis were seen in the left tarsal joints of the animals receiving 150 and 250  $\mu\text{g}$  *M. tuberculosis*. Animals receiving 250  $\mu\text{g}$  showed a significant arthritis of the contralateral tarsal joint. There was a significant correlation (Spearman rank correlation coefficient) between the histological score and (i) the increase in joint circumference ( $n = 37$ ,  $r = 0.71$ ,  $P < 0.0005$ ) (Fig. 2A) and (ii) the inflammation score ( $n = 25$ ,  $r = 0.86$ ,  $P < 0.0005$ ) (Fig. 2B). The response to a subjectively scored mild noxious stimulus (squeeze) did not correlate significantly with joint histology ( $n = 23$ ,  $r = 0.36$ ,  $P > 0.05$ ; not shown), whereas when this parameter was subsequently assessed objectively, a highly significant correlation was seen ( $n = 30$ ,  $r = -0.699$ ,  $P < 0.0001$ ) (Fig. 2C).

## Discussion

Most models of inflammatory arthritis involve lesions in multiple joints, including spontaneous arthritis in MRL/lpr mice, arthritis induced by antigens (adjuvant, streptococcal cell wall, type-II collagen) or by overproduction of tumour necrosis factor in transgenic mice (Keffer et al., 1991); all tend to exhibit similar incidences, time courses and immune reactions to the antigen involved (Magilavy, 1990). Very few models of chronic monoarthritis have been described; indeed the validity of monoarthritic models has been questioned since many human arthritides are noted for their symmetry (Kozin et al., 1976) which is a diagnostic criterion for rheumatoid arthritis in the United States (Mitchell and Fries, 1982). Nevertheless, monoarthritic models have yielded much useful data on mechanisms specific to the arthritic/inflammatory process and its spread, and clinical inflammation and arthritis may be asymmetrical or confined to a single joint.

We now demonstrate a rat model of arthritis in which the induction and, more particularly,



spread of arthritis is dependent on the dose of adjuvant used; vehicle and sub-threshold doses of adjuvant produce only minimal local inflammation without arthritis, intermediate doses of adjuvant ( $150 \mu\text{g}$  *M. tuberculosis*) cause a rapid initial local inflammatory response which is followed, after 2 weeks, by histological evidence of chronic monoarthritis, and higher doses ( $\geq 250 \mu\text{g}$  *M. tuberculosis*) result in much more widespread inflammation. These increases in dose of *M. tuberculosis* lead to localised initial inflammation followed by bilateral or more widespread arthritis by 14 days, suggesting that when unilateral neural activation exceeds a 'threshold', contralateral nerves are excited and may exert efferent as well as afferent effects sufficient to induce inflammatory and destructive changes. This model might be used to define the respective roles of neural and immune systems in the development and spread of inflammatory arthritis. Thus, use of the higher ( $250 \mu\text{g}$ ) dose will allow study of the role of neuropeptide genes and their products in symmetrically spreading disease, away from the site of local immune stimulation. Similarly, the effects of lesions (chemical, physical) or pharmacological manipulation of peripheral nerves on the development and spread of arthritis can be investigated. The contribution of neurogenic inflammation to the inflammatory state has been previously investigated using total sympathectomy or extensive surgical lesions (Levine et al., 1985, 1986), but our model has the advantage that more specific lesions may be performed without compromising the well-being of the animal, as inflammation and manipulations can be confined to a single limb. We have previously demonstrated that adjuvant injection ( $150 \mu\text{g}$  dose) causes very early (8 h) and sustained increases in expression of genes encoding specific sensory neuropeptides in dorsal root ganglia innervating the affected joint, but not the contralateral side, in this monoarthritis (Donaldson et al., 1992). Further-

Fig. 2. Correlation curves (Spearman rank) for relationship between histological score and (A) increase in joint circumference, (B) inflammation score and (C) pressure causing paw withdrawal. (Several points are coincident.)



more, changes in the neurophysiology of primary afferents (Guilbaud et al., 1985), inflammatory mediators and their effects on peripheral nociceptors (Grubb et al., 1988), and neuropeptide content of dorsal root ganglia (Hanesch et al., 1992; Smith et al., 1992) have been localised to the affected side during both the initial inflammation and when chronic monoarthritis occurs. Thus this model has produced data specific to the pathogenesis and maintenance of the arthritic state per se rather than more generalised inflammation.

In the assessment of chronic arthritis, limb or joint swelling and/or response to noxious stimulus have been employed as indirect estimates of disease state. However, the subjective nature of these measures has been criticised (Rainsford, 1982). We now show that joint circumference, indices of superficial inflammation and measurement of nociceptive threshold correlate closely with the underlying arthritic histopathology, thus validating their use.

In conclusion, we describe a model of adjuvant arthritis which is easily manipulated to produce either a monoarthritis or more extensive disease. The severity of chronic arthritis is dose-dependent and higher doses of adjuvant cause dissemination of disease, which then resembles an attenuated form of the classical adjuvant polyarthritis (Pearson and Wood, 1959). Ethically, this model has the advantage of showing contralateral disease spread without the more unpleasant effects seen in classical adjuvant disease. The easy manipulation of the arthritic state by alteration of the injected dose of *M. tuberculosis* facilitates the investigation of the pathogenesis of adjuvant-mediated neurogenic inflammation and its spread.

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