Immunocytochemical investigation of caries-induced neural, vascular and leucocyte responses in human primary and permanent tooth pulp

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Publications and presentations

The following papers have been prepared from work arising from this thesis:

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Summary

This immunocytochemical study investigated nerve density and morphology, neuropeptide expression, vascular status and leucocyte accumulation within the human tooth pulp. It specifically examined differences between the primary and permanent dentition, and explored the effect of caries on the above parameters. The study also sought to correlate quantitative findings with a reported pain history.

Mandibular first permanent molars and second primary molars were obtained from children requiring dental extractions under general anaesthesia. A simple pain history was elicited for each patient. Following exodontia, teeth were split longitudinally, placed in fixative and were categorised as intact, moderately carious or grossly carious. The coronal pulps were removed and serial frozen sections were processed for indirect immunofluorescence. Triple-labelling regimes were employed using combinations of the following antisera: i) protein gene product 9.5 (a general neuronal marker; ii) the neuropeptides calcitonin gene-related peptide, substance P, vasoactive intestinal polypeptide or neuropeptide Y; iii) *Ulex europeaus* I lectin (a label for vascular endothelium) and iv) leucocyte common antigen (a general leucocyte marker). Image analysis was then used to determine the percentage area of immunostaining for each label within different anatomical regions of the coronal pulp.

The findings revealed that there were significant inter-dentition differences for the biological variables under investigation. Essentially, in intact samples, innervation density and neuropeptide expression were greater in permanent teeth but primary tooth pulps were more vascular and contained a greater number of leucocytes. With caries progression, both dentitions demonstrated significant increases in neural density, neuropeptide expression and leucocyte accumulation. However, changes in pulpal vascularity were limited to the pulp horn regions. The only factors found to correlate with the reported pain history were substance P and vasoactive intestinal polypeptide expression. These peptides were significantly upregulated in painful pulpitis. Finally, there was evidence to suggest that changes in neuropeptide expression were associated with changes in vascular status and leucocyte accumulation within the inflamed pulp.

In conclusion, this study has established that significant inter-dentition differences exist in pulpal biology. Furthermore, dynamic changes in pulpal neural density and neuropeptide expression seem to occur with caries progression. These findings are likely to have functional importance in terms of pain experience, inflammation and healing, and thus may help to direct the development of novel therapeutic strategies for the compromised dental pulp.

Contents

1. General introduction

1.1 Introduction

Throughout the centuries, the tooth's abundant innervation and extreme sensitivity have stimulated much interest and investigation. Even today, new knowledge from other biological systems continues to fuel efforts to more fully understand the nature of dental innervation. In particular, neuropeptide-related research is providing a considerable impetus for novel neuroanatomical investigation within the pulp-dentine complex. The purpose of this introduction section is to review a number of well-established aspects of dental innervation and to introduce some areas where knowledge is still in its infancy. Emphasis will be placed on the morphological and cytochemical features of intradental innervation under different developmental and pathological conditions. Finally, the rationale for undertaking this study will be discussed together with the overall aims and objectives of the proposed research.

1.2 Overview of pulp/dentine innervation

1.2.1 Anatomical distribution of intradental nerves

The dense innervation of the dental pulp has long been recognised, generating extensive anatomical investigation throughout the 20th century. Numerous studies have described a pattern of innervation that is common to a number of species including: man (Bradlaw, 1936; Bernick, 1948; Johnsen, 1985); monkey (Bueltmann *et al.*, 1972); dog (Hirvonen, 1987); cat (Byers and Matthews, 1981); rat (Byers and Kish, 1976; Sato *et al.*, 1988); ferret (Jacobsen *et al.*, 1998) and mouse (Avery *et al.*, 1980; Mohamed and Atkinson, 1982). However, it has been shown that the distribution of intradental nerves within continuously growing teeth, such as rodent incisors, does differ from that found in teeth of limited growth (for review, see Byers, 1984).

Nerve fibres enter the tooth via one or more apical foramina or accessory canals and ascend through the radicular pulp (Bernick, 1948; Rapp *et al.*, 1957). Within the radicular region, myelinated and unmyelinated fibres co-exist within nerve trunks and

are often closely associated with blood vessels. The large radicular trunks tend to be located centrally and show very little terminal branching (Engström and Öhman, 1960; Byers and Matthews, 1981). As nerves pass through the coronal pulp and extend towards the pulp-dentine border, marked aborisation of these nerve fibres is seen (Brashear, 1937; Dahl and Mjör, 1973). Ultrastructural investigations have revealed that, as nerves ascend coronally, myelinated axons lose their myelin sheath resulting in a predominance of unmyelinated fibres (Harris and Griffin, 1968).

An extensive plexus of nerves, the subodontoblastic nerve plexus (plexus of Raschkow), is formed in the cell free zone just below the odontoblastic layer. This plexus is not seen within radicular pulp (Maeda *et al.*, 1994) nor within the coronal pulp of continuously erupting animal teeth (for review see, Byers, 1984). A large number of nerve fibres terminate as free nerve endings in the subodontoblastic nerve plexus (SOP), cell-free zone or odontoblast layer but some fibres may extend into dentine for distances of up to 200 μm (Byers and Kish, 1976; Lilja, 1979). Quantitative studies have shown that the density of these intratubular nerves is greatest in the pulp horn region (Lilja, 1979; Holland, 1981; Holland *et al.*, 1987).

1.2.2 Morphology of intradental nerves

Electron microscopic investigation at the tooth apex has revealed that, on average, myelinated (sensory) axons have a diameter 4 μm (Graf and Björlin, 1951; Nair *et al.*, 1992) whereas unmyelinated (sensory and sympathetic) axons mostly have an axonal diameter of less than 1 μm (Fried and Hildebrand, 1981a; Nair and Schroeder, 1995). Electron microscopy has also been employed to study the anatomical characteristics of intradental nerve terminals. These have been well-described both in association blood vessels (for review see, Byers, 1984) and at the pulp-dentine border (Gunji, 1982).

1.2.3 Origin of dental nerves

The tooth and its supporting structures are predominantly innervated by sensory neurons whose cell bodies lie in the trigeminal (Gasserian) ganglion (Arwill *et al.*, 1973; Fehér *et al.*, 1977; Chiego *et al.*, 1980; Fried *et al.*, 1989). A number of

neuroanatomical approaches have shown that sympathetic nerves, however, contribute minimally to overall pulpal innervation (Anneroth and Norberg, 1968; Pohto and Antila, 1968; Casasco *et al.*, 1995). Surgical or chemical sympathectomy has demonstrated that these fibres originate principally from the superior cervical ganglion (Larsson and Linde, 1971; Arwill *et al.*, 1973; Matthews and Robinson, 1980).

Support for a parasympathetic contribution to dental innervation remains controversial. A parasympathetic supply to teeth has been indirectly demonstrated in primates (Chiego *et al.*, 1980) and guinea pig (Segade and Suarez-Quintanilla, 1988), however, other studies have strongly disputed its existence (Sasano *et al.*, 1995; Parker *et al.*, 1998).

1.2.4 Physiological characteristics of dental nerves

Electrophysiological studies have demonstrated that the majority of tooth pulp afferents have conduction velocities in the A-delta and C-fibre range (Matthews, 1977; Lisney, 1978; Närhi *et al.*, 1982a). There is also evidence to suggest that a few tooth pulp afferents may have conduction velocities similar to those of A-beta fibres (Cadden *et al.*, 1983).

1.3 Cytochemistry of dental innervation

1.3.1 Cholinergic and adrenergic innervation

Over the past few decades, there has been a growing appreciation of the cytochemical diversity displayed by dental nerves. Several different subpopulations of intradental nerve fibres have been identified on the basis of their individual neurochemistry. However, the earliest efforts to categorise intradental nerves relied solely on the identification of the classical neurotransmitters, noradrenaline and acetylcholine. It was considered that postganglionic sympathetic axons contained high concentrations of noradrenaline, whereas postganglionic parasympathetic axons contained acetylcholine. Adrenergic (catecholamine) innervation was demonstrated by aldehyde-induced fluorescence techniques and cholinergic innervation was identified indirectly

by performing histochemical staining for acetycholinesterase activity (Anneroth and Norberg, 1968; Pohto and Antila, 1968; Larsson and Linde, 1971; Arwill *et al.*, 1973). These studies, performed in a variety of species including man, revealed that adrenergic fibres were present mostly as delicate beaded networks on the surface of blood vessels, whereas cholinergic fibres were more widespread, occurring within nerve trunks and the SOP as well as in association with some blood vessels.

Later studies have employed specific immunocytochemical markers to identify intradental adrenergic (sympathetic) nerve fibres. Avery and colleagues injected mice, prior to sacrifice, with 5-hydroxydopamine (5-OH-DA) in order to label adrenergic nerve terminals (Avery *et al.*, 1980). They subsequently quantified the 5-OH-DA-labelled nerve endings within decalcified molar teeth and found that the majority of terminals were associated with blood vessels. Immunoreactivity for tyrosine hydroxylase (TH) has also been used to identify adrenergic intradental nerve fibres. Casasco's study in human pulp tissue revealed that TH-immunoreactivity was predominantly confined to a subpopulation of fine varicose nerve fibres localised around the pulpal vasculature (Casasco *et al.*, 1995).

1.3.2 Peptidergic innervation

As previously mentioned, up until the 1970's, intradental nerves were simply categorised as sensory or autonomic, depending on their catecholamine or acetylcholine content. However, over the last three decades, considerable advances in immunohistochemical techniques have facilitated the identification of numerous novel neuroregulators within both the central and peripheral nervous systems. These substances, known as neuropeptides, are biologically active proteins which are synthesised in nerve cell bodies and are transported through the nerve axon. On release they act as neurotransmitters or neuromodulators, and also take part in a wide range of regulatory mechanisms (for reviews see, Dalsgaard *et al.*, 1989; Hökfelt, 1991; Strand, 1999).

Several neuropeptides have now been demonstrated within the teeth of a variety of species, including man, and have provided a valuable insight into the cytochemical

diversity of intradental nerve subpopulations (for reviews see, Akai and Wakisaka, 1990; Wakisaka, 1990). A detailed discussion of the distribution, origin and functional significance of neuropeptide-containing pulpal nerves is provided in chapter five but a brief summary is provided below.

Substance P (SP) was the first neuropeptide to be identified in dental tissue (Olgart *et al.*, 1977a; 1977b). Subsequently, numerous investigators have demonstrated that SP is expressed by a large proportion of intradental nerves, including those which are associated with the pulpal vasculature (Mohamed and Atkinson, 1982; Grönblad *et al.*, 1984; Wakisaka *et al.*, 1985). The extension of SP-immunoreactive (ir) fibres into dentine has also been established (Wakisaka *et al.*, 1984).

Immunocytochemical localisation of calcitonin gene-related peptide (CGRP) within the tooth pulp was first reported in 1986 (Uddman *et al.*, 1986a; Wakisaka *et al.*, 1987a). The distribution of CGRP-ir fibres appears to correlate closely with that of SP, although CGRP-ir fibres are generally more abundant (Luthman *et al.*, 1992). Both SP and CGRP are believed to be present in sensory afferents of trigeminal origin (Wakisaka *et al.*, 1985; Silverman and Kruger, 1987) and it is likely that they actually co-exist within some fibres (Wakisaka *et al.*, 1987a).

Vasoactive intestinal polypeptide (VIP) has also been clearly demonstrated within intradental nerve fibres (Uddman *et al.*, 1980; Wakisaka *et al.*, 1987b; Casasco *et al.*, 1990a). Fibres expressing this peptide are mainly localised around blood vessels although some VIP-ir free fibres have been identified extending towards the odontoblast region (Uddman *et al.*, 1980). The origin of VIP-containing dental nerves, under normal conditions, is uncertain. However, a parasympathetic origin has been proposed since VIP is associated with parasympathetic nerves in other tissues (Lundberg and Hökfelt, 1986). Furthermore, VIP-ir fibres have been reported to persist in the dental pulp following inferior alveolar nerve section and sympathectomy (Akai and Wakisaka, 1990).

Another neuropeptide that has been subject to dental investigation is neuropeptide Y (NPY). This peptide was first identified in teeth by Uddman and colleagues in 1984 and was shown to be mostly localised around blood vessels (Uddman *et al.*, 1984). The same study also demonstrated that NPY-ir fibres were likely to be of sympathetic origin.

Numerous other neuropeptides have also been identified in pulpal nerves and these have included: leucine-enkephalin (leu-ENK) (Grönblad *et al.*, 1984); neuropeptide K (NPK) (Casasco *et al.*, 1990b); somatostatin (SOM) (Casasco *et al.*, 1990a); peptide with N-terminal histidine and C-terminal isoleucine (PHI) (Casasco *et al.*, 1990a); C-terminal flanking peptide of NPY (C-PON) (Casasco *et al.*, 1990a); methionine-enkephalin (met-ENK) (Casasco *et al.*, 1990a); Galanin (GAL) (Wakisaka *et al.*, 1996a) and secretoneurin (SEC) (Pertl *et al.*, 1998).

1.4 Factors affecting dental innervation

1.4.1 Differences between primary and permanent teeth

Very few studies have sought to compare primary and permanent tooth innervation and data have been mainly descriptive. It has been suggested that the overall distribution of intradental nerves within human primary teeth is similar to that in permanent teeth, although the density of innervation may be less (Rapp *et al.*, 1967b).

Johnsen and Karlsson (1974) undertook a quantitative comparison of apical myelinated and unmyelinated axons in feline primary and permanent teeth and devised an innervation index based on tooth weight. They then estimated that innervation density was approximately the same for both dentitions, however, primary teeth were found to contain a greater proportion of unmyelinated fibres. Johnsen and Johns (1978) later quantified apical myelinated and unmyelinated fibres in human teeth. They reported that fully-developed primary canines had significantly more myelinated axons than permanent canines but the number of unmyelinated axons was similar. However, permanent incisors contained significantly more myelinated fibres than their predecessors.

On the basis of these inter-dentition differences in the relative proportions of myelinated and unmyelinated fibres, some investigators have concluded that permanent teeth may have a greater potential to conduct sharp pain (Johnsen and Karlsson, 1974). However, attempts to compare the sensory function of the two dentitions with the use of electrical pulp testing have proved inconclusive (Johnsen *et al.*, 1979; Asfour *et al.*, 1996).

Comparative data relating to the peptidergic innervation of primary and permanent teeth are sparse. Fried and Risling (1991) compared SP-, CGRP- and NPY-expression in developing primary and permanent cat canine teeth. They reported that SP- and CGRP-ir fibres were less common in primary pulps as compared to their successors, and primary teeth appeared devoid of any NPY-ir fibres.

1.4.2 Dental development

Following tooth eruption, both dentitions show marked neural changes with continued dental development (Johnsen and Karlsson, 1977; Fried and Hildebrand, 1981a; Fried and Hildebrand, 1981b). An important developmental factor, specific to the primary dentition, is the natural exfoliation of these teeth following physiological root resorption. The fate of neural elements is of particular interest during this process. Studies in humans and cats have demonstrated a substantial reduction in pulpal innervation following the onset of physiological root resorption (Mohiuddin, 1950; Rapp *et al.*, 1967b; Fried and Hildebrand, 1981b). Some investigators have reported that neural changes are only evident when resorption has involved at least half of the root length whilst others have described considerable neural degeneration at a much earlier stage of root resorption.

A number of post-eruptive neural changes have also been described for the permanent dentition. In a study of human premolars, the number of apical myelinated axons was found to increase with continued root development but fibre size remained fairly constant. In contrast, unmyelinated axon size and group configuration changed following complete root development but axon numbers remained the same (Johnsen *et al.*, 1983). Another important developmental change appears to involve the SOP.

During permanent tooth eruption, there is a rapid organisation of intradental sensory fibres to form this neural plexus and this appears to become more established with continued root development (Bernick, 1964; Peckham *et al.*, 1991; Luthman *et al.*, 1992).

Finally, the effect of ageing on the innervation status of the adult dentition has also been investigated. Bernick's study (1967) reported a degeneration of pulpal nerves within intact teeth obtained from older patients. These changes were believed to stem from progressive pulp tissue calcification. Fried and Hildebrand (1981a) also observed an overall reduction in nerve density in ageing feline teeth, attributed to progressive hard tissue occlusion of the pulp chamber.

It has frequently been hypothesised that qualitative and quantitative changes in nerve fibres during dental development may have implications relating to function. Investigators have thus attempted to compare tooth sensitivity at different stages of dental maturity. It would indeed appear that immature teeth demonstrate higher thresholds to electrical stimuli than do fully developed teeth (Fulling and Andreasen, 1976; Johnsen *et al.*, 1983).

1.4.3 Injury and inflammation

Pulpal injury appears to evoke profound changes in intradental nerve morphology and distribution. Placková (1966) was one of the first investigators to describe the pathological changes in human pulpal innervation following caries. Using a silver impregnation technique he noted intense argyrophilia of neural elements closest to the carious lesion. He also reported conspicuous neural branching adjacent to the inflammatory focus, often extending into the odontoblast layer and predentine. Subsequently, several other investigators have also described the appearance of nerve fibres within the inflamed human tooth pulp (Bernick, 1972; Almeida and Bozzo, 1973; England *et al.*, 1974; Torneck, 1974a; Torneck, 1977). Findings from these studies seem to concur that pulpal nerves undergo a variety of degenerative changes including axonal fragmentation and the formation of varicosities or vacuoles. In

addition it has been noted that, in comparison to other structures, nerve fibres appear relatively resistant to necrosis (Mullaney *et al.*, 1970; England *et al.*, 1974).

Yamaura (1987) used silver impregnation as well as an antiserum to neurofilament protein (NFP) to visualise histological changes in dental nerve fibres following cavity preparation in human third molars. Early changes were seen within the SOP after one day and comprised a reduction in fine nerve fibres and a predominance of thick fibres. However, by 14 days following injury, the delicate fibres had reappeared within the nerve plexus. Yamaura and colleagues also demonstrated an increase in the proportion of fine nerve fibres beneath carious cavities in extracted teeth which had reportedly been hypersensitive (Yamaura *et al.*, 1988).

Overall, Byers and her collaborators have probably made the greatest contribution to our present understanding of the response of pulpal nerves to injury and inflammation. Their studies in rats have demonstrated a marked, but temporary, sprouting of pulpal nerves following injury (Taylor *et al.*, 1988; Khayat *et al.*, 1988; Kimberly and Byers, 1988; Taylor and Byers, 1990). In one series of experiments (Byers *et al.*, 1990b) a pulpal injury model was devised by preparing cavities within the cervical dentine of rat molars. At various time periods following injury (1 to 35 days), immunocytochemical investigation of pulpal nerves was performed using antiserum to CGRP. This subpopulation of nerve fibres was seen to undergo sprouting into the adjacent odontoblast layer but had returned to their normal distribution by the third week. Interestingly, the degree of injury-induced neural sprouting was shown to be age-dependant, being less extensive in old teeth (Swift and Byers, 1992).

In addition to the profound morphological changes evident within dental nerves following injury, it is also apparent that there are concurrent changes in neuropeptide expression (for reviews see, Byers, 1994; Fristad, 1997; Byers and Närhi, 1999). However, whereas our understanding of injury-evoked morphological changes is reasonably comprehensive, our knowledge of these subtle cytochemical changes is much more limited.

Nonetheless, several investigators have demonstrated dynamic changes in neuropeptide expression following experimental dental injury. Grutzner and colleagues (Grutzner *et al.*, 1992) conducted a quantitative study, using radioimmunoassays, to evaluate injury-related changes in levels of CGRP and SP in rat pulps. Following pulpal exposure they found a significant decrease in the pulpal content of both CGRP and SP. Fascinating data have also been obtained using pulp deafferentation models: following inferior alveolar nerve (IAN) section, rat mandibular molars have been found to contain an increased number of both NPY- and VIP-ir fibres (Fristad *et al.*, 1996; Wakisaka *et al.*, 1996c; Fristad *et al.*, 1998).

1.5 Functional implications of dental innervation

It is likely that many of us could testify as to the exquisite sensitivity of dentine. Not surprisingly, the functional significance of the tooth's abundant innervation has principally been related to pain. However, new research is demonstrating the functional importance of sensory neurons and their neuropeptides in other biological and pathological mechanisms. The following sections will highlight the role of intradental innervation in a range of sensory and effector processes.

1.5.1 Sensitivity

Data from human experiments have generally indicated that pain is the only sensation perceived following the application of thermal, mechanical, chemical or osmotic stimuli to the dentine or pulp (Brännström, 1962; Anderson and Matthews, 1967; Ahlquist *et al.*, 1984; Närhi, 1985). However, there is some evidence to suggest that electrical stimulation at threshold can result in a sensation which is not necessarily described as painful (Matthews *et al.*, 1976; Mumford and Stanley, 1981; McGrath *et al.*, 1983; Virtanen *et al.*, 1987).

Dental pain experience can be extremely variable but essentially two modalities are recognised: a short-lasting, sharp and well-localised pain associated with dentinal stimulation which is probably mediated by A-delta and A-beta fibres, and a prolonged, dull, throbbing and poorly-localised sensation thought to be elicited by C-fibre

activation (Närhi *et al.*, 1982; Edwall, 1986; Trowbridge, 1986; Närhi *et al.*, 1992a; Figdor, 1994; Olgart and Kerezoudis, 1994). The most likely mechanism for A-fibre activation, following dentinal stimulation, is via the hydrodynamic effect (Brännström, 1966; Brännström *et al.*, 1967; Matthews and Vongsavan, 1994). This type of sensitivity is therefore greatly dependent on dentinal porosity (Addy and Dowell, 1983; Pashley, 1996). In contrast, it appears that C-fibres only respond to noxious stimuli that directly affect the pulp. Thus C-fibres are considered to respond to stimuli potentially capable of causing tissue injury and are generally associated with symptoms arising from pulpal inflammation (Närhi *et al.*, 1982b; Jyvasjarvi and Kniffki, 1987; Närhi *et al.*, 1992b; Panopoulos, 1992). A further difference between A- and C-fibre activity relates to the finding that C-fibres are better able to function in hypoxic conditions (for review see, Trowbridge, 1986).

The predominance of SP- and CGRP-ir fibres within the dental pulp, has led many investigators to postulate that these peptides are specifically involved in dental pain processing. Certainly SP has a well-accepted role as a nociceptive transmitter and is believed to be important in the regulation of inflammatory pain (Henry *et al.*, 1980; Lembeck *et al.*, 1981; Woolf and Wiesenfeld-Hallin, 1986; Ma and Woolf, 1995; Neumann *et al.*, 1996; Allen *et al.*, 1999). Interestingly, experimental work in human subjects with painful pulpitis has revealed a marked increase in the pulpal content of SP (Bowles *et al.*, 2000).

1.5.2 Blood flow regulation

Both sensory and autonomic nervous systems are fundamentally involved in pulpal blood flow regulation (for reviews see, Kim *et al.*, 1989; Olgart, 1996a). There is now substantial physiological evidence to support the role of neuropeptides, namely SP, CGRP, VIP and NPY, in the control of pulpal vascular tone (Rosell *et al.*, 1981; Edwall *et al.*, 1985; Gazelius *et al.*, 1987; Olgart *et al.*, 1989; Heyeraas *et al.*, 1994). The anatomical association of intradental nerve fibres and the pulpal vasculature will be reviewed in chapter six, together with a more detailed discussion of neural control of pulpal blood flow.

1.5.3 Neurogenic inflammation

It has been suggested that changes in neural density following injury may have evolved as a defence mechanism to facilitate inflammatory reactions and healing. Thus any subsequent changes in tooth sensitivity may, in fact, be considered of secondary importance. Certainly, sensory nerves and their regulatory peptides have been implicated in a variety of inflammation-related functions including vasodilation, plasma extravasation and immune cell regulation (for reviews see, Payan *et al.*, 1987; Walsh *et al.*, 1992).

Kerezoudis and colleagues (1994) were the first to demonstrate the role of SP in afferent nerve-induced plasma extravasation in rat lip and tooth pulp. More recently, Fristad's denervation study in rat showed that an intact sensory innervation was necessary for the recruitment of immunocompetent cells into pulp tissue following dentinal injury (Fristad *et al.*, 1995b). Further detail regarding the possible interactions between neuropeptides and immune cells is provided in chapter seven.

1.5.4 Trophic tissue effects

There is growing evidence to suggest that sensory nerves can exert trophic effects within their target tissues (Hsieh *et al.*, 1996; Chen *et al.*, 1999). However, the efferent role of sensory nerves in dentine formation it still a matter of some debate. Following their observations in injured rat teeth, Byers and collaborators speculated that peptidergic intradental fibres may promote reparative dentine formation (Byers et al., 1990b). More recently, ultrastructural observations in pulpotomised human and dog teeth have revealed a close association between nerve terminals, odontoblasts and predentine, considered suggestive of a functional relationship (Inoue *et al.*, 1995; Inoue *et al.*, 1997; Zhang and Fukuyama, 1999). Jacobsen and Heyeraas (1996) investigated the effect of a decreased sensory innervation on dentine formation in rat molars and, following capsaicin treatment or IAN axotomy, they found that dentine formation was reduced although not abolished. Conversely, it has also been reported that dentine formation is not dependant on an intact sensory innervation (Olgart *et al.*, 1995).

Additional evidence to support the role of SP and CGRP in the regulation of tissue growth within the tooth pulp has been derived from in vitro studies. It has been shown that both of these peptides exert stimulatory effects on pulp fibroblasts, and may therefore help to promote tissue healing (Bongenhielm *et al.*, 1995; Trantor *et al.*, 1995).

1.6 Rationale for study

Clinical observations have led to the general view that primary teeth are less sensitive to noxious stimuli than permanent teeth. This, largely unsubstantiated, belief has no doubt contributed to the widespread practice of performing restorative treatment in the primary dentition without the use of local analgesia (Martin, 1994; Vaughan-Jones, 1994). However, there appears to be little biological evidence to support any innervation differences between the two dentitions. Overall, it would appear that the innervation characteristics of the primary dentition have received little attention over the years. This apparent lack of scientific interest is clearly illustrated by the fact that nerves were only conclusively reported within the dentine of human primary teeth in 1996, many years after dentinal innervation had been well established for the permanent dentition (Egan *et al.*, 1996).

Another popular concept is that primary teeth undergo a more rapid pulpal response to injury (caries) than do their successors, although few studies have attempted to substantiate this theory. Nonetheless, this perceived "unfavourable" inflammatory response appears to be the rationale for performing formocresol pulpotomies in grossly carious primary teeth rather than attempting to maintain pulpal vitality by adopting a more biologically compatible approach (Magnusson, 1980; Kopel, 1992; Mathewson and Primosch, 1995).

In this current climate of evidence-based practice it seems reprehensible that our knowledge and understanding of primary tooth innervation and response to injury is so elementary. It is hoped that this study will help to clarify some of the issues relating to primary tooth innervation and inflammation. In addition, these findings may help to

direct future research towards developing less empirical and potentially harmful treatment modalities for the carious primary dentition.

Dental caries probably represents the most common disease within our society today. Furthermore, there is evidence to suggest that the prevalence of caries has recently started to increase in British children (Pitts and Palmer, 1995). Thus caries and its sequelae will continue to pose considerable demands on health care services in the foreseeable future. A particularly challenging aspect of clinical dentistry relates to the diagnosis and management of dental pain. Carious teeth vary widely in their presenting symptoms making clinical diagnosis more difficult. To date it has proved unreliable to correlate reported patient symptoms with the histopathological state of the pulp (McDonald, 1956; Tyldesley and Mumford, 1970; Garfunkel *et al.*, 1973). However, few studies have attempted to relate reported symptoms with changes in intradental nerve morphology or neurochemistry. Indeed very little is known about potential caries-induced changes in neuropeptide expression in human teeth. The use of human material in this study provides an excellent opportunity to correlate the experimental findings with a known clinical history. Findings from the proposed study may therefore provide further insight into the neurobiology of pain. It may then be appropriate to speculate about novel therapeutic strategies for the more effective management of dental pain.

The dental pulp not only provides an important nociceptive model but also offers a valuable system in which to study peripheral inflammation. There is a growing interest in the interactions between neural and inflammatory mechanisms and this study provides a unique opportunity to investigate changes in intradental neural morphology and neuropeptide expression in relation to inflammation in human subjects. The neuropeptides selected for investigation have been included on the basis of previous work outlining their importance in haemoregulation, immunoregulation and healing as well as nociception. Findings from this study may therefore help to elucidate the role of neuropeptides in pulpal inflammation and may highlight any inter-dentition differences that could be of clinical relevance.

Some of the above areas of investigation have already been pursued in animal models but the use of human tissue in the present study will ensure that the findings are more directly relevant to our clinical practice. Another important justification for undertaking this investigation is the quantitative nature of the experimental design as the vast majority of previous related dental studies have been simply descriptive. It is only with the increasing sophistication and accessibility of computer software that quantitative analysis of complex histological material has become feasible. The adoption of a more reproducible and objective method for the quantification of biological variables should undoubtedly provide more meaningful and valid data.

1.7 Aims and objectives of study

The overall aim of this study was to further our understanding of the role of neuropeptides within the normal and inflamed dental pulp in man, thus gaining insight into their significance in pain and inflammation. The specific objectives of the study were to:

- determine whether there are any differences between primary and permanent tooth pulps in terms of their overall innervation density, expression of neuropeptides, vascular status, vascular innervation characteristics or leucocyte infiltrates;
- investigate the effect of caries progression on each of the above parameters;
- identify any interactions between neuropeptide expression and vascularity or leucocyte accumulation;
- seek a correlation between quantitative changes in innervation, neuropeptide expression, vascularity or leucocyte accumulation and a reported pain history.

1.8 Overall approach to study

Children presenting to the Sheffield dental hospital who required dental extractions under general anaesthesia provided the clinical data and experimental material for this study. Following tooth extraction, coronal pulps were removed and processed for indirect immunofluorescence using combinations of the following neurochemical markers: protein-gene product 9.5 (a non-specific marker of nerve fibres); antisera to the neuropeptides SP, CGRP, NPY, VIP, GAL, SOM and ENK; leucocyte common

antigen (a universal leucocyte marker) and *Ulex europeaus* I Lectin (a label for vascular endothelium). Qualitative and quantitative analysis of immunocytochemical labelling in different pulp regions was undertaken using fluorescence microscopy and computer-assisted image analysis. Findings were then correlated with clinical variables including tooth type, degree of caries and reported pain experience.

2. Materials and methods

2.1 Experimental material

2.1.1 Study population

Mandibular second primary molars and first permanent molars were selected as the experimental material for this study. Teeth were obtained from children requiring dental extractions under general anaesthesia and who attended the out-patient general anaesthetic department of the Charles Clifford Dental Hospital, Sheffield. Prior to each theatre session, patient records and radiographs were examined to identify potential tooth samples. Following this initial screening, the investigator attended the predetermined clinical sessions to examine and interview patients and to collect the teeth. Ethical approval for the study was granted by the South Sheffield Research Ethics Committee and informed consent was obtained from accompanying guardians to sanction the use of extracted teeth for research purposes.

2.1.2 Patient history

Age, gender and ethnic group were recorded for each patient. In addition, the child and accompanying guardian were interviewed to ascertain a simple pain history for teeth included in the study. A positive pain history was recorded either when the child personally reported that they had experienced some spontaneous dental pain during the previous few days or when the parent stated that the child had suffered disturbed sleep or eating, attributable to toothache, over the past week. Patients were excluded from the study if a reliable pain history could not be established. Furthermore, each patient was asked to point to the tooth which they thought had been hurting them and if unable to do so, were also excluded from the study.

Prior to surgery, a clinical examination was performed and teeth were omitted from the study if there was any clinical or radiographic evidence of advanced tooth wear, root resorption, enamel or dentine anomalies, restorative intervention, or associated pathology other than occlusal caries. If a patient presented with more than one tooth that could have been the cause of the reported toothache, the subject was not included

in the investigation. Furthermore, no subjects with a significant medical history were eligible for inclusion in the study.

2.1.3 Tooth collection

All subjects received an inhalation induction using the anaesthetic agent Sevofluorane (8%). Anaesthesia was maintained using 2% Sevofluorane and a 50:50 mixture of oxygen and nitrous oxide. Simple forceps extractions were performed by staff from the Oral and Maxillofacial Surgery Department. Normal surgical protocol was followed: the symptomatic side was usually operated upon first and mandibular extractions were performed prior to maxillary extractions. In cases of multiple extractions, only the first tooth to be extracted in either the left or right mandibular quadrant was retained for subsequent investigation. Furthermore, the order of the experimental tooth extraction in the overall extraction sequence was noted (first to fourth quadrant). The rationale for noting the extraction sequence was based on findings from previous investigations which have showed that the pulpal content of met-ENK and SP was progressively reduced from the first to the third extracted tooth (Walker *et al.*, 1987; Parris *et al.*, 1989; Robinson *et al.*, 1989).

Immediately following exodontia, a longitudinal groove was prepared on the buccal surface of the tooth, using a diamond disc in a portable electric straight handpiece. An assistant sprayed phosphate buffered saline (0.2M PBS, pH 7.4, 4°C) to cool the disc during drilling. The tooth was then split by placing a 5 mm osteotome in the buccal groove and applying a blow with a surgical mallet. In carious samples, the tooth split bisected the occlusal lesion and the mesial half of the tooth was retained and placed in Zamboni's fixative (4% paraformaldehyde and 0.2% picric acid in 0.1 M phosphate buffer, pH 7.4, 4°C) (Zamboni and de Martino, 1967). The time taken from initial forceps application until immersion of the specimen in fixative was recorded in seconds using a stop watch. Teeth were rejected from the study at this stage if this time exceeded 60 seconds. This was a somewhat arbitrary cut-off point as there have been no previous studies correlating extraction time with the neuropeptide content of pulpal nerves. However it was hypothesised that prolonged surgical trauma could

evoke an increased release of peptides from trigeminal nerve endings and hence result in a reduced neural peptide content (Pertl *et al.*, 1997).

In view of the neural degeneration that is known to accompany the process of primary tooth exfoliation (see, section 1.4.2), primary samples with any physiological root resorption were excluded from the investigation. Furthermore, primary and permanent tooth samples were rejected from the study if they showed any pathological root resorption or if the tooth split was unsuccessful or the pulp was seen to be obviously necrotic. The reason for sample rejection was recorded.

Following collection of teeth for pilot studies, experimental material for the main study was gathered during the period November 1997-May 1999. The aim was to collect 60 primary and 60 permanent teeth, with an equal distribution of intact, moderately carious and grossly carious specimens. Each tooth was given a sample number so that subsequent analysis could be performed blind.

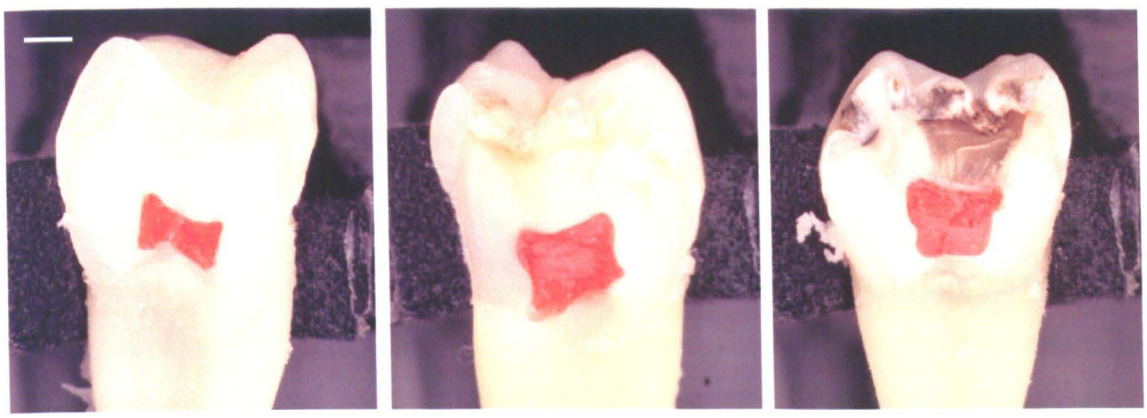
2.1.4 Pulp removal

Teeth were left in fixative for 24 hours at 4°C and were then washed thoroughly in 0.2M PBS. The coronal pulp was carefully removed from the pulp chamber with a small dental excavator and the use of a Nikon dissection microscope at x20 magnification. Samples were rejected if the pulp was not retrieved intact with well-defined pulp horns and a record was kept of this event. Pulp tissue was placed in 0.2M PBS and the pulpless tooth half was replaced in Zamboni's fixative in a labelled 7 ml container for long-term storage at 4°C.

2.1.5 Assessment of caries

Following tooth splitting and fixation, each mesial tooth portion was dried and the degree of caries assessed visually using the Nikon dissection microscope at x20 magnification. The extent of the carious lesion was assessed on the basis of colour changes. Colour change took into account both the obvious brown/orange/black pigmentation of some lesions and the white appearance of demineralised hard tissue. The tooth half was classified as being intact (no colour change within dentine but with

possible staining confined to enamel), moderately carious (colour changes did not extend beyond half the dentine thickness) and grossly carious (colour changes extended beyond half the dentinal thickness). Examples of these categories are shown in Figure 2.1.



(A) (B) (C)

Figure 2.1 Digital images of tooth samples which have been split longitudinally and assessed visually according to the depth of the carious lesion within dentine: **(A)** an intact sample, **(B)** a moderately carious sample and **(C)** a grossly carious sample. Red wax has been placed in the coronal pulp chamber to enhance photographic visualisation of this region. Scale bar = 2.1 mm.

2.1.6 Tissue sectioning

The coronal pulps were left in PBS for 24 hours at 4°C before placing in 0.1M PBS containing 30% sucrose solution for cryoprotection (5 hours at 4°C). The pulp tissue, with the cut surface uppermost, was then embedded in Tissue-Tek OCT compound (Bayer Diagnostics, Basingstoke, UK). Longitudinal sections were cut at 10 μm using a microtome cryotostat (Microm HM 500 OM, Waldorf, Germany) and sections were collected on poly-D-lysine-coated glass slides (Sigma, Poole, UK). Sixty sections were obtained from each tooth pulp. Pilot studies, using both primary (n=6) and permanent (n=6) teeth had established that there was a large variation in the number of 10 μm sections that could be obtained from different pulp samples (n=80-170).

Sections were collected serially on 20 slides so that the 1st, 21st and 41st sections were placed on slide 1, the 2nd, 22nd and 42nd sections were collected on slide 2 and so forth with the 20th, 40th and 60th sections being collected on slide 20. Slides were numbered 1-20 according to the number of the first section to be collected on each

slide. In addition, slides were labelled with the tooth sample number and the extraction side (left or right). Slides were left for 60 min at room temperature to air dry and were then stored at -70°C for up to 21 months.

2.2 Immunocytochemistry

2.2.1 Primary antisera

Details of the primary antisera employed in this study are shown in Table 2.1. Antiserum to protein gene product 9.5 (PGP 9.5), a non-specific neuronal marker, was employed in order to visualise the entire nerve population (Thompson *et al.*, 1983; Maeda *et al.*, 1994; Lin *et al.*, 1997). Immunostaining for the neuropeptides calcitonin gene-related peptide (CGRP); substance P (SP); vasoactive intestinal polypeptide (VIP); neuropeptide Y (NPY); enkephalin (ENK); somatostatin (SOM) and galanin (GAL) was also carried out. Labelling of pulpal leucocytes was achieved by using antiserum to leucocyte common antigen (LCA), a glycoprotein found on the cell membrane of all leucocytes (Kurtin and Pinkus, 1985; Pulido *et al.*, 1988; Streuli *et al.*, 1988).

Table 2.1. Primary antibodies used in the study

Antigen	Host species	Type	Source	Dilution
PGP 9.5	mouse	monoclonal (human)	Ultraclone, Isle of Wight, UK	1:1000
PGP 9.5	rabbit	polyclonal (human)	Ultraclone, Isle of Wight, UK	1:1200
CGRP (II)	rabbit	polyclonal (human)	Peninsula, Merseyside, UK	1:800
SP	rabbit	polyclonal (human)	Genosys, Cambridge, UK	1:800
NPY	rabbit	polyclonal (human)	Genosys, Cambridge, UK	1:800
VIP	rabbit	Polyclonal (human, porcine, rat)	Peninsula, Merseyside, UK	1:800
GAL	rabbit	polyclonal (human)	Peninsula, Merseyside, UK	1:800
[Leu]-ENK	rabbit	polyclonal (human)	Prof J. M Polak, Hammersmith Hospital, London, UK (gift)	1:400 - 1:3,200
[Leu^5]-ENK	rabbit	polyclonal (human)	Genosys, Cambridge, UK	1:400 - 1:3200
[Met^5]-ENK	rabbit	polyclonal (human)	Genosys, Cambridge, UK	1:400 - 1:3,200
SOM	rat	monoclonal (rat)	ams Biotechnology, Oxon, UK	1:50
LCA	mouse	monoclonal (human)	Dako, Bucks, UK	1:1000

2.2.2 Lectin histochemistry

Biotinylated *Ulex europaeus* agglutinin I lectin (UEIL) (20 µg/ml, Vector Laboratories, Peterborough, UK) was employed to label the pulpal vasculature. This lectin, derived from the gorseplant, is specific for α-L-fucose-containing glycocompounds and is considered an excellent marker of human vascular endothelium (Holthöfer *et al.*, 1982; Little *et al.*, 1986).

2.2.3 Fluorescent labels

Two fluorescent secondary antibodies were employed in the study: goat anti-rabbit IgG conjugated to fluorescein isothiocyanate (FITC) which emits a green fluorescence (maximum excitation at 490-500 nm) (Riggs *et al.*, 1958) and horse anti-mouse IgG conjugated to Texas red (TR) which emits a red fluorescence (maximum excitation at 595-604 nm) (Titus *et al.*, 1982). In addition, a blue fluorochrome, 7-amino-4-methyl-coumarin-3-acetic acid (AMCA)-conjugated streptavidin, was employed to visualise the lectin reaction (maximum excitation at 345-355 nm) (Khalfan *et al.*, 1986). Details of these fluorescent labels are given in Table 2.2.

Table 2.2. Fluorescent labels used in the study

Fluorochrome	Host species	Conjugate	Source	Dilution
FITC	goat	anti-rabbit IgG (H+L)	Vector Laboratories, Peterborough, UK	1:20
TR	horse	anti-mouse IgG (H+L)	Vector Laboratories, Peterborough, UK	1:100
AMCA	not applicable	Streptavidin	Vector Laboratories, Peterborough, UK	1:25

2.2.4 Preliminary immunocytochemical investigations

Prior to staining the archive tissue, it was necessary to establish the optimal dilution of each immunoreagent and to conduct specificity tests for all the primary antibodies. Additional pulp samples were collected for this purpose. A range of dilutions for both primary and secondary antibodies was employed to determine which combination

achieved the best staining quality (Polak and Van Noorden, 1997). Steps taken to rectify poor or absent immunostaining included using a variety of different primary antisera or performing heat-mediated antigen-retrieval (Boon and Kok, 1994).

2.2.5 Specificity controls

Immunohistochemical controls were performed by incubating sections with the primary antibody which had been preabsorbed over a 24 h period at 4°C with an excess (10 nmol/ml) of the respective hapten (Polak and Van Noorden, 1997). Additional specificity controls were performed by omitting the primary antibody and incubating the tissue with the antibody dilutent, normal serum and secondary antibody only.

The specificity of the lectin reaction was tested by inhibiting the lectin binding. This was accomplished by pre-incubating the lectin conjugate with 0.2 M α-L-fucose (Vector Laboratories) dissolved in PBS containing 0.2% Triton X-100 (PBST) for 60 min at room temperature prior to applying this mixture to tissue sections (Jowett *et al.*, 1992).

2.2.6 Staining protocols

Triple immunostaining was employed to label the following combinations of structures:

- entire nerve population + neuropeptide (CGRP/SP/VIP/NPY/GAL/SOM/ENK) + vascular endothelium

- entire nerve population or neuropeptide (as above) + leucocytes + vascular endothelium.

Thus, in total, 15 different immunostaining regimes were proposed and each regime was randomly allocated a number (1-15) which corresponded with the slide number as shown in Table 2.3. Slides numbered 16-20 were reserved as spares.

Table 2.3. Immunostaining regimes

Regime	Slide number	Regime	Slide number
CGRP + PGP 9.5^{m} + UEIL	2	SP + LCA + UEIL	7
SP + PGP 9.5^{m} + UEIL	10	NPY + LCA + UEIL	3
NPY + PGP 9.5^{m} + UEIL	1	VIP + LCA + UEIL	14
VIP + PGP 9.5^{m} + UEIL	11	GAL + LCA + UEIL	9
GAL + PGP 9.5^{m} + UEIL	15	SOM + LCA + UEIL	5
SOM + PGP 9.5^{m} + UEIL	12	ENK + LCA + UEIL	13
ENK + PGP 9.5^{m} + UEIL	6	PGP 9.5^{p} + LCA + UEIL	8
CGRP + LCA + UEIL	4	Spare slides	16,17,18,19,20

^{m} = monoclonal antibody ^{p} = polyclonal antibody

2.2.7 Immunocytochemical method

Immunostaining was performed using an indirect immunofluorescence method (Coons *et al.*, 1955). Slides were removed, as required, from storage at -70°C and were left to air dry at room temperature (20°C) for 60 min. Slides were then washed in PBST (2 x 10 min). To reduce any non-specific background staining and to increase the permeability of cell membranes to antibodies, sections were first incubated in PBST containing 10% normal goat serum (Vector Laboratories) for 30 min at room temperature. Following this, sections were incubated with a mixture of the primary antisera and lectin (Table 2.3) diluted in PBST and 5% normal goat serum for 24 h at 4°C in a humid atmosphere.

Slides were subsequently washed in PBS (2 x 10 min) and were incubated with a mixture of three fluorescent secondary markers diluted in PBST and containing 2% normal goat serum: FITC-labelled goat anti-rabbit IgG (1:20), TR-labelled donkey anti-mouse IgG (1:100) and streptavidin AMCA (1:25). In the majority of cases, FITC was employed to visualise the labelling for the various neuropeptides; TR was used to visualise the labelling for LCA or PGP 9.5, and AMCA was used to visualise the labelling for UEIL. However, for the immunostaining regime involving the polyclonal PGP 9.5 antibody (PGP 9.5 + LCA + UEIL), FITC was employed to demonstrate the PGP 9.5-labelling. Secondary antibodies were applied for 90 min at room temperature in a humid atmosphere. Slides were finally washed again in PBS

(2 x 10 min) and sections were carefully dried and mounted in Vectashield (Vector Laboratories).

Due to the large number of slides involved and the length of time required to establish the tissue archive, it was necessary to carry out each staining regime on two separate occasions. The first staining group comprised slides from tooth samples 1-50, and 12 - 21 months later, slides from the remaining samples were processed. However, exactly the same immunoreagents were used for both groups of slides.

2.3 Microscopy and photography

2.3.1 Fluorescence microscopy

Sections were viewed using an Axioplan universal microscope (Carl Zeiss, Oberkochen, Germany) fitted with a x10 eyepiece and an HBO 50 mercury lamp. A different excitation filter was employed for each of the three fluorescent labels, each different filter set comprised a 25 mm diameter exciter and barrier filter. When different filters were employed, there was no apparent "flow through" of the signal generated by one fluorescent marker onto the image created by another.

2.3.2 Photomicroscopy

Sections were photographed using a Carl Zeiss camera (MC 100) mounted on the fluorescence microscope. The same sequence of filter changes was routinely adopted for multiple exposure photomicrographs (FITC-TR-AMCA). Colour slide film (35 mm Kodak Select Elite Chrome, 400 ASA) was used for all photography. Slide film was processed by the Medical Illustration Department, Royal Hallamshire Hospital, Sheffield. A selection of the original 35 mm slides were then scanned using a flatbed scanner (Nikon Cool Scan) at 300 DPI to create digital images for inclusion in this thesis.

2.4 Image analysis

2.4.1 Image analysis system

The system employed for the analysis of immunolabelled tissue is shown in schematic form in Figure 2.2. Fluorescence images were captured using a low light level chilled charge-coupled device (CCD) monochrome camera (Hamamatsu C5985-10) and were displayed on a video monitor (Sony PVM1442QM). The monitor was coupled to a personal computer (Viglen GP4DX33) which was installed with dedicated image-processing software, Image-Pro Plus v3.0 (IPP) and a frame-grabber (Media Cybernetics, Maryland, USA). A zip-drive (Iomega Corporation, Utah, USA) was linked to the PC to permit the storage of images (as TIFF files) on 100 MB disks. Each image required 433 KB of memory.

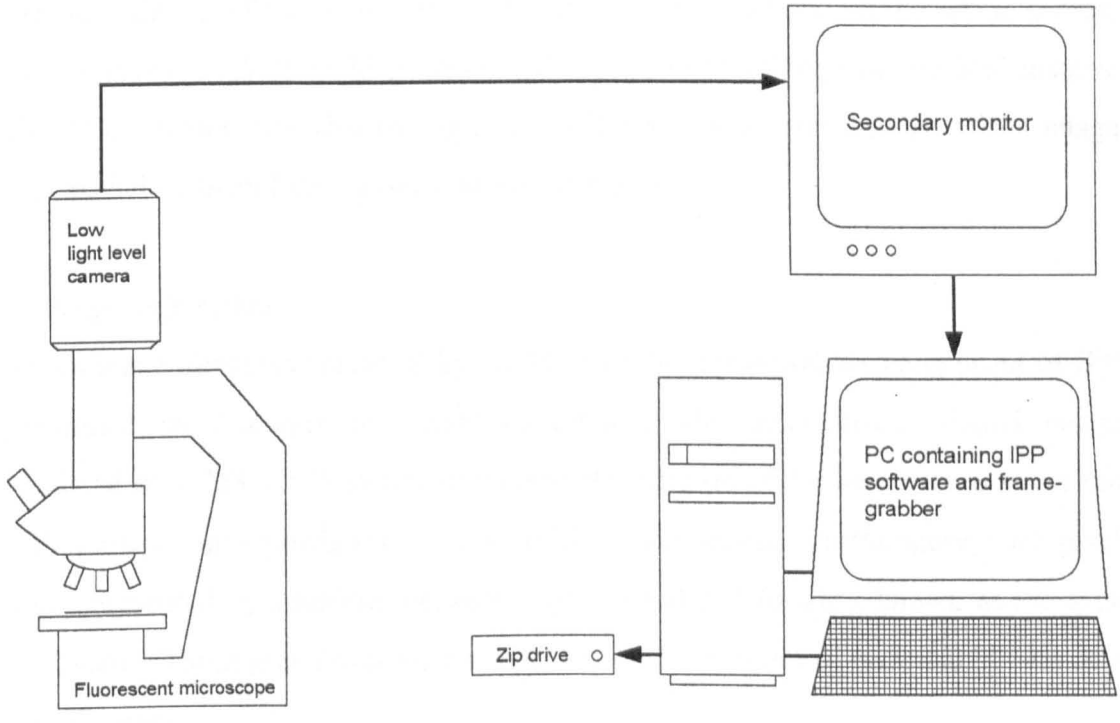


Figure 2.2. Schematic diagram of the system used for image analysis.

2.4.2 Image processing

Image processing involved three stages: field selection, digitisation and segmentation. Each of these steps is briefly described.

- Field selection

The microscopic field was selected (see, section 2.5) and viewed using a x20 objective. By re-directing the light source through the CCD camera, the microscopic image was displayed on the secondary monitor and image re-positioning or focusing was performed as necessary. The video signal was optimised by adjusting the integration period over which the image was captured thus reducing random noise in the video signal.

Specific areas of the image could be isolated for subsequent analysis using the area-of-interest (AOI) tools within the IPP software. This function was necessary when analysing the tip of the pulp horn as the tissue section did not fill the whole screen. Once created, an AOI could be saved and re-applied to subsequent captured images. The AOI function was also employed to isolate artefacts from the rest of the image thus excluding them from subsequent measurements.

- Image digitisation

An analogue signal was received by the PC, and the frame-grabber component of IPP performed an analogue to digital conversion. The monochrome digital image consisted of a 768 x 576 matrix of picture elements (pixels) where each pixel in the digital image corresponded to 0.5 μm^2 of the tissue section. Furthermore, each pixel was represented by a number between 0 (dark) and 255 (bright), known as the grey level value. These grey levels values represented the intensity of transmitted light at a specific point.

- Image segmentation

Regions of the digital image which were positively labelled appeared white and the background was black. Interactive thresholding of this monochrome image was then performed. This involved setting the grey values in order to define the range of

intensities of the objects which were to be included in subsequent automatic area measurements (image segmentation). The upper grey level value was always set at 255 and the lower value was set subjectively according to the intensity of the immunolabelling. By altering the grey values, the white immunostained regions of the digital image could be interactively highlighted in colour until the coloured areas correlated as closely as possible with the positively-labelled structures of the analogue image, displayed on the secondary monitor. All highlighted areas, representing positive staining, were then automatically calculated and recorded.

2.4.3 Image measurement

Multiple automatic measurements were obtained for each image and comprised:

- total area (μm^2) of the AOI in cases where the AOI did not fill the entire frame;
- total area (μm^2) of positive staining (TAS) for all immunolabelled structures;
- percentage area of positive staining (PAS) for all immunolabelled structures within the AOI;
- total number of blood vessels within each AOI.

Additional image processing was required when measuring the TAS and PAS of blood vessels. The lumen of those vessels sectioned obliquely did not exhibit fluorescence staining therefore it was necessary to edit the image by using a "fill" tool. The resultant area measurements thus included both the vessel lumen and the surrounding immunolabelled endothelium.

2.5 Tissue sampling

2.5.1 Fields of interest

Figure 2.3 illustrates the four different fields employed for quantitative analysis. Using the x20 objective, each digital image corresponded to 0.22 mm^2 of tissue. The fields were selected so as to include a variety of distinct intradental nerve configurations:

- field 1, the tip of the mesio-buccal pulp horn which contained an abundance of nerve terminals;

- field 2, the region directly subjacent to field 1 which contained a number of fine intradental nerve fibres;

- field 3, the mesio-buccal aspect of the coronal pulp which contained a section of the subodontoblastic nerve plexus;

- field 4, the mid-coronal pulp region which contained large nerve trunks and neurovascular bundles.

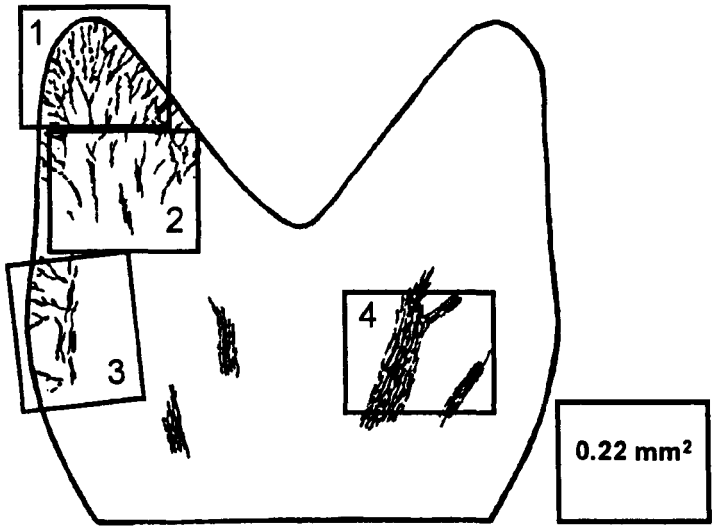


Figure 2.3. Schematic diagram of the coronal pulp showing the four different fields employed for quantitative analysis. Field 1=tip of the mesio-buccal pulp horn; Field 2=region subjacent to field 1; Field 3=buccal subodontoblastic plexus; Field 4=mid-coronal region.

2.5.2 Sampling method

Pilot studies were undertaken to develop a reproducible and objective method of tissue sampling. Fields 1 and 2 were well defined and could readily be identified. Using the secondary monitor, field 1 was selected by positioning the tip of the mesio-buccal pulp horn at the mid-point of the upper extremity of the frame. Field 1 was excluded from quantitative analysis if the pulp horn tip was incomplete. The specimen stage was then simply moved vertically to display the subjacent field 2 whilst viewing the active analogue image on the secondary monitor.

Field 3 was selected by using the fluorescence microscope drawing tube attachment (camera lucida) and 1 cm^2 graph paper. The microscope image was projected onto the graph paper with the with the cross-hair of the x20 objective placed on the outer border of the pulp section at the mid-point between the two pulp horns. The projected image was moved 10 cm vertically (a distance of 1 cm on the graph paper was equivalent to 56 μm on the pulp section) such that the cross-hair appeared within the mid-coronal pulp tissue region. The section was then moved in a horizontal plane until the mesio-buccal periphery of the pulp section was reached. Finally, the specimen stage was rotated so that the maximum length of the pulp periphery would be subject to quantitative analysis. In cases where this area of tissue was not suitable for analysis, a field was selected at a minimal distance above or below this predetermined point. The odontoblast layer and large nerve trunks were excluded from field 3 quantitative analysis.

A pilot study was undertaken to determine whether field 4 could be objectively selected. A maximum of eight fields could be defined within the mid-coronal pulp region, depending on the section size (Figure 2.4). Each of these fields could be reliably selected using the drawing tube and graph paper method. The microscopic image was re-positioned so that the cross-hair of the x20 objective was located on the outer border of the pulp section at the mid-point between the two pulp horns. The image was then moved 10 cm in a vertical direction and 5 cm horizontally to reach the first field. Subsequent fields were identified by moving the projected microscope image in a clockwise direction on the grid for either 10 cm in a horizontal direction to reach the mid-point of the adjacent field or 8 cm in a vertical direction to reach the mid-point of the field directly above or below. Using this systematic approach, PAS for PGP 9.5-labelled tissue was determined in up to eight fields for six primary and six permanent teeth. The location of large nerve trunks (regions of greatest PAS for PGP 9.5) was found to be highly variable between different tooth samples (data not shown). It was therefore felt that predetermined field selection for the mid-coronal pulp could be misrepresentative as large nerve trunks may on occasions be totally excluded from analysis. Thus it was decided that field 4 should be selected subjectively to include the region of greatest neural density.

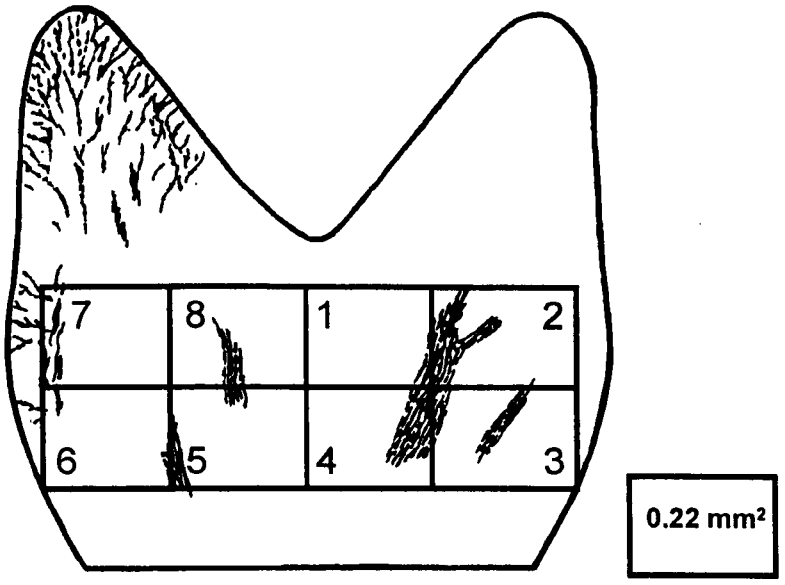


Figure 2.4. Schematic diagram illustrating potential field 4 selection within the mid-coronal pulp region.

2.5.3 Selection bias

Fields 1, 2 and 3 were identified using the excitation filter for the blue fluorescent marker, AMCA. Immunostaining of pulpal vasculature was uniformly intense and blood vessels were evenly distributed throughout the section. This approach therefore avoided the tendency to favour regions of greatest or brightest immunostaining for either PGP 9.5, neuropeptides or leucocytes. These labelled objects were not evenly distributed throughout the section and thus it was important to avoid selection bias. However, as stated previously, field 4 identification relied on a subjective approach and the excitation filter for PGP 9.5-labelled tissue was employed for the selection of this field.

2.5.4 Differences between sections

The study protocol demanded that, whenever possible, the first section on the slide should be used for quantitative assessment. This section was the nearest to the cut surface of the tooth half. However, it was anticipated that occasionally it would be necessary to employ the second or third section for quantitative assessment. Thus,

pilot studies were undertaken to determine whether section number had any effect on immunocytochemical measurements. Furthermore, in carious samples, it was possible that the relationship of the occlusal lesion to the pulp tissue could differ from that seen at the cut surface. Thus, the PAS for PGP 9.5, CGRP, UEIL and LCA was investigated in intact (n=4), moderately carious (n=4), and grossly carious (n=4) teeth, to determine whether the PAS of the various structures changed significantly according to which section was analysed and the degree of caries observed at the split surface. The percentage area of staining for each of the labelled structures was measured in all four fields for three 10 μm thick sections each cut 200 μm apart. One-way analysis of variance was used to test for any significant differences in the PAS for each immunolabelled structure according to the section number.

2.6 Additional analysis

2.6.1 Assessment of blood vessel innervation

Non-automated measurements were necessary to determine the percentage of vessels which were associated with nerve fibres. The mid-point of the pulp section was identified and an image showing the pulpal vasculature in the mid-coronal pulp region was captured on the secondary monitor using the x10 objective. The percentage of blood vessel profiles showing apparent innervation was recorded. This was determined by examining the spatial relationship between the wall of each blood vessel and closely aligned PGP 9.5-ir fibres. The x40 objective was employed for this purpose with fine adjustment of the focal plane. Positive innervation was only recorded when PGP 9.5-ir fibres actually appeared to intersect the vessel wall (Figure 2.5A). Close spatial alignment between PGP 9.5-ir fibres and the vessel wall was not recorded as a positive innervation (Figure 2.5B). The percentage of "innervated" blood vessels which also demonstrated a peptidergic innervation was then determined for each neuropeptide using the same criteria.

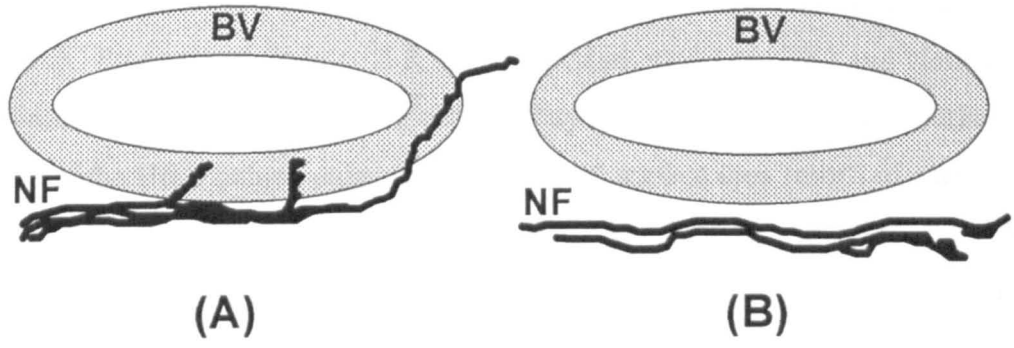


Figure 2.5. Schematic diagram showing pulpal blood vessels classified as having a positive innervation (A) and an absent innervation (B). BV = blood vessel wall, NF = nerve fibre.

2.6.2 Qualitative assessment

Following quantitative assessment, each of the three sections on every slide was examined systematically using x10, x20 and x40 objectives. Sections were viewed using all three excitation filters. Leucocyte distribution, blood vessel morphology, spatial associations between the various labelled structures as well as the distribution of overall and peptidergic innervation were all closely investigated. A descriptive record was kept for each sample describing the salient anatomical features. Photomicrographs were then taken to provide a permanent record of the most notable observations.

2.6.3 Measurement of tissue section size

In order to determine the proportion of tissue from each sample that was subject to quantitative analysis, it was necessary to measure the surface area of the whole coronal pulp section. Following full analysis and photography, slides that had been previously immunolabelled for SP + PGP 9.5 + UEIL were placed in 0.1M PBS for 60 min to facilitate the removal of the coverslips. The slides were then washed again in PBS for 30 min and were stained for haematoxylin and eosin (H&E) using an automatic Shandon Linear Stainer. Slides were then dried and coverslipped using coverslipping resin (Medim UK, Godstone, UK).

Sections were viewed using a Nikon Optiphot-2 microscope (X2T-Ph-21N, Nikon) with the x2 objective. The image was directed through an RGB colour camera (TK-1070E, JVC Professional, Industrial & Scientific Marketing Ltd, UK) and viewed on the secondary monitor. The free-hand draw facility of IPP was employed to outline to periphery of the H&E stained pulp section. The total surface area (mm^2) of each pulp section could then be calculated by the computer software. Using these data, the overall proportion of pulp tissue that had been subject to analysis could be estimated for each sample as follows:

$$\text{Percentage of tooth pulp analysed} = \frac{\text{sum of area of all four fields}}{\text{total section area}} \times 100$$

2.7 Assessment of reproducibility

2.7.1 Caries assessment

Four weeks following initial caries assessment, all specimens were re-examined by the same investigator and the degree of caries categorised for a second time. The level of intra-rater agreement between the initial and repeat assessments was measured using unweighted kappa statistics. In cases where there was a disagreement, the specimen was carefully re-examined and a final caries rating ascribed.

2.7.2 Measurement of tissue section size

Intra-operator repeatability for measuring the tissue section area was addressed by the same investigator re-measuring 12 H&E sections four weeks after the initial measurement had taken place. The initial and repeat area measurements were first plotted on scatter graphs to visualise the linear association between the two measurements. Pearson correlation coefficients were then calculated to determine the strength of the agreement between the initial and repeat measurements. In addition, the mean percentage difference, or percentage error (d_p) between the initial measurements (m_1) and the replicate measurements (m_2) was determined as follows:

- the difference (d) between each set of repeat measurements was calculated as, ($m_1 - m_2$)

- each difference was taken as a positive value and the sum of the differences was determined as, Σd

- the sum of both the first measurements, $\Sigma(m_1)$, and repeat measurements, $\Sigma(m_2)$, were calculated

- the mean percentage difference between the first and repeat measurement (d_p) was calculated as, $d_p = \frac{\Sigma d}{(\Sigma m_1 + \Sigma m_2) \div 2} \times 100\%$

A working example of this methodology is given in section 3.5.2.

2.7.3 Quantification of percentage area of staining

Pilot studies were also undertaken to determine the combined repeatability of field selection and image analysis methodology for the quantification of the PAS for PGP 9.5, CGRP, UEIL and LCA. The tissue employed for these investigations was labelled either for PGP 9.5 + CGRP + UEIL or for PGP 9.5 + LCA + UEIL. A total of 12 sections were analysed as outlined in section 2.4 and the PAS for each marker was recorded in all four fields of analysis.

The same slides were then re-assessed by the same investigator four weeks after the initial assessment. Field selection and image thresholding were performed again in order to obtain a repeat measurement for the PAS for PGP 9.5, CGRP, UEIL and LCA. Thus, in total, there were 16 different sets of 12 repeat measurements. The methodology employed to assess the repeatability of these quantifications was the same as described above in section 2.7.2.

2.7.4 Blood vessel counts

The repeatability of the method for counting blood vessels was determined using the same approach described for the section size data. Repeat vessel counts were undertaken for all four fields using 12 sections, one month after the initial count. The percentage error between the initial and replicate counts was determined as described previously.

2.7.5 Assessment of blood vessel innervation

Reproducibility for the evaluation of vascular innervation was also determined by re-examining 12 sections four weeks after the initial assessment. Slides stained for PGP 9.5 + CGRP + UEIL were employed. Saved x10 images of the previously analysed sections were used as a reference point in order to select approximately the same area for analysis. The repeatability of the method was determined using the same approach as described above.

2.8 Data processing

2.8.1 Data processing software

All data were entered into a PC (Viglen Dossier 486 SD) using the software package Statistical Package for Social Sciences v6.1 (SPSS Inc. Chicago, USA). Graphs and charts were produced using SPSS or Microsoft Excel v5.0 (Microsoft Corporation, USA). References were entered into a designated bibliographic database (Reference Manager v6, Research Information Systems, Carlsbad, USA).

2.8.2 Data handling

Prior to data entry, some preliminary calculations were necessary. Total counts for blood vessels had to be given per standard field size (0.22 mm^2) in order to allow a valid comparison between areas of interest of different sizes. Therefore, blood vessel counts were calculated in proportion to the ratio between the standard field size (0.22 mm^2) and the actual AOI, using the following formula:

$$\text{corrected vessel count} = \frac{0.22 \times \text{actual vessel count}}{\text{AOI}}$$

Data were entered manually from data collection sheets and numbers were rounded up to two decimal places. In the case of missing data, SPSS applied the system-missing value which was automatically excluded from subsequent statistical analysis. A database was set up for each staining regime. The "compute" function within SPSS was employed to transform certain values: the percentage area of PGP 9.5-ir tissue

each neuropeptide by the TAS of PGP 9.5. In addition, logarithmic transformations of some data were performed where necessary (see, section 2.8.4).

Extreme values or outliers were identified by obtaining a list of the five largest and smallest values for each variable. These were then re-validated to ensure that no errors had been made in data recording or entry.

2.8.3 Data presentation

All data have been presented in tables and graphs in their raw form including cases where logarithmic transformations were undertaken prior to statistical analysis.

2.8.4 Data screening

All data were initially screened to assess their distribution prior to embarking on any statistical analysis. Continuous data were plotted as frequency histograms to determine Normality. In cases where a skewed distribution was observed, the data were transformed using a Log_{10} transformation. All percentage data were assumed to have a skewed distribution and logarithmic transformations were performed accordingly. In all cases, logarithmic transformation of the data produced approximate Normality, as illustrated in Figures 2.6 and 2.7.

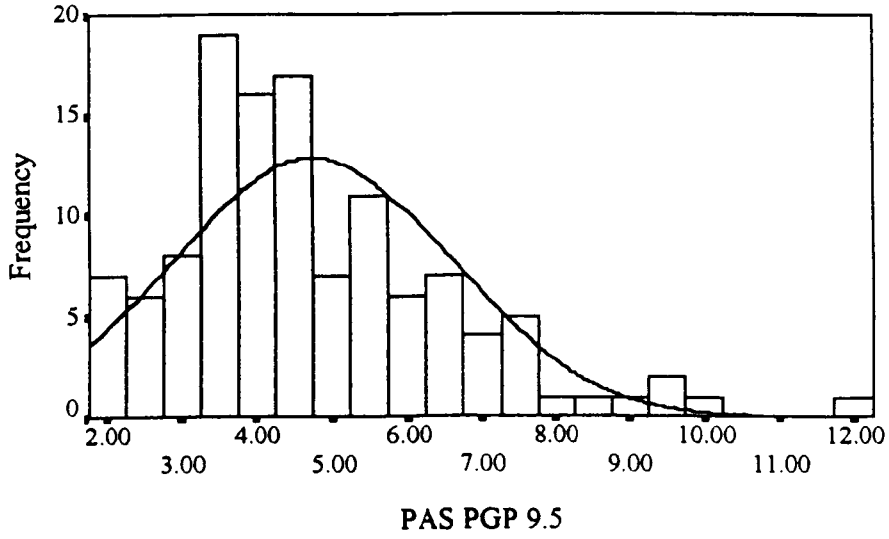


Figure 2.6. Frequency histogram showing percentage areas of staining for PGP 9.5 in field 3 of 124 samples.

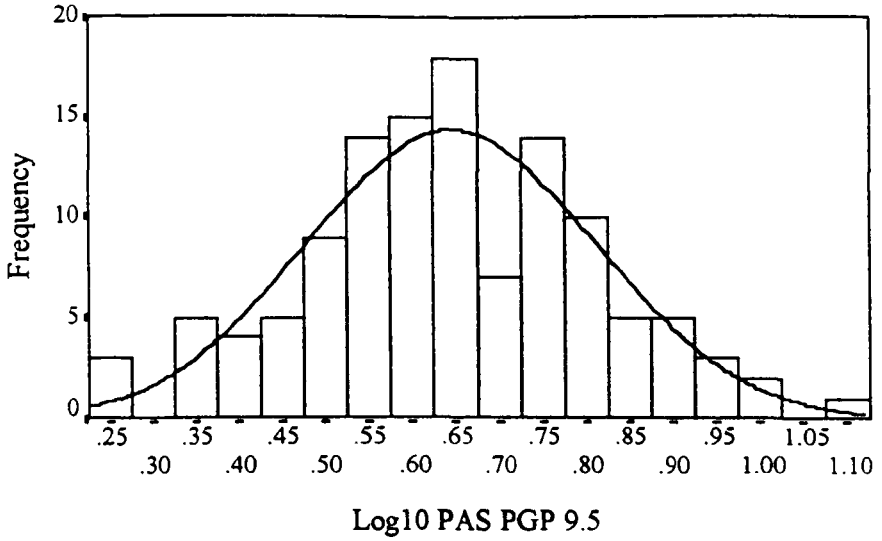


Figure 2.7. Frequency histogram showing percentage areas of staining for PGP 9.5 in field 3 of 124 samples after $\log_{10}$ transformation.

2.8.5 Preliminary statistical analysis

It was hypothesised that a number of method- and tooth-related variables could potentially influence quantitative findings. These included the effect of extraction sequence and the delay until immersion fixation on neuropeptide expression (see, section 2.1.3). In addition, it was considered that overall neural density could be dependent on the age of the subject (see, section 1.4.2). Therefore, some preliminary analyses were undertaken to determine whether these factors had influenced PAS data to any degree.

In order to determine whether the extraction order had any effect on neuropeptide expression, the mean percentage area of PGP 9.5-labelled tissue that was also labelled for CGRP, SP, VIP or NPY in sound permanent teeth from first-quadrant extractions was compared with mean data obtained for sound permanent teeth from third-quadrant extractions. An independent sample *t*-test on log-transformed data was used to test for statistically significant differences in PAS data between the two extraction groups, using field 1 data.

The effect of the time period between forceps application and immersion fixation on neuropeptide expression was investigated by correlating the mean percentage area of

The effect of the time period between forceps application and immersion fixation on neuropeptide expression was investigated by correlating the mean percentage area of PGP 9.5-labelled tissue that was also labelled for CGRP, SP, VIP or NPY in field 1 of intact permanent teeth with the time taken from forceps application until immersion fixation. Data for the PAS for each neuropeptide were plotted against time and a Pearson correlation coefficient was determined to measure the strength of this association.

In order to determine if age had any significant effect on overall neural density, the association between age and PAS for PGP 9.5 was investigated for intact primary and permanent teeth. Data for the PAS for PGP 9.5 were plotted against age, and this was carried out separately for primary and permanent samples using data from each of the four fields of analysis. In addition, Pearson correlation coefficients were determined for each association.

2.8.6 Statistical analysis of main results

Throughout the project statistical advice was sought from the Statistical Services Department of Sheffield University, the same statistician was consulted throughout the project. Parametric methods of analysis were employed whenever possible, as these are generally considered more satisfactory than non-parametric tests (Altman, 1991), hence the need for Normally distributed data. The main statistical analyses performed are outlined below but further detail is given in the relevant text.

Two-way analysis of variance was employed to test for statistically significant differences for the dependant variables (mean PAS for PGP 9.5, neuropeptides, UEIL and LCA) according to the two independent variables: tooth type and the degree of caries. Where appropriate, this was followed by Tukey's test for multiple pairwise comparisons of mean values in order to determine whether there were any significant differences between specific subgroups (Lownie *et al.*, 1998). The significance levels were set at $P<0.05$.

Associations between neuropeptide expression and vascularity or leucocyte accumulation were determined by correlating the PAS for neuropeptides with the PAS for UEIL or LCA respectively. In addition, associations between neuropeptide expression and blood vessel number were determined by correlating the PAS for neuropeptides with the blood vessel count. Logarithmically transformed data sets were plotted as scatter graphs and Pearson correlation coefficients were determined for each association.

Finally, an independent sample *t*-test was used to test for any statistically significant difference in the mean PAS for PGP 9.5, neuropeptides, UEIL or LCA according to a positive or negative history of pain ($P<0.05$, on log-transformed data).

2.8.7 Null hypotheses

The null hypotheses to be tested by this investigation are as follows:

- there are no significant differences in overall pulpal innervation, neuropeptide expression, pulpal vascularity, vessel number, vascular innervation characteristics or leucocyte accumulation between primary and permanent teeth;
- there are no significant changes in any of the above parameters with the progression of caries;
- there is no significant correlation between neuropeptide expression and changes in vascularity, vessel number or leucocyte accumulation within carious samples;
- there is no significant difference in overall pulpal innervation density, neuropeptide expression, pulpal vascularity, or leucocyte accumulation between symptomatic carious teeth and asymptomatic carious teeth.

3. General methodological results

3.1 Experimental material

3.1.1 Sample rejection

A total of 176 second primary and first permanent mandibular molars, from 176 patients, were selected for study inclusion following preliminary clinical and radiographic assessment. Informed consent was granted in all cases. However, 28 primary and 24 permanent teeth (29.5% of the original sample) were subsequently rejected from the study. Table 3.1 outlines the reasons for exclusion of these samples.

Overall, the most common reason for sample rejection was the failure to remove an intact coronal pulp (13, 25%). This was a particular problem in primary molars. Other reasons for specimen rejection included, in decreasing order of frequency: pulp necrosis evident on tooth splitting (11, 21.2%); pulp necrosis evident following microscopic examination (10, 19.2%); unsuccessful tooth splitting (7, 13.5%); poor tissue sectioning (5, 9.6%); an extraction time in excess of 60 sec (4, 7.7%), and failure to adhere to the protocol for tissue fixation and processing (2, 3.9%).

Table 3.1. Reason for sample rejection

Reason for rejection	Primary tooth n (%)	Permanent tooth n (%)	Total n (%)
Prolonged extraction time	0 (0)	4 (7.7)	4 (7.7)
Unsuccessful splitting	4 (7.7)	3 (5.8)	7 (13.5)
Clinical necrosis	7 (13.5)	4 (7.7)	11 (21.2)
Poor coronal pulp removal	11 (21.2)	2 (3.9)	13 (25.0)
Poor tissue section	3 (5.8)	2 (3.9)	5 (9.6)
Failure to adhere to protocol	1 (1.9)	1 (1.9)	2 (3.9)
Microscopic necrosis	2 (3.9)	8 (15.4)	10 (19.2)
Total	28 (53.8)	24 (46.2)	52 (100)

3.1.2 Experimental subgroups

In total, 62 primary and 62 permanent teeth were analysed. There was a similar number of specimens within each of the three caries subgroups as shown in Table 3.2.

Table 3.2. Number of samples according to the dentition and degree of caries

	Degree of caries			
Dentition	None (n)	Moderate (n)	Gross (n)	Total (n)
Primary	22	20	20	62
Permanent	19	22	21	62
Total	41	42	41	124

3.1.3 Patient-related variables

The main patient variables included age, gender and reported pain history. These are presented in Table 3.3 according to the dentition and degree of caries. The mean age of children from whom a primary molar was obtained was 4.9 years (±0.88) and ages ranged from 3.3 to 7.0 years. The mean age of children from whom a permanent molar was obtained was 9.7 years (±2.02) and ages ranged from 6.1 to 14.1 years. Age distributions for both primary and permanent teeth were similar within each of the three caries subgroups.

Thirty nine (62.9%) primary teeth and 33 (53.2%) permanent teeth were from female subjects. The majority of patients providing material for this study were white Caucasians, ethnic minority subjects provided less than 10% of the experimental material.

Pain was reportedly associated with 10 grossly carious primary teeth and 10 grossly carious permanent teeth. Thus, approximately half of all grossly carious samples from both dentitions were associated with a positive pain history. Very few moderately carious primary (n=2) or permanent (n=1) molars were reportedly painful. All intact teeth were asymptomatic.

Table 3.3. Patient-related variables according to the degree of caries

Variable	Degree of caries			
	None	Moderate	Gross	Total
Mean age (±SD) in years				
primary teeth	5.0 (±0.49)	4.8 (±0.76)	4.8 (±1.28)	4.9 (±0.88)
permanent teeth	9.8 (±1.77)	9.8 (±2.18)	9.6 (±2.16)	9.7 (±2.02)
Range (yrs)				
primary teeth	4.3-5.9	3.3-5.7	3.3-7.0	3.3-7.0
permanent teeth	7.3-12.3	6.1-14.0	6.8-14.1	6.1-14.1
Gender (n)				
Males				
primary teeth	12	6	5	23
permanent teeth	11	8	10	29
Females				
primary teeth	10	14	15	39
permanent teeth	8	14	11	33
Pain history (n)				
Negative				
primary teeth	22	18	10	50
permanent teeth	19	21	11	51
Positive				
primary teeth	0	2	10	12
permanent teeth	0	1	10	11

3.1.4 Tooth-related variables

The results for the extraction sequence (1st to 4th quadrant) and the time taken from forceps application until immersion fixation are presented in Table 3.4. Primary (34, 54.8%) and permanent (38, 61.3%) tooth samples were most commonly obtained as first quadrant extractions. However, third-quadrant extractions also comprised a large proportion of the experimental material. For both dentitions, there was a similar distribution of first- and third-order quadrant extractions within each caries subgroup. No fourth-quadrant extractions were included in the study and there was only one case of a second-quadrant extraction for each dentition.

The mean time from forceps application until immersion fixation for a primary tooth was 28.5 sec (±8.5, range 15-60) as compared to 35.2 sec (±13.8, range 20-60) for a permanent tooth. Statistical analysis showed that, whereas tooth type (dentition) had a significant effect on this time (P=0.001, ANOVA), the degree of caries did not have a significant effect (P=0.808, ANOVA).

Table 3.4. Tooth-related variables according to the degree of caries

	Degree of Caries			
Variable	None	Moderate	Gross	Total
Extraction sequence				
Primary teeth (n)				
first quadrant	12	10	12	34
second quadrant	0	0	1	1
third quadrant	9	10	8	27
fourth quadrant	0	0	0	0
Permanent teeth (n)				
first quadrant	13	11	14	38
second quadrant	0	1	0	1
third quadrant	6	9	8	23
fourth quadrant	0	0	0	0
Mean (±SD) time to fixation (sec)				
Primary teeth	25.2(±3.4)	31.9(±12.4)	28.4(±6.5)	28.5(±8.5)
Permanent teeth	40.5(±13.6)	31.4(±13.6)	34.4(±13.6)	35.2(±13.8)
Range of times to fixation (sec)				
Primary teeth	20-60	20-30	15-40	15-60
Permanent teeth	25-60	20-60	20-60	20-60

3.2 Immunocytochemistry

3.2.1 Weak or absent immunostaining

No positive staining could be obtained for ENK despite the use of several different antisera to this peptide. Furthermore, Professor J.M. Polak, co-author of a paper reporting the immunocytochemical identification of ENK in human tooth pulp, was contacted for advice (Casasco *et al.*, 1990a). She kindly provided a sample of the

antiserum employed in the original study (Leu-ENK raised in rabbit against human enkephalin) and incubation was performed using the suggested dilutions. However, specific staining for ENK was still not obtained thus analysis of this peptide could not be undertaken.

Positive labelling was seen for GAL and SOM, but immunoreactivity was very faint despite the use of high antibody concentrations. Preliminary staining of approximately 50 slides revealed only traces of positive immunolabelling for these two peptides which subsequently proved difficult to quantify. Therefore these neuropeptides were also omitted from the study.

3.2.2 Specificity controls

Findings relating to the various specificity controls are presented in the relevant chapters (see, sections 4.3.1, 5.3.1, 6.3.1 and 7.3.1). However, some representative photomicrographs showing positive staining for UEIL, LCA and CGRP and the corresponding immunohistochemical controls (showing no specfic staining) can be seen in Figure 3.1. A photomicrograph demonstrating an immunohistochemical control section for PGP 9.5 is provided in the next chapter (Fig. 4.3F).

3.2.3 Quality of immunostaining

Although the overall quality of immunostaining was excellent throughout the study, there was usually a small percentage of slides (1-3%) where one or more of the fluorescent reactions was of poor quality and presented either as very faint staining or with a large amount of autofluorescent debris on the specimen surface. If this occurred during the first staining run, spare tissue sections for the affected slides were stained during the second staining run. However, poorly stained slides from the second immunostaining batch had to be omitted from subsequent quantitative analysis.

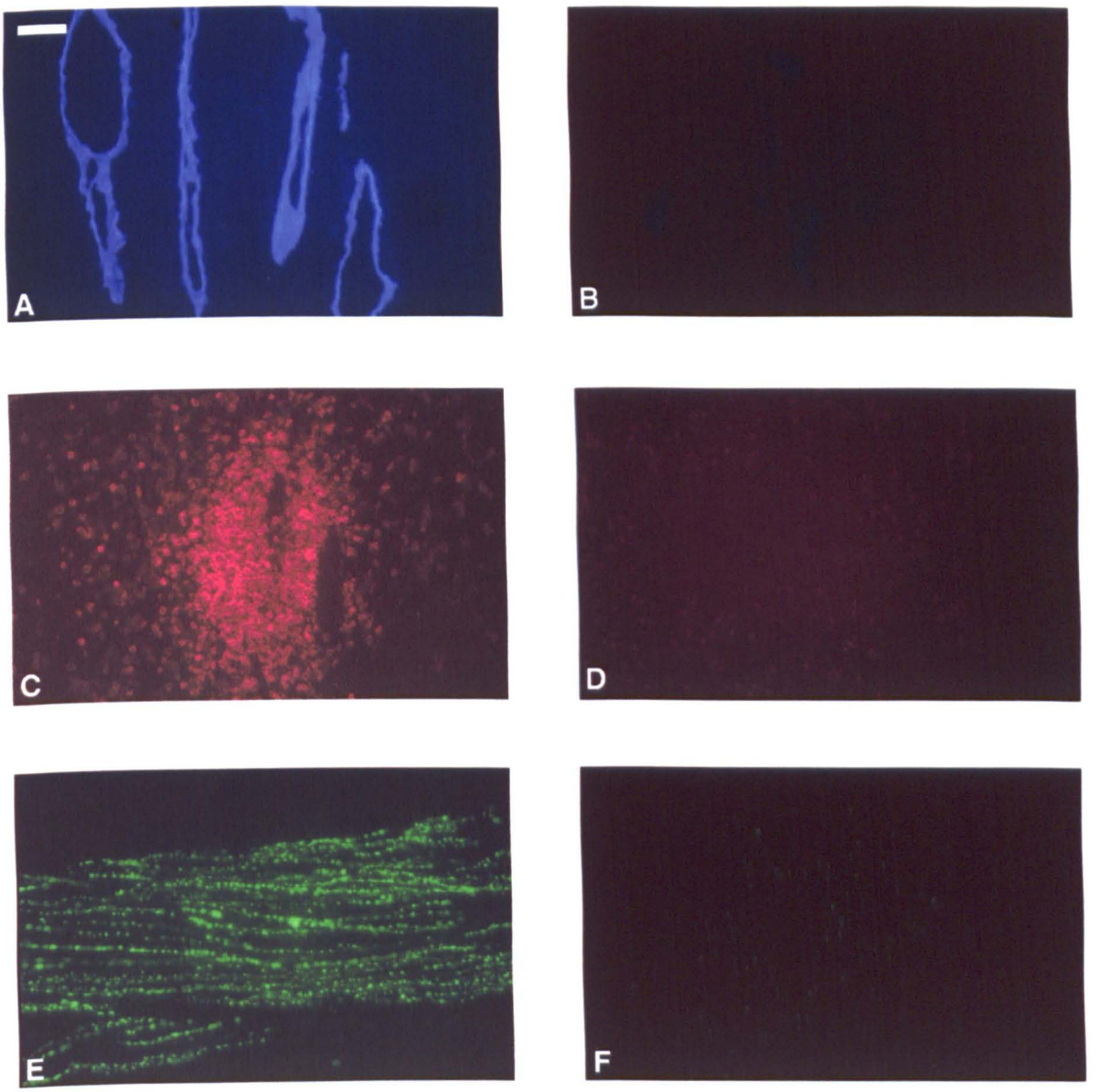


Figure 3.1. Photomicrographs showing pulp sections with positive labelling **(A,C,E)**, and corresponding histochemical controls with no specific labelling **(B,D,F)** for antibodies used in the study. **(A)** Positive labelling for UEIL (blue) and **(B)** corresponding control. **(C)** Positive labelling for LCA (red) and **(D)**corresponding control. **(E)** Positive labelling for CGRP (green) and **(F)** corresponding control. Scale bar = 15 μm **(E,F)**, 30 μm **(A-D)**.

3.3 Effect of methodological variables on staining measurements

3.3.1 Effect of section number

Three sections, each 200 μm apart, were analysed to determine whether the distance from the cut surface of the tooth sample had a significant effect on the PAS of any of the different immunolabelled structures (see, section 2.5.4). Data from four teeth from each of the three caries subgroups were included in the analysis. It was found that there were no statistically significant differences in mean PAS measurements according to section number ($P>0.05$, ANOVA on log transformed data). This was true for each caries subgroup, all four fields of analysis and every staining regime. Representative data are given in Table 3.6, which shows the mean (±SD) PAS, according to section number, for all labelled structures within field 1.

Table 3.6. Mean (±SD) percentage area of staining for different labelled structures within field 1 according to the section number and degree of caries

	Mean PAS (±SD)			
Immunolabelling/ degree of caries	First section (n=4)	Second section (n=4)	Third section (n=4)	*P*
PGP 9.5				
None	4.40 (±1.64)	4.72 (±1.22)	4.23 (±2.13)	0.92
Moderate	6.71 (±4.07)	6.79 (±4.11)	5.69 (±2.28)	0.89
Gross	8.21 (±1.69)	7.39 (±1.99)	8.01 (±2.45)	0.84
CGRP				
None	1.07 (±0.66)	0.94 (±0.71)	0.95 (±0.47)	0.95
Moderate	0.69 (±0.33)	0.48 (±0.22)	0.64 (±0.26)	0.56
Gross	2.12 (±1.21)	1.76 (±1.01)	1.94 (±1.13)	0.90
UEIL				
None	1.78 (±0.24)	2.09 (±0.54)	1.73 (±0.43)	0.45
Moderate	2.51 (±0.83)	2.88 (±0.92)	2.46 (±0.64)	0.73
Gross	2.65 (±1.10)	2.69 (±1.51)	3.12 (±1.71)	0.88
LCA				
None	0.10 (±0.09)	0.07 (±0.02)	0.13 (±0.09)	0.52
Moderate	0.24 (±0.05)	0.21 (±0.07)	0.28 (±0.18)	0.72
Gross	1.05 (±0.83)	0.92 (±1.14)	1.26 (±1.85)	0.43

P=ANOVA on log-transformed data

3.3.2 Effect of extraction sequence on neuropeptide expression

There were no significant differences in CGRP, SP, VIP or NPY expression between samples which were either first- or third-order extractions ($P>0.05$, independent sample *t*-test on log-transformed data) (see, section 2.8.5). Table 3.7 shows the mean PAS for neuropeptides in permanent teeth from both first (n=12) and third (n=6) quadrant extractions.

Table 3.7. Percentage area of PGP 9.5-labelled tissue that was also labelled for neuropeptides in field 1 of intact permanent teeth according to extraction sequence

Neuropeptide	Mean (±SD) PAS		
	First-quadrant extractions (n=12)	Third-quadrant extractions (n=6)	*P*
CGRP	27.14 (±21.40)	19.89 (±16.55)	0.319
SP	13.32 (±9.45)	12.73 (±12.26)	0.603
VIP	9.41 (±6.77)	6.70 (±9.84)	0.151
NPY	0.41 (±0.82)	0.32 (±0.77)	0.289

P=independent sample *t*-test on log-transformed data

3.3.3 Effect of delay until fixation on neuropeptide expression

No significant correlation was found between neuropeptide expression (the percentage of PGP 9.5-labelled tissue that was also labelled for peptide) and the time taken from extraction until immersion fixation (see, section 2.8.5). This was true for all neuropeptides. Pearson correlation coefficients were all very low, indicating a poor association between the two variables ($r = -0.09$ to 0.08; $P>0.05$). A representative scatter plot showing the relationship between SP expression in field 1 and time from extraction to immersion fixation for intact permanent teeth is shown in Figure 3.2 ($r= - 0.09$, $P=0.702$).

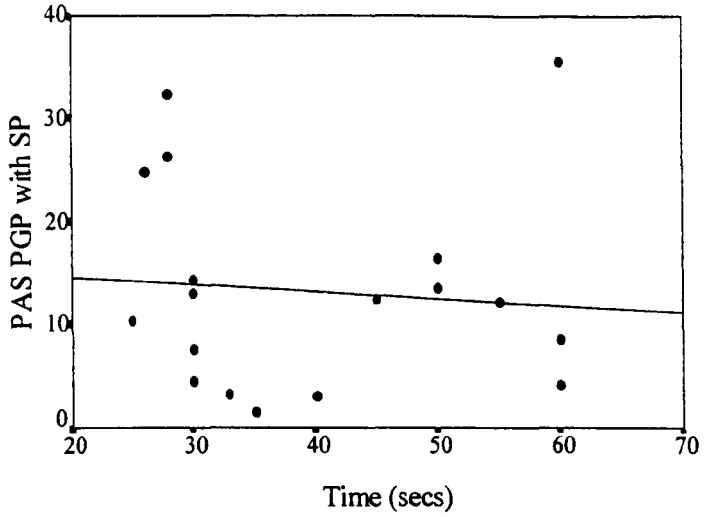


Figure 3.2. Scatter plot with regression line to show the relationship between substance P expression and time taken from extraction until immersion fixation (data from field 1 of 18 intact permanent teeth).

3.3.4 Effect of age on neural density

No significant correlation was found between age and neural density (PAS for PGP 9.5) in any of the four fields analysed (see, section 2.8.5). This was true for both intact primary and intact permanent teeth. Pearson correlation coefficients were low, indicating a poor association between the two variables (r = -0.26 to 0.23; $P>0.05$). A representative scatter plot showing the relationship between the PAS for PGP 9.5 (field 3) and age for both dentitions is shown in Figure 3.3 (r = 0.06, $P=0.799$, primary teeth; r = -0.03, $P=0.887$, permanent teeth).

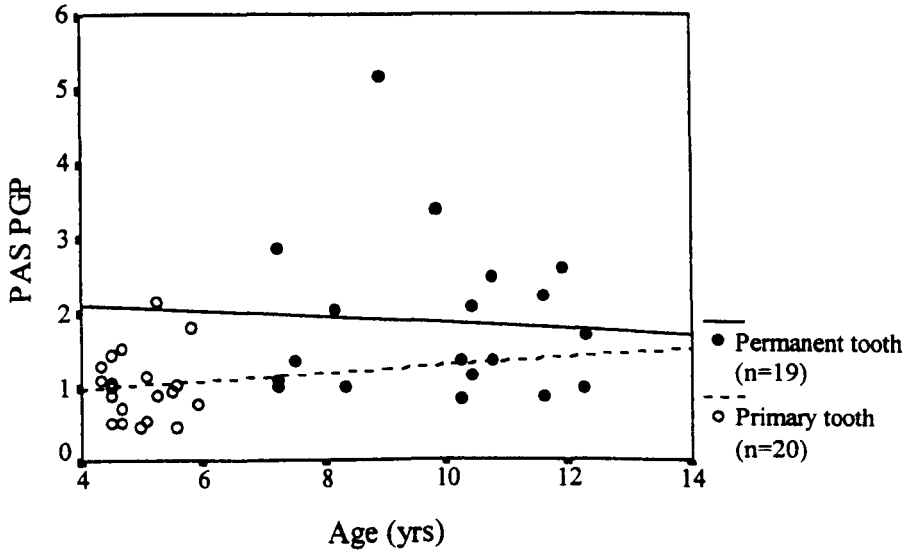


Figure 3.3. Scatter plot with regression lines to show the relationship between neural density (PAS for PGP 9.5) and patient age in field 3 of intact primary and permanent teeth.

3.4 Tissue section size

The mean coronal pulp section area for a primary tooth was 7.55 mm^2 (±2.08; range, 4.39-13.12) and was 9.30 mm^2 (±2.27; range, 4.80-14.00) for a permanent tooth. The difference in mean pulp section size between the two dentitions was found to be statistically significant ($P<0.001$, independent sample *t*-test).

The mean section area for the pulp tip region (field 1) was found to be 0.16 mm^2 (±0.03; range, 0.11-0.22 mm^2) and there was no significant difference between the two dentitions ($P>0.05$, independent sample *t*-test). Therefore, on average, 10.86% of a primary coronal pulp, and 8.82% of a permanent coronal pulp, were subject to quantitative analysis. This was calculated as shown below:

- Percentage of primary tooth pulp analysed = $\frac{\text{sum of area of all four fields}}{\text{total section area}} \times 100$

$$= \frac{0.16 + 3(0.22)}{7.55} \times 100$$

$$= 10.86\%$$

- Percentage of permanent tooth pulp analysed = $\frac{0.16 + 3(0.22)}{9.30} \times 100$

$$= 8.82\%$$

3.5 Assessment of reproducibility

3.5.1 Caries assessment

Table 3.8 shows the initial and repeated assessment for the degree of caries in 124 samples (see, section 2.7.1). There was total agreement between the two assessments in 121 cases (97.6%). The measure of agreement, using the unweighted kappa, was found to be very high indicating that this approach was very reproducible ($K = 0.97$, unweighted Kappa value) (Altman, 1991).

Table 3.8. Number of samples in each caries subgroup at initial and repeat assessment of the degree of caries

Repeat assessment	Initial assessment			
	No caries	Moderate caries	Gross caries	Total
No caries	40	0	0	40
Moderate caries	1	40	0	41
Gross caries	0	2	41	43
Total	41	42	41	124

3.5.2 Measurement of tissue section size

The total surface area of 12 H&E stained sections was measured twice using the image analysis free-hand draw facility (see, section 2.6.3). Table 3.9 gives the data for these 12 sets of initial (m_1) and repeat (m_2) area measurements and the difference between the two measurements.

Table 3.9. Data for initial (m_1) and repeat (m_2) measurements of tissue section size

Sample no.	m_1 (mm^2)	m_2 (mm^2)	$m_1 - m_1$ (mm^2)
1	6.26	6.40	-0.14
2	8.05	7.95	0.10
3	6.10	6.13	-0.03
4	5.80	5.76	0.04
5	7.05	6.98	0.07
6	4.69	4.65	0.04
7	5.80	5.74	0.06
8	5.42	5.58	-0.16
9	6.75	6.48	-0.09
10	6.03	5.88	0.15
11	6.16	6.22	-0.06
12	6.96	6.79	0.17

Using the method described in section 2.7.2, the mean percentage difference (d_p) between the initial and repeat measurements was determined as follows:

- $d_p = \frac{\sum d}{(\sum m_1 + \sum m_2) \div 2} \times 100\%$
- $d_p = \frac{1.11}{(75.07+74.56) \div 2} \times 100\%$
- $d_p = \frac{1.11}{74.82} \times 100\%$
- $d_p = 1.48\%$

Therefore the mean percentage difference between the initial and repeat section size measurements was ±1.48%. This low figure, taken together with the high Pearson correlation coefficient, calculated as being 0.99, indicated that this technique was highly repeatable.

3.5.3 Quantification of percentage area of staining

Data for the initial and repeat measurements of the PAS for several different immunolabelled structures are shown in Table 3.10 (see, section 2.7.3). Twelve sections, from each staining regime, were re-analysed and the findings are presented separately for each of the four different fields of analysis. In addition, the Pearson correlation coefficient and the mean percentage difference between the initial and repeat measurements of PAS are given. Representative scatter plots, showing the linear relationship between the initial and repeat PAS measurements for both PGP 9.5 and CGRP in field 1 are shown in Figures 3.4 and 3.5 respectively. It can be seen that, overall, there were only small differences between the first and the replicate PAS measurements. These differences ranged from ±0.82% to 7.47% of the mean of the two measurements. Reproducibility was the most constant for the analysis of the PAS for PGP 9.5, as a similar percentage error was seen in all four fields. Generally, reproducibility appeared to be optimum for field 1 analysis, and the poorest reproducibility was seen for field 4 analysis.

Table 3.10. Data for 12 initial and repeat measurements of percentage area of staining according to staining regime and field

Staining regime/ field	Initial PAS mean (±SD)	Repeat PAS mean (±SD)	Mean % difference	r
PGP 9.5 + TR				
field 1	7.73 (±2.80)	7.85 (±2.34)	±4.27%	0.94
field 2	3.85 (±1.77)	3.34 (±1.56)	±4.29%	0.87
field 3	1.69 (±0.66)	1.64 (±0.94)	±4.18%	0.94
field 4	4.50 (±1.80)	4.35 (±2.04)	±4.44%	0.96
CGRP + FITC				
field 1	1.29 (±0.97)	1.16 (±0.86)	±3.26%	0.96
field 2	0.82 (±0.66)	0.80 (±0.66)	±4.32%	0.95
field 3	0.41 (±0.20)	0.39 (±0.20)	±7.41%	0.67
field 4	0.58 (±0.35)	0.60 (±0.34)	±7.47%	0.96
UEIL + AMCA				
field 1	2.31 (±0.90)	2.38 (±1.05)	±2.90%	0.83
field 2	1.66 (±0.72)	1.77 (±0.77)	±4.49%	0.86
field 3	2.10 (±0.89)	2.05 (±0.84)	±2.77%	0.98
field 4	2.91 (±1.21)	2.67 (±1.49)	±5.42%	0.84
LCA + TR				
field 1	0.14 (±0.08)	0.14 (±0.11)	±0.82%	0.99
field 2	0.36 (±0.55)	0.34 (±0.54)	±2.69%	0.98
field 3	0.12 (±0.31)	0.13 (±0.32)	±4.07%	0.94
field 4	0.14 (±0.37)	0.12 (±0.34)	±5.54%	0.97

r=Pearson correlation coefficient

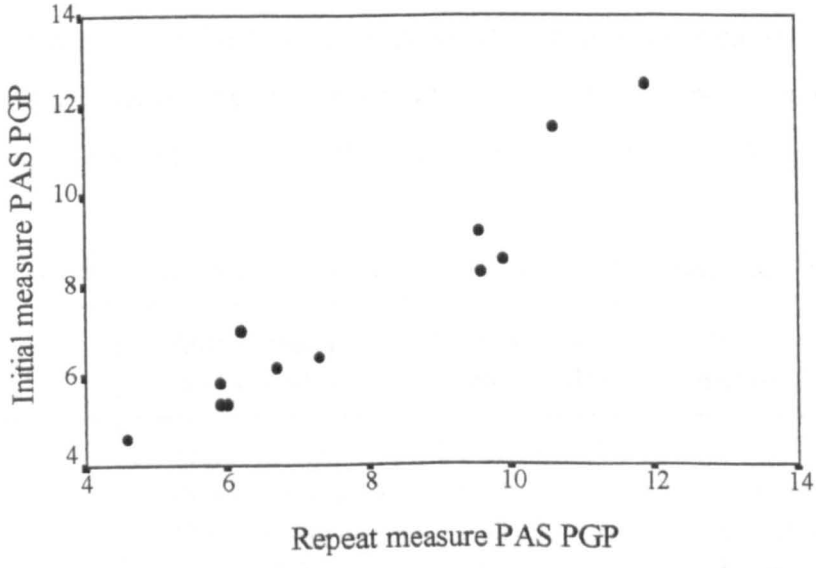


Figure 3.4. Scatter plot showing the relationship between 12 initial and repeat measurements of percentage area staining for protein gene product 9.5 in field 1.

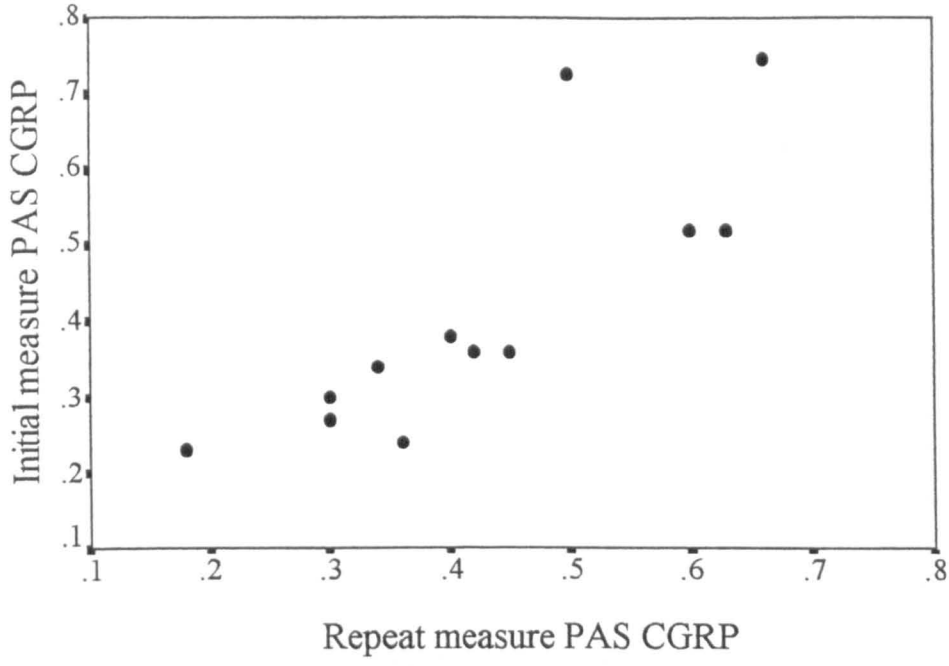


Figure 3.5. Scatter plot showing the relationship between 12 initial and repeat measurements of percentage area staining for calcitonin gene-related peptide in field 1

3.5.4 Blood vessel counts

Table 3.11 gives the results for 12 initial and replicate counts of blood vessels in each of the four fields. Figure 3.6 is a representative scatter plot showing the linear relationship between initial and repeat blood vessel counts within field 2. It can be seen that, overall, there was very little difference between the initial and repeat vessel counts, as indicated by the low mean percentage difference data and high Pearson correlation coefficients. The least error between initial and repeat counts was found for field 1 data (percentage mean difference between counts = ±1.33%).

Table 3.11. Data for 12 initial and repeat counts of blood vessels according to field

Field	Initial count mean (±SD)	Repeat count mean (±SD)	Mean % difference	r
Field 1	20.08 (±5.18)	20.17 (±5.52)	±1.33%	0.99
Field 2	16.55 (±5.66)	16.83 (±5.78)	±2.07%	0.98
Field 3	20.45 (±4.48)	20.82 (±4.45)	±2.13%	0.98
Field 4	6.90 (±2.02)	6.90 (±2.38)	±2.04%	1.00

r=Pearson correlation coefficient

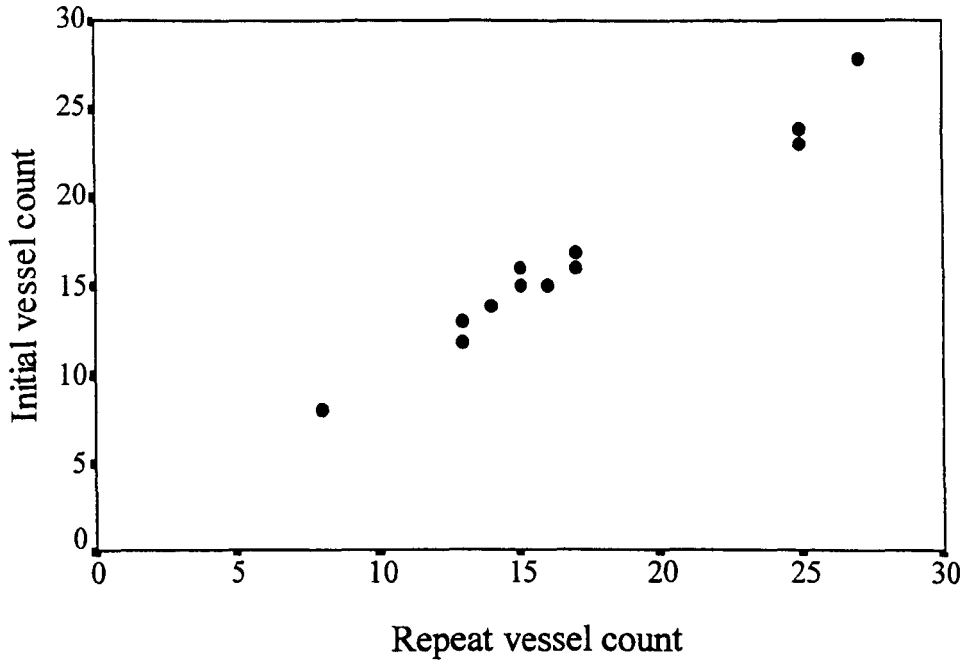


Figure 3.6. Scatter plot showing the relationship between 12 initial and repeat blood vessel counts in field 2.

3.5.5 Assessment of blood vessel innervation

Twelve pulp sections were re-assessed to determine intra-operator reproducibility for the assessment of blood vessel innervation using predetermined criteria (see section 2.6.1). From the initial examination, 13.2% (±3.80) of blood vessel profiles were considered to be innervated by PGP 9.5-ir fibres and 100% of these were determined to be associated with CGRP-ir fibres. Following a repeat examination, 13.8% (±4.50) of blood vessels were assessed as being innervated by PGP 9.5-ir fibres and again 100% of these vessels were recorded as having a positive association with CGRP-ir nerve fibres. The mean percentage difference between the initial and repeat assessment for PGP 9.5-related vascular innervation was ±1.15% and the Pearson correlation coefficient was 0.98. There was total agreement between initial and repeat assessments of CGRP innervation thus the mean percentage difference was 0% and the Pearson correlation coefficient was 1.0.

4. Investigation of overall pulpal innervation

4.1 Introduction

4.1.1 Visualisation of intradental innervation

Early anatomical investigations of intradental innervation relied on a variety of silver impregnation techniques (Powers, 1952; Philipp and Chicago, 1955; Rapp *et al.*, 1957; Fearnhead and Linder, 1956). These studies clearly demonstrated the overall distribution of pulpal nerves but, due to methodological limitations, they failed to show fine nerve fibres or to conclusively reveal the presence of nerves within calcified tissue. Axonal transport studies and autoradiography, although not applicable in humans, later superseded silver impregnation methods and provided a more comprehensive picture of pulp/dentine innervation (Byers and Kish, 1976; Byers and Matthews, 1981; Ibuki *et al.*, 1996).

Over the past two decades, advances in a variety of immunocytochemical techniques have greatly facilitated the investigation of intradental nerve morphology and distribution. Attempts to visualise the entire nerve population have employed a range of markers including antisera to S-100 protein (Maeda *et al.*, 1985; Sato *et al.*, 1988; Lombardi and Castellucci, 1989; Sato *et al.*, 1989; Lin *et al.*, 1994; Atsumi *et al.*, 1999), neuron specific enolase (Fristad *et al.*, 1994) and neurofilament protein (Maeda *et al.*, 1986; Sato *et al.*, 1988; Sato *et al.*, 1989). More recently, the use of protein gene product 9.5 (PGP 9.5) has been favoured (Ramieri *et al.*, 1990; Heyeraas *et al.*, 1993; Maeda *et al.*, 1994).

4.1.2 Protein gene product 9.5

Protein gene product 9.5 was first identified as a new brain-specific protein following high resolution two-dimensional mapping of soluble proteins from different human organs (Jackson and Thompson, 1981). It has a molecular weight of 27,000 and constitutes 1-2% of soluble brain proteins (Doran *et al.*, 1983). Biochemical characterisation of PGP 9.5 has indicated a sequence homologous to ubiquitin

carboxyl-terminal hydrolase, implying a role in the ubiquitin-proteasome pathway (Wilkinson *et al.*, 1989).

As a major soluble protein component of neuronal cytoplasm, PGP 9.5 is widely distributed throughout the neuroendocrine system of several different species. It is present in neurons, nerve axons and all neuroendocrine cell types except epithelial endocrine cells of the gut (Thompson *et al.*, 1983). More recent studies have also reported PGP 9.5 immunoreactivity in other structures including epidermal Langerhans cells (Hsieh *et al.*, 1996), Schwann cells (Rice *et al.*, 1993) and pulpal odontoblasts (Christensen *et al.*, 1993). Interestingly, despite the abundance of PGP 9.5, its function remains enigmatic.

Antiserum to PGP 9.5 has been widely applied for immunocytochemical demonstration of nerve fibres and has proved an excellent general neuronal marker (Levy *et al.*, 1989; Wang *et al.*, 1990; Rice *et al.*, 1993; Astbäck *et al.*, 1997). Comparative studies, in a variety of tissues and species, have shown immunostaining for PGP 9.5 to be qualitatively and quantitatively superior to the staining achieved with other "general" neuronal markers including neuron specfic enolase, neurofilament protein and S-100 protein (Rode *et al.*, 1985; Gulbenkian *et al.*, 1987; Karanth *et al.*, 1991; Fristad *et al.*, 1994). In particular, visualisation of small varicose nerve terminals has proved more consistent with PGP 9.5-labelling than with other neuronal markers. Thus PGP 9.5 is generally considered the optimal universal axonal marker.

Ramieri and co-workers were the first to describe the use of PGP 9.5 within human oral and dental tissues (Ramieri *et al.*, 1990). A later investigation using human foetal teeth reported that labelling for PGP 9.5 closely correlated with that for neuron specific enolase and neurofilament protein (Christensen *et al.*, 1993). In addition to human studies, antiserum to PGP 9.5 has been used to demonstrate the pulpal innervation of cat (Heyeraas *et al.*, 1993), and rat (Fristad *et al.*, 1994).

4.1.3 Rationale for study

A review of the literature has revealed a paucity of information regarding some anatomical features of pulpal innervation. In particular, quantitative data about primary tooth pulp innervation are sparse and there have been few comparative studies with the permanent dentition (see, section 1.4.). It is conjectured that inter-dentition differences in overall neural density and distribution of pulpal nerves may have some biological and clinical relevance.

Another area of interest is the effect of caries on intradental nerve morphology. To date, caries-related neural changes within the primary dentition have received little attention. However, recent immunocytochemical investigations of human permanent teeth have revealed marked changes in nerve morphology with the progression of caries (Yoshiba *et al.*, 1998; Sakurai *et al.*, 1999). It is clinically recognised that some carious teeth are more sensitive than sound teeth, yet there have been few attempts to correlate neural changes with the degree of caries or reported symptoms.

4.1.4 Aims and objectives

The overall aim of the present study was to investigate the distribution of nerves in human tooth pulp with the use of antiserum to PGP 9.5. The specific objectives were as follows:

- to carry out a qualitative and quantitative comparison of overall pulpal innervation between primary and permanent teeth in different anatomical regions;
- to investigate the effect of caries progression on pulpal nerve morphology and distribution in both dentitions;
- to seek a correlation between neural density and the reported pain history.

4.2 Materials and methods

Details regarding the overall experimental approach have been previously outlined in chapter two. Therefore only a few points, specific to the use of PGP 9.5, will be addressed in this section.

Specificity controls were performed by incubating spare tissue sections with the polyclonal PGP 9.5 antibody which had been preabsorbed with the respective peptide (10 nmol/ml). The specificity of the monoclonal antibody was determined by incubating the tissue with the antibody dilutent, non-immune serum and secondary antibody only as there was no commercially available PGP 9.5 peptide (section 2.2.5).

Descriptive findings for the distribution of PGP 9.5-labelled nerves were based on the analysis of 15 sections from each tooth sample. Twelve of these sections were stained using a monoclonal PGP 9.5 antibody (plus antisera to CGRP/SP/VIP/NPY + UEIL), and three sections were stained using a polyclonal PGP 9.5 antibody (plus antisera to LCA + UEIL) (for details of multiple staining regimes see, section 2.2.6).

Quantitative analysis for PGP 9.5-labelling (PAS for PGP 9.5) was undertaken on a total of four sections from each tooth sample, one section being selected from each of the above four neuropeptide staining regimes. One way analysis of variance was then carried out to determine whether there were any significant differences, in neural density, between these four sections. Throughout the text, the phrase "innervation density" is used synonymously with the term "PAS for PGP 9.5."

4.3 Results

4.3.1 Specificity controls

The reaction was considered specific as no positive immunolabelling was obtained either with the use of the primary antibody preabsorbed with the pure peptide or with the replacement of the primary antibody with non-immune serum (Fig. 4.3F). However, occasional non-specific staining was seen within isolated round cells within blood vessels. These were considered to be erythrocytes which are known to autofluoresce, and were thus excluded from any quantitative analysis.

4.3.2 Comparison between primary antisera

Immunostaining with the polyclonal PGP 9.5 antibody and FITC generally produced a brighter and denser immunoreactivity than that achieved using the monoclonal antibody and TR. However both regimes clearly revealed the overall distribution of

nerve fibres within the coronal pulp and provided good labelling for subsequent image analysis and photomicroscopy.

4.3.3 Comparison between different sections

There were no significant differences in the mean PAS for PGP 9.5 recorded for each of the four different sections obtained from every pulp sample ($P>0.05$, ANOVA). This was true for all fields analysed and for each caries subgroup. Thus the means of the four measurements were employed for subsequent statistical analysis and data presentation.

4.3.4 Qualitative observations in intact teeth

The tip of the pulp horn was the most densely innervated region in both dentitions but nerve fibres appeared slightly more abundant within permanent teeth (Fig. 4.1A,B). Nerve fibres were mostly aligned parallel to the pulp horns and these appeared to aborise on reaching the pulp periphery. Fibres in this region were predominantly fine and beaded although some smooth-surfaced fibres were also present. In the region below the pulp horn tip, PGP 9.5-ir fibres were aligned approximately parallel to the longitudinal axis of the tooth and presented as thin bundles or single varicose or smooth fibres. Permanent teeth also appeared more densely innervated than primary teeth in this part of the pulp.

Throughout the coronal pulp, the SOP appeared more dense in permanent teeth than in primary teeth (Fig. 4.1C,D). However, both dentitions showed an abundance of fine, varicose and branching PGP 9.5-ir fibres extending across the cell-free zone towards the odontoblast layer. In tissue sections where the odontoblast layer had been retained, nerve fibres could be seen passing through these cells. In addition, the odontoblasts themselves showed some immunoreactivity for PGP 9.5 (Fig. 4.1C).

The most marked difference between the dentitions was observed within the mid-coronal region: in permanent teeth two or three very large nerve trunks were seen passing up through the pulp stroma, in contrast, smaller, more numerous and comparatively more spaced nerve trunks were evident in primary teeth (Fig. 4.1E,F). As these nerve bundles approached the pulp horns, occasional branching of the main

trunks occurred to form smaller twigs. Within the nerve trunks, PGP 9.5-ir fibres generally exhibited a uniform smooth appearance, however, some finer fibres with axonal swellings were also evident. In both dentitions, nerve bundles were often seen closely associated with pulpal blood vessels. A detailed description of the spatial relationship between PGP 9.5-ir fibres and blood vessels is given in chapter six. Another observation, common to both dentitions, was the presence of smaller nerve bundles or even apparently single fibres towards the peripheral pulp stroma. The total absence of any large nerve bundles was noted in a few sections.

4.3.5 Qualitative observations in carious teeth

The most marked caries-related changes in PGP 9.5-labelling were seen within the pulp horns. Both dentitions appeared to show a prolific increase in PGP 9.5-ir fibres with the progression of caries, and these fibres arborised extensively to form complex neural networks (Fig. 4.2A,B). Indeed this increase in neural branching was occasionally seen to extend some distance into the subjacent pulpal stroma (Fig. 4.2C,D). In addition, a variety of other morphological changes were also observed within carious samples from both dentitions and these included:

- thickening of small nerve bundles to produce denser bands of neural tissue (Fig. 4.2E,F);
- proliferation of nerve fibres to surround intra-pulpal abscesses (Fig. 4.3A,B);
- retraction of neural tissue away from regions of apparent necrosis (Fig. 4.3C);
- loss of overall nerve morphology to produce an area of dense PGP 9.5-labelling with a predominantly varicose appearance (Fig. 4.3D).

However, neural changes were not always evident even within grossly carious teeth. Furthermore, the overall innervation density actually seemed reduced in some carious samples with PGP 9.5-ir fibres appearing sparse and fragmented (Fig. 4.3E). Caries-related changes in neural morphology were also evident within the buccal and lingual SOP throughout the coronal pulp. These generally comprised a marked branching and thickening of nerve fibres. However, no obvious structural changes were evident within the large nerve trunks that coursed through the mid-coronal region of either dentition.

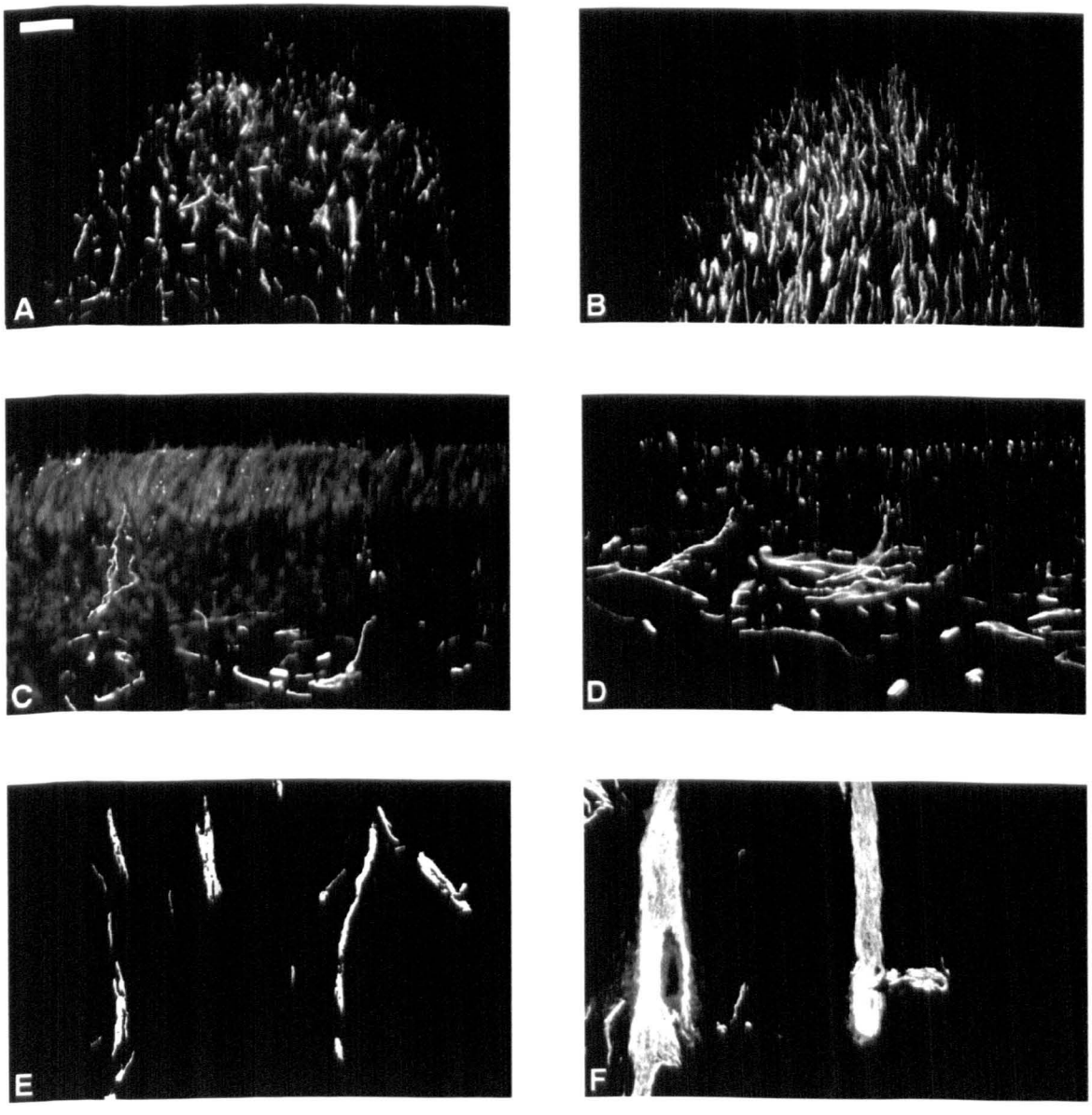


Figure 4.1. Photomicrographs demonstrating differences in the distribution of PGP 9.5-ir fibres in the coronal pulp of intact primary **(A,C,E)** and permanent **(B,D,F)** teeth. **(A)** PGP 9.5-labelling showing the overall innervation in the pulp horn of a primary tooth and **(B)** the overall greater density of innervation seen in a permanent tooth. **(C)** The buccal subodontoblastic nerve plexus in a primary tooth and **(D)** a more dense nerve plexus seen in a permanent tooth. **(E)** Small nerve trunks in the mid-coronal region of a primary tooth and **(F)** comparatively larger nerve trunks in the mid-coronal region of a permanent tooth. Scale bar = 15 μm **(C,D),** 30μm **(A,B),** 60μm **(E,F).**

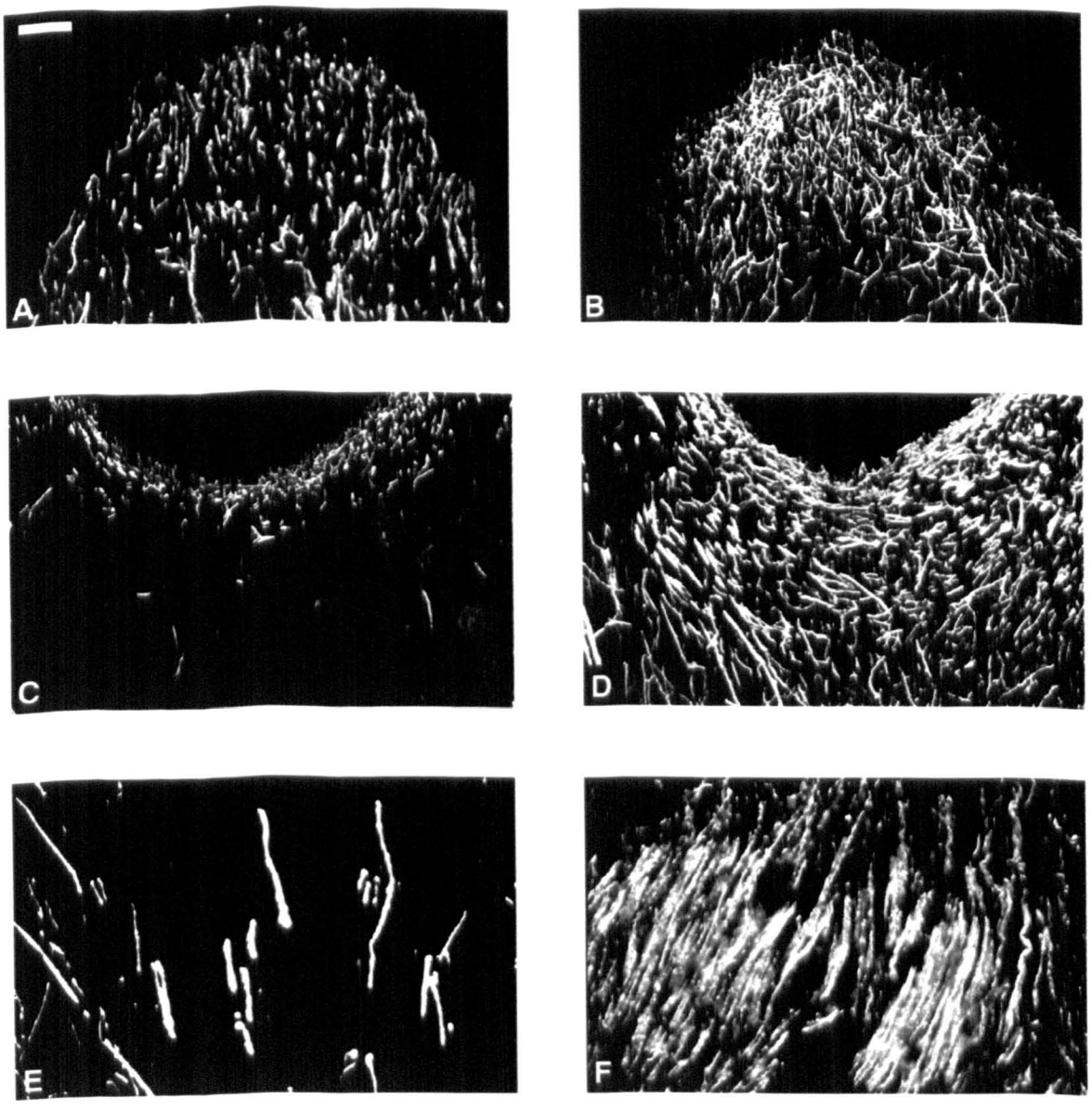


Figure 4.2. Photomicrographs demonstrating differences in the distribution and morphology of PGP 9.5-ir fibres in the coronal pulp of intact **(A,C,E)** and carious **(B,D,F)** teeth. **(A)** PGP 9.5-labelling in the pulp horn of an intact primary tooth and **(B)** a marked increase in the density of labelling seen in a carious primary tooth. **(C)** Region between the pulp horns in an intact permanent tooth showing a normal distribution of PGP-ir fibres and **(D)** a marked increase in PGP 9.5-ir fibre density and distribution within a carious permanent tooth. **(E)** Small PGP 9.5-ir fibre bundles in the pulp horn region of an intact primary tooth and **(F)** thicker PGP 9.5-ir nerve bundles seen in the pulp horn region of a carious primary tooth. Scale bar = 15μm **(E,F)**, 30μm **(A,B)**, 60 μm **(C,D)**.

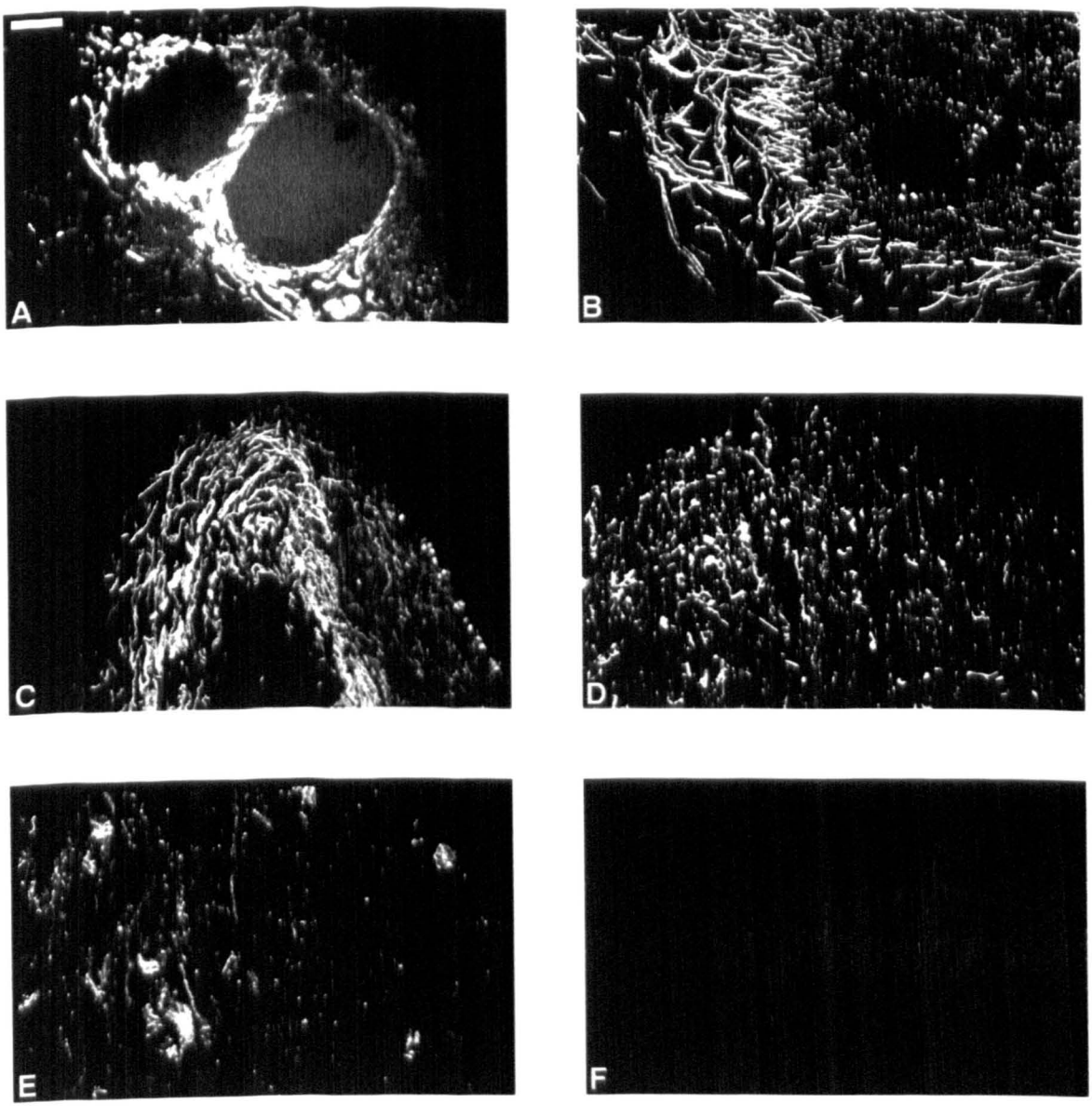


Figure 4.3. Photomicrographs showing caries-related changes in the distribution and morphology of PGP 9.5-ir pulpal nerve fibres **(A-E)** and an immunocytochemical control **(F)**. **(A,B)** PGP 9.5-ir nerve fibres surrounding intra-pulpal abscesses in the pulp horn of a carious permanent tooth. **(C)** Retraction of PGP 9.5-ir fibres away from necrotic tissue in the pulp horn of a carious permanent tooth. **(D)** Intensely varicose PGP 9.5-ir fibres seen in the pulp horn of a carious primary tooth. **(E)** An overall reduction and fragmentation of PGP 9.5-ir fibres seen in the pulp horn region of a grossly carious primary tooth. **(F)** Immunocytochemical control showing no staining for PGP 9.5. Scale bar = 15 μm **(D,E)**, 30 μm **(A,B,C,F)**.

4.3.6 Quantitative analysis

Table 4.1A shows the mean (±SD, range) PAS for PGP 9.5-labelled tissue within each dentition according to the field of analysis and the degree of caries and Table 4.1B provides the corresponding statistical data. Bar charts showing pooled data are presented in Figure 4.4.

Key findings arising from the quantitative analysis of PGP 9.5 are outlined below.

- The greatest mean PAS for PGP 9.5 within intact teeth was seen in field 1 (primary teeth = 5.62%, permanent teeth = 6.20%). For grossly carious samples the greatest mean PAS for PGP 9.5 was also seen in field 1 (primary teeth = 7.70%, permanent teeth = 8.24%).

- The mean PAS for PGP 9.5 was significantly greater in permanent teeth than in primary teeth in all four fields of analysis ($P \leq 0.015$, ANOVA). As an example, in field 3, the mean PAS for PGP 9.5 in intact primary teeth was approximately half that recorded for intact permanent teeth (primary teeth = 1.00%, permanent teeth = 1.79%). However, further pairwise comparisons did not reveal any significant inter-dentition differences within any of the three caries subgroups ($P > 0.05$, Tukey's test).

- Caries was only determined to have a significant effect on mean PAS for PGP 9.5 in the pulp horn (fields 1 and 2) with an overall increase in neural density with caries progression ($P < 0.05$, ANOVA). However, further pairwise comparisons did not reveal any significant differences between the three specific caries subgroups for either dentition ($P > 0.05$, Tukey's test).

Table 4.1A. Mean (±SD, range) percentage area of staining for protein gene product 9.5 according to the tooth type, field of analysis and degree of caries

Field/tooth(n)	Degree of caries		
	None	Moderate	Gross
Field 1			
Primary tooth (n=21,20,18)	5.62 (±2.19, 1.34-5.57)	6.05 (±2.42, 2.56-9.96)	7.70 (±3.62, 2.48-13.87)
Permanent tooth (n=18,20,20)	6.20 (±1.56, 4.39-10.11)	7.54 (±2.23, 3.07-11.30)	8.24 (±2.80, 3.39-13.89)
Field 2			
Primary tooth (n=21,20,18)	2.33 (±0.89, 0.85-5.13)	2.91 (±1.14, 1.34-5.57)	3.82 (±1.89, 1.49-7.84)
Permanent tooth (n=17,20,20)	3.44 (±0.76, 2.60-5.07)	3.81 (±1.18, 1.79-5.55)	4.28 (±2.05, 1.19-8.02)
Field 3			
Primary tooth (21,20,19)	1.00 (±0.24, 0.38-1.52)	1.31 (±0.57, 0.51-2.50)	1.24 (±0.53, 0.49-2.63)
Permanent tooth (n=17,20,19)	1.79 (±0.51, 0.87-2.70)	2.19 (±0.94, 1.20-4.85)	2.18 (±0.88, 0.92-4.12)
Field 4			
Primary tooth (n=19,20,20)	3.74 (±0.96, 1.85-5.03)	3.54 (±1.04, 1.77-5.65)	3.61 (±0.86, 2.16-5.32)
Permanent tooth (n=18,20,18)	5.38 (±1.27, 2.75-7.51)	6.18 (±1.71, 3.39-9.68)	6.19 (±2.34, 3.32-11.91)

Table 4.1B. Significance values for effect of dentition type and degree of caries on the PAS for PGP 9.5 (two-way analysis of variance on log-transformed data)

Main effects	Field 1	Field 2	Field 3	Field 4
Dentition type	P=0.015	P=0.0005	P=0.0005	P=0.0005
Degree of caries	P=0.011	P=0.013	P=0.076	P=0.807

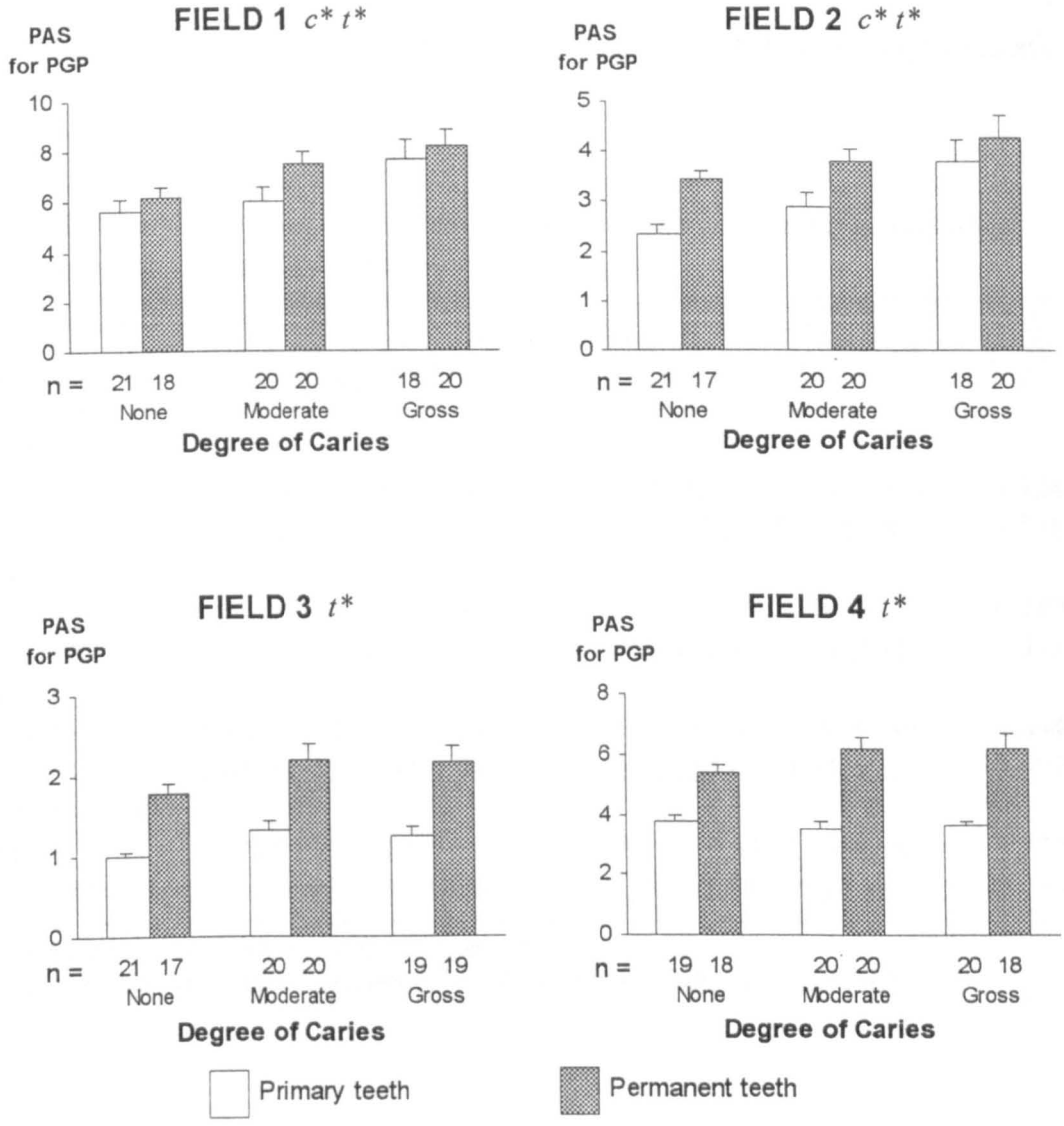


Figure 4.4. Bar charts showing mean (±SEM) percentage area of staining (PAS) for protein gene product 9.5 (PGP 9.5) labelled tissue for primary and permanent teeth according to the degree of caries. Field 1=tip of pulp horn; Field 2=region subjacent to field 1; Field 3=buccal subodontoblastic plexus; Field 4=mid-coronal region.

c^* = significant difference in mean PAS for PGP 9.5 according to the degree of caries, t^* = significant difference in mean PAS for PGP 9.5 between primary and permanent teeth ($P<0.05$, ANOVA, using log transformed data).

4.3.7 Quantitative findings relating to pain history

The mean (±SD, range) PAS for PGP 9.5 within grossly carious teeth, according to the reported pain history, is presented in Table 4.2. It can be seen that, for both dentitions, there was no significant difference in overall innervation density within any field between samples that were reportedly painful or that were asymptomatic ($P>0.05$, Student *t*-test on log-transformed data).

Table 4.2. Mean (±SD, range) percentage area of staining for protein gene product 9.5 according to the reported pain history

Field/tooth	Pain history		
	Asymptomatic[a]	Painful[b]	*P*
Field 1			
Primary	8.72 (±4.26, 2.48-13.87)	6.77 (±2.80, 3.54-13.53)	0.424
Permanent	7.68 (±2.14, 4.30-10.59)	8.86 (±3.39, 3.39-13.87)	0.548
Field 2			
Primary	4.38 (±2.45, 1.51-7.84)	3.35 (±1.22, 1.49-5.53)	0.389
Permanent	4.59 (±1.73, 2.05-7.11)	3.97 (±2.39, 1.19-8.02)	0.313
Field 3			
Primary	1.42 (±0.54, 0.87-2.63)	1.05 (±0.48, 0.49-1.79)	0.084
Permanent	2.04 (±0.73, 0.92-2.94)	2.32 (±1.05, 0.94-4.12)	0.599
Field 4			
Primary	3.83 (±0.79, 2.88-5.32)	3.40 (±0.91, 2.16-4.68)	0.232
Permanent	5.30 (±1.77, 3.32-10.59)	7.08 (±2.58, 3.78-11.91)	0.096

P= independent sample *t*-test on log-transformed data
[a] n=10,11 (primary teeth, permanent teeth); [b] n=10,10 (primary teeth, permanent teeth)

4.4 Discussion

4.4.1 Experimental approach

A comprehensive appraisal of the overall experimental approach is provided in chapter eight. Thus only matters specific to the analysis of PGP 9.5 will be considered in this section.

Numerous investigators have employed antiserum to PGP 9.5 in order to visualise the entire intradental innervation. The excellent immunolabelling achieved for all specimens in this study would further support the use of PGP 9.5 as a general

neuronal marker. Furthermore, the overall distribution pattern of intradental nerves seen for both dentitions was comparable with previous descriptions of human tooth pulp innervation (Bernick, 1948; Philipp and Chicago, 1955; Rapp *et al.*, 1967b; Johnsen, 1985; Ramieri *et al.*, 1990).

Consistent with other reports, odontoblast cell bodies were also seen to demonstrate labelling for PGP 9.5, albeit to a much lesser intensity than that seen for the neuronal staining (Christensen *et al.*, 1993; Fristad *et al.*, 1994). This feature has been attributed to the possible neural crest origin of odontoblasts and their matrix-secreting function. By using very high antibody dilutions (1:15.000-30,000), it has been reportedly possible to abolish PGP 9.5 immunoreactivity within odontoblasts thus facilitating the identification of PGP 9.5-ir nerve fibres in this region (Maeda *et al.*, 1994). However, this was not necessary for the purposes of the present investigation.

It has been shown that marked neural changes accompany continued dental development in permanent teeth including the formation of the SOP (Bernick, 1964) and an increase in the number of myelinated apical nerve fibres (Johnsen and Karlsson, 1977; Fried and Hildebrand, 1981a; Johnsen *et al.*, 1983). It was therefore important to establish that any increases in neural density within carious samples could not be attributed to the stage of dental development rather than the degree of caries. However, it was clearly established that there was no significant association between age and the PAS for PGP 9.5 in permanent teeth (see, section 3.3.4). Thus caries was considered to have a real effect on neural density.

Furthermore, as the age of the primary teeth included the present study did not have any effect on the PAS for PGP 9.5 (see, section 3.3.4) it can be reasonably concluded that "physiological" neural degeneration had not yet commenced to any significant degree in the selected samples (Mohiuddin, 1950; Rapp *et al.*, 1967b; Fried and Hildebrand, 1981b). However, it should be borne in mind that this conclusion is based on the assumption that an intact PGP 9.5 labelling indicates that no neural degeneration is taking place. This would seem to be a reasonable hypothesis although no previous work appears to have been conducted in this area.

A limitation of the present study was that nerves within the predentine and dentine could not be subject to quantitative analysis as the pulp had been separated from the hard tissues. This had been undertaken for ease of tissue processing and to obviate the need for demineralisation procedures which can have a detrimental effect on antigenicity (Jonsson *et al.*, 1986; Wakisaka *et al.*, 1986; Frank *et al.*, 1993). Thus a potentially interesting inter-dentition comparison remains unexplored.

Another acknowledged limitation of the investigation was that no data could be obtained regarding specific nerve subpopulations. The relative contribution made by sensory and autonomic nerve populations and myelinated or unmyelinated fibres may all be functionally important in terms of differences in sensitivity between the two dentitions. However, concurrent investigations of some specific peptidergic nerve subpopulations were undertaken and the findings are discussed in chapter five.

4.4.2 Differences between primary and permanent teeth

Subjective clinical observations appear to be the basis for the commonly held view that primary teeth are less sensitive to nociceptive stimuli than their successors (Rapp *et al.*, 1967b; Egan *et al.*, 1996). The few attempts to determine a biological explanation for this phenomenon have naturally focused on the neuroanatomical features of the two dentitions. Early descriptive studies concluded that the distribution of intradental nerves in primary teeth was similar, although less dense, than that found in permanent teeth (Rapp *et al.*, 1967a; 1967b).

Quantitative comparative studies between the dentitions, both for cat and man, have mostly explored differences in apical nerve number and ultrastructure (Johnsen and Karlsson, 1974; Johnsen and Karlsson, 1977; Johnsen and Johns, 1978). Findings from these investigations have indicated that, generally, permanent teeth possess a greater proportion of myelinated fibres. Investigators have therefore hypothesised that permanent teeth may be better equipped to conduct "sharp" pain impulses.

The present study has clearly shown, on the basis of findings for PGP 9.5-labelling, that there are quantitative differences in neural density between the two dentitions.

Permanent teeth demonstrated a significantly denser innervation in all regions investigated which was most marked within the SOP and the mid-coronal region.

One may question whether these data represent a true difference in innervation density or simply reflect the comparatively larger size of the permanent tooth pulp (see, section 3.4). Johnson and Karlsson (1974) acknowledged the size differential between feline primary and permanent teeth and devised an innervation index based on tooth weight. They were then able to conclude that there was no significant difference in overall innervation density (total number of apical axons) between the two dentitions. In the present investigation, the pulp horn regions of both dentitions were of similar dimensions thus innervation densities were considered directly comparable. It was also felt that differences within the SOP were a true reflection of tooth type differences rather than simply a feature of pulp size. But it was conceivable that, within the mid-coronal pulp region, the larger nerve trunks present in permanent teeth could have simply been an anatomical feature of the greater pulp size. Thus the proportional difference in pulp sizes was compared with the proportional difference in mid-coronal neural density. It was found that, on average the permanent tooth pulp was 23% larger than the primary tooth pulp (see, section 3.4) whereas the PAS for PGP 9.5 was 63% greater in the mid-coronal region of the permanent tooth as compared to the primary tooth (see, Table 4.2). Therefore one may conclude that permanent teeth are relatively more densely innervated throughout the coronal pulp than their predecessors.

Having established a quantitative neuroanatomical difference between the two dentitions it is tempting to speculate that this may have functional significance in terms of nociceptive potential. Early physiological studies support the concept that sensation is related to the number and type of associated nerve fibres (Erlanger and Gasser, 1937) and in some organs, notably the cornea and tympanic membrane, a dense innervation is clearly associated with an increased sensitivity. Permanent teeth, by virtue of their denser innervation, may indeed be more sensitive to nociceptive stimuli than primary teeth. However, the degree of neural density can not be considered the sole indicator of a tooth's capacity to perform a sensory function. The

relative contribution made by sensory and autonomic nerve subpopulations, myelinated or unmyelinated fibres and specific pain-related peptidergic fibre subpopulations may all have functional importance in terms of inter-dentition differences in sensitivity. Furthermore, the complexity of pain perception should be acknowledged. Pain perception involves reception, transmission and perception, and further multi-disciplinary research approaches will be necessary to more fully understand any sensory differences between primary and permanent teeth.

4.4.3 The effect of caries on pulpal innervaton

Caries-induced pulpal inflammation, a reaction to the invasion of microbial organisms and their metabolic products, comprises a complex series of events (for reviews see, Langeland, 1987; Watts and Paterson, 1981). These include vasodilation and increased vessel permeability, plasma extravasation and a host of humoral and cell-mediated immunological responses. The pathological sequelae to caries, both within the pulp and calcified tissues, have been of considerable clinical and scientific interest over the years. However, the response of pulpal nerves to caries or injury appears to have assumed relatively little importance. Indeed many histopathological assessments of the dental pulp have made no reference at all to the status of intradental nerves (McDonald, 1956; Baume, 1970a; Stanley, 1970; Seltzer, 1972; Sayegh and Reed, 1974; Rayner and Southam, 1979; Browne *et al.*, 1980).

Nonetheless, a few early investigators have described a variety of caries-related neural changes comprising nerve fibre fragmentation, beading, irregular thickening, and dense intertwining close to the inflammatory focus (Bradlaw, 1936; Placková, 1966; Bernick, 1972). However, no changes have been observed within the main nerve trunks. Although these studies employed different histological methods to identify intradental nerves, their findings agree closely with those of the present study.

More recently, researchers have employed antiserum to PGP 9.5 to investigate caries-induced neural changes in human teeth (Yoshiba *et al.*, 1998; Sakurai *et al.*, 1999). These studies have reported an increased density of PGP 9.5-ir pulpal fibres in teeth with gross caries. In addition, PGP 9.5-labelling was seen to display a "dotted"

appearance in some carious samples. These descriptive findings are also consistent with those of the present investigation.

Byers and collaborators have provided a particularly valuable insight into the morphological changes affecting intradental nerves following pulpal inflammation (Khayat *et al.*, 1988; Kimberly and Byers, 1988; Taylor *et al.*, 1988; Byers *et al.*, 1990b; Taylor and Byers, 1990). Although these studies have been conducted in rats, following an experimentally-induced pulpal injury, they are still considered relevant to the present study. These investigations have provided important data regarding the timing of intradental sensory nerve responses to different degrees of injury. In her excellent review articles (Byers, 1992a; 1994), Byers refers to immediate, early and late changes in pulpal nerve morphology. The immediate changes, sometimes within 18-24 hours following injury, see an initial destruction of nerve fibres. A few days later, nerve fibres undergo a prolific sprouting response, particularly at the border of surviving vital tissue. The late responses comprise a subsiding of nerve branching coincident with tissue healing.

Interestingly, many of the morphological features described in the above animal experiments, such as pulpal nerve sprouting adjacent to necrotic tissue and around abscesses and a predominance of beaded nerve fibres, were also seen in the present study. However, no indication of the timing of the neural responses to inflammation could be determined from the present human caries model.

4.4.4 Pulpal innervation in relation to pain

Caries-induced acute pulpal inflammation may result in spontaneous pain and increased sensitivity to external stimuli but, in contrast, pain is not a common feature of chronic pulpitis (Trowbridge, 1986; Ahlquist and Franzén, 1994). However, there is a wide spectrum of caries-related symptoms, indeed pulpitis can proceed to complete pulp destruction without accompanying symptoms or conversely be associated with extreme pain. This phenomenon remains unexplained and frequently presents a diagnostic problem for the clinician.

Some investigators have speculated that dynamic changes in neural density may have functional significance in terms of sensitivity (Yamaura *et al.*, 1988; Byers, 1992). However, other investigators have failed to find any correlation between patient-reported symptoms and pulpal nerve status (England *et al.*, 1974; Torneck, 1977; Mendoza *et al.*, 1987). In order to investigate this further, the present study attempted to correlate neural density with reported pain histories in children. However, no significant difference in overall innervation density, within any pulpal region, was found between grossly carious asymptomatic and symptomatic samples. It was interesting to note that, although the differences were not significant, asymptomatic primary teeth tended to be more densely innervated than reportedly painful teeth, but the reverse was true for permanent teeth. It would therefore seem that there is no direct correlation between the density of pulpal innervation and reported symptoms in carious teeth within this subject group.

There may be several reasons why a correlation was not found between neural density and reported symptoms. Firstly, the reliability of the reported pain history in young subjects may be open to question and this issue is addressed in chapter eight. (see, section 8.1.1). Furthermore, a variety of factors may modify intradental nerve function (Mumford and Bowsher, 1976; Sessle, 1987; Närhi, 1989; Närhi *et al.*, 1992b) thus it may be inappropriate to try to correlate neural density, which is simply a morphological feature, with a reported pain history. Some factors believed to modify dental pain experience are briefly considered below.

- Sensitisation of nociceptive afferents

There is conclusive evidence to suggest that numerous inflammatory mediators have a direct or indirect effect on nerve excitability. These may include bradykinin, nerve growth factor, substance P, serotonin, histamine, adenosine, prostaglandins, hydrogen ions, cytokines and nitric oxide (for review see, Levine and Reichling, 1999). It is of particular relevance to the present study that bacterial metabolites from carious lesions have also been shown to modify intradental nerve excitability (Olgart, 1986; Panopoulos, 1992). In addition, local microcirculatory changes in pulpal blood flow can have profound effects on sensory nerve activity (for review, see Kim, 1990).

Therefore a variety of factors may cause the sensitisation of primary afferent nociceptors thus leading to the development of allodynia and hyperalgesia, which are characterised by a lowered pain threshold and an increased pain to suprathreshold stimulation.

- Role of silent afferents

A further mechanism purported to contribute to pain experience is the presence of a certain type of sensory afferent fibre known as a "silent afferent," "sleeping nociceptor" or "mechanically insensitive afferent." These have been identified in several types of tissue including skin, joint and viscera (for review see, McMahon and Koltzenburg, 1990). In intact tissue this fibre population is insensitive to conventional noxious stimulation but in inflamed tissue these fibres respond vigorously to mechanical stimuli (Habler *et al.*, 1990; Schaible and Schmidt, 1988). There is now some evidence to suggest that in normal teeth there are considerable numbers of these "silent nociceptors" which become activated following pulpal inflammation and thus may contribute to painful pulpitis (for review see, Byers and Närhi, 1999).

- Central sensitisation

Our understanding of the neurobiology of dental pain in man is incomplete as studies are limited to the peripheral nervous system. However, innovative animal studies have demonstrated that central sensitisation may be induced by peripheral dental inflammation or injury (Shortland *et al.*, 1995; Sunakawa *et al.*, 1996; Chiang *et al.*, 1998). In light of these findings, it has been hypothesised that central changes may have relevance to the problem of hyperalgesia and referred pain that can accompany acute pulpitis in some patients. There is therefore a need to further elucidate the effect of pulpal pathology on the excitability of second- and higher-order projection neurons.

4.4.5 Mechanisms for neural sprouting

It has been well established that nerve growth factor (NGF) is essential for the development and maintenance of sensory and sympathetic peripheral nerves (Levi-Montalcini, 1987; Isaacson and Crutcher, 1998; Lee *et al.*, 1998). Furthermore, NGF

has an important role in neural regeneration or sprouting following nerve injury (Clarke and Richardson, 1994; Chen *et al.*, 1999). It would therefore seem a likely candidate for the stimulation of nerve branching seen in the present study.

Indeed, Byers and collaborators have sought to quantify NGF in injured teeth in order to establish its role in intradental nerve sprouting (Byers *et al.*, 1990a; 1992b). In situ hybridisation revealed an enhanced expression of NGF mRNA within pulpal fibroblasts six hours following injury, which preceded neural sprouting. Additional indirect evidence to support NGF-related nerve sprouting in the dental pulp stems from the immunocytochemical detection of NGF receptor immunoreactivity within intradental nerve fibres and fibroblasts (Byers *et al.*, 1990a; Fried and Risling, 1991; Maeda *et al.*, 1992). Furthermore, Sakurai and colleagues recently demonstrated a significant increase in NGF receptor immunoreactivity within carious human teeth as compared to intact samples (Sakurai *et al.*, 1999). The action of other growth factors within the pulp are less clear but their contribution to nerve sprouting should not be discounted (Partanen and Thesleff, 1989).

4.4.6 Innervation changes in other models of inflammation

Orthodontic tooth movement is known to evoke a series of inflammatory changes and has therefore provided an additional dental model in which to investigate inflammation-related neural changes. Vandevska-Radunovic and colleagues have clearly demonstrated that experimental tooth movement in rats induces dynamic changes in periodontal and pulpal nerve fibres resulting in an increased sprouting and neural density (Vandevska-Radunovic *et al.*, 1997b; 1999).

Structural and cytochemical neural changes in arthritis-affected tissue have also been identified. However, in contrast to the above findings, immunocytochemical investigation of PGP 9.5-ir fibres within the synovial tissue of patients with rheumatoid arthritis, revealed an overall reduction in innervation density as compared to healthy controls (Mapp *et al.*, 1990).

4.4.7 Clinical significance of findings

This present study has clearly demonstrated that the primary tooth pulp receives a prolific innervation although this is significantly less dense than that seen in permanent teeth. Nonetheless, both dentitions exhibit a similar pattern of intradental nerve distribution and both show a similar degree of increased neural density following caries. On the basis of these findings alone, it is not possible to categorically say that primary teeth should be less sensitive to potentially painful stimuli than their successors. Further work, looking at the specific physiological and cytochemical characteristics of the respective dental innervations is thus indicated. It is possible that clinical impressions regarding the reduced sensitivity of primary teeth may have been based on observations in older children. In such cases, neural degeneration, accompanying physiological root resorption, may have contributed to an apparent reduced response to nociceptive stimuli.

Although this investigation did not identify a significant difference in neural density between grossly carious asymptomatic and painful teeth, it is still speculated that dynamic increases in neural density may have some significance in terms of nociception. It is possible that increases in neural tissue may be accompanied by cytochemical changes which may be more relevant to pain states and this theory is explored in the next chapter.

However, the marked changes in neural distribution and density revealed by the present study may have functional significance in addition to that of nociceptive processing. The traditional concept of the tooth being richly innervated to serve a purely nociceptive role, is now being challenged by increasing evidence that sensory nerves also serve important effector functions (Maggi, 1991; Chen *et al.*, 1999). This aspect is also discussed further in the next chapter.

4.4.8 Summary of findings

In response to the original objectives (see, section 4.1.4) this investigation has shown that:

- Permanent teeth are significantly more densely innervated than primary teeth throughout the coronal pulp. However, both dentitions show a similar distribution pattern of pulpal nerve fibres.

- Both dentitions show marked changes in neural morphology and overall innervation density with the progression of caries.

- There is no significant difference in innervation density between asymptomatic and reportedly painful grossly carious samples from either dentition.

5. Expression of neuropeptides within pulpal nerves

5.1 Introduction

5.1.1 The neuropeptide concept

Neuropeptides are biologically active proteins which essentially act as chemical messengers and serve to integrate the functions of the brain and other organ systems. They appear to be ubiquitous, being present in almost all tissues, but are especially concentrated within the central and peripheral nervous systems (for reviews see, Hökfelt, 1991; Strand, 1999). They are produced by non-neural as well as neural tissues (Jakab *et al.*, 1993), however, this overview will be limited to the expression of neuropeptides within the peripheral nervous system.

Neuropeptides are made in neuronal cell bodies and are transported via axonal flow both centrally and peripherally (Brodin *et al.*, 1981; Harmar and Keen, 1982). Neurons may express more than one type of neuropeptide in addition to the classical transmitters: acetylcholine, noradrenaline, serotonin and GABA (for reviews see, Lundberg and Hökfelt, 1986; Hökfelt, 1991). The picture is further complicated by the finding that neuropeptide expression may be profoundly affected by a wide range of physiological or pathophysiological conditions (Levy *et al.*, 1989; Mapp *et al.*, 1990; Villar *et al.*, 1991; Benarroch, 1994; Long *et al.*, 1998).

Following nerve stimulation, neuropeptides are selectively released from large dense-core vesicles at the nerve terminals (Lundberg *et al.*, 1981). However, it is also likely that there is some spontaneous secretion of neuropeptides at low basal levels (Strand, 1999). On release, neuropeptides may act as neurohormones, neurotransmitters or neuromodulators and they exert their effects by interacting with a variety of cell surface receptors (Villar *et al.*, 1991; Wahlestedt *et al.*, 1990; Fleetwood-Walker, 1995). There appears to be little or no re-uptake of peptides following their release from nerve terminals, and subsequent peptide inactivation occurs by both diffusion and enzymatic degradation (for review see, Maggi, 1991).

5.1.2 Key neuropeptides and their regulatory functions

- Substance P

Substance P belongs to a family of neuropeptides, known as the tachykinins, which also includes neurokinin A and neurokinin B. It is present in many areas of the nervous system and has been clearly demonstrated within a subpopulation of small-diameter unmyelinated C-type fibres (Jessell, 1983; Lundberg and Hökfelt, 1986; Fleetwood-Walker, 1995). The biological actions of SP have been studied widely, and within the central nervous system SP is involved in processing visual, olfactory, auditory and nociceptive input (for review see, Strand, 1999). In terms of pain processing, SP is thought to play a major role in the neurotransmission of nociceptive input and is believed to exert its effects via the NK-1 receptor at the level of the spinal cord (Woolf and Wiesenfeld-Hallin, 1986; De Felipe *et al.*, 1998; Yaksh, 1999). Peripherally, SP is a well known mediator of neurogenic inflammation and induces vasodilation and plasma extravasation following antidromic stimulation of sensory nerves (Lembeck and Holzer, 1979; Baluk *et al.*, 1992). In addition, there is growing support for SP-related modulation of various immune cell functions (McGillis *et al.*, 1987; Payan and Goetzl, 1987a; Lotz *et al.*, 1988). Finally, SP has been implicated as a trophic factor as it stimulates endothelial cell growth (Nilsson *et al.*, 1985; Ziche *et al.*, 1990).

- Calcitonin gene-related peptide

Calcitonin gene-related peptide is a 37 amino acid encoded by the calcitonin gene (Amara *et al.*, 1982). It is widely distributed in the body being present in the thyroid gland and throughout the nervous system. Within the peripheral nervous system, CGRP is expressed in a large proportion of small to medium diameter sensory neurons (for review see, Poyner, 1992). Furthermore, it has been shown that many of these fibres coexpress both CGRP and SP (Franco-Cereceda *et al.*, 1987). There is some evidence to implicate CGRP in the modulation of nociceptive transmission. However, it is unlikely that CGRP has a direct effect on pain processing but is believed to enhance the effect of SP (Pedersen-Bjergaard *et al.*, 1991; Fleetwood-Walker, 1995). Calcitonin gene-related peptide has several other biological actions which principally involve the cardiovascular, gastrointestinal and endocrine systems

(for reviews see, Goodman and Iversen, 1986; Wimalawansa, 1996). In addition, CGRP has been recognised as a potential growth factor due to its reported role in angiogenesis (Kjartansson and Dalsgaard, 1987), bone formation (Bernard and Shih, 1990) and cell proliferation (Hægerstrand *et al.*, 1990; Trantor *et al.*, 1995). It would appear that CGRP also has important effects in terms of vasodilation (Brain *et al.*, 1985) and immune cell regulation (Nong *et al.*, 1989).

- Vasoactive intestinal polypeptide

Vasoactive intestinal polypeptide was originally considered to be a gut hormone but immunohistochemical studies have revealed its extensive distribution throughout the nervous system (for reviews see, Fahrenkrug, 1979; Strand, 1999). Morphological, biochemical and functional investigations would suggest that, in the normal state, VIP is present together with acetylcholine in parasympathetic neurons (Lundberg *et al.*, 1981; 1984). In common with other neuropeptides, VIP has a wide range of biological activities with well-established vasodilatory and smooth muscle relaxant effects (for review see, Strand, 1999). In addition, VIP has purported roles in immune cell function (Ottaway, 1988; Segura *et al.*, 1991; Litwin *et al.*, 1992) and may act as a trophic factor stimulating nerve regeneration following injury (Shehab and Atkinson, 1986; Rayan *et al.*, 1995).

- Neuropeptide Y

Neuropeptide Y is a member of the pancreatic polypeptide family and is widely distributed throughout the central, peripheral and enteric nervous systems (for review see, Gibson *et al.*, 1984). Under normal physiological conditions it is expressed by noradrenergic sympathetic neurons (Lundberg *et al.*, 1990; Benarroch, 1994). Over the years, NPY has been attributed with many physiological roles particularly those relating to the endocrine, metabolic and behavioural regulation of nutrient and energy homeostasis (for review see, Strand, 1999). In addition, NPY has been shown to play an important role in sympathetic-mediated vasoconstriction (Lundberg and Tatemoto, 1982; Wharton and Polak, 1990). There is now recent evidence to suggest that, following nerve injury, NPY may even exert some antinociceptive effects (Mantyh *et al.*, 1994).

5.1.3 Presence and distribution of neuropeptides in teeth

Since the 1970's, numerous investigators have sought to demonstrate the presence of neuropeptides within oral and dental tissues. A large number of peptides have now been identified in the tooth pulp of man and several other mammalian and non-mammalian species (for reviews see, Akai and Wakisaka, 1990; Wakisaka, 1990; Tuisku and Hildebrand, 1997). A variety of experimental approaches have been used to identify neuropeptides in teeth which have mainly comprised immunocytochemistry (indirect immunofluorescence and immunoperoxidase techniques) and radioimmunoassay. Novel techniques have also been developed to measure the release of neuropeptides from the pulp under experimental conditions (Brodin *et al.*, 1981; Hargreaves *et al.*, 1992; Hargreaves *et al.*, 1994).

Substance P was the first neuropeptide to be identified in tooth pulp (Olgart *et al.*, 1977a; 1977b). Subsequent immunocytochemical studies have shown that SP-ir nerve fibres are relatively abundant in the tooth pulp of most species and show a common pattern of intradental distribution (Grönblad *et al.*, 1984; Wakisaka *et al.*, 1985; Casasco *et al.*, 1990a; Luthman *et al.*, 1992). It would appear that, in the radicular and mid-coronal pulp, SP is expressed within nerve trunks and by some fibres located around blood vessels. Within the pulp horn regions, numerous varicose fibres are seen without any obvious relationship to other structures. Substance P-ir fibres have also been demonstrated within the SOP and the use of demineralised sections has confirmed that these fibres may extend some way into predentine and dentine (Wakisaka *et al.*, 1984; 1985). Following the identification of SP, other members of the tachykinin family have also been demonstrated within teeth and these have included, neurokinin A (Wakisaka *et al.*, 1988; Goodis and Saeki, 1997) and neuropeptide K (Casasco *et al.*, 1990b).

Calcitonin gene-related peptide is generally considered to be the most prolific neuropeptide within pulpal nerves and was first identified in tooth pulp in 1986 (Uddman *et al.*, 1986a). Subsequently, several investigators have reported that numerous CGRP-ir fibres are present within the pulp horn regions, coronal SOP and dentine (Silverman and Kruger, 1987; Wakisaka *et al.*, 1987a; Casasco *et al.*, 1990a;

Luthman *et al.*, 1992; Egan *et al.*, 1996; Uddman *et al.*, 1999). In addition, CGRP-ir fibres are found in radicular nerve trunks and around some blood vessels. Essentially, CGRP shares a similar intradental distribution pattern to that of SP, and double-labelling experiments have indicated that SP and CGRP may coexist within a subpopulation of dental nerve fibres (Wakisaka *et al.*, 1987a). However, CGRP-ir fibres are the more abundant.

Uddman and colleagues were the first to report the presence of both VIP (1980) and NPY (1984) in the tooth pulp of a variety of species including man. Vasoactive intestinal polypeptide-ir fibres are found mostly in association with the pulpal vasculature, but some free fibres have been observed in the pulp stroma with no obvious relationship to blood vessels (Wakisaka *et al.*, 1987b; Casasco *et al.*, 1990a). Neuropeptide Y-ir fibres are also predominantly confined to the pulpal vasculature, with only sparse beaded free fibres being present in the pulp stroma (Heyeraas *et al.*, 1993).

A comprehensive immunocytochemical study undertaken by Casasco and co-workers demonstrated eleven different neuropeptides within the human tooth pulp (Casasco *et al.*, 1990a). Some of these peptides had not been previously identified in teeth and included: cholecystokinin; C-terminal flanking peptide of neuropeptide tyrosine, somatostatin, peptide with N-terminal histidine and C-terminal isoleucine. A later publication, by the same authors, described the intradental distribution of SOM in more detail: SOM-ir fibres were seen as fine axonal varicosities within large nerve trunks, around some blood vessels and were sparsely present within the SOP (Casasco *et al.*, 1991).

Grönblad and colleagues were the first to report positive immunolabelling for Leu- and Met-ENK within human tooth pulp (Grönblad *et al.*, 1984). The majority of these immunoreactive fibres were seen to have a non-varicose morphology and Leu-ENK-ir fibres were most prominent below the odontoblast layer. Radioimmunoassay studies subsequently corroborated the presence of enkephalins within dental structures (Tanzer *et al.*, 1988; Robinson *et al.*, 1989).

During the past decade, the diverse cytochemistry of intradental nerves has been further elucidated by the continued identification of novel neuropeptides. Galanin was first demonstrated in the tooth pulps of man, dog, cat and rat by Wakisaka and his team in 1996 (Wakisaka *et al.*, 1996a). Galanin-ir fibres were seen mostly as thin varicose fibres in close proximity to blood vessels. In addition, some fibres appeared to pass towards the odontoblastic layer but did not form part of the SOP. In 1998, Pertl and colleagues used immunocytochemistry and radioimmunoassay to demonstrate the presence of secretoneurin in human tooth pulp (Pertl *et al.*, 1998). This neuropeptide was expressed by varicose axons within nerve trunks and by some vascular-related nerve fibres.

5.1.4 Origin of intradental neuropeptides

Our knowledge of the origin of various intradental peptidergic nerve subpopulations has been largely derived from denervation studies comprising section of the inferior alveolar nerve and surgical or chemical sympathectomy of the superior cervical ganglion (for review see, Wakisaka, 1990). Supporting evidence has been provided by immunocytochemical identification of neuropeptide distribution within both the trigeminal ganglion (Uddman *et al.*, 1986a; Quartu and Del Fiacco, 1994) and superior cervical ganglion (Uddman *et al.*, 1986a; Baffi *et al.*, 1992). Furthermore, retrograde tracing techniques have provided an important adjunct to this line of investigation (Fristad *et al.*, 1996; Wakisaka *et al.*, 1996b; Tuisku and Hildebrand, 1997).

The trigeminal origin of CGRP, SP, NKA and GAL has been well-established: it has been shown that IAN section abolishes intradental expression of these neuropeptides whereas cervical sympathectomy has no apparent effect on peptide expression (Wakisaka *et al.*, 1985; Uddman *et al.*, 1986a; Wakisaka *et al.*, 1987a; 1996a). Thus, it is considered that nerve fibres containing these neuropeptides are sensory afferents originating from the trigeminal ganglion. Furthermore, administration of capsaicin, a selective irritant of sensory unmyelinated fibres, attenuates immunostaining for CGRP and SP in rat molars, indicating that these two peptides are mostly located in chemosensitive unmyelinated fibres (Jacobsen and Heyeraas, 1996).

In contrast, cervical sympathectomy results in a marked reduction, if not total absence, of immunolabelling for NPY, but IAN section appears to have no effect on the expression of this peptide (Uddman *et al.*, 1984; Jacobsen *et al.*, 1998). In addition, it has been shown that the distribution of NPY correlates closely with that of dopamine-β-hydroxylase (Uddman *et al.*, 1984) and tyrosine hydroxylase (Luthman *et al.*, 1992), findings which are consistent with the view that intradental NPY is expressed by sympathetic fibres originating from the superior cervical ganglion.

The origin of VIP, under normal physiological conditions, remains uncertain as neither sympathectomy nor IAN denervation seem to have any marked effect on pulpal VIP expression (Akai and Wakisaka, 1990). Thus it is speculated, by some, that VIP-containing fibres may represent a parasympathetic component to dental innervation. This opinion is further supported by the finding that the distribution of VIP-ir closely resembles that of peptide histidine isoleucine-ir fibres and both these neuroactive substances are known to coexist with acetylcholine in cholinergic parasympathetic fibres in other tissues (Lundberg *et al.*, 1984).

5.1.5 Functional role of neuropeptides in teeth

As previously outlined in section 5.1.2, neuropeptides are known to participate in a diverse range of regulatory mechanisms throughout the body. Following the identification of high levels of some peptides within dental structures, investigators have sought to understand the functional significance of an intradental peptidergic innervation (for review see, Akai and Wakisaka, 1986). Evidently, various stimuli may promote the release of neuropeptides into the peripheral dental tissues, upon which the peptides may exert direct or indirect effects. There is good evidence to suggest that specific neuropeptides are involved in pulpal haemoregulation (for review see, Olgart, 1996a) and immunoregulation (Fristad *et al.*, 1995b), which will be discussed in detail in chapters six and seven respectively. It is also likely that pulpal neuropeptides have regulatory roles in pain processing and tissue healing as outlined below.

- Nociception

The predominance of SP- and CGRP-ir fibres within both the pulp and calcified tissues, and the specific central anatomical projections of these peptidergic nerves, have led to the general consensus that these neuropeptides may play a regulatory role in dental pain processing (Sessle, 1987; Wakisaka, 1990; Byers, 1994; Hargreaves *et al.*, 1994). However, there is little direct pharmacological or physiological evidence to confirm that SP and CGRP subserve a nociceptive role within teeth.

Nonetheless, there is some indirect evidence to support the interrelationship of SP and CGRP and dental pain. An immunocytochemical investigation of teeth from a child with hereditary sensory and autonomic neuropathy, a condition associated with severe sensory loss of the extremities, revealed a total absence of intradental CGRP- and SP-ir fibres (Rodd *et al.*, 1998). Furthermore, teeth subjected to experimental forces have demonstrated significant increases in pulpal levels of SP and CGRP, which may be consistent with the pain symptoms commonly reported by patients undergoing active orthodontic treatment (Vandevska-Radunovic, 1999). Of recent clinical interest was the finding that extracellular levels of SP were five times higher in (symptomatic) pulps with irreversible pulpitis than in normal tooth pulps (Bowles *et al.*, 2000).

In contrast to the purported nociceptive activity of SP and CGRP, other neuropeptides have been considered to act in an analgesic capacity within tooth pulp. These have included enkephalins (Walker *et al.*, 1987), GAL (Wakisaka *et al.*, 1996a) and SOM (Casasco *et al.*, 1991).

- Tissue healing

The role of neuropeptides as growth factors is another area of interest (for review, see Dalsgaard *et al.*, 1989). There is in vitro evidence to suggest that CGRP may stimulate gingival and pulpal fibroblast cell proliferation and therefore it is conceivable that this peptide may play a regulatory role in healing following pulp injury (Trantor *et al.*, 1995; Bongenhielm *et al.*, 1995; Kawase *et al.*, 1999).

Byers and her colleagues considered that CGRP was likely be involved in reparative dentine formation due to the close anatomical relationship of CGRP-ir fibres and reparative dentine in injured rat molars (Byers *et al.*, 1990b). This opinion would appear to be supported by a recent study which found that CGRP-ir fibres were closely affiliated with dentine bridge formation following experimental rat molar pulpotomy (Zhang and Fukuyama, 1999). Furthermore, Jacobsen and Heyeraas (1996) reported that both IAN resection and capsaicin treatment resulted in reduced dentine formation in rat teeth, thus indicating that CGRP and SP may play a part in hard tissue formation within the dental pulp. However, a neural contribution to dentine formation has been disputed by other investigators (Torneck *et al.*, 1972).

5.1.6 Factors affecting intradental neuropeptide expression

It is evident that a number of physiological and pathological events can markedly effect the intradental expression of neuropeptides. The tooth pulp, along with other peripheral tissues, demonstrates a considerable plasticity of its peptidergic innervation. The main factors known to influence peptide expression within teeth are discussed below.

- Dental development

A few studies have attempted to evaluate neuropeptide expression during early tooth development (Mohamed and Atkinson, 1982; Nagata *et al.*, 1994). The progressive ingrowth of SP- and CGRP-ir fibres has been shown at the advanced bell stage which is prior to the appearance of any innervation by NPY-ir fibres (Nagata *et al.*, 1994). Less is known about changes in neuropeptide expression post eruption. Luthman and colleagues did not observe any differences in the immunocytochemical distribution of CGRP-, SP-, VIP- or NPY-ir fibres within human teeth at different stages of dental maturity (Luthman *et al.*, 1992). This finding was corroborated by a later study which found no difference in the expression of SP or CGRP between immature and fully formed third molars (Pertl *et al.*, 1997).

- Differences between primary and permanent teeth

As previously discussed in chapter one, there has been limited investigation of neuropeptide expression in either animal or human primary teeth (Fried and Risling,

1991; Egan *et al.*, 1996; Rodd and Boissonade, 1999a; 2000). Thus, little is known about possible differences between the two dentitions.

- Species differences

There are semi-quantitative data to suggest that there is some variation in neuropeptide expression between different species (Uddman *et al.*, 1980; 1984; 1986b). Notably, under normal conditions, there appears to be no intradental expression of VIP in rat tooth pulp (Uddman *et al.*, 1980). Furthermore, there are even marked differences in peptidergic nerve distribution between different tooth types within the same species, as has been clearly demonstrated for rat (Silverman and Kruger, 1987) and ferret (Jacobsen *et al.*, 1998).

- Pulpal injury and inflammation

There is unequivocal evidence that intradental peptidergic nerves undergo extensive morphological and neurochemical changes following pulpal injury (for reviews see, Byers, 1992; 1994). Immunocytochemical studies conducted by Byers and co-workers have specifically investigated the changes in the distribution of CGRP- and SP-ir nerve fibres following experimental pulpal injuries in rat molars (Khayat *et al.*, 1988; Kimberly and Byers, 1988; Taylor and Byers, 1990; Swift and Byers, 1992). These studies have all reported a marked sprouting of CGRP-ir nerve fibres which subsides following reparative dentine formation and pulpal healing. In addition, there was a reported increase in SP expression with pulpal inflammation. In contrast to the findings for SP and CGRP, no morphological changes have been detected for NPY-ir fibres following pulp injury under the same experimental conditions (Oswald and Byers, 1993).

Radioimmunoassay studies have attempted to quantify changes in peptide expression following pulpal injury. Grutzner and colleagues found that SP and CGRP expression fell to 10% and 45% of baseline levels respectively, following pulpal exposure in rat teeth, which was attributed to an increased release of these neuropeptides from the nerve terminals (Grutzner *et al.*, 1992). However, a similar study by Hargreaves and co-workers reported a two fold increase in the pulpal levels of SP and CGRP

(Hargreaves *et al.*, 1994). These conflicting results were attributed to the smaller pulpal exposures prepared by the latter investigators. Thus it is likely that changes in peptide expression are dependant on the degree of pulpal injury and inflammation.

- Pulpal deafferation

Considerable effort has been directed towards assessing the dynamic changes in neuropeptide expression within the trigeminal ganglion following IAN injury in experimental animal models (Wakisaka *et al.*, 1993; Sasaki *et al.*, 1994; Fristad *et al.*, 1996; Wakisaka *et al.*, 1996b). Less attention has been given to changes in the pulpal expression of neuropeptides subsequent to nerve injuries or deafferation. However, Fristad and collaborators eloquently demonstrated that, following IAN axotomy and reinnervation in rats, there was a change in intradental NPY-ir fibre distribution (Fristad *et al.*, 1996). In contrast to control animals, experimental animals showed a temporary increase in pulpal NPY-ir fibres and some fibres could even be seen within the odontoblast layers of the pulp horn. Furthermore, double-labelling and retrograde tracing revealed that these NPY-ir fibres had a trigeminal origin and were thus considered to be regenerating sensory afferents demonstrating de novo synthesis of NPY. A study by Wakisaka's group demonstrated a similar increase in intradental NPY expression following a chronic constriction type injury of the IAN in rat (Wakisaka *et al.*, 1996c). A more recent investigation by Fristad and colleagues, in the same experimental animal, showed that IAN axotomy also induced a marked increase in pulpal VIP-ir fibres (Fristad *et al.*, 1998).

5.1.7 Rationale for study

Numerous investigators have speculated that the regulation of intradental neuropeptide secretion may provide new therapeutic approaches for the management of pain, inflammation or healing. (Grutzner *et al.*, 1992; Hargreaves *et al.*, 1994; Hargreaves *et al.*, 1996). Whilst this may be a valid premise, future pharmacological intervention can not be considered without first gaining a sound understanding of intradental neuropeptide expression within human teeth, both in health and disease.

To date, there have been no large scale quantitative studies of peptide expression in human teeth and there has been little investigation of caries-induced changes in peptidergic innervation. Some comparable studies have been carried out in animals but their direct relevance to humans in less clear. Furthermore, it is not possible to correlate changes in neuropeptide expression with known pain histories when using an animal model.

There is sparse information about peptidergic innervation within primary teeth thus we have no appreciation of how primary teeth compare with their successors in terms of peptide expression. It is speculated that inter-dentition differences in peptide expression may have important clinical implications and may help to explain observed differences in sensitivity. A comprehensive investigation of neuropeptide expression in healthy and carious human primary and permanent teeth would therefore seem to be justified.

5.1.8 Aims and objectives

The aim of the present study was to further our understanding of the role of neuropeptides within human teeth both in health and disease. The specific objectives were as follows:

- to carry out a qualitative and quantitative comparison of intradental neural expression of CGRP, SP, VIP and NPY between primary and permanent teeth in different pulpal regions;
- to investigate the effect of caries progression on peptidergic nerve morphology and neuropeptide expression in both dentitions;
- to seek a correlation between neuropeptide expression and the reported pain history.

5.2 Materials and methods

Details regarding the overall experimental approach have been previously outlined in chapter two. In summary, qualitative and quantitative analysis was performed on tissue sections which had been triple-labelled using antisera to PGP 9.5, UEIL and

one of each of the four neuropeptides: CGRP; SP; VIP and NPY. Qualitative analysis for each peptide was based on the examination of three pulp sections from every tooth sample and quantitative data were derived from the analysis of one section. Neuropeptide expression was calculated as being the percentage area of PGP 9.5-labelled tissue that was also positively labelled for each specific neuropeptide (see, section 2.4.3):

$$\text{Neuropeptide expression} = \frac{\text{TAS for neuropeptide}}{\text{TAS for PGP 9.5}} \times 100\%$$

5.3 Results

5.3.1 Specificity controls

The reactions were considered specific as no positive immunolabelling was obtained either with the use of the primary antibody preabsorbed with the pure peptide or with the replacement of the primary antibody with non-immune serum (see, section 2.2.5). This was the case for all neuropeptides under investigation (Fig. 3.1E,F, section 3.2.2). However, in a few sections with dense inflammatory infiltrates, some non-specific labelling was evident in some round cells. The morphological appearance of these cells was consistent with that of lymphocytes and the labelling was considered to result from non-specific binding of the secondary fluorescent antisera to basic proteins within certain immune cells (Polak and Van Noorden, 1997). Particular care was therefore exercised during quantitative analysis to exclude these non-specifically stained structures from overall area measurements of neuropeptide immunolabelling.

It is acknowledged that cross-reactivity of specific antiserum with other structurally related peptides and proteins can not be absolutely excluded. Thus it is generally considered more appropriate to refer to immunoreactive material as demonstrating, for instance, "CGRP-like immunoreactivity." Whilst this point is recognised, the present study will for brevity simply refer to the labelling as, for example, "CGRP-immunoreactivity."

5.3.2 Findings relating to calcitonin gene-related peptide

- Qualitative observations

In intact samples, labelling for CGRP was abundant throughout the coronal pulp and appeared in a large proportion of fine, varicose nerve fibres. Calcitonin gene-related peptide-ir fibres were particularly prolific in the pulp horn region and the SOP. Furthermore, numerous branching immunoreactive fibres extended across the cell free zone to penetrate the odontoblast layer. Overall, CGRP-ir fibres appeared to be more abundant in permanent teeth than in primary teeth (Fig. 5.1A,B).

Most nerve trunks were seen to contain a moderate number of CGRP-ir fibres which demonstrated either a varicose or a smooth-surfaced morphology. Within the mid-coronal pulp stroma, CGRP-ir fibres were also seen to have close spatial arrangements with blood vessels (see, chapter six). Individual smooth-surfaced CGRP-ir fibres were occasionally present towards the peripheral regions of the mid-coronal pulp with no obvious relation to any other structures.

Carious specimens, from both dentitions, demonstrated several marked changes in the distribution of CGRP-ir fibres as compared to intact specimens. The most obvious changes were seen within the pulp horn regions where intense aborisation of CGRP-ir fibres was observed. Within this neural network, CGRP appeared to be expressed by the majority of PGP 9.5-ir fibres and displayed an intensely fluorescent appearance (Fig. 5.1C,D).

In the regions below the pulp tip, CGRP-ir fibres were often seen within small nerve trunks or in dense bands of PGP 9.5-labelled tissue. Immunoreactivity within the SOP was also increased, with CGRP-ir fibres frequently presenting as thickened nerve bundles. In areas where PGP 9.5-ir fibres surrounded intrapulpal abscesses or acted as an "interface" between necrotic and vital tissue, CGRP-labelling within the PGP-labelled tissue was also very predominant. Another observation, made for some carious samples, was that CGRP expression appeared to be greatly increased within the nerve trunks (Fig. 5.1E,F).

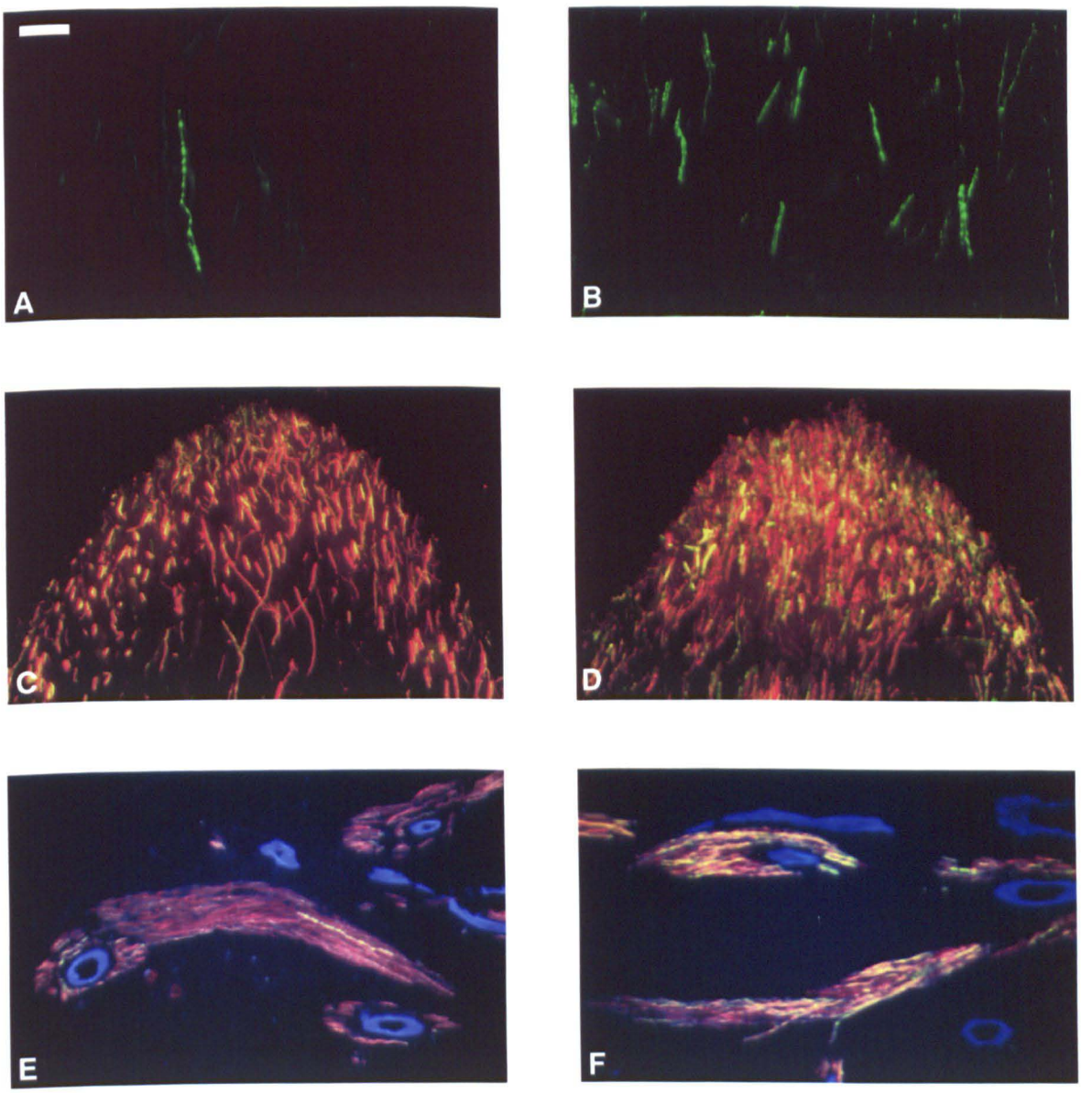


Figure 5.1. Photomicrographs demonstrating differences in the distribution of CGRP-ir fibres in the coronal pulp of intact and carious primary and permanent teeth. **(A)** The pulp horn region of an intact primary tooth showing a single CGRP-ir nerve fibre (green) and **(B)** a greater number of CGRP-ir fibres seen in an intact permanent tooth. **(C)** Double-labelling for PGP 9.5 (red) and CGRP (yellow) in the pulp horn of an intact primary tooth showing moderate CGRP expression and **(D)** an overall increased CGRP expression in the pulp horn of a carious primary tooth. **(E)** Triple-labelling for PGP 9.5 (red), CGRP (yellow) and UEIL (blue) in the mid-coronal region of an intact permanent tooth showing few CGRP-ir fibres within the nerve trunks and **(F)** a marked increase in CGRP expression in the nerve trunks of a grossly carious permanent tooth. Scale bar = 15μm **(A,B)**, 30μm **(C-E)**.

- Quantitative analysis

Table 5.1A shows the mean (±SD, range) percentage area of PGP 9.5-labelled tissue that was also labelled for CGRP within each dentition and according to the field of analysis and degree of caries. Table 5.1B provides the corresponding statistical data. Bar charts showing pooled data are presented in Figure 5.2. Key findings arising from the quantitative analysis of CGRP expression are outlined below.

- The greatest mean CGRP expression within intact teeth was seen in field 3 (primary teeth = 26.79%, permanent teeth = 34.59%). Field 3 also demonstrated the highest CGRP expression in both moderately carious (primary teeth = 41.17%, permanent teeth = 27.34%) and grossly carious samples (primary teeth = 41.96%, permanent teeth = 48.51%).

- Mean CGRP expression was significantly different between the two dentitions ($P \leq 0.009$, ANOVA) with CGRP expression generally being greater in permanent teeth in all four fields of analysis. As an example, mean CGRP expression in field 1 of intact primary teeth was approximately half that found for intact permanent samples (primary teeth = 10.29%, permanent teeth = 24.73%). However, further pairwise comparisons (see, Fig. 5.2) only revealed significant inter-dentition differences for field 1 of intact samples and field 4 of grossly carious samples ($P<0.05$, Tukey's test).

- Caries was determined to have a significant effect on mean CGRP expression in all four fields of analysis ($P \leq 0.017$, ANOVA) with the highest CGRP expression being seen in grossly carious samples. As an example, CGRP expression in grossly carious samples was approximately double that found for intact samples, except in field 3 where the difference was not so great. However, further pairwise comparisons (see, Fig. 5.2) revealed that, for primary teeth, significant differences only existed between grossly carious and intact samples (fields 1, 2 and 4) but for permanent samples, significant differences existed between both grossly carious and intact samples and grossly carious and moderately carious samples (fields 1, 2 and 4) ($P<0.05$, Tukey's test).

Table 5.1A. Mean (±SD, range) percentage area of PGP 9.5-labelled tissue that was also labelled for CGRP according to the tooth type, field of analysis and degree of caries

Field/tooth(n)	Degree of caries		
	None	Moderate	Gross
Field 1			
Primary tooth (n=21,20,18)	10.29 (±7.61, 1.15-31.40)	18.15 (±10.93, 2.91-38.00)	25.28 (±14.22, 10.70-56.60)
Permanent tooth (n=18,20,20)	24.73 (±19.73, 1.45-70.20)	15.13 (±12.01, 3.43-51.30)	43.25 (±21.11, 10.27-80.46)
Field 2			
Primary tooth (n=21,20,18)	14.49 (±9.58, 2.60-36.80)	25.42 (±13.66, 1.80-46.30)	27.58 (±13.92, 8.88-59.70)
Permanent tooth (n=17,20,20)	20.68 (±15.85, 1.96-55.43)	17.83 (±10.14, 30.20-36.80)	40.02 (±32.01, 5.30-85.60)
Field 3			
Primary tooth (n=21,20,19)	26.79 (±17.10, 5.17-65.70)	41.17 (±20.93, 5.00-84.40)	41.96 (±18.04, 21.5-78.20)
Permanent tooth (n=17,20,19)	34.59 (±20.49, 6.36-65.00)	27.34 (±17.49, 3.20-76.0)	48.51 (±19.61, 21.21-95.20)
Field 4			
Primary tooth (n=19,20,20)	11.23 (±5.41, 4.76-21.30)	17.10 (±9.51, 1.0-37.60)	20.48 (±7.84, 8.10-38.90)
Permanent tooth (n=18,20,18)	15.80 (±11.99, 3.70-55.20)	17.34 (±13.94, 1.35-45.5)	28.04 (±14.91, 6.17-61.97)

Table 5.1B. Significance values for effect of dentition type and degree of caries on CGRP expression (two-way analysis of variance on log-transformed data)

Main effects	Field 1	Field 2	Field 3	Field 4
Dentition type	P=0.009	P=0.003	P=0.001	P=0.001
Degree of caries	P=0.0001	P=0.0001	P=0.017	P=0.0001

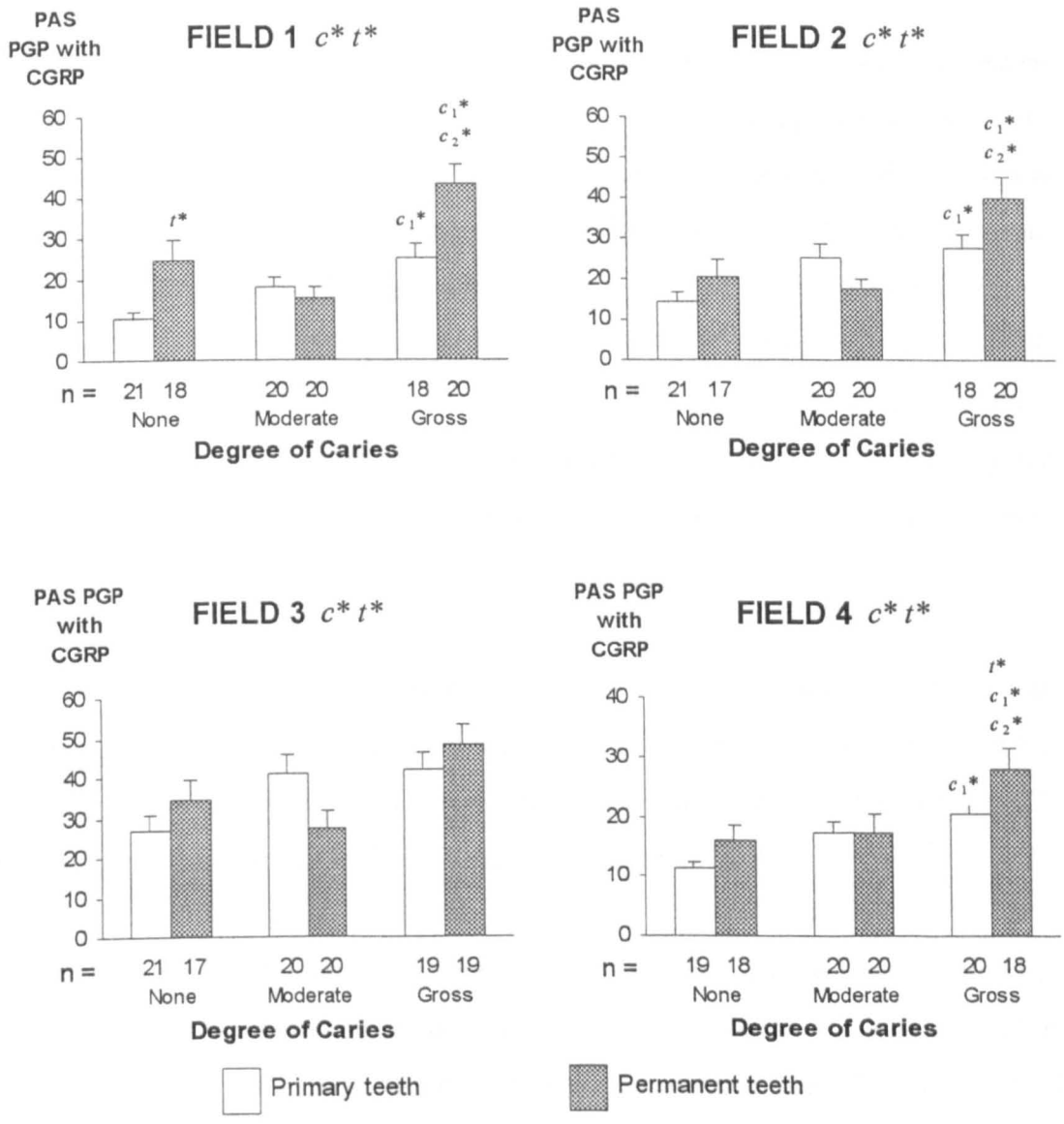


Figure 5.2. Bar charts showing mean (±SEM) percentage area of PGP 9.5-labelled tissue that was also labelled for CGRP in primary and permanent teeth according to the degree of caries. Field 1=tip of pulp horn; Field 2=region subjacent to field 1; Field 3=buccal subodontoblastic plexus; Field 4=mid-coronal region.

Main titles: c^* = significant difference in mean CGRP expression according to the degree of caries; t^* = significant difference between primary and permanent teeth ($P<0.05$, ANOVA, log transformed data).

Bar titles: c_1^* = significantly different from intact samples of the same dentition; c_2^* = significantly different from moderately carious samples of the same dentition; t^* = significantly different from primary teeth with same degree of caries ($P<0.05$, Tukey's test).

5.3.3 Findings relating to substance P

- Qualitative observations

In pulps taken from intact teeth, SP-labelling was visualised as bright fluorescent varicosities within a moderate number of PGP 9.5-ir fibres throughout the pulp horn region and the SOP. In some areas, SP-ir fibres could be seen extending into the odontoblast layer. Nerve trunks also demonstrated SP expression which was apparent within a small number of fibres with either a beaded or a smooth-surfaced morphology. There was a close spatial relationship between some pulpal blood vessels and SP-ir fibres and this aspect will be discussed in the next chapter. Generally, there appeared to be a similar proportion of SP-expressing nerve fibres within primary and permanent teeth, except in the tip of the pulp horn region, where permanent teeth demonstrated a greater proportion of SP-ir fibres (Fig. 5.3A,B).

Overall, there appeared to be an increased SP expression in carious teeth from both dentitions (Fig. 5.3C,D), although there was often a wide variability between samples. In particular, some samples with a known pain history were seen to demonstrate SP-labelling in practically every PGP 9.5-ir nerve fibre (Fig. 5.3E,F).

In addition, some morphological changes in SP-ir fibre distribution were observed in carious samples from both dentitions. These included the dense localisation of SP-ir fibres around intrapulpal abscesses and the formation of mesh-like SP-positive neural "barriers" between areas of dense inflammation and areas of apparently normal pulp tissue.

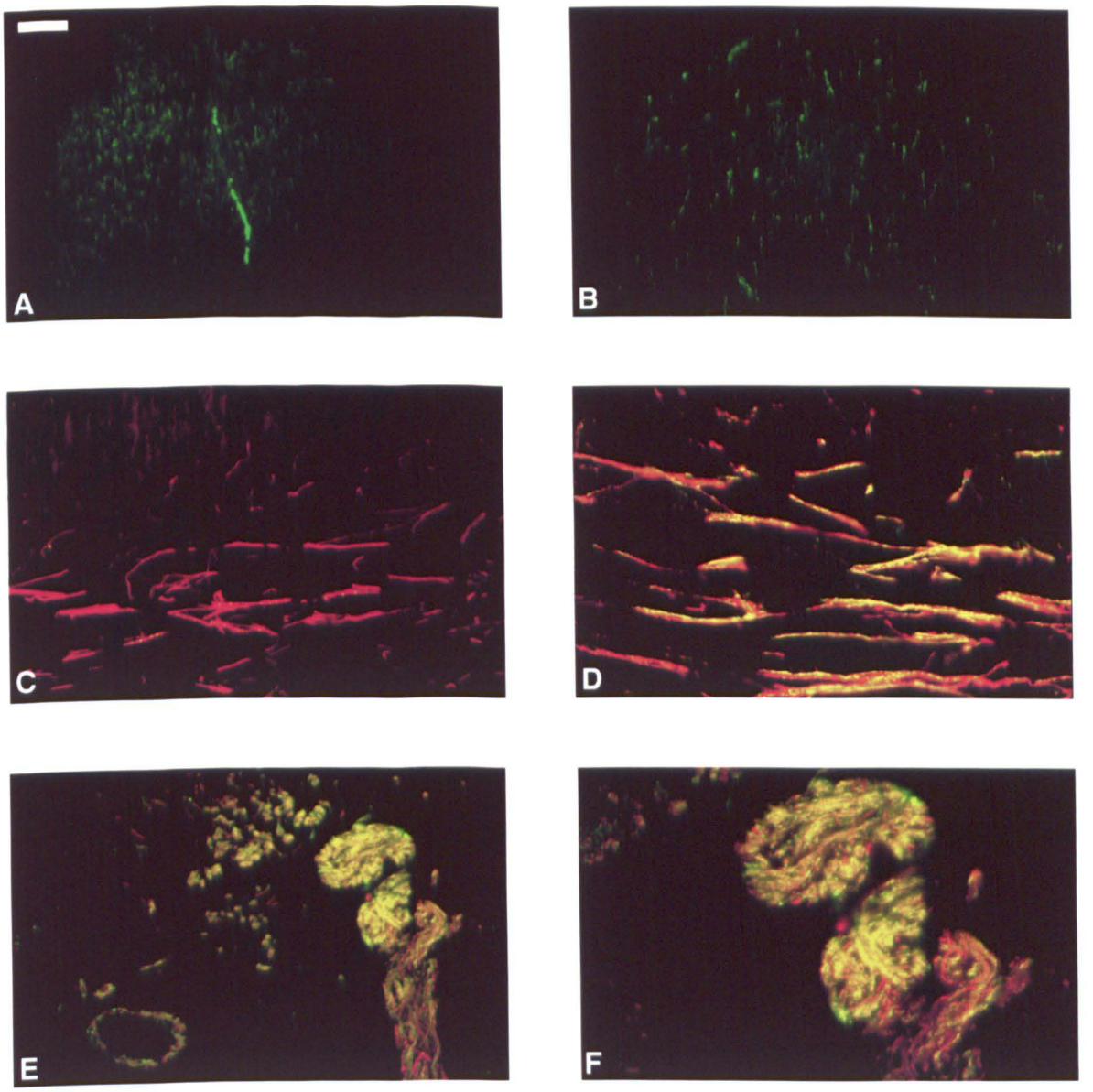


Figure 5.3. Photomicrographs demonstrating differences in the distribution of SP-ir fibres in the coronal pulp of intact and carious primary and permanent teeth. **(A)** The pulp horn region of an intact primary tooth showing a few SP-ir nerve fibre (green) and **(B)** a greater number of SP-ir fibres seen in an intact permanent tooth. **(C)** Double-labelling for PGP 9.5 (red) and SP (yellow) in the subodontoblastic nerve plexus of an intact permanent tooth showing minimal SP expression and **(D)** a marked increase in SP expression within the subodontoblastic nerve plexus of a carious permanent tooth. **(E)** Low and **(F)** high magnification views showing double-labelling for PGP 9.5 (red) and SP (yellow) in the pulp horn region of a grossly carious symptomatic permanent tooth with a marked upregulation of SP expression Scale bar =15μm **(C,D,F)**, 30μm **(A,B,E)**.

- Quantitative analysis

Table 5.2A shows the mean (±SD, range) percentage area of PGP 9.5-labelled tissue that was also labelled for SP within each dentition and according to the field of analysis and degree of caries. Table 5.2B provides the corresponding statistical data. Bar charts showing pooled data are presented in Figure 5.4.

Key findings arising from the quantitative analysis of SP expression are outlined below.

- The greatest mean SP expression within intact teeth was seen in field 4 (primary teeth = 10.39%, permanent teeth = 14.34%). For grossly carious samples the greatest mean SP expression was seen in field 2 for primary teeth (18.27%), and field 1 for permanent teeth (33.64%).

- Mean SP expression was only significantly different between the two dentitions in field 1 (P=0.001, ANOVA). However, further pairwise comparisons (see Fig. 5.4) revealed that, in fact, there was only a significant inter-dentition difference in the absence of caries (P<0.05, Tukey's test). However, within these intact samples, SP expression was over three times greater in permanent teeth than in corresponding primary teeth (primary teeth = 4.26%, permanent teeth = 13.14%).

- Caries was determined to have a significant effect on mean SP expression in all four fields of analysis (P≤0.007, ANOVA) with the highest SP expression being seen in grossly carious samples. As an example, SP expression in field 1 of grossly carious primary samples was approximately four times greater than that seen in intact samples (intact samples = 4.26%, grossly carious samples = 16.50%). However, further pairwise comparisons (see Fig. 5.4) revealed that, for primary teeth, significant differences only existed between grossly carious and intact samples and grossly carious and moderately carious samples in field 1. The same was true for permanent teeth but they also showed a significant difference in SP expression between grossly carious and intact samples in field 3 (P<0.05, Tukey's test).

Table 5.2A. Mean (±SD, range) percentage area of PGP 9.5-labelled tissue that was also labelled for SP according to the tooth type, field of analysis and degree of caries

Field/tooth(n)	Degree of caries		
	None	Moderate	Gross
Field 1			
Primary tooth (n=18,20,20)	4.26 (±3.18, 0.89-14.00)	6.56 (±5.34, 1.05-16.88)	16.50 (±14.26, 5.09-57.23)
Permanent tooth (n=19,19,19)	13.14 (±10.07, 1.66-35.80)	9.95 (±6.94, 2.27-27.73)	33.64 (±26.14, 5.70-83.70)
Field 2			
Primary tooth (n=19,20,20)	9.62 (±7.53, 1.00-28.30)	9.69 (±6.39, 1.26-25.22)	18.27 (±15.93, 4.25-71.25)
Permanent tooth (n=19,19,18)	11.68 (±9.89, 3.00-39.37)	13.40 (±9.34, 2.30-38.56)	23.12 (±20.61, 2.98-93.50)
Field 3			
Primary tooth (n=18,20,20)	10.06 (±11.59, 0.00-43.90)	14.23 (±10.60, 1.02-44.40)	13.96 (±12.26, 0.00-48.00)
Permanent tooth (n=18,19,18)	9.44 (±8.67, 1.49-30.30)	14.20 (±8.83, 2.680-27.10)	20.97 (±19.78, 0.00-69.20)
Field 4			
Primary tooth (n=19,20,20)	10.39 (±8.22, 2.12-35.60)	14.40 (±9.53, 1.18-38.88)	17.42 (±8.75, 8.29-45.60)
Permanent tooth (n=18,19,19)	14.34 (±12.36, 1.03-39.67)	13.31 (±6.00, 6.34-25.29)	24.45 (±20.47, 2.25-95.60)

Table 5.2 B. Significance values for effect of dentition type and degree of caries on SP expression (two-way analysis of variance on log-transformed data)

Main effects	Field 1	Field 2	Field 3	Field 4
Dentition type	P=0.0001	P=0.075	P=0.821	P=0.312
Degree of caries	P=0.0001	P=0.001	P=0.007	P=0.001

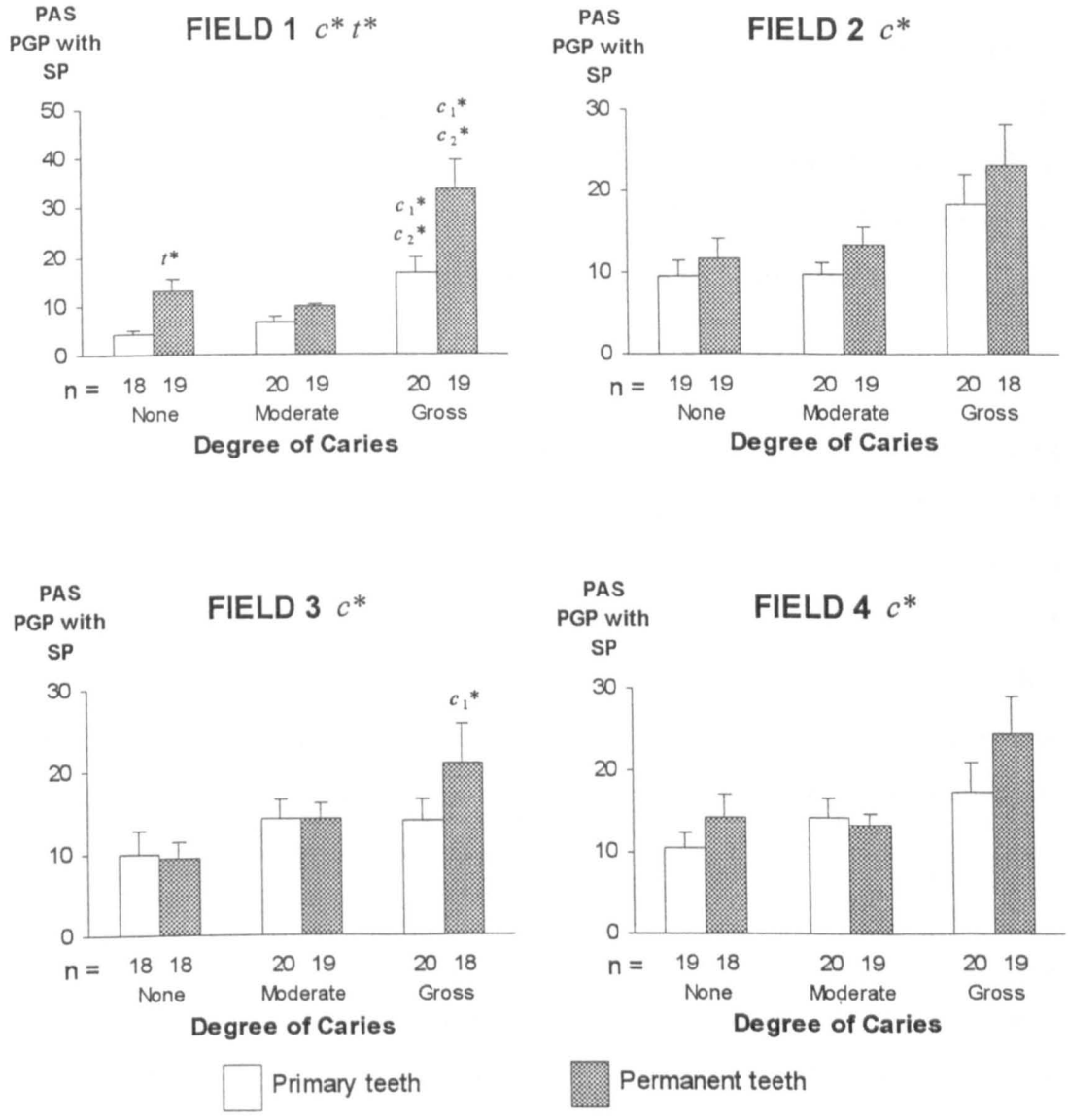


Figure 5.4. Bar charts showing mean (±SEM) percentage area of PGP 9.5-labelled tissue that was also labelled for SP in primary and permanent teeth according to the degree of caries. Field 1=tip of pulp horn; Field 2=region subjacent to field 1; Field 3=buccal subodontoblastic plexus; Field 4=mid-coronal region.

Main titles: c^* = significant difference in mean SP expression according to the degree of caries; t^* = significant difference between primary and permanent teeth ($P<0.05$, ANOVA, log transformed data).

Bar titles: c_1^* = significantly different from intact samples of the same dentition; c_2^* = significantly different from moderately carious samples of the same dentition; t^* = significantly different from primary teeth with same degree of caries ($P<0.05$, Tukey's test).

5.3.4 Findings relating to vasoactive intestinal polypeptide

- Qualitative observations

There was generally little VIP expression in intact primary teeth and many samples lacked any VIP-ir fibres either within the pulp horn region (Fig 5.5A,C) or within the SOP. Nerve trunks in the mid-coronal region usually demonstrated 3 to 4 VIP-ir beaded or smooth-surfaced fibres, although not all trunks showed VIP expression. In addition, labelling for VIP was seen in close association with some pulpal vessels (see chapter six for further detail).

In contrast, intact permanent teeth often showed numerous fine beaded VIP-ir fibres within the pulp horn regions (Fig. 5.5B), which ran parallel to the pulp horn and did not appear to be related to any other structures. Labelling for VIP was also seen in reasonable amounts within the SOP between the two pulp horns, but was not present to any great extent within the buccal or lingual SOP. There was no evidence of any VIP-ir fibres extending into the odontoblast layer. Consistent with observations made for the primary dentition, most large nerve trunks in permanent tooth pulps also contained 3 to 4 VIP-ir fibres (Fig. 5.5E).

Several interesting observations were made for VIP expression within carious samples from both dentitions. In carious primary teeth there was often a marked increase in the number of VIP-ir fibres within the pulp horn regions (Fig. 5.5D). These fibres had a beaded morphology and occasionally were seen to extend into the odontoblast layer. In the permanent dentition, there actually appeared to be a decrease in the number of VIP-ir fibres within moderately carious samples, but there was an overall increase in grossly carious samples. Occasionally a dramatic increase in VIP expression was evident within the nerve trunks of some grossly carious samples where almost every nerve fibre was seen to express VIP (Fig. 5.5F). Interestingly, these samples were often found to be associated with a positive pain history.

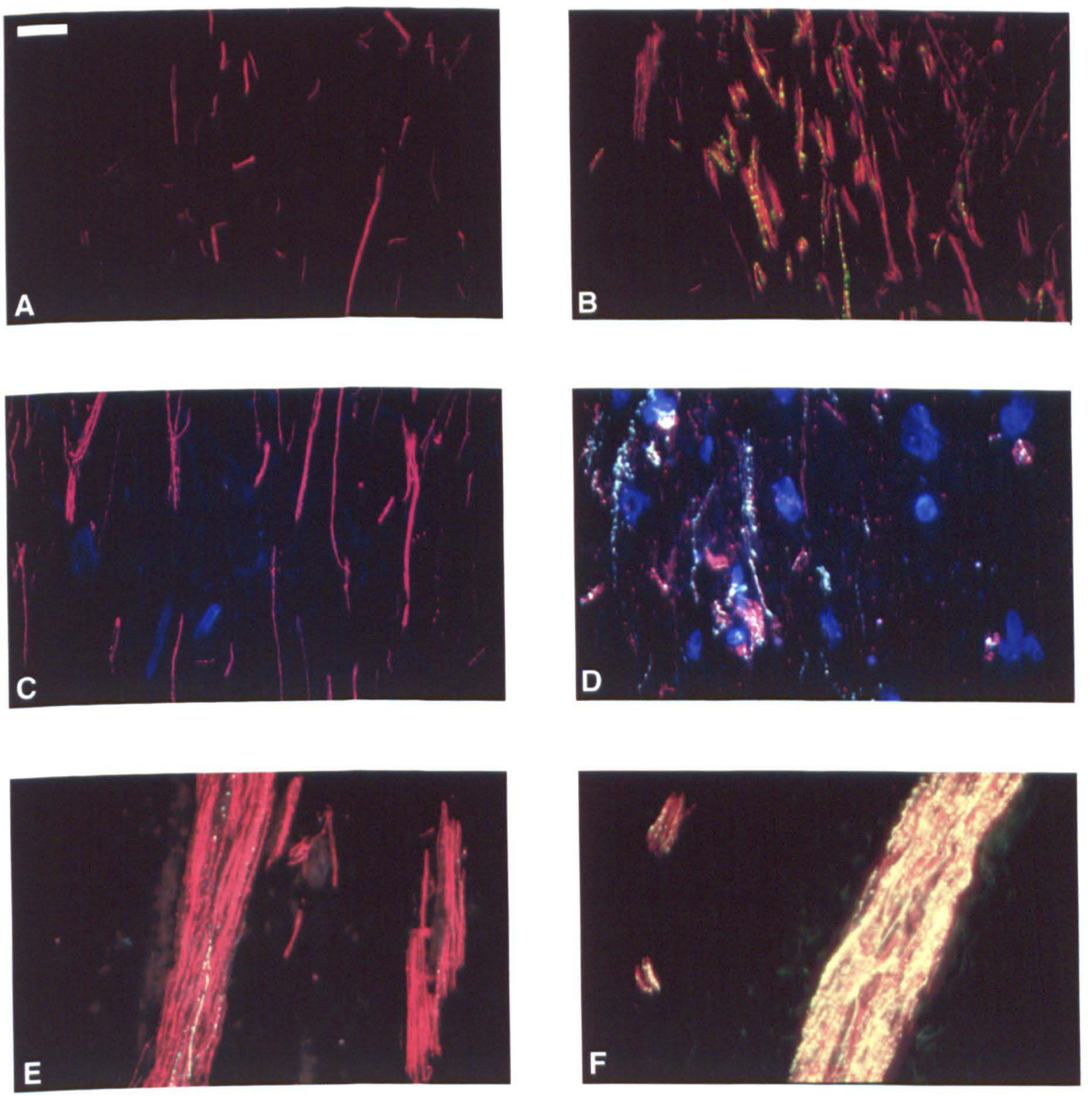


Figure 5.5. Photomicrographs demonstrating differences in the distribution of VIP-ir fibres in the coronal pulp of intact and carious primary and permanent teeth. **(A)** Double labelling for PGP 9.5 (red) and VIP (yellow) in the pulp horn region of an intact primary tooth showing no VIP-ir fibres and **(B)** numerous VIP-ir fibres in the pulp horn of an intact permanent tooth. **(C)** Triple-labelling for PGP 9.5 (red), VIP (green) and UEIL (blue) in the pulp horn region of an intact primary tooth showing no VIP-ir fibres and **(D)** numerous VIP-ir fibres in the pulp horn of a carious primary tooth. **(E)** Double-labelling for PGP 9.5 (red) and VIP (yellow) in the nerve trunk of an intact permanent tooth showing few VIP-ir fibres and **(F)** a marked increase in VIP expression in a grossly carious symptomatic permanent tooth. Scale bar = 15 μm **(A-F)**.

- Quantitative analysis

Table 5.3A shows the mean (±SD, range) percentage area of PGP 9.5-labelled tissue that was also labelled for VIP within each dentition and according to the field of analysis and degree of caries. Table 5.3B provides the corresponding statistical data. Bar charts showing pooled data are presented in Figure 5.6.

Key findings arising from the quantitative analysis of VIP expression are outlined below.

- The greatest mean VIP expression within intact primary teeth was seen in field 2 (1.05%) and for intact permanent teeth was seen in field 1 (8.56%). For grossly carious primary samples the greatest mean VIP expression was seen in field 2 (7.22%) and for permanent teeth was seen in field 1 (11.86%).

- Mean VIP expression was significantly different between the two dentitions in fields 1, 2 and 4 ($P \leq 0.003$, ANOVA). As an example, the greatest difference between the two dentitions was seen in field 1 of intact samples (primary teeth = 0.63%, permanent teeth = 8.56%). Further pairwise comparisons (see, Fig. 5.6) revealed that there were significant inter-dentition differences in VIP expression within intact samples and moderately carious samples for field 2, but only within intact samples for fields 1 and 4 ($P<0.05$, Tukey's test).

- Caries was determined to have a highly significant effect on mean VIP expression in fields 1 and 2 ($P=0.0001$, ANOVA) with the highest VIP expression being seen in grossly carious samples. As an example, VIP expression in field 1 of grossly carious primary samples was approximately ten times greater than that seen in corresponding intact samples (intact samples = 0.63%, grossly carious samples = 6.17%). However, further pairwise comparisons (see, Fig. 5.6) revealed that, significant differences only existed between grossly carious and moderately carious permanent teeth in field 1, and between grossly carious and intact, and grossly carious and moderately carious primary teeth in field 2 ($P<0.05$, Tukey's test).

Table 5.3A. Mean (±SD, range) percentage area of PGP 9.5-labelled tissue that was also labelled for VIP according to the tooth type, field of analysis and degree of caries

Field/tooth(n)	Degree of caries		
	None	Moderate	Gross
Field 1			
Primary tooth (n=21,20,20)	0.63 (±0.75, 0.00-2.10)	1.42 (±2.54, 0.00-11.50)	6.17 (±7.44, 0.00-27.17)
Permanent tooth (n=19,19,21)	8.56 (±7.69, 0.52-28.70)	3.35 (±3.56, 0.00-14.37)	11.86 (±10.34, 1.75-42.10)
Field 2			
Primary tooth (n=20,20,20)	1.05 (±1.28, 0.00-4.80)	1.82 (±2.62, 0.00-11.50)	7.22 (±8.14, 0.00-26.29)
Permanent tooth (n=19,20,21)	6.38 (±5.23, 0.92-19.60)	4.15 (±4.65, 0.00-18.50)	8.51 (±5.92, 1.70-25.00)
Field 3			
Primary tooth (n=21,20,20)	0.39 (±0.68, 0.00-2.40)	1.20 (±3.28, 0.00-14.70)	2.79 (±5.08, 0.00-19.13)
Permanent tooth (n=19,20,19)	2.77 (±4.38,0.00-17.70)	1.67 (±3.07, 0.00-13.28)	2.32 (±3.01, 0.00-12.60)
Field 4			
Primary tooth (n=21,20,20)	0.91 (±0.84, 0.00-3.00)	1.84 (±1.54, 0.18-6.58)	2.62 (±3.02, 0.03-13.41
Permanent tooth (n=18,20,21)	2.68 (±2.73, 0.49-9.48)	2.30 (±3.07, 0.00-13.28)	4.86 (±8.82, 0.63-41.10)

Table 5.3B. Significance values for effect of dentition type and degree of caries on VIP expression (two-way analysis of variance on log-transformed data)

Main effects	Field 1	Field 2	Field 3	Field 4
Dentition type	P=0.0001	P=0.0001	P=0.315	P=0.003
Degree of caries	P=0.0001	P=0.0001	P=0.089	P=0.092

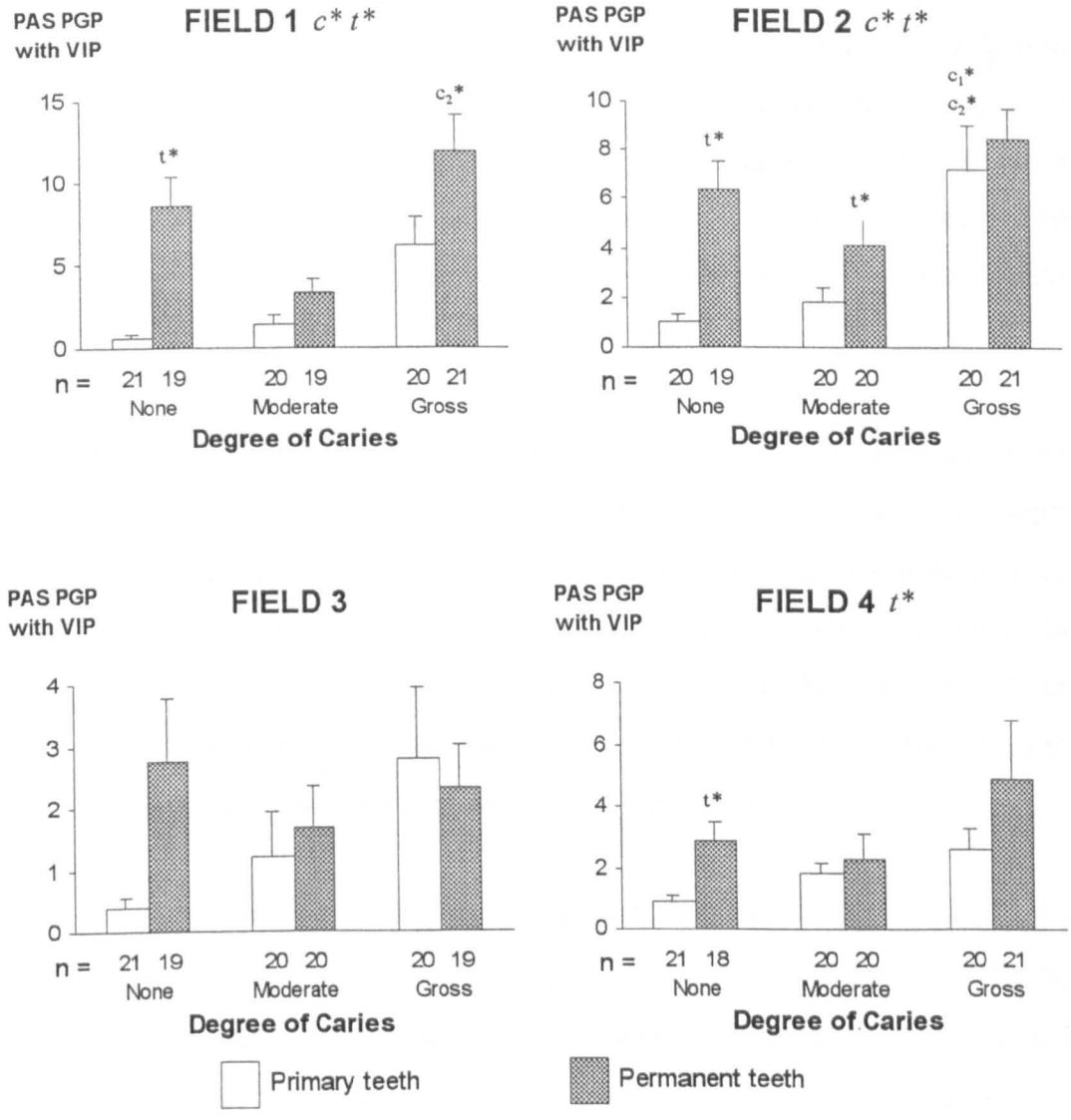


Figure 5.6. Bar charts showing mean (±SEM) percentage area of PGP 9.5-labelled tissue that was also labelled for VIP in primary and permanent teeth according to the degree of caries. Field 1=tip of pulp horn; Field 2=region subjacent to field 1; Field 3=buccal subodontoblastic plexus; Field 4=mid-coronal region.

Main titles: c^* = significant difference in mean VIP expression according to the degree of caries; t^* = significant difference between primary and permanent teeth ($P<0.05$, ANOVA, log transformed data).

Bar titles: c_1^* = significantly different from intact samples of the same dentition; c_2^* = significantly different from moderately carious samples of the same dentition; t^* = significantly different from primary teeth with same degree of caries ($P<0.05$, Tukey's test).

5.3.5 Findings relating to neuropeptide Y

- Qualitative observations

The majority of intact teeth showed no NPY labelling within either the pulp horn regions (Fig. 5.7E) or the SOP. However, a few primary and permanent samples did demonstrate a limited number of small beaded NPY-ir nerves in the pulp horns, although these were not seen at the pulp horn tip nor anywhere in the vicinity of the odontoblast layer. In addition, a few small blood vessels appeared to be associated with NPY-ir fibres in the pulp horn regions (Fig. 5.7A).

The most pronounced NPY-labelling in both primary and permanent teeth was seen in association with blood vessels within the mid-coronal region (Fig. 5.7B) (see, chapter six). However, both dentitions demonstrated a few NPY-ir fibres within some nerve trunks (Fig. 5.7C). Not all trunks contained NPY-ir fibres, and NPY expression generally seemed most abundant within neurovascular bundles (Fig. 5.7D). Furthermore, a few smooth-surfaced NPY-ir fibres were seen towards the peripheral pulp stroma without any obvious relation to any other structures.

With caries, the number of NPY-ir fibres within the pulp horn regions appeared to increase in samples from both dentitions although this was not a universal finding. Very occasionally NPY-ir fibres could be seen extending right to the tip of the pulp horns, although they were not seen to pass into the odontoblast layer (Fig. 5.7F). These NPY-ir fibres displayed either a beaded or a smooth-surfaced morphology. There appeared to be no NPY expression within dense areas of PGP 9.5-labelling in the pulp horn regions. Very occasionally, carious samples demonstrated some NPY-ir fibres within the buccal or lingual SOP.

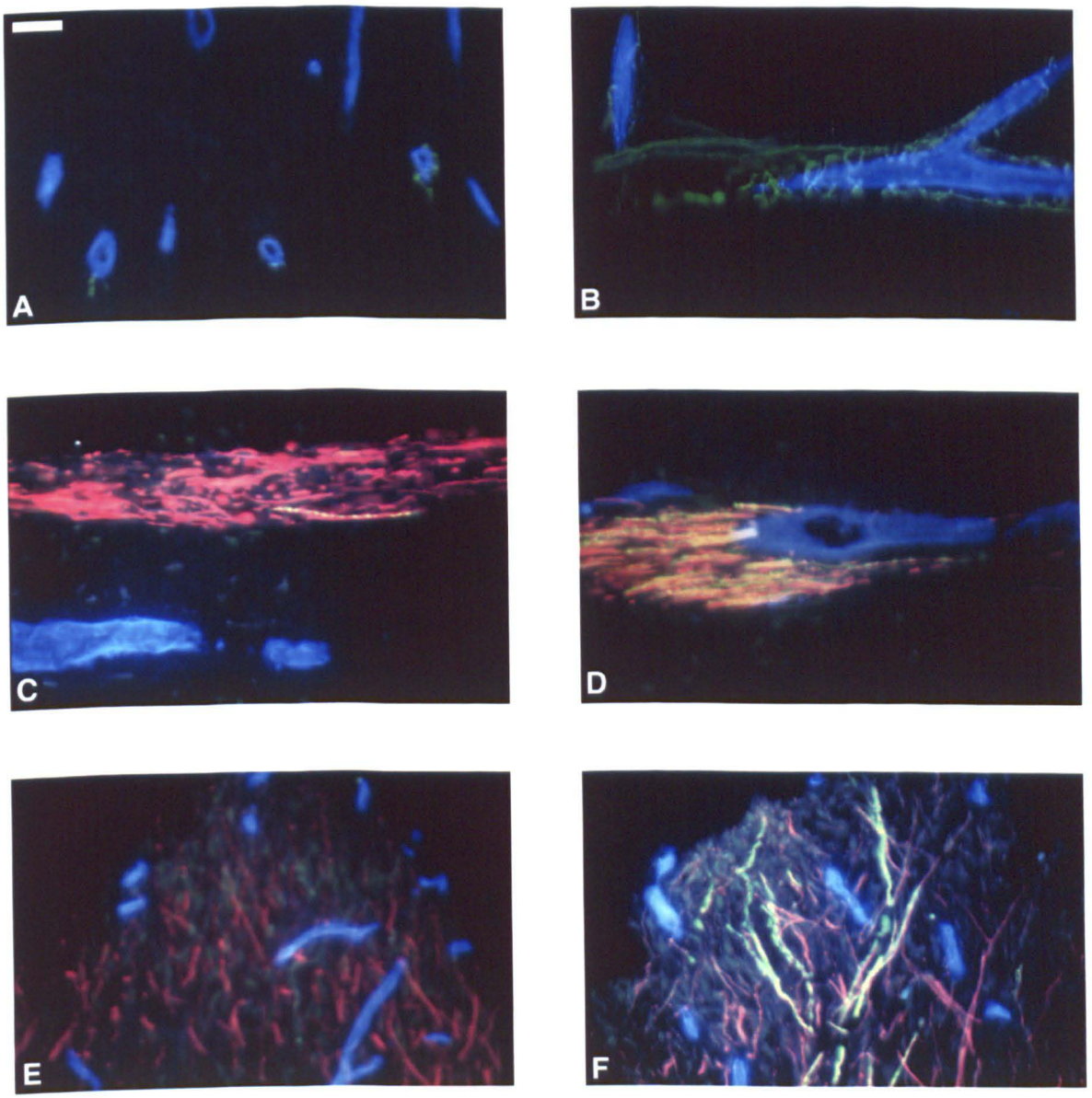


Figure 5.7. Photomicrographs demonstrating differences in the distribution of NPY-ir fibres in the coronal pulp of intact and carious primary and permanent teeth. **(A)** Double-labelling for NPY (green) and UEIL (blue) to show the close spatial relationship between NPY-ir fibres and blood vessels in the pulp horn and **(B)** the mid-coronal region. **(C)** Triple-labelling for PGP 9.5 (red), NPY (yellow) and UEIL (blue) in the mid-coronal region of an intact permanent tooth showing minimal NPY expression in a nerve trunk and **(D)** greater NPY expression within a neurovascular bundle. **(E)** Triple-labelling for PGP 9.5 (red), NPY (yellow/green) and UEIL (blue) in the pulp horn of an intact primary tooth showing no NPY-ir fibres and **(F)** the presence of several NPY-ir fibres in the same region of a carious primary tooth. Scale bar = 15μm **(A-F)**.

- Quantitative analysis

Table 5.4A shows the mean (±SD, range) percentage area of PGP 9.5-labelled tissue that was also labelled for NPY within each dentition and according to the field of analysis and degree of caries. Table 5.4B provides the corresponding statistical data. Bar charts showing pooled data are presented in Figure 5.8.

Key findings arising from the quantitative analysis of NPY expression are outlined below.

- The greatest mean NPY expression within intact primary and permanent teeth was seen in field 4 (primary teeth = 8.15%, permanent teeth = 6.06%). For grossly carious samples the greatest mean NPY expression was also seen in field 4 (primary teeth = 7.87%, permanent teeth = 6.16%).

- There was no significant difference in mean NPY expression between the two dentitions in any field of analysis ($P>0.05$, ANOVA).

- Caries was only determined to have a significant effect on mean NPY expression in field 1 ($P=0.011$, ANOVA) with the highest NPY expression being seen in grossly carious samples. As an example, NPY expression in field 1 of grossly carious primary samples was approximately nine times greater than that seen in intact samples (intact samples = 0.15%, grossly carious samples = 1.36%). However, further pairwise comparisons (see, Fig. 5.8) revealed that, in field 1, significant differences only existed between grossly carious and moderately carious permanent teeth ($P<0.05$, Tukey's test).

Table 5.4A. Mean (±SD, range) percentage area of PGP 9.5-labelled tissue that was also labelled for NPY according to the tooth type, field of analysis and degree of caries.

Field/tooth(n)	Degree of caries		
	None	Moderate	Gross
Field 1			
Primary tooth (n=21,19,18)	0.15 (±0.25, 0.00-0.80)	0.40 (±0.70, 0.00-2.20)	1.36 (±3.04, 0.00-13.00)
Permanent tooth (n=17,18,21)	0.36 (±0.72, 0.00-2.30)	0.32 (±0.45, 0.00-1.60)	1.42 (±2.15, 0.00-9.10)
Field 2			
Primary tooth (n=20,19,18)	1.50 (±2.15, 0.00-7.30)	3.39 (±3.89, 0.00-12.50)	1.81 (±2.12, 0.00-6.40)
Permanent tooth (n=17,19,21)	1.44 (±1.43, 0.00-5.00)	1.33 (±1.41, 0.00-4.80)	3.23 (±4.89, 0.00-22.70)
Field 3			
Primary tooth (n=21,18,18)	0.18 (±0.81, 0.00-3.70)	0.50 (±1.42, 0.00-5.70)	1.67 (±3.58, 0.00-13.30)
Permanent tooth (n=17,19,21)	0.24 (±0.97, 0.00-4.00)	0.51 (±0.92, 0.00-2.70)	0.59 (±1.46, 0.00-5.00)
Field 4			
Primary tooth (n=20,18,18)	8.15 (±6.93, 0.70-23.20)	11.96 (±7.37, 2.78-26.00)	7.87 (±7.01, 2.84-31.00
Permanent tooth (n=17,19,21)	6.06 (±4.75, 1.15-18.40)	7.70 (±5.91, 1.07-20.00)	6.16 (±4.40, 1.35-17.50)

Table 5.4B. Significance values for effect of dentition type and degree of caries on NPY expression (two-way analysis of variance on log-transformed data)

Main effects	Field 1	Field 2	Field 3	Field 4
Dentition type	P=0.515	P=0.459	P=0.469	P=0.211
Degree of caries	P=0.011	P=0.632	P=0.472	P=0.384

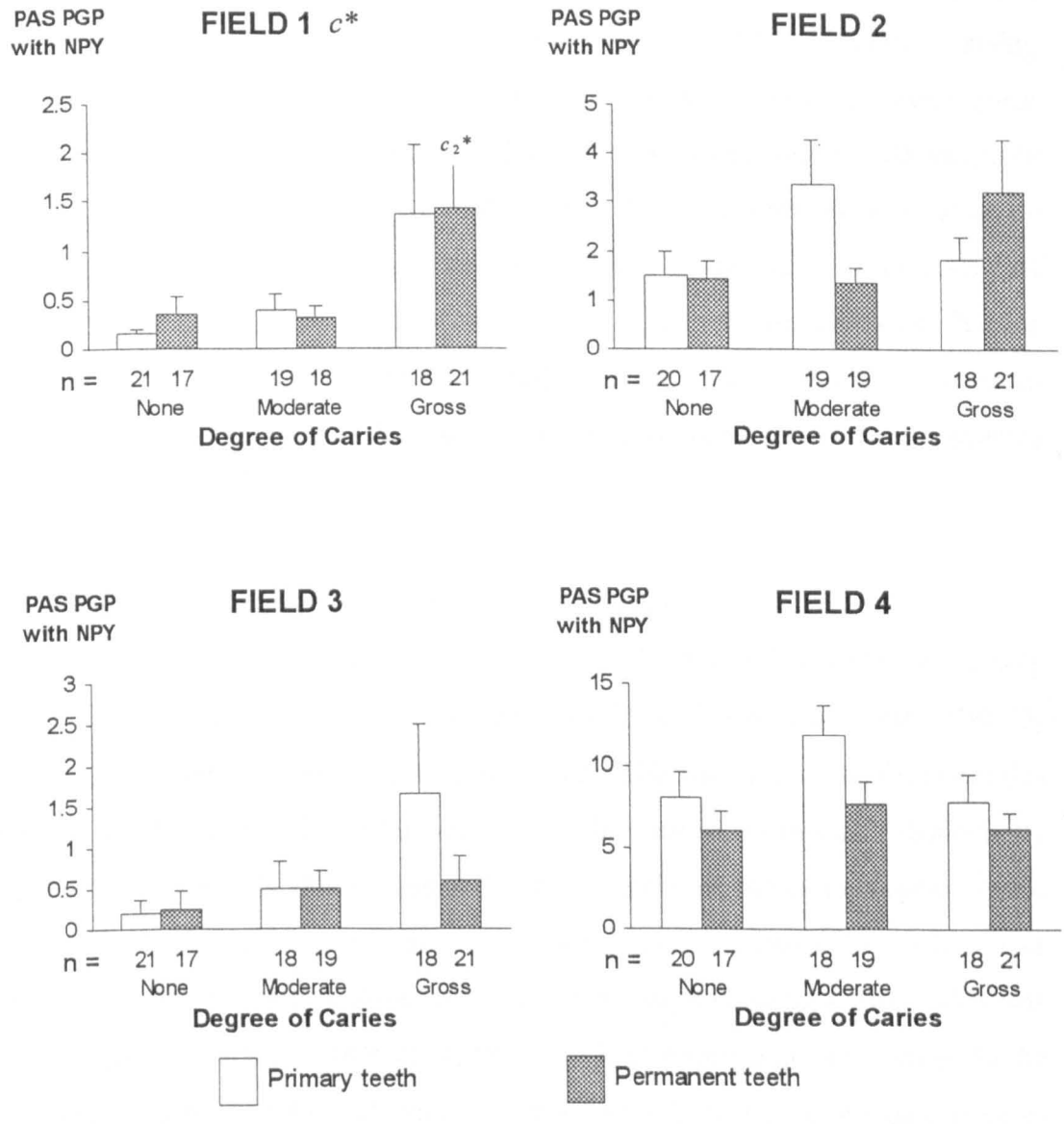


Figure 5.8. Bar charts showing mean (±SEM) percentage area of PGP 9.5-labelled tissue that was also labelled for NPY in primary and permanent teeth according to the degree of caries. Field 1=tip of pulp horn; Field 2=region subjacent to field 1; Field 3=buccal subodontoblastic plexus; Field 4=mid-coronal region.

Main titles: c^* = significant difference in mean NPY expression according to the degree of caries; t^* = significant difference between primary and permanent teeth ($P<0.05$, ANOVA, log transformed data).

Bar titles: c_1^* = significantly different from intact samples of the same dentition; c_2^* = significantly different from moderately carious samples of the same dentition; t^* = significantly different from primary teeth with same degree of caries ($P<0.05$, Tukey's test).

5.3.6 Additional observations relating to neuropeptide labelling

An additional finding was that, on some double exposure photomicrographs demonstrating immunostaining for both a neuropeptide and PGP 9.5, peptide staining appeared a green colour (see, Figs. 5.5D and 6.4F). However, in cases where green peptide labelling is co-localised within red PGP 9.5-ir fibres, one would normally expect to see peptide expression represented by a yellow colour on dual exposure photomicrographs. This apparent anomaly is explained by the fact that, in some fine nerve terminals, PGP 9.5-ir staining was extremely faint, yet varicosities labelled for neuropeptides were comparatively bright. Thus, on double exposure photomicrographs, the positive labelling for peptide assumed a greener appearance than usual.

5.3.7 Neuropeptide expression in relation to pain history

There was no significant difference in neuropeptide expression between grossly carious asymptomatic (n=10) and reportedly painful (n=10) primary teeth ($P>0.05$, independent sample *t*-test on log-transformed data). This was true for all four peptides within all four fields of analysis (data not shown). In contrast, there was a significantly greater SP expression, in all four fields of analysis, within grossly carious symptomatic permanent teeth (n=10) as compared to grossly carious asymptomatic permanent teeth (n=11) ($P<0.05$, independent sample *t*-test on log-transformed data). This was not the case for either CGRP or NPY, but, VIP expression was found to be significantly greater in field 1 of symptomatic samples than in asymptomatic samples ($P=0.001$, independent sample *t*-test on log-transformed data). Table 5.5 shows the mean (±SD, range) percentage area of PGP 9.5-labelled tissue that was also labelled for SP or VIP within asymptomatic and reportedly painful grossly carious permanent teeth. Plots of the data are presented in Figure 5.9.

Table 5.5. Mean (±SD, range) percentage area of PGP 9.5-labelled tissue that was also labelled for SP or VIP in asymptomatic and painful grossly carious permanent teeth

Neuropeptide and field	Pain history		
	Asymptomatic (n=11)	Painful (n=10)	*P*
SP - field 1	17.08 (±12.56, 5.70-45.80)	48.54 (±26.56, 12.60-83.70)	0.002
SP - field 2	14.09 (±9.53, 2.98-34.90)	30.35 (±24.52, 7.46-93.50)	0.043
SP - field 3	11.03 (±6.39, 0.00-19.39)	30.91 (±23.83, 1.22-69.20)	0.019
SP - field 4	12.45 (±5.12, 2.25-18.49)	37.79 (±23.10, 15.77-95.60)	0.001
VIP - field 1	6.17 (±4.18, 1.75-16.00)	18.12 (±11.62, 6.80-42.10)	0.001
VIP - field 2	6.52 (±4.85, 1.70-18.71)	10.70 (±6.45, 2.28-25.00)	0.089
VIP - field 3	1.78 (±199, 0.00-7.03)	3.07 (±4.06, 0.00-12.60)	0.320
VIP - field 4	3.23 (±3.84, 0.96-14.16)	6.65 (±12.24, 0.63-41.10)	0.553

P=independent sample *t*-test on log transformed data

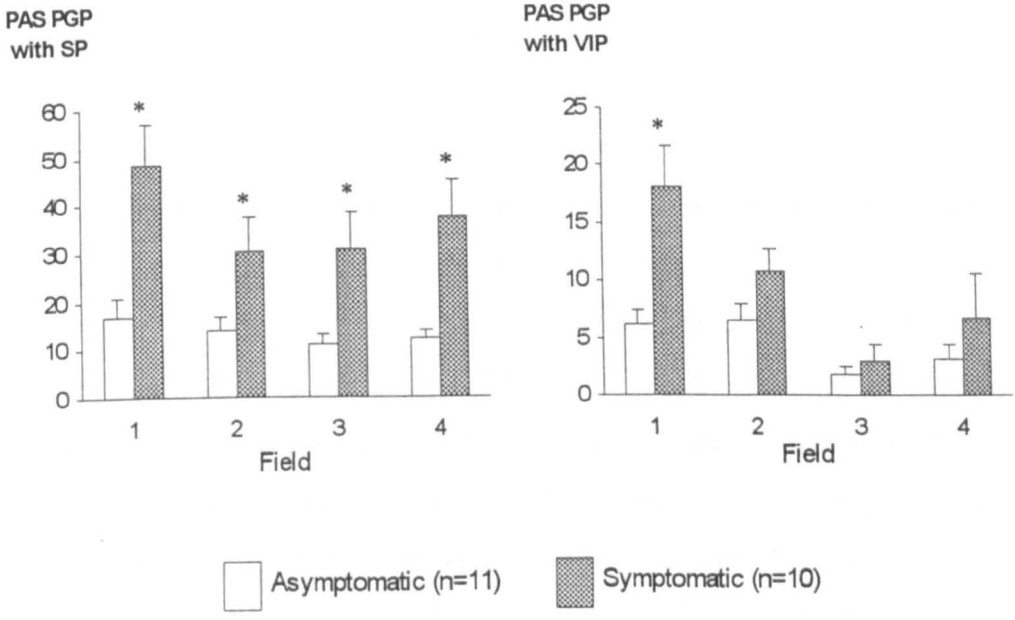


Figure 5.9. Bar charts showing mean (±SEM) percentage area of PGP 9.5-labelled tissue that was also labelled for SP or VIP in grossly carious permanent teeth according to the reported pain history. Field 1=tip of pulp horn; Field 2=region subjacent to field 1; Field 3=buccal subodontoblastic plexus; Field 4=mid-coronal region.
* = significant difference in mean peptide expression between asymptomatic and painful samples ($P<0.05$, independent sample *t*-test on log-transformed data).

5.4 Discussion

5.4.1 Experimental approach

- Immunocytochemistry

Originally, the study also aimed to investigate ENK, GAL and SOM expression within the dental pulp, but labelling for these three peptides was either absent or sparse (see, section 3.2.1). Although a small number of investigators have identified ENK-ir nerve fibres in human tooth pulp, using indirect immunofluorescence (Grönblad *et al.*, 1984; Casasco *et al.*, 1990a), other investigators have been unable to demonstrate any labelling for this peptide (Luthman *et al.*, 1988; Astbäck *et al.*, 1997). Furthermore, it would appear that investigators have not always been able to identify any GAL immunoreactivity in human teeth (Luthman *et al.*, 1988). Somatostatin-ir fibres have been described within the human dental pulp but they would seem to be relatively sparse (Casasco *et al.*, 1991). Thus, more sensitive techniques than those used by the present study may be necessary to investigate these less abundant intradental neuropeptides.

- Experimental variables

It was important to ensure that the experimental methods used in this study did not themselves significantly effect the quantitative findings. Previous investigators have reported that the extraction sequence, the degree of surgical trauma and the use of local anaesthetic (in association with GA) may all modify quantitative measurements of neuropeptide expression within extracted teeth (Walker *et al.*, 1987; Parris *et al.*, 1989; Robinson *et al.*, 1989; Pertl *et al.*, 1997). However, this study found that there were no significant differences in neuropeptide expression between first- and third-order extractions (see, section 3.3.2). Furthermore, within a period of 60 seconds, the delay until specimen placement in fixative appeared to have no significant effect on neural peptide expression (see, section 3.3.3). These findings appear to conflict with previous data, but may be explained by the fact that the extractions in the present study were carried out very rapidly and atraumatically under GA, as compared to the extractions in the previously mentioned studies which either involved local analgesia or surgical exodontia under GA and thus took a much longer time.

Nonetheless, the precise effects of extraction trauma and tooth cutting on neuropeptide content within pulpal nerves could not be determined in this experimental model. It would, however, be possible to investigate the influence of these experimental variables in animal teeth, which could be perfused prior to extraction and tooth splitting.

- Limitations of the study

Three specific limitations of this study should be mentioned. Firstly, the protocol employed for multiple immunostaining regimes did not permit the visualisation of more than one neuropeptide within the same tissue section. Thus the study was not able to investigate the colocalisation of different peptides within the same fibre population or identify any changes in peptide co-expression patterns following caries. Secondly, in samples where an increase in peptide expression was found, the method of quantification could not decipher whether this change was due to an increase in the proportion of fibres expressing the peptide or due to an increase in the amount of peptide within the same proportion of fibres. However, subjective observations were able to provide some clarification in this matter.

5.4.2 Comparison with previous dental studies

- Intact teeth

The overall distribution pattern of CGRP-, SP-, VIP- and NPY-ir fibres within intact teeth was consistent with previous immunocytochemical investigations of human permanent teeth (Grönblad *et al.*, 1984; Wakisaka *et al.*, 1984; Uddman *et al.*, 1984; 1986a; Silverman and Kruger, 1987; Casasco *et al.*, 1990a; Luthman *et al.*, 1992). The only previous report relating to peptidergic innervation in human primary teeth has been for CGRP (Egan *et al.*, 1996) and this was in also in agreement with the present study.

In terms of quantitative findings, this investigation showed that CGRP was the most abundant neuropeptide in the healthy tooth pulp which is in agreement with quantitative radioimmunoassay data (Hargreaves *et al.*, 1992; Pertl *et al.*, 1997). In addition, the present study identified a high inter-individual variation in peptide

expression, particularly for CGRP and SP (see, Tables 5.1A and 5.2A respectively). This feature has also been reported by Pertl and colleagues who, using radioimmunoassays, actually found an inter-individual difference of x85 for CGRP and x100 for SP within the pulps of surgically extracted non-carious third molars (Pertl *et al.*, 1997).

The relative contribution of NPY-containing fibres to the overall innervation was very small. This is consistent with previous quantitative assessments of the sympathetic contribution to pulpal innervation, which has been estimated to comprise no more than 10% of the overall innervation (Noga and Holland, 1983; Johnsen, 1985). It would appear from the literature, that no previous quantitative assessments of VIP expression within the tooth pulp have been undertaken in any species thus results from the present study can not be compared with any pre-existing data.

- Carious teeth

To date, there have been few quantitative investigations of neuropeptide expression in carious human teeth thus there are little data with which to compare the findings from the present study. However, Bowles and colleagues reported a five fold increase in the pulpal levels of SP in teeth diagnosed with irreversible pulpitis (Bowles *et al.*, 2000), which is not dissimilar to the 2-4 fold increase in neural SP expression, within grossly carious samples, found by the present study (see, Table 5.2A).

It is also of interest to compare the morphological findings of the present study with those reported following experimental pulpal injuries in animal models. However, it should be borne in mind that the sequelae following experimentally-induced pulpal injuries may not be directly comparable with those subsequent to caries. Nonetheless, the anatomical findings from the present study demonstrated many features comparable with those seen in Byer's investigations of injured rat pulps (Kimberly and Byers, 1988; Taylor *et al.*, 1988; Byers *et al.*, 1990b; Swift and Byers, 1992). In essence, it is apparent that CGRP- and SP-ir fibres are not static during the inflammatory process but undergo dynamic structural changes, arborising extensively and forming dense networks around inflammatory foci or between degenerative and

vital tissue zones. Another morphological change described by Byers and co-workers, was the predominantly knobbly and beaded appearance displayed by CGRP-ir fibres following pulpal inflammation, a finding which was commonly seen in the present study.

A notable observation made by the present investigation was the significant caries-related increase in the number of VIP-ir fibres within the pulp horn regions, particularly for primary teeth. There appear to have been no previous studies looking at changes in VIP expression following pulpal injury. However, Fristad's group investigated the change in VIP-ir fibre distribution within rat pulps following IAN axotomy and regeneration (Fristad *et al.*, 1998). They reported that, in control teeth, there were very few VIP-ir fibres, which in the main were limited to apical pulpal tissue. However, three weeks post-axotomy, VIP-ir fibres were evident within the coronal pulp and coarse VIP-ir fibres were frequently directed towards the odontoblast layer. Interestingly, it has been suggested that the retraction of intradental nerves away from the pulp/dentine border following dentine injury or caries, may in fact constitute a nerve injury, albeit at its most terminal extension. Thus the findings from Fristad's study, and other similar pulp deafferentation studies, may be more relevant to the present investigation than might be apparent on first inspection.

Caries-induced changes in NPY expression were also noted in this investigation but increases in the number of NPY-ir fibres were limited to the tip of the pulp horns. This finding would seem to conflict with observations made by Oswold and Byers who did not identify any change in NPY expression following experimentally-induced inflammation in their rat model (Oswald and Byers, 1993). However, there have been reports of an increase in pulpal NPY-ir fibres following IAN axotomy (Fristad *et al.*, 1996; Wakisaka *et al.*, 1996c).

5.4.3 Comparison with other models of inflammation

In addition to the tooth pulp, several other models of inflammation have been developed to assess morphological and cytochemical neural changes. Studies, mostly involving experimentally-induced arthritis in various animal models, have conclusively

shown that during chronic inflammation there is a local increase of neuropeptides, notably SP and CGRP, within sensory nerves innervating the affected tissue (Lembeck *et al.*, 1981; Donnerer *et al.*, 1992; Carleson *et al.*, 1996; Luber-Narod *et al.*, 1997). Conversely, in the presence of acute inflammation, a decrease in sensory nerve neuropeptide expression has been reported (Eysselein *et al.*, 1991; Polgár *et al.*, 1998). It would therefore appear that variations in peptide expression may due to differences in the severity of the inflammatory reaction (for review see, Walsh *et al.*, 1992). Of particular relevance to the trigeminal system is the finding that SP expression is markedly increased within the crevicular fluid of patients with gingival and periodontal disease (Linden *et al.*, 1997). Thus the overall increase in neuropeptide expression found in grossly carious tooth pulps by the present study is consistent with peptide increases reported for other chronic inflammatory models.

5.4.4 The interrelationship between neuropeptides and pain

Numerous investigators have attempted, and failed, to find any correlation between reported patient symptoms and the histopathological status of pulpal innervation (England *et al.*, 1974; Torneck, 1977; Mendoza *et al.*, 1987). Indeed the present study did not identify any correlation between overall neural density and reported pain experience (see, section 4.3.7). Thus it was very interesting that, at a more biochemical level, there was a significantly greater SP (and VIP) expression in the pulpal nerves of grossly carious painful teeth than in corresponding asymptomatic samples.

There is now a growing consensus, based on several lines of experimental evidence, that the upregulation of SP in injured tissues may be associated with the development of hyperalgesia (Woolf and Wiesenfeld-Hallin, 1986; Polgár *et al.*, 1998; Yaksh, 1999). There is evidence to suggest that SP may act on peripheral unmyelinated afferent fibres both directly via NK1 receptors (Carlton *et al.*, 1998) and indirectly via its numerous vasodilatory and pro-inflammatory interactions (Cohen, 1990; Pedersen-Bjergaard *et al.*, 1991; Woolf and Wiesenfeld-Hallin, 1986). Thus it is conceivable that SP may be involved in the development of dental hyperalgesia following pulpal injury.

Furthermore, there is evidence to suggest that SP is also involved in the induction of central sensitisation in the setting of inflammation (Ma and Woolf, 1995; De Felipe *et al.*, 1998). Following peripheral inflammation, there is clearly an increase in SP expression (Donaldson *et al.*, 1992; Smith *et al.*, 1992; Polgár *et al.*, 1998), together with an upregulation of SP receptors (Abbadie *et al.*, 1997), at the level of the dorsal horn. Interestingly, an increase in SP immunoreactivity has been demonstrated in the rat trigeminal ganglion and brainstem following adjuvant-induced inflammation of the temperomandibular joint (Hutchins *et al.*, 2000). A number of studies have been undertaken to explore central changes following experimentally-induced tooth pulp inflammation in animal models, but these have not specifically looked at SP expression (Torneck *et al.*, 1996; Chiang *et al.*, 1998; Funahashi *et al.*, 1999; Westenbroek and Byers, 1999). However, a recent radioimmunoassay study in rat actually found a reduction in the level of SP (and CGRP) within the rat trigeminal ganglion for up to 28 days following pulpal exposure (Buck *et al.*, 1999). Therefore, the role of SP in central sensitisation following pulpal injury is not yet clear but, on the strength of data derived from most models of inflammation, it would seem to be implicated.

Interestingly, no correlation between SP expression and reported pain history was determined for primary teeth. Certainly, there was evidence of marked increases in SP expression in the pulps of some grossly carious samples, but there was not always an associated positive pain history. There may be several explanations for this apparent inconsistency. Firstly the validity of a pain history in a very young child may be questionable (Gaffney and Dunne, 1986; Mathewson and Primosch, 1995). Secondly, as younger children may be less able to verbalise their pain symptoms to carers, there may be a delayed presentation for treatment, thus transient changes in SP expression may be missed. Finally, there may be complex developmental and neurobiological differences between children of different ages which may have modulating effects on overall pain perception (McGrath, 1987).

The other peptide that was significantly increased in painful (permanent) tooth pulps was VIP. However, the role of VIP in pain processing is not well established although it has a purported role in chronic pain states or neuropathic pain and is thought to

take over the excitatory effect of downregulated SP (Dickinson and Fleetwood-Walker, 1999). On the other hand, VIP has very well recognised neurotrophic effects and is known to promote axonal extension (Muller *et al.*, 1995; Rayan *et al.*, 1995). Thus the functional significance of an increased VIP expression in painful tooth pulps may not necessarily be related to pain experience but may be associated with the promotion of neural growth and regeneration.

Finally, it should be appreciated that pain-mediating neurons are generally considered to comprise both peptidergic and non-peptidergic subpopulations. However, the present study only explored the former group. It is possible that non-peptidergic fibre subtypes may also be of significance in dental pain experience and thus provide an interesting area for further investigation.

5.4.5 Mechanisms for dynamic changes in neuropeptide expression

This study has shown that, in the presence of gross caries and ensuing pulpal inflammation, there is a marked increase in the neuropeptide content of pulpal nerves. However, these increases could theoretically be due to any number of different mechanisms, including an increase in the synthesis or axonal transport of the peptides or a reduced release of the peptide at the axonal terminals. Firstly, a reduced peptide release seems very unlikely as numerous studies have clearly established that there is in fact an increased secretion of neuropeptides from nerves innervating inflamed peripheral tissues (Donnerer and Stein, 1992; Hargreaves *et al.*, 1994; Carleson *et al.*, 1996; Polgár *et al.*, 1998).

An increase in neural peptide content simply as a result of an increased axonal flow also seems unlikely in view of the experimental work conducted by Donnerer and colleagues (Donnerer *et al.*, 1993). These investigators found that there was no significant difference in the velocity of axoplasmic flow in rat sciatic nerve between the side innervating an inflamed hindpaw and the unaffected control side.

It would therefore seem most likely that the increase in neural peptide expression seen during chronic inflammation is attributable to an overall increase in peptide synthesis

and flow. However, two very different regulatory mechanisms may underlie this change. There may simply be an enhanced biosynthesis of certain peptides by the nerve subpopulations that normally express those peptides or there may be a de novo synthesis of certain peptides by nerve subpopulations that do not normally express those neuropeptides. There is, in fact, good experimental evidence to support the role of both of these mechanisms.

Firstly, in situ hybridisation has shown that, soon after the onset of acute inflammation, there is a marked increase in the expression of preprotachykinin and CGRP mRNA in dorsal root ganglions innervating rat arthritic joints but there is not an increase in the proportion of neurons which express these peptides (Donaldson *et al.*, 1992). Secondly, it is clear that following injury or inflammation, neurons may also undergo phenotypic switching and start producing peptides that they do not normally express in the intact state (Shehab and Atkinson, 1986; Doughty *et al.*, 1991; Kashiba *et al.*, 1994; Neumann *et al.*, 1996).

The plasticity of peptide transcription within the trigeminal system has been investigated by evaluating changes in neuropeptide expression following injury to the IAN (Wakisaka *et al.*, 1993; Sasaki *et al.*, 1994; Fristad *et al.*, 1996; Wakisaka *et al.*, 1996b; Fristad *et al.*, 1998). These studies have reported an increase in the expression of NPY and VIP and a reduction in CGRP- and SP-expression within trigeminal neurons following nerve injuries. Furthermore, the increases in NPY and VIP expression were attributed to the de novo synthesis of these two peptides by subsets of medium to large and small to medium sized sensory cells respectively (Wakisaka *et al.*, 1996b; Fristad *et al.*, 1998). Thus with reference to the present study, it is likely that the significant increases seen in peptide expression within pulpal nerves may have resulted from a combination of increased peptide biosynthesis, and an induction of de novo peptide synthesis.

Finally, the role of NGF in mediating some of the above changes in peptide synthesis warrants some mention. It is evident that levels of NGF are markedly increased within inflamed tissues and this change is believed to underlie the associated increases seen in

the peptide content of sensory afferents (for review see, Woolf, 1996). In vitro studies have shown that NGF is able to promote the synthesis and release of CGRP and SP in sensory neurons (Lindsay *et al.*, 1989; Malcangio *et al.*, 1997). Furthermore NGF administration appears to selectively regulate peptide expression in injured rat primary sensory neurons in vivo, causing a suppression of the normal upregulation of VIP, NPY, GAL and cholecystokinin and a reduction in the decreased synthesis of CGRP and SP (Verge *et al.*, 1995). In addition, treatment with anti-nerve growth factor serum appears to prevent inflammation-induced increases in the neural content of CGRP and SP (Donnerer *et al.*, 1992). Interestingly, Byers and her colleagues showed that an increase in the production of NGF by pulpal fibroblasts preceded injury-evoked CGRP-ir fibre sprouting and thus believed that NGF was the most likely candidate to effect this change (Byers *et al.*, 1992b).

5.4.6 Other inflammation-induced neural changes

It is important to recognise that changes in peptide expression represent just one of a host of inflammation-induced neural changes. It is increasingly evident that peripheral inflammation or nerve injury are associated with several profound cytochemical changes in dorsal root ganglion neurons, which may include an upregulation in the expression of nerve growth factor receptors (Torneck *et al.*, 1996), SP receptors (Abbadie *et al.*, 1997; Allen *et al.*, 1999), capsaicin-sensitive receptors (Nicholas *et al.*, 1999) and various cation channels (Eriksson *et al.*, 1998; Waxman and Wood, 1999; Westenbroek and Byers, 1999). Thus it is highly likely that these neural changes may also have important relevance in terms of altered sensory nerve excitability following tissue injury and inflammation within different systems.

5.4.7 Clinical significance of findings

One of the rationales for undertaking this investigation was to seek a biological basis for the apparent differences in sensitivity seen in primary and permanent teeth. The findings from the present study have now established that significant inter-dentition differences exist for the expression of CGRP and SP within pulpal nerves. These two peptides are present within a subpopulation of nociceptive afferent fibres hence inter-

dentition differences in their relative contribution to overall innervation may, in part, have some bearing on the tooth's potential to process nociceptive input.

The marked difference in VIP expression between the two dentitions was also a notable finding, but is thought to have less relevance in terms of nociceptive potential. However, in view of this peptide's known roles in supporting neuron survival and its involvement in glycogenolysis and cAMP production (Hökfelt *et al.*, 1994; Fu and Gordon, 1997), one may speculate that VIP may be important for maintaining the intradental neural population. Thus the comparatively low VIP expression within pulpal nerves of the primary tooth pulp may in some way relate to the subsequent neural degeneration that accompanies the exfoliation of the primary dentition. The biological mechanisms that underlie this neural degeneration in primary teeth are not known, hence the observations relating to VIP expression may be of potential biological interest and warrant further investigation.

Another finding from the present study, which may have clinical relevance, relates to the positive correlation between increased SP expression and dental pain experience. It is tempting to speculate that the regulation of intradental SP expression, release or receptor-mediated effects may provide novel therapeutic approaches for the management of pulpitic pain. Whilst this may be a reasonable hypothesis, the complex nature of dental pain should be recognised and although SP is likely to be an important modifier of pain experience, there may be a host of other chemical mediators which may also modify nociceptive input (Närhi, 1985; Olgart, 1985; Olgart and Kerezoudis, 1994). Nonetheless, pharmacological investigations are attempting to explore the value of specific SP-receptor antagonists in the management of inflammatory pain and hyperalgesia in other sites (Cohen, 1990; Ma and Woolf, 1995; Fleetwood-Walker, 1995; Besson, 1999).

Finally, it should be stressed that the marked caries-induced changes in peptide expression revealed by the present study may have functional significance in addition to that of nociceptive processing. The traditional concept of the tooth being richly innervated to serve a purely nociceptive role is now being reconsidered in light of

increasing evidence that sensory nerves also serve important effector functions (Maggi, 1991; Chen *et al.*, 1999). Certainly, CGRP is believed to play a role in fibroblast proliferation and hard tissue formation. Thus it is conceivable that future clinical therapies could be aimed at manipulating neuropeptide expression in order to enhance healing responses. The next two chapters will also consider the roles of neuropeptides in vasoregulation and immune cell responses within the dental pulp.

Researchers are now striving to identify a host of receptors through which neuropeptides exert their effects. Indeed, NPY-, CGRP- and SP-receptor expression have recently been identified in human tooth pulp and may provide novel targets for the pharmacological modification of neuropeptide effects (Uddman *et al.*, 1998; 1999; Fristad *et al.*, 2000). It is therefore evident that further neuropeptide-related research within the human tooth pulp may help to direct the development of novel and biologically-compatible therapies for the compromised dentition.

5.4.8 Summary of findings

In response to the original objectives (see, section 5.1.8), this investigation has shown that:

- Permanent teeth demonstrate a significantly greater expression of CGRP, SP and VIP than do primary teeth, and inter-dentition differences are most marked in the pulp horn region of intact samples. However, the distribution of NPY-ir pulpal fibres and the neural content of this peptide are similar for both dentitions.
- Both dentitions show marked changes in peptidergic nerve morphology with caries progression. Furthermore, the expression of neuropeptides is significantly increased in grossly carious samples, particularly within the pulp horn region.
- Substance P expression is significantly greater throughout the coronal pulp of reportedly painful grossly carious permanent teeth than in similarly carious asymptomatic samples. In addition, the expression of VIP is also significantly greater within the pulp horn region of symptomatic samples. There appears to be no correlation between neuropeptide expression and reported pain history for primary teeth.

6. Investigation of the pulpal vascular system and its associated innervation

6.1 Introduction

6.1.1 Overview of the pulpal microvasculature

In addition to being densely innervated, the tooth pulp is a highly vascular tissue. The pulpal microvascular system is complex and comprises a hierarchy of feeding arterioles, precapillary arterioles, capillaries, postcapillary venules and muscular venules (Okamura *et al.*, 1994; Zhang *et al.*, 1998). Essentially, large arterioles enter the tooth via the apical foramina and ascend towards the coronal region, giving off numerous branches en route, and terminating in a rich subodontoblastic capillary plexus. Ultrastructural investigations have revealed that the capillaries are mostly fenestrated and capillary loops may extend into the odontoblast layer (Corpron *et al.*, 1973; Yoshida and Ohshima, 1996). Capillary drainage is via venules, which run centrally through the pulp stroma, often in close association with arterioles. Lymphatics have also been identified within tooth pulp (Bishop and Malhotra, 1990; Matsumoto *et al.*, 1997).

Takahashi (1990) provided a novel insight into the morphology and distribution of pulpal vessels by producing three-dimensional resin corrosion casts of the entire vascular architecture. Examination of these casts, under a scanning electron microscope, revealed a number of interesting anatomical features including the presence of arterio-venous anastomoses, venous-venous anastomoses and U-turn arterioles.

6.1.2 Pulpal vascularity

Although pulpal "vascularity" has been investigated using a variety of physiological techniques (for review see, Kim and Dörscher-Kim, 1990), there have been very few histological assessments of vascularity. Vongsavan and Matthews (1992) estimated the vascularity of feline dental pulp by measuring the cross-sectional area of all blood vessels within a complete transverse pulp section. They calculated that approximately

14% of the pulp volume was occupied by vessels, thus confirming that the pulp was a relatively vascular tissue. Other investigators have undertaken a comparative analysis of pulpal vascularity in primates by counting the overall number of blood vessels within longitudinal histological sections (Lownie *et al.*, 1998).

However, there appears to be little quantitative histological data relating to human pulpal vascularity. Interestingly, the human primary tooth pulp has been frequently described as being a more vascular tissue than the permanent tooth pulp, yet this statement does not seem to have been supported by any evidence (Cohen and Massler, 1967; Kopel, 1992). Indeed, this "apparent" greater vascularity within primary tooth pulps is cited as being a possible reason for the more rapid and widespread inflammatory responses observed in carious primary teeth (Cohen and Massler, 1967; Rayner and Southam, 1979; Mathewson and Primosch, 1995).

6.1.3 Vascular responses to pulpal injury

Vascular changes following injury comprise a major component of the overall inflammatory response and are essential for maintaining an adequate blood supply to the compromised tissue (for review see, Woolf, 1986). This section will review the key histopathological and pathophysiological vascular changes occurring within the tooth pulp following caries or other insults.

Depending on the severity of the dental injury, a variety of abnormal vascular features have been seen within inflamed pulp tissue which may include: dilated capillaries, widely enlarged arterioles and venules, occluded or partly occluded arterioles, and a proliferation of small vessels (Cohen and Massler, 1967; Baume, 1970b; Harris and Griffin, 1973; Trowbridge, 1981; Tønder, 1983; Fristad *et al.*, 1995b). It has also been shown that lymphatic vessels undergo morphological changes in pulpitis and appear markedly distended whereas they are not normally evident within the healthy pulp (Bernick, 1977; Marchetti *et al.*, 1992).

Histological findings provide little indication of the actual dynamics of the vascular response to injury thus functional studies have been necessary to further elucidate

these changes (Kim, 1990). Within most tissues, the normal physiological response to injury comprises an immediate vasodilation and an increased capillary and venular permeability. However, the vasomotor response to injury within the tooth pulp differs to that seen in other tissues by virtue of its low compliance environment. Interestingly, within the pulp, a biphasic response occurs: an initial vasodilation and increased pulpal blood flow (PBF) is followed by a rapid decrease in PBF (Kim and Dörscher-Kim, 1990).

Tønder (1983) measured pulpal blood flow in inflamed and normal dog pulp using radioisotope-labelled microspheres and confirmed that, overall, there was very little increase in blood flow within an inflamed pulp and this increase tended to be well localised. The pulpal vascular response to injury appears to be dominated by fluid exudation rather than a significant increase in blood flow thus limiting any changes in tissue pressure which would otherwise seriously compromise the perfusion of blood to this organ. Indeed it has been shown that the pulp has a very efficient draining system for the removal of excess interstitial fluid, via a network of lymphatic vessels.

6.1.4 Anatomical relationship between pulpal vessels and nerves

Early histochemical investigations clearly demonstrated the presence of delicate adrenergic nerve fibre networks around a number of large pulpal blood vessels (Pohto and Antila, 1968; Larsson and Linde, 1971; Pohto and Antila, 1972; Dahl and Mjör, 1973). Findings from these studies generally concurred that most arterioles were accompanied by nerve fibres whereas venules and capillaries appeared to lack an associated innervation. Evidence for a cholinergic innervation of vessels appears to be more controversial: Rapp and colleagues reported that, in human primary tooth pulp, neural elements stained for cholinesterase were evident around blood vessels (Rapp *et al.*, 1967a), however, a more recent study, undertaken in permanent teeth, was unable to find any cholinesterase-positive nerve fibres (Okamura *et al.*, 1994).

Okamura and colleagues employed antisera to S-100 protein and neuron specific enolase to visualise nerves and examine their vascular relationships within the human dental pulp (Okamura *et al.*, 1995). Using electron microscopy, they demonstrated

two types of vascular-related nerve endings: free endings, which were associated with all types of vessels and the less frequent varicose endings which were located only around arterioles. Following connective tissue digestion, Tabata and colleagues used scanning electron microscopy to further clarify the spatial relationship of pulpal nerve fibres to blood vessels (Tabata *et al.*, 1998b). They reported that unmyelinated nerve fibres passed through the tunica adventitia of some arterioles and occasionally entered the tunica media. These nerve fibres then ramified and terminated on the surface of smooth muscle and endothelial cells. Other ultrastructural investigations have attempted to estimate the distances between nerves and the microvasculature and have shown that some neuromuscular gaps may be as small as 0.3 μm (Ekblom and Hansson, 1984; Lyroudia *et al.*, 1995). These observations were considered suggestive of an intimate functional relationship between neural and vascular elements within the tooth pulp.

Over the past three decades, immunocytochemical investigation of neuropeptide-containing nerves has revealed that pulpal blood vessels are actually supplied by a variety of peptidergic nerve subpopulations. Numerous investigators have described a close spatial relationship between blood vessels and nerve fibres immunoreactive for: SP (Wakisaka *et al.*, 1985); neuropeptide K (Casasco *et al.*, 1990b); NKA (Wakisaka and Akai, 1989); CGRP (Wakisaka *et al.*, 1987a); VIP (Uddman *et al.*, 1980); NPY (Uddman *et al.*, 1984); SOM (Casasco *et al.*, 1991); GAL (Wakisaka *et al.*, 1996a) and secretoneurin (Pertl *et al.*, 1998). Double-labelling techniques have shown that certain blood vessels receive a dual peptidergic innervation. It would appear that SP, NKA and CGRP exhibit a similar distribution pattern around some large pulpal vessels (Wakisaka and Akai, 1989). However, double immunofluorescence has revealed that SP- and VIP-ir fibres display a different pattern of vascular innervation to the same blood vessels (Wakisaka *et al.*, 1987b).

Immunoelectron microscopy has also been employed to more closely investigate the localisation of peptidergic fibres around the pulpal vasculature: Tabata and co-workers identified several SP-ir varicose fibres within the adventitia of large centrally-positioned arterioles and observed a small number of SP-ir fibres within a few microns

from the subodontoblastic capillaries (Tabata *et al.*, 1998a). Zhang and colleagues studied the three dimensional relationship of pulpal blood vessels and CGRP- and NPY-ir perivascular plexuses (Zhang *et al.*, 1998). They reported that there were no marked differences in the pattern of innervation demonstrated by these two peptides, although it was subjectively felt that NPY-ir fibres demonstrated the denser innervation. These investigators also found that large arterioles (40 μm or more in diameter) tended to receive a prolific peptidergic innervation whereas the smaller arterioles received a much sparser innervation which tended to run spirally or longitudinally to the vessel axis. Furthermore, capillaries and venules appeared to be devoid of any innervation.

6.1.5 Neural control pulpal haemodynamics

Haemodynamic regulation within the tooth pulp is considered to be multi-factorial and may involve humoral, cell-mediated and neural mechanisms (for reviews see, Kim and Dörscher-Kim, 1990; Olgart, 1996a). A variety of chemical mediators such as bradykinin, histamine, prostaglandins and nitric oxide have been shown to exert a major effect on PBF and vascular tone (Edwall *et al.*, 1973; Olgart, 1992; Lohinai *et al.*, 1995), however, this section will be limited to the neural regulation of the pulpal microcirculation.

There is extensive physiological evidence to suggest that the pulpal microcirculation is under sympathetic control. At rest, the influence of sympathetic activity on pulpal circulation appears to be low, but under conditions of physiological stress, a profound sympathetic-mediated vasoconstriction may be triggered (Kim *et al.*, 1989). Furthermore, electrical stimulation of the sympathetic nerve supply to the tooth pulp has been shown to cause pulpal arteriolar constriction and a marked reduction in arteriolar and venular flow rate (Ogilvie, 1969; Kim *et al.*, 1989).

The sympathetic influence on the vascular system results, in part, from the action of noradrenaline (for review see, Benarroch, 1994). This neurotransmitter is released from postganglionic noradrenergic terminals and exerts its effect via α_2-type receptors located on vascular smooth muscle cells (Edwall and Kindlova, 1971; Olgart *et al.*,

1989). However, it is now known that noradrenaline coexists with NPY within sympathetic fibres, and it is likely that both of these transmitters play a complementary role in the sympathetic control of pulpal blood flow (Edwall *et al.*, 1985; Olgart *et al.*, 1989; Kim *et al.*, 1996a). Furthermore, NPY appears to evoke a more sustained vasoconstriction than is seen with noradrenaline and its effects are resistant to adrenoreceptor antagonists (Lundberg and Tatemoto, 1982; Kim *et al.*, 1996b).

Whereas sympathetic-mediated pulpal haemoregulation has been well established, the possible contribution of the parasympathetic system remains controversial. Although the parasympathetic system has an established role in mediating vasodilation within many visceral and oro-facial tissues (Fahrenkrug, 1979; Lundberg and Hökfelt, 1986), its vasoregulatory effects in the tooth pulp have been disputed (Matthews and Vongsavan, 1994; Sasano *et al.*, 1995). In Olgart's excellent review (1996a), he concluded that many of the prerequisites for a parasympathetic control of PBF had been identified, but any circulatory effects were likely to be weak and of little physiological significance.

Much of the indirect evidence for a possible parasympathetic influence on pulpal vasoregulation, stems from findings relating to VIP. As outlined earlier (see, section 5.1.2), VIP is normally expressed by parasympathetic neurons, thus its presence within intradental nerves is considered, by some, as evidence for a parasympathetic innervation. The release of VIP following parasympathetic stimulation induces vasodilation in several different tissues (Lundberg *et al.*, 1984), and intra-arterial injection of synthetic VIP has been shown to cause vasodilation in cat pulp (for review see, Olgart, 1996a).

Finally, the vasoregulatory role of the sensory nervous system will be considered. It has been conclusively shown that stimulation of the sensory nerve supply causes an increase in PBF (Gazelius and Olgart, 1980; Heyeraas *et al.*, 1996). In addition, the application of excitatory stimuli to denervated teeth fails to induce the normal vasodilatory response (Olgart, 1992; Olgart *et al.*, 1993). It has also been demonstrated that changes in PBF are likely to be mediated by C- rather that A-delta

nerve fibres (Kim, 1990). Furthermore, it is considered that a subpopulation of sensory nerves which express SP, NKA and CGRP may actually mediate these haemodynamic changes (Heyeraas *et al.*, 1994). Following chemical, mechanical or electrical stimulation of sensory nerves there is an antidromic release of these peptides via an axon-reflex mediated response. The secreted neuropeptides subsequently regulate a number of vascular events, collectively termed "neurogenic inflammation" (for review see, Walsh *et al.*, 1992; Olgart, 1996b).

Substance P has the most established role in neurogenic inflammation. It has been shown that, following noxious stimulation, sensory nerves release SP to evoke vasodilation and plasma extravasation in many tissues, including the dental pulp in some species (Lembeck and Holzer, 1979; Dalsgaard *et al.*, 1987; Olgart *et al.*, 1993; Bester *et al.*, 1988). Supporting evidence has been provided by experimental studies showing that antidromic-induced pulpal vasodilation can be blocked by the use of SP-receptor antagonists (Rosell *et al.*, 1981; Romerio *et al.*, 1999).

Calcitonin gene-related peptide is also known to act as a very potent vasodilator throughout the body and plays an important role in the control of vascular tone and blood flow (Brain *et al.*, 1985; Zaidi *et al.*, 1985). In vivo studies in the cat pulp have revealed that SP and CGRP act synergistically although CGRP appears to evoke a more prolonged vasodilation than SP (Gazelius *et al.*, 1987). Furthermore, the use of CGRP-antagonists results in a significant reduction in a stimulus-induced vasodilation (Olgart, 1996b). Although the peripheral release of SP and CGRP is likely to follow activation of sensory nerve fibres by nociceptive stimuli, there is some indirect evidence to suggest that these peptides can also be released without obvious stimulation of the tooth (Olgart, 1992). Thus it is speculated that afferent nerves may also play a part in the normal homeostatic control of PBF.

In summary, it is clear that both the sensory and autonomic (sympathetic) nervous systems play important and interrelated roles in the regulation of the pulpal microcirculation under different physiological and pathophysiological conditions. In

particular, vascular regulation is subject to a number of neuropeptide-mediated effects.

6.1.6 Neuropeptide and endothelial cell interactions

It is evident that some neuropeptides not only evoke changes in blood vessel tone and permeability but may also have other important vascular effects. Firstly, CGRP and SP may play a role in angiogenesis or neovascularisation, as they have both been shown to stimulate endothelial cell growth (Hægerstrand *et al.*, 1990; Ziche *et al.*, 1990). And secondly, peptides are purported to have direct effects on endothelial cell functions, including altering their surface adhesiveness to leucocytes (for reviews see, Walsh *et al.*, 1992; Smith *et al.*, 1993).

6.1.7 Immunocytochemical identification of the vascular system

Endothelial cells constitute the lining of the entire vascular system thus immunocytochemical localisation of a number of different antigens within these cells has proved a useful approach to the identification of vascular structures. Commonly used vascular markers, within both oral and other tissues, have included a pan-endothelium antibody (Sims, 1999), and antibodies to von Willebrand's factor (factor VIII) (Jacoby *et al.*, 1991; Derringer and Linden, 1998), laminin (Vandevska-Radunovic *et al.*, 1997b) and blood group specific antigens (Little *et al.*, 1986). In addition, the use of the lectin, *Ulex europaeus,* has proved a very reliable non-immunological marker for vascular endothelium (Holthöfer *et al.*, 1982; Ordóñez *et al.*, 1987; Hoyle *et al.*, 1996).

Lectins are plant or animal proteins that can attach to tissue carbohydrates with a high degree of specificity. *Ulex europaeus* Agglutinin I Lectin (UEIL) is a plant protein that is specific for α-L-fucose, which is characteristically contained within human vascular endothelium (Polak and Van Noorden, 1997). Thus biotinylated UEIL provides an excellent label for this tissue. Furthermore, this non-antibody method of marking tissue components has important application in multiple immunostaining regimes as it does not cross-react with antigen-antibody reactions.

6.1.8 Rationale for study

The maintenance of an adequate PBF is critical to meet the functional demands of the tooth both in health and disease, and it has been shown that certain neuropeptides play an important role in regulating some vascular mechanisms, notably as part of the inflammatory response. To date there have been no quantitative assessments of the relative contributions made by different peptidergic subpopulations to the innervation of the human pulpal microvasculature. Furthermore, even descriptive data are largely lacking for the primary dentition. It is hoped that a knowledge of the innervation characteristics of blood vessels within the human tooth pulp may provide a greater insight into neurovascular control mechanisms. Undoubtedly, the neural and vascular systems play important and interrelated roles in the development of inflammation, and the tooth pulp provides a useful human model in which to investigate these parameters.

Another area that warrants further investigation is the actual vascular status of the tooth pulp. Marked changes in vascularity, either by increased vessel size or number, could have significant sequelae in terms of pain or tissue necrosis due to the pulp's low compliance environment. The primary tooth pulp is considered to be more vascular than the permanent tooth pulp and this factor has been purported to be unfavourable during inflammation. However, there are sparse data to support this commonly-held view thus a comprehensive investigation would seem to be justified. It is speculated that inter-dentition differences in pulpal vascularity, particularly in response to caries may be of clinical importance. Furthermore, the correlations between peptide expression and angiogenesis or vasodilation have not previously been investigated within the inflamed tooth pulp. As neovascularisation forms an integral part of the healing process, it would be interesting to explore this process in more detail.

6.1.9 Aims and objectives

The aims of the present study were to investigate pulpal vascularity and to assess the innervation characteristics of pulpal blood vessels using an immunocytochemcial approach. The specific objectives were as follows:

- to compare the overall pulpal vascularity of primary and permanent teeth in health and disease;
- to carry out a qualitative and quantitative comparison of the overall and peptidergic innervation of blood vessels within primary and permanent teeth and to investigate the effect of caries on these interrelationships
- to explore any relationship between neuropeptide expression and changes in vascularity or vessel number within the tooth pulp;
- to seek a correlation between pulpal vascularity and reported symptoms.

6.2 Materials and methods

Details regarding the overall experimental approach have been previously outlined in chapter two. However, some points specific to the analysis of the pulpal vasculature will now be addressed. In summary, three sections from each tooth sample were triple-labelled for PGP 9.5, UEIL and for each of the four neuropeptides, CGRP, SP, VIP and NPY.

6.2.1 Descriptive analysis

A total of 12 sections from each tooth were subject to descriptive analysis. On the basis of previous descriptive data, pulpal vessels were classified as: arterioles (thick-walled vessels with a diameter of 10-50 μm); venules (vessels with thin or absent muscular layers with a diameter of 10-40 μm); capillaries (small vessels, usually with an undetectable lumen, and with a diameter of 4-10 μm), and lymphatics (irregular-shaped vessels, 20-50 μm in diameter, and displaying numerous abluminal endothelial projections) (Dahl and Mjör, 1973; Okamura *et al.*, 1994; Ekblom and Hansson, 1984; Bishop and Malhotra, 1990; Matsumoto *et al.*, 1997).

6.2.2 Analysis of vascular innervation

Specific assessment criteria for blood vessel innervation have been given previously in chapter two (see, section 2.6.1). An assessment of vascular innervation was only undertaken in the mid-coronal pulp as this region contains the greatest proportion of

arterioles, which are the vessels most likely to be innervated (Okamura *et al.*, 1994; 1995). Quantitative vascular innervation data were derived from the evaluation of a total of four sections from every tooth, each section being labelled for one of the four different neuropeptides. Whilst it is acknowledged that it was the number of vessel profiles, rather than the number of vessels *per se,* that were assessed for their innervation characteristics, for brevity the term "vascular innervation" is used.

6.2.3 Quantitative analysis of immunostaining

Quantitative analysis of the PAS for UEIL, PGP 9.5 and each neuropeptide was undertaken for one section from each of the above four staining regimes. In addition, the number of blood vessels was counted in each field of analysis. One way analysis of variance was then employed to determine whether there were any statistically significant differences in the PAS for UEIL or vessel number between the four measurements recorded for each tooth sample.

The expression "PAS for UEIL" is used synonymously with the term "vascularity" throughout the subsequent text. Strictly speaking, the blood vessel count could also be considered to constitute a measure of pulpal "vascularity" (Vongsavan and Matthews, 1992; Lownie *et al.*, 1998). However, in the present study, the vessel count was employed primarily as an indicator of new vessel formation (angiogenesis) rather than as an indicator of changes in vascularity.

6.2.4 Relationships between neural and vascular factors

Possible interrelationships between neural density/peptide expression and vasodilation or angiogenesis were assessed by correlating the PAS for PGP 9.5, CGRP, SP and VIP with the PAS for UEIL or the total number of blood vessels respectively (using log-transformed data). Analysis was only performed for field 1 data as this region receives a dense innervation, demonstrates significant caries-related changes in peptide expression (see, sections 5.3.3, 5.3.4 and 5.3.5) and contains a large number of both capillaries and arterioles. Analysis was not undertaken for NPY as previous data had revealed that this peptide was only sparsely represented within the pulp horn (see, section 5.3.6). Furthermore, absolute data for peptide expression were employed

(PAS for peptide) rather than previously-employed data which related to the relative amount of neural tissue that contained peptide (PAS of PGP 9.5 that was also labelled for peptide). These data were selected as it was felt that the overall amount of potentially-available peptide was more pertinent when considering the degree of it biological effects than was the relative expression of the peptide.

Data were pooled for all carious samples but were subdivided according to the dentition. Intact teeth were not included in the analysis. It was felt that attempting to correlate peptide expression with increases in vascularity or vessel number would be inexpedient in non-carious samples as vascular changes were not likely to occur in the healthy state.

Scatter plots were first constructed to observe the relationship between the two variables and the strength of the association was determined by calculating the Pearson correlation coefficients (r). The association between the two variables was considered significant when $P<0.05$.

6.3 Results

6.3.1 Specificity controls

The specificity of the immunolabelling reaction for PGP 9.5 and each of the neuropeptides has been previously described in sections 4.3.1 and 5.3.1 respectively. Control sections which were incubated with a mixture of UEIL and 0.2 M α-L-fucose (see, section 2.2.5) did not show any positive fluorescent labelling, thus the reaction was considered to be specific (Fig. 3.1.A,B, section 3.2.2).

6.3.2 Comparison between different sections

Statistical analysis confirmed that there was no significant difference either for the mean PAS for UEIL or for vessel number between the four different sections analysed for each tooth pulp ($P>0.05$, ANOVA). Therefore, the means of the four measurements for pulpal vascularity and vessel number were employed for subsequent statistical analysis and data presentation.

6.3.3 Qualitative observations

Labelling for UEIL clearly revealed the overall distribution and varying morphology of the pulpal microvasculature. In the pulp horn of non-carious samples, numerous capillaries were seen to be evenly distributed around the peripheral regions and small arterioles were more centrally located (Fig. 6.1A,B). Small arterioles were also observed running parallel to the subodontoblastic nerve plexus. In the mid-coronal pulp, a number of large arterioles and thin-walled venules were evident, and vessel size generally increased towards the radicular pulp region. The most obvious difference between primary and permanent teeth was seen in the mid-coronal region where primary teeth appeared more vascular due to the presence of a greater number of small blood vessels (Fig. 6.1C,D). Lymphatic vessels, distinguishable by their numerous endothelial sac-like configurations, were infrequently seen in either dentition.

A variety of caries-related vascular changes were observed in both primary and permanent teeth. However, changes were not evident in every carious sample. Firstly, in the pulp horn region, small arterioles and capillaries often appeared to be dilated, and localised areas containing an increased number of small vessels were occasionally noted (Fig. 6.1E,F). Vascular changes were sometimes observed within the buccal and lingual SOP where capillary proliferation and enlarged arterioles were evident. A common finding was the presence of prominent lymphatic vessels (identifiable by the distinctive "vacuolar" appearance of their lectin-labelled endothelium) running throughout the central pulp regions (Fig. 6.4B).

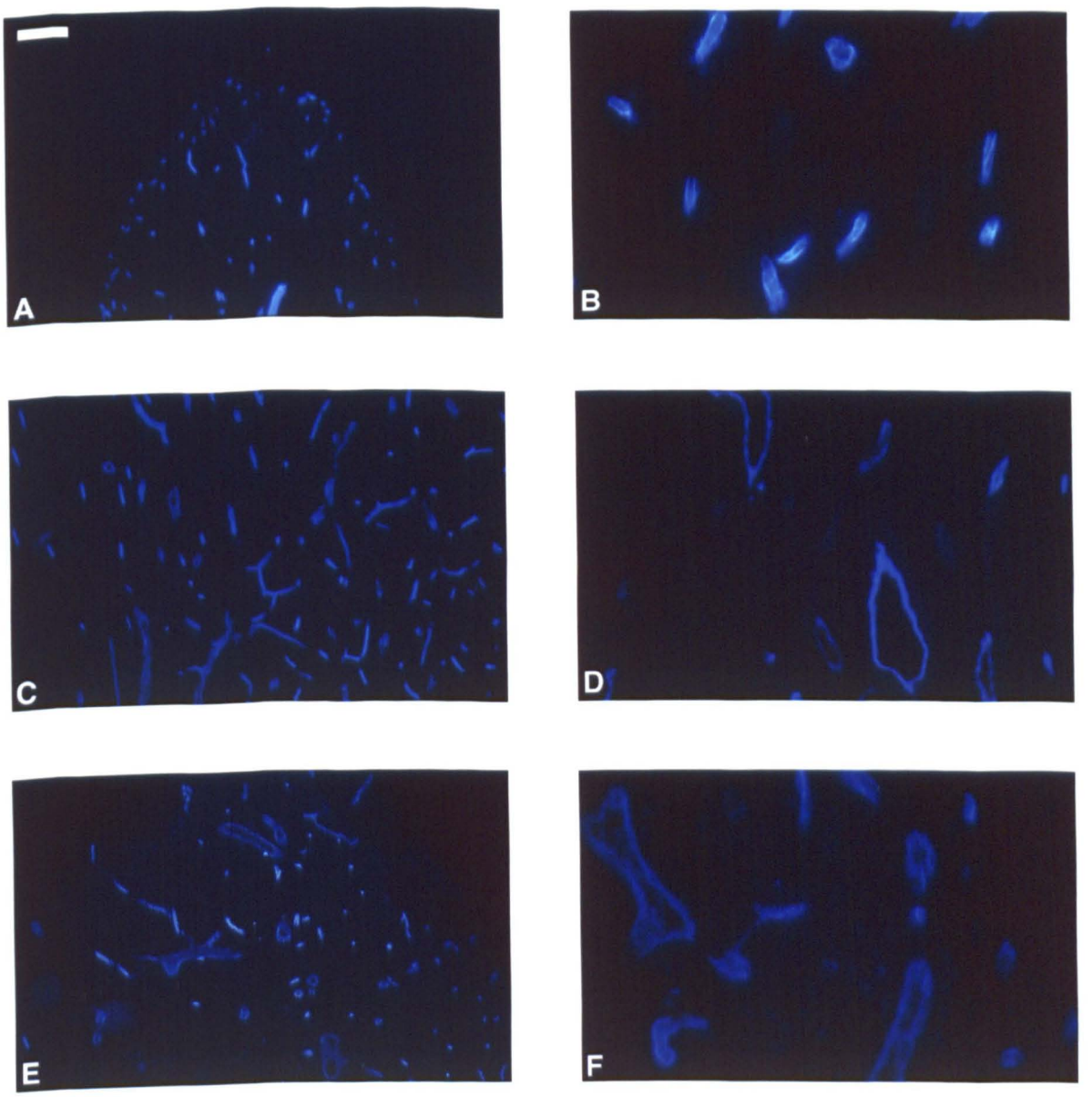


Figure 6.1. Photomicrographs demonstrating differences in the morphology and distribution of UEIL-labelled blood vessels (blue) in the coronal pulp of intact and carious primary and permanent teeth. **(A)** The normal distribution of blood vessels seen within the pulp horn and **(B)** the central pulp horn region of an intact primary tooth.**(C)** The mid-coronal region of an intact primary tooth showing a larger number of small vessels and greater vascularity than seen in **(D)** the same region of an intact permanent tooth. **(E)** An increase in both the number and size of blood vessels seen in the pulp horn and **(F)** the central pulp horn region of a carious primary tooth. Scale bar = 15 μm **(B,F)**, 60 μm **(A,C,D,E)**.

6.3.4 Quantitative analysis

- Pulpal vascularity

Table 6.1A shows the mean (±SD, range) PAS for UEIL-labelled tissue within each dentition according to the field of analysis and the degree of caries and Table 6.1B provides the corresponding statistical data. Bar charts showing pooled data are presented in Figure 6.2.

Key findings arising from the quantitative analysis of pulpal vascularity (PAS for UEIL) are outlined below.

- The greatest mean pulpal vascularity for intact teeth was seen in field 4 (primary teeth = 2.51%, permanent teeth = 1.96%). However, the greatest mean vascularity for grossly carious samples was seen in field 1 (primary teeth = 3.25%, permanent teeth = 2.54%).

- Mean pulpal vascularity was only significantly different between the dentitions in field 4 (P=0.011, ANOVA) but tended towards a significant difference in field 2 (P=0.054, ANOVA). However, further pairwise comparisons for field 4 data (see, Fig. 6.2) did not reveal any significant inter-dentition differences for any specific caries subgroups (P>0.05, Tukey's test).

- Caries was determined to have a highly significant effect on mean pulpal vascularity in fields 1 and 2, with an overall increase in pulpal vascularity with caries progression (P=0.0001, ANOVA). As an example, there was an approximate 30-50% increase in the overall vascularity of grossly carious samples as compared to intact samples. Further pairwise comparisons of mean values (see, Fig. 6.2) revealed that, in field 1, grossly carious primary teeth were significantly more vascular than corresponding intact or moderately carious samples, whereas this was not the case for permanent teeth. However, in field 2, grossly carious samples from both dentitions exhibited a significantly higher vascularity than corresponding intact samples (P<0.05, Tukey's test).

Table 6.1A. Mean (±SD, range) percentage area of staining for *Ulex europeaus* I Lectin according to dentition, field of analysis and degree of caries

Field/tooth(n)	Degree of caries		
	None	Moderate	Gross
Field 1			
Primary	2.06	2.21	3.25
(n=22,20,20)	(±0.63, 0.73-3.18)	(±0.46, 1.30-3.03)	(±0.86, 1.49-5.41)
Permanent	1.94	2.32	2.54
(n=19,21,20)	(±0.54, 1.15-3.00)	(±0.86, 0.86-4.00)	(±1.11, 0.55-5.55)
Field 2			
Primary	1.97	2.17	2.78
(n=22,20,20)	(±0.83, 0.60-3.46)	(±0.61, 1.24-3.76)	(±1.34, 1.08-7.47)
Permanent	1.62	2.02	2.35
(n=19,21,20)	(±0.75, 0.77-3.63)	(±0.55, 1.02-3.10)	(±1.05, 0.98-5.37)
Field 3			
Primary	1.84	1.89	2.02
(n=22,20,20)	(±0.79, 0.61-3.29)	(±0.62, 0.96-3.58)	(±0.78, 0.99-3.20)
Permanent	1.74	2.15	1.83
(n=19,21,20)	(±0.72, 0.66-3.34)	(±0.84, 0.91-4.20)	(±0.69, 0.42-3.24)
Field 4			
Primary	2.51	2.59	2.56
(n=22,20,20)	(±0.95, 0.88-4.50)	(±0.93, 0.80-4.08)	(±1.25, 1.38-5.54)
Permanent	1.96	2.35	2.08
(n=19,21,20)	(±1.08, 0.73-5.20)	(±1.03, 0.70-4.39)	(±0.92, 0.88-4.34)

Table 6.1B. Significance values for effect of dentition and degree of caries on the PAS for UEIL (two-way analysis of variance on log-transformed data)

Main effects	Field 1	Field 2	Field 3	Field 4
Dentition type	P=0.072	P=0.054	P=0.881	P=0.011
Degree of caries	P=0.0001	P=0.0001	P=0.284	P=0.541

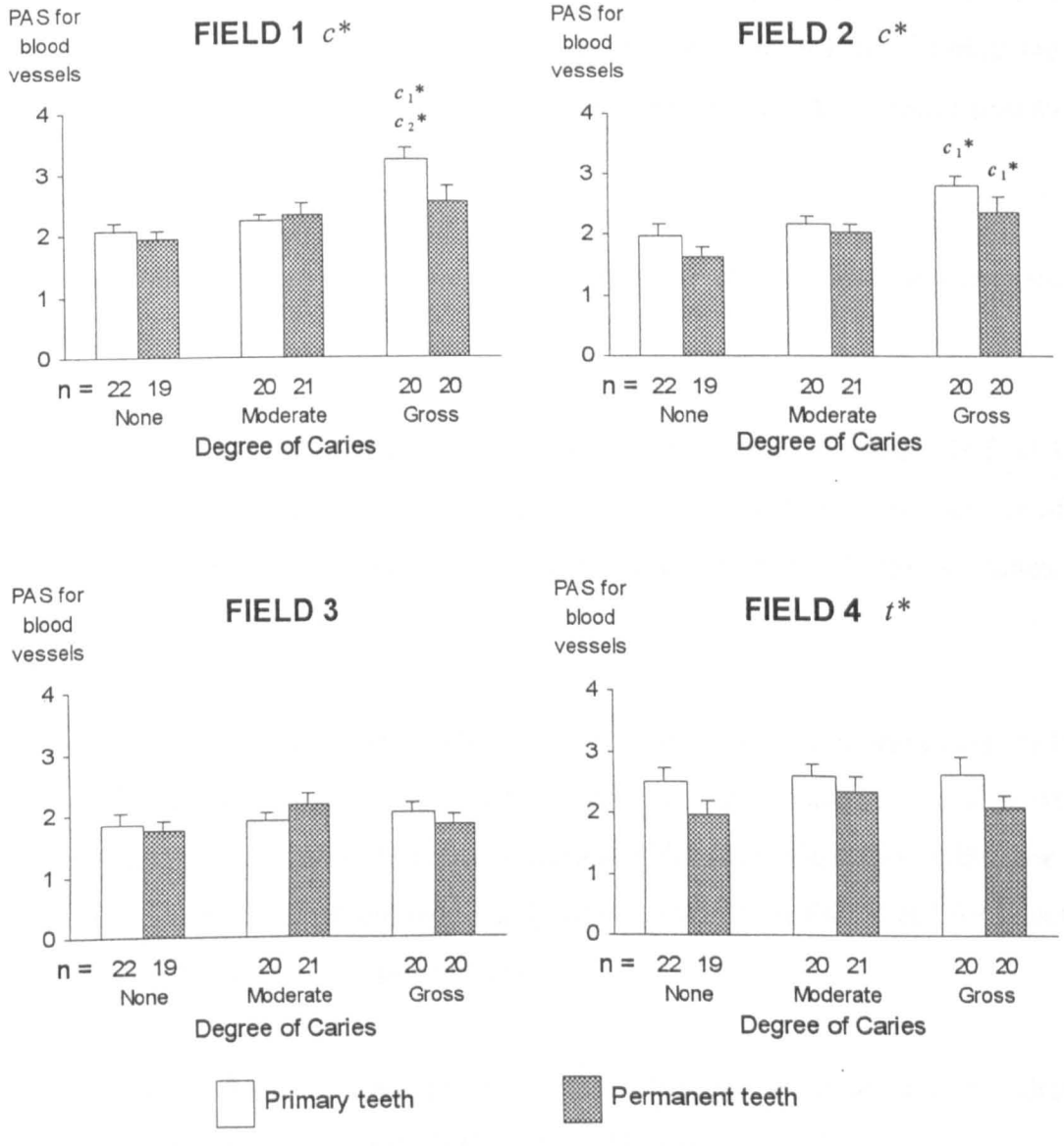


Figure 6.2. Bar charts showing mean (±SEM) percentage area of staining (PAS) for *Ulex europeaus* I lectin-labelled tissue in primary and permanent teeth according to the degree of caries. Field 1=tip of pulp horn; Field 2=region subjacent to field 1; Field 3=buccal subodontoblastic plexus; Field 4=mid-coronal region.

Main titles: c^* = significant difference in mean PAS for UEIL according to the degree of caries and t^* = significant difference between primary and permanent teeth ($P<0.05$, ANOVA, log transformed data).

Bar titles: c_1^* = significantly different from intact samples of the same dentition; c_2^* = significantly different from moderately carious samples of the same dentition; t^* = significantly different from primary teeth with same degree of caries ($P<0.05$, Tukey's test).

- Vessel number

Table 6.2A shows the mean (±SD, range) number of vessels, per 0.22 mm^2 area, within each dentition according to the field of analysis and the degree of caries and Table 6.2B provides the corresponding statistical data. Bar charts showing pooled data are presented in Figure 6.3.

Key findings arising from the quantitative analysis of pulpal vessel number are outlined below.

- The greatest mean number of vessels estimated for intact teeth was found in field 1 (primary teeth = 30.18, permanent teeth = 31.93). The greatest mean number of vessels in carious teeth was also found in field 1 (primary teeth = 39.00, permanent teeth = 33.30).

- Mean vessel number was significantly greater in primary teeth than permanent teeth in fields 2 and 4 (P=0.0001, ANOVA). Further pairwise comparisons of mean values (see, Fig. 6.3) revealed that there were significant inter-dentition differences in vessel number for intact and moderately carious samples in field 4 and for intact samples in field 2 (P<0.05, Tukey's test).

- Caries was not found to have any significant effect on vessel number in either dentition, or in any field of analysis (P<0.05, ANOVA).

Table 6.2A. Mean (±SD, range) number of blood vessels according to tooth type, field of analysis and degree of caries.

Field/tooth(n)	Degree of caries		
	None	Moderate	Gross
Field 1			
Primary	30.18	35.98	39.00
(n=22,20,20)	(±5.64, 17.0-38.50)	(±16.65, 22.0-105.25)	(±7.78, 28.50-55.25)
Permanent	31.93	29.76	33.30
(n=19,21,20)	(±6.69, 19.50-42.25)	(±6.79, 18.50-46.75)	(±12.89 16.00-75.50)
Field 2			
Primary	21.97	21.60	22.75
(n=22,20,20)	(±4.61, 14.50-30.50)	(±0.61, 4.53-32.50)	(±6.0, 13.75-34.25)
Permanent	16.85	17.68	18.89
(n=19,21,20)	(±4.74, 11.50-26.00)	(±4.27, 8.75-24.25)	(±6.34, 8.00-32.75)
Field 3			
Primary	18.61	19.77	18.53
(n=22,20,20)	(±4.01, 11.0-25.0)	(±4.33, 12.25-28.0)	(±3.66, 12.75-25.50)
Permanent	18.04	20.01	18.34
(n=19,21,20)	(±3.64, 13.25-24.25)	(±3.38, 14.67-25.75)	(±3.20, 12.50-24.00)
Field 4			
Primary	7.79	7.38	6.90
(n=22,20,20)	(±2.50, 4.0-13.25)	(±2.00, 3.33-10.50)	(±2.10, 3.25-10.38)
Permanent	5.19	5.36	5.50
(n=19,21,20)	(±1.37, 3.33-9.50)	(±1.64, 2.67-8.88)	(±1.23, 3.33-8.00)

Table 6.2B. Significance values for effect of dentition and degree of caries on vessel number (two-way analysis of variance on log-transformed data)

Main effects	Field 1	Field 2	Field 3	Field 4
Dentition type	P=0.074	P=0.0001	P=0.799	P=0.0001
Degree of caries	P=0.088	P=0.428	P=0.115	P=0.782

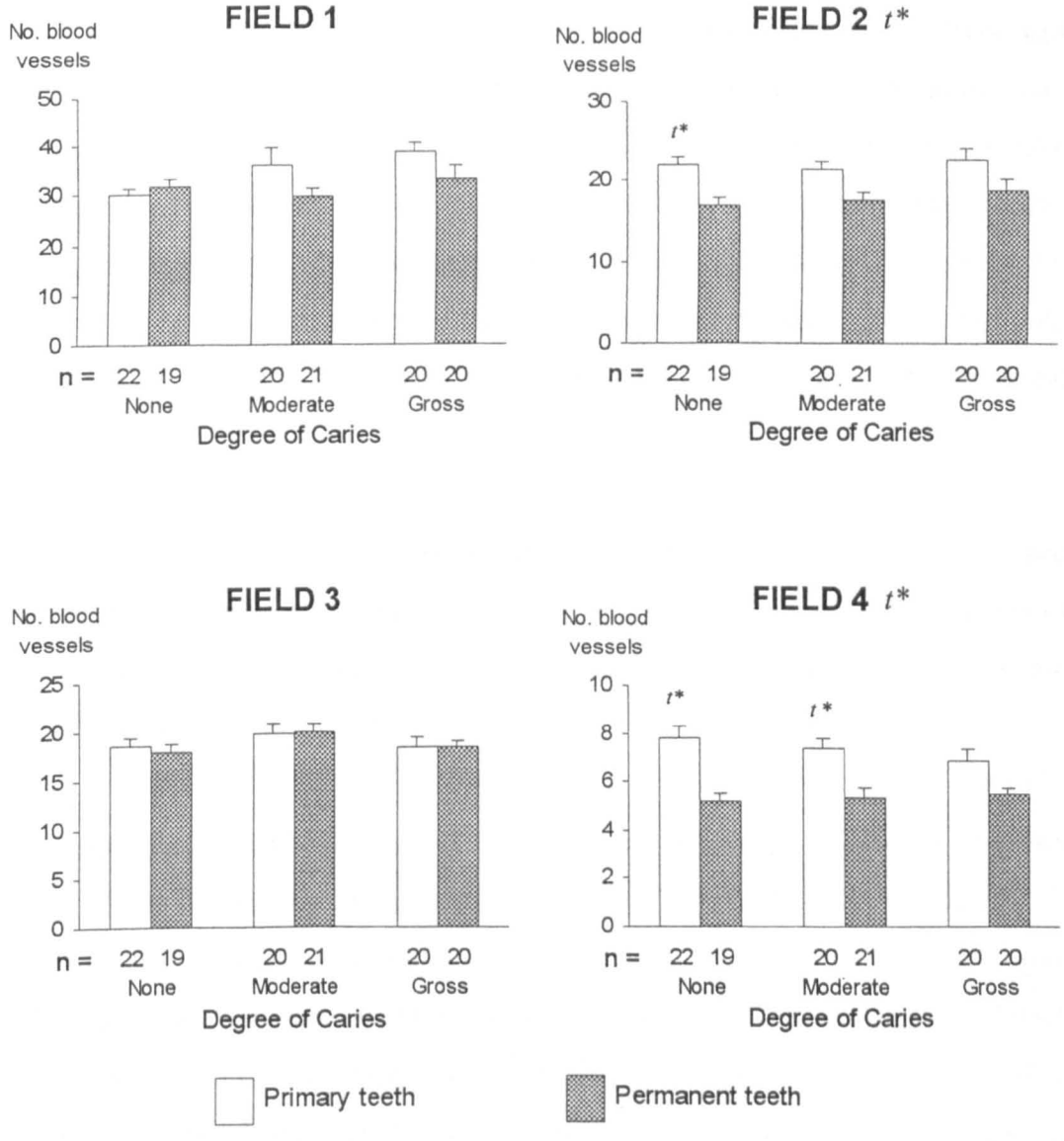


Figure 6.3. Bar charts showing mean (±SEM) number of blood vessels in primary and permanent teeth according to the degree of caries. Field 1=tip of pulp horn; Field 2=region subjacent to field 1; Field 3=buccal subodontoblastic plexus; Field 4=mid-coronal region.

Main titles: c^* = significant difference in mean pulpal vascularity according to the degree of caries and t^* = significant difference between primary and permanent teeth ($P<0.05$, ANOVA, log transformed data)

Bar titles: c_1^* = significantly different from intact samples of the same dentition; c_2^* = significantly different from moderately carious samples of the same dentition; t^* = significantly different from primary teeth with same degree of caries ($P<0.05$, Tukey's test).

6.3.5 Findings related to vessel innervation

- Qualitative observations

A close spatial relationship was clearly evident between PGP 9.5-ir fibres and arterioles within the mid-coronal region. Indeed, the majority of arterioles were intimately associated with varicose or smooth-surfaced PGP 9.5-ir fibres (Fig. 6.4A). These vascular-related nerve fibres presented either as a prominent perivascular plexus or as single fibres which appeared to penetrate the vessel wall. However, no PGP 9.5-ir fibres were seen to extend as far as the endothelial layer. Within the pulp horn regions, PGP 9.5-ir fibres were also seen to be closely associated with some small arterioles.

There was no clear evidence of any direct contact between PGP 9.5-ir fibres and pulpal capillaries, although nerve fibres were often seen to be closely approximated to these vessels. Furthermore, venules and lymphatic vessels also appeared to be devoid of any neural contacts (Fig. 6.4B).

Findings for vascular-related peptidergic innervation will now be presented. The vast majority vessels (arterioles) which were categorised as being "innervated" by PGP-ir fibres, were also seen to be supplied by a peptidergic innervation. These fibres were all characteristically varicose, intensely fluorescent and made a variable contribution to the overall innervation of each vessel (Fig. 6.4C-F). It was also noted that there were no appreciable differences in the vascular innervation patterns demonstrated by primary and permanent samples or intact and carious samples.

The majority of those vessels which were innervated, were seen to receive a subpopulation of CGRP-ir fibres. However, vessels appeared to be associated with a variable proportion of CGRP-ir nerves, ranging from a single fibre (Fig. 6.4C) to a complete perivascular plexus.

Substance P-ir fibres demonstrated a similar vascular relationship to that seen for CGRP. Essentially, the majority of thick-walled arterioles appeared to be innervated by varicose SP-ir fibres. However, the density of innervation varied, with some

vessels exhibiting a dense neural network (Fig. 6.4D) and others showing only a single SP-ir fibre actually extending into the vessel wall.

The contribution of VIP-containing nerves to vascular innervation appeared to be less than that seen for the other three neuropeptides. In a number of sections, no VIP-innervated vessels could be identified (Fig. 6.4E). However, some sections demonstrated vessels which were clearly innervated by VIP-ir fibres.

Finally, pulpal blood vessels were commonly seen to demonstrate a prominent and varicose perivascular network of NPY-ir fibres. Dense innervation was usually associated with thick-walled arterioles, however, smaller arterioles within the pulp horn regions were also seen to be associated with NPY-ir fibres (Fig. 6.4F).

- Quantitative analysis

Table 6.3 shows the mean percentage of mid-coronal pulpal vessels that were categorised as being innervated. In addition, data are given for the percentage of innervated vessels that also received a peptidergic innervation. There were no significant differences in these results according to the dentition type or the degree of caries ($P>0.05$, ANOVA). Data have been pooled for the caries subgroups, but for interest have been presented separately for primary and permanent teeth.

It can be seen that just under 20% of pulpal blood vessels within the mid-coronal region were closely associated with PGP 9.5-ir fibres. Just over 90% of these vessels were supplied by CGRP-ir fibres, and over 80% were supplied with SP-ir fibres. Similarly, NPY-ir fibres were associated with approximately 80% of innervated vessels. However, a wide variation was noted between samples with 17.4% to 100% of vessels demonstrating a close spatial relationship with NPY-ir fibres. Only about 15% of innervated vessels appeared to be supplied by VIP-ir fibres.

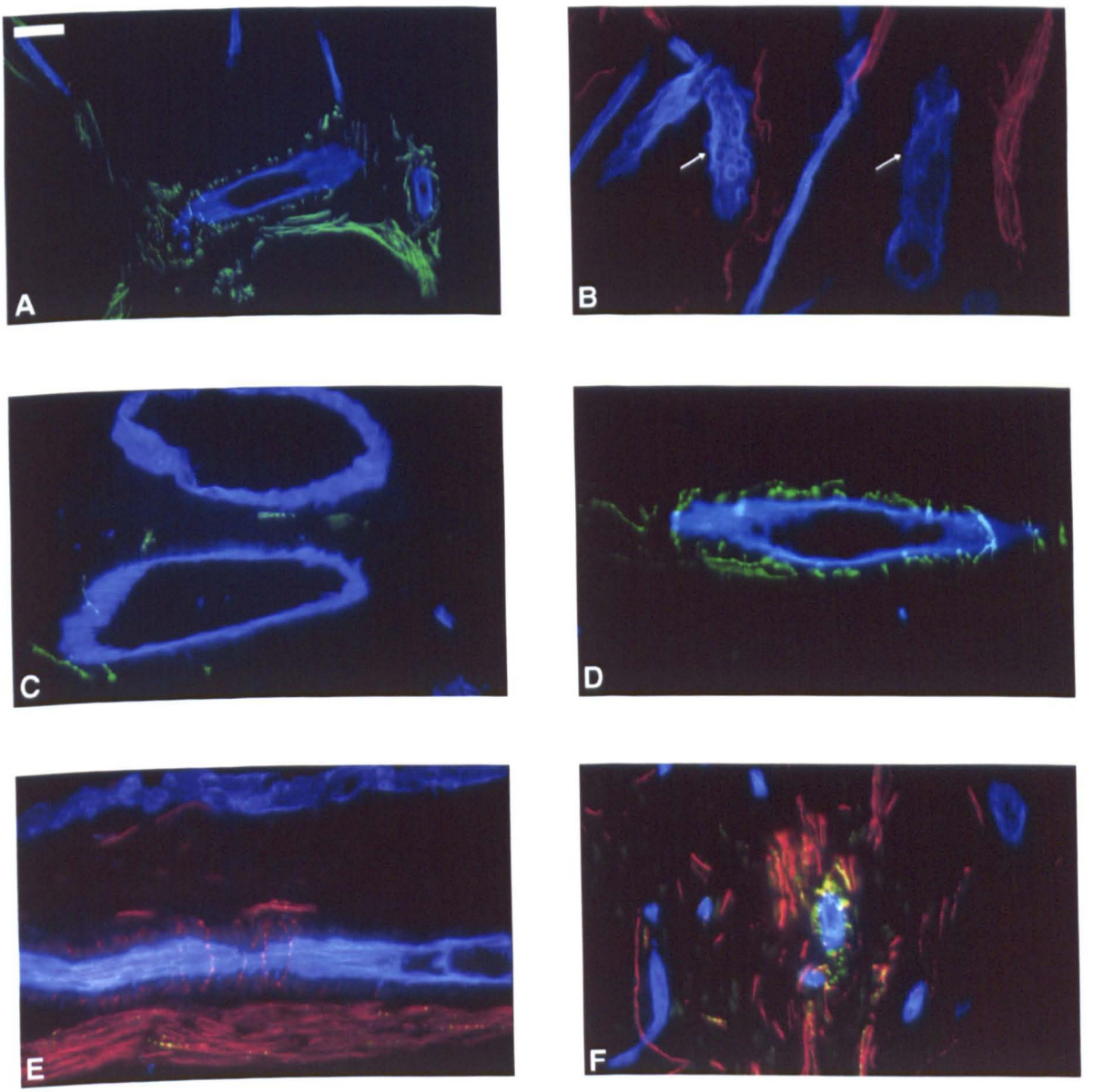


Figure 6.4. Photomicrographs showing the spatial relationships between pulpal nerves and blood vessels in the coronal pulp. **(A)** Double-labelling for PGP 9.5 (green) and UEIL (blue) showing the close relationship between PGP 9.5-ir fibres and arterioles in the mid-coronal region. **(B)** Double-labelling for PGP 9.5 (red) and UEIL (blue) showing lymphatic vessels (arrows) devoid of any neural associations. **(C)** Double-labelling for UEIL and CGRP-ir fibres (green) showing a minimal vascular innervation. **(D)** Double-labelling for UEIL and SP-ir fibres (green) showing a perivascular plexus of SP-ir fibres. **(E)** Triple-labelling for PGP 9.5 (red), UEIL and VIP-ir fibres (yellow) showing perivascular PGP 9.5-ir fibres but an absence of vascular-related VIP-ir fibres. **(F)** Triple-labelling for PGP 9.5 (red), UEIL and NPY (yellow/green) in the pulp horn region showing some NPY-ir fibres closely associated with a small blood vessel. Scale bar = 15 μm **(B-F)**, 30μm **(A)**.

Table 6.3. Percentage of mid-coronal pulpal vessels innervated by protein gene product 9.5 (PGP 9.5) and the percentage of PGP 9.5-innervated vessels also supplied by a peptidergic innervation.

Innervation/dentition	Percentage of vessels innervated		
	Mean	SD	Range
PGP 9.5 (mean)			
Primary	17.2	8.57	14.2-20.3
Permanent	18.2	8.17	15.4-20.9
CGRP			
Primary	92.5	20.48	85.9-99.2
Permanent	90.8	19.98	83.9-97.7
SP			
Primary	84.7	21.42	78.8-90.5
Permanent	88.7	24.32	81.7-95.7
VIP			
Primary	14.0	8.01	0.0-40.0
Permanent	15.7	6.59	4.0-27.2
NPY			
Primary	78.2	27.40	17.4-27.2
Permanent	80.7	21.55	25.0-100

Data are given for 62 primary and 62 permanent teeth.

6.3.6 Correlation between neural factors and vascularity or vessel number

Scatter plots of the PAS for PGP 9.5, CGRP, SP and VIP against the PAS for UEIL or blood vessel number within field 1 of carious primary and permanent teeth are shown in the figures 6.6 to 6.12. The corresponding Pearson correlation coefficients and significance values for the associations are given in Table 6.4. Firstly, it can be seen that innvervation density (PAS for PGP 9.5) correlated very poorly with vascularity (PAS for UEIL) (Fig. 6.5) and vessel number (Fig. 6.6). This was particularly evident for the permanent dentition where correlation coefficients were close to zero.

Primary and permanent teeth demonstrated contrasting data for the association between CGRP expression and vascularity (Fig. 6.7). In primary teeth vascularity tended to increase with increased CGRP expression ($r=0.17$) whereas converse was

true for permanent teeth (r= -0.18). However, neither of these associations were significant (P>0.05). Both dentitions showed a similar degree of positive correlation between CGRP expression and vessel number (Fig. 6.8) (r=0.18, primary teeth; r=0.22, permanent teeth), but again these associations were not significant.

Substance P expression was seen to demonstrate a positive association with vascularity (Fig. 6.9) and vessel number (Fig. 6.10) in both primary and permanent teeth. However the only significant association was found between SP expression and vessel number in primary teeth (r=0.39, P=0.011).

Interesting data were obtained for VIP for both the association with vascularity (Fig. 6.11) and vessel number (Fig. 6.12). In permanent teeth there was little association between VIP and either of these two variables (r=0.04, vascularity; r=0.10 vessel number). However, there was a significant correlation between VIP and these vascular factors in the primary dentition (r=0.37, P=0.023, vascularity; r=0.38, P=0.021, vessel number).

Table 6.4. Pearson correlation coefficients for the association between the percentage area of staining for PGP 9.5, CGRP, SP or VIP and the PAS for *Ulex europeaus* I Lectin (UEIL) and vessel number (N) in the pulp horn (field 1) of carious primary and permanent teeth.

	Correlation coefficient (*P*)			
Dentition	PGP 9.5/UEIL	CGRP/UEIL	SP/UEIL	VIP/UEIL
Primary	0.18 (0.251)	0.17 (0.315)	0.25 (0.111)	0.37 (0.023)
Permanent	0.03 (0.847)	-0.18 (0.294)	0.18 (0.301)	0.04 (0.825)
	PGP 9.5/N	CGRP/N	SP/N	VIP/N
Primary	-0.15 (0.386)	0.18 (0.280)	0.39 (0.011)	0.38 (0.021)
Permanent	-0.01 (0.869)	0.22 (0.196)	0.22 (0.207)	0.10 (0.519)

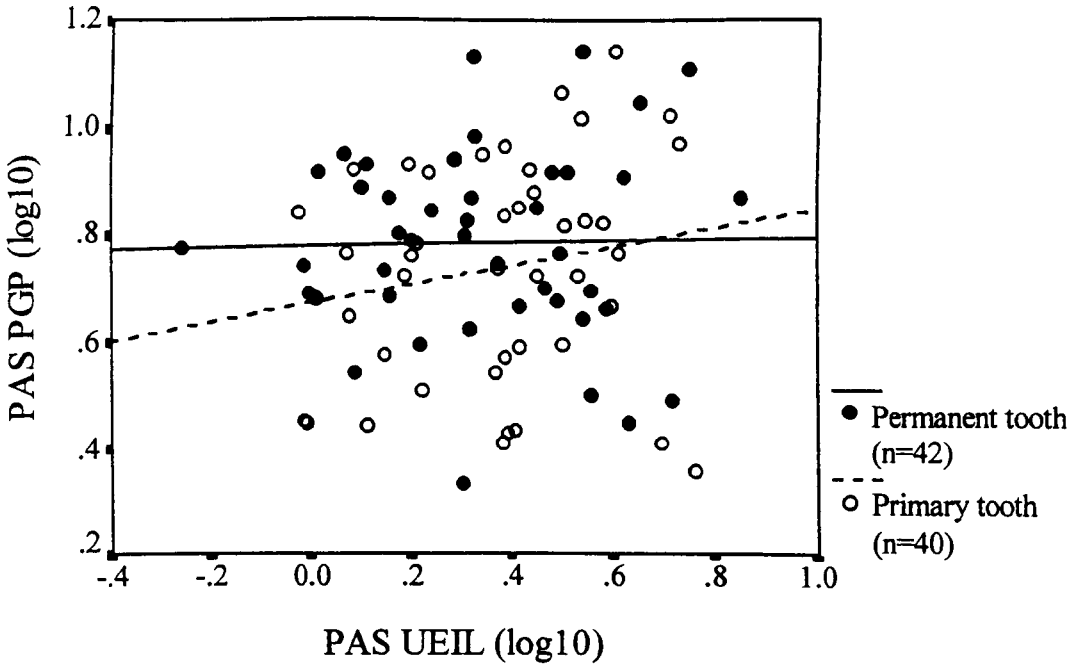


Figure 6.5. Scatter plots with regression lines to show the relationship between the percentage area of staining for protein gene product 9.5 (PAS PGP 9.5) and *Ulex europeaus* I Lectin (UEIL) in the pulp horn of carious primary and permanent teeth.

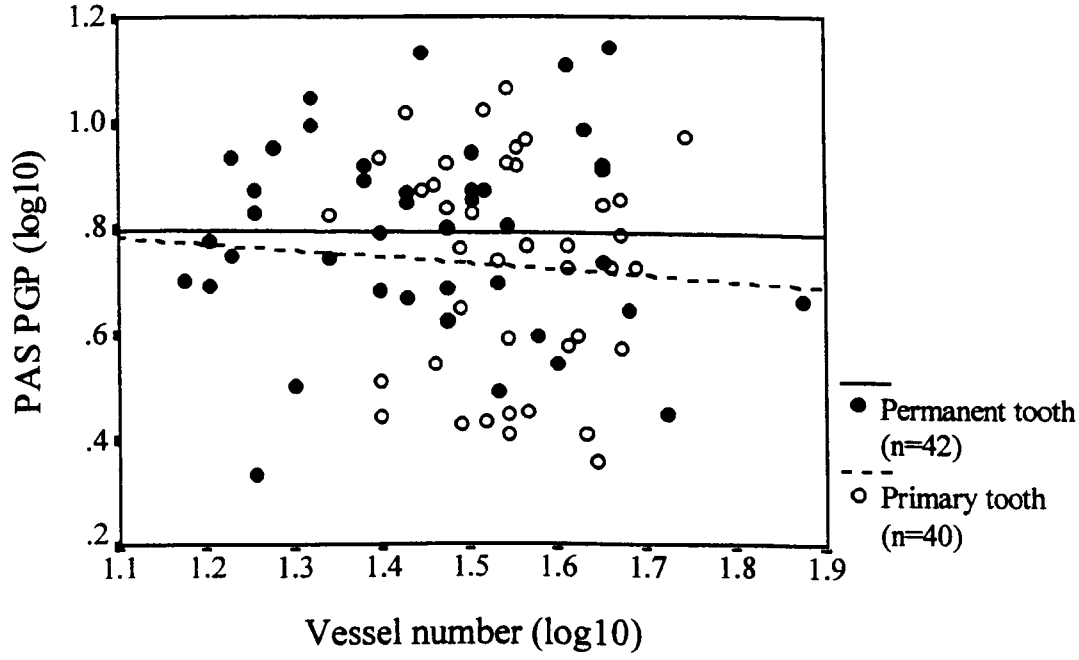


Figure 6.6. Scatter plot with regression lines to show the relationship between the percentage area of staining for protein gene product 9.5 (PAS PGP 9.5) and blood vessel number in the pulp horn of carious primary and permanent teeth.

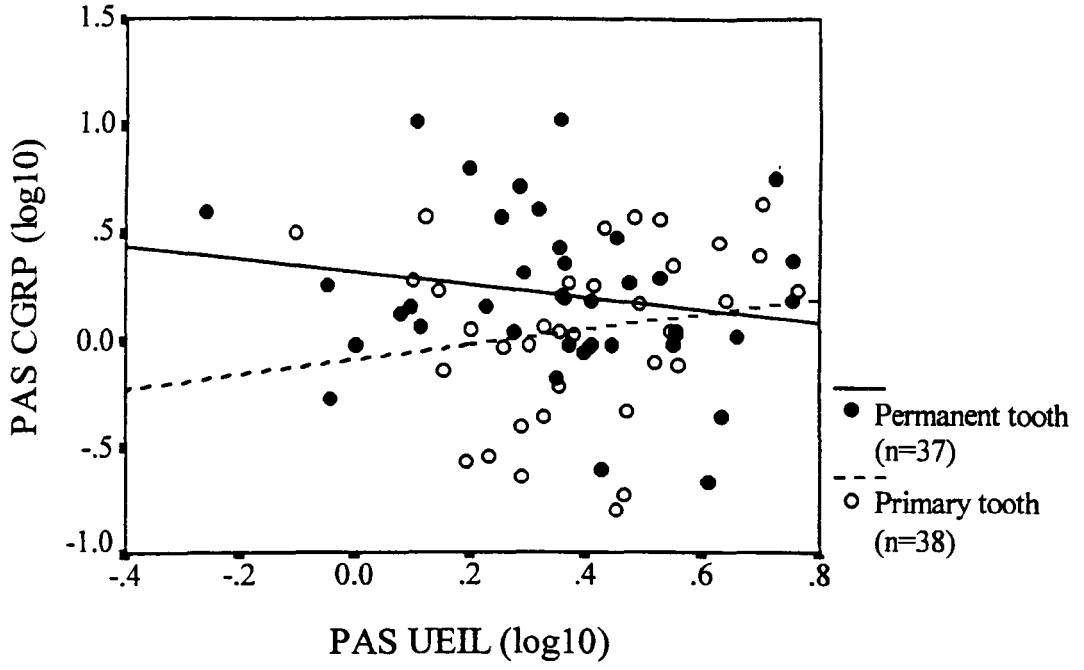


Figure 6.7. Scatter plot with regression lines to show the relationship between the percentage area of staining for calcitonin gene-related product (PAS CGRP) and *Ulex europeaus* I Lectin (UEIL) in the pulp horn of carious primary and permanent teeth.

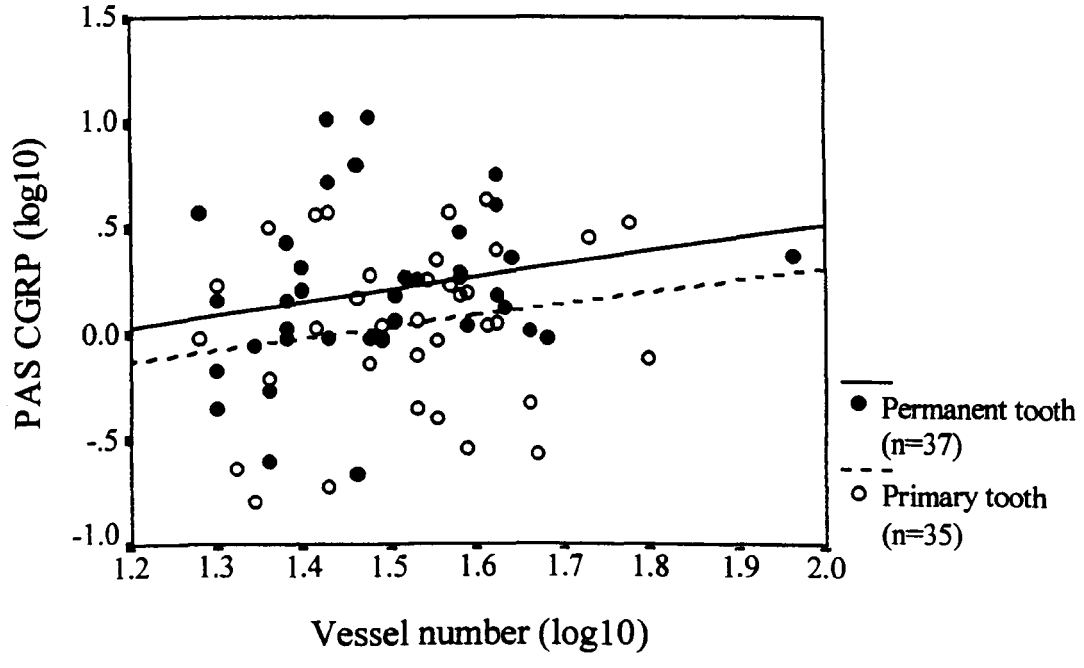


Figure 6.8. Scatter plot with regression lines to show the relationship between the percentage area of staining for calcitonin gene-related peptide (PAS CGRP) and blood vessel number in the pulp horn of carious primary and permanent teeth.

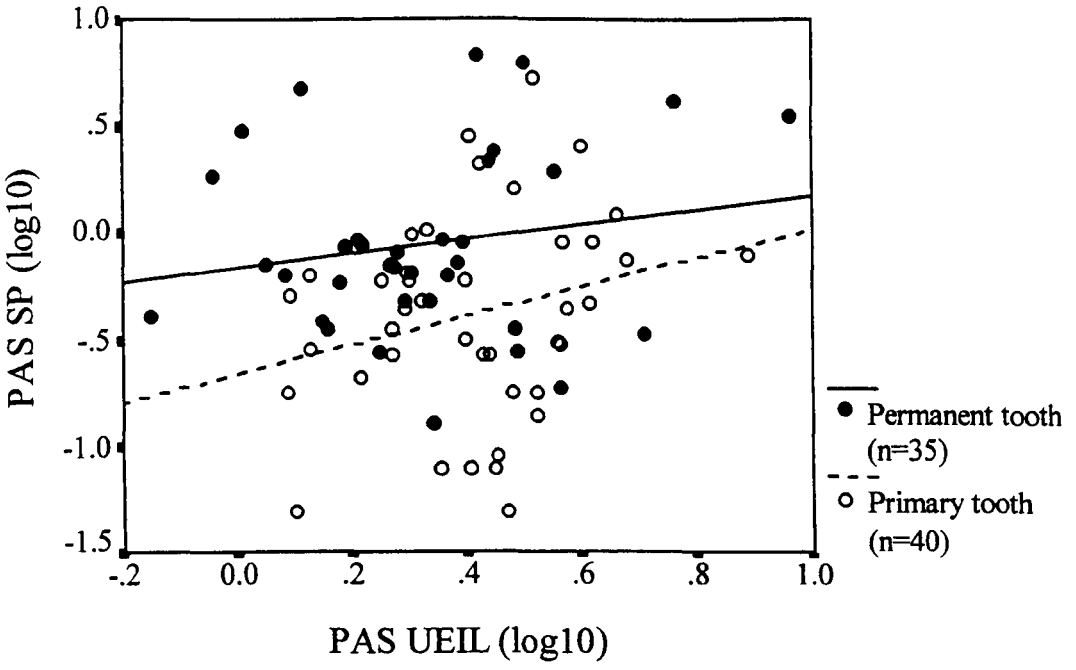


Figure 6.9. Scatter plot with regression lines to show the relationship between the percentage area of staining for substance P (PAS SP) and *Ulex europeaus* I Lectin (UEIL) in the pulp horn of carious primary and permanent teeth.

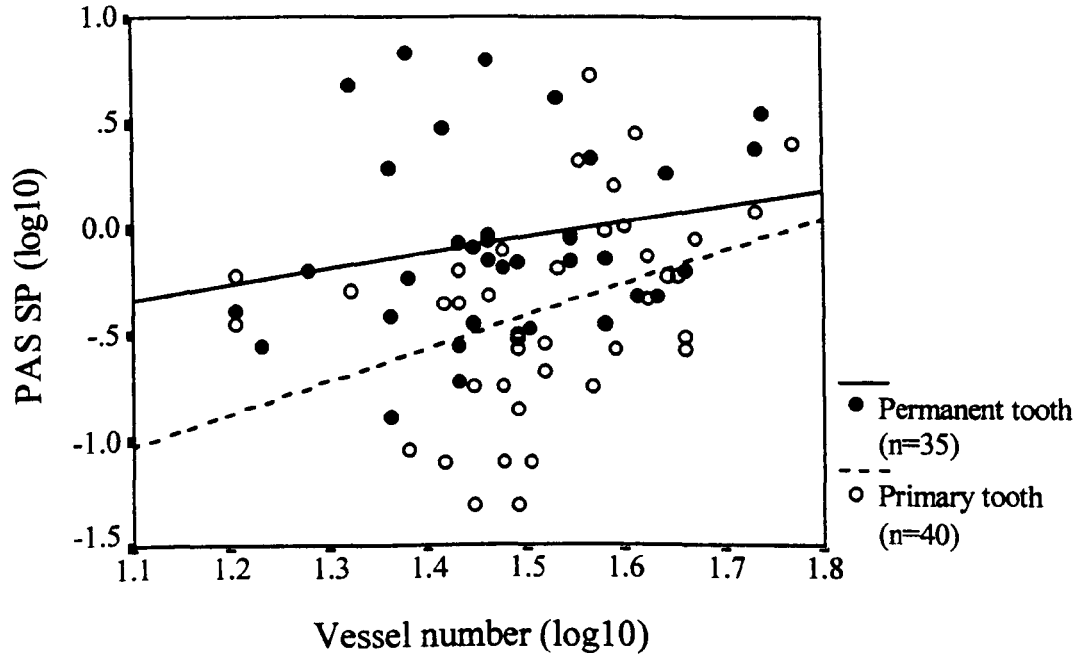


Figure 6.10. Scatter plot with regression lines to show the relationship between the percentage area of staining for substance P (PAS SP) and blood vessel number in the pulp horn of carious primary and permanent teeth.

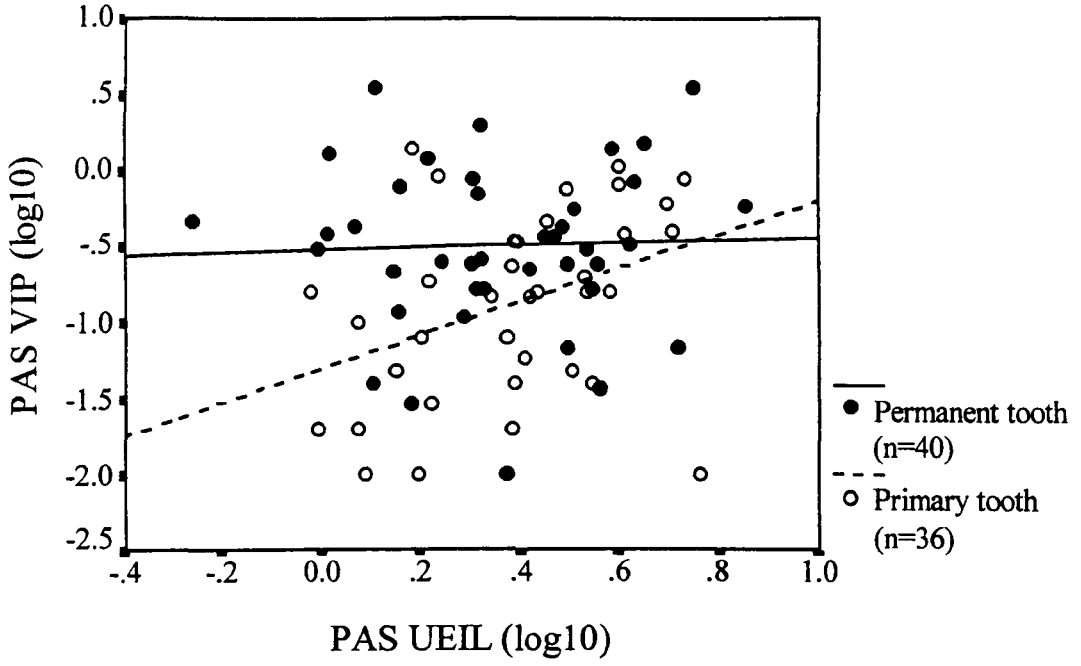


Figure 6.11. Scatter plot with regression lines to show the relationship between the percentage area of staining for vasoactive intestinal polypeptide (PAS VIP) and *Ulex europeaus* I Lectin (UEIL) in the pulp horn of carious primary and permanent teeth.

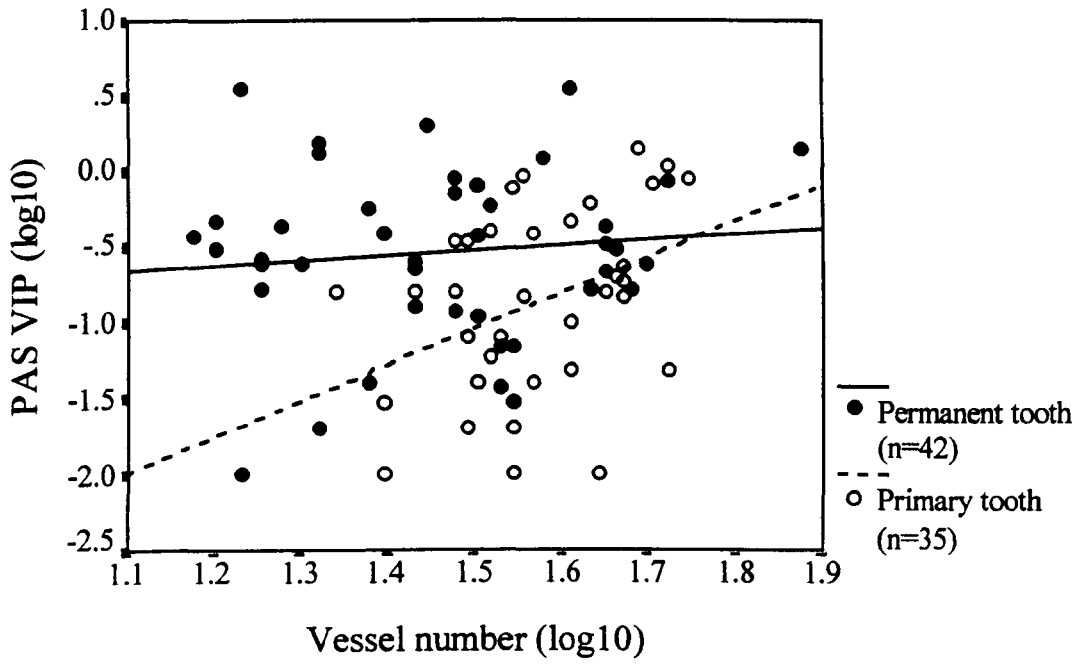


Figure 6.12. Scatter plot with regression lines to show the relationship between the percentage area of staining for vasoactive intestinal polypeptide (PAS VIP) and blood vessel number in the pulp horn of carious primary and permanent teeth.

6.3.7 Vascularity in relation to pain history

The mean (±SD, range) PAS for UEIL within grossly carious teeth according to the reported pain history is presented in Table 6.5. It can be seen that, for both dentitions, there was no significant difference in overall vascularity within any field between samples that were reportedly painful or asymptomatic ($P>0.05$, independent sample *t*-test on log-transformed data). However, there was a trend for painful permanent teeth to be more vascular than asymptomatic permanent teeth within field 4 ($P=0.06$, independent sample *t*-test).

Table 6.5. Mean (±SD, range) percentage area of staining for *Ulex europeaus* I Lectin according to the reported pain history

Field/dentition	Pain history		
	Asymptomatic[a]	Painful[b]	*P*
Field 1			
Primary	3.14 (±0.96, 1.49-4.16)	3.34 (±0.82, 2.43-5.41)	0.510
Permanent	2.47 (±1.30, 0.55-5.55)	2.64 (±0.88, 1.62-3.92)	0.532
Field 2			
Primary	2.63 (±0.98, 1.08-4.52)	2.90 (±1.61, 1.70-7.47)	0.689
Permanent	2.19 (±1.30, 0.98-5.37)	2.55 (±0.63, 1.77-3.54)	0.221
Field 3			
Primary	2.18 (±0.82, 1.15-3.20)	1.89 (±0.76, 0.99-2.86)	0.425
Permanent	1.79 (±0.82, 0.42-3.24)	1.89 (±0.53, 1.26-2.48)	0.536
Field 4			
Primary	2.90 (±1.43, 1.38-5.54)	2.44 (±1.10, 1.48-5.28)	0.464
Permanent	1.76 (±0.84, 0.88-3.61)	2.48 (±0.91, 1.72-4.34)	0.060

P=independent sample *t*-test on log-transformed data
[a] n=10,11 (primary teeth, permanent teeth); [b] n=10,10 (primary teeth, permanent teeth)

6.4 Discussion

6.4.1 Experimental approach

One of the objectives cited for the present study was to compare the pulpal vascularity of primary and permanent teeth of and intact and carious teeth. This objective was met by quantifying the PAS for the endothelial marker UEIL within different tooth samples. Although this approach was considered to provide valid comparative data, it should be borne in mind that these data do not constitute absolute

measures of vascularity due to inherent methodological problems (Sayegh and Reed, 1974; Mjör, 1999). Firstly, it is possible that pulpal vessels may have become dilated following exodontia-induced trauma. Secondly, a variable degree of tissue shrinkage may have occurred during subsequent tissue processing. Finally, surface area measurements are also affected by the orientation of the tissue. The complexity of the pulpal microvascular system is not conducive to obtaining standardised transverse or longitudinal sections of the entire vascular system and this factor may therefore also influence histological estimates of vascularity. Notwithstanding these factors, the immunocytochemical approach adopted was considered to provide reproducible and objective data for a comparative analysis of pulpal vascularity.

Another aspect of the experimental approach that warrants discussion, is the assessment of vascular innervation. The criteria employed (see, section 2.7.4) were stringent as only vessels demonstrating a definite intersection with an associated nerve fibre were classified as being innervated. Thus vessels which were closely aligned to nerve fibres, but did not demonstrate an apparent contact, were categorised as non-innervated. Neurochemicals can diffuse over some distance to exert their effects and thus it is likely that a greater proportion of pulpal vessels are under neural control than has been reported by the present investigation. This level of evaluation is acknowledged to be elementary but a more definitive assessment of vascular innervation would necessitate further ultrastructural and physiological investigations. Furthermore, the overall approach was highly reproducible (see, section 3.5.4) and thus provided valid comparative data.

Finally, there has been some previous work reporting the binding of UEIL to dental primary afferent projections in the spinal trigeminal complex of some animal species (Matthews et al., 1989). However, no colocalisation of UEIL-immunoreactivity within neural-stained tissue was seen in the present investigation. Thus there is no evidence that UEIL labels anything other than the vascular endothelium within the dental tissues of man.

6.4.2 Vascular differences between primary and permanent teeth

This study found that primary teeth were significantly more vascular than permanent teeth within the mid-coronal pulp. Furthermore, this increased vascularity would appear to be due to the presence of a higher number of vessels in this region. There was also a significantly greater number of vessels within the pulp horn region (field 2) of primary teeth although overall vascularity was not found to be greater.

These data would appear to substantiate the commonly-held view that primary teeth are more vascular than their successors. The biological significance of this higher vascularity remains speculative, but may reflect the increased functional demands of the primary tooth pulp. However, the increased vascularity may also simply be attributed to the presence of wider apical foramina that are found in primary teeth. It has been suggested by some authorities that this higher vascularity may prove detrimental during the inflammatory process. There is some descriptive data to suggest that primary teeth undergo extensive inflammation at an earlier stage of caries than permanent teeth, are reportedly less able to localise infection and are more prone to internal resorption (Rayner and Southam, 1979; Magnusson, 1980; Kopel, 1992). However, on the basis of finding from this study, it would not appear that a higher vascularity contributes to a more unfavourable pulpal inflammatory response as both dentitions showed similar caries-induced changes. However, further research is necessary to explore the actual haemodynamics of PBF in order to more fully explore any potential vascular differences between the two dentitions under normal and pathological conditions.

Primary and permanent tooth pulps were found to have a remarkably similar prevalence of innervated blood vessels. Furthermore, the proportion of vessels which were associated with each of the four different peptidergic subpopulations was almost identical. Although this study did not attempt to quantify the density of vascular innervation, subjective observation did not reveal any marked differences in the overall density or pattern of vascular innervation between the two dentitions. It is therefore hypothesised that primary and permanent tooth pulps are likely to be subject to very similar neurovascular control mechanisms.

6.4.3 Comparison with other vascular innervation studies

The overall descriptive findings relating to the association between neuropeptide-containing pulpal nerves and the microvasculature were entirely consistent with those of previous immunocytochemical studies in human permanent teeth (Uddman *et al.*, 1980; Wakisaka *et al.*, 1985; Uddman *et al.*, 1984). However, the relationship of peptidergic nerve fibres and pulpal blood vessels has not been previously reported for the primary tooth pulp, therefore this study provides some important new data.

Little quantitative or comparative data exist regarding the proportion of pulpal vessels that receive a peptidergic innervation. Furthermore, previous studies have been mostly limited to the rat and cat. In their study of rat incisors, Zhang and colleagues reported that a similar number of vessels were associated with both CGRP- and NPY-ir fibres, although it was felt that the latter were the most prolific within the vessel wall (Zhang *et al.*, 1998). Wakisaka and Akai (1989) performed double-immunostaining experiments to more fully explore the distribution of CGRP-, SP-, NPY- and VIP-ir fibres around blood vessels in the feline dental pulp. Firstly they reported that not all blood vessels were accompanied by peptidergic fibres. They also found that the distribution of vascular-related SP- and CGRP-ir fibres was very similar. In addition, they reported that a greater number of vessels were innervated by SP than by VIP. However some vessels demonstrated a dual innervation for both these peptides, although the innervation pattern was different. It would appear, therefore, that there were no major differences between the present study and previous animal studies.

Investigation of peptidergic vascular innervation has also been performed in other human tissues including buccal mucosa, vaginal tissue and temporal or occipital skin (Uddman *et al.*, 1986b; Hilliges *et al.*, 1994; Hoyle *et al.*, 1996). It is apparent from these studies that the distribution of peptidergic nerve fibres around blood vessels varies in different tissues. A notable finding was that, in vaginal tissue, the majority of vessels were associated with NPY- or VIP-ir fibres as compared to other tissue where CGRP- and SP-innervated vessels were the most well-represented.

6.4.4 Caries-induced vascular changes

A common finding in carious pulp samples was the presence of numerous distended lymphatic vessels, which was in contrast to the findings for intact samples. This increased prominence of lymphatic vessels has been previously reported by other investigators (Bernick, 1977; Marchetti *et al.*, 1992). The presence of these enlarged vessels is purported to provide indirect evidence for the important role played by these structures in removing excess interstitial fluid during pulpal inflammation.

Vasodilation and capillary proliferation are established features of the inflammatory process (Trowbridge, 1981). It was therefore not surprising that this study found a significant increase in pulpal vascularity in grossly carious samples. However, this increase in vascularity was limited to the pulp horn regions. It would therefore seem that widespread caries-related vascular changes do not generally occur throughout the tooth pulp of either dentition. This finding is consistent with both physiological and ultrastructural investigations (Tønder, 1983; Takahashi, 1990). However, these results would not tend to support the commonly-held view that primary tooth pulps undergo widespread vascular changes following caries.

If we look at the corresponding data, for the actual number of vessels present, it would appear that the observed increase in vascularity in the pulp horn was not associated with a significant increase in vessel number. Thus indicating, that an increase in vessel size (vasodilation) mostly contributed to the change in vascularity. This was an unexpected finding as capillary proliferation is known to accompany chronic inflammation and healing (Trowbridge, 1990). Interestingly, Vandevska-Radunovic and colleagues reported a significant increase in blood vessel number within the periodontal ligament of orthodontically-moved teeth, but did not find any changes within the pulpal tissue (Vandevska-Radunovic *et al.*, 1997b). However, investigators have reported the formation of formation of new capillary structures within cultured tooth pulps which had been previously subject to experimental tooth movement (Derringer *et al.*, 1996; Vandevska-Radunovic *et al.*, 1997b). It would appear, however, that quantitative assessments of pulpal blood vessel numbers in carious teeth have not been previously undertaken.

A closer examination of the data (Table 6.2A) shows that there was a large variation in vessel number within carious teeth, with some samples demonstrating extremely high numbers of vessels within field 1. Furthermore, anatomical observation revealed a number of sections with localised areas of densely packed small vessels. Thus it may be concluded that capillary proliferation may occur at some stage of caries-induced pulpal inflammation but was not detectable by the methods used in this study.

6.4.5 Correlation between neural and vascular factors

As discussed earlier (see, section 6.1.5), CGRP, SP and VIP have well-established vasodilatory effects. Thus it was anticipated that there would be a positive correlation between increased peptide amounts and increased vascularity. An increase in pulpal vascularity was found with a greater PAS for SP for both dentitions, although this correlation was not significant. Interestingly, primary and permanent teeth demonstrated contrasting results for VIP and CGRP. Both of these peptides demonstrated a positive correlation with vascularity in the primary dentition, but in the permanent dentition an increase in CGRP seemed to be related to a decreased vascularity and PAS for VIP appeared to have no correlation at all with the degree of vascularity.

On first inspection these data appear puzzling as it would seem that VIP and CGRP exert completely different vascular effects in primary teeth as compared to permanent teeth. A number of factors could account for these apparent differences such as inter-dentition variation in microvascular peptide-receptor subtypes or the occurrence of complex neuropeptide interactions which could modify overall vascular responses (for review see, Poyner, 1992).

Calcitonin gene-related peptide and SP have been shown to induce endothelial cell growth under experimental conditions and thus may play a key role in angiogenesis during tissue regeneration and healing (see, section 6.1.6). Notably, CGRP has been shown to increase the survival of ischaemic tissue in an experimental musculocutaneous model in the rat, partly by promoting the synthesis of new vessels within the tissue (Kjartansson and Dalsgaard, 1987). In contrast, VIP does not appear

to stimulate the proliferation of human endothelial cells under experimental conditions (Hægerstrand *et al.*, 1990). It was therefore interesting to discover that, in the primary dentition, there was a positive correlation between an increase in VIP and an increase in vessel number. This finding was in contrast to that in permanent teeth and indicated that, like CGRP, VIP may exert difference effects within each dentition. Vasoactive intestinal polypeptide has a well-accepted role in neuronal growth and survival and there is now increasing evidence for stimulatory effects on the growth of other tissues, particularly during early stages of development (Muller *et al.*, 1995; Waschek, 1995). Thus it is possible that under certain pathological conditions, VIP may also modulate angiogenesis.

In keeping with previous findings, an increase in SP and CGRP were shown to have a positive correlation with increased vessel number. This is consistent with the concept that these peptides may act as neovascular growth factors within both primary and permanent teeth during pulpal inflammation and healing.

It is appreciated that the above attempts to correlate the amount of peptide contained within pulpal nerves with vascular changes could be open to some criticism. Admittedly, an assumption has been made that, in these carious samples, an increased neural content of peptide was likely to reflect an increased release and thus increased amount of biologically available peptide within the target tissue. However, the actual degree or rate of peptide release or whether there are any inter-dentition differences in the dynamics of this mechanism are not known. Furthermore, in the present model, the effects of each peptide can not be considered in isolation as is the case for controlled in vitro experiments. Thus potential associations for one peptide may have been "masked" by the overriding effects of another peptide. In conclusion, these findings are not considered definitive but have been presented as a point of interest and may be of potential value in helping to direct future research relating to the vascular-related peptide effects in the inflamed tooth pulp.

6.4.6 Implications of findings in relation to dental pain

It was initially hypothesised that painful grossly carious samples may be associated with a high pulpal vascularity. In a low compliance environment one would expect that any increase in blood vessel volume may result in an increased tissue pressure, which in turn could affect pain experience. Furthermore, electrophysiological studies have shown that changes in blood microcirculation and intrapulpal hydrostatic pressure may profoundly influence the excitability of intradental nerves (Olgart, 1986; Ahlquist and Franzén, 1999). However, the present investigation did not find any difference in pulpal vascularity between painful and asymptomatic grossly carious teeth for either dentition. This result would appear to be consistent with numerous previous studies that have been unable to correlate the histopathological status of the pulp with reported symptoms (see, section 4.4.4). Furthermore, morphological studies of the pulpal vasculature, such as the present investigation, are limited as they give no indication of the dynamics of pulpal blood flow, filtration and resultant tissue pressure.

6.4.7 Clinical significance of findings

This study has shown that pulpal arterioles appear to be well innervated by a range of peptidergic fibre subpopulations. Thus it is likely that a variety of neuropeptides may profoundly effect vascular functions. Research in both oral and other body tissues is now being directed towards the identification and localisation of peptide receptor subtypes on vascular structures (Walsh *et al.*, 1992; Fristad *et al.*, 2000). An understanding of these ligand-receptor actions offers exciting possibilities for future therapeutic interventions. It is speculated that specific peptide analogues may have useful dental applications, for instance the local use of a SP antagonist may help to reduce unwanted pulpal hyperaemia during certain endodontic procedures.

To date, there has been a general reluctance to advocate the practice of direct pulp capping in cariously-exposed primary molars, despite this being a well-accepted approach for the permanent dentition (Kopel, 1992). Undoubtedly, concerns regarding unfavourable inflammatory responses within the primary tooth pulp have fostered this philosophy. Although this study found an overall higher vascularity

within the mid-coronal region of the primary tooth pulp as compared to the permanent tooth pulp, there were no marked inter-dentition differences in caries-induced vascular changes. Indeed there was no indication that primary tooth pulps underwent widespread vascular changes with caries progression. However, a marked inter-dentition difference was noted in the correlation between VIP and vascular changes. It is tempting to speculate that VIP antagonists may prove of specific therapeutic benefit within the inflamed primary tooth pulp by suppressing local increases in vascularity.

6.4.8 Summary of findings

In response to the initial objectives (see, section 6.1.9), this investigation has shown that:

- Primary teeth are only significantly more vascular than their successors within the mid-coronal pulp region. Caries-related increases in vascularity are limited to the pulp horn regions of both dentitions.

- Almost 20% of mid-coronal blood vessels appear to be innervated. The majority of these vessels (arterioles) are closely associated with CGRP-, SP- or NPY-ir nerve fibres. A much smaller proportion of vessels appear to be associated with VIP-ir fibres. Both dentitions show a remarkably similar pattern of vascular innervation which is not affected by caries.

- Neuropeptides appear to exert some local effects on vascularity and angiogenesis within the pulp horn of carious teeth, although these interrelationships seem to differ between primary and permanent teeth.

- There is no significant difference in the overall vascularity of asymptomatic and reportedly painful grossly carious samples from either dentition.

7. Neuropeptide and leucocyte interactions within the dental pulp

7.1 Introduction

7.1.1 Leucocytes and their functional roles

The body's white blood cells, also known as leucocytes, play an essential part in host defence mechanisms against microbial or foreign body invasion (for reviews see, Trowbridge, 1990; Stevens and Lowe, 1997). They may be involved in non-specific inflammatory responses as well as cell-mediated or humoral immune reactions. The functions of the five main leucocyte cell types will be briefly discussed.

Firstly the granulocytes, which include neutrophils, eosinophils and basophils. The most predominant cell types are the neutrophils and these play important roles in phagocytosis and bacterial killing. Phagocytic activity is also demonstrated by eosinophils in addition to their involvement in allergic-type reactions. Basophils (mast cell precursors) are the least numerous of the granulocytes and may induce hypersensitivity responses by releasing histamine and other vasoactive substances.

Lymphocytes and monocytes are collectively known as mononuclear cells and comprise the second group of leucocytes. The three main types of lymphocytes are called B cells, T cells and natural killer (NK) cells. Following antigen stimulation, B lymphocytes proliferate and convert to plasma cells which are able to secrete immunoglobulins (antibodies) and are involved in humoral immunity. The T lymphocytes form three functional subsets: cytotoxic cells, helper cells and suppressor cells which all play a combined role in cell-mediated immunity. Finally, NK cells are primarily involved in protection against virally-infected cells and cancer cells, and do not require prior antigen sensitisation. Macrophages are derived from monocytes and are also fundamental to host defence mechanisms. The essential role of macrophages is to carry out phagocytosis at the later stages of inflammation. In addition, they secrete a number of cytokines and participate in antigen processing.

Different cell populations tend to be associated with different stages of the inflammatory process. The first cells to be involved in an acute inflammatory reaction are the neutrophils. In contrast, macrophages, lymphocytes and, occasionally, plasma cells comprise the chronic inflammatory cell population.

7.1.2 Immunocytochemcial identification of leucocytes

Immunocytochemical identification of different leucocyte subsets relies on their individual expression of specific cell surface proteins, known as cluster designation (CD) antigens (Hogg and Horton, 1987). Continued efforts to develop antibodies to these characteristic markers have greatly facilitated the identification of specific immune cell subpopulations in different pathological conditions (Habtemariam *et al.*, 1998; Mizuma *et al.*, 1999).

The leucocyte common antigen (LCA), also known as CD45, is a cell surface glycoprotein complex that is selectively expressed on all haemopoietic cells, excluding mature erythroid cells. Thus, the use of an antibody to LCA is generally considered a valuable marker for all leucocyte cell types. However, it has been shown that monoclonal antibodies directed against LCA may exhibit some differences in specificity, but most are capable of labelling almost all mononuclear cells and granulocytes (Kurtin and Pinkus, 1985; Pulido *et al.*, 1988; Facchetti *et al.*, 1988; Streuli *et al.*, 1988).

7.1.3 Leucocyte responses to dental caries

Dental caries is the predominant cause of pulpal inflammation whereby the products of bacterial metabolism evoke inflammatory and immunological reactions. Carious lesions may progress over a period of months or years thus it is not surprising that caries-induced pulpal inflammation usually begins as a chronic low-grade reaction, rather than an acute inflammatory response. However, with advanced caries progression, there may be a spectrum of acute, chronic and reparative type inflammatory changes (Seltzer, 1972). As previously described, the pulpal response to caries or injury comprises a number of neural and vascular changes (see, sections 4.4.3, 5.1.6, 6.1.3). In addition, the migration and accumulation of a variety of

leucocyte subpopulations are considered a fundamental part of the inflammatory process.

A number of histological investigations have described the presence of inflammatory cells within the pulp of carious primary and permanent teeth (Reeves and Stanley, 1966; Cohen and Massler, 1967; Baume, 1970b; Lin and Langeland, 1981; Eidelman *et al.*, 1992). The initial caries-evoked inflammatory infiltrate consists principally of mononuclear cell types including lymphocytes, macrophages and plasma cells (Torneck, 1974b; Trowbridge, 1990). This is in contrast to an acute inflammatory response, which may follow cavity preparation, where the inflammatory cell population comprises mostly neutrophils. However, during the later stages of dental caries, when pulpal suppuration may occur, the number of neutrophils increases markedly (Trowbridge, 1990).

Two important accessory cell types have recently been identified within the intact and carious tooth pulp and are believed to contribute significantly to pulpal defence mechanisms against microbial insults (Jontell and Bergenholtz, 1992; Izumi *et al.*, 1996; Vandevska-Radunovic *et al.*, 1997a; Kannari *et al.*, 1998). One cell line has a highly dendritic appearance, is localised at the pulp periphery, and is likely to be involved in immunosurveillance. The second cell type has a macrophage-like appearance, is located more centrally and is considered to have an important role in phagocytosis.

7.1.4 Neural and immune cell interactions

Over the last decade, the concept of a functional interaction between the neural and immune system has gained universal acceptance (for reviews see, Payan *et al.*, 1986; Ottaway, 1988). It would appear that these two systems are able to communicate bi-directionally via a number of shared signals and receptors. Of particular interest is the finding that neuropeptides can modulate a variety of immune cell functions under different pathophysiological conditions. Some of these important regulatory effects will now be reviewed.

Fundamental to the overall inflammatory process is the initial adhesion of leucocytes to vascular endothelial cells, which must occur prior to leucocyte extravasation into the surrounding tissues (Kogushi *et al.*, 1988). It is now evident that certain neuropeptides, mainly SP, CGRP and VIP, are able to induce the upregulation of adhesion molecule expression, thus promoting leucocyte extravasation (Smith *et al.*, 1993).

Substance P is known to mediate a number of pro-inflammatory responses via its effects on several leucocyte subpopulations. These may include: acting as a chemotactic factor for neutrophils, macrophages and T lymphocytes; stimulating immune cell proliferation (particularly of T lymphocytes); modulating macrophage phagocytosis and regulating the release of inflammatory mediators (cytokines) from both macrophages and lymphocytes (for reviews see, Stanisz *et al.*, 1986; McGillis *et al.*, 1987; Nong *et al.*, 1989). In addition, SP-ir nerve terminals have been shown to have very close spatial contacts with mast cells and SP appears to induce the release of histamine and other inflammatory mediators (Fewtrell *et al.*, 1981; Botchkarev *et al.*, 1997).

Somewhat in contrast to the overall effects of SP, CGRP and VIP are considered to have a number of anti-inflammatory actions. Calcitonin gene-related peptide has been shown to inhibit T lymphocyte proliferation and may impair antigen-processing and presentation (Umeda *et al.*, 1988; Poyner, 1992; Scholzen *et al.*, 1999). Vasoactive intestinal polypeptide appears to inhibit lymphocyte migration, proliferation and production of immunoglobulins, and may also inhibit macrophage chemotaxis, phagocytosis and release of cytokines (Stanisz *et al.*, 1986; Litwin *et al.*, 1992; Delgado *et al.*, 1999).

The identification of specific neuropeptide receptors on many leucocytes has provided strong support for the existence of functional interactions between peptides and immune cells. Receptor sites for CGRP, SP and VIP have been identified on both lymphocytes and macrophages (Hartung *et al.*, 1986; McGillis *et al.*, 1987; Ottaway,

1988; Umeda, 1989; Segura *et al.*, 1991). Furthermore, the use of certain neuropeptide antagonists has been shown to block a number of peptide-mediated immune cell functions (McGillis *et al.*, 1987; Lotz *et al.*, 1988).

7.1.5 Neural and immune cell interactions in the dental pulp

Morphological observations have provided considerable indirect evidence for neural and immune cell interactions within the dental pulp. Using immunocytochemical techniques, intimate anatomical relationships between intradental nerve fibres and certain immune cell populations have been clearly shown (Jontell *et al.*, 1996; Okiji *et al.*, 1997; Yoshiba *et al.*, 1998). In addition, investigators have described immune cell accumulations in areas of increased neural sprouting in both experimentally-induced pulpitis (Suzuki *et al.*, 1996; Toriya *et al.*, 1997) and caries-related pulpitis (Yoshiba *et al.*, 1998; Sakurai *et al.*, 1999).

More direct evidence for functional neural and immune cell interactions has been provided by combined physiological and immunocytochemical approaches. Heyeraas and colleagues electrically stimulated the crowns of innervated and sensory-denervated rat teeth, and found that, following this stimulation, there was a significantly greater number of lymphocytes, plasma cells and granulocytes within innervated tooth pulps (Heyeraas *et al.*, 1996). The investigators therefore concluded that sensory nerves were likely to influence leucocyte migration and proliferation during pulpal inflammation. Fristad and colleagues found that, following dentine injury, there was a significantly higher influx of immunocompetent cells in the pulp of innervated rat molars as compared to those where an IAN axotomy had been performed (Fristad *et al.*, 1995b). Comparable studies were conducted by Vandevska-Radunovic and co-workers who showed that, following IAN axotomy, there was a delayed recruitment of macrophages and class II major histocompatability complex-expressing cells within the periodontal ligament of orthodontically-moved rat molars (Vandevska-Radunovic *et al.*, 1999). Thus the mobilisation and recruitment of immunocompetent cells would appear to be enhanced in tissues with an intact sensory nerve supply.

7.1.6 Rationale for study

As outlined at the beginning of this thesis (see, section 1.6), we have little appreciation of the ways in which primary and permanent teeth may differ in their response to caries. The clinical management of the compromised primary and permanent tooth pulp is fundamentally different yet our practices do not appear to have an established biological rationale (Magnusson, 1980; Schröder *et al.*, 1987; Stanley, 1989; Ketley and Goodman, 1991; Kopel, 1992). Indeed, generalisations about excessive and poorly localised inflammatory reactions in the primary tooth pulp seem to have been founded on subjective observations. Pulpal defence mechanisms essentially involve neural, vascular and immune cell interactions, thus the final stage of this experimental work will focus on pulpal leucocytes. An understanding of leucocyte responses to caries within both the primary and permanent tooth pulp may provide further insight into any basic differences that may exist between the two dentitions.

There is growing evidence that a number of neural and immune cell interactions occur within the inflamed tooth pulp. However, reports of a correlation between neural sprouting and immune cell accumulation have been simply descriptive and limited to the permanent dentition. A more objective evaluation of neural and leucocyte interactions following caries would therefore appear to be warranted.

Finally, there is a growing interest in the potential of peptide antagonists to attenuate immune cell responses in a number of pathological conditions (Foreman, 1987; Eedy *et al.*, 1991; Smith *et al.*, 1993; Wimalawansa, 1996). It is hoped that the findings from the present study may give some indication as to whether the mediation of neuropeptide expression could offer a possible therapeutic option for the suppression of pulpal inflammation.

7.1.7 Aims and objectives

The aims of the present study were to investigate the accumulation of leucocytes within the dental pulp and to explore any relationship between these cells and intradental nerves. The specific objectives were as follows:

- to compare the prevalence of leucocytes in primary and permanent tooth pulps in health and disease;

- to describe the anatomical relationships between leucocytes and intradental nerve fibres;
- to evaluate any association between changes in neural density or neuropeptide expression and leucocyte accumulation;
- to seek a correlation between leucocyte accumulation and the reported pain history.

7.2 Materials and methods

Details regarding the overall experimental approach have been previously outlined in chapter two. However, some points specific to the analysis of pulpal leucocytes will now be addressed. In summary, three sections from each tooth sample were triple labelled for LCA, UEIL and each of the following: PGP 9.5, CGRP, SP, VIP and NPY. Thus a total of 15 sections from each tooth were subject to descriptive analysis.

7.2.1 Quantitative analysis of immunostaining

Quantitative analysis of the PAS for LCA (leucocyte accumulation), PGP 9.5 and each neuropeptide was undertaken for one section from each of the above five staining regimes. One way analysis of variance was then employed to determine whether there were any statistically significant differences in the PAS for LCA between the five measurements recorded for each tooth sample.

7.2.2 Relationships between neural factors and pulpal leucocytes

Possible interrelationships between neural density/peptide expression and leucocyte accumulation were assessed by correlating the PAS for PGP 9.5, CGRP, SP and VIP with the PAS for LCA (using log-transformed data). Analysis was only performed for field 1 data as this region demonstrates significant caries-related changes in neural density and peptide expression (see, sections 4.3.6, 5.3.3, 5.3.4 and 5.3.5). Analysis was not undertaken for NPY as previous data had revealed that this peptide was only sparsely represented within the pulp horn (see, section 5.3.6). Furthermore, absolute data for peptide expression were employed (PAS for peptide) rather than previously-employed data which related to the relative amount of neural tissue that contained

peptide (PAS of PGP 9.5 that was also labelled for peptide). These data were selected as it was felt that the overall amount of potentially-available peptide was more pertinent when considering the degree of it biological effects than was the relative expression of the peptide.

Data were pooled for all carious samples but were subdivided according to the dentition. Intact teeth were not included in the analysis. It was felt that attempting to correlate peptide expression with increases in leucocyte accumulation would be inexpedient in non-carious samples as a leucocyte influx was not likely to occur in the healthy state.

Scatter plots were first constructed to observe the relationship between the two variables and the strength of the association was determined by calculating the Pearson correlation coefficients (r). The association between the two variables was considered significant when $P<0.05$.

7.3 Results

7.3.1 Specificity controls

The specificity of the immunolabelling for PGP 9.5, each of the neuropeptides and UEIL has been previously described in sections 4.3.1, 5.3.1 and 6.3.1 respectively. However, it is worth re-iterating that, in grossly inflamed tissue, a number of small cells demonstrated a granular non-specific FITC labelling. No positive labelling for leucocytes was obtained where the primary antibody to LCA had been omitted and replaced by non-immune serum (Fig. 3.1C,D, section 3.2.2). Thus the reaction for LCA was considered to be specific.

7.3.2 Comparison between different sections

Statistical analysis confirmed that there was no significant difference for the mean PAS for LCA between the five different sections analysed for each tooth pulp ($P>0.05$, ANOVA). This was true for all fields and for each caries subgroup.

Therefore, the means of the five measurements for PAS for LCA were employed for subsequent statistical analysis and data presentation.

7.3.3 Qualitative observations

Labelling for LCA provided excellent visualisation of pulpal leucocytes. Essentially, two different cell types were recognisable by their distinctive morphology. The most abundant was an intensely labelled round cell which was seen throughout the coronal pulp (Fig. 7.1A). The diameter of this cell was found to be in the region of 5-6 μm and the overall surface area was approximately 50 μm^2. The second cell type demonstrated paler immunolabelling, and was generally localised at the pulp periphery. This cell had an irregular outline with small dendritic extensions and had an estimated surface area of 150-175 μm^2 (Fig. 7.1B).

In the intact primary tooth pulp, a moderate number of round LCA-ir cells were seen scattered throughout the coronal pulp. However, small dense clusters of both round and irregular cells were also occasionally seen, particularly in the pulp horn regions (Fig. 7.1C). In contrast, only a sparse number of round LCA-ir cells were evident within the pulps of intact permanent teeth (Fig. 7.1D).

There was an obvious increase in the number of both round and irregular LCA-ir cells within carious primary and permanent pulp sections (Fig. 7.1E). However, round cells were by far the most predominant subpopulation. Generally, accumulations of LCA-ir cells were localised within the pulp horn regions (Fig. 7.1F) although small dense foci of round cells were sometimes seen in the mid-coronal region.

- Leucocyte relationships with the pulpal vasculature

The vast majority of LCA-ir cells were seen to be extravascular (Fig. 7.2A), only a few round cells were ever seen within the blood vessels or the lymphatic vessels (Fig. 7.2B). A further observation was that, in areas of a dense inflammatory cell infiltrate, there was often a corresponding increase in the number of small blood vessels (Fig. 7.1F).

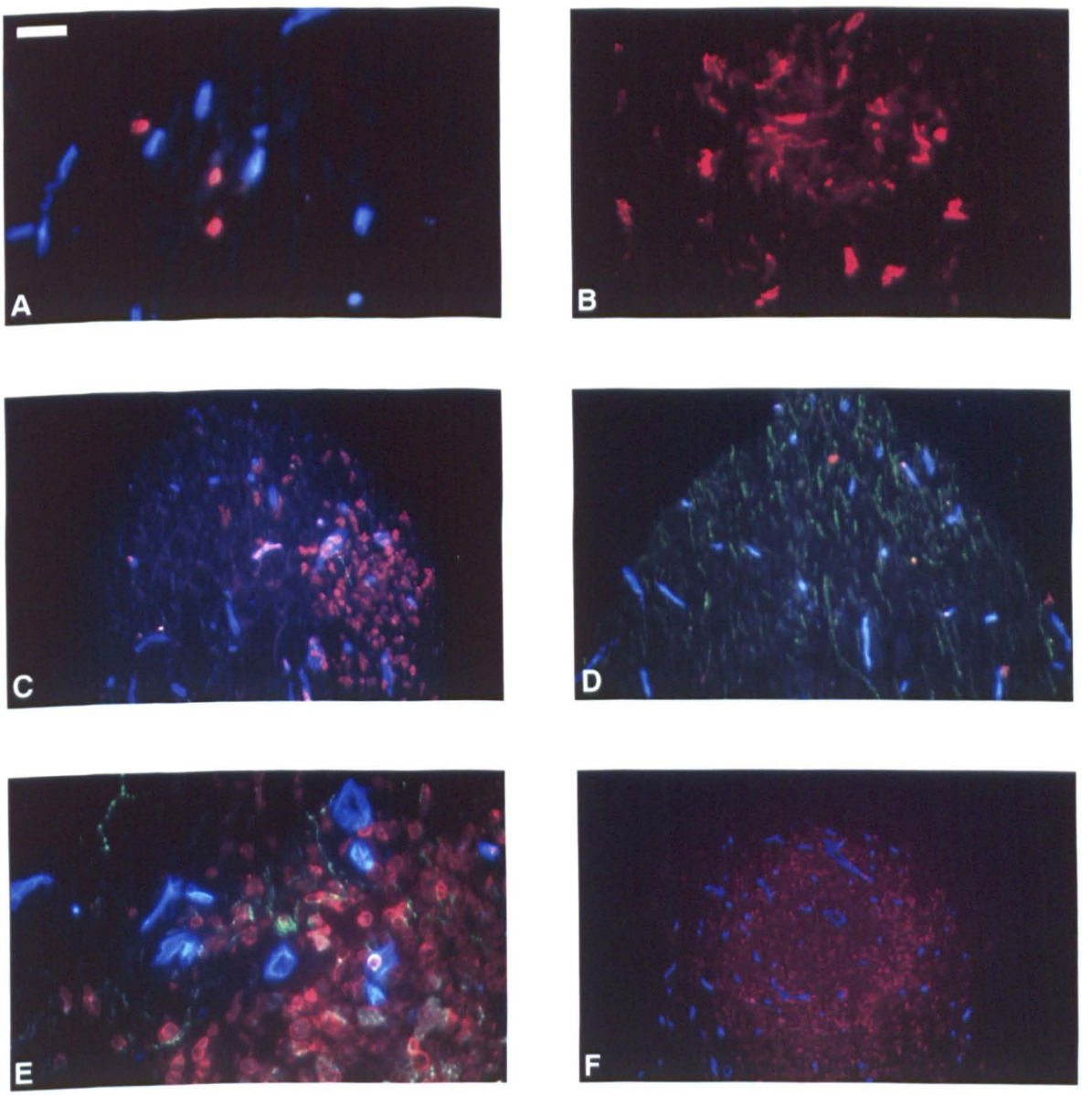


Figure 7.1. Photomicrographs demonstrating differences in the distribution and morphology of LCA-labelled cells (red) in the coronal pulp of intact and carious primary and permanent teeth. **(A)** Double-labelling for LCA and UEIL (blue) in the pulp horn region of an intact primary molar to demonstrate round LCA-ir cells. **(B)** LCA-labelling in the pulp horn region of an intact primary molar to demonstrate some irregular LCA-ir cells. **(C)** Triple-labelling for CGRP (green), UEIL and LCA showing a large number of LCA-ir cells in the pulp horn of an intact primary tooth and **(D)** fewer LCA-ir cells in the pulp horn of an intact permanent tooth. **(E)** Triple-labelling for CGRP (green), UEIL and LCA to show an accumulation of LCA-ir cells in the central pulp horn region of a carious primary tooth. **(F)** Double-labelling for LCA and UEIL demonstrating a dense but localised accumulation of LCA-ir cells in the pulp horn of a carious permanent molar. Scale bar = 15 μm **(A,B,E)**, 30 μm **(C,D)**, 60 μm **(F)**.

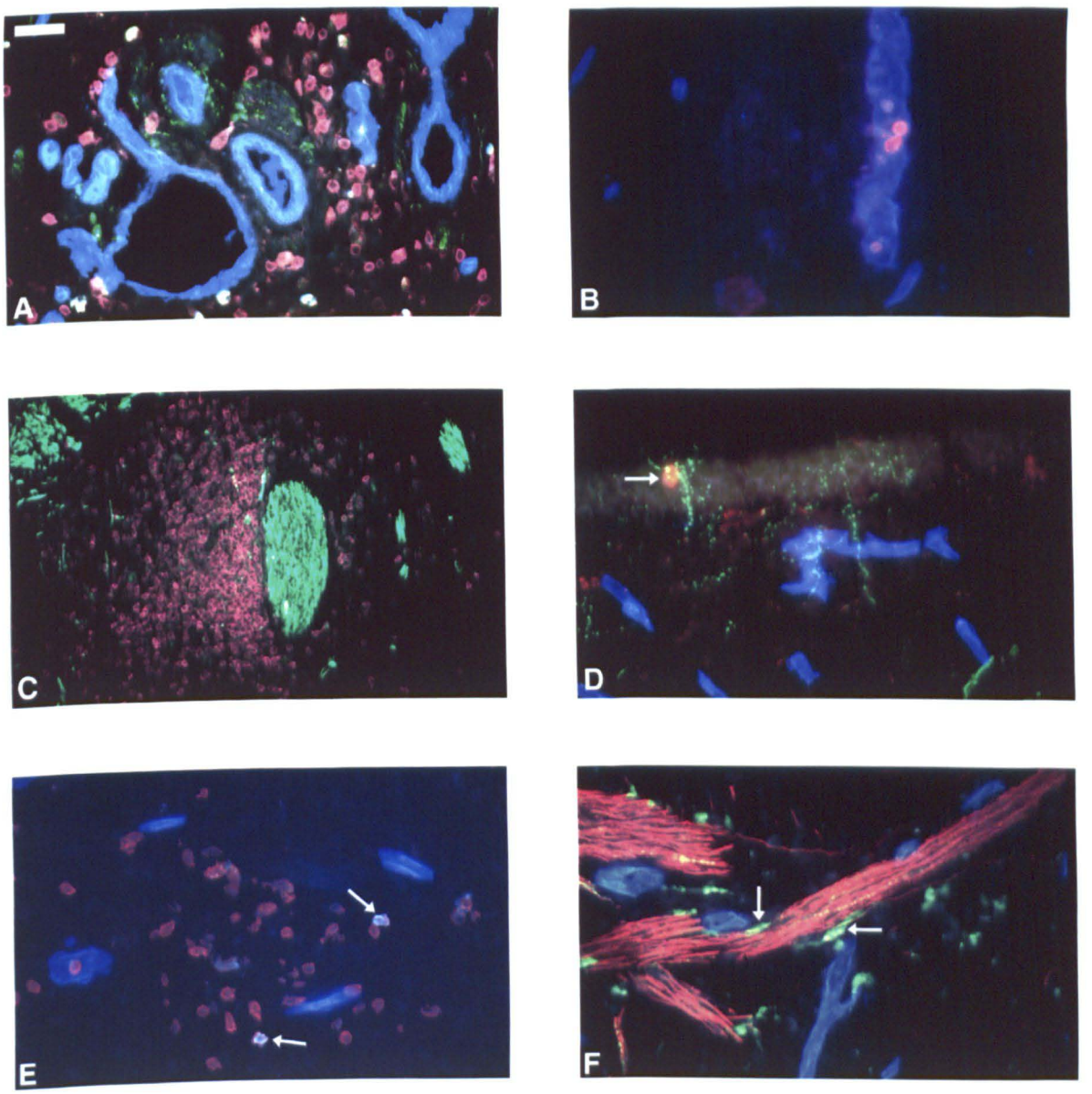


Figure 7.2. Photomicrographs demonstrating the anatomical relationships between LCA-labelled cells (red) and neural and vascular elements in the coronal tooth pulp. **(A)** Triple-labelling for SP (green), UEIL (blue) and LCA in the pulp horn region of a carious primary molar showing an extravascular accumulation of LCA-ir cells. **(B)** Double-labelling for LCA and UEIL to demonstrate the presence of LCA-ir cells within a lymphatic vessel. **(C)** Double-labelling for PGP 9.5 (green) and LCA showing the accumulation of LCA-ir cells alongside a nerve trunk in the mid-coronal region of a carious permanent tooth. **(D)** Triple-labelling for CGRP (green), UEIL and LCA in the subodontoblastic region showing the close contact between a CGRP-ir nerve fibre and a round LCA-labelled cell (arrow) in the odontoblast layer. **(E)** Triple-labelling for VIP, UEIL and LCA to show the presence of VIP immunoreactivity (white) within LCA-labelled cells (arrows). **(F)** Triple-labelling for PGP 9.5 (red), NPY (green) and UEIL to demonstrate the presence of irregular NPY-ir cells (arrows) alongside a nerve trunk. Scale bar = 15μm **(A,B,D,E,F)**, 30 μm **(C)**.

- Leucocyte relationships with neural elements

Some specific anatomical relationships were observed between PGP 9.5- or neuropeptide-ir structures and LCA-ir cells. Firstly, in carious samples showing an increased number of leucocytes, these cells were sometimes localised along a nerve trunk (Fig. 7.2C). Very occasionally, small beaded PGP 9.5-ir or peptidergic nerve fibres were seen to pass in close proximity to round LCA-ir cells and sometimes the nerve fibres appeared to give off a small branch towards these cells (Fig. 7.2D). However, in areas of dense neural branching and leucocyte infiltration it was not possible to reliably identify such close spatial relationships.

A notable finding was that a small proportion of irregular LCA-ir cells within the mid-coronal pulp region were also labelled for VIP (Fig. 7.2E). This immunostaining appeared as multiple small fluorescent dots throughout the cell body but this specific labelling was not seen for PGP 9.5 or the other neuropeptides. In addition, labelling for NPY was observed in what appeared to be some elongated cells which were usually aligned along nerve trunks (Fig. 7.2F).

Another interesting anatomical association between neural tissue and leucocytes was observed in areas of dense leucocyte accumulations. In these regions, PGP 9.5-, CGRP- , SP- and VIP-ir nerve fibres were seen to form "barriers" between the LCA-ir cells and the relatively normal pulp tissue (Fig. 7.3A-C). Although prolific neural branching and LCA-ir cell accumulations were thus seen in close proximity, there appeared to be little migration of leucocytes into the areas of neural tissue (Fig. 7.3D). Furthermore, marked increases in PGP 9.5-ir fibres or neuropeptide expression within the pulp horn region were not always coincident with a local leucocyte accumulation, as was particularly the case for CGRP and VIP (Fig. 7.3E). A further observation was that nerve trunks passing through areas of dense leucocyte accumulation still demonstrated positive labelling for PGP 9.5 and neuropeptides (Fig. 7.3F).

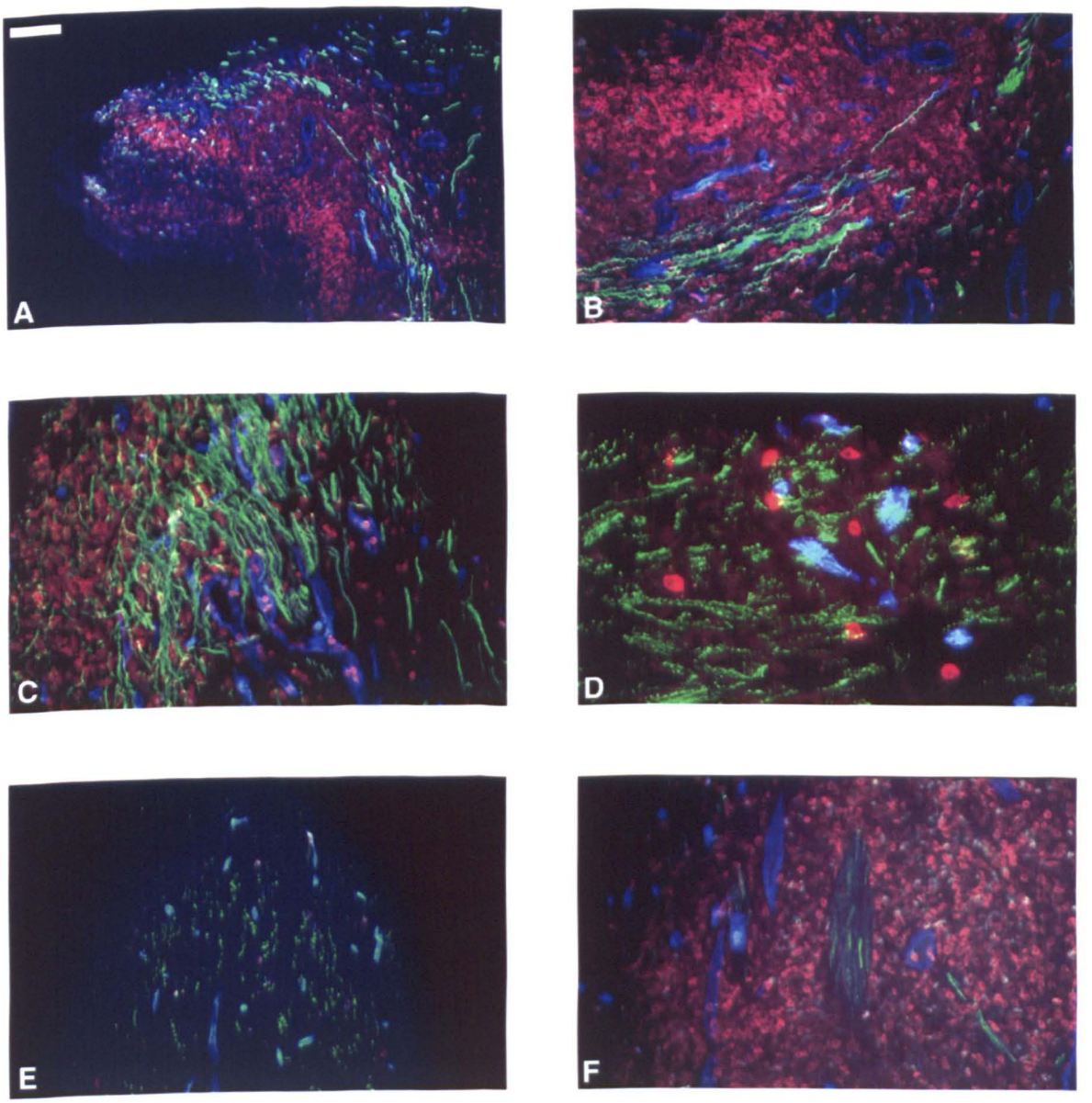


Figure 7.3. Photomicrographs demonstrating the anatomical relationships between LCA-labelled cells (red) and neural and vascular elements in the carious tooth pulp. **(A)** Low and **(B)** high magnification views showing triple-labelling for PGP 9.5 (green), UEIL (blue) and LCA in the pulp horn region of a carious permanent molar to demonstrate the presence of a "neural barrier" between areas of dense LCA-ir cell accumulation and relatively normal pulp tissue. **(C)** Triple-labelling for PGP 9.5 (green), UEIL (blue) and LCA in the pulp horn region of a carious primary molar showing the presence of dense PGP-ir fibres between an area of LCA-ir cell accumulation and relatively normal pulp tissue. **(D)** Triple-labelling for CGRP (green), UEIL and LCA in the subodontoblastic region showing relatively few LCA-labelled cells within the region of CGRP-ir tissue. **(E)** Triple-labelling for VIP (green), UEIL and LCA showing an increase in VIP-ir fibres in the pulp horn of a carious primary molar but relatively few LCA-labelled cells. **(F)** Triple-labelling for SP (green), UEIL and LCA to demonstrate the persistence of SP-ir fibres in a region of dense LCA-ir cell accumulation. Scale bar = 15 μm **(D)**, 30 μm **(B,C,F)**, 60 μm **(A,E)**.

7.3.4 Quantitative analysis

Table 7.1A shows the mean (±SD, range) PAS for LCA-labelled tissue within each dentition according to the field of analysis and the degree of caries and Table 7.1B provides the corresponding statistical data. Bar charts showing plots for pooled data are presented in Figure 7.4.

Key findings arising from the quantitative analysis of pulpal leucocytes are outlined below.

- The greatest mean PAS for LCA within intact teeth was seen in field 2 (primary teeth = 0.55%, permanent teeth = 0.22%). For grossly carious samples the greatest mean PAS for LCA was seen in field 2 for permanent teeth (1.60%) and in field 1 for primary samples (2.37%).

- With the exception of field 3, the mean PAS for LCA was significantly greater in primary teeth than in permanent teeth ($P \leq 0.005$, ANOVA). As an example, mean PAS for LCA in intact primary teeth was approximately twice that recorded for intact permanent teeth. However, further pairwise comparisons (see, Fig. 7.4) did not reveal any significant inter-dentition differences for any specific caries subgroup ($P > 0.05$, Tukey's test).

- Caries was determined to have a highly significant effect on mean PAS for LCA in all four fields of analysis, with an overall increase in immunolabelling with caries progression ($P = 0.001$, ANOVA). Further pairwise comparisons (see, Fig. 7.4) revealed that grossly carious permanent samples demonstrated a significantly greater PAS for LCA than corresponding intact or moderately carious samples in all four fields of analysis ($P < 0.05$, Tukey's test). However, primary teeth only demonstrated significant differences between specific caries subgroups in fields 1 and 2 ($P < 0.05$, Tukey's test).

Table 7.1A. Mean (±SD, range) percentage area of staining for leucocyte common antigen according to tooth type, field of analysis and degree of caries.

Field/tooth(n)	Degree of caries		
	None	Moderate	Gross
Field 1			
Primary	0.40	0.60	2.37
(n=22,20,20)	(±0.68, 0.06-3.21)	(±0.99, 0.02-4.10)	(±3.86, 0.05-15.56)
Permanent	0.17	0.16	1.50
(n=19,22,21)	(±0.42, 0.00-1.99)	(±0.21, 0.00-0.70)	(±2.33, 0.05-8.53)
Field 2			
Primary	0.55	0.90	2.15
(n=22,20,20)	(±1.22, 0.04-5.76)	(±1.45, 0.03-6.10)	(±2.61, 0.03-8.33)
Permanent	0.22	0.25	1.60
(n=19,22,21)	(±0.49, 0.00-2.21)	(±0.56, 0.01-2.50)	(±1.84, 0.02-6.18)
Field 3			
Primary	0.15	0.11	0.35
(n=22,20,20)	(±0.28, 0.01-1.30)	(±0.11, 0.02-0.53)	(±0.70, 0.03-3.21)
Permanent	0.16	0.05	0.69
(n=19,22,21)	(±0.45, 0.00-2.09)	(±0.04, 0.00-0.17)	(±1.84, 0.02-8.34)
Field 4			
Primary	0.27	0.17	0.85
(n=22,20,20)	(±0.33, 0.03-1.35)	(±0.16, 0.04-0.81)	(±1.73, 0.04-7.69)
Permanent	0.12	0.10	0.41
(n=19,22,21)	(±0.17, 0.02-0.80)	(±0.09, 0.01-0.40)	(±0.50, 0.06-1.77)

Table 7.1B. Significance values for effect of dentition type and degree of caries on the PAS for LCA (two-way analysis of variance on log-transformed data)

Main effects	Field 1	Field 2	Field 3	Field 4
Dentition type	*P*=0.001	*P*=0.001	*P*=0.089	*P*=0.005
Degree of caries	*P*=0.001	*P*=0.001	*P*=0.001	*P*=0.001

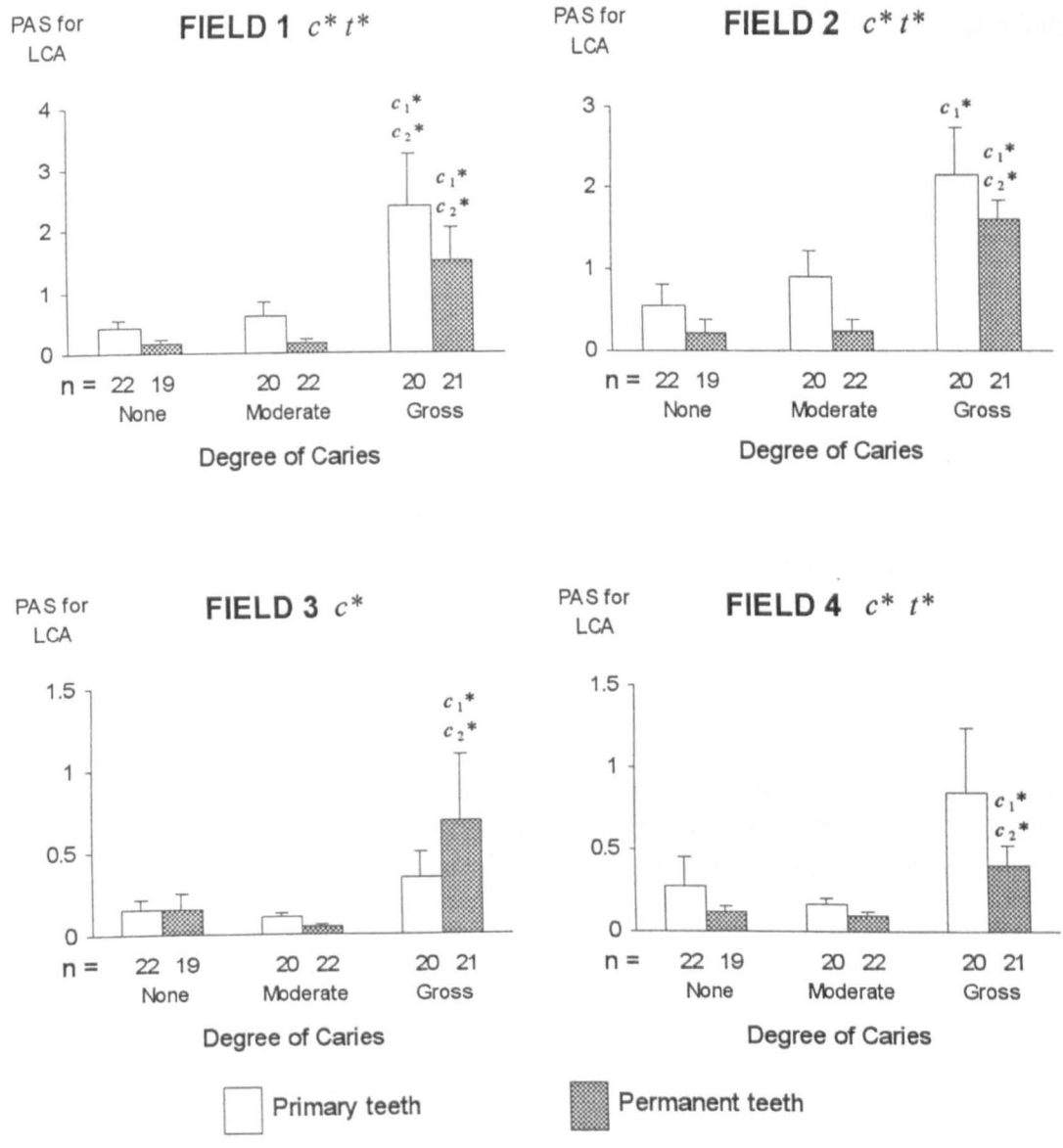


Figure 7.4. Bar charts showing mean (±SEM) percentage area of staining (PAS) for leucocyte common antigen (LCA) labelled tissue in primary and permanent teeth according to the degree of caries. Field 1=tip of pulp horn; Field 2=region subjacent to field 1; Field 3=buccal subodontoblastic plexus; Field 4=mid-coronal region.

Main titles: c^* = significant difference in mean PAS for LCA according to the degree of caries; t^* = significant difference between primary and permanent teeth ($P<0.05$, ANOVA, log transformed data).

Bar titles: c_1^* = significantly different from intact samples of the same dentition; c_2^* = significantly different from moderately carious samples of the same dentition; t^* = significantly different from primary teeth with same degree of caries ($P<0.05$, Tukey's test).

7.3.5 Correlation between neural factors and leucocytes

Scatter plots showing the relationship between the PAS of PGP 9.5 or neuropeptide and LCA within field 1 are seen below in Figures 7.5 -7.8, and the corresponding Pearson's correlation coefficients are given in Table 7.2.

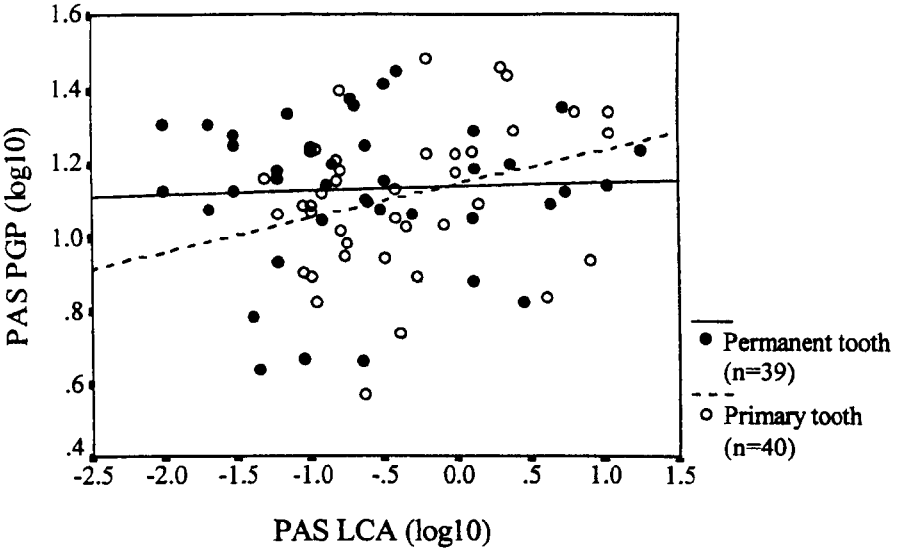


Figure 7.5. Scatter plot with regression lines to show the relationship between the percentage area of staining for protein gene product 9.5 (PAS PGP 9.5) and leucocyte common antigen (LCA) in the pulp horn of carious primary and permanent teeth.

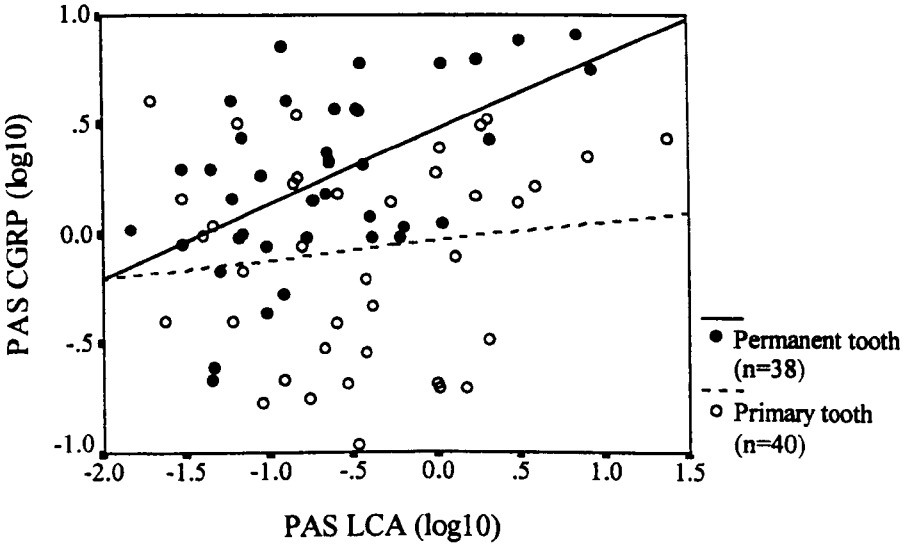


Figure 7.6. Scatter plot with regression lines to show the relationship between the percentage area of staining for calcitonin gene-related peptide (PAS CGRP) and leucocyte common antigen (LCA) in the pulp horn of carious primary and permanent teeth.

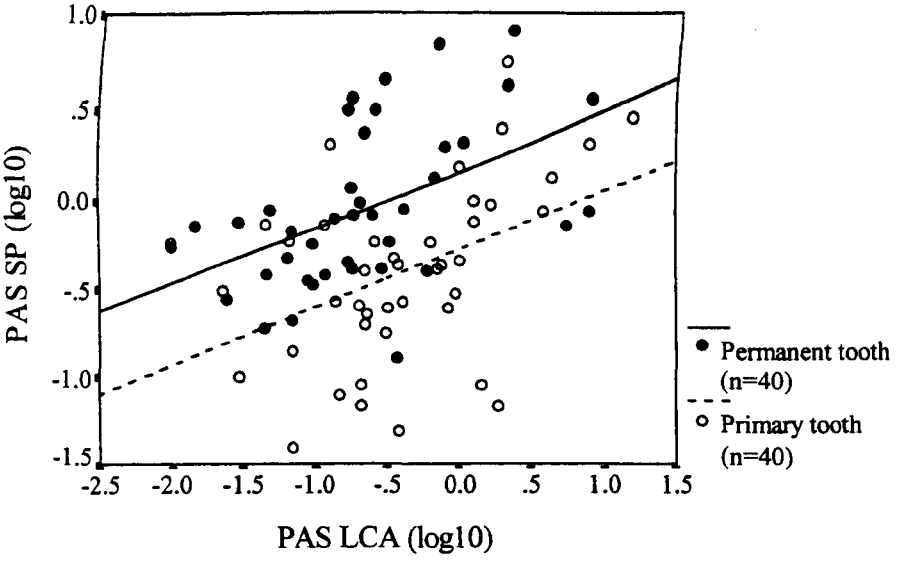


Figure 7.7. Scatter plot with regression lines to show the relationship between the percentage area of staining for substance P (PAS SP) and leucocyte common antigen (LCA) in the pulp horn of carious primary and permanent teeth.

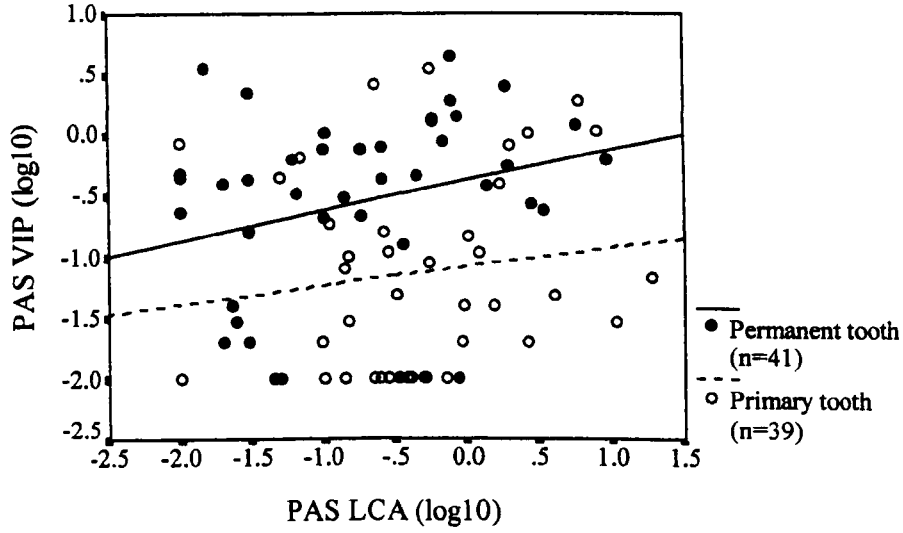


Figure 7.8. Scatter plot with regression lines to the show relationship between the percentage area of staining for vasoactive intestinal polypeptide (PAS VIP) and leucocyte common antigen (LCA) in the pulp horn of carious primary and permanent teeth.

Table 7.2. Pearson correlation coefficients for the association between the percentage area of staining for PGP 9.5, CGRP, SP or VIP and the PAS for LCA in the pulp horn (field 1) of carious primary and permanent teeth.

	Correlation coefficient (*P*)			
Dentition	PGP 9.5/LCA	CGRP/LCA	SP/LCA	VIP/LCA
Primary (n=40,40,40,39)	0.30 (0.053)	0.13 (0.408)	0.45 (0.003)	0.16 (0.322)
Permanent (n=39,38,40,41)	0.05 (0.772)	0.56 (0.001)	0.49 (0.001)	0.26 (0.093)

It can be seen from the above scatter plot for PGP 9.5 and LCA (Fig. 7.5), that the data for permanent teeth demonstrated a considerable spread with little evidence of a linear association. This visual impression was confirmed by the low Pearson coefficient determined for the correlation between the PAS for PGP 9.5 and LCA (r=0.05, *P*=0.772). In contrast, primary teeth did appear to show some evidence for a local correlation between increased neural density and leucocyte accumulation (r=0.30, *P*=0.053).

Interesting results were obtained for CGRP (Fig. 7.6), as permanent teeth showed a significant positive association between increased CGRP expression and leucocyte accumulation (r=0.56, *P*=0.001), but this correlation was not apparent for primary teeth (r=0.13, *P*=0.408). However, both dentitions showed a significant correlation between increased SP expression and an increase in leucocyte accumulation (r=0.45, *P*=0.003, primary teeth; r=0.49, *P*=0.001, permanent teeth) (Fig. 7.7). Finally, as can be seen from Figure 7.8, data for VIP and LCA demonstrated a wide spread and neither dentition showed a significant correlation between these two variables (r=0.16, *P*=0.322, primary teeth; r=0.26, *P*=0.093, permanent teeth).

7.3.6 Prevalence of leucocytes in relation to pain history

The mean (±SD, range) PAS for LCA within grossly carious teeth according to the reported pain history is presented in Table 7.3. It can be seen that, for both dentitions, there was no significant difference in overall leucocyte accumulation within any field

between samples that were reportedly painful or asymptomatic ($P>0.05$, independent sample *t*-test on log-transformed data).

Table 7.3. Mean (±SD, range) percentage area of staining for leucocyte common antigen according to the reported pain history

Field/tooth	Pain history		
	Asymptomatic[a]	Painful[b]	*P*
Field 1			
Primary	1.92 (±2.42, 0.10-7.46)	2.73 (±4.82, 0.05-15.56)	0.950
Permanent	1.40 (±2.78, 0.05-8.53)	1.63 (±1.80, 0.10-5.87)	0.187
Field 2			
Primary	2.51 (±2.91, 0.06-8.33)	1.85 (±2.45, 0.03-7.66)	0.838
Permanent	1.32 (±1.64, 0.02-4.50)	1.94 (±2.10, 0.07-6.18)	0.467
Field 3			
Primary	0.26 (±0.31, 0.03-0.88)	0.20 (±0.21, 0.04-0.76)	0.364
Permanent	1.79 (±0.82, 0.42-3.24)	1.21 (±2.72, 0.02-8.34)	0.507
Field 4			
Primary	1.38 (±2.45, 0.09-7.69)	0.41 (±0.62, 0.04-1.84)	0.121
Permanent	0.31 (±0.51, 0.06-1.77)	0.52 (±0.48, 0.13-1.47)	0.078

P=independent sample *t*-test on log-transformed data
[a] n=10,11 (primary teeth, permanent teeth); [b] n=10,10 (primary teeth, permanent teeth)

7.4 Discussion

7.4.1 Experimental approach

The presence and distribution of inflammatory cells are important parameters in the histopathological assessment of pulpal inflammation (Browne *et al.*, 1980; Watts and Paterson, 1981). The present study sought to identify the total leucocyte population within intact and carious tooth pulps by employing antiserum to the general leucocyte marker, LCA. Immunolabelling for this antigen permitted good visualisation of pulpal leucocytes and revealed the presence of two different subpopulations, distinguishable by their distinct morphologies.

The predominant cell type was a small round cell which demonstrated morphological characteristics consistent with those described for T lymphocytes (Stevens and Lowe, 1997). The morphology, prevalence and distribution of the irregular-shaped cells

would seem to be comparable with those ascribed to macrophages (Stevens and Lowe, 1997). Using immunocytochemical labelling, pulpal macrophages have been described as being larger cells and having short processes extending from their cell body (Jontell *et al.*, 1987). They may be found along nerve trunks, in the vicinity of the odontoblasts or within the mid-coronal pulp stroma (Fristad *et al.*, 1995a).

An obvious limitation of the present study was that the use of a general leucocyte marker precluded the identification of specific leucocyte subsets such as T helper or cytotoxic cells, B lymphocytes and neutrophils. The data from the present study provide a basis for inter-dentition differences in the overall prevalence of leucocytes within the dental pulp, but considerable scope exists for further investigation of differences in the actual composition of inflammatory cell infiltrates within the healthy and compromised tooth pulp.

7.4.2 Inter-dentition differences in relation to pulpal leucocytes

The present study has shown that, with the exception of the subodontoblastic region, leucocyte accumulation was greater in the primary tooth pulp than in the permanent tooth pulp. However, multiple pairwise comparisons failed to identify a statistically significant difference between the two dentitions within any of the different caries subgroups. This result may be explained by the conservative significance levels imposed by tests involving multiple comparisons of means in order to correct for type I errors (see, section 8.1.5). Nonetheless the study established, using analysis of variance, that the overall PAS for LCA was significantly greater in primary teeth than permanent teeth. This finding may be attributed to a number of biological factors. Firstly, previous studies have reported the presence of numerous chronic inflammatory cells within the pulps of primary teeth which are undergoing physiological resorption (Hobson, 1970; Kannari *et al.*, 1998). Although, there was no macroscopic evidence of root resorption affecting the present sample, it is possible that subtle biochemical changes were already occurring. In addition, the calcified tissues of primary teeth be less resistant to the influx of antigenic substances as compared to their successors. Thus, early immune cell reactions may take place in the primary tooth pulp even in the absence of caries.

It is therefore feasible that an inflammatory cell response may be more rapidly mounted in the primary tooth than in its successor, by virtue of the greater number of resident leucocytes present in primary tooth pulps. However, anatomical observations made by this study did not support the theory that primary teeth are unable to localise inflammatory reactions. Carious samples from both dentitions demonstrated evidence of localised inflammatory cell infiltrates which were predominantly restricted to the pulp horn regions.

7.4.3 Caries-induced changes in pulpal leucocytes

Both dentitions showed an overall significant increase in pulpal leucocytes with the progression of caries. This increase was most marked in grossly carious samples. Additional statistical analysis revealed that the PAS for LCA in moderately carious samples was not significantly different to that found in intact samples. Immune cell proliferation and migration are fundamental to the body's defence mechanisms and have been well described in the tooth pulp following caries.

Izumi and colleagues investigated inflammatory cell changes according to the depth of the carious lesion in human permanent molars (Izumi *et al.*, 1995). They reported that a few T lymphocytes were present in the pulp core of intact teeth but there were significant increases in the number of both T helper and T suppressor cells with caries progression. There were no B cells, neutrophils or macrophages in intact or minimally carious teeth but there was a significant increase of these cells in moderately and grossly carious samples. The investigators suggested that the increase in T lymphocytes, at an early stage of caries, provided evidence that cell-mediated immunity took place prior to humoral immunity within the injured tooth pulp. Other studies, also in human teeth, have identified significant increases in numbers of antigen-presenting cells with caries progression (Izumi *et al.*, 1996; Sakurai *et al.*, 1999). Thus the overall findings of the present study would appear to be consistent with previous data.

7.4.4 Findings in relation to pain history

The finding that there was no significant difference in leucocyte accumulation between asymptomatic and reportedly painful grossly carious samples was not unexpected. A

number of previous studies have also failed to establish any correlation between patient symptoms and the histopathological status of the pulp (Baume, 1970a; Tyldesley and Mumford, 1970; Dummer *et al.*, 1980). Indeed, extensive pulpal invasion by inflammatory cells has frequently been seen in the absence of any symptoms in both primary and permanent teeth (McDonald, 1956; Langeland, 1987).

7.4.5 Neural and leucocyte interactions

Anatomical observations made by this study appear to support the existence of close neural and leucocyte relationships within the dental pulp. The most notable being the presence of neural "barriers" between regions of dense leucocyte accumulation and tissue that was relatively free of LCA-ir cells. A similar spatial relationship has been identified between CGRP-ir fibres and "zones of inflammation" in experimental models of rat pulp inflammation (Kimberly and Byers, 1988; Byers *et al.*, 1990b; Swift and Byers, 1992).

There may be several regulatory mechanisms underlying this phenomenon. Byers and her colleagues speculated that proliferating fibroblasts, contained within the outer inflammatory zone, were able to exert stimulatory effects on neural tissue via the release of various regulatory factors (Kimberly and Byers, 1988). In turn, the release of peptides such as CGRP was purported to stimulate reparative dentine formation in this region. In addition, other investigators have reported a close anatomical relationship between SP-ir fibres and immune cells within inflamed human gingival tissue (Bartold *et al.*, 1994). This association led the investigators to propose that SP was responsible for inducing such leucocyte accumulations. A further possible functional interaction between immune cells and intradental nerves may involve NGF (Sakurai *et al.*, 1999). It has been recently shown that activated lymphocytes can synthesise and release biologically active NGF, thus these cells may play an important role in stimulating axonal growth responses during inflammation (for review see, Aloe *et al.*, 1999).

Interpretation of the actual correlation data is somewhat more difficult. Firstly, it would seem that there was little association between changes in overall nerve density

and leucocyte accumulation, particularly in permanent teeth. However, there was a significant correlation between increased SP expression and leucocyte accumulation within both dentitions. Nonetheless, one can only speculate as to whether this association actually represented a true functional interaction. It is not known whether an increased peptide secretion exerted a chemoattractant effect on the leucocytes, or conversely, whether the leucocytes themselves released factors which induced an upregulation of peptide expression. Furthermore, it is possible that changes in peptide expression or leucocyte accumulation occurred independently of each other and were each stimulated by other external factors. Interestingly, NGF has been found to have significant biological effects on both immune cell proliferation (Bracci-Laudiero *et al.*, 1996; Schuligoi, 1998) and peptide expression (see section 5.4.5) and thus may play a key role in the overall cycle of events.

Nonetheless, previous studies have established that SP acts as a chemoattractant for mononuclear and polymorphonuclear leucocytes and is able to stimulate human T lymphocyte proliferation via receptor-mediated mechanisms (McGillis *et al.*, 1987; Payan and Goetzl, 1987b). In addition, SP has been shown to inhibit inflammatory cell aptosis thus prolonging the survival and viability of monocytes and macrophages (Azuma *et al.*, 1996). It would therefore seem reasonable to assume that an increase in the expression (and secretion) of SP may have been involved in the accumulation of leucocytes seen within the carious tooth pulp.

Conflicting results were obtained for CGRP as there was a significant correlation between the neural content of this peptide and leucocyte accumulation in the permanent tooth but not in the primary tooth. Firstly, previous data would suggest that CGRP has an inhibitory effect on (murine) T lymphocyte proliferation (Poyner, 1992). Specific evidence for the anti-inflammatory effect of CGRP in dental tissue was deduced following the culture of rat pulpal cells and T lymphocytes. In this system the addition of CGRP had no effect on T cell proliferation whereas SP was shown to have a significant stimulatory effect (Jontell *et al.*, 1996; Okiji *et al.*, 1997). It is possible that the pro-inflammatory effects of SP are greater than the anti-inflammatory effects of CGRP thus overriding the anti-inflammatory effects of CGRP.

However, there is evidence to suggest that CGRP has a chemoattractant effect on both neutrophils (Smith *et al.*, 1993) and some macrophages (Toriya *et al.*, 1997). Thus the actual composition of the inflammatory cell infiltrate is likely to have a fundamental effect on findings for potential neural and immune cell interactions.

Inter-dentition differences in CGRP/LCA associations may be accounted for by two possibilities: either the composition of the immune cell infiltrate differed such that the majority of cells lacked the necessary CGRP receptors to enable a functional interaction or, the lower CGRP content in primary teeth as compared to permanent teeth was insufficient to exert any biological effects.

Subjective anatomical observations seemed to suggest that there was an inverse relationship between VIP expression and LCA accumulation. However, this impression was not supported by the quantitative data which showed an overall (although non significant) positive correlation between the PAS for VIP and LCA. This finding would, at first sight, appear to conflict with those of other studies where VIP has been shown to exert an inhibitory effect on leucocyte chemotaxis and proliferation (Stanisz *et al.*, 1986). However, it is evident that subpopulations of lymphocytes demonstrate major differences in their VIP recognition capabilities and considerable heterogeneity exists even within given subtypes (Ottaway, 1988). Furthermore, VIP has actually been shown to evoke neutrophil accumulation in human skin (Smith *et al.*, 1993). Future studies should therefore be aimed at exploring correlations between VIP expression and the influx of specific leucocyte populations to gain a clearer insight into the functional interactions of these two systems.

Although these data would seem to suggest that a significant association exists between an increased neural content of SP or CGRP (in permanent teeth) and leucocyte accumulation, caution should be exercised in the assumption of a causal relationship. Furthermore, as outlined previously (see, section 6.4.5), the association between an increased neural peptide content and the amount of biologically-available peptide within the target tissue can not be determined from the present study. Also, as mentioned in the preceding chapter, a limitation of the experimental design was that

possible interactions of each peptide could not be explored independently of other peptide effects. Thus the overriding pro-inflammatory effects of SP, could for example, have masked any anti-inflammatory effects of VIP. Controlled animal experiments involving the use of knockout strains (De Felipe *et al.*, 1998) or selective peptide receptor antagonists would therefore be necessary to investigate individual peptide effects on pulpal leucocytes.

An observation that also deserves further comment was the presence of positive immunostaining for both VIP and NPY in a small proportion of pulpal leucocytes with a macrophage-like appearance. Staining was believed to be specific for both peptides as it was not seen in preabsorption control sections. Admittedly, better visualisation of peptide immunoreactivity within leucocytes would have been gained from confocal microscopy or immunoelectron microscopy. However, the production of NPY and VIP by some immune cell populations has been previously reported and serves to corroborate this novel finding in the tooth pulp (Bracci-Laudiero *et al.*, 1996; Delgado *et al.*, 1999). It is also possible that the labelling seen may have represented neuronally-released peptide that had bound to the immune cell. Certainly there is evidence to support the presence of functional VIP receptors on the cell surface of macrophages (Segura *et al.*, 1991). Interestingly, Kimberley and Byers (1988) demonstrated specific labelling for CGRP within macrophage-like cells in the rat pulp, but this was not seen in the present study.

7.4.6 Clinical significance of findings

It was hoped that this study would elucidate the effects of neuropeptides on leucocyte accumulation within the dental pulp thus indicating possible strategies for immune cell regulation. The presence of an overwhelming inflammatory infiltrate is likely to be detrimental to pulpal survival and healing and the suppression of inflammatory cell influx may prove a beneficial therapeutic regimen (Shovelton, 1972). Certainly, there is some expectation that neuropeptide analogues may offer novel approaches for the management of a number of disorders with an immunological aetiology such as asthma, arthritis, allergic rhinitis, septic shock syndrome and certain dermatological conditions (Foreman, 1987; Eedy *et al.*, 1991; Delgado *et al.*, 1999).

Although the results from the present study are obviously only very preliminary, it would appear that SP may play a role in leucocyte accumulation within the dental pulp of both dentitions whereas CGRP may influence leucocyte influx only within the permanent tooth pulp. However, further research is indicated to more fully understand the pro-inflammatory roles of these peptides and their sites of action, before therapeutic interventions can be considered.

Fundamental differences in the clinical management of carious primary and permanent teeth do not appear to be supported by the present study. It was evident that both dentitions are able to localise inflammatory cell reactions. However, it would appear that inflammatory cell infiltrates may be greater in the primary coronal pulp thus efforts should be made to instigate treatment at the earliest possible stage. Furthermore, anti-inflammatory treatment modalities may prove particularly beneficial in the management of the compromised primary tooth pulp.

Finally, the identification of positive labelling for PGP 9.5 and all four neuropeptides in areas of extensive inflammatory infiltrate may also have some clinical relevance. It is conceivable that dental instrumentation could elicit painful responses in teeth that might have been clinically diagnosed as non-vital. The relative resistance of pulpal nerves (C-fibres in particular) to adverse conditions has also been reported by previous investigators who identified intact pulpal nerves even in the presence of overwhelming inflammation and periapical infection (Torneck, 1977; Khayat *et al.*, 1988; Mendoza *et al.*, 1987).

7.4.7 Summary of findings

In response to the original objectives (see, section 7.1.7), this investigation has shown that:

- Leucocytes are significantly more abundant within the primary tooth pulp than in the permanent tooth pulp, except in the region of the cervical subodontoblastic nerve plexus, and there is a significant increase in leucocyte accumulation within grossly carious samples of both dentitions.

- In areas of dense leucocyte accumulation, pulpal nerves often show a marked aborisation and may form neural "barriers" between areas of gross inflammatory cell infiltrates and relatively normal pulpal tissue.

- There appears to be a positive correlation between an increase in SP expression and increased leucocyte accumulation within carious primary and permanent teeth and a similar association exists for CGRP in permanent teeth.

- There is no significant difference in the prevalence of leucocytes within asymptomatic and reportedly painful grossly carious samples from either dentition.

8. General Discussion

8.1 Experimental approach

Although some consideration has already been given to the methodology employed in the present study (see, sections 4.4.1, 5.4.1, 6.4.1, 7.4.1), a number of general points deserve further discussion. These relate to the assessment of pain and caries, image processing, method reproducibility and statistical analysis.

8.1.1 Dental pain assessment

A young child's understanding of the meaning of pain is dependant on a host of developmental and environmental factors (Gaffney and Dunne, 1986). Accurate pain assessment in children thus presents a considerable clinical challenge and a variety of behavioural, physiological and psychological approaches have been developed for this purpose (for review see, McGrath, 1987). Little work has been undertaken in the area of paediatric dental pain assessment although a few studies have reported the use of visual analogue scales (Jones and Blinkhorn, 1996), picture scales such as faces varying in emotional expression (Fung *et al.*, 1993) and verbal descriptors (Mason *et al.*, 1997).

In view of the recognised problems relating to validity, sensitivity and reproducibility when conducting pain assessments in children, this study sought only to elicit a simple positive or negative history of past dental pain experience. This relied on self-reporting for older children and parental reporting for young subjects. A history of disturbed sleep, disturbed eating or being sent home from school or nursery with suspected toothache were all considered positive indicators of a painful pulpitis (Trowbridge, 1986; Närhi *et al.*, 1992b; Figdor, 1994).

No attempt was made to further elucidate the nature of the dental pain or the presence of any exacerbating factors. Furthermore, sensitivity testing with hot or cold stimuli was not undertaken as discrimination of pain is difficult for children and the validity of any positive responses could be questionable in such a young age group.

8.1.2 Caries assessment

A principal objective of the present study was to evaluate the effect of caries progression on a number of biological variables within the dental pulp. Samples were simply categorised as intact, moderately carious or grossly carious on the basis of colour changes within the hard tissues. This approach to caries assessment has been widely used in previous studies (Izumi *et al.*, 1995; Kobayashi *et al.*, 1996; Kamal *et al.*, 1997; Yoshiba *et al.*, 1998; Sakurai *et al.*, 1999) and also proved to be highly reproducible (see, section 3.5.1).

Other techniques have been employed to assess caries depth including the use of dyes, stereomicroscopy, microradiography, fluorescence and histological assessment (van de Rijke, 1991; Hietala *et al.*, 1993; Ekstrand *et al.*, 1995; Hintze *et al.*, 1995). However, each of these methods has its own limitations and were not considered advantageous in any way.

Despite the ease and reliability of the adopted method for caries assessment there were, however, a number of accepted limitations. Firstly, as there were only three categories of caries, each subgroup encompassed considerable inter-sample variation in actual caries depth. Furthermore, since the average enamel and dentine thickness in primary teeth is less than half that found in permanent teeth (Rayner and Southam, 1979), a "moderately carious" primary tooth was likely to have a smaller distance between the carious lesion and the pulp tissue than that seen in a "moderately carious" permanent tooth. There is evidence to suggest that the amount of residual dentine or remaining dentine thickness between the injurious agent and the pulp tissue is an important factor in predicting pulpal status (Sayegh and Reed, 1974; Stanley *et al.*, 1975; Hamid and Hume, 1997). Therefore, in future studies it may prove informative to explore the correlation between this continuous variable and the associated pulpal response.

Finally, the present study did not evaluate any other characteristics of the carious lesion apart from depth. Caries is known to demonstrate considerable variability in terms of activity and pathogenicity which may greatly influence the nature of the

pulpal response (Massler, 1967). Host-related factors such as age, tooth composition, dietary intake, salivary flow and buffering capacity may also modify the degree of pulpal inflammation following caries (Kim and Trowbridge, 1994). It is therefore likely that a number of factors, other than caries depth per se, may have contributed to the wide range of biological changes observed between different samples. It may have been possible to limit the effects of these modifying factors by comparing a series of paired tooth samples taken from the same individuals. However, to establish such a specific tissue archive would have undoubtedly taken an unrealistic length of time.

8.1.3 Image analysis

Computer-assisted image analysis has found increasing application in quantitative histology. It has enabled greater objectivity, reliability, accuracy and efficiency than was possible with previous manual counting techniques or semi-quantitative assessment (Jagoe *et al.*, 1991). Neuroscience-based research has particularly benefited from this approach due to the complexity of the material often under investigation. (Cowen *et al.*, 1982; Springall *et al.*, 1995; Sapp *et al.*, 1995).

Numerous approaches have been adopted to quantify neuropeptide labelling, the most widely used technique involves an assessment of the total area of fluorescence staining (Ma and Bisby, 1998; Polgár *et al.*, 1998; Long *et al.*, 1998). This measurement is often expressed as a percentage of the total area subject to analysis. In addition, investigators have employed methods based on intercept density, intensity of staining and measurement of immunofluorescent varicosities (Cowen *et al.*, 1982; Cowen, 1984; Amenta *et al.*, 1987; Robinson *et al.*, 1994). Previous immunocytochemical studies of neuropeptide expression within the dental pulp have, in the main, been descriptive or semi-quantitative and it is only relatively recently that digital image analysis has been employed (Swift and Byers, 1992; Hong *et al.*, 1993; Toriya *et al.*, 1997; Sakurai *et al.*, 1999). The present study quantified the total area of specific fluorescent staining as this parameter was appropriate to all structures under investigation and thus enabled meaningful comparison between the different biological data.

Quantitative image analysis is known to be affected by a number of factors (Willemse *et al.*, 1993), the most critical of which being the quality of the immunostaining itself (Sallinen *et al.*, 1994; Mosedale *et al.*, 1996). A major problem associated with quantitative immunofluorescence is label fading following exposure to ultraviolet light and prolonged storage. However, there is unlikely to be a significant reduction in the overall area of fluorescent labelling with up to 3-weeks storage and 20 minutes of UV light exposure (Cowen, 1984). Thus the present study analysed each batch of labelled slides within a three to four-week period and photography was only undertaken after quantitative analysis had been completed.

The use of interactive thresholding is considered the weak link in a chain of otherwise objective methodology and remains an unresolved problem. Although fixed thresholds may be appropriate for some immunohistochemical techniques, they are generally not considered appropriate for neural immunocytochemistry (Springall *et al.*, 1995). Reproducibility therefore relies greatly on the operator's skill and consistency in setting the grey values which in turn determine the overall PAS. Surprisingly, few investigators appear to have investigated this potential source of method error (Rooryck and Klinge, 1995; Fristad *et al.*, 1995a). The repeatability of PAS measurements was, however, considered fundamental to the overall reproducibility of the present methodology and is discussed in the next section.

8.1.4 Method reproducibility

The analysis and interpretation of repeatability data is an area of some controversy (Bland and Altman, 1986; 1999). Caution should be exercised when employing correlation coefficients to assess the repeatability of taking replicate measurements as a high coefficient value is often interpreted as signifying a good agreement when it is in fact a measure of the strength of a linear association (Altman, 1991). This feature is well-illustrated by looking at the data for repeat blood vessels counts in field four (see, Table 3.11). A correlation coefficient of 1.00 was obtained, which may be interpreted by some as signifying a total agreement between the two counts, yet the two counts were actually found to differ by a mean of 2.04%. Nonetheless, correlation coefficients are frequently employed to determine method reproducibility

for image analysis-based studies (Sallinen *et al.*, 1994; Willemse *et al.*, 1994) and for the sake of simplicity, were also employed by the present study. However, all data were first plotted so that visual assessments could be made and data interpretation was not solely reliant on a correlation coefficient. Furthermore, to allow more meaningful comparisons, a mean percentage difference between replicate measures was determined for each staining regime. A comparison of the means of the repeat measurements using a paired-sample *t*-test was not undertaken as this is considered an entirely erroneous approach (Altman, 1991).

Unfortunately, it was not feasible to consider the reproducibility of field selection and PAS measurements individually thus reproducibility data related to the combined effects of these two factors. Furthermore, PAS measurements could have been affected by both the variable integration period over which the signal was obtained, and by the subjective thresholding. However, by looking at the repeatability data for blood vessel counts (see, Table 3.10), where thresholding was not a modifying factor, it would appear that the least difference between the replicate counts was seen in field one. These data confirm the investigator's subjective opinion that field one selection was the most reproducible of the four fields. This impression would also seem to be supported by the PAS data (see, Table 3.9) as the smallest mean percentage difference between the two measurements was generally found for field one analysis.

The greatest disparity between replicate measures of PAS was seen for CGRP in fields three and four. This may reflect the greater imprecision in re-selecting these fields as compared to fields one and two. Furthermore, the overall smaller CGRP content in these two regions meant that even small differences between the repeat measures of PAS were expressed as proportionately quite large percentage differences. Nonetheless, the overall method was considered to demonstrate a satisfactory intra-operator reproducibility for field selection and PAS measurement for all parameters under investigation.

8.1.5 Statistical analysis

Statistical advice was sought throughout this study from the University of Sheffield Statistical Services Unit, thus ensuring that appropriate data analysis was undertaken. Initial plots of the data quickly confirmed that they were not Normally distributed thus straight forward logarithmic transformations were undertaken so that parametric statistical tests could then be employed.

Although a two-way analysis of variance was able to determine whether the tooth type or degree of caries had a significant effect on the independent variable under investigation, this form of analysis was not able to identify significant differences between the six individual subgroups. It was therefore necessary to undertake additional pairwise comparisons of means to determine where significant inter-group differences occurred. Multiple significance testing entails a high probability of finding a significant difference just by chance as each test performed carries a 5% risk of a false positive result (a Type I error) and thus is not advisable. A number of alternative methods have therefore been developed to deal with this problem (Altman, 1991).

Tukey's honestly statistically significant test is one such approach and requires that, to achieve significance, any pairwise difference must exceed a critical value which is determined partly by the size of the entire array of the means (Kinnear and Gray, 1997). However, this is considered a rather "conservative" test in that it errs on the side of non-significance. Thus throughout the study, it was disconcerting to find that, although a statistically significant difference between the dentitions or the caries subgroups was identified by a two-way analysis of variance, no pair of means were found to be significantly different at the 5% level of significance following Tukey's test (for example, see Fig. 4.4). This lack of significant differences was also likely to have been compounded by the high inter-specimen variation found within each subgroup.

8.1.6 Null hypotheses

Four null hypotheses were established at the beginning of this study (see, section 2.8.7) and for completeness the acceptance or rejection of each hypothesis, on the basis of the experimental findings, is stated below.

- The null hypothesis that there are no significant differences in overall pulpal innervation, neuropeptide expression, pulpal vascularity or leucocyte distribution between primary and permanent teeth is rejected.
- The null hypothesis that there are no significant changes in any of the above parameters with caries progression is also rejected.
- The null hypothesis that there is no significant correlation between neuropeptide expression and vascularity, vessel number or leucocyte accumulation in carious samples can only be rejected in part as correlations were not found for all parameters under investigation (see, sections 6.3.6 and 7.3.5).
- The null hypothesis that there is no significant difference in pulpal innervation density, neuropeptide expression, pulpal vascularity or leucocyte accumulation between symptomatic and asymptomatic carious teeth is accepted, with the exception of findings for SP and VIP in permanent teeth which serve to reject the null hypothesis.

8.2 Biological differences between primary and permanent teeth

Primary and permanent teeth demonstrate a number of gross anatomical differences. These have been well-described and relate to overall size, crown and root morphology and hard tissue dimensions (Berkovitz *et al.*, 1992). This study has now provided considerable insight into the variation in pulpal biology that exists between the two dentitions. In summary, it has been shown that permanent teeth are significantly more densely innervated than primary teeth and intradental nerve fibres within the pulp horn region express significantly greater amounts of CGRP, SP and VIP. Furthermore, interrelationships between VIP expression and vascular status appear to be different in the two dentitions with neural VIP showing a significant correlation with vascularity

and vessel number in the carious primary dentition but not in the permanent dentition. Finally, primary teeth are more vascular than their successors within the mid-coronal region and they generally contain a greater number of leucocytes throughout the coronal pulp.

On the other hand, there also appear to be some remarkable similarities between the two dentitions, particularly with respect to NPY expression and the nature of the anatomical associations present between intradental nerves and blood vessels. In addition, correlations between SP expression and vascular status or leucocyte accumulation are similar in both primary and permanent teeth. And most importantly, both dentitions demonstrate comparable pulpal responses to caries progression.

The challenge that now presents itself, is to assimilate all these data, and to speculate as to how they might relate to our clinical practice. Firstly, it is possible that the lower neural expression of SP found in the primary tooth pulp may equate to a reduced potential to process nociceptive information. However, this finding alone can not be considered a sound biological basis for not routinely giving local analgesia when performing restorative procedures in the primary dentition.

Secondly, what can we conclude about the primary tooth pulp's purported tendency to undergo rapid and widespread inflammatory responses? It would appear that this tooth is well-equipped to mount a vigorous inflammatory response by virtue of its considerable vascularity and number of resident immune cells, however, it is not known whether these characteristics are actually detrimental to subsequent healing. From the large number of observations made, the primary tooth pulp appears to respond to caries in a similar way to its successor and demonstrated a comparable ability to localise inflammatory changes. Overall, it is considered that poor clinical results following certain endodontic procedures in the primary teeth of young children are more likely to be due to inadequate technique rather than fundamental differences in pulp biology. However, this may not be the case in older children where degenerative neural changes, coincident with physiological root resorption, may have

occurred. In such cases, caries-related morphological and cytochemical neural changes may not be evoked thus compromising the tooth's healing capabilities.

It is conceivable that CGRP expression may, in part, determine the tooth's ability to form reparative dentine following dental injury since this peptide regulates the maintenance of other calcified tissues. Calcitonin gene-related peptide has been shown to have a dose-dependant osteogenic stimulating effect on bone colonies in vitro (Bernard and Shih, 1990) and also acts directly on osteoclasts to inhibit bone resorption (Zaidi *et al.*, 1987). Interestingly, Fristad and colleagues recently demonstrated the presence of CGRP-receptors on some pulpal odontoblasts, giving further credence to the concept of ligand-receptor mediated effects of CGRP on hard tissue formation (Fristad *et al.*, 2000). As primary teeth have been shown to have a significantly lower CGRP content than their successors, it would be interesting to determine whether this has any detrimental effect on reparative dentine formation following dental injury.

Finally, the significant inter-dentition differences found for VIP expression may be of biological importance with respect to the neural degeneration that is known to accompany physiological resorption in primary teeth (see, section 5.4.7). It would be fascinating to examine a range of intact primary teeth at different stages of root resorption and to correlate overall intradental neural density with VIP expression. Thus further insight may be gained into the mechanisms underlying this little-understood process of apparently "programmed" neural degeneration.

8.3 Findings relating to dental pain

There has been much speculation as to why, despite a prolific innervation, many teeth can reportedly remain symptom-free following extensive caries and pathological changes within the pulpal tissue. In her excellent review, Byers (1999) suggests that the tooth pulp is supplied with a number of effective local regulatory mechanisms that work to inhibit peripheral nerve sensitisation. These may include interactions with endogenous opioids, somatostatin, adrenergic sympathetic and nitric oxide systems. It

is also considered that a dense pulpal innervation serves greater importance in mediating local trophic and regulatory functions than in conveying afferent pain sensations.

It was therefore most interesting that the present study showed SP expression to be significantly greater in painful permanent teeth than in asymptomatic samples with a similar degree of caries. Taken in isolation, these data do not provide conclusive evidence for the direct or indirect role of SP in the mediation of pulpal pain. However, there is a wealth of evidence from other experimental systems to support the role of SP in the chemical transmission of nociceptive information, and development of hyperalgesia following peripheral inflammation (Henry *et al.*, 1980; Lembeck *et al.*, 1981; Woolf and Wiesenfeld-Hallin, 1986; Ma and Woolf, 1995; Neumann *et al.*, 1996; Allen *et al.*, 1999; Levine and Reichling, 1999). Thus it would appear likely that an increased SP expression in pulpal inflammation could contribute to dental pain experience.

The present study also found VIP expression to be significantly higher within the pulp horn region of symptomatic carious permanent teeth than in corresponding asymptomatic teeth. The contributory role of VIP to pain experience is unclear although there is some evidence to suggest that VIP may act as a transmitter of abnormal sensations in neuropathic conditions (Dickinson and Fleetwood-Walker, 1999). Overall, it is felt that, in this model, an increased VIP expression is more likely to have functional significance in terms of neurotrophism than in nociception. However, it does seem surprising that, despite a significantly greater VIP expression in the pulp horn region of symptomatic teeth, there was not a comparable increase in neural density within the same samples (see, section 4.3.7). Further research relating to VIP expression in painful pulpitis would therefore seem to be indicated. In the first instance it would be interesting to conduct double-labelling experiments to determine whether SP and VIP are co-expressed within the same fibre population following caries, or whether they are each upregulated in separate fibre subsets. Evidence from studies involving nerve injuries to the IAN would suggest that, although SP and VIP are colocalised in some fibres following injury, there tends to be little co-expression of

these peptides in neurons showing strong SP- or VIP immunoreactivity (Fristad *et al.*, 1998).

8.4 Future research directions

Undoubtedly, the main area of interest to arise from this experimental work, relates to the finding that SP (and VIP) are significantly upregulated in painful pulpitis. It is naturally tempting to hypothesise that inhibition of peptide expression and activity could have an overall analgesic effect. However, a more comprehensive understanding of the likely mechanisms by which these peptides exert their effects is essential before any therapeutic intervention is considered.

Further research efforts should thus be directed towards identifying specific peptide receptor sites within the normal and inflamed human tooth pulp. Preliminary immunocytochemical work undertaken by Fristad and colleagues has already demonstrated the presence of several SP (NK-1, NK-2 and NK3) receptors in rat dental tissues (Fristad *et al.*, 2000). The prevalence, nature and distribution of peptide receptor sites in human pulp, both in health and disease, would help clarify through which systems SP and VIP are likely to mediate their effects. The next major step would then be to determine what functional effects could be evoked in dental tissues following the experimental administration of selected peptide analogues. Pharmacological interventions in other tissues have shown that a reduction in SP expression or activity can markedly attenuate the inflammatory process (Foreman, 1987), although the use of SP antagonists to reduce inflammatory hyperalgesia has been less successful (Cohen, 1990; Fleetwood-Walker, 1995; Iversen, 1998).

However, it should be appreciated that a number of other chemical mediators may also contribute to the development of painful pulpitis (Närhi, 1985; Olgart, 1985; Olgart and Kerezoudis, 1994) and it would be inexpedient to uphold SP as the sole contributory factor. Further research should be particularly directed towards elucidating the role of NGF in painful pulpitis as this substance has known importance in inflammatory pain mechanisms and is likely to play a key role in stimulating an

increase in SP expression (Lindsay *et al.*, 1989; Woolf *et al.*, 1994; Ma and Woolf, 1997; Woolf, 1996; Woolf *et al.*, 1997; Koltzenburg *et al.*, 1999). In addition, complimentary investigations of those intrinsic analgesic systems which work within the dental pulp may also help to direct the development of effective topical analgesic preparations.

Another possible therapeutic application for peptide regulation may relate to the stimulation of dentinogenesis. The ideal treatment strategy for a cariously or traumatically exposed tooth pulp is to maintain the vitality of this tissue, and the formation of a dentine bridge to protect the exposed pulp is critical for tissue survival. However, it would appear that we have made little progress over the years in developing new therapies to encourage tertiary or reparative dentine formation. Currently, the accepted technique for inducing dentinal bridge formation involves the use of calcium hydroxide preparations. These do not specifically initiate dentinogenesis but in fact cause localised areas of tissue necrosis and inflammation which subsequently heal with the formation of new hard tissue (Phaneuf *et al.*, 1968; Tziafas, 1994). It would therefore be of enormous clinical benefit to develop more regenerative types of treatment whereby the pulp's own healing abilities could be enhanced. Further research is therefore indicated to investigate the potential stimulatory effects of CGRP preparations on reparative dentine formation.

Calcitonin gene-related peptide analogues may not only prove useful in stimulating dentine bridge formation but may help to induce apexification of immature roots in non-vital teeth prior to root canal obturation. Another possible clinical application may be the inhibition of problematic inflammatory root resorption that commonly follows trauma. Further research is certainly indicated to explore some of these potentially valuable therapeutic applications.

8.5 Conclusion

In summary, this study has clearly shown that the pulpal response to caries comprises a host of dynamic structural and cytochemical neural changes which appear to have

significant interactions with vascular and immune cell systems. Undoubtedly, human primary and permanent tooth pulps possess a remarkable potential to counteract tissue injury. Surely we should be striving to more fully understand these responses and thereby direct the development of more effective and biologically compatible treatment strategies. It seems reprehensible that we can find ourselves in the 21st century still using pulp fixation as a means of "treating" the cariously exposed primary tooth pulp.

Undoubtedly, the primary treatment objective for any disease should be its prevention. However, little progress has been made in recent years in terms of caries prevention, particularly with respect to instigating public water fluoridation schemes. Dental decay thus looks likely to pose considerable demands on the dental profession for the foreseeable future. The preservation of both the primary and permanent dentition clearly contributes to the overall health and well-being of our society. It is hoped that continued investigation of human pulpal biology, particularly with respect to neuropeptide effects and interactions, may ultimately help us to more effectively manage the pain and loss of function caused by dental decay.

Finally, this study has demonstrated the usefulness of the human dental pulp as model in which to investigate peripheral changes associated with inflammation and to correlate them with a known pain history. The accessibility of this tissue raises exciting possibilities for future investigations into other aspects of the inflammatory process and mechanisms of inflammatory pain

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