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The Morphology and Innervation Characteristics of Spinal Ligaments: Their Involvement in Spinal Stability and Adolescent Idiopathic Scoliosis

By

HONGXING JIANG 

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DEDICATION

This thesis is dedicated to my best friend and wife Yuling Fu, for her commitment and continued support throughout our years. Without her understanding and love this would not have been possible. And, to our son Wen and daughter Jenny from whom I have taken time to complete my thesis. Thank you for your patience.

ABSTRACT

This thesis focusses on the mechanism by which lateral stability of the spine is maintained and the possible relationship of this mechanism to the development of adolescent idiopathic scoliosis (AIS). In the first of a series of projects it was found that the lateral spinal ligaments, particularly the superior costotransverse ligaments (SCTLs), have the major influence on lateral stability. Subsequently it was found that the lateral spinal ligaments are well developed only in bipedal animals and are absent in quadrupeds which further supports the concept that the lateral spinal ligaments are most important for the maintenance of an erect spine. Based on these findings, the chicken was selected as an appropriate animal model when adequate supplies of human material were not available. To establish appropriate methodology for studying nerve elements in ligaments, immunohistochemical techniques were used to confirm that the central spinal ligaments in humans are richly innervated by Ruffini corpuscles, Pacinian corpuscles, free nerve endings and small and large nerve bundles. This raises questions about the function of these nerve elements and supports the concept of a neurological feedback mechanism involving ligaments which is vital to the maintenance and integrity of specific joints. Further studies showed that there were significant changes in the innervation characteristics of spinal ligaments occurring during growth in chickens, particularly during the critical period surrounding puberty when damage might have long-term effects. In a subsequent study, the pathway taken by proprioceptive information following mechanical stretching of a single ligament was explored and was found to involve local centres in the spinal cord and higher centres in the brain. Understanding this pathway is an essential step toward fully understanding the neuromuscular feedback mechanism that maintains the spine as erect. In another study, the innervation pattern and morphology of the nerve elements in the lateral spinal ligaments were found to be similar between normal subjects and patients with AIS but the innervation density of spinal ligaments from patients with AIS was significantly less than that from

normal subjects. These results suggest that patients with AIS might have deficient neuromuscular feedback control of their spine. Further study of the neuromuscular control of the spine with AIS is warranted.

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CHAPTER 1

INTRODUCTION

The mechanical aspects of spinal ligaments

The human spine is one of the most important supporting structures concerned with the erect posture of the human body. The spine consists of a segmented column of vertebrae which are stabilized by spinal ligaments (Williams et al. 1989; White and Panjabi, 1990; Gunzburg et al. 1992). These ligaments surround the vertebrae and connect adjacent vertebral bodies, the vertebral arches, the transverse processes, and the spinous processes. Traditionally, spinal ligaments have been viewed as purely mechanical constraints which prevent or protect the spine from excessive motion when the ligaments are stretched and under tension (Dumas et al. 1987; Williams et al.1989; White and Panjabi, 1990). Therefore characterization of the mechanical features of the spinal ligaments has been a major topic of interest in studies of spinal ligaments (Chazal et al. 1985; Myklebust et al. 1986; Hindle et al. 1990). Such mechanical studies have shown that the ligaments allow smooth joint motion with minimal energy expenditure as well as add stiffness to the joint to produce forceful constraint when the joint approaches its maximum range of motion (White and Panjabi, 1990). This is facilitated by the non-linear elastic properties of the ligament (Chazal et al. 1985). The mechanical strength of the ligaments is considered fundamental for the stability and maintenance of the erect human spine (White and Panjabi, 1990).

It has been suggested that improper mechanical function of spinal ligaments might lead to the development of spinal deformities such as is found in idiopathic scoliosis (Lindahl and Raeder 1962; Schultz et al 1972; Waters and Morris, 1973). The mechanical properties of supraspinal ligaments from scoliotic patients and normal subjects were studied by Waters and Morris (1973) to investigate the relationship between the mechanical features of the ligaments and the development of idiopathic scoliosis. Although these workers were unable to find any correlation between the mechanical properties and the development of idiopathic scoliosis, other spinal ligaments still warranted further study because it has been shown that lateral spinal ligaments are more important to lateral stability of the spine and possibly more important to the development of idiopathic scoliosis with its prominent lateral curvature

(Schultz et al. 1972; Schultz and Hirsh, 1973; Panjabi and Goel, 1982; Thomas and Dave, 1985). Panjabi and Goel (1982) found that the supraspinal ligament located in the midline and posterior to the rotation center of the vertebrae was subjected to more strain than other spinal ligaments when forward flexion load was applied to the spine. In contrast, Schultz et al. (1972, 1973) found that changes in the lengths of the ligaments which span the transverse processes (lateral spinal ligaments) were necessary to reproduce the geometrical configuration of scoliotic spines in a mathematical kinetic analog of the human vertebral column. The importance of lateral ligaments in maintaining the integrity of the vertebral column was also illustrated experimentally by the development of scoliosis in animal models after division of the costotransverse (lateral) ligaments in rabbits and monkeys (Pal, 1991, Thomas and Dave, 1985, Michelsson, 1965). Examination of lateral ligaments of the spine would seem more likely to produce information related to the maintenance of lateral stability of the spine than studies focusing on central spinal ligaments. However, characteristics of the lateral spinal ligaments have never been reported in detail and will be studied intensively in this thesis. The data collected will lead to further understanding of the role of lateral spinal ligaments in the lateral balancing of the spine in terms of its mechanical support and will also allow the development of an appropriate rationale as to which specific lateral spinal ligaments should be studied further in cases of spinal deformity.

The innervation of ligaments

Although ligaments have been viewed traditionally as simple mechanical constraints across joints by virtue of their ability to provide tension when stretched, it has been known for a long time that they are well innervated (Freeman and Wyke 1967; Halata, 1977; Cavanaugh et al. 1989). Recent research has focused attention on this innervation and the concept has been advanced that ligaments also provide important proprioceptive feedback as part of a neurological protective mechanism both for the ligaments themselves and the joints they span. This is a significant concept and will result in new insights into common problems such as joint instability following injury, spinal deformities such as scoliosis, and lower back pain.

In 1967, Freeman and Wyke hypothesized that an important role for joint receptors might be to contribute to the regulation of the coordination of the muscle tone around the joint by way of the muscle spindle system. This hypothesis was subsequently supported by results from a series of electrophysiological experiments investigating the

reflex control of gamma-motor neurons which innervate the muscle spindles (Appelberg et al. 1983a; 1983b; Sojka et al. 1983; Johansson and Sojka, 1985). Johansson et al. (1986) further showed that natural stimulation of the joint receptor afferent (e.g. pressure on the knee joint capsule) could evoke changes in the responses of primary muscle spindle afferents. This showed that the neurological pathway between joint afferent, fusimotor neurons, and muscle spindles was sufficiently potent to influence the firings of the muscle spindle afferent. Other studies examined the individual afferents of the knee joint and found that different regions had different properties (Burgess and Clark, 1969; Clark and Wyke, 1975; Grigg, 1976; Ferrell, 1980) and that many joint afferents in the posterior region of the knee capsule were sensitive to extension movements (Burgess and Clark, 1969; Grigg, 1976; Ferrell, 1980). In light of these results, the concept of ligaments providing proprioceptive feedback to maintain the integrity of joints is rapidly replacing the traditional idea that ligaments are simply mechanical links between bones which restrict excessive movements of joints.

Most of the research related to the innervation of ligaments has focused on the knee (Kennedy et al. 1982; Sjölander et al. 1989; Katonis et al. 1991; Frank et al. 1991) and there have only been a few studies on the innervation of spinal ligaments (Giles and Harvey, 1987; Yahia et al. 1988; Kontinnen et al. 1990, 1992; Gronblad et al. 1991; Ahmed et al. 1993; Rhalmi et al. 1993). The results from these spinal studies have often been conflicting possibly reflecting the variety of techniques employed although generally, these results are similar to those found in studies of the knee. While most of the studies on the spinal ligaments were focused on the innervation of midline spinal ligaments, there is no report in the literature which has focused on the innervation of lateral spinal ligaments. The lateral spinal ligaments are of significant interest because they have been related to the lateral stability of the spine and development of idiopathic scoliosis (Schultz et al. 1972; Panjabi and Goel, 1982). Understanding the characteristics and pattern of innervation of the lateral spinal ligaments will provide insight for their involvement in the lateral balancing of the spine especially in the coronal plane. A new technique which labels neurofilament protein by immunohistochemistry will be used in studies described in this thesis to investigate the innervation of spinal ligaments.

Overview of Idiopathic Scoliosis

Scoliosis is primarily a disease of the growing child or adolescent. It has been defined simply as a lateral curvature of the spine but true scoliosis also includes rotational deformities of the vertebral axis (Robin, 1990). About 90% of the patients suffer from what is apparently a nonspecific condition known as idiopathic scoliosis, which was defined by Whitman (1922) as the type of scoliosis that could not be ascribed to any known cause. Despite the enormous amount of research and knowledge that has accumulated over the past 70 years, no one has yet been able to discover what is (or are) the cause(s) of this condition. However, idiopathic scoliosis is sufficiently specific in its clinical behavior to be considered by many to be a single disease entity presenting a fairly constant clinical picture (Robin, 1990).

Idiopathic scoliosis may appear in any one of three age groups; in infancy, during the mid-years of childhood, or in relation to the onset of puberty and adolescence (Robin, 1990). The focus of this thesis is directed towards adolescent idiopathic scoliosis (AIS) to avoid unnecessary confusion and because the deformity at this age seems to be well defined. In AIS the more progressive curves are far more common amongst girls and the clinical and radiological patterns are very specific. Recent research has suggested that AIS is probably a specific disease, genetically induced and related to a host of pathologically interacting factors that include hormonal, neurological, biochemical, and possibly biomechanical aspects (Robin, 1990).

There have been many studies related to finding the cause for idiopathic scoliosis. These have included growth studies (Taylor and Slinger, 1980; Dickson et al. 1984; Taylor, 1986;), hormonal studies (Robin, 1990), growth process studies (Haas, 1939; Arkin and Simon, 1950; Arkin et al. 1950; Ottander, 1963; Piggott, 1968; Beguiristain et al. 1980; Machida et al. 1993), biochemical studies (Zaleske et al. 1980; Shapiro and Eyre, 1981; Taylor and Giles, 1984), muscle studies (Khosla et al. 1980; Lucy, 1980; Zetterberg et al. 1983; Blatt et al. 1984) and biomechanical studies (Schultz and Hirsch, 1973; Meade et al. 1987; Pool, 1987). Unfortunately, all these studies have met with only limited success (Robin, 1990).

Perhaps the area of interest that shows the greatest potential for adding significant information to the understanding of AIS is that of neurological pathophysiology. Considerable differences between the patient with AIS and the unaffected adolescent of

the same age and sex have already been shown and the relationship between many neurological diseases and secondary scoliosis has long been recognised (Bernard et al. 1985; Robin, 1990). In those cases where motor paralysis is not a major factor, the regions of the cord most often affected are the postero-lateral columns and the posterior horn of the gray matter (Robin, 1990) which are concerned mainly with transmission of proprioceptive information. In this respect it is interesting to note that Smith and Dickson (1987) and Barrios et al. (1987) have shown that focal damage to these areas of the rabbit spinal cord can give rise to a progressive scoliosis that resembles the development of AIS in humans. Summarising this area of research, Yamada et al. (1984) proposed that virtually any disruption of the postural reflex system can result in scoliosis and indicated that there is clinical and experimental evidence that brain stem dysfunction may contribute to the aetiology of scoliosis. This mounting evidence clearly suggests that a primary defect of CNS function - a defect of posture, proprioception, or equilibrium control - is mainly responsible for production of the spinal curvature. In particular, an abnormality of the brain stem which integrates and transmits impulses that are responsible for the maintenance of posture, proprioception, and equilibrium is thought to result in some of the characteristic features seen in association with AIS (Robin, 1990). Many neurological disorders associated with the development of scoliosis are often associated with nystagmus which, among other areas, is also suggestive of the brain stem being involved in the pathological process in formation of a scoliotic curve. Studies of limb proprioception have shown signs of asymmetry in joint position and in weight discrimination and these have suggested that the defects may relate to peripheral input loss rather than central proprioceptive transmission where no measurable defect has yet been shown. Most recently, local proprioceptive dysfunction in the spinal ligaments has also been proposed as a possible cause of AIS (Yahia et al. 1992).

An increasing body of evidence also suggests that ligaments have an important neurosensory role, supplying important proprioceptive information, or serving as important transducers for dynamic neuromuscular balance and maintenance of an erect spine (Kojima et al. 1990; Katonis et al. 1991). Yahia et al. (1992) investigated the innervation of midline spinal ligaments both from normal and scoliotic subjects. He suggested that a defective receptor system could lead to loss of protective muscle function and instability of the spine. Since other studies (Lindahl and Raeder, 1962, Schultz et al. 1972; Schultz and Hirch, 1974) have shown that the lateral spinal ligament is more important in the lateral balancing of the spine, proprioceptive

dysfunction of the lateral spinal ligaments may be more likely to lead to lateral instability of the spine and the subsequent development of scoliosis. Therefore, in this thesis it is proposed that the lateral spinal ligaments are legitimate candidates for the study of proprioceptive innervation in searching for the mechanism of development of AIS.

Hypotheses:

1. The lateral spinal ligaments provide significant lateral stiffness to the spine and are more sensitive than central spinal ligaments to lateral bending of the spine.
2. The spinal ligaments are innervated by proprioceptors which are connected to a neuromuscular feedback mechanism to fulfill their functions.
3. The development of the innervation of spinal ligaments is not complete at birth. Significant postnatal development of the innervation in spinal ligaments occurs and may not be in harmony with the growth of the ligament itself.
4. Proprioceptive information from spinal ligaments contributes to the maintenance of an erect posture and an upright and balanced spine. Under-development or damage to the innervation of spinal ligaments at an early stage of development results in impairment to this mechanism and ultimately leads to spinal imbalance such as is so prominent in scoliosis.

These hypotheses are wide ranging but they are focused on one idea. the role of spinal ligaments in the lateral balancing of the spine and the mechanisms involved. Each of the hypotheses guides the way towards collecting supporting data and developing a rationale for the next hypothesis. In the following projects, attempts were made to collect data to test some aspects of the hypotheses stated above.

Objectives and Approaches

The objectives of this study are:

- to examine the nature of the mechanics and the innervation of spinal ligaments
- to evaluate their contribution to the maintenance and balancing of the spine and erect posture
- to examine whether the innervation of spinal ligaments is involved in the development of AIS.

These objectives will be explored by the following six projects:

1. The first study is designed to determine which specific lateral spinal ligament in humans is playing a main role in providing lateral stability to the spine. The morphology of spinal ligaments in a series of human cadavers will be analysed.

2. The second study is designed to identify an appropriate animal model because obtaining adequate supplies of human lateral spinal ligament is very difficult. Several different types of animal will be dissected and the morphology of their lateral ligaments will be assessed and compared with humans. The most appropriate model will be selected for further study.

3. The third study will use central ligaments from human patients undergoing back surgery to set up the methodology required to identify neural elements in ligaments. Analysis of the innervation characteristics of these ligaments will be made and used for comparison with later studies of the lateral ligaments. It is anticipated that immunohistochemical techniques will be used to stain the neural elements and confocal microscopy as well as ordinary fluorescence microscopy will be used to obtain data. A method for quantifying the innervation density and distribution will also be developed in this study.

4. The fourth study will examine the development of the innervation of spinal ligaments and particularly the changes that occur during puberty. It is possible that periods of particular vulnerability to ligament injury will be identified. The morphology, distribution and number of proprioceptors in the lateral spinal ligament in chickens will be investigated using the methodology developed in the previous study.

5. The fifth study will identify the neurological pathways and centres involved following

mechanical stimulation of a lateral spinal ligament. c-fos is a highly regulated proto-oncogene located in neurons which can start transcription if the neuron is subjected to stimulation. Since this transcription can occur in a cascade of neuronal pathways, the method of tracing Fos production following stimulation of neurons has been widely used in mapping neural connections in the central nervous system. In this project, evidence of c-fos transcription will be searched for in selected areas of the central nervous system following mechanical stimulation of a lateral spinal ligament in chickens.

6. The sixth and final study will characterize the innervation of lateral spinal ligaments in specimens obtained from both normal human subjects and scoliotic patients. These data will be analysed to determine whether there is any abnormality in the innervation of lateral ligaments from patients with scoliosis. Analysis will focus on the characteristics and symmetry of the innervation of the SCTL in normal humans, comparison of the innervation density between normal and scoliotic subjects, and the symmetry of innervation of the SCTL from patients with idiopathic scoliosis.

In general, this series of projects is designed to determine the role of spinal ligaments in the balancing and maintenance of the erect spine by studying their mechanics, morphology, development and innervation. The characteristics of the innervation of spinal ligaments and the contribution of this innervation to a proprioceptive feedback mechanism particularly in relation to the maintenance of an erect, straight spine will be especially assessed. There will be direct clinical application of the results from these projects for they will provide basic information related to understanding the mechanism of active balancing of the spine and contribute further understanding of the mechanism of diseases such as back pain, unstable spines, and especially idiopathic scoliosis.

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CHAPTER 2

Quantitative Morphology of the Lateral Ligaments of the Spine. Assessment of Their Importance in Maintaining Lateral Stability.

INTRODUCTION

The normal relation of vertebrae to each other is maintained by a variety of structures, but spinal ligaments appear to be the most important in maintaining spinal stability[4,11,13]. This is particularly so during normal motion as the spinal ligaments allow bending to occur with little energy expenditure and then protect the spine from excessive motion as it reaches its limits of movement[18]. However, mechanical constraint may not be the only mechanism used by ligaments to stabilize the spine. Katonis and Assimakopoulos[6] reported that ligaments elsewhere in the body can serve as important proprioceptors in a neuromuscular feedback control loop of joint position. As some spinal ligaments are also well innervated with proprioceptive nerves[7,20], it is quite possible that they contribute to the control of spinal stability in a way similar to that described by Katonis and Assimakopoulos[6] for other regions of the body.

Some aspects of the spinal ligaments, especially those in the midline, have been well studied. For example, Hindle *et al.*[5] found that interspinous and supraspinous ligaments could provide useful assistance in restraining passive flexion of the spine, while Chazal *et al.*[1], Myklebust *et al.*[9] and Yahia *et al.*[20] comprehensively studied the mechanical properties and function of midline spinal ligaments. In contrast, the lateral ligaments of the spine have received less attention, particularly in relation to abnormalities such as scoliosis. This is in spite of the work of Panjabi and Goel[11] who found that the more lateral ligaments (transverse and capsular ligaments) were subjected to the highest strain during lateral bending while the midline ligaments (supraspinous and interspinous ligaments) were more strained only in sagittal movements such as forward flexion.

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It would appear that the ligaments further from the rotation center in the coronal plane are more involved in lateral bending than those in the midline and so might be more involved in the development of lateral deformities such as those found in scoliosis. Surprisingly, most studies investigating lateral instability of the spine (as is found in cases of scoliosis) have focused on the midline ligaments (for example: Yahia *et al.*[20]; Waters and Morris[17]; Venn *et al.*,[16]). It is possible that the lateral ligaments of the spine could be more important for the stability of the spine in the coronal plane but little attention has been paid to them despite some clearly supportive evidence in relation to scoliosis. Schultz *et al.*[14] using a mathematical model to simulate the scoliotic spine concluded that changes in the lengths of the anatomical structures associated with the transverse processes such as the lateral ligaments were necessary to reproduce the geometrical configuration of scoliotic spines. More specifically, Lindahl and Raeder[8] analyzed the forces of the spine involved in idiopathic scoliosis and concluded that scoliosis was due to growth restriction of the intertransverse ligaments or muscles on one side. Clearly, the lateral ligaments are legitimate candidates for study when looking at maintenance of the lateral stability of the spine; however, their characteristics and mechanical properties have not been adequately documented in the literature.

Accordingly, this study was designed to describe the characteristics and configurations of the prominent lateral ligaments of the spine and to determine their possible effectiveness in maintaining lateral stability. The three lateral ligaments selected were the superior costotransverse ligament (SCTL), the lateral costotransverse ligament (LCTL), and the intertransverse ligament (ITL). The positions of a multitude of landmarks on the vertebrae and configurations of these associated ligaments were measured and transposed into a computerized 3-D coordinate system. These data were then used to assess the contributions of these ligaments to the lateral stability of the human spine.

MATERIALS AND METHODS

Complete spinal segments from thoracic level (T) 7 to T11 were removed from 32 embalmed cadavers. There were 18 females and 14 males with a mean age of 75 years (S.D.= 13 years). There was no significant difference ($p>0.05$) between the mean ages of the male (74 ± 13 years) and female (75 ± 9 years) groups.

The LCTL and the ITL were exposed at each vertebral level by detailed posterior

dissection. This was achieved by initially removing the skin and subcutaneous soft tissue followed by removal of segments of trapezius, latissimus dorsi, spinalis thoracis and longissimus thoracis muscles. This dissection allowed sparing and identification of any ligaments that were encountered. A thin layer of loose connective tissue between the ligaments and the muscles made it easier to expose and identify separately the LCTL and ITL. Special care was taken not to damage the intertransverse ligaments while semispinalis thoracis and levatores costarum longi muscles were being removed. To expose the SCTL at each vertebral level, the pleura was first resected around the costovertebral junctions using an anterior approach to the spine. The SCTL and the intercostal membrane were then exposed at each level by blunt dissection of the underlying adipose tissue, intercostal nerve and blood vessels.

Using this methodology, the ligaments were exposed at each of four vertebral levels in the intervals between the 5 vertebrae in each specimen. Ligaments found at T7 and also between T7 and T8 were arbitrarily designated as being from T7. Other ligaments at other vertebral levels were labeled accordingly. A general description of the structure of each ligament was made based on its appearance. The thickness of each ligament was measured at the midpoint between its upper and lower attachments using a microcaliper taking care to avoid compression.

The spinal segments were rigidly fixed to a Microval coordinate measuring system (Brown and Sharp Manufacturing Co., North Kingston, RI) to digitize the position of the ligaments and selected points (see below) on the spine and to translate these data to three dimensional coordinates (Figure 2.1). The linear accuracy of the Microval was specified to be 0.006 mm.

Specialized spinal end caps[3] were used to secure the specimen to the digitizing table. Landmarks on the spinal column, selected to represent the geometry of the vertebrae and ligaments, were identified and marked with ink using a hypodermic syringe and needle prior to being digitized. The landmarks at each vertebral level were:
- five points on the lower endplate of each vertebra; the anterior center point, the two anterolateral corners, and the two posterolateral corners.
- five points on the upper endplate of each vertebra; the anterior center point, the two anterolateral corners, and the two posterolateral corners.
- the middle point of the tip of the spinous process.
- the eight corner attachment points for each pair of SCTL and LCTL (Figure 2.2a, b).

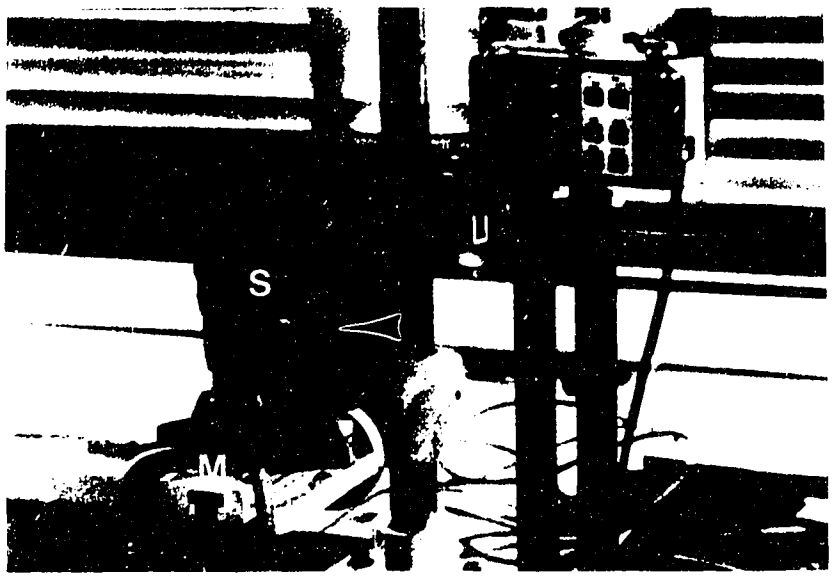

Figure 2.1 This photograph shows the layout of the digitizing system. A segment of human spine (S) has been fixed on the measuring table (M). The position of the tip of the probe (arrow head) relative to the coordinate system is transformed into electronic signals, sent on-line to a computer and recorded for each of the x, y and z axes. From this positional information, the dimensions and positions of SCTL and LCTL relative to vertebrae will be evaluated.

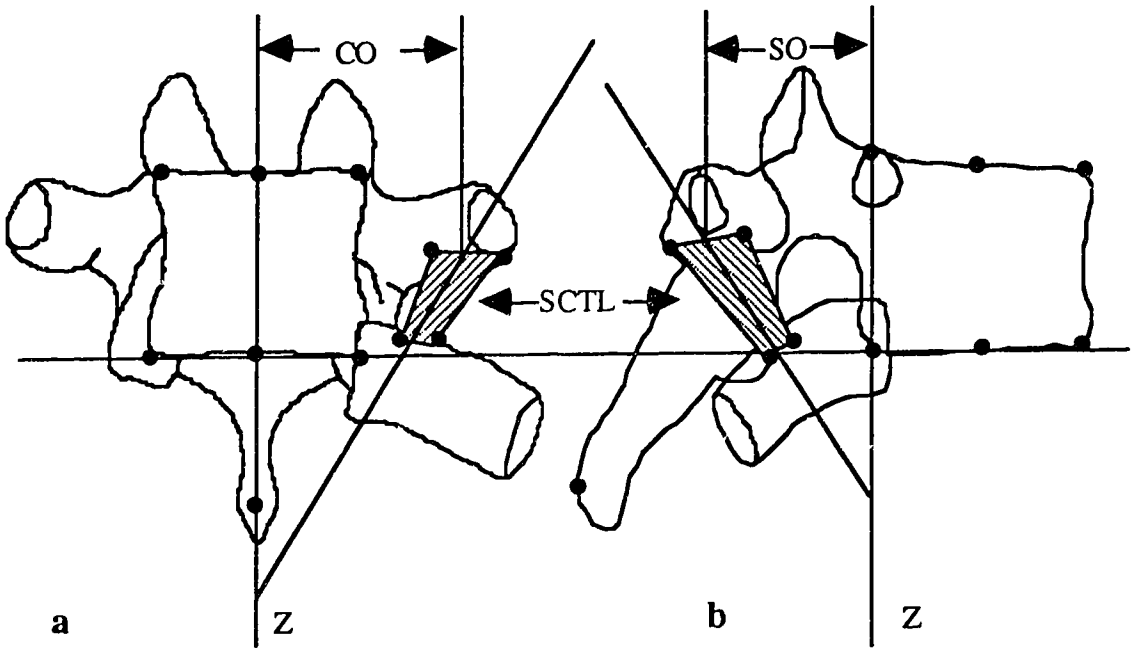

Figure 2.2 This diagram shows both frontal (a) and sagittal (b) views of a thoracic vertebra with SCTL(▨) in position. The landmarks (shown as •) for each vertebra were digitized to form a local three-dimensional coordinate system. The measured 3 dimensional data of SCTL were then fitted into this coordinate system. The coronal offset (CO) is defined as the distance between the middle of the upper border of SCTL and the Z axis in the coronal plane. The sagittal offset (SO) is defined as the distance between the middle of the upper border of SCTL and Z axis in the sagittal plane.

Ligaments that were not distinguishable because of their membrane-like nature were marked as missing. To examine intra-observer error in using the digitizer, the same four corners of a single SCTL were digitized twenty times. The 95% confidence level was ±0.5 mm in height and ±1.0 mm in the coronal and sagittal directions.

The data from each of the digitized points were transferred to a Hewlett Packard 9000 computer (Hewlett-Packard Co., Fort Collins, Colorado) for further analysis. The transverse plane (x, y) was defined as the best-fit plane through the five points digitized on the lower endplate of each vertebra using the least squares method similar to that used by Panjabi and Takata[12]. The sagittal plane (y, z) was defined by a plane through the anterior center point of the lower endplate of the vertebra and the tip of the spinous process of the same vertebra. This plane was perpendicular to the transverse plane. The coronal plane (x, z) was defined by a plane passing through the four posterolateral corner points of a vertebra. This plane was perpendicular to the sagittal plane. The location and orientation of the ligaments relative to this local coordinate system were then calculated (Figure 2.2a, 2.2b).

To minimize complications due to postmortem deformity of the spine (such as rotation and flexion), the positions of individual ligaments were determined relative to their own vertebra as defined above. The length of the ligaments was defined as the distance between the midpoints of the upper and lower attachments. The width of the ligaments was measured midway between the upper and lower attachments. The cross-sectional area of the ligaments was calculated using the thickness and width measurements made earlier and based on the assumption that the ligaments were rectangular in cross-section. The distribution of the different ligament types was analyzed using contingency-table analysis with a significance level of $p \leq 0.05$. Differences between sex, sides and vertebral levels were analyzed using ANOVA, again with $p \leq 0.05$.

Strain provides mechanical stimulation to whatever receptors are present in the ligaments. Accordingly, the strain induced in the SCTL by 1° of lateral bending was calculated from the digitized data (see notes). The assessment of strain also helps to determine the force generated by the SCTL during lateral bending. This is important to evaluate because the generated force would strengthen the lateral stiffness of the spine. To complete this evaluation it is necessary to have a value for Young's modulus (a measurement of elasticity) but unfortunately, the published data for Young's modulus vary greatly[2]. Consequently, for the purposes of this study, several estimates of

Young's modulus from published data[1,2,17] were used and the force developed by the SCTL in response to 1° of lateral flexion was calculated (see notes). The mechanical effectiveness in resisting lateral bending of the spine was also calculated from the force developed by the SCTL in response to 1° of lateral flexion (see notes). A sensitivity analysis of the effect of the SCTL on the bending stiffness of the spine was completed by comparing the differences of stiffness calculated from the different published values of Young's modulus.

RESULTS

Descriptive

The SCTL was a clearly defined tissue that passed laterally from the sharp crest on the superior border of the neck of the rib to the lower border of the transverse process of the vertebra immediately above. The ligament blended laterally with the internal intercostal membrane while the medial border formed a free edge. The orientation of most of the ligament fascicles was from superolateral to inferomedial except for a small bundle of fascicles that ran inferolaterally to the rib below. However, no obvious posterior layer was identified as described in Gray's Anatomy[19]. There were variations in appearance of the SCTL which allowed a classification system to be devised: ligaments consisting of continuous dense fibers were classified as 'tendon-like'(Figure 2.3A); ligaments consisting of bundles of fibers interwoven with adipose-like tissue were classified as 'woven' (Figure 2.3B); and ligaments consisting of thin membrane-like structures which blended into the internal intercostal membrane were classified as 'membranous' (Figure 2.3C). The distribution of each kind of ligament is shown in Table 2.1. Of the 252 SCTLs observed, 71% were tendon-like, 25% were woven and 4% membranous. Interestingly, ligaments classified as woven were found more frequently in females than in males, whereas the other types of ligaments could be found equally distributed between males and females.

The LCTLs were also clearly defined and each formed a thick, dense white band connecting the superior lateral tip of the transverse process of the vertebra to the corresponding rib neck. Each ligament then reached superiorly and laterally from the transverse process forming an acute angle with the vertical line of the spine. The texture and appearance of each LCTL was similar at all vertebral levels (Figure 2.4).

Table 2.1. Frequency Distribution Of SCTL Types.

	T7	T8	T9	T10	Female	Male
Tendon-like	49	47	41	42	89	90
Woven	5	15	21	21	46*	16*
Membrane	9	1	1	0	5	6

* $P < 0.01$ (between male and female)

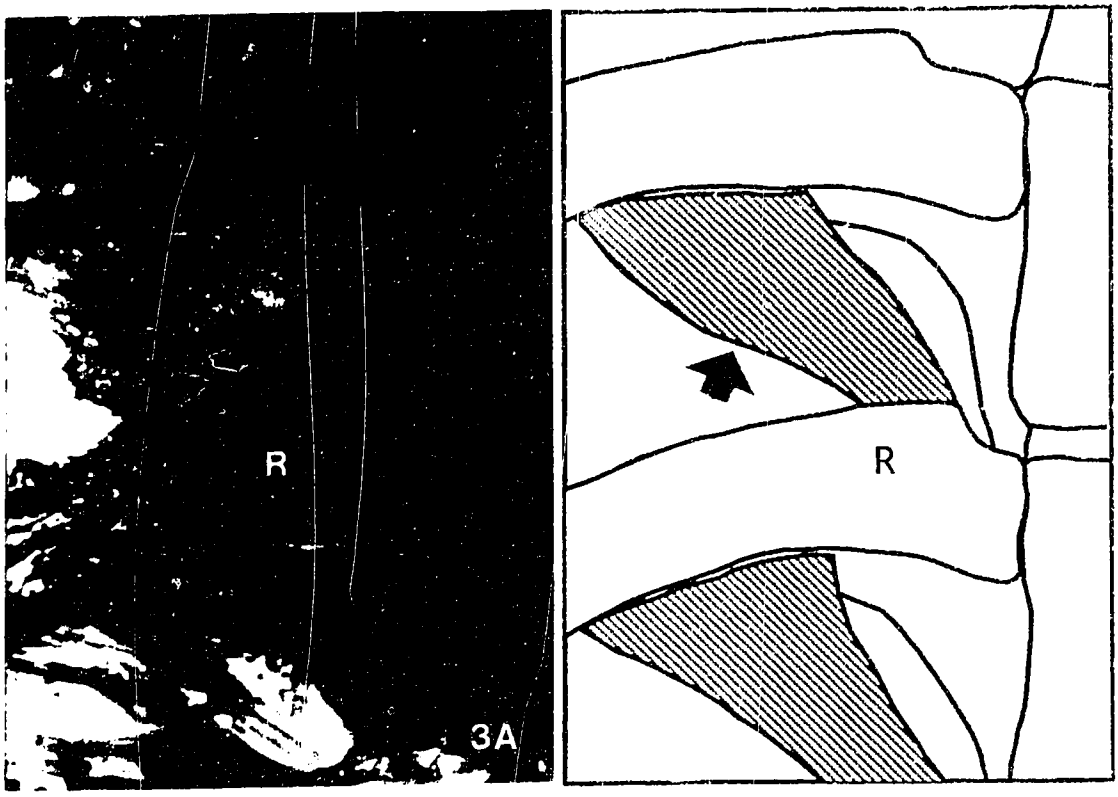

Figure 2.3A The photograph and the explanatory accompanying drawing are ventral views of the human spine in which there has been deep dissection to reveal the SCTL. SCTL is indicated by an arrow and a rib is indicated by "R". Ligaments consisting o continuous dense fibers were classified as 'tendon-like'.

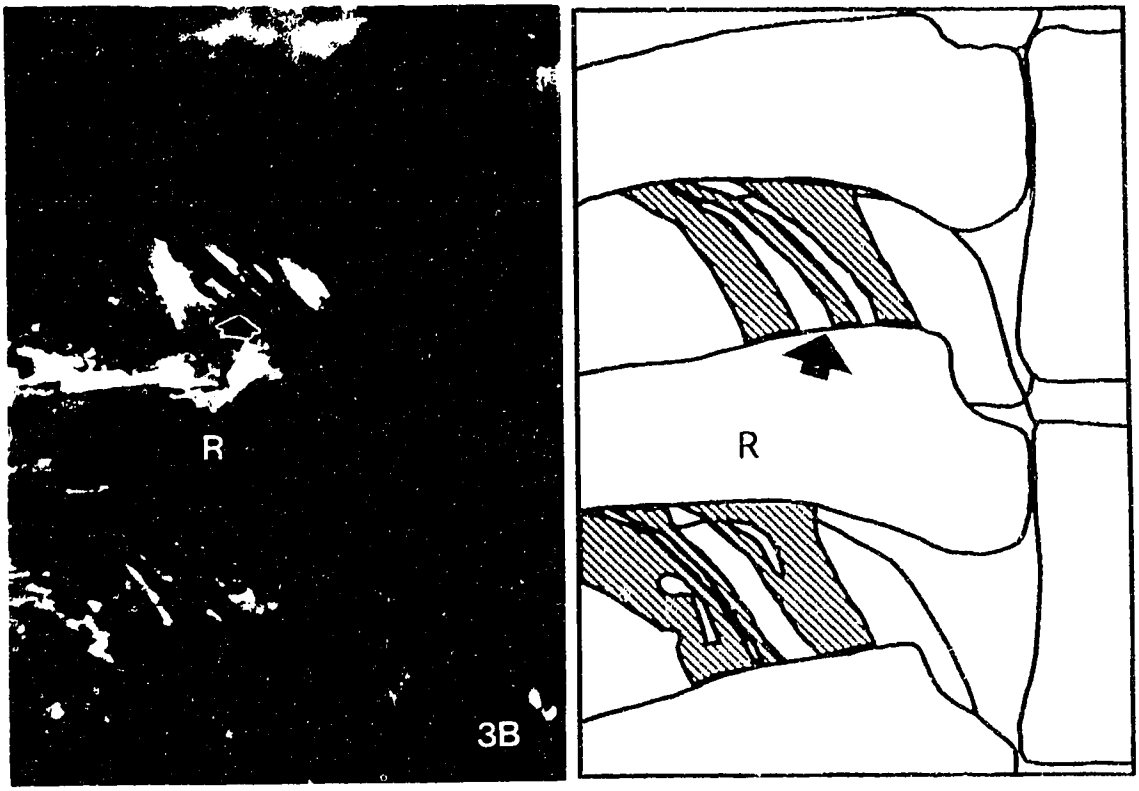

Figure 2.3B. The photograph and the explanatory accompanying drawing are ventral views of the human spine in which there has been deep dissection to reveal the SCTL. SCTL is indicated by an arrow and a rib is indicated by "R". Ligaments consisting of bundles of fibers were classified as 'woven'.

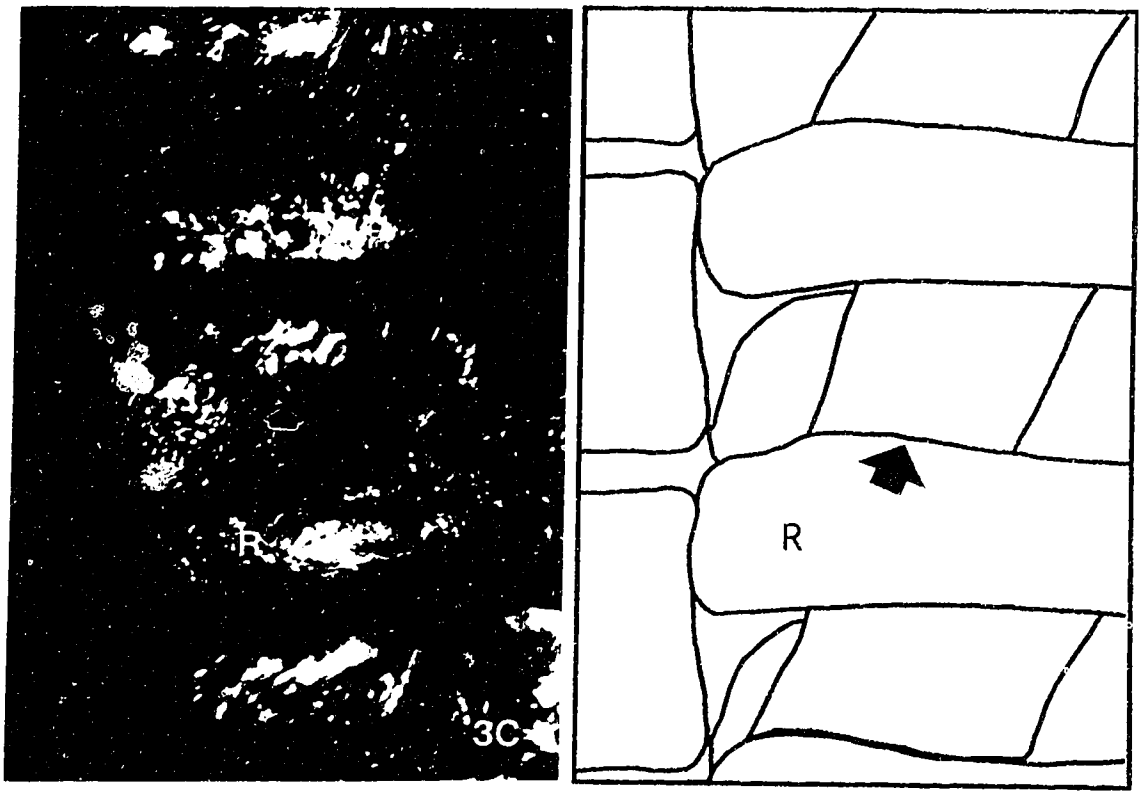

Figure 2.3C The photograph and the explanatory accompanying drawing are ventral views of the human spine in which there has been deep dissection to reveal the SCTL. SCTL is indicated by an arrow and a rib is indicated by "R". Ligaments consisting of thin membrane-like structures were classified as 'membranous'.

The ITL was difficult to recognize as a true ligament because the fibrous fascicles between the transverse processes of the vertebrae did not attach directly to the upper and lower transverse processes. Instead, the ITL consisted of tendinous fascicles of semispinalis thoracis muscle and tendons of the levatores costarum longi muscle. The former arose from the transverse processes and inserted, by tendons, into the spinous processes. The latter arose from the ends of the transverse processes and passed obliquely downward and laterally eventually inserting into the outer surface of the rib caudal to the vertebra from which it had originated. The fibrous fascicles were inseparable from the muscle fibers during dissection and were removed along with the muscle if the muscle was peeled away. The appearance of a 'ligament' was produced by the interweaving of the muscle tendons between the adjacent transverse processes and not by the presence of any actual ligamentous material (Figure 2.4). No further analysis of ITL was made as it was not considered to be a true ligament.

Quantitative

The average length of the SCTL did not differ significantly ($p>0.05$) with sex and side but increased significantly ($p<0.001$) between the upper and lower vertebral levels going from 8±2 mm at T7 to 13±3 mm at T10 (Table 2.2). Similarly, the average cross-sectional area of the SCTL did not differ significantly between sex and side ($p>0.05$) but increased significantly ($p<0.01$) between the upper and lower vertebral levels going from 5±2 mm^2 at T7 to 8±5 mm^2 at T10 (Table 2.2). In contrast, there was no significant difference between sex, side and vertebral level ($p>0.05$) in relation to length, width and offset (distance from midline) of the LCTL. However, the average cross-sectional area of the LCTL decreased significantly ($p<0.001$) between upper and lower vertebral levels going from 13±5 mm^2 at T7 to 5±2 mm^2 at T10.

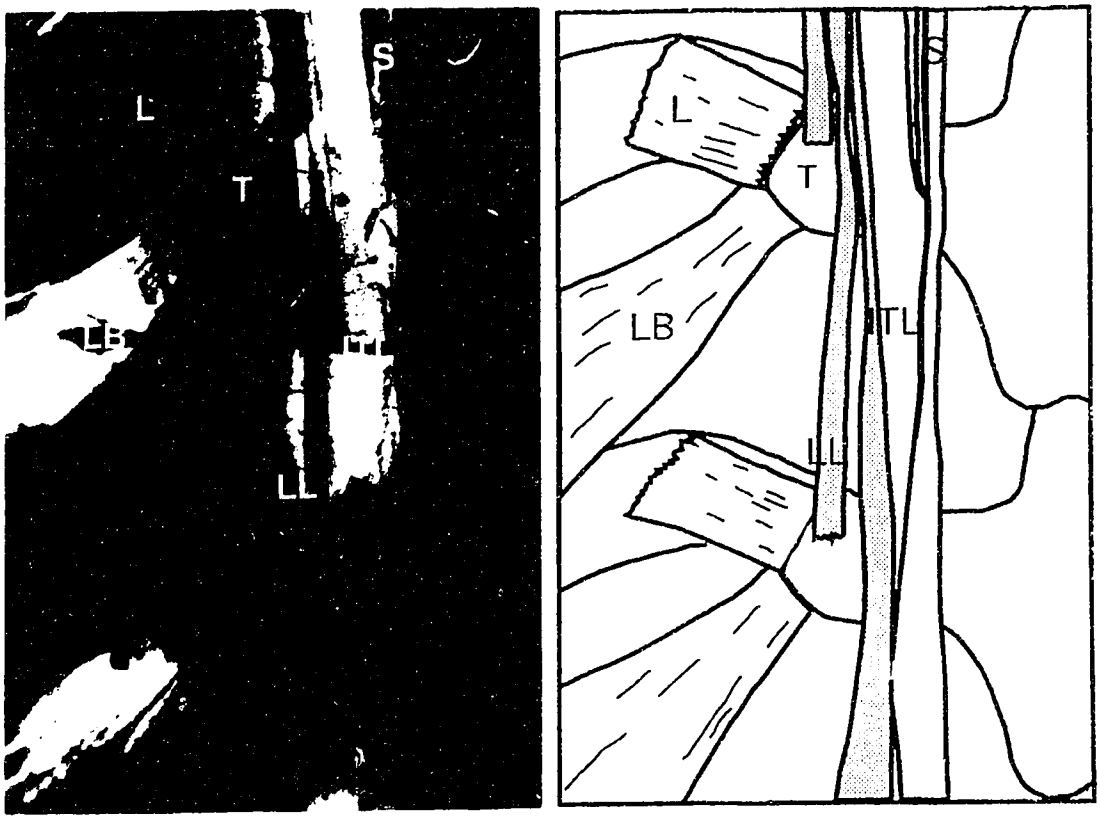

Figure 2.4 This figure and its accompanying explanatory drawing shows a dorsal view of 3 spinal segments where there has been deep dissection. It shows the intertransverse ligament (ITL) at each level consisting of bundles of fibers connected to semispinalis thoracis muscle (S) and levatores costarum longi muscle (LL). From this analysis, it was determined that ITL was not a true ligament. The lateral costotransverse ligament at one level is indicated by L, the transverse process at one level indicated by T and levatores costarum brevis by LB.

Table 2.2. The configurations of SCTL and SCTL

	Levels	Length (mm)	Width (mm)	Thickness (mm)	Area (mm2)
SCTL	T 7	8 ± 2	7 ± 2	0.7 ± 0.3	5 ± 2
	T 8	9 ± 2	7 ± 2	0.7 ± 0.4	6 ± 3
	T 9	10 ± 3	7 ± 3	0.9 ± 0.4	6 ± 5
	T 10	13 ± 3	7 ± 2	1.2 ± 0.4	8 ± 5*
LCTL	T 7	6 ± 1	8 ± 2	1.6 ± 0.4	13 ± 5
	T 8	6 ± 1	8 ± 2	1.7 ± 0.4	13 ± 4
	T 9	5 ± 1	7 ± 2	1.3 ± 0.4	10 ± 4
	T 10	5 ± 1	7 ± 1	0.7 ± 0.3	5 ± 2*

* $P < 0.01$ (compare with other segments)

The vertical axis of rotation of the spine is located at the middle of the vertebral body[21]; both the SCTL and the LCTL are positioned posterolateral to this axis. Since the aim of this study was to assess the contribution made by ligaments to the lateral stability of the spine, data analyses concentrated on the lateral offsets and orientations of the SCTL. The average offsets of the SCTL to the axis of the spine in the coronal plane averaged 34±5 mm with no significant difference between sex, level and side. There was also no apparent difference in relation to sides and levels of the average angles of the plane of the SCTL to the vertical axis of rotation of the vertebra in the sagittal plane. Similarly, from Table 2.3, it can be seen that the angles of the plane of the SCTL to the vertical axis of the vertebra in the coronal plane (Figure 2.2a) averaged between 41±11° at T7 and 31±12° at T10 although there was no significant difference in these values (Table 2.3).

Table 2.3. The average angles of SCTL to the axis of rotation of the vertebra. (Left and right have been combined since there was no significant difference between the respective values.) AP = anteroposterior view LAT = lateral view

Levels	Angle (degree)		Offset (mm)
	AP	LAT	
T 7	41 ± 11	32 ± 12	34 ± 5
T 8	36 ± 12	32 ± 11	34 ± 5
T 9	36 ± 12	36 ± 10	33 ± 7
T 10	31 ± 12	36 ± 9	34 ± 4

Assuming that the connection between the rib and transverse process/vertebra is rigid, the theoretical strain of the SCTL for each degree of lateral bending of the spine calculated from equation 2.0 (see notes) would range from 3.8% at the level of T10 to 5.6% at the level of T7 (Table 2.4). To estimate the bending stiffness provided by the SCTL, Young's modulus was assumed to be similar to that reported for other spinal ligaments. Unfortunately the reported values[2] vary greatly (7.5 - 123 N/mm^2). For a moderate value of 16.3 N/mm^2 of the posterior longitudinal ligaments from an age group of 50-80 years[1], the theoretical stiffness of the SCTL at the level of T7 in lateral

bending of the spine calculated according to equation 5.0 (see notes) would be 0.11 NM/degree. Alternatively, if a Young's modulus value of 123 N/mm^2 was applied[17], the stiffness of the SCTL in T7 in lateral bending of the spine would be as high as 0.83 NM/degree (Table 2.4).

Table 2.4. Theoretical strain and stiffness of SCTL in 1° of lateral bending of the spine

Levels	Strain (%)	Moderate stiffness (NM/degree) when E = 16.3 N/mm^2	High stiffness (NM/degree) When E = 123 N/mm^2
T7	5.6	0.11	0.83
T8	5.3	0.13	1.02
T9	4.6	0.13	0.99
10	3.8	0.15	1.13

DISCUSSION

The results of this study showed that some of the lateral ligaments of the spine may be more important than previously assumed in the balancing of the spine particularly in the coronal plane and in the maintenance of lateral stability. Further study of these ligaments is warranted because they might contribute to disorders such as scoliosis where lateral stability appears to be one of the major factors that is disturbed.

The results of this study showed that the ITL is not a 'true' ligament. The appearance of a 'ligament' was produced simply by the interweaving of muscle tendons. It is possible that ligamentous fibers are present as this conclusion was based on morphological observation and not histological examination. However, if ligamentous fibers are present they must be very small and presumably insignificant. This description disagrees with that given in Gray's Anatomy[19], which states that the intertransverse ligaments are interposed between the transverse processes and are rounded cords intimately connected with the deep muscles of the back in the thoracic region. While the intimacy with the muscle tendons is not denied, the results from this study strongly suggest that an independent ligament does not actually exist.

In sharp contrast to the ITL, the SCTL was a prominent ligamentous structure which varied in texture ranging from being membranous to tendon-like. It is possible that membrane-like or woven SCTL cannot provide as much force as tendon-like ligaments since the cross-sectional area or density of the woven and membranous SCTL would be smaller than that of tendon-like ligaments. The presence of woven-type SCTL therefore may leave the thoracic spine vulnerable to lateral instability. Such speculation, however, requires further study. Interestingly, females had a higher frequency of woven ligaments and this higher frequency coincides with the higher incidence of idiopathic scoliosis in girls. However, the discrepancy in age between the two groups makes impractical any further linkage between these two phenomena in this study but, nevertheless, is interesting to note. Significantly, the SCTL was found to be the only spinal ligament connecting adjacent vertebrae on the lateral aspect of the spine, since the ITL was found not to be a true ligament and the LCTL only connects the transverse process to its own rib. The SCTL connects the transverse process of the upper segment and rib neck of the lower segment which was rigidly connected to its own transverse process/vertebra by two joints, the LCTL and two other ligaments (costotransverse and radiate ligaments). This solitude of the SCTL confirms its

importance in maintaining lateral stability between adjacent vertebrae. However, the lack of supplementary supporting structures for the SCTL coupled with its smaller cross-sectional area, possibly makes the SCTL susceptible to damage from excessive strain with the consequence of instability in lateral balance. In this respect, it is interesting to note that in one of our parallel studies, the spinal ligaments on a series of quadrupeds (dog, rat, mouse and hamster of ten specimens each) were examined. Surprisingly, none of these species appears to have the SCTL or its equivalent (unpublished data). It might be argued that as the spine of a quadruped does not have to be balanced laterally to the same extent as a biped because of its recumbent position, there might be no mechanical need for development of the SCTL in the quadruped. This finding also supports the suggestion that the SCTL is important for the lateral balance of the human spine associated with its bipedal stance.

The LCTL was also a well defined ligament connecting the transverse process of a vertebra to its corresponding rib. The LCTL may provide a strong connection between these two attachments by its dense, short and thick structure. This connection is strengthened by the costotransverse and costovertebral joints and the radiate and costotransverse ligaments. However, the connection between ribs and transverse processes/vertebrae was rigid compared with the connection between the upper transverse process and the lower rib made by the SCTL and did not appear to allow much flexibility. Therefore, it is unlikely that the LCTL will be strained during lateral bending of the spine and probably would not be involved to any great extent with active control of spinal balance.

The stiffness of the thoracic spine has been reported by Panjabi *et al.*[10] to be 2.9 NM per degree at a load of 5 NM in lateral bending. In their model, Panjabi *et al.*[10] measured the spine with the rib head and associated ligaments attached; therefore, the stiffness of the spine included the SCTL. Our theoretical stiffness of the SCTL accounted for 3.7% to 28% of the total lateral stiffness of the spine. However, the theoretical stiffness is quite sensitive to Young's modulus and this variation was a result of differing values for Young's modulus cited in the published literature. The accuracy of Young's modulus for the SCTL is critical to the study of its mechanical effectiveness and requires further study to be more reliable and accurate. The great variation in Young's modulus for spinal ligaments reported in the literature could be due to many factors, including strain rate, strain level, measurement technique and differences in the testing environment. In addition, this study has shown that the SCTL

can be categorized into three types. Since the mechanical properties of ligaments are determined by their structure, Young's modulus could be different for each type and the bending stiffness could also vary because of this. Further work is needed to establish the mechanical properties of SCTL and ITL particularly in relation to Young's modulus to clarify their contribution in maintaining the lateral stability of the spine.

In this study, the theoretical strain in the SCTL due to 1° of lateral bending of the spine ranged from 3.8% at vertebral level T10 to 5.6% at vertebral level T7. In a study by Panjabi and Goel[11], the strains of midline ligaments, such as supraspinous and interspinous ligaments, were found to be about 1% for each degree of lateral bending. Therefore the strains of the SCTL are much higher than that of midline ligaments during lateral bending. Proprioceptors respond to mechanical stimulation such as strain and, if proprioceptors are present in the spinal ligaments to the extent that they are being found in other ligaments[7,20], then the SCTL may be the most sensitive proprioceptive sensor for lateral bending of the spine. As disturbances of spinal balance are considered important factors in the etiology of idiopathic scoliosis[8,14,15,20], this study provides a rationale to look further into the significance of the SCTL in maintaining the balance of the spine and, from there, its possible role in the etiology of idiopathic scoliosis.

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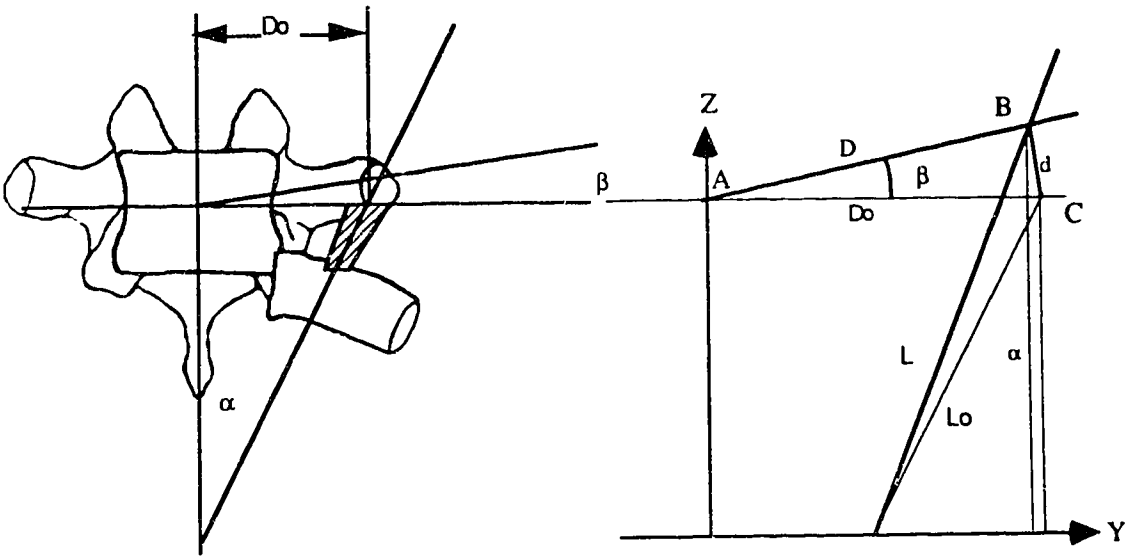
Notes



Nomenclature

d = displacement of SCTL attachment point

D_0 = offset of SCTL from neutral axis of the spine

L_0 = Original length of SCTL

L = Length of SCTL after lateral bending

α = coronal angle of SCTL to the vertical axis

β = Lateral bending angle

ε = strain of SCTL

area = cross-sectional area of SCTL

F = total force

E = Young's modulus

Due to lateral bending

1.1 $d = 2D \sin \beta/2$

1.2 $-ABC = -ACB = 90° - \beta/2$

1.3 $\Delta y = d * \cos(-ABC)$

1.4 $\Delta z = d * \sin(-ACB)$

1.5 $L_0 z = L_0 \cos \alpha$

1.6 $L_0 y = L_0 * \sin \alpha$

1.7 $Ly = L_0 y - Dy$

1.8 $Lz = L_0 z + D z$

1.9 $L = \sqrt{Ly^2 + Lz^2}$

The strain of SCTL for each degree of lateral bending of the spine was calculated as follows:

2.0 $$\varepsilon = \frac{(L - L_0)}{L_0}$$

Total force generated by SCTL was calculated by:

3.0 $F = E * \varepsilon * \text{area}$

Vertical portion of force (F vertical) was calculated by:

4.0 $F_{\text{vertical}} = F * \cos \alpha$

And finally the stiffness of bending moment per degree of lateral flexion was calculated by:

5.0 $\text{Stiffness} = F_{\text{vertical}} * D$

CHAPTER 3

A comparison of spinal ligaments - differences between bipeds and quadrupeds

INTRODUCTION

Spinal ligaments are important structures for maintaining the stability of the spine as they provide both simple mechanical constraint and neuromuscular feedback (Yahia et al. 1988; Rhalmi et al. 1993). As a mechanical structure, a ligament has to be stretched before it can provide support and consequently the mechanical function of a spinal ligament depends on its attachments and their position relative to the centre of rotation of the spine. For example, the supraspinous and interspinous ligaments which are located in the midline and posterior to the rotation axis control flexion of the spine (Panjabi and Goel 1982) whereas the superior costotransverse ligaments (SCTL), located in the lateral region of the spine, are found to be more important in maintaining balance of the spine in the coronal plane. Mechanically, the SCTL with its lateral attachments,has been shown to be more effective in producing lateral force than those ligaments that are attached solely in the midline and which are unable to produce lateral force (Jiang et al. 1994). Furthermore, it has been shown that severing of the SCTL in the rhesus monkey results in development of scoliosis (Thomas and Dave 1985) which accords well with concepts of scoliosis development dependent on ligament involvement according to methods of mechanical modelling of the spine (Lindahl and Raeder 1962; Shultz et al. 1972). Consequently, it has been hypothesized that asymmetry in the mechanics of the lateral spinal ligaments could be related to the aetiology of diseases such as idiopathic scoliosis where lateral curvature and vertebral rotation are prominent features (Jiang et al. 1994).

A version of this chapter has been accepted for publication.

Jiang H, Moreau MJ, Raso VJ, Russell GG, Bagnall KM 1995. A comparison of spinal ligaments: differences between bipeds and quadrupeds. J Anatomy

Having recently demonstrated the importance of the lateral ligaments of the spine by mechanical modelling, particularly SCTL, to the maintenance of an erect posture in humans and its potential significance in scoliosis (Jiang et al. 1994), attempts were made to identify an appropriate animal model to use as the basis for further study. A review of the literature revealed only sparse details of the lateral spinal ligaments for only a few individual animals. From some initial dissections of a variety of species it quickly became evident that there was great variation in these ligaments between different animals. Accordingly a detailed comparative study of spinal ligaments was undertaken. The results showed that while all the animals studied possessed the central spinal ligaments such as the supraspinous and interspinous ligaments, they did not all possess the lateral spinal ligaments such as SCTL. Specifically, the quadrupedal animals (e.g. dog, rat, cat) did not possess the lateral ligaments whereas the bipedal (human) and pseudo-bipedal (e.g. chicken, duck, turkey) possessed well-defined lateral ligaments. It is suggested that the development of the lateral ligaments are a consequence of an upright posture.

MATERIALS AND METHODS

Spines from a variety of different animal species, including human, were obtained either from the Department of Anatomy and Cell Biology (human spines) or from the University Farm, University of Alberta (Table 3.1). Most of the spines were from mature animals but in some cases (for example, chickens) immature spines were also collected for comparison. Similar results were observed in both immature and mature specimens and further comparison was discontinued in this study. The spinal ligaments from thoracic level 7 (T7) to T 11 for the mammals and from T3 to T6 for the birds were exposed by careful dissection. These levels represent the lower thoracic regions in these species and are where the lateral spinal ligaments are most prominent. They also represent regions of the spine in which there is a high incidence of human scoliosis development. The central spinal ligaments, consisting of the supraspinous and interspinous ligaments (SSL/ISL), were exposed posteriorly by cutting the skin and removing the subcutaneous tissue. Similarly, the intertransverse ligaments were exposed by further dissection of the trapezius, latissimus dorsi, semispinalis thoracis and levatores costarum muscles. Special care was taken to notice any other ligamentous tissue between the transverse processes. The SCTL were exposed through an anterior approach in which the pleura was first removed as well as the loose connective tissue underneath.

Having exposed the spinal ligaments, their absence or presence was noted as well as a brief description made of their morphology as they appeared under a dissecting microscope. In cases where ligaments were found to be absent on initial observation, further dissection was performed to ensure that no other extraneous ligamentous tissue was present.

Initially, the results from the dissections of the individual species were kept separate but it became apparent that a pattern developed if they were grouped in accordance with their method of pedal locomotion: bipedal (human), pseudo-bipedal (avians) (Thorp, 1989), or quadrupedal (pig, dog, cat, rat, mouse, hamster).

RESULTS

The presence or absence of the various ligaments and the descriptions of their morphology were consistent within each of the separate species with little individual variation. Similarly, there was consistency within the groupings as shown in Table 3.1. Therefore, the results are described in relation to the groupings shown.

Observations from bipeds (humans):

In all cases, the SSL/ISL complex, which represents the central spinal ligaments, was very prominent in the midline, firmly connecting the adjacent spinous processes. The ligament was extremely tough and extended along and between the full length of the superior and inferior borders of the spinous processes as well as being thickened between the tips of the processes. It was difficult to determine a precise border between the SSL and ISL as there was a gradual merging of the tissues in these regions but the free edge of the ligament, between the tips of the spinous processes, was thickened and was taken to represent the SSL. In contrast, it was found that the only lateral spinal ligament connecting adjacent vertebrae was the superior costotransverse ligament (SCTL). The SCTL passed laterally from the sharp crest on the superior border of the neck of the rib (costal process of the vertebra) below to the lower border of the transverse process of the vertebra immediately above (Figure 3.1). This was a clearly defined, tough tissue clearly separate from adjacent tissues and was very prominent. The ligament blended laterally with the internal intercostal membrane while

the medial border formed a free edge. The orientation of most of the ligament fascicles was from superolateral to inferomedial except for a small bundle of fascicles that ran inferolaterally to the rib below. No obvious posterior layer was identified. The intertransverse ligament (ITL), a commonly described ligament (Williams et al. 1989) in the lumbar region, was found not to be a ligament at all in the thoracic region but was simply the interweaving of the tendons of adjacent muscles. The results relating to the SCTL and ITL in humans have been presented in more detail in an earlier paper (Jiang et al. 1994).

Observations from pseudo-bipeds (avians):

The central spinal ligaments (SSL/ISL complex) were found to be prominent in all species and was similar to that already described for the true bipeds (humans). Further detailed description is unnecessary and so has been omitted. However, two lateral spinal ligaments were found in the avians, an anterior intertransverse ligament (AITL) and an intertransverse ligament (ITL). The AITL was a membrane-like structure with thickening in the lateral aspect (Figure 3.2). The collagenous fascicles were oriented obliquely from the lateral part of the transverse process of the upper vertebra to the middle part of the transverse process of the lower vertebra. This orientation of the ligament is similar to that of the SCTL described for the humans and therefore was considered the equivalent to the SCTL in humans. The ITL was a dense, band-like structure which was oriented parallel to the long axis of the spine and was attached between the posterior lateral part of the upper and lower transverse processes of adjacent vertebrae (Figure 3.3). It too was a prominent structure. It connected to the AITL anteriorly and was surrounded by paraspinal muscles in other aspects.

Observations from quadrupeds (pig, dog, cat, rat, mouse, hamster):

The central spinal ligaments (the SSL/ISL complex) were clearly defined in quadrupeds. The SSL/ISL were attached to adjacent supraspinal processes and were of a similar nature to those already described above for both the bipeds (humans) and pseudo-bipeds (avians). However, these ligaments were less thick and tough compared with those of human or avians. In sharp contrast, no lateral spinal ligaments were found to connect adjacent vertebrae or their transverse processes in any of these species although abundant intercostal muscles were found (Figure 3.4).

In summary, it is clear from Table 3.1 that all species possessed very clear and prominent central spinal ligaments (SSL/ISL) but that the presence of lateral spinal ligaments (SCTL or ITL) was restricted to bipedal and pseudo-bipedal species.

Table 3.1. Comparison of spinal ligaments between different species. Although ligaments were simply identified with "+" for present and "-" for absent, the SSL/ISL in the quadrupeds were generally less well developed.

Pedal Locomotion	Animal	Number	SSL/ISL	ITL	SCTL
Bipedal	Human	32	+	-	+
	Chick	10	+	+	+
	Turkey	3	+	+	+
	Duck	1	+	+	+
Quadrupedal	Pig	10	+	-	-
	Dog	2	+	-	-
	Cat	2	+	-	-
	Rat	8	+	-	-
	Mouse	5	+	-	-
	Hamster	10	+	-	-

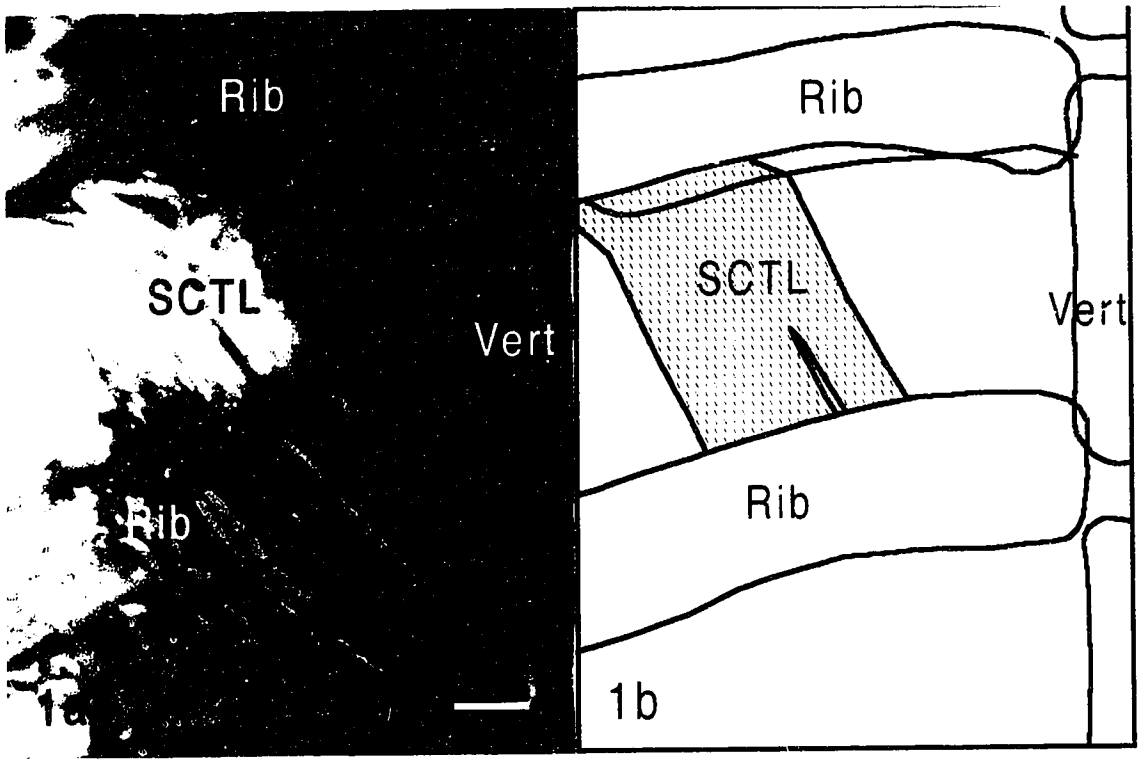


Fig. 3.1. (Bar = 2.5mm) a. A photomicrograph (anterior view) showing a typical superior costotransverse ligament in a human specimen. The ligament is a thick and dense band on the lateral side of the vertebrae. b. an explanatory drawing of the same. Vert = vertebral body SCTL = superior costotransverse ligament

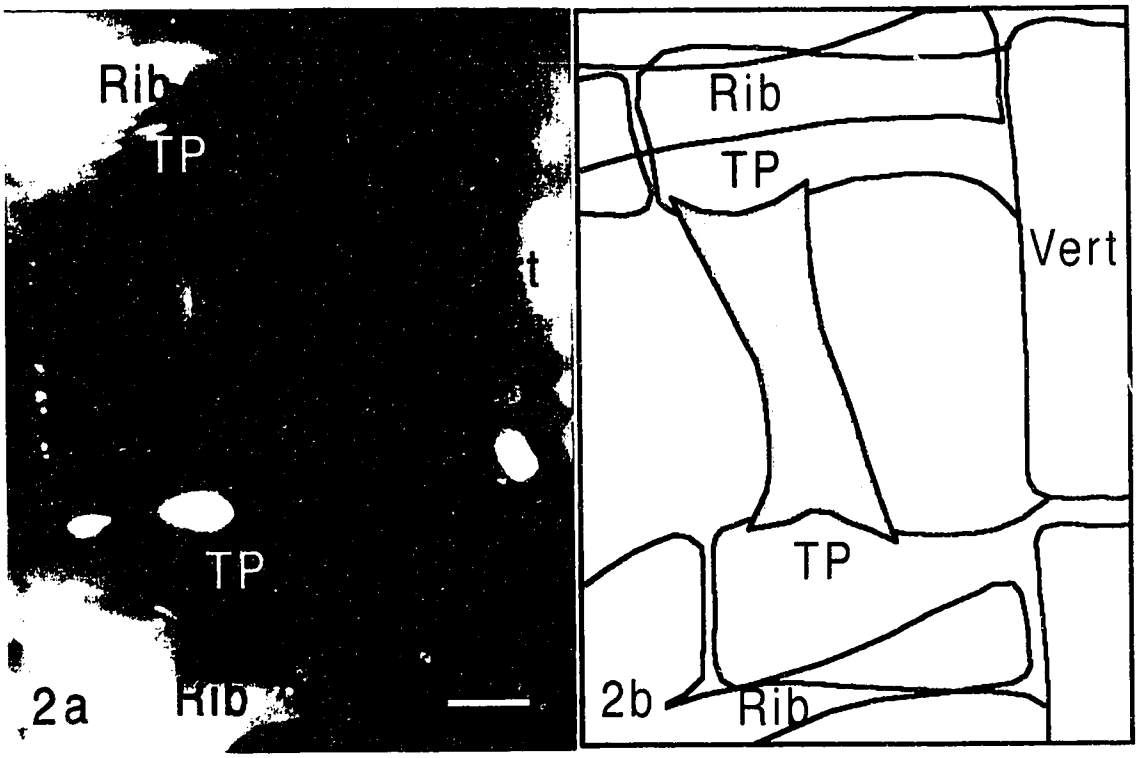


Fig. 3.2. (Bar = 1mm) a. A photomicrograph (anterior view) showing the anterior intertransverse ligament found in a young chicken. This ligament has similar sites of attachment as the SCTL in humans. The ligament becomes more prominent as the chicken gets older but is already well pronounced even at this young age. b. an explanatory drawing of the same. TP = transverse process AITL = anterior intertransverse ligament Vert = vertebral body

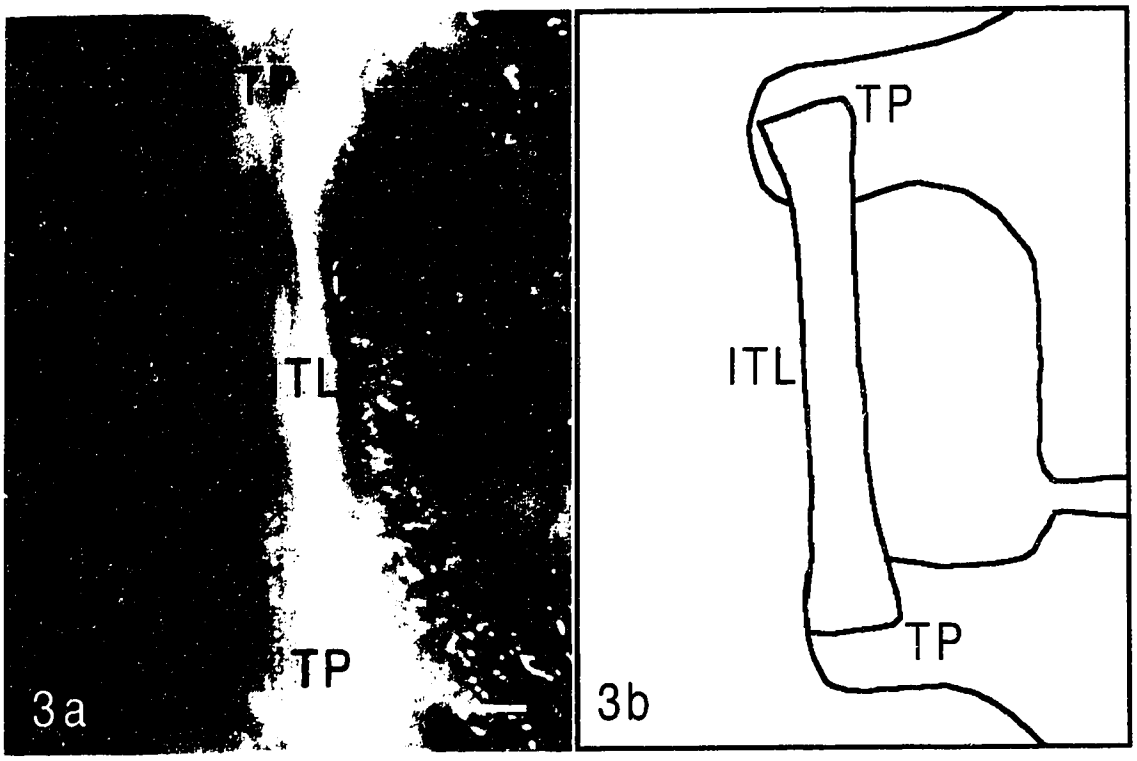


Fig. 3.3. (Bar = 1mm) a. A photomicrograph of the posterior region of the spine in a young chicken. The intertransverse ligament is a well defined ligament which connects two transverse processes. This ligament is also absent in quadrupedal animals. b. an explanatory drawing of the same. TP = transverse process ITL = intertransverse ligament

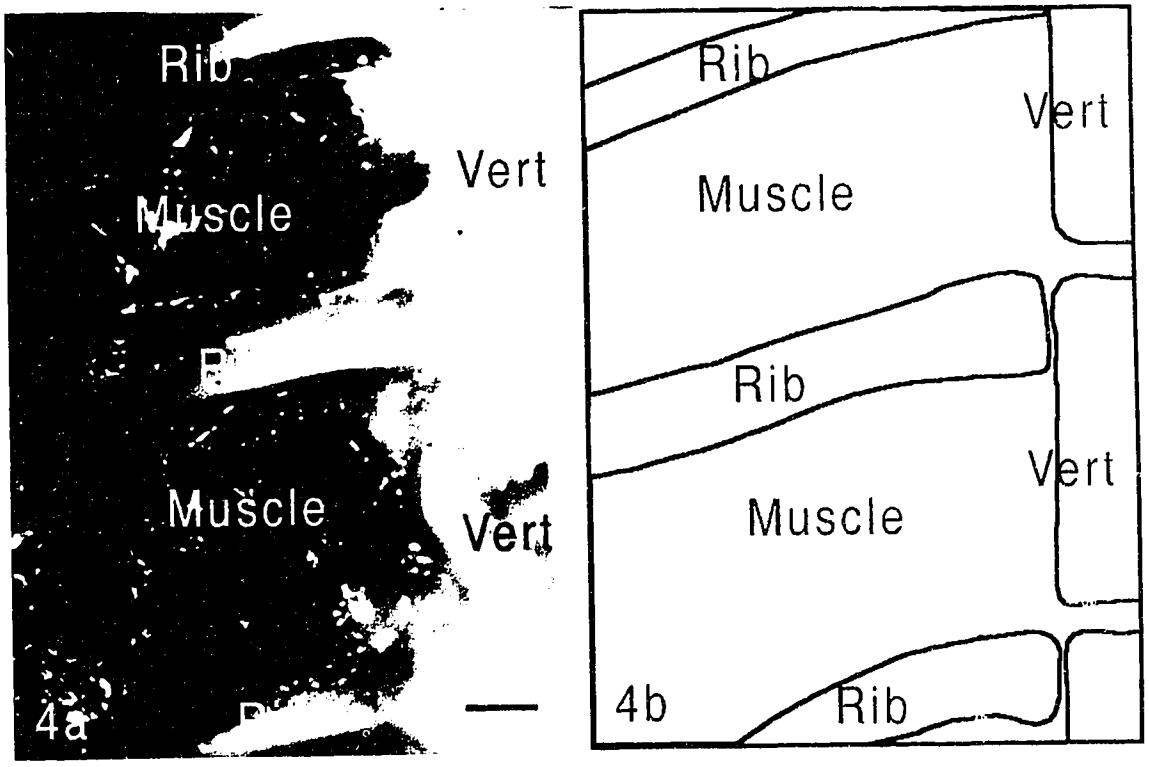


Fig. 3.4. (Bar = 1mm) a. A photomicrograph of the costovertebral region of a rat. No ligamentous tissue could be found among the abundant muscles. b. an explanatory drawing of the same. Vert = vertebral body

DISCUSSION

Although spinal ligaments have been well studied in humans (Williams et al. 1989), descriptions of the spinal ligaments in other animals have apparently not been well documented other than for a brief study of the SSL/ISL in dog, cat and baboon (Heylings, 1980) and a comparison of the iliolumbar ligament in both bipeds and quadrupeds (Pun et al. 1987). In particular, there does not appear to have been any study dedicated to the lateral thoracic spinal ligaments. In this study, we found that all species studied possessed prominent central spinous ligaments although they were less well developed in the quadrupeds. In contrast, only the bipeds (humans) and pseudo-bipeds (avians) possessed lateral spinal ligaments. In the quadrupeds, the absence of lateral spinal ligaments was conspicuous. The appearance of lateral spinal ligaments only in bipedal animals suggests that they might be important in supporting the spinal column for a bipedal stance although other functions are also possible and should be considered.

It is significant to note that the SCTL was well developed in humans and bipedal animals but was absent in the quadrupeds. It has been suggested that the erect posture of the human spine requires considerable ligamentous support and that lateral support is fundamental in keeping the spine straight in the saggital plane (Lindahl and Raeder, 1962; Schultz et al. 1972). Furthermore, the bipedal gait of the human constantly rotates the trunk and tilts the pelvis thereby requiring increased lateral support to remain balanced (Gracovesky, 1985; Townsend and Seireg, 1972; Marks, 1987). In particular, this form of locomotion will disturb the balance of the spine laterally and cause stress in the lateral structures of the spine. Conversely, the spine would be more unstable and prone to development of deformity if it was forced into maintaining an erect posture without proper structural adaptation. Such development of spinal deformity was reported by Tanaka et al. (1982) who observed both bipedal and quadrupedal rats fed with semicarbazide. He found that the frequency of occurrence of scoliosis was 82% in the bipedal rats and 13% in the quadrupedal rats. This significant increase in the development of scoliosis was attributed to the forced erect posture in the bipedal rats for a spine which was designed for a prone posture although it is possible that the experimental procedure used to create the bipedal rats would have made the spine inherantly unstable without the evolutionary changes accompanying the development of an erect posture. Similarly, the costotransverse ligament has been reported to be present in the rhesus monkey which appears to have a dual method of

locomotion. However, cutting of the costotransverse ligaments on one side results in the development of scoliosis (Thomas and Dave, 1985) presumably through removal of the lateral supportive forces for the vertebral column. The finding in this study that the lateral spinal ligaments are found only in those species that walk in a bipedal fashion with an erect posture suggests strongly that these ligaments are critical for the maintenance of an erect spine and that their absence or malfunction could lead to the development of spinal deformity such as is found in scoliosis where lateral instability and abnormal vertebral rotation are prominent features.

The evolution of spinal ligaments has been attributed to the biomechanical load of the spine (Pun et al. 1987) and therefore analysis of the load pattern of the spine could help in understanding the function of the spinal ligaments. The load pattern on the spine is dramatically changed from the prone posture to erect posture. In a bipedal stance, the spine acts as a column which is more liable to instability and needs to be balanced in both the saggital and coronal planes (Lindahl and Raeder, 1962; Schultz et al. 1972; Cappozzo, 1983) whereas in a quadrupedal stance the spine functions as a suspension mechanism. Based on these models, some developmental adaptations of the spinal ligaments have been suggested and identified. For example, Heilings (1980) reported that in the lumbar region, the dog and cat had only thin and poorly defined SSL/ISL. It was suggested that instead of needing to stabilize their spines, these quadrupeds needed to flex and extend greatly their lumbar spines to provide and accommodate for their running gait. In contrast, the strong collagenous SSL/ISL of the human can give good control of flexion of the spine during movement in its bipedal stance (Heilings, 1980). The results of the present study suggest that similar adaptations might have occurred in the erect spine of the bipeds in regards to the stabilizing functions of the lateral spinal ligaments.

It is interesting to note that the iliolumbar ligament also was found only in bipeds and was absent in quadrupeds (Luk et al. 1986; 1987), a finding similar to the lateral spinal ligaments investigated in this study. Specifically, the iliolumbar ligament was absent at birth in humans and only attained "full" differentiation in the second decade after birth (Luk et al. 1986). It was suggested that the stress across the lumbosacral junction as a result of the erect posture played an important role in stimulating the formation of the iliolumbar ligament and was not complete until puberty. The results of the current study suggest that an upright posture in the bipedal and pseudobipedal stance is related to the development of lateral spinal ligaments which are so conspicuously absent in

quadrupeds. Development of these ligaments during growth particularly in relation to acquisition of the upright stance warrants further study.

While Marks (1987) suggested that hominid bipedalism is a learned behavior, this study and those of others (Luk et al. 1986; Heilings 1980; Pun et al. 1987) have described several structural adaptations of the human spine that appear to be related to the maintenance of an upright posture. Clearly, failure to balance the erect spine may cause problems such as idiopathic scoliosis. From reviewing the literature, it is significant to note that quadrupeds do not appear to develop idiopathic type scoliosis naturally while there are many reports of avian models for idiopathic scoliosis and adolescent idiopathic scoliosis is not uncommon among humans (Rogala and Drummond, 1978). This difference might be attributed to the prone posture and locomotion pattern of the quadrupeds which does not apply the type of loads that challenge the lateral stability of their spine. In contrast, weight bearing and the dynamics of bipedal locomotion lend the spine liable to lateral instability. In this regard, the chicken with its pseudo-bipedal stance and form of walking has been reported to develop idiopathic type scoliosis (Machida et al. 1994; Rigdon and Mack, 1968) and, coupled with the presence of well-defined, prominent lateral spinal ligaments, could be an ideal animal model to examine the role of lateral spinal ligament in the lateral balancing of the spine and the development of idiopathic scoliosis.

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CHAPTER 4

The Nature and Distribution of the Innervation of Human Supraspinal and Interspinal Ligaments.

INTRODUCTION

Although ligament has been traditionally considered only as a mechanical structure[3,10,24], there is increasing evidence to suggest that ligaments are innervated and can participate in active neuromuscular reflexes[14,2,22]. The majority of studies on the innervation of ligaments have been carried out on knee ligaments and have shown that they are innervated by both encapsulated and unencapsulated nerve endings[5,13,14,19,21]. By comparison, there have been few studies on the innervation of spinal ligaments[1,6,7,15,16]. Quite surprisingly, these few studies have often found only nerve fibers and not specialized nerve endings. As an exception, Yahia et al.[25], using a gold chloride method, observed free nerve endings, Pacinian corpuscles, and Ruffini corpuscles in human supraspinous and interspinous ligament (SSL/ISL). However, since blood vessels and elastic fibers can also be stained by the gold chloride method there is the possibility of confusion in the recognition of some of these elements. To avoid such ambiguity, many researchers prefer to use precise antibodies against the neurofilament proteins (NFP) or neuropeptides to investigate nerve structure and function. For example, Rhalmi et al.[20] studied the SSL/ISL complex from seven subjects using an antibody against NFP but found only free nerve endings and nerve bundles and no end organs such as Ruffini or Pacinian corpuscles. This is surprising as these end organs have been found in other ligaments at other sites in the body using similar methodology[12,21] and contrast sharply with the results of Yahia et al[25].

A version of this chapter has been published.

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It is possible that end organs such as Ruffini corpuscles in the spinal ligaments are not NFP-immunoreactive (NFP-IR) but this would seem unlikely considering their positive reaction elsewhere in the body. Therefore, in this study, it is proposed to reinvestigate the innervation of the midline spinal ligaments and, in particular, to confirm whether or not these ligaments are innervated by specific nerve endings. Furthermore, as a systematic and quantitative study of the innervation of spinal ligaments has not yet been reported, it was proposed to describe the characteristics and distribution of the innervation of these ligaments in detail.

Knowledge of the overall distribution of nerve endings in SSL/ISL would be helpful because it would provide some indication of the mechanisms involved in maintaining the integrity of the ligaments, especially in relation to the control of its blood supply and the distribution of the collagen bundles. It would also provide information relating to the possible presence of a neurological feedback mechanism involved in protection of the spine from injury as well as a feedback mechanism that might be involved in maintaining the stability of the spine. This would be particularly relevant to the development of diseases such as scoliosis where lack of such stability appears to be a major component. Consequently, in this study, a monoclonal antibody against NFP was used to study the innervation of the SSL/ISL complex on ten human subjects undergoing surgery for spinal decompression. The main purpose of this study was to characterize the innervation of the SSL/ISL complex of the lumbar spine both morphologically and quantitatively and relate the findings to mechanisms involved with maintenance of the ligaments, and protection and stability of the spine.

MATERIALS AND METHODS

The SSL/ISL from ten patients undergoing surgery for spinal decompression were studied. The age of the patients averaged 44 ± 15 years with a range from 35 to 63 years. There were 2 cases of lumbar disc herniation and 8 cases of lumbar stenosis. The patients appeared completely healthy other than for these problems. The ligaments were collected immediately after detachment either from segment L4 to L5 or from L5 to S1 as shown in Figure 4.1. The complete suprapinous ligament was harvested along with a variable portion of the interspinous ligament, not including any portion of the ligamentum flavum. The two ligaments were treated as one complex (SSL/ISL) in this study because there was no clearly defined border between them and they could not be distinguished either morphologically or histologically.

The specimens were removed from the patients at the time of surgery along with a small portion of their bony attachment to the spinous processes using a scalpel since cauterization was found to destroy the antigeneity of the NFP and also the structure of neurovascular bundles inside the ligaments. The right and cranial sides of the specimen were labeled with ink using a hypodermic needle to maintain the orientation of the specimens during subsequent processing and observation. Following removal, the specimens were immediately put into an ice-box and stored for up to 30 minutes. Further dissection was then performed to remove as much as possible of the paraligament (extraneous loose connective tissue that is not part of the ligament) and excess bone tissue and expose the ligaments clearly. The specimens were embedded in O.C.T. (*o*-chlorotoluene - Miles, Elkhart), frozen in isopentane cooled by liquid nitrogen and stored at -80° C in an air-tight container. Using a cryotome (International Equipment Co., Needhan), transverse, serial sections of 50 μm thickness were made of five areas in each of the specimens. The areas were selected to represent the whole ligament (Figure 4.1).

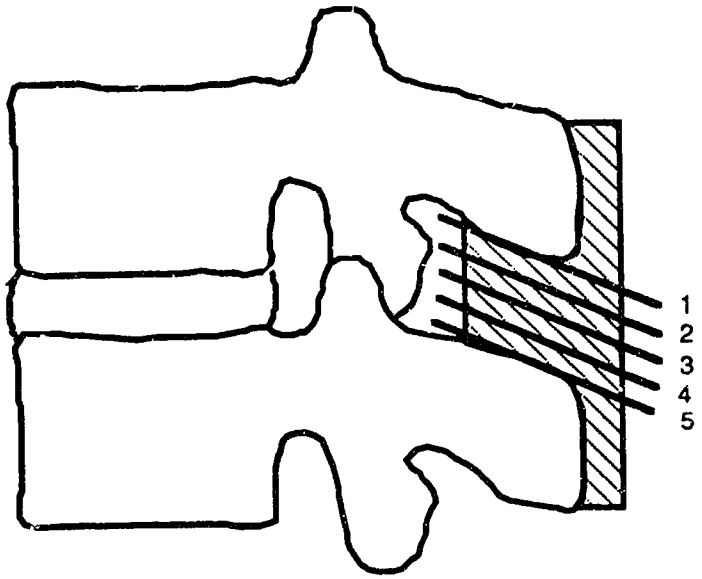


Figure 4.1. A diagram which shows a lateral view of the SSL/ISL complex between the spinous processes of two adjacent vertebrae. Five areas for study were selected as shown: at the upper and lower borders close to the bony attachments; the middle of the ligaments; and two areas inbetween to observe the distribution pattern of NFP-IR nerve elements.

The sections were thaw-mounted onto gelatin-coated slides and post-fixed with acetone for 10 minutes at 4° C. Before further staining, the sections were magnified (50x) and the image projected onto a sheet of white paper. The outline of each section was traced together with the similar projection of a 2 mm glass scale for magnification accuracy. The sections were washed with phosphate buffered saline (PBS), pH 7.4, and pretreated with 0.4% Triton X-100/PBS for 10 minutes. The specimens were washed with PBS and incubated with mouse monoclonal antibody against 200 kD subunits of NFP (SIGMA, St. Louis) for two hours in a humidified chamber at 20° C. The antibody was prediluted 1:100 with PBS containing 3% bovine serum albumin. The sections were then washed again with PBS and treated with goat antimouse antibody conjugated with fluorescein isothiocyanate (FITC) for 1 hour in a dark, humid chamber at 20° C. The secondary antibody was prediluted to 1:150 with PBS. The sections were covered with a coverslip held in place with fluoromount, a quench-preventing mountant (BDH, Poole).

Rat sciatic nerve and human intercostal nerve were used as positive controls (based on results from previous experiments) and underwent the same staining procedure as described previously for the spinal ligaments. Samples of similar tissue were used as negative controls and underwent the same staining procedure except that no primary antibody was applied. The positive controls showed strong labeling of axons and the negative controls showed no fluorescence except for a little intermediate-level background autofluorescence of blood vessels.

Observations of each section were made using a Zeiss ORTHOPLAN fluorescence microscope (Carl Zeiss, Oberkochen) which utilizes UV light. The characteristics and distribution of nerve elements in the ligaments were observed and documented while nerve elements in the adjacent surrounding paraligaments were disregarded. Photographs of appropriate examples were taken using TMAX black and white film (KODAK, Rochester). Ruffini and Pacinian corpuscles are relatively large and cannot be focused in total using an ordinary microscope. Therefore, a confocal microscope (Leica Lasertechnik, Heidelberg) equipped with laser UV light was used to improve focussing ability by optically sectioning the specimens and creating images at specific depths. These images were used to reconstruct a three dimensional image by using a Voxel View (Vital Images Inc., Fairfield. Iowa) software package on a Silicon Graphics computer. The sizes of various nerve elements were also measured using the confocal microscope. The reconstructed 3-dimensional images were then photographed

using a digital film recorder.

To facilitate documentation of the nerve investment pattern, a morphological identification system was developed and employed. The NFP-IR nerve tissues were identified as Ruffini corpuscle (Figure 4.2A, 4.2B), Pacinian corpuscle (Figure 4.3A, 4.3B), free nerve ending (Figure 4.4) or single nerve fiber (Figure 4.5A, 4.5B), small nerve bundles containing less than 10 axons (Figure 4.6A) and large nerve bundles containing more than 10 axons (Figure 4.6B). To distinguish between free nerve endings (Figure 4.4) and single nerve fibers (Fig 4.5A), three consecutive serial sections were observed. Free nerve endings were identified if they were not continuous in all 3 sections, whereas single nerve fibers were identified if they were present in all sections. The site of the observed nerve tissues, their identification and presence of blood vessels were then transferred to their corresponding sites on the predrawing of their own section (see above). The positions of the edge of the sections as well as all the nerve tissues and blood vessels were digitized from the predrawing using an HP digitizer (Hewlett-Packard Co., Fort Collins, Colorado).

To document the distribution of the NFP-IR nerve tissues on sections of the SSL/ISL, the location of each nerve tissue was normalized with respect to its distance from the periphery. An anteroposterior line was drawn in the midline on the digitized outline of the section. This divided the ligament section into left and right portions. The distances of each nerve element to the midline and lateral edge of the ligament were calculated. The normalized distance was then defined as the distance to the midline divided by the sum of the two distances. This allowed comparisons to be made of the nerve element distribution in relation to the midline of the ligament between different subjects, sections and sites. The normalized distribution pattern of the ligament innervation was then presented using histograms (Figure 4.7 A-F). Peripheral areas were considered to be at a distance greater than 70% from the midline to the edge of the ligament.

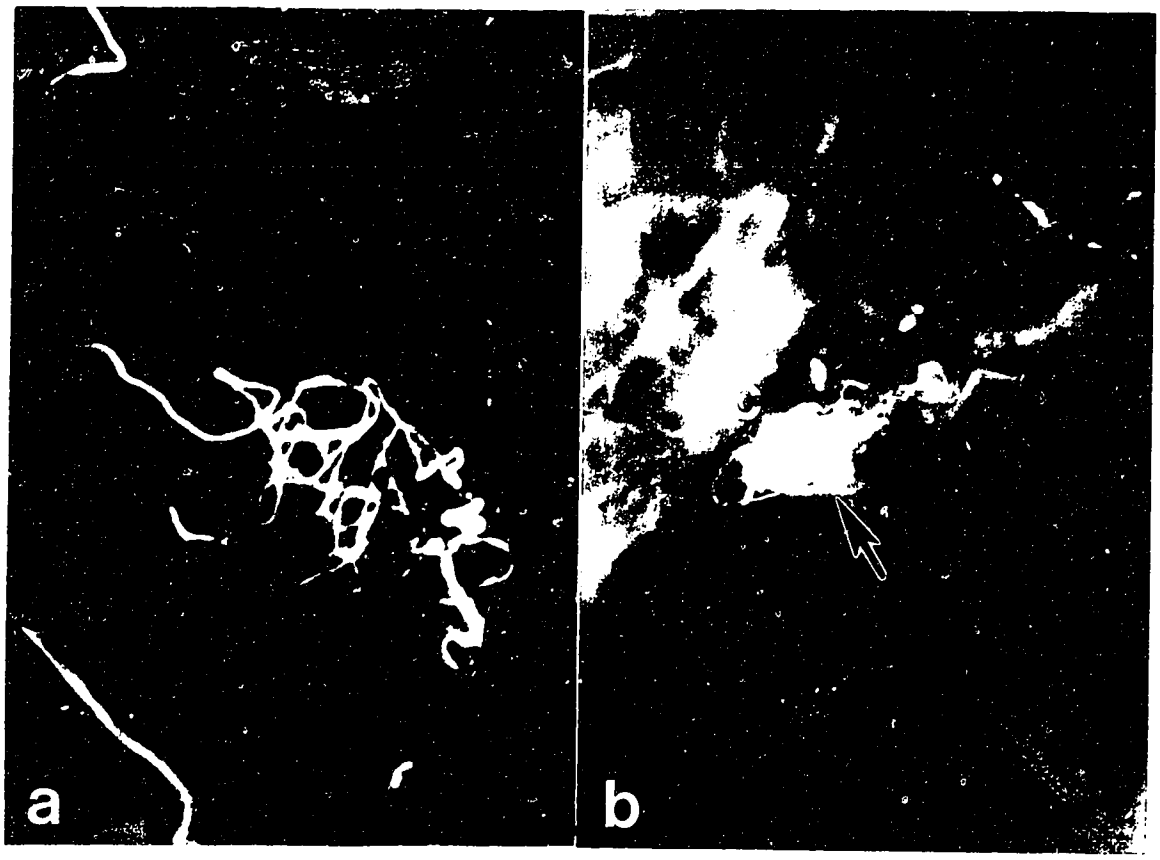


Figure 4.2. a. A reconstructed image of Ruffini corpuscles using confocal microscopy (X450). The Ruffini corpuscles were NFP-IR and innervated by multiple sensory axons; the axons branched and coiled to form the corpuscle. No NFP-IR lamellar cells were observed. b. (X250) shows a Ruffini corpuscle (arrow) using ordinary microscopy. The "C" indicates a cross-sectional view of dense collagen bundles.

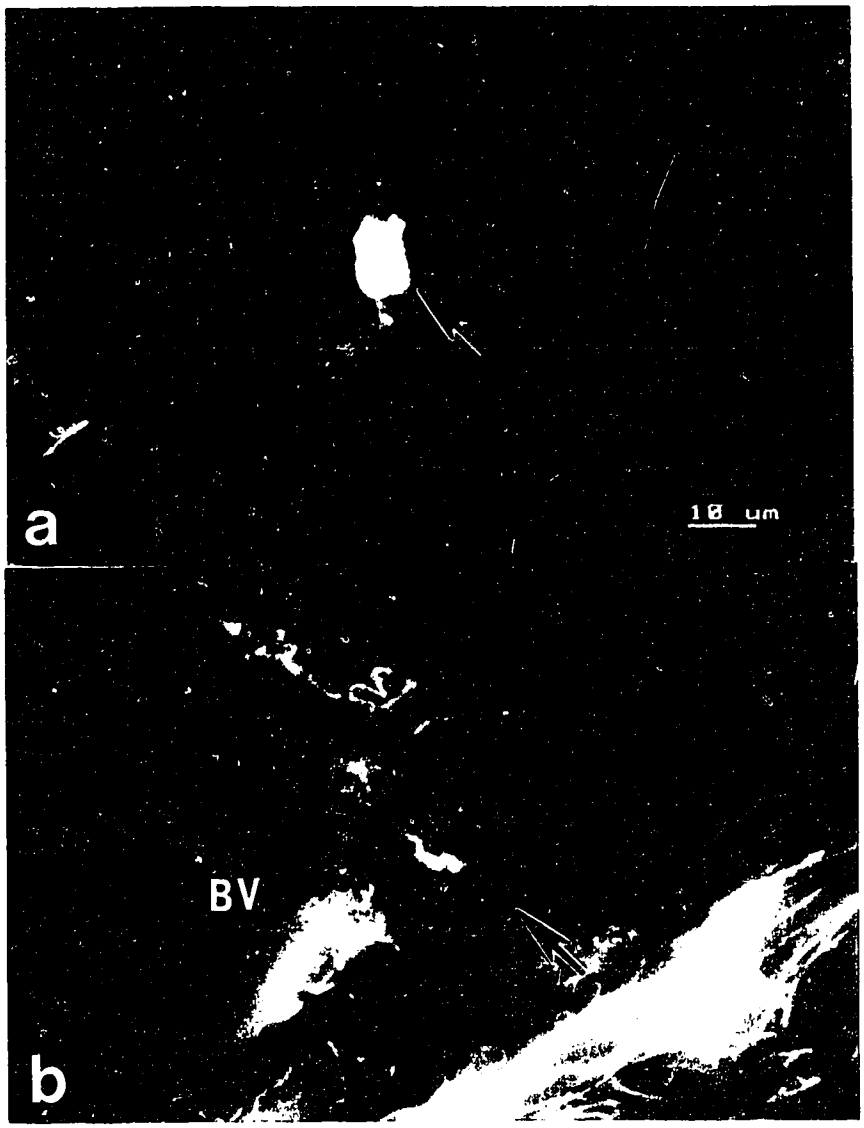


Figure 4.3. a. (X450) shows a Pacinian corpuscle reconstructed from confocal microscopy images. The Pacinian corpuscle was innervated by a thick NFP-IR axon (arrow) that is surrounded by a multi-layer of weakly labeled lamellar cells. b. (X250) an ordinary photomicrograph showing another Pacinian corpuscle (arrow) that is close to blood vessels (BV).

Figure 4.4. An NFP-IR sensory axon (arrow) is parallel to the collagen bundles. The sensory axon ramified and faded into dense collagen bundles and there was no specified end organ (X250).

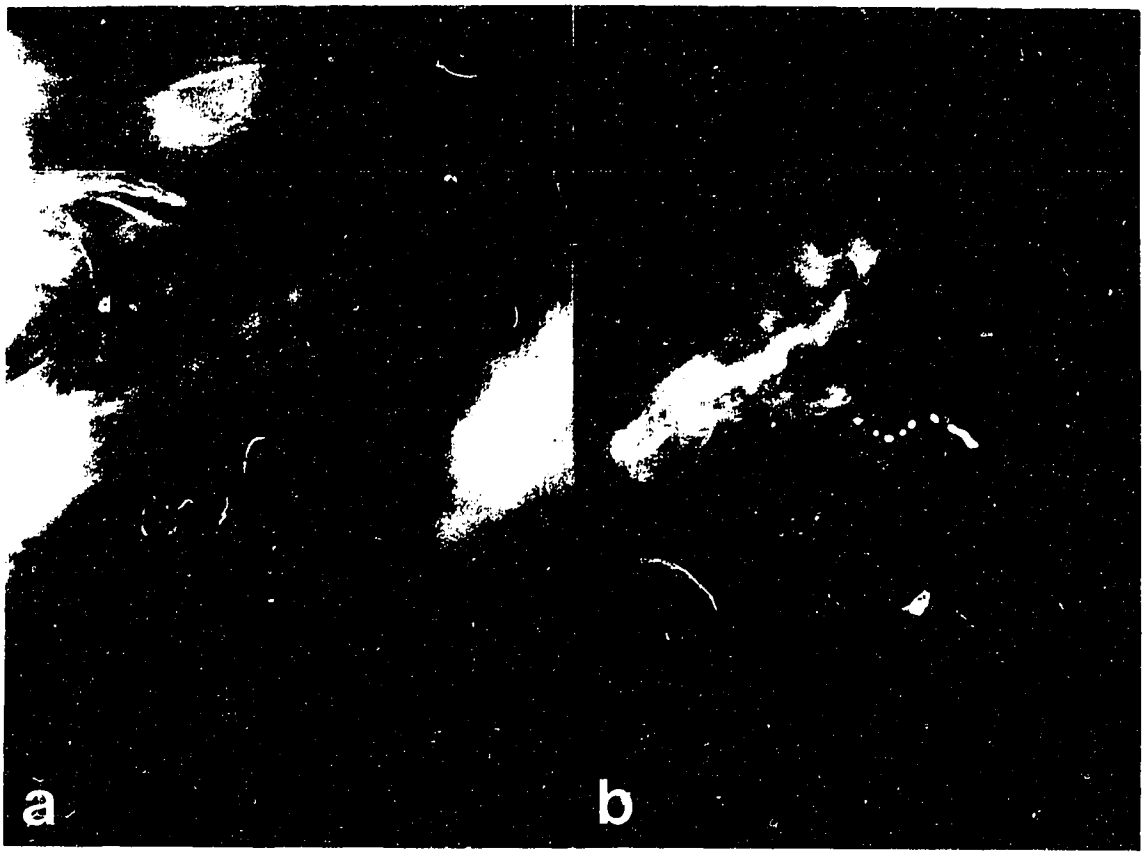


Figure 4.5. a. (x250) shows a single nerve fiber investing dense collagen bundles. The NFP-IR fiber was observed in three consecutive sections and did not ramify. Because the section was thick (50 μm) and the whole fiber could not be brought into focus, the end segments of the fiber are unclear. b. (X250) shows a varicose single fiber which had both continuous NFP-IR labeling and bead-like labeling. These fibers usually do not occur in the vicinity of blood vessels. The length of the beads did not match that of the nodes of Ranvier.

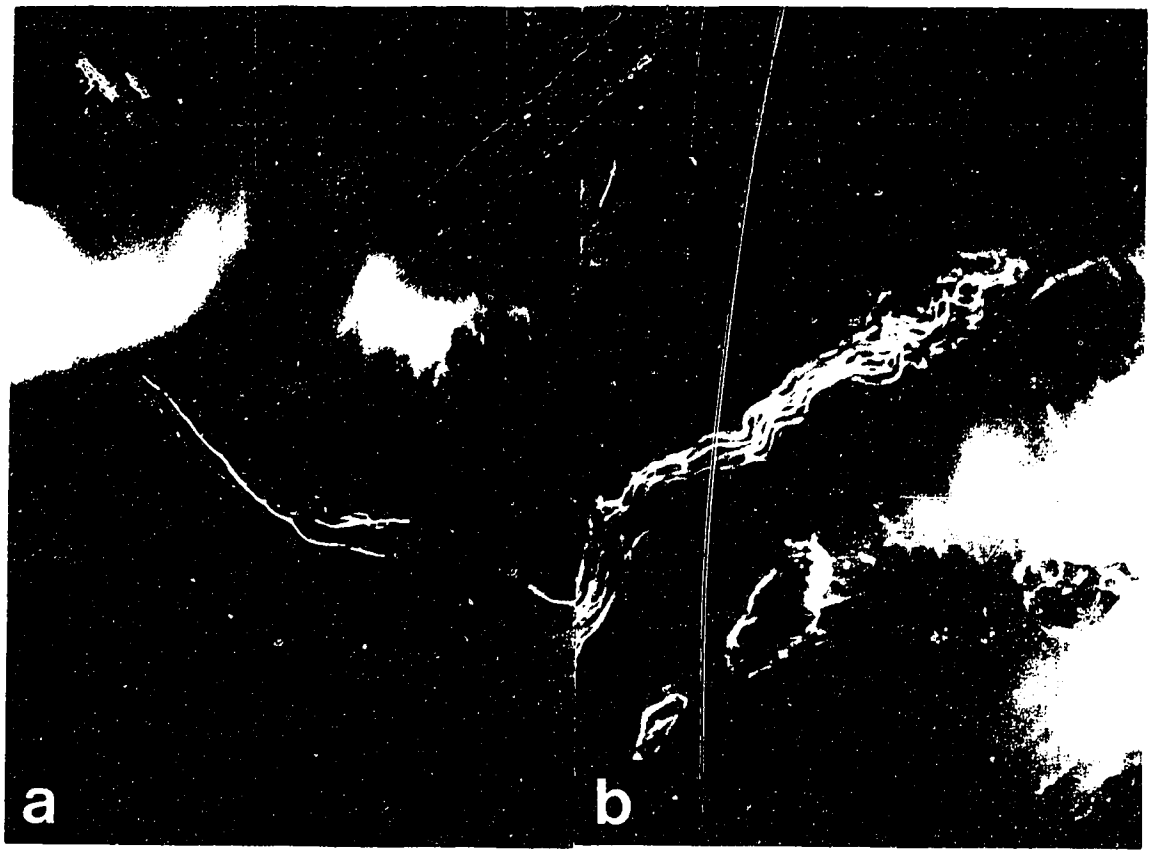


Figure 4.6. A small nerve bundle and a large nerve bundle in collagen bundles are shown in a. and b. respectively (X250). The nerve bundles consisted of relatively thick and thin fibers. Thick fibers sometimes had a varicose nature in certain segments. Note that the large nerve bundle expands in diameter inside the collagen bundles. Since there was usually no ramifying in adjacent sections, it is suggested that this is a specific nerve ending capable of detecting tension in ligaments.

To provide a superficial assessment of quantitation, the innervation density was defined as the pooled number of nerve elements per square centimeter of the sections. Specific comparisons of innervation densities were made between the five areas of the SSL/ISL defined previously (Figure 4.1) to evaluate the variation of innervation between the ends of the ligaments at their bony attachments and the areas in between. The analysis used ANOVA with a significance level of P=0.05. Comparisons were also made among the 10 individual subjects again using ANOVA. The analysis also focused on whether the nerve elements were symmetrically distributed on the left and right sides of the SSL/ISL. Each nerve element was assigned either left or right according to the midline previously described. The innervation densities between left and right sides were compared using Student's t-test.

RESULTS

Observations

The SSL/ISL complex consisted of dense collagen bundles interwoven with areas of relatively loose connective tissue which was usually close to blood vessels and it was in these areas of loose connective tissue that the majority of nerve elements were observed. Nerve elements were found in both the dense collagenous bundles and the loose connective tissue. and were easily visible and clearly distinguishable from any background autofluorescence.

The Ruffini corpuscles were invested by both NFP-IR thick (3 - 7μm) and thin fibers (<3μm) (Figure 4.2A, 4.2B). The fibers were highly branched and coiled forming a cluster. No surrounding NFP-IR capsular cells were observed. The nerve fibers to the Ruffini corpuscles usually originated from the paraligament or paravascular tissue, while the Ruffini corpuscles themselves usually were close to dense collagenous tissue.

The Pacinian corpuscles were invested by NFP-IR single, thick fibers which were encircled by multi-layers of lamellar cells (Figure 4.3A, 4.3B). The lamellar cells were weakly NFP-IR. Most of the Pacinian corpuscles were close to blood vessels.

The SSL/ISL was found generally to be innervated by NFP-IR thin, single nerve fibers (Figure 4.5A) which invested both blood vessels and dense collagen tissue. NFP-IR

thick and varicose single fibers (Figure 4.5B) were less frequently seen in the SSL/ISL and usually did not have a close relationship with blood vessels. Free nerve endings (Figure 4) were rarely observed, usually ramifying from a single nerve fiber and fading into the collagen bundles. Since it was difficult to distinguish technically the single nerve fibers from free nerve endings using the NFP-IR method, the incidence of these two structures were pooled in the following analysis.

Small and large nerve bundles (Figure 4.6A, 4.6B) were found more peripherally in the ligaments sometimes in accompaniment with blood vessels. They usually appeared in the relatively loose tissue within the ligaments or between thick collagen bundles, but they also penetrated into the collagen bundles (Figure 4.6B).

Distribution pattern of nerve elements in the SSL/ISL

The overall, average innervation density for the sections of 50 μm was 19.2 ± 5.1 nerve elements per square centimeter, ranging from 12.3 to 28.3 among subjects. There was no significant difference in the overall innervation density of the SSL/ISL from patient to patient ($P>0.05$). The innervation density among the five areas (Table 4.1) was also not significantly different ($P>0.05$). This indicates that the nerve density is the same throughout the ligaments longitudinally. It was also found that there was no significant difference ($P>0.05$) between left and right sides of the ligament in terms of nerve elements per subject for each of the five different kinds of nerve tissues (Table 4.2).

Table 4.1. Comparison of the average overall innervation density per square centimeter of ligament sections (n=10)

Section 1	Section 2	Section 3	Section 4	Section 5
18.2 ± 10.5	22.1 ± 8.1	18.6 ± 6.9	18.7 ± 8.2	18.5 ± 11.9

$P > 0.05$

Table 4.2. Comparison of observed nerve elements per subject between left and right sides (n=10).

	Large nerve bundle	Small nerve bundle	Single nerve fiber	Ruffini corpuscle	Pacinian corpuscle
Left	5.9 ± 5.8	11.9 ± 8.5	5.2 ±2.5	6.0 ± 4.3	3.2 ±3.4
Right	5.2 ± 5.2	10.3 ± 5.9	6.5 ± 2.8	5.6 ± 3.4	2.6 ± 1.7

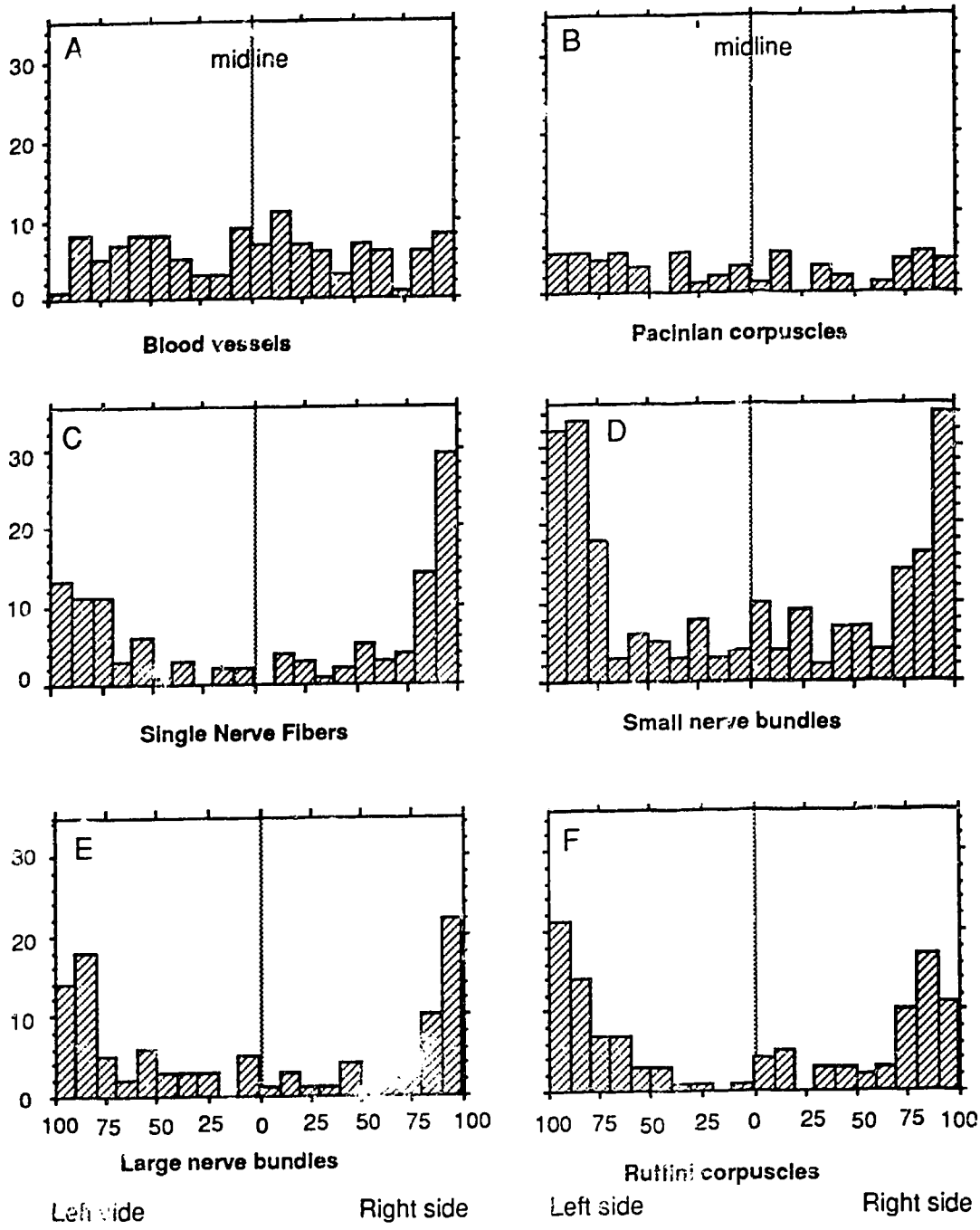
$P > 0.05$

Blood vessels were scattered randomly throughout the ligaments (Figure 4.7A) and from left to right with no apparent specific distribution pattern. Interestingly, the Pacinian corpuscles were also distributed randomly (Figure 4.7B) most often in close association with blood vessels. The majority of large nerve bundles, small nerve bundles and single nerve fiber/free nerve endings were mostly located symmetrically in the periphery of the SSL/ISL complex and their densities gradually reduced as they reached the midline (Figure 4.7C, 4.7D, 4.7E). Within the outer 30% of the peripheral area of the ligament sections, 66% of large nerve bundles, 67% of small nerve bundles and 70% of single nerve fibers were located. Ruffini corpuscles were also found to be distributed more densely (71%) in the periphery of the ligaments (Figure 4.7F) which is a different distribution to that of Pacinian corpuscles (Figure 4.7B). The average relative densities of each nerve element are shown in Table 4.3.

Table 4.3. The average density of nerve elements per square centimeter of ligament section (n=10).

large nerve bundle	small nerve bundle	single nerve fiber	Ruffini corpuscle	Pacinian corpuscle
3.3 ± 1.1	6.6 ± 1.1	3.8 ± 0.4	3.7 ± 1.2	1.8 ± 1.0

Figure 4.7. Histograms to show the normalized regional nerve density from left to right of the SSL/ISL complex. The approximate width of the total ligament is 6mm. A. shows that the blood vessels were distributed randomly from the left to the right of the SSL/ISL. B. shows that the Pacinian corpuscles were also randomly distributed. C., D., and E. show that the single nerve fiber, small and large nerve bundles have the same distribution pattern from side to side. They were more densely distributed in the periphery of the ligament and more sparsely distributed towards the middle. F. shows that the distribution pattern of Ruffini corpuscles is different to that of Pacinian corpuscles being predominantly in the periphery of the SSL/ISL.



Relative distance of nerve elements from the midline to the edge of SSL/ISL.

DISCUSSION

The results from this study have shown that the SSL/ISL complex is richly innervated as nerve elements were found throughout the ligament. Nerve elements were found equally in all areas along the length of the ligament and were equally distributed between left and right sides. However, there were differences in the distribution patterns of the specific nerve endings. Pacinian corpuscles were found equally spread throughout the ligament and were equally distributed between peripheral and deep regions. They were always closely associated with blood vessels. In contrast, the Ruffini corpuscles were found more frequently in the periphery of the ligament unassociated with the blood vessels although they too were found equally throughout the complete length of the ligament. These results contrast sharply with the results of Konttinen *et al.*[16] who suggested that ligament in general is practically aneural. However the results are in accord with the findings of El-Bohy *et al.*[4] and Rhalmi *et al.*[20] who briefly reported that nerve elements could be found within the SSL/ISL, but who did not elaborate upon these findings in any great detail.

Traditional staining techniques that identify nerves and nerve endings such as those based on gold chloride or silver nitrate, have been used extensively to establish an identification system for recognition of specific nerve fibers and endings[5]. However, these techniques stain both the myelin sheaths as well as the axons. Antibodies against neural filament proteins, as used in this study, are very specific and precise identification can be made, including distribution of specific molecules and detailed structure of the axons. In particular, nerve fibers as small as 0.5µm in diameter can be identified using the confocal microscope which is sufficient to include most if not all nerve fibers. The morphology and appearance of nerve elements are different when compared with the more established and well documented descriptions[5]. A new identification system using these types of antibodies needs to be developed so that recognition of the various nerve elements is consistent and no confusion arises. This current study was started to develop such a system which can be perfected as more information is collected. In particular, correlation between the diameter of specific axons and the diameter of the surrounding myelin sheaths needs further study.

The results presented in this study showed the SSL/ISL to be richly innervated, but the actual, total innervation of the ligament was probably underestimated. The nerve elements that were identified in the loose connective tissue could be traced into the

dense collagen bundles but further tracing was often difficult because of the strong autofluorescence associated with the dense collagen bundles. Although this autofluorescence was partially filtered by use of the confocal microscope which often revealed nerve tissue inside the dense collagen bundles, it was assumed that this autofluorescence sometimes masked the fluorescence of additional nerve elements. This would affect the observation of nerve endings in the collagen bundles in particular.

Most nerve bundles were seen at the periphery of the ligament and then were observed to taper into the dense collagen bundles where continued tracing was difficult. However, in those cases where tracing could be continued, many of the small nerve bundles terminated in the dense collagen bundles with axons that curled and appeared to spiral, forming small Ruffini-like organs (Figure 4.6). These structures may be an adaptive form of Ruffini corpuscle in the dense collagen bundles since the structure of Ruffini corpuscles has been reported to be modified by surrounding connective tissues[9]. Similarly, further division of small nerve bundles was seen rarely. While this supports similar observations made by Rhalmi *et al.*[20] it requires further study if any suggestion of function is to be made.

Pacinian corpuscles are fast adaptive corpuscles and have been reported to be capable of sensing fast motion and acceleration[5,9,20]. In this study, Pacinian corpuscles were observed throughout the SSL/ISL complex. They consisted of a thick central axon and several layers of lamellar cells, similar to those reported elsewhere in studies that have also used immunohistochemistry[23]. They were always found in close association with blood vessels and were not associated or correlated with the collagen bundles. This distribution is similar to that for Pacinian corpuscles found in the cruciate ligaments of the knee-joint[18]. With this distribution, it is suggested that Pacinian corpuscles function as sympathetic afferent endings that initiate reflexes concerned with the local control of blood flow[18]. The results of the current study would support such a suggestion. However, Yahia et al.[25] from the results of their histological studies proposed that Pacinian corpuscles in the SSL/ISL complex may act as a safety signal for ligament overload during fast rates of load application. While this might be possible, the distribution of Pacinian corpuscles and their close association with blood vessels also strongly suggests a vascular function for these end organs. Whether Pacinian corpuscles participate in sensing the mechanical load applied to the SSL/ISL complex as suggested by Yahia et al.[25] is unclear.

Ruffini corpuscles are often classified as encapsulated nerve end organs[5,17], but many studies have failed to show any apparent capsular structure of Ruffini corpuscles in the ligaments[9,25]. This current study too failed to showed any NFP-IR capsular cells around Ruffini corpuscles which suggests that there might indeed be none although it is possible that the capsular cells simply might not be responsive to the NFP antibody. Halata[9] commented that absence of a capsular cell does not necessarily alter the function of the corpuscle, it only decreases the area working as a receptive field for the individual corpuscle. The results of the current study would support the suggestion that Ruffini corpuscles may exist in different forms according to the surrounding tissue.

Ruffini corpuscles are slow adaptive nerve end organs and sensitive to static motion especially to stretch and providing awareness of joint position and movement[5,8]. They are particularly common in articulations where static position sense is necessary for the control of posture[14,22]. In the present study, Ruffini corpuscles were frequently observed close to dense collagen bundles and showed a different distribution pattern to that of blood vessels and Pacinian corpuscles. The distribution pattern of the Ruffini corpuscles perhaps reflects their non-vascular associated nature. In contrast, the closeness of Ruffini corpuscles to dense collagen bundles supports the suggestion that they are involved in the detection of mechanical load carried by the SSL/ISL complex[25]. In this respect, the significance of Ruffini corpuscles could be similar to those in the cruciate ligament where it was found that the triceps-plantaris and the posterior biceps-semitendinosus muscle spindles could be activated by stretching the posterior cruciate ligament of the knee which stimulates Ruffini corpuscles[22]. However, it has now been suggested that the actual innervation might be located in the fibrous capsule rather than in the ligaments themselves. Nevertheless, this is an example of a protective neurological feedback mechanism and might be duplicated in a similar mechanism associated with protection and stability of the spine.

Ruffini corpuscles were found to be distributed symmetrically between the two sides of the SSL/ISL. They were also more densely distributed in the periphery of the ligament. This suggests that the Ruffini corpuscles might be innervated by their own ipsilateral pathway reflecting the symmetrical system present throughout the human body. Such a system might allow discrimination of unbalanced stretching of the SSL/ISL in the coronal plane. When the spine is bent laterally, the part of the ligament on the convex side of the spine would be stretched more than the concave side. While both sides have the same innervation density, the difference of the stretching may allow an awareness

of unbalanced posture of the spine. This suggests that the innervation of the SSL/ISL may participate in active balancing of the spine laterally and its dysfunction may be an important element in the production of scoliosis where lateral instability of the spine is so apparent.

Both the large and small nerve bundles were found to consist of both relatively thin and thick fibers. In addition, there were also varicose fibers which appeared as small, interconnected areas of bright fluorescence, suggestive of small vesicles or regional concentration of NFP. These were similar to varicose nerve fibers reported elsewhere[1]. It is interesting to note that the length of the NFP-IR beads did not match that of the nodes of Ranvier and leaves their function yet to be determined.

The distribution patterns of the various nerve elements possibly reflect their function to some extent. Pacinian corpuscles were always found in close association with blood vessels which suggests that they are very much involved with local blood flow control. In contrast, Ruffini corpuscles were found more frequently in the periphery of the ligaments, closely associated with the dense collagen bundles. This reflects their non-vascular nature and suggests that they are more involved in the detection of mechanical load within the SSL/ISL complex. Similarly, the close relationship of the large and small nerve bundles with the dense collagen tissue also supports the idea that their endings are mainly involved in sensing the mechanical loads being applied to the collagen bundles during movement.

The extent of the innervation and the distribution pattern of the nerve elements found in this study lend support to the concept of ligaments being part of neurological feedback mechanisms for the protection and stability of the spine. While being important for understanding the possible pathology of lower back diseases, these mechanisms might also be significant in the development of other diseases such as scoliosis where spinal instability is so evident. For the future, it is intended that the current study will be extended to include the lateral spinal ligaments which appear to be more suited for the maintenance of lateral spinal stability[11].

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CHAPTER 5

The spatiotemporal development of innervation in spinal ligaments of chickens.

INTRODUCTION

Although ligaments in general have been traditionally viewed simply as mechanical structures (Hindle et al. 1990; White and Panjabi, 1990) there is much evidence to show that they are well innervated with both simple free nerve endings and encapsulated mechanoreceptors (Freeman and Wyke, 1967; Sojka et al. 1983; Sjölander et al. Katonis et al. 1991; Biedert et al. 1992; Anmed et al. 1993; Michelson and Hutchins, 1989, 1995). It is thought that this innervation provides proprioceptive information which ultimately contributes to muscle coordination around joints designed to increase stability and prevent damage (Freeman and Wyke, 1967; Johansson et al. 1991a, b; Michelson and Hutchins, 1995). Although the precise mechanism by which the mechanoreceptors in the ligaments accomplish this function is not known, it is generally thought that the sensory input influences gamma motor neuron output and subsequently affects the length of muscle spindle fibres (Freeman and Wyke, 1967; Johansson et al. 1991a, b; Michelson and Hutchins, 1995).

Most of the studies of ligament innervation have focused on the knee joint but studies of spinal ligaments have also provided evidence to support the concept of ligaments being involved in a protective, proprioceptive feedback mechanism. For example, it has been shown that spinal ligaments are mechanically important for maintaining stability of the spine especially in an erect posture (White and Panjabi, 1990; Jiang et al. 1994, 1995b) and they have been shown to be richly innervated by a variety of nerve endings (Wyke, 1975; Yahia et al. 1988; Sojka et al. 1983; Cavanaugh et al. 1989; Sjölander et al. 1989; Gronblad et al. 1991; Pedersen et al. 1993; Yamashita et al. a, 1993b; Jiang et al. 1995a). Consequently, it has been suggested that spinal ligaments participate in the active control of spinal balance by providing proprioceptive feedback

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through a neuromuscular reflex mechanism (Ferrell and Hart, 1980; Avramov et al. 1992; McGlashen et al. 1991; Ashton et al. 1992; Jiang et al. 1994, 1995a; Michelson and Hutchins, 1995) similar to that being investigated and described in the knee joint, particularly in relation to the cruciate ligaments (Grigg et al. 1978; Ferrell and Hart, 1980; Grigg and Hoffman, 1982; Sojka et al. 1983; Sjölander et al. 1989; Beard et al. 1993, 1994). Presumably, localised reflex activity along the spine would be directed through muscle responses designed to maintain adjacent vertebrae in close alignment and prevent associated joints from being damaged particularly during extensive stretching (El-Bohy et al. 1988; Konttinen et al. 1990; Gronblad et al. 1991; Yamashita et al. 1993b; Jiang et al. 1995a; Michelson and Hutchings, 1995).

There are many morphological descriptions of nerve endings in the literature but most refer only to the adult or mature stage of development (Freeman and Wyke, 1967; Giles and Harvey, 1987; Halata, 1988; Vega et al. 1989; Biedert et al. 1992; Jiang et al. 1995). There is much less literature available describing the development of nerve elements, especially the development of the distribution of the nerve elements within the tissues of the structure they are supplying. There have been studies of postnatal muscle spindle development (Maeda et al. 1985), general nerve development (Wheeler and Plummer, 1989), development of Pacinian corpuscles (Zelena, 1978), and the postnatal development of Ruffini corpuscles in the peridontal ligament (Nakakura-Ohshima et al. 1993), but there has been little description of the development of spinal ligament innervation particularly in relation to the changes that take place during the accelerated growth period surrounding puberty.

We are currently interested in the pathogenesis of adolescent idiopathic scoliosis (AIS) and the relationship of the innervation of spinal ligaments to the maintenance of a straight spine and an erect posture. Previous studies have suggested that spinal ligaments, especially the lateral spinal ligaments, are important in the stability and dynamic balancing of the spine (Jiang et al. 1994, 1995a, b). Since AIS usually develops at puberty when there is accelerated growth, it would be interesting to observe the dynamic changes that occur in the innervation of spinal ligaments during this time. This is important because lack of development of peripheral sense organs has been related to loss of proprioceptive afferents (Ernfors et al. 1994) and it has been reported that there is a correlation between innervation density of a tissue and the sensitivity of its neurological function (Zelena, 1976; Zimny, 1988; Abdel-Rahman et al. 1992; Ernfors et al. 1994). Accordingly, this study was designed to describe and characterize

the changes in innervation that take place in spinal ligaments during growth. Specifically we studied the types of innervation present, their distribution throughout the ligaments, and the changes that occurred in relation to the growth of the ligament. We were also interested in determining whether periods of particular vulnerability could be identified during growth in which damage to the innervation of the ligament would have maximum effect.

MATERIALS AND METHODS

Thirty six White Leghorn chickens in four groups of nine at 0, 2, 7, and 13 weeks of age were obtained from the Poultry Unit at the University of Alberta. The chicken was selected as an appropriate animal model for the human because it has a pseudo-bipedal stance with a semi-erect spine as well as having been found to possess characteristics of the spinal ligaments which are similar to those of the human (Jiang et al. 1995b). The rapid rate of growth of chickens also means that the entire growth period can be observed in 13 weeks of age including the important period of accelerated growth during puberty. Furthermore, the chicken has been reported to be a reliable animal model for the production of scoliosis, again similar to AIS in the human (Machida et al. 1994, 1995).

Each group of chickens was sacrificed using an overdose of Somnatol given peritoneally. The supraspinous and interspinous ligaments (SSL) as one unit and intertransverse (ITL) ligaments from both sides of the spine were dissected immediately from thoracic (T) levels 4-5 on both sides and fixed in 0.5% Zamboni's solution for 3 hours. The morphology of these ligaments has been described in detail in a previous study (Jiang et al. 1995a) and are representative of both central and lateral spinal ligaments. The orientation of the ligaments was maintained throughout the following procedures. The specimens were washed in phosphate buffered saline (PBS) for 1 hour and immersed in a solution of 30% sucrose/PBS overnight before being embedded in O.C.T. and flash frozen in isopentane previously cooled in liquid nitrogen. Serial sections of 32 μm thickness were made using a cryostat. The sections were thaw mounted onto glass slides precoated with gelatin and stored at -20°C.

Counterstain was first applied using 1% pontamine sky blue (Cowen et al. 1985) for 5 minutes followed by immersion in acetone at 4°C for 10 minutes. The sections were

washed in PBS/0.3% Triton X-100 for 10 minutes between each of the following steps in the staining procedure. A PAP-smear pen was used to a draw a hydrophobic circle around individual sections to restrict the spread of overlying solutions. Mouse antibodies to neurofilament protein subunits of 68 KD, 160 KD and 200 KD were pooled and use ingle primary antibody after being diluted 1:100 with PBS/0.3 Triton X-100. The sections were incubated with the primary antibody in a humid chamber for 2 hours (10 µl/section) at room temperature. This was followed by further incubation with anti-mouse antibody conjugated with FITC for 1 hour in a dark humid chamber. Flouromount was used as an adhesive for coverslips to prolong the fluorescence.

Positive controls consisted of staining sections of skin and sciatic nerve which had been collected at the same time and subjected to same procedures. Negative controls consisted of staining the same tissues using the same protocol but substituting the antibody against neurofilament protein with PBS or nonspecific antibodies.

The shape of each section was traced onto paper using a magnifying projector. A micrometer was also projected for scaling purposes. The slides were observed using a microscope equipped with UV light and the sites of neural elements and their characteristics documente the pretraced drawing of the section. The shape of the section and the neural elements on each section were digitized into a HP computer (Hewlett-Packard Co., Fort Collins, Colorado) for further data processing. The area of each section was measured by tra ng the perimeter. The location of each nerve element was digitized onto the computer image. By totaling the areas of the ligament on each section and including the thickness of each section, the volume of each ligament was also obtained. The neural elements were identified as Ruffini corpuscles (highly coiled nerve ending), free nerve endings (single nerve ending), small nerve bundles (10 fibres or less), and large nerve bundles (more than 10 fibres) and examples are shown in Figure 5.1A - D.

Descriptive statistics were applied to summarize the data and a Student's t-test with $p=0.05$ was applied to analyse the differences of similar measurements made for the different age groups.

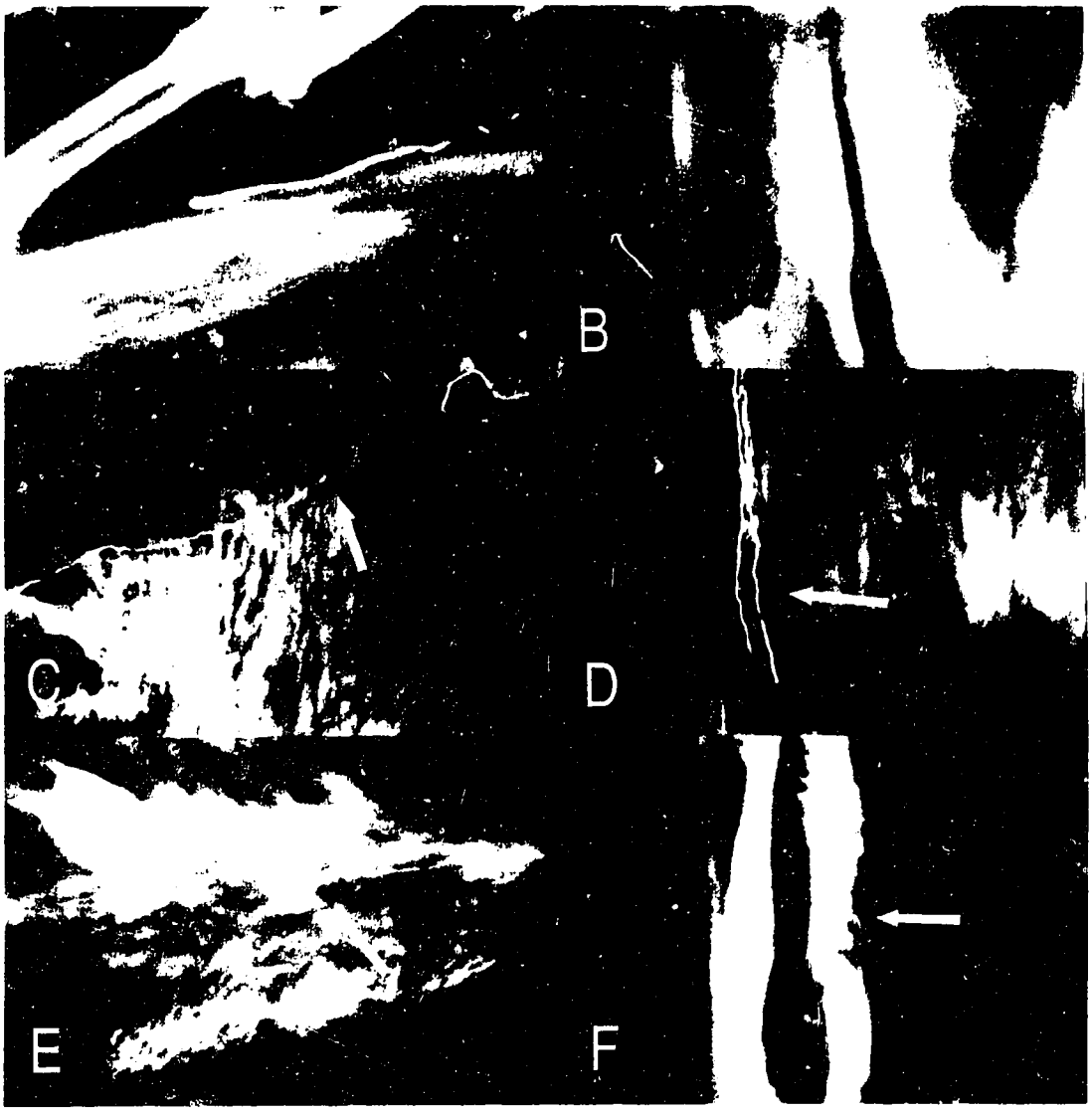

Figure 5.1 (X250) **A.** shows a single nerve fibre (arrow) on the periphery of ITL from a 0 weeks old chicken. **B.** shows a Ruffini corpuscle (arrow) which is not fully developed in the dense collagen bundles of ITL from a 2 weeks old chicken. This Ruffini corpuscle is small and has only few branches. **C.** shows free nerve endings (arrows) which penetrate deeper into the dense collagen bundles of an ITL from a 2 weeks old chicken. **D.** shows a small nerve bundle (arrow) within the dense collagen bundle of ITL from a 7 weeks old chicken. **E.** shows a Ruffini corpuscle in the ITL from a 7 weeks old chicken. **F.** shows that by the age of 13 weeks the ITL has started to ossify commencing at the middle of the ligaments (arrow).

RESULTS

Nerve axons in the chicken skin and sciatic nerve from the control material showed strong immunofluorescent reaction to the NFP antibody and there was no immunofluorescent reaction found in either of the negative control groups.

At 0 weeks of development (immediately after hatching), very few nerve elements were found in either the SSL or ITL ligaments. Only occasional single nerve fibres and small nerve bundles could be found in each of the ligaments and these were always located in the periphery of the ligaments (Figure 5.1A). There were no large nerve bundles nor Ruffini corpuscles found in this age group for both SSLs and ITLs.

In general there were many more nerve elements observed in both of the ligaments at the age of 2 weeks. Large nerve bundles appeared in the ITLs with increasing numbers of single nerve fibres and small nerve bundles. Although a few Ruffini corpuscles were observed at this stage in the ITLs, they were smaller and ' mplicated than those seen at later stages (c.f. Figure 5.1B and 5.1E). The nerve ı. also penetrated further into the ligaments at this age (c.f. Figure 5.1A and 5.1C).

There was a statistically significant increase ($P < 0.001$) in the total number of nerve elements in both SSL and ITL for the 7-weeks old age group when compared to the younger age groups (Figure 5.2). When the elements were identified, this increase was shown to be restricted to single nerve fibres and small nerve bundles (Figure 5.3 and Figure 5.4). In contrast, the number of large nerve bundles remained unchanged in both ligaments. The Ruffini corpuscles were larger and appeared to have a more complicated nerve terminal with increased intertwining of the end fibre (Figure 5.1E). Most of the Ruffini corpuscles were still located in the periphery of the spinal ligaments (Figure 5.1E). There were no Pacinian corpuscles or blood vessels observed within any of the spinal ligaments.

Interestingly, the total number of nerve elements in the spinal ligaments decreased in the 13-weeks old group (Figure 5.2). This reduction was found to coincide with the ossification of both the ITL and SSL which had started to appear in the middle of the ligament with apparent trabecular bone (Figure 5.1F). This ossification clearly reduced the actual volume of collagenous ligament available for consideration.

It was also found that there was uneven distribution of the nerve elements between the two ligaments. In the SSL the nerve elements were equally distributed along the length of the ligament whereas in the ITL, significantly more of the nerve elements appeared in the cranial third when compared to the middle and caudal thirds (Figure 5.5). The changes in innervation distribution that were observed occurred mainly in this cranial third. The innervation in the middle and caudal thirds remained relatively unchanged during the developmental period studied.

Relative innervation density was further analysed in terms of the number of nerve elements per unit of ligament volume (mm^3). Figure 5.6 shows that the innervation density increased significantly between 0 and 2 weeks of age but then significantly decreased by 7 weeks of age. Innervation density is dependent on an increase in the number of nerve elements as well as any increase in ligament volume. Although there was a significant increase in total number of nerve elements, this was overshadowed by the relatively larger increase in ligament volume during this time shown in Figure 5.7. This combination of total nerve elements and ligament volume resulted in a significant decrease in innervation density as the ligament grew.

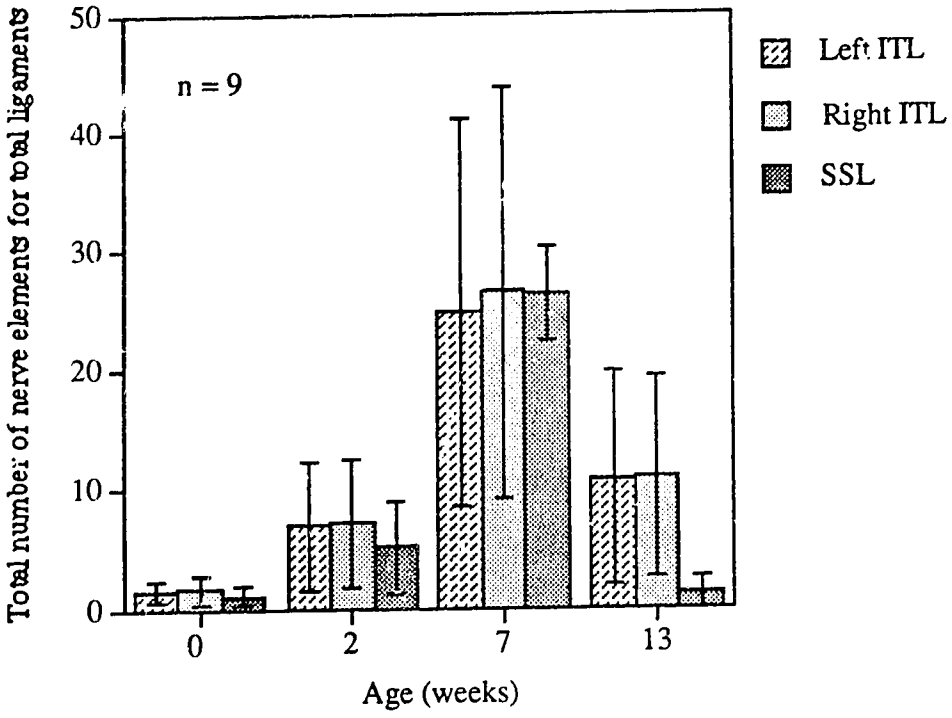

Figure 5.2 A histogram to show that the total numbers of nerve elements in different age groups increased between 0 weeks and 7 weeks old, but the numbers were reduced at 13 weeks old. The actual counts of all nerve elements were not significantly different between left and right sides at all ages ($P>0.05$).
ITL = intertransverse ligament, SSL = supraspinal ligament

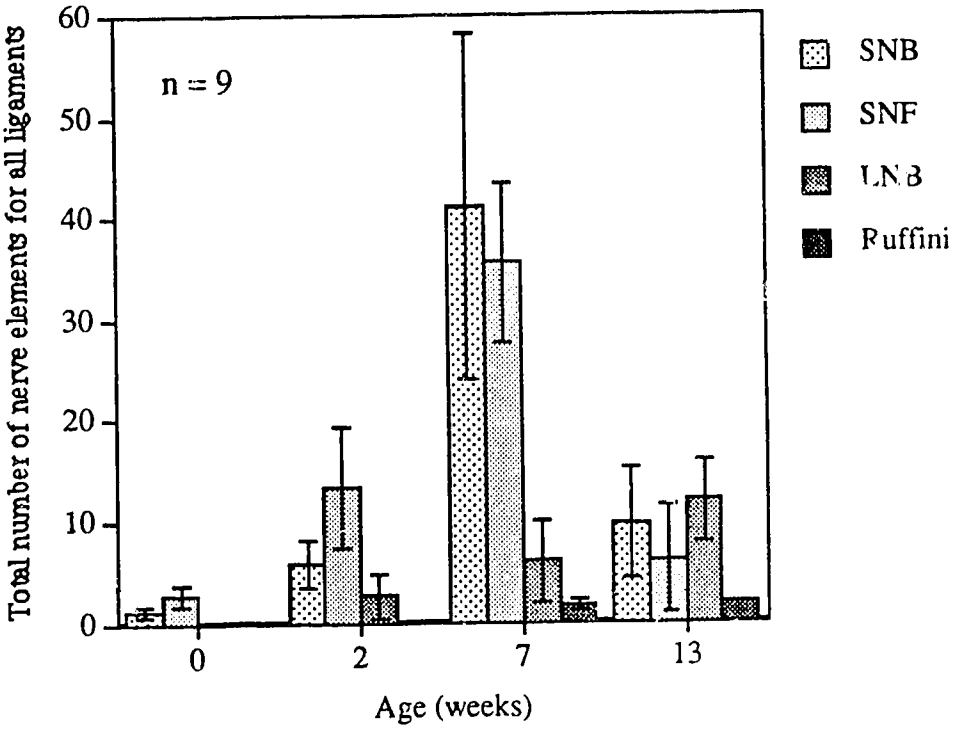

Figure 5.3 A histogram to show the total numbers of different nerve elements in SSL. Note that the increases and decreases in numbers was due to changes in SNB and SNF and that the numbers of LNB and Ruffini corpuscles remained relatively constant. SNB = small nerve bundle, SNF = single nerve fibre, LNB = large nerve bundle, SSL = supraspinal ligament

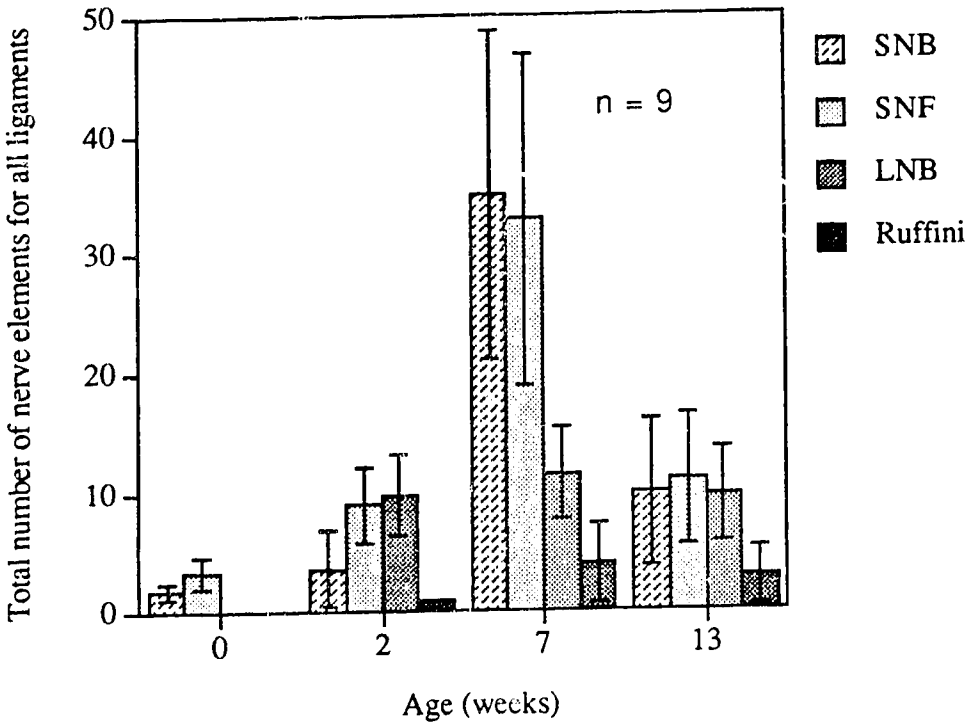

Figure 5.4 A histogram to show the total numbers of different nerve elements in ITL. Note that the increases and decreases in numbers was due to changes in SNB and SNF and that the numbers of LNB and Ruffini corpuscles remained relatively constant. ITL = intertransverse ligament, SNB = small nerve bundle, SNF = single nerve fibre, LNB = large nerve bundle

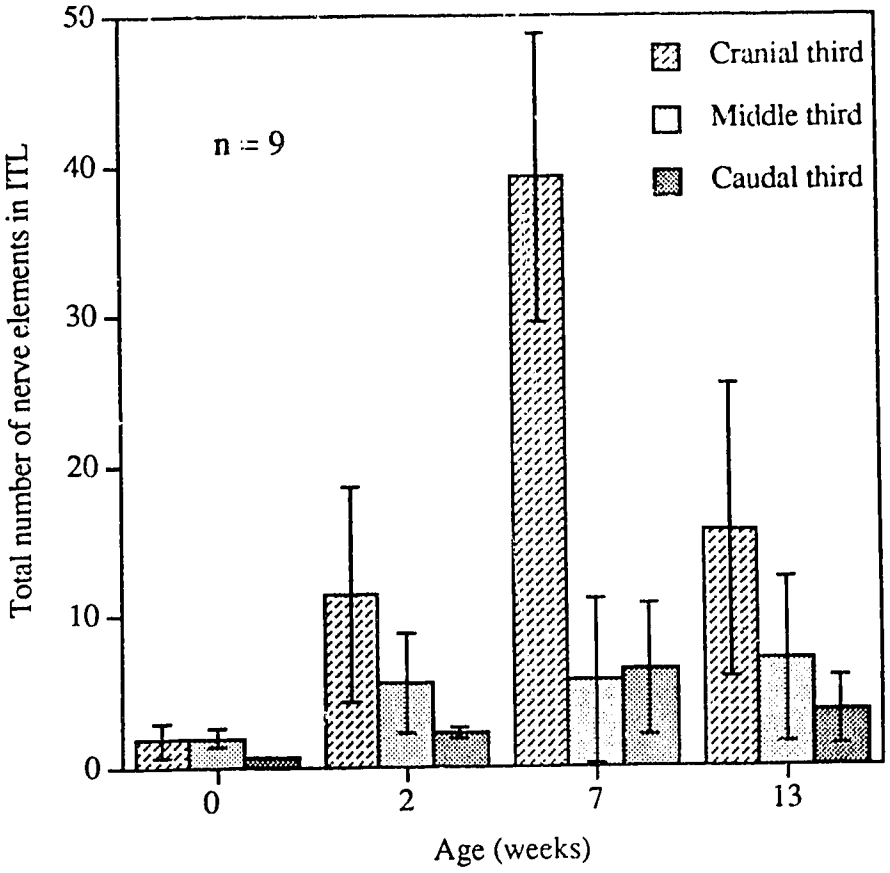

Figure 5.5 A histogram to show the change in the total numbers of nerve elements between different areas of the intertransverse ligaments. Note that significant changes occurred along the cranial third of the ligament

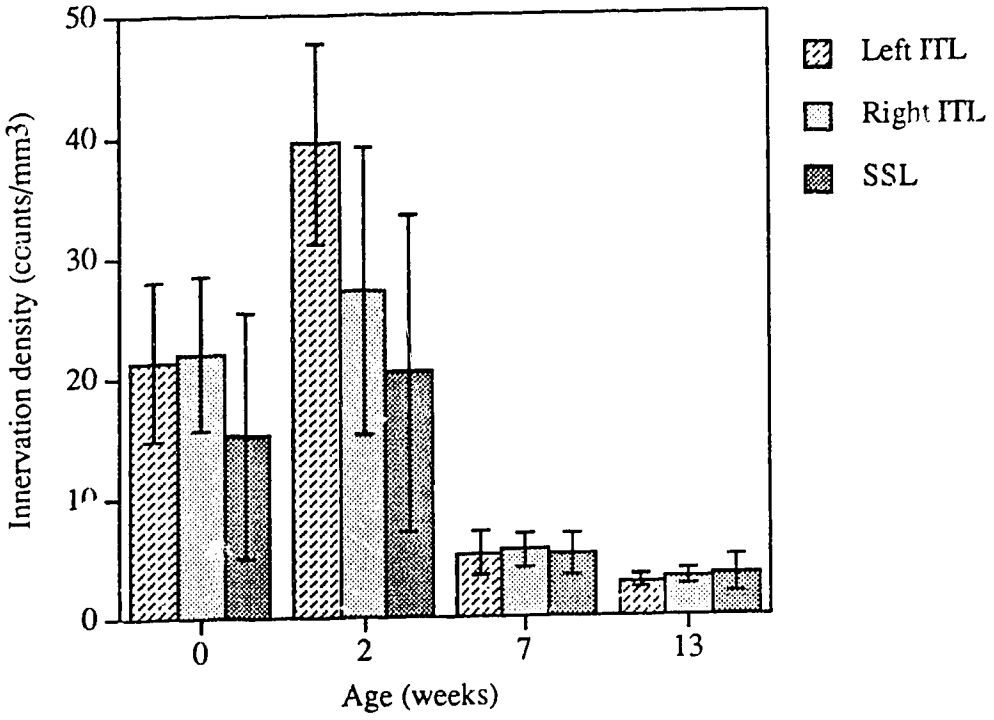

Figure 5.6 Although the absolute number of nerve elements increased during growth, relative innervation density of the spinal ligaments increased between 0 and 2 weeks and then decreased. The increase in nerve elements was overshadowed by a greater increase in ligament volume. ITL = intertransverse ligament, SSL = supraspinal ligament

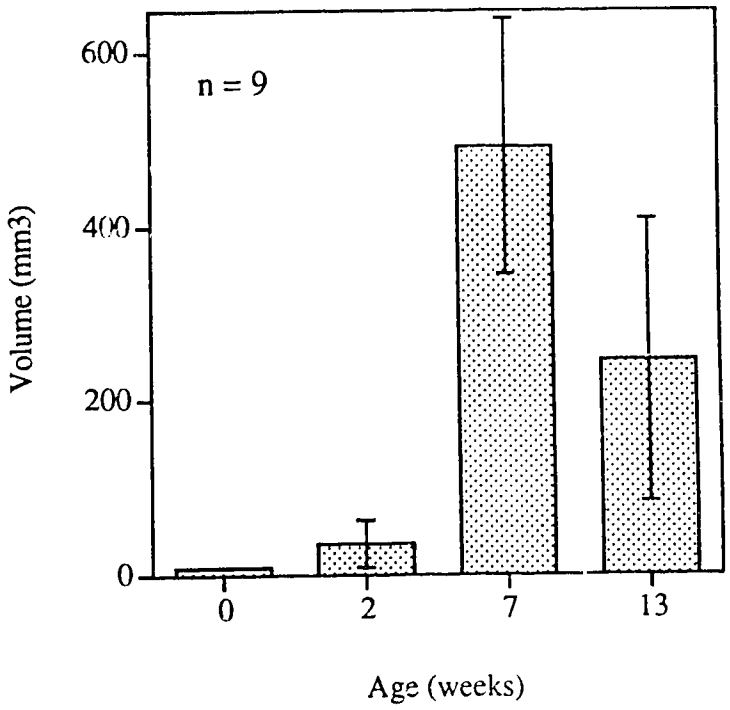

Figure 5.7 A histogram to show the volume change of ITL during growth period of chickens. ITL = intertransverse ligament

DISCUSSION

The results of this study have shown that there is considerable postnatal development of the innervation in spinal ligaments of the chickens. In both the central (SSL) and lateral (ITL) spinal ligaments at the time of hatching there were very few nerve elements visible and these were restricted to single nerve fibres and small nerve bundles. After 2 weeks, the numbers of both single nerve fibres and small nerve bundles had increased significantly. These nerve elements were now accompanied by large nerve bundles. Ruffini corpuscles could only be observed in the ITL but there are very few to be found. Between 2 and 7 weeks after hatching, there were significant increases in the numbers of large and small nerve bundles as well as single nerve fibres and Ruffini corpuscles could be seen frequently in both ligaments. During this time, the significant increase in numbers of nerve elements was overshadowed by the relatively larger increase in size of the ligament and the innervation density actually decreased. By 13 weeks, ossification of the ligaments had started and the amount of collagenous material forming the ligament had been significantly reduced. There were correspondingly significant decreases in the number of single nerve fibres and small nerve bundles but the numbers of large nerve bundles and Ruffini corpuscles remained the same as those found at 7 weeks after hatching. Throughout this period of growth from the time of hatching to 13 weeks later there were significantly more nerve elements in the cranial one third of the ITL when compared to the middle and caudal thirds but there were no differences between ITL on the left and those examined on the right. These substantial and significant changes in innervation of these ligaments after hatching suggest that this may be a critical period of development and that damage to the ligament at this time may affect the innervation which could result in detriments to coordinated and proper development.

It is interesting to note that most of the increase in nerve elements is found in the single nerve fibres and small nerve bundles and that the numbers of Ruffini corpuscles and large nerve bundles remains relatively constant. This is re-emphasised between 7 and 13 weeks when there are significant decreases in nerve elements restricted again to the single nerve fibres and small nerve bundles. This suggests that the changes that occur in numbers of nerve elements are due to branching of the major nerves. At the time of hatching, increases would be due to branching after the nerves have penetrated the ligament. Such branching would lead to an increase in the number of single nerve fibres as well as small nerve bundles as is observed. Retween 7 and 13 weeks after

hatching, when ossification is starting, the reduction in nerve elements would be due to these smaller branches being eliminated. Using this model, the major innervation consisting of the large nerve bundles would remain constant and provide a source for the branching mechanism. The observation that the number of Ruffini corpuscles remains constant during development suggests that there are a fixed number of these end organs and that damage to them might be critical in relation to development. Their absence during the first 2 weeks after hatching probably reflects the methodology used for identification rather than their later appearance. The NFP-antibody attaches to epitopes on filaments in the nerve axons rather than at the end of the nerve fibre (Nakakura-Ohshima et al. 1993). If the fibre innervating or forming the end organ has not yet penetrated sufficiently to reach the area where the corpuscle will form then the Ruffini corpuscle will not be identified. Similar patterns of delayed development have been reported for the postnatal development of Pacinian corpuscles (Zelena, 1978). Problems associated with denervation and reinnervation of spinal ligaments during development and their effects on spinal stability await further studies.

The chicken is a good animal model for studying development of the innervation in spinal ligaments because it has ligaments that are morphologically and histochemically similar to the human (Jiang et al. 1995). The chicken also has a pseudo-bipedal stance in which the spine is partially erect which has been shown to be a prerequisite for the development of lateral spinal ligaments (Luk and Leong,1987; Luk et al. 1986; Jiang et al. 1995). Furthermore, development occurs rapidly in the chicken which passes through puberty at approximately 10 weeks and is mature in relation to size at 13 weeks. However, unlike the human, ossification of many spinal ligaments occurs after this time which makes the chicken spine more rigid (Robinson, 1970). Nevertheless the development between hatching and 13 weeks can be compared to the human especially in terms of distribution of the nerve elements and development of the neural characteristics. Significantly, the innervation density decreases with development in spite of significant increases in numbers of neural elements. This is due to the much larger relative increase in size of the ligament. At the time of hatching, the volume of the ligament is relatively small and during development there is growth in all dimensions. However, most growth occurs in thickness of the ligament as shown by an increase in the number of serial sections associated with each ligament. Presumably this is in response to the increased demands placed on the ligament as the animal develops greater mobility and size and emphasises the importance of these lateral ligaments in maintaining a stable spine as has been shown in other studies (Luk et al,

1986, Luk and Leong, 1987).

Other studies have suggested that innervation density is related to the functional sensitivity of the innervation. For example, Zimny (1988) reported that the concentration of mechanoreceptors appears greater in areas related to the extremes of movement and probably represents the first line of defense in sensing these extremes. Similarly, Vallbo and Johansson (1984) found that the discrimination sensitivity of fingers correlated very well with the density of the mechanoreceptors. In this respect it is interesting to note that most of the neural elements contained in ITL were always found in the cranial one-third of the ligaments. Perhaps this reflects the most sensitive area of the lateral ligament or merely reflects the proximity of the major innervation pathway as a branch from the intercostal nerve and the restriction in subsequent branching because of this placement. The overall decrease in innervation density during growth is difficult to explain as it might be expected that with a larger mass to control the chicken would require greater mechanical sensitivity. This would not be supplied by a decrease in innervation density as was found in this study.

In summary, this study showed that while both central and lateral spinal ligaments in the chicken were present at the time of hatching they were limited in their innervation. The number of nerve elements rapidly increases and the morphology of nerve endings becomes more mature with development. Although the actual number of nerve elements increases during development, the relative innervation density decreases by the age of 7 weeks because of an overwhelming increasing in ligament volume. Most of the innervation is located in the cranial third of the ITL and there is no apparent difference in the innervation between ligaments on the left and those on the right sides of the spine. There is considerable development of the innervation in the ligaments during growth prior to puberty and change to the innervation during this period possibly has long term effects to the function of the proprioceptive innervation.

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CHAPTER 6

Identification of the location, extent and pathway of sensory neurological feedback following mechanical stimulation of a lateral spinal ligament in chickens.

INTRODUCTION

Traditionally, ligaments have been viewed as simple mechanical constraints across joints which provide tension when stretched. Freeman and Wyke (1967) proposed a possible additional function for ligaments when they hypothesised that an important role for joint receptors might be to contribute to the coordination of muscle tone around joints and provide a neurological feedback mechanism to enhance joint stability. This hypothesis has been supported subsequently by electrophysiological (Clark and Wyke, 1975; Grigg, 1976; Grigg and Greenspen, 1977, 1978; Grigg et al. 1978; Ferrell and Hart, 1980; Ferrell, 1980; Baxendale and Ferrell, 1981, 1982, 1983; Grigg and Hoffman, 1982; Johansson et al. 1991a, b; Sojka et al. 1991; Rampersaud et al. 1995) and morphological (Burgess and Clark, 1969; Ferrell, 1980; Rossi and Grigg, 1982; Sojka et al. 1983; Halata, 1988; Sjölander et al. 1989; Gronblad et al. 1991) studies of ligaments which have shown them to be well innervated and responsive to various forms of stimulation. In light of these results it is now thought that ligaments do more than act as simple mechanical links between bones by also providing important proprioceptive feedback as part of a neurological protective mechanism both for the ligaments themselves and the joints they span.

While much of the work in this area has centered on the ligaments of the knee joint (Kennedy et al. 1982; Sojka et al. 1983; Sjölander et al. 1989, Katonis et al. 1991, Biedert et al. 1992, Beard et al. 1994, 1993), our attention is focused on the spinal ligaments and their importance in the maintenance of an erect posture and upright spine.

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Recent studies by ourselves (Jiang et al. 1995a, b) and others (Yahia et al. 1988; Cavanaugh et al. 1989; Ahmed et al. 1993; Rhalmi et al. 1993; Rivard et al. 1993; Yamashita et al. 1993a, b) have shown that spinal ligaments are richly innervated in a way similar to the innervation of ligaments around the knee joint (Jiang et al. 1995a). The innervation consists of Pacinian corpuscles, Ruffini corpuscles, small and large nerve bundles, and free nerve endings. However, the precise feedback pathway for these nerve endings in spinal ligaments is unknown. Such information would be important for it would highlight the areas of the central nervous system involved in the feedback mechanism and would provide focus for further studies, particularly in diseases where malfunction of the mechanism is suggested to be of primary importance. For example, patients with adolescent idiopathic scoliosis (AIS) have been shown to have deficiencies of sensory input associated with the dorsal columns (Barrios et al. 1987; Smith and Dickson, 1987; Robin 1990) and it has been suggested that this might be related to improper perception of proprioceptive information originating in the lateral spinal ligaments (Machida et al. 1993, 1994, 1995; Jiang et al. 1994). Unfortunately, precise identification of the areas of the central nervous system involved in such perception is currently unknown.

Accordingly, this study was designed to trace the path taken by proprioceptive information from the intertransverse ligament in chickens following extensive mechanical loading. Stretching the ligament stimulates the sensory nerve endings and activates the transcription of c-fos proto-oncogene in the neuron with subsequent production of Fos protein in the nerve cell body (Curran, 1988). Continued transfer of the stimulus by way of synapses to interneurons and neural nuclei activates the production of Fos in their cell bodies (Nagao et al. 1993; Bullit et al. 1992; Mack and Mack, 1992; Bullitt, 1989, 1990). The proprioceptive pathway can be determined by identifying the chain of neurons containing Fos protein. This phenomenon has been used in several previous studies to determine a variety of proprioceptive and functional pathways in the central nervous system (Morgan et al. 1987; Sagar et al. 1988; Krokoff et al. 1993; Jasmin et al. 1994a, b). The results of this current study showed that mechanically stretching an important lateral spinal ligament in chickens produced a barrage of proprioceptive feedback from several levels of the spinal cord as well as responses from the contralateral side of the cord at equivalent levels. Furthermore, the pathway of the sensory input can be traced to include nerve cell bodies in the dorsal root ganglia, the sympathetic ganglia, the intermediate horn of the spinal cord, the cuneatus and gracilis nuclei of the medulla oblongata, the vestibular nuclei, and the

thalamus. Identification of these areas provides specific focus for future studies.

MATERIALS AND METHODS

Four-week old White Leghorn chickens were obtained from the Poultry Unit at the University of Alberta. The chicken was selected as the experimental model because it is a good example of a bipedal animal with well developed lateral spinal ligaments similar to the human (Machida et al. 1995; Jiang et al. 1995b). The chickens were conditioned to their new surroundings for a period of 48 hours before the experiment to eliminate any excess emotional stress which might affect Fos production (Krukoff, 1993). This conditioning included gentle holding four times a day by the personnel conducting the experiment. Food and water were withheld for 6 hours before surgery to prevent subsequent respiratory problems.

Control experiments:

A. To establish a positive control for c-fos production, stimulation was achieved using nociception. Three conditioned 4-week old chickens were anaesthetized using Somnotol (100 mg per 100g body weight) by intra-peritoneal injection. An additional dose (50 mg per 100 g body weight) was given after 1 hour so that the chickens remained anaesthetised for approximately 2 hours. Following induction of anaesthesia the chickens were placed in the left recumbent position and a light was placed overhead to provide heat and maintain the body temperature. The right side of the skin covering thoracic levels (T)1 to T6 was pinched three times every 2 minutes for 2 hours using a pair of rat-toothed forceps (Tavares et al. 1993). The chickens were then sacrificed using an overdose of Somnotol and the dorsal root ganglia and spinal cord between levels T1-6 were processed for identification of Fos protein using the protocol described below in the main experiment.

B. Several control experiments were performed to establish a methodology which produced minimal background Fos levels since c-fos can be activated by a variety of different methods of stimulation (Krukoff, 1993) including anaesthesia and surgical procedure.

1. Conditioned 4-week old chickens were anaesthetized using Somnotol. Following induction of anaesthesia, a 1 cm-long, midline incision was made in the skin of the back followed by gentle, blunt dissection to expose the intertransverse ligament

between T3-4 on the right side. The wound was covered with a sponge soaked in sterile phosphate buffered saline (PBS) and kept moist at all times. When there was evidence that the anaesthesia was becoming lighter, a further injection of Somnotol at a reduced dosage was given to keep the chicken anaesthetised for a total of 2 hours. However, the vital status of the chickens became extremely fragile after this length of time using Somnotol and the results showed that c-fos activity also remained high in both the dorsal root ganglia and spinal cord.

2. To lengthen the time of stable anaesthesia and to reduce the c-fos activity in response to the initial surgery, we investigated the use of Halothane coupled with subcutaneous injection of a local anaesthetic, Lidocaine. Twenty chickens, each 4-weeks old, were used. Ten of the chickens were anaesthetized with 2% Halothane only while the remaining ten chickens were anaesthetized initially with 2% Halothane followed by a local, subcutaneous injection of 0.5 - 1.0 ml of 1% Lidocaine in the area of the impending incision. Care was taken to inject Lidocaine only in the cutaneous and subcutaneous regions and not to invade the area of the lateral spinal ligaments. Assistance here was given by a thick layer of paraspinal muscle including deep fascia between the subcutaneous tissue and the ligaments which provided an extra barrier to prevent the spread of anaesthetic. The right intertransverse ligament at T3-4 was exposed as described above but no further stimulation was applied to the ligament. The wound was kept moist with a sponge soaked in sterile PBS. For each group, two chickens were sacrificed at the following time intervals after exposure of the ligament: immediately, 60, 90, 120, and 180 minutes. The dorsal root ganglia and spinal cord between levels T1-5 were processed for identification of Fos protein using the protocol described below in the main experiment. The combination of Halothane and Lidocaine proved to be the most satisfactory anaesthetic and so was used in all subsequent experiments.

Main experiment:

Five chickens each 4-weeks old were anaesthetized initially using 2% Halothane followed by a local subcutaneous injection of 0.5-1.0 ml of 1% Lidocaine in the area of the impending in sion. The right intertransverse ligament at the level of T3-4 was exposed following a small midline incision and subsequent blunt dissection. The exposed ligament was kept moist by covering with a sponge soaked in sterile PBS. Based on the results of the previous control experiment, each chicken was kept anaesthetised and quiet for one and a half hours following surgery before any further

stimulation, for it had been shown that the Fos produced by the initial surgery would have been reduced to almost zero by this time. A simple, hand-held mechanical load of 300 g was applied for one minute every other minute vertically to the ligament for one hour followed by a period of no stimulation for 30 min. The weight stretched the ligament extensively (approximately 5.5% in length - unpublished data) each time it was applied. The chickens were sacrificed using an overdose of Somnotol and were immediately perfused with 100 ml of PBS introduced through the left ventricle followed by 200 ml of 4% paraformaldehyde. The dorsal root ganglia and sympathetic ganglia in connection with spinal cord levels from T1 to T5, the brain stem and the diencephalon were removed and immersed in the same fixative for 4 hours at 4°C. The specimens were then washed with PBS and transferred into a solution of 30% sucrose/PBS and left overnight at 4°C after which they were embedded in O.C.T. (*o*-chlorotoluene - Miles, Elkhart) and frozen in isopentane cooled with liquid nitrogen. Serial, transverse, frozen sections, 16 µm thick, of the spinal cord and brain were cut using a cryostat and thaw-mounted onto gelatin-coated glass slides. The sampling locations in the brain are shown in Figure 6.1. The sections were first incubated with sheep anti-Fos antibody (Cambridge Research Biochemicals, UK) prediluted to 1:500 in 0.4% Triton/PBS (10 µl per section) overnight at room temperature followed by incubation with biotinylated anti-sheep antibody diluted 1:200 in 0.4% Triton/PBS (10 µl per section) for one hour. The sections were finally incubated with Avidin-Texas red diluted 1:200 with 0.4% Triton/PBS (10 µl per section) for 45 minutes in a dark, humidified box. The sections were washed twice in PBS for 10 minutes each time between incubations.

Assessment of Fos production was made by observing each section using a Zeiss ORTHOPLAN fluorescence microscope (Carl Zeiss, Oberkochen) which utilizes UV light. The number of fluorescent nuclei in each section was counted and their position in the tissue was noted. The degree of fluorescence for a particular region was determined by adding together the number of fluorescent cells in adjacent serial sections which formed any particular area. Black and white and colour photographs were taken of selected areas of each section for each of the dorsal root ganglia, sympathetic ganglia, spinal cord, nucleus cuneatus and gracilis, vestibular nucleus and thalamus using a x25 objective lens.

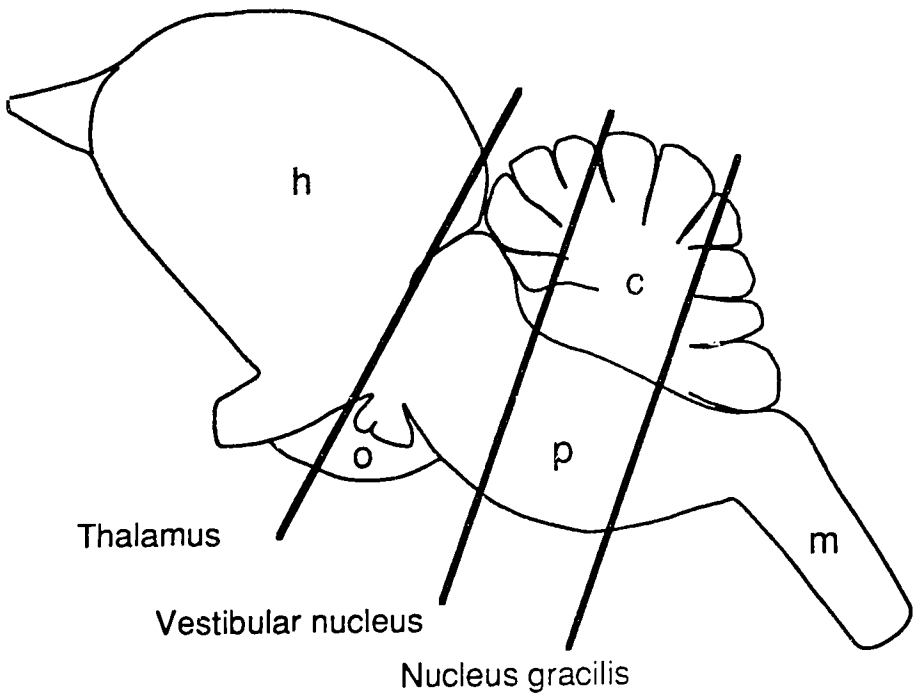


Figure 6.1. A lateral view of the brain in the chicken. The sample sites for sectioning in the brain stem are indicated by the lines.
c=cerebellum p=pons m=medulla oblongata o=optic lobe h=cerebral hemisphere

To establish the background level of autofluorescence using this methodology, some of the sections were processed using the protocol described above but with PBS substituting for the primary antibody to Fos. Similarly, several sections were stained with a similar but non-specific antibody to determine the specificity of the antibody being used.

RESULTS

Control experiments:

Positive Fos expression was found in the dorsal root ganglia and areas of the spinal cord in the control experiment in which the chickens were subjected simply to nociceptive stimulation (Figure 6.2a). There were fewer cells exhibiting positive Fos expression in both the dorsal root ganglia and spinal cord on the contralateral side when compared with observations taken on the side ipsilateral to the stimulation. No positive fluorescence was found in the neuronal nuclei when the anti-Fos primary antibody was substituted by either PBS or the non-specific antibody in the staining protocol (Figure 6.2b).

When anaesthesia was achieved with Somnotol only, Halothane only, or Halothane and Lidocaine combined, and no other stimulation was applied, there was no Fos-positive reaction in any of the tissues examined following several hours of anaesthesia. However, following surgical exposure of the intertransverse ligament at T3-4 using Somnotol anaesthetic alone, high Fos levels (many fluorescent nuclei) were still evident after more than 2 hours. of anaesthesia. Fluorescent cells were seen in the ipsilateral dorsal root ganglia, and in the intermediate and occasionally ventral horns of the spinal cord. Similar areas of fluorescence were also seen on the contralateral side but there were fewer numbers of fluorescent cells in these regions. Coupled with the difficulties in maintaining anaesthesia using Somnotol over long periods of time in the chickens, it was decided to use Halothane as the main anaesthetic in the other experiments.

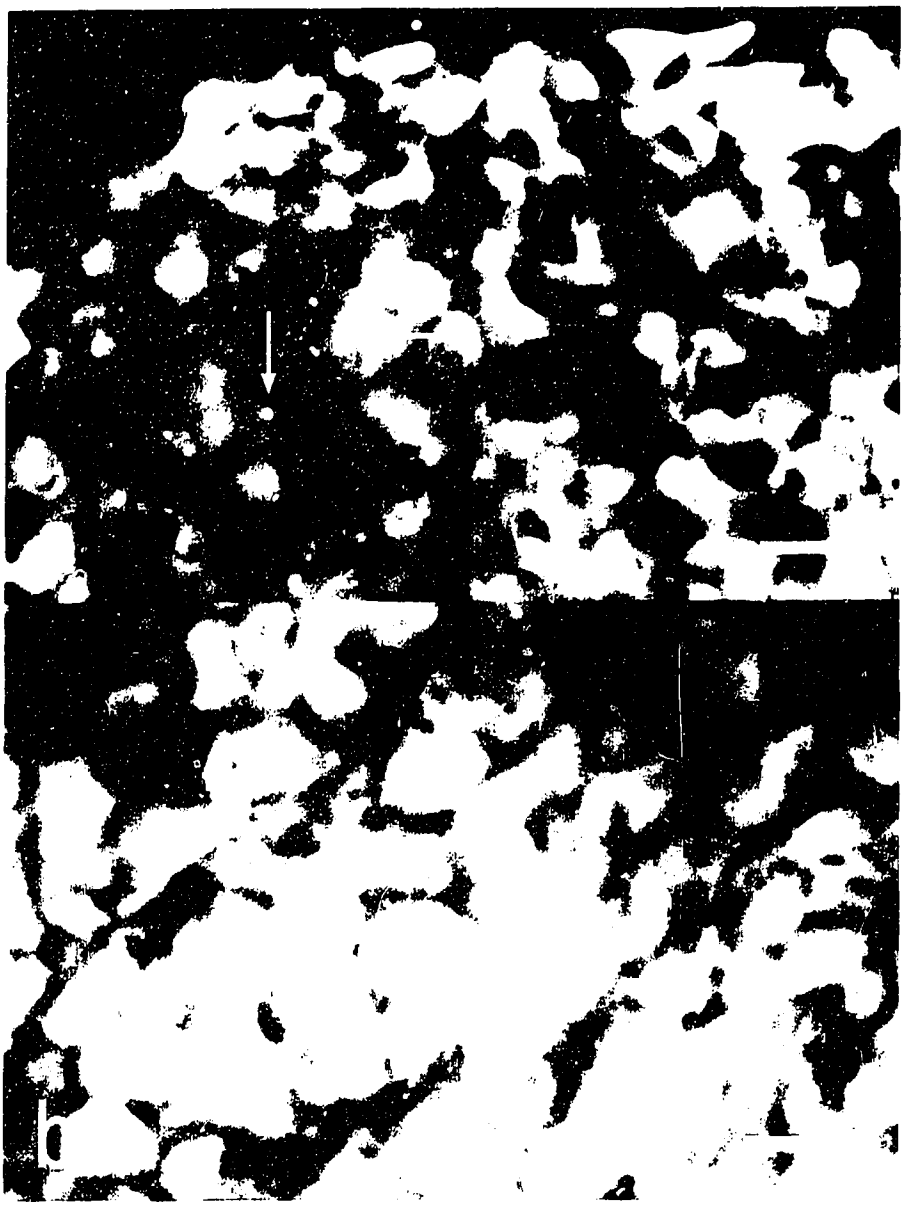


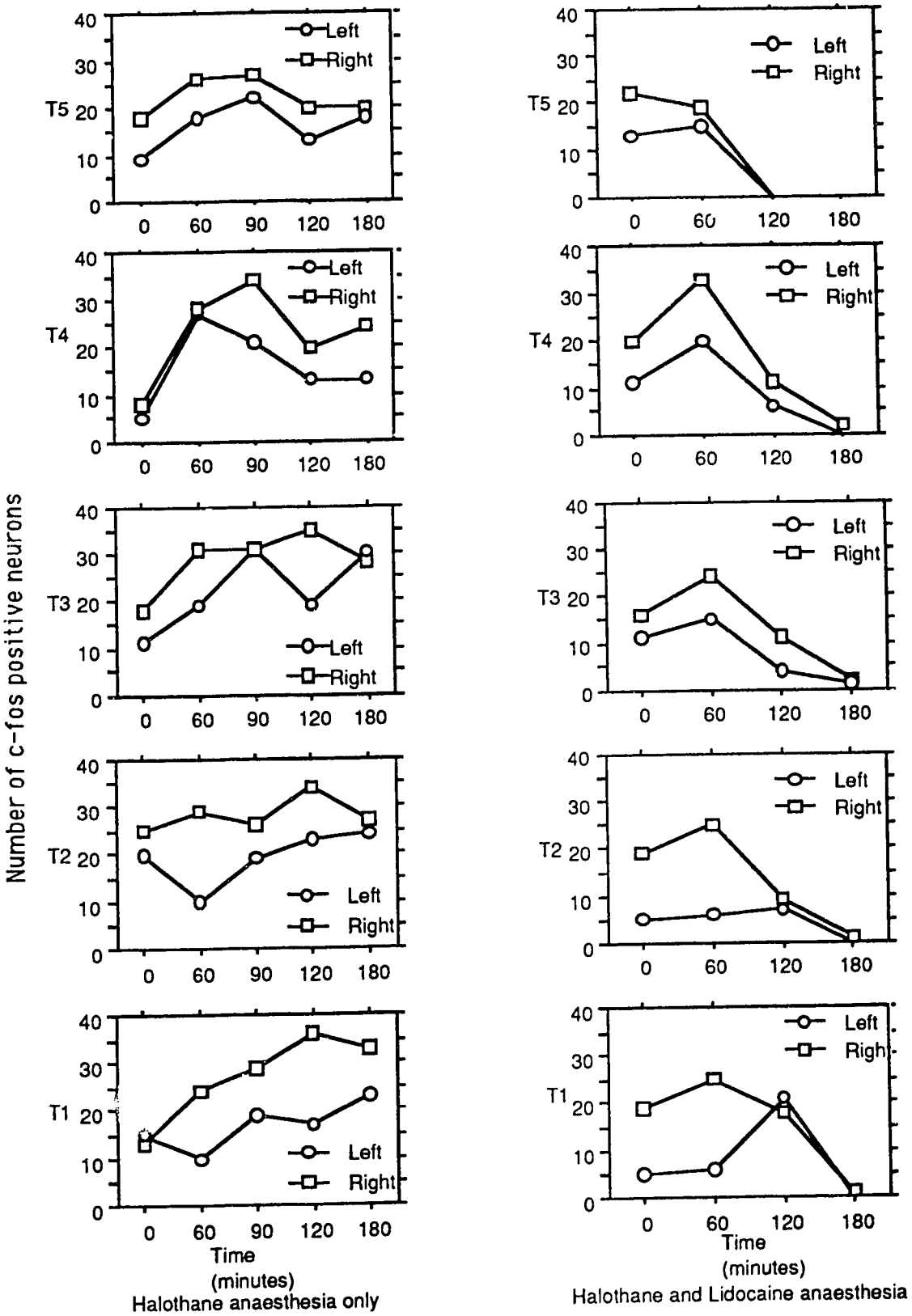
Figure 6.2. (Bar=20μm) a) Micrograph of a typical dorsal root ganglion following nociceptive stimulation used as a positive control. Note the many fluorescent cell nuclei (arrows). b) Micrograph of a typical dorsal root ganglion used as a negative control. Note the absence of fluorescent cell nuclei.

When only Halothane was used as the anaesthetic prior to exposure of the intertransverse ligament, Fos expression remained high throughout the 3 hours of the experiment (Figure 6.3). When Halothane and Lidocaine were used in combination as anaesthetics, Fos expression declined until the number of fluorescent cells was insignificant after 180 minutes (Figure 6.3). It was decided that Halothane and Lidocaine would be used in combination as anaesthetics for the other experiments and that stimulation of the ligament would commence 90 minutes after exposure. In this way, Fos production due to the surgery would be low by the time stimulation started and virtually eliminated by the end of the experiment. Any Fos observed at the end of the experiment would be due to the stimulation of the ligament and not to the surgery.

Main experiment:

Mechanical stimulation of the right intertransverse ligament at the level of T3-4 following the production of anaesthesia using Halothane and Lidocaine produced consistent results. Fluorescent cells were found in the intermediate horns of the gray matter in the spinal cord at levels T2-T4 on both sides as well as in the corresponding sympathetic ganglia. Similarly, fluorescent cells were also found in higher centres of the brain in the nucleus cuneatus and gracilis and the vestibular nuclei and thalamus. Examples are shown in Figure 6.4. Detailed examination revealed that there were many cells showing Fos expression in the dorsal root ganglia (Figure 6.4a) from T1 to T5 on the ipsilateral side (between 14 and 17 Fos-positive cells per section) as well as on the contralateral side but there were fewer fluorescent cells on the contralateral side (between 9 and 12 fluorescent cells). There were also Fos-positive cells in the sympathetic ganglia (Figure 6.4b) but again the fluorescence was more prominent on the ipsilateral side. In the spinal cord (Figure 6.4c), Fos-positive cells were found only in the intermediate horn in the gray matter between the levels from T2 to T4. Fluorescent cells were also observed in the combined nucleus gracilis and cuneatus (Figure 6.4d) with an average of 24 fluorescent cells per section being found on the ipsilateral side and only 17 on the contralateral side. A few cells with Fos-positive expression in the nuclei were equally distributed between both centres of the vestibular nuclei (Figure 6.4e) and the thalamus (Figure 6.4f). An example of a typical distribution of the fluorescent areas throughout the central nervous system in an experimental chick is shown in Figure 6.5.

Figure 6.3. Fos production in the dorsal root ganglia at various times following exposure of the lateral spinal ligament on the right at the level of T3-4. Two different methods of anaesthesia have been used as indicated. Note that when Lidocaine is used in conjunction with Halothane, Fos levels are at zero after 180 minutes whereas the levels remain high when Halothane alone is used. Note that in all cases, the fluorescent cells are more numerous on the side of surgery.


Number of c-fos positive neurons
T5
T4
T3
T2
T1
Left
Right
0
10
20
30
40
0
60
90
120
180
Time
(minutes)
Halothane anaesthesia only
Halothane and Lidocaine anaesthesia

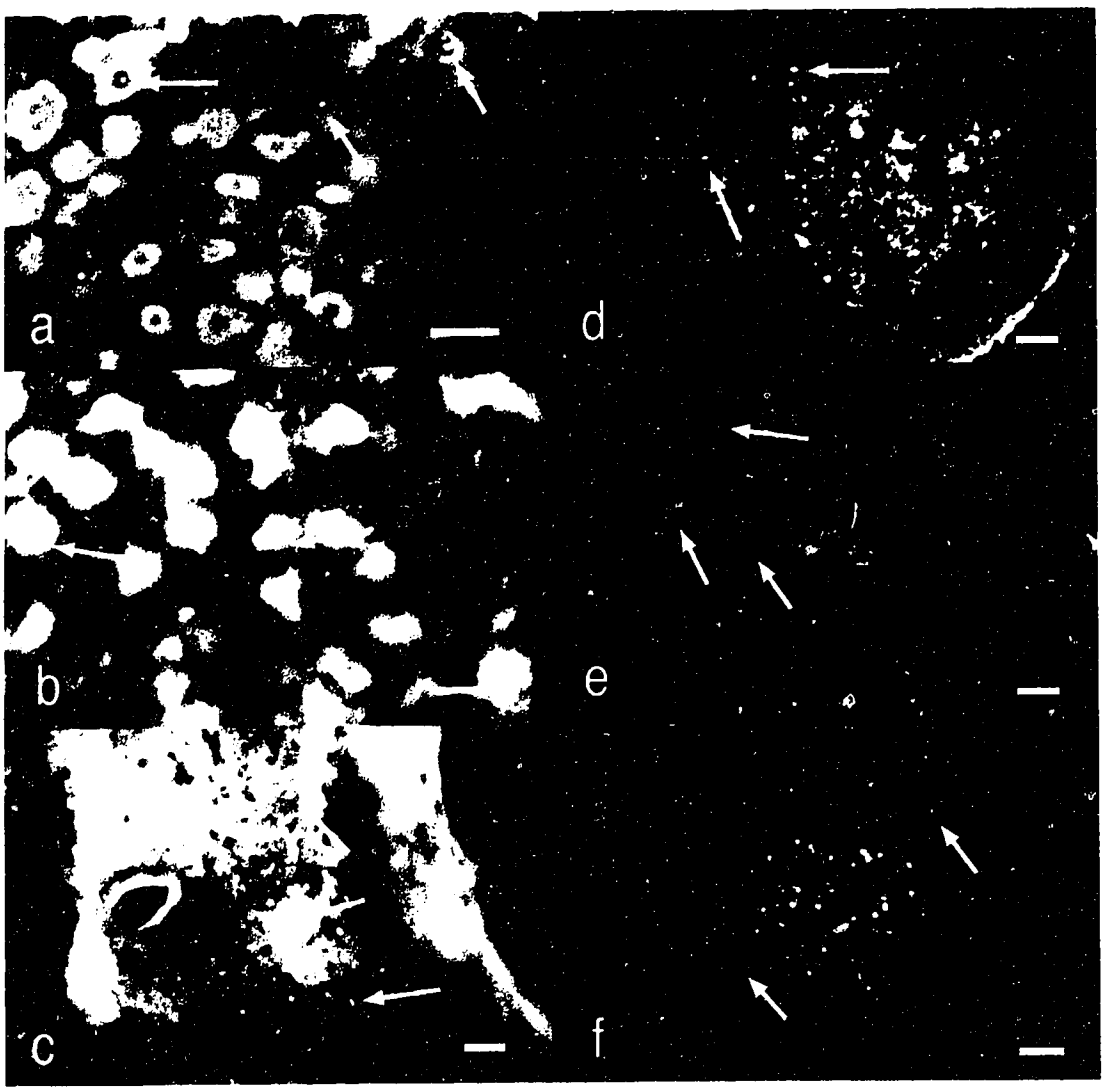


Figure 6.4. (Bar=20μm) Examples of fluorescent nuclei (arrows) indicative of Fos production. a) dorsal root ganglion b) sympathetic ganglion c) spinal cord (the dorsal surface is to the left and the large black circle is the central canal) d) nucleus cuneatus e) vestibular nucleus f) thalamus

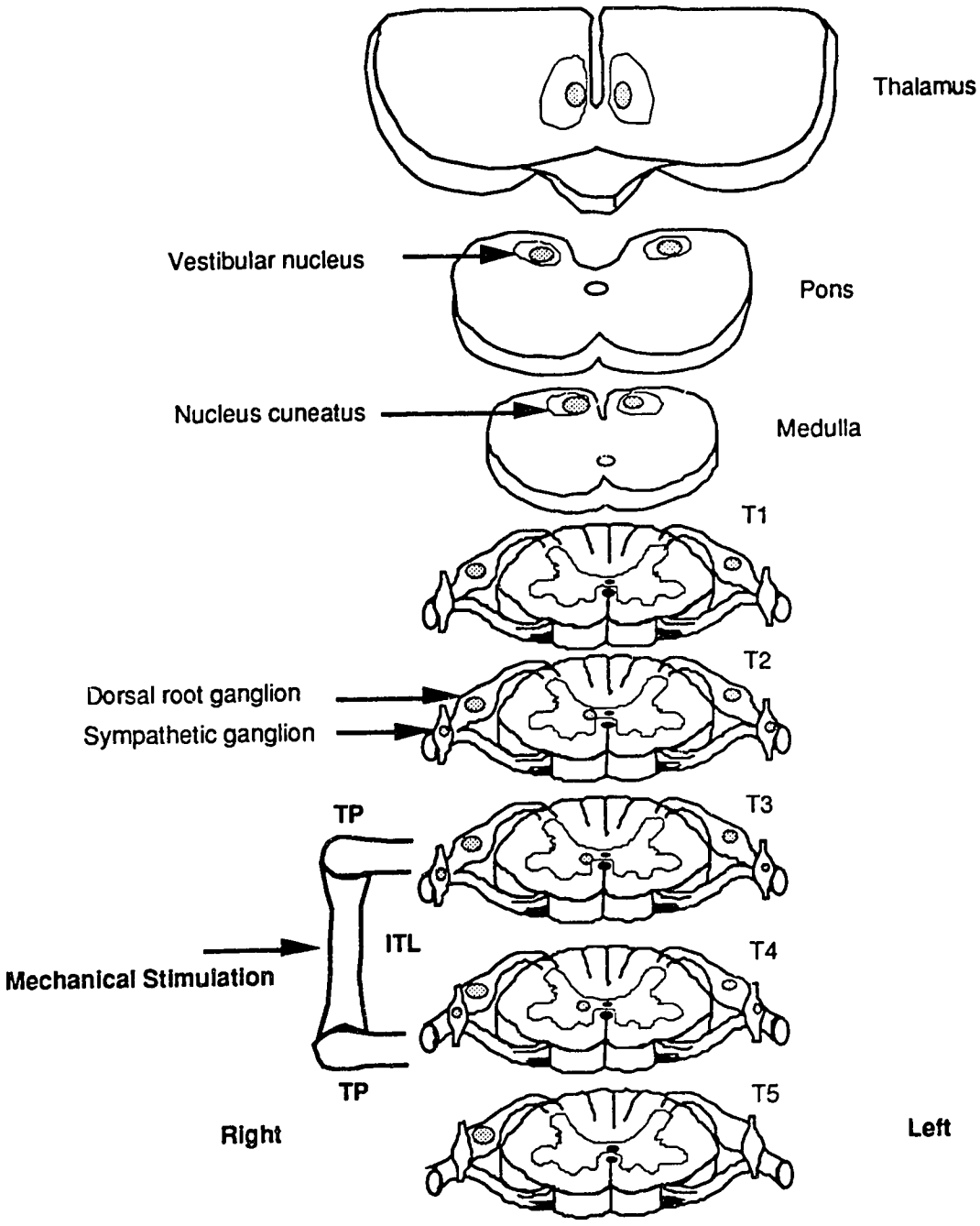


Figure 6.5. Typical results from one of the experimental chickens showing the distribution of Fos in the spinal cord and the brain following stimulation of the spinal ligament. The ligament was exposed at the T3-4 level and Fos-positive nuclei were observed in several adjacent levels of the spinal cord as well as centres in the brain as indicated.

DISCUSSION

The results from the current study showed that mechanical stimulation of a single intertransverse ligament in the chicken by extensive and repeated stretching led to widespread neuronal activity in several well-established sensory areas of the nervous system. Fos-positive particles were found in nerve cell bodies in the dorsal root ganglia, sympathetic ganglia, and areas of the intermediate gray matter of the spinal cord on both ipsilateral and contralateral sides at and around the level of stimulation. Other fluorescent cells, indicative of associated activity, were found in the nucleus cuneatus and gracilis, the vestibular nuclei and the thalamus which represent other proprioceptive centres in the higher centres of the brain. These results strongly support the suggestion that spinal ligaments provide more than simple structural support for the spine by contributing to muscle coordination around the joints and increasing joint stability as suggested for other joints (Freeman and Wyke, 1967; Johansson et al. 1991a, b; Michelson and Hutchins, 1995).

Identification of those areas containing Fos-positive particles allows a first attempt to be made at identifying the possible pathway for proprioceptive information following stretching of the lateral ligaments of the spine. Fos-positive cells in the ipsilateral dorsal root ganglion and the intermediate gray matter of the spinal cord at the same level as the stimulated ligament indicate the first part of the neurological feedback pathway. Stimulation of the nerve ending in the spinal ligament would result in Fos production in the nerve cell body in the dorsal root ganglion. Continued transmission of the stimulus along the axon to the nerve terminal would result in synaptic activity in the intermediate horn of the spinal cord where the stimulation would be conveyed to dendrites of an interneuron. In turn, Fos would be produced in its cell body situated in the intermediate horn of the spinal cord. Fos-positive cells in the intermediate horn suggest association with the dorsal columns which are known to convey impulses concerned with discriminative tactile sense (touch and pressure) and kinaesthetic sense (sense of position and movement) (Carpenter, 1976). Association with this pathway is supported by the presence of Fos-positive particles in neurons in the nucleus cuneatus and gracilis as well as the thalamus. These higher centres in the brain stem contain neurons associated with relay sites subsequent to the dorsal columns (Carpenter, 1976). Fos-positive neurons in the medial portion of the intermediate gray matter of the spinal cord are also in position to connect with neurons associated with both the anterior and posterior spinocerebellar tracts (Clarke's column) which have been shown

to be associated with relaying impulses from stretch receptors such as Golgi tendon organs (Carpenter, 1976). It has also been suggested that such impulses mediated along the spinocerebellar tracts are utilised in the fine coordination of posture and movement (Carpenter, 1976). Identification of these specific sites of neuronal activity associated with stretching of spinal ligaments allows focus to be centred on these areas and tracts in future studies relating to perception of proprioceptive information from lateral spinal ligaments. The close association with the spinocerebellar tracts and the thalamus also suggests that areas of the cerebellum as well as the sensory portions of the cerebral cortex be included in future studies as these are established extensions of the sensory tracts identified.

The presence of fluorescent cells in the ipsilateral dorsal root ganglia and intermediate horns at spinal cord levels adjacent to the level of stimulation might be explained in several ways. First, afferent fibres from the single dorsal root may supply impulses to neurons in Clarke's nucleus in as many as six or seven spinal ligaments as has been previously determined (Carpenter, 1976). We were unable to verify this in the current experiment but while it may explain some of the fluorescence in the multiple levels of the spinal cord it does not explain fluorescence in either the dorsal root ganglia at these other levels or at sites on the contralateral side. Second, it is possible that single lateral spinal ligaments are innervated by several spinal cord levels. While this is possible and would explain the presence of fluorescent cells in both the dorsal root ganglia and intermediate horns at these other levels, it does not explain the presence of fluorescent cells in sites on the contralateral side of the spinal cord. Crossover action of the stimulus through interneurons might be possible in the spinal cord, but it would seem more likely that the multiple sites of fluorescence, particularly on the contralateral side, are indicative of the widespread, mechanical response to stretching of a single ligament. Such stimulation is not an isolated action, for many adjacent structures are mechanically linked to the ligament and are affected by the action of stretching. The simple but effective method of stretching the ligament employed in this study was designed to stretch the ligament close to its maximum length but in doing so probably also affected other adjacent tissues on both the ipsilateral and contralateral sides and triggered similar, related proprioceptive responses. This would have resulted in increased Fos production indistinguishable from that directly emanating from the isolated stretching of the ligament and would have resulted in other levels and areas of the spinal cord also becoming involved in Fos production. While there is always the possibility of multilevel innervation of spinal ligaments, this study shows that stretching of a lateral

spinal ligament results in a relatively massive and widespread input of neurological information from several levels of the spinal cord which is displayed on both the ipsilateral and contralateral sides. Future experiments in which the lateral spinal ligament is isolated from other structures during stretching will reveal a more direct pathway for the proprioceptive information from a single ligament.

The wide spread of the proprioceptive input to several vertebral levels also suggests the possibility of a reflex response to such stretching of the spinal ligaments which is centred on the local area of the spinal cord. Presumably such localised reflex activity would be directed through muscle responses designed to maintain the adjacent vertebrae in close alignment and prevent the associated joints from being damaged (Michelson and Hutchings, 1995). The involvement of the higher centres in the brain such as the vestibular nuclei and thalamus suggests a more central involvement of reflex activity in response to the proprioceptive information possibly designed to maintain general balance and an overall upright posture similar to the mechanisms described for this purpose by Roberts (1967). This separation of the possible reflex activity into separate components might be important because of the potential for their separate involvement in the development of abnormalities in the balancing of the spine such as is found in scoliosis. Any abnormality might be related to misperception of information associated with either local reflex activity of the spinal cord or central reflex activity associated with centres in the brain but possibly not both. This concept of improper proprioceptive feedback being associated with the production of scoliosis has been suggested previously by other workers. For example, Yamada et al. (1984) proposed that virtually any disruption of the postural reflex system could result in scoliosis and indicated that there was clinical and experimental evidence that brain stem dysfunction could contribute to the aetiology of scoliosis. Similarly, Barrios et al. (1987) and Smith and Dickson (1987) have shown that damage to the postero-lateral columns in the spinal cords of rabbits can give rise to a progressive scoliosis that resembles the development of adolescent idiopathic scoliosis in humans. Robin (1990) also concluded that there is mounting evidence to suggest that a primary defect of CNS function - a defect of posture, proprioception, or equilibrium control - is mainly responsible for production of the spinal curvature in scoliosis. In particular, Robin (1990) concluded that an abnormality of the brain stem which integrates and transmits impulses that are responsible for the maintenance of posture, proprioception, and equilibrium is thought to result in some of the characteristic features seen in association with adolescent idiopathic scoliosis. This concept of improper proprioceptive feedback

being involved with the development of scoliosis and its link with ligament support and posture control clearly warrants further study.

The results of the control experiments support the work of earlier studies which have shown that c-fos is a highly regulated gene in the nuclei of neurons whose transcription is elevated for a short time after the application of a stimulus (Curran, 1988). This elevation in transcription apparently occurs within 5 minutes and continues for 15-20 minutes (Greenberg and Ziff, 1984; Greenberg et al. 1985). The accumulation of mRNA peaks at 30-45 minutes (Muller et al. 1984) and the synthesised protein, Fos, has been shown to have a half life of about 2 hours (Curran et al. 1984; Muller et al. 1984). The elevation of Fos levels following surgery in all control experiments to expose the ligament supports these findings and justifies the delay of 90 minutes between exposure of the ligament and initiation of the mechanical stimulation. The high levels of Fos resulting from the surgery would have returned to normal by the time the stimulation had finished and any Fos observed would have been due only to the mechanical stimulation of stretching the ligament.

It is interesting to note that following anaesthesia with Halothane no nuclei of somatic motor neurons in the ventral horn of the grey matter in the spinal cord contained Fos-positive particles although visceral motor neurons in the sympathetic ganglia were prominently fluorescent. While it is possible that motor pathways are not stimulated following stretching of a lateral spinal ligament, this seems unlikely. It is more probable that the Halothane anaesthetic affected the motor pathways such that the motor neurons remained unstimulated and did not produce Fos protein. This would not be surprising for Halothane is used to prevent motor activity during surgery and has been shown to affect Fos production (Krukoff, 1993). In contrast, the fluorescent autonomic motor neurons seen in the sympathetic ganglia show that visceral motor activity at the various spinal cord levels is not affected by Halothane and that normal visceral activity is presumably maintained during anaesthesia. The brief use of Somnotol in the control experiments sometimes produced areas of the ventral horn in the grey matter of the spinal cord that contained Fos-positive nerve cell bodies indicative of somatic motor activity but these were not consistent or well defined. It is unfortunate that avians do not tolerate Somnotol well for the associated motor pathway that might be expected following the stretching of lateral spinal ligaments might possibly be traced using Somnotol as the anaesthetic. In a small pilot study, the use of other anaesthetics such as Ketamine produced involuntary somatic motor activity in the

chickens while under anaesthesia and the Fos produced by this movement completely masked the effects of the designed mechanical stimulation even in the sensory areas of grey matter. At present the only data available in the literature in which somatic motor activity has been identified by the presence of Fos protein in the cell bodies of motor neurons in the ventral horn of the spinal cord has been restricted to experiments in which no anaesthetic has been used (Jasmin et al. 1994a, b). Although the precise mechanisms by which mechanoreceptors accomplish their suggested role in reflex activity are not known, it is generally thought that they influence gamma motor neuron output from the gray matter of the spinal cord which subsequently affects the length of muscle spindle fibres (Freeman and Wyke, 1967; Johansson et al. 1991a; Michelson and Hutchins, 1995). This study was unable to verify such an active motor response and the associated motor pathway in response to the stretching of a spinal ligament therefore remains unexplored using this methodology because of the unavailability of an appropriate anaesthetic.

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CHAPTER 7

A comparison of the innervation characteristics of the lateral spinal ligaments between normal subjects and patients with adolescent idiopathic scoliosis.

INTRODUCTION

Although the aetiology of adolescent idiopathic scoliosis (AIS) has been studied intensively during the past seventy years (Robin 1990), the true cause of AIS is still not known. The development of the lateral spinal curvature associated with AIS has been attributed to many factors related to growth of the vertebrae themselves including the asymmetrical growth of the vertebral bodies (Roaf, 1960) and asymmetrical growth at the paired neurocentral junctions (Knutsson 1966). The validity of these proposed mechanisms has been subsequently challenged. Similarly, because AIS appears to be initiated and most progressive during the rapid growth period associated with puberty in girls, hormonal abnormalities have also been investigated as possible causative factors. In this respect, growth hormone (GH) levels have been examined by Misol et al. (1971) and Willner et al. (1976) and somatomedin levels by Willner et al. (1976) and Skogland et al. (1981) but no consistent results were observed. Other areas of study have included biochemical properties of the tissues involved, particularly collagen (Pedrini et al. 1973; Bushell et al. 1979; Ghosh et al. 1980; Venn et al. 1983a, b). Again inconsistent results have been reported although Taylor et al. (1981) and Burwell (1981) both found that patients with AIS had more joint laxity than normal subjects. Therefore many researchers have proposed theories which explore potential mechanisms of development but no single theory has been shown to be satisfactory and universally accepted.

Recently, general proprioceptive dysfunction has been proposed as a major contributing factor in the development of AIS (Sahlstrand and Petruson, 1979; Gregoric et al. 1981; Lidström et al. 1981; Yekutiel et al. 1981; Barrack et al. 1984; Herman et al. 1984; Keessen et al. 1992). The hypothesis suggests that a proprioceptive sensory rearrangement or re–calibration of the internal representation of the body in space is present in AIS patients resulting in a non–erect vertebral alignment being erroneously

perceived as straight. It has been suggested that such proprioceptive dysfunction can occur at either a local level in the spinal cord or at a central level higher up in the brain (Alexander et al. 1972; Chin et al. 1984; Pincott et al. 1984; Yahia et al. 1992; Jiang et al. 1995a).

It has also been suggested that ligaments might play a part in this sensory dysfunction as there is an increasing body of evidence which shows that they have an important neurosensory role in supplying proprioceptive information or serving as important transducers for dynamic neuromuscular balance and maintenance of an erect spine (Schultz, 1972, 1974; Chin et al. 1984; Kojima et al. 1990; Yahia et al. 1992; Jiang et al. 1994, 1995b). Accordingly, this study wa designed to examine one aspect of the proprioceptive system and compared the innervation characteristics of the superior costotransverse ligaments (SCTL) of the spine between patients with AIS and normal controls. The types of nerve endings present in the ligaments, their quantitative density, and their distribution pattern were compared.

MATERIALS AND METHODS

Permission to obtain lateral spinal ligaments (LSLs) from both fresh human cadavers which represented normal controls and scoliosis patients was obtained from the Ethical Committee of The University of Alberta Hospitals. However, fresh, lateral spinal ligaments, especially the SCTLs, are not easily obtained for they are not often exposed or accessible at surgery and lie close to the somatic pleura. Fresh human material for the control group was collected through the HOPE (Human Organ Procurement and Exchange) Program and normal, fresh, human SCTLs were harvested within 12 hours after cardiac arrest. LSLs were also obtained from scoliosis patients who were undergoing spinal fusion surgery for curve correction as these ligaments can be safely obtained during this procedure. In all cases of scoliosis the SCTLs were obtained from both sides of the spine at the level of the apex of the primary curvature. The SCTLs in the control group were obtained at thoracic level (T) 9 to approximate the levels in the scoliosis group. The details of the patients in both normal and scoliotic groups are summarized in Tables 7.1 and 7.2. These details were obtained with permission from medical charts. Even with the necessary permission, collection of the material for this project took over 2 years because of the scarcity of appropriate patients.

Table 7.1. Demographic data of normal subjects

Subject	Age (years)	Sex	Cause of death	Spinal disease	level
1	44	Female	MVA	No	T9
2	45	Female	MVA	No	T9
3	43	Female	MVA	No	T9
4	17	Male	Gun shot	No	T9
5	22	Male	MVA	No	T9

MVA = Motor Vehicle Accident

Table 7.2. Demographic data of the patients with scoliosis

subject	age (years)	sex	diagnosis	Cobb angle at surgery	Apex	convex	progressive
1	14	F	AIS	90	T10	Right	Yes
2	12	F	AIS	50	T7	Right	Yes
3	13	F	AIS	60	T9	Right	Yes
4	13	F	AIS	62	T9	Right	Yes
5	15	F	AIS	50	T9	Right	Yes

The specimens from both groups were immediately immersed in Zamboni's fixative contained in a small bottle directly in the operating room. The bottle was placed in an ice box at 0°C for easy and immediate transfer to the laboratory. The orientation of each of the SCTLs from the control group was identified and maintained during subsequent processing. Unfortunately, the restrictions in the operating room prevented the identification of orientation information for the specimens from the scoliosis patients.

The ligaments were fixed for at least 6 hours and then transferred to a solution of 30% sucrose/phosphate buffered saline (PBS) in which they were stored overnight at 4°C. Each specimen was surrounded and supported with O.C.T. (*o*-chlorotoluene - Miles, Elkhart) on a small cork block and flash frozen by immersion in isopentane cooled by liquid nitrogen. Serial sections of 48 μm were cut using a cryostat and the sections were thaw mounted onto glass slides pre–coated with gelatin before being stored for a short time at -20°C.

The staining procedure commenced with immersing each section in acetone for ten minutes and then incubating them with a pooled, single solution of mouse monoclonal antibodies against neurofilament protein subunits 68, 160 and 200 KD (SIGMA, St. Louis) in a humid chamber for 2 hours. The antibodies were prediluted to 1:300 with PBS containing 0.4% Triton X-100. Rabbit, anti–mouse IgG conjugated with FITC (SIGMA, St. Louis) was used as the secondary antibody to visualise the reaction sites. This reaction was completed in a dark humid chamber for 1 hour with the antibody prediluted to 1:100 using the same diluent as before. The sections were dried and mounted with Fluoromount to preserve the fluorescence (BDH, Poole). During the procedure, the sections were washed twice using PBS containing 0.4% Triton X-100. Positive controls were obtained from samples of human skin and intercostal nerve collected at the same time as the initial surgery. Negative controls were obtained by using the same protocol except that the PBS was substituted for the primary antibody to assess background autofluorescence or a nonspecific antibody was used to determine specificity.

Observation of the neural elements in the SCTLs was performed using an Orthoplan fluororescent microscope (Carl Zeiss, Oberkochen). The position and types of neural elements were documented on an enlarged drawing made of each section as described in an earlier study (Jiang et al. 1994). The relatively high levels of autofluororescence produced by the dense collagen bundles occasionally made observation of detailed structures difficult. When there was any doubt in terms of recognition of a neural element a confocal microscope equipped with laser UV light (Leica Lasertechnik, Heidelberg) was used. This reduced the background fluorescence and made identification of neural elements more easy. The observed neural elements together with the predrawn picture of the section were digitized into a HP computer (Hewlett-Packard Co., Fort Collins, Colorado) for subsequent data analysis.

The size of the nerve elements was determined directly from either the fluorescence microscope or from the confocal microscope after calibration with a graticule. The volume of each ligament was calculated by measuring the area of each section using a computer program, multiplying by the thickness of the section, and totalling the results. The orientation of the SCTLs from the controls was retained throughout the analysis and the geometric centre of each of the sections was determined visually so that dorsal and ventral compartments could be defined. A computer program was developed to calculate the number and location of nerve elements relative to the dorsal and ventral compartments. The number of nerve elements located in the cranial, middle and caudal thirds of the ligaments was also calculated using the same computer program. The total number of nerve elements per ligament was also calculated. The innervation density was defined as the average number of nerve elements per cubic mm of ligament. A Student's t–test was used to compare the differences between ligaments on the left and right sides as well as differences between data from control and scoliotic patients. The significance level was set at $p < 0.05$. The distribution pattern of the innervation in the normal subjects was also analyzed and described. No attempt was made to analyze the distribution pattern in ligament from the scoliosis patients because the orientation of the ligament was not maintained. Because of the relatively small number of samples studied in this project, a statistical power test was also performed.

RESULTS

The innervation of SCTL in the normal group.

The SCTLs consisted of well organized collagen bundles oriented parallel to the long axis of the ligament. However, many septa of relatively loose connective tissue penetrated between the dense collagen bundles and lay in the same direction. A thin layer of loose connective tissue was also found on the ventral surface of the ligament and it was here that a prominent nerve could be observed. Gross dissection identified this nerve as a branch of the intercostal nerve. On the dorsal surface, a thick layer of adipose tissue was found between the ligament and the overlying muscular tissue.

The positive controls of skin and intercostal nerve showed strong fluorescent reactions from which neural tissue could be clearly seen. In the negative controls where the antibody against neurofilament protein had been replaced by either PBS or nonspecific

antibody, no immunofluorescent reactions other than autofluorescence could be seen in the tissue sections.

The ligaments from the normal group were found to be richly innervated. In this study, only the nerve elements inside the ligament were analyzed while those in the paraligament, a thin layer of connective tissue surrounding the ligament, were disregarded. The nerve elements inside the ligaments included free nerve endings (Figure 7.1), small nerve bundles (bundles containing less than 10 fibres) (Figure 7.1 and Figure 7.2A), large nerve bundles (more than 10 fibres) (Figure 7.2B) and, less commonly, Ruffini corpuscles (Figures 7.3 and 7.4). Interestingly, neither Pacinian corpuscles nor Golgi end organs were observed in the SCTL.

The free nerve endings and the small nerve bundles were located in collagen bundles at the periphery of the ligament as well as within the dense collagen bundles deep within the ligaments. However, these types of nerve elements were more commonly found in the relatively loose connective tissue or septa which penetrated the dense collagen bundles (Figure 7.2A and 7.2B). These nerve elements consisted of a variety of NFP positive axons which included both thin fibres of <3 μm, thick fibres of >3 μm, and varicose fibres which had regular bead-like expansions along their course. No attempts were made to categorize or analyze these fibers using normal standards since the thickness of the fibers might not coincide with traditional classification standards which are based on silver nitrate staining techniques (Jiang et al. 1995a).

The Ruffini corpuscles were also located mostly in the periphery of the ligaments and were always enclosed by the dense collagen bundles. The size of the Ruffini corpuscles varied from 20 micrometers to 100 micrometers and consisted of highly branched and coiled axons (Figures 7.3 and 7.4). Although Ruffini corpuscles are classified as encapsulated mechanoreceptors, no apparent capsule was observed in this study. This is perhaps due to the anti–neurofilament protein antibody not having any affinity to the capsular Schwann cells.

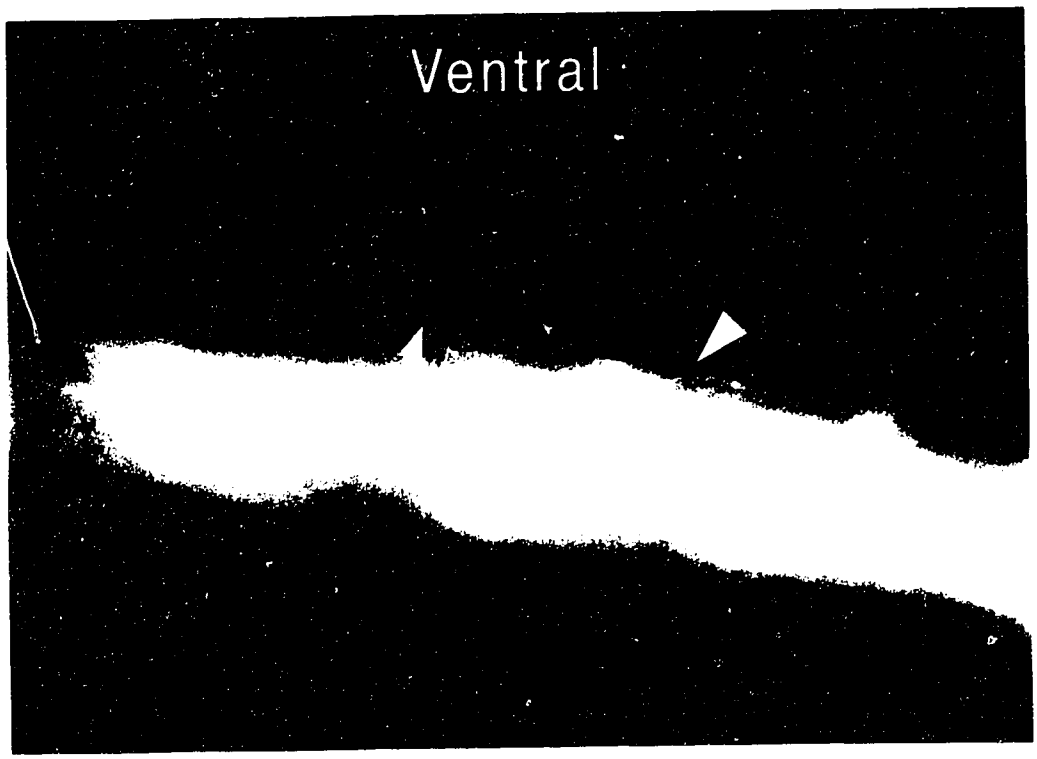

Figure 7.1. A photomicrograph (X250) showing a single nerve fibre (arrowhead) and a small nerve bundle (arrow) located in the periphery of the dense ligament tissue. Ventral = ventral side of the SCTL.

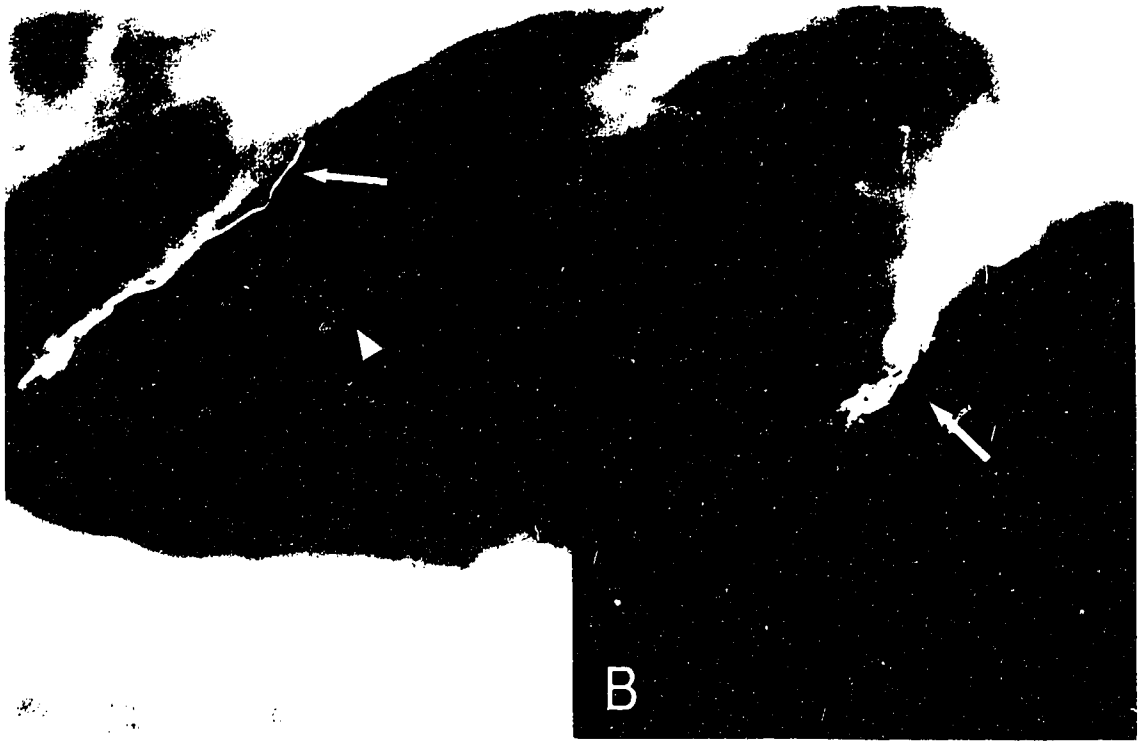

Figure 7.2. A. A photomicrograph (X250) showing a single nerve fibre (arrow) and a small nerve bundle (arrowhead) which were located in the middle of the SCTL. B. A photomicrograph showing a large nerve bundle (arrow) which is located in the middle of the SCTL.

Figure 7.3. A photomicrograph (X250) showing a Ruffini corpuscle (arrow) which is innervated by two branched axons. The end structure is not clearly seen because the corpuscle is relatively large and cannot be focused properly by ordinary fluorescence microscopy.

Figure 7.4. An image of a Ruffini corpuscle (arrow) which was obtained using a confocal microscope coupled with reconstruction on an SGI system using VoxelView. Multiple, optical sections had been made through a thick section and these images were reconstructed 3-dimensionally.

The innervation distribution pattern in the cranial, middle and caudal thirds of the ligaments is shown in Table 7.3. Note that no difference in distribution was found between any of the elements and their sites along the ligament ($P > 0.05$). The distribution pattern relative to dorsal and ventral areas of the ligament are also shown in Table 7.3. In contrast, significant differences in distribution were found in all elements ($P < 0.05$) between the dorsal and ventral areas of the ligament. The distribution of nerve elements in the SCTL of both left and right sides of the body is presented in Figure 7.5 and clearly shows no differences in numbers of elements between these two sites ($P > 0.05$).

Table 7.3. Distribution of the average number of nerve elements in the SCTL in relation to position within the ligament (N=5).

Structures	Cranial	Middle	Caudal	Ventral	Dorsal
		$P > 0.05$		$p < 0.05$	
Ruffini	13 ± 5	12 ± 4	14 ± 5	29 ± 7	11 ± 8
FNE	89 ± 20	82 ± 30	84 ± 13	182 ± 26	71 ± 28
SNB	84 ± 10	97 ± 31	85 ± 32	200 ± 33	68 ± 25
LNB	12 ± 7	12 ± 13	6 ± 3	24 ± 9	6 ± 6
Total	194 ± 40	204 ±60	193 ± 26	438 ± 51	154 ± 55

The innervation of SCTL in the scoliosis group

There was no apparent difference in the organization of collagen and connective tissue bundles between the ligaments from patients with AIS when compared with those from the normal control group. There was also no apparent difference in the morphology of the free nerve endings, small nerve bundles and Ruffini corpuscles. There were insufficient data for reliable analysis of the large nerve bundles due to the small number of patients and so the data were ignored. However, significant differences were found in the numbers of nerve elements when the ligaments from the two groups were

compared. As there was some uncertainty as to whether or not the complete SCTLs were taken from the patients in the scoliosis group at the time of surgery and the difference in size of the ligaments between the two groups, only innervation densities (the number of nerve elements per cubic millimeter) were compared rather than the total number of nerve structures in each of SCTL. The results (Figure 7.6) show that the total innervation density of the SCTLs from the patients with scoliosis averaged 2.68 ± 0.8 per mm^3 which was significantly lower ($P = 0.01$) than similar measurements acquired from the normal group (4.2 ± 0.6 per $mm^{3)}$. Similarly, the number of Ruffini corpuscles in the SCTLs from patients with scoliosis averaged 0.09 ± 0.08 which is also significantly lower than the numbers from the normal group of 0.3 ± 0.1 ($P = 0.004$). However, there was no statistical difference found in the total innervation densities of any of the nerve elements when figures obtained from the left and right sides of the spine in the scoliosis group were compared ($p > 0.05$).

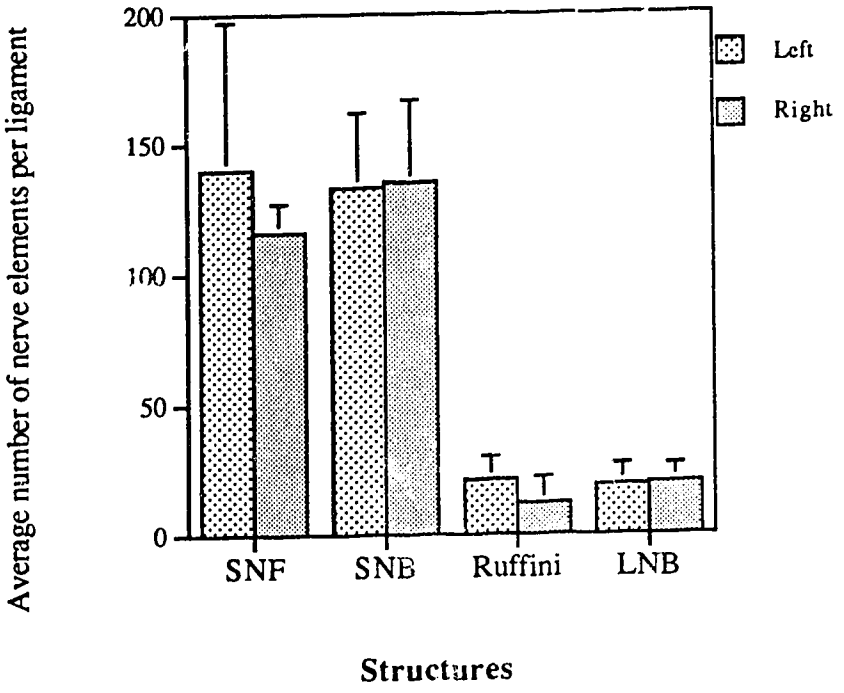

Figure 7.5. A histogram to show the number of each specific nerve element found in the SCTLs from normal subjects. Note that no significant difference was found between left side and right side for all of the different nerve elements.

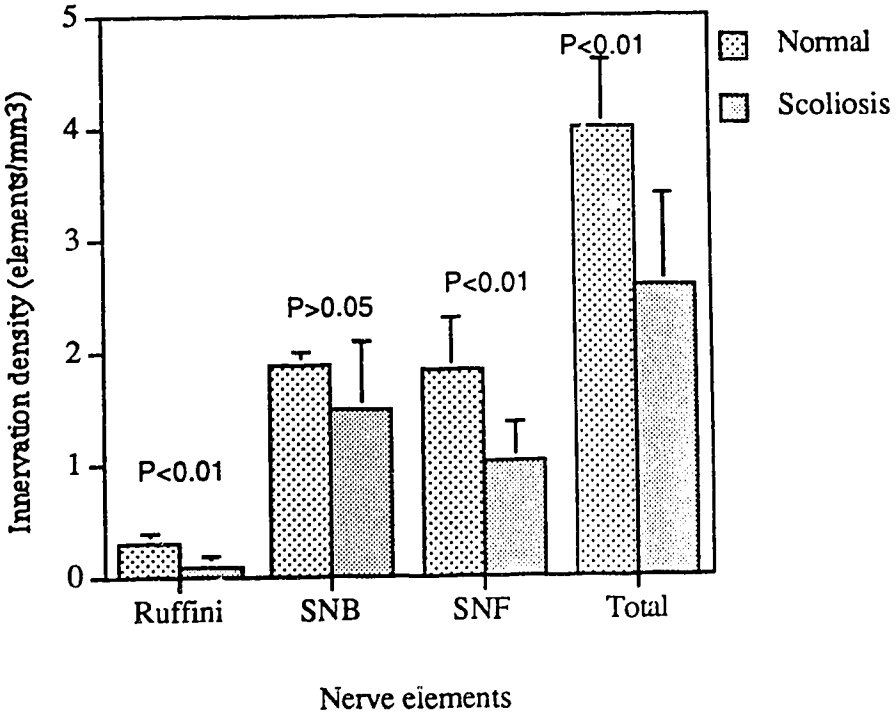

Figure 7.6. A histogram to show that the innervation densities of Ruffini corpuscles and single nerve fibres were significantly lower in the scoliosis group. In comparison, no significant difference was found between the normal and scoliosis groups for the small nerve bundles. When the total numbers are compared there are significantly more elements in the ligaments from normal subjects.

DISCUSSION

The concept of a proprioceptive function for ligaments has been gathering attention in recent years. The innervation contained within ligaments has been shown to be able to send afferent neuronal signals capable of affecting the tension of related muscles (Beard et al. 1994). In particular, the innervation of spinal ligaments has also been related to the active maintenance of an erect posture and a defect in the perception of the proprioceptive information has been proposed as a possible cause in the development of scoliosis (Yahia et al. 1992). In this study, the lateral spinal ligaments which have been shown to be more important than the central spinal ligaments in the lateral balancing of the spine (Jiang et al. 1994, 1995b) were found to be richly innervated with free nerve endings, small and large nerve bundles as well as Ruffini corpuscles. In the normal control group, the innervation was found to be symmetrical between left and right sides of the spine but was more concentrated in the ventral portion of each ligament. No apparent morphological defect of the innervation was found in the lateral spinal ligaments from the scoliosis group when compared with similar data collected from the control group. However, the innervation density was significantly lower in the ligaments from the scoliosis group when compared with those in the normal group. The lower innervation density perhaps indicates a subnormal ability to send correct proprioceptive signals to the central nervous system and therefore compromises the neuromuscular protection and maintenance mechanism necessary for lateral balancing of the spine. These findings might be related to the development of AIS particularly when there are such dramatic changes occuring during the rapid growth period associated with puberty.

Labeling and observing nerve tissue by immunohistochemistry and fluororescent markers is hindered by the dense nature of the surrounding collagen bundles and their autofluororescence. To enhance the sensitivity of our study, both acetone and Triton X-100 were used to facilitate the mobilization of antibodies into the ligament tissue. Using pooled monoclonal antibodies against the three subunits of neurofilament protein rather than a single antibody seemed also to magnify the signal. The sensitivity and specificity in recognising the nerve elements was greatly enhanced by using the confocal microscope for some of the nerve endings which are relatively large and embedded in the dense collagen bundles. Ordinary fluorescence microscopy is unable to reveal the precise structure of many of these elements. In contrast, a confocal

microscope is able to section optically through the thick sections of ligament tissue and reveal the embedded nerve elements free of the surrounding autofluorescence and allow subsequent three-dimensional reconstruction of the complete structure. This methodology increased our reliability in identification. Interestingly we were unable to find any Pacinian corpuscles in these ligaments contrary to other published data referring to central spinal ligaments (Yahia et al. 1988; Jiang et al. 1995a). It is possible, although doubtful, that the antibody we used failed to reveal the characteristic lamellar cells of the Pacinian corpuscles especially as we have used it with success previously when studying the central spinal ligaments (Jiang et al. 1995a). The absence of Pacinian corpuscles in the lateral spinal ligaments perhaps reveals more about their function and warrants further study.

The difficulties experienced in obtaining material for this study resulted in both the control and experimental groups each consisting of only 5 subjects. As such small numbers might compromise the validity of the analysis a power test was completed. This showed that with a type I error threshold set at 0.05, type II error threshold set at 0.1 and the sample size of five in each group, the data analysis would be able to detect a difference of 1.2 nerve elements per mm^3. This is the difference of the mean between the two groups or twice their average standard deviation. More samples would be needed to detect smaller differences between the two groups in any further studies.

The erectness of the human spine is believed to be a learned behaviour and evolutionary adaptation may not be adequate for the dramatic change of load pattern and constant challenge to the balance of the spine in both sagittal and coronal planes consequently resulting in deformation and abnormality (Lindahl and Raeder, 1962; Luk et al. 1986; Barrios et al. 1987; Luk and Leong, 1987). The stability or maintenance of the erect spine can only partially be supported by the mechanical constraints of spinal ligaments (White and Panjabi, 1990) and so the concept of a proprioceptive neuromuscular pathway emanating from the ligaments playing a major role in spinal structure is very appealing (Yahia et al. 1988; Rhalmi et al. 1993; Yamashita et al. 1993a, b). Evidence in support of the existence of such a pathway and mechanism similar to that being established for other areas of the body (Ferrell and Hart, 1980; Sojka et al. 1983; Baxendale and Ferrell, 1981; Beard et al. 1994) is rapidly accumulating and must be considered very seriously (Cavanaugh et al. 1989; Beard et al. 1994). The functions of the proprioceptors contained within the ligaments, especially the Ruffini corpuscles, has been well established (Freeman and Wyke, 1967; Kennedy et al. 1982; Halata,

1988; Kannari et al. 1992). Ruffini corpuscles have been reported to be low-threshold, slowly adapting mechanoreceptors which are most sensitive to the static position of the joint in which they are located (Kennedy et al. 1982). Studies have shown that the proprioceptive afferent signal does not directly control the alpha motor system but rather influences the gamma motor system, ultimately affecting the firing of muscle spindles (Sojka et al. 1983). This mechanism usually controls the tone of related muscles rather than their active contraction (Johansson et al. 1991a, b; Michelson and Hutchins, 1995) but perhaps muscle tone plays a major role in the maintence of an erect spine in addition to specific muscle contractions. The results of this study showed that both the number of nerve elements and the innervation density were similar in the lateral spinal ligaments on both sides of the spine in both the normal controls and the scoliotic patients. While this supports the concept of a balance of proprioceptive information being essential for maintaining an erect spine in the normal group, the equal numbers on both sides of the spine found in the scoliotic patients is difficult to explain other than to suggest that interpretation of the proprioceptive information is most important rather than simple numbers of nerve elements.

The reduced innervation density found in the ligaments from the scoliotic patients is very important for it implies a compromise in the normal proprioceptive protective mechanism. A patient with AIS might not be receiving appropriate and adequate proprioceptive information to be fully aware, either consciously or subconsciously, of their actual body postures and position of the spinal elements (Yahia et al. 1992). Consequent adjustments might be inappropriate and lead to imbalance. It might be argued that the difference in age and maturity between the two groups would negate any differences found but the results from another study (unpublished) of the changes in innervation characteristics experienced during puberty in chickens have shown that innervation density decreases with age. Therefore the lower figures shown by the scoliotic patients for innervation density might be expected to decrease even further as the patients mature but this would only increase the size of the discrepancy. Whether or not any discrepancy is a primary or secondary factor associated with scoliosis must also be considered but it is difficult to imagine why such numbers would be lower especially as they are symmetrical. This area too warrants further study perhaps initially by increasing the sample size to confirm the findings of this current study.

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CHAPTER 8

General discussion and conclusions

Review of thesis hypothesis and objectives

Although spinal ligaments have been recognized as important in the maintenance of balance of an erect spine in the human (Gunzburg et al. 1992; White and Panjabi, 1990; Dumas et al. 1987), the mechanism by which this is achieved and the consequences of any pathology are still not fully understood. Maintenance of a straight and erect spine in the coronal plane appears dependent on the integrity of the lateral spinal ligaments (Smith and Dickson, 1987; Thomas and Dave, 1985) and therefore these ligaments would appear to be pivotal in the development of AIS (Schultz et al. 1972; Schultz and Hirsch, 1974; Lindahl and Raeder, 1962). The first project in this thesis established the relative importance of each of the spinal ligaments in the lateral balancing of the spine and confirmed that the lateral ligaments, especially the SCTL, were the major influencing factor. This defined the focus for subsequent studies which were concentrated on the characteristics of the SCTL or lateral ligaments.

To confirm further the importance of the lateral spinal ligaments for the maintenance of an erect spine, the lateral spinal ligaments from both bipedal and quadrupedal animals were observed and analyzed. The results showed that the lateral spinal ligaments are present or very well developed only in the bipedal animals. As the development of a structure in evolutionary terms usually means that the structure is necessary for appropriate body function (Luk and Leong, 1987; Luk et al. 1986) this confirmed the important status assigned to the lateral ligaments in relation to the maintenance of an erect posture .

The third project in this thesis established that lateral spinal ligaments were richly innervated and provided support for the hypothesis that proprioceptive feedback played an important role in joint stability. Complete testing of the entire hypothesis that the innervation was part of a neurological feedback mechanism which actively balanced and maintained an erect spine in humans was beyond the scope of this thesis but the results provided significant evidence that such a mechanism may exist.

Some spinal deformities develop during the period of rapid growth surrounding puberty and this study has also produced evidence to show that significant changes in innervation characteristics within ligaments occurs during development particularly around puberty. The significance of these changes and their relationship to the development of AIS has yet to be established but there is clearly the potential for damage to the innervation or incorrect development at this critical time and the production of longterm abnormalities.

An initial attempt at defining the precise pathway for the proprioceptive feedback has also been made based on the tracing of Fos production following mechanical stimulation of a single ligament. The results have shown that the information is focussed at both a local level in the spinal cord as well as at a more central level in higher centres of the brain and brainstem. It has been suggested that individual joint stability is possibly dependent on reflex action at the local level in the spinal cord whereas overall balance and the maintenance of an erect spine might be dependent on more central areas of control in the brainstem and brain.

Finally, significant differences in innervation of the lateral spinal ligaments between normal subjects and patients with AIS have been found. These results suggest a possible deficiency in proprioceptive feedback in patients with AIS and provide a basis for future studies.

Discussion of the overall results

It was found that the lateral spinal ligaments (LSL) which connect the upper and lower vertebrae on the lateral side of the spine in humans are important for the lateral balancing of the spine, while superior costotransverse ligament (SCTL) is the most important. The stiffness provided by the SCTLs accounted for 4% to 28% of total stiffness of the ligamentous spine. More importantly, the SCTL is most sensitive to lateral bending of the spine and is subjected to more strain during lateral bending. Therefore the SCTLs could be a very sensitive sensor for the active neuromuscular control of the lateral stability of the spine if it is innervated by proprioceptors. The SCTLs were studied further in the subsequent projects to confirm the nature of their rich innervation. It was also found that the lateral spinal ligament is well developed

only in bipedal and pseudo-bipedal animals while it is absent in quadrupeds. The presence of lateral spinal ligaments in only the bipedal and pseudo-bipedal animals further supports the concept that the lateral ligaments of the spine are fundamental to the maintenance of an erect spine.

It was confirmed that the spinal ligaments are richly innervated by Ruffini corpuscles, Pacinian corpuscles, free nerve endings and nerve bundles using immunohistochemical techniques involving monoclonal antibodies against neurofilament protein, an intermediate cellular filament universally present in all nerve tissues. Methodology was also developed to quantify the innervation density in the spinal ligament. A confocal microscope was used to increase the sensitivity of the observations. The results showed that most of the nerve elements in the supraspinal and interspinal ligament (SSL/ISL) in the lumber region were located in the periphery of the SSL/ISL complex while Ruffini corpuscles were located close to the dense collagen bundles and Pacinian corpuscles were located close to blood vessels. The proximity of the Ruffini corpuscles to the dense collagen bundles together with the findings of its slow adaptive and low threshold nature (Halata, 1988; Zimny, 1988; Freeman and Wyke, 1967) suggested that these corpuscles play an important role in the detection of tension load on the spinal ligaments and probably is the initial structure which starts activation of the neuromuscular feedback control for the stability of the erect spine (Yahia et al. 1992). The closeness of the Pacinian corpuscles to blood vessels suggests that these corpuscles are involved in the vascular sensory afferent pathway control mechanism.

Although the innervation of spinal ligaments was characterized in adults (Rhalmi et al. 1993; Yahia et al. 1992; 1988), understanding the nature of innervation during development could be more significant to the development of AIS since the deformities develop during growth periods. It was found that the proprioceptive nerve endings were not fully developed until the chickens were 7-weeks old. The total number of nerve elements increased during growth of the chickens but, interestingly, the relative innervation density in terms of number of nerve elements per volume of ligament decreased during rapid growth periods. These results suggested that postnatal development of the proprioceptors is an important part of the whole developmental process and that abnormal proprioceptive function, possibly due to damage or improper coordination, might lead to abnormal development (Nakakura-Ohshima et al. 1993). Specifically, abnormalities affecting the relative decrease in the innervation density and the proprioceptive response to stimulation during rapid growth may cause the erect

spine to become unstable.

Fos protein was found in the dorsal root ganglia, sympathetic ganglia, intermediate horns of the spinal cord, the cuneatus and gracilis nuclei, vestibular nucleus and thalamus following mechanical stimulation of the lateral spinal ligament. Since c-fos transcription is activated only by stimulation conducted to the neurons (Krukoff, 1993), the locations of neurons with positive c-fos reaction indicated the neurological pathway of the innervation of the lateral spinal ligaments. Understanding this pathway would be another step toward fully understanding the neuromuscular feedback mechanism in the active maintenance of an erect spine.

The clinical focus of this thesis was to gain understanding of the mechanism for the development of AIS characterized by lateral bending of the spine. The innervation densities of SCTLs from both normal subjects and patients with AIS were analyzed. Although the innervation pattern and morphology of the nerve structures in the SCTLs were similar in both the normal and scoliosis groups, the innervation density of SCTLs from patients with scoliosis was found to be significantly less than that from normal subjects. Studies have shown that lower than normal innervation density could cause poor proprioceptive ability (Zimny, 1988; Vallbo and Johansson et al. 1984). The lower innervation density in the scoliosis group could imply that these patients have poorer local neuromuscular control of their spine. These results warrant further study to confirm the implication of lower innervation density and the possible causative relation to the development of AIS.

Future studies

1. Development of an animal model for scoliosis based on the hypothesis that the lateral spinal ligaments play an integral role in providing proprioceptive feedback for the maintence of an erect spine. The hypothesis implies that improper interpretation of the information occurs following stretching of the ligament. A model could be made whereby a ligament would be overstretched following normal lateral bending. This overstretching would be interpreted as excessive and over-compensation produced when, in fact, the spine was in a stable position.

2. Examination of the effects of denervation and reinnervation of the spinal ligaments

especially during the growth period surrounding puberty. Development of the innervation is dependent on an intact nervous system. Denervation might prevent subsequent proper development of the innervation to the ligaments even after reinnervation has occurred as the window for correct development might have been missed. Injury to the ligaments during development coupled with improper subsequent development might have enormous consequences in relation to the maintence of an erect spine as well as the development of an unstable spine and back pain.

3. Isolation of an intact spinal ligament would allow better identification of the proprioceptive pathway. In this thesis the effects of mechanically stretching a lateral spinal ligament were not restricted to a single spinal level as many attached structures became affected also. Isolation of a ligament would allow more precise and limited stretching to occur. The precise pathways from a single ligament could then be determined without the extraneous confusing signals developing from adjacent structures. Identification of the receptive areas would allow focus for other studies. Electrophysiological studies of an isolated ligament would allow motor activity to be examined, particularly the direct confirmation of specific reflex activity, and possible identification of the motor pathways involved.

4. Development of external tests for the prediction of clinical development of AIS. These tests would be based on the proprioceptive abilities of the spinal ligaments and their manifestation in active processes. Such tests might include balance abilities, position awareness facilities, and subtle yet inappropriate muscle responses to movements related to balance.

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