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Reduced Substance P-like Immunoreactivity in Hereditary Sensory Neuropathy of Pointer Dogs*

J. F. Cummings[1], A. de Lahunta[1], S. T. Simpson[2], and J. M. McDonald[2]

[1] Dept. of Anatomy, New York State College of Veterinary Medicine, Cornell University, Ithaca, NY 14853, USA
[2] Dept. of Small Animal Surgery and Medicine, School of Veterinary Medicine, Auburn University, Auburn, AL 36830, USA

Summary. Two unrelated Pointer dogs, each from a breeding of normal parents which produced three affected pups in a litter of nine, began to bite their paws at 3 and 5 months of age. Insensitivity to painful stimuli was marked in the distal parts of the limbs and receded proximally. The affected dogs were euthanatized at 5 and 20 months because of acral mutilation and infection. Changes affecting the primary sensory neurons included: small spinal ganglia with reduced numbers of cell bodies, degeneration of unmyelinated and myelinated fibers in dorsal roots and peripheral nerves, and reduced fiber density in the dorsolateral fasciculus (dlf).

Since nociceptive loss was the salient deficit in a neuropathy affecting primary sensory neurons, immunohistochemical studies focused on substance P, the undecapeptide imputed to mediate nociception at the first synapse in the spinal cord and brain. The localization and density of substance P-like (SPL) immunoreactivity was studied in three control dogs and the two Pointers by the indirect antibody peroxidase-antiperoxidase method. The spinal intumescences of the control dogs contained dense SPL-immunoreactivity in fibers of the dlf and the superficial laminae of the dorsal horn (i.e., laminae I, II, and the dorsal part of III). Immunoreactive fascicles on the lateral aspect of the dorsal horn and in the reticular process sent contributions medially to a plexiform fiber arrangement in lamina V. Medially, SPL-immunoreactive fibers were more loosely arranged in the internal third of laminae VI and VII and in lamina X. In the Pointers, the loss of primary sensory neurons was associated with notable reduction of SPL staining in the dlf and superficial laminae of the dorsal horn. In the lumbar intumescence of the older Pointer greater preservation of SPL staining in the lateral third of laminae I and II was consistent with somatotopic termination of trunkal afferents in this region.

In both Pointers there was no detectable decrease in trigeminal sensitivity and the SPL immunoreactivity in the spinal nucleus of the trigeminal nerve of the younger Pointer and the corresponding control dog appeared equivalent. In the older Pointer, however, the immunoreactivity in this nucleus was decreased relative to the control. This decrease and appearance of scattered fiber degeneration in the dorsal columns of the mature Pointer suggested that fiber degeneration progresses slowly with age to include sensory systems not affected in early postnatal life.

The findings in the Pointers were compared with those made in immunohistochemical studies of SP in familial dysautonomia and the mutilated foot rat.

Key words: Hereditary sensory neuropathy – Substance P – Immunohistochemistry

Introduction

The nociceptive loss and acral mutilation found in hereditary sensory neuropathy of English Pointer dogs has been associated with marked deficiency of the primary sensory neurons (Cummings et al. 1981). Although blunting of digital pain sensation may be detected prior to weaning, the condition becomes overt at 3–5 months when affected dogs suddenly begin to lick and bite their paws. Acral changes include swollen reddened paws, ulcerations, lacerations, paronychia, subluxations, painless fractures, and autoamputations.

A previous study of the pathologic changes in a 5-month-old pup revealed small spinal ganglia with reduced numbers of nerve cell bodies. There was clear evidence of ongoing and prior degeneration of myelin-

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Offprint requests to: Dr. J. F. Cummings (address see above)

ated and unmyelinated nerves in the dorsal roots and peripheral nerves. However, since the peripheral axonal loss appeared substantially less than the numerical reduction in ganglionic cell bodies, it was concluded that the deficit resulted largely from insufficient neuronal development and secondarily from postnatal degeneration of sensory neurons.

The clinical findings in the original patient and four dogs subsequently examined suggested that primary nociceptive neurons were predominantly affected. Acral loss of sensitivity to painful stimuli was not accompanied by depression of tendon reflexes, proprioceptive loss, or ataxia.

Previous immunocytochemical studies have clearly demonstrated the undecapeptide, substance P (SP) in small primary sensory neurons and in their central and peripheral endings (Hökfelt et al. 1975a, b, 1976). Evidence has been presented in support of the theory that SP in primary sensory neurons serves as a neurotransmitter or modulator for nociceptive stimuli (Henry 1976, 1982). Pearson et al. (1982) recently demonstrated depletion of SP axons in the substantia gelatinosa of the spinal cord and spinal nucleus of the trigeminal nerve in patients with familial dysautonomia, and they correlated this reduction with the diminished temperature and pain sensitivity that occurs in this syndrome. In the present study, tissues from an affected Pointer pup and an adult Pointer were examined to determine if the observed clinical and pathologic deficits were correlated with anatomically defined reductions in SP-like immunoreactivity.

Case Reports

Pointer 1

This 4.5-month-old, female pup's history and clinical course have been recorded in more detail previously (Cummings et al. 1981). This pup and two others were smaller than their six littermates at 12 weeks of age when they began to lick and bite their paws. Acral mutilation progressed rapidly in all four paws which were profoundly hypalgesic or analgesic. Acral changes included: swelling, paronychia, plantar and palmar ulceration, nail loss, and amputation of the digits. Tendon reflexes remained intact, and there was no detectable gait abnormality or weakness when the pup was euthanatized at 5 months of age.

Pointer 2

A five-generation pedigree of this 20-month-old, female English Pointer contained no ancestors in common with Pointer 1. This dog and two other females in a litter of nine born to clinically normal parents began to lick and bite their digits at 5 months of age. The paws were mutilated extensively at 6 months when this dog was referred to the School of Veterinary Medicine, Auburn University. Acral analgesia extended proximal to the carpus and tarsus. Although the dog's gait was compromised by digital amputations, a proprioceptive deficit was not discernible and tendon reflexes remained intact. Tibial and plantar nerve biopsies taken shortly after admission contained degenerating myelinated and unmyelinated axons. In an effort to prevent further mutilation and infection, the paws were bandaged, a muzzle was applied, and the dog was treated with broad spectrum antibiotics. During 14 months of hospitalization the intensity with which the dog chewed on the extremities declined, but never to the point where the animal could be left unattended without a muzzle. Chronic acral infections and osteomyelitis grew progressively worse and the dog was euthanatized at 20 months.

Materials and Methods

Tissue Preparation

Pointer 1 was euthanatized by an i.v. overdose of pentobarbital sodium. Six liters of a chilled fixative solution containing 1% glutaraldehyde-1% paraformaldehyde phosphate-buffered to pH 7.2 were perfused at 120 mm Hg via the left cardiac ventricle. Brain, spinal cord with roots, and peripheral nerves were removed and placed in fixative for 3 days and then transferred to refrigerated buffer. The tissues remained stored in buffer for 3 months and were then returned to fixative for 33 months prior to the application of immunohistochemical procedures.

As it was impossible to obtain unaffected littermates or pure-bred Pointers of comparable ages, we were constrained to use dogs of other breeds for controls. We attempted, however, to match each of the Pointers with a dog of similar age and weight (see control dogs 1 and 2 below).

Control dog 1, a clinically normal English Setter dog, 3 weeks younger than Pointer 1, but comparable in weight and confirmation, was necropsied according to the same procedures used for Pointer 1. Tissues were stored in an identical fashion for 3 years.

Control dog 1A, a clinically normal 8-month-old Beagle, was euthanatized by an i.v. overdose of pentobarbital sodium and perfused via the left cardiac ventricle at 120 mm of Hg with 2 l of chilled phosphate-buffered saline solution (PBS) followed by 6 l of 4% paraformaldehyde in 0.1 M phosphate buffer. Brain and spinal cord were removed and immersed in fixative for 90 min at 4° C and then transferred to a refrigerated 5% sucrose solution prepared in 0.1 M phosphate buffer.

Pointer 2 was given an i.v. overdose of pentobarbital sodium and was transcardially perfused at 120 mm Hg with chilled PBS (2 l) followed by 6 l of 4% paraformaldehyde-0.2 glutaraldehyde in 0.1 M phosphate buffer. The brain and spinal cord were removed, immersed in fixative for 90 min at 4° C and transferred to a refrigerated 5% sucrose solution prepared in 0.1 M phosphate buffer.

Control dog 2, a clinically normal Beagle comparable in age (20 months) and weight (29 lb) to Pointer 2 was necropsied according to the same procedures used for Pointer 2.

Immunohistochemical Procedures

Ten-micrometer frozen sections were cut transversely from the cervical and lumbar intumescences of the spinal cord and from the caudal brain stem and the first cervical spinal segment of the two Pointer and three control dogs. These sections were mounted on gelatinized slides. Some 20-μm spinal cord sections were also cut and these were processed as free sections. Attached and free sections were washed in PBS and incubated at 4° C with anti-SP. Antibody to SP (Immuno Nuclear Corp., Stillwater, MN, USA) was raised in rabbits and was used in the unlabeled antibody peroxidase-antiperoxidase (PAP) technique of Sternberger (1979). The primary antibody was diluted 1:500 with PBS, pH 7.4, containing 1% normal sheep serum and 0.3% Triton X100. After incubating for 16–24 h, excess primary antibody was removed by rinsing and immersing in cold PBS. Sheep-antirabbit serum (Cappel Laboratories, Inc., Cochranville, PA,

USA) was diluted 1:100 and applied to sections for 1 h at room temperature. The sections were then rinsed in PBS and the PAP complex (Cappel Laboratories), at a dilution 1:100, was applied to the sections for 1 h at room temperature and then rinsed away in two changes of PBS. The sections were then incubated in 3,3-diaminobenzidine hydrochloride and hydrogen peroxidase solution for 10–30 min. The sections were then rinsed, dehydrated, cleared, and coverslipped.

As a control procedure, some sections were treated with primary antibody which had been pre-absorbed with excess substance P.

Results

It was suspected that the immunoreactivity in the CNS of Pointer 1 and control dog 1 might be suppressed by the 3-year period of wet tissue storage. However, since the tissues of these two dogs had been treated by identical procedures, a comparative analysis of substance P-like (SPL) immunoreactivity was conducted. As a means to assess any deleterious effect of prolonged storage on SPL immunoreactivity, tissue from control dog 1A was fixed and stored briefly in accordance with standard PAP technique (Sternberger 1979). The SPL immunoreactivity in this tissue was compared with that found in corresponding sections from control dog 1.

The intensity of the SP immunoreactivity in the intumescence of the spinal cord of control dog 1A exceeded that found in control dog 1. In control 1 A, densest SPL staining appeared in the dorsolateral fasciculus (Lissauer's tract), spinal gray lamina I or Waldeyer's zone, lamina II or substantia gelatinosa and the dorsal part of lamina III (Fig. 1). Granular SPL staining appeared in fibers that extended along the lateral border of the dorsal horn and these blended with SP-immunoreactive fibers in the reticular extensions of laminae IV, V, and VI into the lateral funiculus, i.e., the processus reticularis. Some of these lateral fibers swept medially into the neck of the dorsal horn to form a plexiform arrangement in lamina V along with scattered, vertical fibers that projected ventrally from the superficial laminae of the dorsal horn. The plexiform arrangement of the positively stained fibers in the central and lateral regions of lamina V often extended into the adjoining parts of laminae IV and VI. A few SPL-immunoreactive fibers ran along the medial aspect of the dorsal horn. Ventrally, however, SPL-immunoreactive fibers formed more expansive arrangements in the medial third of laminae VI and VII which included the nucleus intermediomedialis and in lamina X, the substantia grisea centralis. Widely scattered, varicose, SPL-positive fibers were also found among motor neurons in the ventral horn.

Except for a lack of SPL immunoreactivity in the ventral horn, the general distribution of SPL staining in control dog 1 was similar to that observed in control dog 1A. The density of SPL immunoreactivity, however, appeared reduced in control dog 1 in which less than optimal tissue techniques were used. The suppression of SPL immunoreactivity was seen when the superficial laminae of the dorsal horns from cervical or lumbar segments of control dogs 1 and 1A were compared (Figs. 1, 2A).

Fig. 1. SPL immunoreactivity demonstrated by the PAP technique in the dorsolateral fasciculus and the dorsal horn of the C_7 spinal segment of control dog 1A. ×46

In Pointer 1, the profile of the dorsal horn appeared smaller and the head of the horn more rounded than in control dog 1. The SPL immunoreactivity in the spinal cord of Pointer 1 was depleted and stood in marked contrast to the staining observed in control dog 1. The dorsolateral fasciculus (dlf) in Pointer 1 was virtually devoid of SPL immunoreactivity, and the staining in laminae I and II was greatly reduced relative to that observed in control dog 1 (Figs. 2a, b). The SPL immunoreactivity in the reticular process and the medial parts of laminae VI and VII in Pointer 1 appeared to be better preserved.

Although depletion of SPL immunoreactivity in spinal laminae I and II clearly distinguished Pointer 1 from control dog 1, comparable differential staining was not found on comparing the spinal nuclei of the trigeminal nerve in these two animals (Figs. 3a, b). The extent and density of immunoreactivity in this nucleus appeared essentially the same in both animals.

Except for the addition of 0.2% glutaraldehyde to the fixative solution, the tissues from Pointer 2 and control dog 2 were processed as those of control dog 1A. The addition of the small amount of glutaral-

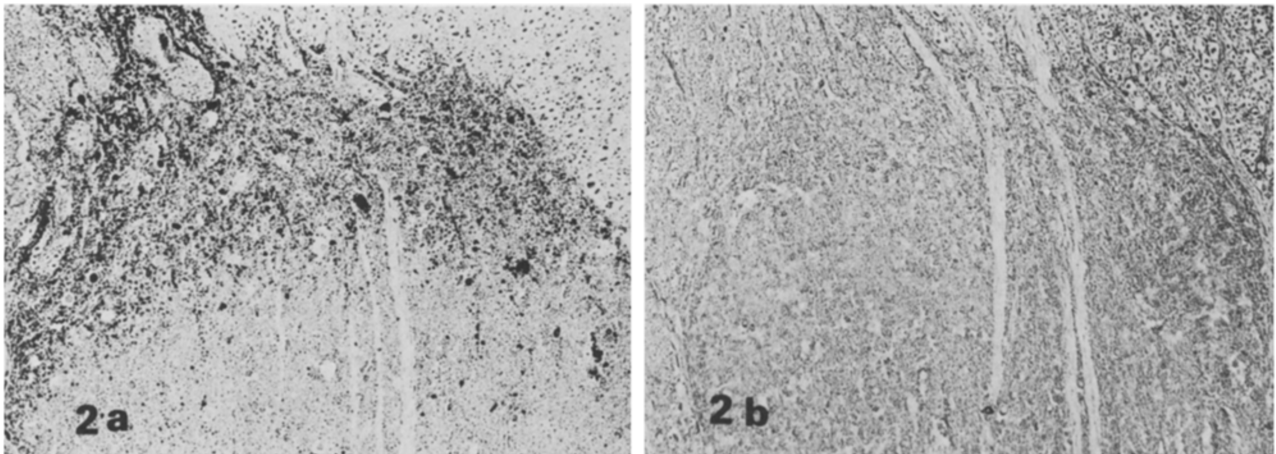

Fig. 2. SPL immunoreactivity in dorsal horn and the adjoining part of the dorsolateral fasciculus of the C_7 spinal segment of control dog 1 (**a**) and Pointer 1 (**b**). ×120

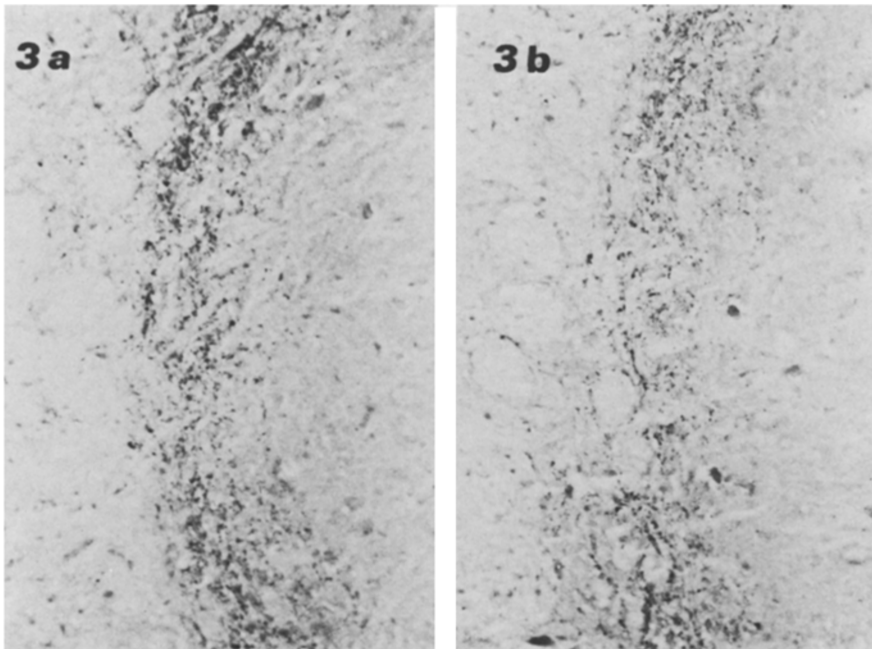

Fig. 3. SPL immunoreactivity in the spinal nucleus of the trigeminal nerve (pars caudalis) in Pointer 1 (**a**) and control dog 1 (**b**). ×125

dehyde had no discernible effect since the SPL immunoreactivity in control dog 2 appeared equal in intensity and distribution to that found in control tissue fixed in 4% paraformaldehyde alone (i.e., in control dog 1 A).

Comparison of corresponding spinal cord sections from control dog 2 and Pointer 2 revealed smaller and rounder dorsal horn profiles and reduced SPL immunoreactivity in the latter. This reduction in immunoreactivity was obvious in the dlf and in the superficial laminae of the dorsal horns (Fig. 4a, b), and it appeared more marked in cervical than lumbar spinal segments. Despite the clear-cut reduction in the dlf and laminae I and II, SPL immunoreactivity in the processus reticularis, the lateral and central parts of lamina V, the medial parts of laminae VI and VII and lamina X of Pointer 2 usually appeared comparable to that observed on corresponding sections from control dog 2 (Figs. 5a, b). Examination of 20-μm-thick free sections from the lumbar intumescence of Pointer 2 indicated that the loss of immunoreactivity was not uniformly distributed through laminae I and II. There appeared to be better preservation of SPL-immunoreactivity in the lateral one-third of laminae I and II than in the medial two-thirds (Figs. 6a, b). This differential preservation

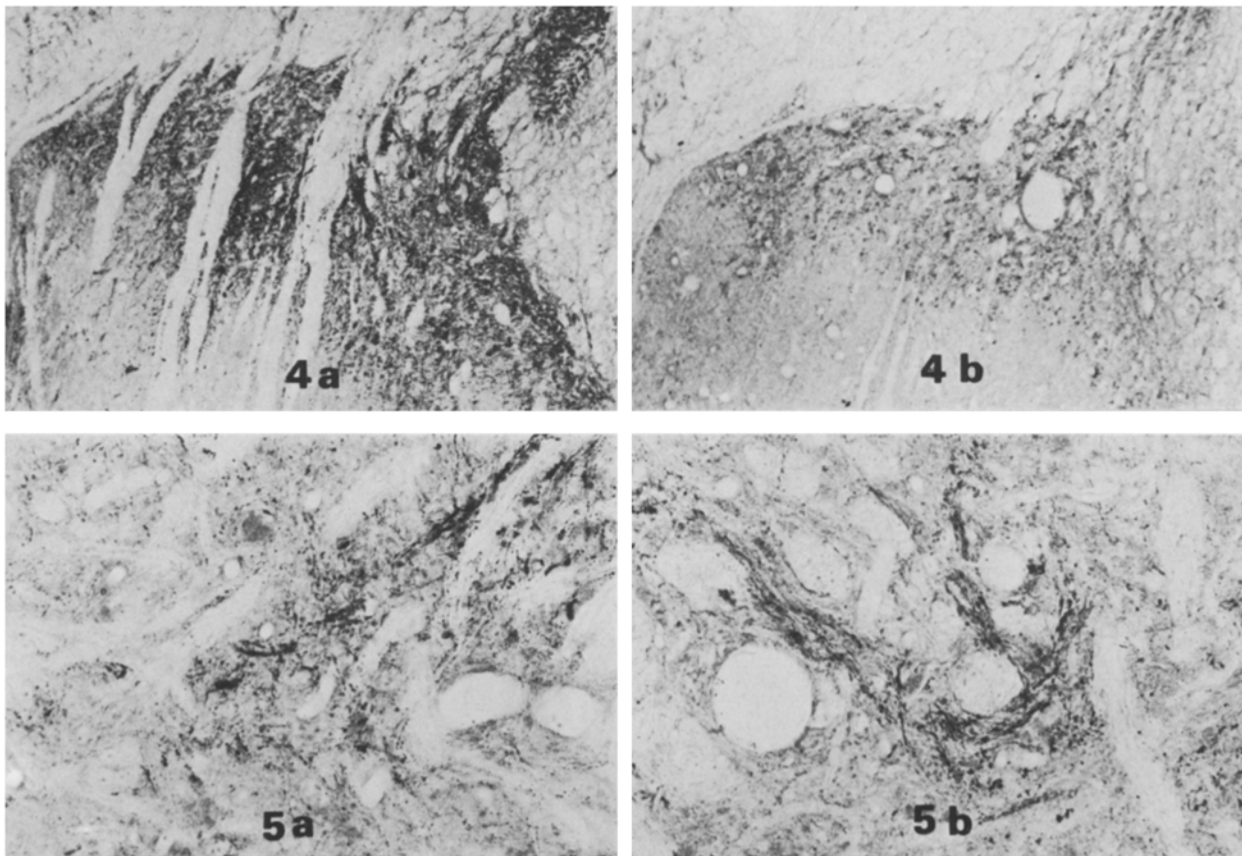

Fig. 4a, b. SPL immunoreactivity in the superficial laminae of the dorsal horn and the adjoining portion of the dorsolateral fasciculus in the C_8 spinal segment. **a** Control dog 2, ×105. **b** Pointer 2, ×125

Fig. 5. SPL-immunoreactive fibers in lamina V of the dorsal horn of the L_5 spinal segment of control dog 2 (**a**) and Pointer dog 2 (**b**). ×160

was not obvious in thick sections from the cervical segments where the depletion of SPL immunoreactivity appeared more extensive.

In contrast to the findings in Pointer 1 and control dog 1, the SPL staining in the spinal nucleus of the trigeminal nerve in Pointer 2 was reduced relative to that found in control dog 2 (Figs. 7a, b). The extent of this reduction, however, did not appear as great as the depletion of SPL immunoreactivity in the superficial laminae of the cervical spinal cord.

When adjacent spinal cord sections from the two Pointer dogs and 3 control dogs were incubated with primary antibody which had been preabsorbed with excess substance P, SPL staining was totally inhibited.

Discussion

The localization of immunoreactivity in the cervical and lumbar spinal segments of the control dogs (1 A and 2) in this study corresponded closely with the distribution of SP reported in the cat (Hökfelt et al. 1975a, b; Tessler et al. 1980) and man (de Lanerolle and La Motte 1982). Studies of staining depletion after dorsal rhizotomy have established that much of the SP immunoreactivity in the superficial laminae of spinal cord (LI–III) is contained in terminals of primary afferent fibers (Hökfelt et al. 1975a, b; Barber et al. 1979). Evidence has accumulated to support the proposal that SP is an excitatory agent mediating nociception at the first synapse in the spinal cord and brain stem (Henry 1982). Recent immunohistochemical studies on two forms of hereditary sensory neuropathy (HSN) have provided clinicopathologic support for this proposal.

In familial dysautonomia, Riley-Day syndrome or HSN Type III of Dyck and Ohta (1975), autonomic abnormalities are accompanied by decreased pain and temperature sensation (Pearson et al. 1974). The diminished pain sensitivity in familial dysautonomia has been correlated with a loss of small diameter, sensory axons and cell bodies (Pearson et al. 1978) and, more recently,

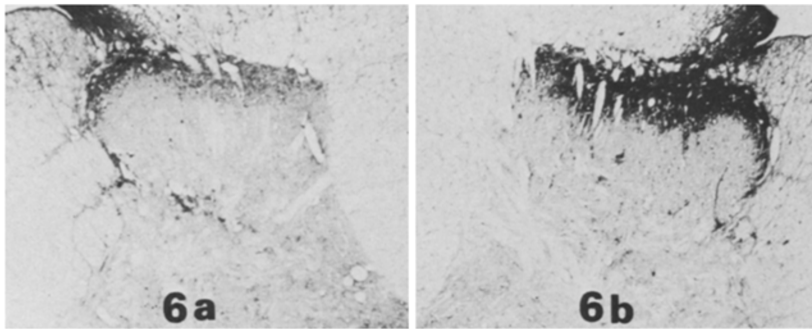

Fig. 6. SPL immunoreactivity in the dorsal horn and dorsolateral fasciculus of the L_6 spinal segment of Pointer 2 (**a**) and control dog 2 (**b**). Note that the reduction in SPL staining is greatest in the medial two-thirds of laminae I and II in **a**. **a** ×27; **b** ×23

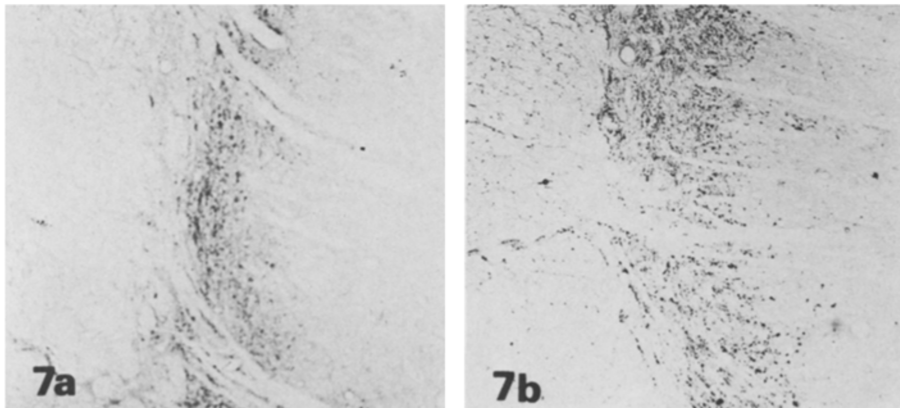

Fig. 7. SPL immunoreactivity in the spinal nucleus of the trigeminal nerve (pars caudalis) in Pointer 2 (**a**) and control dog 2 (**b**). ×98

with depletion of SP immunoreactivity in the substantia gelatinosa of the spinal cord and spinal nucleus of the trigeminal nerve (Pearson et al. 1982). In the mutilated foot (mf) rat, a recessively inherited sensory neuropathy presents early in postnatal life with ataxia, ulceration of the feet, and decreased response to painful stimuli. Quantitative studies have revealed marked deficiency of spinal ganglia cells and dorsal root nerve fibers (Jacobs et al. 1981). In a recent immunocytochemical study, the decreased pain sensitivity was associated with reduced SPL staining in Lissauer's tract and spinal laminae I and II of the dorsal horn (Scaravilli 1983).

On standard neurologic examination (de Lahunta 1983) of affected Pointer dogs, nociceptive loss was the most salient sensory deficit. This loss in the limbs receded from distal to proximal so that acral analgesia seemed to give way to hypalgesia proximal to the carpus and tarsus. No nociceptive loss, however, was detected about the face. Tendon reflexes remained intact, and there was no evidence of ataxia. In an earlier pathologic study of a Pointer pup (i.e., Pointer I), reduced numbers of cell bodies in the spinal ganglia and loss of myelinated and unmyelinated sensory fibers were associated with diminished density of axons and fiber degeneration in the dorsolateral fasciculus (Cummings et al. 1981). Comparable changes were not found in the larger myelinated axons in the medial division of the dorsal roots or the dorsal funiculus. Thus, the clinical and pathologic findings were consistent with preferential or, at least, prominent involvement of primary nociceptive afferents. The reduction of SPL immunoreactivity in the dlf and superficial laminae of the dorsal horn in this dog closely resembled the depletion found in association with pain and temperature insensitivity in familial dysautonomia (Pearson et al. 1982). In contrast to the findings in familial dysautonomia, however, there was neither detectable loss of pain sensitivity in the distribution of the trigeminal nerve nor discernible reduction of SP immunoreactivity below control levels in the spinal nucleus of this nerve.

Although the clinical findings in Pointers 1 and 2 were very similar, the ages of the dogs at the time of death differed as did the methods of tissue processing.

As in Pointer 1, there was fiber loss in the dlf of Pointer 2, and the dorsal horns also appeared small and more rounded than in the corresponding control. Scaravilli (1983) has associated similar dorsal horn changes in the mf rat with loss of primary afferent fibers. Pointer 2 differed from Pointer 1 in that there was some degeneration of large myelinated fibers in the dorsal funiculus. The dorsal funicular degeneration was

not manifested clinically, but its presence in this older dog suggested that, as in familial dysautonomia (Pearson et al. 1974), the dorsal column fibers become affected as degeneration of primary sensory neurons continues into adulthood.

In Pointer 2 there also was reduction in SPL immunoreactivity below control levels in the dlf and the superficial laminae of the dorsal horn. In this dog with a longer survival, however, the residual immunoreactivity was greater than in Pointer 1 especially in the lumbar segments. The greater residual to some extent might reflect the improved tissue techniques employed for Pointer 2. Since three sources of SP fibers to laminae I and II have been identified, i.e., the spinal ganglia, spinal neurons and the raphe nuclei of the brain stem (Jessell 1982), it was problematic to what extent surviving primary afferent fibers contributed to the residual immunoreactivity. There was in the lumbar spinal segments, however, evidence that the primary afferent neurons subserving the trunk and, perhaps the proximal portion of the limbs were better preserved than those innervating the distal portions of the limbs. This evidence was provided by the better preservation of SPL immunoreactivity in the lateral third of laminae I and II as opposed to the medial two-thirds. Experimental neuroanatomic studies (Devor and Claman 1980) have revealed a transverse somatotopic organization of afferent endings within the substantia gelatinosa wherein sensory fibers from the distal parts of the limbs project medially, while those from the trunk end laterally. Thus, the lateral retention of SPL immunoreactivity within the superficial laminae appeared to correlate with the distal to proximal regression of nociceptive loss.

In Pointer 2, as in Pointer 1, there was no clinically discernible reduction in sensitivity in the distribution of the trigeminal nerve. However, in contrast the Pointer 1, the SPL immunoreactivity in the spinal nucleus of the trigeminal nerve of Pointer 2 was reduced below that observed in the control. Our failure to detect a reduction in facial pain sensitivity in Pointer 2 was not surprising because of the imprecision of clinical evaluation of nociception in the dog. Since the reduction in SPL staining occurred in the adult Pointer, but not in the pup, it was suspected that trigeminal axon loss, like dorsal column degeneration, began postnatally and progressed with age.

The pathogenesis of the defect that affects the prenatal and postnatal survival of primary sensory neurons in Pointer dogs remains a matter of speculation. A defect in nerve growth factor (NGF) or NGF receptors has been considered in the pathogenesis of the peripheral autonomic and sensory deficits that occur in familial dysautonomia (Pearson 1979). An abnormality in NGF function also has been considered in the pathogenesis of the hereditary sensory neuropathy of the mf rat. This consideration, in the case of the mf rat, was based on demonstrations of a dependence of SP-containing sensory neurons upon NGF during prenatal and early postnatal development (Otten et al. 1980; Ross et al. 1981)

Studies on the chronologic progression and distribution of the pathologic changes in the Pointer dog neuropathy have been limited by the number of affected individuals. Results, to date, suggest that the changes in affected Pointers are more restricted in their distribution than those occurring in familial dysautonomia or in the mf rat. Clinical and pathologic studies indicate selective loss of primary sensory neurons with major but not exclusive involvement of nociceptive neurons. As in familial dysautonomia, the insufficient prenatal survival of sensory neurons and their slowly progressive postnatal degeneration suggest a deficiency in the activity of a trophic or maintenance factor (Pearson 1979). Thus, a localized deficiency of a trophic factor (e.g. NGF) or its receptors might accentuate and prolong the neuronal death that occurs naturally in the prenatal development of the spinal ganglia (Hamburger and Oppenheim 1982).

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