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# Shaking pups: a disorder of central myelination in the spaniel dog. II. Ultrastructural observations on the white matter of the cervical spinal cord

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**Summary**

The ultrastructure of the cervical cord is described in a new canine mutant with severe hypomyelination of the C.N.S. Axons were either non-myelinated or surrounded by a myelin sheath that was markedly reduced in both its thickness and length of internode. Myelinated and non-myelinated zones were present on a single axon. There was no paucity of oligodendrocytes but many of those present contained empty or granular vacuoles within the cytoplasm. Features suggesting immaturity of myelination were commonly found at paranodes and along the internode. Abnormal inter-relationships of oligodendrocytes and astrocytes were present at many paranodes. These observations suggest an intrinsic defect of oligodendrocyte metabolism such that they are incapable of normal extension of their plasma membranes, while the cytoplasmic vacuoles may represent breakdown of defective lipids.

## Introduction

In a previous paper we described a new inherited disorder of myelination of the C.N.S. in male Springer Spaniel pups (Griffiths *et al.*, 1981). Affected animals were characterized by the development of a severe generalized tremor at 10–12 days of age. Light microscopical examination revealed severe hypomyelination throughout the C.N.S. which was more marked in the brain and optic nerves than in the spinal cord. The P.N.S. was normal. Within the C.N.S., axonal diameters appeared similar to controls but axons were either naked or surrounded by disproportionately thin myelin sheaths which frequently terminated at heminodes. Preliminary cell counts revealed no paucity of oligodendrocytes but vacuoles were present adjacent to myelin sheaths and within glial cells. It was postulated that a defect in oligodendrocyte metabolism

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prevented normal radial or longitudinal extension of their plasma membranes and that the vacuoles might represent breakdown of defective lipid. The present electron microscopy (EM) was undertaken (a) to study the character of the oligodendrocytes and vacuoles, (b) to examine the myelin for any structural defect as seen, for example, in the Shiverer mouse (Privat *et al.*, 1979) and (c) to compare the morphology of the nodal and heminodal areas with abnormalities described in these regions in the C.N.S. and P.N.S. of various murine mutants (Rosenbluth, 1979a, b; Suzuki & Zagoren, 1977).

## Materials and methods

Three affected pups (2 at 1 month and 1 at 2 months of age) were available for EM studies. Three normal dogs, including a 2-month-old unaffected male littermate, were studied at the same time intervals. This report concentrates on the morphological appearance of the cervical spinal cord of the 2-month-old control and affected pups.

The animals were anaesthetized and heparinized, and the respiration was maintained with a positive pressure while the descending thoracic aorta was cannulated retrogradely. The origin of the aorta was clamped so that the perfusate was directed to the cervical cord and brain and drained from a further cannula inserted into the right ventricle. A two-stage fixation procedure similar to that of Karnovsky (1965) was used. An initial perfusate of 500 ml of 2% paraformaldehyde and 2% glutaraldehyde in a 0.08 M cacodylate buffer (pH 7.2) and 0.05% calcium chloride at 37° C was followed by 2 l of 2% paraformaldehyde and 5% glutaraldehyde in a similar vehicle at 4° C. The brain and cervical cord were left *in situ* for 2 h, removed and further fixed overnight in 2.5% glutaraldehyde. Selected blocks from the cord, brain and optic nerves were post-fixed in either 1% osmium or in Karnovsky's (1971) ferrocyanide–osmium mixture for 1.5 h, dehydrated in alcohols and embedded in Araldite. 1 $\mu$m sections were stained with toluidine blue and thin sections were cut with a diamond knife and left unstained, or were stained with lead citrate or with both lead citrate and uranyl acetate.

## Results

*Control pups*

At 2 months, myelin was well developed (Fig. 1) and conformed to that accepted as normal in various mammalian species (Peters *et al.*, 1970). Evidence of immaturity was, however, frequent. Oligodendrocyte cytoplasm in the outer tongue and occasionally within the myelin lamellae was uncompacted and contained numerous organelles (Fig. 2). Oligodendrocyte processes were commonly present between myelinated fibres (Fig. 1) and were occasionally seen in continuity with myelin sheaths. The periodicity of the myelin major dense line (MDL) was $10.9 \pm 0.2$ nm (mean ± S.E.M.) and a double intraperiod line was present. The extra-cellular space was larger and more irregular than in mature dogs. Numerous gap junctions as described by Morales & Duncan (1975) in the cat spinal cord and occasionally puncta adherentia were seen between oligodendrocytes and astrocytes (Fig. 3). At nodes, virtually all lateral loops ended in an inward direction (Fig. 3) but not all (particularly in larger fibres) made contact with the axolemma. The expected orderly successive termination of loops on the axolemma was observed. The paranodal peri-axonal space was $2.8 \pm 0.2$ nm and bounded by parallel

membranes of the myelin loops and axolemma. The space under the innermost loop was commonly less regular and slightly wider. Axolemmal transverse bands were present at a perodicity of 22.4 ± 1.5 nm. Successive lateral loops were attached by tight junctions. The sub-axolemmal dense zone was present at the nodes (Fig. 4).

Only a limited examination was made of 1-month-old pups but it was established that the majority of lateral myelin loops terminated in an inward direction and the transverse bands were present at the paranode. Features of immaturity such as uncompacted lamellae, large lateral loops and occasional internodal loops were present in somewhat greater frequency than in the 2-month-old pups.

*Hypomyelinated pups*

The predominant features were the large number of axons with either no myelin or disproportionately thin myelin sheaths (Figs. 5 and 6). There was no obvious correlation between the state of myelination or non-myelination and the axonal calibre as reported previously (Griffiths *et al.*, 1981). Axonal diameters were apparently similar to those of the age-matched normal pups and no change in their axonal diameter was evident between an unensheathed zone and a hypomyelinated zone of a single fibre.

As reported previously (Griffiths, *et al.*, 1981) there was no apparent reduction in the number of glial cells, and oligodendrocytes were commonly observed. Many oligodendrocytes contained membrane-bound vacuoles within the cytoplasm of their cell bodies or processes, particularly in the inner and outer tongues (Figs. 7 and 8). Some vacuoles were empty and others contained electron-dense granular material. Lipid droplets and myelin figures were also seen within some oligodendrocytes. Fewer oligodendrocyte processes were observed between the nerve fibres while the number of astrocytic processes appeared increased compared to control pups. Macrophages were not seen within the parenchyma. A number of myelin sheaths showed non-compaction of their lamellae in addition to the increased cytoplasm of the outer tongues. Many fibres in which extrusion of oligodendrocyte cytoplasm had occurred showed an increased 'looseness' of the lamellae, with undulation around the axon rather than the tight circular profiles of the controls. The remaining myelin contained a MDL with a periodicity of 11.4 ± 0.5 nm and a double intraperiod line. Many myelinated internodes were very short for the axonal diameter (Figs. 9 and 17).

Variations of paranodal and internodal morphology were found. Nodes, bounded by two paranodes were found, but more common was the heminode where only one myelinated paranode was present. The term heminode is used to indicate the portion of a fibre where one myelinated internode terminates at a paranode adjacent to a non-myelinated length of axon which is the situation in dystrophic mice (Stirling, 1975; Ellisman, 1979). In both instances the lateral loops terminated with successive loops apposed to the axolemma, although the cytoplasm of many loops was more voluminous than the control pups. Variations in the width of the periaxonal space and absence of parallelism in apposing glial and axonal membranes were seen at many paranodes (Fig. 11). Successive glial loops were joined by tight junctions as in the control

animals. The transverse bands also showed marked variation, a few paranodes having a normal complement of bands while the majority were completely or partially deficient. At certain nodes or heminodes no sub-axolemmal dense layer was seen. Occasionally, instances were found where the inner myelin lamellae terminated internodally on the axolemma while the outer lamellae were continuous (Fig. 10). Oligodendrocyte

**Fig. 1.** Area of dorsal column from two-month-old normal littermate showing well-developed myelination. An oligodendrocyte process runs across the centre of the field. The boxed area is shown in Fig. 2. Scale bar: 2 $\mu$m.

**Fig. 2.** Myelination is still immature as shown by the uncompacted outer tongues of oligodendrocyte cytoplasm containing various organelles. Scale bar: 1 $\mu$m.

**Fig. 3.** Longitudinal section of dorsal columns from two-month-old normal littermate. The paranodes demonstrate the 'normal' arrangement of termination of myelin lamellae at the lateral loops although the amount of cytoplasm is still slightly greater than in the adult. The sub-axolemmal dense layer is present. The oligodendrocyte shows two specialized areas of contact with adjacent astrocytic processes; a gap junction (arrow) and a punctum adherens (arrowhead). Scale bar: 1 $\mu$m. Inset: A separate area from that shown above demonstrating a gap junction between oligodendrocyte and astrocyte processes. Scale bar 0.1 $\mu$m.

**Fig. 4.** Nodal area from a two-month-old normal pup. On the right, successive myelin lamellae terminate on the axolemma as lateral loops. Successive loops are joined by tight junctions. The paranodal periaxonal space is regular with parallel membranes of the loops and axolemma and regular transverse bands (arrowheads). On the left, the adjacent paranode shows several lateral loops and regular transverse bands. Scale bar: 0.5 $\mu$m.

**Fig. 5.** Fig. 5 and subsequent figures are from spinal cord of two-month-old affected pup. Area of dorsal columns showing many naked or hypomyelinated axons. Myelination is not related to axon size. The intervening area contains astrocytic processes. Scale bar: 3 $\mu$m.

**Fig. 6.** An oligodendrocyte adjacent to two naked axons shows no evidence of attempted myelination. Scale bar: 3 $\mu$m.

**Fig. 7.** An oligodendrocyte cell body, with several cytoplasmic vacuoles, some containing granular material (arrows). Scale bar: 1 $\mu$m.

**Fig. 8.** The inner tongue of the oligodendrocyte contains numerous vacuoles, some of which are attached to the myelin sheath. Scale bar: 2 $\mu$m.

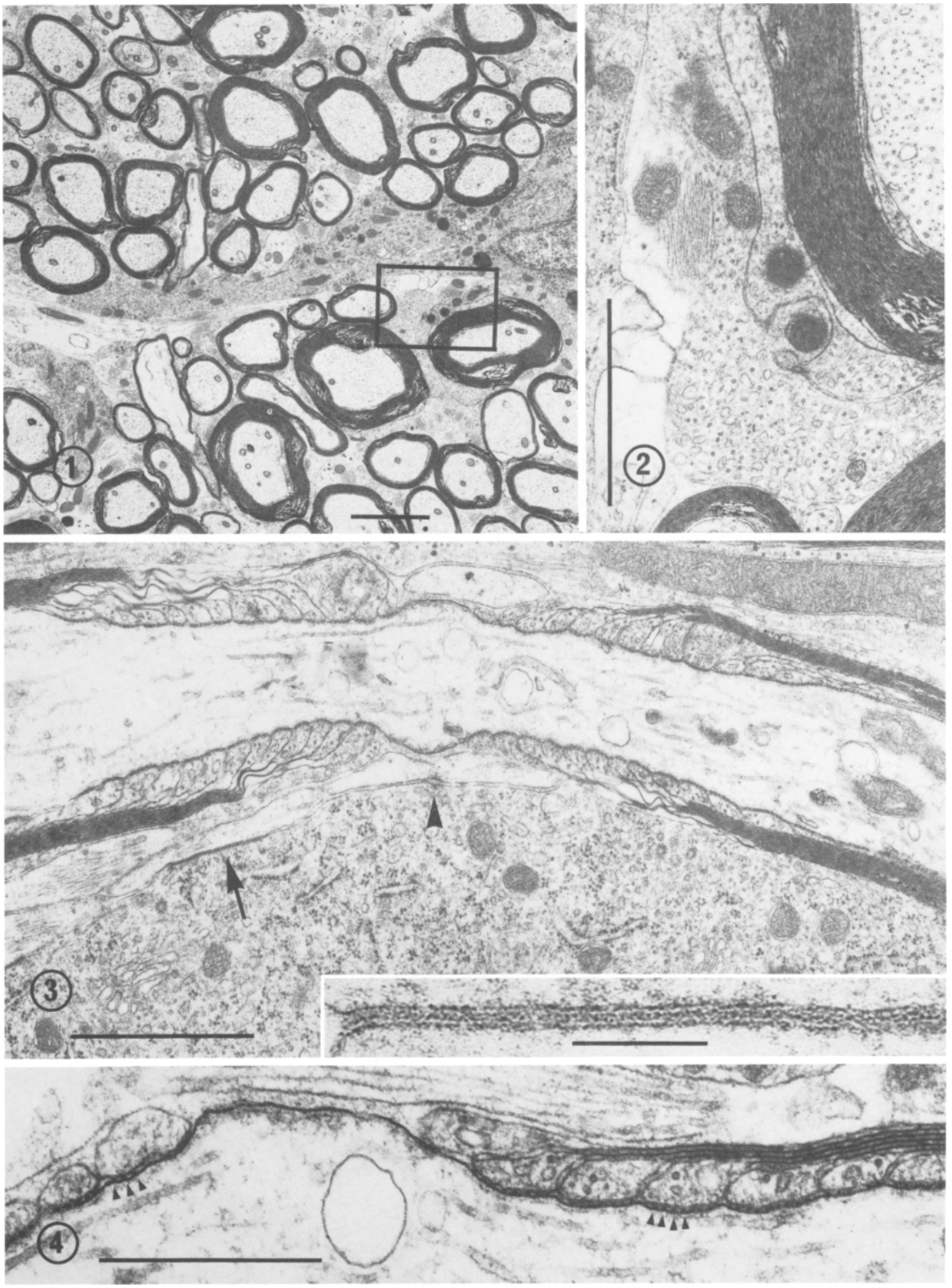
**Fig. 9.** A short, hypomyelinated internode terminates at heminodes (arrowheads). The internode also contains a closed node (pseudonode) (arrow) which is shown in Fig. 10. Scale bar: 5 $\mu$m.

**Fig. 10.** Closed or pseudonode showing the termination of the inner lamellae on the axolemma. The periaxonal space is wide and irregular and contains no transverse bands. Scale bar: 0.5 $\mu$m.

**Fig. 11.** A paranode contains successive lateral loops ending in an inward direction on the axolemma (ax, axon). At only one lateral loop is a normal periaxonal space, with parallel membranes, present (arrowheads). The remaining space is widened and irregular. Tight junctions join successive lateral loops (arrows). Scale bar: 0.2 $\mu$m.

**Fig. 12.** A heminode showing both inward and outward (ol) termination of lateral loops. The outward loops end in the extracellular space or on astrocytic processes (as). The paranodal periaxonal space is again abnormal (ax, axon). Scale bar: 1 $\mu$m.

**Fig. 13.** A heminode showing only outward terminating lateral loops and detached loops. Part of a Schmidt–Lanterman incisure is present (sl). Scale bar: 1 $\mu$m.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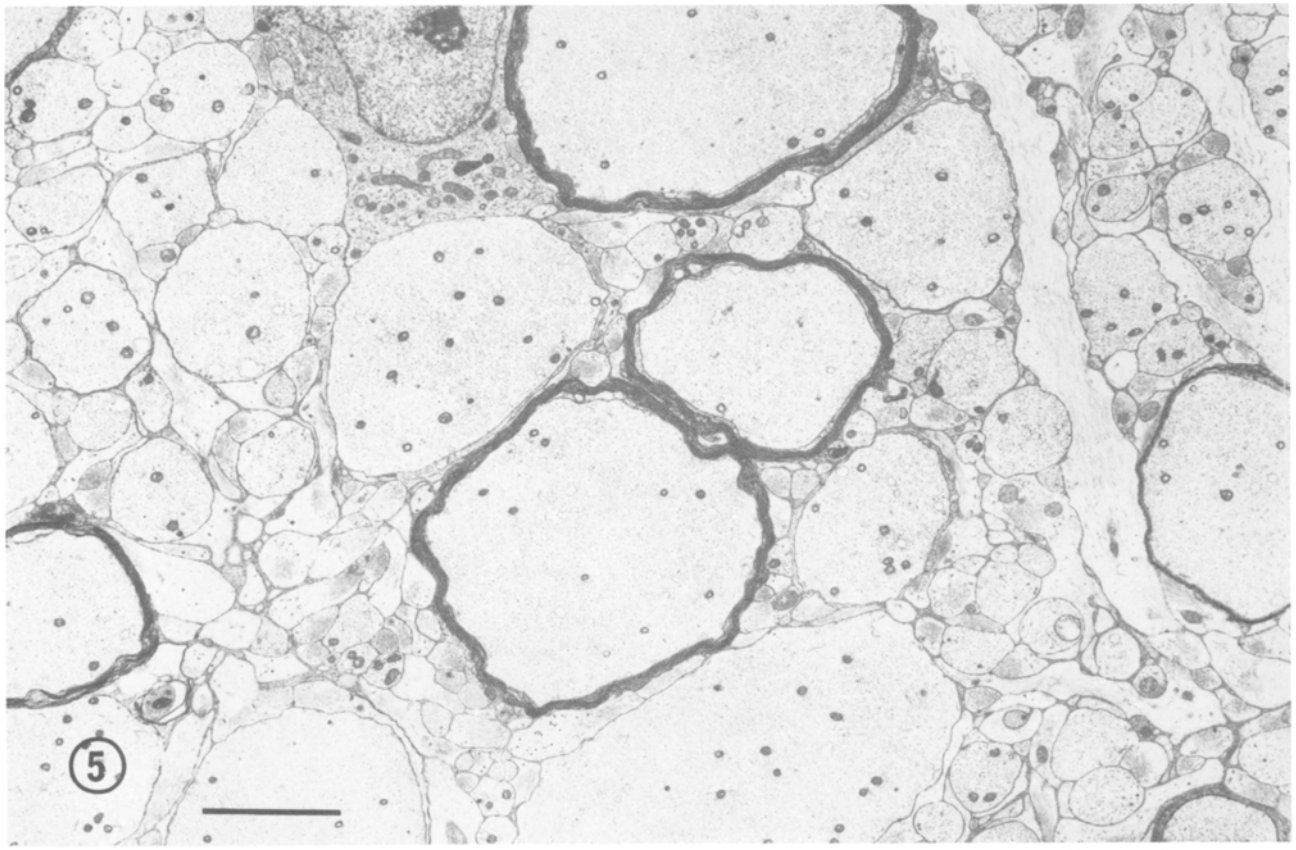
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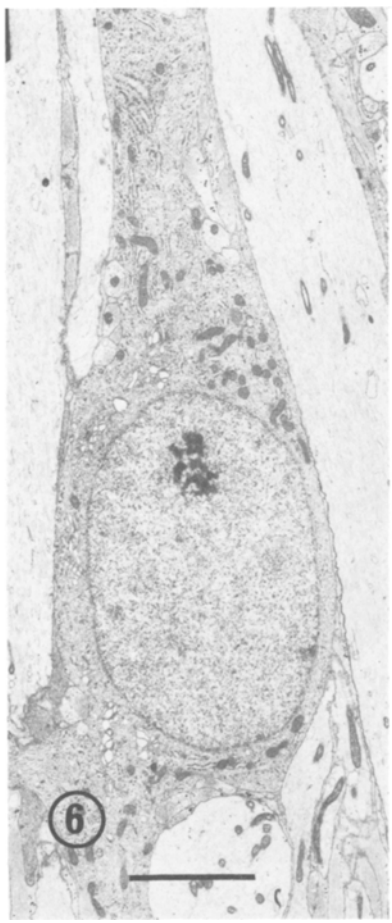
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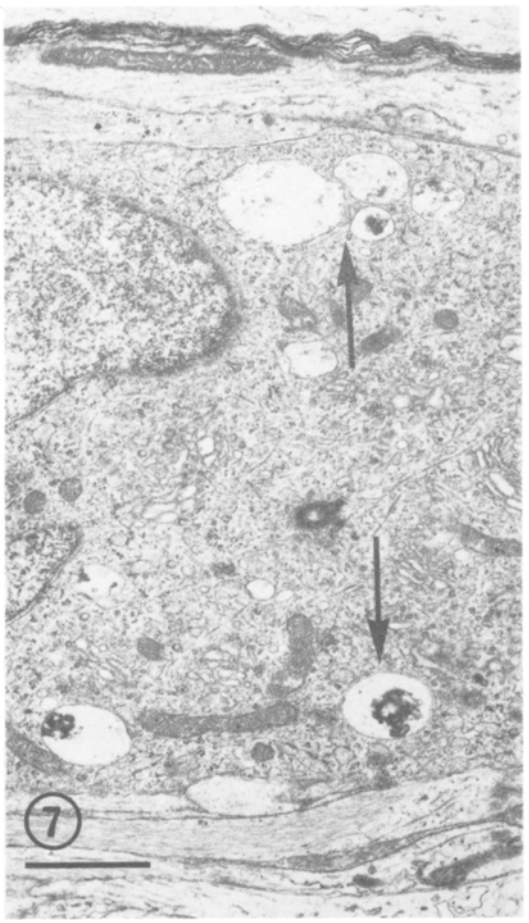
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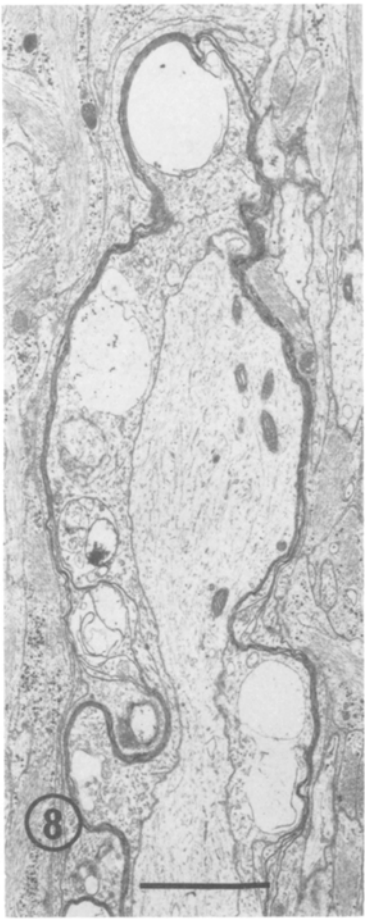
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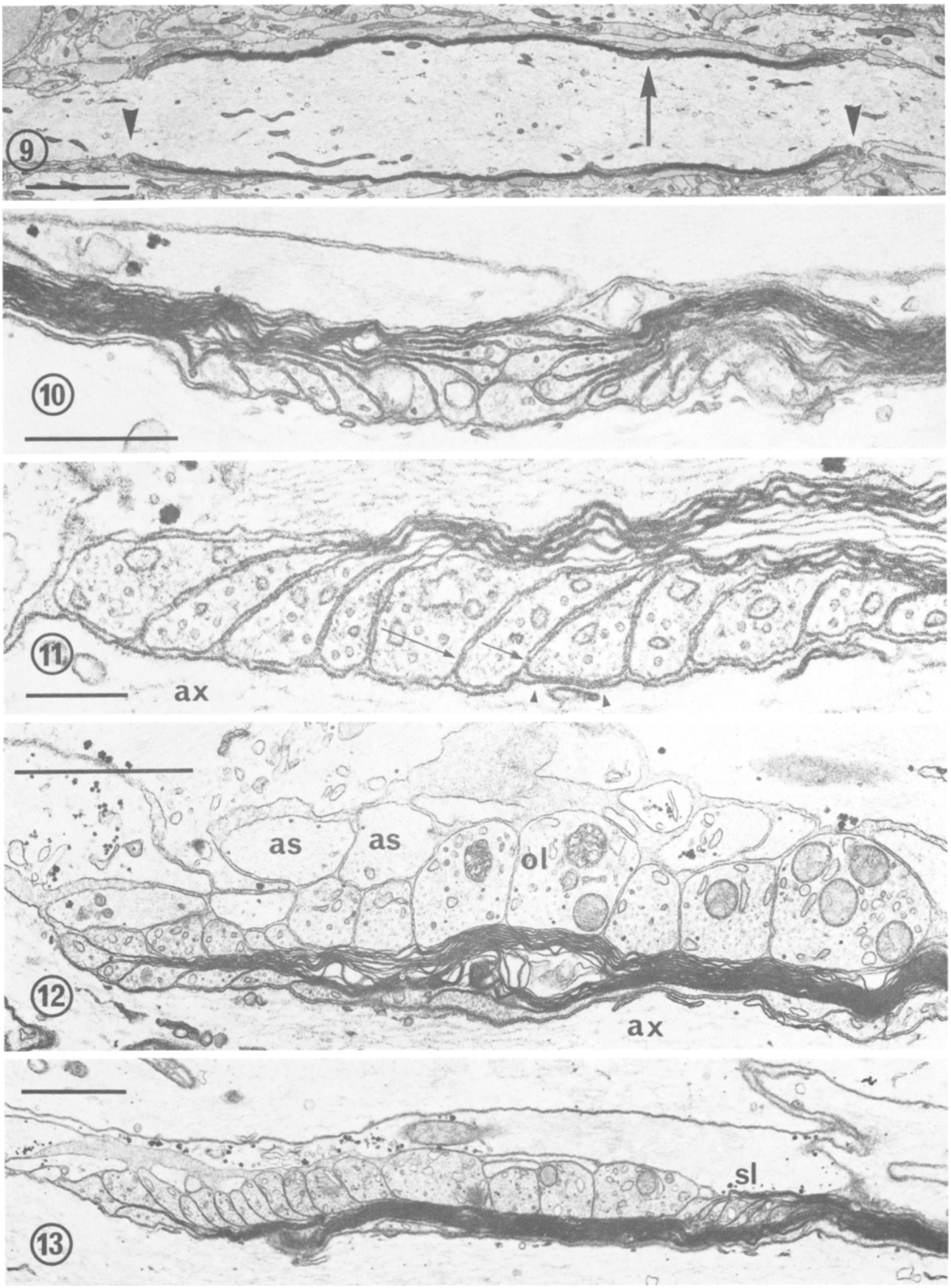
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cytoplasmic loops joined by tight junctions, were found internodally and adjacent to the paranodal lateral loops (Figs. 12, 13 and 16).

Another common finding was outward termination of the lateral loops, usually at a heminode, so that the loops ended on the extracellular space or on astrocytic processes (Figs. 12 and 13). Frequently, there were specialized areas of contact resembling tight junctions between these astrocytic processes and the outward-terminating oligodendrocyte loops (Figs. 14 and 15).

Occasionally the lateral loops of the myelin sheath terminated on an astrocytic process inserted between the myelin sheath and the axon (Figs. 16 and 17). The apposed membranes of the lateral loops and astrocytes were separated by an irregular space varying from 2.7 to 6 nm in width. Only very occasionally did the membranes show any degree of parallelism and the overall appearance was very similar to that described for the heminodes of many axons (Fig. 18). No evidence of any structure resembling the transverse bands of normal paranodal axolemma was found at the lateral loops terminating on astrocytic processes inserted between the axon and myelin sheath.

## Discussion

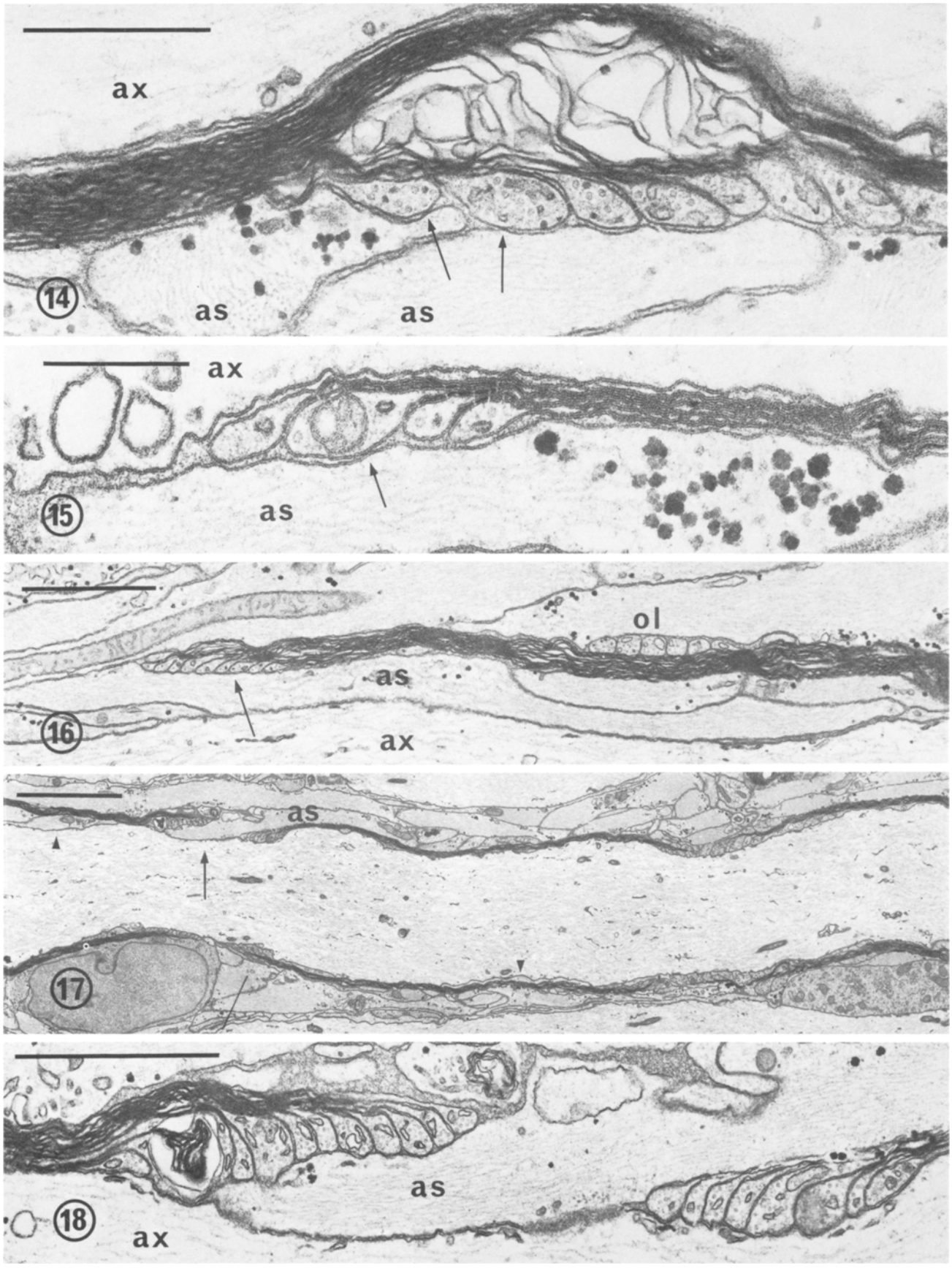
This ultrastructural study confirms the findings of the earlier light microscopical study (Griffiths *et al.*, 1981). The majority of axons in the spinal cord are either completely devoid of myelin or are surrounded by a minimal number of lamellae. The myelin that is present has both MDL and intraperiod lines at normal periodicities. The defect is therefore not similar to that in the Shiverer mouse in which a defect of the MDL occurs (Privat *et al.*, 1979).

A number of morphological configurations seen in the 2-month-old affected pup have been seen in the 1-month-old controls or described in other species during early myelination. The increased amounts of oligodendrocyte cytoplasm in lateral loops, internodal or paranodal oligodendrocyte cytoplasmic pockets and outward-terminating lateral loops are features seen in immature myelin sheaths (Hildebrand, 1971; Raine,

**Figs. 14 and 15.** Two heminodes with outward terminating lateral loops ending in association with astrocytic processes (as). In several areas the extracellular space is absent and a tight junction-like specialization is present between the lateral loop and the astrocyte (arrows). The lateral loops are joined by tight junctions (ax, axon). Scale bars: Fig. 14, 0.5 $\mu$m; Fig. 15, 0.2 $\mu$m.

**Figs. 16 and 17.** Two paranodal areas (arrows) at which the lateral loops end on an astrocytic process (as) inserted between the axon (ax) and the myelin sheath. In Fig. 16 several internodal oligodendrocyte loops are also present (ol). In Fig. 17 the internode is disproportionately short and several further astrocytic processes are present adjacent to the axon within the myelin sheath (arrowheads). Scale bars: Fig. 16, 1 $\mu$m; Fig. 17, 3 $\mu$m.

**Fig. 18.** 'Pseudoparanodal' area from Fig. 17 showing the lateral loops of one myelin sheath ending on an astrocytic process (as). The adjacent loops terminate on the axolemma. No obvious paranodal specializations are seen in either location (ax, axon). Scale bar: 1 $\mu$m.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1977; Fraher, 1978). The internodal termination of myelin lamellae has been described in the C.N.S. of normal, myelinating, one-week-old kittens by Hildebrand (1971) who described them as 'closed nodes'. Suzuki & Zagoren (1977) demonstrated such structures in the peripheral nerves of quaking mice. Several workers have described their occurrence during remyelination of the P.N.S. following experimental demyelination (Hall, 1973; King *et al.*, 1975; Bonnaud-Toulze & Raine, 1980), and Hall (1973) suggested that these 'pseudonodes', as she termed them, developed into Schmidt–Lanterman incisures. No evidence of such transformation was found in the pups but their frequency was too low to allow definite conclusions on this point.

The transverse bands are a specialization of the paranodal axolemma (Schnapp *et al.*, 1976) which occur with a periodicity of about 250 Å and develop as myelination progresses (Hildebrand, 1971; Raine, 1977). Their partial or complete absence at the majority of paranodes in these pups is similar to the situation in quaking mice (Rosenbluth, 1979a). Studies of normal mice and a number of murine mutants indicate that glial contact is necessary for the development of these paranodal specializations of the axolemma (Rosenbluth, 1979a, b). The abnormalities in the pups are presumably another manifestation of oligodendrocyte dysfunction.

These various features suggest that the state of myelination is immature for the age of the animal, an observation which has also been made in quaking mice (Wisniewski & Morell, 1971). Indeed the maturity of myelination in 2-month-old affected pups is less than that of 1-month-old controls. Despite some evidence of increased maturity at 2 months it seems highly unlikely that normal, mature myelination would ever occur in these pups.

Oligodendrocytes contained empty vacuoles, granular vacuoles and myelin figures within their cytoplasm. Similar granulomembranous bodies containing acid phosphatase and identified as secondary lysosomes have been demonstrated in quaking mice (Watanabe & Bingle, 1972). Blakemore & Harding (1974) also described numerous such vacuoles in congenital tremor type A IV, a hereditary, hypomyelinating disease of pigs. Our interpretation of this finding in the shaking pups is similar to that of previous authors, i.e. there is degradation of abnormal or excessively fragile myelin within these vacuoles. This would imply an error in oligodendrocyte metabolism with the production of defective myelin lipids or proteins, or an abnormality in their normal ratios, so that normal myelination does not proceed and some myelin is catabolized. However, the morphological studies suggest that the rate of catabolism is not great.

Numerous examples of abnormal inter-relationships between astrocytes and oligodendrocytes were evident where outward-ending myelin loops terminated on astrocytes internodally and at nodes. In addition there were the 'pseudoparanodal' terminations of an array of lateral loops on astrocytic processes inserted between the myelin sheath and axon. No evidence of any astrocytic membrane specialization similar to that of the normal paranodal axon was seen. This could be because similar specializations do not develop in such locations or because of immaturity, bearing in mind that the axonal paranodes themselves were immature. It will be interesting to

study similar areas in animals surviving for longer periods. The significance of the tight junction-like attachments between the myelin loops and astrocytes is also unknown. These interesting and unusual oligodendrocyte/astrocyte interactions will be studied further with freeze fracture.

The cellular basis of the abnormality of C.N.S. myelination in this new canine mutant requires further ultrastructural and quantitative study which is currently in progress. Results to date suggest that it will prove an interesting model in which to study abnormal axon–glial interactions and the effects that these have on the development of membrane specializations.

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