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Ballooning of myelin sheaths in normally aged macaques

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Summary

In aged animal brains, a variety of "holes" are formed in the neuropil. One type of hole, here designated as the *myelin balloon*, is an abnormality of the myelin sheath and is found in a number of diverse sites in the brain. Profiles of myelin balloons display rather smoothly rounded peripheral contours and typically range up to 10 μm in diameter, although exceptionally large examples may be twice this size. The balloons are bounded by lamellae of myelin, and to accommodate the contents of the balloon, the myelin sheath becomes split at the intraperiod line. Since the intraperiod line is formed by the apposition of the outer faces of the myelin-forming plasma membrane, the contents of the myelin balloons are, in effect, in continuity with the extracellular space, and it is suggested that the contents of the balloons are fluid, with the fluid exerting an outward pressure on the walls of the balloons to produce their spherical shapes. Myelin balloons are not only produced during aging but also occur in a number of genetic strains of mice and in a number of human disease states. They thus represent a non-specific, though distinctive and common, alteration of the myelin sheath and are a reflection of the fact that under a variety of conditions, including normal aging, oligodendrocytes are unable to maintain the integrity of their sheaths.

Introduction

A broad array of morphological abnormalities may be encountered in specimens of neural tissue from humans and animals of advanced age. Among these abnormalities are various types of voids, or holes, in the neuropil. Two regions in which these have been described are, first, the auditory brainstem of the aged monkey, rat, and gerbil, where they have been termed *holes* by Hoeffding & Feldman (1988) and by Feldman (1994) and *spongiform* lesions by Faddis and McGinn (1997), and second, the aged monkey primary visual cortex, where they have been termed *vacuoles* or *vesicles* by Peters (1991, 1996). However, careful study reveals that the holes do not constitute a uniform population of structures. They vary, for example, in size and shape and in the appearance of their boundaries, and the boundary of one type is formed of myelin-like lamellae. It is this type of hole that is the subject of the present article.

It has been known for many years (Kaes, 1907) that myelinated axons are vulnerable to a wide variety of disease and other pathological processes, with more recent studies of topics such as genetic abnormalities and compromise of the immune system further widening our appreciation of the extent of axonal vulnerability. Aging also degrades myelinated axons, so that in normally aging human brains, prominent among the findings of age-dependent change are reports of progressive pallor of myelin staining (Kemper, 1994). However, although this pallor is consistent with evidence of myelin degeneration (Miller *et al.*, 1980; Lintl & Braak, 1983), the problems associated with obtaining well-preserved human postmortem tissue make it difficult to assess how normal aging affects myelinated fibers in the human brain, but this is possible using animal models. In one such animal study, carried out on the aging rat auditory nerve, Hoeffding and Feldman (1988) found a progressive loss of nerve fibers that was accompanied by light microscopic evidence of myelin sheath abnormalities. In some cases, this appeared to take the form of a total degeneration of the sheath and its axon, and in other cases a thickening of the myelin sheath. This latter change had previously been observed by Feldman and Vaughan (1979), who also illustrated further changes, such as areas of degenerative vacuolization appearing at the periphery of the axon in the region of the innermost lamella of the myelin sheath.

The present investigation of neuropil holes bounded

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by myelin-like lamellae was carried out on perfusion-fixed CNS tissue from normally aging rhesus monkeys of varying ages. At the outset of the study, it was hypothesized that holes of this type represented cross-sectioned myelinated nerve fibers in which the axon had totally degenerated, leaving an empty fiber interior invested by the fiber's original myelin sheath, swollen considerably beyond its normal diameter. It is now evident that this view is not accurate. The structures are actually localized myelin sacculations, or balloons, extending from a myelin sheath that may contain a relatively intact axon, which is normal in appearance at levels proximal and distal to the balloon.

Materials and methods

ANIMALS

Tissue from five young adult macaque monkeys (*M. mulatta*) between 5 and 12 years of age and eight aged animals between 25 and 35 years of age was used in this study. An analysis of the lifespan in this species (Tigges *et al.,* 1988) indicates that about 25% of individuals attain an age of 25 years and only 6% an age greater than 30 years. The animals used were from an aging colony maintained at Yerkes Regional Primate Research Center at Emory University and at Boston University School of Medicine. Both sites are fully accredited. The colony is maintained for the purpose of research on the effects of normal aging on the brain and behavior and is monitored to screen out individuals with known pathology that might adversely affect normal aging. All animals in the colony are cared for under professional veterinary supervision in accordance with the *Guide for the Care and Use of Laboratory Animals* (N.I.H. publication 86-23).

TISSUE PREPARATION

Tissue fixation was carried out by perfusion under deep anesthesia, as described by Peters *et al.* (1994), and in full accordance with approved Institutional Animal Care and Use Committee regulations. After preanesthetization with Ketamine, a Ketamine/Rompun mixture was administered, I.V., to a state of areflexia. Animals were then tracheally intubated, placed on CO_2/O_2 artificial respiration, and transaortically perfused with a warm solution of 1% paraformaldehyde and 1.25% glutaraldehyde in either 0.1 M cacodylate buffer or 0.1 M phosphate buffer at pH 7.4. Immediately following cessation of the perfusion, the brains were removed and tissue samples placed in a solution of 2% paraformaldehyde and 2.5% glutaraldehyde in the same buffer. The samples studied were from the ventral cochlear nucleus, medial superior olivary nucleus, inferior colliculus, and substantia nigra of the brain stem, from the cerebellum, and from the neocortex. The neocortical areas were area 17 (from the occipital operculum), area 41 (from the superior temporal gyrus), and area 46 (from the floor of the sulcus principalis in the prefrontal cortex). The tissue blocks were osmicated, dehydrated in an ascending ethanol series, and embedded in Araldite. During processing the cortical blocks were *en bloc* stained with uranyl acetate. Semithin sections, 2 μm in thickness, were stained with toluidine blue and pyronin-B for light microscopic examination, and thin sections were stained with uranyl acetate and lead citrate for electron microscopic examination.

Results

LIGHT MICROSCOPY

Light microscopic examination of semithick plastic sections revealed examples of neuropil voids, or holes, of varying types in all brain regions studied and in every aged animal examined. Similar holes were not encountered in the young monkeys. In all the regions from the aged monkeys, one specific type of hole exhibited an apparently empty interior, with a smoothly contoured and densely stained marginal ring of uniform thickness (Fig. 1 *asterisk*). This ring, an important distinguishing feature of these structures, distinguishes them from other types of holes (Fig.1 *h*) and from capillaries (Fig. 1 *c*). Since most profiles of these ring-bound holes are rounded, it may be assumed that their three-dimensional form approximates a sphere. Holes of this type are present in small or moderate numbers in all aged animals, and they are scattered throughout the neuropil. Most of the round holes with thick walls range in diameter from 5 to 10 μm, but others may be as small as 2 μm, and unusually large examples (e.g., Fig. 3) can be 20 μm in diameter.

ELECTRON MICROSCOPY

In electron microscopic preparations, it is evident that the dark margins encircling the profiles of the round holes are lamellae of myelin. For this reason, the holes will be referred to as *myelin balloons.* Examples of the ultrastructural appearance of myelin balloons are presented in Figures 2 through 7. At all but the very lowest magnifications, the lamellar nature of the marginal ring is evident, and at sufficiently high resolution it is evident that the lamellae have the alternating major dense and intraperiod lines typical of myelin sheaths and that they have the same periodicity as myelin (Figs. 5 and 7).

The interiors of the myelin balloons are largely devoid of stainable material, although some inclusions may be present. Among the inclusions encountered are loosely dispersed arrays of flocculent material, or thin membranous partitions (Figs. 2 and 3), which in some instances seem to be derived from degenerating cytoplasmic debris that may be adjacent to the inner borders of the myelin balloons (Figs 2 and 4).

Due to the frequency with which these structures exhibit a circular profile and show no evidence of an enclosed axon, our initial impression was that the myelin ballons represented cross sections of degenerated myelinated nerve fibers that had lost their axons. With continuing study, it became evident that this conclusion was wrong. It is now clear that the myelin balloons are formed by localized sacculations that balloon out from the myelin sheaths of some nerve fibers in aged animals. Examples of the evidence for this conclusion are presented in Figures 4 and 6. Figure 4 shows a myelin balloon protruding from the side of an obliquely sectioned nerve fiber, and the margin of this balloon is clearly in continuity with the myelin sheath enveloping the axon. A point to be emphasized is that at the site of the balloon the lamellae

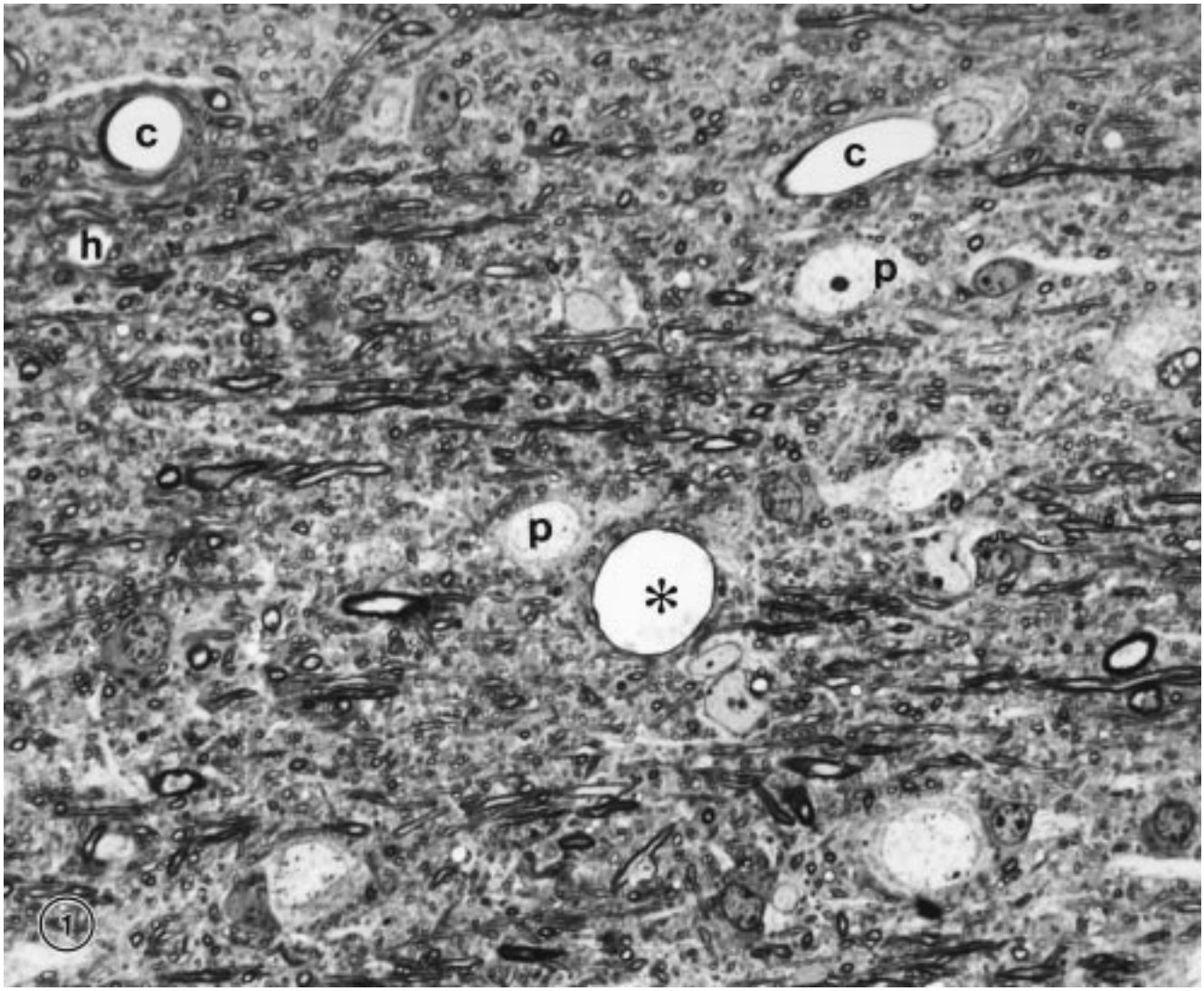
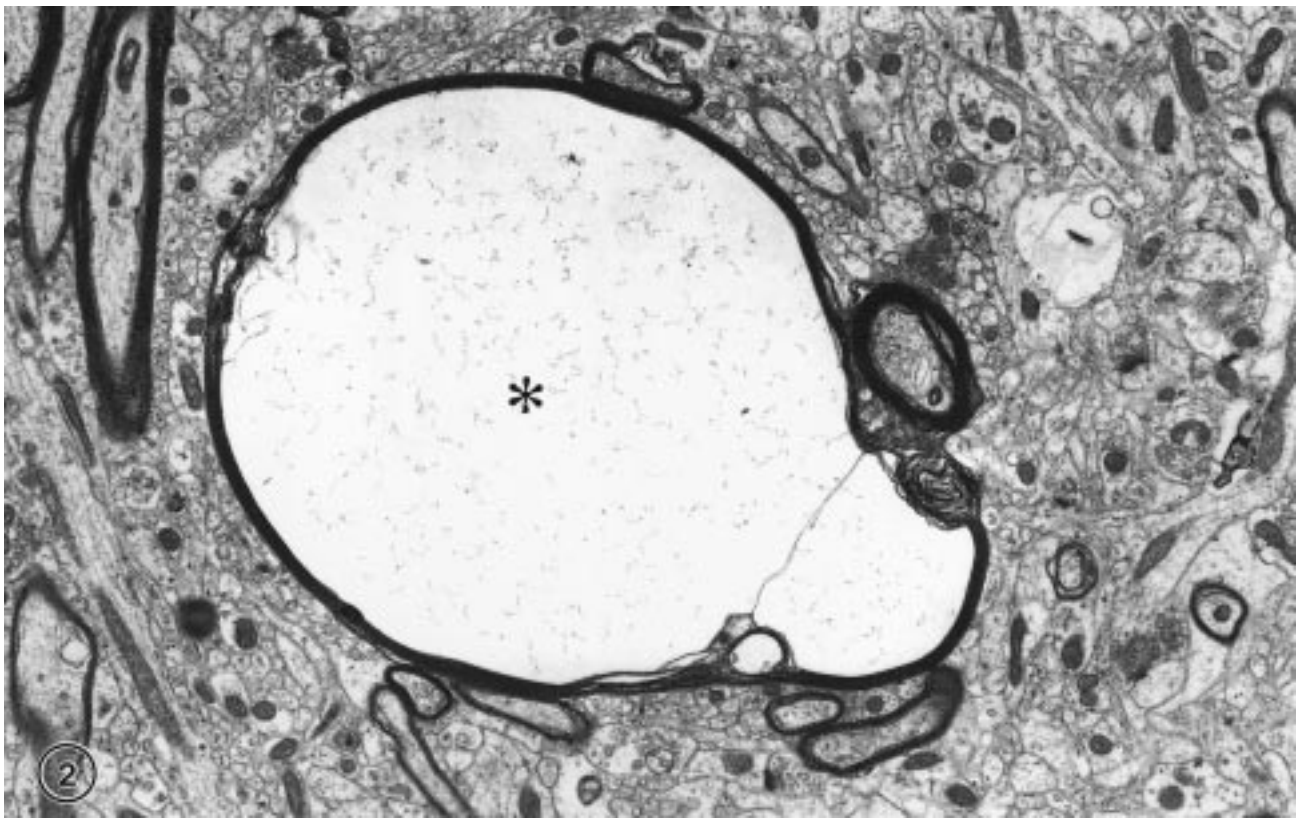


Fig. 1. Light microscopic appearance of a neuropil void (asterisk) in layer 6 of the primary visual cortex of a 35-year-old nacaque. The void has a relatively empty-appearing center, a smoothly rounded contour, and a peripheral margin consisting of a densely stained ring of rather uniform diameter. This structure is a relatively large myelin balloon, whose diameter exceeds that of the neighboring neuronal perikarya (P). Along the right edge of the figure are two capillaries (c), and a small neuropil "hole" (h) of a different type from the myelin balloon. Also present in the field are numerous profiles of transversely and longitudinally sectioned nerve fibers. × 1000.

on the side of the myelin sheath are split to accommodate both the dark and apparently degenerating cytoplasm at the base of the balloon and the contents of the balloon it self. There is an enormous expansion of the myelin lamellae of the sheath to form the wall of the balloon, and despite the breakdown of its sheath, the axon appears to be relatively normal (Fig. 6).

It is evident in Figures 5 and 7, which show the wall of the balloon at high magnification, that to accomodate the balloon, the myelin sheath splits at the intraperiod line, since the inner face of the balloon is lined by the outer leaflet of the plasma membrane forming the myelin sheath. The intraperiod line is formed by apposition of the outer faces of the oligodendrocyte membrane forming the myelin (Peters, 1960), and at high resolution it can be seen that the intraperiod line is composed of two thinner lines, which represent the outer leaflets of the plasma membrane separated by a gap of about 2 nm (see Peters *et al.*, 1991). In effect, therefore, in myelin there is a space in the intraperiod line, and it has been shown experimentally that this space can be penetrated by various markers (see Peters *et al.*, 1991). Consequently, through the space in the intraperiod line, the contents of the balloons are in continuity with the extracellular space in the surrounding neuropil.

An additional point that should be recognized concerning delamination, or splitting of the lamellae of the sheath, is that although the splitting that accommodates the lumen of the balloon occurs at the intraperiod line, this is not true of other splits that accommodate degenerating cytoplasm, or debris. When a split in the sheath is occupied by degenerated cyto-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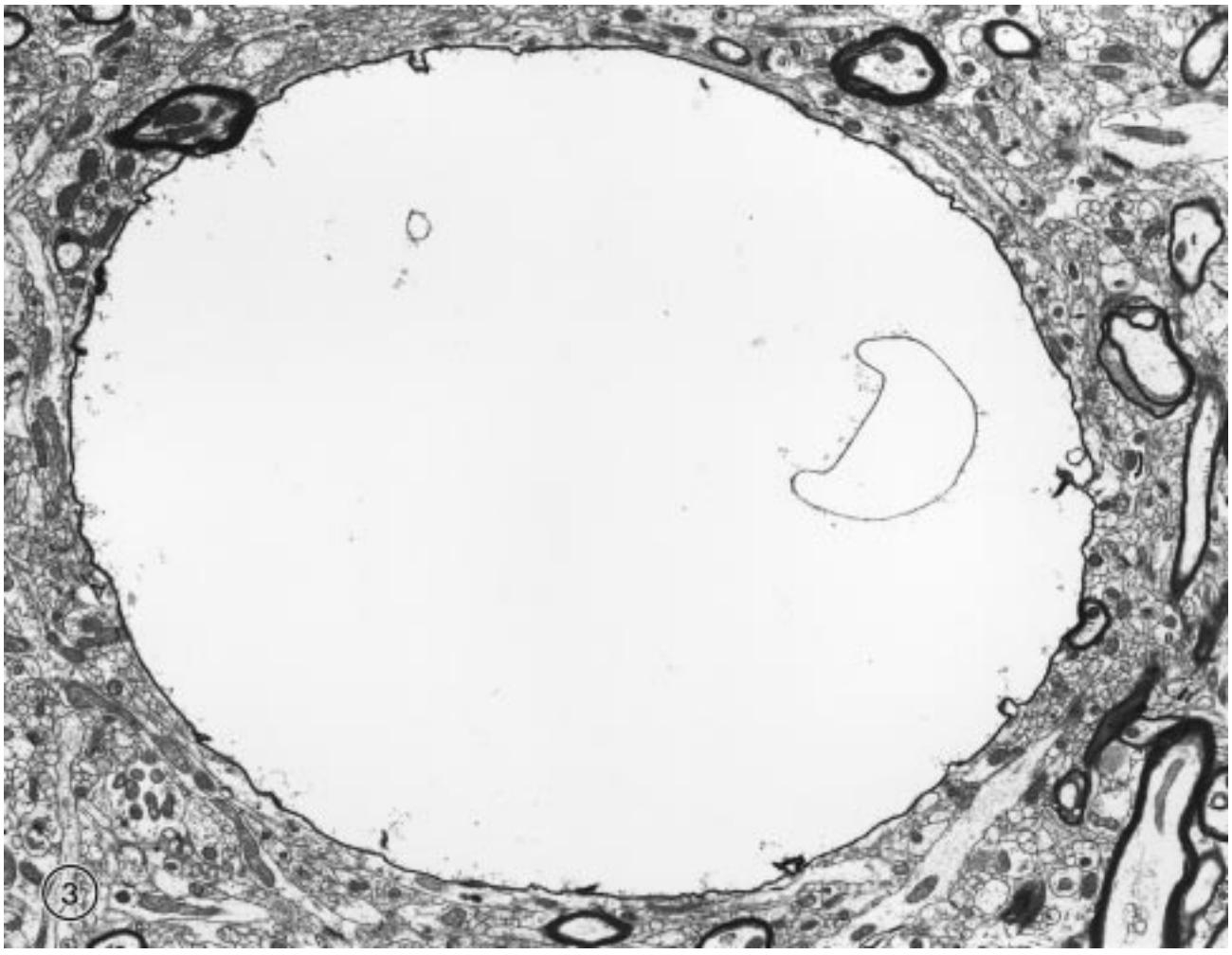
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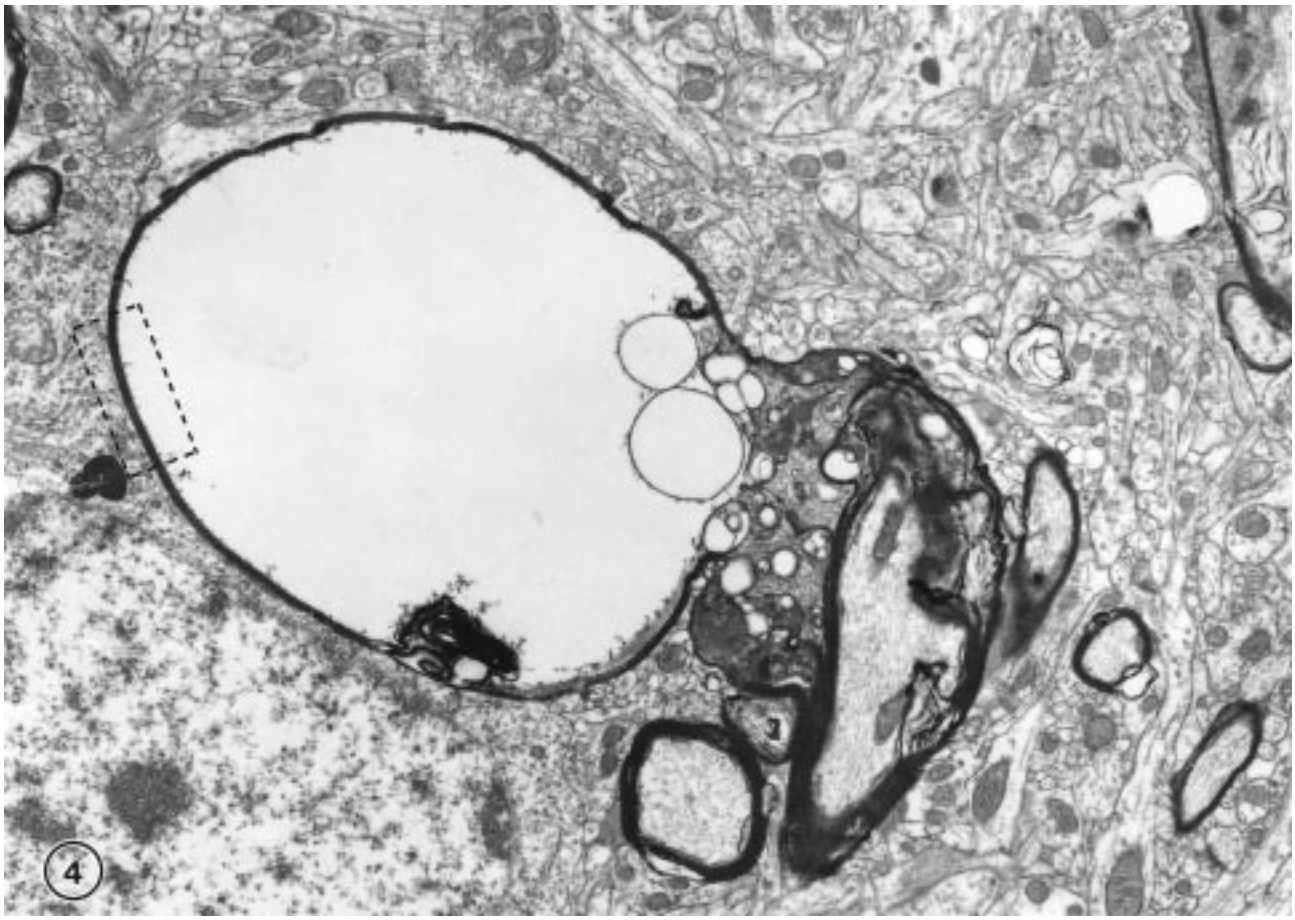

Fig. 4. A profile of a myelin balloon of intermediate size, which reveals the direct continuity between the lamellate margin of a balloon and the myelin sheath that gives rise to it. The myelin sheath splits to enclose first the degenerating cytoplasm, and then, at the left of the figure, the interior space of the myelin balloon. The portion of the margin of the myelin enclosed in the rectangle is shown at a higher magnification in Fig. 5. From a 28-year-old macaque, area 17, layer 6. × 11,000.

plasm, the split ocurs at the major dense line, indicating that the dark, degenerated cytoplasm originates from the oligodendrocyte forming the sheath.

Discussion

The observations presented above show that myelin balloons are protrusions from the sides of myelinated axons. This association is not widely appreciated, and the reason for this has to do with the low probability of obtaining an appropriate section plane in which the localized point of attachment to the myelin sheath is evident. Thus, the most commonly encountered profiles of balloons simply show a rounded vacuole enveloped by myelin.

While the present article focuses on a myelin change associated with advanced age in the macaque, it is important to recognize that this lesion, the myelin balloon, is relatively non-specific. It is widely distributed in many brain regions, it is found in a variety of species, and it is not exclusively associated with old age (Raine, 1984; Ostapoff & Morest, 1989; Yagi *et al.*, 1989, Ludwin, 1995; McGinn & Faddis, 1997). One of the earliest definitive accounts, utilizing electron micros-

Fig. 2. Ultrastructural appearance of a myelin balloon (asterisk). It has a relatively empty-appearing center, a generally rounded contour, and a dark peripheral margin that at higher magnification can be seen to be composed of myelin lamellae. This balloon profile is approximately 10 μm in diameter, and its interior has a thin membranous partition. From a 32-year-old macaque, area 46 of prefrontal cortex, layer 5. × 11,000.

Fig. 3. An example of an unusually large myelin balloon. The major axis of the profile measures approximately 20 μm. Although the magnification is not high enough to enable visualization of the lamellar nature of the marginal boundary, its uniform thickness is evident. From a 25-year-old macaque, area 17, layer 6. × 2700.

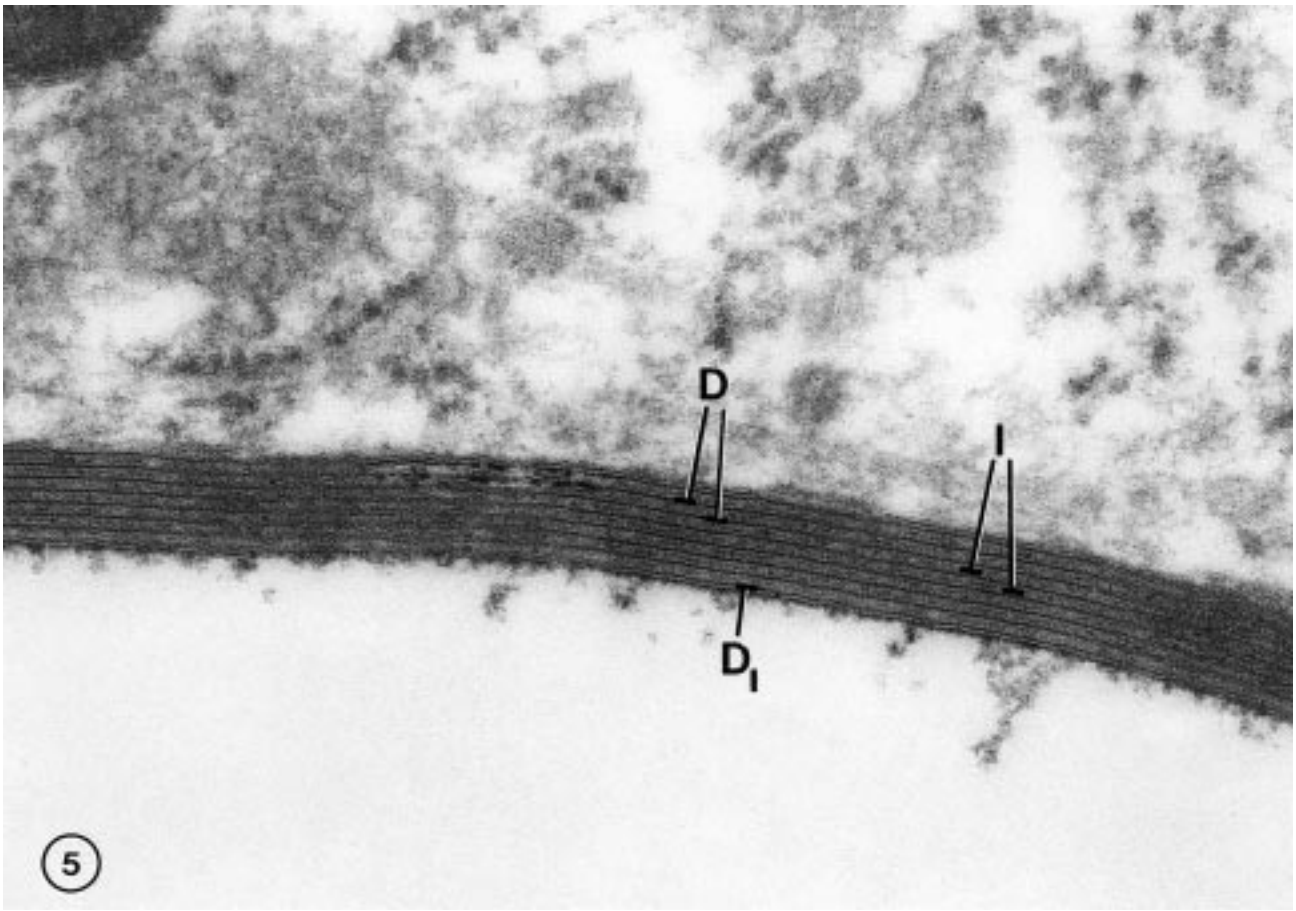

Fig. 5. Higher magnification of a portion of the lamellate margin of the balloon shown in Fig. 4. In the bounding myelin sheath, the major dense line (D) and the paired intraperiod line (I) are visible. On the side of the sheath facing the lumen, the major dense line is obvious (D_1), and below it is a single thickness of plasma membrane, the outer leaflet of which lines the lumen of the balloon. From a 28-year-old macaque, area 17, layer 6. × 120,000.

copy to document structural details and elucidate the intramyelinic origin of the lesions, was that of Hirano (1969), who observed the balloons in rats subjected to experimental triethyl-tin intoxication.

Myelin balloons, though a constant feature of the aged macaque brain, are not found in great abundance. For instance, a 3 mm-wide semithin section through the thickness of the cortex may show only one or two examples. This contrasts markedly with another type of pathological change that also produces light microscopically evident voids, or holes, in the neuropil, namely, spongiform degeneration. Spongiform lesions, which have also been referred to by alternative names such as *microcysts*, are characteristically observed in specific areas and circumstances, such as in the cochlear nucleus of the gerbil (McGinn & Faddis, 1987; Czibulka & Schwartz, 1993; Faddis & McGinn, 1997), in the zitter rat (Kondo *et al.*, 1995), and in specific human pathological states such as Creutzfeldt–Jakob disease (Hirano, 1981). However, microcysts differ from myelin balloons in that when they occur the cysts are common and they are not bounded by myelin lamellae. A further distinction is based on the genesis of the lesions: myelin balloons clearly arise within axonal myelin sheaths, whereas lesions of the traditional spongiform type, at least in the gerbil, appear to arise chiefly from dendrites (Faddis & McGinn, 1997). In view of these clear-cut differences, it is unfortunate that accounts of spongiform degeneration often extend the range of structures defined under that heading to include structures that are clearly myelin balloons. Faddis and McGinn (1997), for example, illustrate axonally originating *myelin splits* of the type illustrated in the present article and indicate that they form about 8% of the *spongiform* lesions in their material. It would appear to better serve our understanding of the forms of brain pathology to keep the two types of lesions clearly separate.

The myelin balloons also appear to be different from the myeloid bodies described by Hildebrand (1982) in normal spinal cord white matter. These Gomori-positive bodies, which appear as rings when sectioned, are composed of shells of lamellated material that lie inside microglial cells, and consequently they appear to be

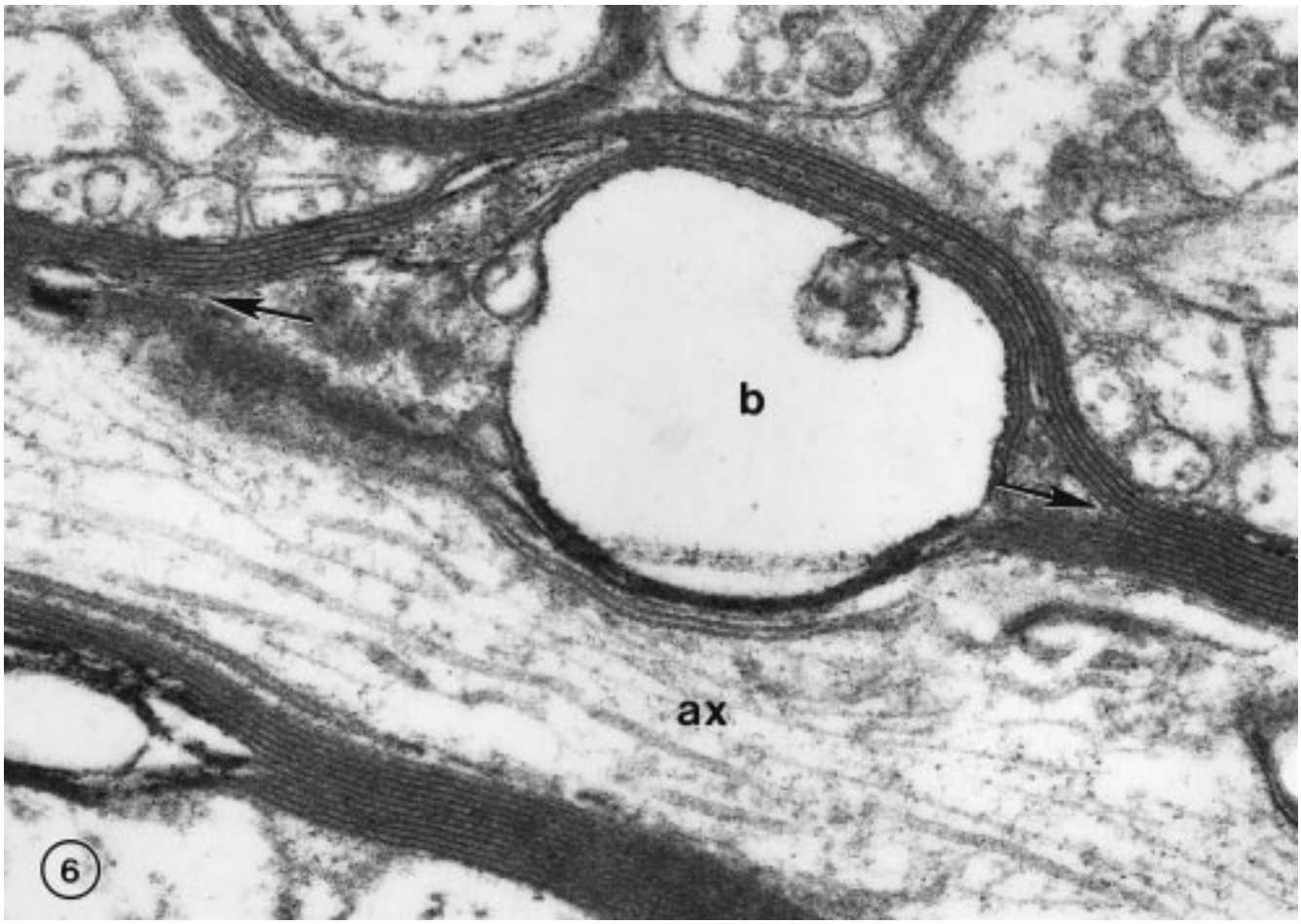

Fig. 6. Longitudinal section through a nerve fiber in layer 4 of area 46 of a 35-year-old monkey. The lamellae of the sheath bounding the axon (ax) have split (arrows) to accommodate a small balloon (b). Part of the sheath bounding the balloon is shown at higher magnification in Fig. 7. × 80,000.

formed from material, perhaps myelin, that has been phagocytozed by the microglial cells.

The most salient feature of the balloons is the large vacuolar space enclosed by the lamellae. This space generally appears to be empty of stainable contents, although occasional wisps of material and poorly organized membranous components can be present. As shown above the lamellar split enveloping the balloon occurs at the intraperiod line. This may allow fluid to enter the gap in the intraperiod line, and indeed the smooth contours of the balloons and their spherical shape suggest that their contents are fluid and that the fluid exerts a positive pressure inside the balloon to make it swell. The integrity of the intraperiod line is believed to be maintained by proteolipid protein (PLP) (Duncan *et al.,* 1987), and it may be that during aging the production of PLP by some oligodendrocytes is defective, leading to a split in the intraperiod line. Whether splits could be produced by an over- or an underproduction of PLP is not clear, since any defect in the Plp gene appears to lead to myelin defects (see Monuki & Lemke, 1995), so a range of normally occurring mutants of the Plp gene affect myelination, and an increase dosage of the Plp gene can also lead to dysmyelination in mice (Anderson *et al.,* 1998). Interestingly, in the late-onset degeneration produced in these mice, myelin balloons are common, and the range of myelin breakdown exhibited is very similar to that which occurs in aging. A splitting of the intraperiod line has also been reported in mice that are lacking the galactolipid galactocerebroside (Coetzee *et al.,* 1996), which is another constituent of central myelin, although its exact function is not known. In these mice, which do not survive for more than a few months, the myelin breaks down and large myelin balloons appear in the white matter (see Coetzee *et al.,* 1998, for a review). Other instances of myelin delamination occur, for example, in diabetes (Tamura & Parry, 1994) and in experimental toxicity produced by triethyl tin (Malamud & Hirano, 1973) and by lysolecithin (Blakemore, 1978).

The sequence of events that triggers the formation of

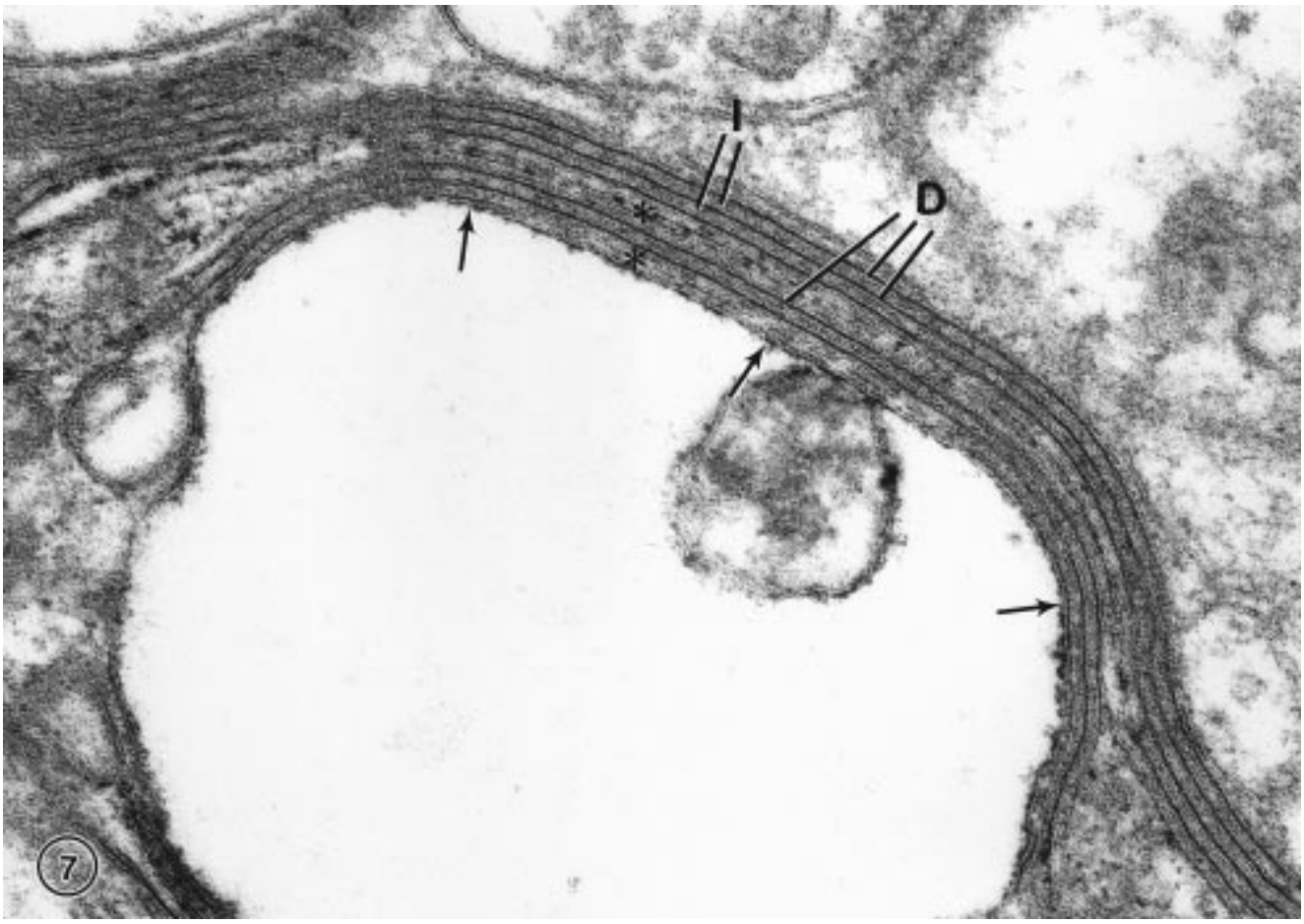

Fig. 7. In this enlarged portion of the sheath bounding the balloon shown in Fig. 6, the major dense line (D) and the intraperiod line (I) are evident. In two locations (*), the major dense line is split. In the areas indicated by arrows, it is evident that the outer leaflet of the myelin-forming membrane faces the lumen of the balloon. × 160,000.

myelin balloons in the normally aging macaque brain is not known. But since the balloons originate within myelin sheaths, they may, in a fundamental sense, be considered abnormalities of oligodendrocytes. In the aging macaque brain, the oligodendrocytes accumulate electron-dense inclusions both within their perikaryal cytoplasm and within swellings of the processes that extend to the sheats they form (Peters, 1996). Since there is no evidence that oligodendrocytes engage in phagocytosis, the inclusions may represent cytoplasmic degeneration products. Alternatively, the material may represent accumulations of protein and lipids, perhaps even proteolipid protein synthesized by the oligodendrocyte, that are normally utilized in myelin maintenance and turnover but cannot be properly utilized in old age. Such excess material might then accumulate in oligodendrocyte processes or be passed into the myelin sheath, where it may form intralamellar deposits of electron-dense cytoplasm such as that shown in Figure 4.

In the case of large balloons, such as those shown in Figures 3 and 4, it is evident that to form the margin of the balloon the surface area of the sheath must expand extensively. One way to account for this increase in surface area is to assume that the myelin is elastic and stretches to accomodate the fluid in the balloon. This process can be envisioned as being similar to the expansion of the wall of a toy balloon as it is inflated. But such elastic expansion would presumably entail a thinning of the lamellae, which is not found. Alternatively, the myelin may shift into the balloon from adjacent areas of the sheath. If, on the other hand, the increased amount of myelin is supplied by the oligodendroglial cell, balloon formation would represent an example of oligodendrocyte reactivation in advanced age, a process consistent with the thickening of the myelin sheaths that occurs in the auditory nerve of the aged rat (Hoeffding & Feldman, 1988).

The formation of a myelin balloon along the course of a myelinated axon represents a localized asymmetry of the periaxonal myelin sheath, a region of myelin delamination and debris accumulation. The formation

may have functional consequences for physiological conduction along the axon, since it is known, for example, that decompaction of CNS myelin reduces conduction velocity (Gutierrez *et al.*, 1995) and that partially demyelinated axons show a temporal dispersion of transmitted nerve impulses (Waxman, 1977), disrupting the synchrony that characterizes the normal transmission of volleys of neural information. In studies of segmentally demyelinated central axons in rats, Felts *et al.* (1997) compared conduction in demyelinated versus myelinated segments of individual axons and found that the refractory period of transmission in the former segments was as much as 34 times the values observed in the myelinated segments. In the auditory system—a region investigated in the present study and clearly subject to the formation of myelin balloons—synchrony of transmission is critical for accurate analysis of many types of sound stimuli such as tones of varying frequencies. It is therefore of interest to note that in studies of genetic dysmyelination in mice (Zhou *et al.*, 1995), decreases in myelin thickness were observed to correlate strongly with increases in auditory evoked response thresholds, response latencies, and other fundamental measures of hearing.

In summary, during aging in the macaque, in a number of mutants of other species, and in a variety of experimental conditions, myelin balloons can be generated. These balloons, which can become very large, are protrusions from the sides of myelin sheaths, and they appear to be filled by fluid that occupies space produced by a splitting of the intraperiod line. It is speculated that the formation of myelin balloons might impair axonal conduction in advanced age.

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