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What's Behind the Decline? The Role of White Matter in Brain Aging

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Abstract The specific molecular events that underlie the age-related loss of cognitive function are poorly understood. Although not experimentally substantiated, age-dependent neuronal loss has long been considered central to age-related cognitive decline. More recently, age-related changes in brain white matter have taken precedence in explaining the steady decline in cognitive domains seen in non-diseased elderly. Characteristic alterations in the ultrastructure of myelin coupled with evidence of inflammatory processes present in the white matter of several different species suggest that specific molecular events within brain white matter may better explain observed pathological changes and cognitive deficits. This review focuses on recent evidence highlighting the importance of white matter in deciphering the course of ''normal'' brain aging.

Keywords Age-related cognitive decline · Myelin · Oligodendrocyte · Calpain · CNP

Abbreviations

ARCD	Age-related cognitive decline
AD	Alzheimer's disease
PD	Parkinson's disease
MRI	Magnetic resonance imaging
FA	Fractional anisotropy
R_2	Transverse relaxation rates
CNS	Central nervous system
NFT	Neurofibrillary tangle
iNOS	Inducible nitric oxide synthase
MHC	Major histocompatibility complex
CAOs	Complement activated oligodendrocytes
C3aR	Complement C3a receptor
PNS	Peripheral nervous system
GFAP	Glial acidic fibrillary protein
ACT	α1-Antichymotrypsin
MBP	Myelin basic protein
PLP	Proteolipid protein
MAG	Myelin-associated glycoprotein
CNP	2',3' Cyclic nucleotide phosphodiesterase
MOSP	Myelin oligodendrocyte specific protein
EAE	Experimental allergic encephalomyelitis
MS	Multiple sclerosis

Special issue in honor of Naren Banik.

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Age-related cognitive decline (ARCD)

Severe impairment of memory and executive function, otherwise known as dementia, can be the result of a number of pathologic processes, including several neurodegenerative diseases, the most prevalent of which is Alzheimer's disease (AD). In addition, even in the absence of a clear pathologic process, normal healthy adults also experience a steady decline in cognitive ability whether measured longitudinally [64] or cross-sectionally [83, 90]. This age-related cognitive decline (ARCD) is not evenly distributed amongst cognitive tasks, such that vocabulary and tasks requiring semantic knowledge as well as autobiographical and emotional memory remain relatively intact while processing speed, working memory, and learning decline. These studies are fraught with issues of individual variability, by necessity are correlational and

potentially confounded by the considerable incidence of age-related diseases known to adversely affect cognitive performance [33]. Yet somewhat remarkably, a consistent decline in cognitive ability within certain domains is maintained from population to population [4, 11, 12, 17, 19, 64, 90].

Although the pathophysiology behind the major causes of dementia is comparatively well-understood, the biology underlying ARCD is only recently being elucidated. In most general terms, ARCD has been attributed to changes in prefrontal cortex and the integrity of anterior white matter circuits leading to non-selective recruitment of irrelevant brain regions in impaired individuals [33, 50]. Our group, as part of a larger program project, has focused on understanding the molecular changes underlying ARCD, using the rhesus monkey as a model, with the most intriguing findings being those changes present in brain white matter. It is our belief that age-related changes in white matter have not received adequate attention in the context of playing a potentially causative role in cognitive decline and hence, the discussion to follow will focus on the importance of changes in brain white matter, beginning with those at the gross level and culminating with those occurring at the molecular level, as key events in understanding ARCD.

Imaging age-related changes in brain volume

Magnetic resonance imaging (MRI) has provided a useful non-invasive tool for analyzing gross age-related changes in brain volume. In aged human subjects, several studies demonstrate a decline in the volume of brain white matter, particularly in frontal lobe areas [8, 30, 61, 76]. Recent advances in MRI technology allow for more accurate volumetric measurements by better distinguishing between gray and white matter as well as capitalizing on the unique features of compact white matter regions. The use of diffusion tensor MRI to measure fractional anisotropy (FA), the degree to which water molecules in a tissue are allowed to diffuse, is particularly useful in assessment of white matter tract integrity, as increased anisotropy correlates with markers of myelination [45, 101]. In healthy elderly adults, FA is reduced compared to young, particularly in the anterior or frontal regions of the brain including the corpus callosum [32, 61, 79, 80]. In a more elegant analysis, a group of behaviorally tested monkeys showing impaired executive function with age also show significant reductions in FA in the anterior corpus callosum, the superior longitudinal fasciculus II and the cingulum bundle; all corticocortical pathways vital for executive function [52]. Using a different methodology, by transformation of the T2 relaxation times into transverse relaxation rates (R_2), a more sensitive measure of brain myelination differences is generated [25] such that myelination increases R_2 while myelin breakdown decreases R_2. In a large population ($n = 252$) of healthy adults ranging from 19 to 82 years of age, frontal lobe white matter R_2 shows a quadratic regression relationship with age, indicating that white matter volume increases until the fifth decade of life, after which it declines steadily [7, 8], implying that myelination is highly sensitive to time-dependent changes. These age-dependent decreases in white matter volume generally occur without significant change in gray matter volume [30].

The aged neuron: lost or diseased?

The importance of age-dependent neuronal loss is obvious and its potential role in ARCD should also be evident. As a result, many early studies, particularly prior to the advent of MRI, focused on neuronal counts and morphology changes, for the most part ignoring changes in glial cell number or morphology [13, 20, 35]. However, with the advent of precise stereologic methodology [98] and better preservation of brain tissue, studies reporting no loss of neurons with age began to appear [5, 16, 96]. Newer methods were designed to account for the fact that young brains tend to shrink more during preparation thus leading to a conclusion of greater neuronal density in young subjects. Accounting for the difference in brain shrinkage, volumetric estimates of gray and white matter in human post-mortem brain tissue suggest no significant loss of neurons with age and a significant age-related loss of up to 11% in white matter [31]. This paradigm shift was supported in part by work in the aged rhesus monkey, indicating changes in the myelinated axons of white matter might be more important to aging [68, 71]. Indeed, detailed counts of neurons from primary visual cortex (Brodmann area 17) and prefrontal cortex (area 46) in the monkey demonstrate there is no change in neuron number with age (Fig. 1) [67, 70]. Furthermore, in the hippocampus, the center of working memory, the number of CA1 pyramidal cell neurons remains stable across age [99]. One recent report indicated that in a cognitively relevant location in the frontal cortex (area 8A), focal neuronal death occurs with age [91], however, this report has yet to be confirmed. Overall, simple age-related neuronal loss is not an adequate suspect in explaining ARCD [26].

On the whole, other than in Layer I of the cortex, where neurons lose apical dendritic branches and occasionally synapses [62, 63], neuronal number and structure are largely unaffected by age. It is important to note that in prefrontal cortex area 46 of aged rhesus monkeys, the

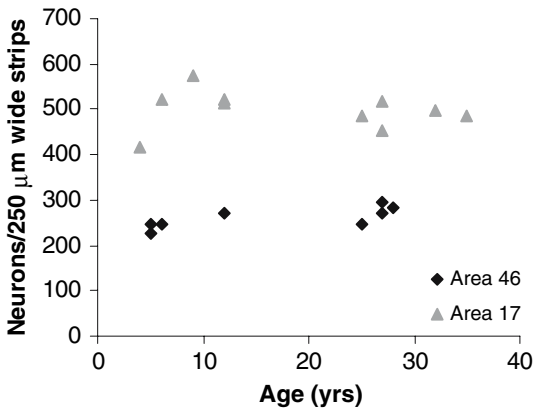

Fig. 1 Lack of evidence for neuronal loss in the aged rhesus monkey. Comparative analysis of mean neuronal counts from 250 μm strips of primary visual cortex (area 17) and prefrontal cortex (area 46) in monkeys of varying ages reveals no significant change in total neuronal number per unit volume. Adapted from Peters et al. [67, 70]

observed dendritic and synaptic loss correlate with the degree of cognitive impairment [74]. Furthermore, in the monkey, neurons do show age-related changes in their expression of neurotransmitters and receptors [78]. Recent microarray analysis of gene expression in area 46 in aged monkeys, indicates widespread transcriptional changes occur in neurons, including the degree to which they are susceptible to apoptosis [14]. Thus, while overall neuronal number may not change with age, various alterations in the health of neurons should be expected and cannot be discounted in any discussion of age-related changes in the central nervous system (CNS).

The contribution of amyloid burden

In humans, ~50% of those over the age of 85 develop profound memory impairment as a result of AD pathology, including the formation of senile amyloid plaques and neurofibrillary tangles (NFTs) [18]. The high incidence of AD in humans coupled with the virtual absence of AD-like pathology in wild-type laboratory animals such as the mouse, rat, and rhesus monkey, make it difficult to discern which age-related changes are associated with ARCD versus AD. While amyloid plaques can be found in rhesus monkeys, they are generally of the diffuse, non-pathogenic form and their distribution is markedly different from that seen in AD [34, 92]. Furthermore, plaque density in behaviorally characterized monkeys does not correlate with cognitive deficits [15, 89]. Additionally, NFTs are not typically found in aged monkeys and the degree of age-related cognitive impairment is not consistent with AD [47]. For these reasons, AD-like phenomena have not been a focus of understanding mechanisms of ARCD and can be considered a disparate pathologic process.

Evidence of age-related white matter inflammation

In the monkey, the number of activated microglial cells, identified by their up-regulation of the major histocompatibility complex (MHC) class II proteins, increases dramatically with age specifically in regions of brain white matter and the increase correlates with cognitive impairment [87]. Similar increases in activated microglia have been noted in mice [56]. These phagocytic cells are known to produce a multitude of pro-inflammatory molecules [57], among them inducible nitric oxide synthase (iNOS). The resulting increase in nitric oxide presumably accounts for the concomitant increase in detection of nitrotyrosine residues in the white matter of aged monkeys [87]. Activated microglia are known to be active and phagocytic during phases of demyelination in the Long Evans shaker rat [103], illustrating the consequences of an increasing number of these cells with age. In the optic nerve of aged monkeys, increased numbers of microglia and hypertrophied astrocytes can be detected under the electron microscope, and the microglia cells appear to have phagocytosed myelin debris [81].

Interestingly, recent work in the rhesus monkey indicates that early components of the complement cascade (C3 and C4) can be found covalently bound to the myelin membrane in both young and old animals (J. A. Duce et al. 2006, submitted). This binding is associated with a significant increase in aged animals in the number of complement activated oligodendrocytes (CAOs), a pathological hallmark of white matter inflammation seen in multiple sclerosis (MS) [84] and other neurodegenerative diseases [102]. Complement component 3a (C3a) is a potent chemotactic factor and presumably as a result of robust expression of the C3a receptor (C3aR), activated microglia can be found in close association with these CAOs and in mice lacking C3aR expression, white matter inflammation is attenuated [10]. Thus, fixation of complement components to myelin may be a critical inflammatory trigger in the aging brain.

In addition to the activation of microglia, reactive astrocytosis can be found in white matter regions throughout the aged monkey brain, evidenced by an increased cell size of glial fibrillary acidic protein (GFAP)-positive cells [88]. An increase in GFAP mRNA and protein expression can also be detected in the brains of aged humans and rodents, though the total number of astrocytes does not change appreciably [27, 59]. Reactive astrocytes, like microglia, are known to produce abundant pro-inflammatory molecules including various proteases and inhibitors [22]. Of particular interest is the protease inhibitor α1-antichymotrypsin (ACT), a serine protease inhibitor and acute phase protein. In AD, ACT expression is highly up-regulated at the RNA level [2] with reactive astrocytes being the major

source of ACT in the brain [1]. Microarray analysis of brain white matter from young and old rhesus monkeys reveals that ACT is also up-regulated during normal aging (Duce and Abraham, unpublished results).

Myelin changes in the aged brain

In part, based on the volume of evidence from gross pathology, imaging studies, and histologic assessments implicating changes in white matter as central to the mechanism of ARCD, a number of studies focus on microscopic examination of aged myelin. In the peripheral nervous system (PNS) of aged Fischer 344 rats, no change in the number of myelinated fibers was observed, though the ultrastructure of the myelin sheath, evidenced by ballooning and infolding, was disrupted with age [46]. Using histologic stains for myelin content [41], demonstrated diffuse myelin pallor throughout the human brain, particularly in the intracortical fibers thought to be responsible for high-level cognitive function. Tang et al. [95] and Pakkenberg and Gundersen [62] have both demonstrated a loss of white matter from the human brain with age, of up to 15%, as well as a decrease in the length of myelinated fibers. In a population of Danish individuals, the total length of myelinated fibers was seen to decrease by 10% per decade and 45% across the lifetime [54]. This decrease in fiber length indicates a much greater degree of myelin loss than indicated by studies of white matter volume.

Disruption of the myelin sheath, either by activated pro-inflammatory cells or by inappropriate protein maintenance within the oligodendrocyte (or a combination of both), has been described in aging rat [46, 93], monkey [66, 86], and human [3] brains. Four distinct phenomena are known to occur as myelin ages and these have been best described at the ultrastructural level as occurring throughout the CNS of the aged monkey (Fig. 2) [65]. First, localized splitting of the major denseline to accommodate dense cytoplasm from the oligodendrocyte can be seen in some sheaths (A). Second, myelin sheaths can balloon out, separating the intraperiod line, which then surrounds a fluid-filled space (B). Some thick myelin sheaths demonstrate a double sheath (C), with one compact set of lamellae surrounded by another. Finally, in both primates and rodents, so-called redundant myelin sheaths have been described [65, 69, 73] (D). These sheaths have far too many lamellae in proportion to the caliber of the axon. The significance of these ultrastructural changes on the function of the axon remains unclear, although the changes correlate with deficits in cognitive function in both primary visual cortex [69] and prefrontal cortex [72], suggesting that the disruption may interrupt normal axonal conduction and thus impair cognition.

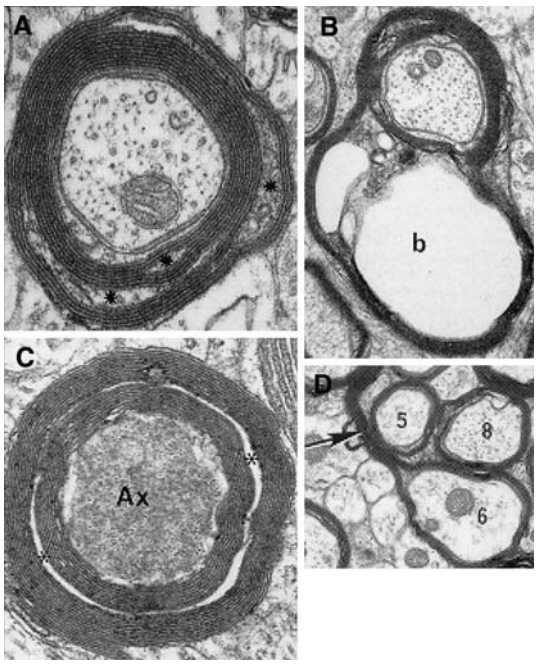

Fig. 2 Age-related changes in myelin structure. There are four important features of age-related change in myelin ultrastructure [72]. These include, localized splitting of the MDL to accommodate dense cytoplasm of the oligodendrocyte (**A**), ballooning of myelin sheaths at the IPL forming a fluid-filled space (**B**), double myelin sheaths (**C**), and so-called ''redundant'' myelin sheaths (**D**). Adapted from Peters et al. [69, 73] and Peters and Sethares [72]

Recent studies have suggested that these age-related changes in myelin membranes alter axonal protein organization at or near the node of Ranvier. In both aged Fischer 344 rats and rhesus monkeys, voltage-gated potassium channels of the *Shaker* family (specifically Kv1.2), normally localized to the juxtaparanode beneath compact myelin, are seen to mislocalize into the paranodal region with age [37]. This reorganization occurs together with the presence of thick myelin sheaths with excessive paranodal loops of myelin. It is not clear from this study whether these changes are detrimental or restorative to axonal conduction, as the functionality of Kv1 channels in myelin is not well characterized. However it does demonstrate the ability of myelin to have retrograde effects on neuronal health.

The role of inappropriate protein degradation

Another common consequence of brain aging is the increased detection of ubiquitin. Particularly notable in age-related neurodegenerative diseases, ubiquitin becomes associated with the NFTof AD, the Lewy body of Parkinson's disease (PD), the inclusions of amyotrophic lateral sclerosis, etc. [51]. In fact, one of the genes associated with

autosomal recessive juvenile PD, parkin, codes for an enzyme in the ubiquitin pathway [44, 85]. Ubiquitin, a small, highly conserved 8 kDa protein modifier is attached to proteins destined for degradation by the proteasomal pathway (reviewed by Fang and Weissman [23]). Likewise, ubiquitin can be removed by the action of deubiquitinases and the addition of ubiquitin may serve multiple cellular functions besides protein degradation [29].

In the aging brain, immunohistochemical staining for ubiquitin reveals localization to myelin and lysosome-related bodies in neurons [51]. Using the electron microscope together with immunolabeling, age-related ubiquitin deposits in humans and dogs can be seen within focal enlargements of myelin sheaths [55]. Likewise [21], report dense ubiquitin-positive inclusions within glial cells and ubiquitin staining in swollen myelin sheaths as well as documenting significantly more ubiquitin immunoreactivity in white matter than in gray. In aged dogs, white matter degeneration is marked by deposits of non-degraded ubiquitin-positive conjugates in myelin [24]. Interestingly, though ubiquitin conjugation serves primarily to target proteins for degradation, ubiquitin-protein conjugates are consistently found in the aged brain and in neurodegenerative diseases, suggesting a failure of proteasomal function with age, leaving large non-degraded, and potentially non-functional protein complexes to accumulate. The toxicity of these complexes may be particularly relevant in white matter and myelin, where the normal rate of protein turnover is already considerably lower than that seen in whole brain [48].

Age-related changes in myelin biochemistry

The ultimate effect of age-related increases in white matter inflammation and myelin disruption must trickle down to observable alterations in myelin lipids and protein expression. On this topic, a number of reports spanning several decades exist, demonstrating evidence of both age-related increases and decreases in various proteins and lipids that comprise myelin. At this time, the lack of well-controlled studies prevents a thorough discussion of age-related changes in myelin lipids. It is essential to add, however that sulfatide-null mice demonstrate an increasing prevalence of redundant, uncompacted or degenerating myelin as well as alterations in nodal and paranodal ion channel localization as they age [38, 53]. These changes are very similar to changes observed in aged rhesus monkey myelin ultrastructure and axonal reorganization. There has been no focused analysis of changes in CNS sulfatide over time, though in a global analysis of myelin lipids sulfatide was among the myelin components that were decreased in frontal and temporal white matter regions in a cohort of human brains aged 20-100 [94].

With respect to total amounts of myelin, measured either with histologic stains or by various purification methods, there is a consensus of significant myelin loss over time [9, 86, 100]. A majority of these older studies indicate no change in the individual protein composition of myelin but were carried out prior to clarification of the myelin proteome [9, 100]. Myelin basic protein (MBP) has shown to be decreased in elderly with no cognitive impairment (NCI) [6, 97] as well as in AD [77], though in a study of behaviorally characterized rhesus monkeys, MBP, proteolipid protein (PLP), and myelin-associated glycoprotein (MAG) were unchanged while the levels of myelin oligodendrocyte specific protein (MOSP) and 2′3′-cyclic nucleotide phosphodiesterase (CNP) were increased with age [86].

In addition to examining the age-related changes in the total amount of myelin proteins, exploring what post-translational modifications occur to these proteins with age is equally important. As an example, MBP isolated from MS human brain and demyelinating mouse models indicate that multiple post-translational modifications, including deimination (resulting in citrullinated proteins) and dephosphorylation, may be essential to altering myelin structure and function [42] and are commonly observed in age-related neurodegenerative disease [39, 58]. In the case of CNP, there is evidence from a cohort of aged rhesus monkeys that CNP accumulation is related to the increased detection of ubiquitinated species of CNP in myelin from aged animals (Hinman, Chen, Hollander, and Abraham in preparation). Additionally, both CNP and MOSP showed evidence of limited proteolysis, which in the case of CNP was attributable to an age-dependent increase in activated calpain-1 [36, 86]. This calpain-mediated limited proteolysis of CNP can also be observed in vitro by incubation of white matter homogenates from a young monkey prepared in the absence of protease inhibitors. The degradation is enhanced by the addition of 500 μM calcium and can be inhibited by a specific calpain inhibitor (Fig. 3a). Interestingly, the appearance of the major 40 kDa proteolytic fragment of CNP appears to correlate with age in a limited sample of aged rhesus monkeys (Fig. 3b). Already implicated in myelin breakdown in MS and its animal model, experimental allergic encephalomyelitis (EAE) [82], the exact proteolytic mechanism of calpain in the degradation of myelin proteins has yet to be determined, however its role in limited proteolysis is likely essential to understanding myelin breakdown. Further work is necessary to understand the potential significance of limited proteolytic fragments of CNP and other myelin proteins such as MOSP.

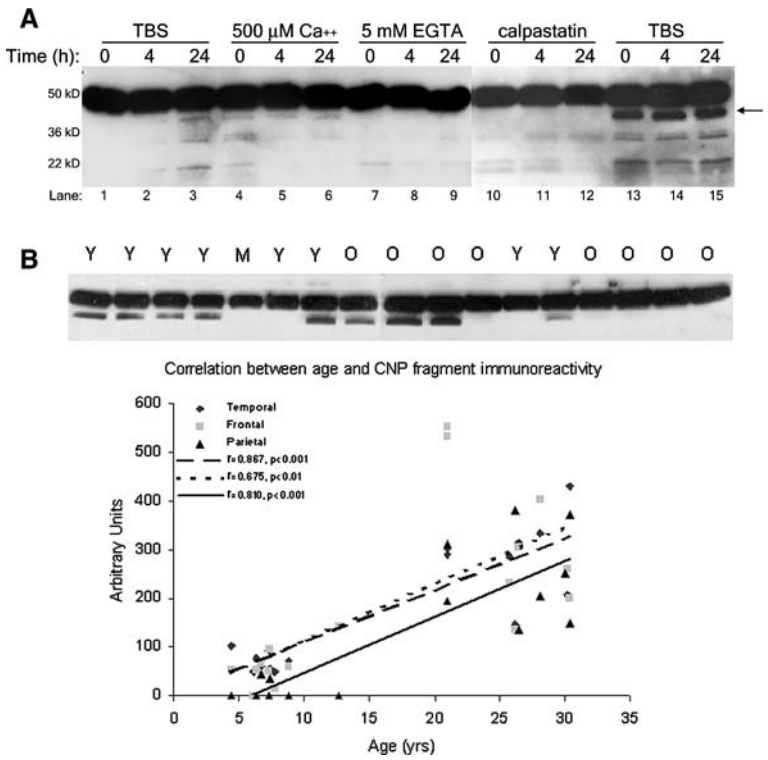

Fig. 3 Age-dependent limited proteolysis of CNP by calpain-1. Incubation of TX-100 soluble homogenate from a 9-year-old (young) monkey for 24 h at 37°C in the presence of TBS (lane 3) results in the same pattern of CNP proteolysis as that seen in a 26-year-old monkey at time zero (lane 13 from *left*; *arrow* indicates major 40 kDa CNP fragment). This pattern can be induced at time zero by the addition of 500 µM Ca^{++} (lane 4) and abolished by the addition of 5 mM EGTA (lanes 7–9) or 0.35 U of calpastatin (lanes 10–12) (**A**). Long exposures of immunoblots for CNP in TX-100 soluble fractions of temporal lobe subcortical white matter reveals the presence of a limited proteolytic fragment of CNP migrating at ~40 kDa. Quantitative assessment of the level of this major 40 kDa fragment observed in aged monkeys demonstrates a statistically significant correlation with age throughout brain white matter (**B**)

The apparent paradox of simultaneous protein accumulation and increased proteolysis can be explained by considering the relationship between failed proteasomal degradation and the recruitment of alternative proteolytic mechanisms such as calpain. This relationship has been described in the context of P-glycoprotein and p107 such that both ubiquitination and calpain activation are necessary for the degradation of the target molecules [40, 60]. In the nervous system, the parkin gene codes for a ubiquitin ligase which appears to induce calpain-mediated degradation of alpha-synuclein suggesting further that cytotoxic states such as alpha-synuclein accumulation is a stimulus for cooperation between ubiquitin and calpain-mediated proteolysis [43].

Changes such as those described in the rhesus monkey, occurring in critical lower abundance myelin proteins (e.g., CNP and MOSP), may be triggered by the activation of inflammatory processes and underlie myelin disruption. Interestingly, mice overexpressing CNP show evidence of redundant myelin and intramyelinic vacuoles similar to those seen in aged monkeys [28], while mice lacking CNP show an age-dependent reactive gliosis and disorganized paranodal profiles [49, 75], features also noted in aged monkeys. While the precise role of CNP in myelin has yet to be determined, these similarities argue strongly for a prominent role for CNP (and the microtubule cytoskeleton) in the long-term maintenance of the myelin membrane.

Summary

The study of ARCD has matured recently, moving from a focus on age-related neuronal loss as being directly causative, to a greater understanding of the contribution of molecular changes in brain white matter. In recent years, research of this nature has shifted focus from characterizing gross or morphologic changes with age to understanding what molecular events underlie age-related white matter inflammation and myelin membrane disruption. Considering the abundance of evidence from both humans, rodents, as well as the monkey, declines in white matter integrity appear at least as important as neuronal changes in understanding ARCD. In particular, age-related changes in myelin protein degradation and the recruitment of

alternative mechanisms of proteolysis appear as principal events in the brain aging process, occurring in advance of age-related changes in neurons. This evidence should help to guide future research in brain aging and influence potential interventional targets.

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