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Age-Related Fibrillar Deposits in Brains of C57BL/6 Mice

A Review of Localization, Staining Characteristics, and Strain Specificity

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Abstract

The present article reviews findings regarding the age-related occurrence of clusters of unusual granules in the brains of C57BL/6 (B6) mice and discusses the potential relevance of this phenomenon as a model of specific aspects of brain aging in humans. The granules occur predominantly in the hippocampus of B6 mice and represent aggregations of fibrillar material that are mostly associated with astrocytes. The deposits become evident at about 4 to 6 mo of age, and increase markedly in both number and size thereafter. Similar structures have been observed in adult senescence accelerated mice (SAM) and have been noted, although very rarely, in older mice from other strains. The deposits appear to manifest dominant genetic heritability. Heparan sulfate proteoglycan and laminin or related molecules have been identified as components of the granular material. Although the deposits do not represent senile plaques with β-amyloid deposition, they might mimic the deposition of extracellular matrix molecules that is thought to be an early event in amyloidogenesis in the aged brain and in Alzheimer's disease.

Index Entries: Aging; amyloid; corpora amylacea; transgenic mice; senescence accelerated mouse; genetics; proteoglycan; laminin; astrocytes; inclusions; learning and memory.

Introduction

Among the many notable morphological changes observed in the brains of aged mice *(1–7)* Lamar et al. *(8)* and Mandybur et al. *(4)* reported clusters of granular deposits in the hippocampus of C57BL/6 (B6) and immunologically deficient athymic nude mice, respectively, that were positive for the periodic-acid Schiff (PAS) stain. Similar PAS-positive material was reported in the brains of the senescence accelerated prone mouse strain (SAM-P), a murine strain purported to present early and broad

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manifestations of aging in several physiological systems *(9)*. Wirak et al. *(10)* described similar deposits of granular material in the hippocampus of a transgenic hybrid cross with a B6 background that had received a transgene construct coding for human amyloid β-peptide (Aβ). The granules in these transgenic mice reacted positively to polyclonal antibodies to Aβ, and thus appeared to demonstrate the potential application of transgenic technology to model, in mice, Aβ deposition similar to that occurring in Alzheimer's disease (AD).

Using a polyclonal antibody to a 110-kDa laminin binding protein (LBP110), Jucker et al. *(11)* noted similar granules in the brains of normal adult B6 mice over the age of 6 mo, primarily in the hippocampus. The granules were positive for PAS, consistent with the earlier reports in B6 mice *(8)*, athymic nude mice *(4)*, and SAM-P mice *(9)*. The deposits in B6 mice apparently were also immunoreactive with a polyclonal antibody to Aβ, and a subpopulation of granules showed fluorescence after staining with thioflavin-S, suggesting the possibility of age-related Aβ deposition in this normal, inbred mouse strain. However, the positive staining for Aβ and other antigens with polyclonal antibodies could not be blocked by preincubation with the corresponding antigens, thus precluding definitive identification of the deposits. Moreover, ultrastructural analyses of the granules revealed fibrillar-like material with little morphological similarity to Aβ deposits, and additional immunohistochemical studies with monoclonal antibodies to Aβ were negative *(11,12)*. In conjunction with the Jucker et al. *(11)* report, Wirak and coworkers reported that the granules in their transgenic lines had been found subsequently in their control lines, a finding suggesting that the deposits were not the result of the Aβ transgene, but occur naturally in this hybrid strain with a B6 background. Thus, the question remained as to the etiology and significance of this unique, age-related deposition of fibrillar material in mouse brain.

The objective of this article is to provide an overview of findings regarding these granular deposits and to consider their potential relevance as a model of specific aspects of cerebral aging in humans. Only rare reports have been made of AD-like pathological features in the brains of aged rodents *(13,14)*. Although the granular deposits observed in mouse brain do not represent Aβ plaques, we are pursuing the hypothesis that the granules may mimic a precursor condition for Aβ deposition in humans. This possibility was sparked by our recent observation that the deposits are positive for monoclonal antibodies to heparan sulfate proteoglycan (HSPG) and laminin *(12)*. Extracellular matrix (ECM) molecules, in particular HSPG, coexist with Aβ in senile plaques found in AD brain *(15,16)*, and deposition of ECM molecules has been suggested to be a precondition for amyloidogenesis and to play an important role in the pathogenesis of AD *(17)*.

Another significant feature of the deposits in mouse brain is their possible relationship to corpora amylacea in humans *(18)*. Although important differences were noted *(19)*, the murine granules have some similarities with corpora amylacea, and thus, might also provide clues to the pathophysiological significance and development of corpora amylacea in humans.

Appearance and CNS Distribution of the Fibrillar Deposits

Antibody to LBP110 was found to be a reliable marker for the granules in brains of adult B6 mice (Fig. 1). The distribution of the granules was confined to specific brain regions *(12)*. Figure 1A depicts granules in hippocampus where they formed clusters (20–60 μm in diameter, each granule 1–5 μm in diameter). In B6 mice over 6 mo of age the granules occur primarily in the CA1/CA2 region, but granules also have been observed in all hippocampal subfields, including the molecular layer of the dentate gyrus. The only cortical regions where granules were observed consistently included entorhinal and piriform cortices, where they formed clusters similar to those observed in hippocampus. Granules were also found in cerebellum, but were more diffusely distributed among granule cells and Purkinje cells rather than aggregating in well-defined clusters. Only in mice of advanced age (>24 mo) were individual clusters occasionally noted in the striatum, diencephalon, amygdala, and brain stem.

Incidence of Fibrillar Deposits with Aging

The incidence of LBP110-positive granules in the hippocampus of B6 mice changes markedly with age (Figs. 1, 2). The clusters of granules could not be stained by anti-LBP110 until about 4–6 mo of age, but then their incidence increases dramatically up to 18 mo (Fig. 2). Although considerable interindividual variability was apparent among animals older than 18 mo, we have noted a decline in the

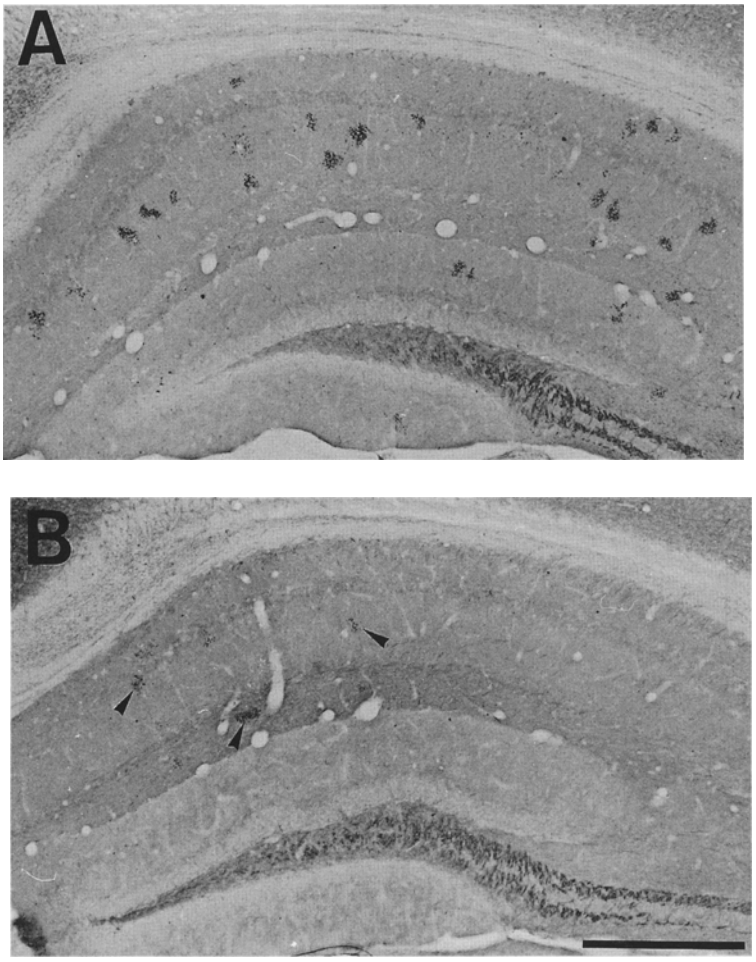

Fig. 1. Clusters of granules in coronal sections through the dorsal hippocampus of a male 18-mo-old B6 mouse **(A)** and a male 6-mo-old B6 mouse **(B)**. Clusters were visualized with an antibody to LBP110. Note the infrequent occurrence of clusters, each with only a few granules (arrowheads) in the 6-mo-old mouse. Bar = 400 μm.

mean number of clusters at 24 and 30 mo of age, i.e., near or beyond the median life-span of 26–27 mo estimated for this strain *(20)*. Nonetheless, every aged B6 mouse that we have examined has numerous LBP110-positive granules in hippocampus. Oversized granules were consistently noted only in mice 24 mo of age and older *(12)*.

Histochemical and Immunohistochemical Characterization

In addition to being positive for PAS, the granules also stained with Gomori's methenamine silver stain. Although some of the granules were fluorescent following staining with thioflavin-S, they were not positive for staining with Congo red. The granules were not stained with thionin, Cresyl violet, Luxol fast blue, Bodian's Protargol, hematoxylin and eosin (H & E), or acetylcholinesterase histochemistry.

Although the granules showed distinct staining with several polyclonal antibodies, including one to Aβ, the staining proved to be nonspecific *(11)*. More recently, immunohistochemical analyses using monoclonal antibodies (MAb) have been applied in an attempt to characterize the composition of the granules. To date, the most robust labeling of the granular material has been obtained with MAbs to HSPG core protein and to laminin B2 chain. Less distinctive staining was observed with MAbs to merosin, an A chain variant of laminin, and to chicken chondroitin sulfate proteoglycan. The granules did not stain with MAb to glial fibrillary acidic protein (GFAP), cell adhesion molecules L1 and

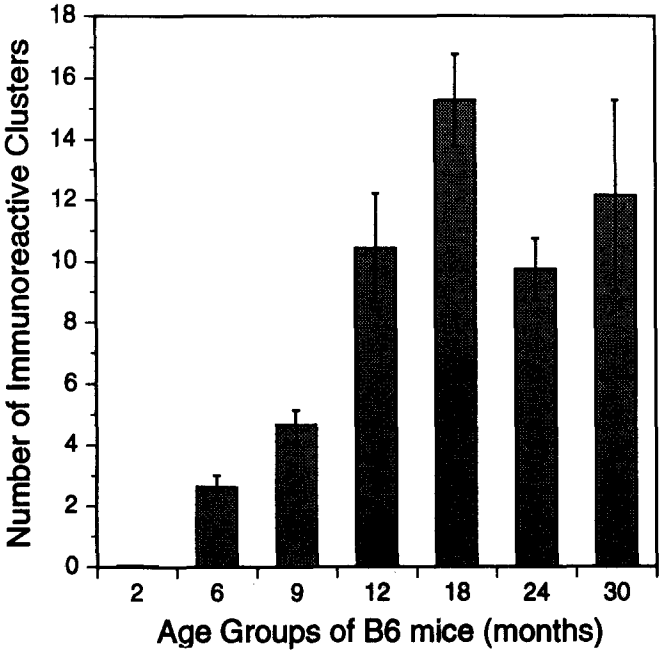

Fig. 2. Relative density of hippocampal clusters in male B6 mice of various ages. Indicated is the mean number (± SEM) of clusters per unilateral hippocampus in 25 μm-thick sections through the dorsal hippocampus (Adapted from ref. *12*).

NCAM, tenascin, janusin, L2/HNK-1 carbohydrate epitope, low-affinity NGF receptor, microtubule associated protein tau and MAP2, synaptophysin, or Aβ. A subpopulation of the granules stained with an MAb to high-mol-wt neurofilament epitopes (for antibody specifications *see* ref. *12*). The results of MAb analyses suggest that the granules are composed of HSPG- and laminin-like molecules, and confirm the polysaccharide nature of the granular material indicated by the histological stains.

Association with Astrocytes

By applying double-labeling techniques and confocal laser microscopy using antibodies to LBP110 and to GFAP, the association of the granules with astrocytes was established *(12)*. Individual clusters stained with PAS or visualized by anti-LBP110 often can be seen associated with the processes of a single astrocyte stained with anti-GFAP (Fig. 3). Extensive analyses of double-labeled brain slices has indicated that 60–80% of the clusters appear to be associated with glial elements, whereas others are apparently independent of GFAP-stained astrocytes. As further evidence for a glial association, granules were often found around blood vessels in a pattern suggestive of astrocytic endfeet.

Ultrastructural Analysis

Electron microscopic analysis of hippocampal slices from adult B6 mice (>6 mo) revealed the fibrillar material that comprises individual granules (Fig. 4A). In general, the fibrillar material resides in the neuropil, is surrounded by a discontinuous membrane, and generally lacks organelles. Bundles of glial filaments often appeared to pass around the fibrillar granules. The fibrillar material could be found within astrocytic somata occasionally, and thus, the granules have been referred to as astrocytic inclusions *(11)*.

Strain-Specific Incidence

We are conducting a broad survey of mouse strains to determine the genotypic specificity of the fibrillar deposits. The results shown in Table 1 reveal that the deposits are generally uncommon among inbred mouse strains, and their occurrence appears to be confined to only a few strains. However, it is important to note that the occurrence is not exclusively restricted to B6, SAM-P8, and AKR/J (founder line for SAM, ref. *22*), and to 129/Sv. Similar granules have been observed—although very rarely—in older mice from other strains with little relationship to the B6 and SAM line *(12)*. Moreover, the B6 and SAM strains appear to come from very different genetic backgrounds. Thus, certain strains are clearly more susceptible to the granules suggesting a genetic predisposition for deposition of the material.

Recent analysis revealed that 12-mo-old virgin female B6 mice have a significantly greater incidence of deposits in the hippocampus compared to virgin B6 males ($t[14] = 3.42$, $p < 0.01$). This result confirms the nonquantitative observation of a gender difference in previous studies *(8,12)*. Compared to B6, the SAM-P8 strain appears to have a greater incidence of clusters, and the deposits appear to emerge somewhat earlier in life *(9)*. After analyzing histological and immunohistochemical staining properties, astrocytic association, morphology, distribution, and ultrastructure (Fig. 4), we conclude that the structures in SAM-P8 and B6 mice are likely identical *(9,12,21)*. The SAM-R1 (senescence accelerated resistant) strain, which does not manifest the phenotypic pattern of aging observed in the P8 strain, exhibits none or very few fibrillar deposits at

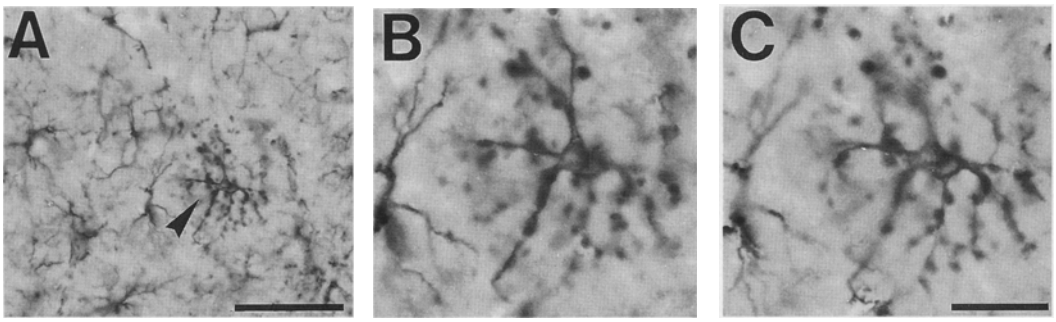

Fig. 3. Double-immunostained sections showing the relationship between an immunoreactive cluster visualized with anti-LBP110 and a GFAP immunoreactive astrocyte. **(A)** Low-magnification view; **(B, C)** High-magnification view at two different focal planes. Note the close association between individual granules and astrocytic processes. Bars = 50 μm (A), 20 μm (C).

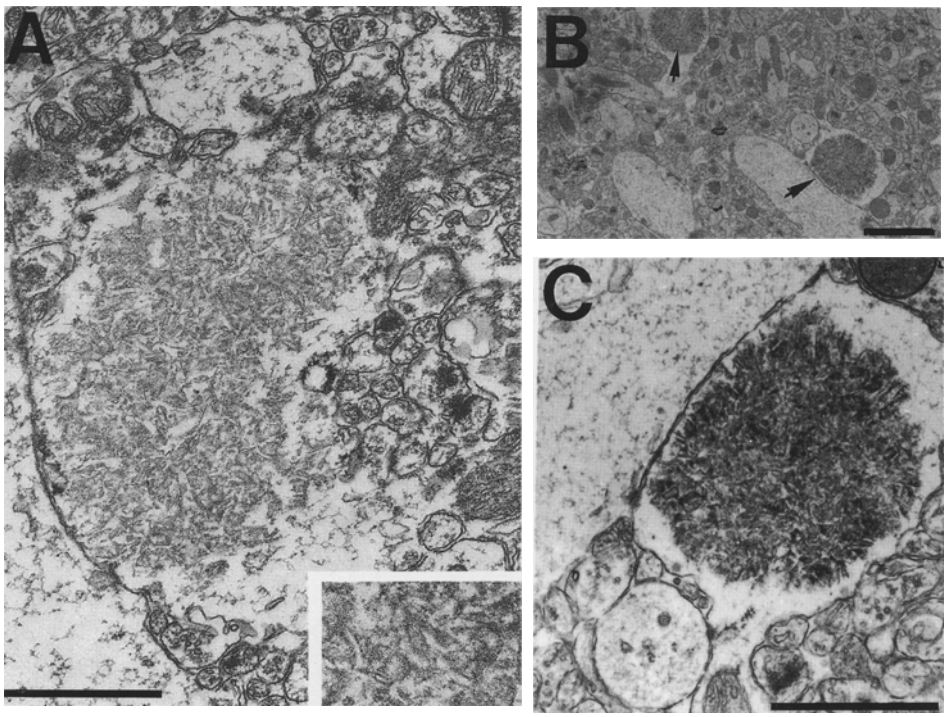

Fig. 4. **(A)** Electron micrograph of a fibrillar inclusion in the hippocampus of a 12-mo-old B6 mouse. The insert shows the ultrastructure of the fibrillar material at higher magnification. **(B)** Similar material has been identified in the hippocampus of a 12-mo-old SAM-P8. One of the two clusters (indicated by arrows) is shown at higher magnification in **(C)**. Differences in staining intensity of the fibrillar material are probably the result of methodological differences in the two laboratories in which the specimens were prepared. Bars = 1 μm (A, C) 2 μm (B).

least up to 9 mo of age, which is the oldest group we have examined to date.

Analysis of F1 hybrid strains revealed an apparent dominant heritability of the deposit incidence (Table 1). All three hybrids that we have examined so far, $B6D2F_1$, $B6C3F_1$, and $CB6F_1$ strains showed LBP110-positive granules in hippocampus although with variable incidence. Up to now we have examined only males in these hybrid strains. $B6D2F_1$ hybrid mice, like 129/Sv mice, are used frequently to develop transgenic mice. The awareness of the deposition of this material as a possible con-

Table 1
Incidence of Inclusions in Inbred and One Outbred (CRL:CFW(SW)BR) Mouse Strain[a]

Strain	Supplier	Sex	Age	Clusters in hippocampus
C57BL/6J	Jackson	M	7–9 mo	++
C57BL/6J	Jackson	F	7–9 mo	+++
C57BL/6J	GRC, NIA	M	2–30 mo	(*see* Fig. 2)
C57BL/6NCrlBR	Charles River	M	7–9 mo	+
C57BL/6NCrlBR	Charles River	F	7–9 mo	++
C57BL/6Zur	Univ. Zürich	M	10–12 mo	++
BALB/cJ	Jackson	M	7–9 mo	–
BALB/cJ	Jackson	F	7–9 mo	–
BALB/cAnNCrlBR	Charles River	M	7–9 mo	–
BALB/cAnNCrlBR	Charles River	F	7–9 mo	–
BALB/cNCrlNia2	Charles River	M	17 mo	–
C3H/HeJ	Jackson	M	7–9 mo	–[b]
C3H/HeJ	Jackson	F	7–9 mo	–[b]
C3H/HeNCrlBR	Charles River	M	7–9 mo	–[b]
C3H/HeNCrlBR	Charles River	F	7–9 mo	–[b]
DBA/2J	Jackson	M	10–12 mo	–
DBA/2NCrlNia2	Charles River	M	17 mo	–
CBA/J	Jackson	M	7 mo	–
CBA/NCrlNia2	Charles River	M	17 mo	–
SAMP8/Ta/Nia	GRC, NIA	M	9 mo	++++
SAMR1/Ta/Nia	GRC, NIA	M	9 mo	–[b]
AKR/J	Jackson	M	7 mo	+++
A/J	Jackson	M	7–17 mo	–
Crl:CFW(SW)BR	Charles River	M	17 mo	–
129Sv(ec)	Univ. Zürich	F	10 mo	++
B6D2F$_1$/CrlNia	Charles River	M	17 mo	+
B6C3F$_1$/CrlNia	Charles River	M	17 mo	+++
CB6F$_1$/CrlNia	Charles River	M	17 mo	+

[a]Extended version of Table 1 in ref. *12*. Some mice were retired breeders. M and F indicate male and female, respectively. Mean number of clusters in the hippocampus was determined and scored as follows: –, no clusters or <1 cluster/unilateral hippocampus in 8 coronal sections through the dorsal hippocampi; +, ++, +++, ++++ indicates increasing numbers. [b]Granules very rarely observed but might increase with aging.

founding variable in certain mouse strains might help to prevent future misdirections.

Interestingly we have recently observed similar deposits in a 5½-yr-old deer mouse *(Peromyscus)*, and thus, the fibrillar deposits have been identified in a second species.

Functional Significance

The pathophysiological significance of the granular material has yet to be established. If the granules occurred exclusively in the SAM-P8 line, then their incidence would have obvious significance. The SAM-P8 strain has a reduced maximal life-span, rarely surviving past 16 mo of age *(9)*, and exhibits impaired cognitive function compared to the SAM-R1 and B6 strains *(23)*. However, the granular deposits in the B6 strain and B6-derived hybrid strains are associated with no known functional abnormality. The life-span of the B6 and its hybrids is relatively long compared to that of other inbred strains *(24)*. Moreover, although the B6 strain exhibits age-related impairments in performance in some cognitive tasks *(23,25–28)*, its performance in other learning and memory tasks at advanced ages appears unimpaired *(29)* or relatively less impaired *(30)* compared to other strains.

We have attempted to relate the incidence of hippocampal clusters to cognitive performance in aged

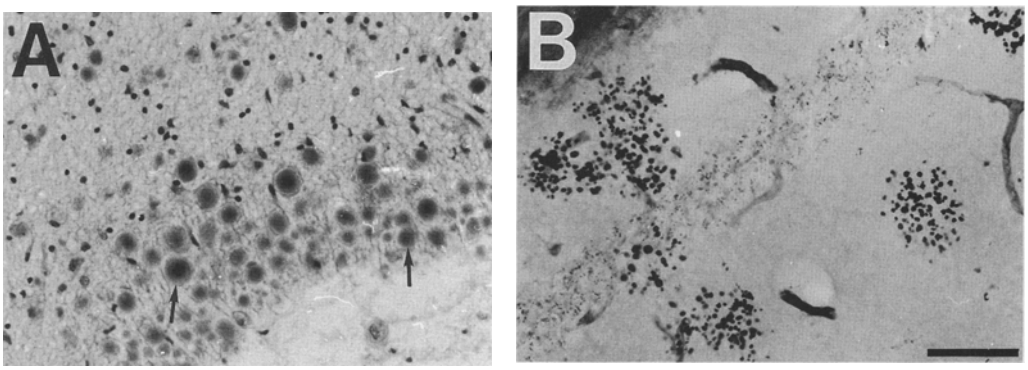

Fig. 5. Comparison of **(A)** corpora amylacea (two bodies are indicated by arrows) in hippocampus of a 90-yr-old human (H & E stain) and **(B)** clusters of granules in hippocampus of a 12-mo-old B6 mouse (Gomori's methenamine silver stain). Bar = 50 μm.

B6 mice *(12)*. In the Morris water maze task, which has proven to be highly sensitive to aging *(31)* and to hippocampal damage *(32)*, no significant correlation was found between performance and the number of deposits in hippocampus of 24-mo-old male B6 mice. Before we conclude that the depositions do not have any functional significance, several other experiments should be conducted. For example, we have not yet examined whether there is a relationship between incidence of granules and cognitive performance at younger ages, e.g., at 18 mo of age, the time-point with the highest incidence of the structures. Moreover, we have not examined the relationship of the clusters in cerebellum to motor performance in the B6 strain, which shows marked age-related decline in several motor tasks *(33)*.

Discussion and Future Directions

Our current understanding of the murine granular deposits can be summarized as follows:

1. The incidence of the granules is age-related;
2. They occur in specific brain regions, primarily hippocampus, in selected mouse strains, most prominently in B6 and SAM-P8 strains;
3. They appear to manifest dominant genetic heritability;
4. They have a strong association with astrocytes; and
5. They are composed of fibrillar-like material with antigenic similarities to ECM molecules, in particular HSPG and laminin-like molecules.

Interest in neuropathological changes in aged rodents is driven by the need to identify possible animal models of human cerebral degenerative diseases, in particular AD *(34,35)*. Recently, considerable effort has been devoted to producing transgenic lines that express various Aβ gene constructs. However, at the present time, none of the transgenic lines develops AD-like pathological features *(36)*. The fibrillar deposits in adult and aged B6 mice do not resemble classic amyloid fibrils, nor do they stain with monoclonal antibodies to Aβ; therefore, the deposits clearly are not a model of Aβ deposition *per se*. Nevertheless, the deposits in B6 mice might provide a useful model to study aspects of Aβ-plaque formation. Following the hypothesis of Snow and Wight *(17)*, we are pursuing the possibility that the granules may represent the focal deposition of ECM molecules that could function as an initial step in the pathogenesis of amyloidosis. HSPG and laminin have been immunolocalized to senile plaques *(15,16,37,38)*, and astrocytes in close proximity to the plaques have been suggested to be involved in the deposition of HSPG *(15)*. HSPG in particular, but also laminin, have significant binding affinities to the β-amyloid precursor protein and/or Aβ *(39–44)*. Therefore, one future direction of our research is to determine the potential of the murine fibrillar deposits to model aspects of plaque formation in AD, e.g., whether the mouse deposits are capable of sequestering Aβ delivered in vivo.

There are some intriguing similarities between the murine deposits and corpora amylacea in humans. However, there are also important differences, including ultrastructure, staining properties, size, and distribution *(19)*. For example, the murine granules appear in clusters in the parenchyma of the abovementioned brain areas, whereas corpora amylacea in the aged human brain are more widely distributed and occur most frequently in the subpial region (Fig. 5). Thus, there is presently not enough

evidence to consider the granules the murine equivalent of corpora amylacea in aged humans. Nevertheless the murine granules might help to clarify aspects of the development and the nature of corpora amylacea.

Given the strain-specific incidence of granules and the apparent dominant genetic heritability, a primary goal of future experiments will be to use genetic and molecular mapping procedures to identify the genetic contribution to the early and abundant accumulation of the fibrillar maternal in B6 mice and SAM. Another direction for future research is indicated by the observation that female B6 mice have a higher incidence of the deposits compared to males. Significant effects of steroid hormones on brain aging and age-related neuropathological changes have been reported *(5,45–47)*. Thus, the incidence of granules may be influenced by steroid hormones. Accordingly, sexual maturation might be the starting point of the accumulation of the fibrillar material, and the lower incidence of clusters in the last third of the life-span of B6 mice might be linked to age-related changes in the reproductive neuroendocrine axis.

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