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Agrin in Alzheimer's disease: Altered solubility and abnormal distribution within microvasculature and brain parenchyma

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ABSTRACT **Agrin is a heparan sulfate proteoglycan that is widely expressed in neurons and microvascular basal lamina in the rodent and avian central nervous system. Agrin induces the differentiation of nerve-muscle synapses, but its function in either normal or diseased brains is not known. Alzheimer's disease (AD) is characterized by loss of synapses, changes in microvascular architecture, and formation of neurofibrillary tangles and senile plaques. Here we have asked whether AD causes changes in the distribution and biochemical properties of agrin. Immunostaining of normal, aged human central nervous system revealed that agrin is expressed in neurons in multiple brain areas. Robust agrin immunoreactivity was observed uniformly in the microvascular basal lamina. In AD brains, agrin is highly concentrated in both diffuse and neuritic plaques as well as neurofibrillary tangles; neuronal expression of agrin also was observed. Furthermore, patients with AD had microvascular alterations characterized by thinning and fragmentation of the basal lamina. Detergent extraction and Western blotting showed that virtually all the agrin in normal brain is soluble in 1% SDS. In contrast, a large fraction of the agrin in AD brains is insoluble under these conditions, suggesting that it is tightly associated with β-amyloid. Together, these data indicate that the agrin abnormalities observed in AD are closely linked to β-amyloid deposition. These observations suggest that altered agrin expression in the microvasculature and the brain parenchyma contribute to the pathogenesis of AD.**

Agrin is a large, multidomain heparan sulfate proteoglycan (HSPG) (1) originally discovered as a synapse-organizing molecule at the neuromuscular junction, where it is highly concentrated in the synaptic basal lamina. Agrin's best understood biological activity is to induce the aggregation of acetylcholine receptors and other postsynaptic elements on the myotube surface (2–4). Agrin also is necessary for the differentiation of the presynaptic apparatus (5). More recently, agrin has been shown to be widely expressed in the rodent and avian central nervous system (1, 6, 7), with its highest expression observed during peak periods of synaptogenesis (8). It is localized within neurons (9) as well as in the basal lamina of the microvasculature (10) and in the developing optic tract (7, 11). Agrin levels are transiently increased after seizure activity (12), and exogenous agrin induces cAMP response element-binding protein phosphorylation in cultured neurons (9). Together these observations suggest that agrin may play an important role in the basement membrane of the microvasculature as well as in synaptic plasticity.

Alzheimer's disease (AD) is a neurodegenerative disorder characterized clinically by progressive dementia and memory loss (13). Pathologically, the disease is associated with neuronal and synaptic loss (14–16), as well as the appearance of β-amyloid-containing diffuse and neuritic plaques (senile plaques), neurofibrillary tangles within neurons, and cerebral amyloid angiopathy (13). Many studies have shown that HSPGs are localized within these characteristic lesions of Alzheimer's disease (17, 18). Moreover, the amyloid precursor protein (APP) binds heparan sulfate, suggesting that the interaction of APP with HSPG in the extracellular matrix may stimulate the effects of APP on neurite outgrowth. It has been suggested that APP–proteoglycan interactions may disturb normal APP function and contribute to the neuritic outgrowth surrounding the cores of senile plaques (19, 20). HSPG levels are elevated in AD brains (21).

AD thus is marked by synaptic loss, and many of its pathologic features are associated with HSPG function (21, 22). Further, those brain regions containing some of the highest levels of agrin, e.g., hippocampus and amygdala, are characterized by lifelong synaptic plasticity (23) and are affected severely in AD (24). Unlike other basal lamina elements that have been implicated in the pathogenesis of AD, such as laminin (19, 25) and perlecan (26–28), agrin is widely expressed in neurons, suggesting that it has a role in the brain parenchyma. Therefore, we asked whether alterations in the distribution and biochemical properties of agrin may be linked to AD.

MATERIALS AND METHODS

Human Tissues. All brains (AD, $n = 16$, mean age = 76 ± 6 years, eight male, eight female; normal aged, $n = 11$, mean age = 65 ± 7 years, five male, six female) were obtained at autopsy (postmortem interval, 2–24 hr). Fourteen brain areas were sampled routinely in our protocol. These representative areas were selected carefully on the basis of their suitability for diagnosing AD, as well as the other currently classified neurodegenerative diseases. The diagnosis of AD was made in accordance with widely accepted National Institute on Aging criteria (29) as well as the Braak and Braak neuropathological staging of Alzheimer-related changes (24). Control brains were obtained from aged hospital patients with no history of neurological disease and no pathologic evidence of AD or other degenerative brain diseases.

Samples of prefrontal cortex (Broadmann A10), primary visual cortex (A17), visual inferotemporal area (A20), amyg-

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Abbreviations: AD, Alzheimer's disease; APP, amyloid precursor protein; HSPG, heparan sulfate proteoglycan; BBB, blood–brain barrier.

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dala, and hippocampus were fixed by immersion in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 24 hr and cryoprotected with 30% sucrose in 0.1 M phosphate buffer (pH 7.4). Samples then were snap-frozen in liquid nitrogen and stored at −70°C until they were used. The sample of normal muscle used for agrin control studies was biopsied from a 17-month-old female infant with a clinical history of "delayed motor milestone development" and immediately snap-frozen.

Immunohistochemistry. An antihuman agrin antisera (F606051) was raised in rabbits by immunization with a recombinant C-terminal 50-kDa fragment of agrin 4,8 produced in *Pichia pastoris* comprising the G2 and G3 domains (30). Frozen tissue samples were embedded in OCT compound and sectioned at a thickness of 8 μm and mounted on glass slides. Muscle sections were fixed in 95% ethanol. The tissue was pretreated with 3% hydrogen peroxide and 10% normal goat serum. A group of slides also was pretreated with 2 units/ml of Heparanase III (Sigma) for 2 hr at 37°C before undergoing the standard pretreatment described above. The serum was used at a dilution of 1:500 in 50 mM Tris, pH 7.4/150 mM NaCl/0.2% Triton X-100/3% BSA. After incubation in primary antibody for 24–48 hr, tissue sections were washed and stained with either a modified ABC technique using the Vectastain Elite ABC Peroxidase system (Vector Laboratories) with diaminobenzidine as the chromagen or a goat anti-rabbit Ig conjugated to CY3 (Jackson ImmunoResearch). To eliminate autofluorescence from tissue lipofuscin, sections were posttreated with 0.3% Sudan Black B in 70% ethanol for 10 min followed by washing in distilled water.

Agrin Protein Analysis. For analysis of total agrin levels, frozen cerebral cortical gray matter was stripped of the meninges and 0.4-g samples were homogenized in 1× PBS on ice by using a bench-top homogenizer (Contorque, Eberbach, Ann Arbor, MI). To quantitatively solubilize the samples, they were resuspended in 100 μl of 0.2 M NaOH and heated for 15 min at 37°C (31). Samples then were neutralized with equivalent amounts of HCl to bring sample pH to 7.0, heated for 10 min at 60°C in reducing SDS sample buffer (Pierce), separated by SDS/PAGE (4.5–15% gel), and transferred to nitrocellulose. Blots were blocked with 1% BSA in minimal essential medium/Hepes, rinsed with 1× PBS/0.1% Tween, and incubated overnight at 4°C with antihuman agrin antiserum (F606051; 1:3,000), followed by alkaline phosphatase-conjugated goat anti-rabbit Ig (1:8,000; Boehringer Mannheim).

For analysis of detergent solubility of agrin tissue, 0.4 g was homogenized in 1× PBS/1% SDS on ice by using a bench-top homogenizer and spun for 30 min at 10,000 × *g* at 4°C. Pellets were solubilized in 100 μl of 0.2 M NaOH and analyzed as described above.

RESULTS

Localization of Agrin in Normal Aged and AD Brain. We first examined the distribution of agrin in normal aged human brain. Agrin immunoreactivity uniformly invested the walls and outer surfaces of all microvessels, consistent with previous studies demonstrating its presence in basal laminae (10) (Fig. 1*A*). Agrin staining also was present within the cytoplasm of selected neurons in all brain areas examined, including the neocortex (A10, A17, A20), amygdala, and hippocampus (Fig. 1 *A* and *B*). Among the areas studied, agrin-immunoreactive neurons were most abundant in the amygdala and hippocampus (not shown).

The distribution of agrin immunoreactivity in AD brains was markedly different than that exhibited in control brains. Although staining of microvessels was evident in all AD brains, close examination of the capillaries revealed ragged and irregular outer walls and attenuated diameters when compared with controls (Fig. 1 *C* and *F*). Agrin immunoreactivity was concentrated within both diffuse and neuritic plaques in all AD samples (Fig. 1 *C*, *D*, and *F–H*). There was intense agrin immunoreactivity within the amyloid core, surrounded by a halo composed of punctate immunoreactivity. Puncta often were associated with microvessels embedded in plaques, suggesting that these abnormal agrin deposits may arise from damaged basal lamina (Fig. 1*D*). They were less commonly observed in the neuropil adjacent to microvessels in aged control cases (Fig. 1*B*). Agrin immunoreactivity was seen in association with some neurofibrillary tangles (Fig. 1*H*), which may account in part for the increased neuronal agrin immunoreactivity that was observed in AD cases. Agrin immunostaining also was observed in the reactive gemistocytic astrocytes of AD cases (Fig. 1*G*), but was not detected in astrocytes in aged control brains. Of the regions examined, overall agrin immunoreactivity was, by far, the highest in those areas exhibiting the most robust synaptic plasticity, the amygdala and hippocampus. This finding is consistent with the fact that AD affects these areas earliest and most severely (24).

Because agrin itself is a large HSPG and is likely to be associated with other proteoglycans, it is possible that the protein core of agrin could be masked by heparan sulfate side chains. Pretreatment of brain samples with Heparanase III resulted in a subtle increase in staining intensity; however, no additional immunoreactive structures were revealed.

We performed two experiments to confirm the specificity of the anti-agrin immunoreactivity. First, we used the antibody to immunostain human infant muscle. In agreement with results in immature rat and avian muscle (8, 32), agrin immunoreactivity was concentrated in the basement membranes of individual myofibers (Fig. 1*E*). Second, staining in both muscle and in human brain was abolished when primary or secondary antibodies were omitted from the staining procedure (not shown) or when the primary antibody was preabsorbed with 10^{-6} M agrin protein for 24 hr (Fig. 1 *C* and *E Insets*).

Agrin Protein Analysis. We next characterized the size and solubility properties of the agrin protein expressed in normal aged and AD brains. In Western blots of homogenates from prefrontal cortex (A10), antiagrin antibodies labeled a polypeptide with a molecular mass of approximately 500 kDa in both control (Fig. 2*A*, lanes 1–4) and AD brains (Fig. 2*A*, lanes 5–8). No additional immunoreactive bands were observed in AD brains (not shown). This mobility is similar to that observed for agrin isolated from avian and rodent brain (1, 6). This polypeptide was not detected when an irrelevant antiserum was used in the first layer (Fig. 2, lanes 4* and 8*). In an effort to determine whether the overall levels of agrin present in control vs. AD brains were comparable, we analyzed homogenates from eight patients. Although there was some variability of levels among the samples, we found that overall the quantity of agrin was similar in both groups.

A hallmark of β-amyloid-containing structures in AD brains is their very limited solubility (31, 33). The solubility properties of agrin were strikingly different in control and AD brains. Virtually all of the agrin from normal brains was solubilized efficiently in 1% SDS at neutral pH (Fig. 2*B*, lanes 1–4). However, a significant fraction (≈50%, not shown) of the agrin from AD brains was refractory to this treatment and could be solubilized only in 0.2 M NaOH (Fig. 2*B*, lanes 5–8). These results are in agreement with our immunohistochemical findings that agrin is present in senile plaques. Further, these comparable solubility properties raise the possibility that agrin may be an integral component of β-amyloid-containing structures in AD brain.

DISCUSSION

We have demonstrated the widespread presence of agrin in the human central nervous system and identified a clear association between the distribution of this protein and the pattern of

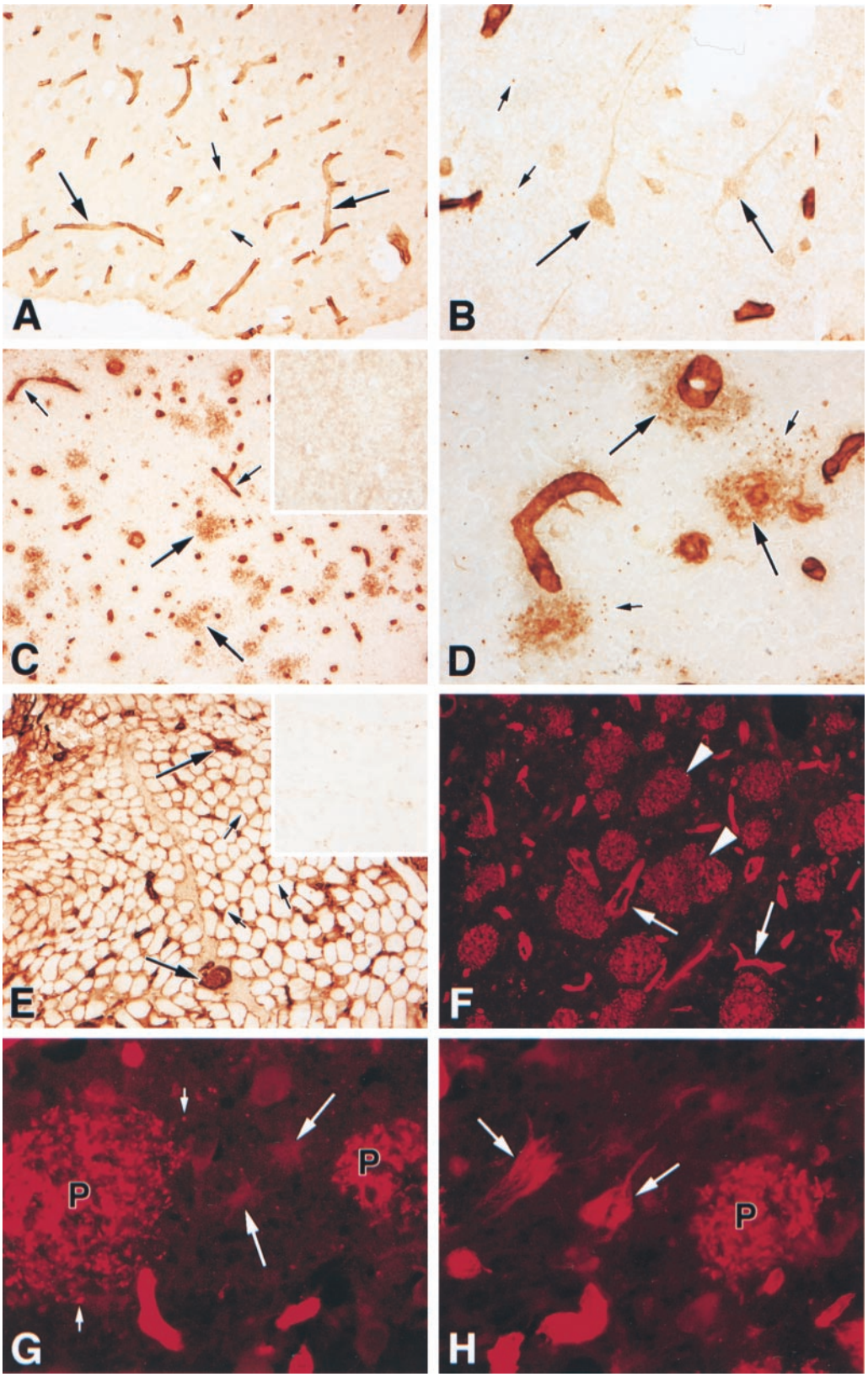

FIG. 1. Localization of agrin in aged normal and AD brain. Sections were immunohistochemically stained with either diaminobenzidine-labeled tertiary antibody (*A–E*) or immunofluorescence-stained with CY3-labeled secondary antibody (*F–H*) as described in *Materials and Methods*. (*A*) Aged control brain section labeled with antiagrin antibody. Agrin immunoreactivity is prominent within the cerebral microvasculature (large arrows) and also is evident in selected neurons (small arrows). Prefrontal cortex, A10, ×200. (*B*) A higher magnification of the control brain section in *A*. Agrin immunoreactivity is evident within the cytoplasm of the neuronal soma and processes (large arrows). Occasional neurons also demonstrate staining of the nucleus. Note the presence of rare, agrin-immunoreactive puncta (small arrows) in the neuropil, which often are adjacent to blood vessels. (A10, ×600.) (*C*) Prefrontal cortex (A10) of a patient with AD immunostained with anti-agrin antibody. Note the robust staining of neuritic and diffuse plaques (large arrows) and blood vessels. In contrast to aged control cases (e.g., *A*), blood vessels in AD had attenuated diameters and a more ragged profile (small arrows). No immunoreactivity was observed if the antisera was preabsorbed with 10^{-6} M agrin protein (*Inset*). (×200.)

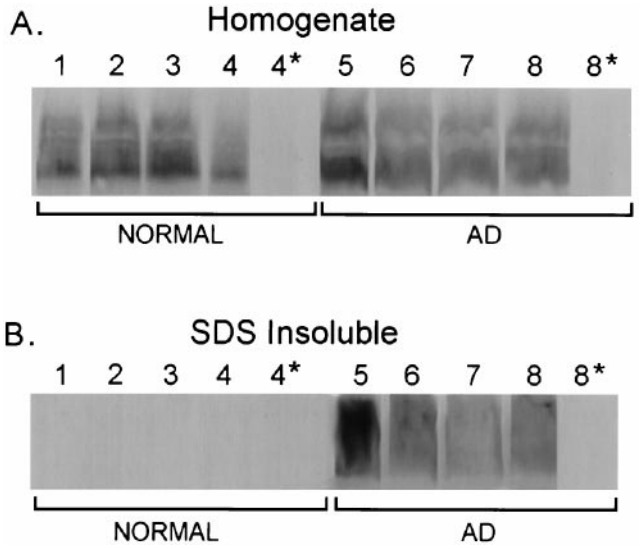

FIG. 2. Western blot analysis of agrin expression in normal aged and AD) brain. Equal volumes of total homogenates or of the SDS-insoluble fractions from normal (1–4) or AD brain (5–8) prefrontal cortex (area A10) were probed with anti-agrin antisera. All samples were solubilized in 0.2 M NaOH before electrophoresis (see *Materials and Methods*). (*A*) The anti-agrin antibody recognized a polypeptide with an apparent mobility of ≈500 kDa in both normal and AD brains. No polypeptide was detected when normal rabbit IgG was substituted for the first layer (lanes 4* and 8*). (*B*) Virtually all of the agrin from normal brains was efficiently solubilized in 1% SDS at neutral pH (lanes 1–4). However, a portion (see *Results*) of the agrin from AD brains was insoluble under these conditions (lanes 5–8). No polypeptide was detected when normal rabbit IgG was substituted for the first layer (lanes 4* and 8*).

neurodegenerative lesions characteristic of AD. We also have shown that AD brains contain a significant pool of detergent-insoluble agrin. These observations are meaningful, both in the context of discerning the function of brain agrin and in determining the mechanisms of AD pathogenesis.

Several lines of evidence indicate that the antiserum used here is specific for agrin, and that our results are not a result of the recognition of a cross-reactive protein. The characteristics of agrin in normal human brain are similar to those that have been described in other species. The expression of agrin within neurons and in the microvasculature has been reported in rodent and chicken (3, 6). Further, the immunoreactivity we observed in human muscle is similar to that reported in other species. Human agrin, like its rodent and chicken counterparts, migrates as a polydisperse, ≈500-kDa band on a SDS-polyacrylamide gel. Further, all immunoreactivity is abolished if the antiserum is preabsorbed with purified agrin. As discussed below, agrin from AD brain has altered distribution and solubility properties, but shows a similar mobility on SDS gels. Moreover, no additional immunoreactive polypeptides are observed when blots of AD brain are analyzed. Together, these data provide strong support for the conclusion that agrin is a component of senile plaques in AD brain.

Abnormalities in synaptic structure and function are the earliest manifestations of AD pathology (14–16). Numerous studies have demonstrated alterations in the distribution of synaptic elements, including neurotransmitters, vesicles, and receptors (34–36). These changes precede both the deposition of extracellular fibrillary β-amyloid and the accumulation of APP within neurites, astrocytes, and microglia. Alzheimer's pathology is most severe in those brain areas exhibiting the highest degree of synaptic plasticity, i.e., the entorhinal region and limbic cortex (38). Further, adaptive growth responses may accompany degenerative events in diseased hippocampus.

Our findings demonstrate striking parallels between AD pathology and agrin expression and function. For example, we observed the highest levels of neuronal agrin expression in the amygdala and hippocampus in both aged normal and AD brains. Moreover, several studies have implicated a role for agrin in hippocampal synaptic plasticity (9, 39). The increased agrin immunoreactivity that was observed in the AD hippocampus could reflect the compensatory increase in plasticity. Agrin is essential for presynaptic differentiation at nerve–muscle synapses (5) and also has been suggested to play a role in axonal outgrowth in the central nervous system (7). Therefore, agrin could function in the neuritic-sprouting response characteristic of AD (40).

Our immunohistochemical results show that agrin is an integral component of senile plaques in AD. Several lines of evidence suggest that this association may reflect an interaction with β-amyloid. The very limited solubility of agrin in AD brains suggests that it may be bound tightly to fibrillar β-amyloid, which shows similar solubility properties (31, 33). This interaction may be through the heparan sulfate side chains of agrin. Such glycosaminoglycans have been shown to bind fibrillar but not nonfibrillar β-amyloid with high affinity (41, 42). Further, this binding inhibits degradation of fibrillar β-amyloid, suggesting that agrin also could be involved in plaque formation (41). However, it should be noted that the current results do not provide direct evidence for an association of agrin with fibrillar β-amyloid. It is also possible that the insolubility of agrin in AD brains occurs independently of a β-amyloid association. Future experiments will be needed to resolve this question, as well as to determine whether alterations in the distribution and solubility of agrin contribute to the synaptic loss in AD.

Agrin is a component of the microvascular network of both AD and non-AD brains, suggesting that it may be involved in blood–brain barrier (BBB) formation and/or function (10). Abnormalities were seen in the blood vessels of AD brains, consistent with the findings in other studies (43, 44). In particular, senile plaques, with positive agrin staining, were seen frequently in the vicinity of capillaries. These observations led to the hypothesis that breakdown of the BBB may be a necessary step in plaque formation. An intriguing finding in our study was the halo of punctate agrin immunoreactivity encircling the cores of senile plaques. The possibility that these puncta arise from the basal lamina of diseased blood vessels is suggested by their presence in close proximity to capillaries in normal aged controls. It is tempting to speculate that such

(*D*) A higher magnification of AD brain illustrating two neuritic plaques with surrounding puncta of agrin immunoreactivity (large arrows). Circumferential puncta of immunoreactivity also can be seen in plaques surrounding and adjacent to cerebral capillaries (small arrows). (A10, ×600.) (*E*) Normal infant skeletal muscle labeled with anti-agrin antibody. Note the uniform agrin immunoreactivity of the basement membranes surrounding individual muscle fibers (small arrows) and capillaries (large arrows). (*Inset*) The same skeletal muscle after the primary antibody was preabsorbed with 10^{-6} M agrin protein. There is essentially complete abolishment of agrin immunoreactivity. (Quadriceps muscle, ×200.) (*F*) Amygdala of a patient with AD labeled with anti-agrin antibody. Note the robust staining of neuritic and diffuse plaques (arrowheads) and blood vessels. Blood vessels in this AD case have attenuated diameters and ragged profiles (arrows). (×200.) (*G*) A higher magnification of the AD amygdala seen in *F* illustrating two neuritic plaques (P) with surrounding puncta of agrin immunoreactivity (small arrows). Agrin immunoreactivity also may be seen in reactive gemistocytic astrocytes and their stellate processes (large arrows). (×600.) (*H*) Another high-magnification photomicrograph of the AD amygdala seen in *F* showing agrin immunoreactivity within two neurofibrillary tangles (arrows). Note the fine wisps of paired helical filaments conforming to the shape of the neurons they are within. An agrin-stained neuritic plaque (P) also is present. (×600.)

agrin deposits could initiate AD pathology, perhaps by altering synaptic plasticity or promoting the aggregation of β-amyloid.

Other studies have shown that extracellular matrix elements, including HSPGs, are concentrated within pathologic lesions in AD (17, 18, 21, 25, 45). The HSPGs include perlecan, as well as an unidentified molecule(s) recognized by an anti-HSPG antibody (46). The HSPGs are of particular interest in view of the interactions between heparan sulfate side chains and β-amyloid noted above. Moreover, perlecan has been shown to accelerate fibrillar β-amyloid deposition when infused into adult rat brain (26, 28). However, although perlecan is a component of senile plaques and the microvasculature, it has not been observed in abundance in the normal brain parenchyma. On the other hand, agrin is expressed in normal neurons and is known to be important for synaptic formation and, possibly, plasticity in the periphery (8, 23). Together, these observations suggest that agrin is likely to play a distinctive role both in the early and later stages of Alzheimer's pathogenesis.

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