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Neurobiology of Aging xxx (2004) xxx–xxx

NEUROBIOLOGY OF AGING

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Reversible age impairments in neurite outgrowth by manipulations of astrocytic GFAP

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Received 17 March 2004; received in revised form 26 May 2004; accepted 16 June 2004

Abstract

Aging is associated with neuron atrophy and impaired sprouting after lesions. In contrast during normal aging without neurodegenerative diseases, astrocytes display increasing activation, with progressive increases of glial fibrillary acidic protein (GFAP) beginning before midlife. Because many neuronal functions depend on astrocytic support, we developed a heterochronic co-culture system to study influences of aging astrocytes on neurons. Neurite outgrowth by embryonic neurons (E18) was markedly less when co-cultured with confluent astrocytes derived from old (24 mo) versus young (3 mo) cortex. These impairments were reversible. Diminishing the GFAP levels of old astrocytes by RNAi restored neurite outgrowth, whereas overexpression of GFAP in young astrocytes modeled these effects of aging by reducing neurite outgrowth. Quantitative relationships were found such that neurites were co-localized with high intensity laminin, which both varied inversely with GFAP. These results implicate increased astrocytic GFAP expression as a proximal cause of neuron atrophy during normal aging.

Keywords: Aging; Astrocytes; GFAP; Neuronal sprouting; Laminin

1. Introduction

The role of normal age changes in astrocytes on age changes in neurite outgrowth and neuronal plasticity has not been considered. During normal aging in the absence of neurodegenerative diseases or experimental lesions, synaptic atrophy begins soon after maturation in some brain regions [1,7,15,21,24,36]. Moreover, the aging brain supports less neuronal sprouting in response to experimental lesions [8,32,33,35]. A potentially related phenomenon is the smaller induction of long term potentiation (LTP) in aging rodent brains, which is considered to indicate impairments in synaptic plasticity [1,27]. These impairments could be due to putative intrinsic neuronal aging, such as the accumulation of mitochondrial DNA deletions. Alternatively, neuronal dysfunctions could reflect factors extrinsic to neurons in the local cellular environment. Consistent with this suggestion, grafted fetal neurons show less outgrowth in aging brains in two models, locus ceruleus grafts into the hippocampus [13] and dopaminergic neurons into the striatum [5].

In particular, we hypothesize that astrocytic aging changes contribute to impaired neuronal outgrowth. Concurrently with the slowly progressing neuronal changes, astrocytes in the cerebral cortex and most brain regions become hyperactive during aging. The best validated astrocytic aging marker is glial fibrillary acidic protein (GFAP), an astrocyte-specific intermediate filament. Beginning soon after maturation GFAP expression increases progressively during aging as assayed per cell by GFAP transcription and GFAP cell content in humans and rodent models [8,25,26]. Because GFAP is also widely used as a marker of neurodegeneration, it is pertinent that the early-onset age changes in astrocytes arise without provocation by injury or neurodegenerative disease.

In vivo studies can not easily resolve the primacy of neuronal atrophy or astrocytic hyperactivity in brain cell pathways of aging, because neurons and astrocytes interact at

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doi:10.1016/j.neurobiolaging.2004.06.009

many levels. GFAP expression, for example, is highly sensitive to neuronal impairments as shown by astrocytic hypertrophy after local neuron death and by rapid induction of GFAP-containing astrocytic processes after pharmacological blockade of neuronal impulses [4]. Reciprocally, lesion-induced astrocytic hyperactivity is implicated in impaired neuronal outgrowth, through the formation of GFAP-rich glial scars. Neuronal outgrowth is impaired at sites of astrocytic hyperactivity, e.g. after spinal cord lesions [22] or grafts of neurons into the retina [17]. Glial scars may inhibit neurite outgrowth by forming physical or diffusion barriers, or by altering the production of neurotrophic factors or inhibitory factors [2,22]. Other feed-forward models can be considered, in which deficits of neurons activate glial cells, which in turn accelerate further neuronal impairments.

As an approach to resolving the circularity of feed-forward pathways in aging, we developed a heterochronic culture model in which embryonic neurons (E18) were co-cultured with primary astrocytes derived from young (3 mo) or old (24 mo) brain donors. Age increases in GFAP transcription is a robust phenotype that persists in primary monotypic culture in the absence of interactions with microglia or neurons [28]. Note that "aging" in this primary culture model does not refer to the Hayflick phenomenon of replicative senescence, which is an outcome of serial culture. This co-culture model allows study of astrocyte aging apart from effects of microglia, which also become activated during normal aging [8,25]. In "wounding-in-a-dish" model with neonatal astrocytes and E18 neurons, the secretion of extracellular laminin and neuronal outgrowth were minimal. Antisense-GFAP markedly increased laminin secretion and neuronal outgrowth, although decreases in GFAP were modest [6,18]. More robust effects on GFAP were obtained by lowering GFAP through estradiol, which decreases GFAP transcription and protein, and enhanced laminin reorganization and sprouting [29]. These findings implicate elevated GFAP in attenuating astrocytic production of laminin and other factors that support neurite outgrowth independently in lesioned co-cultures.

We now extend these findings to unlesioned co-cultures without initiation of glial scarring. GFAP was bidirectionally manipulated in these co-cultures, either lowered by small interfering RNA (GFAP-RNAi) in old co-cultures, or increased by transfection of GFAP-cDNA in young co-cultures. Vimentin was also manipulated by SiRNA to evaluate the specificity of GFAP effects and because of evidence that GFAP-vimentin double knockouts support sprouting better than GFAP knockouts alone [17].

2. Methods

2.1. Cell culture

Primary cultures of astrocytes were originated from cerebral cortex of young (3 mo) and old (24 mo) male F344 rats (19) and plated at 200,000 cells/cm^2 in DMEM/F12 medium (Cellgro, Herndon, VA) with 10% of FBS (Life Technologies Inc., Rockville, MD), 100 u/ml penicillin, and 50 u/ml streptomycin (Sigma, St. Louis, MO) at 37 °C/5% CO_2. Media were refreshed every 3 d until confluence by day 30, when microglia and oligodendrocytes were removed by shaking [28]. Because most prior studies of primary glial cultures are derived from neonates, we characterized the number of microglia. In test cultures: using OX-42 as a marker, there was no difference in percentage of microglia after shaking: 11.9 ± 1.2% (young) and 10.8 ± 1.0% (old); not significant. Although this level of microglia is several-fold higher than in neonatal derived cultures **[**29]**),** the numbers of astrocytes are probably underestimated because of their high density. For astrocyte–neuron co-cultures, neurons from fetal (E18) cerebral cortex were seeded onto confluent monolayer of astrocytes at 1:3 ratio (neuron:astrocyte).

Neuron cell numbers were not influenced by astrocyte age. NeuN immunostaining of neuronal nuclei showed slightly lower neuron density in old versus young-derived astrocyte co-cultures: 85 ± 11% (day 2) and 88 ± 15% (day 5), old, relative to young co-cultures (mean ± S.E.M., two independent test cultures, 6–8 images each; not significant).

2.2. Transfection of RNAi and cDNA

Astrocytes were plated onto poly-D-lysine coated four chamber slides (Nalge Nunc International, IL) at 200,000 cells/chamber. The next day, cells were transfected with RNAi (30 nM) using SiPORT™ (Ambion, Austin, TX). RNAi to rat GFAP (GI:8393430) and rat vimentin (GI:14389298) were synthesized by Silencer™ (Ambion, Austin, TX):

GFAP RNAi-1115 (RNAi): AAAACCGCATCACCATTCCTG;
GFAP mutRNAi-1115 (mutRNAi): AAAACCGACTCACCATTCCTG;
GFAP RNAi-461: AAGATGAAACCAACCTGAGGC;
GFAP mutRNAi-461: AAGATGACCAAAACCTGAGGC;
Vimentin RNAi-1048: AATTTTGCCCTTGAAGCTGCT;

Numbering: nucleotide position from start codon. These experiments used RNAi-1115; RNAi-461 gave similar results.

For GFAP overexpression, cells were transfected with pcDNA-huGFAP-wt (kindly provided by Dr. Michael Brenner) using Effectene™ (Qiagen, Valencia, CA). The cells transfected with pcDNA3.1 without insert were used as control.

For studies on neurite outgrowth in the absence of lesion, E18 neurons were plated on confluent monolayer of young or old astrocytes (N:A ratio = 1:3). Neurite outgrowth and neuron numbers were analyzed at 1, 2 and 5 days in co-culture. For RNAi or cDNA treatment, astrocytes were transfected with RNAi or GFAP cDNA (see above), and 2 days later E18 neurons were plated at the same ratio. For "wounding-in-a-

dish", co-cultures were scratch-wounded after two days in co-culture [29]. Forty-eight hours after wounding, co-cultures were fixed and examined by immunochemistry. E2 treatment was started at the time of wounding. In "wounding-in-a-dish", E18 neurons were plated into RNAi treated astrocytes of both ages.

2.3. Immunocytochemistry and image analysis

Number of microglial cell in aged cultures was estimated using microglial marker MRC OX-42 (CR3 complement receptor), (1:100; Serotec, Oxford, UK).

Astrocyte–neuron co-cultures were double immunostained with polyclonal rabbit anti-GFAP (1:500, DAKO Corp., Carpinteria, CA) or with polyclonal rabbit anti-laminin-1 (1:25, Sigma) and monoclonal anti-MAP-5 (1:100, Sigma); fluorescent secondary antibodies, Alexa Fluor 488 goat anti-mouse (green), for MAP 5 and Alexa Fluor 594 goat anti-rabbit (red) for GFAP or laminin-1, both 1:400 (Molecular Probes, Eugene, OR). Laminin immunoreactivity was analyzed as total cell-associated laminin signal by fixing with methanol (5 min, −20 °C) before immunostaining. Neuron number was estimated by immunostaining for NeuN (monoclonal antibodies, 1:100; Chemicon International, Temecula, CA). Immunopositive cells were counted in 6–8 images per chamber. Neurite outgrowth (without wounding) was measured as the area covered by MAP-5 immunopositive neurites, defined with a modified Sobel edge detection algorithm (IPLab, Scanalytics). Captured micrographs of stained culture (double immunostained for GFAP and MAP-5) were digitized to separate the two fluorescent channels. Area covered by MAP-5 immunopositive neurites was calculated using the following strategy: we developed a semi-automated program using IPLab imaging software to define the edge profile of the neurites, exclusive of cell bodies. This filtered image was further processed (skeletonized) to generate a single pixel line. From the one pixel width line, the total neurite length was calculated.

Neuronal sprouting 2 d after lesion was expressed as number and length of MAP-5 immunopositive neurites extending into the wound zone, measured in eight random areas (100 mm diameter) per chamber [29].

2.4. Co-localization analysis

Laminin (red) and MAP-5 (green) were extracted from double-immunostaining images (1560 × 1080 pixels) (Fig. 4). The area covered by MAP-5 positive neurites was defined by the Sobel edge detection algorithm. Red laminin staining was stratified into three levels of signal intensity: low, intermediate, and high. Using the binarized MAP-5 and the laminin images, a co-localization image was generated and quantified. The co-localization score was defined as percentage of laminin-positive area (of each signal intensity) that is covered by MAP-5-positive neurites (see details in Fig. 4 legend).

2.5. Western blotting

Cellular proteins were obtained by lysing co-cultures in 10 mM Tris, 2% SDS, 10% β-mercaptoethanol, and 0.5 mM EDTA, followed by boiling for 5 min. Laminin secreted by astrocytes was analyzed in cell-conditioned medium. Equal amount of proteins were electrophoresed on 10% acrylamide gels. After transfer to membranes, immunoblotting was done with GFAP (1:1000) or laminin (1:25) antibodies, followed by peroxidase conjugated secondary antibodies and Super-Signal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL). Relative optical density was measured by IPLab gel (Signal analytics Corp., Vienna, VA). GFAP signal was normalized to β-tubulin (1:2000; ICN, Aurora, OH), and laminin signal was quantified using laminin standard (purified EHS laminin; Sigma).

2.6. Statistical analysis

Experiments were repeated four to five times. Data were collected blindly and analyzed by ANOVA, with statistical tests (Fisher's) before data normalization.

3. Results

3.1. Old astrocytes support less neurite outgrowth

Heterochronic cultures were formed with E18 neurons overlain on confluent layers of astrocytes derived from adult rat cortex of either of two ages: old, 24 mo, at average life span; young, 3 mo, at maturity. During 5 days of co-culture, old astrocytes supported 50% less neurite outgrowth (Fig. 1A and B). Neuronal cell body size (perikaryal area) showed transient effects of astrocyte age: at day 1, neuronal perikarya in co-culture with old-derived astrocytes were smaller by 10 ± 1% ($P < 0.05$; mean ± S.E.M., four experiments), but age effects did not persist to day 5. Neuronal density did not differ by astrocyte age (see Section 2).

We also verified the persistence of the age effect on astrocytic GFAP in astrocyte–neuron co-cultures, because GFAP expression in neonatal-derived astrocytes is sensitive to the presence of neurons [29]. In these co-cultures, GFAP protein per cell was higher in old versus young astrocytes by 78 ± 6% ($P < 0.04$; mean ± S.E.M., four experiments), consistent with in vivo aging changes (see Section 1). However, old-astrocyte cultures did not contain any very large hypertrophic cells which could be classified as "reactive", as observed after lesions in vitro [18,29].

3.2. Bidirectional manipulation of GFAP and neurite outgrowth

We manipulated GFAP levels to evaluate effects on neurite outgrowth in unlesioned co-cultures. RNAi reversed the age impairment of neurite outgrowth (Fig. 2A). Controls included GFAP mutRNAi (Fig. 2A) and vimentin-RNAi (not

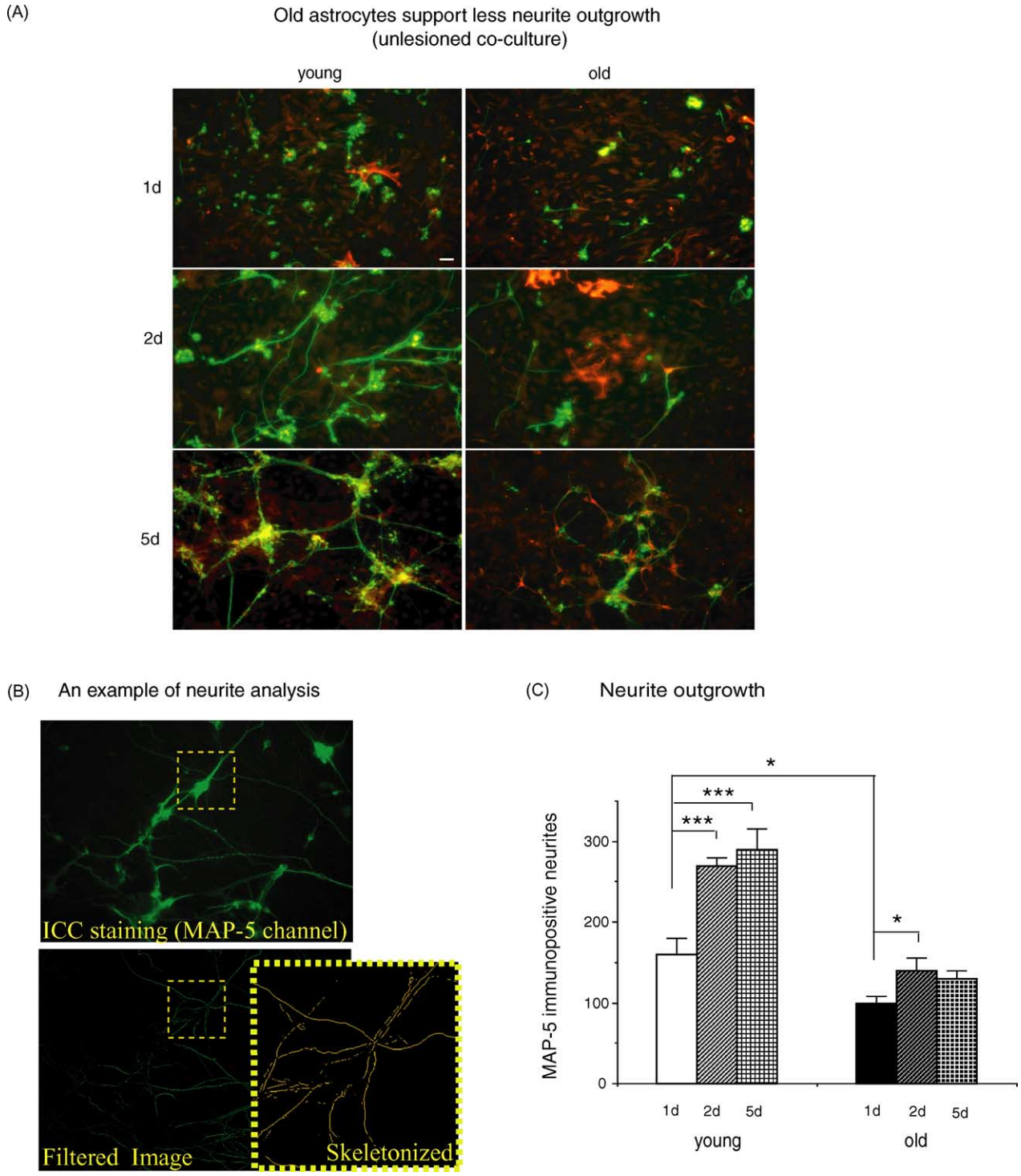

Fig. 1. Neurite outgrowth in E18 neurons co-cultured with confluent astrocytes from young adult (3 mo) or old adult (20 mo) cortex. At times indicated, co-cultures were double immunostained for GFAP and MAP-5. (A) Photomicrographs of immunostaining for MAP-5 (green) and GFAP (red) at 1, 2 and 5 d in co-cultures; scale bar, 10 μm. (B) An example of neurite analysis. Micrographs of culture (double immunostained for GFAP and MAP-5) were digitized into two fluorescent channels. The area covered by MAP-5 immunopositive neurites was calculated using the following strategy: a semi-automated program using IPLab imaging software was developed to define the edge of the neurites exclusive of cell bodies. This filtered image was further processed (skeletonized) to generate a single pixel line. From the one pixel width line, the total neurite length was calculated as shown as MAP-5 immunopositive neurites in (C). (C) During 5 days of co-culture, old astrocytes supported 50% less neurite outgrowth. Neurite outgrowth was measured as the area covered by MAP-5 immunopositive neurites (Sobel edge detection algorithm); mean ± S.E.M., as % of old at 1d; five experiments; $^{*}P < 0.05$; $^{***}P < 0.001$.

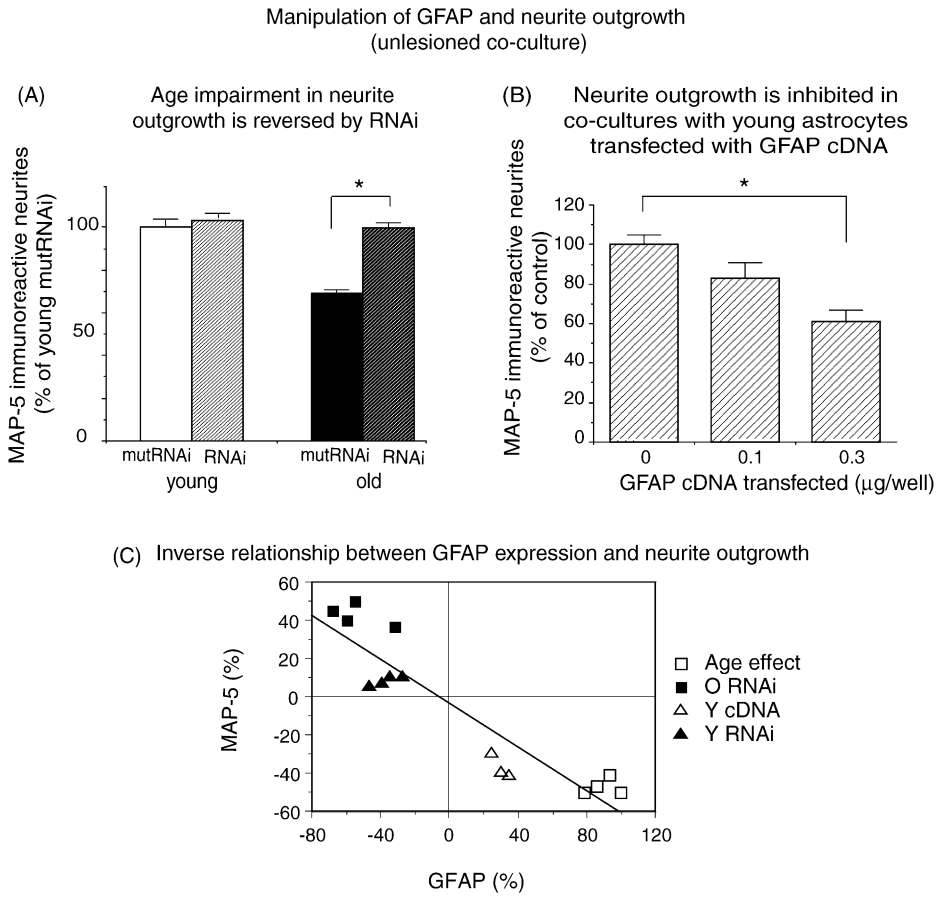

Fig. 2. Manipulation of astrocytic GFAP. Astrocytes were transfected with RNAi (30 nM) or human GFAP cDNA (0.1 or 0.3 μg/well). E18 neurons were seeded 2 d later. Controls include mutated GFAP RNAi (mutRNAi) and vimentin RNAi (not shown), which did not change GFAP. Double immunostaining for GFAP and MAP-5 (neurite outgrowth) was performed for analysis. (A) Transfection with RNAi increased neurite outgrowth in co-cultures with old astrocytes; mean ± S.E.M., four experiments, as % of young mutRNAi; $^*P < 0.05$. (B) Transfection with GFAP cDNA decreased neurite outgrowth in co-cultures with young astrocytes; mean ± S.E.M., three experiments; $^*P < 0.05$. (C) Relationships of MAP-5 and GFAP fit to a linear model with slope 0.58 and $R^2 = 0.85$; points, means of independent experiments. Changes in both MAP-5 and GFAP are defined as follows: age effect, old as % young; O RNAi, old astrocytes treated with GFAP-RNAi vs. mutGFAP-RNAi (% of mut RNAi) in co-cultures with old astrocytes; Y cDNA, cDNA-GFAP vs. empty vector with young astrocytes (% of empty vector). Y RNAi (% of mut RNAi). Note that Y RNAi group showed a minimal change in MAP-5 while GFAP was decreased probably because neurite outgrowth was maximum at that condition.

shown), which did not modify GFAP or neurite outgrowth with astrocytes of either age. Vimentin-RNAi in combination with GFAP-RNAi did not have additional effects (not shown).

Total cellular GFAP was decreased by RNAi (27 ± 3%, $P < 0.05$; mean ± S.E.M. of three experiments, relative to mutRNAi; normalized to β-tubulin; Western blots) in co-cultures with astrocytes of either age, consistent with responses of neonatal astrocytes to antisense-GFAP [18]. In co-cultures of E18 neurons with young adult astrocytes, RNAi did not improve neurite outgrowth, which was probably already maximal in these conditions.

As a reciprocal experiment, we increased GFAP in young astrocytes by transfection with human GFAP-cDNA, which increased GFAP by 31 ± 2% ($P < 0.05$; mean ± S.E.M., three experiments) and decreased neurite outgrowth (Fig. 2B). Thus, modest changes in astrocytic GFAP resulted in either induced or inhibited neuronal sprouting.

These bidirectional manipulations of GFAP (Fig. 2C) show strong inverse relationships to neurite outgrowth (MAP5) with similar scaling across a two-fold range of GFAP values.

3.3. Neurites are co-localized with high intensity laminin

The "wounding-in-a-dish" paradigm with neonatal-derived astrocytes was used to show a general association of neurite outgrowth with laminin. The experimental decreases of GFAP enhance neurite outgrowth after lesions in association with a reorganization of laminin into extracellular network, which is a permissive substrate for neurite sprouting [6,18,29]. We extend these findings to unlesioned cultures of adult, young and old-derived, astrocytes with quantitative evaluation of co-localization of laminin and neurite densities. Laminin-1 expression showed striking age changes (laminin, red; neuronal MAP-5, green; Fig. 3A). In

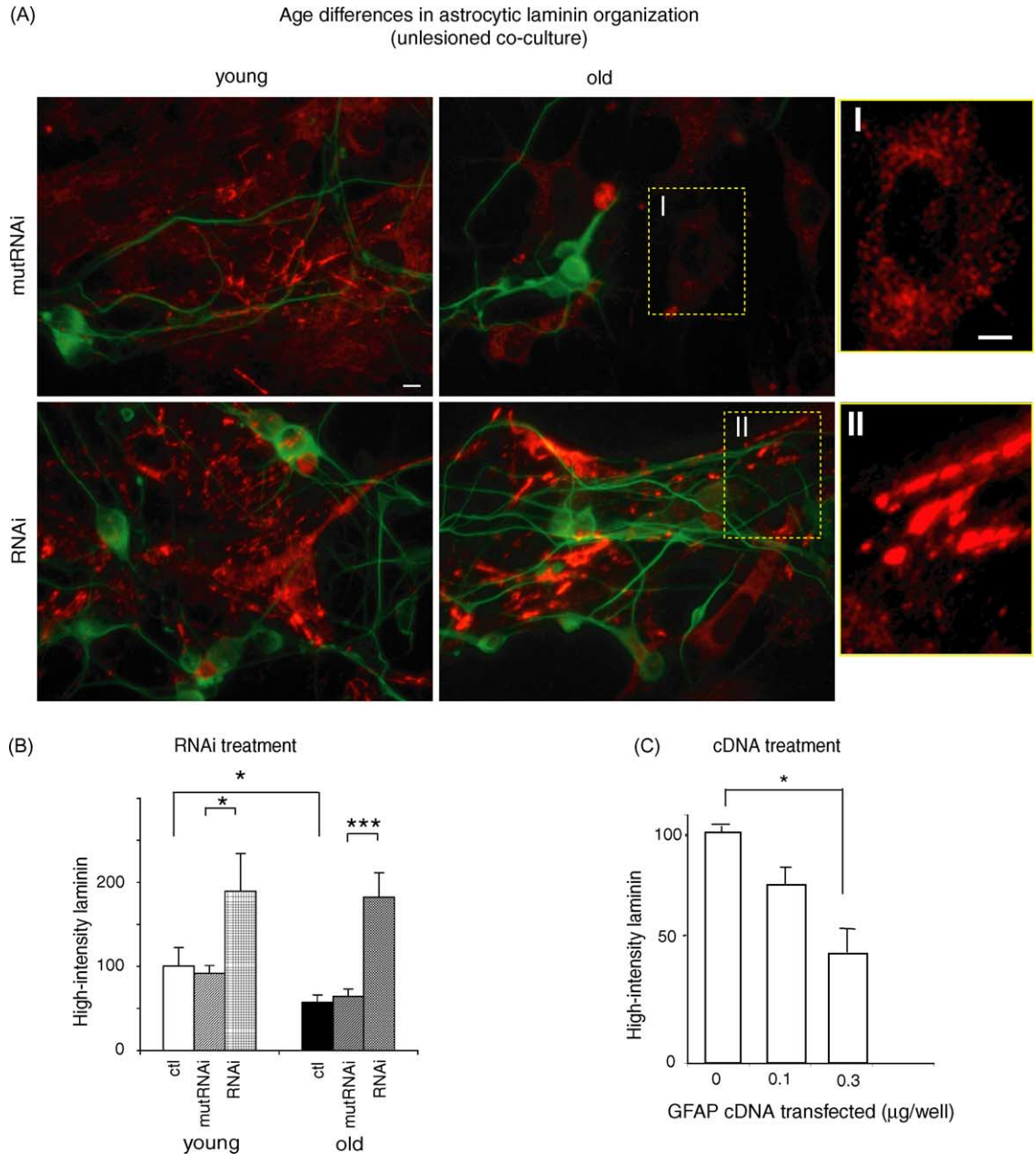

Fig. 3. Age differences in astrocytic laminin organization: effect of RNAi and co-localization of MAP-5 immunopositive neuronal processes with high intensity laminin. Co-cultures with astrocytes from both ages treated with RNAi were immunostained for MAP-5 (green) and laminin (red). Square I, low intensity laminin (cytoplasmic); square II, high intensity laminin (extracellular linear arrays); scale bar, 10 μm. (B) Effect of RNAi on high intensity laminin; mean ± S.E.M., four independent experiments, as % young controls, $^{*}P < 0.05$; $^{***}P < 0.001$. (C) Effect of DNA trasfection into young astrocytes on high intensity laminin, mean ± S.E.M., three independent experiments, as % of empty plasmid; $^{*}P < 0.05$.

co-cultures with E18 neurons, old astrocytes had markedly less laminin, which is visualized as punctate perinuclear staining (square I, Fig. 3A). This age change was also found in monotypic astrocyte cultures without neurons: old astrocytes secreted 34 ± 4% less laminin ($P < 0.05$, five experiments; Western blots of conditioned media).

RNAi reversed these age changes in laminin organization and yielded arrays of extracellular high density laminin (square II, Fig. 3A) (wild-type GFAP RNAi versus GFAP mutRNAi). These laminin arrays had higher density and staining intensity than in controls (untreated and mutRNAi) of both astrocyte ages (Fig. 3B). Consistent with the inhibitory effect on neurite outgrowth, treatment of young astrocytes with GFAP cDNA down-regulated high density laminin (Fig. 3C).

The co-localization of laminin and MAP-5 was analyzed at three levels of laminin staining intensity (low, intermediate, high), e.g. panel 4 Fig. 4A shows the highest laminin inten-

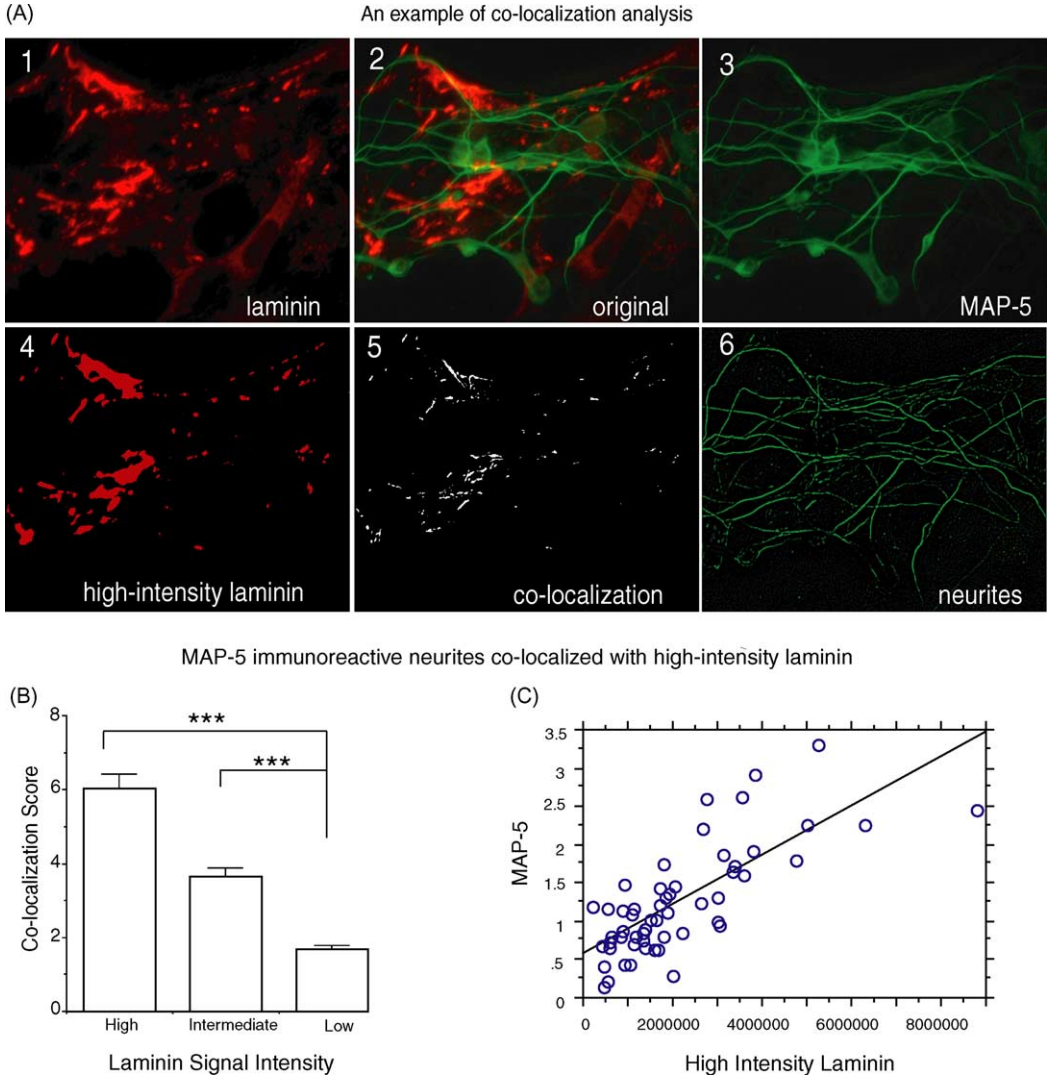

Fig. 4. Co-localization of MAP-5 immunopositive neurites with high intensity laminin. (A) Example of co-localization analysis (see Section 2). Each image of double immunostaining (A2, 1560 × 1080 pixels) was split into red, laminin signal (A1) and green, MAP-5 signal (A3). The area covered by MAP-5 (Sobel edge detection algorithm) as neurites (panel D6, below). Red laminin staining was stratified into three levels of signal intensity: high (A4), intermediate, low (not shown). From co-localized image (A5) the co-localization score was defined as a percentage of laminin-positive (segmented) area covered by MAP-5-positive neurites. We note that the stratified laminin signals occupy different total areas: high < intermediate < low. The co-localization score represents the % of laminin in each category (high, intermediate, low) that is covered by MAP-5 staining. (B) Co-localization score summarizing all age and treatment groups. Co-localization of neurites vs. laminin signal intensities; mean ± S.E.M., five experiments, expressed as a co-localization score; $^{***}P < 0.001$. (C) Correlation analysis of MAP-5 immunopositive neurites and laminin demonstrated that neurites most strongly associated with high intensity laminin across age and treatment groups. Each dot represents a single image; $R^2 = 0.56$, $P < 0.0001$.

sity. The co-localization score represents a percentage of the laminin coverage by MAP-5-positive neurites at these three levels of laminin. MAP-5 most strongly co-localized with the highest intensity laminin across all age and treatment groups (Fig. 4B and C), consistent with the hypothesis that laminin is a factor in neurite guidance.

3.4. Neuronal sprouting is impaired in "wounding-in-a-dish" with old astrocytes

In the wounding model with neonatal astrocytes, a modest reduction of GFAP by antisense or by estradiol (E2) enhanced sprouting of E18 neurons (see Section 1). We extended these studies to young and old astrocytes. Co-cultures were given scratch wounding with or without E2. In the presence of 0.1 nM E2, young adult astrocytes supported neuronal sprouting, as measured by number and length of neurites extending into the wound zone (Fig. 5A and B). As was observed in co-cultures with neonatal astrocytes, E2 attenuated the increase of GFAP after lesioning (Fig. 5C). With old-derived astrocytes, E2 did not stimulate sprouting (Fig. 5A and B) nor attenuate the wounding-induced increases of GFAP (Fig. 5C). Wounding alone increased GFAP to higher levels in old than young astrocytes (Fig. 5C), as observed in vivo [11,35].

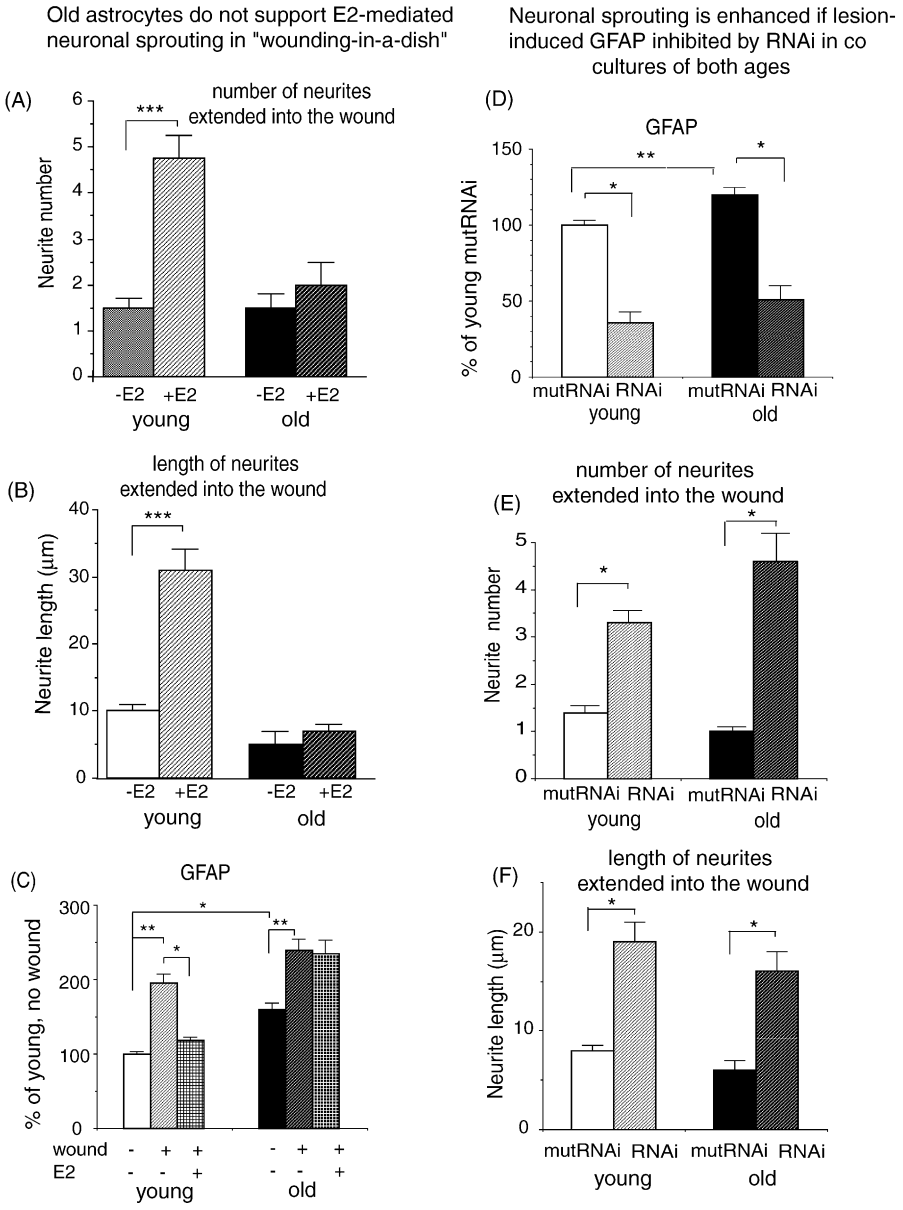

Fig. 5. (A–C) Age impairs astrocytic support of neuron sprouting in presence of estradiol (E2, 100 pM) examined 2 d after scratch wounding ± E2 for MAP-5 and GFAP, relative to vehicle (0.08% ethanol). Neurite outgrowth 2 d after wounding is expressed as number (A) and length (B) of MAP-5 immunopositive neurites extended into the wound zone. In co-cultures with young but not old astrocytes, E2 stimulated sprouting. Neurite number in wound zone; mean ± S.E.M. of four experiments; $^{***}P < 0.001$. (C) Wounding increased GFAP to higher levels in old than young. In co-cultures with young astrocytes, the addition of E2 attenuated the increase of GFAP after lesioning. However, with old astrocytes, E2 did not decrease GFAP; data mean ± S.E.M., four experiments; expressed as a percentage of young; $^{*}P < 0.05$; $^{**}P < 0.01$; (D–F) RNAi enhances neuronal sprouting in wounded astrocyte–neuron co-cultures of both ages. Effects were compared between RNAi containing wild-type GFAP (RNAi) or mutant GFAP (mutRNAi) 2 d after wounding. Astrocytes were transfected with RNAi (30 nM). Two days after transfection, embryonic neurons were plated on astrocytes. After 2 d, co-cultures were wounded and 2 d later examined for neurite outgrowth (double immunostained for GFAP and MAP-5). Controls included mutated GFAP RNAi (mutRNAi) and vimentin RNAi (not shown) neither of which changed GFAP levels. (D) Wounding increased GFAP to higher levels in old than young astrocytes. GFAP RNAi reduced cellular GFAP in co-cultures of both ages; GFAP immunoreactivity; mean ± S.E.M., four experiments, as % of young wound ± mutRNAi; $^{*}P < 0.05$. (E and F) Neurite sprouting was enhanced, measured by number of MAP-5 immunopositive neurites and length of neurites extended into the wound; mean ± S.E.M., four experiments; $^{*}P < 0.05$.

3.5. Manipulation of lesion-induced GFAP with RNAi

RNAi was used to evaluate the potential of old astrocytes to support neurite outgrowth after wounding by decreasing cellular GFAP. As expected, lesion-induced GFAP was decreased in co-cultures of both ages (Fig. 5D), while neurite sprouting into the wound zone was enhanced (Fig. 5E and F). Controls included mutated GFAP-RNAi (Fig. 5D–F) and

vimentin-RNAi (not shown), which did not change GFAP levels or neurite outgrowth. The combination of GFAP-RNAi and E2 enhanced the wounding-induced neuronal sprouting to the same extent with astrocytes of both ages (not shown).

4. Discussion

Old astrocytes consistently supported less neurite outgrowth, which was associated with age increases in GFAP expression. In particular, we observed symmetric and inverse relationships between GFAP expression and the astrocytic support of neurite outgrowth in unlesioned cultures.

First, the age-related impairment in the astrocytic support of neurite outgrowth was reversed by down-regulating GFAP with RNAi. Second, age-like impairments of astrocytic support of neurite growth were induced by transfecting young adult astrocytes with GFAP cDNA. While a general relationship between GFAP in reactive gliosis and neuronal sprouting is widely recognized during lesion response, our studies show that increased GFAP expression during aging is an independent factor in impaired neurite outgrowth in the absence of lesions and of astrocytes with reactive morphology. This point is emphasized, because the negative associations of neurite growth with GFAP expression have been observed after experimental lesions, which induce large reactive astrocytes not found in the unlesioned cultures. These findings raise the possibility that increased GFAP during aging is not only secondary to neurodegeneration and allows the hypothesis that the spontaneous age-related increase of GFAP is a factor in impaired synaptic plasticity during aging.

What causes the early-onset and slowly progressing elevations of GFAP during aging? One factor may be diet-sensitive changes in oxidative load. Caloric restriction, which slows many aging changes in rodents [20,26], also attenuates the increase of GFAP expression during aging at the RNA and protein levels [25]. GFAP transcription is sensitive to oxidative stress, as mediated by an NF-1 response element (unpublished). Because caloric restriction decreases the load of oxidized proteins and lipids and increases the redox balance (GSH:GSSG), we can then consider a pathway of cellular aging in the brain in which the early-onset and slowly progressing accumulations of oxidative changes stimulate GFAP transcription and, gradually, or at some threshold level, reduce the astrocytic support of neuronal functions.

These studies of adult-derived astrocytes differ from most prior work that employed neonatal astrocytes. The age of origin is cogent because adult- and neonatal-derived astrocytes differ in secreted growth factors [37]. However, young adult (shown here) and neonatal astrocytes [29] have similar E2-dependent wounding responses in vitro which model the in vivo repression of GFAP by E2 after lesion [9,35].

The reversibility of impaired neurite outgrowth by GFAP manipulations may be a basis for therapeutic interventions. Neurite outgrowth after spinal cord injury was improved only in mice carrying the double GFAP- and vimentin-knockout, whereas the single knockout did not differ from wild-type [22]. However, a 30% reduction in GFAP induced by oxysterol pre-treatment promoted reinnervation after spinal cord lesions [10]. This is consistent with enhanced neurite outgrowth in in vitro models with relatively modest reduction of GFAP by RNAi (this study), antisense [18], or E2 [29]. Astrocytic age changes may be targets of therapeutic interventions to enhance the poor outgrowth of fetal neuron grafts in aging brain [5,13].

These findings extend reports that astrocytes from GFAP-KO mice support more neurite outgrowth in association with increased laminin [23]. However, GFAP-KO did not alter glial scaring or enhance the impaired neurite outgrowth after lesions [22]. Double GFAP-vimentin-KO did not develop glial scars and had improved neuronal outgrowth and neurological recovery [23]. Similarly, in a retinal transplant model, the double GFAP-vimentin-KO lacked reactive glia at the transplantation site and showed enhanced sprouting [17]. Contrary to in vivo findings, GFAP-KO astrocytes are as good in support of sprouting as double GFAP-vimentin-KO alone [23].

The strong enhancement of neurite outgrowth by lowering GFAP was associated with increased expression of astrocytic laminin. In particular, neurites were strongly co-localized with high-density laminin in all experimental groups (Fig. 4A–C). These findings extend the qualitative associations between GFAP, laminin and neurite outgrowth [18,19,23,29] by defining the first quantitative relationships between cellular GFAP, local laminin density and neurite outgrowth (Figs. 2C and 3F). As further evidence for the direct role of laminin in neurite outgrowth, in “wounding-in-a-dish”, the immunoneutralization of laminin blocked sprouting, whereas the addition of laminin enhanced sprouting [6]. Similarly, in brain slice cultures, antisense laminin-1 attenuated sprouting [14].

The subcellular mechanisms behind the reciprocal relationships of GFAP and laminin expression are obscure. GFAP expression can induce phosphorylation of focal adhesion kinase [31], implying interactions between the GFAP cytoskeleton and the extracellular matrix (ECM). Moreover, actin organization influences the ECM in astrocytes [23,30]. Integrins as the primary cell surface receptors for the ECM and a site of laminin binding might mediate these effects of the cytoskeleton. Other astrocyte factors that mediate sprouting are also sensitive to GFAP manipulation, e.g. metalloproteinases [6]. We do not know how GFAP levels or organization influence the astrocyte secretion of apolipoprotein E, which mediates sprouting [34]. However, secreted soluble factors in conditioned media may be less important than ECM [23].

The age increase of GFAP may also be a factor in Alzheimer disease (AD), because aging is the major risk factor for AD, with the risk of AD doubling every 5 years after 65 [16]. In AD, synapse loss showed inverse correlations with astrocyte volume [3]. According to our hypothesis, synapse loss during aging could be intensified by GFAP-driven as-

trocytic changes during the progression of AD. The greater induction of GFAP in lesions of old brains [11,12,35] and in the present culture models are consistent with feed-forward cascades of neurodegeneration and astrocytosis.

Acknowledgements

This work was supported by NIA Grants AG-14751 (C.E. Finch) and AG-13499 (C.E. Finch and T.E. Morgan).

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