Introductory remarks on the significance of plaque deposition and severity of dementia

INTRODUCTION

Amyloid-β (Aβ) is the major constituent of senile plaques in Alzheimer disease (AD). Mutations in the β-amyloid precursor protein (APP) and in presenilin, both of which affect Aβ processing, lead to autosomal dominant early onset AD (1). Similarly, transgenic mice over-expressing human APP containing pathogenic mutations develop senile plaques (2–4) and behavioral deficits (5, 6).

Despite the evidence for Aβ’s central role in AD, the mechanisms linking Aβ deposition with dementia remain unclear. For example, the severity of dementia is not well correlated with the overall number of Aβ deposits (7). We have hypothesized that a subset of senile plaques, those that are thioflavine-S-positive, morphologically compact Aβ deposits, are most damaging to the neuropil, because the density of SMI-32-positive processes is greatly diminished within them (8). This subpopulation of plaques may correlate better with neuronal loss and dementia than total Aβ (8).

Compact thioflavine-S-positive plaques have also been associated with local synapse loss (9) and neuronal death (10), as well as neuritic dystrophy and glial activation (3, 4). Golgi studies have documented multiple neuronal abnormalities as well: bulbous swellings (11), axonal and dendritic sprouting (12), and a decrease in dendritic arborization (13) in human AD cases. Recent work has also demonstrated an increase in curvature of morphologically otherwise normal neuronal processes near thioflavine-S-positive plaques in both human AD and in transgenic models (14–15).

In the current study, we used in vivo multiphoton imaging of fluorescently labeled neuronal processes to study the effect of plaque morphology on neurites as they pass near or through diffuse and compact Aβ plaques. The technique allows observation of a set of neuronal processes from projection neurons within a relatively large volume of cortex surrounding a plaque, providing information on neurite features that would be beyond the scope of usual histological preparations. Increased process curvature and neuronal dystrophy were noted in about one half of the processes that passed near thioflavine-S plaques. Moreover, we identify marked changes in neurite trajectory as a new feature of neuropathological alteration due to thioflavine-S plaques. These data reinforce the possibility that thioflavine-S plaques impact both their local microenvironment and potentially disrupt distributed neural systems by altering neurite structure.

MATERIALS AND METHODS

Animal Models

Control mice (strain C57 BL6) were used in the development of neuronal imaging techniques in vivo. Two age groups were studied: 2 to 3 months and 15 to 18 months. Transgenic mice (strain PDAPP) expressed human APP with a point mutation of valine to phenylalanine at amino acid 717 (2). This mutation co-segregates with familial AD and causes an overproduction of Aβ with 42 amino acids. PDAPP mice develop Aβ-associated neuropathology starting at 8 to 10 months and their plaque burden becomes progressively more severe with age. The
PDAPP mice used in this research were aged 19 to 22 months. In total, 11 control and 12 transgenic mice were used.

Surgical Procedures

All surgical protocols were conducted under the approval of the MGH animal care committee. The mice were first anesthetized with Avertin (1.3% 2,2,2-tribromoethanol, 0.8% tert-pentylalcohol; 250 mg/kg; administered i.p.) until the toe pinch reflex was completely suppressed. The scalp was then shaved, sterilized, and removed, providing dorsal access to the skull. The mice were then placed in a custom-made stereotaxic apparatus, and 4 small holes were bored with a high-speed drill (Fine Science Tools, Foster City, CA) centered on the midline-bregma suture under a dissecting microscope (Leica). This provided access for the injections of fluorescent dextran, which were made with a 33-gauge needle attached to a 10-μL Hamilton syringe. The holes were filled with sterile bone wax and the animals were allowed to recover on a heating pad before being returned to their cages.

Four days later, a round incision, 6 to 7 mm in diameter, circumscribed the injection holes. The skull was removed and dural tissue was gently peeled from the cortical surface. Amyloid-β stains (thioflavine-S or anti-Aβ antibodies directly labeled with fluorescein) were applied directly to the exposed brain. After rinsing with calcium-magnesium-enriched PBS, an 8-mm glass coverslip was placed over the incision site to eliminate motion artifact induced by blood circulation and breathing. After cementing the coverslip in place, the mice were imaged and later allowed to recover. In cases of repeated imaging, coverslips were removed and replaced to allow the reapplication of fluorescent probes. Table 1 summarizes the surgical and imaging procedures used in this study.

Fluorescent Markers

Dextran-lysine conjugated AlexaFluor 594 (10 kDa, 50 mg/ml; Molecular Probes, Eugene, OR) was injected via Hamilton syringe 200 to 1,000 μl below the cortical surface, 1.5 μL per injection. This dye fluoresces red at about 620 nm. Monoclonal antibodies 10D5 and 3D6, directed against amino terminal of APP, were labeled with fluorescein, which emits in the green range around 522 nm. After skull and dura removal, 20 μL of antibody (1 mg/mL) was applied to the brain surface for 30 minutes and then rinsed. Thioflavine-S (1 mg/mL, Sigma, St. Louis, MO), a histological stain for compact plaques, was subsequently applied to the brain surface for 10 minutes and then rinsed (19). Thioflavine-S emits blue light near 450 nm when excited by multiphoton imaging at 750 μm.

Multiphoton Microscopy

Under anesthesia, mice were imaged with multiphoton fluorescence (16–18). This was generated from a 1.2 W femtosecond pulse mode-locked Ti:Sapphire laser (Tsunami, Spectra-Physics, Mountain View, CA). The output laser was tuned to 750 nm and powered by a 10 W pump laser (Millennium Xs, Spectra-Physics). The self-contained Radiance system (Bio-Rad, Hercules, CA) digitally controlled laser output power, line scanning speed, and axial position. Light was separated through 2 dichroic mirrors into 3 ranges: 380 to 480 nm (thioflavine-S); 500 to 540 nm (fluorescein); and 560 to 650 nm (AlexaFluor594). Signals were amplified through the use of 3 integrated photon-multiplying tubes.

Three objectives, ×10, ×40, and ×60 (0.30, 0.80, and 0.90 numerical apertures, respectively; Olympus, Melville, NY) were used. The axial resolution was slightly less than 5 μm on the higher magnification objectives, which corresponds to the depth of the focal plane for multiphoton fluorescence; the resolution in the x-y plane was ~1 μm. The axial direction was oversampled in 1-μm steps by an automated focus motor while the x and y coordinates were manually controlled.

Images were acquired with LaserSharp2000 software (Bio-Rad). All images were stored as 512 by 512 arrays of 8-bit pixels, collected at a line speed of 166 lines per second. Laser power and photon multiplying tube gain settings were modulated manually to adjust for tissue depth and fluorescence intensity. Images were acquired in z-series stacks from the cortical surface downwards. Image processing was performed using Scion Image.

Ratio Analysis

Neuronal curvature can be quantified by comparing its end-to-end distance to the overall process length (14). We measured and analyzed this with two-dimensional projections generated in Adobe Photoshop. Processes for analysis were identified manually on the basis of 3 criteria: discernible contrast from background, continuity, and end-to-end distance of at least 20 μm. Processes that did not meet all of these requirements were not measured. Overall process length was determined by summing the lengths of straight lines of 3 μm or more overlaying the process. Endpoints of processes were determined either by the distance from plaque edge (defined below), or if the process proceeded outside the image field. Plaques were identified by immunofluorescence and considered “thioflavine-positive” if they possessed a thioflavine-S-positive core and were at least 15 μm in diameter. Controls were generated by superimposing, in random locations, circles the size of a plaque (20 μm in...
Histological Analysis

After final imaging, animals were administered a lethal dose of Avertin (≈1,500 mg/kg). Whole brains were removed from the skull and placed in 4% paraformaldehyde for 24 hours at 4°C. Afterward they were placed in 10% glycerol solution in TBS to prepare for sectioning. Fifty-μm-thick sections were examined for thioflavine-S and dextran-AlexaFluor 594 and counterstained for GFAP immunoreactivity (Sigma) to detect astrocytes or with tomato lectin (Sigma) to detect activated microglia.

Statistical Analysis

All statistical analyses were performed using SAS statistical software (SAS Institute, Cary, NC). Significance levels for statistical tests were set at $p \leq 0.01$.

RESULTS

Imaging of Diffuse Amyloid and Dense-Core Plaques

Thioflavine-S-positive dense-core plaques were successfully imaged in vivo from 12 PDAPP mice, aged from 19 to 22 months. Although the overall plaque number varied among animals, PDAPP mice displayed plaques in all cortical areas accessible for imaging, ranging from the brain surface to 150 μm in depth. Plaques were usually spherical in appearance with occasional asymmetries. One hundred twenty plaques were imaged with a diameter of 27 μm ± 8 μm (mean ± SD). In vivo immunofluorescence was also performed using fluorescein-conjugated Aβ antibodies 10D5 and 3D6. This staining revealed both diffuse and compact plaques in superficial layers of the cortex, providing a broader spectrum of morphological Aβ deposits than thioflavine-S staining alone.

Fluorescent Dextrans and Neuronal Imaging

A discrete set of (presumably random) neuronal cell bodies and processes were labeled by AlexaFluor 594 in the immediate vicinity of each injection site of the dextran. Neuronal uptake of the fluorescent dextran was comparable between control and transgenic mice (Fig. 1A, B). Processes were discernible from background 2 days after injection and best labeling occurred at 4 to 14 days postinjection. The red AlexaFluor 594 dye was readily differentiated from the green immunofluorescence of fluorescein 10D5 and the blue-green thioflavine-S in triple stained preparations. Neuronal processes labeled with AlexaFluor 594 in control animals resemble Golgi preparations and could frequently be observed to course over 250 μm within the 3-dimensional image stacks. Young PDAPP mice were indistinguishable from nontransgenic mice.

Dextran-Labeled Neurites Occur in the Vicinity of Diffuse Plaques in PDAPP Mice

In PDAPP mice, in addition to numerous normal appearing neurons and neurites, we found many examples of neurites colocalizing with diffuse Aβ deposits. Eighteen instances of an AlexaFluor 594-labeled neuronal
process were identified partially or entirely within immunofluorescent diffuse Aβ deposits. These neurites showed either no noticeable morphological abnormalities deposits or very slight curvature in the vicinity of the diffuse Aβ (Fig. 2). While these changes were modest in degree and number compared to neurites near thioflavine-S-positive plaques, neurite trajectories appeared to be altered in diffuse plaques compared to nontransgenic controls. Several examples of processes directly penetrating diffuse amyloid were noted, with minimal consequence on neurite morphology at the site that they intersected the diffuse plaque.

Thioflavine-S Plaque-Associated Neuritic Dystrophy is Observed in Dextran-Labeled Processes In Vivo

Dextran-labeled neurons and neurites were also observed in the vicinity of thioflavine-S plaques in PDAPP mice. The neuritic processes in this instance frequently had abnormal morphological features, unlike neurites in control mice. For example, bulbous outgrowths were often observed near the plaques (Fig. 3). Similar varicosities have been observed with Golgi staining near senile plaques (11), but may also be associated with abnormal dendritic sprouting (12). These AlexaFluor 594-positive features resemble dystrophic neurites as observed with conventional silver staining in PDAPP mice and human AD cases.

In addition, thioflavine-S-positive plaques were often associated with rather large aggregates of the dextran-linked dye. In Figures 3 and 4 this is seen as red superimposed on the blue image of the thioflavine-S-positive plaques. Absolute identification of the identity of these aggregates in the in vivo imaging was not possible, because in many instances they were not continuous with labeled neuronal processes. Postmortem conventional histological analysis using standard confocal analysis of histological sections prepared from these mice was consistent with microglial uptake of the dextran because the AlexaFluor aggregates colocalize with tomato lectin staining near thioflavine-S plaques (but not with GFAP immunostaining). However, a great deal of the aggregated AlexaFluor dye was washed off the plaques during the immuno-labeling protocols. This suggests that some of the AlexaFluor signal associated with thioflavine-S-positive plaques in vivo was due to weak and nonspecific binding of the dextran-fluorophore conjugate to the surface of the thioflavine-S plaques.

Thioflavine-S Plaques are Associated with Both Proximal and Distal Changes in Neurite Trajectories and Architecture

We identified 92 instances of AlexaFluor 594 filled neuronal processes near (within 50 μm) thioflavine-S-positive compact plaques. In contrast to our observations on diffuse plaques, none of these processes clearly traversed through a thioflavine-S plaque. Moreover, many processes within 5 μm of plaques appeared to curve around rather than penetrating them. This could take the form of a very smooth bend (Fig. 4a) or a very curved process (Fig. 4b). Both types resemble Golgi-stained processes near plaques in the hippocampus of human AD cases (11). In either case, many processes appear to resume their former direction after coursing along the plaque borders. This phenomenon appears to correspond...
Fig. 3. Four examples of cellular distortions (red) in processes proximal to dense-core plaques labeled with thioflavine-S (blue). A: A process with a high curvature near a plaque. B: As a process approaches plaque it appears to possess bulbous outgrowths while being deflected. C: Two processes intersect with a dextran clump adjacent to a plaque. D: As process goes around plaque it intersects with another clump and appears distorted in its lower segment. Scale bar = 10 μm.

Other neuronal processes, however, did not assume their former direction. Instead they appeared to continue toward a different trajectory after coming close to a plaque (Fig. 4c). This morphology appears to correspond to “proximal plaque deflections” observed in Golgi preparations of human AD (11). Finally, neuronal disruptions were also observed in processes that were distal to a dense-core plaque (>15 μm from plaque edge). Although many processes that were outside the periphery of a plaque showed no significant distortions, a few seemed to exhibit significant directional shifts to avoid plaques (Fig. 4d). These correspond to “distal arches” or “distal deflections” described in AD postmortem material (11). The frequencies of processes in all categories are summarized in Table 2.

Quantification of Plaque-Associated Neuritic Changes

A measure of the curvature of neuronal processes was used in order to compare these results to earlier postmortem measures (14, 15). The curvature measure is a
Fig. 4. Thioflavine-S-positive dense-core plaques (blue) occlude neuronal processes imaged in vivo (red). A, B: Two examples of a process passing very close and avoiding a dense-core plaque. C: A process that appears to be deflected by a senile plaque. D: A more distal example of a neuronal process bending around a plaque. Scale bar = 25 μm.

A comparison of end-to-end (straight line) distance to the actual distance traversed by a neurite. An absolutely straight line would have a ratio of 1.0; the ratio decreases in proportion to the extent that a process’ trajectory deviates from straight. The ratio of end-to-end distance to overall length has been observed to significantly decrease in both human AD cases and in the PSAPP and Tg2576 transgenic mouse models of AD (14, 15).

In control mice, neuronal processes are quite straight with values of 0.95 or greater. If compact plaques disrupt processes, however, this ratio would decrease in regions near plaques.

Two measurements were performed overall on 92 processes within 50 μm of 45 thioflavine-S-positive plaques. First, the end-points were designated and the straight-line distance between the ends was compared to the actual distance traversed by the process. Secondly, this measurement was repeated for the 57 of the 92 processes that came within 15 μm of the edge of thioflavine-S plaque. New endpoints were designated 15 μm from the plaque edge and the measurement repeated using these endpoints (Fig. 5).

Curvature measurements were performed in 2 types of controls. First, neuronal processes within the same
TABLE 2
Frequencies of Characterized Neuronal Processes within 50 μm of a Dense-Core Plaque

<table>
<thead>
<tr>
<th>Proximal arch*</th>
<th>Proximal deflection†</th>
<th>Distal arch‡</th>
<th>Distal deflection</th>
<th>Neuronal dystrophy§</th>
<th>Unaffected process</th>
</tr>
</thead>
<tbody>
<tr>
<td>22%</td>
<td>11%</td>
<td>7%</td>
<td>2%</td>
<td>8%</td>
<td>51%</td>
</tr>
</tbody>
</table>

Note: Proximal refers to disruptions within 15 μm of a plaque edge. Distal refers to disruptions between 15 μm and 50 μm of plaque edge.

* See Figure 1a, b for example.
† See Figure 1c for example.
‡ See Figure 1d for example.
§ See Figure 2 for example.

Fig. 5. Comparison of neuronal processes close to (15 μm), surrounding (50 μm), and distant to (over 100 μm) dense-core plaques along with controls. Ratios of end-to-end distance to overall length measure the curvature of the processes within a given region. Note that the distributions are significantly more right-skewed for the 15-μm and 50-μm populations compared with distant and control groups.

images were examined if their distance was greater than 100 μm from any plaque edge. Sixty-nine processes were found and examined in this fashion. Secondly, 20-μm-diameter spheres representing “phantom plaques” were overlaid in random locations onto the collapsed three-dimensional images of processes from nontransgenic mice. For these processes, an examination identical to the 50-μm category above was performed. The distributions of ratios of end-to-end distance are illustrated in Figure 5 and summarized in Table 3.

The population of processes within 50 μm of a plaque edge was significantly different from those in nontransgenic controls (ANOVA; F = 10.31; df = 1,133; p ≤ 0.01). Since the 15-μm measurements were a subset of the 50-μm measurements, statistical significance was not recalculated, but the difference is obvious from inspection. The 15-μm group mean (R = 0.93) and standard deviation (σ = 0.07) appear similar to postmortem measurements made from postmortem analyses in 2 other transgenic models of AD, PSAPP, and Tg2576 mice (R = 0.92 and 0.93, σ = 0.10 and 0.07, respectively (14).

Although processes closer to plaques generally displayed higher curvatures, a substantial fraction (17%) of processes more than 15 μm from any plaque were more than 2 standard deviations from the control mean.

DISCUSSION

Senile plaques are primary neuropathological features of AD and mouse models of AD, but their relationship to neural system dysfunction remains uncertain. Early Golgi studies revealed a variety of dendritic and axonal alterations in AD, including varicosities near plaques, proximal plaque deflections, and distal arches (11). In reference to amyloid deposition, interpretation of these changes is confounded by the presence of neurofibrillary changes and neuronal death in AD, as well as the capricious nature of Golgi impregnation. More recent immunohistochemical studies of postmortem tissue in AD and transgenic mice (14, 15) allowed observation of only a thin histological section, limiting studies of neurite trajectories and geometry to a thin plane. Our current results suggest that impressions based on traditional neuropathological techniques, which demonstrate dystrophic neurites immediately surrounding plaques, may underestimate the impact of thioflavine-S-positive plaques on projection neurons in their vicinity because effects on neurite trajectory are difficult to appreciate in thin sections.

Our current experiments provide a technique to visualize, with exquisite subcellular detail, neuritic processes in vivo. We have used this technique to examine the effect of plaques on processes from projection neurons.

TABLE 3
Ratio of End-to-End Distance to Overall Length for Processes in Control and Transgenic Mice

<table>
<thead>
<tr>
<th></th>
<th>Nontransgenic (control)</th>
<th>Outside 100 μm of plaque edge</th>
<th>Within 50 μm of plaque edge</th>
<th>Within 15 μm of plaque edge</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean (n)</td>
<td>0.97 (43)</td>
<td>0.97 (69)</td>
<td>0.94 (92)*</td>
<td>0.93 (57)*</td>
</tr>
<tr>
<td>SD</td>
<td>0.03</td>
<td>0.03</td>
<td>0.06</td>
<td>0.07</td>
</tr>
<tr>
<td>% over 2 SD from control mean</td>
<td>2%</td>
<td>4%</td>
<td>17%</td>
<td>25%</td>
</tr>
</tbody>
</table>

* p ≤ 0.01 as analyzed through ANOVA. SD = standard deviation.
Since the neurites that take up the fluorescent dextran dye are random and independent of plaque proximity, they can be taken as representative of processes from projection neurons that are coursing through the neuropil. We find that processes that pass through diffuse Aβ deposits show only subtle effects on their morphology or trajectories. This is consistent with similar observations made in histological material (15).

By contrast, we find an impressive array of alterations that occur in neurites in the immediate vicinity of thioflavine-S-positive plaques. Our in vivo observations suggest 2 major findings. First, it appears that processes of projection neurons tend not to pass through compact thioflavine-S-positive plaques, but rather are altered from their normal course by the presence of a plaque. Previous evaluations using SMI-32 immunostaining as a marker of projection neuron processes came to a similar conclusion in a study of postmortem AD tissue (14). Secondly, quantitation of the types of geometric changes of neurites surrounding plaques suggest a marked increase in the “cavernness” of neurites even when they do not appear overtly dystrophic. The increase in cavernness, measured as a ratio of linear end-to-end distance to distance traversed by the neurite, shows that many neurites take a circuitous route in the vicinity of plaques. The exact degree of changes measured from our in vivo images have PDAPP mice match almost exactly with analogous measures obtained for neuritic changes around thioflavine-S-positive plaques in TG2576 and PSAPP mice (15). The results also match well with measures of non-phospho tau containing processes near plaques in human AD (14). Thus, our in vivo measures appear to be closely aligned with expectations based on prior neuropathological studies.

In addition to an exclusion zone in the core of thioflavine-S plaques, there appears to be a region surrounding thioflavine-S plaques of about 50 μm in which the geometry or trajectory of nearly half of all imaged processes near plaques in human AD (14). Thus, our in vivo images have PDAPP mice that occur in neurites in the immediate vicinity of thioflavine-S-positive plaques. Previously we had noted that the effects on curvilinear distance alone would predict, based on theoretical models using cable theory, a change in the spike timing parameters of neural systems (14). The additional changes noted here would no doubt further disrupt communication between linked populations of projection neurons, and would be predicted to disrupt synchronous electrophysiologic responses to stimuli, and thus normal mechanisms of neural system plasticity. Taken together, these data support a model in which thioflavine-S-positive plaques cause a marked disruption of the architecture of the neuropil, even in the absence of prominent phospho tau alterations, and/or neurodegeneration.

These experiments also highlight the power of multiphoton microscopy to provide high resolution in vivo imaging. Dextran-labeled dyes for retrograde transport are available in multiple colors and, as demonstrated here, can be used to obtain in vivo images with detailed dendritic structure. This type of high-resolution tool to allow examination of neuronal and neurite structure in vivo may have broad application in studies of both neuropathology and development in experimental systems.

REFERENCES


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