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Multicolor time-stamp reveals the dynamics and toxicity of amyloid deposition

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The pathogenic role of amyloid plaques in Alzheimer's disease (AD) remains controversial given poor correlation between plaque burden and cognitive status in clinicopathological studies. However, these postmortem studies cannot provide information about the dynamics of plaque expansion and consequent neurotoxicity. We developed a novel method for plaque birth-dating and growth analysis using sequential labeling with amyloid-binding dyes and postmortem quantitative confocal imaging. Using this technique in an AD mouse model, we find that plaques grow gradually over months with growth slowing in older animals. The degree of neuritic dystrophy correlates with the speed and extent of plaque enlargement suggesting a causal relationship. Surprisingly, new plaques induce a disproportionately large area of neuritic dystrophy whereas with older plaques the degree of injury plateaus despite continued growth. Our results suggest that the kinetics of amyloid deposition is a critical determinant of neurotoxicity, which is completely overlooked by traditional measures of plaque burden.

Although the precise pathological substrate in Alzheimer's disease (AD) is likely to be complex, the amyloid plaque remains an important hallmark that is associated with structural¹ and functional synaptic and glial abnormalities^{2,3}. Clinicopathologic studies, however, have shown that the number of amyloid plaques does not always correlate well with the degree of cognitive decline⁴. This has led many to conclude that the amyloid plaque is not a major contributor to AD neuropathology. However, there is a scarcity of studies which quantify the cumulative damage of amyloid plaques on the surrounding cellular structures. Furthermore, the speed and extent of amyloid plaque growth are understudied factors that may be critical determinants of the severity of neuronal network disruption.

Longitudinal imaging with positron emission tomography (PET) has revealed patterns of regional β -amyloid (A β) accumulation⁵. However, due to limited spatial resolution, PET cannot provide information about the growth of individual amyloid plaques nor the disruption of the microenvironment. More recently, *in vivo* imaging studies in transgenic mice with two photon microscopy (TPM) have provided insights into the dynamics of individual plaque growth. It has been suggested that fibrillar amyloid deposits develop rapidly, reaching their full size within a day⁶, or gradually over weeks⁷ to months^{8,9}. The reasons for these discrepancies are unclear but could be due to differences in animal age, experimental conditions such as the type of cranial window for *in vivo* imaging^{7,10} or potential artifacts associated with incomplete labeling with amyloid-binding dyes¹¹. Thus, the precise time course of amyloid plaque growth and its role in neuronal injury remains controversial.

Here we report the development of a novel technique that uses sequential dye labeling of amyloid plaques (multicolor time-stamp) to study the timing of development and growth pattern of individual amyloid deposits in postmortem tissue. This simple but potentially powerful technique allows for high-throughput analysis of the kinetics of amyloid plaque expansion in any brain region as opposed to just the superficial cortex as in TPM studies. More importantly, this method allows the concomitant use of molecular and structural markers for detailed studies of the effects of amyloid deposition kinetics on the local neuroglial microenvironment.

Using this technique we made several novel observations about the dynamics and toxicity of amyloid plaques. Our results suggest that although plaques grow gradually over many months, neurotoxicity is most prominent in early stages of plaque formation and plateaus as plaques grow larger. This suggests that the commonly used static plaque counting methods in clinicopathologic studies provide an incomplete picture of the contribution of plaques to neuronal disruption and cognitive decline. Our data could provide important information for generating more accurate models of the process of neuronal network disruption associated with amyloid deposition, without the technical limitations of current *in vivo* imaging methodologies.

Finally, we demonstrate that the time-stamp technique could also be used to study the progression of other AD pathological hallmarks such as cerebral amyloid angiopathy and intracellular neurofibrillary tangles. Future availability of suitable molecular labels may make it possible to apply this technique to human subjects.

Results

Designing a novel method to study the dynamics of amyloid deposition in postmortem tissues. Neuropathology studies have provided a wealth of information about amyloid plaques and changes in the surrounding neurons and glia. However, these studies are only able to capture a single moment in plaque development rather than describe the timing of amyloid plaque appearance and growth. Recently, it has become feasible to obtain time lapse images of individual plaques in transgenic mouse models using two photon microscopy (TPM)^{1,12}. These studies, however, are

limited to the superficial cortex and suffer from potential artifacts associated with the invasiveness of the cranial window used for imaging¹⁰, which may preclude precise quantitative analysis of plaque growth. Furthermore, performing histological or other molecular analysis of the microenvironment of plaques that have previously been imaged *in vivo* with TPM is challenging.

To overcome these limitations, we developed a method for plaque birth dating and growth quantification in postmortem tissues of AD mouse models. We reasoned that we could obtain dynamic information by sequentially labeling amyloid plaques at various time intervals using amyloid-binding dyes with distinct fluorescence emissions. Mice would be sacrificed after any desired time interval and brain slices imaged with high resolution confocal microscopy for subsequent quantitative analysis. The difference in fluorescent area between the dye labels would reflect the amount of plaque growth (Fig. 1), while the presence or absence of labeling with the dye

Figure 1 | Sequential multicolor labeling reveals plaque growth in postmortem tissues. (a–c) Diagrammatic timeline of sequential plaque labeling in AD mouse models. Fluorescent amyloid-binding dye methoxy-X04 (MX) is administered *in vivo* by systemic injection at time point 1 (T₁). To allow for complete dye labeling we wait 48 hours post-injection. The brain is then collected at any desired time interval. A second fluorescent amyloid-binding dye, Thiazin red (TR) is applied to brain slices at time point 2 (T₂) and then imaged by high resolution confocal microscopy. (b,c) Low magnification view of a portion of the cortex reveals numerous labeled plaques at T₁ (b). The differential overlap between T₁ and T₂ (60 days) reveals new plaques and the expansion of pre-existing ones (white area represents overlap between cyan and red) (c). Scale bar, 100 μ m. (d–f) High-resolution confocal microscopy (d–e) followed by image thresholding (f) clearly demonstrates plaque growth over a 60 day interval. Images are z projections of three optical slices through the center of the plaque. Scale bar, 5 μ m.

injected at the initial time point (T_1) would serve as a marker for birth dating (Fig. 1 and Fig. 3a–c).

Selection of dyes with long-term binding stability and no significant competitive displacement or quenching. A variety of amyloid binding dyes were tested for plaque labeling at T_1 . We found that methoxy-XO4 (MX)¹³ and fluoro-styrylbenzene (FSB)¹⁴, two Congo red derivatives, were the best available candidates given their blood brain barrier (BBB) permeability and relatively pure blue fluorescence emission. Importantly, once administered and after complete plaque labeling has occurred (see **Supplementary Fig. 1** and methods), these dyes remain stably bound to amyloid plaques for at least 90 days as demonstrated by the lack of change in mean plaque fluorescence intensity (Fig. 2a) and size (Fig. 2b). In addition, we have previously shown using two-photon microscopy that plaques labeled once with MX and re-imaged over multiple intervals do not become smaller or change in shape¹⁵. This strongly suggests that MX does not dissociate from the plaque, redistribute within the plaque, or lose its fluorescence over time. When selecting dyes for the second time point (T_2) we took into account the possibility of fluorescence “bleed-through”, resonance energy transfer (FRET) and quenching between colocalized dyes. Therefore, we avoided green/yellow-emitting dyes and looked for suitable red-emitting ones. However, BBB permeable red-emitting dyes are not readily available. Thus, rather than injecting dyes *in vivo* as in T_1 , we applied them directly to postmortem brain slices at T_2 . We tested both Congo red (CR) and Thiazin red (TR) and found that CR was insufficiently bright. Furthermore, when titrated to higher concentrations, CR caused loss of MX fluorescence possibly due to quenching or competitive displacement (data not shown). Alternatively, TR was bright at low concentrations and when applied sequentially did not cause any loss of MX fluorescence (Fig. 2c) or change in plaque size (Fig. 2d). The long-term stability of MX labeling coupled with the lack of competitive displacement or quenching by TR makes this dye-pair suitable for sequential plaque labeling.

Baseline calibration of fluorescence intensity at initial time point (T_1). Change in plaque area over time is ultimately calculated by subtracting absolute plaque sizes measured after thresholding both MX and TR fluorescence images. For proper implementation of the time-stamp technique, the calculated plaque area from MX and TR labeling must match well at T_1 , when no plaque growth has yet occurred. We first injected MX into a subset of transgenic mice, allowed complete plaque labeling (48 hours), then collected the brains and applied TR. In this control experiment, the MX and TR label should overlap completely on each plaque. However, even after carefully adjusting dye concentrations and staining duration, we found that there were intrinsic properties of the dyes that led to small differences in labeling brightness between MX and TR in control tissue (T_1) (Fig. 2e,h). Thus, using the same pixel intensity threshold for both MX and TR would lead to a small but systematic mismatch in calculated plaque areas (Fig. 2f,i,k).

To correct this mismatch, we developed a fluorescence calibration step aimed at equating plaque areas measured from MX and TR. First, we set out to determine how brightness differs at baseline (no growth) between plaques fully labeled with both dyes. An intermediate threshold pixel intensity value of 1000 was applied to both TR and MX images at T_1 (Fig. 2f,i and methods) and the resulting areas were compared (Fig. 2k). Using this threshold with our control dataset, we observed an overall tendency for the TR area to appear smaller than that of MX (Fig. 2i,k). In order to compensate for this small bias, we developed macros on *NIH ImageJ* software to automatically find the precise pixel intensity threshold for TR to render its area equal to MX (Fig. 2g,j and **Supplementary Movie 1**). After analyzing many plaques in this way, we calculated the average threshold correction necessary for TR to match the MX area. The

area mismatch, however, did not follow a linear distribution given that larger plaques had a greater TR to MX disparity than smaller ones (Fig. 2k). Therefore instead of selecting the same threshold for all plaques regardless of their size, we divided plaques in groups based on size and applied a different threshold to each group to optimize TR to MX matching (Fig. 2l and methods). Using this method we were able to virtually eliminate the bias observed in the absence of threshold correction (Fig. 2g,m). The thresholds obtained from control mice were then applied to all data generated with experimental animals. Confocal imaging parameters such as laser intensity, detector gain and pinhole size were kept identical for all experimental and calibration tissues.

Multicolor time stamp demonstrates that amyloid plaque growth is a protracted process that slows with aging. We examined the rates of amyloid plaque formation and growth over various time intervals ranging from days to months in a mouse model of AD harboring mutations in the APP gene (CRND8)¹⁶. We saw no evidence of explosive plaque formation and growth arrest over days as has been reported with *in vivo* two photon imaging experiments⁶. Instead, we observed that new (Fig. 3) and pre-existing (Fig. 4) plaques continued to grow gradually over many months (**Supplementary Fig. 2**).

We utilized the time-stamp technique to selectively identify new born plaques at various time intervals ranging from 8 to 90 days. We examined the total plaque population in a given field of view and quantified the number of plaques labeled with both TR and MX (pre-existing plaques) and those only labeled with TR (new plaques) (Fig. 3a). We found that the percentage of new plaques (Fig. 3b) and their average size (Fig. 3c) steadily increased with longer time intervals between T_1 and T_2 . This provides further evidence that new plaques continue to grow gradually after they are born, and argues against the rapid plaque formation and growth arrest hypothesis⁶.

Next, we measured cross-sectional areas through the middle of plaques to determine the absolute area growth over time. The diameter of plaques was extrapolated from the measured area and found to increase gradually by very similar amounts in all plaque size groups (Fig. 4a,b and **Supplementary Fig. 2**). This suggests that the rate of amyloid addition per unit of plaque surface is a constant and that soluble A β binding affinity is independent of plaque size at least in this mouse model. We also found that the rate of plaque growth slowed with aging, and interestingly, this occurred evenly in all plaque size groups (Fig. 4c). One explanation could be that as plaque number increases with age the amount of A β available to bind individual plaques decreases uniformly. Thus plaque number more than individual plaque size may determine the timing of the eventual growth plateau.

In parallel studies, we used *in vivo* transcranial two-photon imaging in a transgenic mouse model to track individual plaque growth over weeks to months (Fig. 4d). It should be noted that a far smaller number of plaques per mouse can be imaged *in vivo* than with the time-stamp technique, and that imaging is limited to the superficial cortex. Consistent with our time-stamp results, we found that individual plaques continued to grow over months (Fig. 4e) and plaque growth was much slower in older mice (Fig. 4e) as recently described^{8,9}.

The time-stamp technique can be used to study the effects of plaque expansion on the surrounding brain microenvironment.

To study the consequences of plaque formation and growth on adjacent neuronal structures, we combined the amyloid time-stamp with immunohistochemistry for protein markers found within dystrophic neurites. Antibodies for synaptic and neurofilament structures like synaptophysin and internexin- α effectively reveal the area of abnormal neuronal processes around plaques (Fig. 5a–e). We used these antibodies to quantify the size of the area of neuritic dystrophy for correlation with the pattern of

Figure 2 | Testing amyloid-binding dye stability and calibrating fluorescence intensity. (a) Comparison of mean MX-labeled plaque fluorescence intensity at 2 and 90 days post-injection. ($n = 301$ plaques from 4 mice; $p = 0.1830$). All mice were 4 months old at time of injection. (b) Mean diameter of MX-labeled plaques at 2 and 90 days post-injection (blue bars; $p = 0.9657$). MX labeled plaque diameter remained stable despite significant growth as evidenced by TR labeling at 90 days (red bars; *** $p < 0.0005$). (c) Comparison of mean MX-labeled plaque fluorescence intensity with and without addition of TR. ($n = 234$ plaques from 2 mice; $p = 0.1684$). (d) Mean MX-labeled plaque diameter with and without TR labeling. ($n = 234$ plaques from 2 mice; $p = 0.3207$). (e–j) Prior to plaque analysis, slices from control animals at T₁ are analyzed. An example of plaque fluorescence calibration shows that fluorescence intensity of MX and TR plaque label at T₁ can have small discrepancies (e,h), which can lead to error in plaque size measurement following thresholding (f,i). To address this potential problem, threshold values are adjusted to equate MX and TR areas at T₁ (g,j). Using an arbitrary threshold of 1000 for both MX and TR labels at T₁ shows that the mismatch distribution is not linear (k). To compensate for this, plaques are classified by size and different threshold values are applied to each size group (see methods for details) (l), resulting in elimination of the systematic mismatch bias (m). Data shown = mean $\pm$ s.e.m.

Figure 3 | Newly born amyloid plaques are small and continue to grow gradually over months. (a) Thresholded plaque areas (z-projection of confocal optical sections). Plaques newly formed during the 60 day interval (TR-labeled only; yellow arrowheads) appear significantly smaller on average than pre-existing plaques (TR and MX labeled). (b) The ratio of new plaques to total plaque number gradually increases over 8 to 90 days ($n = 1727$ plaques from 3 mice per time interval). (c) The size of new plaques gradually increases over time ($n = 619$ plaques from 3 mice per time interval; * p values between all intervals < 0.05). All mice were 4 months old at time of MX injection. Data shown = mean $\pm$ s.e.m.

plaque growth. However, these markers also label the adjacent normal synapses and neuronal processes, therefore it is laborious to distinguish and manually trace the precise boundaries of the area with abnormal neuronal structures (Fig. 5a–e). To increase the throughput of our analysis we looked for proteins that would be highly enriched in dystrophic neurites (DNs) while being nearly absent in normal adjacent structures. This would allow automated detection and thresholding of the dystrophic area boundaries for higher throughput quantification. A variety of intracellular vesicles and organelles are known to accumulate within dystrophic neurites¹⁷. We found that the lysosomal associated membrane protein-1 (Lamp1) was highly enriched in DNs as evidenced by its specific colocalization with the neuronal proteins synaptophysin and internexin- α and its virtual absence from other cellular structures around plaques (Fig. 5d–f, d'–f' and Supplementary Fig. 3). Thus we used Lamp1 labeling as a tool for detecting the boundaries of DNs and examining the relationship between plaque expansion and neuronal injury. A similar labeling strategy using a variety of other molecular markers could be combined with the time-stamp technique to study diverse aspects of the pathology around fibrillar amyloid deposits.

Rapid induction of neuronal dystrophy by newly formed plaques is determined by the speed and extent of amyloid deposition. Amyloid plaques are almost invariably surrounded by dystrophic axonal processes and degenerating dendrites with reduced spine density^{1,18}. However, the lack of a strong statistical correlation between cognitive performance and the number of plaques found in postmortem human studies¹⁹ has put into question the relevance of amyloid deposits in the pathogenesis of AD. An alternative explanation is that simple quantification of plaque density in postmortem tissues overlooks potentially important variables such as the time course, speed, magnitude and location of amyloid deposition and the degree of neuritic dystrophy.

We therefore implemented the time-stamp method to systematically study these variables. We first examined plaque evolution in the cortex over a 16 day interval to determine the timing of neuronal damage in relation to plaque formation. Surprisingly, we observed that small new plaques were surrounded by a disproportionately large area of dystrophy (Fig. 6a and Supplementary Movie 2). Given that the majority of new plaques appearing over a 16 day interval ranged between 2–8 μm in diameter, we compared new and pre-existing plaques only within this size range. Within this narrow range, however, the size distributions were different such that the average plaque size in the newly formed group was significantly smaller (Fig. 6b). Interestingly, despite their smaller average size and more recent formation, new plaques had an equivalent area of neuritic dystrophy around them as larger pre-existing plaques (Fig. 6c,d). Furthermore, there was a strong effect of plaque location on the degree of damage such that plaques in the white matter were associated with a markedly larger area of neuritic dystrophy than in the cortex (Fig. 6e and Supplementary Fig. 4). Altogether, this suggests that substantial neuritic damage occurs rapidly during the initial stages of plaque formation and can be influenced by their location in the brain.

We then examined the effect of gradual plaque growth on the degree of neuritic dystrophy. We reasoned that if there was a causal connection between ongoing amyloid deposition and neuritic damage, these two variables should have a strong quantitative correlation. We implemented the time-stamp technique over an interval of 60 days, then selected all pre-existing plaques (TR and MX-labeled) and classified them into groups by their initial size (based on MX label). Using this strategy we were able to calculate the absolute amount of amyloid added over a fixed time interval and correlate it with the resulting size of the neuritic area. We found that for plaques 2–18 μm in diameter there was a strong correlation between the absolute amyloid addition and the amount of neuritic dystrophy. Despite substantial amyloid addition, however, larger plaques (18–28 μm) exhibited only marginal increases in the size of the dystrophic area (Fig. 7a), suggesting that amyloid plaque toxicity stabilizes with time.

Next, we asked whether the speed of amyloid accumulation rather than just the absolute amount of deposition also influenced the degree of neuritic damage. To study this, we took the same group of plaques analyzed above and classified them by their final size (TR label) instead of the initial one. Subtracting the initial size from the final one, we were able to determine for each plaque, how fast amyloid deposition occurred (Fig. 7b). Surprisingly, we observed for smaller plaques (5–14 μm) that the more rapidly they grew the larger the extent of neuritic damage (Fig. 7b). However, these effects eventually plateau, such that the pace of amyloid accumulation in larger plaques has a much more limited influence on the degree of damage. The reason for this phenomenon is unclear but it may be related to changes in A β conformation as plaques become denser making these deposits gradually less toxic²⁰.

Our findings suggest that the magnitude of toxicity is not simply governed by plaque size but is differentially regulated by the kinetics of growth throughout the lifetime of a plaque. Specifically, we dem-

Figure 4 | Gradual growth occurs at a constant rate for all plaque sizes and slows with aging. (a) Distribution of plaque growth measurements in 4 month old mice over various time intervals shows gradual increase in size. Note that the diameter of plaques of all sizes grew by the same amount for each time interval ($n = 1108$ plaques from 3 mice per interval; $F = 91.6$, $p = 1.5 \times 10^{-126}$, *Bonferroni-Holm post hoc tests). (b) Plaque diameter growth over 60 days plotted against initial plaque size (MX diameter). Regression analysis shows no significant correlation ($p = 0.23$, $R^2 = 0.006$). This indicates that the average increase in plaque diameter over time is constant for all plaque sizes. (c) Comparison of growth patterns over 32 days in 4 and 14 month old mice. In aged mice, plaques continue to grow gradually but at a significantly slower rate. This rate continues to be constant for all plaque size groups as in younger mice. Note that plaques with diameters $>30 \mu\text{m}$ (blue bars) are only present in 14 month old mice ($n = 653$ plaques from 3 mice per group; $F = 131.3$, $p = 3.83 \times 10^{-26}$, *Bonferroni-Holm post hoc tests). (d) Time lapse two photon microscopy (TPM) over 90 days shows gradual growth of adjacent small and medium sized plaques. Scale bar, $15 \mu\text{m}$. (e) Plaque growth measured from *in vivo* TPM images in 4 and 15 month old mice ($n = 113$ plaques from 8 mice; * p values between intervals < 0.05). Data shown = mean $\pm$ s.e.m.

onstrate that the speed and extent of plaque expansion in the early stages of growth have a significant impact on the degree of injury. This supports a causal relationship between amyloid accumulation and neuritic dystrophy. Furthermore, the gradual slowing of the expansion of neuritic dystrophy observed, suggests that early plaque growth is the most damaging event while the long-term plaque presence may be much less detrimental.

Implementation of the time-stamp technique for probing other protein misfolding disorders and human Alzheimer's disease. We first tested whether the multicolor time-stamp strategy could be used to study other hallmarks of AD such as cerebral amyloid angiopathy (CAA). We found that methoxy-XO4 (MX) and thiazin red (TR)

were both reliable labels of CAA in transgenic mice (Fig. 8a). Furthermore, when applied in a sequential fashion as with amyloid plaques, they could be used to follow the progression of amyloid deposition over intervals of months. High-resolution confocal microscopy of cerebral microvessels immunolabeled for the endothelial protein PECAM1, coupled with the time-stamp technique, revealed in detail the process of expansion of pre-existing amyloid deposits and the formation of new ones around the vascular wall (Fig. 8a). Therefore, when combined with a variety of vascular, pericyte, astrocytic and microglial markers, the time-stamp technique could be used for studying the impact of ongoing cerebrovascular amyloid deposition on the integrity of the neurovascular unit²¹.

Figure 5 | The effects of plaque kinetics on neurotoxicity can be studied by combining the time-stamp technique with various markers of neuritic dystrophy. (a) The boundary between synaptophysin immunoreactive dystrophic neurites (DNs) and normal synaptic puncta (green) around plaques can be differentiated by their morphology (yellow arrowheads). (b) The border of the dystrophic area can be manually traced. Here we show a DN area that we manually traced and pseudo-colored (grey). (c) The pseudo-colored area can be isolated and combined with the time stamp label (red and cyan thresholds) for correlation of plaque expansion with DN area. Scale bar, 15 μ m. (d–f) Identification of a protein marker exclusively enriched in DNs. Synaptophysin (d) and internexin- α (e) immunoreactivities within the dystrophic area (yellow dotted outline) are highly colocalized but provide poor contrast with adjacent normal neuronal structures. (f) The lysosome-associated membrane protein-1 (Lamp1) (green) is highly enriched in the DN area and provides excellent contrast (yellow outline). Scale bar, 10 μ m. (d'–f') Zoomed images provide clear evidence of colocalization between synaptophysin, internexin- α and Lamp1 within the DNs boundaries (yellow dotted line). Scale bar, 15 μ m.

We then tested whether in addition to extracellular amyloid deposits the time-stamp technique could be applied to track the dynamics of intracellular neurofibrillary tangles (NFTs). We utilized mice expressing mutant human microtubule-associated protein *tau* (MAPT P301S) that produce NFTs throughout the central nervous system²². We found that the amyloid binding dyes FSB (an MX alternative) and TR, both had strong affinity for NFTs. Furthermore, *in vivo* administration of FSB led to robust and diffuse NFT labeling, despite their intracellular location¹⁴ (Fig. 8b). Using sequential labeling with FSB and TR, we demonstrate that it is possible to detect pre-existing NFTs as well as newly formed ones (Fig. 8b,c). Thus the time-stamp technique could be applied to study different aspects of cellular pathology associated with the formation of intracellular NFTs.

Finally, there are currently no methods to study the dynamics and toxicity of amyloid plaque and NFT formation and growth at high-resolution in humans. However, with the ongoing development of β -sheet binding dyes²³ and other molecular markers for clinical imaging, we postulate that a strategy similar to the time-stamp technique may become feasible in postmortem human tissue from brain donors. To explore this possibility, we tested FSB and TR to sequentially label amyloid plaques and NFTs in human AD tissue. High-resolution confocal imaging demonstrates that both dyes directly overlap at T₁ (Fig. 8d,f) and could thus be calibrated for quantitative analysis. Furthermore, we show the potential for combining the time-stamp technique with markers of dystrophic neurites (Fig. 8e) to study the effects of plaque kinetics on the surrounding cellular microenvironment in postmortem human tissue.

Figure 6 | Neurotoxicity occurs rapidly after plaque development. (a) Lamp1 immunoreactive DNs (green) surrounding a pre-existing plaque (blue and red thresholds) and a new plaque (red threshold) that formed within the 16 day interval. Scale bar, 10 μ m. (b) Despite choosing for analysis new and pre-existing plaques within the same size range (2–8 μ m diameter), new plaques had a tendency to be of a smaller average size. (n = 307 plaques from 3 mice; ** p < 0.001). (c,d) Comparison of Lamp1 (c) and Synaptophysin (d) areas between new and pre-existing plaques ranging 2–8 μ m in diameter. (n = 307 plaques, p = 0.5063, or 129 plaques, p = 0.7352, from 3 mice, respectively). (e) Quantification of Lamp1 area surrounding new and pre-existing plaques in grey and white matter over a 16 day interval (both groups are 2–8 μ m in diameter; n = 361 plaques from 3 mice; p < 0.005). All mice were 4 months old at time of MX injection. Data shown = mean $\pm$ s.e.m.

Given that several other neurodegenerative conditions are characterized by protein misfolding and aggregation, it is possible that dyes targeting different protein aggregates could be used to implement the time-stamp technique in conditions such as Huntington's, prion and other diseases.

Discussion

We have developed a simple and potentially powerful technique to study the initial appearance and growth kinetics of abnormal brain protein aggregates in postmortem specimens. This method, which relies on sequential labeling with fluorescent β -sheet binding dyes and imaging with high-resolution confocal microscopy, provides quantitative dynamic information about the aggregation process in unperturbed brain tissue. Large datasets of amyloid plaques and neurofibrillary tangles located in any area of the brain can be acquired and analyzed in an unbiased and highly efficient fashion. Coupled with immunohistochemistry or other molecular techniques, this method can be used to study the toxicity of protein aggregation on the surrounding cellular microenvironment and to probe the therapeutic potential of a variety of interventions.

Using this technique we have provided new insights into the kinetics of amyloid plaque growth in an AD mouse model. We have shown that amyloid deposits grow gradually and reach their final size over many months. Given the extent of A β overproduction in transgenic mice, our findings suggest that in humans, the time interval between initial amyloid plaque appearance and growth plateau must be in the order of years.

A large number of clinicopathological studies have shown that the density of amyloid plaques is not a good correlate of the degree of cognitive decline⁴. This has led many to suggest that fibrillar amyloid does not play a substantial role in AD pathogenesis despite clear evidence of extensive neuritic damage around amyloid deposits^{1,18}. Nevertheless, the amyloid hypothesis remains a cornerstone of AD research given strong genetic evidence of abnormal amyloid precursor protein (APP) processing²⁴. Furthermore, a large number of drugs in current development are aimed at preventing plaque formation or promoting their removal²⁵. Therefore, to properly assess the impact of specific treatments on amyloid-induced neuropathology it would be essential to develop accurate models of the kinetics of amyloid accumulation in relation to neuronal injury.

The time-stamp technique allowed us to examine the precise temporal sequence of fibrillar amyloid plaque formation, expansion and surrounding neurotoxicity in transgenic mice. It has been shown *in vitro* that amyloid fibrils form via nucleation-dependent polymerization by binding of monomeric and oligomeric A β to growing fibril ends²⁶. The nucleation-dependent polymerization model would predict that once nucleation occurs, amyloid plaque growth accelerates quickly, like a seeded crystal²⁶. Our results suggest that following amyloid nucleation, subsequent polymerization occurs gradually over many months. Thus nucleation-dependent polymerization may not fully explain the process of plaque expansion.

In aging, we observed that plaques grew at a significantly slower rate. Interestingly, similar to young mice, this rate was equal for all plaques regardless of their initial size. One potential explanation is that, as the overall number of plaques increases with age, they could collectively act as a large sink for soluble A β , leading to an equivalent reduction in its availability for binding individual plaques of all sizes. This is consistent with findings in humans showing that A β in the cerebrospinal fluid drops with conversion to symptomatic AD²⁷. In addition to this global reduction in A β availability, the density of fibrillar amyloid within plaques increases overtime, potentially leading to a gradual reduction in binding affinity for soluble A β . There might be factors at the individual plaque level that lead to differential changes in the binding affinity and growth rates such as the glial microenvironment¹⁵. Although in our mouse model, the growth rate of all plaques appeared to decline proportionally regardless of plaque size, the kinetics of plaque evolution may vary in different AD mouse models or in more advanced age. Differential plaque growth decline between plaques of different sizes at later stages, would suggest that in addition to global availability of A β , local factors in the microenvironment also influence the rate of plaque expansion.

Figure 7 | The speed and extent of plaque expansion determines the degree of neurotoxicity. (a,b) Lamp1-immunopositive dystrophic neurite area was plotted against added plaque area (TR-MX areas) over a 60 day interval. Diagrammatic representation of analysis shows that plaques were binned in various groups by their initial (a, blue circles) or final (b, red circles) diameter. Note that in larger plaques (red and blue lines) the expansion of Lamp1 immunopositive area plateaus despite continued growth. ($n = 122$ plaques from 3 mice).

The specific factors that determine how plaques become toxic and contribute to cognitive impairment remain poorly understood. Our data shows that initial plaque formation and early expansion induce rapid and robust neurotoxicity, but the extent of this damage ultimately plateaus in later stages of progression. In addition, the speed of initial plaque deposition rather than just the absolute amount of amyloid addition influences the degree of neuritic injury. Interestingly, a PET study in AD patients with and without ApoE4 genotype showed that despite similar amounts of amyloid binding as measured by the Pittsburgh B compound (PiB), ApoE4 patients had lower brain metabolism²⁸. Given that ApoE4 patients experience earlier onset pathology, this along with our data suggests that the speed of amyloid accumulation is an important factor in AD progression. Consistent with this, very old individuals can have a large number of plaques and yet remain cognitively stable suggesting that slowly developing amyloid deposits are less damaging²⁹.

These observations are consistent with a model where the kinetics of fibrillization is an important determinant of toxicity^{30,31}. The gradual stabilization of toxicity as plaques grow and become more densely packed with fibrillar material, suggests that the peripheral halo made of prefibrillar A β rather than the core itself is the most toxic substance³². The small but persistent plaque growth observed in aged AD mice raises the possibility that late-stage plaques act as a “central

sink” for soluble A β , leading to reduced expansion of new highly-toxic deposits. Given the limited neurotoxicity we found with large plaques, they could provide an unforeseen neuroprotective effect. Thus, the rationale for using therapies to remove plaques could potentially be harmful and should instead be aimed at preventing the formation and early growth of new deposits.

Aside from growth kinetics, our data suggests that the degree of neuritic damage is determined in part by the location of amyloid deposits. We find that plaques in the white matter induce significantly larger areas of neuronal injury compared to similar-sized plaques in the cortical gray matter. Although the mechanism explaining this finding remains unclear, it may have important implications given that disruption of inter-hemispheric axonal tracts could be more functionally devastating than the focal disruption of neuronal connections in the cortex.

Taken together, the multicolor time-stamp method has shown that unappreciated factors such as the initial plaque appearance, growth rate and location are important determinants of the overall degree of injury. This may provide a partial explanation for the apparent dissociation between the magnitude of amyloid deposition and degree of cognitive decline observed in autopsy series.

Although our studies were limited to mouse models of neurodegeneration, the time stamp technique could in theory be applied to

Figure 8 | Application of the time-stamp technique to other pathological hallmarks of AD in mice and humans. (a) Z projection of a confocal stack shows a PECAM1-immunolabeled microvessel (green) with cerebral amyloid angiopathy (CAA) in a CRND8 transgenic mouse. Single optical sections demonstrate the differential labeling of amyloid (yellow arrowheads) with MX (T_1) and TR (T_2) over a 60 day interval. (b,c) A mutant *tau* transgenic mouse (P301S) labeled with fluoro-styrylbenzene (FSB) (T_1) and TR (T_2) demonstrates the feasibility of detecting pre-existing (b) and new intracellular neurofibrillary tangles (NFT) (c). (d) Images of human tissue from an Alzheimer's patient labeled with both FSB and TR (T_1) demonstrate strong overlap between the plaque labels. (e) The area of synaptophysin immunoreactive dystrophy was traced (pseudo-color grey) and plaque labels at T_1 were thresholded (cyan and red lines) to simulate different time points. Scale bar, 10 μm . (f) Labeling of NFTs with FSB and TR in human AD tissue demonstrates colocalization at T_1 .

humans. A variety of molecular labels of amyloid plaques such as near infrared fluorescent amyloid-binding dyes²³ have recently been developed for noninvasive optical imaging of AD progression. When approved for clinical use, these compounds may make it feasible to apply the time-stamp strategy to study the time course of plaque, CAA and neurofibrillary tangle development and toxicity in AD subjects. A similar strategy could eventually be implemented to study other abnormal protein aggregation disorders and to test the efficacy of specific therapeutic interventions.

Methods

Mice. CRND8 [APP KM670/671NL1 (Swedish) + V717F (London)] mice were used for all time stamp experiments. For NFT imaging, MAPT*P301S mice were used (Jackson Laboratories). Experimental protocols were approved by the Northwestern University Feinberg School of Medicine Institutional Animal Care and Use Committee.

Chemicals. Methoxy-X04 (MX) was obtained from the laboratory of Dr. William Klunk (University of Pittsburgh). Thiazin Red (TR) and Thioflavin S (TS) were obtained from Sigma Chemicals (St. Louis, MO). Fluoro-styrylbenzene (FSB)¹⁴ was obtained from Dojindo Laboratories (Japan).

Sequential dye labeling of plaques. Alzheimer's-like mice were injected with 100 μl of [5 mg/ml] MX at time point one (T_1). At the second time point (T_2), mice were fully anesthetized with Ketamine/Xylazine, and transcardially perfused with saline and then 4% paraformaldehyde. Brains were extracted and post-fixed in 4%

paraformaldehyde and cryoprotected in 30% sucrose/PBS overnight. Brains were frozen, embedded in OCT and sliced into 40 μm thick coronal sections using a cryotome. Floating sections were incubated in a 0.02 μM TR solution for 20 minutes shaking at room temperature, and washed 3 times in PBS for 5 minutes each.

Confocal Imaging. Sampling was standardized by using coronal brain sections from the same stereotaxic coordinate range (interaural 3.58 mm/bregma 0.22 mm to interaural 1.86 mm/bregma -1.94 mm). Only plaques located in the cortical grey matter whose full volume resided well within the slice thickness were selected for imaging. Two- or three-channel 12-bit image stacks were acquired in a sequential mode at 1 μm z-steps using a Zeiss LSM-510 confocal microscope (40 $\times$ oil immersion lens. NA:1.25). Fluorescence excitation and emission filters were as follows: MX: 405nm laser/420–480 nm filter, TR: 543nm laser/ 560 nm long pass filter, Alexa Fluor-488: 488nm laser/505–550 nm filter. Uniform pinhole, laser power and PMT detector gain settings were used in all time-stamp experiments.

Identification of new plaques. New plaques were defined as Thiazin Red (TR)-only labeled structures which had an area greater than 5 μm^2 and were present in at least two consecutive 1 μm z slices.

Quantification of plaque growth and Lamp1 area in fixed tissues. Images were analyzed using NIH ImageJ and custom semi-automated macros. Each 12-bit image had a pixel range from 0 to 4095. Plaque areas from both MX and TR labels were determined by thresholding and measuring with ImageJ. After screening hundreds of individual plaques, a pixel intensity threshold value of 1000 was chosen because it was distant from pixel saturation and background values and gave good delineation of plaque edge as determined by visual inspection. The TR pixel intensity threshold was calculated from a quantitative dye calibration process (see below). Plaques measuring

less than $5\ \mu\text{m}^2$ were excluded to avoid background noise contamination. MX- and TR-labeled plaque areas were measured in consecutive $1\ \mu\text{m}$ z-slices through the middle of the plaque volume, and then averaged together for each plaque. Diameters were calculated from the average areas of both MX and TR assuming each area to be a perfect circle. For older CRND8 mice, there was presence of small ($1\text{--}10\ \mu\text{m}^2$) lipofuscin autofluorescent granules which were very bright in the TR channel and dim in the MX channel. A custom ImageJ macro was written to mask these autofluorescent areas identified by their small size and very high red/blue pixel intensity ratio. Some plaques were eliminated from quantification because two or more plaques merged over the time interval which would bias quantifications of individual plaque growth. In brain sections also containing Lamp1 immunopositive dystrophic neurites, a fluorescence threshold of 1000 was selected and incorporated into the custom macro. The measured Lamp1 area includes the plaque area, which is subtracted out so that only the dystrophic halo of Lamp1 area is used for quantitative analysis.

Calibration of Thiazin Red threshold. From our observations, MX takes 2 days to label plaques completely (data not shown), so we considered 48 hours postinjection to be our initial time point (T_1). Thus, a subset of mice in each experimental group were sacrificed 2 days after MX injection and brain slices were stained with TR as described above. In these control tissues there should be no difference in plaque size between the TR and MX areas. MX area was determined by thresholding at a pixel intensity of 1000 as above. A custom ImageJ macro was written which adjusts the TR threshold for each plaque until the TR area exactly matches the MX area. The calculated thresholds were divided into multiple bins according to plaque size and averaged (see also Fig. 2). This thresholds and bins were then applied for all experimental quantifications.

Two-photon microscopy (TPM) plaque imaging. MX or Thioflavin-S (TS)-labeled plaques were imaged through a thinned skull preparation as previously described (Xu et al., 2007). Briefly, transgenic mice were anesthetized with Ketamine/Xylazine and the skull was exposed with a midline scalp incision. About a $1\ \text{mm}$ diameter skull region over the somatosensory cortex was thinned with a high speed drill and scraped with a microsurgical blade to a final thickness of $\sim 30\ \mu\text{m}$. The skull was attached to a custom-made steel plate to stabilize the head while imaging. A mode-locked Ti-sapphire laser (Coherent Inc. and Prairie technologies) was used for two-photon excitation and tuned to $835\ \text{nm}$ for MX or $850\ \text{nm}$ for TS. Emission wavelengths of MX and TS were collected in the $350\text{--}490\ \text{nm}$ range. Images were taken using a water immersion objective (Olympus $40\times 0.8\ \text{N.A.}$) at z-steps of $1\text{--}2\ \mu\text{m}$ and zooms of $1\text{--}3\times$. In most cases images were taken at depths up to $\sim 100\ \mu\text{m}$ below the pial surface. The same regions were relocated at different time points using the unchanging pattern of pial blood vessels as reference as well as registration of X,Y,Z coordinates of each scanning area at the initial time point and relocation of the same coordinates using a precision motorized stage at subsequent time points. The precise identity of the areas is confirmed by the presence of unambiguous microvascular shadows or plaque positions.

Quantification of plaque area from TPM images. Mice were injected intraperitoneally with $50\ \mu\text{l}$ of 1% Thioflavin S four days before each imaging session. Image stacks containing plaques were run through a despeckle filter to remove noise. A maximal projection of each plaque using a constant number of slices per time point was generated with NIH ImageJ. To achieve accurate plaque area measurements regardless of brightness differences across time points a threshold had to be calculated for each time point. This threshold was obtained by measuring the pixel intensity in the same region of interest (ROI) within each plaque across time points and averaging that value with the background pixel intensity.

Immunohistochemistry. Antibodies for Synaptophysin (Millipore), Lamp1 (Developmental Studies Hybridoma Bank clone 1D4B), internexin- α (Novus Biologicals) and PECAM/CD-31 (BD Pharmingen) were diluted $1:250$ in $1\times$ PBS solution containing 3% goat serum and 0.2% Triton X-100. Sections were incubated for 72 hours at 4°C in the primary antibody solution and washed 3 times for 20 minutes each in $1\times$ PBS. Alexa-fluor secondary antibodies were diluted $1:500$ in a $1\times$ PBS solution and incubated with tissues for 6 hours at room temperature and subsequently washed 3 times for 20 minutes each in $1\times$ PBS.

Quantification of synaptophysin area. $1\ \mu\text{m}$ z-step confocal images of plaques were analyzed using NIH ImageJ and custom semi-automated macros. The boundary of the dystrophic neurite area is easily identified as the transition between normal small puncta and large bulbous structures. This boundary is then manually traced in 4 optical sections near the center of the plaque. The reported area of neuronal dystrophy per individual plaque is derived from the mean value of the 4 optical sections minus the respective TR plaque area.

Serial labeling of neurofibrillary tangles (NFTs). At T_1 , 6 month-old mutant *tau* mice received an intravenous injection of $200\ \mu\text{l}$ of $2.4\ \text{mM}$ FSB in $1\times$ PBS. At T_2 (either day 8 or 16), brains were collected and processed as described in the Methods section. Floating sections were incubated with $0.02\ \mu\text{M}$ TR for 20 minutes at room temperature and subsequently washed 3 times for 5 minutes each in $1\times$ PBS.

Dual-labeling of plaques and tangles in human tissue. postmortem human AD specimens were obtained from the brain bank in the Cognitive Neurology and

Alzheimer's disease Center (CNADC) at Northwestern University. Tissue was cryoprotected in 30% sucrose in $1\times$ PBS solution and $40\ \mu\text{m}$ cryotome sections were prepared. Floating slices were incubated for 20 minutes with $1\ \mu\text{M}$ FSB and $0.4\ \mu\text{M}$ TR to label amyloid plaques and $100\ \mu\text{M}$ FSB and $0.4\ \mu\text{M}$ TR to label NFTs. Sections were washed 3 times for 5 minutes each in $1\times$ PBS.

Statistical analysis. Statistical analysis was performed using two-tailed Student's t-test or linear regression analysis. $P < 0.05$ was considered significant. For multiple comparisons a one-way ANOVA with Bonferroni-Holm post hoc tests were used.

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Author contributions

C.C., A.S. and J.G. designed the study; C.C. and A.S. performed experiments; C.C., A.S. and J.G. wrote the manuscript; J.G. supervised the study.

Additional information

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