Neuronal loss is the ultimate outcome in a variety of neurodegenerative diseases and central nerve system disorders. Understanding the sequelae of events that leads to cell death would provide insight into neuroprotective approaches. We imaged neurons in the living brain of a mouse model of Alzheimer’s disease that overexpresses mutant human amyloid precursor protein and presenilin 1 and followed the death of individual neurons in real time. This mouse model exhibited limited neurodegeneration and atrophy, but we were able to identify a small fraction of vulnerable cells that would not have been detectable by using standard approaches. By exploiting a genetically encoded reporter of oxidative stress, we identified susceptible neurons by their increased redox potential. The oxidative stress was most dramatic in neurites near plaques, propagated to cell bodies, and preceded activation of caspases that led to cell death within 24 h. Thus, local oxidative stress surrounding plaques contributes to long-range toxicity and selective neuronal death in Alzheimer’s disease.

In vivo imaging | reduction-oxidation sensitive GFP

Alzheimer’s disease (AD) is underscored by neurodegeneration and is the most common form of dementia. The pathological hallmarks of this disease include amyloid plaques, neurofibrillary tangles, and neuronal loss. Early-onset familial AD is caused by genetic mutations of amyloid precursor protein (APP) or presenilin 1 and 2 (PS1 and PS2). Although recent genetic studies have revealed risk factors for late onset AD, the pathogenic pathways for sporadic AD remain largely unknown. The development of mouse models of AD that develop senile plaques similar to those found in AD patients was a critical step in identifying the role of amyloid β (Aβ) on neuronal function. A major disappointment of most of the mouse models is the lack of overt neuronal loss that is a hallmark of the human disease. Many, in fact, have used this lack of neuronal death as evidence that amyloid is not relevant to dementia in AD. We and others (1–4) have identified structural and functional alterations of neurons in the brains of APP mice that implicate amyloid-mediated toxicity, but we have never detected neuronal death. The ability to monitor cell death in an experimental model provides the opportunity to intervene with neuroprotective agents that could be applied to the spectrum of neurodegenerative diseases and CNS disorders. We were able to identify vulnerable cells by quantitatively imaging the redox potential of neurons in the living brain. Our hypothesis was that amyloid-mediated increases in oxidative stress are the initiators of the toxic cascade that leads to cell loss. Accumulating evidence supports a role for oxidative stress in the pathogenesis of neuronal degeneration and death in AD (5–8). The evidence supporting oxidative stress in AD comes largely from postmortem samples and includes increased lipid peroxidation, decreased polyunsaturated fatty acids (9–12), increased protein oxidation (13, 14), and DNA oxidation (15, 16). The addition of exogenous Aβ also increases formation of free radicals and oxidative injury in in vitro studies (17, 18). However, there is no direct in vivo evidence of increased cellular oxidative stress in AD. Therefore, we translated a fluorescent indicator for oxidative stress for use with in vivo multiphoton microscopy imaging to address directly the question of whether oxidative stress is increased in living AD mouse brain and, if so, whether the oxidative stress is linked to neuronal degeneration.

Results

Redox-sensitive variants of the green fluorescent protein (roGFP) have been developed that allow the monitoring of oxidation/reduction potentials in cells by using ratiometric methods (19, 20). The probes were constructed by placing two cysteine residues onto neighboring strands of the β-barrel of GFP in positions favorable for disulfide bond formation. The formation of the Cys147-Cys204 disulfide caused by oxidants leads to a fluorescence increase when excited at 395 nm and a decrease when excited at 475 nm (19, 20). We mimicked these dual excitation wavelengths by using multiphoton excitation at 800 nm and 900 nm, respectively. Thus, the fluorescence (emission measured at 515 nm) ratio of excitation at 800 nm and 900 nm was used to monitor the oxidation/reduction status of roGFP in cells (Fig. 1A). We generated adenovirus-associated virus (AAV, stereotype 2/8) expressing roGFP1 and infected cultured mouse neocortical neurons. To calibrate the sensitivity of roGFP1 to various cellular redox conditions, we incubated primary neurons with the reducing agent DTT, and the oxidant, 4,4’-dithiodipyridine (DTDP), at varying concentration for 30 min. In neurons, the roGFP1 ratio decreased from 0.98 ± 0.03 to 0.59 ± 0.01 with application of 10 mM DTT (n ≥ 246 neurons), whereas the ratio increased to 5.21 ± 0.15 (n ≥ 287 neurons) when treated with 30 μM DTDP (Fig. 1B). Thus, the sensitivity of roGFP1 to redox changes in cultured neocortical neurons is similar to the published reports (19, 20) and confirms our roGFP1-AAV construct serves as an accurate indicator for intracellular redox potential.

The sensitivity of roGFP1 was further examined in the somatosensory cortex of mice in vivo. Two months after AAV injection, we observed roGFP1 fluorescence in both apical tufts in layers I/II and neuronal soma in layers II–IV (Movie S1). DTT or DTDP were topically applied to the cortex, and fluorescence ratio changes of roGFP1 were examined. Similar to cultured neurons, the changes in roGFP1 ratio in both neuronal soma and neurites were correlated with DTT or DTDP treatment in the brain (Fig. S1A). The maximal ratio change using 2 mM DTDP increased the roGFP1 ratio from 1.01 ± 0.01 to 1.78 ± 0.04 (n ≥ 59 imaging sites from three mice); whereas 2 mM DTT decreased the resting roGFP1 ratio (0.90 ± 0.01, n ≥ 20 imaging sites from three mice). Although the dynamic range of roGFP is reduced in the living brain compared with in vitro preparations, as with other genetically encoded reporters (2), it remains a sensitive reporter of changes in redox potential. Thus, this approach provides a quantitative

Author contributions: H.X. and B.J.B. designed research; H.X. performed research; H.X. contributed new reagents/analytic tools; H.X., S.H., J.J., M.S., and J.K. analyzed data; and H.X. and B.J.B. wrote the paper. The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1217938110/-DCSupplemental.
functional readout of intracellular oxidative stress in cortical neurons in living mouse brain.

We next examined the intracellular redox status in APP/PS1 transgenic mice of both apical tufts in layers I/II and neuronal soma in layer II–IV. We used age-matched (7–9 mo) littermates as controls. To avoid the effects of senile plaques and address the question of whether soluble oligomeric Aβ species would result in oxidative stress in neurons in vivo, only cell bodies and neurites that were 20 μm or more away from plaques were measured in APP/PS1 mice. No significant differences in the average roGFP ratio was detected in either neuronal soma or neurites (Fig. S1B), suggesting that soluble Aβ did not significantly increase oxidative stress in most of the neurons in the APP/PS1 mice. Detailed analysis, however, showed that a small fraction of somas and neurites showed increased oxidation (R800/900 ≥ 1.5) in both control and APP/PS1 mice. Importantly, APP/PS1 mice showed many more neurons and neurites with increased redox potential than control mice: 0.13% neurons in control mice (Fig. 1C; n = 20,625 neurons from eight mice) and 0.42% neurons in APP/PS1 mice (Fig. 1C; n = 53,543 neurons from 13 mice) showed oxidative stress in the soma. A much larger fraction of neurites with elevated roGFP ratios in the brain were detected (Fig. 1D; 0.88%, in control mice, n = 1,469 neurites from six mice; 3.12% in APP/PS1 mice, n = 2,440 neurites from eight mice). Together, these results suggest that the increased levels of soluble Aβ in the APP/PS1 mice did not significantly alter the redox status in the majority of neurons, but led to the emergence of a small population of neuronal cell bodies and neurites in the brain with increased oxidative stress.

Next, the effect of individual senile plaques on oxidative stress was investigated in APP/PS1 mice. Strikingly, in contrast to the neurites away from Aβ plaques, the majority of neurites close to Aβ plaques (<20 μm) showed evidence of oxidative stress (64.9 ± 9.7%, Fig. 1E and F and Movie S2). Most of these oxidized neurites surrounding Aβ plaques formed dystrophic varicosities, which are likely to be axonal abnormalities (21). Oxidation in dystrophic neurites has been reported by using immunohistochemistry and includes lipid peroxidation and the accumulation of a variety of mitochondrial stress markers (22, 23). Furthermore, structural alteration of dendrites that included a beaded appearance was also observed in oxidized neurites near Aβ plaques (Fig. S2). The finding of intracellular oxidative stress localized to the immediate vicinity of plaques suggests that Aβ plaques might be a focal source of oxidative stress. In rare cases, we found oxidative stress in long neurite segments near plaques that extended up to 100 μm away from individual senile plaques (Fig. 1E; n = 9...
neurites from eight mice), suggesting a spatial influence much larger than the plaque itself.

To quantitatively analyze the spatial impact of Aβ plaques on oxidative stress, the roGFP ratio in concentric bands spaced at 4-μm intervals from the border of individual plaques was measured. The oxidative stress “burden” within the range of 16 μm from the boundary of plaques was significantly increased compared with that in the areas far from the plaques (n = 12 plaques from three mice; Fig. 1F). In contrast to Aβ plaques, cerebrovascular amyloid angiopathy showed no effects on the redox potential in both neuronal soma and neurites close to affected vessels (Fig. S3). Taken together, and in comparison with other brain areas, the rich accumulation of oxidized neurites surrounding Aβ plaques demonstrates the role of Aβ plaques as a central source for oxidative stress and accompanied neuritic degeneration.

We next sought to examine the cause of the oxidative stress. We topically applied the reducing agent DTT (10 mM, 30 min) to the cortex of APP/PS1 mice. We observed that the roGFP ratio in oxidized neurites decreased dramatically from 2.52 ± 0.01 to 1.56 ± 0.06 after DTT treatment (n = 6 plaques from three mice; Fig. S4A), suggesting that the cellular oxidation can be at least partially reversed with a strong reducing agent. This experiment also demonstrates that oxidation of the roGFP probe is reversible in vivo. Our previous reports demonstrated that N-tert-butyl-phenylnitrone (PBN), a free radical spin trap, could reduce the extracellular free radicals associated with Aβ plaques and reduce extracellular plaque associated oxidative stress (24). However, the cellular oxidation in both oxidized neurites and normal neurites was not affected by 1- to 4-wk treatment with PBN (100 mg/kg, i.p. daily; Fig. S4B), suggesting that extracellular free radicals associated with Aβ plaques might not contribute to the cellular oxidation in surrounding neurites or that PBN is not a strong enough antioxidant to overcome the intracellular stress.

Next, we examined the involvement of soluble Aβ aggregates in the generation of cellular oxidation. We imaged roGFP in primary neuronal cultures obtained from Tg2576 mice. At 14–18 d in vitro (DIV), we were not able to detect oxidative stress in neurites (Fig. S5A–C). We also applied conditioned media harvested from primary neuronal cultures derived from Tg2576 mice to naive neuronal cultures. We were not able to detect intracellular oxidative stress (Fig. S5D). roGFP was imaged in 2.5- to 3-mo-old APP/PS1 mice, at an age with soluble Aβ species, but before plaque deposition. Although rare examples of oxidized neurites were detected, there was no difference between transgenic and nontransgenic animals at this age (Fig. S6). Finally, we used the monoclonal antibody 3D6, recognizing amino acids 1–5 of Aβ, that has been shown to lead to the clearance of amyloid deposits in vivo (25, 26). We applied 3D6 directly to the cortical surface of APP/PS1 mouse brain and compared its effect acutely at 1 h and 1 wk after application. No significant changes in the roGFP ratio in both oxidized and normal neurites (Fig. S4C) were observed at either time point. Although it is inappropriate to overinterpret this negative result, it is known that 3D6 binds to both soluble and aggregated Aβ, and we would predict that acute application would “neutralize” all species of amyloids to some extent. Together with the observation that the average cellular redox potential far from plaques was not elevated in APP/PS1 mice, these results further suggest that soluble Aβ in the APP/PS1 mouse brain may not directly induce cellular oxidative stress. Thus, clearance of extracellular Aβ by 3D6 or reactive oxygen species by PBN did not rescue the cellular oxidative stress in neurites surrounding Aβ plaques in APP/PS1 mouse. This non-rescue event suggests that once the redox potential is increased within cells that it no longer needs the continued presence of Aβ, and is severe enough that extracellular antioxidants are ineffective at buffering the oxidative stress. This non-rescue event implies that prevention therapies will be more effective than treatment therapies or that longer durations of treatment will be necessary.

We next explored the relationship between the generation of Aβ plaques and oxidative stress by injecting AAV-roGFP in younger (4-mo-old) APP/PS1 mice, when Aβ plaques begin to accumulate. Cranial windows were implanted shortly (2 wk) after the injection of virus and mice were imaged 2 wk after surgery. Thus, imaging sessions began at 5 mo of age and were repeated monthly (Fig. 2B). First, we found that the cellular oxidative stress in neurites surrounding Aβ plaques propagated to larger areas month by month (Fig. 2A). The oxidized neurites surrounding Aβ plaques survived for weeks, in some cases, longer than 1 mo (Fig. 2F and Fig. S7B). By following images of 30 Aβ plaques with oxidized neurites longitudinally, we found that in the APP/PS1 mice, the total...
amount of oxidized neurites increased over sevenfold within 5 mo (Fig. 2B). Imaging over these long intervals also revealed a tendency for some plaques to increase in size (Fig. 2B). Fig. 2C reflects the observation that the number of oxidized neurites increased with the size of amyloid plaques. Interestingly, during the 5 mo of imaging, Aβ plaques always preceded the detection of oxidative stress in surrounding neurites (Fig. 2D and Fig. S7A). A total of 33 newly formed Aβ plaques were identified in four mice. The time delay between the emergence of Aβ plaques and the oxidation of surrounding neurites was approximately 1–3 mo (Fig. 2D). Finally, during the accumulation of plaques, we were surprised to observe neuronal death in rare situations (n = 6 cells from four mice) in areas adjacent to Aβ plaques, which were surrounded by oxidized neurites (Fig. 2E).

We looked at this neuronal death more closely, using longitudinal imaging over a 24-h period. We stereotaxically injected AAV-roGFPI in 5-mo-old transgenic mice. A cranial window was implanted 1 mo later, and mice were allowed to recover for 1 mo after the surgery before the beginning of imaging. In the initial imaging session, we observed a small fraction of neurons with an increased redox potential (R<sub>800/900</sub> ≥ 1.5) in both soma and surrounding dendrites (n = 34 neurons from five mice) (Fig. 3A and B) as described above. At subsequent time points, we observed that in those identified neurons, the roGFPI ratio increased from 1.86 ± 0.05 to 2.98 ± 0.15 within 2 h; 38.24% of those oxidized neurons died within 6 h; 97.06% of which died within 24 h (Fig. 3B), whereas none of the neurons with low redox potential indicated by roGFPI1 disappeared. This result demonstrates a strong correlation between oxidative stress and neuronal death. Interestingly, we observed that some neurons (n = 13) first showed morphological alterations in dendrites followed by the formation of apoptotic body-like structures in the soma at approximately 4–6 h after oxidation was detected (Fig. 3A and Fig. S8A). As a control, the remaining neurons with a roGFPI ratio of 1.01 ± 0.01 (n ≥ 11,334 neurons from five mice) did not show any changes within 24 h (Fig. 3B).

To further confirm the death of the oxidized neurons, the nuclear dye Hoechst 33342 was topically applied to the mouse brain. We found that in the oxidized neurons (n = 18 from four mice), nuclear condensation detected with Hoechst 33342 was found 9 h after the detection of oxidative stress in the soma (Fig. 3C). After 24 h, the condensed nucleus disappeared. In contrast, in neurons without oxidative stress (n ≥ 8,000 from four mice), we never observed nuclear condensation or loss within the same 24-h period (Fig. 3C).

To exclude the phototoxic effects of repeated laser scanning in the generation of oxidative stress and the acceleration of cell death, we minimized the imaging sessions to only image at 12 or 24 h (Fig. S8B). Consistent with previous results, all of the neurons (n = 14 neurons from four mice) identified with an increased redox potential proceeded to die within 24 h. To further exclude the surgical procedure as a source of oxidative stress, we imaged six mice at 0, 1, 2, and 4 mo after implanting the cranial window. Despite this large range of recovery times after the surgical procedure, the frequency of neurons identified as exhibiting an increased redox potential measured with roGFPI1 remained constant (Fig. S9).

Next, we asked whether the neuronal death that was preceded by an increased redox potential followed a pathway of programmed cell death. To this end, a fluorescent indicator of caspase activation (FLICA) (27) was applied topically to the cortex of the brain in vivo and imaged with multiphoton microscopy. We found that most oxidized neurons were FLICA positive (39 of 44), suggesting a caspase-dependent programmed cell death in most of the neurons with oxidative stress (Fig. 4A; n = 8 mice). The five neurons without oxidative stress showed no increase in FLICA positive neurons after 4 h (Fig. 4B).

Fig. 3. Oxidative stress precedes neuronal death in living APP/PS1 transgenic mice. (A) Time-lapse images showing oxidative stress preceding neuronal death in an APP/PS1 mouse. Arrowheads show the dynamics of oxidative stress and neuronal death. Arrowheads show oxidative stress in the neuronal somas and neurites, both of which became fragmented around 4 h later. (B) Plot (Upper) shows the time course of neuronal death of oxidized neurons (n = 34 neurons) and nonoxidized neurons (n ≥ 11,334 neurons from five mice). Column bar (Lower) shows the percentage of cell died with 24 h in both oxidized and nonoxidized neurons (n = 34 mice). (C) Loss of neurons after the appearance of oxidative stress (n = 18 neurons from four mice). Red circle shows the occurrence of oxidative stress. Note that the loss of roGFPI signal is accompanied with nuclear condensation at 12 h, and the same nucleus has disappeared within 24 h. (Scale bars: A, 30 μm; C, 20 μm.)
oxidized neurons that were FLICA negative recovered within 1 d with the roGFP ratio returning to normal levels, confirming that it is the activation of caspases that leads to ultimate cell death. With more frequent imaging, we found examples of caspase activation that preceded the oxidative stress by ∼1 h (Fig. S10A; n = 6 neurons from three mice), but we interpret this delay of observed oxidative stress as a limitation of the speed and sensitivity of the roGFP1 reporter (19, 28). Furthermore, caspase activation could persist along with the increased oxidative stress in the soma for up to 12 h with neuronal death being the ultimate outcome (Fig. S10B; n = 21 neurons from three mice). Taken together, we identified oxidative stress in a small number of neurons (~0.4% of total neurons), the majority of which were undergoing apoptotic (caspase-dependent) programmed cell death within 24 h. Interestingly, no evidence for caspase activation (FLICA) was detected in the persistently oxidized neurites near plaques, which suggests that oxidation in the neurites alone was not sufficient for caspase activation and subsequent cell death (Fig. 4B).

Discussion

Longitudinal in vivo imaging using roGFP in APP/PS1 transgenic mice allowed the determination of the temporal relationship among Aβ plaque deposition, oxidative stress, and cell death. Our data demonstrate that plaques precede (∼1–3 mo) and lead to oxidative stress in surrounding neurites (Fig. 4C). The oxidative stress in neurites surrounding Aβ plaques propagates spatially over time, which ultimately leads to oxidation in neuronal soma. In contrast to the oxidized neurites, which survive for several weeks, oxidation in neuronal soma was associated with caspase-dependent apoptosis. Degeneration of neuronal soma was rapid and occurred within 24 h once the cellular oxidation propagated into the cell body and triggered caspase activation (Fig. 4C). These results implicate Aβ as the mediator of oxidative stress and subsequent neurodegeneration; however, it does not necessarily demonstrate that Aβ has a direct effect on neuronal toxicity. It is possible that intermediate cellular players, including astrocytes or microglia, respond to amyloid deposits with chemokine or cytokine signaling that, in turn, leads to oxidation in neurites.

Our experiments do not support a role of soluble, oligomeric Aβ in the generation of intracellular oxidative stress. Acute experiments in primary neuronal cultures, in young mice before plaques are present, or in brains treated with an anti-Aβ antibody failed to implicate nonfibrillar amyloid as a mediator of oxidative stress. These results, however, should be tempered with the caveats that the concentration and duration of soluble amyloid exposure may have been insufficient. Our chronic imaging experiments demonstrated that oxidative stress builds slowly in neurites in the proximiy of senile plaques and eventually propagates to neuronal cell bodies.

Using in vivo imaging in a transgenic mouse model, we followed the death of individual neurons in real time. Although these results should be verified in other transgenic mouse models, we were able to identify vulnerable neurons by an increase in redox potential in the cell soma. Thus, we show direct evidence of the involvement of cellular oxidative stress in neuronal degeneration in living animals. Although the number of cells that died were a small fraction of neurons in the brain, the use of the roGFP indicator allowed us to identify and monitor the time course of cell death even though these events were rare. The delineation of this sequence of events leading to neuronal death highlights several potential avenues for therapeutic intervention to prevent or reverse the progression of AD. An effective treatment will likely include a combination of drugs that reduce oxidative stress and prevent amyloid accumulation. Our results also suggest that a preventative approach will be
more effective than a treatment approach. Additionally, it is likely that the mechanisms of cell death are common to other neurodegenerative diseases or CNS disorders, allowing a generalization of neuroprotective strategies.

Materials and Methods

Animals and Surgery. Animal experiments were performed under the guidelines of Institutional Animal Care and Use Committee (IACUC, protocol 2004N0000047). We used APP/PS1 double transgenic mice expressing mutant human APPswe/human PS1-ΔE9 along with age-matched nontransgenic littermate controls (29). Four- to 6-month-old mice received intracranial injections of roGFp-AAV (sterotype 2/8) and cranial window implantation 2 wk, 1 mo, or 2 mo later. Intracranial injections and cranial window implantation have been described (26, 30). Time-lapse images of roGFp were taken monthly. For surgeries and imaging sessions, mice were anesthetized with 1.5% (vol/vol) isoflurane.

Neuronal Culture Preparation. Primary neuronal cultures were prepared as described (2). In brief, mixed cortical neurons were generated from CD1 or Tg2576 at embryonic day 15, and plated 15 days later. The neurons were maintained in Neurobasal medium containing 2% B27 supplement (Invitrogen) for 6 DIV before roGFp-AAV infection.

roGFp-AAV. roGFp1 (C48S/T65S/S147C/Q204C), obtained from J. Remington (University of Oregon, Eugene, OR), was introduced into the construct pAAV-CBA-roGFp1-woodchuck hepatitis virus posttranscriptional regulatory element (WPRE) (30). The plasmid contained the AAV terminal repeats (TRs), the only remaining feature of the wild-type AAV genome. Flanked by the TRs, the expression cassette included the following components: (i) a 1.7-kb sequence containing hybrid cytomegalovirus immediate-early enhancer/chicken β-actin promoter/exon1/Intron; (ii) roGFp1; and (iii) WPRE. The virus titer was 1.0 × 10^{12} viral genomes per milliliter.

Labeling Procedures. Methoxy-XO was injected (4 mg/kg i.p.) 1 d before the imaging to label amyloid plaques. To facilitate image alignment from section to section, Dextran Texas Red (Dex Red; 70,000 molecular weight; Invitrogen) was injected into a lateral tail vein to create a fluorescent angiogram. Caspase indicator (FLICA; Invitrogen) was applied topically (5x FLICA solution in sterile PBS) before sealing the cranioptery with a coverslip. Hoechst 33342 (10 μg/ml; Invitrogen) was also applied topically to label nuclei.

In Vivo Imaging. Images of roGFp expressing neurons, amyloid pathology, Texas Red Dextran filled angiograms, FLICA, and Hoechst staining were obtained by using one of two microscopes: (i) Bio-Rad 1024ES multiphoton microscope (Bio-Rad), mounted on an Olympus BX50WI upright microscope; (ii) Olympus Fluoview 1000MPE with preir optical and a fast AOM mounted on an Olympus BX51WI upright microscope. A wax ring was placed on the edges of the coverslip of the cortical window and filled with distilled water to create a well for an Olympus Optical 20x dipping objective (numerical aperture, 0.95). A mode-locked titanium/sapphire laser (Tsunami; Spectra-Physics) generated two-photon fluorescence with 700- to 960-nm excitation, and three photomultiplier tubes (Hamamatsu) collected emitted light in the range of 380–480, 500–540, and 560–650 nm (26). Neurites were typically sampled 0–100 μm below the surface of the brain, and cell soma were sampled 100–500 μm below the surface. With single-photon excitation, roGFp has been reported to have two absorbance maxima at ~395 and 475 nm. We mimicked these two excitation wavelengths by using multiphoton excitation at 800 nm and 900 nm, respectively. Thus, the fluorescence (emission measured at 515 nm) ratio of excitation at 800 nm and 900 nm was used to monitor the oxidation/reduction status of roGFp. The excitation power of each wavelength was adjusted so that the resting ratio of live cells in vitro and in vivo was ~1. Overall, the R800/900 was approximately 1.0 in both wild-type and transgenic mice.

Image Analysis. Three-dimensional image stacks were processed by using ImageJ and IDL software (Research Systems). The boundary of dense-core plaques was determined as the position in which the fluorescence intensity was the half-maximum of methoxy-XO fluorescence intensity. Data are reported as mean ± S.E. Statistical significance was determined by Student’s t test, ANOVA or χ2 test where appropriate. Images presented in the figures are single slices or 2D maximum intensity image projections of the 3D volumes.

ACKNOWLEDGMENTS. We thank James Remington (University of Oregon) for providing the roGFp construct (proGFp-N1). This study was supported by National Institutes of Health Grants AG024688 (to B.J.E.), EB000708 (to B.J.E.), and NCSF31100776 (to H.X.).

Supporting Information

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Fig. S1. Redox characteristics of redox-sensitive variants of the green fluorescent protein (roGFP) and distribution of roGFP ratio in neuronal soma in living mouse brain. (A) Redox characteristics of roGFP in living mouse brain. roGFP was calibrated in vivo by using oxidant and antioxidant compounds applied topically to the brain. (A Left) Representative images show ratiometric changes in redox potential after addition of various concentrations of dithiothreitol (DTT) and 4,4′-dithiodipyridine (DTDP) (n ≥ 20–59 imaging sites from 3 mice for each condition) (Scale bar: 15 μm.) (A Right) The summary data for each treatment. (B) Distribution of roGFP ratio in neuronal soma in living mouse brain. Increased oxidation (R_{800/900} ≥ 1.5, red bar) in amyloid precursor protein (APP)/presenilin 1 (PS1) transgenic mice is compared with control wild-type mice (n = 20,625 neurons from 8 control mice and n = 53,543 neurons from 13 APP/PS1 mice).

Fig. S2. An example of amyloid β (Aβ) plaque caused morphological changes and oxidative stress in nearby dendrites. A dendrite crossing above an Aβ plaque exhibited oxidation simultaneously with a beaded morphology in the part close to the plaques. White masks in Left and Center show a plaque right under the oxidized (green color) dendrite; white mask in Right shows the magnified dendrite. (Scale bar: 24 μm.)
Fig. S3. Cerebrovascular amyloid angiopathy (CAA) did not result in oxidative stress in surrounding neuritis. CAA did not affect redox status in surrounding neurites \((n \geq 3\) mice). (Scale bar: 30 μm.) n.s., nonsignificant.

**Fig. S4.** Effects of DTT, N-tert-butyl-phenylnitrone (PBN), and 3D6 on the oxidized neurites surrounding Aβ plaques. (A) Reduction of oxidative stress in neurites surrounding Aβ plaques by DTT (10 mM, 30 min) treatment \((n = 6\) plaques from three mice). (B) One week of daily injection or 4 wk of every other day injection of PBN, a free radical spin trap, did not rescue the oxidative stress in neurites surrounding Aβ plaques \((n = 46\) plaques from four mice). (C) 3D6, an anti-Aβ antibody, did not rescue the oxidative stress surrounding Aβ plaques \((n = 12\) plaques from three mice). (Scale bars: A and C, 12 μm; B, 16 μm.)
Fig. S5. Oxidative stress in primary neuronal cultures. Primary neuronal cultures from APP/PS1 mice at 14 days in vitro (DIV) (A), and 18 DIV (B) or primary cultures from Tg2576 mice at 14 DIV (C) did not show oxidative stress as measured with roGFP (n ≥ 162 cells from ≥ 3 cultures). (D) Conditioned media harvested from Tg2576 cultures (tgCM) or from WT control cultures (wtCM) at 14 DIV was applied to wt control cultures and did not cause oxidative stress change (n ≥ 163 cells from ≥ 3 cultures).

Fig. S6. Oxidative stress in young (2.5- to 3-mo-old) mice. roGFP ratios were measured in young APP/PS1 mice (2.5–3 mo old), an age that precedes plaque deposition. Rare examples of oxidized cell bodies (Left) and neurites (Right) were observed, however, no differences between wild-type (wt) and transgenic (tg) mice were detected (n = 3 mice).

Fig. S7. Example of Aβ plaques preceded the occurrence of oxidative stress in surrounding neurites and example of an oxidized neurite with long-term survival. (A) Example of Aβ plaques preceded the occurrence of oxidative stress in surrounding neurites. Arrows show the oxidized neurites formed 5 mo after the appearance of Aβ plaques. (B) Example of Aβ plaques preceded the occurrence of oxidative stress in surrounding neurites. Arrowheads showing an oxidized neurite that survived at least 1 mo. (Scale bars: A, 30 μm; B, 16 μm.)
Fig. S8. Examples of formation of apoptotic body-like structure in oxidized neurons and time course of death of oxidized neurons imaged longitudinally. (A) Formation of apoptotic body-like structure was observed at approximately 6 h after the oxidative stress occurred in neuron soma (green color, \( n = 13 \) cells from four mice). All detected neurons with elevated redox potential died within 24 h. (B) Examples of time course of death of oxidized neurons imaged longitudinally. Arrowheads show the dynamics of oxidative stress and neuronal death. Once oxidative stress occurred in the soma, all of the neurons except one died within 1 d (\( n \geq 48 \) neurons from eight mice). Thus, repeated laser scanning did not accelerate the process of neuron death. Unlike somas, the highly oxidized neurite (arrow in Bottom) surrounding plaques were observed for a few days and even weeks. (Scale bars: 30 \( \mu m \)).

Fig. S9. The number of neurons detected with elevated redox potential did not depend on the interval after the cranial surgery, demonstrating that the surgery per se did not lead to oxidative stress and subsequent neuronal death.
Fig. S10. Examples of caspase activation co-occurred with oxidative stress. (A) Caspase activation [fluorescent indicator of caspase activation (FLICA) positive] preceded oxidative stress in some neuronal cell bodies (0 h). At subsequent time points, oxidative stress was detected in the FLICA positive neuron \((n = 6)\) neurons from three mice). (B) Continued caspase activation was maintained while oxidative stress in a neuron cell body developed over time \((n = 21)\) neurons from three mice). (Scale bars: 30 μm.)

Movie S1. Example of images in a z-stack of AAV-roGFP in living APP/PS1 transgenic mouse brain. Z-stack of roGFP from cortical surface to a depth of 300 μm in a living mouse brain. Red, excited at 900 nm; green, excited at 800 nm; blue, methoxy-XO4 stained Aβ plaques; gray, Texas Red dextran (70 kDa) angiogram; red circles, oxidized neurons.

Movie S1
Movie S2. Three-dimensional reconstruction of an Aβ plaque surrounded by oxidized neurites in APP/PS1 transgenic mouse. Red, excited at 900 nm; green, excited at 800 nm; blue, methoxy-XO4 stained plaques.

Movie S2