



A peephole into the brain: neuropathological features of Alzheimer's disease revealed by *in vivo* two-photon imaging

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Alzheimer's disease (AD) is a protein conformational disorder characterized by two major neuropathological features: extracellular accumulations of amyloid- β peptides in the form of plaques and intracellular tangles, consisting of hyperphosphorylated tau proteins. Several morphological and functional changes are associated with these lesions in the diseased brain, such as dendritic and synaptic alterations, as well as microglial and astroglial recruitment and their activation. The availability of transgenic mouse models that mimic key aspects of the disease in conjunction with recent advances in two-photon imaging facilitate the study of fundamental aspects of AD pathogenesis and allow for longitudinally monitoring the efficacy of therapeutic interventions. Here, we review the ambitious efforts to understand the relationship between the main neuropathological hallmarks of AD and their associated structural and functional abnormalities by means of *in vivo* two-photon imaging.

Keywords: amyloid plaques, neurofibrillary tangles, dendritic spines, glial cells, multiphoton imaging, microglia, calcium imaging, CAA

INTRODUCTION

Longevity is a long-cherished dream of mankind, but the downside of an aging population is currently one of the big challenges society is facing. Alzheimer's disease (AD), as the most common form of dementia in the elderly, currently affects ~35 million people worldwide (Prince et al., 2011). It is a fatal, progressive, neurodegenerative disease characterized by memory impairment and cognitive decline. When Alois Alzheimer first described the disease in 1906/07 (Alzheimer, 1907), he had already identified the pathological hallmarks that still, to this day, constitute the major diagnostic criteria for AD (Jack et al., 2011). The most prominent of which are senile plaques, representing extracellular proteinaceous aggregates that mainly consist of the amyloid- β peptide (A β ; Glenner and Wong, 1984; Masters et al., 1985). A β is derived from the sequential cleavage of the amyloid- β precursor protein (APP) by the β - and γ -secretases (Haass, 2004; Haass and Selkoe, 2007; Lichtenthaler et al., 2011). Neurofibrillary tangles (NFT) constitute the second major hallmark of AD (Kosik et al., 1986; Nukina and Ihara, 1986). These intracellular aggregates of the hyperphosphorylated microtubule-associated protein tau form intrasomal inclusions as well as neuropil threads, and are thought to be causally related to neuronal apoptosis (Dickson, 2004; Spires-Jones et al., 2009b; Wakasaya et al., 2011).

Interestingly, although plaques and tangles pathologically define the disease, it is the loss of synapses that correlates strongly with the dementia typical of AD (Masliah et al., 1991; Terry et al., 1991; Scheff et al., 2006). Research over the past decade has thus focused on the mechanisms causing synaptic deterioration, including the abnormal accumulation of A β and tau. The

resulting decrease of synapse density is believed to eventually lead to neuronal network disruption and cognitive decline.

Traditional histological analyses of affected brains only allow for a static assessment of pathology. The advent of two-photon microscopy (Denk et al., 1990) has enabled *in vivo* long-term imaging studies (Holtmaat et al., 2009), from which the field of AD research has benefited tremendously. *In vivo* imaging of transgenic mice through a cranial window not only allows to monitor general neuropathological features of AD-like lesions (Table 1; Figure 1), but also to address questions regarding the kinetics and temporal sequence of events (Figure 2; Bacskai et al., 2001; Spires et al., 2005; Spires-Jones et al., 2011). Two-photon microscopy furthermore facilitates direct observation of the effects of new treatment approaches. Although none of the mouse models available to date perfectly recapitulates the human AD neuropathology in its entirety, they still represent a valuable tool to precisely study key aspects of the disease, such as the occurrence and growth of amyloid plaques, the onset and progression of dendritic spine pathology, or the induction of neuronal apoptosis (Radde et al., 2008).

We here review some of the key findings and concepts that have emerged from the rapidly growing body of literature, on structural and functional alterations in transgenic mouse models of AD as revealed by *in vivo* two-photon microscopy.

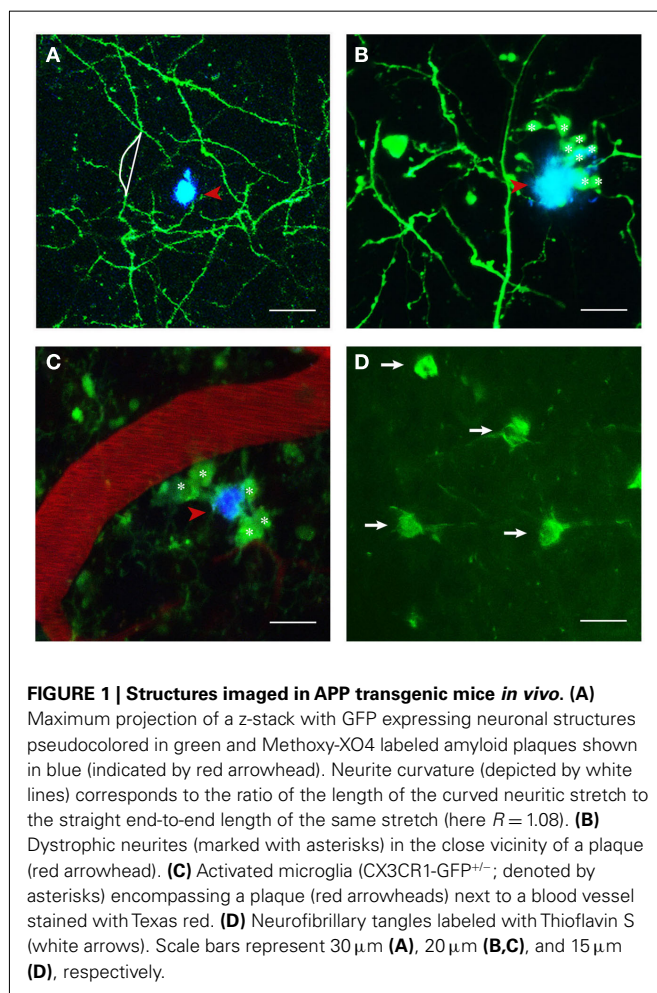
STRUCTURAL *IN VIVO* IMAGING

IMAGING AMYLOID DEPOSITS IN APP TRANSGENIC MICE

Amyloid- β plaques have been studied extensively as one of the major hallmarks of AD, and as a consequence many treatment

Table 1 | Structures analyzed in mouse models of Alzheimer's disease by *in vivo* two-photon microscopy.

Structure analyzed	Transgenic mouse model	Dyes	Reference
Amyloid- β plaques	APP _{swe} /PS1 _{dE9} ,	Thioflavin S,	Christie et al. (2001b), Bolmont et al. (2008), Meyer-Luehmann et al. (2008), Yan et al. (2009), Burgold et al. (2011), Hefendehl et al. (2011)
Cerebral amyloid angiopathy	APP _{swe} /PS1 _{L166P} , PDAPP, Tg2576	Methoxy-XO4	Garcia-Alloza et al. (2006b), Robbins et al. (2006), Prada et al. (2007)
Neurofibrillary tangles	APP _{swe} /PS1 _{dE9} , PDAPP, Tg2576	Thioflavin S, Methoxy-XO4	
Neurite structures (dendritic spines, dystrophic neurites, neurite curvature)	rTg4510	Thioflavin S	Spires-Jones et al. (2008), de Calignon et al. (2009), de Calignon et al. (2010)
Microglia	GFP-M, YFP-H	–	Tsai et al. (2004), Spires et al. (2005), Grutzendler et al. (2007), Spires-Jones et al. (2007), Meyer-Luehmann et al. (2008), Bittner et al. (2010), Jung et al. (2011)
	CX3CR1-GFP, Iba1-GFP	–	Bolmont et al. (2008), Koenigsknecht-Talboo et al. (2008), Meyer-Luehmann et al. (2008), Fuhrmann et al. (2010), Liu et al. (2010)

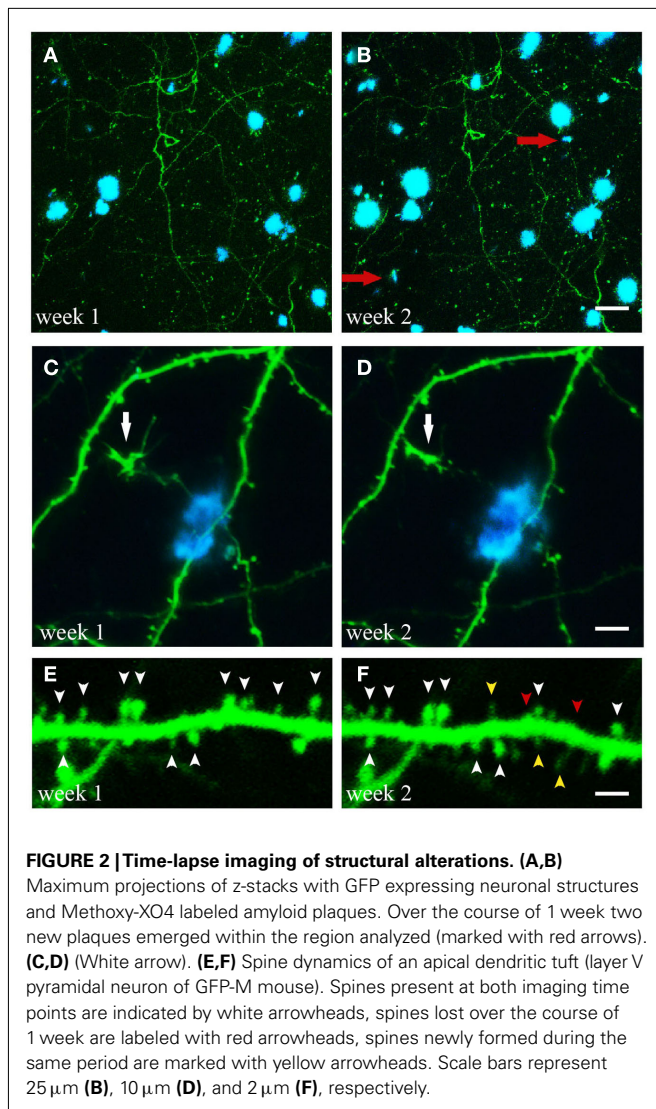


studies are aimed at reducing the formation of plaques (Shah et al., 2008; Chopra et al., 2011; Kurz and Perneczky, 2011). Extracellular amyloid plaques mainly consist of aggregated A β peptides that are derived from a larger APP. To release A β , APP is first cleaved at its ectodomain by β -secretase, and subsequently is cleaved intramembranously by γ -secretase (Haass, 2004; Steiner, 2004; Lichtenthaler et al., 2011). Depending on the

exact cleavage site, the released A β peptide can vary in length, with longer A β peptides being more prone to aggregation (Yan and Wang, 2006). According to *in vitro* models, A β aggregation follows a nucleation-polymerization model, where A β monomers form higher molecular-weight oligomers and protofibrils, before they finally aggregate into insoluble amyloid plaques (Harper and Lansbury, 1997).

In order to monitor amyloid plaques *in vivo*, dyes binding to beta-pleated sheet protein structures, such as Thioflavin S or Methoxy-XO4, are applied either topically or peripherally, with the latter agent being able to cross the blood-brain-barrier (Christie et al., 2001b; Klunk et al., 2002; Bacskai et al., 2003). As opposed to dyes binding to beta-pleated sheet protein structures, which only allow for the visualization of the compact forms of plaques, fluorescently labeled antibodies against A β additionally mark diffuse plaques.

Crucial aspects addressed by longitudinal two-photon imaging are the process of *de novo* plaque formation as well as the kinetics of cerebral amyloidosis. The appearance of new plaques is a rare, but rapid event that is age-dependent (Meyer-Luehmann et al., 2008; Burgold et al., 2011; Hefendehl et al., 2011; Figures 2A,B). Previous studies assessing the kinetics of plaque growth have yielded somewhat inconsistent findings. Whereas some studies reported no significant change in plaque size when imaged over the course of 14 days (Meyer-Luehmann et al., 2008) and up to 150 days (Christie et al., 2001b), others found an increase in plaque size only for small plaques (<10 μm radius) over the course of 4 weeks (Bolmont et al., 2008). More recent studies observed growth for all plaques analyzed, and reported on either growth rates independent of plaque size (Hefendehl et al., 2011) or a higher growth rate for newly deposited (i.e., small) compared to pre-existing plaques (Yan et al., 2009; Burgold et al., 2011). Factors potentially contributing to these inconsistencies could be of biological or methodological nature. Among others, the type of the transgenic mouse model as well as sex and age of the animals differed between the studies (Table 2). Furthermore, the dye applied in order to label amyloid plaques, Methoxy-XO4, is in itself known to interfere with the process of plaque deposition (Cohen et al., 2009). Therefore the imaging frequency, i.e., the frequency of dye re-application, is likely to contribute to the variability in observed plaque growth (Burgold et al., 2011).



It has also been suggested that the type of cranial window, used to perform *in vivo* imaging, might be an additional confounding variable. Yan et al. (2009) compared thinned – vs. open skull preparations and only detected significant plaque growth with the thinned skull preparation. Two recent studies, however, observed clear plaque growth with the open skull preparation, suggesting the equivalence of both approaches (Burgold et al., 2011; Hefendehl et al., 2011). Furthermore it should be mentioned that image processing and the applied detection stringency, i.e., the set detection threshold, can also affect the results. Notably, plaque pathology reaches a ceiling phase, lacking apparent growth, and *de novo* plaque formation (Burgold et al., 2011). In summary, longitudinal *in vivo* two-photon imaging revealed the rapid appearance and slow, but steady growth of plaques with varying growth rates influenced by such factors, as the transgenic mouse models, age of the animals, initial size of plaques (pre-existing vs. newborn plaques), or type of cranial window. These studies shed light on the kinetics of plaque pathology progression, thereby providing basic knowledge for further therapeutic intervention studies.

In addition to amyloid plaques, extracellular A β deposits often occur within the walls of cerebral and leptomeningeal blood vessels, called cerebral amyloid angiopathy (CAA). CAA results in a loss of vascular smooth muscle cells and induces microhemorrhages (Van Dorpe et al., 2000; Christie et al., 2001a; Winkler et al., 2001; Fryer et al., 2003). Many of the APP transgenic mouse models develop not only A β deposits in the parenchyma but also CAA (Calhoun et al., 1999; Van Dorpe et al., 2000; Christie et al., 2001a; Robbins et al., 2006). However, some APP transgenic mice, e.g., APP_{swe}/PS1_{dE9}, are not an ideal model to study CAA, as these mice develop CAA at a much slower rate when compared to other APP_{swe} transgenic mice (Garcia-Alloza et al., 2006b).

The temporal progression of CAA was first approximated by imaging postmortem intact whole brains of transgenic mice of different ages, revealing a consistent, and stereotyped age-dependent spatial distribution of CAA (Domnitz et al., 2005). A far more precise approach to analyze the kinetics of CAA progression is longitudinal two-photon imaging of the same individual vessel segment. Thus *in vivo* imaging of APP transgenic mice at weekly intervals, starting at the age of 8–11 months, showed that the emergence of CAA is a multifocal event in form of band-like A β deposits (Robbins et al., 2006). Furthermore, in contrast to the accumulation of amyloid plaques in the parenchyma, CAA progression tends to propagate from already existing deposits (Robbins et al., 2006). Therapeutic interventions, such as passive immunization, was able to halt CAA progression and even to reduce vascular amyloid in APP transgenic mice, as demonstrated by immunohistochemical analysis (Schroeter et al., 2008; Catepoel et al., 2011). While chronic antibody treatment over 14 days monitored by two-photon microscopy resulted in a robust CAA regression, a single antibody exposure only led to moderate CAA regression, thus corroborating histological studies (Prada et al., 2007).

IMAGING NEURITES IN THE VICINITY OF AMYLOID PLAQUES

Albeit pathologically defining the disease and being a diagnostic criterion, the occurrence of amyloid plaques only poorly correlates with the cognitive decline characteristic of AD (Terry et al., 1991; Guillozet et al., 2003). A much better correlate is the loss of synapses (Terry et al., 1991; Masliah et al., 1994). Amyloid plaques are associated with numerous structural and functional pathological features, such as neurite curvature (D'Amore et al., 2003; Garcia-Alloza et al., 2006a; Meyer-Luehmann et al., 2008; Figure 1A) and breakage (Tsai et al., 2004; Figures 2C,D), neuritic dystrophies (D'Amore et al., 2003; Tsai et al., 2004; Figure 1B), synapse loss (Tsai et al., 2004; Spires et al., 2005), accumulation of glial cells (Bolmont et al., 2008; Figure 1C), and altered neuronal response properties (Busche et al., 2008). Conventional immunohistochemical techniques have been extensively used to elucidate the spatial relationship between amyloid plaques and synaptic markers and found a decrease in synaptic density, both in AD patients and in transgenic animal models, with synapse loss being most pronounced in the immediate vicinity of amyloid plaques (DeKosky et al., 1996; Grutzendler et al., 2007; Knobloch and Mansuy, 2008; Penzes et al., 2011). These studies however do not allow for a detailed analysis of the dynamics of synaptic changes (reviewed by Giannakopoulos et al., 2009).

Table 2 | Studies analyzing amyloid plaque formation and growth *in vivo*.

Reference	Transgenic mouse model	Sex, age at first imaging session	Observation period	Dye	Type of cranial window	Main finding
Christie et al. (2001b)	Tg2576	♂, 18.6 months	2–150 days	Thioflavin S (0.005% in ACSF)	Thinned skull	Plaque growth arrest, plaque size unchanged
Meyer-Luehmann et al. (2008)	APP _{swe} /PS1 _{dE9} X _{YFP}	♂, ♀, 5–6 months	14 days	Methoxy-XO4 (5 mg/kg)	Open skull	Rapid plaque formation (within 24 h), stable plaque size up to 2 weeks
	Tg2576	♂, ♀, 11 months	14 days			Rapid plaque formation within 1 week
	PDAPPx _{CX3CR1} -GFP	♂, ♀, 18 months	3 days			Rapid plaque formation within 24 h
Bolmont et al. (2008)	APP _{swe} /PS1 _{L166P} × lba1-GFP	♂, ♀, 3–4 months	1 month	Methoxy-XO4 (10 mg/kg)	Open skull	Stable plaque volume, difference between size categories: small plaques (radius < 10 μm) grew (by 84%), large plaques (radius > 15 μm) slightly shrank (by 12%)
Yan et al. (2009)	APP _{swe} /PS1 _{dE9}	♂, ♀, 6 and 10 months	7, 28, 90 days	Methoxy-XO4 (5 mg/ml)	Thinned skull	6 months: plaque growth (growth rate: small > large plaques); 10 months: no plaque growth
		Open skull			6 months: no plaque growth	
Burgold et al. (2011)	Tg2576x _{YFP}	♂, ♀, 12 and 18 months	6 weeks	Methoxy-XO4 (loading dose 2.0 mg/kg, maintenance dose 0.4 mg/kg)	Open skull	12 months: growth rate inversely proportional to volume; 18 months: lack of <i>de novo</i> plaque formation and plaque growth
Hefendehl et al. (2011)	APP _{swe} /PS1 _{L166P} × lba1-GFP	♂, ♀, 3–4 months	6 months	Methoxy-XO4 (~5 mg/kg)	Open skull	Peak of <i>de novo</i> plaque formation at 4–5 months, similar growth rates for newly formed and pre-existing plaques

In vivo two-photon microscopy has therefore been employed to unravel the temporal sequence of pathological events as well as their dynamics. To this end, mouse models of AD were crossbred with mice expressing variants of GFP in neuronal subsets, such as the GFP-M or YFP-H line (Feng et al., 2000; Tsai et al., 2004; Meyer-Luehmann et al., 2008). Fluorophore expression in these mice is regulated by the Thy-1 promoter, which in the cerebral cortex results in a sparse labeling of mainly layer V neurons (Feng et al., 2000). Alternatively, neuronal structures can be labeled by the injection of viral constructs, inducing fluorophore expression (Spires et al., 2005).

Since A β is known to bind to molecular components of the postsynapse (NMDA-, EphB receptors; Lacor et al., 2007; Cisse et al., 2011), imaging studies primarily focused on the analysis of dendritic spines, which are the main site of excitatory synaptic input to cortical pyramidal cells (Tsai et al., 2004; Spires et al., 2005; Spires-Jones et al., 2007). Typically, the distal tufts of apical dendrites of layer V pyramidal neurons are repeatedly imaged. Despite being relatively stable in adulthood when compared to development, dendritic spines are generally flexible and motile structures, thereby allowing for continuous remodeling of neuronal circuits (Majewska et al., 2006; Bhatt et al., 2009). Even under baseline conditions (i.e., without any particular sensory deprivation paradigm, often employed to study synaptic plasticity) a certain fraction of

spines is constantly added and removed, while overall spine density remains mostly unaffected (Trachtenberg et al., 2002; Holtmaat et al., 2005, 2009; see however Hofer et al., 2009).

In APP_{swe}/PS1_{M146L} mice, Tsai et al. (2004) found an ongoing enhanced spine formation and elimination within the peri-plaque region (i.e., within 15 μm of the plaque border) when imaging over the course of 2 days for up to 5 weeks. In an APP transgenic mouse model (Tg2576) only older animals (18–24 months) showed increased spine elimination and decreased formation compared to age-matched wildtype control animals (Spires-Jones et al., 2007). Spine elimination rate exceeded the formation rate, measured over the relatively short time of 1 h, thereby presumably resulting in the described spine loss (Spires-Jones et al., 2007). A recent longitudinal imaging study (Bittner et al., 2010) for the first time analyzed spine changes in a triple transgenic mouse model of AD (3xTg-AD), bearing in addition to the APP_{swe} and PS1_{M146V}, also the tau_{P301L} mutation (Oddo et al., 2003). Amyloid plaque-dependent as well as – independent events were found to underlie dendritic spine loss (Bittner et al., 2010). Bittner et al. found that spine loss in hippocampus and frontal cortex only became apparent after plaques were deposited, and was most pronounced in the vicinity of plaques as observed by immunohistochemistry. In contrast, somatosensory cortex in the 3xTg-AD mouse model is devoid of amyloid plaques even in old mice. Longitudinal *in vivo*

two-photon imaging of apical dendrites in somatosensory cortex also revealed the loss of spines, but exclusively on dystrophic dendrites, which were positive for both hyperphosphorylated tau as well as intraneuronal A β (Bittner et al., 2010).

Transgenic mouse models, bearing PS1 mutations only, do not exhibit plaque pathology, hence allow for the separate analysis of the impact of PS1 mutations on dendritic spines independent of amyloid pathology. The impact of additional overexpression of either wildtype PS1 or the familial AD-PS1_{A246E} (FAD-PS1) mutation on dendritic spines was analyzed by another study (Jung et al., 2011). Whereas apical tuft dendrites showed an increased spine density for both types of PS1 overexpression, basal dendrites only exhibited a higher spine density in the FAD-PS1 mice (Jung et al., 2011). Interestingly, neither spine dynamics nor shape was affected by the different PS1 overexpression, thus the authors suggest a gain-of-function role for PS1 on dendritic spine plasticity (Jung et al., 2011).

Besides the manifold observations of dendritic spine alterations in AD mouse models, additional structural changes were characterized by *in vivo* two-photon imaging, such as neuritic dystrophies, neurite curvature, and – breakage or dendritic shaft atrophy (Tsai et al., 2004; Garcia-Alloza et al., 2006a). Axonal dystrophies, seen in the immediate vicinity of plaques, also undergo constant formation and elimination over the course of days to weeks (Tsai et al., 2004). Interestingly, plaque pathology (i.e., A β deposition) precedes neuritic pathology, as curved as well as dystrophic neurites only occurred days to weeks after a plaque was newly formed (Meyer-Luehmann et al., 2008). These results further support the notion of amyloid plaques acting as a mediator of neurite and spine pathology, which is most likely caused by the combination of a high oligomeric A β concentration associated with plaques as well as their space occupying nature (Koffie et al., 2009) and is at least partially due to calcineurin activation (Wu et al., 2010).

Knowing the kinetics of plaque pathology and the above-mentioned associated structural changes allows for monitoring the effects of treatment approaches aiming at the reduction of amyloid burden. Immunotherapy has been proven to rapidly clear plaques (Bacskai et al., 2001), reduce the number and size of plaque-associated neuritic dystrophies (Brendza et al., 2005), and to acutely increase spine formation in APP transgenic mice (Spires-Jones et al., 2009a). Curved neurites around a plaque seem to be positively affected by antioxidants (Garcia-Alloza et al., 2006a, 2010), but not by γ -secretase inhibition (Garcia-Alloza et al., 2009). Unfortunately, previous treatment studies have limited their analysis to a small subset of neuropathological features seen in APP transgenic mice, thereby hindering the evaluation of the efficacy of the treatment approach *per se*. Future imaging studies should therefore aim at monitoring multiple neuropathological events simultaneously.

IMAGING MICROGLIA IN APP TRANSGENIC MICE

Apart from the above-mentioned neuritic alterations associated with amyloid plaque deposition, the immediate plaque vicinity is characterized by an accumulation of glial cells. Resting microglia are uniformly distributed throughout the brain. They are considered the surveillants of the brain, as their processes are highly motile, constantly sampling the microenvironment, whereas the

soma itself remains stationary (Nimmerjahn et al., 2005). Once microglia detect cues of a brain insult, they rapidly become activated in a multistaged process, allowing them to migrate to the lesion site, release cytokines, and phagocytose material (Kettenmann et al., 2011). In AD, as well as in mouse models of AD, microglial cells are associated with amyloid plaques (Itagaki et al., 1989; Stalder et al., 1999). A recent *in vivo* imaging study demonstrated that microglia migrate rapidly to newly formed plaques (Meyer-Luehmann et al., 2008), a process which is enhanced by immunotherapy targeting aggregated A β (Koenigsknecht-Talboo et al., 2008). Their precise contribution to AD pathophysiology, however, remains controversial, as they are ascribed both a protective and a detrimental role (Morgan et al., 2005; Johnston et al., 2011).

Multiple studies have addressed the question, whether microglia cells are of crucial relevance in the formation and growth of amyloid plaques. There is some evidence from immunohistochemical – and *in vivo* imaging experiments, suggesting that microglia are able to phagocytose A β , thus restricting plaque growth (Simard et al., 2006; Bolmont et al., 2008). However, this is in contrast to an earlier electron microscopy study, in which the authors failed to detect any phagocytosed A β within microglia (Stalder et al., 2001) and a previous *in vivo* imaging study, where microglia lacked intracellular dense core plaque material (labeled with Methoxy-XO4), but were positive for protofibrillar A β (Liu et al., 2010). Surprisingly, microglial depletion was found to neither affect plaque count nor plaque size, thereby questioning a causal involvement of microglia at least in the pathogenesis of amyloid- β deposition (Grathwohl et al., 2009).

A number of *in vivo* studies have tried to unravel the influence of microglial activation, mediated by the chemokine receptor CX3CR1, on amyloid pathology, and associated neurotoxicity (Cardona et al., 2006; Fuhrmann et al., 2010; Liu et al., 2010). Notably, CX3CR1-deficient APP transgenic mice showed gene dose dependent reduction of amyloid deposition (Lee et al., 2010). Disruption of neuron–microglia interaction via fractalkine signaling seems to alter microglia activation and phagocytic capacity, thereby resulting in an enhanced phagocytosis of protofibrillar A β (Lee et al., 2010; Liu et al., 2010).

In a separate *in vivo* imaging study, triple transgenic mice (3xTg-AD; Oddo et al., 2003), crossbred to mice lacking the CX3CR1 receptor, showed no neuron loss, indicating a crucial role of the microglial chemokine receptor in mediating neuronal apoptosis (Fuhrmann et al., 2010). Interestingly, at the age of 4–6 months, 3xTg-AD animals do neither exhibit neurofibrillary tangle nor amyloid plaque pathology. This gives rise to the question of what the causing agent inducing the expression of fractalkine in subsequent apoptotic neurons is. One likely candidate is intracellular A β , as mice at the age analyzed do not yet possess hyperphosphorylated tau proteins. But why are other mouse models, which also exhibit intracellular A β , devoid of neuronal apoptosis? And furthermore, it remains unsolved whether microglia are causally involved in the apoptotic process or just recruited to the site of insult. In other words can interference with the CX3CR1 pathway prevent neuronal loss? Taken together, the role of microglia cells in the course of AD pathology is far from clear. Hopefully chronic two-photon imaging studies will help to

resolve some of the open questions regarding the precise nature of microglial involvement in AD pathology.

IMAGING tau PATHOLOGY

Whereas *in vivo* imaging of amyloid- β pathology has been feasible for quite some time, monitoring the second pathological hallmark of AD, namely neurofibrillary tangles *in vivo*, was only accomplished recently (Spires-Jones et al., 2008). NFT are thought to occur and spread downstream of abnormalities of A β during the course of AD (Gotz et al., 2004; Oddo et al., 2004; Santacruz et al., 2005; Tanzi, 2005; Sperling et al., 2011), but their correlation to the cognitive decline is much better than that observed for amyloid- β pathology, arguing for the importance of NFT in the degenerative process (Grober et al., 1999; Giannakopoulos et al., 2003). Aggregation of hyperphosphorylated tau protein into NFT has been suggested to contribute to neuronal loss in tau transgenic mice (Andorfer et al., 2005; Ramsden et al., 2005; Santacruz et al., 2005). The timing of hyperphosphorylated tau protein aggregation into tangles and the question whether NFT directly lead to neuronal cell death are fundamental issues that are ideally suited for *in vivo* two-photon imaging techniques.

The rTg4510 transgenic mouse model, which reversibly expresses human tau_{P301L}, is characterized by the development of NFT, neuronal loss, and memory impairment (Ramsden et al., 2005; Santacruz et al., 2005; Spires et al., 2006). These mice start to build up NFT at 2.5 months of age. Spires-Jones and co-workers repeatedly imaged rTg4510 mice to study the kinetics of NFT formation as well as the causal relationship between NFT and neuronal cell death. Surprisingly, they found that neurofibrillary tangle formation, caspase activation, and even the disruption of membrane integrity do not immediately induce neuronal cell death (Spires-Jones et al., 2008; de Calignon et al., 2009). Instead, neurofibrillary tangle formation occurred within 1 day after caspase activation, and the respective neurons remained healthy for several days (de Calignon et al., 2010). The authors thus propose that tangles may not be harmful or toxic *per se* (as opposed to soluble hyperphosphorylated tau), but rather serve a protective role against caspase activation and subsequent apoptosis. However, given the short follow-up period of 5 days, this hypothesis needs further evaluation in order to rule out a slower process of apoptosis. Moreover, it is unclear whether tangle bearing neurons maintain their physiological functional properties, although some evidence from *in vitro* experiments of rTg4510 cortical slice cultures points to no apparent functional differences between tangle – and non-tangle bearing neurons (Rocher et al., 2010).

These studies challenge our current view on the causal involvement of one of the main neuropathological hallmarks of AD and ultimately raise the question as to which neurons actually undergo apoptosis in mouse models of tauopathies including the rTg4510 transgenic or the 3xTg-AD mice. Do these neurons represent rare cases in which caspase activation does not result in the induction of tangle formation? And if so, how can the occurrence of NFT correlate with the degree of cognitive decline in AD? Can tangles be regarded as proxy for the concentration of soluble tau? Besides all these open questions the recent findings unraveled the temporal sequence of events, clearly establishing caspase activation as the initiating event in the process of tangle formation.

FUNCTIONAL *IN VIVO* IMAGING IMAGING NEURONAL ACTIVITY

Given the structural changes accompanying the deposition of amyloid plaques, it does not come as a surprise that neuronal function is also altered in neuronal structures neighboring plaques. Conventional electrophysiological methods to study the functional properties of neurons either yield a low spatial resolution (recordings of groups of neurons, e.g., field recordings or EEG) or only allow for single cell analysis (e.g., patch clamp). One elegant way of analyzing the spatio-temporal dynamics of cell populations, with single cell resolution, is *in vivo* two-photon imaging using calcium indicators. Calcium indicators take advantage of the fact that electrical activity of an excitable cell is tightly coupled to changes in intracellular calcium concentration (Tsien, 1981; Stosiek et al., 2003). Two main classes of indicators are available: synthetic dyes (e.g., OGB-1, Fluo-4, Rhod-2), which are applied by the bolus loading technique (Stosiek et al., 2003; Garaschuk et al., 2006), and genetically encoded calcium indicators (e.g., GCaMP, yellow chameleon, troponin C-based indicators), which are delivered to the brain by viral vectors or electroporation (Grewe and Helmchen, 2009). Genetically encoded calcium indicators allow for chronic functional imaging. In addition to being a proxy of neuronal activity, calcium plays an important role as intracellular signaling molecule in a number of processes, such as synaptic plasticity (transmitter release, excitability) and development (dendritic development, synaptogenesis, growth cone behavior; Ryglewski et al., 2007).

Calcium homeostasis and signaling are known to be disrupted in AD (Berridge, 2011). Hence, *in vivo* calcium imaging can be employed to address both the detection of altered calcium concentrations under baseline (i.e., resting) conditions, as well as changes in neuronal/glial activity. Two papers recently addressed the relationship between amyloid plaques and either intracellular calcium concentration or neuronal activity (Busche et al., 2008; Kuchibhotla et al., 2008). Garaschuk and co-workers for the first time described aberrant neuronal activity in the immediate vicinity of amyloid plaques in APP23xPS45 mice (Busche et al., 2008). Using Oregon Green BAPTA-1 AM (OGB-1 AM) as a calcium indicator, they found significantly more cells (50% in APP23xPS45 vs. 12% in WT) with extremely low or very high activity levels in frontal cortex (layer II/III) of APP23xPS45 compared to non-transgenic wildtype mice. When analyzing the spatial distribution of these neurons, they observed hyperactive cells exclusively within a 60 μ m radius from the next amyloid plaque. Silent cells and such with regular firing rates were distributed throughout the cortex, with a relative increase of silent cells further away from plaques. Pre-depositing mice on the other hand did not show any abnormal neuronal activity patterns. In order to disentangle the mechanism underlying the hyperactivity, the authors used a pharmacological approach and increased or decreased GABAergic inhibition. Hyperactive cells responded to both treatments, but exhibited a lower relative frequency increase after application of a GABA_A receptor antagonist compared to silent or regularly firing cells. Hence, the authors suggest that impaired synaptic inhibition underlies the observed hyperactivity (Busche et al., 2008).

A nicely complementing study more specifically investigated calcium concentrations within dendrites and spines using

the ratiometric genetically encoded calcium indicator yellow chameleon (YC3.6; Kuchibhotla et al., 2008). A large proportion of dendrites in the immediate vicinity of amyloid plaques exhibited an overload of resting calcium concentration (Kuchibhotla et al., 2008). As a consequence, a disruption of spino-dendritic compartmentalization, a feature thought to be necessary for proper synaptic network integration (Augustine et al., 2003; Bloodgood and Sabatini, 2007), was observed. Furthermore, the study provided a link between structural and functional alterations, since more than half of the neurites with elevated calcium concentration were beaded, i.e., dystrophic (Kuchibhotla et al., 2008).

Taken together, these studies pinpoint amyloid plaques as mediators of functional alterations observed in AD mouse models, both on the cellular as well as on the subcellular level. Furthermore, they link structural changes to altered function, which presumably underlies the neuronal network dysfunction prominent in AD. To date, no study has addressed chronic changes in neuronal activity. Recently developed genetically encoded calcium indicators allow for repeated functional imaging, thereby providing a valuable tool to monitor the induction and progression of the above-mentioned functional neuronal alterations.

IMAGING ASTROCYTIC ACTIVITY

Another cell type known to exhibit large calcium signals are astrocytes. Astrocytes are involved in numerous physiological processes, such as neurovascular coupling, provision of nutrients to neurons, balance of extracellular ion concentration, and the uptake and release of neurotransmitters ("tripartite synapse"; Araque et al., 1999; Verkhratsky, 2010; Verkhratsky and Parpura, 2010). They also play a crucial role under pathological conditions, such as stroke, epilepsy, traumatic brain injuries, multiple sclerosis, and neurodegeneration (Verkhratsky et al., 2010; Sidoryk-Wegrzynowicz et al., 2011). Astrocytes are specifically implicated in AD pathology as they are, along with microglia, recruited to amyloid plaques (Itagaki et al., 1989; Nagele et al., 2004; Tuppo and Arias, 2005). Reactive astrogliosis may be triggered by factors released by affected cells as well as amyloid- β deposits, and results in an enhanced gap-junction coupling between cells (Pihlaja et al., 2008; Peters et al., 2009). The particular role of astrocytes is not yet fully understood. Whereas active glial cells might convey detrimental effects on neurons by releasing cytokines, interleukins, reactive oxygen species, or TNF α (Johnstone et al., 1999; Akiyama et al., 2000; Koistinaho et al., 2002), astrocytes are also linked to the clearance of plaques, as they can phagocytose deposited A β (Wyss-Coray et al., 2003; Koistinaho et al., 2004; Pihlaja et al., 2008).

To investigate the functional response of astrocytes to amyloid plaques in APP_{swE}/PS1_{DE9} mice *in vivo*, Kuchibhotla et al. (2009) co-injected the calcium indicator OGB-1 AM and sulforhodamine 101(SR 101) in order to counterstain astrocytes (Nimmerjahn

et al., 2004). They observed a globally elevated resting calcium concentration within the astrocytic network, independent of amyloid plaque proximity. Synchronous calcium transients were much more frequent in APP/PS1 than in non-transgenic control mice, and appeared in a coordinated fashion across long distances, but were uncoupled from neuronal activity (Kuchibhotla et al., 2009). Furthermore, the authors succeeded in detecting calcium waves between astrocytes *in vivo*, exclusively in mice already bearing amyloid plaques. These waves seem to originate in the plaque vicinity and travel radially over $\sim 200 \mu\text{m}$, thereby indicating a focal neuropathological insult (Scemes and Giaume, 2006). In conjunction with *in vitro* studies, these findings emphasize the active contribution of astrocytes to AD neuropathology (Rodriguez et al., 2009).

CONCLUSION

While great progress towards understanding the molecular mechanisms and genetic pre-dispositions of AD has been achieved within the past two decades, therapeutic options that halt or reverse the disease are still not available to date. Further research, unraveling underlying cellular and molecular mechanisms, and validating newly developed treatment approaches is thus needed. Performing transcranial *in vivo* two-photon microscopy on transgenic mouse models of AD has shed much light upon the kinetics and temporal sequence of neuropathological AD hallmarks and their associated structural and functional alterations in the living intact brain. *In vivo* imaging data stress the relevance of amyloid plaques as a focal source of neurotoxicity, as numerous pathological features, such as the occurrence of dystrophic neurites, neurite breakage, spine loss, clusters of hyperactive neurons as well as the initiation of intracellular calcium waves among astrocytes, are tightly spatially and temporally linked to plaque deposition. Furthermore, recent studies challenge our current understanding of the toxic nature of NFT by proposing a protective role for these intracellular aggregates. Future research will hopefully provide more insight into the causality of the observed pathological features as well as the efficacy of newly developed treatment approaches by, e.g., combining and correlating longitudinal imaging approaches and behavioral studies.

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