The Structural Plasticity of Dendritic Spines in Amyloid Precursor Protein Transgenic and Knockout Mouse Models

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Table of Contents

Summary

Dynamic synapses are the structural basis of brain to respond to pathological or physiological changes in internal or external environment. Synapse formation, elimination and morphological alterations rewire neural circuits by establishing new connections, abolishing and strengthening or weakening preexisting ones. Excitatory glutamatergic synapses in mammalian brain normally reside at dendritic spines. The structural parameters of dendritic spines are tightly regulated in normal brain and changed in an array of neurodegenerative diseases.

Being the most common neurodegenerative disease, Alzheimer's disease (AD) exhibits progressive neuropathology that lasts more than decades. The pathogenesis of AD is widely believed to be initiated by amyloid deposition, which is composed of amyloid β (Aβ) peptides. Aβ is the proteolytic fragment of amyloid precursor protein (APP) that contains a large extracellular ectodomain and a short cytoplasmic tail. After the discovery of APP mutations in early-onset familial AD that increase Aβ levels in brain, transgenic mouse models overexpressing mutated APP have been created to recapitulate AD pathogenesis. Besides the neurotoxicity of Aβ, physiological functions of APP may also participate in the pathogenesis of AD as the regulation of APP proteolysis into Aβ modulates the expression of APP and other APP fragments. To investigate its physiological functions, APP knockout (APP-KO) mice have been generated. In this dissertation, spine density, morphology and plasticity of APP transgenic and knockout mouse models were extensively examined by chronic in vivo two photon microscopy.

In Paper One, decreased spine density of apical tufts originated from layer 5 pyramidal neurons was observed in 4-5-month-old APP23 mice, which overexpress APP with Swedish mutation, before amyloid deposition. In age-matched APPswe/PS1deltaE9 (deltaE9) mice with mutant APP and presenilin-1, spine loss was found only on the dendrites that were localized close to amyloid plaques. The reduced spine density was due to decrease spine formation, while spine elimination remained unchanged. Also, these two AD mouse models displayed distinct patterns of morphological alterations in dendritic spines. In APP23 mice, the content of intraneuronal APP was inversely correlated with spine density and the fraction of mushroom spines. In deltaE9 mice, no intraneuronal APP was detected, while spine loss and

alterations of spine morphology were accompanied with the growth of amyloid plaques. These results suggest intracellular APP accumulation and extracellular Aβ deposits contribute to spine pathology in young adult APP23 and deltaE9 mice, respectively.

In Manuscript One, the impaired adaptive plasticity of young adult deltaE9 mice was demonstrated by their failures to gain more dendritic spines and form novel neural circuits when housed under enriched environment (EE). Interestingly, elimination of Aβ deposits by reducing β-secretase activity restored the increase of spine density in detaE9 mice upon EE, but did not recover neural network remodeling. However, anti-inflammatory treatment by the administration of pioglitazone or interleukin 1 receptor antagonist successfully rescued the deficiencies of increasing spine density and remodeling neural networks in deltaE9 mice upon EE. These data imply that neuroinflammation thwarts experience-dependent structural plasticity of dendritic spines in young adult deltaE9 mice, which recapitulate the preclinical stages of AD with amyloid deposition in brain before the onset of dementia.

In Manuscript Two, spine dynamics was found to be reduced in 4-5-month-old APP-KO mice illustrated by decreased spine formation and elimination. Additionally, APP-KO mice failed to increase spine density when housed under EE. These observations also prevailed in APPsα knockin (APPsα-KI) mice, which express APPsα but lack full length APP. Meanwhile, the distributions of dendritic spine subtypes classified by their morphologies were also changed in APP-KO mice accompanied with reduced N-methyl-D-aspartate (NMDA) receptor-mediated miniature excitatory post-synaptic currents (mEPSCs) and decreased postsynaptic NMDA receptor expression. Strikingly, potentiation of NMDA receptor responses by administering D-serine restored the morphology, dynamics and adaptive plasticity of dendritic spines in APP-KO mice. These results indicate constitutive and adaptive spine plasticity is maintained by the functional cooperation between APP and NMDA receptor.

Collectively, this dissertation confirms that different spine abnormalities occur in APP transgenic and knockout mouse models. These distinct pathological alterations of dendritic spines suggest APP and its proteolytic fragment Aβ may both participate in the pathogenesis of AD in their own ways.

1 Introduction

Structural plasticity of dendritic spines

The basis of cognition

“Men ought to know that from nothing else but the brain come joys, delights, laughter and sports, and sorrows, griefs, despondency, and lamentations [86].” Associated with mind, brain is the most special and complex organ. In the long history of neural science, brain and mind were thought to be separated. The disclosure of aphasia since the 19th century leads to the development of cognitive neurosciences [48]. It firstly addressed how cognitive functions are produced by the brain. One of the ultimate challenges of science nowadays is to understand how the brain processes what we feel, act, learn and remember.

The brain is primarily composed of glial cells and neurons. Glial cells, which outnumber neurons by tenfold, perform a number of critical functions for supporting neurons, including insulation, nourishment, structural and metabolic support [108]. The various supporting functions are reflected in the different subtypes of glial cells, including astrocytes (ion and metabolic homeostasis), microglia (active immune defense) and oligodendrocytes (axon insulation) [43]. Also, these glial subtypes have characteristic morphologies: astrocytes have a star-shaped appearance while microglial cells are highly branched.

Differed from glial cells morphologically and functionally, neurons are the signaling components and execute the bulk of information processing in the brain[8, 104]. Neurons typically consist of four regions, including the soma, the axon, axon terminals and dendrites. Different regions have distinct functions in generating neural signals and communicating in the neural network. The soma or the cell body separated by the plasma membrane from the outside contains organelles that are similar with other animal cells and works as the metabolic center of the neuron. Arising from the site of cell body called axon hillock, an axon surrounded by myelin sheath extend and often branch to convey electrical impulses. The end of an axon is called axon terminal or the presynaptic terminal. They are the sites where the axon contacts with and sends information to other neurons. The contact point is named the synapse. The synapse consists of two sides: presynaptic, which is generally an axon terminal, and postsynaptic. The postsynaptic side is usually the cell body of other neuron or the

dendrite. Dendrites also arise from the soma and resemble the branches of the tree. In most cases, neural signals transit from the axon to a dendrite of other neuron.

Cognitive information that transits within neurons in brain is carried by electrical and chemical signals. Ion channels embedded in the cell membrane are responsible for the maintaining of resting membrane potential. Changes that make the membrane electrical potential differ from the resting value produce transient electrical signals, including receptor potential, action potential and synaptic potential. Among them, the action potential enables the electrical signals to be carried over long distances in neurons. After the electrical signals are triggered and propagated, they are conducted to the presynaptic axon terminals and transmitted to the other neurons electrically or chemically. At electrical synapses, the currents originated in the presynaptic neurons go through gap junction channels and then enter into postsynaptic neurons. At chemical synapses, the presynaptic neurons release chemical transmitters at axonal terminals induced by action potentials. The transmitters travel through the synaptic cleft and bind to the postsynaptic receptors. The activated receptors regulate associated ion channels and change membrane potentials on postsynaptic neurons. Based on the signaling transductions among interconnected neurons, the organized neural circuits in functionally specific regions of cerebral cortex give rise to the cognitive functions.

The synaptic plasticity

At chemical synapses, the effectiveness of signaling transduction can be strengthened or weakened during short and long periods. This synaptic property is called synaptic plasticity [225]. Synaptic strength can be altered by the changes in the presynaptic release of neurotransmitters and/or modulating postsynaptic response to transmitters [61]. Activity-dependent control of synaptic plasticity is thought to contribute to many diverse cognitive processes, including memory and learning, developmental synaptic pruning and formation, and the symptom of pathological conditions [130].

To study activity-dependent synaptic plasticity, long-term potentiation (LTP) and long-term depression (LTD) are two classical models. LTP was firstly reported in 1973 [18] and represents the increase of synaptic strength that follows a brief and high frequent electrical stimulation. In several mammalian brain regions, such as neocortex [9], hippocampus [18] and amygdala [133], LTP has been detected. It is even suggested that LTP may occur at all excitatory synapses [130]. Contrary to LTP, LTD is the reduction in the effectiveness of

synaptic signaling transduction. Due to the absolute significance of synaptic plasticity, extensive efforts have been made to demonstrate the underlying mechanisms.

N-methyl-D-aspartate receptors (NMDARs) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs) are two ionotropic glutamate receptors that directly participate in the synaptic plasticity of excitatory synapses. Activation of these receptors leads to the depolarization of plasma membrane by strong influx of sodium ions and a small efflux of potassium ions. Basal glutamatergic transmission relies on AMPARs while NMDARs mainly serve as the regulator of synaptic transmission. In LTP, glutamate released from the presynaptic terminals relieves the magnesium block of NMADRs when the postsynaptic neuron is depolarized. Glutamate binding and depolarization lead to the maximal calcium influx of NMADRs, which triggers multiple intracellular signaling cascades to alter synaptic efficiency. On the contrary, repeated occurrence of smaller calcium influx through NMDARs triggers LTD following low-frequency synaptic stimulation. Although LTP and LTD are both induced by NMDARs-mediated calcium influx, it is accepted that strong increases in postsynaptic calcium lead by strong activations of NMDARs trigger LTP, while mild increases in postsynaptic calcium lead to LTD [126, 129]. Quantitative characteristics of calcium signals cause the insertion or removal of AMPARs in the synapses leading to LTP or LTD. The maintenance of LTP or LTD requires protein synthesis and synaptic structural changes.

Dendritic spines

After being detected by Ramon y Cajal [26], dendritic spines have been expected to be the locus for neuronal plasticity. Dendritic spines are the membranous protrusions that arise from dendrites to receive informational input from axonal terminals [152, 235]. Dendritic spines provide isolations for chemical and electrical signaling transduction in postsynaptic compartments (Fig. 1).

As functioned as synaptic transmission, dendritic spines are morphologically specialized. They classically contain a bulbous head (0.001-1 μm^3) linked to the dendritic shaft by a thin spine neck (<0.1 μm) [83]. The spine head, where molecular signals are compartmentalized after synaptic activation, consists of the post-synaptic density (PSD), a membrane-attached plate of electron dense thickening that is close and directly opposite to the presynaptic terminals [20]. The PSDs contain hundreds of proteins to serve as the devices of collecting synaptic signals, including neurotransmitter receptors, coupled signaling molecules and

scaffolding proteins [92, 155, 214]. Smooth endoplasmic reticulum (SER) has also been found within many dendritic spines, which is known to play a role in regulating calcium [5]. The released calcium from SER promotes the remodeling of actin cytoskeleton [154]. Actin filaments, instead of microtubules, are concentrated in spines to form organized bundles [28, 135]. In addition, local protein synthesis and degradation occur in dendritic spines. Polyribosomes, the devices that are essential for translating proteins, are distributed in dendritic spines along with lysosomes and multi-vesicular bodies [189, 194]. Recycling endosomes in dendritic spines facilitate the processes of exo- and endocytosis [116, 161]. The quantities of compositions in dendritic spines vary greatly as their size and shape.

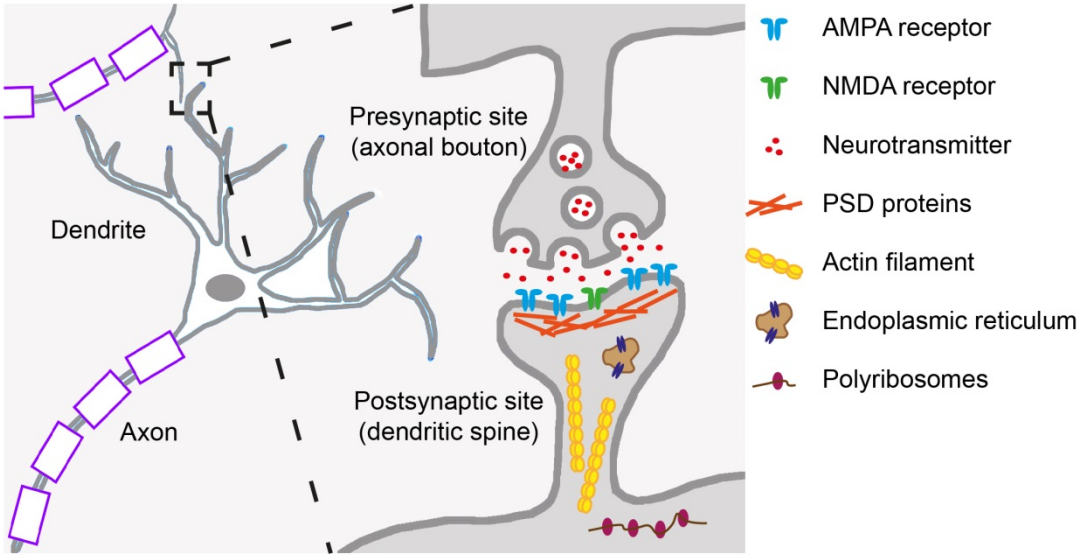

Figure 1 Diagram of a synapse that is composed of a presynaptic bouton and a postsynaptic spine. The presynaptic bouton contains transmitter vesicles with glutamate, which is released into synaptic cleft and binds to neurotransmitter receptors located in dendritic spine head.

Dendritic spines are characterized with their morphological diversity. During the development of brain, dendritic spines are relatively elongated and thin, while they gradually exhibit a prominent spine head and thus obtain a mushroom-like structure when the brain matures [157, 237]. In adult brain, most dendritic spines contain thin necks and either big heads (>0.6 μm in diameter) or smaller heads [82]. Based on the relative sizes of spine heads and necks, dendritic spines have been divided into three main subtypes [164]. Spines with large heads and narrow necks are categorized as mushroom spines. Thin spines contain smaller spine heads and thin necks, while stubby spines are short and have no obvious spine necks. These categories provide measurably distinct spine shapes that might indicate different synaptic functions. Mushroom spines are found to be enriched in actin filaments [28] and most likely to have larger PSD with more neurotransmitter receptors, polyribosomes, SER and endosomes

[82, 159, 162, 189]. In contrast, thin spines contain less spine apparatuses, while they are more flexible to change the morphology when responding to increases or decreases in synaptic activity [19]. While the intrinsic mechanisms underlying the relationship between morphology and functions of dendritic spines are not fully understood, it is important to unveil how structural plasticity of dendritic spines is regulated and how its alterations modify synaptic transmissions in pathophysiological processes.

Dendrites of neonatal mammalian pyramidal neurons barely have spines [166]. During the first few weeks after birth, the density of dendritic protrusions greatly increases and synaptogenesis boost up [138, 223]. The subsequent pruning of over-produced dendritic spines occurs during juvenile stages and thus facilitates the refinement of neural circuits [168, 239]. In adult brain, the rate of spine pruning is dramatically declined and dendritic spines are more stable [89]. Apart from the absolute spine numbers, spine morphology also changes during development. Although stubby spines are the most abundant subtypes of dendritic spines in the early stages of development, filopodia, the elongated dendritic protrusions without distinctive spine heads, are prominent in the developing brain which are infrequently observed in adulthood [139]. Filopodia are regarded as the precursors of mature dendritic spines as their high motilities promote the hunting of presynaptic partners in the developing brain [237].

In adult brain, dendritic spines are also maintained in a dynamic state. Individual spines form and eliminate over time, as well as morphological changes occur [45, 46, 81, 188]. Synaptic input from the external environment modulates formation, elimination and morphology of dendritic spines, which provides the structural basis of learning and memory. Many studies have addressed that LTP, representing the enhanced excitatory synaptic strength, can change spine number and morphology. Electron microscopy (EM) analysis followed by induction of LTP has revealed increased size and number of dendritic spines [95]. The new spines after LTP stimulation sprout from the dendrites rather than through splitting existing spines [54]. These results based on experimental protocols of enhancing synaptic strength suggest that morphological changes in dendritic spines may occur with enhancement of neural activities through learning and sensory experience.

Numerous learning paradigms have been reported to induce changes in the density and morphology of dendritic spines. In adult motor cortex, training on motor skills increases the

number of synapses [113]. Also, increase of spine density after spatial learning tasks or induced by associative memory formation has been reported in hippocampus [50, 124, 144]. In addition, the size of dendritic spines changes with learning [64]. Besides learning, novel sensory experience has been applied to influence the spine number and morphology. Housing animals in enriched environment (EE) provides increased sensory experience and thus causes an increase in spine density on dendrites [42, 101]. Whisker stimulation in freely moving animals also gives rise to increased spine number [115]. On the contrary, deprivation of sensory experience by dark rearing leads to a decrease in spine density and creates spines with shorter length but larger heads in visual cortex, which are partially reversible with exposure to light [215]. These changes in spine number and morphology, induced by the stimulation of external environment, possibly provoke the remodeling of established neural circuits and then strengthen or weaken the synaptic connectivity in order to alter the efficiency of synaptic communication.

In vivo two photon microscopy

The evidence demonstrating the fact that synaptic activity modifies the structure of dendritic spines firstly arose from EM studies in 1970 to 1980 [18, 56, 57, 206]. In these pioneering studies, the enlargement of dendritic spines was observed after the induction of LTP. However, the results obtained from EM could not reveal that if the enlarged spines existed before or were just newly formed during LTP induction, as EM is not time-lapse imaging. Thus, it was difficult to tell whether the enlargement of dendritic spines is directly caused by LTP or if this phenomenon just occurs in parallel with LTP.

To solve this problem, the first attempt to realize time-lapse imaging of dendritic spines during LTP induction was done in 1995 with confocal microscopy [90]. This study imaged individual dendritic spines of hippocampus neurons in acute brain slices before and after the induction of LTP and found increased spine length in a subpopulation of small spines. Furthermore, filopodia-like dendritic protrusions were found newly formed and existing spines went lost after LTP induction [131]. Although these observations provided direct evidence on the relationship between the enhancement of synaptic strength and the morphological changes of dendritic spines, ex vivo studies have limitations in illustrating if the observed phenomena in slices are consistent in intact brain or physiological stimulation on synaptic inputs, instead of artificial electronic stimulation, also facilitates the structural plasticity of dendritic spines.

A major technical advancement of imaging spine morphology is the application of two photon laser scanning microscopy, which has been adopted for the in vivo imaging of dendritic spines nowadays [41, 84]. In this microscopy, two photons of low energy are released from the laser and then collaborate to induce the electronic transition of higher energy in a fluorescent molecule [196]. The excitation of two photons is a nonlinear process and the long-wavelength excitation light is less scattering in tissues as to allow deeper penetration. Moreover, the intensity of focused excitation light is highest in the focal point and diminishes quadratically in the surrounding volume (Fig. 2). Consequently, fluorophores are mostly excited in a limited volume and thus the three dimensional contrasts and resolution are comparable to confocal microscopy even without spatial filters in the path of detection [41]. Compared to standard one photon microscopy, photo-toxicity is also greatly reduced in two photon microscopy as the energy of excitation is strongly decreased outside the focal point. Collectively, the advent of two photon microscopy provides a great opportunity to study the structure and structural plasticity of dendritic spines in vivo.

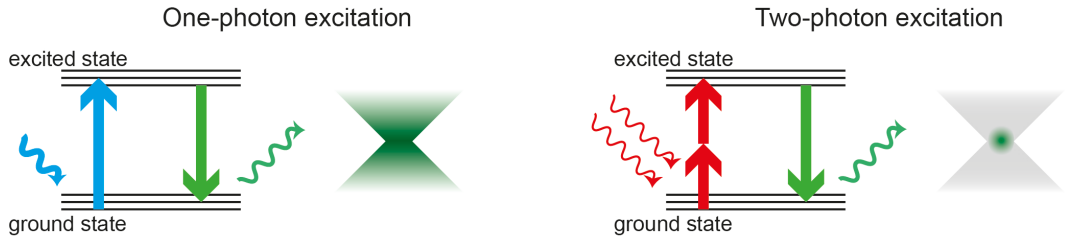

Figure 2 Diagrams of one-photon and two photon excitation. Two simultaneous photons with lower energy are absorbed to excite a fluorescent molecular, which emits a photon in the visible wavelength.

In vivo remodeling of dendritic spines

Combined with chronic in vivo two photon microscopy, transgenic mouse models expressing green fluorescent protein (GFP) or yellow fluorescent protein (YFP) in neurons of interest have been utilized to explore the morphological changes of dendritic spines in vivo [52] (Fig. 3). Dendritic spines are found to be highly dynamic at early postnatal stages and the rate of spine turnover rate decreases during postnatal development [123, 238]. In mature brain, the total number of dendritic spines becomes relatively stable with matched spine elimination and formation [88, 229]. However, the comparative stability of neural circuits in adults is able to be remodeled by novel experience.

The structural changes of dendritic spines have been examined in several sensory cortical regions in adult brain. In the somatosensory cortex, environmental enrichment upsets the

balance between spine formation and elimination and thus increases spine density in layer 3 and 5 pyramidal neurons [101]. Also, whisker potentiation stabilizes new-formed spines in neurons at the border between spared and deprived barrel columns, which may be mediated by alphaCaMKII auto-phosphorylation [224]. In the visual cortex, monocular deprivation increases spine density of layer 5 pyramidal neurons and decreases the number of inhibitory synapses that present on dendritic spines [88, 207]. In the motor cortex, motor skill learning enhances spine formation, while increased spine elimination follows up [229, 233]. Interestingly, the new formed dendritic spines after learning come up in clusters that are enriched in neighboring spine pairs [60]. In the frontal association cortex, fear conditioning increases spine elimination, while fear extinction increases spine formation, which occurs close to the positions of spine elimination when mice exposed to fear conditioning [120].

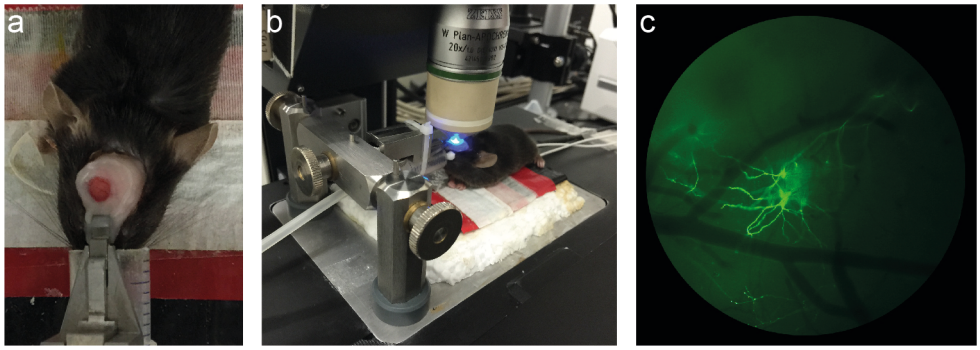

Figure 3 In vivo imaging of GFP-labeled dendrites. (a) a cranial window above somatosensory cortex. (b) transgenic mouse is anesthetized and placed under the two-photon microscope. (c) GFP-labeled dendrites in the cortex of transgenic mouse.

Besides the morphological changes of dendritic spines in physiological conditions, chronic in vivo two photon imaging has also been applied to investigate structural spine plasticity in pathological conditions. After stroke, peri-infarct dendrites demonstrate increased spine formation over weeks [23]. After spinal cord injury, spine density decreases with spine morphology changed in the motor cortex [110]. After a retinal lesion, spine formation and elimination both increase massively in adult mouse visual cortex [107]. In a transgenic mouse model of fragile X syndrome, the down-regulation of spine turnover rate and the transition of spine subtypes during postnatal development are delayed and transient spines are overproduced [34, 160]. Importantly, in transgenic mouse models of Alzheimer's disease, loss of dendritic spines has been shown [16, 192, 205].

The studies into the structural plasticity of dendritic spines in the intact brain with the development of imaging technologies have definitely broadened our knowledge of organization and remodeling of neural networks in physiological and pathological conditions. The mechanisms underlying experience-dependent spine plasticity in behaviorally relevant learning conditions and the changes developing in pathological conditions need to be further investigated in details.

Dendritic spine alterations in pathological conditions

Dendritic spines undergo pathological alterations resulted from a number of insults and diseases. Pathological alterations of dendritic spines mainly refer to the changes in spine distribution and morphology [55]. Pathology of spine distribution is mediated by a dramatic decrease or increase in spine density. Spine loss is seen in neurodegenerative disorders, malnutrition and toxin exposure, which may be caused by the degeneration of axon after neuronal loss [24, 76]. On the other hand, an increase in spine density is reported in patients with fragile X syndrome or some neuropsychiatric diseases [94, 163]. Besides the structural integrity of afferent axons that affect spine density, their functional integrity is ascribed to the alteration in spine morphology. Reduced dendritic spine size is found in the striatum of schizophrenics [176] or in visual cortex after visual deprivation from birth [59, 204]. Mutations that lead to mental retardation usually disturb spine shapes. Long and tortuous spines have been observed in fragile-X syndrome, Down' syndrome, fetal alcohol syndrome and maple syrup urine disease [53, 103, 200, 226].

Spine or associated synaptic pathology may contribute to cognitive deficits, especially in neurodegenerative disorders. Being the most common neurodegenerative disorder, Alzheimer's disease (AD) is associated with synaptic loss. Patients with AD exhibit a significant loss in synapses and synaptic density correlates with cognitive capacities [40]. Also, a progressive alteration of dendritic spines is observed in brains of AD patients [137]. Decreased neurotransmitter receptors further confirm the loss of synaptic function. The expression of nicotinic acetylcholine receptor α4β2 is reduced in the medial frontal cortex and nucleus basalis magnocellularis, which implies an impairment in cholinergic synapses [137]. In addtion, 5-hydroxytryptamine (5-HT)4 receptor is upregulted in early AD, while 5-HT1 receptor is decreased in advanced stages of AD [128, 140]. The cause of spine pathology in AD needs to be studies in details for successfully tackling this disease.

Alzheimer's disease

The discovery of the disease

In 1906 at the 37th meeting of the Society of Southwest German Psychiatrists, a Bavarian psychiatrist, Alois Alzheimer, presented a pathological syndrome that was subsequently named after him [29, 69, 182]. In Alzheimer's report, his patient, a woman referred as Auguste D., exhibited progressive cognitive decline, gradual loss of language function, and altered social behaviors such as delusions and paranoia. The patient maintained normal motor skills and sensory functions in the beginning, while she continued to lose cognitions and showed motor disorders as the disease progressed [4]. After the death of the patient who survived no more than five years after the onset of the disease, Alzheimer carried out an autopsy and found out specific alterations in her brain. First of all, the brain weight was reduced with enlarged ventricles. Secondly, extracellular plaques of dense material were detected in the brain sections. Thirdly, stained by silver solution, neurofibrillary tangles were found in normal-looking cells. These features are still observed in most patients of Alzheimer's disease (AD) nowadays. In 2010, 21 to 35 million people worldwide suffered from AD and there is no effective pharmacological treatments until now [167]. Thus, it is still crucial to investigate the pathological processes of AD, even though it has been discovered more than one hundred years.

Clinical symptoms of AD

Being the most common cause of dementia, AD usually undergoes a typical clinical course that exhibits progressive neuropathology. The progression of AD from preclinical stage to the stage of dementia lasts more than decades [171, 191]. The long preclinical stage of AD refers to the period when Alzheimer's pathology can be determined in normal cognitive conditions [190]. With positron emission tomography (PET) imaging, amyloid deposits (one of the neuropathological markers in AD) have been detected in a considerable fraction of people with intact cognitive functions [1, 165]. In agreement with these observations, reduced expression of amyloid β $(A\beta)_{42}$ in cerebrospinal fluid (CSF), which is inversely related with amyloid imaging load, is also found in preclinical AD [51, 210]. The subjects that have been diagnosed in preclinical stage of AD are at risk for future cognitive decline [213].

Between the pathological alterations in cognitively intact elderly and those observed in typical AD, there exists an intermediate stage of cognitive impairment named mild cognitive

impairment (MCI). Patients with MCI comprise a population at high risk for developing AD [209]. The clinical criteria for diagnosing MCI include the concern to the decline in cognition, impairment in one or more cognitive functions, independence in performing complex functional tasks and no dementia [3]. Typically, MCI patients who display the impairment in episodic memory are most likely to progress into AD.

With the progression of cognitive decline, AD patients suffer from severe impairment in recent memory [10]. The abilities of reasoning, planning and organizing are also impaired. Reading and writing skills start to deteriorate [35, 150]. The understanding of texts and completeness of spelling become difficult. A substantial fraction of patients develop delusional symptoms induced by cognitive deficits [169]. Also, patients become easy to lose emotional control with aggressive physical or verbal activities [58].

At the late age of AD, the severe impairments are observed in almost all cognitive functions [44]. Patients are only able to speak simple phrases or single words. After the loss of language abilities, many patients can still respond to emotional signals. The life expectancy of AD patients is no more than a decade [119].

Neuropathological markers

By silver staining, Alzheimer identified neuritic plaques and neurofibrillary tangles in the brain sections of Auguste D. [4] (Fig. 4). These two neuropathological characteristics bring a starting point for understanding the molecular mechanisms underlying the pathogenesis of AD. Although it remains controversial that the AD related pathological events and their temporal sequences due to the biochemical complexity of the disease, there is no doubt that substantial progress in elucidating AD biology has been achieved from deciphering the compositions of the histological hallmarks [181].

Neurite plaques are microscopic foci of extracellular amyloid deposits [66, 134]. Such plaques usually contain fibrillar cores which are composed of fibrillar Aβ. The fibrillar core can be stained by Congo red or thioflavin S in brain sections. In vivo imaging of fibrillar Aβ is achieved by either radiolabelled derivatives of the dyes in PET imaging or fluorescent derivatives such as Methoxy-X04 [15, 17, 208]. Within and surrounding the amyloid deposits, dystrophic neurites have been observed [62, 145, 186]. These aberrant neurites are dilated with ultrastructural abnormalities, such as enlarged lysosomes, abundant mitochondria and

helical filaments [182]. The pathological relation between dystrophic neurites and cognitive impairments has been suggested in AD transgenic mice that exhibit neuronal dystrophy and deficient cognitive tasks without neuronal loss [73, 93]. Also, neurite plaques are correlated with activated glial cells. The activated astrocytes often encircle the outside of plaques with their processes protruding inside the cores of amyloid plaques, while the activated microglial cells are located near the amyloid cores [136, 211]. The activation of microglia follows the formation of fibrillar amyloid plaques [102]. The most fibrillar Aβ in neurite plaques are the combination of Aβ species cleaved at amino acid 42 and 40 ($A\beta_{42}$ and $A\beta_{40}$). $A\beta_{42}$ is more hydrophobic and principally inclined to aggregation, while $A\beta_{40}$ is produced more abundantly and normally co-localized with $A\beta_{42}$ in the deposits [97, 98].

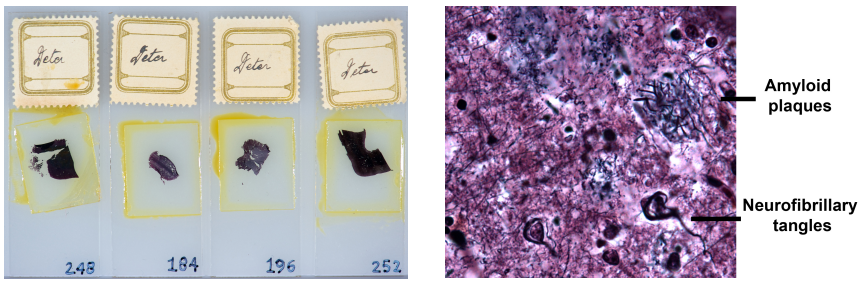

Fig. 4 Microscopic brain preparation of the first AD case. The amyloid plaques and neurofibrillary tangles in brain autopsies of Auguste D., the first AD case described by Alois Alzheimer (Source: archives of Center of Neuropathology and Prion Research, LMU, Munich)

When the protein subunits of amyloid deposits have been identified as Aβ peptides [66, 134], antibodies against endogenous or synthetic Aβ were developed. With these antibodies, immunochemical staining reveals extensive number of Aβ deposits, which cannot be examined by the dyes that are specific for fibrillar aggregates. Also, these plaque-like deposits seem to be not surrounded by dystrophic neurites and activated glial cells. Such Aβ deposits are referred as diffuse or pre-amyloid plaques [99, 198, 231]. Unlike the mixed deposits of $A\beta_{42}$ and $A\beta_{40}$ in fibrillar plaques, the diffuse plaques are largely composed of $A\beta_{42}$ with little $A\beta_{40}$ [96, 181]. It has been speculated that the diffuse plaques may be the precursors of fibrillar plaques [143, 156, 220]. In brain regions that are not clearly involved in clinical symptoms of AD, such as cerebellum and striatum, and do not strongly exhibit neuronal dystrophy and activation of glial cells, diffuse Aβ deposits are mostly found. Also, diffuse plaques are often detected in normal elderly people in brain regions that fibrillar plaques are shown in AD patients. Transgenic mouse models of AD also develop diffuse deposits before fibrillar ones [32, 193]. In addition, patients with Down's syndrome display diffuse plaques when they are teenagers and fibrillar despoits decades later [122]. These

results collectively support the hypothesis that diffuse plaques are the immature plaques that precedes the formation of fibrillar plaques with surrounding neuritic and glial cytopathology.

Neurofibrillary tangles are intraneuronal cytoplasmic bundles of abnormal fibers that usually occur in brain regions typically disturbed in AD progressions, such as entorhinal cortex, hippocampus, amygdala and parietal lobes [21, 22]. These fibers contain pairs of filaments that are curved into helices (PHFs) as revealed by electron microscopy. PHFs are also sometimes interspersed with straight filaments [181, 182]. Biochemical analyses show that the subunit protein of the fibers is the microtubule-associated protein tau [75, 118, 153]. The tau is mainly located in axons in physiological conditions and its phosphorylation pattern regulates the subcellular localization. In PHFs, this soluble cytosolic protein is hyper-phosphorylated and becomes relatively insoluble. The aggregates of hyper-phosphorylated tau are usually mixed with ubiquitin, which may represent an attempt to degrade this intraneuronal protein inclusion in neurons. The formation of mature neurofibrillary tangles can be defined in four stages [7]. At stage 0, diffuse or granular tau staining is observed in pyramidal neurons with normal morphology. It represents the beginning of tau aggregation. At stage 1, with antibodies against tau, elongate inclusions are stained as early tangles. At stage 2, classical neurofibrillary tangles are detected in the somas with tau antibodies. At stage 3, the host neurons die and ghost tangles appear which are identified by anti-ubiquitin staining. The aggregates of hyper-phosphorylated tau may be a secondary effect of Aβ in AD. Knockout of tau in transgenic mice prevents the neurons from the damages caused by Aβ [174, 175]. Also, tau tangles are observed in other brain insults, such as epilepsy, focal cortical dysplasia and Niemann-Pick disease type C [149, 184, 195, 236]. Interestingly, tau pathology correlates better with cognitive decline than amyloid pathology [2, 14, 65].

These two neuropathological markers of AD, neurite plaques and neurofibrillary tangles, can independently develop in human cases. The biochemical characteristics of tau aggregates are similar in AD and other brain disorders that do not exhibit neurite plaques. On the other hand, in brains of cognitive normal elderly adults, neurite plaques can be detected without the appearance of neurofibrillary tangles. In some cases of AD, only a few neurofibrillary tangles can be detected in the neocortex although neurite plaques are abundant [203].

The amyloid hypothesis

More than twenty years, the amyloid hypothesis has dominated studies on the pathogenesis of AD [79, 80, 183]. This hypothesis proposes that amyloid deposition plays a central role in AD and implies elimination of Aβ will cure AD. The advent of the hypothesis has extensively promoted AD research. Also, the amyloid hypothesis itself has undergone revolutions during these years. Initially, the local toxic effects of amyloid plaques on neighboring cells were assumed as the cause of AD. However, soluble oligomers of Aβ are now supposed to contribute to the onset of the disease [78]. The most solid proof for the amyloid hypothesis is the discovery of AD causative genes

As early as in 1906, amyloid plaques were reported in the neocortex and hippocampus of AD patients and thus they are inevitably related with this disease [4]. In 1980s, biochemical analysis isolated the amyloid proteins and identified Aβ as the subunit protein of amyloid deposits [67, 72, 134]. Also, similar neuropathological markers are observed in patients with Down syndrome and the amino acid sequence of amyloid deposit in this disease is identical to the ones in AD patients [66, 132]. These results suggest AD and Down syndrome may share common pathological processes. As Down syndrome is due to the trisomy of the 21st chromosome, it means that increased expression of genes on the 21st chromosome may cause AD. In the process of isolating the gene encoding Aβ, amyloid precursor protein (APP) has been identified as the precursor to Aβ [70, 173, 201]. It is appealing that the gene of APP is on the 21st chromosome, implying the overexpression of this gene in Down syndrome may lead to the cognitive decline [105]. Based on these findings, the gene of APP became a target for researchers to investigate if its mutilations cause AD. In a Dutch family with hereditary cerebral hemorrhage with amyloidosis, the first APP mutation related with the pathogenesis of AD has been discovered [125]. Later, several different APP mutations were reported in families with early-onset AD [30, 68, 146]. All these AD causing mutations increase the production of Aβ. Recently, a mutation in APP decreasing Aβ production was found to be protective against AD and age-related cognitive decline [100].

Interestingly, some mutations that also result in early-onset AD are not localized in APP gene, or even on 21st chromosome. However, these mutations, presenilin 1 or 2 mutations (PS1 or PS2), were reported to elevate Aβ expression, implying they are likely to influence APP metabolism [180]. In 1997, presenilins were firstly found to interact with APP directly by co-

immunoprecipitation [228]. The following studies demonstrated that presenilins are the catalytic component of a protein complex that contributes to APP proteolysis [37, 227]. In addition, carriers of apolipoprotein E ε4 (ApoE4) are inclined to accumulate Aβ and have a strong risk for developing AD [142]. Taken together, all the AD causing or risk mutations identified in human cases induce the increases in Aβ levels or changes in Aβ ratio.

As the imbalance between Aβ production and clearance is believed to be causative for AD pathogenesis, this peptide should directly or indirectly contributes to the decline of cognition in AD patients, which means abnormal Aβ species need to be neurotoxic. Amyloid plaques, which are composed of fibrillar Aβ, are typically surrounded by dystrophic neurites, implying Aβ aggregates might cause local synaptic abnormalities [205]. Recent studies also indicate soluble Aβ oligomers may contribute to neuronal dysfunctions in AD [78]. Soluble Aβ oligomers range from dimers to dodecamers [216]. These oligomers are detected in human brain and CSF and exist in AD brain at a higher level [71]. The facts that the oligomers bind particularly to synapses and inhibit LTP provide evidence for their roles in cognitive impairment [114, 216, 219]. As Aβ oligomers exist in the surrounding area of amyloid plaques, it is difficult to ascertain if the pathology observed in the vicinity of plaques is caused by insoluble deposits, soluble oligomers or a combination of them.

The proteolysis of APP

Being the precursor protein of Aβ, APP contains a group of polypeptides which include alternative slicing isoforms of 695, 751 and 770 residues with a variety of posttranslational modifications [91, 217, 222]. The 751 and 770 residue isoforms usually present in both non-neuronal and neuronal cells, while 695 isoform is highly overexpressed in neurons other than non-neuronal cells [77]. Compared to 751 or 770 amino acids, 695 isoform lacks a 56-amino acid motif, which is similar to the sequence of Kunitz-type of serine protease inhibitors (KPI) [179]. Actually, APP belongs to a large gene family, which is called the amyloid precursor proteins (APLPs). APLPs share considerable homology with ectodomain and cytoplasmic tail, but they are quite different in the Aβ domain [47, 117].

APP is a single transmembrane protein that contains a large extracellular ectodomain and a short cytoplasmic tail. Proteolytic cleavages of APP release secreted derivatives into extracellular space and vesicle lumens. These processes are initiated either by an activity of α-secretase, which occurs at 12 residues NH_2-terminal to the transmembrane domain and

releases soluble ectodomain termed APPsα, or by an activity of β-secretase that mainly cuts 28 amino acids NH_2-terminal to the APP transmembrane domain and releases APPsβ [49, 185]. In these ways, 83-residue and 99-residue COOH-terminal fragments (CTF) are generated in the membrane, irrespectively. CTF99 other than CTF83 contains the domain of Aβ. Following the subsequent cleavage by γ-secretase, p3, Aβ and APP intracellular domain (AICD) are produced [147] (Fig. 5).

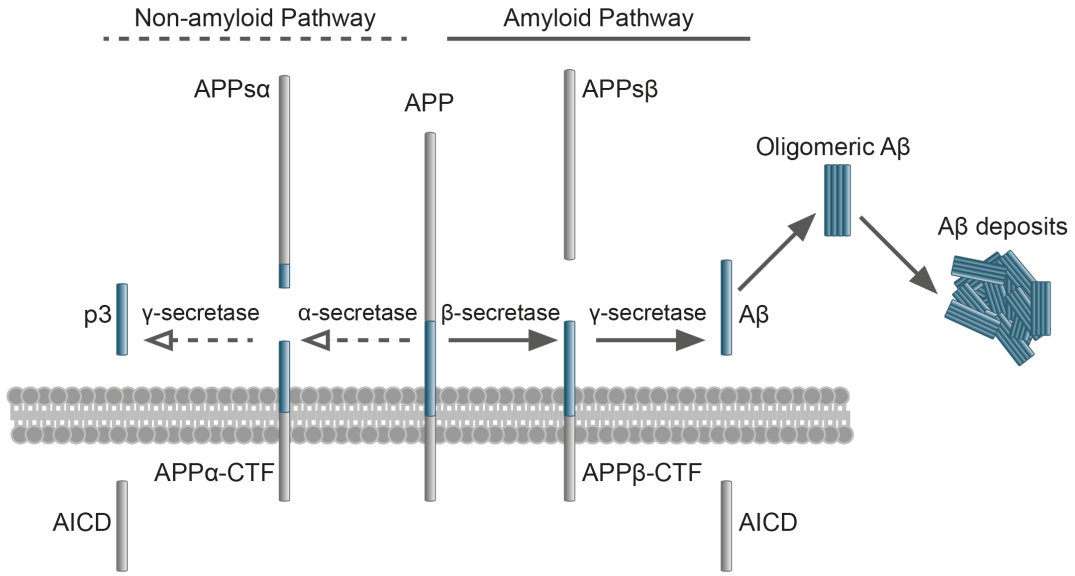

Figure 5 The proteolysis of APP. APP is degraded initiated by α or β-secretase. In amyloid pathway, Aβ is produced following the subsequent cleavage of γ-secretase.

The ratio of Aβ peptides is dependent on the activity of γ-secretase or APP sequence [106]. The γ-secretase is a complex that contains four proteins, including PS1 or PS2, nicastrin, anterior pharynx defective 1 (APH1) and PS enhancer 2 [38]. PS1 and PS2 provide the catalytic site for the proteolysis of CTF [39, 227]. As PS2 γ-secretase dose not mainly participate in Aβ production, only a few PS2 mutations are found to be contribute to the early-onset AD [85]. In wild-type PS1, $Aβ_{40}$ peptide is the major product of CTF cleavage mediated by γ-secretase. $Aβ_{50}$/ $Aβ_{49}$ is firstly cleaved by γ-secretase and then degraded into a shorter form [199, 230]. In most PS1 mutations that lead to early-onset AD, total amount of Aβ is reduced, while the ratio of $Aβ_{42}$/ $Aβ_{40}$ is enhanced [13]. The familial AD mutations in APP gene that locate at β-secretase cleavage site increase the production of all Aβ species, while the ones in γ-secretase cleavage site are in favor of $Aβ_{42}$ formation [180]. These results imply that the ratio of Aβ may be more crucial than the absolute amount of Aβ, at least in the pathogenesis caused by familial AD PS mutations.

Physiological functions of APP

Although it is widely believed that the proteolytic peptide of APP, Aβ, plays a central role in AD pathogenesis, the question of whether loss of APP due to the enhanced proteolytic process into Aβ also participates in the pathogenesis of AD remains unclear. Thus, the physiological functions of APP need to be unraveled.

APP is found to be highly expressed in neurons and localize in soma, dendrites and axons [87, 232]. The expression of APP is upregulated along with increased neuronal activity [197]. It undergoes anterograde transport with vesicles after being synthesized in the endoplasmic reticulum of cell soma [148]. Post-translationally modified by glycosylation and phosphorylation, APP associates with cytoplasmic proteins that facilitate APP transport into pre- and postsynaptic compartments [218]. The synaptic interaction of APP may form membrane tethers to modulate synaptic function [221]. Indeed, the extracellular domain of APP induces its trans-synaptic dimerization, which may be mediated by heparin [36, 74]. While cis-dimerization of APP modulates the proteolytic cleavage by γ-secretase, trans-dimerization promotes the adhesion between cells [170, 187]. The APP extracellular domain also interacts with extracellular matrix proteins and thus contributes to cell-matrix adhesion [11, 109, 141]. In addition, APP may be a modulatory protein for other adhesion molecules, as it is found to co-localize with them at the sites of adhesion [6, 127, 158, 234]. The role of APP in adhesion induces synaptogenesis [221] and it raises the question that whether APP-meditated synaptic adhesion is involved in AD, which is characterized by impaired synaptic functions.

Besides the neurotrophic effects of full-length APP due to its adhesion properties, growing evidence points out that α-secretase released APP soluble fragment, APPsα, is also involved in physiological functions of APP. Enhanced APPsα levels induce an increase in synaptic density [12, 177], while antibodies against APPsα inhibits LTP and spatial memory [202].Physiological deficits in APP knockout mice are fully restored by APPsα [172]. APPsα may enhance the phosphorylation of extracellular regulated protein kinases to promote neurite growth and adult neurogenesis [33, 178]. Also, there is evidence that APPsα stimulates the proliferation of neural stem cells in adult rodent brain through co-working with epidermal growth factor [25]. On the other hand, APPsβ undergoes further cleavage that binds to death receptor 6 mediating axonal pruning and degeneration [151]. AICD, the APP intracellular domain after γ-secretase cleavage, translocate into the nucleus to initiate

intracellular signaling cascades [63, 112]. Combined with Fe65 and Tip60, AICD form a transcriptionally active complex [27], of which the downstream targets have been identified [31, 111, 121, 212].

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2 Paper One

Intraneuronal APP and extracellular Aβ independently cause spine pathology in transgenic mouse models of Alzheimer's disease (Acta Neuropathol, 2015)

Title page

Intraneuronal APP and extracellular Aβ independently cause spine pathology in transgenic mouse models of Alzheimer's disease

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Abstract

Alzheimer's disease (AD) is thought to be caused by accumulation of amyloid-β protein (Aβ), which is a cleavage product of amyloid precursor protein (APP). Transgenic mice overexpressing APP have been used to recapitulate amyloid-β pathology. Among them, APP23 and APPswe/PS1deltaE9 (deltaE9) mice are extensively studied. APP23 mice express APP with Swedish mutation and develop amyloid plaques late in their life, while cognitive deficits are observed in young age. In contrast, deltaE9 mice with mutant APP and mutant presenilin-1 develop amyloid plaques early but show typical cognitive deficits in old age. To unveil the reasons for different progressions of cognitive decline in these commonly used mouse models, we analyzed the number and turnover of dendritic spines, as an important structural correlate for learning and memory. Chronic in vivo two photon imaging in apical layer V pyramidal neuron dendrites revealed a decreased spine density in 4-5-month-old APP23 mice. In age-matched deltaE9 mice, in contrast, spine loss was only observed on cortical dendrites that were in close proximity to amyloid plaques. In both cases the reduced spine density was caused by decreased spine formation. Interestingly, the patterns of alterations in spine morphology differed between these two transgenic mouse models. Moreover, in APP23 mice, APP was found to accumulate intracellularly and its content was inversely correlated with the absolute spine density and the relative number of mushroom spines. Collectively, our results suggest different pathological mechanisms, namely an intracellular accumulation of APP or extracellular amyloid plaques, may lead to spine abnormalities in young adult APP23 and deltaE9 mice, respectively. These distinct features, which may represent very different mechanisms of synaptic failure in AD, have to be taken into consideration when translating results from animal studies to the human disease.

Keywords: Alzheimer's disease, Intraneuronal APP, Extracellular Aβ, Dendritic spines, Two photon *in vivo* imaging

Introduction

Alzheimer's disease (AD) is the most prevalent cause of dementia and currently no effective treatment exists. Multiple strands of evidence suggest that amyloid precursor protein (APP) and its proteolytic fragment, amyloid β-protein (Aβ), play a crucial role in the pathogenesis of AD [62]. APP is a single-pass transmembrane protein enriched at synapses [19]. The highly conserved APP gene is located on chromosome 21 and overexpression of APP in Down's syndrome (Trisomy 21) causes accumulation of amyloid plaques early in life [21]. Through sequential enzymatic cleavage by β and γ-secretases, full-length APP is processed to yield amyloid beta (Aβ) as well as other fragments. Accumulation of fibrillar Aβ leads to formation of senile plaques, the typical neuropathological hallmark of AD. Soluble oligomeric Aβ, in contrast, is thought to mediate synapse dysfunction and loss, which strongly correlate with cognitive decline in AD [20, 32]. The amyloid hypothesis takes the imbalance between Aβ production and clearance as the primary cause of AD [20]. Based on this hypothesis and the discovery of familial AD mutations that facilitate Aβ production, transgenic mouse models overexpressing mutant APP and/or presenilins (PS), which form part of the γ-secretase complex, have been created to recapitulate AD pathology.

Among the APP transgenic mouse models, APP23 and APPswe/PS1deltaE9 (deltaE9) mice have been extensively used for exploring AD related pathology and drug development [63]. To recapitulate the pathogenesis of human AD, APP23 mouse model overexpresses human APP with the Swedish mutation under the murine Thy1 promoter [57], while deltaE9 mice express APP with the Swedish mutation controlled by mouse prion protein promoter elements together with mutant human PS1 lacking exon 9, which is associated with familial AD [29, 52]. Although these two transgenic mouse models display neuronal loss, cholinergic deficit, cognitive impairments, amyloid plaques and neuroinflammation in old age, the onsets of amyloid plaque formation and cognitive decline between them are very different in early adulthood [5, 8, 9, 30, 38, 56]. Aβ deposits are not observed in APP23 mice younger than 6 months, but age-matched deltaE9 mice have already developed plaques [28]. Despite of the slower progress of amyloid plaque formation, APP23 mice show faster cognitive decline than deltaE9 mice. APP23 mice begin to develop cognitive deficits at three months, while deltaE9 mice do not have typical impaired memory until one year of age [60, 61]. Uncovering and understanding the discrepancies between them are important for the utility of particular animal models to deepen our knowledge of synaptic failure in AD.

Using *in vivo* two-photon imaging of cortical layer V pyramidal neurons, we found reduced dendritic spine density in 4-5-month-old APP23 mice. In age-matched deltaE9 mice, loss of dendritic spines was only observed in close proximity to plaques. Furthermore, chronic in vivo imaging revealed that spine loss in AD transgenic mouse models was the consequence of decreased spine formation. Also, morphologies of dendritic spines in APP23 and deltaE9 mice were altered differently. Immunostaining showed accumulated intracellular APP in APP23 mice. The amount of intracellular APP was negatively correlated with spine density and morphology. These results suggest that spine abnormalities in young adult APP23 and deltaE9 mice might be caused by intracellular APP and extracellular Aβ deposits, respectively.

Materials and Methods

Animals

APP23 (Novartis) and APPswe/PS1deltaE9 mice (Jackson Laboratory) [29, 52, 57] were crossed with GPF-M mice (Jackson Laboratory) [13] to obtain double transgenic offspring heterozygous for the corresponding genes. All transgenic lines were kept on C57BL/6 background. eGFP positive littermates without mutant APP and PS1 transgenes were used as controls. Only female mice at the age of 4-5 months were used in this study. All protocols and procedures were conducted according to the animal protocol approved by the Ludwig-Maximilian University Munich and the government of Upper Bavaria.

Cranial window implantation and *in vivo* two-photon imaging

As previously described [24], mice were anesthetized by intraperitoneal injection of ketamine/xylazine (130/10 µg/g body weight). Subsequently, dexamethasone (6 µg/g body weight) was injected intraperitoneally to prevent development of cerebral edema. A piece of skull above the somatosensory cortex was removed and replaced with a 4 mm diameter coverslip. After a 4-week recovery period, apical dendrites originating from layer V pyramidal neurons were imaged using a LSM 7MP microscope (Zeiss) equipped with a 20x water-immersion objective (1.0 NA, Zeiss). Mice were anesthetized with isoflurane and placed on a heating pad to maintain the body temperature. Any single imaging session lasted no longer than one hour. In subsequent imaging sessions, imaged regions were re-localized based on the unique pattern of blood vessels. To stain amyloid plaques in vivo, methoxy-X04 (1 mg/kg) was intraperitoneally injected 24 h before imaging. For overview images, 424 x 424 x 350 μm^3

z-stacks (0.83 µm/pixel) were taken. Higher resolution images (0.138 µm/pixel) were used for counting dendritic spines.

Spine analysis

Spines were counted manually in ZEN 2011 (Zeiss). Due to limitations in resolution in the Z-direction, only laterally protruding spines were taken into account, as only those could be identified with certainty and classified morphologically. Spines that had emerged or disappeared since the previous imaging session were classified as formed or eliminated, respectively. Spine turnover rate (TOR) was calculated as follows: $(N_f + N_e)/(2 \times N_t \times D)$, N_f = formed spines, N_e = eliminated spines, N_t = total spines, D= interval days between imaging sessions. For morphological analysis, maximum intensity projections from *in vivo* two-photon stacks were used. The length of each spine was measured from the tip of the spine head to the bottom of the spine neck. Spine head width was defined as the length between the left edge of spine head to the right edge. Spines were classified into mushroom, stubby and thin spines based on their appearances as described before [22, 24].

Immunohisochemistry

Following transcardial perfusion with phosphate buffered saline (PBS) and 4% paraformaldehyde (PFA), mouse brains were fixed in 4% PFA overnight at 4 °C and then cut into 65 µm thick free-floating frontal sections at the level of the somatosensory cortex. β amyloid (4G8, Covance, 1:200), beta-amyloid 40 (139-5, Covance, 1:100), and beta-amyloid 42 (11-1-3, Covance, 1:100) and anti-APP 22C11 (Millipore, 1:20) antibodies were used for APP and Aβ staining. Anti-mouse or rabbit Alexa 647 antibody (Life technologies, 1:1000) was used as the secondary antibody. For spine imaging, sections were incubated with anti-GFP coupled with Alexa 488 (Life technologies, 1:300) and then mounted on glass coverslips using fluorescence mounting medium (Dako). For the microscopy of cortical areas, LSM 780 confocal microscope (Zeiss) was equipped with a 10x/0.3 objective. To image pyramidal neurons and dendrites, a 40x/1.4 objective was used and 212 x 212 x 80 μm^3 z-stacks (0.415 µm/pixel) were taken for overview images and APP quantification. To quantify the relative APP amount, custom-written Matlab software was applied to correct for the depth-dependent changes inherent to data obtained from brain slices immunostained with fluorophor-coupled antibodies. Exponential fitting was applied to correct for the reduction in fluorescence intensity toward the center of the brain slice due to decreasing antibody penetration as well as the

additional reduction imposed by light scattering and light absorption over the complete depth of the slice. Higher resolution images (0.069 µm/pixel) were used for counting dendritic spines.

Statistics

Analyses were performed blinded with respect to mouse genotype. The numbers of mice for *in vivo* two photon imaging were 5-6 per group. 7-12 dendrites were imaged in each mouse; the length of each dendrite was 25-35 µm. The data are presented as the means for every mouse (round symbols) and the means of the means (horizontal line with error bars), except for the data shown in figure 3, where the data from 13 dendrites out of 5 mice, which were located in proximity to nascent plaques (50-80 µm), are shown. More than 30 neurons from 5 mice were imaged in *ex vivo* imaging. Results are presented as mean ± S.E.M and compared with controls by one-way ANOVA with Dunnett's test. Kolmogorov-Smirnov test was used for comparing cumulative frequency distributions. Extra sum-of squares F test was used when data were fitted a straight line with nonlinear regression. $p<0.05$ was defined as statistically significant with * $p<0.05$, ** $p<0.01$, N.S.: not significant.

Results

Dendritic spine density of layer V pyramidal neurons is reduced differently in young adult APP23 and deltaE9 mice

In this study, we used APP23 and deltaE9 mouse models, which both express human APP with the Swedish mutation. In deltaE9 mice, mutant human PS1 lacking exon 9 is co-expressed [29, 52, 57]. These two mouse models develop neuropathological hallmarks of AD differently in young adulthood. APP23 mice show cognitive deficits before amyloid plaque formation while deltaE9 mice develop memory loss after Aβ deposition [28, 60, 61].

To examine whether and how AD transgenic mouse models develop synaptic pathology in young adulthood, we crossed APP23 and deltaE9 mice with GFP-M transgenic mice to visualize apical dendrites of layer V pyramidal neurons by *in vivo* two-photon microscopy (Fig. 1a). We found a significant decrease of spine density in APP23 mice at the age of 4-5 months (0.28±0.01 spines/µm, vs. WT 0.38±0.03 spines/µm, Fig. 1b). Because Aβ deposits emerge in deltaE9 mice as early as 4 months of age and amyloid plaques disturb dendritic spine stability [3, 16], we analyzed dendrites in deltaE9 mice that were close and far from plaques. Dendrites that were chosen from plaque-free overview images (>100 µm from plaques,

supplementary Figure 1a) did not show spine loss (0.36±0.01 spines/µm, Fig. 1b), but the ones that were in close proximity to plaques (<30 µm from plaques, supplementary Figure 1b) displayed a strong decrease in spine density (0.27±0.02 spines/µm, Fig. 1b).

Imbalance between spine formation and elimination causes spine loss

To determine whether spine dynamics are altered in APP23 and deltaE9 mice, we repeatedly imaged apical dendrites one week apart in the somatosensory cortex. While the spine turnover rate in both AD models did not differ from WT animals (0.038±0.003 vs. 0.042±0.003 vs. 0.039±0.004 vs. 0.045±0.003, Fig. 1c), we found that significantly fewer new spines emerged in APP23 mice (0.05±0.004 spines/µm, vs. WT 0.11±0.01 spines/µm, Fig. 1e). In deltaE9 mice, spine formation was also decreased on dendrites that were in proximity to plaques (<30 µm, 0.065±0.003 spines/µm, Fig. 1e), but not on dendrites far away from plaques (>100 µm, 0.099±0.01 spines/µm, Fig. 1e). The spine eliminations among WT, APP23 and deltaE9 mice (>100 µm and <30 µm) were comparable (0.098±0.004 spines/µm vs. 0.087±0.008 spines/µm vs. 0.097±0.005 spines/µm vs. 0.087±0.008 spines/µm, Figure 1d). These results suggest that the decrease in the spine density of young adult AD mice is as a consequence of an imbalance between spine formation and elimination.

Alterations in spine morphology differ between APP23 and deltaE9 mice

Besides absolute spine density, spine morphology also correlates with dendritic spine function and thus impacts cognitive performance [51]. To examine whether the spine morphology of these AD transgenic mouse models is altered, we measured spine length and spine head width of the *in vivo* imaged dendritic spines**.** Spine lengths of dendritic spines from APP23 and deltaE9 mice (<30 µm) were significantly decreased, while spines from deltaE9 mice (>100 µm) showed decreased spine head width (Fig. 2a, b). Moreover, we classified the spines according to their morphological appearance into mushroom, stubby and thin spines [24]. APP23 and deltaE9 mice (>100 µm and <30 µm) showed a reduced fraction of mushroom spines (35.0±6.9%, 38.2±5.7% and 44.3±2.7% vs. WT 59.6±3.5%, Fig. 2c). Furthermore, in APP23 mice and deltaE9 mice (<30 µm), the decreases of mushroom spines were accompanied with strong increases in the stubby spines (48.6±6.0% and 42.5±3.7% vs. WT 19.4±5.2%, Fig. 2d). However, thin spines, but not stubby spines, were increased in deltaE9 mice (>100 µm, 36.7±5.7% vs. WT 20.9±2.3%, Fig. 2e). Collectively, these results show that morphological alterations of dendritic spines in APP23 and deltaE9 mice, of deltaE9

mice, are distinct. In addition, these alterations even differ between different distances to fibrillar plaques within deltaE9 mice.

Spine loss and alterations in spine morphology are associated with amyloid plaque growth in deltaE9 mice

In young adult deltaE9 mice, dendrites that were located near (<30 μm) and far (>100 μm) away from plaques displayed two different patterns of spine abnormalities. Close to plaques (<30 μm) a decrease in spine density and increase in the fraction of stubby spines were observed. Dendrites far away from plaques (>100 μm) did not develop spine loss but showed increased fraction of thin spines. To investigate whether the alterations of dendritic spine abnormalities are correlated with the distance between dendrites and plaques, we imaged dendrites that resided 50-80 μm away from plaques. With amyloid plaque growth over one month, the distance between dendrites and plaques became smaller (from 59.9±2.7 μm to 52.6±2.6 μm, Fig. 3a). Meanwhile, dendrites started to develop spine loss (Fig. 3b, c). The decrease of spine density was caused by reduced spine formation (Fig. 3d). Moreover, the fraction of mushroom spines remained unchanged (Fig. 3e), while the fraction of stubby spines increased along with the decrease of thin spine fraction (Fig. 3f, g). Taken together, these results indicate amyloid plaques cause manifold dendritic spine alterations in deltaE9 mice.

APP accumulates intracellularly in APP23 mice

To exclude the possibility that the decreased spine density which we observed in APP23 mice was not caused by the close vicinity to amyloid plaques [4], we used methoxy-X04 to label fibrillar amyloid deposits *in vivo* [7] and no plaque was found in the imaged volumes in APP23 mice at the age of 4-5 months(data not shown). *Ex vivo* immunohistochemical staining further confirmed that APP23 mice had not yet developed amyloid plaques (data not shown). Furthermore, we stained brain sections using an antibody that recognizes both APP and Aβ (4G8). Surprisingly, a strong APP/Aβ somatic staining was observed in the cortex of 4-5-month-old APP23 mice (Fig. 4a). To further clarify the identity of the intracellular immunoreactivity, we used antibodies specific to detect Aβ40, Aβ42 and APP. No intracellular immunoreactivity was detected by Aβ specific antibodies in APP23 mice (Fig. 4c, d). The ability of these antibodies to bind Aβ peptides was verified by the detection of extracellular Aβ deposits in deltaE9 mice (Fig 4c, d). In contrast, intracellular APP immunoreactivity was also observed with the APP specific antibody 22C11 in APP23 mice (Fig. 4b). Western blot

analysis further confirmed APP23 mice mainly overexpressed full-length APP, but not Aβ, in young adulthood (Supplementary Figure 2). Notably, the expression of APP in APP23 mice was higher than in deltaE9 mice (Supplementary Figure 2), which is in line with previous reports [28, 57]. These results suggest that the intracellular accumulations in APP23 mice consist of APP, rather than Aβ.

The amount of intracellular APP correlates with dendritic spine alterations

In young adult APP23 mice, spine density of cortical pyramidal neurons was reduced and spine morphology was also changed. To assess if these structural alterations were caused by the observed intracellular APP accumulation, the amount of APP in the soma of eGFP labeled cortical layer V pyramidal neurons was quantified from brain sections (Fig. 5a). Along with the increase of intracellular APP, spine densities on apical and basal dendrites of pyramidal neurons declined (Fig. 5b, c, f). In addition, the fractions of mushroom spines were decreased (Fig. 5d, g), while stubby spine fractions were increased (Fig. 5e, h). Besides, the accumulation of intracellular APP in CA1 pyramidal neurons also coincided with the decrease of spine density and alterations of spine morphology (Supplementary Figure3). Altogether, these results suggest that intracellular accumulation of APP may be responsible for the spine alterations in 4-5-month-old APP23 mice.

Discussion

Extracellular Aβ is accepted to be in the center of AD pathogenesis due to its neurotoxicity that disrupts multiple physiological processes [53]. Guided by the amyloid hypothesis, AD mouse models have been created to recapitulate the cognitive impairments seen in AD patients. These mouse models typically express human APP with our without PS1 with familial AD mutations, which both cause familial forms of AD. Although most of the mouse models develop typical amyloid plaques and cognitive deficits with age, the pathophysiology in young transgenic mice, reflecting preclinical forms of AD, is less well understood [63]. APP23 mice display cognitive impairments before plaque formation, while deltaE9 mice develop abundant plaques before the decline of cognitive performance. The underlying mechanisms of these discrepancies are still not clear.

The major correlate of cognitive impairment is synapse loss, which is closely associated with spine loss as excitatory glutamatergic synapses normally reside at dendritic spines in the mammalian brain [43]. In addition to absolute spine density, the dynamic turnover of spines,

termed structural plasticity, is also involved with learning and memory: the formation and elimination of dendritic spines rewire neural circuits by establishing or abolishing connections in the brain during learning experiences [15]. Thus, it is plausible to examine alterations of dendritic spines as readout for structural correlate of cognitive decline in AD transgenic mouse models.

In this study, we found that 4-5-month-old APP23 mice displayed reduced spine density of cortical layer V pyramidal neurons. In deltaE9 mice, spine loss was only evident on dendrites that were located close to plaques. We found similar results in the APPswe/PS1L166P mouse model [48], which accumulates plaques faster than the deltaE9 model: here, spines were lost only in the vicinity (<50 μm) of plaques, while spines were not altered distant (>50 μm) to plaques or before plaques had appeared [3]. These results suggest that spine loss mediated by fibrillar amyloid plaques occurs only in the immediate vicinity of extracellular Aβ deposits in deltaE9 and APPswe/PS1L166P mice.

The decreased spine densities observed in APP23 and deltaE9 mice were caused by reduced spine formation as revealed by chronic repetitive *in vivo* two-photon imaging. Interestingly, we found two different patterns of spine morphological alterations in these two transgenic mouse models. In APP23 mice, the spine length was reduced and the relative proportion of stubby spines was increased. In deltaE9 mice, in dendrites close to plaques, the findings were identical. In contrast, the dendrites that were far away from plaques in deltaE9 mice showed decreased spine head width and elevated thin spine fraction. With amyloid plaque growth in deltaE9 mice, dendrites, that were originally located 50-80 μm away from plaques, became closer to plaques and started to lose spines. This effect was accompanied with an increase in the fraction of stubby spines. In APP23 mice, APP accumulated intracellularly. A higher content of APP was inversely correlated with spine density. Furthermore, an increased fraction of mushroom spines and decreased fraction of stubby spines were observed in neurons, which contained higher levels of intracellular APP. In summary, our data suggested different pathological mechanisms, intracellular APP and extracellular amyloid plaques, might lead to spine abnormalities in young adult APP23 and deltaE9 mice, respectively.

Dendritic spines are the small bulbous postsynaptic elements of the majority of excitatory synapses and serve as the basic units for learning and memory [22]. Loss of dendritic spines

is the major correlate of cognitive impairment in human AD [59]. In agreement with the spine loss described before, APP23 mice younger than 6 months show memory impairments in multiple cognitive tests, including Morris-type water maze test, Y-maze test, Barnes-maze test and novel-object recognition test [12, 25, 31, 60]. On the other hand, the performance of deltaE9 mice at the same age is normal in most cognitive tests (T-maze test, Y-maze test, Morris-type water maze test, novel taste neophobia test, response acquisition test, Barnes-maze spatial memory task with hidden-target strategies), with the exception of impairments observed in Barnes-maze spatial memory task with cued-target strategies and modified radial-arm water maze test [18, 34, 45, 49, 61]. The specific spatial learning deficit described in young deltaE9 mice may depend on dendritic spine shape, rather than a reduced spine number, considering spine loss is only observed on dendrites that are localized very close to amyloid plaques, which just start to emerge in 4-5-month-old deltaE9 mice [6, 16, 28]. With aging, Aβ deposits grow in size. Amyloid plaques mice are abundant in hippocampus and cortex of one-year-old deltaE9 mice. At this age general axon degeneration and synapse loss are observed, along with impaired cognitive performance [18, 46]. Thus, loss of synapses coincides with decline in cognitive performance in these models.

Indeed, there is convincing evidence that not only the absolute spine number contributes to cognitive performance. In fact, dendritic spine size and shape are known to affect various functional parameters relevant for cognition, including spine motility, neurotransmitter receptor numbers and organelle abundance [33, 51]. Growing evidence shows that morphological changes of dendritic spines are associated with long-term synaptic plasticity (LTP) [68]. LTP increases spine head volume while shortening and widening spine neck [67]. This morphological plasticity allows generating changes in electrical properties of dendritic spines, which serve as isolated electrical compartments. For instance, it has been shown that shorter spine necks lead to larger depolarization while longer necks generate smaller somatic potentials [1]. It is believed that different types of memories need to obey different electrophysiological rules, and thus require morphological diversities of spines [51]. Along with changes in spine density, distinct alterations of spine morphology in APP23 mice and deltaE9 mice might also result in the different cognitive impairments described before [12, 18, 25, 31, 34, 45, 49, 60, 61]. Layer V pyramidal neurons in the somatosensory cortex are involved in motor learning [14, 64-66] and the formation of new dendritic spines correlates with the performance after learning [66]. While most behavioral tests focus on hippocampus dependent memory tasks, the resulting behavior results from a complex interplay of various

brain regions, in which somatosensory cortex neurons may play crucial roles. Thus, the alterations of dendritic spines which we found may well reflect part of the behavioral phenotype observed in these mice. Yet the susceptibility of spines to the various toxic insults due to the overexpression of APP and its cleavage products may differ between brain regions, between different functional locations within a neuron (e.g. between apical and basal dendrites) or with the age of the experimental animals. Therefore, the relation of dendritic spine loss in layer V pyramidal neurons to cognitive dysfunction is not certain.

Compared to APP23 mice, deltaE9 mice harbor an additional transgene of a familial AD mutation in PS1 with a deletion of exon 9, accelerating the cleavage of APP and thereby Aβ formation. In consistence with previous studies [10, 16], extracellular amyloid plaques have developed in 4-5-month-old deltaE9 mice but not APP23 mice. Being the abnormal protein aggregates that characterize human AD, Aβ deposits are one of the biomarkers for AD neuropathologic assessment [26]. Aβ production and aggregation might initiate serial molecular cascades, thus lead to clinical AD [20]. This amyloid cascade hypothesis seems to be feasible in early onset AD, which is known to be caused by mutations of genes that increase Aβ accumulation [27]. However, as early onset AD only accounts for a few percent of AD cases and the correlation between cognitive decline and Aβ deposits is weak [2, 17], alternative explanations for the pathogenesis of AD have emerged[36, 40].

In contrast to age-matched deltaE9 mice only a minor soluble Aβ burden was found in the brains of young APP23 mice [10, 37, 61]. Overexpressed APP in APP23 mice is predominantly localized intracellularly and the mechanisms of this aberrant accumulation and its relevance in sporadic AD need to be further investigated. Interestingly, a number of studies have reported increased amount of APP mRNA in AD patients [39, 41, 47], indicating that up-regulated transcriptional activity of APP may also contribute to AD pathophysiology. Moreover, accumulated APP has been found in dystrophic neuritis of AD [11, 54]. It is therefore tempting to speculate that intraneuronal accumulation of APP and/or its cleavage products including Aβ in AD may also contribute to synaptic damage [44, 58]. Indeed, an extra copy of the APP gene can cause neuronal dysfunction and symptoms similar to those seen in AD [42]. APP gene triplication in Down's syndrome and APP locus duplication in rare families lead into clinical AD-like pathology in adults and result in early-onset dementia [21, 50]. The neurotoxicity of APP is largely thought to be caused by its proteolytic fragments. Besides Aβ, other proteolytic APP fragments, such as C83, C99 and APP intracellular domain, could also

be involved in AD pathogenesis [55]. By regulating gene expression, these derivatives may give rise to neuronal degeneration [35, 55]. Additionally, through the direct interaction between APP and N-methyl-d-aspartate receptors (NMDARs), overexpressed APP up-regulates the expression of NMDARs and thus may contribute to neuronal toxicity by disrupting synaptic homeostasis [23].

To conclude, despite the fact that APP23 and deltaE9 mice show similar cognitive impairments and neuropathology in advanced age, our data clearly show different dendritic spine abnormalities in these two transgenic mouse models in young adulthood. Our findings imply that synaptic failure in these mouse models may be caused by different mechanisms in an age dependent manner. Since the mechanisms underlying the development of sporadic AD are still uncertain, this study has significant implications for the analysis of distinct AD transgenic mouse models during preclinical drug evaluation for treatment of early-stage AD.

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Conflicts of Interest

The authors declare that they have no conflict of interests.

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Figure legends

Figure 1. Decreased spine density in dendrites of APP23 mice and deltaE9 mice.

(**a**) By in vivo two-photon imaging, the same apical dendrites from layer V pyramidal neurons in the somatosensory cortex were repeatedly imaged one week apart. Each image is a maximum intensity projection of serial sections. White arrowheads point at spines formed over one week and empty arrowheads point at eliminated spines. Scale bar represents 10 μm.

(**b**) Spine densities of layer V pyramidal neuron apical dendrites in WT, APP23 and deltaE9 mice. In deltaE9 mice, dendrites that were localized at plaque-free overview images are classified as deltaE9 (>100 μm) and the ones in close proximity to plaques are named as deltaE9 (<30 μm).

(**c**)Turnover rates of apical dendrites in WT, APP23 and deltaE9 (>100 μm and <30 μm) mice.

(**d, e**) Spines that were eliminated (d) and newly formed (e) over one week in WT, APP23 and deltE9 (>100 μm and <30 μm) mice.

In WT group, n=6. In APP23 group, n=6. In deltaE9 (>100 μm) group, n=5. In deltaE9 (<30 μm) group, n=5. **, $p<0.01$ (ANOVA with Dunnett's post-hoc test).

Figure 2. Dendritic spine morphology changes differently in APP23 and deltaE9 mice.

(**a, b**) Cumulative distributions of spine length (a) and spine head width (b) in WT, APP23 and deltaE9 (>100 μm and <30 μm) mice. (a-b) **, $p<0.01$ (Komogornov-Smirnov test).

(**c-e**) Fractions of mushroom (c), stubby (d) and thin spines (e). Representative classified spines are on the top-left corner.

In WT group, n=6. In APP23 group, n=6. In deltE9 (>100 μm) group, n=5. In deltE9 (<30 μm) group, n=5. (c-e) *, $p<0.05$; **, $p<0.01$ (ANOVA with Dunnett's post-hoc test).

Figure 3. Spine loss and morphological alterations are accompanied by amyloid plaque growth in deltaE9 mice

(**a**) Maximum intensity projections of two-photon in vivo images of GFP-labeled dendrites (white) and methoxy-X04 labeled plaques (blue) are shown. The distance from dendrite to plaque (red arrow line) is reduced after one month due to plaque growth. Scale bar represents 40 μm

(**b**) Maximum intensity projected dendrites from a (arrowhead pointed, near plaque) and from plaque-free overview images (Without plaque) in deltaE9 mice. Scale bar represents 10 μm.

(**c**) Spine densities of the dendrites that were near plaque or in plaque-free area over one month. Each dashed line represents one dendrite.

(**d**) Newly formed and eliminated spine densities of the dendrites that were near plaque or in plaque-free area over one month.

(**e-g**) Fractions of mushroom (e), stubby (f) and thin spines (g) in these two different dendrites over one month. Each dashed line represents one dendrite.

Paired t test was used for plaque growth mediated spine alterations and unpaired t test was used to compare spine formation and elimination between groups. n=13 in each group. *, $p<0.05$; **, $p<0.01$.

Figure 4. Intracellular accumulation of APP in APP23 mice

(**a-d**) Immunohistochemical labeling of intracellular APP/Aβ (4G8, a), intracellular APP (22c11, b), Aβ42 deposits (11-1-3, c) and Aβ40 deposits (139-5, d) in WT, APP23 and deltaE9 mice. Scale bar represents 100 μm.

Figure 5. Increased intracellular APP accumulation is accompanied with decreased spine density and altered spine morphologies in the somatosensory cortex of APP23 mice

(**a**) Maximum intensity projections of ex vivo images of GFP-labeled neurons (white, A and B) and intracellular APP accumulation in layer V pyramidal neurons (black). Green dashed circle indicates the area of soma from GFP-labeled neurons. Arrows and arrow heads point to basal and apical dendrites, respectively. Scale bar represents 20 μm.

(**b**) Maximum intensity projected basal and apical dendrites from A and B. Scale bar represents 10 μm.

(**c-e**) The dot plots are the intensity of intracellular APP in basal dendrites from layer V pyramidal neurons versus spine density, mushroom and stubby fractions separately. Straight lines are fitted by nonlinear regression. Each dot represents one neuron.

(**f-h**) The dot plots are the intensity of intracellular APP in apical dendrites from layer V pyramidal neurons versus spine density, mushroom and stubby fractions separately. Straight lines are fitted by nonlinear regression. Each dot represents one neuron.

In basal dendrite group, n=38. In apical dendrite group, n=33 (c-h) *, $p<0.05$; **, $p<0.01$ (F test).

Figures

Figure 1

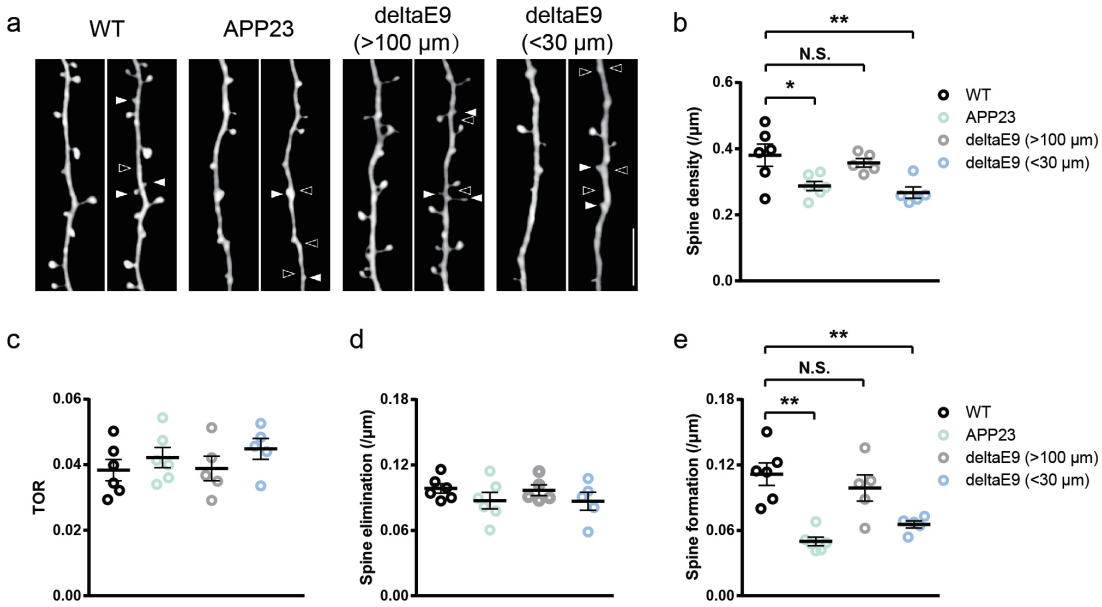


Figure 2

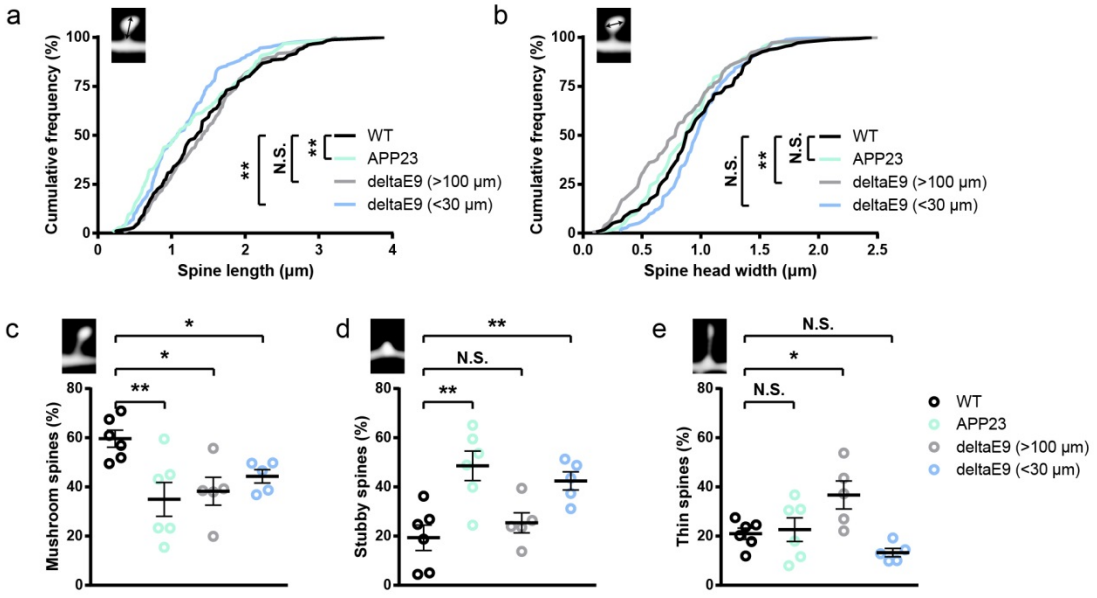


Figure 3

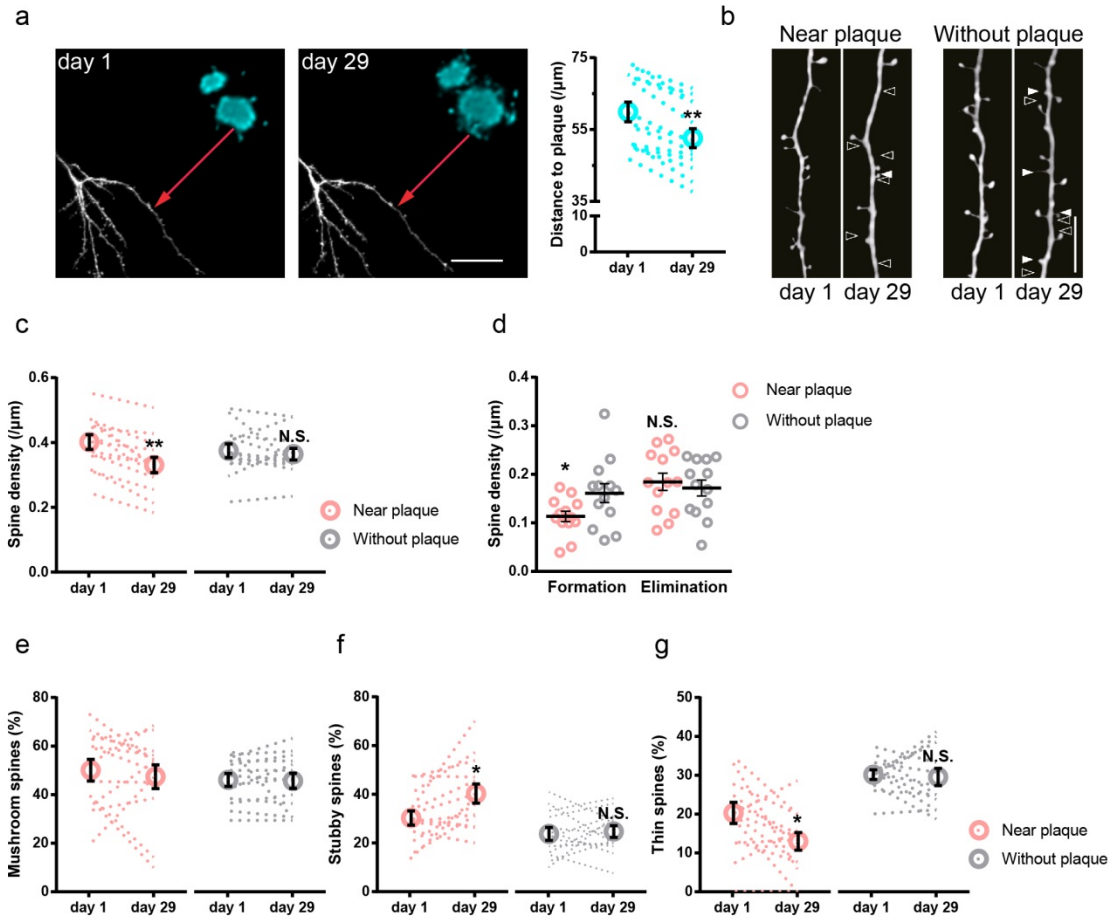


Figure 4

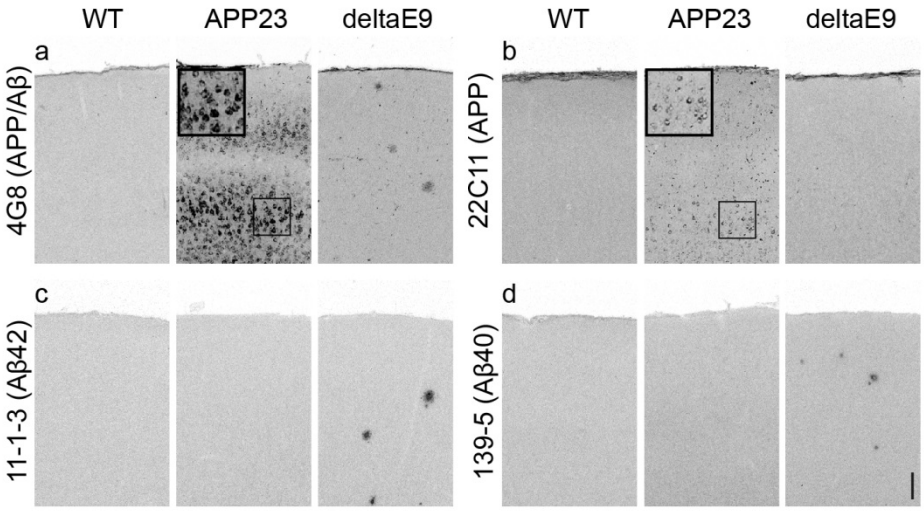
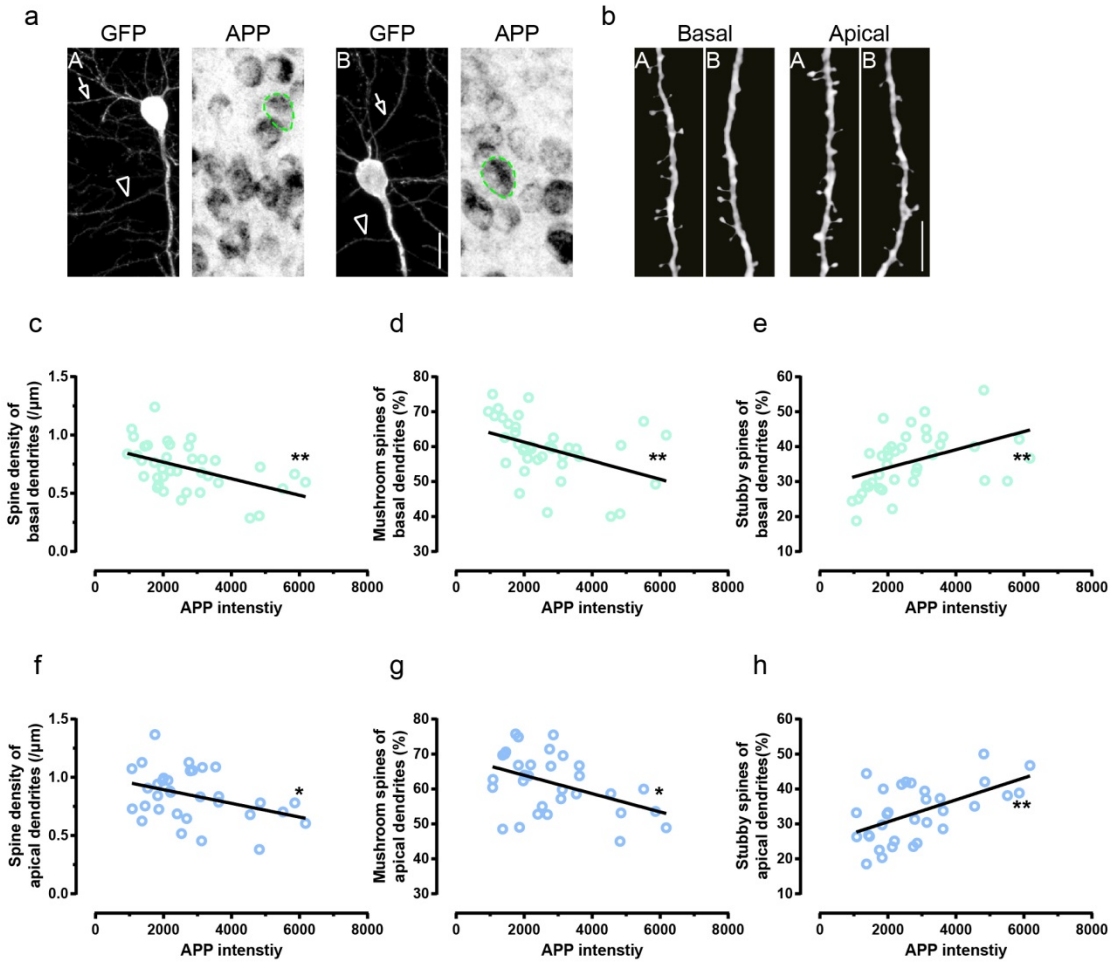


Figure 5


a
GFP
APP
GFP
APP
A
B
b
Basal
Apical
A
B
A
B
c
d
e
Spine density of basal dendrites (/μm)
Mushroom spines of basal dendrites (%)
Stubby spines of basal dendrites (%)
APP intenstiy
APP intenstiy
APP intenstiy
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**
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f
g
h
Spine density of apical dendrites (/μm)
Mushroom spines of apical dendrites (%)
Stubby spines of apical dendrites(%)
APP intenstiy
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Supplementary materials

Supplementary methods

Western blot: Tricine-SDS-PAGE was used in western blot as described before [1]. Briefly, 10% cortical tissues (w/v) were homogenized in lysis buffer supplemented with protease inhibitors (Roche), followed by centrifugation at 500 rpm for 1 min. The supernatant was collected and protein concentrations were adjusted by Bradford assay (Sigma-Aldrich) to ensure the same amount of protein being loaded for each sample (100 µg). Samples were mixed with SDS-containing sample buffers and incubated at 37 °C for 20 min. After electrophoresis in 15% sample gel, proteins were transferred to a polyvinylidene difluoride membrane (Millipore). The APP/Aβ primary antibody, 6E10 (Convance), was used at 1:500 concentration for immunoblotting. Full-length APP and Aβ oligomers were determined based on the molecular weights [2]. Protein bands were quantified in ImageJ. Results were normalized to control and repeated measures one-way ANOVA was used followed by Newman-Keuls's test.

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Supplementary figure legends

Supplementary Figure 1. Dendrites at different distances from plaques in deltaE9 mice.

(**a, b**) In vivo overview images showing GFP-labeled dendrites (white) and methoxy-X04 labeled plaques (blue). Dendrites that were localized at plaque-free overview images are classified as deltaE9 (>100 µm, a) and the ones in close proximity to plaques are named as deltaE9 (<30 µm, b). Arrowheads point to the chosen dendrites for spine analysis.

Supplementary Figure 2. Young adult APP23 mice overexpress AP

(**a, b**) Western blot examples (a) and quantification of protein band (≈85 kDa, b) reveal an overexpression of APP in the cortex of APP23 mice.

(**c-e**) Quantifications of protein bands (≈23 kDa, ≈56 kDa and ≈115 kDa) reveal overexpressed Aβ in the cortex of deltaE9 mice, but not in APP23 mice. n=5 in each group. (b-e) *, $p<0.05$; **, $p<0.01$ (ANOVA with Dunnett's post-hoc test).

Supplementary Figure 3. Increased intracellular APP accumulation is accompanied with decreased spine density and altered spine morphologies in the CA1 region of APP23 mice

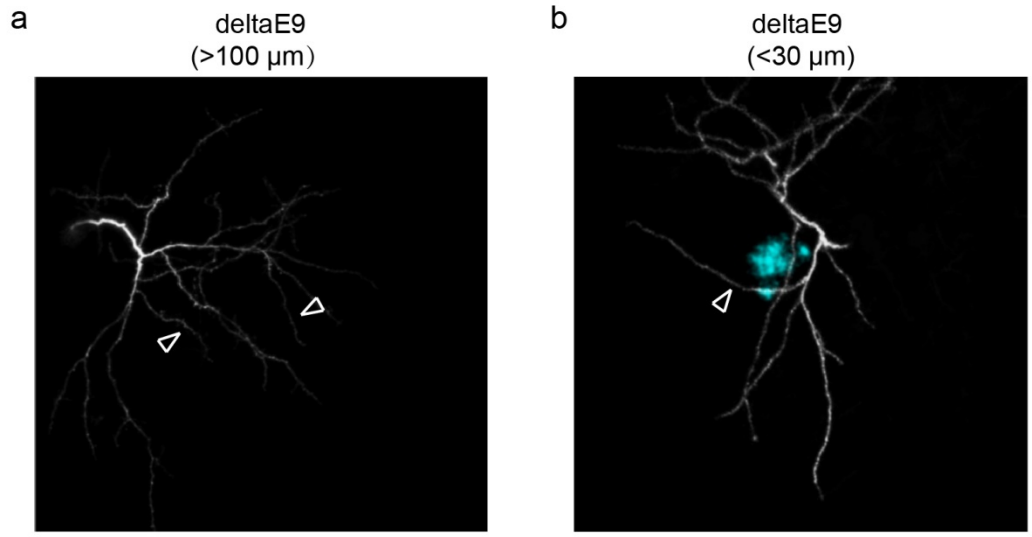
(**a**) Maximum intensity projections of ex vivo images of GFP-labeled neurons (white, A and B) and intracellular APP accumulation in CA1 pyramidal neurons (black). Green dashed circle indicates the area of soma from GFP-labeled neurons. Arrow points at the chosen dendrites for spine analysis. Scale bar represents 20 μm.

(**b**) Maximum intensity projected basal and apical dendrites from A and B. Scale bar represents 5 μm.

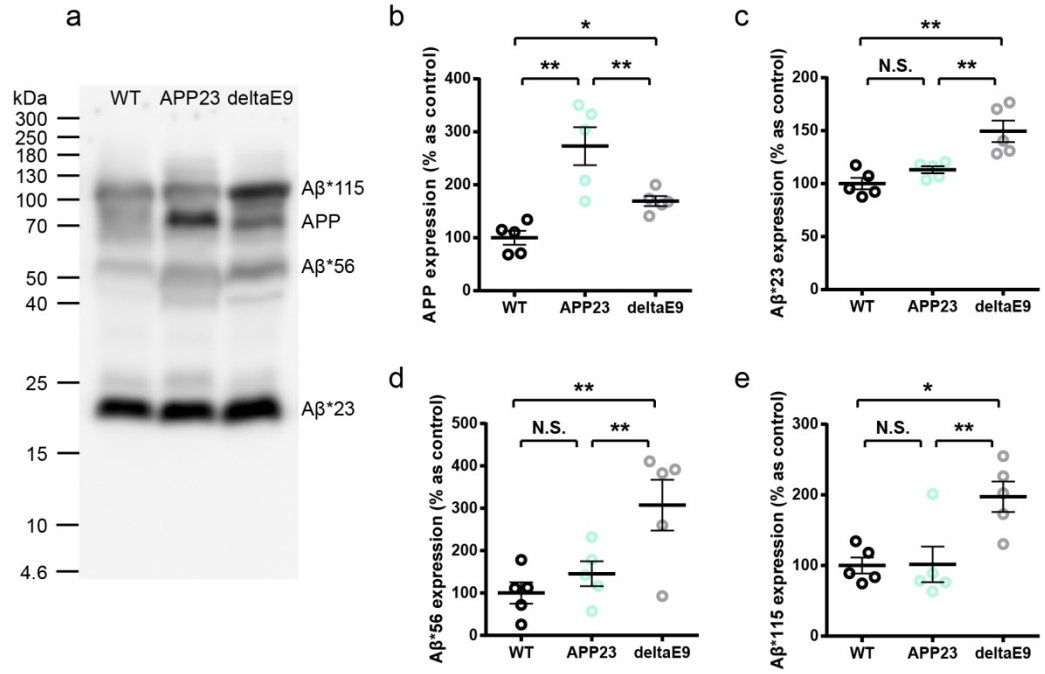
(**c-e**) The dot plots are the intensity of intracellular APP in dendrites from CA1 pyramidal neurons versus spine density, mushroom and stubby fractions separately. Straight lines are fitted by nonlinear regression. Each dot represents one neuron. n=38. (c-e) **, p<0.01 (F test).

Supplementary Figures

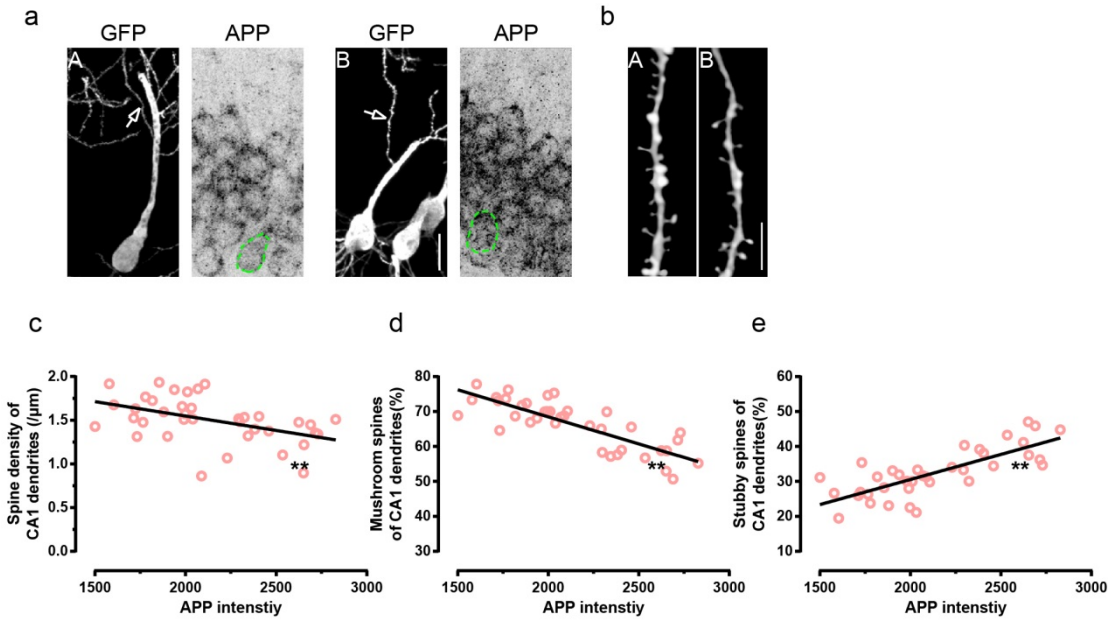
Supplementary figure 1



Supplementary figure 2



Supplementary figure 3



3 Manuscript One

Neuroinflammation impairs adaptive structural plasticity of dendritic spines in a preclinical model of Alzheimer's disease (Submitted)

Title page

Neuroinflammation impairs adaptive structural plasticity of dendritic spines in a preclinical model of Alzheimer's disease

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Abstract

To successfully tackle Alzheimer's disease (AD), pathophysiological events in preclinical stages need to be identified. Preclinical AD refers to the stages that exhibit normal cognitive function and amyloid deposition in the brain, which are replicated in young adult APPswe/PS1deltaE9 (deltaE9) mice. By long-term in vivo two-photon microscopy, we demonstrated the impaired adaptive spine plasticity in these transgenic mice illustrated by their failures to increase dendritic spine density and form novel neural connections when housed in enriched environment (EE). Elimination of amyloid plaques by reducing BACE1 activity restored the gain of spine density upon EE in deltaE9 mice but not the remodel of neural networks. On the other hand, the anti-inflammatory treatment with pioglitazone or interleukin 1 receptor antagonist in deltaE9 mice successfully rescued the impairments in increasing spine density and remodeling neural networks during EE. Our data suggest that neuroinflammation disrupts experience-dependent spine structural plasticity in preclinical stages of AD.

Keywords: Preclinical AD, APPswe/PS1deltaE9 mice, dendritic spines, structural plasticity, neuroinflammation

Introduction

Being the most prevalent cause of dementia, Alzheimer's disease (AD), characterized by progressive cognitive deficits, amyloid plaques, neurofibrillary tangles (NFTs) and neuronal loss, still lacks effective cure at the present time [19, 37]. The failure to develop successful pharmacotherapy may, at least partially, be ascribed to the long pathophysiological process, which starts many years before the stage of symptomatic AD [40]. Therefore, much earlier intervention in the asymptomatic or preclinical stages may be required to successfully treat AD [32, 42].

Preclinical AD has been recently defined as the stages prior to mild cognitive impairment and featured with amyloid deposition in the brain [41]. Subjects in the preclinical stages are at risk for future cognitive decline [47]. Indeed, the lag between the appearance of amyloid plaques and detectable impairment in cognition is more than a decade [34, 41]. Growing evidence supports the notion that amyloid deposition disrupts functional networks in the brain of cognitively normal elderly [15, 30, 39, 43]. To have a better chance of curing AD, it is therefore crucial to identify pathophysiological events occurring in preclinical stages, preceding dementia but with the formation of amyloid deposits.

Transgenic mouse models are essential research tools for uncovering AD pathogenesis as well as validating new therapeutic approaches. To recapitulate AD pathology, transgenic mouse models carry familial AD gene mutations in amyloid precursor protein (APP) and/or presenilins (PS) based on the amyloid hypothesis, which holds the abnormal production of APP proteolytic fragment, amyloid β-protein (Aβ), as the primary cause of AD [14]. The transgenes with APP/PS mutations in mouse models lead into the formation of amyloid plaques and subsequent memory loss, but without the development of NFTs and massive neuronal loss [2]. Although these mice fail to replicate all aspects of the disease, they seem to faithfully imitate pre-dementia stages of AD [1].

Among the APP transgenic mouse models, APPswe/PS1deltaE9 (deltaE9) mice has been widely used and they express APP with the Swedish mutation together with mutant human PS1 with a deletion of exon 9 [21, 36]. Interestingly, in deltaE9 mice, amyloid deposition precedes typical cognitive impairments [20, 46]. Amyloid plaques start to emerge at the age of 4-5 months [4, 12], while the performance of 7-month-old deltaE9 mice is normal in most cognitive tests [25, 31, 46]. The temporal lag between the emergence of amyloid plaques and

the onset of dementia consequently provides a critical period for the study of pathophysiological events related to preclinical AD.

In this study, we used long-term in vivo two-photon microscopy to elucidate the adaptive spine plasticity of 4-5-month-old deltaE9 mice. Our data demonstrated that deltaE9 mice failed to increase spine density and establish novel neural connections when exposed to enriched environment (EE), which we showed to be attributed to amyloid deposition induced neuroinflammation.

Materials and Methods

Animals

APPswe/PS1deltaE9 (deltaE9) mice [20] (Jackson Laboratory) were crossed with GPF-M mice [8] (Jackson Laboratory) to obtain double transgenic offspring which were heterozygous for the corresponding genes (deltaE9 +/- x GFP +/-). GFP positive littermates without APP/PS1 transgenes were used as controls (deltaE9 -/- x GFP +/-). BACE1 knockout mice [5] were also purchased from Jackson Laboratory and deltaE9 +/- x Bace1 +/- x GFP +/- (deltaE9/Bace +/-) were generated by interbreeding. All transgenic mice were maintained on C57BL/6 background. Female mice at the age of 4-5 months were used. Mice were housed and bred in pathogen-free environment in the animal facility at the Centre for Neuropathology and Prion Research of the Ludwig Maximilian University Munich (LMU), with food and water provided *ad libidum* (21 ±1 °C, at 12/12 h light/dark cycle). All mice were either housed singly in standard cages (30×15×20 cm) or in groups in an environmentally enriched (EE) cages (80×50×40 cm) equipped with platforms and variety of toys, which were relocated 3 times per week. Pioglitazone (350 ppm, Actos^{TM}) was supplemented into rodent chow. All protocols and procedures involving animals were approved and conducted in accordance with the regulations of LMU and the government of Upper Bavaria (Az. 55.2-1-54-2532-62-12).

Cranial window implantation and in vivo two-photon imaging

The detailed surgical procedure of cranial window implantation has been described previously [11, 18]. In brief, mice were anesthetized by intraperitoneal injection of ketamine/xylazine (120 and 10 mg/kg, respectively). Subsequently, dexamethasone (6 mg/kg) was injected to prevent development of cerebral edema. A piece of skull above the somatosensory cortex was then removed and replaced with a cranial window (4 mm). Of note, lentivirus (LV) encoding IL-1 RA (LV vector was a gift from Dr. A.M.W. van Dam [45]) was intraparenchymally injected into

the cortex before implanting the coverslip when specified. After 4 weeks of recovery period, mice were imaged by using a LSM 7MP microscope (Zeiss) equipped with a 20x objective (NA 1.0; Zeiss). Mice were anesthetized with isoflurance (1% in 95% O_2 and 5% CO_2) and placed on a heating pad to keep the body temperature at 37°C. Apical dendrites originating from GFP labeled layer V pyramidal neurons were imaged in consecutive sessions (once per week). The imaging session did not last more than 60 min. The unique pattern of blood vessels was used to re-localize the imaged regions in subsequent imaging sessions. GFP was excited by a femtosecond laser (Spectra Physics) at the wavelength of 880 nm. The intensity of laser and settings of data acquisition were kept consistent during the experiments. To ensure the dendrites were chosen in amyloid plaque-free regions, methoxy-X04 (1 mg/kg) was intraperitoneally injected 24 h before imaging in the first and last time points. Overview images were taken as 424 x 424 x 350 μm^3 (0.83 µm/pixel). Higher resolution images (0.138 µm/pixel) were used for counting dendritic spines. For illustration purpose, maximal projection images were deconvolved (AutoQuantX3), with contrast and brightness adjusted.

Spine analysis

Dendritic spines were analyzed manually in ZEN 2011 (Zeiss) by scrolling through the images in z-stacks. As the limitations of resolution in Z-direction, only laterally protruding spines were counted, as only those could be identified with certainty. In consecutive sessions, a dendritic spine was determined as the same if its location did not change within a range of 0.5 µm along the dendrite. Otherwise, spines that disappeared or emerged compared to the previous imaging session were defined as formed or eliminated, respectively. The fate of preexisting spines was calculated as the fraction of dendritic spines in the first imaging session that remained stable during the imaging period. Similarly, the fate of new-gained spines was the fraction of formed spines in the first week of EE or matching week of SC that remained stable during the rest of imaging period. Transient spines were determined as spines that did not survive over one week.

Immunochemistry

Following transcardial perfusion with phosphate buffered saline and 4% paraformaldehyde (PFA), mouse brains were cut into 65 µm thick sections from somatosensory cortex after being fixed in 4% PFA overnight. GFAP (Abcam 1:500) and Iba1 (Wako 1:500) antibodies were used for activated astrocytes and microglial staining. Anit-rabbit Alexa 647 antibody (Invitrogen 1:1000) was used as the secondary antibody. To stain amyloid plaques, sections

were incubated with 145 μM methoxy-X04 in PBS for 30 min and then washed with PBS. After mounting on glass coverslips by fluorescence mounting medium (Dako), sections were imaged using LSM 780 confocal microscope (Zeiss)

Statistics

For statistical analysis and comparison, GraphPad Prism 5 was used. In the longitudinal measurements of spine analysis, extra sum-of-squares F test was used when data were fitted with a line using the nonlinear regression. Comparison among groups was performed using one-way ANOVA followed by Newman-Keuls post-test. Two-tailed Student t-test was used in comparison between two different groups. The numbers of mice were 4-6 per group for in vivo imaging. 8-12 dendrites were imaged in each mouse. The length of each dendrite was 25-35 μm. The data were presented as the means for every mouse. All results were presented as means ± S.E.M. $p<0.05$ was defined as statistically significance. * $p<0.05$, ** $p<0.01$.

Results

Adaptive structural plasticity of dendritic spines is impaired in deltaE9 mice at the age of 4-5 months

As replicating the preclinical stages of AD [1, 41], 4-5-month-old deltaE9 mice develop amyloid deposits without cognitive decline [4, 12, 25, 31, 46]. In agreement with the cognitive normality, our previous study observed normal spine density and dynamics on dendrites that were far away from amyloid plaques in deltaE9 mice at this age[53]. To further examine if activity-induced structural spine plasticity on these dendrites is disturbed in preclinical AD, we housed deltaE9 mice at the age of 4-5 months under enriched environment (EE) over 5 weeks and monitored the apical tufts of layer V pyramidal neurons in the somatosensory cortex (Supplementary Figure 1). EE, which provides a spectrum of synaptic inputs and thus leads to adaptive synaptic alterations within the adult brain [28, 29, 35], induced a steady increase of spine density in control group (Fig. 1a, c). In contrast, EE failed to increase spine density in deltaE9 mice (Fig. 1a, c). Of note, unlike control mice demonstrating gradual decline in dendritic spine elimination upon EE, the rate of spine elimination in deltaE9 mice remained unaltered (Fig. 1d). EE did not change the rate of spine formation in both groups (Fig. 1e). Moreover, during the imaging period, the density and dynamics of dendritic spines remained unchanged when mice were housed under standard conditions (SC, Fig. 1b, c-e). Thus, EE-induced decrease in spine elimination and subsequent increase in spine density were absent in deltaE9 mice.

To find out how preexisting neural networks reacts on the stimulation of EE in preclinical stages of AD, we tracked the fate of dendritic spines that existed in the first imaging session over the whole period of enrichment. Interestingly, in control and deltaE9 genotypes, less preexisting spines survived when mice housed under EE (Fig. 1f, g). This indicated a breakdown of the established neural networks in both groups during EE. Furthermore, the fate of spines that were newly formed in EE or SC was also monitored. A higher number of gained spines remained stable during EE in control mice, but not in deltaE9 mice (Fig. 1 h-j). This result suggested the failure of building up novel neural networks induced by EE in deltaE9 group. Collectively, our data imply the reorganization of neural networks upon EE is impaired in preclinical stages of AD.

Reduction of BACE1 in deltaE9 mice restores the respond with an increase in spine density upon EE

Full-length APP is processed to yield amyloid beta, the principal component of amyloid plaques, through sequential enzymatic cleavage by β and γ-secretases. To confirm if amyloid plaques contribute to the impaired adaptive spine plasticity in deltaE9 mice, we crossed deltaE9 mice with BACE1, the primary β-secretase, knockout mice to obtain deltaE9 genotype containing a heterozygous BACE1 gene knockout (deltaE9/BACE +/-). Partial reduction of BACE1 activity almost abolished amyloid plaques and associated glial cell activation (Figure 2). Of note, the density and dynamics of dendritic spines in deltaE9/BACE +/- genotype remained unchanged (Supplementary Figure 2a-c). Unlike deltaE9 group, deltaE9/BACE +/- mice gained the adaptive increase in spine density housed under EE (Fig. 3a, b). To our surprise, the increase in spine density was caused by boosting spine formation (Fig. 3e) instead of decreasing spine elimination (Fig. 2d), which was opposite to the observations in control group (Fig. 1d, e). In addition, the fate of spines that existed before or newly formed after EE was indistinguishable between different housing conditions (Fig. 3f, g). An increased fraction of transient spines (Fig. 3c) further corroborated the notion that newly gained spines in EE did not incorporate into neural circuits. These deficits in neural network remodeling appear to be caused by the reduction of β-secretase, as no change in transient spine fraction was observed in deltaE9 or control mice housed in EE (data not shown). The restoration of adaptive spine density increase suggests removal of amyloid plaques might ameliorate the impaired adaptive plasticity of dendritic spines in preclinical AD.

Pioglitazone rescues the deficits of adaptive dendritic spine plasticity in deltaE9 mice

As the imaged dendrites were located in amyloid plaque-free brain regions [53], it was plausible to hypothesize that diffusible factors originating from amyloid deposits might contribute to the unaltered spine density upon EE, which was restored by the removal of plaques (Fig. 3b). Of note, amyloid plaques were surrounded by activated glial cells that are known to release pro-inflammatory cytokines [49]. To investigate if these cytokines caused the impaired adaptive plasticity, we treated deltaE9 mice with pioglitazone, a PPAR-gamma agonist, which inhibits the production of pro-inflammatory cytokines [23]. Pioglitazone treatment successfully rehabilitated the steady increase of spine density in deltaE9 mice during exposure to EE (Fig. 4a, b). Like in control mice, the EE-induced spine density increase was resulted from the gradual decline in spine elimination, while the rate of spine formation was unchanged (Fig. 4d, e). Moreover, less preexisting spines and more gained spines were observed during EE when deltaE9 mice were fed with pioglitazone (Fig. 4 f, g). The fraction of transient spines also remained stable (Fig. 4c). These results indicate that the failure of remodeling neural networks upon EE in deltaE9 mice can be attributed to the up-regulation of pro-inflammatory cytokines.

IL-1 RA rehabilitates the impaired adaptive plasticity of dendritic spines in deltaE9 mice

The known deleterious effects of interleukin-1β (IL-1β), a key mediator of the inflammatory response in AD, on synaptic plasticity [44] prompted us to examine whether up-regulated levels of IL-1β undermined the adaptive spine plasticity. The expression of IL-1β was indeed significantly enhanced in deltaE9 mice (Supplementary Figure 3 a). To diminish IL-1β activity, we injected lentivirus (LV) expressing interleukin-1 receptor antagonist (IL-1 RA) [45] into the somatosensory cortex (Supplementary Figure 3 b). IL-1 RA rectified the adaptive gain of spine upon EE accompanied with the gradual decline in spine elimination instead of rising spine formation (Fig. 5a, b and d, e). Also, the fate of spines that existed before or newly formed during EE was normalized in deltaE9 mice administered with IL-1 RA (Fig. 5f, g), while the fraction of transient spines was unchanged (Fig. 5c). Taken together, these data suggest up-regulated IL-1β perturbs EE-induced reorganization of neural networks

Discussion

Being excitatory postsynaptic compartments, dendritic spines are the membranous protrusions that receive and integrate informational input from presynaptic terminals[52]. This

function is supposed to be disturbed at the very early stages of AD pathogenesis, which may explain why synaptic loss is a much better indicator for cognitive impairment in AD than Aβ burden or neuronal loss [38]. With the advent of cognitive decline, irreversible damage may have already occurred. Prevention strategies in the asymptomatic stages of AD are therefore warranted.

Preclinical AD is replicated in young deltaE9 mice that develop amyloid deposits before the onset of cognitive decline [4, 12, 25, 31, 46]. In this study, we found that 4-5-month-old deltaE9 mice did not increase dendritic spine density when housed under EE in contrast to control mice. The novel external environment also failed to remodel neural networks in these transgenic mice. Reduction of BACE1 activity in deltaE9 mice reduced the deposition of Aβ and restored the increase of spine density during EE, but not the impaired reorganization of neural networks. However, anti-inflammatory treatments, pioglitazone and IL-1 RA, successfully rescued the spine density increase and neural network remodeling upon EE in deltaE9 mice. These results suggest that neuroinflammation contributes to impaired adaptive plasticity of dendritic spine in preclinical stages of AD.

Structural plasticity of dendritic spines refers to the change of their distribution in response to experience[10]. Learning and sensory experience have been reported to remodel neural connections through de novo growth and loss of dendritic spines, which provides a structural substrate for adaptive behaviors. Spine density increases after spatial learning tasks or manipulations that intensify sensory inputs [24, 26], while deprivation of sensory experience leads to a decrease in spine density [48]. This structural synaptic plasticity may substantially boost information storage capacity in brain [6]. The failure to increase spine density in young adult deltaE9 mice upon EE suggests an impairment of experience-dependent spine structural plasticity before spine loss in asymptomatic AD stages. In addition, stabilized new spines and destabilized preceding spines in novel experience reflect a rewiring of neural networks, which facilitates a quicker adaption of brain to the same situation in the future [17, 50, 51]. Interestingly, the ability to dismantle the preexisting neural networks in novel external environment remains intact in the preclinical stage. However, deltaE9 mice fall short of the establishment of novel neural networks. These results imply experience-dependent demolition and construction of neural networks are two processes that are independent from each other.

BACE1 initiates the proteolytic process of APP into Aβ, which accumulates to form amyloid plaques. As Aβ is believed to play a central role in AD, BACE1 becomes an attractive drug target. Indeed, partial reduction of BACE1 activity leads to dramatic reductions on amyloid plaque burden and synaptic deficits with a small decrease of Aβ levels in young AD transgenic mice [27]. However, pharmacological inhibition of BACE1 impairs structural and functional synaptic plasticity implying its physiological role in dendritic spines[9]. The boosted transient spines, which contribute to increased spine formation, in deltaE9/BACE +/- mice during EE indicate the maintenance of experience-dependent synaptic rearrangement requires physiological level of BACE1 activity. It still remains unclear that whether BACE1 itself or its substrates are involved in synaptic physiology.

Amyloid deposition is one of neuroinflammation drivers associated with activated glial cells and the release of pro-inflammatory cytokines. These soluble mediators, IL-1β in particular, directly and extensively disturb synaptic transmission and plasticity. IL-1β regulates the expression and phosphorylation of glutamate receptors on dendritic spines[33]. The altered sensitivity of receptors to synaptic glutamate modulates synaptic plasticity. In addition, IL-1β disrupts BDNF signaling cascades and thereby prevents activity driven formation of filamentous actin in spines which is required for spine structural plasticity[44]. The restorative effects of pioglitazone and IL-1 RA demonstrated herein implicate a deleterious role of IL-1β in experience-dependent spine structural plasticity preceding cognitive impairment in AD.

Of note, numerous clinical studies have demonstrated that anti-inflammatory treatment reduces dementia risk or delay the onset of AD [3, 7, 16], although anti-inflammatory drugs in typical AD fail to be proven effective [13, 22]. These trials suggest prevention of inflammatory processes is clinically beneficial at the preclinical stages of AD. Our data confirm that neuroinflammation caused impairments of spine structural plasticity is curable by anti-inflammatory treatment in a preclinical mouse model of AD. This finding implies the normalization of adaptive structural plasticity of dendritic spines may correlate with the beneficial effects of anti-inflammatory treatment in preclinical AD patients.

We conclude that our in vivo dendritic spine analysis reveals that neuroinflammation, caused by amyloid deposition, undermines the adaptive changes of neural networks upon novel external environment before the occurrence of dementia, providing new insights for a possible benefit of anti-inflammatory treatments in preclinical AD.

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Conflicts of Interest

The authors declare that they have no conflict of interests.

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Figure legends

Figure 1. Adaptive plasticity of dendritic spines is impaired in deltaE9 mice

(**a, b**) Two-photon micrographs of GFP-labeled apical dendrites of layer V pyramidal neurons. Mice were housed in standard conditions (SC) and imaged twice in a week apart before put into enriched environment (EE) (A). In (b), mice were housed in SC all along. Empty or dark arrows point to eliminated or formed spines compared to previous imaging session. Blue arrowheads mark spines that existed in the first imaging session and were stable over the entire imaging period while red arrowheads represent gained spines in the first week of EE or matching period of SC that survived over the rest of imaging period.

(**c-e**) Quantifications of relative spine density, fraction of eliminated or formed spines in mice housed under EE (above) or SC (below).

(**f, g**) Fractions of spines from the first imaging session that remained stable during the whole imaging period.

(**h, i**) Fractions of gained spines in the first week of EE or matching period of SC that remained stable during the whole imaging period.

(**j**) The data at day43 from h and j were compared by one-way ANOVA. Scale bar=2 μm.

Figure 2. Partial reduction of BACE1 in deltaE9 mice greatly decreases amyloid plaque load and subsquent glial cell activation

Immunohistochemical labeling of amyloid plaques (blue), activated astrocytes (GFAP, red) and microglias (Iba-1, red) in the cortex. Scale bar=300 μm.

Figure 3. Reduction of BACE1 restores the spine density increase, but not neural circuit remodeling, upon EE in deltaE9 mice

(**a**) Two-photon micrographs of GFP-labeled apical dendrites. DeltaE9/Bace +/- mice were housed under SC (above) or EE (below).

(**b-e**) Quantifications of relative spine density, fraction of transient, eliminated or formed spines.

(**f, g**) Fraction of spines in the first imaging session or gained spines in the first week of EE and matching week of SC that survived over the imaging period. Scale bar=2 μm.

Figure 4. Pioglitazone recovers the observed impairments of spine structural plasticity in deltaE9 mice

(**a**) Two-photon micrographs of GFP-labeled apical dendrites. DeltaE9 mice were fed with pioglitazone during EE or matching period of SC

(**b-e**) Quantifications of relative spine density, fraction of transient, eliminated or formed spines.

(**f, g**) Fraction of spines in the first imaging session or gained spines in the first week of EE and matching week of SC that survived over the imaging period. Scale bar=2 μm.

Figure 5. IL-1 RA rescues the impaired adaptive plasticity of dendritiic spines in deltaE9 mice

(**a**) Two-photon micrographs of GFP-labeled apical dendrites of layer V pyramidal neurons. Mice were housed in SC (above) or EE (below). Empty or dark arrows point to eliminated or formed spines compared to previous imaging session. Blue arrowheads mark spines that existed in the first imaging session and were stable over the entire imaging period while red arrowheads represent gained spines in the first week of EE or matching period of SC that survived over the rest of imaging period.

(**b-e**) Quantifications of relative spine density, fraction of transient, eliminated or formed spines.

(**f, g**) Fraction of spines in the first imaging session or gained spines in the first week of EE and matching week of SC that survived over the imaging period. Scale bar=2 μm. In SC and EE group, mice number was 4 and 6, irrespectively. 8-12 dendrites were imaged in each mouse. The data were presented as the means for every mouse. All results were presented as means ± S.E.M.

Figures

Figure 1

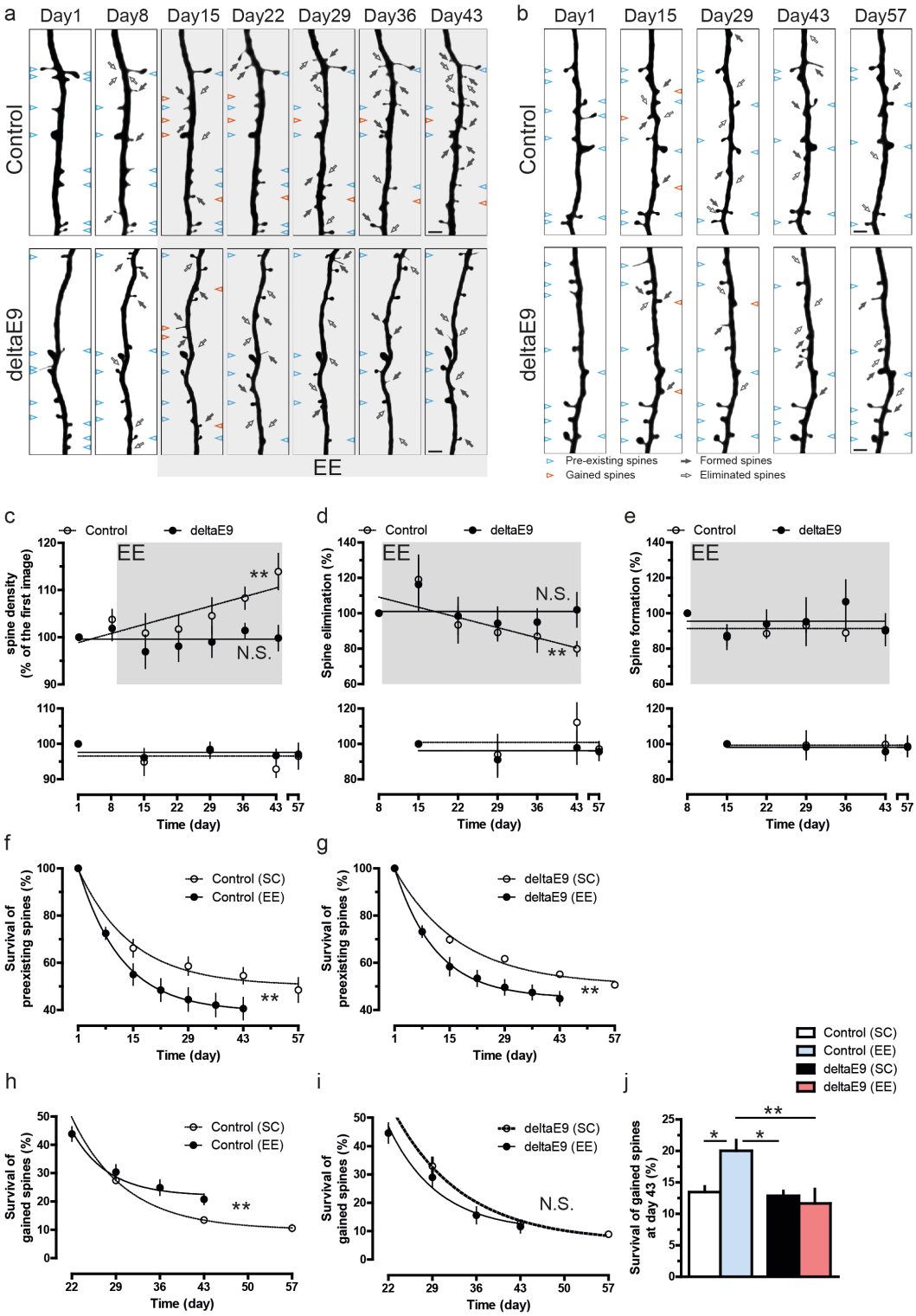


Figure 2

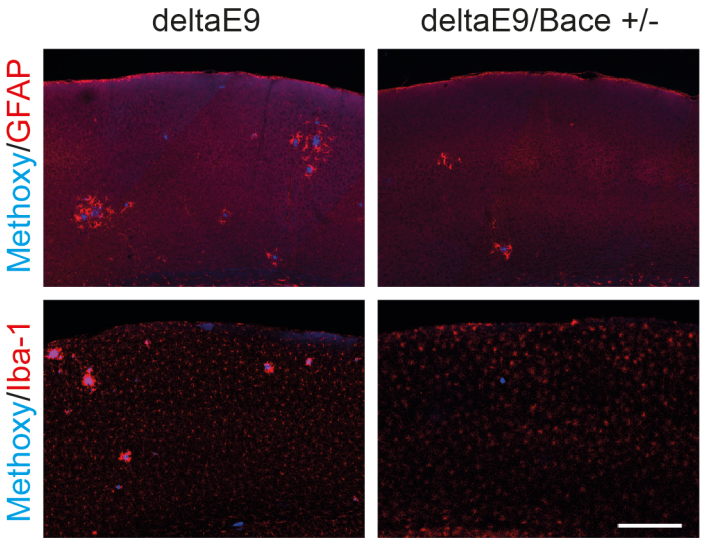
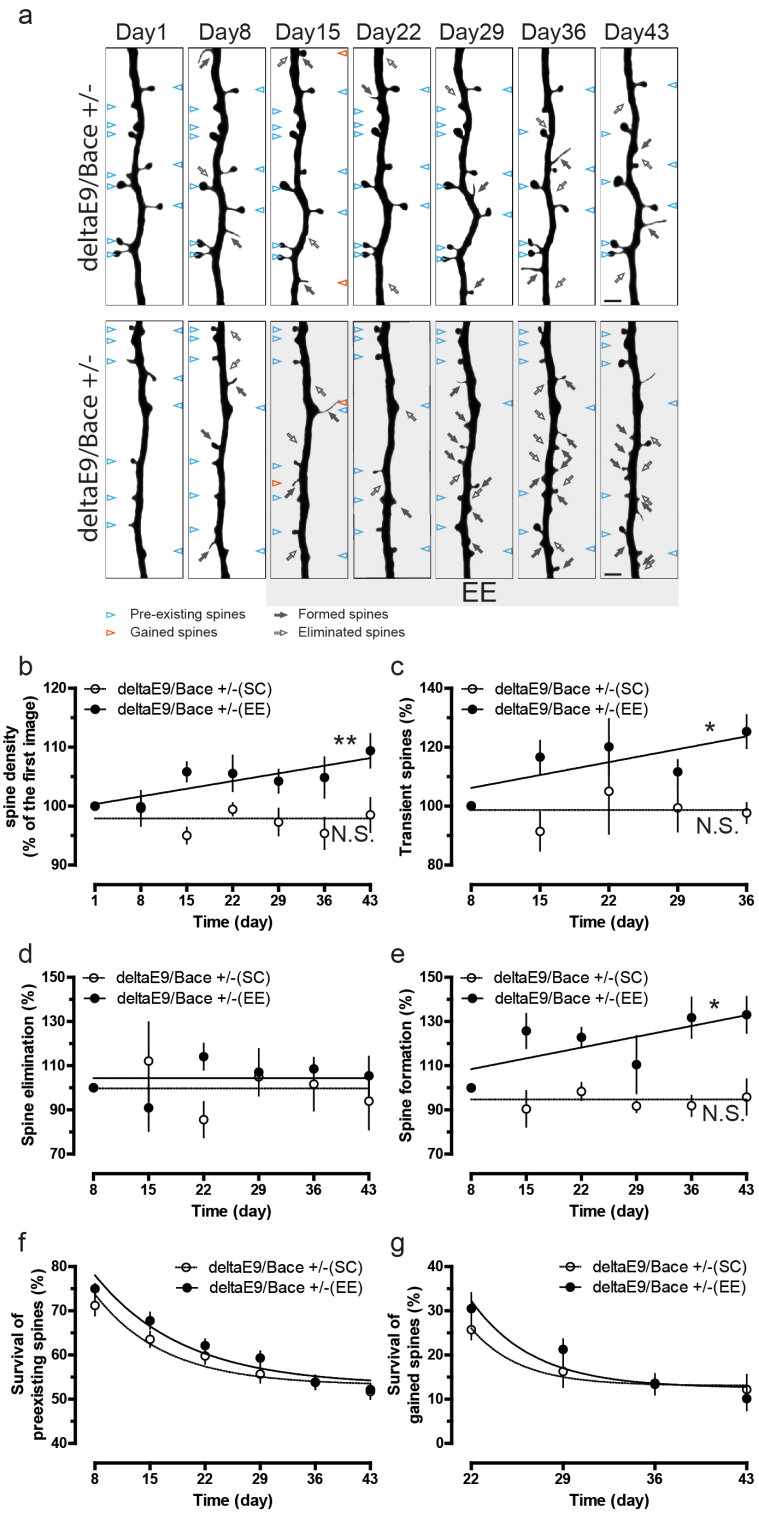


Figure 3


a
Day1
Day8
Day15
Day22
Day29
Day36
Day43
deltaE9/Bace +/-
deltaE9/Bace +/-
EE
Pre-existing spines
Gained spines
Formed spines
Eliminated spines
b
deltaE9/Bace +/-(SC)
deltaE9/Bace +/-(EE)
spine density (% of the first image)
**
N.S.
Time (day)
c
deltaE9/Bace +/-(SC)
deltaE9/Bace +/-(EE)
Transient spines (%)
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N.S.
Time (day)
d
deltaE9/Bace +/-(SC)
deltaE9/Bace +/-(EE)
Spine elimination (%)
Time (day)
e
deltaE9/Bace +/-(SC)
deltaE9/Bace +/-(EE)
Spine formation (%)
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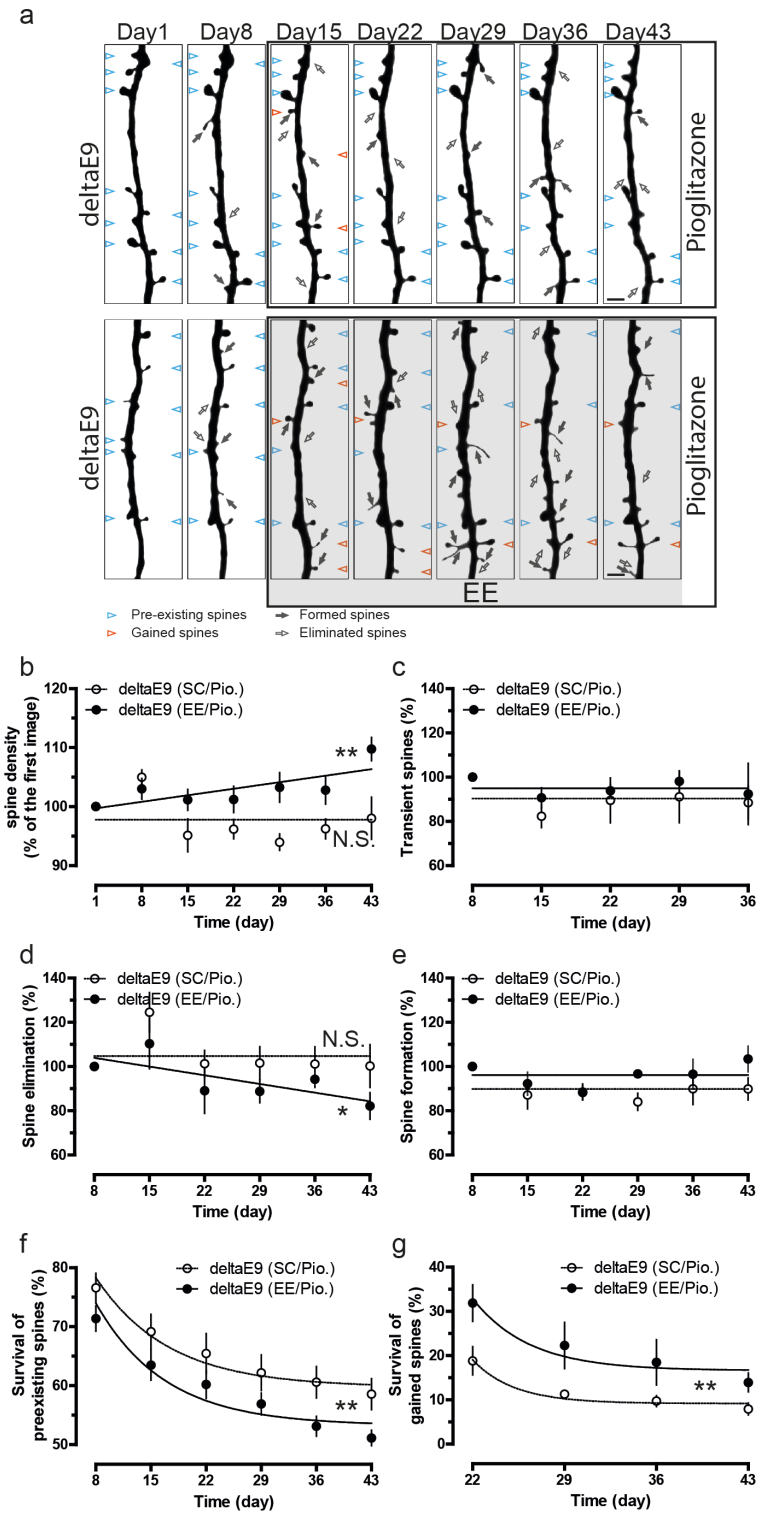
N.S.
Time (day)
f
deltaE9/Bace +/-(SC)
deltaE9/Bace +/-(EE)
Survival of preexisting spines (%)
Time (day)
g
deltaE9/Bace +/-(SC)
deltaE9/Bace +/-(EE)
Survival of gained spines (%)
Time (day)

Figure 4


a
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deltaE9
Pioglitazone
deltaE9
Pioglitazone
EE
Pre-existing spines
Gained spines
Formed spines
Eliminated spines
b
deltaE9 (SC/Pio.)
deltaE9 (EE/Pio.)
spine density (% of the first image)
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N.S.
Time (day)
c
deltaE9 (SC/Pio.)
deltaE9 (EE/Pio.)
Transient spines (%)
Time (day)
d
deltaE9 (SC/Pio.)
deltaE9 (EE/Pio.)
Spine elimination (%)
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Time (day)
e
deltaE9 (SC/Pio.)
deltaE9 (EE/Pio.)
Spine formation (%)
Time (day)
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deltaE9 (SC/Pio.)
deltaE9 (EE/Pio.)
Survival of preexisting spines (%)
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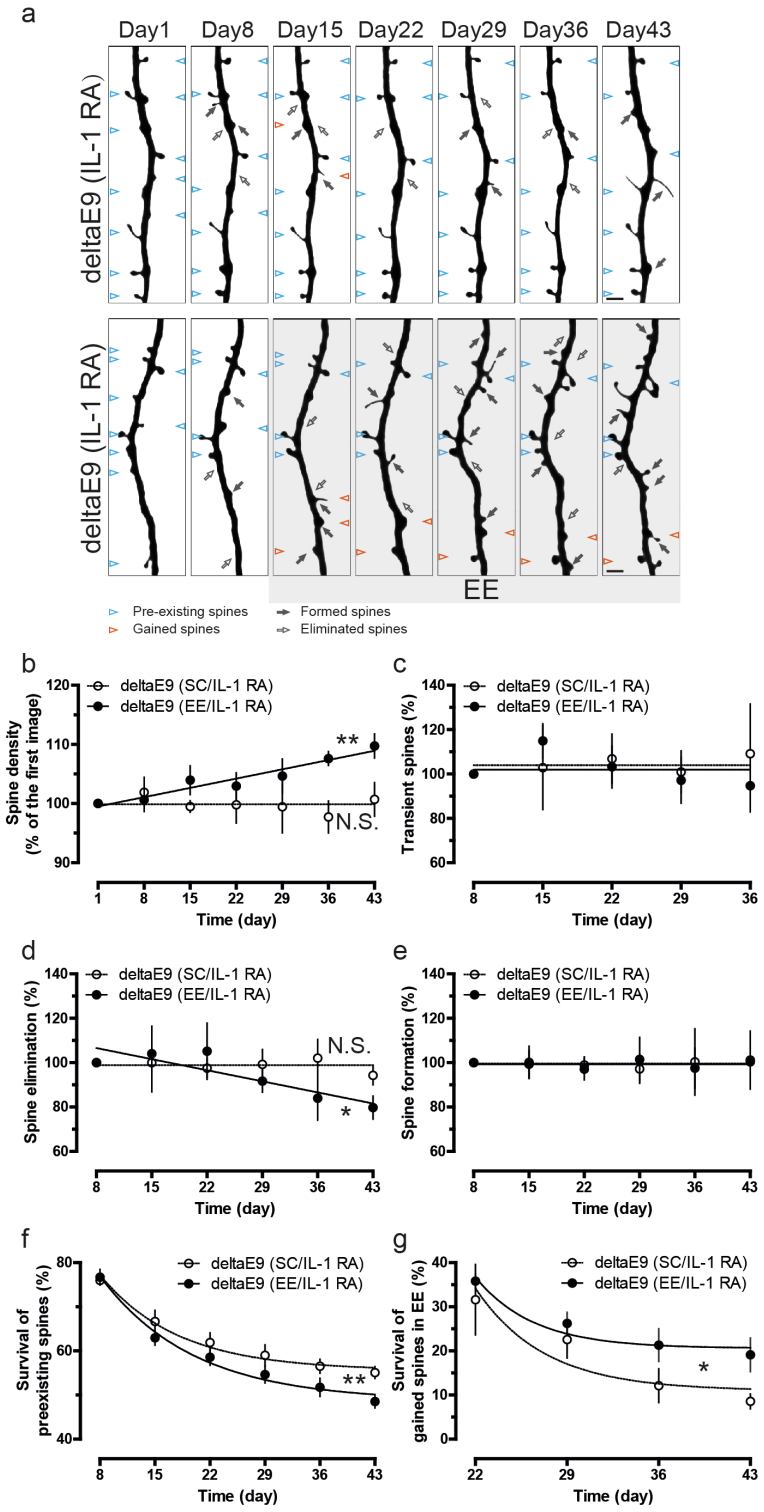
Time (day)
g
deltaE9 (SC/Pio.)
deltaE9 (EE/Pio.)
Survival of gained spines (%)
**
Time (day)

Figure 5


a
Day1
Day8
Day15
Day22
Day29
Day36
Day43
deltaE9 (IL-1 RA)
deltaE9 (IL-1 RA)
EE
Pre-existing spines
Gained spines
Formed spines
Eliminated spines
b
deltaE9 (SC/IL-1 RA)
deltaE9 (EE/IL-1 RA)
Spine density (% of the first image)
**
N.S.
Time (day)
c
Transient spines (%)
d
Spine elimination (%)
N.S.
*
e
Spine formation (%)
f
Survival of preexisting spines (%)
**
g
Survival of gained spines in EE (%)
*

Supplementary materials

Supplementary methods

Western blot

10% cortical tissues (w/v) were homogenized on ice in lysis buffer with protease inhibitors (Roche), followed by centrifugation at 500 rpm for 1 min. The supernatant was collected and protein concentrations were adjusted by the bicinchoninic acid assay to ensure the same amount of protein being loaded for each sample. Samples were mixed with SDS-containing sample buffers and incubated at 100 °C for 20 min. After electrophoresed on 12% sample gel, proteins were transferred into polyvinylidene difluoride membrane (Millipore). The primary antibodies against IL-1β (Cell signaling), IL-1 RA (Thermo Scientific) and tubulin (Santa Cruz) were used at 1: 1000 concentrations for immunoblotting. Protein bands were quantified by ImageJ.

Supplementary figure legends

Supplementary Figure 1. Transcranial in vivo two-photon imaging and housing conditions

(**a**) Transcranial in vivo two-photon imaging was taken in somatosensory cortex (left, black circle). Lateral view of GFP-labeled layer V pyramidal cortical neurons is in the middle. Apical tuft dendrites of layer V neurons were imaged at 20-70 μm depths (right). Scale bar represents 100 μm. (**b**) Schematic drawing of an EE cage (left) and a cage of SC (right).

Supplementary Figure 2. Partial reduction of BACE1 in deltaE9 mice does not change spine density and dynamics

(**a-c**) Quaitfiations of spine denstiy, fraction of eliminated or formed spines in mice at the age of 4-5 months housed under SC.

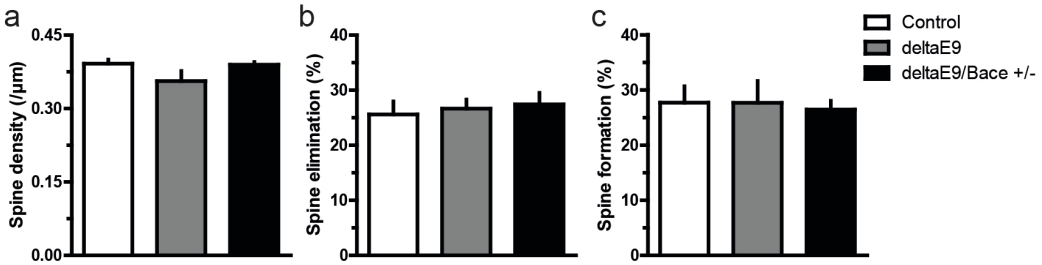
Supplementary Figure 3. Western blots of interleukin-1β and interleukin-1 receptor antagonist

(**a**) Western blot images and quantification show the expression of interleukin-1β (IL-1β) was increased in deltaE9 mice. (**b**) Interleukin-1 receptor antagonist (IL-1 RA) was overexpressed by the injecion of lentivirus (LV), as illustrated by western blot images and quantification.

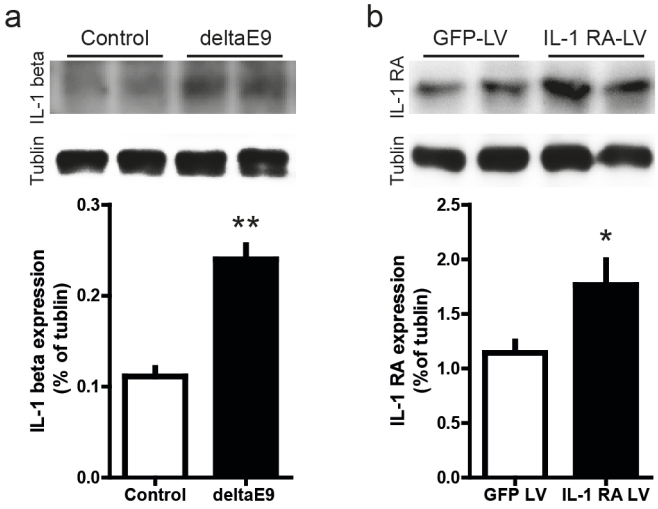
Supplementary figures

Supplementary figure 1

Supplementary figure 2



Supplementary figure 3



4 Manuscript Two

Amyloid Precursor Protein and NMDA Receptor Cooperate to Maintain Constitutive and Adaptive Plasticity of Dendritic Spines in Adult Brain (Submitted)

Title page

Amyloid Precursor Protein and NMDA Receptor Cooperate to Maintain Constitutive and Adaptive Plasticity of Dendritic Spines in Adult Brain

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Abstract

Dynamic synapses facilitate activity-dependent remodeling of neural circuits, thereby providing the structural substrate for adaptive behavior. However, the mechanisms governing dynamic synapses in adult brain are still largely unknown. Here, we demonstrate that in the cortex of adult APP knockout (APP-KO) mice, formation of new and elimination of existing dendritic spines is reduced, with overall spine density remaining unchanged. APP-KO mice also failed to respond with an increase in spine density upon environmental enrichment. These impairments prevailed in APPsα-KI genotype. Comparison of mEPSCs between APP-KO and wild type mice revealed selective reduction in the NMDA receptor mediated synaptic currents. Strikingly, potentiation of NMDA receptor responses by the co-agonist D-serine rescued spine dynamics, adaptive plasticity and morphology in APP-KO mice. These data suggest functional cooperation between APP and NMDA receptors in maintenance of synapses with predominantly NMDA-receptor mediated transmission, prerequisite for constitutive and adaptive synaptic plasticity in the adult brain.

Key words: Amyloid precursor protein, NMDA receptor, Dendritic spine, Two photon *in vivo* imaging, Miniature EPSC

Introduction

Small protrusions of dendrites, known as spines, provide primary sites for excitatory inputs in principal neurons of most brain regions. Harboring the receptive elements of glutamatergic connections, dendritic spines are of major importance for synaptic integration and plasticity, hence a prerequisite for encoding cortical representations and adaptive remodeling of neural circuits [47, 53, 65]. To ensure these functions, the morphology and distribution of dendritic spines are maintained in a highly dynamic state and are tightly regulated [33, 42, 66]. Thus, it is not surprising that the structural parameters of dendritic spines including spine density, morphology and plasticity are affected in an array of neurodegenerative diseases [7, 17, 18, 38]. As such, research into mechanisms governing functions and structural plasticity of dendritic spines, which remain largely unexplored in adult brain, holds important clues not only towards understanding the basic biology of synapses with neural mechanisms of adaptive behavior but may also reveal key areas for therapeutic interventions.

Most of the data concerning the physiology and plasticity of dendritic spines emerged from developing neurons and typically have been acquired *ex vivo* and *in vitro* [6, 13, 40, 49, 50]. While there is no doubt that these studies made major contributions towards elucidating events involved in acute response of synapses to electrical stimulations or pharmacological treatments, rigorous research of spine plasticity *in vivo* has become feasible only recently with the advancement of two photon microscopy [22, 29, 67]. Indeed, the high resolution structural data provides superb opportunity not only for exploring the dynamics of spines but also to identify structural correlates of adaptive related rewiring of neural circuits within the intact brain.

Enduring interest towards amyloid precursor protein (APP), due to its key role for the pathogenesis of Alzheimer's disease, has been refueled by recent evidence indicating its multifaceted role in synaptic physiology and development [24, 27, 44, 46]. While the mechanistic details remain to be elucidated, increasing evidence indicates important trans-synaptic adhesive functions for trans-membrane APP and major neurotrophic roles of secreted ectodomain APPsa in neurons [2, 3, 10, 31, 57]. The high level of APP expression in the developing nervous system with its enrichment at nascent synapses and potent synaptogenic effects of the secreted APPsα have also been implied for the involvement of APP in the formation and stability of synapses during neurodevelopment [9, 11, 25, 27, 61-63]. Moreover, APP and amyloid β-peptide (Aβ) have been implicated in regulation of trafficking

and surface expression or internalization of ion channels and synaptic receptors [26, 56, 58, 64]. Despite of the key relevance of these processes for integrative mechanisms of neurons and synaptic plasticity, the role of APP in governing dendritic spine dynamics and adaptive remodeling of neural circuits in adult brain remains poorly defined.

In this study, we combined long-term *in vivo* two photon imaging and electrophysiology of cortical neurons to elucidate the role of APP in adaptive spine plasticity and synaptic transmission in the adult mouse brain. Our data show that the lack of APP impairs the structural plasticity of dendritic spines and suggest its key role in maintenance of thin spines with predominantly NMDA-receptor mediated transmission, which is a prerequisite for synaptic plasticity in the adult brain.

Materials and methods

Experimental animals

All protocols and procedures involving animals were approved and conducted in accordance with the regulations of LMU and the government of Upper Bavaria (Az. 55.2-1-54-2532-62-12). GFP-M mice [16] were purchased from Jackson Laboratory, USA. APP-KO and APPsα-KI mice were described previously [39, 52]. APP-KO (APP -/-) × GFP-M+/- and APPsα-KI (APPsα +/+) × GFP-M+/- lines were generated by interbreeding. All transgenic mice were maintained on C57BL/6 background. Female transgenic mice at the age of 4 months were used for imaging and electrophysiological recordings, and female age matched wild-type (WT) littermates were used as controls. Mice were housed and bred in pathogen-free environment in the animal facility at the Centre for Neuropathology and Prion Research of the Ludwig Maximilian University Munich (LMU), with food and water provided *ad libidum* (21 ±1 °C, at 12/12 h light/dark cycle). All mice were either housed singly in standard cages (30×15×20 cm) or in groups in an environmentally enriched (EE) cages (80×50×40 cm) equipped with platforms and variety of toys, which were relocated every 2-3 days. In experiments with D-serine treatment, every other day D-serine (Sigma-Aldrich) was prepared freshly and supplemented into drinking water (0.55 mg/mL).

Longitudinal *in vivo* two-photon imaging experiments

The surgical procedure of chronic cranial window implantation and the details of experiments have been described previously [18, 28]. In brief, under anesthesia with kethamine/xylazine

(120 and 10 mg/kg, respectively) (WDT/Bayer Health Care), cranial window (4.0 mm) was implanted above the somato-sensory cortex of mice after open-skull craniotomy. After 4 weeks of recovery period, *in vivo* two photon microscopy was carried out using LSM 7 MP microscope (Carl Zeiss) equipped with 20 × objective (NA 1.0; Carl Zeiss). Mice were anesthetized with isoflurane (1% in 95% O2 and 5% CO2), and body temperature was kept at 37 °C with the heating pad (Fine Science Tools GmbH). Apical dendrites originating from GFP positive layer V pyramidal neurons were imaged in consecutive sessions at specified time points. GFP was excited with a femtosecond laser (Mai Tai DeepSee, Spectra Physics) at a wavelength of 880 nm. The imaging session did not exceed 60 min. Special efforts were made to keep the intensity of laser and data acquisition settings consistent throughout the experiments. Due to limitation in axial resolution, only laterally protruding spines were included into analysis. Emerging or disappearing spines over two consecutive imaging sessions over one week were defined as forming or eliminating spines, with their fractions normalized to the total spine number. Spine turnover rate (TOR) was defined with the following formula: (TOR) = $(N_f + N_e)/ (2 \times N_t \times D)$, where N_f = formed spines, N_e = eliminated spines, N_t = total spines, D = interval days between imaging sessions. For illustration purpose, high resolution (0.138 µm/pixel per frame with 1 µm/pixel z-direction) maximal projection images were deconvolved (AutoQuantX3, Media Cybernetics), with contrast and brightness adjusted.

Electrophysiological recordings

The details of preparation of acute cortical slices and electrophysiological recordings have been described elsewhere (Filser et al., 2014). Chemical and drugs for electrophysiological experiments were purchased from Sigma-Aldrich unless specified otherwise. Mice of both WT and APP-KO (four groups with 3-4 mice in each group) were anaesthetized with isoflurane (1% in 95% O_2 and 5% CO_2) and decapitated after cervical dislocation. Brains were rapidly taken out and placed for 5-6 min in ice-cold bubbled (95% O_2, 5% CO_2) slicing solution (mM): sucrose, 75; NaCl, 85; KCl, 2.5; NaH_2PO_4, 1.25; $NaHCO_3$, 25; $CaCl_2$, 0.5; $MgCl_2$, 4; glucose, 25, pH 7.4. Coronal slices (300 µm) containing the somato-sensory cortex were cut (VT1200S; Leica) in the same solution and transferred into a warming chamber (35 °C) filled with the same media except sucrose was omitted and NaCl increased to 125 mM (30 min). Subsequently, the tissue was transferred into recording artificial cerebrospinal fluid (aCSF, mM): NaCl, 125; KCl, 2.5; NaH_2PO_4, 1.25; $NaHCO_3$, 25; $CaCl_2$, 2; $MgCl_2$, 2; glucose, 25.

Recordings from layer V pyramidal cells were made under continuous perfusion of slices with aCSF (bubbled with 95% O_2, 5% CO_2) at RT in a recording bath fixed to the stage of a BX51 upright microscope (Olympus). Neurons were visualized with differential interference contrast (DIC). Patch pipettes were pulled from borosilicate glass (HEKA Electronics) with P87 puller (Sutter instruments) and filled with internal solution (mM): CsCl, 140; KCl, 10; NaCl, 5; MgATP, 2; EGTA, 0.01; HEPES, 10; 280-290 mOsm, pH 7.3, with in bath resistance of 4-6 MΩ. Analog signals were digitally sampled at 10 kHz and stored for off-line analysis. Recordings were made from holding potential of -65 mV or -45 mV as specified, after correction for the liquid junction potential. Only neurons firing overshooting action potentials immediately after the breaking of the seal were included in current analysis. Selective blocker of $GABA_A$/glycine receptor-channel picrotoxin (100 μM, DMSO) was supplemented routinely to the recording media to isolate the spontaneous excitatory postsynaptic currents , with miniature EPSC (mEPSC) isolated further the blockade of action potential-driven synaptic activity with tetrodotoxin (TTX, 0.5-1.0 μM). Mixed NMDA/AMPA receptor mediated mEPSCs were recorded at -45 mV holding potential under the low extracellular Mg^{2+} (0.5 mM) (Espinosa and Kavalali 2009). The frequency, amplitude and decay time constant of synaptic currents were analyzed using Synaptosoft software (Synaptosoft, Co.), with event detection threshold qualifying set up at 2.5-3.0 times the S.D. of the noise, with graphs generated using IgorPro 6.22 software (Wavemetrics, USA).

Confocal microscopy and spine morphometry

To achieve a better resolution of spine morphologies, *ex vivo* confocal microscopy of GFP positive somatosensory neurons was used. Mice were injected with a lethal dose of ketamine/xylazine (200/14 mg/kg, i.p.), perfused transcardially with phosphate-buffered saline (0.1 M PBS, 50 ml) followed by paraformaldehyde (150 ml, 4% in PBS). Brains were extracted and post-fixed in PFA at 4 °C overnight and cut in coronal plane (60 μm) with the vibratome (VT 1000S, Leica). Sections containing somato-sensory cortex were incubated in 0.1% Triton X-100, 5% normal goat serum (NGS) for 2 h at room temperature and exposed to rabbit anti-GFP antibody tagged with Alexa488 (1:200, Invitrogen) in PBS with 5% NGS for 2 h at room temperature. After three washes with PBS, slices were mounted with fluorescent media and covered for microscopic analysis. Apical dendrites of layer V pyramidal cells were imaged in slices through 40× oil immersion objective (NA 1.3; Carl Zeiss), using LSM780 confocal microscope (Carl Zeiss). Images were deconvoluted (AutoQuantX3, Media

Cybernetics) with dendrites and spines reconstructed using Imaris (Bitplane) at high resolution (0.069 µm/pixel per frame with 0.395 µm/pixel z-direction). Morphological subtypes of dendritic spines were identified as follows: mushroom spine: max_width(head) / min_width(neck) > 1.4 and max_width(head) > 0.2 µm and min_width(neck) > 0 µm; stubby spine: length(spine) / mean_width(neck) <= 3 or min_width(neck) = 0 µm or min_width(neck) > 0.5 µm; thin spine: length(spine) / mean_width(neck) > 3. Fractions of spine sub-types (of total spine number) were assessed and compared.

Western Blots

For NMDA receptors quantification, postsynaptic density (PSD) fraction was prepared as described [30]. Briefly, cortical tissue was homogenized in ice-cold Buffer A (0.32 M sucrose, 1 mM $MgCl_2$, 0.5 mM $CaCl_2$ and 6 mM Tris at pH 8.0) with protease inhibitors (Roche). Brain extract was centrifuged at 1,400 × g for 10 min with supernatant (S1) collected. The pellet was re-homogenized with Buffer A and centrifuged at 710 × g for 10 min. This supernatant was then mixed with S1 and centrifuged at 710 × g for 10 min. The supernatant was then collected and centrifuged at 13,800 × g for 12 min to obtain the pellet (P1). P1 was re-suspended in Buffer B (0.32 M sucrose and 6 mM Tris at pH 8.0) with protease inhibitors (Roche). This solution was then gently loaded onto a discontinuous sucrose gradient (0.85/1/1.15/ in 6 mM Tris at pH 8.0) and centrifuged at 82,500 × g for 2 h. The synaptosome fraction, which condensed between 1 M and 1.15 M sucrose, was collected. The volume of synaptosome fraction was adjusted with Buffer B to 1 mL. Equal volume of Buffer C (1% Triton X-100 and 12 mM Tris at pH 8.0) was added into the synaptosome fraction; the mixture was centrifuged at 50,000 × g for 20 min before collecting the pellet, which was re-suspended into 40 mM Tris (pH 8.0) to obtain PSD fraction. Total protein corrected samples (Bradford assay) were eletrophoretically separated by 10% SDS-PAGE and transferred onto 0.45 mm PVDF membrane (Millipore) and developed with primary antibodies at dilutions: NMDAR1 (1:1000), NR2A (1:1000), NR2B (1:1000) (Cell Signaling) and PSD95 (1:1000, Synaptic Systems). Western blots were quantified with ImageJ (NIH Image).

Statistics

For statistical analysis and comparison, GraphPad Prism 5 was used. Comparison between two different groups was performed using two-tailed Student *t*-test. In the longitudinal measurements of spine analysis, repeated one-way ANOVA was performed followed by Dunnett test. Extra sum-of squares F test was used when data were fitted with a line using

the nonlinear regression. The numbers of mice were 5–6 per group for *in vivo* imaging. 8–12 dendrites were imaged in each mouse; the length of each dendrite was 25–35 µm. The data are presented as the means for every mouse. All results were presented as mean ±S.E.M. with p values less than 0.05 defined as statistically significant. Analysis was performed blind with respect to mouse genotype.

Results

Dendritic spine dynamics and adaptive plasticity are impaired in the absence of APP

APP proved critical in the formation and stabilization of synaptic connections in the developing nervous system [27, 46, 51, 62, 63]. To find out if the dynamics of dendritic spines and activity-dependent synaptic plasticity in adult brain also depend on APP, we monitored and compared the density and turnover rate (TOR) of spines in cortical pyramidal neurons of 4-5 month-old WT and APP-KO mice *in vivo*. Apical tufts of layer V pyramidal neurons were imaged in the somatosensory cortex prior to and during their exposure to environmental enrichment (Fig. 1). While no difference was found between the spine densities of WT and APP-KO mice housed under standard environment (Fig. 1b), both the elimination and formation of new spines were significantly lower in neurons of APP-KO mice compared to controls, resulting in reduced spine TOR (Fig. 1c-e). Thus, the decrease in spine TOR without change in spine density indicates the key role of APP in dendritic spine dynamics.

Environmental enrichment is known to provide a spectrum of synaptic inputs, which activate and lead to adaptive synaptic alterations within the adult brain [43, 48, 54]. To investigate if APP is involved in neural circuit remodeling in adulthood, both WT and APP-KO mice were exposed to environmental enrichment over 5 weeks, with spine density and dynamics monitored (Fig. 1a, f-h). In agreement with earlier reports [5, 32, 37], in WT mice environmental enrichment induced a steady increase of spine density. In sharp contrast, environmental enrichment failed to increase spine density in APP-KO mice (Fig. 1f). Moreover, the TOR of dendritic spines in APP-KO mice was consistently lower compared to WT (Fig. 1g). Of note, unlike WT mice demonstrating gradual decline in dendritic spine elimination upon environmental enrichment, the rate of spine elimination in APP-KO genotype remained unaltered (Fig. 1h). Collectively, these data demonstrate an essential role of APP in constitutive turnover of dendritic spines and their adaptive remodeling in the adult brain.

Structural plasticity of spines in APPsα-KI and APP-KO mice are comparable

As neurotrophic effects of the APP ectodomain APPsα are well documented [9, 11, 35, 46], we examined if the lack of this fragment in APP-KO mice could account for their impaired structural plasticity. We monitored and analyzed dendritic spine dynamics in APPsα knock-in (KI) mice, which express APPα but lack full length APP[52]. As illustrated in Fig. 2 (a-d), both the spine TOR and reactive increase in spine density associated with environmental enrichment in APPsα-KI mice were comparable to those in APP-KO genotype (Fig. 1). These results indicate that constitutive secretion of APPsa is not sufficient for normal spine turnover and suggest that cell surface full length APP maintains spine dynamics and adaptive spine plasticity.

Impaired spine plasticity in APP-KO mice coincides with altered spine morphology

Spine morphology presents a reliable indicator of the developmental state and strength of excitatory synaptic inputs of cortical neurons [8, 21]. Classified in three major groups - stubby, mushroom and thin spines, the relative fraction of various spine types in the brain is regulated by synaptic activity and developmental mechanisms [4, 36]. To find out if impaired plasticity of dendritic spines in APP-KO mice correlates with aberrations in spine morphology, we assessed spine type distribution in adult WT and APP-KO mice housed under standard or enriched conditions (Fig. 3). In APP-KO mice, the fraction of thin spines was reduced while the relative number of mushroom spines was enhanced irrespective of housing conditions (Fig. 3a-c). Counting of stubby spines revealed no differences between two genotypes (not shown). Overall, the reduction in thin spines paralleled by an increased fraction of mushroom spines support impaired dendritic spine plasticity of in APP-KO genotype, and suggest changes in their excitatory synaptic inputs.

NMDA receptor-mediated mEPSCs frequency and time constant are reduced in APP-KO mice

Because miniature excitatory post-synaptic currents (mEPSCs) provide a direct measure of the synaptic weight [19], we compared mEPSCs in cortical pyramidal cells of two genotypes recorded in the presence of picrotoxin (200 μM) and tetrodotoxin (0.5 μM) at -65 mV holding potential. No differences were found between amplitudes (not shown), frequencies or decay time constants of mEPSCs of two groups housed under standard conditions (Fig. 4). Given that the fraction of thin spines, which are known to receive synaptic inputs mediated predominantly via NMDA receptors [34, 41], are notably reduced in APP-KO mice, we

compared the contribution of NMDA receptors to mEPSCs of both genotypes recorded at -45 mV under low extracellular Mg^{2+} (0.5 mM) (Fig. 4a-f). In WT mice housed under standard conditions, mEPSC recorded at -45 mV revealed higher frequency with slower decay time constant, consistent with activation of pure NMDA receptor mediated transmission, compared to those recorded at -65 mV (Fig. 4a-c). In contrary, no significant differences in depolarization-dependent increase in mEPSC frequency or decay time constant were detectable in APP-KO neurons (Fig. 4d-f). Taken together, these data indicate a lower contribution of NMDA receptor mediated currents to the generation of mEPSCs in APP-KO mice, which are known for their slower decay kinetics and voltage-dependence of their activation.

To verify if the differences between the electrophysiological readouts are associated with changes in NMDA receptor expression, we compared postsynaptic NMDA receptor1 (NR1), NMDA receptor2A (NR2A) and NMDA receptor2B (NR2B) subunits between the two genotypes. As illustrated in Fig. 5a-d, NR1 and NR2A expressions in APP-KO mice were significantly lower compared to WT controls. These biochemical data accord with results of electrophysiological experiments and indicate deficiency of NMDA receptor-mediated functions in APP-KO mice.

Activation of NMDA receptor restores the structural plasticity of dendritic spines in APP-KO mice

As NMDA receptors regulate the stability and morphology of dendritic spines [59, 60], we tested if pharmacological activation of NMDA receptors with D-serine could rescue the impaired structural plasticity in APP-KO mice. D-serine was supplemented to the drinking water of APP-KO mice housed under standard or enriched conditions and dendritic spines were monitored over several weeks. Interestingly, as illustrated in Fig. 6a-e, D-serine treatment of APP-KO increased constitutive spine dynamics under standard housing conditions (Fig 6b and c) and also rescued the adaptive gain of spines upon environmental enrichment (Fig 6e). Likewise, treatment of APP-KO mice with D-serine also enhanced the fraction of thin spines and lowered the relative number of spines with mushroom morphology (Fig. 6f and g). These data suggest that constitutive and adaptive structural plasticity of dendritic spines depend on physiological activation of NMDA receptors, which are impaired in the absence of APP.

Discussion

We have shown here that in adult APP-KO mice, dendritic spine dynamics and remodeling are impaired. This finding assigns an important role to APP in governing structural plasticity of dendritic spines, which appears to be independent of APPsα-mediated functions. The compromised structural spine plasticity is associated with reduced NMDA receptor-mediated mEPSCs and postsynaptic NMDA receptor expression. Remarkably, the spine plasticity deficit could be rescued by D-serine, a co-agonist of NMDA receptors. These converging results pinpoint the close functional cooperation between APP and NMDA receptors in maintenance of constitutive and adaptive plasticity of dendritic spines in the adult brain.

As a ubiquitous type I trans-membrane glycoprotein expressed in the brain, APP with its cleavage product Aβ has long been implicated in AD [20, 55]. Produced by β/γ proteolysis of APP, Aβ40/42 represent the main constituents of amyloid plaques in AD brain and are considered as a major cause of neurotoxicity and synapse loss, leading to cognitive decline and memory deficits. At the same time, the role of APP and its fragments in synaptic physiology has been widely recognized with several studies demonstrating the essential role of APP and related APLPs for synaptogenesis [27, 45, 46, 51]. In fact, recent evidence emphasizes the prevalence of protective effects of full-length APP and APPsα on synapses and neurons [25, 52, 64]. Hence, deciphering molecular mechanisms mediating APP functions is essential not only for basic research of synaptic physiology but also for translational neuroscience. Because the morphology and dynamics of dendritic spines correlate with the strength and stability of excitatory synapses, decrease in the fraction of thin spines with reduction in spine turnover in APP-KO mice are consistent with impaired structural plasticity. The lower fraction of dynamic thin spines with an increase in more stable mushroom spines suggest that the excitatory inputs of layer V pyramidal neurons of APP-KO mice are hardwired more rigidly and are less prone to contextual and behavioral remodeling. As lower spine TOR persisted in APPsα-KI mice, which failed to respond with an increase in spine density upon environmental enrichment, it is suggested that APP holoprotein (rather than APPsα) is of critical importance for the maintenance of structural spine plasticity. As a note of caution we should also bear in mind that while full length APP undergoes regulated cleavage with APPsα secretion that correlates with synaptic activity, constitutive production of APPsα in APPsα-KI mice might fall short in both location and timing of APPsα release.

While it remains unclear how precisely APP exerts its physiological effects on excitatory synapses, there is a considerable body of evidence implying APP in regulating the trafficking and surface expression of ion channels and receptors [12, 26, 58, 64]. In the context of our findings, it is important to note that biochemical studies indicate a close interaction of APP with NR1 and NR2A subunits of NMDA receptors, which are of major importance in governing the spine dynamics and plasticity [14, 23, 38, 40]. In agreement with previous *in vitro* reports [12, 26], our measurements in acute brain tissue of APP-KO mice reveal reduced expression (as quantified in PSD fractions) of NR1 and NR2A subunits of NMDA receptor. Lower expression of NR1 and NR2A in APP-KO mice housed under standard or enriched conditions correlated with reduced frequency and decay time constant of mEPSC recorded under low extracellular Mg^{2+} and depolarized potentials, and accord with attenuation of NMDA receptor-mediated inputs [15]. Noteworthy, comparable mEPSC frequency and amplitude in WT and APP-KO mice at close to resting potentials (-65 mV) implies that the AMPA receptor-dependent component of excitatory transmission in APP-KO mice remains largely intact. These electrophysiological measurements are in accordance with our morphological data, which show a lower fraction of thin spines in APP-KO mice. As both, the morphology and stability of dendritic spines are subject to regulation by NMDA receptors, lowered expression of NR1/NR2A subunits in APP-KO would lead to spine plasticity impairments. Of note and in agreement with our observations, data from transgenic mice with deletion of NR1 subunit of NMDA receptor revealed enlarged spine heads in cortical neurons with reduced structural plasticity [59]. Similarly, acute loss of NMDA receptors [1] and their pharmacological inhibition [60, 67] have been reported to impair synaptic plasticity. Although our data cannot rule out the contribution of impaired synaptic and neurotrophic functions of APP in APP-KO mice, the restorative effects of NMDA receptor co-agonist D-serine demonstrated herein implicate close cooperation between NMDA receptors and full-length APP in maintenance of the dynamics and plasticity of dendritic spines.

To conclude, our data imply a major importance of APP in structural plasticity and adaptive remodeling of cortical synapses in the adult brain. They also suggest that deficit of APP holoprotein could lead to synaptic impairments in AD brain independently of its metabolites. Further research of APP mediated functions is likely to provide valuable insights into the biology of dendritic spines and open avenues for discovery of novel therapeutic targets for AD, a scientific investment with immense beneficial potential.

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Conflicts of Interest

The authors declare that they have no conflict of interests.

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Figure legends

Figure 1. Structural plasticity of dendritic spines is impaired in the cortex of adult APP-KO mice.

(**a**) Consecutive *in vivo* imaging of the same apical dendrites from layer V pyramidal neurons in the somatosensory cortex over 46 days reveals formation and elimination of dendritic spines (white and empty arrowheads, respectively) in WT and APP-KO mice. Prior to the exposure to environmental enrichment (EE), all mice were housed in standard conditions. Scale bar - 10 μm.

(**b-e**) Summary graphs of spine density, turnover rate (TOR), elimination and formation. Note that the spine density has been assessed at the first imaging time point (Day 1), while for the measurements of spine dynamics images from the Day 1 and 8 were analyzed.

(**f-h**) Graphical representations of the relative spine density, TOR and elimination over the period of the exposure of mice to EE. Non-linear regression (F test) has been used for fitting the data points. Two-tailed Student *t*-test was used in (a-e) and repeated one-way ANOVA was performed followed by Dunnett test in (f-h). WT n=5 mice and APP-KO n=6 mice; * $p<0.05$, ** $p<0.01$, NS - no significant difference.

Figure 2. APPsα fails to rescue the impaired structural spine plasticity of APP-KO mice

(**a**) Longitudinal *in vivo* imaging of the same apical dendrites from layer V pyramidal neurons in the somatosensory cortex of APPsα-KI mice housed under standard or enriched environments: white and empty arrowheads point to newly formed and eliminated spines, respectively. All mice were initially housed under standard conditions. Scale bar - 10 μm.

(**b, c**) Summary plots of spine density and TOR. Note that the spine density was assessed at the first imaging time point (1 d), while for the spine dynamics measurements, the data from the day 1 and 8 were analyzed.

(**d**) Representation of the relative spine density in WT and APPsα-KI mice under the environmental enrichment. Non-linear regression (F test) has been used for fitting the data points. Two-tailed Student *t*-test was used in (b-c) and repeated one-way ANOVA was performed followed by Dunnett test in (e). WT n=5 mice and APPsα-KI n=5 mice; ** $p<0.01$, NS - no significant difference.

Figure 3. Dendritic spine morphology is altered in APP-KO mice.

(**a**) Typical confocal images of apical dendrites with spines (z-projections) from layer V pyramidal neurons in the somatosensory cortex of WT and APP-KO mice (top and bottom)

housed in standard and enriched environments (left and right). For classification of spine types, 3D reconstructions by Imaris have been applied. Thin, mushroom and stubby spines are encoded in blue, green and red, respectively. Scale bar represents 2 μm.

(**b, c**) Summary plots of thin and mushroom spine fractions in WT and APP-KO mice exposed to standard (SE) and enriched environments (EE). . Two-tailed Student *t*-test was used and n=6 mice in all experimental groups; * $p<0.05$, ** $p<0.01$, NS - no significant difference.

Figure 4. Environmental enrichment fails to enhance the miniature excitatory synaptic currents and reveals reduced contribution of NMDA receptor to mEPCSs in APP-KO mice.

(**a, d**) Representative mEPSCs recorded in pyramidal neurons in slices from WT and APP-KO mice housed under standard and enriched environmental conditions. Note that recordings were made at -65mV and -45 mV holding potentials.

(**b, c** and **e, f**) Summary plots comparing the mEPSC frequency and decay time constants (tau) of mEPSC between mice of WT (b, c) and APP-KO (e, f) mice exposed to two different housing conditions (n=8 and n=9 slices from standard and enriched conditions); Two-tailed Student *t*-test was used; * $p<0.05$.

Figure 5. Quantification of NMDA receptor proteins of WT and APP-KO mice.

(**a-d**) Western blots and quantifications of NR1, NR2A and NR2B proteins from WT and APP-KO mice housed under standard and enriched environments: (a) representative blots with (b-d) summary plots. Note, that all NMDA receptor proteins have been detected from PSD fraction. Two-tailed Student *t*-test was used; n=6 mice in each group; * $p<0.05$, ** $p<0.01$.

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Figure 6. Treatment of APP-KO mice with NMDA receptor co-agonist D-serine restores the structural plasticity and morphology of dendrite spines.

(**a**) Consecutive *in vivo* imaging of the same apical dendrites from layer V pyramidal neurons in the somatosensory cortex of APP-KO mice housed under standard or enriched environment. Note that both groups of mice received D-serine after the second imaging time point (8 d); white and empty arrowheads point to newly formed and eliminated spines, respectively. Scale bar - 10 μm.

(**b**) Spine TOR prior and during continuous D-serine treatment.

(**c, d**) Summary plots of the fraction of spine elimination and formation in APP-KO mice before and after D-serine treatment (8 d and 46 d, respectively).

(**e**) Relative spine densities in D-serine treated APP-KO mice housed under standard and enriched environments. Non-linear regression has been used for fitting the data points.

(**f, g**) Summary plots of the fraction of thin and mushroom spines in control and D-serine treated APP-KO mice. For illustration purpose, the control data from Figure 2b, c are presented also here. Non-linear regression (F test) has been used for fitting the data points. Two-tailed Student *t*-test was used in (c, d and f, g) and repeated one-way ANOVA was performed followed by Dunnett test in (b, e). N=5 mice in each group; * $p<0.05$, ** $p<0.01$, NS - no significant difference.

Figures

Figure 1

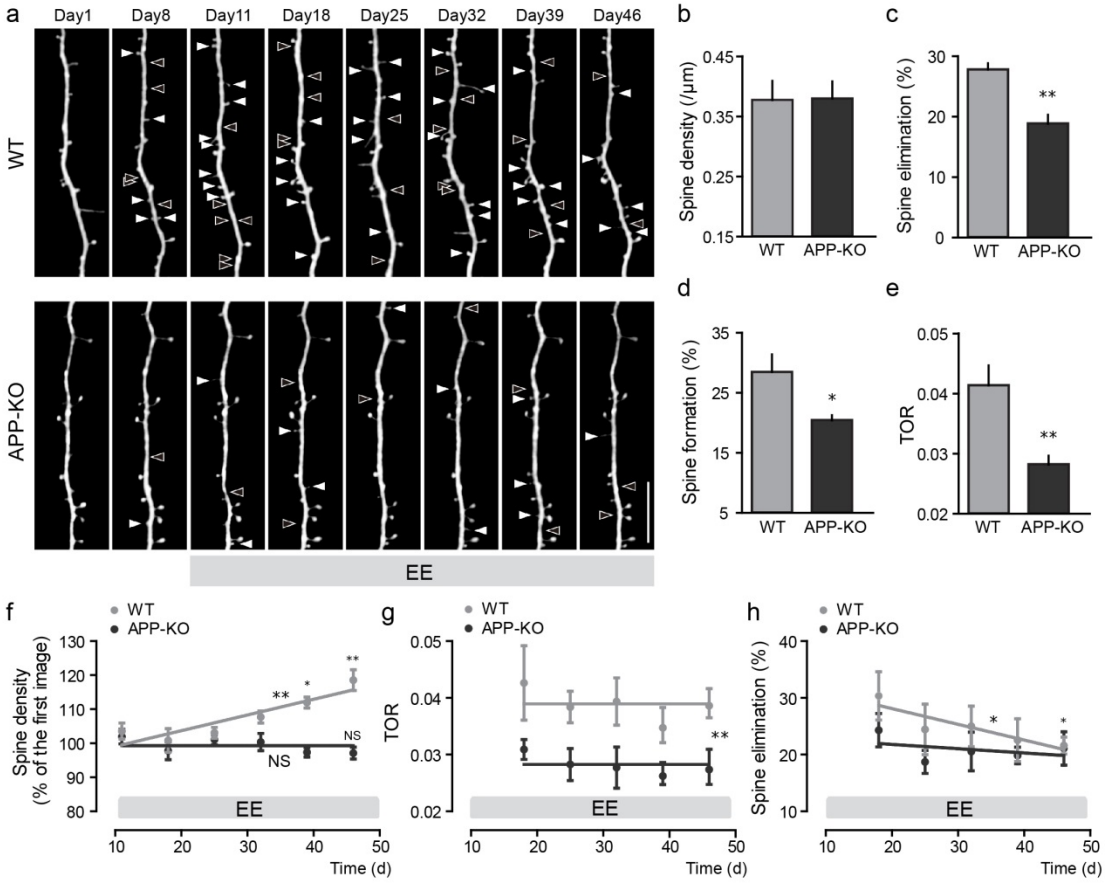


Figure 2

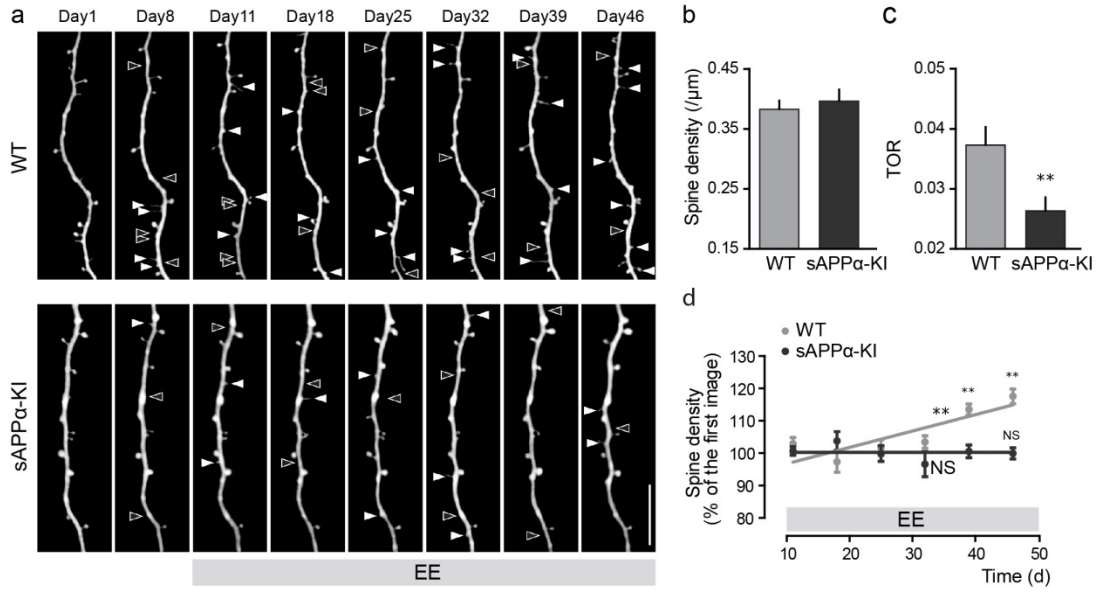


Figure 3

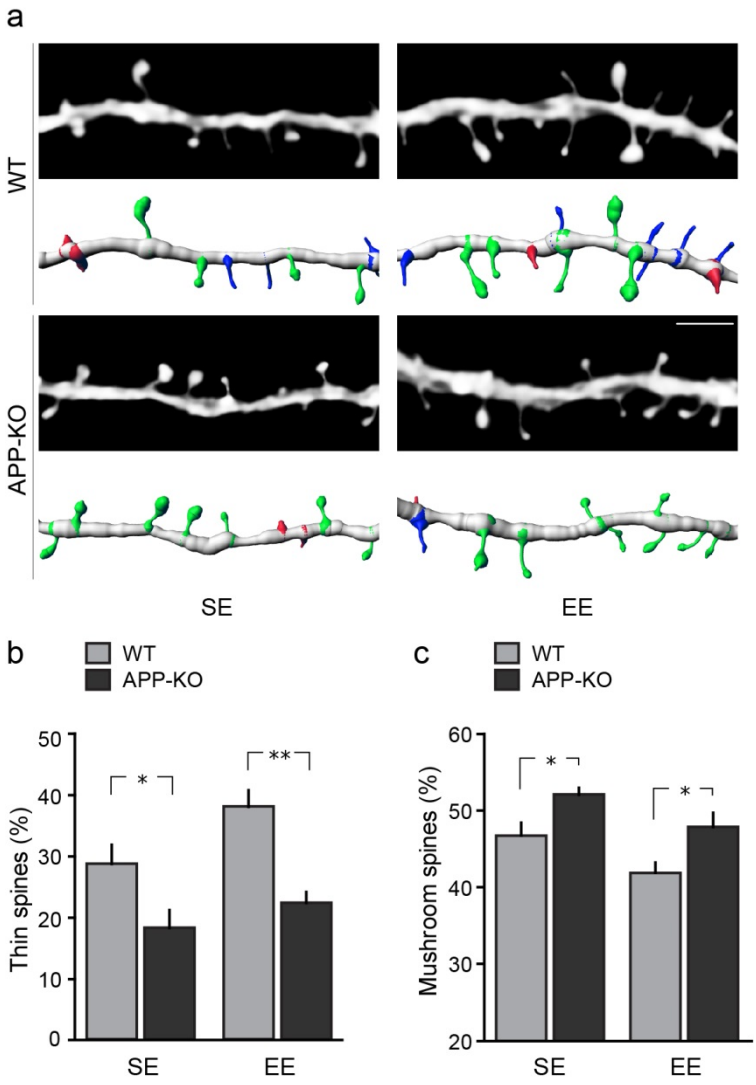

a
WT
APP-KO
SE
EE
b
WT
APP-KO
Thin spines (%)
50
40
30
20
10
0
*
**
SE
EE
c
WT
APP-KO
Mushroom spines (%)
60
50
40
30
20
*
*
SE
EE

Figure 4

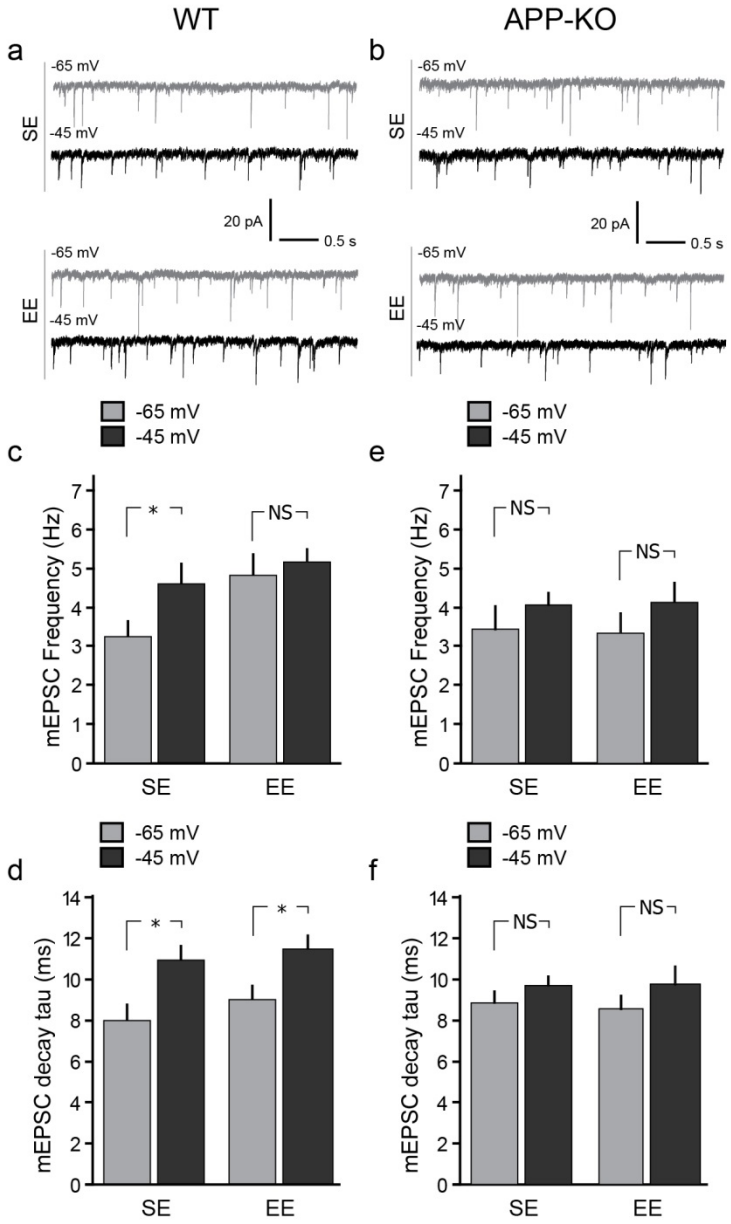


Figure 5

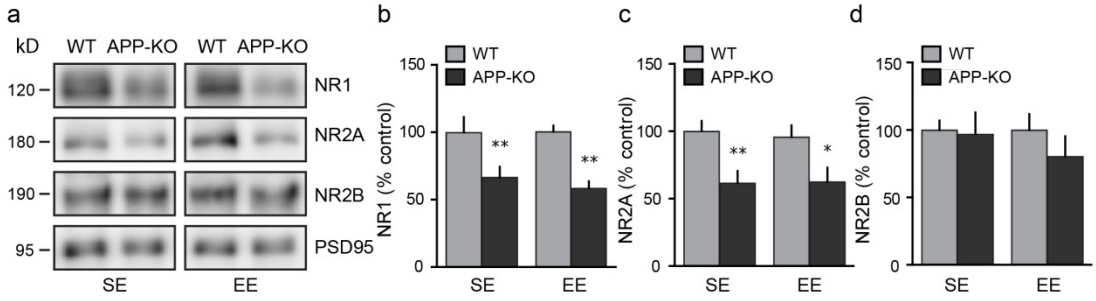
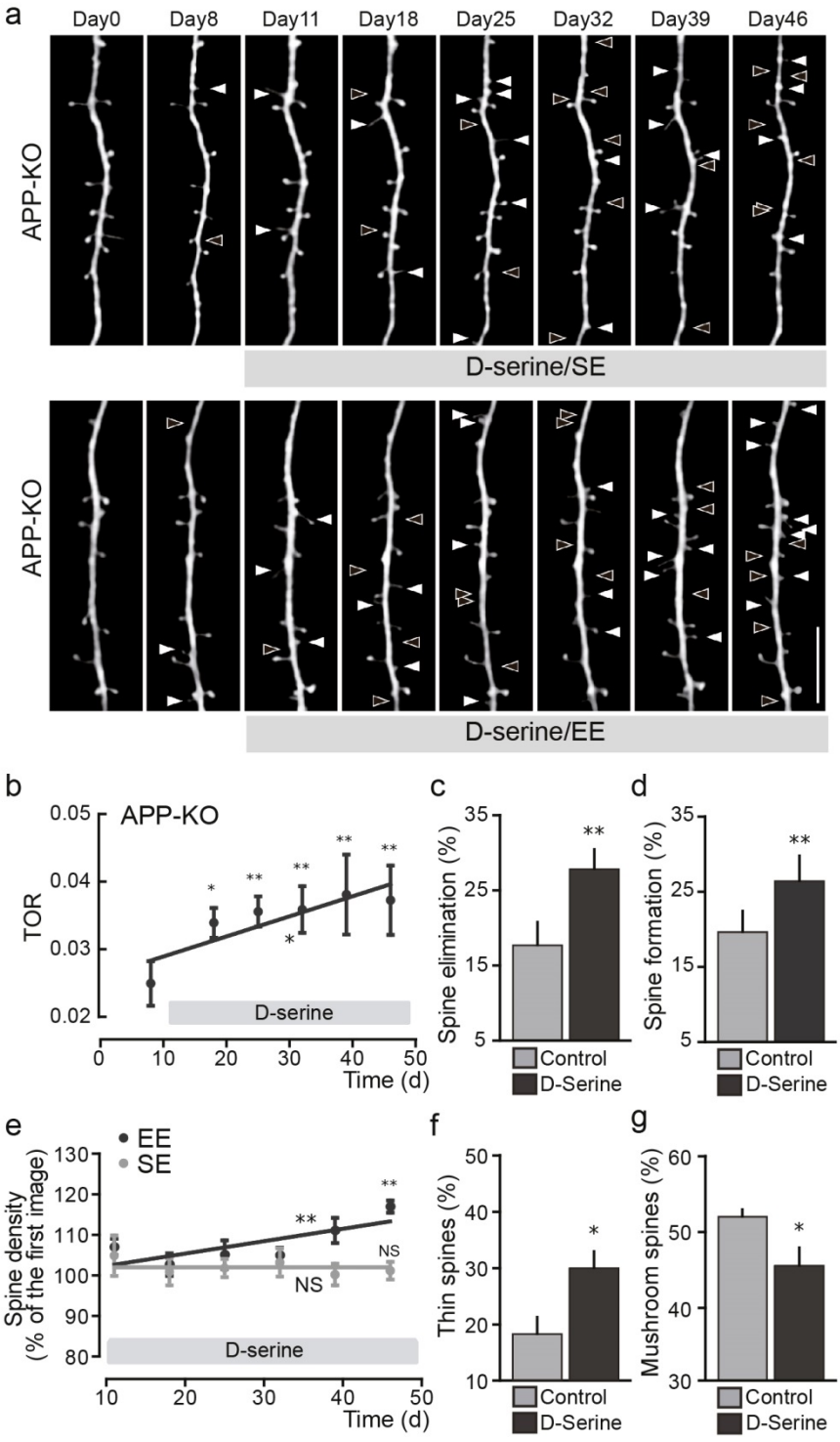


Figure 6


a
Day0
Day8
Day11
Day18
Day25
Day32
Day39
Day46
APP-KO
D-serine/SE
APP-KO
D-serine/EE
b
APP-KO
TOR
D-serine
Time (d)
c
Spine elimination (%)
Control
D-Serine
d
Spine formation (%)
Control
D-Serine
e
EE
SE
Spine density (% of the first image)
NS
NS
D-serine
Time (d)
f
Thin spines (%)
Control
D-Serine
g
Mushroom spines (%)
Control
D-Serine

5 General Discussion

As a degenerative brain disorder, AD accounts for 60 to 80 percent of dementia with an estimated number of more than 35 million cases worldwide [39]. Although there still does not exist effective pharmacological treatment of AD, the accumulation of APP proteolytic fragment, Aβ, is believed to play a central role in AD development [18, 38]. The amyloid hypothesis is strongly supported by the discovery of familial AD gene mutations in APP and presenilins, both of which facilitate Aβ production. On the basis of these findings, transgenic AD mouse models have been created by expressing mutant APP and/or presenilins. These transgenic mice offer an opportunity to study the pathogenic events in the process of AD. Besides the intensive studies on Aβ neurotoxicity, physiological functions of APP also draw attention to AD research [32]. The regulation of producing Aβ from APP proteolysis modulates the expression of APP and other APP fragments that may be physiologically pivotal. To identify the physiological role of APP, APP knockout mice and APP fragment knockin mice in APP null background have been generated [30, 35, 52]. In this dissertation, we used APP23 (overexpress human APP with the Swedish mutation, APPswePS1deltaE9 (deltaE9, overexpress APP with the Swedish mutation together with mutant PS1 lacking exon 9), APP knockout (APP-KO) and APPsα knockin (APPsα-KI, express APPsα but lack full length APP) to study the structural plasticity of dendritic spines during AD related pathophysiological processes.

The structural plasticity of dendritic spines refers to the alterations of spine distribution and morphology in physiological or pathological conditions, which is the structural basis of refinement or impairment of neuronal circuits [28, 48, 51]. In neurodegenerative disorders, the most prominent pathology of dendritic spines is usually seen as decreased spine density that may be contributed by deafferentation resulted from neuronal loss [4, 17]. In particular, AD patients display a remarkable synaptic loss that is correlated with their cognitive capabilities [12, 43]. Besides pathological events, novel sensory experience also affects dendritic spine plasticity in adult brain. Increased spine density has been reported in mice housed under EE, which provides multiple external sensory experiences [13, 24]. These studies investigated the number and morphology of dendritic spines in young adult transgenic mouse models mentioned above. Also, EE was adopted as a behavioral paradigm to further examine the adaptive spine plasticity. Our results disclosed that in different AD transgenic mouse models

(APP23 and deltaE9), different pathological mechanisms resulted in spine abnormalities. Furthermore, neuroinflammation associated with amyloid plaques impaired EE-induced spine plasticity. Last but not least, reduced dendritic spine dynamics and deficient increase in spine density during EE were found in APP-KO and APPsα-KI mice, which might be ascribed to the reduction of NMDARs. Collectively, these results suggest that the structural plasticity of dendritic spines is impaired during AD related pathophysiological processes.

APP23 and deltaE9 mice are two well-studied transgenic mouse models of AD [47]. To increase Aβ levels in brain, APP23 mice overexpress human APP with the Swedish mutation, while deltaE9 mice contain APP with the Swedish mutation together with mutant PS1 lacking exon9 [23, 37, 42]. These two mouse models both successfully recapitulate the AD pathogenesis in old age, such as neuronal loss, cholinergic deficit, cognitive decline and amyloid deposition. However, they display different temporal progress of amyloid plaque formation and cognitive impairment in young adulthood [2, 6, 8, 25, 29, 41]. In APP23 mice, cognitive decline precedes the formation of amyloid deposits. On the contrary, deltaE9 mice develop amyloid plaques before the onset of cognitive decline. In agreement with the previous findings of cognitive performance and amyloid deposition, our results confirmed that the loss of spines in young adult APP23 mice was observable in apical dendrites of layer 5 pyramidal neurons before amyloid deposition. However, dendritic spines of deltaE9 mice were lost only in the vicinity of amyloid plaques, which indicated the total number of spines remained unchanged, as cortical β-amyloid area is quite small in young deltaE9 mice. Moreover, distinct alterations in spine morphology were also found in APP23 and deltaE9 mice. Although it is well known that dendritic spine morphology affects various functional properties of dendritic spines that are associated with cognitive functions [26, 36], it still remains unclear if and how the altered spine morphology correlates with cognitive impairment in AD. More importantly, it is also unknown that whether pathological spine distribution and morphology contribute to specific cognitive impairments and if they function individually or collaboratively in cognitive decline. The different pathological mechanisms, namely intracellular APP accumulation and extracellular amyloid deposits that underlie the spine pathology of APP23 and deltaE9 mice irrespectively, suggest synaptic failure or other AD symptomatic features in mouse models may be ascribed to distinct causes. Thus, it needs to be very careful to compare the results obtained from different AD mouse models and translate them into the human disease.

Being the structural correlate of cognitive capabilities, the spine density on vast majority of dendrites in young deltaE9 mice was comparable to control mice. This finding agrees with the normal performance in most cognitive tests of age-matched deltaE9 mice [27, 34, 45], which starts to develop amyloid plaques [5, 16]. The temporal lag between amyloid deposition and cognitive impairment in AD mice faithfully imitates the preclinical stages of AD that have been recently defined as the asymptomatic period with the emergence of amyloid plaques in brain [40]. As pathological events progress many years before clinical manifestations, irreversible damages may occur in preclinical AD. Therefore, it is crucial to investigate these events and identify effective pharmacological interventions in preclinical stages of AD to prevent or delay the onset of dementia. Our results disclosed that impaired adaptive structural plasticity of dendritic spines occurred in young adult deltaE9 mice, which displayed amyloid deposits but not cognitive decline. The experience-dependent spine plasticity remodels established neural networks that facilitate the brain in adapting to novel external environment [22, 49, 50]. The failure to gain spine density and stabilize new spines in preclinical AD mice suggests the deficiency in dendritic spines already occurs before spine loss and cognitive decline. Accompanied with the appearance of amyloid plaques and subsequent activated glial cells, diffusible pro-inflammatory cytokines are released [46]. These cytokines have been reported to affect synaptic transmission and plasticity [14, 15]. The restoration of adaptive structural spine plasticity in young deltaE9 mice by anti-inflammatory treatments further reveals that pro-inflammatory cytokines may contribute to the deficiency of dendritic spines in preclinical AD. Interestingly, the early administration of anti-inflammatory drugs has been confirmed to be able to decrease dementia risk and delay the onset of AD [3, 9, 19]. It is therefore suggested that impaired adaptive spine plasticity induced by neuroinflammation may precede and play an important role in symptomatic cognitive decline.

Besides increased Aβ levels, loss of APP, which might be caused by its enhanced proteolytic process, may also contribute to the pathogenesis of AD. The synaptic adhesion and synaptogenesis mediated by APP manifest its protective roles in synapses and neurons [21, 31-33]. In these studies, we identified APP is involved in spine plasticity of adult brain. APP-KO mice in adulthood showed decreased spine dynamics, impaired adaptive spine plasticity and altered spine morphology together with reduced NMAD receptor-mediated mEPSCs and NMDA receptor expression in postsynaptic sites. Interestingly, activation of NMDA receptors by D-serine rescued spine pathology in APP-KO mice. APP has been reported to act as a NMDA receptor auxiliary subunit [10, 20]. The interaction between APP and NMDA receptor

facilitates the delivery of NMDA receptor from endoplasmic reticulum to synaptic membranes. APP-NMDA receptor trafficking complexes probably bear on other transmembrane proteins, such as Neuropilin tolloid like 1 [11]. How APP associates with assembled NMDA receptor and whether an intermediary protein is involved need to be further investigated. To date, the role of NMDA receptor in dendritic spine dynamics is still unclear. Physical and ionotropic properties of NMDA receptor may be differently involved in spine elimination and formation. Physical loss of NMDA receptor might disrupt NMDA receptor related protein-protein associations and lead to a great spine loss [1]. However, pharmacological blockade of NMDA receptor decreases the rate of spine elimination during adolescence and increases spine stability after entorhinal denervation [44, 53]. Brain-derived neurotrophic factor (BDNF), the regulator in EE-mediated brain plasticity, shares common cellular signaling molecules with NMDA receptor to modulate synaptic plasticity [26]. As the interactions between BNDF and NMDA receptor signaling cascades are mutual and complicated, it is not clear whether activation of BNDF receptor or NMDA receptor alone is sufficient to induce activity-dependent structural spine plasticity and whether deficiency on one of the receptors hinders the physiological function of the other. Recent studies have shown that activation of NMDA receptor alone is not enough to induce the rapid spine remodeling in LTP and NMDA receptor dysfunction impairs BDNF mediated facilitation hippocampal synaptic transmission [7, 26].

To conclude, this dissertation provides evidence for abnormal structural spine plasticity in APP transgenic and knockout mouse models. Altered spine distribution and morphology are found to be caused by different mechanisms in different AD mouse models overexpressing human APP with the Swedish mutation alone or together with PS1 mutation. Also, adaptive structural plasticity of dendritic spines that precedes cognitive decline is impaired in a preclinical model of AD, which is recovered by anti-inflammatory treatments. Last but not least, decreased spine dynamics and deficient experience-dependent gain of spine density are observed in APP-KO and APPsα-KI mice. All the results of the dissertation facilitate to reveal spine abnormalities in AD related pathophysiological processes.

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Abbreviations

5-HT	5-hydroxytryptamine
Aβ	Amyloid β
AD	Alzheimer's disease
AICD	Amyloid precursor protein intracellular domain
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
APH1	Anterior pharynx defective 1
APLPs	Amyloid precursor-like proteins
ApoE4	Apolipoprotein E ε4
APP	Amyloid precursor protein
APP knockout	APP-KO
APPsα knockin	APPsα-KI
BACE	β-site amyloid precursor protein cleaving enzyme
BDNF	Brain-derived neurotrophic factor
CSF	Cerebrospinal fluid
CTF	COOH-terminal fragments
deltaE9 mice	APPswePS1detlaE9 mice
EE	Enriched Environment
EM	Electron microscopy
GFP	Green fluorescent protein
IL-1β	Interleukin-1β
IL-1 RA	Interleukin-1 receptor antagonist
KPI	Kunitz-type of serine protease inhibitors
LV	Lentivirus
LTD	Long-tern depression
LTP	Long-term potentiation
MCI	Mild cognitive impairment
mEPSCs	Miniature excitatory post-synaptic currents
NMDA	N-methyl-D-aspartate
NFTs	Neurofibrillary tangles
NR1	NMDA receptor 1

NR2A	NMDA receptor 2A
NR2B	NMDA receptor 2B
PET	Positron emission tomography
PHFs	Paired helical fragments
PS1	Presenilin 1
PS2	Presenilin 2
PSD	Post-synaptic density
TOR	Turnover rate
SE	Standard environment
SER	Smooth endoplasmic reticulum
WT	Wild-type
YFP	Yellow fluorescent protein

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List of publications and manuscripts

1. **Zou C**, Montagna E, Shi Y, Peters F, Blazquez-Llorca L, Shi S, Filser S, Dorostkar MM, Herms J. Intraneuronal APP and extracellular Aβ independently cause dendritic spine pathology in transgenic mouse models of Alzheimer's disease. *Acta Neuropathol* (2015) Jun; 129(6): 909-20.

2. **Zou C**, Ovsepian SV, Zhu K, Mueller UC, Herms J. Amyloid precursor protein and NMDA receptor cooperate to maintain constitutive and adaptive plasticity of dendritic spine in adult brain (Submitted).

3. **Zou C** and Herms J. Neuroinflammation impairs activity-dependent structural dendritic spine plasticity in a pre-clinical model of Alzheimer's disease (Submitted).

4. **Zou C**, Luo Q, Qin J, Shi Y, Yang L, Ju B, Song G. Osteopontin promotes mesenchymal stem cell migration and lesses cell stiffness via integrin β1, FAK and ERK pathways. *Cell Biochem Biophys* (2013) Apr; 65(3): 455-62.

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Hiermit versichere ich an Eides statt, dass ich die vorliegende Dissertation The Structural Plasticity of Dendritic Spines in Amyloid Precursor Protein Transgenic and Knockout Mouse Models selbstständig angefertigt habe, mich außer der angegebenen keiner weiteren Hilfsmittel bedient und alle Erkenntnisse, die aus dem Schrifttum ganz oder annähernd übernommen sind, als solche kenntlich gemacht und nach ihrer Herkunft unter Bezeichnung der Fundstelle einzeln nachgewiesen habe.

I hereby confirm that the dissertation The Structural Plasticity of Dendritic Spines in Amyloid Precursor Protein Transgenic and Knockout Mouse Models is the result of my own work and that I have only used sources or materials listed and specified in the dissertation.

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Munich, date Unterschrift signature

Declaration of author contributions

In Paper One, Chengyu Zou conceived, designed and performed the experiments. Besides, Chengyu Zou analyzed the data in Fig. 1, Fig. 3, Fig. 5, Suppl. Fig. 4, built all figures and wrote the manuscript. Elena Montagna analyzed the data in Fig. 2 and performed the experiments in Fig. 5 and Suppl. Fig. 3. Yuan Shi analyzed the data and performed the experiments in Fig. 5 and Suppl. Fig. 3. Mario M. Dorostkar, Jochen Herms and Chengyu Zou revised the manuscript until final publishing.

In Manuscript One, Chengyu Zou designed the study, analyzed the data, prepared the figures and wrote the manuscript. Jochen Herms and Chengyu Zou revised the manuscript.

In Manuscript Two, Chengyu Zou conceived the study, performed the experiments and analyzed the data in Fig. 1, Fig. 2, Fig. 3, Fig. 5, and Fig 6. Saak V. Ovsepian performed the experiments and analyzed the data in Fig. 4. Saak V. Ovsepian and Chengyu Zou built the figures, wrote and revised the manuscript.

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