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review

Dendritic spines: from structure to *in vivo* function

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Dendritic spines arise as small protrusions from the dendritic shaft of various types of neuron and receive inputs from excitatory axons. Ever since dendritic spines were first described in the nineteenth century, questions about their function have spawned many hypotheses. In this review, we introduce understanding of the structural and biochemical properties of dendritic spines with emphasis on components studied with imaging methods. We then explore advances in *in vivo* imaging methods that are allowing spine activity to be studied in living tissue, from super-resolution techniques to calcium imaging. Finally, we review studies on spine structure and function *in vivo*. These new results shed light on the development, integration properties and plasticity of spines.

Keywords: calcium; plasticity; spine; super-resolution imaging; two-photon imaging

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See the Glossary for abbreviations used in this article.

Introduction

In 1888, the legendary neuroanatomist Ramón y Cajal was the first to describe dendritic spines on neurons [1]. Since this first description, technical advances have driven our knowledge of the structural and functional properties of dendritic spines but the function of spines remains the subject of intense study and debate [2,3]. Dendritic spines arise as small protrusions from the dendritic shaft of various types of neuron, including the pyramidal neurons of the neocortex, the medium spiny neurons of the striatum and the Purkinje cells of the cerebellum. Depending on the neuronal type, spines occur at various densities and are found in all vertebrates and in some invertebrates. A striking characteristic of spines is their variety of shapes and sizes, suggesting a high degree of functional diversity. Until relatively recently, it was only possible to study putative functions of the spines *in vitro* and in fixed tissue. In such preparations, much progress has been made on defining the structural and biochemical properties of spines. Now, however, advances in imaging techniques make it possible to investigate spine function and plasticity at high spatial resolution in living tissue.

Dendritic spines: synaptic transmission and plasticity

Spine morphology. The morphology of spines is highly variable and they are commonly classified into three types: thin, mushroom and stubby (Fig 1A; [4]). Thin spines have a thin, long neck and a small bulbous head, whereas mushroom spines have a larger head. Stubby spines are devoid of a neck [5] and are prominent between postnatal development [6]. The size of dendritic spines varies among brain areas, as well as between species. For example, the area of spine heads in the temporal cortex is around 0.37 μm^2 in mice and 0.59 μm^2 in humans, and the length of the spine neck in the same cortical area is on average 0.73 μm in mice and 0.94 μm in humans [7]. Importantly, on the same dendrite a continuum of shapes can be observed, and the morphology of a spine can change rapidly through activity-dependent and -independent mechanisms [8–12]. In addition, thin, hair-like protrusions called filopodia, which lack a bulbous head, are found on dendrites of developing neurons (Fig 1A). They are transient structures that might receive synaptic input and can develop into dendritic spines [13,14].

The morphological changes of spines are tightly linked to biochemical reactions taking place inside the spine. The tiny spine head is biochemically isolated from the dendrites by the spine neck. In this small volume, an astonishingly high number of biochemical reactions take place [15]. Typically, spine heads form an asymmetric excitatory synapse with a presynaptic axon [16]. These synapses are characterized by a 'postsynaptic density' (PSD), which appears as an electron-dense, dark area under the electron microscope. Most proteins in the PSD are directly or indirectly involved in synaptic communication and in the regulation of synaptic strength [15]. Ultrastructural studies have shown a correlation between the size of the PSD, the spine head volume and the number of vesicles in presynaptic terminals in CA1 pyramidal neurons [9], cerebellar Purkinje cells [8] and in the olfactory cortex [10]. These results led to the idea of a causal link hypothesis between spine structure and the function of spine synapses. This was further supported by studies of calcium dynamics in spines, which revealed a close relationship between spine morphology and function [11]. Later, by using glutamate uncaging on single spines (for a review see [12]), it was demonstrated that spine morphology—mushroom compared with thin spines—correlates directly with the number of AMPA receptors [17], and that the spine–neck geometry is an important determinant of NMDA receptor-dependent calcium signalling in spine heads and dendritic shafts [18]. Furthermore, there is evidence that the induction of long-term potentiation (LTP) correlates with spine enlargement [19]. Using imaging to monitor the changes in spine shape is thus a useful way in which to study their function.

The final determinant of spine morphology is the cytoskeleton. Spine heads contain actin filaments that interact with the plasma membrane and the PSD at their barbed ends (Fig 1B). In spine necks,

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Glossary

AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
CaMKII	Ca^{2+}/calmodulin-dependent kinase II
FRET	Förster resonance energy transfer
HVC	hyperstriatum ventrale pars caudale/high vocal centre
NMDA	*N*-methyl-D-aspartate
OGB1	Oregon-Green-BAPTA-1
PMCA	plasma membrane Ca^{2+}-ATPase

actin filaments form long bundles (Fig 1B; [20]). It was shown that actin polymerization occurs within seconds of LTP, underlying the enlargement of dendritic spines [21]. Thus, there is good evidence that, at least for hippocampal synapses, the reorganization of the actin cytoskeleton is tightly linked to synaptic efficacy [12,22].

Calcium imaging assessment of spine function. A powerful way to study spine function is to monitor changes in intracellular calcium concentration in this small neuronal compartment. Calcium is an intracellular secondary messenger that regulates the functional and structural properties of individual synapses, with remarkable spatio-temporal specificity [23,24]. In this section, we focus on the biochemical reactions that involve calcium influx and calcium homeostasis in spines.

A main source of calcium entry in spines is influx through ionotropic glutamate receptors and voltage-gated calcium channels (Fig 1B). NMDA and AMPA glutamate receptors are non-specific cation channels with a variable permeability for calcium ions; NMDA receptor channels are particularly calcium-permeable. Typically, the spine is first depolarized by AMPA receptor activation, which removes the blocking action of extracellular magnesium ions on NMDA receptor channels, and leads to further depolarization and calcium entry. Spine depolarization can then be further amplified by voltage-gated calcium or sodium channels [24]. Thus, spines act as NMDA-receptor-dependent coincidence detectors of pre- and postsynaptic activity [25,26]. It is well accepted that the onset of LTP, spine enlargement and an increase in receptor trafficking are coincident and mechanistically linked processes [27]. LTP induction is associated with exocytosis from endosomes and insertion of AMPA receptors into the plasma membrane [28]. Importantly, AMPA receptors diffuse laterally along the plasma membrane [29,30], reaching PSD proteins on which they can be anchored in an actin-dependent manner [31,32].

Another important form of synaptically mediated spine calcium signalling involves calcium release from internal stores, through either ryanodine receptors [33] or inositol trisphosphate (IP3) receptors (Fig 1B; [34,35]). In the cerebellar Purkinje neurons, metabotropic glutamate type 1 receptors (mGluR1s), through a signal cascade involving activation of G protein and phospholipase C, can produce IP3 and cause calcium release from the endoplasmic reticulum in dendritic spines [34,35]. This calcium release is mediated through calcium-permeable IP3 receptors. These spine calcium signals can act as coincidence detectors [36], having important roles for long-term synaptic depression [37]. Calcium levels inside the endoplasmic reticulum are tightly regulated by the sarco/endoplasmic reticulum calcium ATPase (SERCA) (Fig 1B). Interestingly, mGluR1s are located perisynaptically at some distance from the presynaptic glutamate release sites [38]. Therefore, only repetitive presynaptic activity can efficiently activate them.

Molecular mechanisms in spines assessed by live cell imaging. Live cell imaging experiments involving the use of sensors with a high specificity for target proteins have greatly advanced our understanding of the signalling mechanisms in spines. Of significant importance is the imaging of the action of CaMKII, which is a crucial component of the PSD (Fig 1B; [39,40]). CaMKII is essential for the induction and maintenance of some forms of synaptic plasticity [41–44]. For imaging, CaMKII can be tagged by fluorescent proteins [45,46], for example to directly study the dynamics of CaMKII inside spines in response to synaptic activation [47]. The activity of one of the CaMKII downstream targets, the small GTPase Ras, was also imaged after induction of LTP in the spines of hippocampal neurons [48]. Ras is active when bound to GTP, and inactive when bound to GDP (Fig 1B). This cycle is regulated through interaction with GTPase-activating proteins (GAPs) and guanine nucleotide exchange factors (GEFs)[49]. It is important to note that not only Ras, but also other members of the small GTP-binding protein superfamily, for example, Rho, Rab, Sar1/Arf and Ran, are involved in neuronal function [49]. They have been shown to regulate a wide variety of processes including gene expression, cytoskeletal reorganization and vesicle trafficking [49]. More specifically, Ras is involved in the regulation of dendritic protein synthesis and gene transcription, whereas Rho GTPases have key roles for the regulation of the actin cytoskeleton and thereby spine morphology [50–52]. The activation of Rho GTPases was directly determined in single spines in relation to structural changes induced by LTP [27,53].

Accumulating evidence supports a role for dendritic mRNAs in the regulation of synaptic functions in spines. The classical view is that proteins are synthetized in the soma and then transported to appropriate locations in the dendrites. However, in addition to this mechanism, it has been shown that local translational machinery exists in dendrites, such that mRNAs can be shipped to the dendrites and then translated according to local needs, often in an activity-dependent manner [54,55]. For example, in the developing neurons of the optic tectum in *Xenopus* tadpoles, Bestman and Cline have imaged *in vivo* RNA-binding proteins tagged with fluorescent proteins [56]. They have also shown that these RNA-binding proteins are distributed throughout the developing dendritic tree and can locally regulate branch dynamics [57].

Advances in spine imaging

Recent years have seen the rapid development of molecular and cellular imaging techniques that allow spine morphology to be studied at high resolution, even in the living brain. In addition to imaging of spine morphology and localization, new methods have been developed to functionally analyse molecular interactions and spine activity.

Imaging spine morphology with two-photon microscopy. Since the early work of Ramón y Cajal, the morphological properties of spines have been studied in fixed tissue at various levels of spatial resolution, ranging from light microscopy to ultrastructural studies involving electron microscopy [8,16,58]. In vital preparations, important insights into spine morphology and dynamics were obtained by using camera imaging and confocal microscopy [59-61]. A major step forward was the implementation of two-photon laser scanning microscopy [62], which is nowadays widely used for the imaging of spines in the highly scattering brain tissue (see reviews [63,64]). An advantage of two-photon microscopy is that the use of long

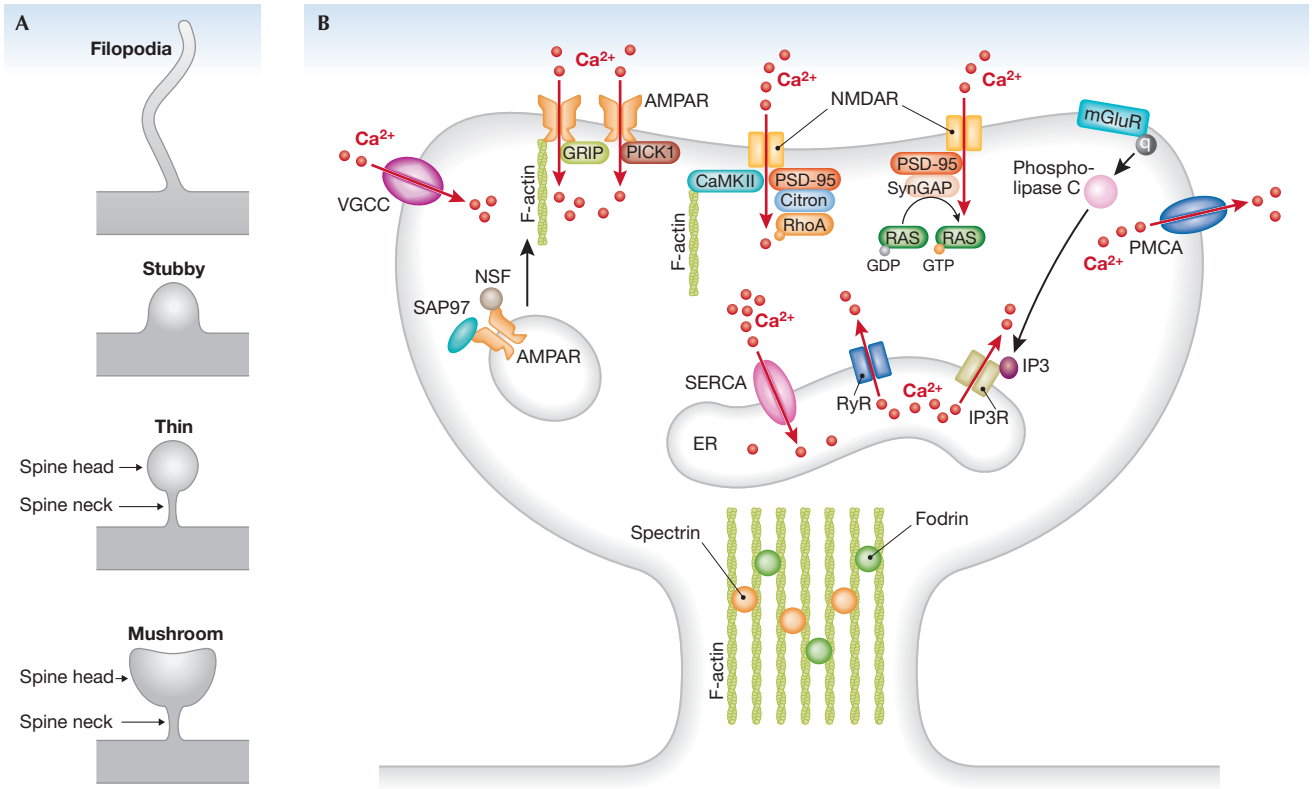
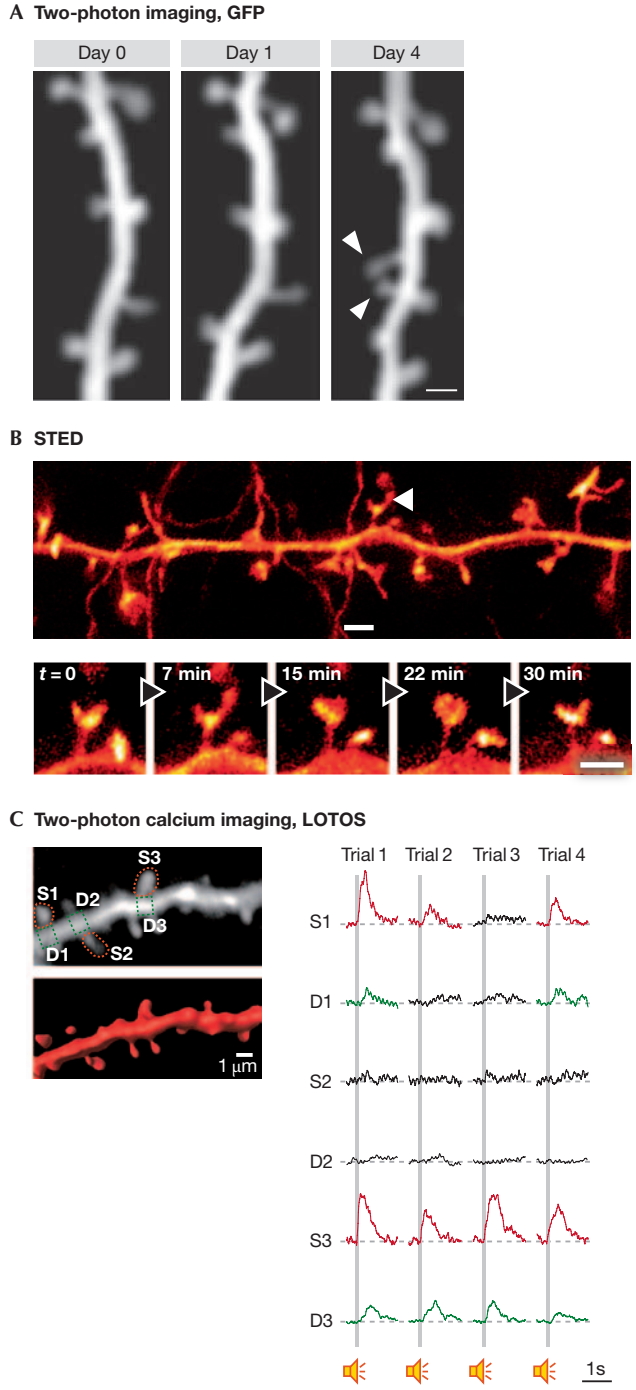


Fig 1 | Structural and molecular organization of spines. (**A**) Schematic drawings of spine morphologies based on the most common four-category classification. Note that on the same dendrite a continuum of shapes can be observed, and that the morphology of a spine can change rapidly. (**B**) Receptors and molecules related to calcium (Ca^{2+}) signalling in spines. Red arrows indicate flux of calcium ions. AMPAR, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor; CaMKII, Ca^{2+}/calmodulin-dependent kinase II; ER, endoplasmic reticulum; GAP, GTPase-activating protein; GRIP, glutamate-receptor-interacting protein; IP3(R), inositol trisphosphate (receptor); mGluR, metabotropic glutamate receptor; NMDA, *N*-methyl-D-aspartate; NSF, N-ethylmaleimide sensitive factor; PICK1, protein interacting with C kinase; PMCA, plasma membrane Ca^{2+}-ATPase; PSD, postsynaptic density; RyR, ryanodine receptor; SAP97, synapse-associated protein 97; SERCA, sarco/endoplasmic reticulum Ca^{2+}-ATPase; VGCC, voltage-gated calcium channel.

wavelength-excitation light provides a depth penetration of several hundred micrometres into the intact nervous tissue. In addition, because excitation is limited to a small focal volume, photo-damage outside the focal plane is strongly reduced compared with standard one-photon microscopy (for a review see [65]). Neurons of interest are typically labelled with fluorescent proteins such as GFP or YFP (Fig 2A), either in transgenic mouse lines or after labelling through viral transduction. It is now possible to image spines *in vivo* up to a depth of about 800 μm [66].

Another area of application of two-photon excitation involves the activation of compounds at precise locations. Thus, it is possible, in a single spine, to activate proteins tagged with photoactivatable GFP to study the dynamics of these proteins after, for example, inducing LTP. This approach has been used to monitor the dynamics of PSD95—tagged with photoactivatable GFP—during activity-dependent synaptic growth [67,68]. Several studies have used two-photon uncaging of a caged glutamate compound to establish the direct structure–function relationship between spine shape and synaptic activity (see review [12]). In addition, the role of the spatial and temporal sequence of synaptic inputs can be studied by uncaging glutamate, at specific locations, in a defined temporal sequence [69–71]. Two-photon glutamate uncaging has also been adapted for *in vivo* studies [72].

Imaging spines at super-resolution. With two-photon imaging, the spatial resolution is typically limited to around 300 nm, due to Abbe's diffraction law stating that features closer than half the wavelength of light cannot be distinguished [73]. Super-resolution techniques have overcome this resolution barrier [73–76]. Two main strategies have been developed to increase spatial resolution. First, saturated stimulated emission depletion (STED) microscopy [73] has been applied to image spines at a nanoscale resolution [75]. Features such as the thickness of the spine neck or subtle changes in the shape of the spine head—for example, from thin to mushroom shape—can be studied with this approach at a resolution smaller than 70 nm. STED has been applied to image spines in the living brain (Fig 2B; [74]). So far, this technique is limited to layer 1 (around 15 μm) in conditions in which the mouse is anaesthetized and paralysed. The second strategy is based on the techniques of photoactivation localization microscopy (PALM) [77] and stochastic optical reconstruction microscopy (STORM) [78]. Both approaches rely on the sequential, statistical excitation of a fraction of fluorophores spaced at distances larger than the diffraction limit [76]. This gives a spatial resolution of around 20 nm. However, for the moment, the time needed to acquire a super-resolution image and the associated bleaching of fluorophores make the two methods suitable mostly for fixed specimens (cultured cells) and limit the temporal resolution.



◀ **Fig 2** | Examples of spine imaging methods *in vivo*. (**A**) *In vivo* chronic two-photon imaging of spine dynamics in apical dendrites of layer 5 neurons in the mouse motor cortex. Repeated imaging of the same dendritic branch revealed two neighbouring new spines (arrowheads) formed between days 1 and 4 of motor-learning task training. Scale bar, 1 µm. Neurons were labelled with YFP. Reprinted by permission from Macmillan Publishers Ltd: *Nature* [107] © 2012. (**B**) *In vivo* STED microscopy in the molecular layer of the somatosensory cortex of a mouse with eYFP-labelled neurons. Projected volumes of dendritic and axonal structures reveal temporal dynamics of spine morphology (lower panel). Scale bars, 1 µm. Reprinted by permission from the American Association for the Advancement of Science (AAAS) [74]. (**C**) *In vivo* two-photon spine calcium imaging using the LOTOS procedure. Left panel, two-photon image (top) and three-dimensional image reconstruction (bottom) of a dendritic segment of a layer 2/3 neuron in the mouse primary auditory cortex. Right panel, four consecutive trials of subthreshold calcium transients evoked by auditory stimulation in spines (red) and corresponding dendritic shaft regions (green), as indicated in the left panel. Reprinted by permission from [82]. LOTOS, low-power temporal oversampling; STED, stimulated emission depletion; eYFP, enhanced yellow fluorescent protein.

Imaging spine activity in vivo. As introduced above, calcium imaging has proven to be a powerful tool for studying neuronal activity at the cellular and subcellular scale. This method has benefited from the development of sensitive fluorescent calcium indicator dyes, including synthetic dyes (e.g. Fura2, OGB1) and, more recently, FRET-based and single fluorophore genetically encoded calcium sensors [79]. In parallel, high-resolution imaging techniques have been developed to monitor changes in the fluorescence of such dyes in neuronal somata, dendrites and spines, *in vivo*.

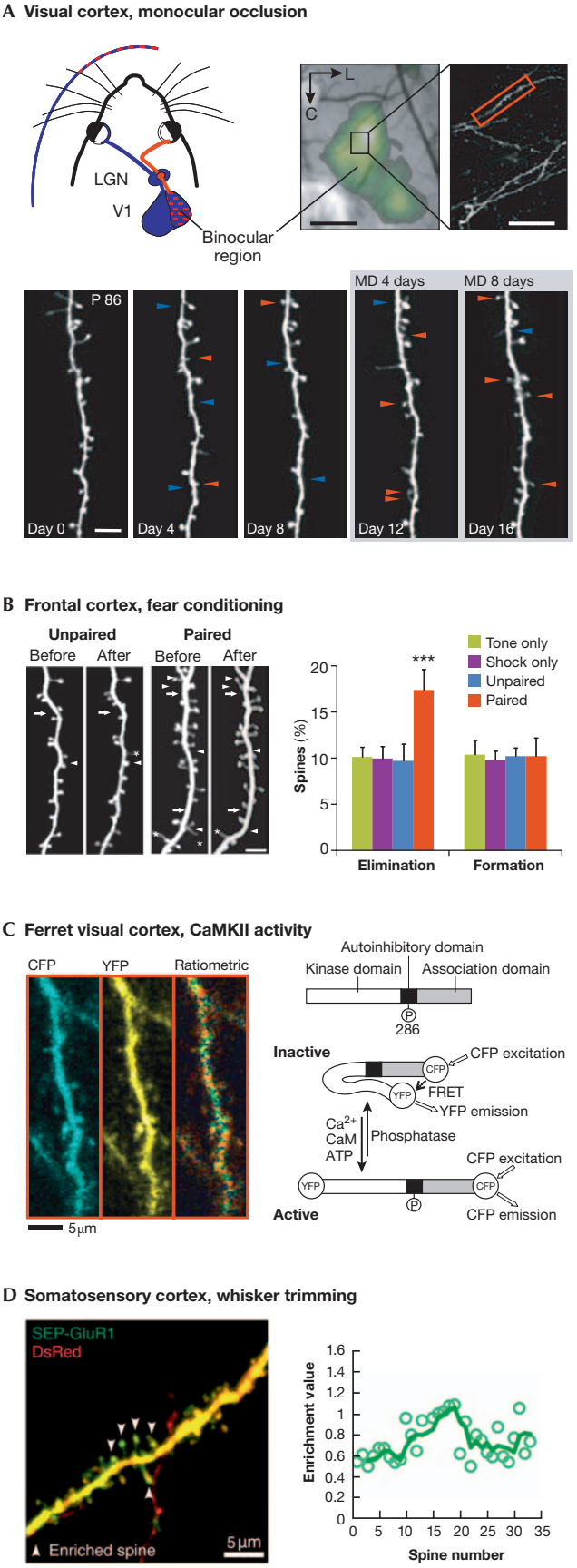
The initial calcium imaging experiments performed in spines [80,81] used a CCD camera to image fluorescence changes of the calcium dye Fura2 in slices. A few years later, the use of two-photon imaging greatly improved spine calcium imaging [25]. However, until just recently, imaging calcium signals in spines was largely restricted to studies performed *in vitro*, mainly because of the phototoxic damages produced by strong laser light *in vivo*. Development of the low-power temporal oversampling (LOTOS) procedure, which uses an acousto-optic deflector-based two-photon microscope [82], minimizes phototoxic damage and allows the imaging of prolonged periods of spine activity. Thus, multiple trials of stimulus-evoked calcium signals can be recorded in the same spiny dendrite (Fig 2C). In brief, the LOTOS-based procedure relies on the acquisition of images at high frame rates (e.g. 1,000 Hz), short pixel dwell-times (50 ns) and low intensities of the excitation laser beam [82]. Another study has shown that LOTOS can also be used in combination with a resonant galvoscanner-based two-photon imaging device [83].

Determining spine structure and function *in vivo*

Knowing how spines are structurally modified by experience, and how single spine synaptic inputs are distributed in dendrites, is crucial for determining how neurons integrate information and generate their output signals. Thus, clustered inputs might bind behaviourally relevant clustered synapses within individual dendrites promoting the generation of local dendritic spikes, as indicated by *in vitro* brain slice experiments (for reviews see [84–86]). Alternatively, inputs with similar features might be widely distributed over multiple dendrites and integrated linearly within the dendritic tree [3,87]. Although these two modes of synaptic input arrangement on dendrites are not necessarily mutually exclusive, a specific knowledge of the functional organization of the input patterns is essential for an understanding of the neuron-type-specific algorithms of input integration.

Activity-dependent spine remodelling. Chronic two-photon imaging has emerged as a powerful tool for the analysis of *in vivo* spine remodelling over time [88,89]. These structural changes have been monitored during postnatal development or in adulthood, and after

Fig 3 | Imaging activity-dependent spine structural changes *in vivo*. (**A**) Spine dynamics in apical dendrites of layer 5 neurons in binocular visual cortex after monocular deprivation in adult mice. Upper panel, schemata of the mouse visual system with intrinsic signal map of the binocular visual cortex (scale bar, 500 μm) and low-magnification image of an apical dendrite (scale bar, 50 μm). Lower panel, high-magnification view of the dendritic stretch shown above (red box), imaged *in vivo* every four days before and after monocular deprivation (MD). Arrows point to spines appearing (red) or disappearing (blue), compared with the previous imaging session. Scale bar, 5 μm. Reprinted by permission from Macmillan Publishers Ltd: *Nature* [94] © 2008. (**B**) Spine elimination after fear conditioning in layer 5 neurons of the mouse frontal association cortex. Left panel, representative *in vivo* images of dendrites before and after fear conditioning with foot shock paired or unpaired with tones. Arrows and arrowheads indicate spine formation and elimination, respectively. Asterisks mark filopodia. Scale bar, 4 μm. Percentage of spine elimination and formation 48 h after conditioning. Only the paired group showed an increase in freezing response and spine elimination. Reprinted by permission from Macmillan Publishers Ltd: *Nature* [109] © 2012. (**C**) *In vivo* imaging of CaMKII activity in layer 2/3 neurons of the ferret visual cortex. Schematic drawing of the conformations of the FRET-based probe for the detection of CaMKII activity (Camui), in the inactive and active form. Left panel, CFP and YFP channel images of a dendritic segment as well as a ratiometric image in intensity-modulated display mode, indicating the CFP–YFP ratio. Warm hue represents high CaMKII activity. Reprinted by permission from [122], © 2011 National Academy of Sciences, USA. (**D**) Left panel, example of clustered synaptic SEP–GluR1 (GluR1 tagged with a pH-sensitive form of green fluorescent protein, Super Ecliptic pHluorin) enrichment in a basal dendrite of a layer 2/3 pyramidal neuron in the somatosensory cortex of a whisker-intact mouse. Scale bar, 5 μm. Right panel, profile of SEP–GluR1 spine enrichment along the dendrite shown on the left. Neighbouring spines showed a significant positive correlation value that was significantly greater than that observed in whisker-trimmed animals. Reprinted by permission from [124], © 2011, with permission from Elsevier. CaMKII, Ca^{2+}/calmodulin-dependent kinase II; CFP, cyan fluorescent protein; FRET, Förster resonance energy transfer; GluR1, glutamate receptor 1; LGN, lateral geniculate nucleus; SEP, super ecliptic pHluorin;YFP, yellow fluorescent protein.



inducing plasticity through various paradigms such as sensory deprivation or motor skill learning. Chronic two-photon imaging has been used to study the development of spine motility in different cortical areas. It was shown that, at early postnatal ages, spines are highly plastic and spine turnover decreases with age [90,91]. In adults, the total spine number is globally stable over time due to comparable rates of spine formation and elimination [92–94]. However, spine remodelling occurs in the adult brain after induction of experience-dependent plasticity. As described below, with some examples, these activity-dependent structural changes in spines were investigated in primary sensory cortical areas, in the motor cortex and in higher cortical areas such as the frontal cortex, in both physiological and pathological conditions (Fig 3).

In the somatosensory cortex, chronic two-photon imaging was used to study structural changes of dendritic spines after whisker potentiation in layer 5 neurons [88,95]. The results show that both structural—stabilization of new spines—and functional—somatic activity—changes were most pronounced in layer 5 neurons located at the border between spared and deprived barrel columns [96]. In the visual cortex, the turnover of spines was studied in the binocular region after monocular deprivation [97]. In adult mice, a monocular deprivation episode of four days was found to double the rate of spine formation in apical dendrites of layer

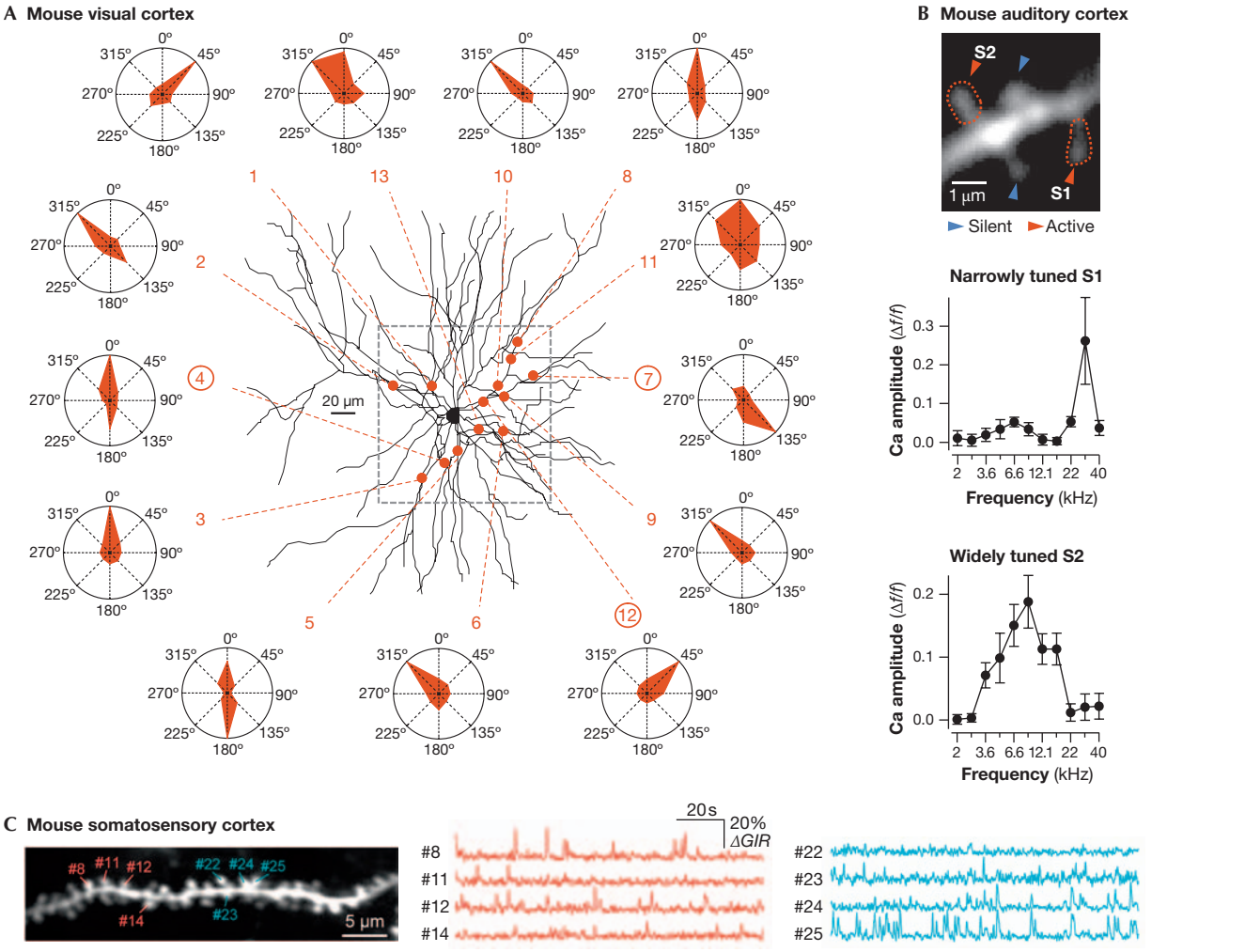


Fig 4 | Imaging spine activity *in vivo*. (**A**) *In vivo* two-photon calcium imaging of synaptic inputs evoked by visual stimulation in a layer 2/3 pyramidal neuron of the mouse visual cortex. Red dots indicate the location of each hotspot of local dendritic calcium signal, on the *Z*-projection of the reconstructed dendritic tree. Red dashed lines point to the polar plot obtained for the corresponding local dendritic calcium signal. The frame (grey dashed line) indicates the area of imaging. Reprinted by permission from Macmillan Publishers Ltd: *Nature* [131] © 2010. (**B**) *In vivo* two-photon calcium imaging of dendritic spines of a layer 2/3 neuron in the mouse auditory cortex, using the LOTOS procedure. Frequency tuning curves of the narrowly tuned spine S1 and of the widely tuned spine S2, shown in the two-photon image in the left panel. Error bars, s.e.m. Reprinted by permission from Macmillan Publishers Ltd: *Nature* [82] © 2011. (**C**) *In vivo* two-photon calcium imaging of dendritic spines using conventional two-photon imaging. Left panel, a stack image of dendrites of a layer 2/3 pyramidal cell in the mouse somatosensory cortex *in vivo*. Right panels, typical traces of spontaneous calcium activity from eight spines detected as functionally clustered and indicated in the left panel. Reprinted by permission from the American Association for the Advancement of Science (AAAS) [127]. LOTOS, low-power temporal oversampling.

5 pyramidal neurons. The resulting increase in spine density was specific to layer 5 cells located in the binocular cortex, in which most neurons increase their responsiveness to the non-deprived eye (Fig 3A; [94]). However, the relationship between structural and functional changes is not always clear. For example, although the output of the layer 2/3 binocular neurons was strongly modified by monocular deprivation [98–100], no structural modification of the spines could be identified in the apical dendrites of these neurons [94]. It could be that structural changes occur in deeper parts of the dendritic trees [101], or that different mechanisms of ocular dominance plasticity take place in the upper layers. Indeed, two studies strongly suggest that inhibition is important in this experience-dependent plasticity. They both used fluorescently tagged gephyrin to label inhibitory synapses in the mouse visual cortex *in vivo*. They found that a short period of monocular deprivation caused the pruning of a significant number of inhibitory synapses, mainly located on dendritic spines [102–104].

Structural changes associated with motor skill learning have been investigated in the motor cortex [105]. Morphological changes were monitored in spines of apical dendrites of layer 5 neurons in the contralateral motor cortex (Fig 2A). New spines were formed within one hour after initiation of a forelimb reaching task or within two days after rotarod running training [92,106]. Spine formation during initial learning was followed by enhanced spine elimination, leading to a total spine count that was the same as control levels [92,106]. In addition, it was shown that one-third of new dendritic spines emerge in clusters during the initial learning phase, and that most of these clusters are neighbouring spine pairs [107]. These findings

suggest that repetitive activation of cortical networks during learning induces clustering of new synapses along dendrites.

Spine dynamics have also been studied in higher order cortical areas. For example, spine dynamics were monitored in the forebrain nucleus HVC of zebra finches during song learning, which revealed that a higher level of spine turnover is correlated with a greater capacity for subsequent song imitation [108]. Another study using the mouse frontal association cortex investigated how spines of layer 5 pyramidal neurons are modified by fear learning and extinction (Fig 3B; [109]). Whereas fear conditioning increased the rate of spine elimination, fear extinction increases the rate of spine formation. In addition, extinction causes the formation of dendritic spines within a distance of 2 μm from spines eliminated after fear conditioning [109].

Finally, two-photon chronic imaging has been used to study synaptic functions in pathological conditions. The impact of strokes was assessed in the somatosensory cortex, revealing increased spine formation in the peri-infarct dendrites [110,111]. Spinal cord injury was shown to decrease spine density in the motor cortex [112], whereas retinal lesions induce massive remodelling of spines in the visual cortex [113]. Several studies have revealed spine loss in mouse models of Alzheimer disease, in hippocampal [114] and cortical pyramidal neurons [115,116]. Finally, a developmental delay in the downregulation of spine turnover and an overproduction of transient spines were revealed in a mouse model of fragile X syndrome [117,118].

To conclude, most studies so far have been performed in the apical tuft of layer 5 pyramidal neurons. Other types of plasticity might occur in other cell types. For example, it was found that a subset of inhibitory neurons carry dendritic spines that form glutamatergic synapses [119]. Given the spine changes observed in these inhibitory neurons after deprivation induced by retinal lesions, it seems that structural changes in inhibitory neurons might precede structural changes in excitatory circuitry. Thus, comprehensive studies of other neuronal cell types are essential, for a more complete understanding of synaptic plasticity, at the level of local networks. Otherwise, despite the correlations between spine dynamics and experience-dependent changes, the causality of this relationship remains unclear.

Molecular mechanisms of synaptic function in vivo. To gain functional insight into experience-dependent synaptic changes, new probes and markers are being developed in rapid sequence to image the expression of proteins known to be involved in synaptic plasticity. For example, a FRET-based CaMKII sensor [44,120] and a FRET reporter of Ras GTPase activation [121] were used for LTP studies in hippocampal cultured slices. For *in vivo* studies, a genetically engineered FRET probe for the detection of CaMKII activity was used to monitor CaMKII activity in single synapses of layer II/III neurons in the ferret visual cortex (Fig 3C). The results suggest that spines lost after monocular deprivation have a low basal concentration of CaMKII, whereas spines that are preserved show increased activation of CaMKII [122,123]. An elegant optical approach was also used for the *in vivo* study of the dynamics of AMPA receptor trafficking, after inducing synaptic plasticity through sensory experience or deprivation (Fig 3D; [124]). This approach revealed that experience-driven GluR1 incorporation into synapses is clustered on portions of dendrites, and that such clusters are eliminated when mice are deprived of sensory experience. By contrast, the incorporation of synaptic GluR2, through sensory deprivation, occurred in a distributed manner with little evidence for clustering [124,125].

In parallel to the development of molecular target-specific fluorescent probes, an increasing number of transgenic mice are available to monitor the expression or localization of synaptic components. For example, adult transgenic mice in which GFP–GluR1 is expressed under control of the c-fos promoter have been used to probe the insertion of newly synthesized AMPA receptors after fear conditioning [126] and behavioural exploration *in vivo* [127]. The results indicate that glutamate receptors are preferentially inserted into neighbouring spines [127].

Functional neuroanatomy in vivo *with calcium imaging. In vivo* two-photon calcium imaging was first used to map functional inputs in dendrites in invertebrates. In the cricket cercal system, simultaneous pre- and postsynaptic calcium imaging revealed different topographical organizations of sensory inputs into interneuron dendrites, underlying different computation processes of these inputs [128]. Further studies in the visual systems of locusts [129] and *Xenopus* tadpoles [130] indicated that the topographical organization of sensory inputs on dendrites has an influence on the integration of visual information. Similar approaches were implemented for the functional analysis of dendrites in the mammalian brain. Studies performed in the mouse visual and somatosensory cortices identified dendritic NMDA-dependent calcium hotspots driven by visual (Fig 4A; [131,132]) or whisker stimulation [83] in layer 2/3 neurons. A common conclusion for both sensory modalities is that sensory input coding for the same feature is heterogeneously distributed throughout the entire dendritic tree, with no evidence for clustering on individual dendrites.

Recently, *in vivo* calcium imaging in single spines in the intact mouse brain became feasible. The first report of this approach involved the LOTOS two-photon imaging procedure [82] to detect sound-evoked responses in single spines in the auditory cortex (Fig 4B). Similarly to the dendritic 'hotspots' mentioned above, these results demonstrated a widespread distribution of afferent sensory inputs throughout the dendritic tree of a given neuron [82]. Although in a few instances spines with similar frequency tuning were locally clustered, most other similarly tuned spines were located on remote sites throughout both apical and basal dendrites. Another study showed that, at least when recording spontaneous activity, conventional two-photon imaging can also be used for *in vivo* spine calcium imaging [127]. The results provide evidence for local clustering of synaptic inputs in the dendrites of layer 2/3 pyramidal neurons in the mouse somatosensory cortex (Fig 4C).

Finally, it should be remembered that synaptically evoked spine calcium signals have been detected unambiguously only in the absence of the back-propagation of action action potentials, or in neurons that had low resting membrane potentials and were not firing [82], or that were hyperpolarized [83,131], or in the presence of intracellular sodium channel blockers [127]. In this last study, the neurons were additionally voltage-clamped at –30–0 mV. These recording conditions might have influenced the probability of occurrence of the spine calcium events that were detected. Thus, improved methods of spine activity detection might reveal synaptic input sites in conditions of spiking activity, which might help to increase our understanding of the mechanisms by which specific inputs drive the output signals.

Sidebar A | In need of answers

(i) In spite of the strong evidence for distributed inputs, how relevant are clustered inputs *in vivo* for neuronal function?
(ii) What is the functional circuit map of the presynaptic neurons contacting dendritic spines?
(iii) How are spine signals integrated by dendrites?
(iv) What is the relation between structural and functional spine plasticity *in vivo*?
(v) What is the molecular machinery underlying spine function and plasticity *in vivo*?

Conclusions

The insights into dendritic spine structure and function, resulting from the use of modern imaging techniques, have significantly increased our knowledge of synaptic function, ranging from a better understanding of the molecular mechanisms of experience-dependent plasticity to the dendritic organization of sensory inputs in the intact brain *in vivo*. As we have described, it is the technical developments that have driven forward studies of spine function, involving both new imaging technology—for example, two-photon microscopy, STED and LOTOS—and the development of a large variety of new fluorescent sensors. The main challenges that remain include questions related to spine function during the formation of circuits *in vivo* (Sidebar A), the mechanisms of single spine-dependent experience plasticity in conditions of behaviourally relevant learning processes and the changes that occur in pathophysiological conditions, such as stroke and Alzheimer disease.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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