GENESIS OF DENDRITIC SPINES: INSIGHTS FROM ULTRASTRUCTURAL AND IMAGING STUDIES

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Dendritic spines are small protrusions from many types of neuron, which receive most of the excitatory inputs to the cell. Spines are thought to have important roles in neural information processing and plasticity, yet we still have a poor understanding of how they emerge during development. Here, we review the developmental generation of dendritic spines, covering recent live imaging experiments and older ultrastructural data. We address the potential role of dendritic filopodia in spine development and recent findings of spinogenesis in adult animals, and conclude by discussing three potential models of spinogenesis.

PURKINJE CELLS
Inhibitory neurons in the cerebellum that use GABA (γ-aminobutyric acid) as their neurotransmitter. Their cell bodies are situated beneath the molecular layer, and their dendrites branch extensively in this layer. Their axons project into the underlying white matter, and they provide the only output from the cerebellar cortex.

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It is more than a hundred years since dendritic spines were first described[1], yet their function is still unclear. As spines mediate most excitatory connections in the central nervous system (CNS), they must be key elements in neuronal circuitry, and speculation about their function has encompassed roles as connecting devices as well as biochemical or electrical compartments[2–7]. Imaging experiments have shown that spines are calcium compartments[8], although calcium compartmentalization can also occur without any morphological specialization[9,10]. Also, the electrical function of the spine has never been directly examined.

How, and under what circumstances, do spines originate during development? Answering this question will not only provide insight into the ontogeny of neural circuitry, but could also shed light on the role of spines in the adult nervous system. In this article, we review evidence from the examination of spine development in several systems, and discuss the features of spinogenesis that are intrinsic to the neuron and the factors that are extrinsic and activity dependent. We start by reviewing data from cerebellar PURKINJE CELLS, as many key issues have been directly addressed in the cerebellum. In fact, Purkinje cells exemplify two different plans of spine development: an intrinsic, 'hardwired' plan and an extrinsic, 'plastic', activity-dependent plan. We then review the development of spines in PYRAMIDAL NEURONS in the neocortex and hippocampus. We discuss the potential role of dendritic FILOPODIA in spinogenesis and some controversial data on spinogenesis in the adult neocortex, and conclude by discussing general models for spinogenesis and their relationships to synaptogenesis.

As most spines are thought to serve as recipients of synaptic inputs[11], synaptogenesis must be related to spinogenesis. However, for several reasons, we prefer to treat spinogenesis as a separate topic. First, there is evidence that the complete developmental programme that leads to the formation of mature spines can occur in the absence of axon terminals. Second, in some systems, synaptogenesis is delayed developmentally with respect to spinogenesis, whereas in others the converse is true. Third, synaptogenesis, at least as understood traditionally, is thought to be a protracted phenomenon, taking days or even weeks to complete, whereas spines can arise in minutes. Last, synaptogenesis is a broad topic that certainly merits its own review, and we recommend previously published articles[12–18] to interested readers.

Our review will not cover the molecular cascades that are associated with spinogenesis or spines in general. We prefer to concentrate on the discussion of phenomenology and models, as we feel that a clear-headed phenomenological understanding is convenient before the detailed molecular analysis is carried out. Indeed, we hope that our integration of several decades

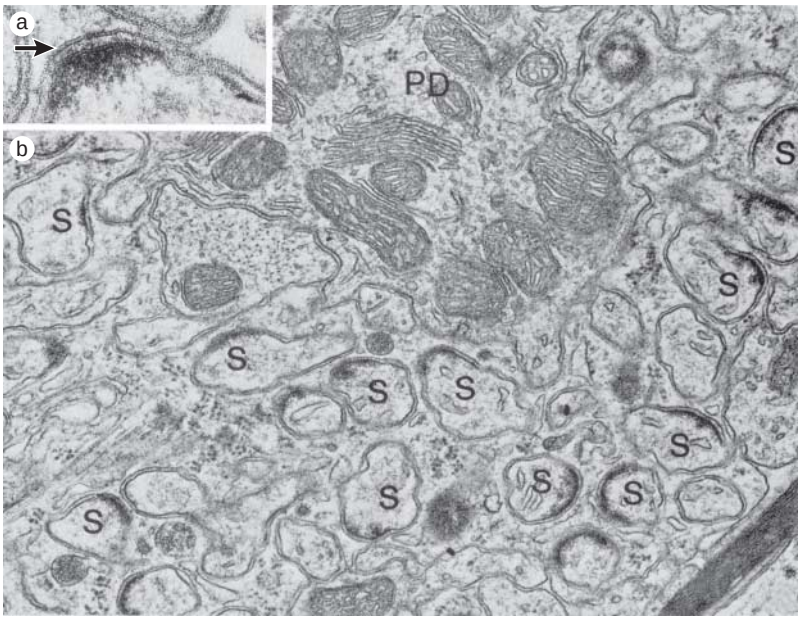

Figure 1 | **Dendritic spines in Purkinje cells without presynaptic input. a** | High power electron micrograph of a 'naked' spine in a Purkinje cell dendrite. Note the postsynaptic differentiation and the extracellular cleft (arrow). **b** | Thick Purkinje cell dendritic profile (PD) surrounded by numerous free spines (S) with normal-looking postsynaptic differentiations. Reproduced, with permission, from REF. 25 © (1975) Elsevier Science.

of descriptive work will be useful to researchers who are interested in more mechanistic aspects. For the molecular aspects of spinogenesis and synaptogenesis, we refer the reader to other recent reviews[19,20].

Spinogenesis in Purkinje cells

Parallel fibre spines: the Sotelo model. Besides having a stereotypical circuit, the mammalian cerebellum has a key advantage for developmental studies — it is not necessary for life. Surgical removal of the entire cerebellum produces motor symptoms without affecting any vital functions[21], so many natural mutations that affect the development of the cerebellum can be isolated. Moreover, cerebellar mutants can be easily identified by their clear phenotypic traits, which involve noticeable deficits in motor coordination. These advantages have been exploited for the study of spinogenesis, and have allowed the detailed analysis of many mutations that affect cerebellar neurons at different developmental stages (reviewed in REF. 22). These studies have helped us to address the issue of how spines first emerge, and show that they can emerge irrespective of the presence of axonal terminals.

Studies in which Purkinje cells develop in the absence of granule cell fibres have provided three lines of evidence to indicate that spinogenesis can be intrinsic to the neuron[22] (FIG. 1). The first comes from *WEAVER* MUTANT MICE. In *weaver* mutants, granule cells — the presynaptic partners of around 90% of Purkinje cell spines — are absent[23]. In these animals, Purkinje cells develop abnormal and atrophic dendrites, which are nevertheless covered with spines. These spines seem to be quite normal, even to the point of having normal postsynaptic specializations[23–26]. A second line of evidence comes from *REELER* MUTANT MICE[27,28], in which the migration of neuronal precursors in the cerebellum is grossly perturbed. In many of these animals, a central region of the cerebellar cortex has a large number of ectopic Purkinje cells with no granule cells. Again, these Purkinje cells develop morphologically normal spines in the absence of their presynaptic partner[29]. Finally, because of the delayed development of granule cells with respect to Purkinje cells, X-irradiation of neonatal rats can selectively ablate granule cells, in some cases in great numbers[30]. In these animals, Purkinje cells develop morphologically normal dendritic spines at roughly normal densities[31].

The normal developmental sequence of events in the cerebellar cortex is also consistent with these observations. According to Larramendi[32], spines in the distal dendritic branches of Purkinje cells develop before they establish synaptic contacts with PARALLEL FIBRES. Also, at early postnatal stages (P0–P12), 'naked' spines without terminals can be identified[33]. However, given the resilience of spines to loss of parallel fibres[23], the possibility that these spines have lost a terminal cannot be completely ruled out.

So, evidence from both the normal and abnormally developed cerebellum indicates that the initial formation of parallel fibre spines on Purkinje cells does not depend on presynaptic axons[34]. In this model, which was proposed by Sotelo[34], spine formation seems to be, at least in some cases, an intrinsic, perhaps even cell-autonomous, property of the neuron. Somehow, each Purkinje cell 'knows' how to build a parallel fibre spine and probably also intrinsically controls the density of spines. In agreement with this, Purkinje cells in electric fish arrange their spines in a spiral along the dendrite (J. O'Brien and N. Unwin, personal communication).

However, closer scrutiny could still reveal differences between innervated and uninnervated spines. Certainly, it seems unlikely that the postsynaptic specialization is completely normal, considering the importance of reciprocal pre- and postsynaptic interactions to synaptogenesis in the neuromuscular junction (NMJ)[35]. Indeed, the development of Purkinje cells in culture is aided by the addition of glial and granule cells[36].

Climbing fibres: activity and spine maintenance. More than one hundred years ago, it was suggested that changes in spines could underlie learning[37–39], and this idea is still one of the main threads in contemporary research on spines[40]. The first observation of spine plasticity was made by Ramón y Cajal. In a seminal observation, he noted that the spine density in pyramidal neurons was higher in early postnatal development than at later stages[39], and he inferred that the circuit must somehow rearrange itself by losing connections. More recently, this initial proliferation of synapses located in spines, followed by a decline in their number, has been confirmed[41,42].

What controls this 'pruning' of spines and their associated synapses? We argue that spine development has two phases: an initial period of spine proliferation, which is probably intrinsic to the neuron, followed by a later decline, which depends on the activity of the synapse and the neuron. To shed light on the second phase, we will

PYRAMIDAL NEURONS
A class of neuron in the cerebral cortex with a pyramid-shaped cell body. These neurons send long axons down the spinal cord and form dendrites that extend laterally through the cortical layer that contains the cell body.

FILOPODIA
Long, thin protrusions that are present at the periphery of migrating cells and growth cones. They are composed largely of F-actin bundles.

WEAVER MUTANT MICE
This mouse strain is characterized by cerebellar abnormalities and ataxia, which are associated with a mutation in an inwardly rectifying potassium channel.

REELER MUTANT MICE
A mouse strain that is characterized by tremors, dystonia and ataxia. These phenotypes are associated with a deficiency in the production of the reelin protein.

Box 1 | Why are there two types of spines in Purkinje cells?

In Purkinje cells, the generation of spines that receive parallel fibre or climbing fibre input seems to be controlled differently. These two spine types also seem to mediate opposite functions: parallel fibres connect with as many Purkinje cells as possible, whereas climbing fibre axons connect with only one. This difference in circuit function might underlie the two different developmental strategies of spines. The intracellular machinery of parallel fibres might build terminals at specific distances along the axon to make connections with the Purkinje cells. The narrow dendritic tree of the Purkinje cells and the orthogonal orientation of the fibres would make it almost impossible for a parallel fibre to contact a given Purkinje cell more than once. Equally, the Purkinje cells might simply produce as many spines as possible to fill these orthogonal surfaces. It is conceivable that the easiest way to achieve this would be using some cell-autonomous, space-filling developmental algorithm[106].

In the proximal compartment, climbing fibres would initially be strongly attracted to Purkinje cells, avoiding other targets like Golgi cells. In a later phase, to ensure that only one climbing fibre contacts each Purkinje cell, activity-based competition could occur, with the result that the 'winning' climbing fibre inhibits spinogenesis in the 'captured' Purkinje cell. This could prevent other climbing fibres (or other axons) from regaining this territory. As in the neuromuscular junction (NMJ)[35,91], winner-take-all algorithms might be the most efficient way of achieving a precise one-to-one match. However, in contrast to the NMJ, some additional rules must also operate in the cerebellum to prevent a climbing fibre capturing more than one target. Perhaps the limiting number of neurotransmitter vesicles that a single inferior olivary axon can sustain or shuttle limits climbing fibres to winning only one 'battle'.

continue our review of the cerebellar system, this time concentrating on spines in the proximal region of the Purkinje cell tree, which are mainly contacted by CLIMBING FIBRES. One of the clearest examples of morphological plasticity in spines and their afferents comes from this second population of spines. Initially, Purkinje cells are innervated by a 'nest' of climbing fibres that contact the soma[43]. These contacts are made on finger-like perisomatic spines[32,44], which cover the soma at this stage and are electrophysiologically functional[45,46]. Later in development, climbing fibres translocate to 'thorns' — large spines located on the proximal dendrites[47]. Shortly afterwards, all but one of the fibres are eliminated, so that each Purkinje cell is innervated by a single climbing fibre[32,43,45]. The process of eliminating perisomatic spines, supernumerary climbing fibres and thorns is thought to be activity dependent, and to involve competition between different afferents, as a similar process occurs during the formation of the NMJ. The molecular mechanisms that underlie this fascinating phenomenon are still unclear.

The activity of climbing fibres and, interestingly, of parallel fibres, seems to regulate spine formation in the proximal region of the Purkinje cell. Evidence for the involvement of climbing fibres comes from two sets of *in vivo* experiments. In an early study, it was found that lesions of the inferior olive (the nucleus that gives rises to the climbing fibre projection) led to the emergence of supernumerary spines in the proximal dendrites of Purkinje cells[48]. This indicated that climbing fibres repress spinogenesis, presumably through the release of neurotransmitters or other factors. A recent experiment, using TETRODOTOXIN (TTX) infusions *in vivo* with minipumps — a manipulation that blocks both pre- and postsynaptic activity — confirmed that the regulation of Purkinje cell spine numbers by climbing fibres depends on neuronal activity[49]. When all neuronal activity was blocked by TTX during development, the spine density in the proximal dendrites of the Purkinje cells reversibly increased by an order of magnitude. In spite of the global effect of TTX on activity, the spine density in the distal dendritic tree, where granule cells make their contacts, was unaffected by TTX perfusion. These data show that neuronal activity is involved in the suppression of spine formation in the proximal region of the dendritic tree, but not in the distal spiny branches. As inferior olive lesions result in similar increases in proximal spines, it seems probable that climbing fibre activity directly or indirectly suppresses spine formation. However, it is also possible that the activity of the Purkinje cells themselves, either spontaneous or evoked by climbing or parallel fibres, is crucial. These possibilities are not mutually exclusive. Indeed, data from *weaver* and *reeler* mice, in which Purkinje cells are deprived of granule cell inputs but innervation by multiple climbing fibres persists[50], indicate that granule cell inputs can influence the fate of the more proximal climbing fibre spines.

Data from two populations of spines in Purkinje cells exemplify two different developmental paradigms (see also BOX 1). Spines on the distal spiny branchlets are intrinsically generated and maintained regardless of the activity of the terminal and the neuron, whereas spines on the proximal dendrites seem to be generated endogenously but are repressed by the activity of climbing fibres. These two types of spine define two developmentally, morphologically and functionally different compartments: the proximal dendrites, which are innervated by climbing fibres, and the distal spiny branches, which receive parallel fibre input[34]. Evidence from another mutant, the *STAGGERER* MOUSE[26], indicates that this compartmental distinction is not academic but is actually recognized by the neuron. In *staggerer* animals, the formation of spiny branchlets is selectively impaired, but proximal dendrites seem to develop normal synapses with climbing fibres, indicating that the *staggerer* gene has a specific effect in the distal compartment.

Spinogenesis in pyramidal neurons

Do these data from Purkinje cells apply to other spiny neurons? We will now focus on the pyramidal neurons in the neocortex and hippocampus. In cortical circuits, it is difficult to disrupt presynaptic axons specifically without affecting postsynaptic cells, as the manipulations that are used fundamentally disrupt brain function. However, transgenic and knockout animals are becoming increasingly useful for disentangling the roles of pre- and postsynaptic structures[51].

Terminals induce spine formation: the Miller/Peters model. In many, but not all, species, spine formation in pyramidal neurons occurs after birth. In rat neocortical pyramidal neurons, spinogenesis begins quite precisely in the middle of postnatal week 1 (REF. 52). Spine density then increases continually during the next four weeks and is subsequently reduced with increasing age[52], reflecting an initial overproduction and later elimination of synapses during early cortical development[39,42].

PARALLEL FIBRES
The axons of cerebellar granule cells. Parallel fibres emerge from the molecular layer of the cerebellar cortex towards the periphery, where they extend branches perpendicular to the main axis of Purkinje neurons and form '*en passant*' synapses with this cell type.

CLIMBING FIBRES
Cerebellar afferents that arise from the inferior olivary nucleus, each of which forms multiple synapses with a single Purkinje cell.

TETRODOTOXIN
(TTX). A potent marine neurotoxin that blocks voltage-gated sodium channels. TTX was originally isolated from the *Tetraodon* pufferfish, and contains a positively charged guanidinium group and a pyrimidine ring.

STAGGERER MOUSE
A mouse strain that has a deletion in the gene that codes for the nuclear hormone receptor RORα. The homozygous mutant mouse shows ataxia, which is associated with atrophy of the cerebellum and loss of Purkinje cells. The heterozygous mutant also shows an age-related loss of Purkinje cells, but seems to be phenotypically normal.

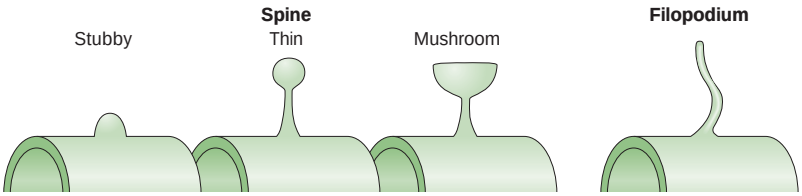

Figure 2 | **Examples of different spine morphologies.** Schematic drawing of spine morphologies, in categories as described in REF. 2.

During the initial stages of spinogenesis, axonogenesis is proceeding rapidly. Axons from pyramidal neurons, which contact most of the spines in the neocortex, develop a short time before dendrites and spines, and axon extension occurs even before neuronal migration is complete[52]. In the rat neocortex, synapse formation does not become extensive until the postnatal period, and it occurs a short time before spine development. In the adult neocortex, all spines are thought to have synapses, with at least one presynaptic terminal impinging on each[11]. Naked spines have only been reported occasionally[53] and could represent an abandoned synaptic site[54], although we still lack systematic studies on their existence.

In addition to a change in density, spines from pyramidal neurons undergo profound morphological rearrangements during postnatal development[52]. Traditionally, spines are classified morphologically as stubby, thin or mushroom[2] (FIG. 2). Early in development, stubby spines (lacking clear necks) are common. In the adult, thin or mushroom spines, which have more prominent necks and heads, are more common[55], although many stubby spines are still present in the adult mouse and human cortex[56].

The development of spines in pyramidal cells could be related to synaptogenesis. Synapses on dendritic shafts predominate in pyramidal cells at early developmental stages[54,57–62], so the traditional hypothesis proposes that the spines of pyramidal neurons arise from shaft synapses by a process of outgrowth. Specifically, on the basis of their data from rat visual cortex, Miller and Peters proposed a three-stage model for spinogenesis in pyramidal neurons[55], and similar 'axonal induction' models have been proposed by Hamori[63], Lund[54] and Braitenberg[11]. First, synapses are made on the dendritic shafts, and immature spines can be recognized by their flocculent material. Most of these spines are 'stubbies' (FIG. 2). In the second stage, the presynaptic region of the axon shows a swelling as synaptic vesicles accumulate. In the third stage, many spines are thin or mushroom-shaped, with a lollipop shape and a clear neck, and axonal terminals have well developed varicosities. Similar data on the development of synapses have been obtained in the mouse cortex — at P12, most synapses occur on dendritic shafts, whereas at P21, most synapses occur on spines[11]. So, the idea is that when a spine emerges, it takes a pre-existing shaft synapse and carries it along as it extends away from the dendritic shaft (FIG. 3). Therefore, a spine is induced through the effect of the terminal on the dendritic shaft.

This hypothesis is different from the Sotelo model, which proposes that the terminal has a minor role in spinogenesis. It is possible that Purkinje cells and pyramidal neurons use completely different spinogenesis strategies, as they are very different cell types with seemingly different circuit functions (for example, excitatory versus inhibitory). At the same time, although there is consensus regarding the time course of events in pyramidal neurons, we feel that the available data on pyramidal spinogenesis are circumstantial and that the Miller/Peters hypothesis needs to be properly demonstrated. For example, direct observation of the time course of generation of individual spines has not been achieved, and the available data rely on comparisons across cells and animals of different ages. Specifically, what is missing from the literature is a live imaging experiment that documents how spines emerge during development. Mature spines probably do not emerge *de novo*, but result from incremental growth of intermediate structures. Imaging experiments should confirm that spines emerge through a serial and protracted process. Moreover, the key aspect of the Miller/Peters hypothesis that needs to be directly demonstrated is the role of presynaptic terminals as inducers of spines in dendritic shafts. It is possible that, as in Purkinje cells, pyramidal spines arise intrinsically without significant influence from axonal terminals, and would subsequently be innervated by the terminals. Another alternative to the Miller/Peters model is that spines arise through transformation of existing filopodial precursors[64] (see later text).

A final objection to the Miller/Peters model arises from the fact that axonal trajectories through the NEUROPIL are largely straight, and most axonal contacts are made *en passant*[65]. If axonal terminals were contacting dendritic shafts and 'pulling out' spines, we would expect axons to have convoluted trajectories, at least during early development, and this has not been reported. However, it is possible that the neuropil is more compact early in development, and that subsequent interstitial growth of the neuropil is responsible for pulling spines away from the shaft synapses, so as not to alter the straight trajectories of axons. We should also mention that the earliest synapses on dendritic shafts constitute a small proportion of the total number of synapses in the adult neuron, and they might not be a representative sample of the final population.

Spontaneous and evoked appearance of spines have been reported in recent imaging experiments in cultures[64], slices[66–68] and even in anaesthetized animals[69,70] showing that the generation of new spines is possible (FIG. 4). However, none of these studies have specifically focused on characterizing normal developmental spinogenesis or have carefully examined the role of the presynaptic terminal. Overall, we conclude that, although current evidence is consistent with the Miller/Peters hypothesis, alternative models of spinogenesis that do not require synaptic terminals or shaft synapses have not been ruled out, and a more critical examination of this question is needed.

NEUROPIL
A felt-like network that is interspersed between the cells of the grey matter in the central nervous system. It consists of neuronal and glial processes and synaptic terminals.

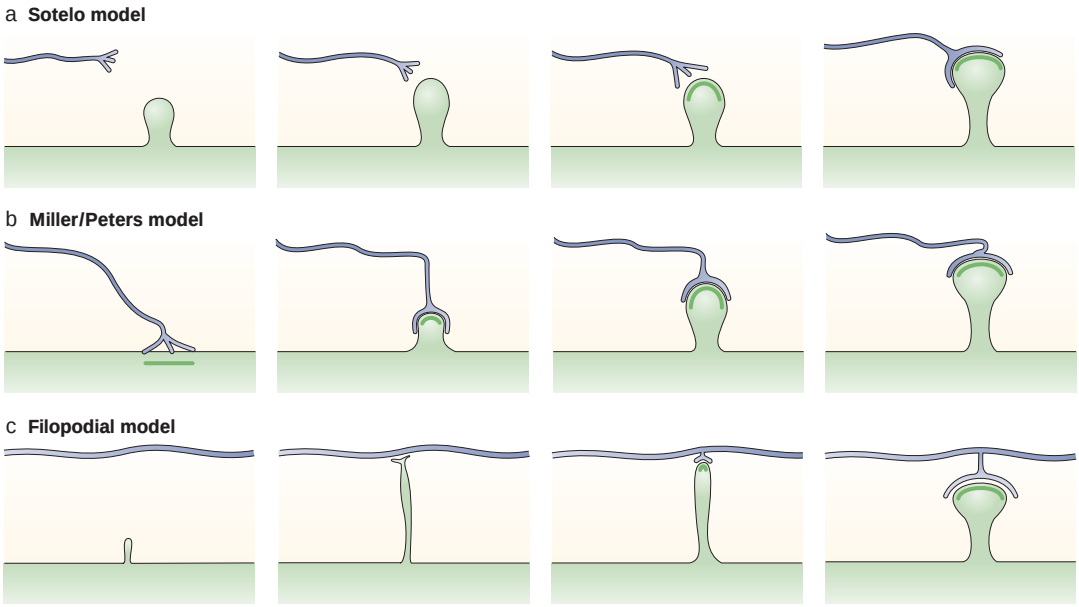

Figure 3 | **Three models for spinogenesis.** This diagram illustrates the essential features of the three models of spinogenesis. In the Sotelo model (**a**), spines emerge independently of the axonal terminal. In the Miller/Peters model (**b**), the terminal actually induces the formation of the spine. Finally, in the filopodial model (**c**), a dendritic filopodium captures an axonal terminal and becomes a spine.

Neuronal activity and pyramidal spine development. What is the influence of neuronal activity on the development of spines from pyramidal neurons? Do pyramidal neuron spines behave like parallel fibre spines or like climbing fibre spines from Purkinje cells? We will first focus on the role of sensory activity, and we will then discuss a potential role for spontaneous activity.

Comparative developmental studies have provided some interesting insights into the role of sensory activity. Some species, such as rats and mice, are born with relatively immature brains (altricial), whereas others are born with more developed brains (precocious). In the guinea-pig, spinogenesis has already occurred by birth, and these animals are born with an essentially mature complement of spines and synapses[71]. This simple fact has an important implication — that in some species at least, spinogenesis and even synaptogenesis occur in the absence of environmental influences[11]. Even in rats and mice, a large proportion of spinogenesis and synaptogenesis in the primary visual cortex occurs before eye opening[52], and the only morphological event that seems to correlate with eye opening is the elongation of the spine neck[55]. Moreover, between different individuals of the same species, there is a less than one-day offset in the overall time course of spinogenesis and synaptogenesis[14]. These observations indicate that many aspects of this programme are genetically determined, and that sensory evoked activity is not essential for ontogenetic spinogenesis in pyramidal neurons.

However, there is increasing evidence that the developing brain *in utero* has robust patterns of spontaneous activity, which are potentially important for circuit rearrangements[72–74]. So, it remains possible that spontaneous activity is necessary for normal spinogenesis in pyramidal neurons. An ideal experiment to test the role of spontaneous activity in spinogenesis would be to block all activity during uterine development. This has recently been achieved in *munc18*-knockout mice[51]. Munc18 is necessary for transmitter release throughout the CNS and peripheral nervous system (PNS), but surprisingly, mice that lack the protein are reported to have relatively normal neocortical synapses and circuits at birth. Unfortunately, these mice die shortly after birth, before spinogenesis has taken place. Interestingly, there is massive apoptosis in many regions of the nervous system (although not in the cortex), implying that neurotransmitter release might be necessary for neuronal survival throughout the CNS. Further analysis of these mice — for instance, by culturing tissue from newborn mice — or other transmitter-release-deficient mice that survive into the period of spinogenesis is likely to be pertinent to the understanding of the role of activity in spine formation.

Neuronal activity and pyramidal spine maintenance. The role of activity in the maintenance of connections has been investigated extensively[14], and we will only touch on this issue as it relates to spinogenesis. As we have already stated, spine density (and synapse density) seems to follow a stereotypical developmental pathway, with initial overproduction followed by a reduction to a plateau level that persists through adulthood[14,15,39,42]. There is ample evidence that, after the overproduction stage, spine density can be affected by sensory deprivation or by experimental paradigms that modify synaptic activity[40,67,75–78]. Spine density can increase as well as decline, implying that spinogenesis can also occur at later stages in development.

Another interesting insight has come from recent molecular studies, which indicate that the Rho family of small GTPases is an important potential contributor to

spinogenesis and spine loss. Overexpression or suppression of these proteins results in the creation of new spines *in vivo* and *in vitro*[79–82]. Such spines can not only be produced in developing neurons, but also in cells with mature phenotypes[82], and spine density, size and length are controlled by different members of the Rho family[82]. These 'designer' spines illustrate that even mature pyramidal neurons have the entire molecular complement that is necessary for extensive spinogenesis.

It is also worth mentioning that spines do not only appear and disappear — their basic morphology also seems to change continuously[83,84], sometimes in an activity-dependent manner[85]. Although these topics are of great importance for understanding spine function, we prefer to restrict the focus of this review to true spinogenesis, and refer the reader to our previous reviews for a proper treatment of this subject matter[40,85,86].

Do pyramidal neurons have two types of spines? On the basis of the comparison with Purkinje cells in which spines from parallel and climbing fibres behave differently, it is interesting to consider whether spines in pyramidal neurons also fall into different categories. Most excitatory connections on pyramidal cells seem to be relatively weak functionally, and are implemented by only a few spines[87–89]. In some ways, this is similar to the case for parallel fibres, and one might propose that spines of pyramidal cells are also 'cell autonomous'. At the same time, some excitatory connections onto pyramidal neurons — such as synapses between mossy fibres and CA3 pyramidal cells in the hippocampus[90] — are strong, so much so that a single presynaptic fibre can fire the postsynaptic neuron. This might resemble the case of the climbing fibre projection more closely. Knowing more about the activity dependence of these two types of spines could reveal whether there is computational logic behind these different spinogenesis paradigms.

Spine stability and spinogenesis in mature animals

The stability of neural circuits in adult nervous systems is an issue of central importance for understanding brain function. It is still not known whether the neuronal circuitry is constantly undergoing rewiring or whether, once developed, it constitutes a relatively stable network. Technical limitations have made it difficult to address this question in the CNS, although significant progress has been made by studying the NMJ. Lichtman and colleagues have shown that in the adult, NMJs are relatively stable over many months[35,91], whereas in young animals, the termination patterns of axons on the muscle fibres undergo substantial and constant remodelling[92].

Purves and colleagues observed substantial dendritic remodelling of neuronal synapses in the PNS, which was thought to reflect, at least in part, the rearrangement of synaptic connections[93,94]. However, these studies were performed in relatively young mice. Recently, Lichtman and colleagues extended these studies to adult mice, and have specifically addressed the change in the rate of synaptic rearrangements over time[95]. The authors used transgenic mice with sparsely fluorescently labelled axons to show that synaptic terminals in the submandibular ganglion undergo constant rearrangement during the first few weeks of life, whereas the rate of synaptic change is considerably decreased in older mice.

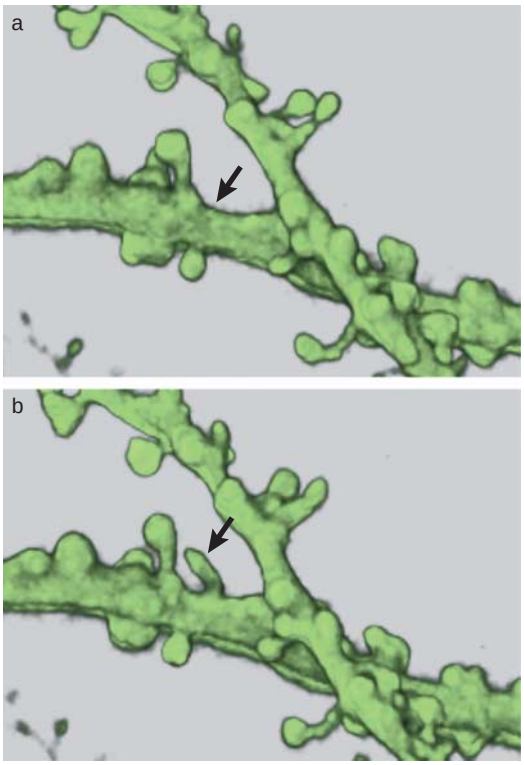

Figure 4 | **Spinogenesis in pyramidal cells.** Images were obtained from CA1 cells in an organotypic hippocampal slice culture using a two-photon microscope. Images were taken at 5 min intervals approximately 60 min after induction of synaptic potentiation. Note the new spine that has emerged in the position marked with an arrowhead. Similar data have been obtained by Engert and Bonhoeffer[67] for spines and Maletic-Savatic *et al.*[77] for filopodia. Images courtesy of V. Nägerl.

Although these pioneer experiments provide a good starting point for understanding the dynamics of spine generation and retraction in the CNS, they have addressed synaptic stability rather than the stability or generation of spines. Until the advent of TWO-PHOTON LASER MICROSCOPY[96], it was practically impossible to image spines repeatedly in living animals. However, two recent studies have overcome this technical obstacle and have investigated the generation (and disappearance) of spines in the cerebral cortex *in vivo*.

Both of these studies[69,70] used *in vivo* two-photon microscopy in adult mice that were transgenically engineered to express fluorescent proteins in a subset of their neurons. Unfortunately, the two studies led to different conclusions, and there are no obvious explanations for this discrepancy. Grutzendler *et al.*[69] addressed the simple yet crucial question of spine stability in the developing and adult visual cortex. They observed that in the adult, the half-life of a spine is approximately 13 months. This implies that even in an old mouse of 24 months of age, approximately 30% of the spines would have been present for the entire adult life of the mouse.

TWO-PHOTON LASER MICROSCOPY
A form of microscopy in which a fluorochrome that would normally be excited by a single photon is stimulated quasi-simultaneously by two photons of lower energy. Under these conditions, fluorescence increases as a function of the square of the light intensity, and decreases as the fourth power of the distance from the focus. Because of this behaviour, only fluorochrome molecules near the plane of focus are excited, greatly reducing light scattering and photodamage of the sample.

The authors also reported that during adolescence, the turnover rate is considerably higher. However, even during this phase, approximately 70% of the spines remain stable for one month or longer. Although the authors showed clear modulation of spinogenesis during the critical period, and that new spines can be generated in the neocortex of young and adult mice, the main message from this work is that spines are remarkably stable throughout life, including the critical period. However, this stability only relates to the *de novo* generation (or loss) of spines, and the authors also addressed the question of whether single spines change shape. They found that, as in the *in vitro* condition[84,97], spines undergo considerable changes in shape and size[85]. They speculated that these size changes might reflect the strengthening and weakening of synapses[40,98–100], but they did not provide any evidence for this interpretation.

The Grutzendler *et al*. paper only studied spine stability under normal circumstances, whereas Trachtenberg *et al.* investigated spinogenesis during the critical period in the intact animal under conditions of sensory deprivation[70]. Unfortunately, their baseline observation of spine stability in a control situation was considerably different from that reported by Grutzendler *et al*.[69]. Trachtenberg *et al.* reported that 6–10 weeks after birth, approximately 20% of spines have a lifespan of less than a day, another 20% less than a week, another 10% less than a month, and the remaining 50% more than a month. This contrasts with the observations of Grutzendler *et al.* who reported that around 90% of spines are stable at this age, although both reports agreed that new spines do emerge.

Trachtenberg *et al.* also observed that the stability of spines is correlated with their morphology. Larger, and in particular, stubby spines seem to be much more stable than longer, thinner ones. This indicates a possible discrepancy between the two studies, which could explain the difference in results. If some of the dendritic protrusions that Grutzendler *et al.* categorized as filopodia (which, as they acknowledge, are very dynamic) were counted as spines by Trachtenberg *et al.* this could give radically different results with regard to stability of the putative 'spines'. Other inconsistencies between the two studies include the exact age of the animals and the cortical area that was imaged (visual versus somatosensory), although in our view these are unlikely to account for the observed differences. Also, the expression of the fluorescent proteins was targeted to different populations of neurons in the strains of mice that were used by the two groups. It is possible that different neuronal populations exhibit different levels of spine stability.

The paper by Trachtenberg *et al.* provided striking serial-section electron microscopic reconstructions of the imaged spines to investigate their synapses. They found that some contained synapses, although others did not, and they concluded that new spines make new synaptic contacts in many instances. The reasoning put forward in the paper, however, relies largely on indirect arguments for spine retraction, and to us it is not convincing. The authors also investigated the role of sensory deprivation on the stability of spines. Trimming every other whisker on the snout of the animal increases the fraction of short-lived spines at the expense of the more stable ones. Although the effect is small, this is an important result, because, in line with earlier studies[101], it indicates that the stability of spines can be modulated by sensory manipulations, and therefore by plastic adaptations in the cortex .

Imaging single spines over periods of weeks and months in living animals is a remarkable achievement, which will undoubtedly considerably help us to understand the roles of spines — and their appearance and disappearance — in the formation and plasticity of neural circuits. At the same time, the basic question of spine stability remains unresolved, although the common denominator of both of the recent studies is that the turnover of spines is higher during the critical period than in adult life and — importantly in the present context — that spinogenesis occurs in neocortical tissue *in vivo*. Moreover, a substantial fraction of spines is stable for many months, corroborating the possibility that these could be used for the storage of information in the brain. Regardless of whether information is stored in individual synapses or in circuit assemblies, the persistence of spines might be essential for long-term information storage.

Dendritic filopodia: structure looking for function

To round off our review, we will consider dendritic filopodia and their potential and controversial role as spine precursors. Like axonal filopodia that emerge from growth cones, dendritic filopodia are long (2–20 μm), thin (<0.3 μm in diameter) structures that are present in developing dendrites[55,102]. Their elongated shape indicates an exploratory function in the extracellular space. Except under pathological circumstances[102,103], it is rare to find filopodia on mature neurons, so their function is probably developmental. Unfortunately, to our knowledge, no marker has been found that can distinguish filopodia from spines. So presently, identification is based on their morphological features, such as the presence of a thin and particularly long stalk and lack of a knobby head[66,104]. This constitutes a serious problem, because the dynamic nature of dendritic spines and filopodia can confound the distinction between the two[68]. Therefore, spines and filopodia might not be different entities, but could be part of a continuum of morphologically plastic structures[105]. On the other hand, they could represent two completely different processes with different functions.

Filopodia in Purkinje cells and the Vaughn model. Hints to the potential function of filopodia might be gained from their developmental profile and distribution. Filopodia occur in most, or perhaps all, developing neurons. In Purkinje cells, two different types of filopodia have been described: 'terminal' filopodia located near the distal tips of developing dendrites, and 'collateral' filopodia that occasionally emerge from dendritic shafts[44,106]. The association of terminal filopodia with the tips of the dendrite raises the possibility that they are

involved in dendritic growth and branching, by interacting with the extracellular environment[44,106,107]. In this view, the final morphology of the dendrite would reflect the history of interactions of terminal filopodia with the environment. The 'choice' of which filopodium proceeds to form a terminal branch might then be determined by the success in making a synaptic contact. This idea was incorporated into Vaughn's 'synaptotropic' hypothesis, which states that filopodia 'catch' axons, and synapses are first formed on the filopodia before being incorporated into the dendritic shaft[13,108,109]. Indeed, in the developing spinal cord, ~70% of synaptic contacts are found on filopodia[108,110]. Collateral filopodia, on the other hand, could have been 'left behind' by the growth of the dendrite. Alternatively, they might represent the incipient growth of a new dendritic branch, or they could be involved in spinogenesis or synaptogenesis[106].

Filopodia in pyramidal neurons. In rat neocortical pyramidal neurons, filopodia of the collateral type are transient and occur mostly in P3–P12 animals[55]. They are elongated and can be directly apposed ('arm against arm') to axons along their full length, although clear synapses between neocortical dendritic filopodia and axons have rarely been described. Neocortical filopodia often occur in groups, as if they emerge from hot spots on the dendrite, similar to axonal filopodia and other developing neurites[105,111,112]. In hippocampal CA1 pyramidal cells, the distribution of filopodia during development has been studied in detail using ultrastructural techniques[104]. Two types of filopodia were described: an elongated type, similar to the collateral filopodia of Purkinje cells, and a sheet-like structure, more similar to a growth cone or a terminal filopodium. The first type often has synapses and supports up to 20% of the total synapses made on pyramidal neurons at this developmental stage. These dendritic filopodia sometimes contact axonal filopodia. The authors interpreted their data as supportive of Vaughn's hypothesis that filopodia contribute to the generation of synapses on the dendritic shaft.

It has also been proposed that filopodia in pyramidal cells serve as precursors to spines[64,66]. In two imaging studies by the Smith laboratory, the dynamics of early dendritic protrusions from hippocampal pyramidal neurons were monitored in cultured slices[66] and dissociated cultures[64]. On the basis of the developmental reduction in motility and disappearance of elongated filopodia, and the appearance of shorter spine protrusions with increasing developmental age, the authors proposed that dendritic filopodia become stabilized by transforming into spines (FIG. 3c). They postulated the existence of a 'protospine' and intermediate morphological structures that represent stabilized filopodia. The authors also noted that most filopodia were transient, and only those that successfully captured an axonal terminal became stabilized. The grabbing of axons by dendritic filopodia, presumably driven by actin networks or other motors, would then produce a spine — a structure that would reflect the tension created by the axonal pull on the dendritic membrane.

A recent two-photon imaging study of filopodia on developing neocortical pyramidal neurons characterized their motility during early postnatal development in acute slices (P2–P12)[105]. On the basis of differences in density, motility, length and response to neural activity, the authors proposed the existence of two populations of filopodia, in growth cones and in shafts. Filopodia in growth cones, analogous to the terminal filopodia of Purkinje cells, could be involved in dendritic growth and branching in an activity-independent manner, whereas shaft (collateral) filopodia might be responsible for activity-dependent synaptogenesis, in some cases becoming dendritic spines.

What is the evidence that filopodia are spine precursors? In favour of such a role are the imaging data that document how filopodia become stabilized[64,66,77,105], even though ultrastructural confirmation of postsynaptic specializations was not done in any of these studies. Also, it is intriguing that there seems to be a link between filopodia and synaptogenesis[13]. The idea that they create a 'virtual' dendrite[64] (that is, a dendrite that samples a radial volume determined by the length of its filopodia) makes sense considering that one of the problems that developing neurons face is the spatial sampling of axons[2,3,7]. Also, the astronomically high numbers of filopodia (50,000 new filopodia per day in developing mouse neocortical neurons[105]), compared with the much smaller number of spines present after spinogenesis (~2,000 total spines for these same cells[113]), could indicate that sampling of potential axons by the dendrite is very extensive. It is difficult to believe that the massive energy expenditure that is required for producing and retracting tens of thousands of filopodia per day is not related to synaptogenesis, which is arguably the biggest problem a developing neuron needs to solve.

However, there are several pieces of evidence against filopodia being spine precursors. First, the time course of the ontogenetic progression in spinogenesis of shaft synapses transforming into stubby and then mushroom spines seems to be incompatible with filopodia directly transforming into longer spines. Moreover, filopodia occur in many neurons that do not have spines at maturity[114–116], and even in many non-neural cells[105,113,117]. In our view, these observations make it unlikely that the only function of filopodia is to generate spines, but this does not exclude the possibility that every spine is preceded by a filopodium.

Further experimental work is necessary to clarify the fate of dendritic filopodia and its relationship with spines. In particular, long-term imaging (for several days) of developing dendrites and axons could directly solve this issue. Finally, manipulations that specifically block the motility or emergence of dendritic filopodia[82,117] will be very helpful in determining their role in spinogenesis and synaptogenesis.

Conclusions and future experiments

There are two different phases in the biological process of spine formation — spine emergence and spine maintenance — that probably have different molecular mechanisms and a different degree of dependence on

neuronal activity. Important differences between the developing and mature CNS encourage us to treat these topics separately.

Three models for spinogenesis. We can summarize most of the evidence relating to the emergence of spines in three models of spinogenesis: the Sotelo, Miller/Peters and 'filopodial' models (FIG. 3). These models are not necessarily mutually exclusive, but for the sake of clarity we will discuss them as different hypotheses. We will concentrate on synthesizing the best evidence for or against each model, and will also point out the gaps in the experimental work as a guide for future experiments.

According to the Sotelo model (FIG. 3a), spine development is intrinsic and independent of the presynaptic terminal. In our view, the ultrastructural evidence from Sotelo, Larramendi and other investigators of Purkinje cell development has sufficiently demonstrated the applicability of this model to the explanation of the development of parallel fibre spines. Experiments such as the *munc18* knockout or genetic lesioning of axonal pathways could test whether this model applies to spines from other cell types.

The Miller/Peters model (FIG. 3b) predicts sequential growth of spines from dendritic shafts, triggered by the axonal terminal. In principle, this model is incompatible with the Sotelo model, and it might apply to a different population of spines, such as those on pyramidal neurons. Although the ultrastructural evidence from neocortical and hippocampal pyramidal cells supports this model, it still needs to be demonstrated using time-lapse imaging of spinogenesis in the presence of terminals, or as a function of the activity of the terminals. We think that these experiments should be possible using simultaneous imaging of pre- and postsynaptic structures, and might require ultrastructural confirmation by electron microscopy.

Finally, the filopodial model (FIG. 3c) is derived from Vaughn's synaptotropic hypothesis. This postulates that dendritic filopodia establish synaptic contacts, which are then brought down to the dendrite[13]. Filopodia would 'reel in'[13] or 'slide'[104] axonal terminals towards the dendritic shaft, where the terminal produces a spine, as in the Miller/Peters model. In favour of this model is the enrichment of synapses found on filopodia[104,108,118] and observations of a filopodium becoming a protospine[64]. Contradicting this model is the observation that many cells have filopodia but do not develop spines[114–116], the lack of synapses on filopodia on some cell types[116], and data that show that spines appear first as stubbies rather than as longer protospines. A further problem of this model is that developing axons would have to follow convoluted routes due to simultaneous reeling in by many different dendrites — this is not observed in slices (C. Portera-Cailliau, unpublished data), although it has been observed in cultures (N. Ziv, unpublished data). This model is particularly interesting because it provides a function for dendritic filopodia, which is to increase the number of axons that the dendrite can sample. To conclusively confirm (or falsify) the filopodial model, live imaging of intact tissue will be of central importance. Imaging of the interactions between filopodia and axonal terminals is now possible[119–121], so it should be feasible to document the function of dendritic filopodia and their role in spinogenesis.

Spine maintenance and stability. In the second phase of the life history of spines, perhaps the most remarkable feature is the large reduction in the number of spines that occurs in the early postnatal period. This pruning of spines probably reflects a similar pruning of synaptic inputs, thereby representing a considerable reduction of connectivity in the developing brain — arguably one of the central steps in neural development — that might be sculpted through learning rules and/or input competition[74].

There is clear evidence that the presynaptic terminal and neuronal activity have important roles in the pruning and maintenance of spines. As discussed earlier in the text, in systems as different as the cerebellar climbing fibres[49] and pyramidal neurons in the mouse visual cortex[75], neuronal activity can have a massive influence on the final number of spines. However, although activity deprivation decreases the number of spines in the visual cortex, it actually increases the spine number in the climbing fibre experiments, so the role of activity varies depending on the specific spine. In fact, there is a vast literature on the different effects of a myriad of experimental manipulations on spine numbers or shapes[40].

The stability and plasticity of spines in the adult nervous system are still unresolved issues, and visualization of cortical spines for periods of many weeks[69,70] has not provided answers to these questions. Instead, it has left us in a rather unsettling situation with two opposing views. We hope that additional studies will provide more definitive data. It would also be desirable to have additional information on the relationship between spine turnover and synaptic turnover.

Heterogeneity of spines. Our final conclusion is to acknowledge the diversity of spines, and presumably also synapses. For each of the topics that we have discussed, conflicting data have emerged from the study of various cell types. The simplest solution to these controversies is to assume that different populations of spines behave differently. Even in a single cell type — the Purkinje cell — there are two populations of spines with completely different dependencies on presynaptic innervation. We think that there are probably many different classes of spines, and that the 'canonical' spine might not exist. Our argument resonates well with the large heterogeneity of calcium compartmentalization[122,123] and receptor localization[124,125], and morphological differences[2], even among spines from the same neuron. Understanding the heterogeneity of spines, with regard not only to their morphology but also to their functional parameters, will be essential for understanding spine function. We feel that we are at an exciting exploratory time where live imaging studies will enable us to understand the biological diversity of dendritic spines, and to resolve the function of these fascinating yet mysterious organelles.

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The authors declare that they have no competing financial interests.

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