



Moving molecules make synapses

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Two groups use a fluorescently tagged postsynaptic density protein and time-lapse imaging to track the location and timing of synapse formation in the developing brain.

What mechanisms determine where and when synapses are made in the developing brain? Asking the question is easy, but finding an answer is complicated by the need not only to establish the molecules involved but also to identify the times and places at which they act. Fortunately, cell biology's molecular magic marker, green fluorescent protein (GFP), has given us a way of labeling a wide range of structural proteins and following their activity in living cells, a strategy that is increasingly being used to study molecular dynamics in primary neurons. One particularly useful target for this approach is PSD-95, the major protein of the postsynaptic density (PSD), a disk-shaped organelle that marshals neurotransmitter receptors and other functional molecules at the postsynaptic sites of excitatory synaptic junctions. Okabe *et al.*¹ recently showed that PSD-95 tagged with GFP (PSD-95-GFP) expressed in hippocampal neurons is an effective marker of the PSD structure. Two new papers, one from the same group² in the *Journal of Neuroscience* and one from Marrs *et al.* in this issue of *Nature Neuroscience*³, now describe the use of PSD-95-GFP to follow the events of synapse formation.

These studies focus on excitatory synapses in the central nervous system, the overwhelming majority of which are made onto dendritic spines, tiny protrusions from the surfaces of dendrites with a thin neck and an expanded head, which act as sites of signal integration and plasticity⁴. During development, spine synapses are fashioned through a series of stereotyped steps. On the dendritic side, the developmental process begins with the emergence of highly motile filopodia. These are progressively replaced by

polymorphic 'protospines,' which then mature into spines of typical adult anatomy^{5,6}. On the axonal side, the differentiation of presynaptic sites is marked by the development of varicosities containing accumulations of synaptic vesicles and by the onset of activity-evoked vesicle recycling^{7,8}. These studies indicate that the establishment of synapses can occur within 1–2 hours following initial contact between axons and dendrites. One outstanding question about this process concerns the order in which the component parts of the synapse are assembled. Which comes first, the presynaptic axon terminal with its complement of neurotransmitter-releasing vesicles or the dendritic specialization with its receptor-containing PSD? Related to this is the question of what signals, from axon or dendrite, initiate and regulate each phase of the process.

Both groups approach these questions using time-lapse recording of hippocampal neurons transfected to express PSD-95-GFP. Marrs *et al.*³ examine pyramidal neurons in slices of hippocampus of 4–7 day-old rats after maintaining them in culture for 6–12 days; at this time, dendrites of the developing cells are undergoing profuse synaptogenesis and spine formation. As expected, uncoupled GFP, expressed alone, works as a soluble, space-filling marker, evenly labeling dendrite shafts and spines. By contrast, PSD-95-GFP forms intensely fluorescent puncta in the heads of dendritic spines within 24 hours of transfection, suggesting that newly synthesized PSD-95-GFP incorporates into existing PSDs. Confirming this synaptic association, Marrs *et al.* use staining with antibodies against synapsin-1, a marker for presynaptic vesicles, to show that the majority of PSD-95-GFP positive spines are contacted by synaptic vesicle-containing axonal boutons. Using hippocampal neurons in dissociated cell cultures, Okabe *et al.*² provide equivalent data

with GFP-based markers and antibodies against another presynaptic protein, synaptophysin.

Armed with this evidence for the fidelity of PSD-95-GFP as a marker for the postsynaptic junctional structure, both groups then use time-lapse recording to follow the fates of dendritic protrusions over periods of 10 hours or more. One important result reported by both groups is that all stable spines—meaning those that persisted throughout the hours of recording with only minor morphological changes—contain PSD-95 hotspots, whereas transient filopodia, which have a mean lifetime of less than 30 minutes, are never associated with PSD-95 clusters. Marrs *et al.* focus on the developmentally intermediate 'protospines' and find them to be correspondingly intermediate in their relationship to PSD-95 clusters. Some 80% of protospines contain PSD-95-GFP clusters that are motile, with individual hotspots moving outward into growing protospines, and in some cases dividing to spin off new, independent hotspots. This behavior is not seen in mature spines, whose PSD-95 clusters remain anchored in the spine head.

These various movements seem related to the origin of PSDs during synaptogenesis. Some 90% of PSD-95 clusters make their first appearance in dendrite shafts or dynamic spine precursors just before migrating to their ultimate position in the heads of the maturing spines. By contrast, less than 10% make their first appearance in spines after they are already established³. Okabe *et al.*² do not make such a clear distinction between filopodia and spines, but their results parallel those of Marrs *et al.* in showing that some 90% of new PSD-95 clusters occur in 'filopodia-spines'. Both groups report a similar time course for the appearance of PSD-95 clusters following the initial appearance of filopodia, with an upper limit of 2–3 hours for the entire process.

Okabe *et al.*² go a step further in analyzing the sequence of events by expressing PSD-95 and synaptophysin tagged with YFP and CFP, spectral variants of GFP that allow the two molecules to be distinguished from one another in the same time-lapse recording. PSD-95-YFP labels the postsynaptic PSD, whereas synaptophysin-CFP labels the presynaptic axon terminal, so that when they are expressed in neighboring neurons, it is possible to visualize developing pre- and postsynaptic elements as they make

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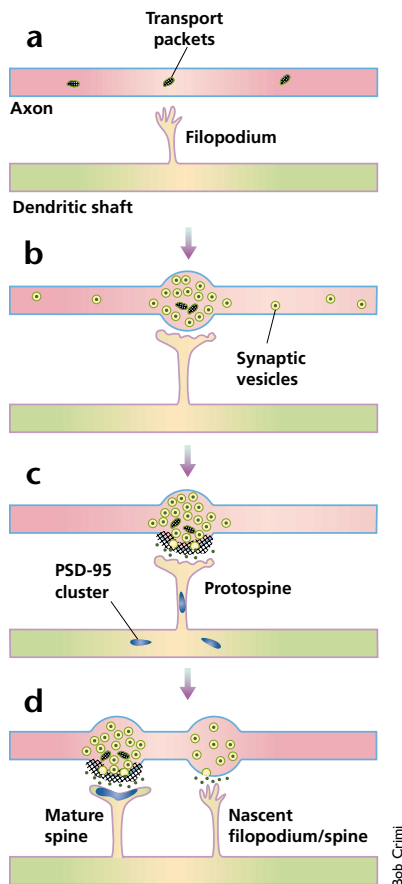


Fig. 1. Hypothetical scheme of events in the formation of spine synapses during nervous system development. **(a)** Before making contact with axons, dendrites send out numerous transient filopodia that extend and contract, rapidly 'feeling out' the space around them; at this stage, axons contain widely distributed polymorphic 'transport packets'. **(b)** Shortly after a dendritic filopodium contacts an axon, vesicles accumulate in varicosities at the same site, suggesting that the dendritic component sends a vesicle recruitment signal to the axon. **(c)** Some of these vesicles are 'transport packets' containing components of the presynaptic junctional complex (indicated by cross-hatching), which become inserted into the surface membrane at the site of contact. Others are synaptic vesicles, which begin releasing neurotransmitter (indicated by black spots) and thus initiate synaptic signaling. **(d)** The presence of an active presynaptic terminal induces the recruitment of PSD-95-containing clusters to postsynaptic junctional sites in the heads of developing spines to form a mature spine synapse. Neurotransmitter release from axon terminals may induce the outgrowth of new filopodia and spines (right).

synaptic connections. Indeed, motile filopodia form on the dendrites of neurons that lack spines in the mature state, such as retinal ganglion cells, supporting this interpretation⁹. Instead, filopodia may represent an early step in which dendrites 'explore' their environment in search of axonal partners ready to form synapses^{5,6}. Dendritic filopodia may be instrumental in triggering the formation of presynaptic specializations, because recent studies show that active vesicle clusters appear at sites of contact between dendrites and axons as soon as 30 minutes after initial contact has been made^{7,8}. The appearance of PSD-95 clusters follows the formation of these synaptic vesicle accumulations^{2,3,8}, suggesting that neurotransmitter signaling may initiate the transformation of filopodia into mature dendritic spines. The exact nature of the signals involved at each stage of this process remains to be demonstrated. Activation of postsynaptic NMDA receptors can lead to the outgrowth of dendritic filopodia and spines^{10,11}, suggesting one mechanism by which neurotransmitter release from nascent axonal boutons may initiate spine formation. However, the possible role of surface molecules involved in junctional contacts, such as neuroligin¹², also deserves careful consideration.

With so many molecules involved in forming a new synapse, it is natural to ask how their coordinate assembly at nascent synaptic sites is achieved. Are the components 'glued' to their predecessors one after another, like the balsawood pieces of a model airplane? In terms of both efficiency and accuracy, such a protein-by-protein mechanism seems unlikely. Perhaps more plausible is a 'pre-packaging' mechanism in which the components

arrive pre-assembled and ready for incorporation into the nascent structure. There is already persuasive evidence that this occurs on the presynaptic side, where axonal transport packets containing an array of presynaptic active zone molecules appear at nascent synaptic sites immediately following contact between developing axons and dendrites^{7,13}. The subsequent appearance in clusters of PSD-95, a molecule that is pivotal in linking together diverse components of the PSD^{14,15}, may represent the existence of a pre-formed multi-molecular complex ready to form the postsynaptic specialization of dendritic spines as they mature into synapses.

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contact and subsequently interact. The small numbers of appropriately double-labeled synaptic partners they were able to record illustrates the difficulty of these experiments. Of 543 PSD-95-YFP-positive clusters and 326 synaptophysin-CFP-positive axonal varicosities examined in recording sessions stretching over many hours, only 7 pairs showed the full sequence of events from initial contact to spine formation. In each case, the generation of PSD-95 accumulations occurred after the appearance of synaptophysin-positive clusters at the same location. Conversely, the authors did not detect the formation of new synaptophysin-positive clusters at the sites of pre-existing PSD-95 accumulations.

Together, the two sets of results suggest the following sequence of events (Fig. 1). Before synapse formation, dendrites generate a profusion of filopodia-like protrusions. Because they are motile and transient, these filopodia are unlikely to form the basis of stable