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Current Opinion in Neurobiology

Assembling the presynaptic active zone

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Rapid neurotransmission depends on the structural and functional integrity of synaptic connections. How synapses assemble is currently being intensely investigated to help our understanding of neuronal development and synaptic plasticity. Here we focus on the assembly of the presynaptic active zone, which regulates the synaptic vesicle exo/endo-cycle and is characterized by ultrastructural specializations and large scaffold proteins. While genetic and biochemical studies from rodents, *Caenorhabditis elegans* and *Drosophila* have started to identify proteins organizing active zone assembly, drawing a coherent picture remains challenging, with genetically established hierarchies and protein–protein interactions still to be placed into spatio-temporal and functional context. Recent advances in light and electron microscopy, together with *in vivo* imaging of protein traffic, will help to tackle this challenge.

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Current Opinion in Neurobiology 2009, **19**:311–318

This review comes from a themed issue on
Signalling mechanisms
Edited by Michael Ehlers and Gina Turrigiano

Available online 22nd April 2009

0959-4388/$ – see front matter

DOI 10.1016/j.conb.2009.03.003

Introduction

During nervous system development, synaptic circuitry must be defined by forming synaptic connections with high-spatio-temporal precision. While developmental synapse formation seems to proceed properly in the absence of neurotransmission, neuronal activity can trigger changes in the molecular composition and functional status of synapses. This synaptic plasticity is considered to underlie neuronal processes, such as learning and memory. In the context of both developmental and plasticity-related changes, a detailed understanding of how the molecular architectures of synapses assemble (and dissemble) is critical [1,2].

Here we review the assembly process of the presynaptic active zone, controlling presynaptic neurotransmitter release. Genetic studies of both vertebrate and invertebrate model synapses have provided first insights into the regulation of active zone formation within the last years. Despite these successes, the dissection of synapse assembly processes has proven difficult due to genetic redundancies. This likely reflects a highly cooperative and regulated nature of synapse assembly, complicating the straightforward deduction of molecular models here. Thus, attempts to genetically define event hierarchies and assembly intermediates should be complemented by biochemical, ultrastructural and dynamic protein trafficking data.

Synaptic modules

For functionality, chemical synapses depend on the proper interplay of several 'modules' (highlighted in *italics*). At the presynaptic site, the *active zone* (AZ) provides the platform for rapid fusion of neurotransmitter-filled synaptic vesicles (SVs) after calcium influx. The AZ membrane is decorated by a proteinacious cytomatrix (cytoplasmic matrix at the active zone – CAZ) which is characterized by a set of specialized proteins [2]. While CAZs display variable morphologies at different synapse types, they are likely generically critical for effective organization of the associated SV *exo/endo-cycle* machinery [3•,4]. How the CAZ interacts with the exo/endo-cycle in detail, for example by recruiting SVs or establishing physical proximity between exocytic proteins, calcium channels [5] and potentially the endocytic machinery, is subject of intense investigation.

At the postsynaptic site, neurotransmitter receptors accumulate within another electron dense compartment, the *postsynaptic density* (PSD), which is critical for the stability and dynamic regulation of neurotransmitter receptor populations [6]. Between the pre- and postsynaptic membrane, a synaptic cleft of defined width is found, which seems to be characterized by the presence of *transsynaptic* pairs of cell adhesion molecules.

Modularity of synapse assembly

Are these synaptic modules independent units of assembly, or does their formation require the presence of a synaptic site assembling the other modules in parallel?

Multiple findings argue in favor of units being able to self-assemble. Vesicle fusion activity can be reconstituted *in vitro*, in the absence of cytomatrix scaffolds and even of calcium [7]. In immature neurons, mobile moving clusters of SVs have been observed [8] exchanging with the neuronal plasma membrane in the absence of postsynaptic differentiation. Furthermore, in genetically engineered

Drosophila embryos, presynaptic AZs can form in the complete absence of postsynaptic partner cells and thus of any postsynaptic specializations [9]. Notably, presynaptic differentiation, including the formation of AZs in cultured neurons, can be induced by the presentation of a single postsynaptic cell adhesion protein (Neuroligin) expressed on non-neuronal cells [10]. *Vice versa*, postsynaptic differentiation is inducible by the Neuroligin-interactor Neurexin [11]. Additionally, postsynaptic differentiation can occur prior to formation of a detectable functional presynaptic AZ in young hippocampal neurons [12].

Thus, vesicle release machinery, AZ matrix and to some degree the postsynaptic specialization can (under certain experimental circumstances) display intrinsic assembly propensities and form 'in isolation'. This intrinsic assembly propensity might become dominating when physiological signals from other modules are missing after genetic intervention. Under physiological conditions, however, it is to be expected that synaptic modules closely communicate to fine tune the synapse assembly process.

Active zone assembly

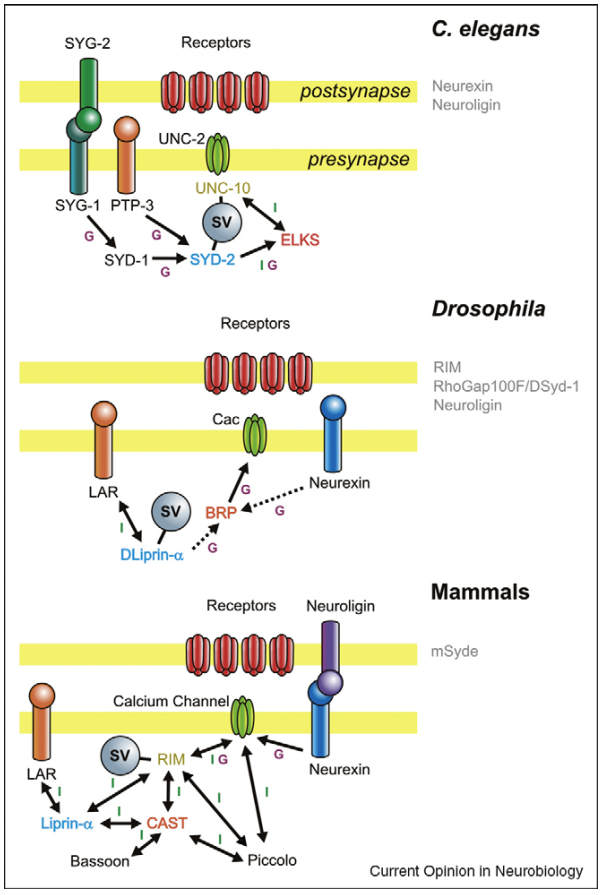
Proteins organizing AZ assembly are likely to be part of the CAZ themselves. Due to the low solubility of AZ material, the biochemical identification of AZ proteins has lagged behind the characterization of ion channels and SV proteins, with fundamental components still awaiting a functional characterization. Recently, however, unbiased genetic approaches (especially studies from the *Caenorhabditis elegans* HSNL synapse and the *Drosophila* NMJ [13,14]) have identified several presynaptic proteins as important for AZ assembly (Figure 1).

Defining the location

Up high in a hierarchy of assembly events, a membrane district suitable for the formation of a new AZ needs to be defined. In *C. elegans*, the immunoglobulin containing cell adhesion molecules UNC-40/DCC, as well as the heterophilic interaction pair SYG-1/Neph1 and SYG-2/Nephrin, have been shown to be important in this regard, though at different synapse populations [15–17]. As other IgCAMs might take over similar functions in *Drosophila* and mice, it appears that cell adhesion codes triggering initial assembly have diverged between synapse types as well as between species (Figure 1).

Along with defining the location where synapses form, pre- and postsynaptic cell adhesion molecules might coordinate the formation of postsynaptic structures, tightly coupled to ongoing clustering of AZ components. The complex formed between Neurexins and Neuroligins is a prototypical candidate for transsynaptic communication. Their role in synapse function and structure has recently been explored in knockout studies of both mice and flies, and in cell culture assays and has been reviewed elsewhere extensively [18].

Figure 1



Proteins implicated in active zone assembly. A summary of proteins considered as important players of AZ assembly. Results from different model organisms are depicted in separated panels. SYD-2/DLiprin-α/Liprin-α family proteins are highlighted in blue, ELKS/BRP/CAST in red and UNC-10/RIM in yellow. Type of interactions are indicated by letters above the arrows: I = direct physical interaction, G = genetic interaction/regulation inferred from genetic findings. Dashed lines indicate indirect evidence of interaction from imaging data. Proteins spelt out in grey have not been studied in the respective systems (e.g. the mammalian and *Drosophila* SYD-1 orthologues RhoGap100F/DSyd-1 and mSyde), but would be of major interest in the authors' eyes.

Downstream of cell adhesion molecules, SYD-2/Liprin-α was found to be crucial for AZ assembly and SV clustering in *C. elegans* and *Drosophila* [19••,20••,21,22]. The SYD-2/Liprin-α family is characterized by coiled coil- and SAM-domains, and has been implicated in both pre- and postsynaptic assembly by recruiting and interacting with a multitude of synaptic proteins and by regulating synaptic cargo transport [23]. Thus, SYD-2/Liprin-α might guide transport of further components to AZs, with its

described binding partners being prime candidates to be relevant cargo (see Figure 1).

In addition to SYD-2/Liprin-α, a C2-domain and putative Rho-GAP-domain containing protein named SYD-1 was found to be essential for AZ assembly at the HSNL synapse of *C. elegans* [19••,20••]. There, SYD-1 seems to help the functional recruitment of SYD-2/Liprin-α, since a gain of function allele of *syd-2* (a missense mutation in a coiled coil domain of SYD-2, *syd-2(gf)*) allows for the suppression of SYD-1 requirement [20••]. Whether homologues of SYD-1 operate similarly during *Drosophila* and mouse AZ assembly is an interesting and yet open question.

Continuing towards maturation

Which other AZ proteins functionally interact with SYD-2/Liprin-α within AZ assembly? Among the SYD-2/Liprin-α binding partners, RIM/UNC-10s are generic AZ proteins. However, RIM/UNC-10s so far appear dispensable for principal AZ assembly in both worm and mouse [19••,24], though they are important to functionally anchor neurotransmitter-containing vesicles in the vicinity of voltage-operated calcium channels [25•] and to target SVs to the AZ membrane [26].

Notably, the activity of SYD-2(gf) in *C. elegans* does not depend on RIM/UNC-10 or calcium channels (UNC-2), but instead requires ELKS-1, a member of the CAST/ERC family [20••]. CAST (CAZ-associated structural protein) originally was identified biochemically as an AZ associated coiled coil domain protein [27], which interacts with Bassoon, Piccolo, RIM-1 and Liprin-α [27–29]. *Drosophila* Bruchpilot (BRP), whose N-terminal half encodes the *Drosophila* CAST homologue [30,31], proved to be crucial for proper clustering of calcium channels within AZs, CAZ formation and efficient neurotransmitter release [31,32]. Thus, this protein which in addition to the CAST/ELKS domains contains long additional coiled domains at its C-terminus, seems to provide a large scaffold involved in assembly as well as functional maturation of *Drosophila* AZs.

Two further large scaffolding molecules, Piccolo and Bassoon, were among the first AZ specific proteins to be identified. Not conserved in *Drosophila* or *C. elegans*, they turned out to be specific to mammalian synapses. While Piccolo seems dispensable for principal AZ assembly at glutamatergic synapses (however important for function) [33], Bassoon is reported to play an important role in the assembly and functioning of various types of synapses [2], and to be amongst the first proteins that appear at newly forming AZs [34].

A digital nature of AZ assembly and structure?

A principal question under investigation is whether preassembled units of AZ proteins are shipped to prospective AZs, or whether AZs assemble *de novo* from diffuse pools of the relevant proteins. In fact, presynaptic proteins have been suggested to be transported in specialized transport vesicles positive for the mammalian AZ markers Piccolo and Bassoon [35]. These 80-nm dense core vesicles were named Piccolo/Bassoon transport vesicles (PTV) and were analyzed using live imaging of GFP-Bassoon combined with fractionation/immuno-labeling [36]. It was suggested that PTVs carry a comprehensive set of AZ materials, providing unitary building blocks for AZs.

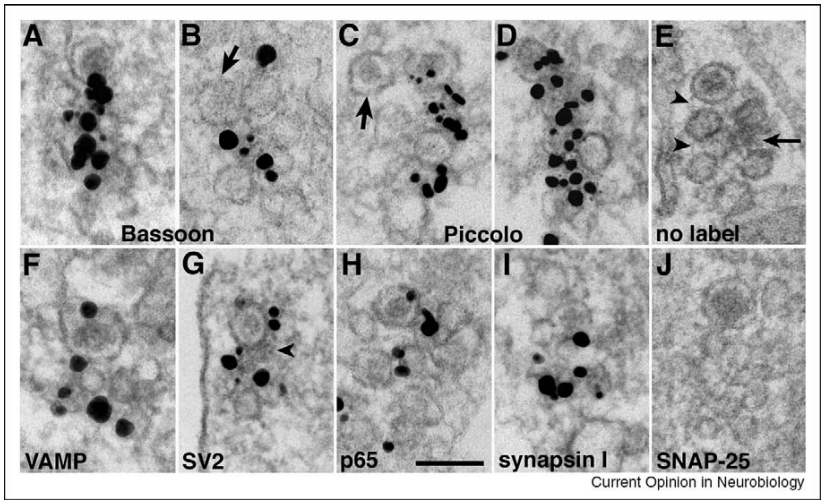
However, the situation appears more complex. Recently, immuno-electron microscopic analysis could validate that in fact Piccolo and Bassoon decorate dense core vesicles, whereas SV proteins are mostly found in small clear SVs (Figure 2) [37••]. Both dense core and SVs seem to be transported in a preassembled multi-vesicle transport aggregate [37••], with the potential to rapidly form functional presynaptic sites. Notably, extra-vesicular electron dense material and filaments connecting SVs are observed at multi-vesicle transport aggregates as well.

Potentially reflecting a modular fashion of synapse assembly, a recent electron tomography study of rat neocortical synapses [38••] has provided evidence that AZ architecture might be arranged from several 'synaptic units'. Polyhedral cages surrounded by a subset of SVs are shown to be associated with cytomatrix filaments. Surprisingly these polyhedral cages ('syndesomes') resemble Clathrin cages (Figure 3), which so far have only been discussed in the context of vesicular endocytosis.

Thus, ways to directly subject assembling AZs to molecularly resolved, high-resolution analysis appear critical for a deeper understanding of the AZ assembly process. In addition, several ultrastructurally defined elements (extra-vesicular electron density, filaments, syndesomes, etc.) are waiting to be defined by genetics and immuno-electron microscopic analysis, to be placed in a coherent assembly scheme and to be functionally evaluated. Moreover, whether aspects of AZ assembly can be reversible, for example whether discrete units leave AZs, will be highly relevant, in particular, for plasticity research.

Interestingly, at mammalian ribbon synapses, AZ components seem to assemble to electron-dense precursors, instead of being transported to AZs by membranous compartments [34]. Here, Bassoon and Piccolo assemble early together with RIBEYE and RIM, whereas other synaptic players such as Munc-13, calcium channels or CAST accumulate late during synapse assembly [34], probably demarking a later maturation process. Thus, at least at this specialized synapse (what synapse is not specialized!) not all players seem preassembled but rather arrive in a sequential fashion. Similarly, the CAST homologue BRP was shown to accumulate late in the synapse

Figure 2



Multivesicular aggregates, putative precursors of active zone assembly. (**A**) Immuno-electron-microscopic images at hippocampal cultures showing multi-vesicular aggregates, likely reflecting AZ precursor structures. Aggregates are characterized by larger dense core vesicles (arrow in **C**), smaller clear, SV-like vesicles, electron dense material (arrow in **E**, arrow head in **G**) and filamentous material connecting vesicles (arrow heads in **E**). AZ proteins Bassoon (**A,B**) and Piccolo (**C,D**) are found at dense material within the cluster center, while label of SV proteins VAMP (**F**), SV2 (**G**) and p65 (**H**) is mostly associated with SV-like vesicles. SNAP-25 labeling is not detected in aggregates (**J**), while synapsin-1 labeling is associated with the vesicles (**I**). Scale bar = 100 nm. Reproduced with permission from [37••].

assembly process by *in vivo* imaging at *Drosophila* NMJ synapses [39••].

Apart from characterizing 'building blocks' for AZ assembly, the signals mediating the precise targeting of AZ proteins to sites of AZ assembly are important. Recently, the serine/threonine kinase Unc-51 was shown to be essential for localization of presynaptic BRP opposite to postsynaptic glutamate receptors by regulating the activity of the MAP kinase ERK [40•].

Providing the back bone

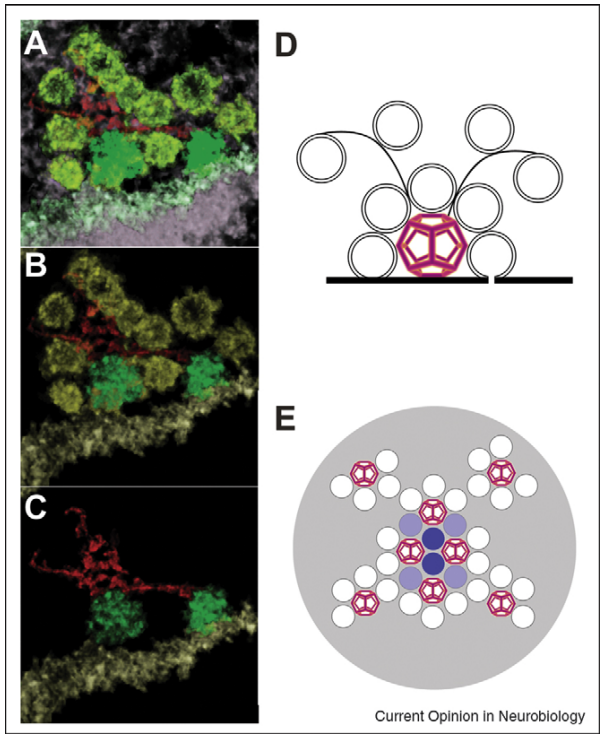
Changes in the synaptic protein composition form a basis for synaptic plasticity. With the advent of live fluorescent imaging of synapses (fluorescence recovery after photobleaching, photo-activation procedures, single molecule imaging), a picture of high-protein flux at synapses [6], with dynamic proteins often exchanging on a minutes time scale, has emerged.

Despite these protein trafficking and diffusion dynamics, mammalian CNS boutons persist for long periods [41,42] and apparently do not eventually equilibrate and become identical in size, composition, or function [43]. What is the basis for this persistence (also referred to as synaptic 'tenacity' [44••]) of synaptic specializations? Are there proteins with a slow turnover providing a basis for this synaptic tenacity, or in other words, if synaptic molecules are in a dynamic equilibrium with extrasynaptic pools, what drives and maintains their high concentrations at synaptic locations?

At *Drosophila* NMJ synapses, two different glutamate receptor complexes (either containing the GluRIIA or the GluRIIB subunit) are co-expressed within individual PSDs. On a genetic level, GluRIIA promotes and GluRIIB limits the number of synapses forming. In fact, GluRIIA incorporates essentially irreversibly at PSDs, and GluRIIA (but not GlluRIIB) incorporation is directly correlated with PSD growth [39••]. Thus, GluRIIA in fact is a good candidate to confer persistence to postsynaptic assemblies in *Drosophila*.

The CAZ is resistive to chemical extraction procedures [45], making it a candidate for a 'core scaffold', that specifies and maintains the position of membrane-associated molecules as well as exo/endocytic machinery and SV clusters [44••]. Interestingly at mammalian AZs, Munc-13 shows rapid exchange [46], while Bassoon exhibits high-retention times in cultured hippocampal neurons [44••] and remains static after stimulation [47]. This indicates that Bassoon might be part of a relatively static core scaffold in contrast to Munc-13, which is directly associated with the process of neurotransmitter exocytosis. Thus, presynaptic tenacity, to a large degree, might be based on the tenacity of the CAZ. The CAZ might thus be constructed of a static backbone (e.g. Bassoon) and mobile machinery (e.g. Munc-13).

Figure 3



Tomographic analysis of AZ structure at rat cortex synapses. (**A**–**C**) Tomographic analysis identifies structural elements at individual AZs of rat cortex. Polyhedral cages (green), vesicles (yellow), and filaments (red) are shown. (**D**) Model of AZ organization picturing AZs as constructed of 'units' comprising a polyhedral cage surrounded by a corona of vesicles. Vesicles deeper within the presynaptic cytoplasm are tethered to the AZ via long filaments. (**E**) Possible arrangement of eight synaptic units on the plane of the AZ seen from on top. Reproduced after modification and with permission from with permission from [38••].

It appears likely to us that such cooperative protein scaffolds with stable interactions provide nucleation zones for the clustering of less static synaptic proteins in a dynamic equilibrium. Final proof whether a low turnover of a given synaptic protein contributes to structural stability of synaptic specializations might be provided by an acute, externally triggered degradation of genetically modified proteins (for an example see Ref. [48]).

Synapse assembly *in vivo*: sequence and timing?

Getting a realistic impression of molecular processes mediating assembly and plastic changes of synapse populations in functional circuits seems inevitable for a deeper understanding of learning and memory. Time course and coordination of synapse assembly in intact nervous systems is thus under intense investigation. Table 1 gives an overview of studies addressing the time course of synapse assembly using different methods and systems.

Somewhat different from expectations based on cell culture experiments suggesting a rapid assembly leading to mature synapses in one to two hours or less, *in vivo* synapse assembly has recently been suggested to protract over many hours (Table 1).

In intact developing *Drosophila* larvae, addition of synaptic sites at neuromuscular junctions has been followed over extended periods, using *in vivo* visualization of glutamate receptors and the AZ protein BRP. New synapses formed over several hours in physical separation from pre-existing synapses and retrieved their glutamate receptors from diffuse, non-synaptic pools [39••,49].

For mammalian brains, retrospective serial section electron microscopy (SSEM) of previously imaged dendritic spines in the adult rodent neocortex showed that spine growth precedes the growth of synapses *in vivo* [50]. In line with this, newly formed spines became functional within a day after induction of long-term plasticity (LTP) in hippocampal slices [51]. In fact, a further SSEM study found that new spines would form synapses, judged by morphological means (such as SV filled boutons and a synaptic cleft) after 15–19 hours after outgrowth, induced by tetanic stimulation [52•]. Recently, however, electrophysiological analysis coupled with electron microscopy (EM) suggested that synapses form within hours after spontaneous spine formation, matching the findings from dissociated cell culture more closely [53•]. However, synaptogenesis might be regulated more strictly in hippocampal slice culture (potentially due to better preserved regulatory cues from the surroundings) than in dissociated neurons. The synapse assembly program might also differ between situations of 'high-activity' (LTP-induction) and 'low activity' (spontaneous).

Outlook

Synapses are small. Individual AZs measure only a few hundred nanometers in diameter, limiting the use of standard light microscopy for studying sub-synaptic processes. The further understanding of how 'synaptic architectures' assemble thus demands further ultrastructural analyses. Here, the use of high-pressure freeze electron microscopy allows the study of synaptic architecture under well-preserved and realistic conditions [54]. Using this technique, SVs were shown to be interlinked by filaments while other filaments linked SVs to the presynaptic AZ [3•]. It will be interesting to revisit further synaptic structures, such as 'synaptic units' [38], with high-pressure freeze EM avoiding aldehyde fixation. In addition, light microscopy, since the advent of stimulated emission depletion microscopy (STED, 'breaking the diffraction barrier') [55] has proven valuable for high-resolution studies of synapse architecture [1,31]. Live cell imaging of fluorescent-protein fusions with STED resolution was achieved recently [56], even unraveling nanoscale dynamics of single lipid molecules at the plasma

Table 1

Recent studies dealing with temporal aspects of synapse assembly in different systems.

Organism	Preparation	Methods	Aspect studied	Proteins	Time for assembly	Reference
Drosophila	NMJ	Imaging	Postsynaptic maturation	DGluRIIA, DGluRIIB, BRP	Several hours	[39••]
Mouse	Hippocampal slice culture	Imaging, electrophysiology	Relation spino-/synaptogenesis without stimulation	AMPAR, NMDAR	Within hours	[53•]
Mouse	Hippocampal slice culture	Imaging, EM	Relation spino-/synaptogenesis after tetanic stimulation		1 day	[52•]
Mouse	Hippocampal slice culture	Imaging, electrophysiology	Functionality of newly formed spines		<1 day	[51]
Mouse	Barrel cortex	EM, imaging	Synaptic differentiation		>1 day	[50]
Rat	Primary hippocampal neurons, microsphere assay	Electrophysiology, imaging	Neurexin induced postsynaptic differentiation	PSD-95, AMPAR	<2 hours	[58]
Rat	Neuronal sandwich	Imaging	PSD-95 accumulation, pre-and postsynaptic calcium imaging	PSD-95	2–3 hours	[59]
Rat	Primary hippocampal culture	Imaging	PSD recruiting presynaptic markers	PSD-95, Synaptophysin	2 hours	[12]
Mouse	Hippocampal neuron culture	Imaging	Morphogenesis of dendritic spines, PSDs and SVs	PSD-95, Synaptophysin	1–2 hours	[60]
Rat	Hippocampal neuron culture	Imaging	Time-course of synaptic molecule recruitment	Bassoon, PSD-95, AMPAR, NMDAR	1–2 hours	[61]

membrane of living cells [57•]. These and other techniques will deliver exciting possibilities for further addressing synapse assembly at high-spatial and/or temporal resolution. Thus, we expect that our insight into *in vivo* synapse assembly will be deepened in the foreseeable future.

Acknowledgements

We would like to thank Omid Khorramshahi for help with the figures and Robert J Kittel along with members from the Sigrist lab for critical comments. This work was supported by the DFG (Exc 257).

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