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Ted Abel *Editors*

Synaptic Tagging and Capture

From Synapses to Behavior

Second Edition

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Chapter 3
Molecular Mechanisms Underlying Synaptic Tagging and Consolidation

Yasunori Hayashi, Miquel Bosch, Pin-Wu Liu, Tomohisa Hosokawa, and Takeo Saneyoshi

Abstract After a synapse undergoes long-term potentiation (LTP), it acquires a newly remodeled molecular and structural organization. This reorganization over time is explained by a hypothetical structure called a synaptic tag, which is specifically formed at potentiated synapses, not at unstimulated ones, and captures newly synthesized proteins to persistently stabilize the potentiated state. However, to date, the molecular identity of the synaptic tag remains unclear. Based on several lines of experimental evidence, we propose that remodeled filamentous (F-) actin and CaMKII together form the synaptic tag by modifying the postsynaptic cytoskeletal structure to capture newly synthesized synaptic proteins. Liquid–liquid phase separation, a biophysical property of biological macromolecules, also plays a key role in this process. F-actin and CaMKII both fulfill the criteria to be the tag: they are specifically enriched at potentiated synapses without requiring new protein synthesis and persist for at least 1 h. Additionally, the intrinsic binding capacity of F-actin and CaMKII is ideal for capturing newly synthesized proteins at the synapse, thereby consolidating the synaptic structure and function and eventually allowing memory persistence.

Keywords LTP · CaMKII · Synaptic tag · F-actin · Liquid-liquid phase separation (LLPS)

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3.1 Introduction: Memory, LTP, and the Tag

One of the most fundamental questions in neuroscience is to understand how memories are stored in the brain. At the cellular level, synaptic plasticity represents the best molecular explanation for the process of learning and memory. When a synapse receives a transient but strong stimulation, subsequent transmission on that synapse is enhanced for a long time, a phenomenon called long-term potentiation (LTP) (Malenka and Bear 2004; Nicoll 2017; Hayashi 2022). Drugs or genetic modifications that interfere with LTP often impair learning and memory in intact animals (Malenka and Bear 2004). In addition, learning is known to induce LTP-like potentiation of synaptic transmission; on the other hand, the artificial generation of LTP can induce memory (Whitlock et al. 2006; Nabavi et al. 2014).

After the initial induction of LTP, the synapse goes through several distinct phases of potentiation, each sensitive to different inhibitors and thus involving different molecular mechanisms. The first phase is post-tetanic potentiation (PTP; approximately <1 min) (Zucker and Regehr 2002), which is the result of presynaptic accumulation of intracellular Ca^{2+} and leads to increased transmitter release but does not require postsynaptic NMDA-type glutamate receptors (NMDAR) (Collingridge et al. 1983; Malinow et al. 1988). The next phase is short-term potentiation (STP; approximately <15 min), which requires postsynaptic NMDARs but is not blocked by either an inhibitor or genetic manipulation of Ca^{2+}-calmodulin-dependent protein kinase II (CaMKII), a serine/threonine kinase strongly implicated in LTP and memory (Collingridge et al. 1983; Malinow et al. 1988). This is followed by LTP, which requires both NMDARs and CaMKII (Malinow et al. 1988; Malenka et al. 1989; Tsien et al. 1990; Silva et al. 1992). LTP can be further subdivided into the early (approximately <2 h) and late (>2 h) phases (Frey et al. 1988; Nayak et al. 1998; Kelleher 3rd et al. 2004; Pang et al. 2004), the major difference being the requirement for protein synthesis. While the early phase of LTP (E-LTP) does not require the synthesis of new proteins, the late phase (L-LTP) does. In agreement with this, both transcriptional and translational inhibitors can inhibit L-LTP, but not E-LTP. Experimentally, a weak tetanic stimulation induces E-LTP, with the potentiated transmission typically returning to baseline within about 2 h or so. However, by giving a stronger tetanic stimulus, L-LTP lasting more than 2 h can be induced (Frey et al. 1988; Kelleher 3rd et al. 2004). As a result, the synaptic changes are consolidated and become less sensitive to depotentiating stimulation (Fujii et al. 1991). Importantly, induction and consolidation of LTP take place selectively at stimulated synapses, not at naïve unstimulated ones, which means that the structural/functional modifications underlying LTP must be synapse-specific.

As the consolidation of E-LTP into L-LTP requires the synthesis of new proteins, this poses an interesting challenge to the neuron. Once new proteins are synthesized in the soma or dendrite, they must find the appropriate synapses that received LTP-inducing stimulation among all the nonstimulated ones. Indeed, the existence of such a mechanism was reported in an elegant study by Morris et al. (Frey and Morris 1997; Redondo and Morris 2011). They first induced E-LTP with a weak tetanus via

a specific synaptic input, which alone would not induce L-LTP. However, when they induced L-LTP by a strong tetanus via a second input pathway using a separate electrode, the first input pathway, which only received E-LTP induction, also underwent L-LTP. They hypothesized that a structure called a synaptic tag is formed specifically at the potentiated synapses but not at the unstimulated ones. This tag captures newly synthesized proteins from the soma or dendrite at the potentiated but not at the nonpotentiated synapses. This synaptic tagging and capture (STC) hypothesis was proposed in the late 1990s, and many following studies confirmed it, at the molecular, cellular, and even behavioral level (Sajikumar and Frey 2004a, b; Sajikumar et al. 2005; Redondo and Morris 2011; Moncada et al. 2015). However, the molecular identity of the synaptic tag and how it leads to the synaptic consolidation still remain to be fully elucidated.

3.2 Synaptic Tag and Trafficking of Synaptic Proteins

There are four criteria that the synaptic tag must satisfy (Fig. 3.1) (Martin and Kosik 2002; Okamoto et al. 2004; Okamoto et al. 2009; Viola et al. 2014). First, it must be formed specifically at potentiated synapses, but not at naïve synapses. Second, it must form without requiring newly synthesized proteins. Third, it must persist for at least 1–2 h, allowing for new proteins to be synthesized and trafficked to the

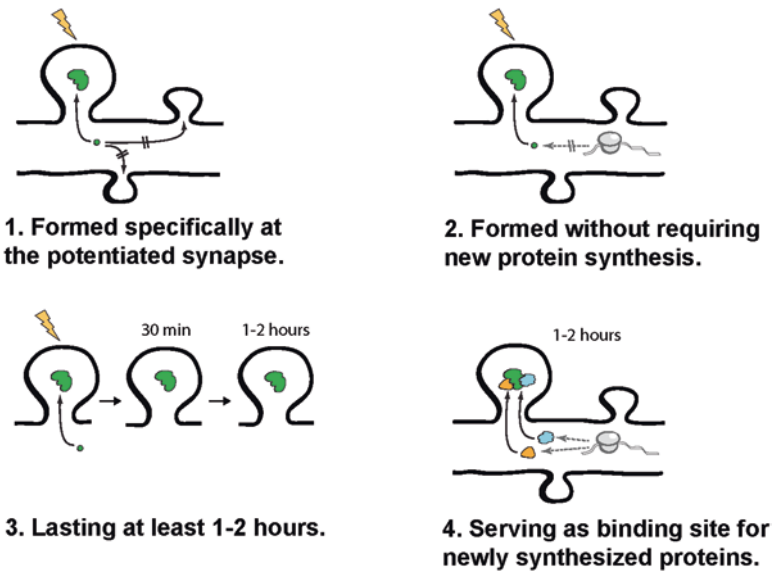

Fig. 3.1 Criteria required for a synaptic tag. (Based on Martin and Kosik 2002; Okamoto et al. 2004; Okamoto et al. 2009; Viola et al. 2014)

potentiated synapses. Finally, it should serve as a binding site to capture newly synthesized proteins.

AMPA-type glutamate receptor molecules (AMPARs) are the main ionotropic glutamate receptor mediating synaptic transmission. The trafficking and trapping of AMPARs at the synaptic site have been considered as the mechanism of LTP expression (Shi et al. 1999; Hayashi et al. 2000; Malinow et al. 2000). AMPARs are anchored at the synapse through the postsynaptic density (PSD), a multimolecular complex of scaffolding proteins, cytoskeletal proteins, and signal transduction molecules (Sheng and Hoogenraad 2007). It follows then that to understand LTP consolidation, it's imperative to know the exact time course of the AMPAR synaptic translocation, the PSD complex remodeling, and the formation of structures that could serve as synaptic tags. To address this question, Bosch et al. tested the translocation of a number of synaptic proteins, each labeled with GFP, upon the induction of LTP in single dendritic spines by photouncaging of caged-glutamate (Bosch et al. 2014) (Fig. 3.2). Glutamate photouncaging induces a rapid expansion of the targeted spine, termed structural LTP (sLTP) (Matsuzaki et al. 2004). At the same time, proteins are translocated to the spine together with AMPARs, but in a distinct order and extent. The first proteins to be concentrated at the spine within a few minutes after the induction of LTP are actin and some of its regulator proteins, including cofilin and the Arp2/3 complex. They are followed by other actin-binding proteins such as profilin, α-actinin 2, drebrin A, and CaMKII (both α and β isoforms), which may stabilize the F-actin. In contrast, PSD scaffold proteins such as PSD-95, Shank, SAP97, and Homer1B do not enter the spine until approximately 60 min later, as they require additional proteins to be newly synthesized (Bosch et al. 2014).

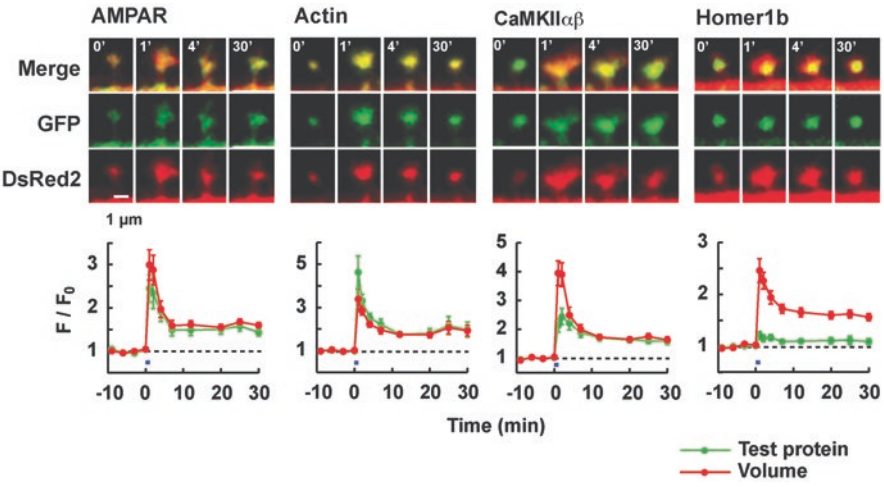

Fig. 3.2 Translocation of AMPAR subunit GluA1, actin, CaMKII α/β, and Homer1B. Each of the test proteins was tagged with GFP (for GluA1, a pH-sensitive superecliptic pHluorin was used to highlight the surface protein) and co-expressed with the red fluorescent protein DsRed2 as a volume filler, in hippocampal CA1 pyramidal neurons. The neuron was observed with a two-photon microscope and sLTP was induced in a single spine by photo-uncaging of caged glutamate. While actin is rapidly translocated to the synapse, Homer1B remains constant. (From Bosch et al. 2014)

3.3 Remodeled F-actin as a Candidate for Synaptic Tag

These results suggest that actin is rapidly regulated within the dendritic spine after LTP induction, which is consistent with the result by Okamoto et al. who used a Förster resonance energy transfer (FRET) approach to visualize the local equilibrium of actin polymerization/depolymerization (Okamoto et al. 2004) (Fig. 3.3). This was achieved by co-expressing donor CFP- and acceptor YFP-labeled actin molecules. When the donor and acceptor actin polymerized to form F-actin, it showed FRET. Indeed, the induction of LTP by a locally implanted electrode induced a sustained (>30 min) increase in FRET, which likely explains the dendritic spine enlargement associated with LTP.

F-actin provides the binding site for a number of postsynaptic proteins (Pollard 2016), including CaMKIIβ subtype, α-actinin, drebrin, myosins, neurabins, cortactin, and synaptopodin, as well as other regulatory proteins, which, in turn, can associate with various other structural or signaling proteins. To test whether the increased F-actin itself is sufficient for synaptic translocation of actin-binding proteins, F-actin was pharmacologically stabilized using phalloidin, and the translocation of CaMKIIβ, which bears an F-actin binding domain, was tested (Okamoto et al. 2004). As a control, CaMKIIα, which has a similar overall structure and molecular weight but lacks the F-actin binding domain, was also tested. As a result, CaMKIIβ was translocated to the synapse by the pharmacological induction of F-actin, but not

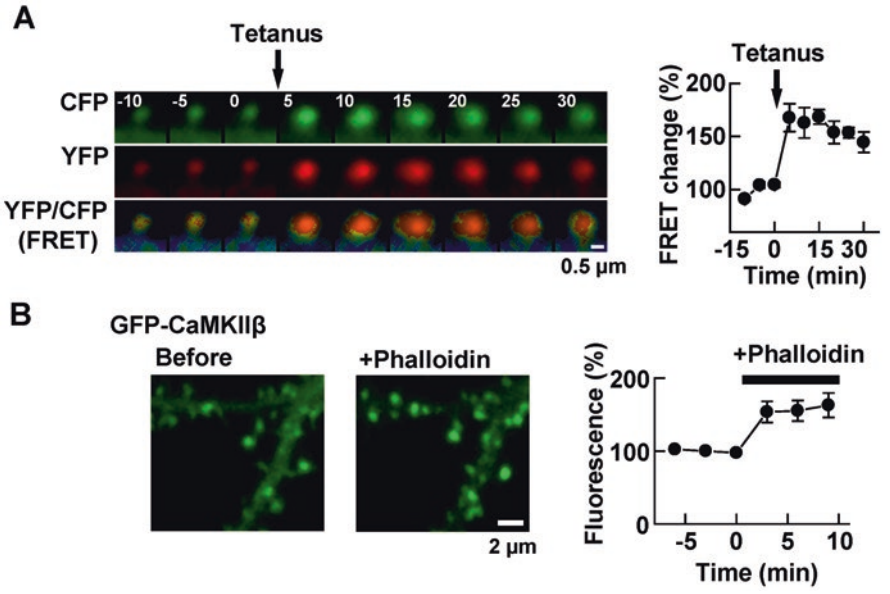

Fig. 3.3 Formation of F-actin is sufficient to induce translocation of an actin-binding protein, CaMKIIβ. (**a**) FRET-based sensor of F-actin formation indicates that LTP induction induces persistent formation of F-actin. (**b**) Pharmacological formation of F-actin by injection of phalloidin was sufficient to synaptically translocate an actin-binding protein, CaMKIIβ, to the synapse. (From Okamoto et al. 2004)

CaMKIIα. CaMKIIα was also translocated when it was co-expressed with CaMKIIβ forming a hetero-oligomer. These results suggest that the increase in F-actin content itself is sufficient for the translocation of CaMKII and most likely other F-actin binding proteins as well.

These properties of newly formed F-actin indeed fulfill the criteria of the synaptic tag (Fig. 3.1) (Okamoto et al. 2004, 2009; Fonseca 2012; Pinho et al. 2020). F-actin is increased only in stimulated spines, but not in other spines. Actin polymerization does not require new protein synthesis. The increased FRET persists for more than 30 min without any sign of decay. Finally, new actin filaments can serve as a binding site for many F-actin binding proteins, which further associate with other postsynaptic proteins. This idea has been tested pharmacologically, with the disruption of F-actin by latrunculin A or cytochalasin D preventing the formation of a synaptic tag in the two-pathway experiments (Ramachandran and Frey 2009; Fonseca 2012).

F-actin can form macrostructures of a higher order, such as bundles and meshes, when actin filaments are cross-linked by specific actin-binding proteins, such as fimbrin, α-actinin, filamin, and also CaMKII (Hotulainen and Hoogenraad 2010; Chazeau et al. 2014; Chazeau and Giannone 2016). Although these structures are already present in naïve spines, the induction of LTP creates new F-actin macrostructures specifically at the potentiated spine with novel properties of higher stability, spine confinement, and different binding capacities (Honkura et al. 2008). One of these macrostructures is the one created by cofilin and F-actin. Although cofilin is an actin depolymerization factor, when present at a high stoichiometric ratio, it is able to stably bind to F-actin and, instead, stabilize the actin filament. Bosch et al. observed the selective formation of such cofilactin filaments at the base of spines that underwent LTP (Andrianantoandro and Pollard 2006; Bosch et al. 2014; Goto et al. 2021). This new structure is not only necessary for the consolidation of sLTP (Bosch et al. 2014) but also for the consolidation of context-specific memory and the sleep-dependent transfer of memory to the cortex (Goto et al. 2021). It can play a crucial role, especially in the early phase of LTP as part of the remodeled actin cytoskeleton that serves as a synaptic tag.

3.4 Liquid–Liquid Phase Separation and CaMKII as Candidate of Synaptic Tag

Another likely and not mutually exclusive candidate of the synaptic tag is CaMKII. It is a highly abundant protein kinase, accounting for 5–20% of all PSD proteins (Kim et al. 2016; Bayer and Schulman 2019; Yasuda et al. 2022). CaMKII has a rotational and planar symmetric structure composed of a hexamer or heptamer of dimers (total 12- or 14-mers) with a central hub domain surrounded by kinase domains. Pharmacological and genetic blockade of this protein significantly impairs LTP as well as learning and memory (Malinow et al. 1988; Malenka et al. 1989; Tsien et al. 1990; Silva et al. 1992). However, it is still not clear why CaMKII exists in such

abundance and why it has such a peculiar structure not seen in other kinases. Furthermore, the amount of CaMKII increases at the dendritic spine after LTP induction and maintains its accumulation over time (Bosch et al. 2014).

Liquid–liquid phase separation (LLPS) is an emerging concept in biology seen in proteins with multivalent weak affinity interactions or those with intrinsically disordered regions, through which the proteins can spontaneously associate with each other and form a liquid-like condensed phase, sometimes referred to as a membrane-less organelle, while excluding other proteins in the noncondensed diluted phase (Hyman et al. 2014; Shin and Brangwynne 2017; Hayashi et al. 2021). The condensate still retains the property of a liquid because it is structurally flexible and the molecules within the condensate are dynamically moving around and exchanged with those in the diluted phase, thereby providing a degree of metastability. The condensate can trap molecules with affinity to the constituent molecules that would not normally have the capacity to undergo LLPS by themselves ("client" proteins). The condensates are not always a homogenous mixture of proteins and can form complex heterogeneous structures such as phase-in-phase architectures, with different functional types of condensate to perform, for example, sequential reactions. From these properties, LLPS is proposed to play an essential role in forming cellular nanodomains of signal transduction and structure by selectively bringing them together.

CaMKII can form stable complexes with substrate proteins such as the carboxyl tail of the NMDAR subtype GluN2B (Bayer et al. 2001). This led to Hosokawa et al. to test if CaMKII undergoes LLPS through its ability to cross-link multiple substrates and pseudosubstrate proteins with its oligomeric structure (Fig. 3.4).

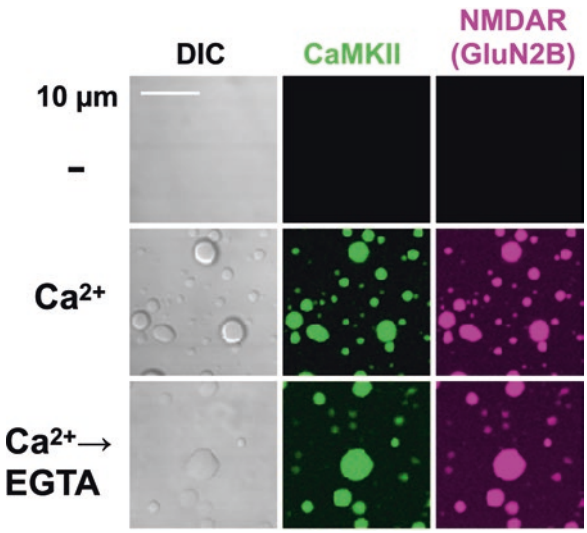

Fig. 3.4 Liquid-liquid phase separation of CaMKII with its substrate GluN2B carboxyl tail. CaMKII and GluN2B carboxyl tail, expressed in and purified from bacteria, fluorescently tagged, were mixed and observed under fluorescent microscopy. (From Hosokawa et al. 2021)

They demonstrated that CaMKII can indeed undergo LLPS with its substrate protein GluN2B (Hosokawa et al. 2021). Interestingly, this is triggered by Ca^{2+}/calmodulin and, once formed, persists even after Ca^{2+} is chelated by EGTA. Further analysis indicates that this is mediated by T286 autophosphorylation, occurs within the autoinhibitory domain, and abolishes the autoinhibition, thereby opening the substrate binding site and allowing persistent binding with substrate proteins. These results indicate that CaMKII is indeed a postsynaptic crosslinker and can retain substrate proteins via an LLPS mechanism. At the same time, the interaction with the carboxyl tail of GluN2B places CaMKII condensates directly beneath the source of Ca^{2+} influx (Bayer et al. 2001).

This CaMKII condensate can also serve as a synaptic tag because it also meets the four criteria (Fig. 3.1): (1) it is specifically formed in synapses that have undergone LTP, (2) without requiring the synthesis of new proteins (CaMKII exists not only in PSD but also in the dendritic cytosol as a freely diffusing fraction, which acts as a reserve pool), (3) lasting >1 h after LTP induction, and (4) serving also as a binding site for many substrate proteins. In a single PSD, there are ~470 dodecamers of CaMKII, which corresponds to ~5600 monomers, whereas only ~20 molecules of GluN2B exist (Sheng and Hoogenraad 2007). Therefore, only a small fraction of CaMKII is occupied by GluN2B, leaving the rest available to interact with other proteins. Besides GluN2B, CaMKII interacts with other substrate proteins such as densin-180, as well as substrate proteins such as Tiam1 (Saneyoshi et al. 2019; Özden et al. 2022; Yasuda et al. 2022). At the same time, there are a myriad of proteins that are phosphorylated by CaMKII (Hudmon and Schulman 2002; Lisman et al. 2002). For example, Stargazin has a CaMKII phosphorylation site at the intracellular carboxyl tail, which inhibits its association to phospholipids in the plasma membrane (Opazo et al. 2010; Sumioka et al. 2010). In addition, Tiam1 and CaMKII form a reciprocal activating kinase effector complex (RAKEC), in which Tiam1 binding maintains CaMKII activity while phosphorylating Tiam1 (Saneyoshi et al. 2019). This interaction is necessary for efficient sLTP (Kojima et al. 2019).

Is CaMKII condensate working independently from F-actin or do they depend on each other? Indeed, the β subtype of CaMKII has an F-actin binding module within the linker sequence between the kinase and association domains, through which CaMKIIβ accumulates with F-actin in neurons (Shen et al. 1998; Okamoto et al. 2007). In contrast, CaMKIIα, which lacks the linker sequence, does not interact with F-actin. Since a single CaMKII oligomer can carry more than one CaMKIIβ, it can interact with F-actin from multiple interfaces. This makes CaMKII an F-actin bundling protein, rather than merely a binding protein (Okamoto et al. 2007). Consistent with this, the overexpression of CaMKIIβ stabilizes F-actin turnover within the dendritic spine by preventing the access of actin-regulating proteins (Okamoto et al. 2007; Kim et al. 2019). Therefore, CaMKII condensate and F-actin are likely to interact with each other at the synapse, together forming a higher order F-actin structure potentially serving as a synaptic tag.

3.5 Role of the Captured Proteins in Synaptic Consolidation

What is the role of the postsynaptic proteins captured by the synaptic tag mechanism? A hint comes from the longitudinal observation of spine protein composition after sLTP induction (Bosch et al. 2014). The major PSD scaffolding proteins, including Homer1B and Shank, are not translocated to the synapse within the first ~30 min. Their total amount at the spine remains the same as before LTP induction. However, their amount starts increasing after around 1 h (Fig. 3.5). Intriguingly, this

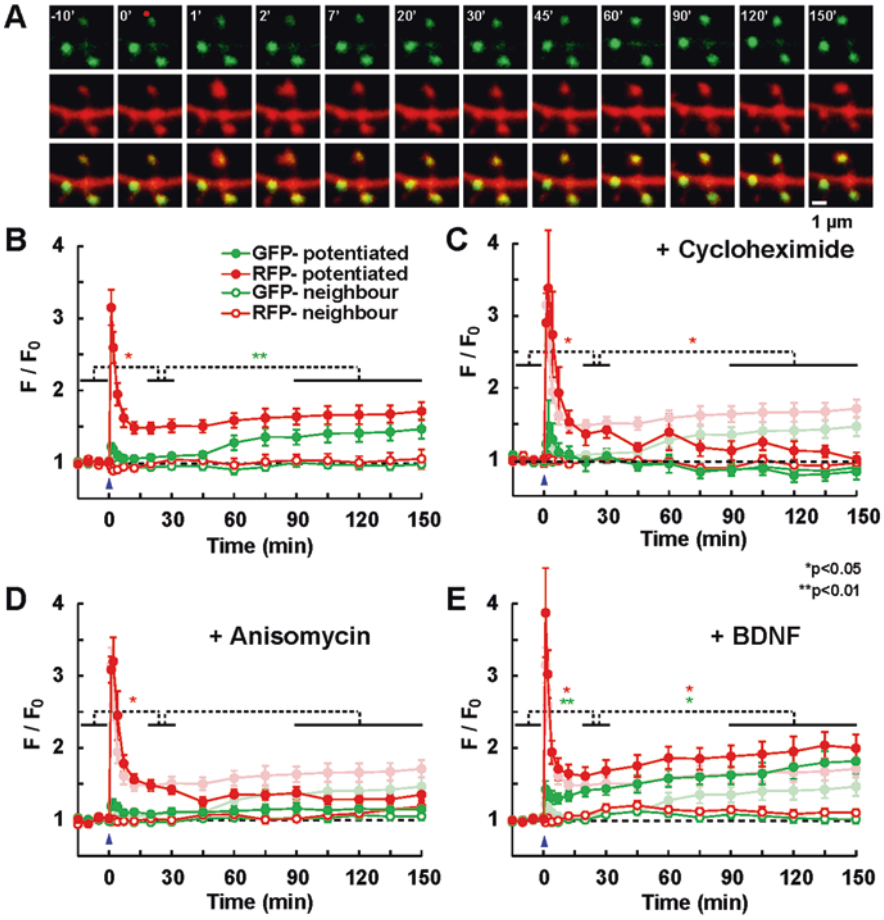

Fig. 3.5 Delayed translocation of PSD scaffolding proteins after LTP induction depending on protein synthesis. (**a**, **b**). Time-lapse images of the dendritic spine expressing GFP-Homer1B (A) and summary data (**b**). Synaptic translocation of GFP-tagged Homer1B was monitored over 150 min after sLTP induction. Translocation did not take place until around 60 min (**c**–**e**). This was blocked by both transcriptional (C. cycloheximide) and translational (D. anisomycin) inhibitors and enhanced by BDNF (**e**), consistent with the property of L-LTP. (From Bosch et al. 2014)

increase can be blocked by the translational inhibitors cycloheximide and anisomycin, which also significantly reduces the extent of sLTP. In contrast, brain-derived neurotrophic factor (BDNF), which is known to promote L-LTP (Pang et al. 2004; Panja and Bramham 2014), enhances both the translocation of Homer1B, as well as sLTP (Tanaka et al. 2008; Bosch et al. 2014). Therefore, the translocation of these proteins is protein synthesis dependent. However, it should be noted that Homer1B and Shank are not the product of neuronal activity-dependent protein synthesis. In this study, these proteins were expressed by using a heterologous promoter (cytomegalovirus (CMV) promoter) and a polyadenylation signal (simian virus 40 (SV40) polyadenylation signal), which are unlikely to be normally modulated by neuronal activity. Indeed, there was no change in the overall brightness of the dendritic branch. Therefore, the translocation of these scaffolding proteins is not dependent on their synthesis, but rather on a yet-to-be-identified newly synthesized protein that serves as an interface between the bona fide synaptic tag and the scaffolding proteins.

What is the consequence of the translocation of PSD scaffolding proteins? It has been demonstrated that the synapse undergoes a synaptic consolidation process after the induction of LTP. Soon after induction (<1 h), the synapse can be easily depotentiated with low-frequency stimulation (Fujii et al. 1991; Huang and Hsu 2001). However, after this initial phase (>1 h), the potentiated synapse becomes more resistant to depotentiating stimulation. This phenomenon is a form of metaplasticity, where plasticity itself is plastic depending on the synapse history. The delayed transport of PSD scaffolding proteins that depend on protein synthesis can explain synaptic lability during E-LTP and synaptic consolidation at L-LTP. Actin polymerization mediates E-LTP, which can be readily reversed by any mechanism that depolymerizes F-actin. However, as the scaffolding proteins are transported to the synapse and added to the PSD, the synapse becomes more resistant. Indeed, many of the PSD scaffolding proteins such as PSD-95, SynGAP, GKAP, Homer1B, and Shank have much slower turnover rates (~30 min), indicating they are much more stable than actin and CaMKII, with turnover rates of ~1 and ~5 mins, respectively (Star et al. 2002; Okamoto et al. 2004; Kuriu et al. 2006; Sharma et al. 2006). The delayed transport of PSD scaffolding proteins can also explain another phenomenon of metaplasticity: LTP is saturated during E-LTP because the PSD cannot grow and accommodate more AMPARs. Only when new scaffolding proteins arrive at the PSD during L-LTP, can more AMPAR be incorporated at the PSD and the synapse can be further potentiated (Frey et al. 1995; Lynch et al. 2013).

3.6 Concluding Remarks

The sequential transport of various postsynaptic molecules to the synapse after LTP induction reasonably explains several synaptic events observed during this period, including synaptic tagging and consolidation. LTP induction triggers a large reorganization of postsynaptic structure and molecular composition in a sequential

manner. Thus, AMPAR trafficking and insertion into the synapse is only the tip of the iceberg of more global events occurring beneath the synapse. The proteins that are transported to the synapse first, without requiring protein synthesis, serve as synaptic tags for proteins that arrive later. We specifically propose that the molecular identity of the synaptic tag is of a cytoskeletal nature, primarily involving F-actin and CaMKII. We propose that the synaptic tag is a remodeled F-actin cytoskeleton, where LTP induces the formation of new actin filaments crossed-linked to form a higher order macromolecule. On one side, cofilin binds to F-actin and stabilizes the whole F-actin macrostructure at the base of the spine. On the other side, close to the PSD, Ca^{2+} influx triggers CaMKII association with GluN2B and forms a cross-linked network with other proteins, such as Tiam1, via a liquid–liquid phase separation mechanism. This meshwork is linked to the new F-actin filaments through the β subunit of CaMKII. This new macrostructure is formed specifically at the potentiated spine within a few minutes, will last for 1–2 h, and its main function will be to capture the appropriate newly synthesized proteins from the dendrite, such as those that will bring the scaffolding proteins and the AMPARs to the PSD.

Although we haven't covered it extensively here, covalent modifications of proteins such as phosphorylation, lipid modification, and ubiquitination also play an important role in this process and should be further elucidated and integrated into the picture described above.

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Conflict of Interest Statement None.

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