JSHC
Japan Society of Histochemistry and Cytochemistry
Established 1959

Acta Histochem. Cytochem. 44 (1): 9–15, 2011
doi:10.1267/ahc.10035

Review

Turnover of Synapse and Dynamic Nature of Synaptic Molecules *In Vitro* and *In Vivo*

Satoru Kondo[1] and Shigeo Okabe[1]

[1]*Department of Cellular Neurobiology, Graduate School of Medicine, University of Tokyo, 7–3–1 Hongo, Bunkyo-ku, Tokyo 113–0033, Japan*

Received November 18, 2010; accepted January 14, 2011; published online February 23, 2011

Recent advances of imaging techniques have enabled us to investigate the dynamics of synapses in living neurons. The synapse is constructed of presynaptic and postsynaptic elements which contain various kinds of structural and functional molecules. The postsynaptic density (PSD) is the most prominent structure among the excitatory postsynaptic elements. One of the main components of PSD is the scaffolding proteins which interact with multiple proteins in the synapse. Scaffolding proteins are suggested to play key roles in the emergence, maintenance, and remodeling of the excitatory synapses. Several kinds of scaffolding proteins are known to be present in the mammalian and also other vertebrate brains. These proteins were labeled with green fluorescent protein (GFP) and expressed in cultured neurons to analyze the dynamics and turnover of molecules in the synapses. In this review we describe how these molecules behave when the synapse is newly added or eliminated in the steady state and also when neuronal activity is changed.

Key words: excitatory synapse, turnover, postsynaptic density, scaffolding proteins, imaging

I. Introduction

It was once believed that synapses formed static and stable structures that were maintained as such for extended periods of time. However, imaging techniques have revealed that the synapse is actually a dynamic structure that appears and disappears repeatedly during development [10] even after maturation of the neuronal circuit [6, 18]. Furthermore it has been shown recently that the some plastic changes of synaptic transmission such as long-term potentiation (LTP) cause the enlargement of spine [11] where most of the excitatory synapses are made. The synapse contains various kinds of molecules that are the bases for the function and construction of synapses [2]. Of the postsynaptic elements the postsynaptic density (PSD) is the most prominent structure comprising adhesion molecules, neurotransmitter receptors, ion channels, scaffolding proteins, and cytoskeletal proteins [13]. Among them the scaffolding proteins are a quite important component, because they interact with the other constituents of the synapse listed above. Adhesion molecules and membrane receptors are located on the plasma membrane, while cytoskeletal proteins are located in the cytoplasm. Scaffolding proteins show the characteristic spatial localization linking the membrane proteins and cytoplasmic cytoskeletal proteins or signaling molecules [13]. Scaffolding proteins function not only as anchors of membrane proteins but also as transducers of signals from plasma membrane to cytoplasm. It should be a fundamental question to ask whether synaptic molecules are also dynamic or static. To this end we developed a new technique to visualize the dynamics of synaptic molecules. We tagged scaffolding proteins with fluorescent proteins so that these fused molecules were exogenously expressed in cultured neurons. By performing time-lapse imaging or fluorescence recovery after photobleaching (FRAP) imaging we investigated the behavior of synaptic molecules during development or while manipulating synaptic activity. We will also review some recent *in vivo* experiments.

Correspondence to: Shigeo Okabe, M.D., Ph.D., Department of Cellular Neurobiology, Graduate School of Medicine, University of Tokyo, 7–3–1 Hongo, Bunkyo-ku, Tokyo 113–0033, Japan.
E-mail: okabe@m.u-tokyo.ac.jp

This paper was presented at the Japan/US Joint Symposium on "New Insight in Bio-imaging", 51st Annual Meeting of Japan Society of Histochemistry and Cytochemistry (Tokyo, 2010)

II. Scaffolding Proteins

Various kinds of scaffolding proteins are known to be enriched in the PSD, but here we just focus on a limited number of them that are relatively abundant in the PSD or have direct interaction with glutamate receptors. These are PSD-95, Shank, Homer, and GKAP proteins [13] (Fig. 1). Each scaffolding protein has the characteristic domain sequences and associates with other molecules through these domains to form heteromeric complexes. Among them PSD-95 is the best studied and most abundant of the molecules. PSD-95 has several domains, PDZ, SH3, and GK domains. The PDZ domain interacts with glutamate receptor (NMDA-type receptor), adhesion molecule (neuroligin), and ion channels (voltage-gated potassium channel). The GK domain binds to GKAP, another type of scaffolding protein. PSD-95 is also indirectly coupled to an AMPA-type glutamate receptor through interaction with the transmembrane AMPA receptor regulatory protein (TARP). Shank has also several domains, PDZ, SH3, and proline-rich domains. The PDZ domain interacts with GKAP, while the proline-rich domain binds to Homer and cortactin, an actin binding protein. Therefore Shank forms multiple complexes with three other scaffolding proteins, PSD-95/GKAP/Shank/Homer. Because this complex can bind to actin filaments through cortactin, there is a possibility that the arrangement of the receptors, adhesion molecules, and ion channels on the plasma membrane are regulated by them. Homer has EVH and coiled-coil domains (except one type of Homer). The EVH domain interacts with metabotropic glutamate receptors, receptors for calcium release (IP3 and ryanodine receptors), and Shank. Homer forms multimerized structures through coiled-coil domain. Quantitative analysis showed that these four proteins are estimated to share about 12% of PSD molecules suggesting the main framework of PSD [16].

Scaffolding proteins form large heteromeric complex structures and have long been regarded as rigid and static molecules from biochemical and histological studies.

III. Synapse Turnover Studies *In Vitro*

In this section we will show that scaffolding proteins are not static but rather fluid molecules that are redistributed by neuronal activity, although they are the main structural components in the synapse. Currently most of the studies regarding the turnover of synaptic molecules are done in *in vitro* culture systems. To analyze the turnover of scaffolding molecules in the synapse we visualized them by adding fluorescent protein tags that were expressed in cultured neurons. This technique enabled us to track molecular behavior in conjunction with the appearance and disappearance of synapses in steady state or under neuronal stimulation.

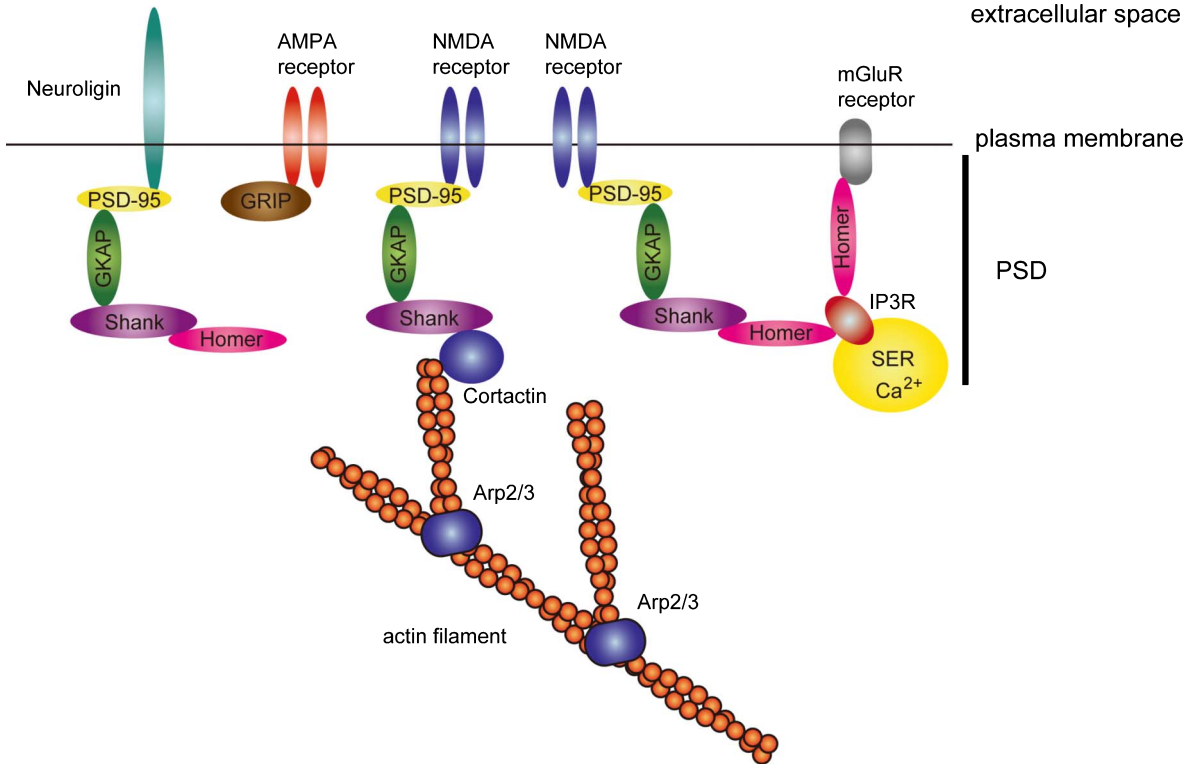

Fig. 1. Molecular construction of the excitatory synapse. Molecules that appear in the text are illustrated. PSD is the most prominent structure of the postsynaptic elements and are localized just beneath the plasma membrane. PSD consists of various kinds of molecules. Scaffolding proteins are the major component of PSD and they interact with both membrane proteins and cytoplasmic proteins. Scaffolding proteins are regarded important for the emergence, maintenance, and remodeling of the synapse.

Hippocampal neuronal culture

Much of the knowledge about the synapse dynamics in the CNS *in vitro* owes to hippocampal dissociated culture [19]. The excitatory synapse formation and elimination can be observed in low-density culture where both axonal and dendritic density is relatively sparse. Functional excitatory synapses start to appear within one week after plating and earlier studies assumed that functional molecules are accumulated slowly long after the morphological synapses are established. It further indicated that functional synapses develop over many days, and that functional and structural molecules are recruited slowly during this time period in a sequential manner [9]. These developmental processes were deduced from static fixed cells. Live imaging is necessary to determine the dynamic properties of structures and molecules. The availability of fluorescent proteins, establishment of transfection techniques of genes into cultured neurons, and development of live imaging systems enabled the investigation of the dynamics of fluorescent protein-labeled molecules in living neurons. Recent imaging approaches from several laboratories, including ours, revealed that, contrary to earlier studies, synaptic molecules are quite dynamic and individual synapses can form within a few hours.

Turnover of PSD-95 proteins

As we have already discussed PSD-95 is the most abundant protein among scaffolding molecules. When we exogenously expressed the GFP-tagged PSD-95 in cultured neurons, we observed that PSD-95 was properly accumulated and localized to the postsynaptic elements [14] (Fig. 2a–d).

Next the time-lapse imaging of GFP-PSD-95 revealed that this accumulation is a dynamic structure and showed that more than 20% of PSD-95 clusters are newly formed or eliminated within 24 hr (Fig. 2e–g). This turnover is a neuronal activity-dependent phenomenon because it is inhibited by applying a blocker for excitatory synaptic transmission [14]. This raises the question whether this turnover of molecules indicates the turnover of the synapse. Synapse formation occurs as a sequence of events, where the attachment of presynaptic and postsynaptic elements is followed by the accumulation of structural and functional molecules. Therefore we labeled the presynaptic element with synaptophysin tagged with cyan fluorescent protein (CFP) and simultaneously imaged the PSD-95 tagged with yellow fluorescent protein (YFP) (Fig. 3a). Time-lapse imaging showed that accumulation of synaptophysin and PSD-95 occurred simultaneously [15] (Fig. 3b, c). In agreement with our observation the synchronized accumulation of presynaptic and postsynaptic proteins was also demonstrated from the other laboratories [1, 5]. Furthermore the accumulation of PSD-95 proteins is correlated with the formation of dendritic spines. These results suggest that the accumulation of pre- and postsynaptic molecules and

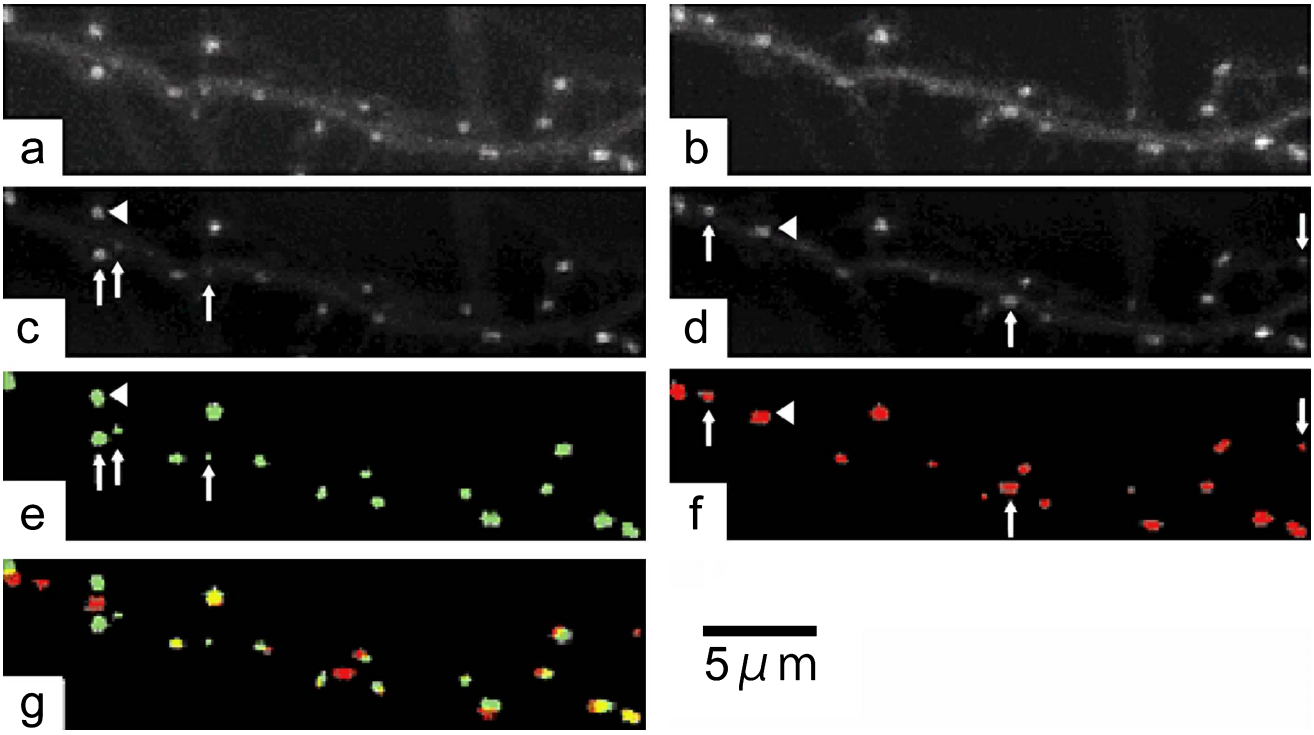

Fig. 2. Turnover of PSD-95 clusters. GFP-tagged PSD-95 molecules are expressed in cultured hippocampal pyramidal neurons. Time-lapse imaging of PSD-clusters was carried out at 24 hr intervals (**a, b**). After the subtraction of background (**c, d**) binary images show the extracted PSD-95 clusters (green: before, red: after) (**e, f**). Superimposition of images (**e**) and (**f**) reveals the newly formed and lost clusters (**g**). Arrows in (**e**) indicate the lost and in (**f**) indicate newly formed clusters. Arrowheads indicate the unmatched clusters potentially generated by the spine movement.

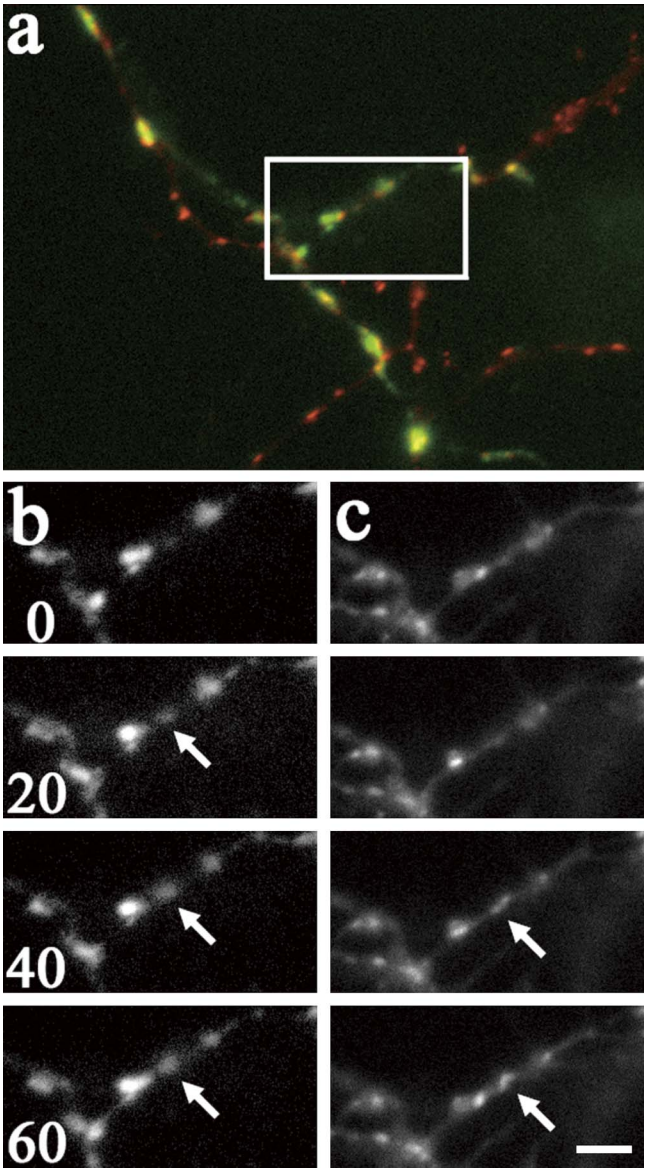

Fig. 3. Simultaneous observation of postsynaptic PSD-95 clusters and presynaptic synaptophysin clusters. YFP-tagged PSD-95 and CFP-tagged synaptophysin, expressed in cultured hippocampal pyramidal neurons, are imaged and analyzed to determine the dynamics of these protein clusters. Superposition of these two clusters on the pyramidal neurons shows the contact of a single axon on a dendritic arbor (**a**). Time-lapse images from the white box in (**a**) are shown in (**b:** synaptophysin) and (**c:** PSD-95). A new cluster of synaptophysin was first detected at t=20 min, and this fluorescence gradually increased. A fluorescence signal of PSD-95 was first detected at the location of the new synaptophysin at t=40 min. The fluorescence signal of this cluster also increased.

synapse formation are correlated, and that the establishment of the formation of functional synapses is completed within a few hours. These live cell imaging approaches indicate that, at least *in vitro*, the formation of individual synaptic connections is rather a rapid process than was previously assumed.

Long-term remodeling of PSD-95 clusters

The next question is whether the synapses that were formed within a short time period are maintained for an extended period of time as a stable structure. Although the gene transfer technique to introduce GFP-tagged synaptic molecules enables the experiments to observe the dynamics of synaptic molecules in relatively short time intervals ranging from seconds to hours or up to a day, prolonged observation over days and weeks is difficult. The problem is mainly derived from the instability of the expression level of the GFP-tagged molecules in individual cells introduced by transfection or virus-mediated gene transfer over a long time scale. To achieve stable expression we generated a transgenic mouse that expresses GFP-tagged PSD-95 (PSD-95-GFP) and solved the problem by preparing neuronal cultures from this animal. Expression of PSD-95-GFP remained stable and the remodeling process could be followed for more than a week. We examined the turnover of PSD-95-GFP clusters by using this stable expression culture system [4]. The turnover within one day was identical to that shown in the transient expression culture system. PSD-95 clusters showed transient lifetime and also rapid increase within 24 hr. Long-term imaging at 1 day intervals over a period of 5 days revealed the unstable structure of the newly formed PSD-95-GFP clusters. The temporal profile of PSD-95 cluster density cannot be described as a simple pattern of increase; some showed a monotonous increase of clusters while others showed complex remodeling. These results indicated that the neuronal circuit formation cannot be described by the simple increase of new synapses. This dynamic structure might be regulated by a kind of homeostatic mechanism maintaining the neuronal activity constant.

Imaging studies combining hippocampal culture and GFP-tagged synaptic molecule provided data that furthered our understanding of the turnover of molecules in living neurons. Similar techniques were applied to NMDA and AMPA glutamate receptors which demonstrated that these molecules are also accumulated at the synapse in a similar time course as PSD-95 after the first contact of pre- and postsynaptic elements [5].

Neuronal activity-dependent redistribution of PSD scaffolding proteins

Changes in synaptic connections are essential for the development and function of the nervous system. The strength and number of connections can undergo rapid and extensive changes after sensory stimulation and learning, indicating these changes are neuronal activity-dependent [18, 20]. It should be quite important to know the properties of dynamics of synaptic molecules during the remodeling of synaptic connections. We considered four types of PSD proteins and examined the changes of localization by adding fluorescent protein tags to the PSD molecules in the hippocampal neuronal culture. We applied FRAP method to examine their turnover [8] (Fig. 4). Under unstimulated conditions where neuronal activity is not manipulated, each molecule showed characteristic behavior. PSD-95 showed

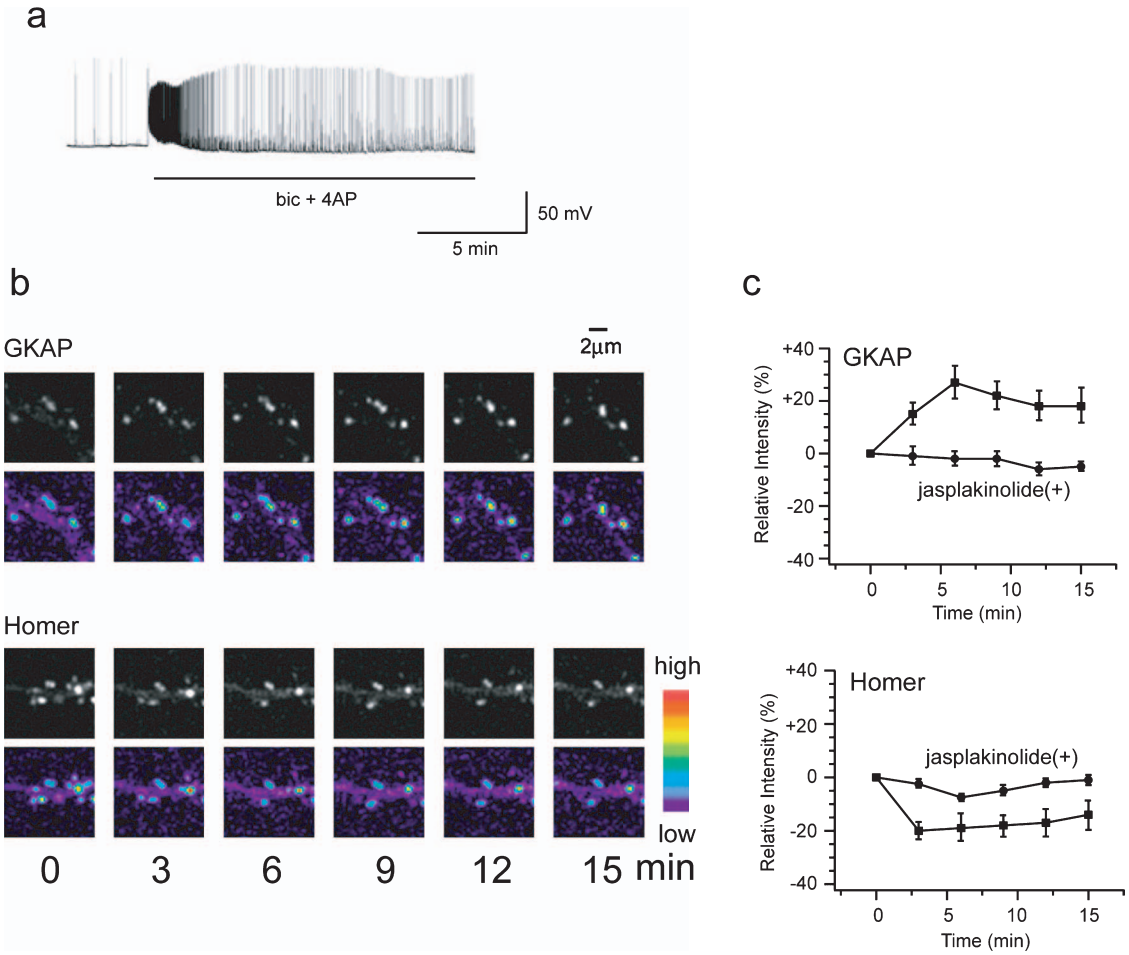

Fig. 4. Activity-dependent redistribution of scaffolding proteins. Bicuculline (50 mM) and 4-AP (500 mM) treatment enhanced the neuronal activities in the cultured hippocampal pyramidal neurons (**a**). Enhancement of neuronal activities by bicuculline and 4-AP induced the redistribution of scaffolding proteins. The intensity of GKAP clusters increased, while the intensity of Homer clusters decreased (**b, c**). The enhanced dynamics of the GKAP and Homer by bicuculline and 4-AP is suppressed by the application of jasplakinolide (an actin-stabilizing compound, 5 mM) (**c**).

the slowest while Homer had the fastest turnover in the synapse. GKAP and Shank were in the middle range between PSD-95 and Homer. We manipulated an increase in synaptic transmission and neuronal activity by adding bicuculline (a GABAA-receptor blocker) and 4-AP (a potassium channel blocker) into the culture medium (Fig. 4a). The localization of PSD molecules was analyzed under this synaptically-evoked condition. Redistribution of PSD-95 was not induced but identical stimulation induced rapid dissociation of Homer and Shank clusters. The response of GKAP was different from other PSD molecules, showing the accumulation of clusters to the postsynaptic sites (Fig. 4b, c). These results indicate that the dynamics and localization of each PSD scaffolding protein is independently modulated under the increase of synaptic transmission. Further pharmacological experiments revealed that these activity-dependent redistributions of Homer, Shank, and GKAP are related with actin cytoskeleton dynamics. Interestingly turnover of PSD-95 is independent of the neuronal activity and actin dynamics and PSD-95 clusters are less dynamic structures in the synapse. Actin dynamics are known to be altered by the increased strength of synaptic connections like long-term potentiation (LTP) and are related with the morphological enlargement of spine structure. Our findings suggest that the molecular components of PSD scaffolding proteins are also regulated by the neuronal activity changes. Because the changes of scaffolding proteins are actin-dependent, actin binding molecules, which mediate the binding between PSD scaffolding proteins and actin, could play important roles for the regulation of redistribution.

IV. Dynamic Aspects of Synaptic Proteins Revealed *In Vivo*

In the previous section we focused on the approaches for applying live optical imaging to the synapse and synaptic molecules in the cultured nervous system. *In vitro* study of cultured neurons provides a simplified system that is particularly useful for understanding the cell biology of individual synapses. Imaging of neurons *in vivo* could

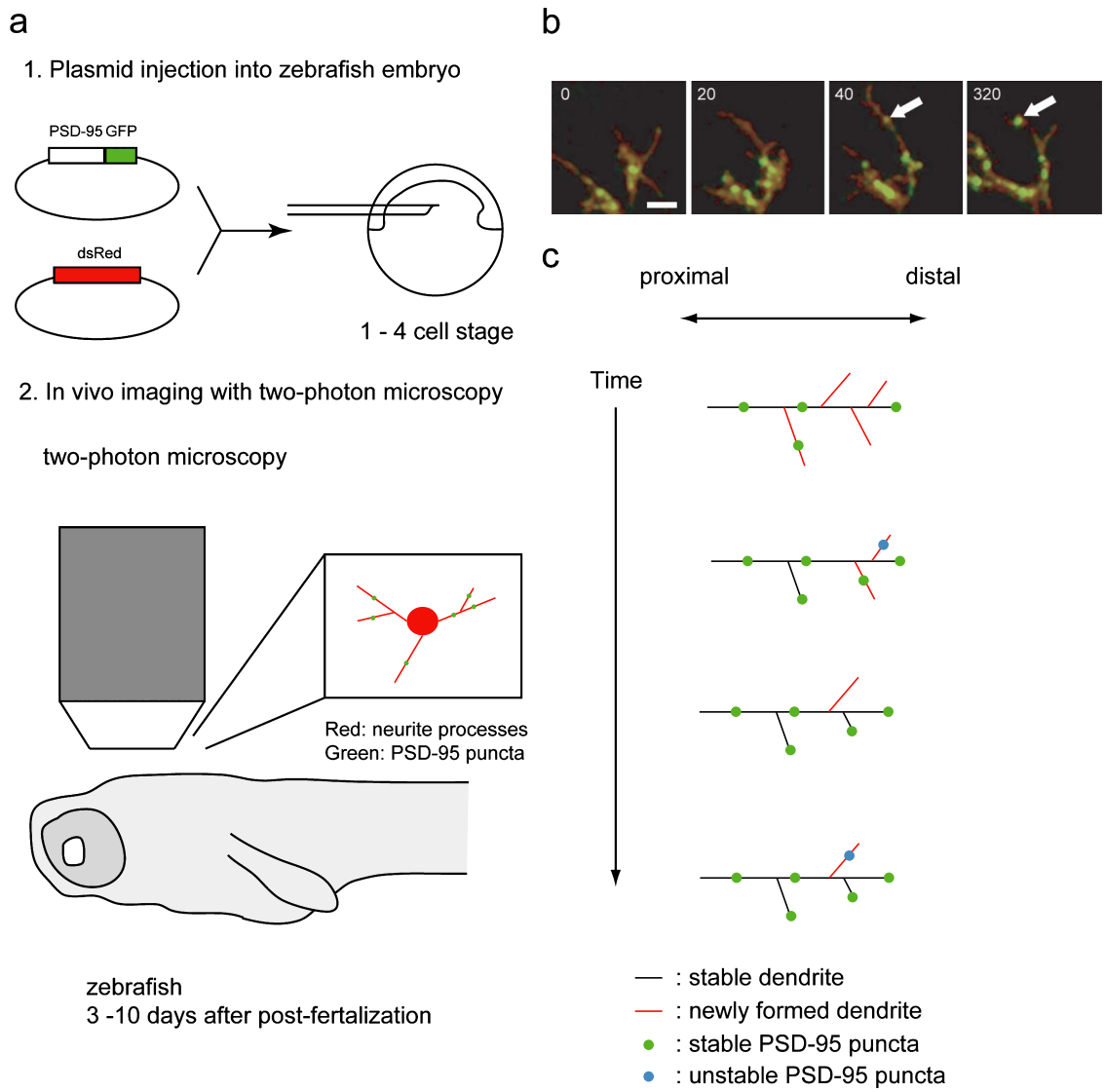

Fig. 5. Dynamics of PSD-95 puncta and dendritic maturation *in vivo*. Two types of vector (PSD-95-GFP and dsRed) are injected into zebrafish embryo at 1–4 cell stage (**a-1**). *In vivo* imagings are performed at 3–10 days after post-fertilization with two-photon microscopy. Neurite processes are visualized with dsRed and localizations of PSD-95 are visualized with GFP (**a-2**). Time-lapse imagings show the growth of dendritic arbor (dsRed) and new PSD-95 puncta (green). The maturation of dendrite is correlated with the maturation of PSD-95 puncta (white arrow) (**b**). The diagram shows that the growth of dendritic arbor is concurrent with PSD-95 punctum formation. The newly formed dendrites that obtained stable PSD-95 puncta become the stable dendrites (**c**). Bar=3 μm.

present a more top-down approach by watching the process of dynamics within its natural context. The reason that not all the experiments are done *in vivo* is related to the difficulty to introduce genes exogenously in the living animals and to acquire deep imaging. A significant advance in *in vivo* imaging is the development of the two-photon microscope which allows three-dimensional imaging of the deep tissues [7]. This *in vivo* imaging technique was applied to the zebrafish together with the gene transfer method to inject plasmid DNA into the early embryo. Zebrafish is an ideal organism, because the larvae are nearly transparent. Niell *et al.* performed the long-term *in vivo* imaging of GFP-tagged PSD-95 proteins in the zebrafish immature tectal neurons [12]. They observed puncta of PSD-95-GFP and classified them into two categories, unstable or stable, depending on their lifetime by setting the threshold at 3 hr. Time-lapse imaging demonstrated that PSD-95 puncta are distributed along the dendritic arbor, and that unstable PSD-95 puncta were associated with the elimination of newly formed filopodial dendrites. On the contrary, stable puncta of PSD-95 were associated with the stabilization of filopodial dendrites and formation of synapses. In the tectal neurons of zebrafish the stabilized synapses with PSD-95 resulted in the stabilization of dendritic arborization and growth (Fig. 5).

V. Conclusions

We have shown that synapses are dynamic structures that repeatedly appear and disappear. Scaffolding proteins were once regarded as rigid structural proteins forming large molecular complexes in the synapse. However, imaging approaches revealed a surprisingly high rate of turnover and mobility of scaffolding proteins. The structure and composition of scaffolding proteins are changeable and modified by neuronal activity. Several genetic studies have suggested that mutations, deficiencies, and reductions of the scaffolding proteins are associated with pathological states such as developmental disorders [3], schizophrenia [17], and drug abuse [21]. These dynamic features of scaffolding proteins should regulate the precise neuronal functions which were not possible to observe by traditional histological analyses.

VI. References

1. Ahmari, S. E., Buchanan, J. and Smith, S. J. (2000) Assembly of presynaptic active zones from cytoplasmic transport packets. *Nat. Neurosci.* 3; 445–451.
2. Chua, J. J., Kindler, S., Boyken, J. and Jahn, R. (2010) The architecture of an excitatory synapse. *J. Cell Sci.* 123; 819–823.
3. Durand, C. M., Betancur, C., Boeckers, T. M., Bockmann, J., Chaste, P., Fauchereau, F., Nygren, G., Rastam, M., Gillberg, I. C., Anckarsäter, H., Sponheim, E., Goubran-Botros, H., Delorme, R., Chabane, N., Mouren-Simeoni, M. C., de Mas, P., Bieth, E., Rogé, B., Héron, D., Burglen, L., Gillberg, C., Leboyer, M. and Bourgeron, T. (2007) Mutations in the gene encoding the synaptic scaffolding protein SHANK3 are associated with autism spectrum disorders. *Nat. Genet.* 39; 25–27.
4. Ebihara, T., Kawabata, I., Usui, S., Sobue, K. and Okabe, S. (2003) Synchronized formation and remodeling of postsynaptic densities: Long-term visualization of hippocampal neurons expressing postsynaptic density proteins tagged with green fluorescent protein. *J. Neurosci.* 23; 2170–2181.
5. Friedman, H. V., Bresler, T., Garner, C. C. and Ziv, N. E. (2000) Assembly of new individual excitatory synapses: time course and temporal order of synaptic molecule recruitment. *Neuron* 27; 57–69.
6. Grutzendler, J., Kasthuri, N. and Gan, W. B. (2002) Long-term dendritic spine stability in the adult cortex. *Nature* 420; 812–816.
7. Helmchen, F. and Denk, W. (2005) Deep tissue two-photon microscopy. *Nat. Methods* 2; 932–940.
8. Kuriu, T., Inoue, A., Bito, H., Sobue, K. and Okabe, S. (2006) Differential control of postsynaptic density scaffolds via actin-dependent and -independent mechanisms. *J. Neurosci.* 26; 7693–7706.
9. Lee, S. H. and Sheng, M. (2000) Development of neuron-neuron synapses. *Curr. Opin. Neurobiol.* 10; 125–131.
10. Lendvai, B., Stern, E. A., Chen, B. and Svoboda, K. (2000) Experience-dependent plasticity of dendritic spines in the developing rat barrel cortex in vivo. *Nature* 404; 876–881.
11. Matsuzaki, M., Honkura, N., Ellis-Davies, G. C. and Kasai, H. (2004) Structural basis of long-term potentiation in single dendritic spines. *Nature* 429; 761–766.
12. Niell, C. M., Meyer, M. P. and Smith, S. J. (2004) In vivo imaging of synapse formation on a growing dendritic arbor. *Nat. Neurosci.* 7; 254–260.
13. Okabe, S. (2007) Molecular anatomy of the postsynaptic density. *Mol. Cell. Neurosci.* 34; 503–518.
14. Okabe, S., Kim, H. D., Miwa, A., Kuriu, T. and Okado, H. (1999) Continual remodeling of postsynaptic density and its regulation by synaptic activity. *Nat. Neurosci.* 2; 804–811.
15. Okabe, S., Miwa, A. and Okado, H. (2001) Spine formation and correlated assembly of presynaptic and postsynaptic molecules. *J. Neurosci.* 21; 6105–6114.
16. Sugiyama, Y., Kawabata, I., Sobue, K. and Okabe, S. (2005) Determination of absolute protein numbers in single synapses by a GFP-based calibration technique. *Nat. Methods* 9; 677–684.
17. Szumlinski, K. K., Lominac, K. D., Kleschen, M. J., Oleson, E. B., Dehoff, M. H., Schwarz, M. K., Seeburg, P. H., Worley, P. F. and Kalivas, P. W. (2005) Behavioral and neurochemical phenotyping of Homer1 mutant mice: possible relevance to schizophrenia. *Genes Brain Behav.* 4; 273–288.
18. Trachtenberg, J. T., Chen, B. E., Knott, G. W., Feng, G., Sanes, J. R., Welker, E. and Svoboda, K. (2002) Long-term in vivo imaging of experience-dependent synaptic plasticity in adult cortex. *Nature* 420; 788–794.
19. Verderio, C., Coco, S., Pravettoni, E., Bacci, A. and Matteoli, M. (1999) Synaptogenesis in hippocampal cultures. *Cell. Mol. Life Sci.* 55; 1448–1462.
20. Xu, T., Yu, X., Perlik, A. J., Tobin, W. F., Zweig, J. A., Tennant, K., Jones, T. and Zuo, Y. (2009) Rapid formation and selective stabilization of synapses for enduring motor memories. *Nature* 462; 915–919.
21. Yao, W. D., Gainetdinov, R. R., Arbuckle, M. I., Sotonikova, T. D., Cyr, M., Beaulieu, J. M., Torres, G. E., Grant, S. G. and Caron, M. G. (2004) Identification of PSD-95 as a regulator of dopamine-mediated synaptic and behavioral plasticity. *Neuron* 41; 625–638.