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Dynamic imaging of AMPA receptor trafficking *in vitro* and *in vivo*

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Abstract

Modulation of synaptic strength through trafficking of AMPA receptors is a fundamental mechanism underlying synaptic plasticity and has been shown to be an important process in higher brain functions such as learning and memory. Many studies have used live time-lapse imaging of fluorescently tagged AMPA receptors to directly monitor their membrane trafficking in the basal state as well as during synaptic plasticity. While most of these studies are performed *in vitro* using neuronal cell cultures, in the past years technological advances have enabled the imaging of synaptic proteins *in vivo* in intact organisms. This has allowed for visualization of synaptic plasticity on a molecular level in living and behaving animals. Here, we discuss key studies and approaches using dynamic imaging to visualize AMPA receptor trafficking *in vitro* as well as imaging synaptic proteins, including AMPA receptors, *in vivo*.

Introduction

The human brain is arguably one of the most intricate structures in the universe. It is composed of over 100 billion neurons and can perform highly complex computations. Importantly, it is able to constantly adapt to new situations, allowing us to form memories of our experiences. In the brain, neurons are connected with one another by as many as 10 000 synapses per neuron. The formation and elimination of synaptic connections and the efficacy of existing synapses can be modified by experience. This dynamic process is called synaptic plasticity and is critical for higher brain function such as learning and memory. Understanding the molecular mechanisms underlying synaptic transmission and plasticity is one of the fundamental questions in neuroscience.

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Synaptic transmission is mediated by the binding of neurotransmitters released from activated presynaptic neurons to postsynaptic receptors. GABA and glycine are the major inhibitory neurotransmitters that bind to inhibitory receptors, glutamate is the principal excitatory neurotransmitter in the mammalian brain. While metabotropic glutamate receptors (mGluRs) and NMDA receptors (NMDARs) play a more regulatory role, AMPA receptors (AMPA) are the workhorse of fast excitatory synaptic transmission in the central nervous system. AMPARs are tetrameric ligand-gated ion channels consisting of different combinations of the four subunits GluA1–4.

Widely regarded as the cellular basis for learning and memory, synaptic plasticity involves long-lasting changes in the molecular and cellular architecture of a neuron that determine how readily it will generate an action potential in response to a particular presynaptic input. Among the most studied forms of synaptic plasticity are long-term potentiation (LTP) and long-term depression (LTD), wherein the strength of synaptic transmission is increased and decreased, respectively. At the molecular level, both LTP and LTD are mediated by sustained changes in the number and activity of AMPARs in the postsynaptic membrane. Thus, regulation of AMPAR membrane trafficking is a key cellular mechanism underlying synaptic plasticity, learning, and memory [1,2].

Trafficking of AMPARs in and out of synapses is a dynamic process involving endosomal trafficking as well as lateral diffusion along the plasma membrane [3]. Disruption of proper AMPAR function or trafficking impairs synaptic plasticity and, consequently, learning and memory. Genetic alterations in AMPARs or proteins that regulate AMPAR trafficking have been implicated in various diseases, including autism spectrum disorders, schizophrenia, Alzheimer's disease, and intellectual disability [4]. Thus, understanding the molecular processes governing experience-dependent changes in AMPAR trafficking is crucial to understanding the cellular basis of behavior as well as many neurological diseases. In this review, we will discuss key findings in the field of AMPAR trafficking with a focus on studies using time-lapse and longitudinal imaging approaches in primary dissociated cultured neurons or organotypic brain slice cultures (*in vitro*) and in living animals (*in vivo*).

Dynamic imaging of AMPA receptor trafficking *in vitro*

Biochemical, cell biological, and electrophysiological tools have been extensively used to study synaptic plasticity and have greatly advanced our knowledge of the underlying cellular and molecular mechanisms. However, optical microscopy allows for direct observation of cells and molecules. Immunohistochemistry in fixed brain slices and dissociated neuronal cultures have revealed the spatial distribution of AMPARs in basal as well as stimulated conditions [5–7]. However, due to the dynamic nature of AMPAR trafficking, only live imaging allows for monitoring of changes in individual synapses in real time.

An important technological advance allowing for time-lapse imaging of AMPAR proteins was the tagging of AMPAR subunits with fluorescent proteins (Figure 1a). After confirming that fusion of green fluorescent protein (GFP) with AMPARs does not affect its channel properties, Shi *et al.* [8**] imaged these GFP-tagged AMPARs in cell culture and brain slices and showed that the pattern of expression mimics the distribution of endogenous protein.

Using this approach, they observed that there are few GluA1 subunit-containing receptors in spines in the basal state, but with tetanic synaptic stimulation, GluA1 accumulated in spines as well as in clusters along the dendrite.

Although useful, GFP-tagged AMPARs cannot be used to distinguish between intracellular receptors and receptors inserted into the plasma membrane. This distinction is critical, since only membrane-bound AMPARs are active and participate in synaptic transmission. To selectively visualize membrane inserted AMPARs, Ashby *et al.* [9**] used super-ecliptic-pHluorin (SEP) rather than GFP to tag AMPARs. SEP is a pH-sensitive version of GFP that is brightly fluorescent at neutral pH values (around 7.4), but quenched in acidic environments [10]. AMPAR insertion into and out of the cell plasma membrane is mediated by exocytosis and endocytosis [11,12]. Intracellularly, AMPARs pass through the acidic environments of the secretory and endocytic pathways. When present on the membrane, they are exposed to the pH-neutral extracellular space. Thus, by inserting SEP in the extracellular or lumen-exposed terminal (N-terminus) of AMPARs, the majority of fluorescent signal will originate from receptors at the cell surface (Figure 1a and c) with a small fraction of fluorescent signal from AMPARs in the pH-neutral lumen of the endoplasmic reticulum [13].

This approach allowed for the study of surface localization of different AMPAR subunits, membrane trafficking of AMPARs following LTP- or LTD-inducing stimuli, as well as visualization of discrete insertion events. In dissociated neuronal cultures and hippocampal slice cultures, synaptic potentiation can be induced pharmacologically by bath applying glycine or forskolin (chemical LTP), or by uncaging glutamate at single spines. These systems have been used to show that surface expression of GluA1 and GluA2 in spines is increased after LTP induction [14–17] (Figure 1b and c). The addition of AMPARs at the spine surface can occur via lateral diffusion from the dendritic shaft or through exocytosis from intracellular compartments (Figure 1c). One way to distinguish between these two mechanisms is to combine time-lapse imaging of SEP-tagged AMPARs with fluorescence recovery after photo-bleaching (FRAP). After photobleaching the surface population of AMPARs in a large region, only newly exocytosed receptors will be visible [18*,19*]. While lateral diffusion is constantly replenishing AMPARs at spines [19*,20*,21], the increase in surface AMPARs after LTP requires exocytosis [15]. However, whether exocytosis of AMPARs occurs directly in spines or at dendrites adjacent to spines, followed by lateral diffusion into spines is still debated [3]. Using total internal reflection fluorescence (TIRF) microscopy, it is possible to directly visualize individual insertion events of AMPARs in primary neuronal cultures [22*,23*,24]. These studies showed that the majority of insertion events happen in the soma and dendritic shafts and that AMPARs released from these insertions sites can diffuse into spines. However, during synaptic activity or LTP, exocytosis directly in spines has also been observed [14,25,26].

Application of the NMDAR agonist NMDA to cultured neurons induces long-lasting depotentiation of synapses (chemical LTD) [27]. Imaging SEP-tagged AMPARs during this process has revealed a rapid cell-wide decrease in surface receptors followed by a slow recovery [9**]. Both LTP and LTD experiments have been used to study the effect of protein-protein interactions and post-translational modification in AMPAR trafficking [28–31].

However, the use of the NMDA-induced recycling assay needs to be carefully controlled since NMDA application also induces rapid acidification of intracellular compartments, thereby complicating the interpretation of changes in fluorescence intensity from SEP-tagged AMPARs [32]. A further concern with imaging receptor trafficking using fluorescent tags is that this usually involves overexpressing a single AMPAR subunit and having a fluorescent protein fused to the N-terminus of the receptor protein. This might affect the subunit composition of synaptic receptors and the fluorescent tag might interfere with interactions either between AMPAR subunits or between AMPARs and auxiliary subunits such as the family of transmembrane AMPAR regulatory proteins (TARPs). A solution to avoid overexpression of tagged receptors will be to generate knock-in mice or neurons in which the endogenous receptor is tagged with a fluorescent protein.

An alternative approach to visualize lateral movement of AMPARs in the neuronal membrane and to avoid the use of overexpressing fluorescent protein fused AMPARs is single-particle tracking. Antibodies against GluA1 or GluA2 are labeled either with fluorescent dyes or Quantum Dots (QDs) and sparsely applied to the surface of cultured neurons. Live tracking of AMPAR movement revealed that single GluA2-containing AMPARs are either stationary or diffusing rapidly [32]. Receptors at synapses display more restricted diffusion compared to extra-synaptic receptors [33]. During synaptic activity, GluA1-containing AMPARs are trapped and accumulated at synaptic sites through a reduction in motility [34] and the lateral exchange of desensitized AMPAR with naïve functional receptors modulates the recovery of synaptic transmission after synaptic depression [20]. These studies using QDs to track AMPAR motility provided great insight into the behavior of individual receptor proteins, however, a major drawback of using this approach is that labeling receptors using QDs via antibodies adds a large complex (~50 nm) to the receptor and can interfere with its trafficking in spatially confined areas such as the synaptic cleft with a width of ~30 nm [35–38]. Indeed, studies have shown that by using small fluorescent dyes [35], reducing the size of the QDs [36,37], or by linking QDs directly to the receptor using biotin and streptavidin [38], receptor dynamics were changed.

Restricted diffusion of AMPARs at synapses was further demonstrated in dissociated neuron cultures by imaging SEP-tagged AMPARs following partial photobleaching of single spines [39]. Moreover, using super-resolution microscopy, it has recently been shown that the postsynaptic density (PSD) is structured in nanoscale subdomains formed by clusters of postsynaptic scaffolding proteins such as PSD-95. AMPARs are concentrated in these clusters which might contribute to regulating synaptic transmission. These studies implicate that not only trafficking of AMPARs in and out of synapses but also within synapses plays an important role in synaptic transmission and plasticity [40].

Besides gaining a better understanding of the dynamics of AMPAR trafficking, live imaging has also provided a way to study the molecular mechanisms involved in regulating AMPAR trafficking. By introducing genetic deletions or mutations on the AMPAR itself or interacting proteins, many studies have demonstrated the importance of phosphorylation on the AMPAR C-tail and the proper binding of scaffolding and trafficking proteins, as well as auxiliary subunits such as TARPs, for proper AMPAR trafficking [16,22,28]. For further review on the mechanism underlying AMPAR trafficking please see [2,41,42].

Longitudinal imaging of neuronal structure and synaptic molecules *in vivo*

As described above, imaging of AMPARs has been achieved in dissociated cultured neurons and slice cultures. However, the *ex vivo* nature of these preparations limits the interpretation of these findings. In order to follow changes in AMPAR levels in individual neurons and individual spines over time during acquisition and maintenance of a learned task, visualization of AMPARs needs to move into intact animals.

Imaging methods, especially two-photon laser scanning microscopy (TPLSM), have been widely employed to study synaptic plasticity in recent years. TPLSM was invented around 25 years ago [43] and has enabled high-resolution and high-sensitivity fluorescence imaging in intact neural tissue. Over the past 20 years, TPLSM has been used to visualize neuronal structure as well as neuronal activity in live animals on a cellular and subcellular level. Many studies used genetically encoded fluorescent calcium indicators such as the GCaMP series to visualize neuronal activity [44]. These studies have contributed greatly to our understanding of functional neuronal networks and their activity during behavior in mice.

Moreover, using TPLSM and transgenic mice expressing fluorescent morphology markers in neurons, studies have looked at structural changes occurring over days to months during development and learning [45]. A large number of studies focused on dendritic spines, the site of post-synaptic excitatory synapses. While it was previously known from post-mortem studies that spine densities can change over time, spine turnover rates could now be directly studied by repeated imaging of the same neurons and tracking the fates of individual spines over time [45–50,51*]. These studies found that spines are indeed dynamic *in vivo*. They appear and disappear over minutes, hours, and days, and become more stable in the adult after an initially plastic period in development. Moreover, sensory deprivation or enrichment as well as learning can alter the rates of spine turnover.

In vivo structural imaging has advanced our understanding of spine turnover and dendritic remodeling, which presumably underlies rewiring of neuronal connections during development and experience. However, to address the detailed mechanisms underlying synaptic plasticity, such as LTP or LTD, we need to turn to molecules. Several groups have visualized molecules *in vivo* by tagging the protein of interest with a fluorescent protein such as GFP. Gray *et al.* investigated the postsynaptic scaffolding protein PSD-95 in young mice and found that PSD-95 clusters at spines can be stable during early development [52*]. Using a PSD-95 tagged with a photoactivatable form of GFP, however, they showed that synaptic PSD-95 is rapidly redistributed and that synaptic retention times of PSD-95 increase with developmental age and decrease following sensory deprivation. It has been further shown that cytosolic spine volumes and PSD-95-eGFP levels in spines co-vary over time, suggesting that the strength of many individual synapses continuously changes in the adult neocortex [52*,53].

Imaging molecules also allows for the study of inhibitory synapses that lack a distinct morphological structure such as the dendritic spines for excitatory synapses. Using fluorescently tagged Gephyrin, a structural protein at inhibitory synapses, studies have shown that inhibitory synapses on dendritic shafts and spines differ in their distribution

across the dendritic arbor and in their remodeling kinetics during normal and altered sensory experience [54,55]. Furthermore, they find inhibitory synapse and dendritic spine remodeling to be spatially clustered and that clustering is influenced by sensory input [56].

Longitudinal imaging of AMPA receptor trafficking *in vivo*

While visualizing spine structure and synaptic structural proteins *in vivo* have revealed the dynamic events reorganizing synaptic inputs to a neuron, information about the functional relevance, molecular composition, and strength of a synapse remain elusive. To achieve a better understanding of how experience and behavior can shape synaptic strength, *in vivo* imaging of synaptic receptors is necessary.

Because of technical difficulties, *in vivo* imaging of AMPARs has not been realized until recently. By combining *in utero* electroporation (Figure 2a) and two-photon imaging (Figure 2b), our group was able to visualize SEP-tagged GluA1 in synapses of layer 2/3 neurons in the somatosensory barrel cortex of living mice (Figure 2c) [57^{**}]. While our study was performed using a microscope based on the Sutter MOM system and ScanImage software [58], most current commercial two-photon microscopes will be able to visualize fluorescently tagged receptors *in vivo*. This approach enabled the longitudinal tracking of the AMPARs at individual synapses of neurons during basal states as well as under stimulated conditions. We found that acute whisker stimulation led to a significant increase in the intensity of surface AMPAR GluA1 subunit (sGluA1) in both spines and the surrounding dendritic shafts while producing only a small change in spine size (Figure 2d). Interestingly, changes in spine sGluA1 intensity were positively correlated but distinct from changes in dendritic shaft sGluA1 intensity following whisker stimulation. The increase in spine sGluA1 intensity evoked by whisker stimulation was long lasting and NMDAR-dependent. Previously, spine structural dynamics have been used as a measure of synaptic plasticity. However, these results indicate that, by investigating spine turnover in isolation, one might miss key plasticity events occurring in preexisting spines. AMPAR imaging permits the visualization of plasticity occurring within spines, in addition to the investigation of spine dynamics and spine size changes. This data showed that plasticity at preexisting spines was much more widespread than spine turnover, and the ability to image receptors *in vivo* opens up a new and more sensitive way to study synaptic plasticity.

A next step will be to study AMPAR dynamics *in vivo* in mice undergoing a learning task. It has been shown that many learning tasks induce synaptic potentiation measured by electrophysiological recordings in task relevant regions of the brain. By imaging AMPARs in neurons of these brain regions, we will learn how synaptic plasticity on a molecular level leads to effects on a cell population level and ultimately behavior.

Conclusion and outlook

Fluorescently tagged AMPARs have been widely used to directly visualize the dynamics of AMPARs in neurons during synaptic plasticity. Imaging of AMPAR trafficking *in vitro* has provided great insight into the regulation of receptor trafficking in and out of synapses and visualizing AMPAR trafficking *in vivo* demonstrated the dynamic nature of receptors during

sensory stimulation. *In vitro* studies provide the great advantage of easy manipulation through pharmacological or electrical stimulations and, thus, is well suited for further investigation of detailed mechanisms underlying AMPAR trafficking and synaptic plasticity. The *in vivo* system allows for monitoring synaptic changes while animals are experiencing or learning new tasks and can provide a direct link between molecular changes at synapses with behavior. Moreover, in contrast to *ex vivo* preparations, the entire brain network is intact *in vivo* and neurons are receiving and integrating the wide variety of physiological signals. Studies combining the visualization of AMPARs at synapses with imaging of optical sensors for intracellular signaling events and calcium imaging will be able to reveal how signaling events in neurons lead to synaptic plasticity and how synaptic plasticity can shape the firing pattern of the neuron. Readouts of molecular events such as neurotransmitter receptor trafficking *in vivo* also provide a tool to study how genetic mutations in proteins involved in synaptic plasticity affect changes in the brain and behavior such as learning and memory.

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Figure 1.

Imaging fluorescently tagged AMPARs *in vitro*. (a) Schematic of a single AMPAR subunit tagged with GFP or SEP at the N-terminus. (b) Time-lapse imaging of cultured neurons expressing SEP-GluA1 (green) and mCherry (magenta) before and after chemical LTP (ChemLTP) using confocal microscopy. Yellow arrows indicate spines with newly inserted SEP-GluA1 upon ChemLTP and blue arrow heads indicate spines without newly inserted SEP-GluA1 upon ChemLTP. Scale bar, 2 μm. Image modified from Ref. [17]. (c) Schematic model of AMPAR trafficking during basal and LTP states. AMPARs can diffuse in and out of synapses through lateral diffusion and are inserted via exocytosis. SEP-tagged AMPARs selectively visualize membrane inserted receptors.

Figure 2.

Imaging fluorescently tagged AMPARs *in vivo*. **(a)** Schematic of expressing SEP-tagged AMPARs in neurons of the mouse cortex using *in utero* electroporation. **(b)** Schematic of a mouse head-fixed during two-photon imaging. **(c)** Example *in vivo* images of mouse cortical neurons. Left, dendritic segments expressing the morphology marker dsRed (magenta) and SEP-GluA1 (green). Right, 3D reconstruction of sparsely labeled layer 2/3 neurons of the mouse somatosensory cortex. **(d)** Acute whisker stimulation induces increases in spine and dendrite surface GluA1. Representative time-lapse images of layer 2/3 apical tuft dendrites

taken with no whisker stimulation (Control) or before (Stimulated, hour 0) and after 1-hour-long acute whisker stimulation (Stimulated, hour 1, 2, and 3). Arrowheads mark spines and arrows mark dendritic shafts (SEP-GluA1 in green, dsRed2 in magenta, overlap in white). Images were upscaled and contrast enhanced, modified from Ref. [57].