Synaptic stability and plasticity in a floating world

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A fundamental feature of membranes is the lateral diffusion of lipids and proteins. Control of lateral diffusion provides a mechanism for regulating the structure and function of synapses. Single-particle tracking (SPT) has emerged as a powerful way to directly visualize these movements. SPT can reveal complex diffusive behaviors, which can be regulated by neuronal activity over time and space. Such is the case for neurotransmitter receptors, which are transiently stabilized at synapses by scaffolding molecules. This regulation provides new insight into mechanisms by which the dynamic equilibrium of receptor–scaffold assembly can be regulated. We will briefly review here recent data on this mechanism, which ultimately tunes the number of receptors at synapses and therefore synaptic strength.

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Introduction

The concept of diffusion, rooted in physical science, is now being applied to biological systems. Diffusion of a molecule in the plasma membrane can be measured by single-particle tracking (SPT) (Box 1). Advances in microscopy and the production of smaller and better probes for SPT have led to a recent explosion in understanding of how diffusion affects numerous neuronal processes. Description of SPT implementation, including molecule labeling, image acquisition, data treatment, and analysis of diffusion properties, has already been addressed in detail [1,2]. Study of the diffusive properties of molecules by SPT, and in particular neurotransmitter receptors, has greatly reshaped our understanding of molecular trafficking in neurons. The number of neurotransmitter receptors at a synapse is a key element determining synaptic transmission efficacy. Exocytosis and endocytosis play a role in determining the number of receptors on the membrane surface and at synapses (reviewed in [3,4]).

This receptor cycling between intracellular compartments and the membrane surface is crucial for maintaining a mobile population of surface receptors that can be delivered to synapses via lateral diffusion [5•]. SPT experiments have demonstrated that receptors can exchange rapidly between the synaptic and extrasynaptic compartments, and that transient stabilization of receptors at synapses can occur by interaction with binding partners, such as scaffold proteins. This phenomenon, referred to as a 'diffusion trap', operates at both excitatory and inhibitory synapses [6,7]. Thus, the regulations of receptor-scaffold and scaffold–scaffold interactions are one of the central mechanisms for the maintenance and plasticity-related changes of receptor number at synapses.

Cohesion by lateral diffusion: synapse stability despite mobility

Though synapses are stable on a long time scale, the individual molecules of a synapse turn over on a shorter time scale, even at steady state. Both receptors and scaffolding proteins can turn over within tens of minutes (reviewed in [8]). Numerous transient interactions between synaptic components maintain a synaptic connection, despite this continual exchange of the individual constitutive elements. This has led to the concept of the synapse as a multimolecular assembly, the dynamics of which is governed by diffusion-reaction processes [6,7]. As a consequence, the various interactions between synaptic molecular components can then be described as akin to chemical reactions, characterized by their K_{on} and K_{off}. The combination of these reactions can account, at least in part, for the residence (dwell) time of a given molecule in the synaptic multimolecular complex. The complexity of this phenomenon relies on the fact that molecular interactions are not straightforward. Multiple association states between molecules exist, defined by different affinities and rates of association/dissociation (Figure 1). These various states on the one hand introduce nonlinearity and complexity to the relationship between affinities and dwell time of a molecule at the synapse, and on the other hand offer multiple levels of regulation to fine tune their association. In such a model, weak interactions would be more biologically relevant than strong ones, since the latter are less likely to be easily modified. Actually, traditional biochemical methods which study protein interaction *ex vivo*, such as affinity chromatography and immunoprecipitation, tend to emphasize these strong affinities in protein interactions. In contrast, study of protein interactions by SPT allows access to molecular biochemistry in living cells, as well as the appreciation of weak interactions that may have profound biological significance. For instance, biochemical methods have shown

Box 1 Principles of diffusion explained for the neurobiologist.

Diffusion is a time-dependent process that results from thermal-dependent random collisions of molecules. The path these molecules take when diffusing is called a 'random walk', and this behavior is referred to as free Brownian diffusion. This concept, rooted in physical science, is now being applied to biological systems. When looking at diffusion in a biological context, such as in the plasma membrane, the diffusion path of the molecule is often no longer random. Other proteins and the heterogeneity of lipids within the membrane restrict diffusion by binding or by creating obstacles or both, thus imposing a bias upon free diffusion. This biased diffusion can then lead to local accumulations, rather than to the dispersion of a molecule [12].
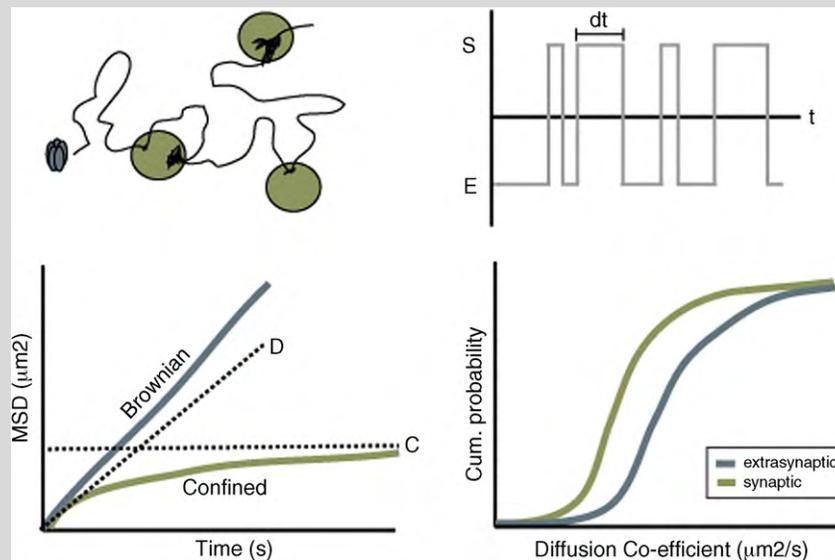
The main interest of SPT is that it allows, in principle, molecules to be tracked one by one and therefore to visualize their interactions with other molecules as a function of time. An important advantage of SPT lies in its high pointing accuracy, which allows localization of the tracked molecule below optical diffraction limits (reviewed in [6]). For instance, the trajectory of a receptor diffusing in the membrane may exchange between the synaptic (S) and extrasynaptic (E) membrane (panel a). From this, the dwell time (dt) of a receptor within the synapse may be calculated (panel b). Simple analysis of diffusion trajectories involves calculation of the mean square displacement (MSD), which is obtained by averaging the distance a molecule travels over time.

$$\text{MSD } (nt) = \frac{1}{(N-n)} \sum_{i=1}^{N-n} [(x_{n+i} - x_i)^2 + (y_{n+i} - y_i)^2]$$

where t is the acquisition time and N the total number of frames acquired. Illustrations of MSD versus time plots (left panel) for a neurotransmitter receptor at synaptic (green line) or extrasynaptic (blue line) sites show the characteristic differences between their diffusive behaviors (panel c). For molecules undergoing free or Brownian diffusion, the MSD versus time plot is linear. For molecules undergoing diffusion within a synapse the MSD versus time plot is negatively bent. The confinement area (C) can be estimated from the asymptote of this plot. The diffusion coefficient (D) for each molecule can be determined by a linear fit of the initial few points of MSD versus time curve. Under typical experimental conditions, the diffusion of a population of molecules has a wide distribution of diffusion coefficients, ranging between 10^{-4} and 10^{-1} $\mu m^2/s$. Therefore, to compare groups, such as proteins at synaptic versus extrasynaptic sites, it is useful to look at the cumulative probability using semi-logarithmic plots (panel d). Shifts of these curves to the left or right represent a decrease or increase of diffusion (respectively).

direct interaction between the inhibitory synaptic scaffold molecule gephyrin and both glycine receptors (GlyRs) and inotropic GABA receptors (GABAARs). The former interaction was easily identified by immunoprecipitation, however the latter is weaker and very sensitive to detergent (Refs. in [8]). These interactions can be monitored by following the diffusion properties: GlyRs diffuse slower due to their 'stable' interaction with gephyrin and the GABAR has a higher diffusion rate reflecting its 'less stable' binding [9••]. A similar phenomena have been shown at excitatory synapses, where AMPA receptors (AMPARs) show faster diffusion within synapses than NMDA receptors (NMDAR) [10].

Particular attention has been spent studying receptor–scaffold interactions, which have been reported for receptors at inhibitory and excitatory synapses (reviewed in [8]). The relatively high speed of diffusion of free mobile receptors, compared with the long characteristic residency time of a receptor in the synapse, makes receptor accumulation at synapses relatively insensitive to diffusion rates [11]. Receptor accumulation is more likely to depend on the density and/or affinity of binding sites, such as those offered by scaffolding proteins [12]. In the framework of a reaction-diffusion process, the molecular concentrations in and out of a synapse, together with the trapping capability of the postsynaptic scaffold assembly,

Figure 1
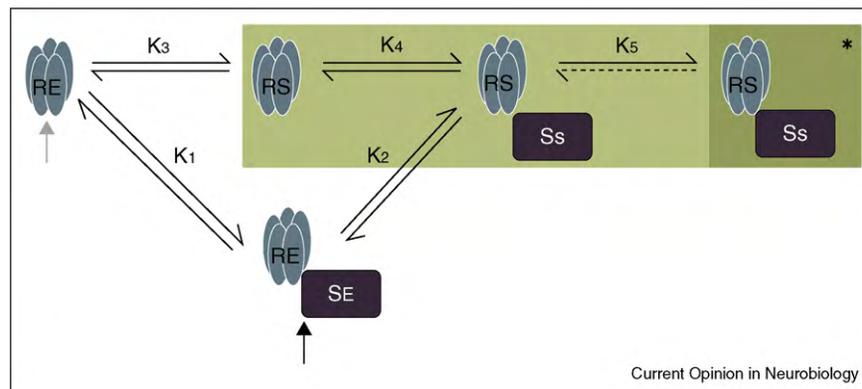
Diffusion-reaction model of neurotransmitter receptor and scaffold trafficking. Schematic illustration of the different paths leading to stabilization of a receptor by scaffolding proteins (adapted from [14]). Intracellular receptors (R) and their scaffolds (S) may be inserted into the plasma membrane either separately (gray arrow) or as receptor scaffold complexes (black arrow). Receptors on the membrane surface can diffuse between different membrane compartments, from extrasynaptic (suffix E) to synaptic (suffix S) areas. Receptor–scaffold complexes may be formed outside (equilibrium 1) or inside scaffold clusters (equilibrium 4). Exchange between extrasynaptic and synaptic compartments can occur for both receptors (equilibrium 3), and receptor–scaffold complexes (equilibrium 2). Within the synapse, receptor–scaffold complexes may reach a higher level of stabilization (*, equilibrium 5).

set the chemical potentials [13•]. Several examples of this have now been described by SPT. GlyRs on the plasma membrane at extrasynaptic sites have characteristic lateral mobility with free Brownian diffusion, and at inhibitory synapses show decreased diffusion, which is confined to a smaller area [14,15]. Within gephyrin clusters, GlyRs have different diffusion characteristics, likely corresponding to different degrees of stabilization with the scaffold gephyrin [16]. GABA$_A$Rs also have characteristic features with free Brownian diffusion at extrasynaptic sites, and when diffusing into inhibitory synaptic sites they display a decreased diffusion rate and increased confinement [9••,17••,18]. Metabotropic glutamate receptors (mGluRs) show confined diffusion when spatially associated with the excitatory synaptic scaffold Homer [19]. Exchange of AMPARs between excitatory synaptic and extrasynaptic sites depends on their interactions with the accessory protein Stargazin and the scaffolding protein PSD-95 [20]. NMDA receptors show subunit-specific diffusion rates outside and inside excitatory synapses [21]. This general mechanism of receptor trapping by scaffolding proteins also accounts for reciprocity, where scaffold stabilization may be influenced by the receptor. This reciprocity within the receptor–scaffold multimolecular complex is the consequence of the combination of the chemical potential generated by extrasynaptic versus synaptic receptors, as well as by synaptic versus bulk scaffold proteins [13•]. This reciprocity can ensure a synchronized and adaptive construction of synapses. Alterations in scaffold–scaffold interactions can also modulate the lateral diffusion of receptors. For example, alternative-splicing of gephyrin can modify the scaffold's affinity for GlyRs [22], leading to incorporation of 'weaker' binding sites within the synapse. Furthermore,

a small amount of gephyrin is linked to GlyRs outside the synapse, and provides a means for receptor recruitment via gephyrin–gephyrin interactions [16,22]. This continual exchange of receptors at the synapse is important for its function, namely recovery from depression, where desensitized receptors are exchanged with naïve functional ones [23••]. In summary, free Brownian diffusion in extrasynaptic domains and confined diffusion at synapses appear to be common features of many neurotransmitter receptors. Each receptor subtype, such as GlyR versus GABA$_A$R, or AMPARs versus NMDARs, shows characteristic differences in its lateral diffusion (Table 1). The regulations of this diffusion, by the cytoskeleton, lipid domains, or neuronal activity, are also receptor-specific.

Control of lateral diffusion: scaffolds and obstacles

Given the large number of synaptic proteins, molecular interactions determining receptor lateral mobility are not due only to the scaffold proteins directly associated with receptors, as already discussed herein. For instance, adhesion proteins also affect the synaptic accumulation of several neurotransmitter receptors. In particular, the adhesion complex of neurexin/neuroligin can recruit AMPARs [24,25]. How adhesion proteins affect neurotransmitter receptor diffusion is complex. They may hinder receptor diffusion behaving as a binding site, either directly or indirectly, by acting on scaffold proteins, and/or by acting as a diffusion barrier restricting their exchange between synaptic and extrasynaptic membrane. All of these mechanisms are likely to play a role in how adhesion complexes affect diffusion and accumulation of receptors at the synapse. Therefore, to fully appreciate the diffusion of neurotransmitter receptors in the plasma

Table 1

Diffusion properties of molecules at inhibitory, excitatory, and extrasynaptic sites. Mean diffusion (D), confinement area and dwell time of neurotransmitter receptors and GM1 (a lipid enriched in lipid rafts). Values for synaptic (bold, right) and extrasynaptic (left) sites are given as derived from SPT experiments. Changes in mean diffusion (D) are noted for conditions where neuronal activity was increased, the actin cytoskeleton was disrupted with latruculin, or cholesterol depletion by COase. Up arrows indicate increases, down arrows indicate decreases, and dashes indicate no change.

	Inhibitory						Excitatory							
	Gly[a,b]		GABA$_A$[c,d]		GM1[d]		AMPA[e,d]		NMDA[e]		mGluR[f]		GM1[d]	
D ($\times10^{-2}$ m²/s)	**0.1**	0.7	**1.2**	2.2	**1.3**	15.4	**2.8**	8.4	**2.1**	3.7	**1.0**	5.0	**2.0**	15.4
Confinement (μm)	**0.15**		**0.26**		**0.33**								0.46	
Dwell time (s)	**15**		**4.9**		**0.66**				0.24				0.45	
Changes in D														
Increased activity	↓		↑		↑		↑		–					
Actin disruption	↑	↑			↑	↑							↑	↑
Cholefsterol depletion			↑	–	↑	↓	–	↓					↑	↓

[a] Charrier *et al.* [39].
[b] Levi et al. [9].
[c] Bannai *et al.* [17••].
[d] Renner *et al.* [32•].
[e] Groc *et al.* [36•,56].
[f] Serge *et al.* [19].

membrane, it is important to take into consideration both binding sites and obstacles.

The plasma membrane is a heterogeneous mixture of proteins and lipids, which forms various domains with different compositions (reviewed in [26]). Therefore, in addition to specific molecular interactions, diffusion of a given molecule can also be modulated by steric hindrance by resulting from other proteins or lipid domains. Below the plasma membrane, the cytoskeleton also contributes to the control of lateral diffusion by creating fences below the membrane that hinder the movement of proteins that protrude into the cytoplasm. Furthermore, the cytoskeleton can anchor transmembrane molecules, which then act as obstacles to lateral diffusion (reviewed in [27]). The resulting compartmentalization of the membrane can temporarily confine proteins and/or lipids, and their movements between these compartments are referred to as 'hop diffusion' [27]. The context, or specific interactions of a molecule, shapes nonspecific influences on diffusion. In addition, not all proteins are equivalent with respect to diffusion. This is due mainly to their sizes and integration into multimolecular complexes (this integration can be biologically regulated). Functionally, hop diffusion is important for dynamic regulation of the spatial distribution of signal transduction in the plasma membrane, by 'fencing in' signaling complexes of activated receptor molecules [28]. Two extreme cases where diffusion is blocked by obstacles are found at the axon initial segment (AIS) and at nodes of Ranvier [29], where special ankyrin G and spectrin isoforms are important for the concentration of proteins such as voltage-gated sodium channels (VGNCs), and the maintenance of polarity of neuronal [30]. Stunningly, even phospholipids cannot escape the diffusion barrier of the AIS. During maturation of the AIS the rate of phospholipid diffusion decreased in the AIS membrane by a factor of more than 800-fold, whereas for other membrane regions such as the dendrites, cell body, or axon, this diffusion rate decrease was only one to threefold [31]. In summary, the cytoskeleton can restrict the diffusion of proteins and lipids to varying degrees, acting as a fence containing signaling complexes, or a maximum security prison in the case of the AIS.

Receptors diffuse slower at synaptic than at extrasynaptic membranes, reflecting their stabilization not only by scaffolding proteins, but also by the presence of obstacles. There will be different consequences when a molecule acts as a binding site or as an obstacle, although both these can reduce diffusion in the case of neurotransmitter receptors [12,13•]. Binding sites provide specific interactions that create a potential well for receptor accumulation. This leads to increased receptor density at steady state. Obstacles cause nonspecific steric hindrance to diffusion that does not lead to receptor accumulation at steady state, but the density of obstacles may tune the time required for receptor concentration to reach steady state (Figure 2). The organization and/or density of obstacles in the membrane may act as a filter, sorting proteins by size, much like the semipermeable membrane in osmosis [32•]. Thus obstacles and binding sites co-operate: by restricting the diffusion of a receptor to a specific area, even temporarily, obstacles can increase the probability of a receptor interacting with its binding partners. Therefore, obstacles introduce additional influence on the diffusive properties of receptors, and contribute to receptor residency times at synapses that result from more than simple association/dissociation reactions between receptors and scaffolds. For instance, the extracellular

Figure 2
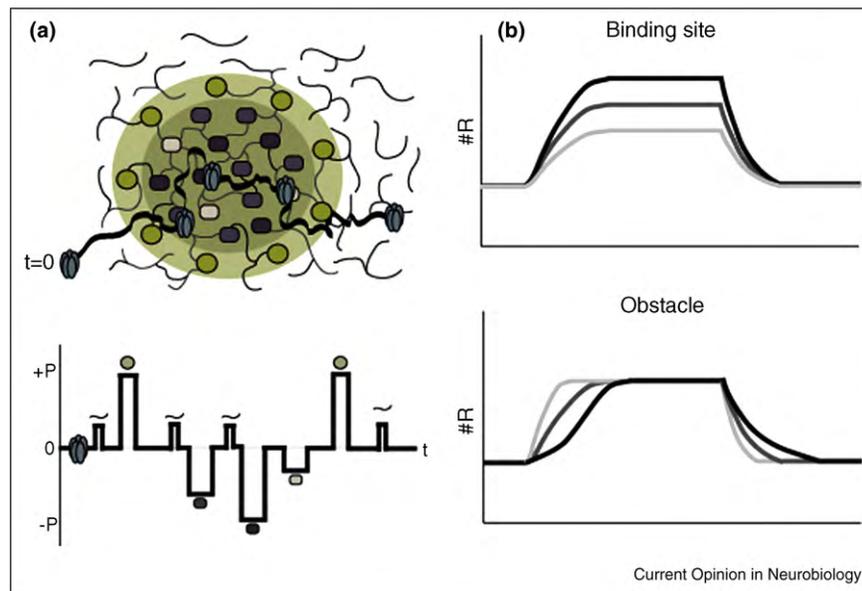
Influence of scaffold proteins and obstacles on diffusion of neurotransmitter receptors. **(a)** Bird's eye model of the postsynaptic membrane (top panel), showing the diffusion path taken by a receptor (in blue) as it goes from extrasynaptic membrane (at $t = 0$), and diffuses into the perisynaptic area (light green) and synaptic area (dark green), and back into the extrasynaptic membrane. Along this path, the receptor may encounter obstacles such as adhesion complexes (green circles) in the perisynaptic area or the cytoskeleton (grey wavy lines) in all areas of the membrane. The receptor may be temporarily bound by scaffolding molecules with different affinities (dark purple denotes strong affinity, light purple denotes weak affinity). The diffusion potential (P) of each of these encounters is plotted over time (lower panel). The receptor sees potential barriers when encountering obstacles, and wells when encountering scaffold binding sites. Modified from Triller [8]. **(b)** Effects of changing the number of binding sites (upper panel) versus obstacles (lower panel) on receptor number at synapses at steady state. Number of receptors is plotted on the *y*-axis, versus space on the *x*-axis. Changing the number of binding sites in the synapse, from a lower number (gray) to higher (black), does not change the time for the receptor to flux between extrasynaptic and synaptic areas, as indicated by the equal slope of the curve. However, the amount of receptor at synaptic sites correlates with the amount of scaffold binding sites, as indicated by a change in the plateau at synaptic spaces. Changing the number of obstacles in the perisynaptic membrane, from a lower number (gray) to higher (black), changes the time for the receptor to flux between extrasynaptic and synaptic areas, as indicated by the change slope of the curve. A greater slope indicates a faster flux and vice versa. However, the amount of receptor is unchanged within the synapse, as indicated by the equal plateau in all cases.

matrix (ECM) forms a dense meshwork around synapses that can act as a barrier reducing the flux of proteins in and out of the synapse. In addition, the ECM provides signals and acts as an extracellular scaffold for signaling proteins such as integrins which control many intracellular metabolic pathways, and in turn may influence receptor lateral diffusion [33,34]. When this matrix is removed, AMPAR diffusion increases, as well as exchange rates between synaptic and extrasynaptic membrane [35]. Such is also the case for the ECM protein reelin, which affects NMDAR diffusion and can coordinate the developmental switch of NMDAR subunits [36•].

Many scaffold proteins are bound by cytoskeleton-related proteins at both excitatory and inhibitory synapses ([37,38] and references within). This connection between scaffold and cytoskeleton may also act to control and organize the distribution and/or density of synaptic proteins, effectively changing the distribution of binding sites and obstacles impinging on receptor lateral diffu-sion. Pharmacological disruption of the actin cytoskeleton can decrease synaptic amounts of GlyRs, and in parallel gephyrin. In terms of diffusion, this increases exchanges between the synaptic and extrasynaptic membranes and decreases receptor dwell time at synapses [39,40]. Even the confinement of lipids within the synapse is decreased by disruption of the actin cytoskeleton [41]. Thus, obstacles can limit the diffusion of both proteins and lipids, corralling them within a synapse [32•,40]. Receptors and lipids encounter the same barriers to diffusion, restricting their diffusion due to steric effects. Since only receptors are enriched at synapses, barriers to diffusion cannot by themselves explain the local enrichment of receptors. It is the interplay of all the synaptic components (both lipid and protein, binding site and obstacle) that influences the residency time of neurotransmitter receptors at the synapse, and ultimately receptor number at steady state (refer to Table 1 for a summary of changes in diffusion of receptors upon disruption of obstacles such as the actin cytoskeleton or lipid rafts).

Membrane geometry may also influence lateral diffusion. A striking example of spatial confinement by membrane geometry occurs at spines, sites where most excitatory synapses are found. Due to their shape, a bulbous head connected through a more or less cylindrical neck to the dendrite, spines are considered a nearly independent compartment. In response to activity, spines can regulate diffusing molecules by changing actin dynamics, and consequently their shape [42]. In addition to restricting the diffusion of calcium and other soluble factors in the cytoplasm [43], modeling of diffusion dynamics indicates that spine shape can restrict membrane-associated proteins. Fluorescence recovery after photobleaching (FRAP) experiments have shown that kinetics of membrane-linked GFP exchange between the dendritic shaft and the spine head compartment are slower in spines with long necks and/or large heads [44]. AMPAR lateral diffusion also depends on spine morphology and is controlled by the spine neck [12,45,46]. Thus, increases in glutamate release that shorten spine length will have functional consequences, by increasing receptor influx into the spine head [12]. Interestingly, the activity-dependent changes in receptor flux at spines may be subunit-specific, favoring the retention of GluR1-containing AMPARs [47•,48••], which have well-documented involvement in synaptic plasticity.

Tuning lateral diffusion: synaptic plasticity

Synaptic plasticity is an important determinant of learning and memory, and changes in receptor number at synapses are one of the main underlying molecular events. The concept of an ever-changing synapse with all constituents turning over around equilibrium provides an attractive framework for the tuning of synaptic strength during plasticity. In this framework, changing the amount of receptors at a given synapse requires but a small change in the rates of association and/or dissociation within the receptor–scaffold complex, causing a shift in the amount of receptors driving the synapse to a new steady state. At steady state, in- and out-synapse flux of receptor equals zero, and a slight imbalance will produce an increase or a decrease of receptor number at synapses. In the synapse, the distribution and fraction of individual scaffolding proteins are thought to be organized in a complex and stereotyped manner (reviewed in [8]). Each molecular interaction within this network can be regulated by activity. Ultimately these regulations will impact on receptor number during synaptic plasticity. The receptor–scaffold multimolecular complex will then reach its new steady state, with a higher or lower number of receptors resulting in potentiation or depression, respectively.

At excitatory synapses, cellular models of plasticity, such as long-term potentiation (LTP) and depression (LTD), can result from the insertion or removal of AMPA receptors (AMPARs), respectively (reviewed in [49]). Activity-dependent insertion and removal of AMPARs occurs at perisynaptic and extrasynaptic sites [47•,50,51]. An activity-induced increase in extrasynaptic AMPARs by increased exocytosis would change the difference in receptor concentration between extrasynaptic and synaptic compartments. This would lead to a concentration-dependent driving force for diffusion of AMPARs into the synapse. The trapping of receptors may furthermore be modulated by activity-dependent changes in scaffold–receptor affinity. Post-translational modifications such as phosphorylation [51,52], on either the receptor or the scaffold, can modify receptor trafficking at synapses. Surface diffusion experiments have shown that AMPARs are mobile at both synaptic and extrasynaptic sites, with many dwelling for only a few seconds at synaptic sites [10,20,45,54,55]. A rise in intracellular calcium, a central element in synaptic plasticity, can decrease AMPAR diffusion at synapses, and increase their dwell time. In consequence, this leads to receptor accumulation at individual synapses [10,55,56]. Local spontaneous activity can influence the diffusion of AMPARs at specific synapses. Inactive synapses contain fewer GluR1-containing AMPARs than their nearby active synapses [57], suggesting a synapse-specific competition for AMPARs based on activity. Consistent with this, at active synapses, the exchange of GluR1-containing AMPARs is reduced compared with neighboring inactive synapses [48••]. In synapses that have undergone LTP, GluR1-containing AMPARs show reduced mobility compared with naïve neighboring synapses [47•]. This input-specific maintenance of AMPARs at distinct synapses is a hallmark of input selective synaptic plasticity, with consequences for learning and memory.

Synaptic plasticity is also present at inhibitory synapses, although the cellular and molecular mechanisms are less well understood (reviewed in [58]). Synaptic plasticity at excitatory and inhibitory synapses are calcium-dependent, however, in general, signals that potentiate excitatory synapses depress inhibitory synapses, and vice versa. Both inhibitory scaffolds and neurotransmitter receptors display changes in synaptic trafficking that are both calcium-dependent and modulated by synaptic activity. Gephyrin clusters show sub-micrometric rapid lateral motion and are continuously moving on the dendritic shaft, and this can be modulated by synaptic activity [59]. Synaptic accumulation and diffusion dynamics of $GABA_A$Rs are controlled by excitatory neuronal activity in the hippocampus. When neuronal activity is decreased by blocking glutamatergic receptors or neurotransmitter release, $GABA_A$R diffusion increases, the confinement of the receptor decreases, and the dwell time at synapses also decreases. Together, these lead to a decrease in the amount of $GABA_A$R at synapses. This activity-dependent regulation is accompanied by a parallel decrease in gephyrin clustering at synapses, suggesting a change in scaffold–scaffold interactions. These modifications are

dependent on calcium and the phosphatase calcineurin [17[••]]. This activity-dependent neuronal disinhibition could be part of the mechanism underlying the induction of LTP at excitatory synapses. In the spinal cord, most inhibitory synapses have a mixed glycine–GABA phenotype. At these mixed synapses, increasing neuronal activity decreases diffusion rate and increases the synaptic accumulation of GlyRs, but not GABA$_A$Rs. This regulation of GlyR synaptic content occurs without modification of the amount of gephyrin, therefore suggesting a change in the affinity between receptor and scaffold, and is dependent on calcium influx via NMDA receptors [9[••]]. This provides a mechanism for the homeostatic regulation of glycinergic transmission in response to increased excitatory transmission in the spinal cord.

Conclusion

SPT is a powerful tool which provides insight into the diffusive properties of many molecules and shapes our understanding of molecular trafficking in neurons. Nevertheless, many fundamental questions remain. The next steps for single-molecule biology are the simultaneous tracking of multiple molecules, and the development of techniques that will allow for tracking in more integrated systems, such as in slices and *in vivo*. Advances in analysis will also provide new insights, through the marriage of evolving diffusion theories and modeling to biological processes. For instance, current analyses are limited to the determination of the translational diffusion coefficient of a symmetric molecule. Diffusion of an asymmetric object, such as many molecules or molecular complexes in biology, is characterized by translational and rotational diffusion coefficients and, as a consequence, the trajectories of asymmetric molecules differ from those of symmetric ones [60]. Taking this into account will for example allow the visualization in real time of coupling between membrane molecules following ligand binding. Beyond diffusion, SPT-based localization has been successfully implemented for sub-diffraction resolution fluorescence imaging. Photo-switching microscopy methods, called stochastic optical reconstruction microscopy (STORM) or photo-activated localization microscopy (PALM), involves reconstitution of images from many thousands of localized individual molecules, much like the modern artistic movement of pointillism. Computational efforts are needed to integrate these data, but real-time imaging of live samples has already been applied to actin dynamics in spines [61]. This approach will allow simultaneous sub-diffraction resolution of the scaffold, and when coupled with SPT to visualize receptor diffusion will lead to exciting new insights into subsynaptic structure and dynamics in living neurons in integrated systems.

Conflict of interest

The authors declare no conflicts of interest that relate to papers accepted for publication in this journal.

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