MODES OF DYNAMIC AMPA RECEPTOR DELIVERY DURING SYNAPTIC PLASTICITY

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ABSTRACT

Synaptic plasticity is thought to be the cellular basis of processes like memory and learning. This regulation of synaptic strength is mediated by the concentration of AMPA type glutamate receptor available in the synaptic membrane. While it is thus undisputed that integration of AMPA receptors into the synapse is crucial, it is currently debated how AMPA receptors are trafficked to the synapse and especially where these receptors are integrated into the plasma membrane. On the one hand, AMPA receptors have been shown to diffuse through the plasma membrane from dendritic shafts to dendritic spines where they get trapped by scaffolding proteins positioned at the synapse. On the other hand, a strong case has been made for involvement of active recycling of AMPA receptors by endosomal transport and storage vesicles, delivering the receptors straight into dendritic spines. This review discusses the experimental evidence of these two pathways and offers a model that combines elements of both active and passive transport of AMPA receptors. Understanding the dynamics of AMPA receptor trafficking may ultimately broaden our knowledge on the cellular mechanisms underlying information storage and by synaptic contacts in the central nervous system.

KEYWORDS

AMPA receptors, endosomal transport, lateral diffusion, synaptic plasticity, post-synaptic density

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INTRODUCTION

Neural networks are thought to save information by means of synaptic contacts. Synapses are the highly specialized interfaces allowing signals to pass from a presynaptic to a postsynaptic neuron. Neurons are highly polarized cells with a somatodendritic compartment, in which input signals are perceived and processed, and the axonal compartment for fast transmission of a stimulus to the presynaptic terminal. At synapses, the presynapse releases neurotransmitters like glutamate, which can bind to ligand-gated ion-channels in the postsynaptic membrane to elicit an electrical current in the postsynaptic cell. The vast majority of excitatory synapses are found on dendritic spines, small mushroom-shaped protrusions on the dendritic shafts. Dendritic spines are connected to the dendritic shaft by a neck of variable diameter, while their heads border on the synaptic cleft **(Figure 1)**. Young spines are unstable filopodia-like structures stabilizing and increasing in size in response to synaptic activity (Harris, 1999; Lippman and Dunaevsky, 2005). Growth of dendritic spines has in turn been reported to depend on scaffolding proteins of the postsynaptic density (PSD) (Sala et al., 2001). The PSD is an electron-dense structure directly under the postsynaptic membrane and thus in the head of the dendritic spine. It is a proteinaceous cluster involved in coupling ion channel activity to intracellular signalling. Its crucial function is highlighted by the neurological pathologies in patients with mutations in PSD genes (Bayés et al., 2011). Interestingly, synaptic activity influences the size and composition of the PSD emphasizing the dynamic intracellular responses to glutamate stimulation (for extensive review on dendritic spines and PSD see Sheng and Hoogenraad, 2007). Dendritic spines have been proposed to establish a separate compartment that can regulate diffusion of signalling molecules between dendritic shaft and synapse, possibly to localize biochemical signals or retain molecules at single synapses (Nimchinsky et al., 2002).

Intriguingly, while many synaptic connections are required to remain stable for considerable times, many of the protein components of the synapse, including glutamate receptors and PSD proteins, show a highly dynamic turnover. The first evidence of dynamic neurotransmitter receptor renewal was found in experiments on rat neuro-muscular junctions and their nicotinic acetylcholine receptors, which were shown to relocate spontaneously or after induction of new junctions and to replace inactivated receptors by diffusion through the membrane (Anderson and Cohen, 1977; Young and Poo, 1983), while the overall number of receptors per junction remained constant throughout a rat's lifetime (Pestronk et al., 1980). Furthermore, these early experiments showed that receptor localization can be perturbed by electrical stimulation and is thus dependent on synaptic activity (Axelrod et al., 1978). Later, also glutamate receptors were shown to move between synapses along the plasma membrane (Tardin et al., 2003; Triller and Choquet, 2005).

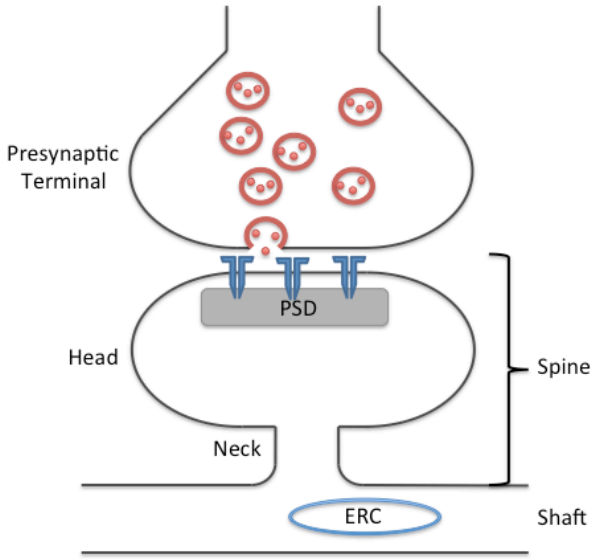

Figure 1. Schematic view of an excitatory synapse. Excitatory synapses are located on top of dendritic spines. These spines are small protrusions, often showing a bulbous head region containing the PSD (grey), that are connected to the dendritic shaft by a narrower neck. Endosomal recycling centres (ERC, blue) are often found at the base of dendritic spines. The presynaptic terminal contains storage vesicles containing glutamate neurotransmitter (red), which can be released by membrane fusion of the vesicles. Glutamate then binds to receptors (blue) on the postsynaptic membrane, which will open their ion channels to initiate depolarization.

The majority of excitatory neurotransmission in vertebrate central nervous system is mediated by the neurotransmitter L-glutamate. During early studies on ion-channels responsive to glutamate, it was discovered that different artificial agonists can activate distinct subsets of glutamate receptors (reviewed in Palmer et al., 2005). Alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) is a low affinity agonist for GluA1-4 receptors, while N-methyl-D-aspartate (NMDA) and kainate are agonists for NR1-3 and GluR5-7 receptors, respectively (Palmer et al., 2005). This review focuses on AMPA type receptors, which are important in the transmission of fast-excitatory signals in the central nervous system, as they initiate the primary depolarization in the postsynaptic membrane in response to glutamate stimulation (Dingledine et al., 1999; Jonas, 2000). In fact, AMPA receptors are found in the majority of excitatory synapses in the brain. Their dynamic accumulation in the synapse underlies synaptic plasticity, which is believed to be the cellular basis for experience-driven learning as described above. The best-studied forms of synaptic plasticity are long-term potentiation (LTP) and depression (LTD). Indeed, LTP inducing stimuli increase AMPA receptor levels in the synapse, whereas they are depleted during LTD (Shepherd and Huganir, 2007). Thus, synaptic activity regulates the amount of AMPA receptors in the synapse, which leads to changes in signalling capacity and stabilization of the particular synaptic connection, possibly explaining how information can be stored in a neuronal network (Martin et al., 2000; Malinow and Malenka, 2002; Shepherd and Huganir, 2007).

In this review, I focus on the modes of transport of AMPA receptors in dendrites. First, the biogenesis of AMPA receptors and their trafficking to the dendritic compartment are summarized. The following sections discuss the experimental evidence for models of regulated AMPA receptor transport in and out of the synaptic membrane. This discussion is split into aspects of lateral diffusion and retention and evidence for a role of active transport through recycling endosomes. In the last section, I propose a model for AMPA receptor transport that incorporates elements of both active and passive transport and end with a hypothesis on the role of dendritic spine morphology for synaptic plasticity and information storage.

Biogenesis & Trafficking to Dendrites

AMPA Receptor Subunits & Modifications

There are four genes encoding the AMPA receptor subunits GluA1-4 (also called GluR1-4 or GluRA-D). After transcription, the messenger RNAs undergo a series of modifications including alternative splicing and RNA editing, further increasing the diversity of AMPA receptor subunits. RNA editing of GluA2 is particularly important as the Q/R editing renders AMPA receptors containing GluA2 impermeable for calcium ions (Seeburg et al., 1998). As all membrane proteins, the peptide folds in the rough endoplasmic reticulum where the future extracellular part, containing the ligand-binding domain, is situated in the ER lumen. The ion channel domain is folded in the ER membrane and continues into the carboxy-terminal cytosolic domain, which later binds subtype specific interacting proteins (**Table 1**) (Greger and Esteban, 2007).

Folding of the peptides is assisted by contacts between individual subunits (Greger et al., 2007) as dimerization of AMPA receptor subunits commences already on the amino-terminal regions, while the carboxy-terminus is still being translated (Greger and Esteban, 2007). After initial dimerization of single AMPA receptor subunits, these dimers form tetramers in a second step (Tichelaar et al., 2004), giving rise to a variety of hetero-tetramers. Importantly, the subunit composition of AMPA receptors has significant effects on receptor trafficking and the postsynaptic current elicited upon stimulation (Greger and Esteban, 2007), because different (and RNA edited) subunits show variation in their ion gating kinetics (Palmer et al., 2005). Differences in cytoplasmic domains allow each subunit to bind a specific set of proteins that are, amongst other tasks, important for targeted transport of the receptor to its

destination. An overview of AMPA receptor binding proteins is summarized in **Table 1**. Most important for mature excitatory neurons of the hippocampus are GluA1/2 and GluA2/3 receptors (Wenthold et al., 1996; Lu et al., 2009), to which this review is limited. Generally, it appears that GluA2/3 are shuttled continuously into synapses, while GluA1/2 receptors are only integrated upon acute stimulation of the synapse (Shi et al., 2001). It is thus important to appreciate the diversity of AMPA receptors, if one is to understand their behaviour and function.

Trafficking of AMPA Receptors to Dendrites

After leaving the endoplasmic reticulum and sorting in the trans-Golgi network, vesicular transport ensures AMPA receptor delivery to the dendritic compartment. Long-range trafficking is most efficient along the microtubule cytoskeleton. In dendrites, microtubules are not as uniformly oriented as in the axon (Baas et al., 1989; Kapitein and Hoogenraad, 2011). Consequently, there have been reports of both minus-end directed dynein and plus-end directed kinesin motors involved in AMPA receptor trafficking. Dynein inhibition results in decreased delivery of GluA2 to dendritic spines (Kapitein et al., 2010). Earlier reports showed that the kinesin KIF5 transports GluA2 to dendrites dependent on the binding of GluA2-Receptor-Interacting-Protein1 (GRIP1) (Setou et al., 2002) and that KIF1a has a similar function but binds to liprin α, which in turn binds the GRIP1-AMPA receptor complex (Shin et al., 2003). Microtubules are thus required for bulk transport of newly synthesized AMPA receptors into the dendrites and may also serve to maintain a pool of vesicles by movement back and forth along anti-parallel fibres (Kapitein et al., 2010). Local transport of AMPA receptors to the membrane is then usually mediated by the actin filaments situated closer to the cortex. Myosin V has recently been identified as the motor responsible for this last step of directed transport (Correia et al., 2008; Wang et al., 2008).

AMPA Receptor Exocytosis

Finally, once the AMPA receptor containing vesicles are close to the plasma membrane, exocytosis results in membrane integration of the receptors. Fusion of the delivery vesicle with the plasma membrane is mediated by the SNARE family of proteins. SNAP23 and Syntaxin 4 have been suggested as the v-SNARE and t-SNARE for AMPA receptor containing vesicles, respectively (Kennedy et al., 2010; Suh et al., 2010). Furthermore, exocytosis is known to take place both under basal and stimulated conditions (Kopec et al., 2006; Opazo and Choquet, 2011). In contrast,

Table 1. AMPA receptor binding proteins and their roles in receptor transport.

Binding Protein	AMPA subunit	Secondary Interactions	Function	Reference
SAP-97	GluA1	Myosin V	Actin transport	(Correia et al., 2008)
		Myosin VI	Actin transport	(Wu et al., 2002)
Protein 4.1N	GluA1	Spectrin	Membrane insertion	(Lin et al., 2009)
GRIP 1/2	GluA2	Kinesin	MT transport & retention	(Setou et al., 2002)
		Liprin α	MT transport & retention	(Wyszynski et al., 2002)
		NEEP 21	Endosomal recycling	(Steiner et al., 2005)
		Sec 8	Membrane insertion	(Mao et al., 2010)
		GRASP-1	Rab4 driven recycling	(Hoogenraad et al., 2010)
PICK 1	GluA2	Arp2/3	Inhibition of actin polymerization	(Rocca et al., 2008)
		KIBRA	Membrane insertion*	(Makuch et al., 2011)
NSF	GluA2	-	Membrane insertion	(Beretta et al., 2005)
		-	Ca^{2+} dependent recycling	(Hanley and Henley, 2005)
AP-2	GluA2	-	NMDA induced internalization	(Lee et al., 2002)
BRAG 2	GluA2	Arf6	mGluA induced internalization	(Scholz et al., 2010)

* Not directly shown, but likely since KIBRA interacts with Sec8 of the exocyst complex.

the location of AMPA receptor exocytosis is heavily debated in the literature.

There are two hypothetical routes for AMPA receptor delivery to the synapse (**Figure 2**). Receptors may be delivered to the plasma membrane of dendritic shafts, from where they are free to diffuse into spinal membranes and finally enter the synapse (Figure 2, Route A). Alternatively, there may be active transport of AMPA receptor containing vessels into dendritic spines, where they fuse with the plasma membrane in close proximity to the synapse (Figure 2, Route B). Originally, it was proposed that integration is likely to happen outside the synapse as vesicular traffic through the PSD, which is positioned directly under the synaptic membrane, might interfere with its function (Harris et al., 1992). Indeed exocytosis has been reported to preferentially take place on the membrane of dendritic shafts rather than in dendritic spines in some articles (Adesnik et al., 2005; Yudowski et al., 2007). However, these findings have been challenged by an increasing amount of publications reporting exocytic events within the dendritic spines (discussed in detail below) (Passafaro et al., 2001; Gerges et al., 2004; Kennedy et al., 2010). In addition, the case for involvement of active transport of AMPA receptors has been supported by reports of decreased AMPA receptor concentration at synapses in response to blockage of either (1) endosomal trafficking factors of the Rab family (Ehlers, 2000; Gerges et al., 2004; Brown et al., 2007), (2) motor-proteins involved in cargo-transport into spines (Correia et al., 2008) or (3) spine localised exocytic machinery (Gerges et al., 2006; Kennedy et al., 2010). The factors mentioned here are discussed in detail below. It is important to keep in mind that most likely both models describe in part the physiological mechanism of AMPA receptor transport and the discussion at the end of this review suggests how these pathways may interact.

The following section reviews the literature on AMPA receptor diffusion through the plasma membrane (Route A) and also summarizes our current understanding on how AMPA receptors are captured by scaffolding proteins in the PSD to retain them in the synaptic membrane. The section thereafter gives an overview on the reports suggesting that diffusion alone does not account for the dynamics of AMPA receptor transport in dendritic spines.

Passive Transport: Lateral Diffusion

The kinetics of membrane-bound proteins have been studied extensively using both bulk and single molecule measurements. Bulk transport is usually examined using Fluorescence Recovery After Photobleaching (FRAP), which allows to calculate diffusion constants of fluorescently tagged proteins (Axelrod et al., 1976a; Reits and Neefjes, 2001) and has from the start been used to analyse receptor mobility at synapses (Axelrod et al., 1976b; 1978). On the other hand, to gain a better understanding of the kinetics of single molecules, single particle tracking has been developed (Schmidt et al., 1995; Saxton and Jacobson, 1997; Triller and Choquet, 2005; 2008) allowing to study the behaviour of different populations or pools of labelled proteins. For example, one can measure the mean squared distance (MSD) a particle has moved in a given time as a measure of how confined the mobility of the

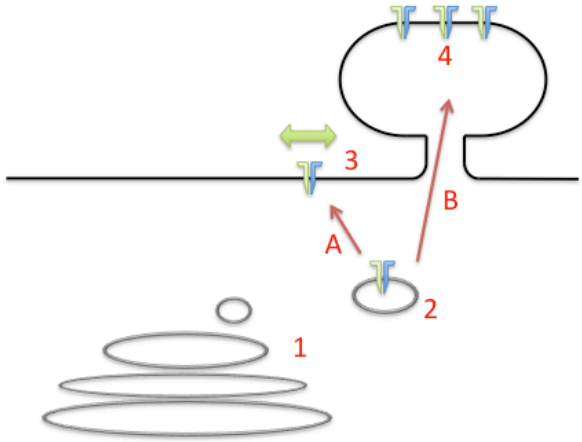

Figure 2 Delivery routes of AMPA receptors to the synapse. AMPA receptors are transmembrane proteins sorted into vesicles (2) by the trans-Golgi network (1). Ultimately, AMPA receptors need to be integrated into synapses situated in the heads of dendritic spines (4). To reach this destination they can either be inserted into the plasma membrane on dendritic shafts (3) and from there diffuse through the membrane (**Route A**), or the vesicles can be transported into the dendritic spine where integration into the membrane takes place in close vicinity to the synapse (**Route B**).

particle is. A freely diffusing particle has a liner function of MSD over time, whereas the function reaches a maximum, if mobility were confined (**Figure 3**). A combination of these and other methods has been employed to formulate the classic model of fluid mosaic membranes, stating that proteins can diffuse rather freely through the phospholipid bilayer (Singer and Nicolson, 1972). Nevertheless, receptor mobility is also influenced by its interactions with other proteins and may be hindered by physical barriers (Kusumi et al., 2005).

Such lateral diffusion of molecules through the plasma membrane may account for dynamic integration of AMPA receptors into synapses if receptors can be retained in the synapse (Triller and Choquet, 2005) and if access to the dendritic spines is regulated by the spine neck (Ashby et al., 2006). Some electron microscopy (EM) based morphological studies of synapses have given the false impression that receptor molecules are nearly exclusively found in the synaptic membrane (Nusser, 2000). However, extrasynaptic receptors and continuous receptor flux in the plasma membrane have been established as a more accurate model of receptor distribution. For instance, it had already been shown in 1983 that acetylcholine receptors in neuromuscular junctions are undergoing rapid lateral diffusion (Young and Poo, 1983). While a chemical inhibitor of acetylcholine receptors could prevent acute receptor-induced depolarisation, over time depolarisations reappeared. By showing that this recovery could be blocked by crosslinking acetylcholine receptors to prevent their diffusion throughout the membrane, the first demonstration of lateral diffusion of receptors was published and led to the hypothesis that diffusing receptors may be trapped by synapses (Young and Poo, 1983).

AMPA Receptor Diffusion in the Plasma Membrane

The first study to distinguish plasma membrane bound AMPA receptors from the intracellular pool followed AMPA receptor exocytosis under basal and stimulated conditions and found subunit-dependent differences in receptor transport (Passafaro et al., 2001). Using overexpression of GluA1 and GluA2 proteins containing a small tag on the extracellular amino-terminus that can be cleaved by thrombin treatment, this study dissected the differences in kinetics and localisation of GluA subunits. GluA2 was identified as integrating slowly but constitutively into the membrane while GluA1 integration was fast and strongly upregulated during glycine induced LTP stimulation (Passafaro et al., 2001). Furthermore, by studying the location of newly integrated AMPA receptors, it was shown that GluA2 integrates directly into spine heads, as they instantly co-localised with staining for Shank, a component of the PSD in dendritic spines. GluA1, on the contrary, was initially detected diffusely along the dendritic membrane and only a slight local enrichment was measured in spines after 5 to 15 minutes following NMDA stimulation (Passafaro et al., 2001). All these differences have been shown to depend on the intracellular carboxy-terminal domain of the subunits, as replacing the GluA1

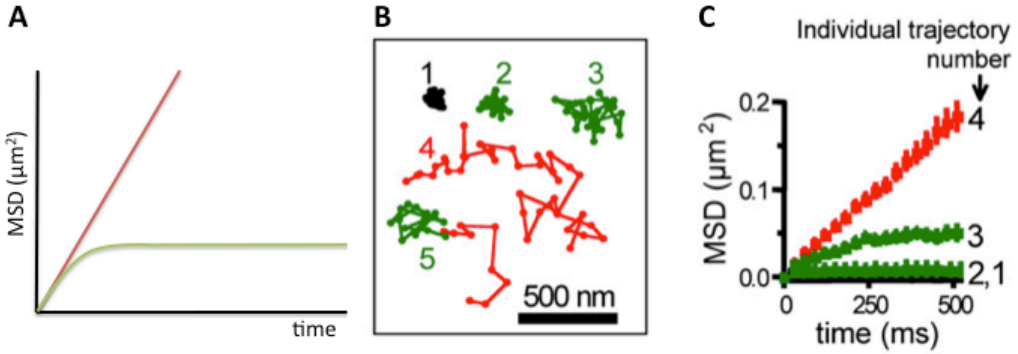

Figure 3 Quantification of mobility of membrane bound proteins. A Plotting mean squared displacement (MSD) against time results in a liner function for freely diffusing particles, while it reaches a maximum, if mobility is confined. **B** Example plots of XY movement of membrane proteins (green: confined, red: free, black: Brownian motion). **C** MSD/time plots as in A for tracks shown in B. Panels B & C are reproduced from Tardin et al. 2003.

carboxy-terminus with the one of GluA2 resulted in GluA2-like transport and vice versa (Passafaro et al., 2001). These results highlight differences in cellular transport of AMPA receptor subunits but did not answer whether different subunits use different transport modes or how the carboxy-terminus regulates their trafficking.

Further support for integration of AMPA receptors at the dendritic shaft came from experiments using superecliptic pHluorin tagged GluA1 (SEP-GluA1), which allows imaging selectively membrane bound GluA1, as the low pH of intracellular vesicles quenches this GFP derived fluorophore (Miesenböck et al., 1998; Yudowski et al., 2007). Imaging this construct after photo-bleaching a region of dendrites (to reduce fluorescence of SEP-GluA1 already present in the membrane), exocytosis could be visualized on the dendritic shaft (Yudowski et al., 2007). Interestingly, some exocytic events resulted in a wave of increased fluorescence spreading along the membrane (up to 5µm) into nearby spines, indicating a possible diffusion of the receptors into surrounding synapses (Yudowski et al., 2007). While current studies challenge the view that exocytosis occurs solely on the dendritic shaft (see below), this study introduced a valuable tool to follow membrane bound GluA subunits and demonstrates lateral diffusion occurring on the dendritic shaft.

Using FRAP on SEP-tagged GluA1 and GluA2, several studies showed diffusion from the dendritic shaft into spines and also in and out of synapses (see e.g. Ashby et al., 2006; Newpher and Ehlers, 2008; Makino and Malinow, 2009; Opazo and Choquet, 2011). A recent study could confirm that, while GluA2 is integrated into synapses continuously irrespective of LTP induction, GluA1 is more efficiently incorporated into synapses upon LTP (Makino and Malinow, 2009). Studying LTP in single spines of hippocampal slices, two-photon glutamate uncaging resulted in increased concentration and immobilization of GluA1 in the spine, an effect not seen for GluA2 (Makino and Malinow, 2009). This led the authors to claim that GluA1 containing AMPA receptors are needed for synaptic potentiation. At least this experiment shows that no changes in GluA2 are required during LTP. Seeing that GluA2 binds both GluA1 and GluA3 and that these heterotetramers are considered to be required for stimulating and basal conditions, respectively (Shi et al., 2001), it might have been of additional value to monitor GluA3 behaviour in this study. Nevertheless, by combining FRAP and electrophysiological readouts with glutamate uncaging at single spines, this report makes a compelling case for a model in which LTP is predominantly mediated by synaptic entrapment of diffusing GluA1 receptors from the immediate extrasynaptic region of the spine. Over the following minutes, the pool of available GluA1 in the membrane is then suggested to replenish by local up-regulation of GluA1 exocytosis into the dendritic membranes close to the spine (Makino and Malinow, 2009). The signalling mechanisms responsible for such a localised exocytosis were not investigated, but others proposed locally restricted calcium signalling (Newpher and Ehlers, 2008; Opazo and Choquet, 2011). Supporting evidence for this model comes from studies showing AMPA receptors shifting in and out of the perisynaptic region upon LTP or LTD induction (Tardin et al., 2003; Ashby et al., 2004). While no exocytic events in dendritic spines were shown in this study, recent reports on exocytic zones in spine heads (Kennedy et al., 2010) may also explain the source of such perisynaptic receptors.

Following Single Receptor Molecules

While analysis of bulk transport of AMPA receptors with techniques like FRAP can indicate overall kinetics of their diffusion, more accurate methods are necessary to determine differences between different pools of AMPA receptors or even individual molecules. This is especially important as the bleached region in FRAP experiments is often larger than a synapse, making the distinction between synaptic and perisynaptic regions difficult. In agreement with the results of FRAP studies (Makino and Malinow, 2009), single particle tracking revealed differences in the mobility of AMPA receptors at synapses compared to extrasynaptic membranes. This mobility of individual receptors is required for their insertion into the synapse by lateral diffusion.

The first publication of single receptor movements was based on GluA2 antibody coated 0.5 µm diameter latex beads, whose displacement was followed with DIC microscopy after allowing them to bind to receptors on the dendritic surface (Borgdorff and Choquet, 2002). With this technique, it was possible to discriminate between periods of rapid and slow AMPA receptor diffusion and to quantify their confinement. During maturation of dissociated neuron cultures, an increase in transient receptor immobilization could be measured as synaptogenesis proceeded (Borgdorff and Choquet, 2002). The authors identified the regions of confinement as likely synaptic regions, judging by staining with the presynaptic marker FM1-43. Functional studies showed that Tetrodotoxin treatment or lowering intracellular calcium levels decreases receptor confinement and shortens retention times. In contrast, uncaging calcium ions locally in the vicinity of these beads triggered receptor retention for longer than 200 seconds in 76% of the observed cases. By this time, the calcium concentration had levelled off to basal state (Borgdorff and Choquet, 2002). These results suggest that a short elevation in calcium concentration leads to longer-lasting downregulation of AMPA receptor mobility in synapses. Lastly, immunostaining of endogenous receptors showed that surface GluA2 levels were strongly increased at sites of repeated calcium uncaging (Borgdorff and Choquet, 2002). While latex beads yielded interesting results on receptor mobility on the dendritic membrane, their size limits detailed studies of AMPA receptor diffusion in the synapse.

Building upon these results, Tardin and co-workers used fluorescently labelled antibodies on live neurons to follow GluA2 diffusion in dendritic spines and particularly in synapses. The GluA2 antibody was directly labelled, e.g. with Cy5, at low concentrations, to avoid having more than one fluorophore per antibody. Subsequently, live neuron cultures were briefly incubated with a low concentration of labelled antibody to tag a subset of GluA2 receptors on the neuron surface. Since most fluorescent spots displayed one-step bleaching, they are likely to correspond to single GluA2 molecules (Tardin et al., 2003). Single molecules could be followed for up to 4 seconds and their trajectories were reconstructed (see Figure 3B, C). Analysis of these trajectories revealed that GluA2 receptors at synapses correlate with a smaller mean-squared displacement and thus a more confined diffusion than extrasynaptic receptors (with a distance of more than 300nm synaptic staining) under non-stimulated conditions. However, at any position there was a considerable variation in AMPA receptor diffusion (Tardin et al., 2003), but overall, the kinetics of AMPA receptor diffusion are comparable with previous measurements based on latex bead tracking (Borgdorff and Choquet, 2002). Using a glutamate bath application, endocytosis of AMPA receptor was induced as described previously (Carroll et al., 1999). Next to increased endocytosis, this treatment also resulted in increased diffusive mobility of AMPA receptors in the synapse and decreased the pool of immobile receptors in the synapse by nearly a third. Conversely, an increase in AMPA receptor concentration was observed in the perisynaptic region. Intriguingly, glycine stimulation and biccuculine application only reduced the mobility of newly exocytosed AMPA receptors for the initial 5 minutes but did not change mobility of AMPA receptors present in the synapse. 40 minutes after the treatment, a striking decrease of AMPA receptor concentration was measured in the perisynaptic region (Tardin et al., 2003). It should be kept in mind that this study exclusively followed GluA2 receptors, not GluA1, which was shown to translocate into synapses upon stimulation (Passafaro et al., 2001; Makino and Malinow, 2009). Also possible diffusion of AMPA receptors from outside the dendritic spine into the synapse was not assessed. Nevertheless, these findings suggest that LTP inducing stimulation increases AMPA receptor uptake and immobilization in the synapse, while LTD increases their mobility and allows them to diffuse out of the synapse.

Yet another approach to follow AMPA receptor diffusion is to use antibody-conjugated semi-conductor Quantum Dots (QD). Their size is a reasonable compromise and is small enough to enter the synapse, while at the same time they are more resistant to photobleaching than

fluorophores like Cy5, allowing longer imaging. Moreover, QDs have previously been used for similar studies on glycine receptors (Dahan et al., 2003). Following GluA1 antibody conjugated QD, AMPA receptors were shown to be immobilized in active synapses, leading to an accumulation of GluA1 at stimulated synapses (Ehlers et al., 2007). This effect is the result of decreased diffusion rates of GluA1 out of active synapses, leading to longer dwell times and less receptor exchange with extrasynaptic membrane. Interestingly, this behaviour was studied with spontaneous synaptic activity of cultured hippocampal neurons that were not artificially stimulated. Under these basal conditions, AMPA receptor retention at synapses was not decreased following acute inhibition of presynaptic neurotransmitter release, indicating that long-term adaptations of active synapses are the underlying factor retaining GluA1 (Ehlers et al., 2007). While following individual QD-tagged receptor molecules, single receptors were observed diffusing out of an inactive synapse and subsequently trapped at an active synapse, showing that diffusion of AMPA receptors between closely spaced spines is possible. Moreover, this shows that the diffusion kinetics of AMPA receptors are determined by the synapses and not exclusively by the specific receptor molecules (Ehlers et al., 2007). It would be interesting to study whether interspinal attraction of AMPA receptors is increased in spines undergoing LTP. However, seeing that such events were monitored only very rarely, it is unlikely that diffusion of AMPA receptors from other dendritic spines is the main effector of receptor accumulation in active synapses. Lastly, this publication reports that in active synapses, the diffusion of individual GluA1 molecules is very confined. While inactive synapses retain GluA1 more than shaft membrane, the receptor molecules are recorded to travel throughout most of the synaptic membrane. In active synapses these movements are more confined to what seems to be microdomains within the postsynaptic membrane (Ehlers et al., 2007).

Taken together, these reports have suggested a model in which AMPA receptors can move in and out of synapses by diffusing through the plasma membrane and that synaptic plasticity can be achieved by a calcium concentration dependent mechanism regulating receptor immobilization at the synapse. However, it remains unclear on which scale such diffusion is relevant for AMPA receptor delivery. Keeping in mind the possible pathways of AMPA receptor delivery to the synapse (Figure 2), lateral movement may be needed for long-range diffusion from the shaft to the synapse (Route A) or for short-range diffusion within the spine head (Route B). Supporting evidence for the former path may be found in the observed exocytosis of AMPA receptors into the shaft membrane (Yudowski et al., 2007; Makino and Malinow, 2009). On the other hand, undirected exocytosis all over the dendritic membrane is counterintuitive to fast activity dependent spine localised accumulation, because receptors would have to diffuse over longer distances and are therefore also more likely to encounter obstacles slowing down this process (Newpher and Ehlers, 2008). Unfortunately, the studies above could not demonstrate the origin of the exocytosed vesicles, i.e. are they vesicles carrying newly synthesized receptors from the Golgi network, or are they recycling endosomal compartments? Moreover, the results on diffusion between synaptic and perisynaptic regions on the spine head (Tardin et al., 2003; Ehlers et al., 2007) are compatible with both delivery routes as spinal endo- and exocytosis takes place laterally of the PSD (see below; Blanpied et al., 2002; Kennedy et al., 2010).

Mechanisms of AMPA Receptor Immobilization

From the studies reviewed above it became apparent that AMPA receptors change their diffusion kinetics upon entry into the synapse. In turn, this immobilization at the synapse is regulated by synaptic stimulation. In addition, clustering of AMPA receptors in the PSD (Ehlers et al., 2007) has been reported in EM studies, showing an accumulation of proteins in specific subdomains of the postsynaptic membrane, while other areas appeared void of these clusters (Petersen et al., 2003). Such clustering of AMPA receptors into microdomains, which are potentially even coupled to presynaptic release sites (MacGillavry et al., 2011) may be necessary to elicit responses from

these receptors as they have been shown to have low affinity to their agonists (Traynelis et al., 2010). Mathematical modelling highlighted the crucial role of receptor positioning, as even small displacements result in decreased response probability (Raghavachari and Lisman, 2004). In line with this hypothesis, NMDA receptors, which have higher affinity to glutamate, are usually found in lower numbers per spine than AMPA receptors and seem to be restricted to the centre of the PSD (Kharazia et al., 1996). It is thus possible that the low affinity of AMPA receptors for glutamate requires their clustering in specific microdomains of the synapse. This section focuses on how receptor immobilization is achieved on a molecular level by the PSD. In particular, interactions between AMPA receptors and proteins in the PSD, molecular crowding, the architecture of the spine compartment and the membrane lipid composition are evaluated for their respective roles in this process.

Protein Interactions in the PSD

AMPA receptors bind to several proteins of the PSD that may regulate their retention. PSD proteins are therefore obvious candidates for AMPA receptor retention. PSD-95 is the best-studied AMPA receptor binding proteins in the PSD. The interaction between these two proteins is indirect as transmembrane AMPAR regulatory proteins (TARPs) are required both to bind PSD-95 and to accumulate AMPA receptors at the synapse (Bats et al., 2007). Interestingly, TARPs remain closely associated with AMPA receptors. If prevented from binding to PSD-95, AMPA receptors were seen to diffuse in complex with TARPs out of the synapse, as measured by FRAP (Bats et al., 2007). Another study on this complex showed that overexpression of TARPs leads to accumulation of AMPA receptors in the extrasynaptic membrane without changing the electrical transmission of the synapse, while overexpression of PSD-95 localises more AMPA receptors directly into the synapse (Schnell et al., 2002). Using FRAP to asses the mobility of PSD-95 within the synapse, it was demonstrated that in contrast to AMPA receptors (Ehlers et al., 2007), PSD-95 is nearly completely immobile in the PSD (Blanpied et al., 2008). However, not only the amount of PSD-95 available in the PSD may determine AMPA receptor retention but also their binding affinity may play a role, as phosphorylation of the TARP stargazin by CaMKII upon LTP induced calcium influx facilitates PSD-95 binding (Sumioka et al., 2010). Intriguingly, CaMKII also phosphorylates PSD-95 during LTP, resulting in its destabilisation within the PSD and loss of other PSD components (Steiner et al., 2008). Since this destabilization is only transient, it may underlie reorganisation and strengthening of the PSD in response to stimulation (MacGillavry et al., 2011). Such behaviour is reminiscent of the short-term increase of AMPA receptor diffusion discussed above and it would be interesting to study whether the two observations may be linked. In summary, these studies suggest that PSD-95 is required for functional insertion and retention of AMPA receptors into the synapse but also show that there may be additional regulation beyond a simple PSD-95 slot model. Current reports also focus on the positioning of vertically oriented PSD-95 filaments and CaMKII holoenzymes within the PSD, which has been suggested to confine AMPA receptors within microdomains (Blanpied et al., 2008; Newpher and Ehlers, 2008). Direct evidence for these models is yet to be reported. The model is further complicated by the fact that the scaffold proteins themselves have a differentially regulated turn-over at the PSD mediated by independent or redundant mechanisms, as recent FRAP studies have shown (Kuriu et al., 2006; Blanpied et al., 2008).

Next to interacting with PSD-95, AMPA receptors also bind to PICK1 (Xia et al., 1999) and GRIP/ABP (Dong et al., 1997), which both contain PDZ domains and compete for binding to the GluA2 carboxy-terminus. It has been speculated that PKC mediated binding to PICK1 targets AMPA receptors for endocytosis because PICK1 contains a BAR domain attracting it to curved membranes as found in endocytic clathrin coated pits (Jin et al., 2006) and because it may regulate actin polymerization at these zones by interacting with Arp2/3 (Rocca et al., 2008). If these proteins are mutated to prevent AMPA receptors binding, synaptic levels of AMPA receptors decline. Initially, this was thought to be a defect of AMPA receptor retention, but recently

this effect was proposed to be due to errors in receptor delivery to the synapse (Osten et al., 2000).

A recent review speculates that other proteins may contribute to the establishment and maintenance of AMPA receptor clusters, namely involvement of the actin skeleton and adhesion molecules like the neuroligin / neurexin complex (MacGillavry et al., 2011). While the latter hypothesis still lacks substantiating evidence, interactions between actin filaments and the PSD have been reported (Capani et al., 2001) but since there is no direct interaction between AMPA receptors and the cytoskeleton in the synapse, the role of actin is further discussed in the following section.

Taken together, there is accumulating evidence that AMPA receptors (including TARPs) interact with a variety of proteins in the PSD that may mediate their retention. It is important to remember that the proteins introduced above are involved in the overall structure of the PSD, which contains additional scaffolding proteins and other components (Okabe, 2007; Sheng and Hoogenraad, 2007). This dense network may position AMPA receptor binding proteins at specific sites and regulate their retention into microdomains at the synapse (MacGillavry et al., 2011). The protein dense structure of the PSD may also have an effect on receptor diffusion dynamics by confining their movement through steric interactions and limitation of available space as discussed below.

Cytoskeleton & Molecular Crowding

Another method to confine AMPA receptors to the synapse could lie in unspecific interactions that prevent diffusion out of the synaptic membrane by forming physical barriers. Such barriers can consist of cytoskeletal proteins of which actin is the most prominent one in dendritic spines. Actin may indeed play a role in AMPA receptor retention at the PSD because (1) the abundance of synaptic receptor clusters decreased by 40% in response to depolymerisation of F-actin (Allison et al., 2000), (2) subcortical actin slows diffusion of membrane bound proteins specifically in dendritic spines, as seen by photobleaching membrane-bound GFP (Richards et al., 2004), and (3) actin has been shown to bind the PSD scaffold (Capani et al., 2001; Okabe, 2007; Sheng and Hoogenraad, 2007). However, many proteins of the PSD, including PSD-95, remain unaffected if actin filaments are destabilized pharmacologically (Kuriu et al., 2006), raising the question whether there are multiple independent scaffold complexes in the PSD. It has also been suggested that actin is responsible for maintaining the deeper layers of the PSD, e.g. GKAP and SHANK, while those complexes closer to the membrane are actin independent; a notion that fits with the current understanding of the laminar build-up of the PSD (Kuriu et al., 2006; Newpher and Ehlers, 2008). Yet these data may also simply reflect the actin dependent transport of synaptic proteins, rather than a role of actin in determining microdomains directly. Especially since no significant actin structures have been found within the PSD or directly surrounding the synaptic membrane by EM and because F-actin is known to regulate protein trafficking from the dendritic shaft into spines (Blanpied et al., 2008), it seems more plausible that actin depolymerisation leads to insufficient turnover of PSD proteins rather than to a direct defect in AMPA receptor positioning. This notion does not conflict with the overall constraint of membrane diffusion by subcortical F-actin that has been shown using membrane anchored GFP exchange in dendritic spines (Richards et al., 2004), but rather questions that F-actin retains AMPA receptors specifically in the PSD. Nevertheless, corralling by PSD-95 may not only define the PSD as a whole but might also be employed to define microdomains within the synaptic membrane (MacGillavry et al., 2011). Such a model is supported by the presence of relatively stable PSD-95 scaffolds that may convey spatial information within the PSD (Blanpied et al., 2008).

Modelling showed that also the sheer crowdedness of proteins in the PSD may retain AMPA receptors in the postsynaptic membrane (Santamaria et al., 2010). The bulkiness of the extracellular domain of AMPA receptors (Sobolevsky et al., 2009) is likely to enhance this effect due to collisions and steric interactions that greatly reduce the diffusion efficiency of the

protein. While some proteins like N-cadherin or neuronal pentraxins are believed to establish a barrier to AMPA diffusion by directly binding to GluA2 and presynaptic membrane (O'Brien et al., 1999; Saglietti et al., 2007), other unspecific factors like lipid rafts and ECM have also been suggested to restrict AMPA diffusion. AMPA receptors bind to mobile lipid rafts on the cell surface after NMDA stimulation (Hou et al., 2008) and this interaction traps AMPA receptors at the synapse. If these rafts are depleted, endocytosis of the receptor is upregulated (Hering et al., 2003) and exocytosis is downregulated (Hou et al., 2008), resulting in lower AMPA receptor concentration at the synapse. Lipid rafts may thus not only have a trapping effect but also regulate endo- and exocytic trafficking of AMPA receptors in the spine membrane. How these barriers organize AMPA receptors in the synapse remains unclear as of yet.

Dendritic Spine Architecture

Lastly, the physical shape of dendritic spines may account for the kinetics of AMPA receptor diffusion in the membrane. Biophysical modelling showed that spine geometry, and the length of the spine neck in particular, strongly influences exchange of AMPA receptors between dendritic shaft and spine head or PSD (Holcman and Triller, 2006). Also experimental results show that the morphology of spines influences the diffusion rate of membrane proteins as spines compartmentalize not only the cytoplasm (Svoboda et al., 1996) but also the plasma membrane. Diffusion of AMPA receptors into mushroom spines, having a narrow neck, was found to be slower compared to stubby spines with a short and wide neck (Ashby et al., 2006). Fluorescence loss in photobleaching (FLIP) showed that GluA2 diffuses throughout the spine membrane and FRAP experiments revealed these differences in diffusion kinetics between different kinds of dendritic spines (Ashby et al., 2006). Unfortunately, the report falls short of elucidating the mechanism behind the diffusion barrier in the spine neck. Moreover, earlier studies showed that membrane bound GFP diffused slower out of dendritic spines than along the shafts, implying that membrane diffusion in spines is generally retarded and that this effect is not specifically retaining glutamate receptors (Richards et al., 2004). In a situation where AMPA receptors are integrated into the plasma membrane of the dendritic shafts, this will clearly have an important effect on AMPA receptor availability at the synapse by regulating its speed of diffusion.

Taken together, the differences in AMPA receptor diffusion between synaptic and extrasynaptic membranes can be explained by a multitude of factors. Probably, it is not a single mechanism governing this process but an intricate interplay between them that facilitates the availability and location of AMPA receptors in the synaptic membrane. These mechanisms are not solely important for AMPA receptor delivery by lateral diffusion but may equally support aspects of the active transport model, which is discussed in the following section.

Active Transport: Endosomal Trafficking

It is thus apparent that lateral diffusion of AMPA receptors can explain some aspects of their basal and activity driven transport. Yet, increasing evidence has been put forward highlighting the role of active endosomal transport to control AMPA receptor availability at the synapse involving several intracellular compartments (Kennedy and Ehlers, 2006). The following part of this review is dedicated to investigate the necessity and role of endosomal transport for AMPA receptor integration. Since neurons are polarized cells, it is important that they can rely on directed transport of proteins into their specialized compartments. An intricate system of the secretory vesicle system originating from the Golgi apparatus and the endosomal compartments take over this form of active transport and ensure that proteins are delivered to their site of activity, e.g. AMPA receptors to the synapses in dendritic spines. The endosomal system of neurons is comparable to the one studied in polarized epithelial cells, but certainly there are also differences in the neuron specific functions of several structures and proteins (for review see Lasiecka and Winckler, 2011).

Overall, many trafficking steps are poorly understood, especially owing to the complexity and dynamics of neuronal endosomes. Nevertheless, several lines of evidence demonstrate the involvement of endosomes in the process of AMPA receptor delivery to the synapse.

Spinal Endo- & Exocytic Zones

Early studies on endocytosis showed that AMPA receptors are internalized upon stimulation of either AMPA or NMDA receptors, which could be visualized by internalization of GluA1 antibodies and subsequent acid wash to prevent detection of membrane bound GluA1 (Carroll et al., 1999). Upon AMPA treatment, GluA1 co-localized within five minutes to AP2 enriched sites, suggesting that activity induced endocytosis is dependent on clathrin coated pits (for review on clathrin coated pits see Brodsky et al., 2001). Indeed, it was demonstrated that inhibition of this pathway by chemical interference or expression of dominant negative Dynamin-2 prevents this AMPA induced internalization of GluA1 (Carroll et al., 1999). Conversely, stimulation of synaptic activity was shown to increase internalization of AMPA receptors into intracellular vesicles (Ehlers, 2000). Moreover, studies in brain slices highlighted the role of continuous endo- and exocytosis for synaptic plasticity using electrophysiological read-outs (Lüscher et al., 1999). Yet, these early studies could not resolve where endocytic events take place and considered the entire somatodendritic area as one compartment. In contrast to the evidence on dendritic shaft localised exocytosis discussed above, there is increasing evidence for spine localized areas of AMPA receptor endocytosis and also, as discussed later, exocytosis.

In the seminal publication by Blanpied *et al*., detailed analysis of the localization of clathrin-GFP fusion proteins in cultured neurons revealed hot-spots of endocytosis in dendritic spines. In fact, 75% of all spines showed endocytic zones in mature neurons, mostly in the heads of the spines and adjacent to the PSD (Blanpied et al., 2002). Moreover, EM on adult hippocampal regions confirmed the lateral position of clathrin and showed that within dendritic spines, AP-2 adaptor proteins are closer to the PSD than are clathrin and dynamin, suggesting that next to the defined structure of the PSD, also the endocytic zone may have a specific molecular architecture facilitating protein transport from the synapse (Rácz et al., 2004). Interestingly, it was also shown that the lifetime of individual clathrin coated pits increased ten-fold during neuronal maturation in cultured neurons as judged by kymograph analysis (Blanpied et al., 2002). Possibly this effect is mediated by anchoring of the endocytic zone by scaffold proteins of the PSD (Lu et al., 2007). Moreover, the morphology of dendritic spines also correlates with this effect: dendritic filopodia usually have weaker endocytic zones compared to mushroom shaped spines, possibly due to the lack of an extensive PSD (Blanpied et al., 2002). No difference in the dynamics of spinal clathrin coated pits could be induced by artificial synaptic stimulation, suggesting that synaptic activity does not influence the formation of the endocytic zone. Strikingly, FRAP experiments on clathrin-GFP revealed that, while cycling is generally rapid, recovery in the spine head is slower and less complete than in the dendritic shaft (Blanpied et al., 2002). This raises the possibility that there is a limited pool of clathrin cycling within a dendritic spine and underlines the differences in spine versus shaft compartments of the dendrites. Unfortunately, the cargo taken up by endocytosis was not investigated in this paper but EM studies show GluA2/3 dimers situated in spinal clathrin coated pits during internalization (Petralia et al., 2003). How the position of endocytic zones is initially specified is not clear. Next to a role of PSD scaffolds (Lu et al., 2007), it is tempting to speculate that the lipid composition of the membrane determines the position of the endocytic zone as Dynamin-3 and AP-2, amongst others, bind specifically to $PI(4,5)P_2$ lipids (Newpher and Ehlers, 2008).

Overexpression of dominant negative Dynamin-3, which is specifically found in postsynaptic specializations, in contrast to the ubiquitous Dynamin-2 discussed above (Carroll et al., 1999), has been shown to disrupt localization of the endocytic zone to the PSD in dendritic spines (Lu et al., 2007). Under basal conditions, this leads to a decrease of electric stimuli in the postsynapse

due to depletion of AMPA receptors (Lu et al., 2007). Moreover, glycine treatment cannot induce the usual LTP like plasticity if endocytic zones are dislocated (Petrini et al., 2009). Rather, it was observed that the mobile pool of AMPA receptors (as determined with FRAP) necessary for LTP is dependent on recycling endosomes that take up GluA1 by endocytosis. Therefore, if the endocytic zone is ablated (Dynamin-3 knockdown or dominant negative expression) or recycling endosomes are inhibited (dominant negative Rab11a), synaptic potentiation is prevented by insufficient mobile GluA1 supply to the synapse (Petrini et al., 2009).

Directed exocytosis of AMPA receptors in dendritic spines is still a topic under debate. While it seems unlikely that AMPA receptors diffuse all along the dendrites into every dendritic spine, a combination of endosomal transport and short range lateral diffusion from the dendritic shaft into the nearby spines is an emerging model (Newpher and Ehlers, 2008; Opazo and Choquet, 2011). Several studies have focused on the kinetics of AMPA receptor insertion and showed that there is a subtype specific regulation of AMPA receptor integration dependent of differences in the carboxy-terminal domains (Passafaro et al., 2001). Localising the precise sites of AMPA receptor integration into the cell membrane has proven more difficult, especially as overexpressed fluorescently tagged fusion protein may undergo different transport and exocytosis than the endogenous AMPA heterodimers (Newpher and Ehlers, 2008).

Using expression of transferrin receptor (TfR) doubly tagged with SEP and mCherry, to visualize plasma membrane bound and total pool of this endosomal membrane marker, respectively, Kennedy *et al.* could follow exocytic events in single spines. Under basal conditions 56% of dendritic spines had stable recycling endosomes in their neck or head, many of which could also been shown to contain GluA1 and to occasionally fuse with the plasma membrane (Kennedy et al., 2010). Upon glycine stimulation, these recycling endosomes fused with the spine's plasma membrane just lateral to but not overlapping with the PSD. This fusion released all GluA1 stored in spinal recycling endosomes into the plasma membrane, from where they were either trapped at the synapse (62%) or diffused out of the spine (38%). This all-or-nothing release depleted recycling endosomes from dendritic spines after exocytosis (Kennedy et al., 2010). Subsequently, it was shown that Syntaxin-4 (Stx4) is a t-SNARE protein, which is enriched in the post-synapse and co-localizes to the exocytic release site in dendritic spines lateral of the PSD. Exocytosis was also directly shown in Stx4 rich areas by following TfR-SEP at Stx4-HA positive sites in COS-7 cells and hippocampal dendritic spines (Kennedy et al., 2010). Depleting Stx4 with shRNA expression or inhibiting Stx4 function by expression of a dominant negative Stx4 protein, led to a specific reduction of exocytic events, showing that Stx4 is required for rapid activity dependent exocytosis. Lastly, it was also shown that Stx4 inhibition prevents LTP expression (Kennedy et al., 2010).

Studies on the exocyst complex, which is involved in exocytosis in different systems including membrane trafficking during neuronal development, shed light on the molecular mechanism of AMPA receptor exocytosis and suggested active delivery of the receptors into dendritic spines (Gerges et al., 2006). In particular, two members of this complex, Sec8 and Exo70, were shown to be required for AMPA receptor induced electrical currents in hippocampal slice cultures and to co-immunoprecipitate with AMPA receptors in hippocampal synaptosomal extracts. Judging by a general transport defect in neurons expressing dominant negative Sec8, this excoyst member has a role in directional trafficking of GluA2 receptors to dendrites. On the other hand, dominant negative Exo70 results in defects specific to the integration of GluA1 and GluA2 into the membrane of spines and led to accumulation of both types of receptors in the interior of the spine, suggesting that its function is directly related to spinal exocytosis (Gerges et al., 2006). This finding is particularly interesting as AMPA receptor accumulation in the interior of the spine is indicative of endosomal transport of AMPA receptors to the spinal membrane. Thus, it appears that AMPA receptors are not only passively

diffusing into spines, but active endosomal transport constitutively recycles a pool of GluA2 and additionally recruits GluA1 receptors in response to CaMKII signalling. Together, these papers demonstrate that AMPA receptors are actively transported into dendritic spines where they are shuttled in and out of the synapse by spinal exocytosis and endocytosis in close proximity to the PSD. Next, endosomal traffic of AMPA receptors in dendritic spines will be reviewed.

Recycling Endosomes

A first indication that intracellular storage vesicles are the source of AMPA receptors during LTP came from a study on glycine stimulated neurons that showed neither LTP nor AMPA receptor integration if treated with tetanustoxin, a potent inhibitor of SNARE dependent exocytosis (Lu et al., 2001). Also, after ligand induced AMPA receptor internalization, receptors were found in endosomal vesicles (Ehlers, 2000). Thus, it appears that endosomal transport of AMPA receptors is essential for regulating the concentration of AMPA receptors at the synapse. Park and co-workers used an elegant system inhibiting trafficking from endosomal compartments to the plasma membrane by expressing dominant negative mutants of Rme1 and Rab11a, two proteins required for endosomal recycling, to study spinal AMPA receptor trafficking. Indeed, expression of these constructs decreased GluA1 antibody staining on the dendritic surface. In particular, it was shown that previously endocytosed GluA1 failed to be re-integrated into the membrane but was instead found in endosomes predominantly located in dendritic shafts (Park et al., 2004). NMDA dependent AMPA receptor potentiation after glycine stimulation of cultured hippocampal neurons or hippocampal slices was inhibited by expression of dominant negative Rme1, Rab11a, Syntaxin-13, (all recycling endosome associated) but left unaffected by dominant negative Rab6a (Golgi associated) or Syntaxin-7 (late endosome associated). Moreover, after pharmacologically blocking protein synthesis, no deficiency in GluA1 integration during LTP could be measured (Park et al., 2004). This supports the notion that specifically recycling endosomes are the source of cycling AMPA receptors during LTP. However, increased endosomal trafficking is not restricted to AMPA receptors. By monitoring fluorescent transferrin, this study demonstrated that the kinetics of overall neuronal endosomal recycling was upregulated two-fold during LTP. Interestingly, it was also shown that basal delivery of newly synthesized receptors was not affected by expression of the dominant negative proteins listed above, indicating that there are at least two pathways of AMPA receptor transport to the synapse (Park et al., 2004). In a follow-up study by the same group, emphasis was put on changes in spine morphology resulting from LTP induced endosomal transport. Recycling endosomes were imaged in dissociated neuron cultures with immunofluorescence microscopy of transferrin cargo and in hippocampal slices with EM. This showed that about 90% of dendritic spines have endosomes in their proximity, mostly at the base of the spine (Park et al., 2006). Blocking recycling endosomes with dominant negative Rme1, Rab11a or Syntaxin-13 (as above), resulted in rapid loss of dendritic spines (Park et al., 2006). LTP induction increased spine size by mobilization of endosomes into the spine head. Time-lapse microscopy showed LTP induced accumulation of endosomes in spine heads, which could be prevented by acutely or genetically blocking endosomal trafficking. It was also shown that exocytosis of these endosomal vesicles occurred preferentially in spine heads, but also at the dendritic shaft. Upon LTP induction, this process was highly upregulated, both in cultured cells and slices. Slices also showed that LTP induced endosome displacement to the spine head was transient, a result of the exocytosis events which deplete the pool of endosomes within 30 minutes (Park et al., 2006).

Another study investigated diffusion kinetics of cytoplasmic proteins through spine necks in cultured slices of hippocampal neurons. Photoactivatable GFP was traced diffusing between spines of different morphology and after induction or inhibition of synaptic potentiation. It was found that the diffusion rate through the neck is dependent on the cross-sectional area of the neck. Furthermore, the neck diameter is in turn dependent on synaptic activity (Bloodgood and

Sabatini, 2005). Thus, the dendritic spines can be seen as compartments regulating the diffusion of cytoplasmic and membrane signalling molecules. Such regulation of cytoplasmic diffusion into dendritic spines is likely to also have effects on transport of endosomes through spine necks.

Other studies focused on the nature of the endosomal vesicles involved in AMPA receptor trafficking. In particular, the Rab family of proteins are common markers for the kind of endosomal compartment they are found in, next to their diverse functions in vesicle formation, motility and fusion (for review see Stenmark, 2009). Rab8 was shown to be localised exclusively to the somatodendritic compartment in neurons (Huber et al., 1993) and found to localise to postsynaptic terminals with EM (Gerges et al., 2004). Studying the effect of dominant negative Rab8 in hippocampal slices with electrophysiological techniques demonstrated that Rab8 is required for constitutive GluA2 and acute GluA1 delivery and their induced currents, under basal and stimulated conditions, respectively (Gerges et al., 2004). Additional studies of GluA2 surface levels determined by fluorescence intensity line plots further revealed that Rab8 inhibition leads to a decrease of GluA2 on the surface of dendritic spines compared to adjacent shafts, while the total amount of receptor remained unchanged (Gerges et al., 2004). These results indicate that Rab8 is not required for long-distance trafficking of newly synthesized AMPA receptors from the trans Golgi network but suggests an involvement in the local delivery of AMPA receptors to the postsynaptic membrane. Additionally, it was later shown in a similar approach that also Rab4 and Rab11 play important roles in endosomal trafficking of AMPA receptors. Similar to Rab8, expression of dominant negative Rab11 led to a virtually complete inhibition of LTP (Brown et al., 2007). Tracing GluA1-GFP localisation with intensity line plots, it could be shown that these two Rab proteins are involved in two distinct trafficking steps: While Rab11 is responsible for AMPA receptor trafficking into dendritic spines, Rab8 is required for membrane integration of the receptor (Brown et al., 2007). In addition, it was found that Rab4 positive endosomes are required to maintain spine size, suggesting that different endosomal trafficking events may be responsible for spine size and AMPA receptor transport and integration (Brown et al., 2007). It would be interesting to perform high resolution microscopy on single GluA1 containing endosomes to study whether Rab11 driven endosomes are directly converted into Rab8 endosomes or if there is overlap with Rab4 activity and to gain more insight into their dynamics and pathways in dendritic spines.

Also actin-based transport plays an important role in AMPA receptor trafficking. Correia *et al.* found by mass spectroscopy that GluA2 is interacting with myosin Va. Biochemical experiments showed further that this interaction is established by globular cargo-binding domain of myosin Va and that GluA2 simultaneously binds to Rab11. Moreover, immunofluorescence microscopy revealed partial co-localization of myosin Va, GluA1/2 and PSD95 both in cultured hippocampal neurons and organotypic slices (Correia et al., 2008). Using a dominant negative form of myosin Va and siRNAs, it could be shown that functional myosin Va is required for postsynaptic currents upon synapse stimulation and LTP expression. While myosin Va was not required for constitutive recruitment of AMPA receptors during basal stimulation, it was shown to be responsible for CaMKII mediated GluA1 accumulation at the synapse during LTP. In particular, myosin Va is not required for trafficking of AMPA receptors to the dendrites - this process was rather dependent on functional microtubules - but for the short-range transport of GluA1 (but not GluA2) from the dendritic shaft into spines (Correia et al., 2008). This trafficking step was known to be dependent on the actin cytoskeleton and to be initiated upon calcium influx during LTP (Shi et al., 1999). Interestingly, Rab11, driving recycling endosomes carrying AMPAR receptors (see above), seems to enhance the interaction between myosin Va and GluA1 and inhibition of myosin Va resulted in reduced amounts of Rab11 in spines (Correia et al., 2008). These findings fit a model, in which high calcium concentration at stimulated spines results in activation of myosin Va on Rab11 positive recycling endosomes, which triggers translocation of these endosomes into the spine heads.

Recycling vs Degradation

A brief review on the balance of recycling and degradation pathways concludes this section on endosomal transport. A study on endocytosis kinetics in cultured neurons using biotin labelling of surface receptors revealed that AMPA receptor endocytosis is regulated by synaptic activity and that under stimulated conditions, AMPA receptors undergo quicker reinsertion compared to basal conditions (Ehlers, 2000). Moreover, synaptic activity influences the percentage of AMPA receptors reinserted into the membrane. The amount of sequestered AMPA receptors varies between 10% under basal conditions and less than 2% after addition of picrotoxin. Interestingly, blocking NMDA receptors during stimulation resulted in a steep increase in AMPA receptor sequestration up to 30% (Ehlers, 2000). This experiment shows that the route of internal AMPA receptor trafficking not only depends on synaptic stimulation but that signalling events downstream of the activated receptors can influence the route of AMPA receptors trafficking. Moreover, immunofluorescence microscopy showed that following AMPA treatment, GluA1 was hardly re-incorporated into the membrane after initially being rapidly internalized into early endosomes. In contrast, NMDA treatment resulted in a strong accumulation of GluA1 in synapses after the initial internalization (Ehlers, 2000). The endosomal trafficking routes of AMPA receptors also differ between these two modes of synaptic stimulation. NMDA triggers internalization of GluA1 into early endosomes (Rab5/EEA1 positive) and gradually accumulation in recycling endosomes (Rab4/TfR positive) from where they can be reinserted into the membrane. In contrast, AMPA stimulation results in quicker passage of GluA1 out of early endosomes and into late endosomes (Lamp1 positive) and lysosomal AMPA receptor degradation was upregulated five-fold (Ehlers, 2000). Subsequently, it was shown that dephosphorylation of serine 845 on GluA1 by PP1 and PP2B during NMDA receptor signalling promotes endocytosis of GluA1. Upon re-phosphorylation of this residue by PKA (which is also activated by NMDA receptor signalling), GluA1 is transported back to the membrane. AMPA treatment does not lead to phosphorylation of serine 845 and consequently AMPA receptors are not shuttled back to the membrane but are trafficked to late endosomes and are ultimately degraded in lysosomes (Ehlers, 2000).

Active transport is thus essential for maintaining adequate levels of AMPA receptors at the synaptic membrane and is regulated directly by synaptic activity. Current reviews suggest that trafficking of recycling endosomes may be confined to single dendritic spines (Greger and Esteban, 2007; Newpher and Ehlers, 2008; Opazo and Choquet, 2011). However, no direct evidence of such a mechanism has yet been reported in the literature. It could theoretically be a mechanism to stabilize the number of available receptors per synapse, as discussed below. Alternatively, endosomal storage compartments at the base of dendritic spines may send out recycling endosomes targeted for exocytosis to multiple spines in the vicinity, rendering the endosomal trafficking even more dynamic than currently appreciated. Super resolution imaging of single endosomes and their cargos may reveal the detailed endosomal dynamics in the near future.

Discussion

Integrative Model for Active and Passive Transport in Synaptic Plasticity

Taking the findings of both active and passive AMPA transport into account, a model can be envisioned that combines both delivery pathways during synaptic plasticity. While it seems plausible that both mechanisms are taking place in dendritic spines, the difficulty lies in assessing the contribution of either to basal and stimulated conditions. Certainly, it is too simplistic to propose that lateral diffusion is sufficient to maintain steady-state distributions of AMPA receptors, while active transport mediates LTP induced accumulation of receptors at the membrane. In fact, it was found that active transport is on-going both in presence or absence of stimulation (Lüscher et al., 1999; Shi et al., 1999; Ehlers, 2000; Gerges et al., 2006; Brown et al., 2007).

Lateral diffusion was proposed to underlie AMPA receptor transport from the dendritic shaft to the synapse on the top of dendritic spines (Passafaro et al., 2001; Ashby et al., 2006; Yudowski et al., 2007). However, data showing that active endosomal transport is required to maintain AMPA receptor levels (Ehlers, 2000; Park et al., 2004; 2006; Correia et al., 2008; Petrini et al., 2009) in combination with specialized endocytic and exocytic zones within the head of dendritic spines (Carroll et al., 1999; Blanpied et al., 2002; Gerges et al., 2006; Kennedy et al., 2010) strongly suggest that at least the majority of AMPA receptors is transported via this active route to the synapse.

Exocytosis of AMPA receptors straight into the synapse, as it was suggested by some (Gerges et al., 2006), is a model that could exclusively rely on active transport, without the need for diffusion of receptors into the synapse. Evidence for such a mechanism is however scarce and relies on EM images showing GluA2/3 accumulation at the distal PSD in a condition that prevents membrane integration by blocking Exo70 of the exocyst complex (Gerges et al., 2006). The limitation here is obviously that these snapshots of AMPA receptor localization are not direct proof for the location of exocytosis. It is, for example, conceivable that Exo70 activity is required to localize endosomes to Stx4 in the exocytic zone. Therefore, direct proof by following exocytic events in live imaging set-up (Kennedy et al., 2010) is likely to be more accurate. Thus, a model in which endo- and exocytic zones are adjacent to the PSD is favourable and also fits with data of AMPA receptor diffusion through the perisynaptic membrane (Tardin et al., 2003; Ehlers et al., 2007). This is not to say that AMPA receptors do not have the potential to diffuse from one spine to the other, as elegantly shown by Ehlers *et al.* (Ehlers et al., 2007), but I favour the view that this is a rarer occurring event, reflecting that trapping of AMPA receptors in endocytic zones is not sufficient to prevent diffusion out of the spine into the shaft. Moreover, such mobile receptors on the surface of dendritic shafts may serve other functions, e.g. formation of novel spines. Moreover, diffusion of AMPA receptors within the spine is also dependent on their retention at the PSD. During LTP, receptor movement is confined to a minimum within the PSD, while perisynaptic receptors are highly mobile so they are able to incorporate into the PSD anchoring system or into clathrin coated pits to undergo endocytosis (Ehlers et al., 2007). In conclusion, active transport accumulates AMPA receptors in the dendritic spines from where lateral diffusion through the plasma membrane leads to their incorporation into the synapse by anchoring and retention at the PSD (**Figure 4**).

It is interesting to speculate that the integration of AMPA receptors into the shaft and spine membrane may stem from different transport events. Delivery of newly synthesized proteins from the Golgi network may happen at membrane domains different from those used by recycling endosomes. Such a model is somewhat reminiscent of the transcytosis model of protein sorting in which vesicles from the trans-Golgi first deliver membrane proteins to the plasma membrane from where they are endocytosed and redirected to their final destination (Lasiecka and Winckler, 2011). Experimental proof for such a model is however not directly reported. It would be interesting to follow membrane integration of recycled versus newly synthesized receptors by following them after photoconversion of their fluorescent tags to address this question.

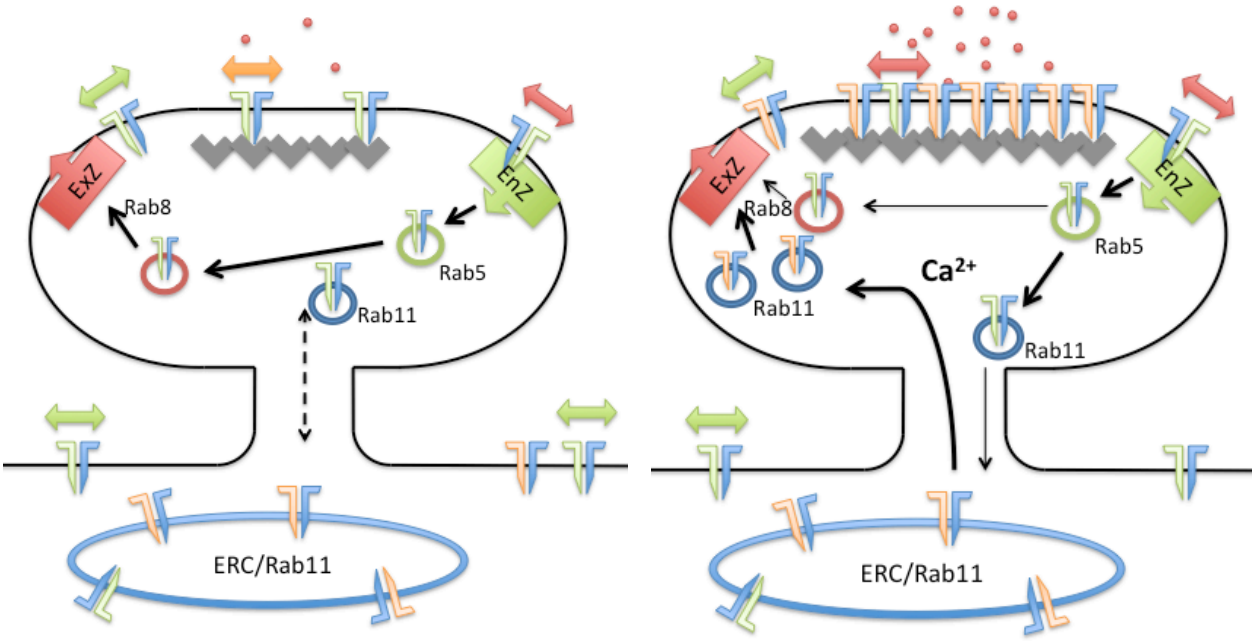

Figure 4 Model of AMPA receptor trafficking routes during synaptic plasticity. Left: In basal conditions, GluR2/3 (blue/green) are continuously cycled between synapse, perisynaptic membrane and intracellular compartments. Exocytosis of fast recycling endosomes (red) at the Stx-4 rich exocytic zone (ExZ) integrates receptors into the postsynaptic membrane in a Rab8 dependent process, where they diffuse into the synapse. PSD (grey) mediated interactions retain AMPA receptors at the synapse. Endocytosis into Rab5 positive early endosomes (green) occurs from clathrin coated pits in the endocytic zone (EnZ) that strongly inhibit further diffusion towards the neck. Additional receptors are stored in recycling endosomes (ERC) at the base of the spine. **Right**: NMDA receptor dependent Ca^{2+} influx leads to accumulation of GluR1/2 (orange/blue) receptors at the strengthened synapse, where their diffusion is very restricted. Integration of ERC stored receptors is mediated by Rab11 positive endosomes, which enter the spine upon calcium-induced myosin V activation and fuse with the plasma membrane in the exocytic zone. Coloured double headed arrows indicate diffusion kinetics of different AMPA receptor populations: green: fast, orange: medium, red: slow diffusion.

Is AMPA receptor recycling confined within single spines? Probably the answer is once more yes and no. The system of recycling endosomes consists of two pathways termed 'fast' and 'slow'. In the fast pathway, cargo is transported from the initial early endosome directly back to the membrane. On the other hand, the slow trafficking involves an additional trafficking step to Rab11 positive endosomal-recycling compartments (ERC) (Stenmark, 2009; Kelly et al., 2011). Such ERCs have been observed in dendrites often situated close to the base of dendritic spines (Cooney et al., 2002; Park et al., 2004). It is thus possible that internalized AMPA receptors are both quickly resurfacing via intraspinal recycling and transported to the ERC, from where they may also enter other spines in the vicinity. Rab4 mediated transport has been suggested to account for much of the steady-state cycling within dendritic spines but its role in AMPA receptor transport has so far remained unclear (Brown et al., 2007). It is tempting to speculate that the Rab11 positive storage compartment may deliver additional AMPA receptors required for LTP. This view is supported by NMDA receptor dependent calcium influx into the spine triggering a conformational change of myosin V, which subsequently transports Rab11 vesicles containing GluA1, but not GluA2, into the spine (Correia et al., 2008; Wang et al., 2008). It remains to be seen whether GluA1 and GluA2 are differentially trafficked between fast and slow pathways. One way to explain the shift from GluA2/3 to GluA1/2 accumulation (Passafaro et al., 2001) could be GluA1 mobilization from the ERC. Interesting but hardly researched is the possibility that active transport through the spine neck to and from the ERC may be regulated by neck constriction, as it has been shown for cytoplasmic proteins (Bloodgood and Sabatini, 2005). Nevertheless, it has been shown that Rab11 is required for maintenance and formation of dendritic spines (Park et al., 2006). Recent

improvements in imaging techniques have revealed that the endosomal vesicle trafficking may in fact be more dynamic than thought before. Especially the conditions in which neurons are imaged, e.g. temperature and buffers, may influence the dynamics of endosomal transport and should be re-evaluated under more physiological conditions (L. Kapitein, personal communication August 2012).

A Cellular Model of Memory Storage?

Both AMPA receptors and PSD scaffolding molecules were shown to be transiently destabilized at the synapse after LTP stimulation (Ehlers, 2000; Steiner et al., 2008). It is tempting to speculate that trafficking may be necessary for reorganization and strengthening of the synapse by mobilising components for a new round of plasticity. In particular, it may be the case that release of Glu2/3 receptors is needed to precede the integration of GluA1/2 receptors (Passafaro et al., 2001). While direct experimental proof of such a mechanism is outstanding such a model does fit with the data currently published.

Intraspinal recycling of AMPA receptors is an attractive model to explain long-term plasticity. By retaining endocytosed AMPA receptors within a single spine, the synapse will be able to integrate them during the next stimulation when they would be re-integrated into the membrane. This way the spine would have an autonomous pool of available receptors defining its maximum plasticity potential. However, there is evidence that endocytosed cargo is also transported to the dendritic shafts (Blanpied et al., 2002) contradicting a "sealed" spinal circulation of cargo. Also, it is not clear what role diffusion of AMPA receptors through the membrane out of the spine plays in this context. Yet, it is interesting to speculate that based on experience spines can constrict their necks to prevent intracellular transport of AMPA receptors and other proteins to stabilize their synaptic potential (**Figure 5**). If spines can in fact regulate such intracellular barriers to transport, it would be additionally important to establish whether such a mechanism can also induce a barrier for the diffusional transport of membrane proteins through the plasma membrane of the spine's neck. Indeed, it has been shown that diffusion of AMPA receptors is slowed down at dendritic spines with mushroom shape compared to stubby spines (Ashby et al., 2006), but regulating mechanisms of this phenomenon have not been reported. If memory and learning are indeed regulated by the strength of synaptic connections, this model may explain the very essence of long-term stabilization of these connections and thus storage of information in a molecular and cellular context.

Many mechanisms of AMPA receptor trafficking have been proposed over the last two decades. Currently, there seems to be growing support for a combination of active and passive transport to target AMPA receptors to the synapse (Opazo and Choquet, 2011). At the same time, it becomes increasingly apparent that inconsistencies in experimental design obscure the identification of underlying cellular mechanisms of AMPA receptor transport. Many studies follow the transport of a single specific subunit and omit comparison to other subunits. It would be desirable to test proposed transport models for their application on all AMPA receptors involved in synaptic plasticity to distinguish subunit specific behaviours from

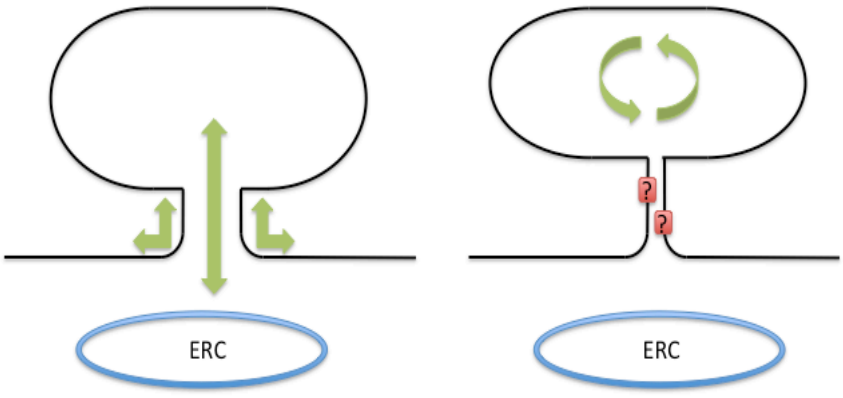

Figure 5 A cellular model of information storage in synapses. By constricting and elongating their necks, dendritic spines may regulate intracellular trafficking and diffusion kinetics through the postsynaptic membrane. The direct mediators of neck constriction are currently unknown but such a mechanism may stabilize the composition of synapses in spines with narrow necks leading to reliably constant postsynaptic responses.

general principles. Another problem of current approaches to identify the mode of AMPA receptor transportation at dendritic spines is that tagged receptor subunits may be trafficked differently than endogenous proteins or in a rather inefficient manner (Newpher and Ehlers, 2008). However, as there are constantly new techniques available to follow single molecules and visualize dendritic spines with super resolution microscopy, the knowledge on the precise dynamics of spinal trafficking will soon expand and answer our current questions.

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