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The excitatory postsynaptic density is a size exclusion diffusion environment

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ABSTRACT

Receptors are concentrated in the postsynaptic membrane but can enter and exit synapses rapidly during both basal turnover and processes of synaptic plasticity. How the exchange of receptors by lateral diffusion between synaptic and extrasynaptic areas is regulated remains largely unknown. We investigated the structural properties of the postsynaptic membrane that allow these movements by addressing the diffusion behaviors of AMPA receptors (AMPARs) and different lipids. Using single molecule tracking we found that not only AMPARs but also lipids, which are not synaptically enriched, display confined diffusion at synapses. Each molecule type displays a different average confinement area, smaller molecules being confined to smaller areas. Glutamate application increases the mobility of all molecules. The structure of the synaptic membrane is thus probably organized as a size exclusion matrix and this controls the rate of exchange of molecules with the extrasynaptic membrane.

1. Introduction

Memory and learning are thought to be mediated by the dynamic regulation of the efficacy of synaptic transmission. Excitatory neurotransmission is mediated in large part by activation of the postsynaptic AMPAR subtype of glutamate receptors. In recent years, evidence has accumulated that composition of the postsynaptic membrane in AMPA receptors (AMPARs) fluctuates rapidly and that this dynamic accounts for the construction and plasticity of excitatory synapses (Bredt and Nicoll, 2003; Malinow and Malenka, 2002; Sheng and Kim, 2002; Shepherd and Huganir, 2007). However, how receptors can enter and leave synapses still remains unclear.

The mechanisms that underlie changes in the number of functional AMPARs at synapses are thought to include exocytosis and endocytosis from or to intracellular pools as well as exchange between synaptic and extrasynaptic areas by lateral diffusion in the plasma membrane (Choquet and Triller, 2003). For instance, sites of endocytosis are located at a distance from synapses (Blanpied et al., 2002; Racz et al., 2004), suggesting that for basal turnover or during depression of synaptic transmission, receptors have to diffuse out of the synapse before being endocytosed. We and others have demonstrated by single particle and single molecule tracking (SMT) that AMPAR, as well as other types of receptors like GlyR, mGluR5 and NMDAR, diffuse in the neuronal membrane (Borgdorff and Choquet, 2002; Dahan et al., 2003; Groc et al., 2004; Meier et al., 2001; Serge et al., 2002; Tardin et al., 2003). Diffusion of AMPARs not only plays a role in regulating receptor numbers at synapses (Triller and Choquet, 2005), but also participates in the control of fast synaptic transmission by allowing fast replacement of desensitized receptors by naïve ones in milliseconds within the glutamate release zone (Heine et al., 2008). AMPARs display a restricted diffusion behavior which is amplified during maturation, in parallel with synaptogenesis, but still diffuse in mature neurons (Borgdorff and Choquet, 2002; Choquet and Triller, 2003). Moreover, the regulation of receptor diffusion could be involved in synaptic plasticity as it is differentially modulated by protocols that mimic neuronal activity (Groc et al., 2004).

Receptors are concentrated and stabilized at PSDs through interactions with subsynaptic scaffold proteins (Scannevin and Huganir, 2000). For AMPARs, these proteins include PDZ-domain-containing proteins (PSD-95, SAP-97, GRIP, PICK) (Dong et al., 1997; Lisman and Zhabotinsky, 2001; Schnell et al., 2002; Terashima et al., 2004) and TARPs (Chen et al., 2000; Fukata et al., 2005). At PSDs, receptors and their associated proteins show a precise organization. This organization must be reconciled with the ability of receptors to rapidly enter and leave synapses. Strikingly, both immobile and mobile receptors are found inside synapses (Tardin et al., 2003), but mobile synaptic receptors are not free to leave the synapse, their diffusion being confined by unknown mechanisms.

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In order to investigate the properties of the diffusion environment at the synapse, we followed by SMT the diffusion of AMPARs and that of the membrane lipid DOPE and cholera toxin (ChTx) which binds to ganglioside G_{M1}. This technique not only provides the high resolution needed to study localization of molecules in sub-micrometer membrane domains like the synapse, but also reveals the heterogeneity in diffusion characteristics of the population of molecules by removing the averaging effect of ensemble measurements. Our results show that mobile AMPARs and lipids are confined at mature synapses, albeit to different degrees. Glutamate increases the concentration of mobile receptors allowing them to leave the postsynaptic density by lateral diffusion.

2. Results

2.1. AMPARs, ChTx and DOPE are differentially distributed inside and outside synapses

We used single molecule fluorescence microscopy to localize and to track the movements of either GluA2-subunit-containing AMPARs or two lipids (G_{M1} and DOPE) in 21–27 DIV cultured hippocampal neurons. Ganglioside G_{M1}, a lipid occurring at the outer leaflet of the membrane, is known to form segregated domains in model membranes and is used as a lipid-raft marker (Dietrich et al., 2002; Samsonov et al., 2001). DOPE was chosen as a non-raft lipid probe. We also performed control experiments with $DiIC_{18}$, an artificial fluorescent lipid probe (Spink et al., 1990).

AMPARs and G_{M1} were detected using ligands coupled to a Cy5 dye (respectively, anti-GluA2 antibodies as in Tardin et al. (2003) and cholera toxin (ChTx)) whereas DOPE was directly coupled to a Cy5 dye (Fig. 1A). At the end of our experiments only ~20–25% of AMPARs (Tardin et al., 2003) and ~15–20% of ChTx (not shown) were internalized, so even if internalized fluorophores cannot be distinguished from surface ones the vast majority of the detected molecules were on the surface of neurites.

In order to identify the synaptic molecules we used rhodamine123 (Rh123) to stain synapses (Tardin et al., 2003) (Fig. 1B). AMPARs were found at a 7-fold higher density in areas of synaptic staining ($p < 0.001$, t test) while both lipids studied here displayed a similar relative density inside and outside synapses (Fig. 1C). On average, the detected AMPAR surface density was distributed homogeneously from the centroid of the synaptic staining up to a distance of 0.3 μm away (Fig. 1D) and decreased between 0.3 and 0.6 μm distance to reach the value of the extrasynaptic density. AMPAR enrichment on Rh123 stains indicates that they do indeed correspond to synaptic sites.

Trajectories of individual molecules were reconstructed from sequential images acquired at 18 Hz (Fig. 1A and B). Fig. 1B displays sample DIC images of recording fields overlaid with the synaptic staining and the detected trajectories. Molecules moved over areas of various sizes, ranging from nearly immobile to highly mobile spreading over several μm during our observations (up to 5 s).

2.2. Mobility is reduced in synapses versus non-synaptic areas for all the molecules studied

For each trajectory we calculated the mean square displacement (MSD) as a function of time. We first studied the mobility of molecules using the instantaneous diffusion coefficient D deduced from the initial slope of the MSD function versus time. Within the resolution of our experimental set-up, molecules with D below 0.005 $\mu m^2/s$ could not be distinguished from immobile molecules. For all molecule types, a large mobile fraction was found inside and outside synapses ($53 \pm 3\%$, $58 \pm 2\%$ and $42 \pm 2\%$ for AMPAR, ChTx and DOPE, respectively in extrasynaptic regions and $60 \pm 5\%$, $67 \pm 5\%$, and $42 \pm 3\%$, respectively within synapses) (Fig. 1E). We insured that diffusion of synaptic molecules was not due to the movement of the synaptic stains, which diffused with a D value below our immobility threshold (not shown).

The instantaneous diffusion of mobile molecules is moderately but significantly reduced in synapses. Indeed, on average and for each molecule type, mobile molecules diffused 1.5–2 times less in synaptic than in extrasynaptic areas ($p < 0.001$, Table 1). Comparing the different types of molecules, the mean D values of AMPAR, ChTx and DOPE were at the same order of magnitude in synaptic and extrasynaptic areas (Table 1). However, distributions of D were broad, spanning several orders of magnitude, which suggests the existence of different sub-populations of mobile molecules (Fig. 1E). We did not pursue classification into sub-populations in this context, as more suitable analytical techniques were employed later.

2.3. AMPARs, ChTx and DOPE explore confinement domains of various sizes in synapses

The comparison of D provides no information on the modes of diffusion. In particular, molecules undergoing rapid Brownian diffusion on short time scales can nevertheless be confined in membrane sub-domains. The dependence of the MSD on time is commonly used to extract this information as well as the size of the domain through which the molecule diffuses. For instance, confined diffusion causes the MSD to reach a plateau over time (Saxton and Jacobson, 1997).

Trajectory lengths of individual molecules are limited in time because of the rapid photobleaching of individual dyes. MSD values for individual molecules display high variability, which renders characterization of the types of movement difficult on individual trajectories. We therefore chose a statistical approach to analyze the mean diffusion behavior of sub-populations (Tardin et al., 2003; Schütz et al., 1997). Probability distributions are analyzed on the square displacements r^2 at a given time interval t, pooling together all the trajectories of a given type of molecule (see Section 4). Using this method, two categories of mobile molecules were found at all values of t characterized by a mean square displacement $\langle r^2(\tau)\rangle$ (Fig. 2A). These categories are termed "*slowly*" and "*rapidly*" mobile groups. A third group, considered immobile, corresponds to molecules that move in domains whose size is below our resolution threshold: these are not represented in the graphs. For each group, generation of the mean square displacement $\langle r^2(t)\rangle$ as a function of t represents the averaged MSD of the population (Fig. 2B). This approach allowed us to analyze multiple diffusion types in each compartment without an arbitrary categorization of each individual molecule. The type of movement was then determined selecting the best fit to the mean MSD between the linear equation describing Brownian motion and the equation proposed by Kusumi et al. (1993) for confined diffusion. We obtained the characteristic size L of the area of diffusion and used this value to compare the relative degrees of confinement of each molecule.

In extrasynaptic membranes, rapidly mobile molecules all displayed similar behavior: their diffusion constant was fast (in the order of 1–2 $\mu m^2/s$) but their area of diffusion was restricted, probably by the geometry of the neurite (Fig. 2B). By contrast, we found major differences in the slowly mobile group: while AMPARs and ChTx displayed confined diffusion to domains of similar sizes ($L = 319 \pm 12$ nm for AMPARs, $L = 322 \pm 6$ nm for ChTx), DOPE displayed free diffusion (Fig. 2B).

At synapses, all molecules displayed confined movements (Fig. 2B). To test whether this behavior was due to our method of analysis, we randomly generated domains of similar sizes to Rh123 stains in the extrasynaptic membrane. Applying the same