### Publishable summary

Molecular organization of the postsynaptic membrane is the core machinery involved in the functioning of an excitatory chemical synapse. Of the multiple components which orchestrate the reception of neurotransmitter release, AMPA receptors are the key molecules involved in the fast synaptic transmission. Over the years efforts have been made to understand the localization and correlate subsequent function of these receptors along with molecules associated with them in neuronal synapses. It has been shown that AMPA receptors are recruited, incorporated and recycled in a transient manner and this mobility has been identified as a decisive step in controlling the synaptic plasticity. Thus the equilibrium between the synaptic and extra synaptic AMPA receptor number is crucial in controlling basal transmission and synaptic response. This balance is known to be regulated by the subunit composition of these receptors and by interacting intracellular scaffold proteins. However, how this machinery is organized in postsynaptic density or in response to the reception of chemical signal is still unclear. The major goal of our study during the funding period was be to analyze with the highest possible spatio-temporal resolution the molecular mechanisms involved in the recruitment and redistribution of AMPARs in the synapses. The major objectives for the funding period were devoted to understand molecular mechanisms of AMPAR stabilization and their mobility and also AMPAR trafficking between different subcellular regions. The technological challenge was to try to acquire this information at the highest possible subcellular resolution from living cells. The focus of the first half of the funding period was the instrumentation and calibration of a new high density single molecule detection system based on photoactivation localization microscopy in combination with single particle tracking. After these the newly developed SPT-PALM microscope was used for routine observation of physiological questions addressed in the proposed project, which will be summarized below.

Aim 1: Molecular mechanisms of AMPA receptor stabilization - Hippocampal neurons were transfected with different AMPA receptor subunits fused to EOS fluorescent protein. These were either HA-mEos2-GluA1, EOS-TEV-HA-GluA1, myc-GluA2-tdEOS alone or in combination with Homer1c Cerulean, HA-GluA1 or Homer1c GFP. The SPT-PALM results indicated that the localization and mobility of the subunits in each cases were markedly different. GluA1 subunit of the AMPA receptors showed very low mobility and was immobilized in several 50-80nm clusters which were named as Nanodomains (Fig1). The number of these clusters varied from spine to spine (Fig2).. However, almost 80% of the spines had atleast one nanodomain. Interestingly the overexpressed of GluA2 subunit showed very high mobility and no or very little confinement at the synapses. On co-expressing untagged GluA1 along with GluA2 we were able to restore the clustering, indicating that the stochiometry between various subunits was indeed important for the receptor clustering at the synapse. Furthermore we showed with complementary high density single molecule technique named Universal Point Acquisition of Nanoscale Topography (uPAINT) that the endogenous AMPA receptors are also organized to Nanodomains. We also used Stimulated Emission Depletion Microscopy (STED) to show that Surface AMPA receptors show similar distribution indicating a functional organization on the postsynaptic membrane. As a result of careful analysis of diffusional behaviour of several single molecules in and out of nandomains, we were able to conclude that these domains are the result of immobilization dynamics of several receptor molecules. We also found that several of these domains are stable for minutes but there are also the formation and deletion of domains along time. It was also observed that there are three possible mechanistic modes of how the receptors are trafficked along the spatial environment of the receptor. We showed the individual receptor molecules can show strong, weak or no confinement before getting immobilized strongly indicating a very short organization controlled by local environment at the posysynaptic density

Fig1. a) Epifluorescence image of a dendritic segment expressing EOS-GluA1. b) PALM image of the corresponding dendritic segment showing non homogenous organization of receptors which is not visible in epifluorescence image.

Aim 2: AMPA receptor trafficking to and from synapses to different subcellular regions - We used a computational approach to extract biophysical properties from individual receptor trajectories, such as average diffusional tensor at the spine head and trapping zones where receptor mobility is greatly reduced. The method is based on combining information obtained from many trajectories passing through each point, possible only through the tracking of single molecules at high density. By this computational approach we identified trapping zones of AMPAR with in specific sub-micro domains. These trapping zones are organized in discreet clusters of large size of about 100nm. This size, evolved from biophysical computation suggests that these cannot be generated by a single interacting molecule such as a scaffolding molecule, but rather by an ensemble of coordinate molecules. Due to the large number of trajectories passing through a small area it was possible to calculate the mobility dynamics for each region of the dendritic segment in observation. From the trajectory reconstruction, it was possible to model the transition between different regions of an already observed dendritic segment. Since the cellular model is obtained from a real data the information coming out from the simulation reflected the mobility kinetics in living neurons. It was observed that, the resident time of receptor in a dendritic spine can take 1 minute, compatible with previous theoretical computations and experimental estimation. Interestingly, we found that dendritic spines could fall into two groups depending on whether the vectorial sum of the moment of the diffusion tensor at the spine neck was inward or outward. We further estimated the mean time for a receptor to reach the spine head from the dendritc shaft is of the order 4 minutes for Type II spines and of the order of hours for Type I. Although dendritic spines are usually classified according to their morphology, we found here two spine classes, based on the AMPAR diffusion tensor in the neck. It would be interesting to unravel the origin of such difference.

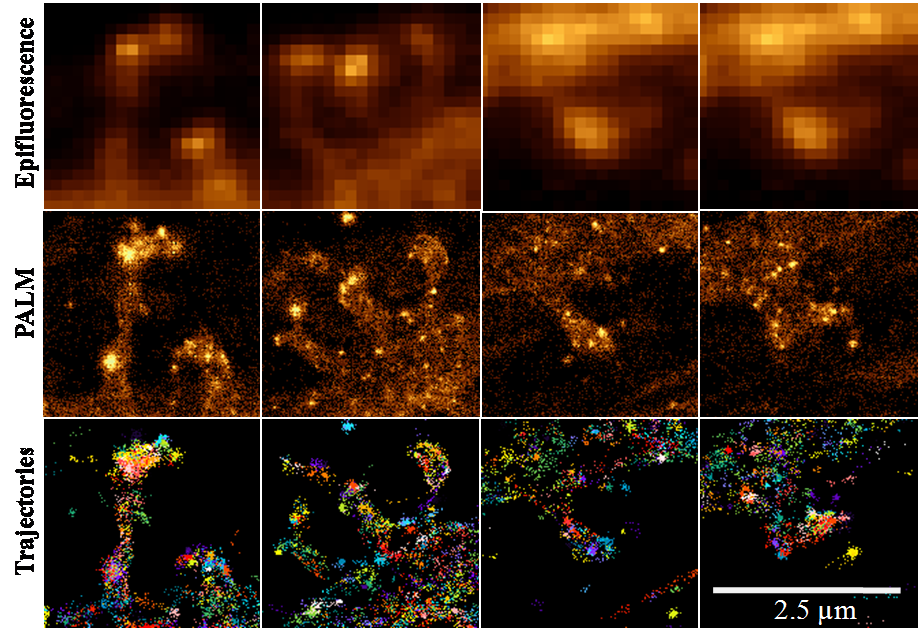


Fig2. Comparison of nanodomain organization in different spines. Epiflourescence images of each spines are different from the super resolution image obtained by PALM. In the super resolution image we can see the sub spine arrangement and that the number of the domains vary from spine to spine. Trajectories indicate the psedocolour coded picture of the trajectories taken by individual GluA1 molecules. Each colour denotes one trajectory.

Aim3: Activity dependant changes in AMPAR mobility – Of various AMPA receptor subunits, GluA1 subunit is thought to respond strongly to synaptic stimulus. With the use of uncaging experiments we were able to see that GluA1 subunit tagged to Phlourin, showed an increase in the fluorescence intensity at synapses after bath application of glycine. However inorder to understand the activity dependant recruitment of AMPA receptors we focused ourselves on the organization of major cytoplasmic scaffolding proteins which are associated with this complex. These proteins included SAP97, PSD95 and Homer1C. Each of these molecules showed distinct localization patterns using SPT-PALM. We have not yet fully utilized the power of high density single molecule tracking to yield more information on the synaptic recruitment and mobility. Currently this is ongoing and we are still developing the dual color detection and tracking of AMPARs with different anchoring proteins as an extension of the project. We hope that these will enable us to study relative dynamics of multiple proteins simultaneously at 30-50nm resolution.

Though the localization of AMPA receptors has astute implications in defining the synaptic response, their organization and fluctuations within the postsynaptic density still remain ambiguous. Considering this to be essential information we devised a paradigm for detection and analysis of AMPA receptor localization and their mobility on neuronal synapses generated by high density stochastic single molecule detection and tracking from living hippocampal neurons. These studies conducted during the funding period revealed several interesting new observations which was highly relevant in the field of molecular neuroscience.