Proc Indian Natn Sci Acad **85** No. 3 September 2019 pp. 577-587
© *Printed in India*. DOI: 10.16943/ptinsa/2019/49583

Review Article

Actin Cytoskeleton and Membrane Interactions: Role in GPCR Function and Organization

SHIKHA PRAKASH, ANJALI KRISHNA and DURBA SENGUPTA*
CSIR-National Chemical Laboratory, Dr. Homi Bhabha Road, Pune 411 008, India

(Received on 21 March 2018; Accepted on 15 September 2018)

Cell signaling networks are generally initiated at the cell membrane and mediated by receptors such as the G protein-coupled receptors (GPCRs). Adjacent to the cell membrane lies a complex network of proteins - the actin cytoskeleton to which several structural and physiological roles have been attributed. An emerging role of the cytoskeleton is to modulate GPCR function and organization, either directly or indirectly. The GPCR-cytoskeleton cross-talk is a complex hierarchical process where each step has its own set of rules and combinations. Due to the inherent complexity involved at each step and the multiple spatio-temporal levels, a complete picture is yet to emerge. In this review article, we provide an overview of actin-membrane interactions and how they modulate GPCR function and organization. In this context, we briefly discuss the structural characterization of actin monomers and filaments together with their interactions with partner proteins, such as the actin binding proteins. The effect of actin interactions on membrane domains is examined that assumes significance in light of membrane modulated effects in GPCR function. We aim to bring together concepts in GPCR signaling and cytoskeletal dynamics towards addressing emerging concepts in GPCR function.

Keywords: Actin; Cytoskeleton; GPCR; Cell-membrane; Pinning Sites; ABPs

Introduction

Cell signaling is the process by which cells relay specific information from the environment to the interior by sensing external cues (Alberts *et al*., 2013; Hancock, 2016). It encompasses pathways mediated by a large number of proteins that interact to form communication networks which extend from the membrane to the nucleus. Recent reports suggest that signal transduction is sensitively dependent on how these molecules are organized and interact collectively. Previous biochemical and physiological studies have identified several players, such as membrane-bound G protein-coupled receptors (GPCRs), intracellular GTP-binding proteins and protein kinases. Several signaling pathways have also been identified, a few of which converge on proteins that directly regulate the behavior and organization of the actin cytoskeleton in the cytoplasm (Alberts *et al*., 2013; Hancock, 2016). The lacuna in our understanding lies in the molecular basis of these cascades, the cross-talk between the players and how the cytoskeleton could in return modulate the function of the receptors that initiate the signal. In this review, we discuss the role of the cytoskeleton in modulating GPCR function and organization. Overall, we observe several points of cross-talk between actin and GPCRs. Reports on direct interactions between GPCRs and the cytoskeleton are limited, but evidence pointing to indirect interactions through actin regulating proteins and the membrane is increasing. We briefly overview structural dynamics of GPCRs and the cytoskeleton, and focus mainly on reports of the interplay between the cytoskeleton and cell membrane embedded GPCRs.

Structural and Functional Characteristics of GPCRs

The GPCR family of proteins are important seven transmembrane receptors (7TM receptors) that perceive extracellular signals and in response propagate a cellular signaling cascade (Pierce *et al*., 2002; Dohlmn, 2015; Thal *et al*., 2018). They are the

*Author for Correspondence: E-mail: d.sengupta@ncl.res.in; s.prakash@ncl.res.in

largest and most diverse class of eukaryotic membrane proteins. The GPCR family includes receptors involved in the recognition of light, taste, odors, hormone, pain, neurotransmitters and several other stimuli (Rosenbaum *et al.*, 2009; Hilger *et al*., 2018). Given the implications of GPCRs in disease and physiology, they form attractive targets for approximately one third of the currently marketed drugs (Campbell and Smrcka, 2018; Hauser *et al.*, 2017; Sid, 2018; Sriram and Insel, 2018).

Advances in crystallography and more recently the advent of cryo-electron microscopy (cryo-EM) has helped uncover several facets of the structure and activation of monomeric GPCRs (Granier and Kobilka, 2012; Boland *et al*., 2017; Venkatakrishnan *et al*., 2013). A representative member of the GPCR family, the serotonin$_{1A}$ receptor (Paila *et al*., 2011; Patra *et al*., 2015) is shown in Fig. 1. It is an important neurotransmitter receptor that has a role in cognitive, behavioral, and developmental functions (Kalipatnapu and Chattopadhyay, 2007; Nautiyal and Hena, 2017). Archetype GPCR signaling involves activation by extracellular ligands (such as serotonin) (Saxena and Chattopadhyay, 2011; Pucadyil and Chattopadhyay, 2004; Wirth *et al*., 2017) and coupling of transducers such as G protein on the intracellular site (Wirth *et al*., 2017; Thal *et al*., 2018). Over time evidence has accumulated that GPCRs not only signal through heterotrimeric G proteins, but also via arrestins and other G-protein-independent pathways (Hilger *et al*., 2018; Latorraca *et al*., 2017; Dwivedi *et al*., 2018; Wisler et al., 2018). Ligand mediated GPCR activation is dynamic in nature, resulting in concerted conformational changes in the transmembrane domains, and facilitating downstream protein interactions (Hilger *et al*., 2018; Dror *et al*., 2011; Nygaard *et al*., 2013; Gether, 2000; Manglik and Kruse, 2017). We direct readers to recent reviews for an overview of structural details and activation mechanisms (Weis and Kobilka, 2018; Erlandson *et al*., 2018; Latorraca *et al*., 2017).

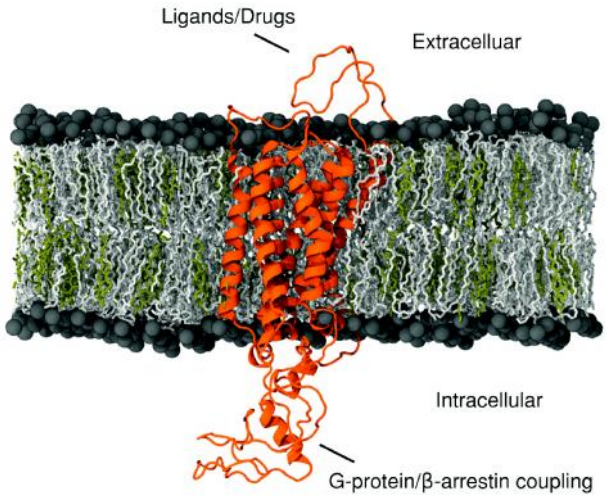

Fig. 1: Structural representation of a representative GPCR, the serotonin $_{1A}$ receptor. The receptor is represented in orange, lipids in gray and cholesterol in olive green color

An emerging concept in the field of GPCR biology is the role of its environment and other proteins in the regulation of GPCR function (Chattopadhyay, 2014). Several modulators of GPCR function have been identified, such as a wide repertoire of lipids, especially cholesterol (Jafurulla and Chattopadhyay, 2013; Oates and Watts, 2011; Gonzalez *et al*., 2017; Gimpl and Gehrig-Burger, 2012). It has been shown that tuning the membrane composition can alter GPCR function and organization. Membrane effects can be specific as well as non-specific in nature (Prasanna *et al*., 2014; Paila and Chattopadhyay, 2009; Jaipuria *et al*., 2018; Periole, 2016). We direct the readers to reviews (Oates and Watts, 2011; Chattopadhyay, 2014; Sengupta and Chattopadhyay, 2015; Sengupta *et al*., 2018) for a comprehensive insight into lipid effects on GPCR function. Furthermore, the association of GPCRs to form dimers and higher order oligomers has been reported (Chakraborty and Chattopadhyay, 2014) and its functional significance continues to be debated. The organization of GPCRs into varying membrane nano domains and oligomers appears to directly modulate GPCR function (Conn, 2013). However, accumulating evidence suggests that the actin cytoskeleton and GPCR function can be related either directly or indirectly (Vazquez-Victorio *et al*., 2016; Haanappel and Salome, 2017). In addition, actin has been reported to regulate related cell signaling pathways through compartmentalization, dynamics and clustering (Mattila *et al*., 2016; Krapf , 2018; Köster *et al*. 2016). Thus, the actin cytoskeleton could prove to be the missing piece of the GPCR jigsaw puzzle.

Actin: The Hidden Player

A schematic representation of membrane-embedded GPCRs along with the underlying cytoskeletal network is shown in Fig. 2. A priori there appear to be no

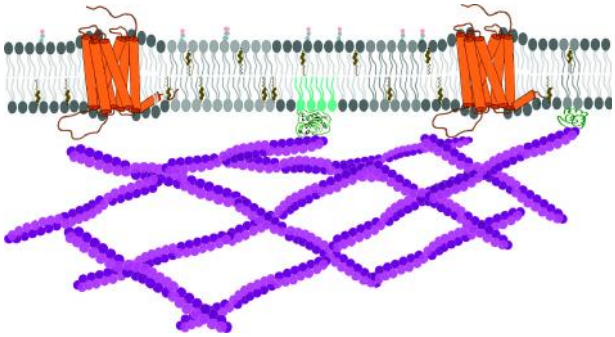

Fig. 2: A schematic representation of membrane-cytoskeleton interactions. The lipid bilayer is shown in gray with gradients to highlight different lipid types. Cholesterol is represented in tan and PIP2 in cyan. The GPCRs have been highlighted in orange, ABPs in green and the underlying actin cytoskeleton in shades of purple

direct links between the GPCR and the actin cytoskeleton. However, Kusumi and co-workers showed that actin influences opioid receptor diffusion using single-molecule techniques (Suzuki *et al*., 2005). Furthermore, a link between signaling and receptor mobility was established in the serotonin$_{1A}$ receptor, thereby implying that actin could play a regulatory role in GPCR signaling (Ganguly *et al*., 2008). Using quantitative fluorescence correlation spectroscopy (FCS), Chattopadhyay and co-workers demonstrated that the serotonin$_{1A}$ receptor is confined to cell membranes, possibly due to its interaction with the actin cytoskeleton (Ganguly and Chattopadhyay, 2010). Interestingly, the reorganization of the actin cytoskeleton upon GPCR signaling has also been reported (Ganguly, Saxena and Chattopadhyay, 2011). Taken together, the data suggests a dual role of actin in modulating GPCR organization as well as function, although the molecular details remains unclear. We review below the structural dynamics of actin, its interactions with the membrane and the crosstalk with GPCRs.

Structural Dynamics of The Actin Cytoskeleton

The actin cytoskeleton is a complex meshwork of proteins extending from the cell membrane to the nucleus that plays an important role in cell shape and motility. Actin filaments are enriched in a narrow region underlying the plasma membrane known as the cortex, where they are organized into a network. Actin occurs in two different forms: monomeric globular actin (G-actin) and polymeric filamentous actin (F-actin). G-actin has been well characterized structurally and several crystal structures are available (Mouilleron *et al*., 2012 ; Kotila *et al*., 2018). Over the years, there has been a gradual increase in both crystal (Holmes *et al*., 1990; Oda *et al*., 2009; von der Ecken *et al*., 2014) and cryo-EM (Fujii *et al*., 2010; Galkin *et al*., 2010, 2015) structural data on F-actin with increasing resolution (Oosawa, 2018). Quite recently, the cryo-EM structures of an actin filament in complex with myosin VI (Gurel *et al.*, 2017) and cofilin (Tanaka *et al.*, 2018) were resolved. For a direct comparison, we have represented the two forms of actin in Fig. 3. It can be discerned from the figure that F-actin is composed of two parallel strands of actin monomers, in which each G-actin protomer rotates 166^{o} to give a double stranded helix appearance. Several structural changes are associated with the inter conversion of monomeric G-actin to filamentous F-actin, including a "flattening" of the protomers. This is achieved by a relative rotation of two of the major domains of G-actin by 20^{o} in a propeller-like (or swing door) manner (Kudryashov and Reisler, 2013). This inter conversion

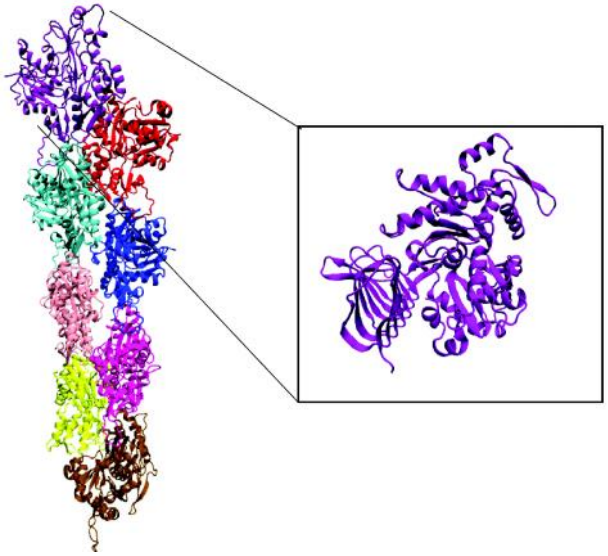

Fig. 3: The structural representation of (inset) actin monomer and (left) actin filament. The actin protomers are shown in cartoon representation and colored individually. The PDB structure of actin monomer (PDB ID: 6FM2) corresponds to the recently deposited crystal structure ADP-G-actin in com- plex with CAP (not shown) (Kotila *et al.*, 2018). The actin filament structure corresponds to the structure determined by cryo EM discussed above (PDB ID: 6BNO) (Gurel *et al.*, 2017)

is an active, cyclic process involving polymerization and de-polymerization, although factors governing G to F actin transition are still being explored (Oda *et al.*,2009; Pfaendtner *et al*., 2010; von der Ecken *et al*., 2014; Galkin *et al*., 2015; von der Ecken and Heissler, 2016). The details of these actin filament networks and the molecular mechanisms controlling actin filament dynamics have been reviewed previously (Pollard *et al*., 2000; Pollard, 2016).

Interactions of The Actin Cytoskeleton with Cell Membranes

The earliest evidence on actin-membrane interactions was reported several from several biochemical studies (Branton *et al*., 1981; Luna and Hitt, 1992; Hitt and Luna, 1994; Cowin and Burke, 1996). In general, actin interacts with the membrane mainly through indirect interactions, although direct interactions have also been reported (Sasaki *et al*., 2014; Ashdown and Burn, 2017). Indirect interactions occur through cytoskeletal elements linked to transmembrane proteins or actin binding proteins (ABPs) that interact with the inner leaflet of the plasma membrane or organelle membrane (Senju and Lappalainen, 2019; Lemiere *et al*., 2016; Fritzsche *et al*., 2017; Papadopulos, 2017). Further details on ABPs is given in the next section. Membrane phospholipids have been shown to modulate the "pinning" of the actin cytoskeleton. Membrane phosphoinositides constitute less than 5% of the membrane lipids, but are able to modulate attachment sites of membrane-actin networks (Liu and Fletcher, 2006; Senju and Lappalainen, 2019). Recently it was shown that although different ABPs interact with membranes through distinct domains, they bind phosphoinositide rich membranes via similar multivalent electrostatic interactions (Senju *et al*., 2017).

Actin-binding Proteins (ABPs)

The role of ABPs in the organization and dynamics of the actin cytoskeleton has been well characterized (Lappalainen, 2016). They perform several roles, such as catalyzing nucleotide exchange in actin monomers, and initiation, elongation, termination and cross-linking of actin filaments (Uribe and Jay, 2007; Pollard, 2016; Tanaka *et al*., 2018). Cofilin is the main player involved in the depolymerization of actin filaments. It does so by preferentially binding to ADP-actin and subsequently sequesters resulting monomers in the ADP-bound form, thus preventing their incorporation into filaments (Pollard, 2016). It controls the contractibility and motility of various cellular and sub-cellular components. Recently, a high resolution cryo-EM structure of cofilin bound to actin (cofilactin) was resolved by Tanaka and co-workers (Tanaka *et al*., 2018). The cofilin-actin interface could be delineated and the authors proposed models for the cooperative binding of cofilin to actin (Tanaka *et al*., 2018). Another ABP, profilin, can reverse the effect of cofilin, thus allowing the incorporation of actin monomers into filaments. Other important ABPs include Thymosin-b4, Gelsolin, Arp2/3 complex, Filamin, Dystrophin and we direct the readers to more extensive reviews on ABPs (Dos Remedios *et al*., 2003; Winder and Ayscough, 2005; Uribeand Jay, 2007; Kristo *et al*., 2016; Pollard, 2016).

ABPs have been reported to act as (virtual) bridges by simultaneously interacting with the membrane and the cytoskeleton, such as annexins (Winder and Ayscough, 2005). Proteins that link the actin cytoskeleton to membranes or membrane proteins include dystrophin, utrophin, talin and vinculin (Winder and Ayscough, 2005). The binding of PI(4,5)P2 via Arp2/3 initiates actin polymerization which is promoted by actin nucleation factors, WAVE and WASP. On the other hand the F-actin severing protein ADF/Cofilin, and the G-actin binding protein profilin, actin-capping protein, are all constrained by binding PI(4,5)P2 (Bezanilla *et al*., 2015).

Effect of Cytoskeletal Pinning Sites on Membrane Organization

The attachment of actin networks to the membrane at the "pinning sites" has been reported to lead to membrane reorganization (Koster *et al*., 2016; Lemiere *et al*., 2016; Fritzsche *et al*., 2017; Papadopulos, 2017). For instance, actin can modulate the formation and size of membrane microdomains which have been proposed to form scaffolds that compartmentalize various signal transducers (Kusumi *et al*., 2011). In addition, it was been shown that the pinning sites could give rise to confined diffusion at the membrane (Kusumi *et al*., 2012). Monte Carlo (MC) simulations of lipid membranes have demonstrated that both phase separation and lateral diffusion in membranes can be strongly affected by actin (Ehrig *et al*., 2011; Machta *et al*., 2011). In fact,

several membrane proteins and lipids exhibit restricted diffusion along the membrane, spanning over micrometer and nanometer length-scales and microsecond and nanosecond time-scales (Kusumi *et al*., 2012; Li *et al*., 2018; Cebecauer *et al*., 2018; Kusumi and Suzuki, 2005; Fujiwara *et al*., 2016; Kusumi *et al*., 2011).

Actin may have opposing effects of organization and phase mixing in membranes (Arumugam *et al*., 2015). Using bacterial cytoskeletal, FtsZ/MinD system, together with giant unilamellar vesicles, it was demonstrated that the artificial membrane-associated cytoskeleton, on the one hand, suppresses large scale phase separation below the phase transition temperature, and, on the other hand, preserves phase separation above the transition temperature (Arumugam *et al*., 2015). Using a combined experimental and modeling approach, it was proposed that membrane composition, the membrane curvature, and the actin pinning sites are coupled (Honigmann *et al*., 2014). An important point to note here is that the membrane demixing was obtained even in the presence of simple pinning sites via biotinylated lipid streptavidin complexes (Honigmann et al., 2014). Interestingly, when the membrane-cytoskeleton interaction is mediated by proteins that preferentially partition into a specific domain type, cytoskeleton meshwork organization and lipid domain distribution are intimately related. Pinning of a specific phase by the preference of pinning molecules has been qualitatively shown in vitro (Zhao *et al*., 2013). Taken together, these studies indicate that actin-membrane interactions could involve a wide range of mechanisms and general principles are emerging despite the lack of a detailed molecular picture.

Cross-talk Between Actin and GPCRs

Despite the first reports on cytoskeleton mediated GPCR diffusion and mobility (Suzuki *et al*., 2005; Ganguly *et al*., 2008), the precise interactions between actin and membrane proteins remain unclear. This is probably due to the spatio-temporal dynamics of both GPCRs and actin and the inherent limitations of analyzing membrane-bound dynamics. Several strategies have been employed to examine the binding of actin to membrane channel proteins, including co-immuno precipitation, surface plasmon resonance, fluorescence resonance energy transfer (FRET), and atomic force microscopy (Sheng and Pak, 2000). Direct interactions between ion channels and actin cytoskeleton has been reported (Sasaki *et al*., 2014), and could be relevant for GPCR interactions as well. However, no direct actin-GPCR structure modules have been observed so far.

There are limited reports on the direct interactions between ABPs and GPCRs. α-actinins (α-actinin-1 to α-actinin-4) are a class of ABPs that not only bind to actin filaments but also modulate the activity of membrane receptors (Foley and Young, 2014). A recent study by Sun *et al*. (Sun *et al*., 2016) showed that the binding of the adenosine A_2 receptor with actinin-1 is destabilized in the presence of two point mutations. Bezanilla and co-workers (Bezanilla *et al*., 2015) characterized the interaction of α-actinin-4 with group1 metabotropic glutamate receptors (mGluR) which in turn governs remodeling of dendritic spines in primary neurons. Similarly, the actin-binding protein 280 (ABP-280) has been shown to associate with dopamine D3 receptors (Li *et al*., 2002). Interestingly, a conserved filamin binding sequence was found in the extracellular domains of > 20% of the 824 GPCRs encoded in the human genome through an exhaustive sequence-based analysis (Tirupula *et al*., 2015). Direct binding between Filamin-A and select GPCRs was confirmed through biochemical experiments (Tirupula *et al*., 2015).

GPCR Organization is Modulated by the Cytoskeleton

Reports suggest that actin modulates GPCR organization in at least three possible (interconnected) ways: confining (corraling) diffusion, altering the oligomerization state and modulating association kinetics. Mechanisms underlying corralled diffusion are discussed above (Suzuki *et al*., 2005; Ganguly *et al*., 2008). Such confined or corralled diffusion could have functional consequences (Ganguly *et al*., 2008).

To investigate the role of actin cytoskeleton in GPCR oligomer size, Chattopadhyay and co-workers utilized homo-FRET approaches in live cells and demonstrated that the actin cytoskeleton could directly modulate the organization of the receptor (Ganguly, Clayton and Chattopadhyay, 2011). The result suggest that destabilization of the actin cytoskeleton by cytochalasin D lead to an increased contribution from higher order oligomers in live cells (Ganguly, Clayton

and Chattopadhyay, 2011). Interestingly, a cell type-dependent clustering of β_2-adrenergic receptor was reported using super resolution microscopy approaches (Scarselli *et al*., 2012). It was shown that the cluster size depended on actin cytoskeleton integrity but not on membrane microdomains (Scarselli *et al*., 2012). In addition, the cluster size and dynamics of the chemokine receptor CXCR4 were reported to be directly modulated by the actin cytoskeleton (Martiýnez-Munoz *et al*., 2018). Interestingly, the effect of actin on GPCR oligomer/cluster size is receptor dependent. GABA-B oligomers were reported to directly interact with the actin cytoskeleton and form large ordered clusters Calebiroa *et al*. (2013). However, disruption of actin filaments lead to reduced cluster sizes in β_2-adrenergic receptor, but not in GABA-B complexes (Scarselli *et al*., 2016; Calebiroa *et al*., 2013). It has been suggested that the actin cytoskeleton could help to confine specific GPCRs to specific domains in order to increase efficiency of them encountering their signaling partners (Scarselli *et al*., 2016). These results imply that the actin cytoskeleton could play a regulatory role in GPCR organization.

Evidence on the altered GPCR association kinetics by actin cytoskeleton comes from mathematical modeling and comparison to single particle tracking measurements. We have previously developed a kinetic Monte Carlo model in which the membrane was represented by a two-dimensional lattice with periodic boundary conditions and the receptors as point particles (Deshpande *et al*., 2017). As shown in Fig. 4, the square lattice was initially randomly populated with a given density of receptors. Each receptor was propagated with displacement steps δ_{ri}, derived from the expression of the mean squared displacement of a two-dimensional random walk, by the relation: $\delta r_i = \sqrt{(4D_i \delta t)}$ where, i = 1, 2 indicates whether the particle is a monomer or dimer, D_i is the diffusion coefficient of the ith particle and δt the time interval (Pawar *et al*., 2015; Athale *et al*., 2014). In the model, the monomers were allowed to associate at adjacent sites with the probability of association (P_{on}) given by $P_{on} \approx k_{on} \delta t$, where k_{on} is the on-rate. Dimer dissociation was based on the probability $P_{off} \approx k_{off}\ \delta t$, where k_{off} is the off rate. Cytoskeletal filaments were modeled as barriers for receptor diffusion, with a hopping probability, P_{hop}, similar to the 'picket fence' or corralled diffusion

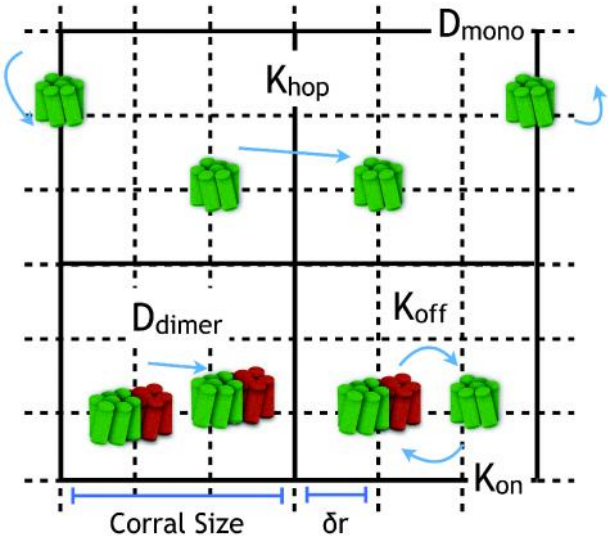

Fig. 4: Schematic representation of the kinetic Monte Carlo model of the classical association-diffusion model. A two-dimensional lattice is defined with periodic boundary conditions to represent the membrane. Receptors are colored in green and orange. The steps taken in the consecutive steps are marked. Cytoskeleton corrals are represented as underlying barriers

model (Suzuki *et al*., 2005). The model predicts a continuous change in the percentage of receptor dimers, with increasing corral size as well as probability of hopping. The monomer-dimer equilibrium of the GPCRs predicted are in line with previous experimental findings (Kasai *et al*., 2011; Calebiroa *et al*., 2013). The model reveals a complex interplay between cytoskeletal components and their influence on receptor association kinetics within the features of the membrane landscape. The results suggest a functional coupling between receptor and actin and a modulation of the dynamics of receptor organization.

Cytoskeleton Mediated Trafficking of GPCRs

Membrane trafficking plays a critical role in regulating cell signaling, including downstream responses mediated by GPCRs. They are sorted and internalized via different endocytotic pathways to be either degraded or recycled (Pons *et al*., 2017). Actin filaments are important players in receptor sorting within endosomes, aided by proteins such as WASH and the Arp 2/3 complex (Derivery *et al*., 2012; Gomez and Billadeau, 2009) and specialized protein interactions. A recent study has shown that phosphorylation of Filamin A (FLNa) governs

chemokine receptor CCR2B recycling through endocytosis (Pons *et al*., 2017). Alternatively, actin binding sequences may be present in GPCRs like the β_2-adrenergic receptor that sequester the receptor in endosomes comprising actin (Puthenveedu *et al*., 2010). This in turn is mediated by the actin-sorting nexin 27-retromer tubule (ASRT) machinery and PDZ proteins that bind to the cortical actin cytoskeleton prior to the recycling of the receptor (Bowman *et al*., 2016; Varandas *et al*., 2016; Bahouth and Nooh, 2017). Similarly, a conserved filamin binding sequence was found across the cytoplasmic domains of several GPCRs, and their direct binding to filamin-A was reported (Tirupula *et al*., 2015). Recent evidence suggests that GPCR signaling can persist in the endosomes (Chaturvedi *et al*., 2018; Irannejad and von Zastrow, 2014), and cytoskeletal coupling could be an important regulatory principle in this context.

Conclusions and Future Perspectives

The understanding of actin cytoskeleton networks and their role in GPCRs signaling has undergone significant advancement due to improved experimental techniques with increased spatio-temporal resolution. It now appears that actin serves as a critical point of integration of receptor signaling such that changes in the cytoskeleton induced by one signal can readily influence the function of other receptors. Ligand binding as well as G protein coupling in GPCRs has been shown to be altered by indirect changes in physico-chemical properties space of the membrane (Prasad *et al*., 2009; Pal *et al*., 2016). Additionally, modulation of the GPCR association has been reported to be directly linked to indirect effects (Prasanna *et al*., 2015; Pawar, Prasanna and Sengupta, 2015). F-actin network assembly, organization, and dynamics are controlled by the spatial and temporal regulation of the activity of ABPs that in turn could affect membrane dynamics and properties. Consequently, actin can indirectly affect GPCRs by altering the physical properties of membrane. In this article, we have highlighted representative experimental and computational approaches to address these questions in GPCR biophysics. These are exciting times for actin cytoskeleton researchers since we are able to use complimentary approaches to address interactions problems at a spatio-temporal resolution that was not possible to achieve even a few years back. Unraveling the molecular basis of GPCR-actin will be an important step towards understanding human health and disease.

Acknowledgement

The authors thank Prof. Amitabha Chattopadhyay and Dr. Xavier Prasanna for discussions. DS gratefully acknowledges the Dept. Science Technology (DST, (Govt. of India) grant: EMR/2016/002294) for funding.

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