Chapter 64

Role of Lipid Rafts and the Underlying Filamentous-Actin Cytoskeleton in Cannabinoid Receptor 1 Signaling

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Abbreviations

CB1 Cannabinoid 1 receptor
DAG Diacyl Glycerol
EGFR Epidermal growth factor receptor
ERK1/2 Extracellular signal-regulated kinases 1/2
FGFR Fibroblast growth factor receptor
GPCR G-Protein-coupled receptor
HPTLC High-performance thin-layer chromatography
MCD Methyl-β-cyclodextrin
PKCε Protein kinase Cε
PLC Pospholipase C
***R*(+)-MA** Methanandamide
RTK Receptor tyrosine kinase

CANNABINOID RECEPTOR 1 SIGNALING: A FORCEFUL REGULATOR OF NEURONAL DEVELOPMENT AND FUNCTION

The endocannabinoid system is a complex signal transduction system, comprising the G-protein-coupled cannabinoid receptors 1 and 2 (CB1 and CB2), the best characterized endogenous ligands 2-arachidonoylglycerol (2-AG) and *N*-arachidonoylethanolamine (anandamide, AEA), and the enzymes necessary for the biosynthesis and degradation of these ligands. Uniquely among neurotransmitters, endocannabinoids are produced from precursor phospholipid molecules in the plasma membrane (PM) upon demand, for example, elevation of intracellular calcium (Cadas, Gaillet, Beltramo, Venance, & Piomelli, 1996). AEA binds to CB1 as a partial agonist, while 2-AG is a full agonist of both CB1 and CB2. The second major difference between the two receptors is that CB1 is prominently found in the central nervous system (CNS), where it mediates the central pharmacological effects of Δ^9-tetrahydrocannabinol, the main psychoactive ingredient of marijuana (Howlett et al., 2002), whereas CB2 is predominantly expressed in peripheral tissues. The abundance of CB1, particularly in the cerebral cortex, striatum, cerebellum, hippocampus, and hypothalamus, and the discovery of several endogenous ligands have further established the CB1 system as an extensive base for the regulation of CNS physiology and function. CB1 is expressed as early as the trophoblast (Sun & Dey, 2008), becomes abundant in the developing brain, and plays important roles in the self-renewal proliferation capacity of neural cells and then in neuronal differentiation, controlling important aspects such as dendritic branching, and synaptic density and transmission (Asimaki & Mangoura, 2011; Diaz-Alonso et al., 2012; Tagliaferro et al., 2006; Takahashi & Castillo, 2006; Xapelli et al., 2013). These neuromodulatory actions of CB1 highlight the effects of cannabinoids in a wide range of systemic responses such as motor activity, memory, fear extinction, analgesia, and euphoria. CB1 is also important for appetite and metabolism regulation. The gut–brain axis that acts to maintain energy homeostasis is controlled by CB1 expression in the hypothalamus, while persistence of higher body weight even after resection of the stomach curvature correlates well with persistently high CB1 expression (Fedonidis et al., 2014) and the fact that certain CB1 genotypes associate with human obesity (Russo et al., 2007). A third difference between CB1 and CB2 has been documented at the cellular and molecular level, which is their association with lipid rafts, that is, the frequent detection of CB1, but not CB2, in these specialized membrane subdomains. This chapter focuses on the importance of lipid rafts as a platform of CB1 signaling.

CB1 SIGNALING TO THE MAJOR EFFECTOR EXTRACELLULAR SIGNAL-REGULATED KINASE

The signaling pathways that elicit the diverse cellular and organismal effects of CB1 have been the focus of extensive research efforts. As with all G-protein-coupled receptors (GPCRs), initiation of signaling cascades upon agonist–receptor binding occurs at the plasma membrane (cell surface). The specific topology of a given GPCR in discrete plasma membrane domains allows access of a liganded receptor to signaling lipids and to specific effectors

available at this topology and therefore instant activation and/or interactions of effector proteins that form signaling complexes. These topologies regulate how long these formed signaling complexes will be maintained and whether they would traverse the endocytic pathway, which additionally contributes to the biological action of a specific agonist (Moore, Milano, & Benovic, 2007). The whole process, as it possesses cell type- and cell state-specific effectors, allows for differences in the magnitude and duration of the specific GPCR signal and therefore of its downstream biological output. More specifically, the signal (1) may modify preexisting proteins and alter the physiological processes in which they are involved, e.g., phosphorylation of cytoskeleton proteins that control cellular process formation; (2) may be a transducer into the nucleus and alter transcription and therefore expression of proteins; and (3) in neurons, may do both at the periphery, where translation of proteins may occur from locally stored mRNAs. A typical paradigm for the importance of the magnitude and duration in the biological outcome of the signaling is the activation of the signaling kinase extracellular signal-regulated kinase (ERK). ERK is the last effector in the three-kinase module consisting of Raf (mitogen-activated protein kinase (MAPK) kinase kinase), MAPK kinase, and ERK (or MAPK). GPCRs activate the ERK cascade utilizing a number of effectors and protein–protein signaling interactions, including protein kinase C (PKC), protein kinase A, Src-family kinases, and the transactivation of membrane receptors with tyrosine kinase activity (RTKs), such as epidermal growth factor receptor (EGFR), to converge onto Ras and Raf activation (Daub, Weiss, Wallasch, & Ullrich, 1996; Jiang, Dong, Trujillo, Miller, & Eberhardt, 2001; Luttrell et al., 1996; Mangoura & Dawson, 1993). The ERK signal is passed on to modifications in the activity of transcription factors, as well as to modifications of peripheral proteins responsible for the acute responses of a cell. Indeed, the magnitude and duration of ERK activation have been causally linked to specific cellular responses in neurons and neural cells, as transient and strong activations (<10 min) associate with cell proliferation, while sustained and milder activations induce differentiation (e.g., Leondaritis, Petrikkos, & Mangoura, 2009) and correlate well with enhancement of memory (e.g., Kelleher, Govindarajan, Jung, Kang, & Tonegawa, 2004; Zisopoulou et al., 2013). ERK activation by CB1 has been demonstrated in a variety of cells in culture (Asimaki & Mangoura, 2011; Bouaboula et al., 1995; Galve-Roperh, Rueda, Gomez del Pulgar, Velasco, & Guzman, 2002; Rubovitch, Gafni, & Sarne, 2004) and in brain slices (Lin, Mao, Su, & Gean, 2009), and the signaling mechanism of the GPCR CB1 to ERK has become a specific research focus.

CB1 SIGNALING TO ERK EMANATES FROM LIPID RAFTS

For several GPCRs, and for CB1 in particular, signaling to ERK depends on lipid rafts, that is, lipid-driven, relatively ordered, fluid domains in plasma membranes (Figure 1). These fluctuating nanoscale membrane assemblies, enriched in lipids like cholesterol,

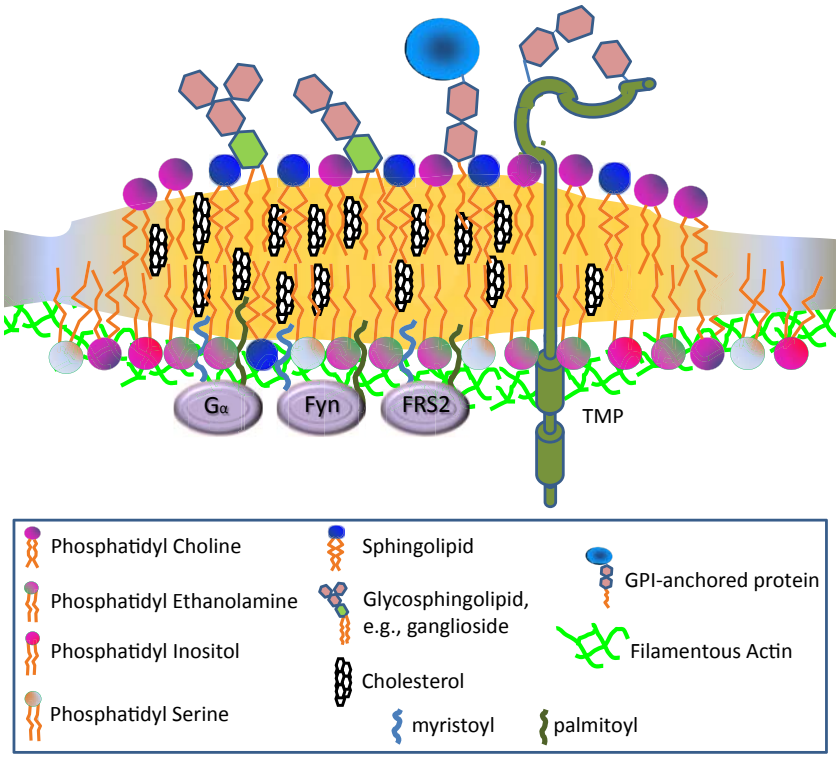

FIGURE 1 Schematic representation of the outer and inner leaflets of a lipid raft. Lipid rafts are nanoscale assemblies of sphingolipids, such as sphingomyelin, and glycosphingolipids, such as gangliosides, cholesterol, glycosylphosphatidylinositol (GPI)-anchored proteins, glycosylated transmembrane proteins, and lipid-modified, membrane-tethered proteins that contribute to membrane heterogeneity and constitute a base for cell type-specific signaling. The underlying cortical filamentous actin cytoskeleton and its associated proteins impose corral-like constraints that control the lateral diffusion of membrane proteins and lipids.

TABLE 1 **Detection of CB1 in Lipid Rafts with Various Methodologies and the Effects of Agonism and/or Methyl-β-Cyclodextrin**

Cell Type	Method of Lipid Raft Isolation	CB1 Detection in Rafts	Effect of Treatment	Effect of MCD	References
BV2 murine microglial cell line, caveolin 1-null	No detergent (OptiPrep fractionation or carbonate extraction)	Yes	Cannabidiol, no	ND	Rimmerman et al. (2011)
C6-2B rat glioma cell line	Carbonate extraction	Yes (caveolin rafts)	ND	ND	Bari et al. (2008)
Primary cortical neurons	No detergent OptiPrep fractionation	Yes	*R*(+)-MA, transiently into rafts	Blocks ERK activation	Asimaki et al. (2011)
MDA-MB-231, human breast cancer cell line	Detergent (Triton X-100-resistant membranes)	Yes	Long-term (24 h AEA), yes, away from rafts	CB1 moves away from rafts	Sarnataro et al. (2005)

glycolipids, or sphingolipids, and in glycosylphosphatidylinositol-anchored proteins and transmembrane receptors (Harder & Simons, 1999; Karnovsky, Kleinfeld, Hoover, & Klausner, 1982; del Pozo et al., 2005), are regulatory for both intracellular signaling and membrane trafficking. As raft lipids are also enriched in intracellular sorting vesicles intended for the PM (Orci et al., 1981), lipid-driven rafts may serve as a mechanism for sorting proteins with transmembrane domains along the secretory pathway from the trans-Golgi network to the PM and for endosomal sorting/recycling (Brown & Rose, 1992; Klemm et al., 2009; Lusa et al., 2001). What regulates the affinity of transmembrane proteins, as receptors are, to rafts is still only partially understood; their propensity to interact with raft lipids or raft-embedded proteins and their lipid modifications are certainly important. Among the lipid modifications, palmitoylation, the only reversible lipid modification and one posted for CB1 (Oddi et al., 2011), does regulate "raftophilicity" (Resh, 2006 and references therein). Studies have shown that, in addition to palmitoylation, transmembrane domain length and transmembrane sequence are also critical determinants of membrane raft association (Diaz-Rohrer, Levental, Simons, & Levental, 2014).

The cytoplasmic face of lipid rafts is enriched in molecules for intracellular signaling, such as Src-family kinases, G proteins, small GTPases, and adapter molecules, tethered there by their post-translational lipid modifications. Again, palmitoylation predicts, as for FRS2 (Kouhara et al., 1997), and double modification by both palmitoylation and myristoylation ascertains, as for G_{α} species (reviewed in Chen & Manning, 2001), Fyn (Alland, Peseckis, Atherton, Berthiaume, & Resh, 1994), or flotillin 1 (Neumann-Giesen et al., 2004), a raft residence (Figure 1). Moreover, several other signaling proteins or transmembrane receptors may conditionally flow into lipid rafts; for example, signaling phosphorylations and resulting protein–protein interactions recruit the adapter protein GRB2 (Ridyard & Robbins, 2003), the tyrosine kinases Src and fibroblast growth factor receptor (FGFR) (Asimaki, Leondaritis, Lois, Sakellaridis, & Mangoura, 2011), or the guanine nucleotide exchange factor SOS (Aronheim et al., 1994). As a consequence lipid rafts facilitate the formation of signaling complexes upon stimulation of the membrane receptors with ligands. In addition to the generation of relatively linear signaling cascades, the formed signaling complexes serve to generate positive or negative signaling feedback loops that may increase the efficiency of the signal by providing both amplification mechanisms and the necessary turn-off switches. These switches, through regulation of the magnitude and duration of the signaling cascade, contribute significantly to the specificity of the signal.

LIPID RAFTS, FILAMENTOUS-ACTIN NETWORKS, AND MICROTUBULES REGULATE CB1 SIGNALING AND LOCALIZATION

Effects of Lipid Raft Cholesterol

As with other cyclodextrins, the water-soluble, cyclic glucose heptamer methyl-β-cyclodextrin (MCD) sequesters cholesterol in its hydrophobic core into soluble inclusion complexes and blocks clathrin- or lipid raft (with or without caveolin)-dependent vesicle trafficking from (or to) the membrane (Puri et al., 2001), all disrupting GPCR signaling. A few studies, using Western blotting analysis of membrane or cell fractions, have postulated the localization of CB1 to rafts (Table 1). Despite the different methodologies used, that is, cell lysis with detergent (preparation of Triton X-100-resistant membranes), with detergent-free solutions, or by carbonate extraction and then fractionation of the lysates in OptiPrep™ or sucrose gradients, CB1 is detected in the rafts. At least for the detergent-free method of Macdonald and Pike, protein raft markers like flotillin 1 show the expected distribution. In addition, we validated the preservation of the native composition of lipid rafts with high-performance thin-layer chromatography of phospholipids to verify enrichment of sphingomyelin (Figure 2). The methodology may account in part for the differences in detection of CB1 movement; however, differences in the time of exposure to CB1 agonist, as well as the different cellular backgrounds, play important roles too. When

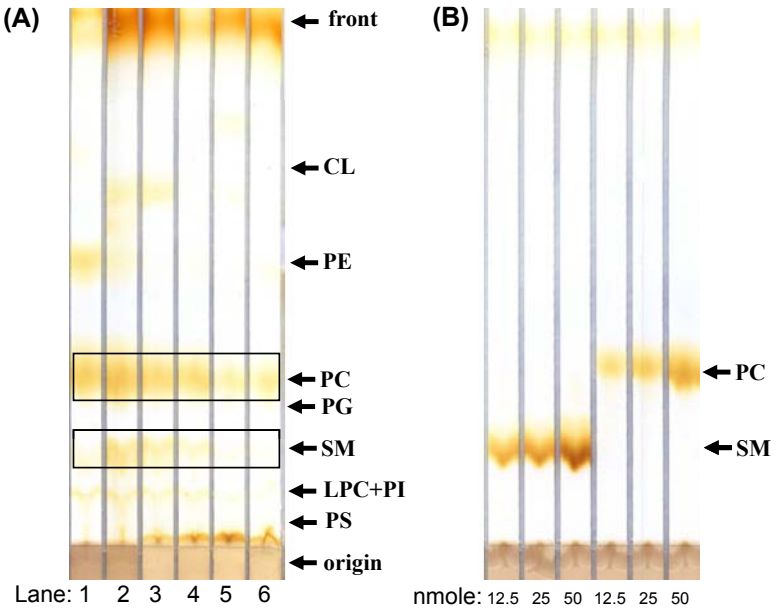

FIGURE 2 Detergent-free lipid-raft preparations preserve native lipid composition and highlight the enrichment in sphingomyelin. (A) Total lipids were extracted using a modified Bligh-Dyer method from the embryonic stem cell line E14T (lane 1) and OptiPrep fractions were combined as follows: lipid rafts, fractions 1–5 (lane 2); nonraft membranes, fractions 6–8 (lane 3); and endoplasmic reticulum–Golgi membranes, fractions 9 and 10 (lane 4), 11 (lane 5), and 12 (lane 6). Equal amounts of each extract were chromatographed on borate-impregnated thin-layer chromatography (TLC) plates with a solvent system consisting of $CHCl_3$:CH_3OH:H_2O:NH_4OH (120:75:6:2) and lipids were visualized by iodine staining. Arrows denote the positions of authentic phospholipid standards chromatographed in separate lanes on the same TLC plate. PS, phosphatidylserine; LPC, lysophosphatidylcholine; PI, phosphatidylinositol; SM, sphingomyelin; PG, phosphatidylglycerol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; CL, cardiolipin. (B) PC and SM standards (12.5–50 nM) were chromatographed as in A.

applied, MCD leads to the disappearance of CB1 from the raft fractions (Asimaki et al., 2011) or the Triton X-100-resistant membranes (Sarnataro et al., 2005).

The effects of MCD on the distribution of CB1 are readily seen with confocal microscopy. The upper row in Figure 3 presents the typical fluorescence confocal microscopic images of the CB1–GFP (green fluorescent protein) subcellular localization in COS-7 cells, which have an ample cytoplasm and a well-developed cell cortex. Seventy-two hours posttransfection and under resting conditions (control), fluorescence contributed by the tagged receptor at the level of the maximum nuclear diameter (equatorial section, column A) is seen at the PMs of lamellipodia (arrows) and filopodia (arrowheads); such distribution becomes evident closer to the adhering surface of the cell (column B). PM expression reflects proper receptor folding and stability, as well as intracellular movement and processing through the endoplasmic reticulum (ER) and Golgi organelles. Indeed the receptor is also seen in a juxtanuclear compartment and paranuclear compartments around the nucleus, consistent with an ER (asterisk) and Golgi cisternae and associated vesicular profile localization (asterisks), as expected for an overexpressed receptor that is constantly synthesized. Some endosomal origin of this intensity should be considered, attributable to cycling of the receptor between PM and endosomes after autocrine or paracrine stimulation of the CB1 itself or through its transactivation by other membrane receptors, again due to paracrine or autocrine stimulation, often characterized as constitutive endocytosis. When cells were incubated with the specific CB1 agonist methanandamide (*R*(+)-MA; 9 nM × 15 min; Figure 3, second row), PM localization becomes minimal, while the intensity of the intracellular juxta- and perinuclear pools of CB1 receptors significantly increases by threefold (inset in first versus second row). This enrichment may reflect the presence of the receptor on recycling endosomes or even a proteasomal localization, as proteasomes are denser in the vicinity of the microtubule organizing center (MTOC) by the nucleus and on the cytosolic side of the ER. Notably, a perinuclear, ring-like enrichment of CB1 appears, consistent with an additional distribution to the ER (inset second row). While this may further argue for a presence in recycling endosomes, it should be noted that endosomal EGFR interacts with the ER phosphatase PTP1B, in an apparently global mechanism of direct interactions between PM receptors and ER molecules (Eden, White, Tsapara, & Futter, 2010). With MCD treatment (10 mM × 15 min, at 37 °C), the receptor appears minimal at the PM and concentrated in some intracellular pools, including some irregular large vesicular structures (arrows), not seen in controls. When MCD incubation is followed by *R*(+)-MA, we do not observe any measurable difference in intracellular densities compared to MCD alone, nor does the overall pattern present with similarities to that with *R*(+)-MA alone, all indicating disruption of the *R*(+)-MA-induced intracellular flow of the receptor. Furthermore, CB1–GFP fluorescence did not show any significant colocalization with that of LysoTracker Red DND-99, used as a marker of lysosomes (Figure 4). After treatment with *R*(+)-MA, and despite the increased intracellular signal, only a small pool of the receptor is seen to colocalize with lysosomes (Figure 4, arrows).

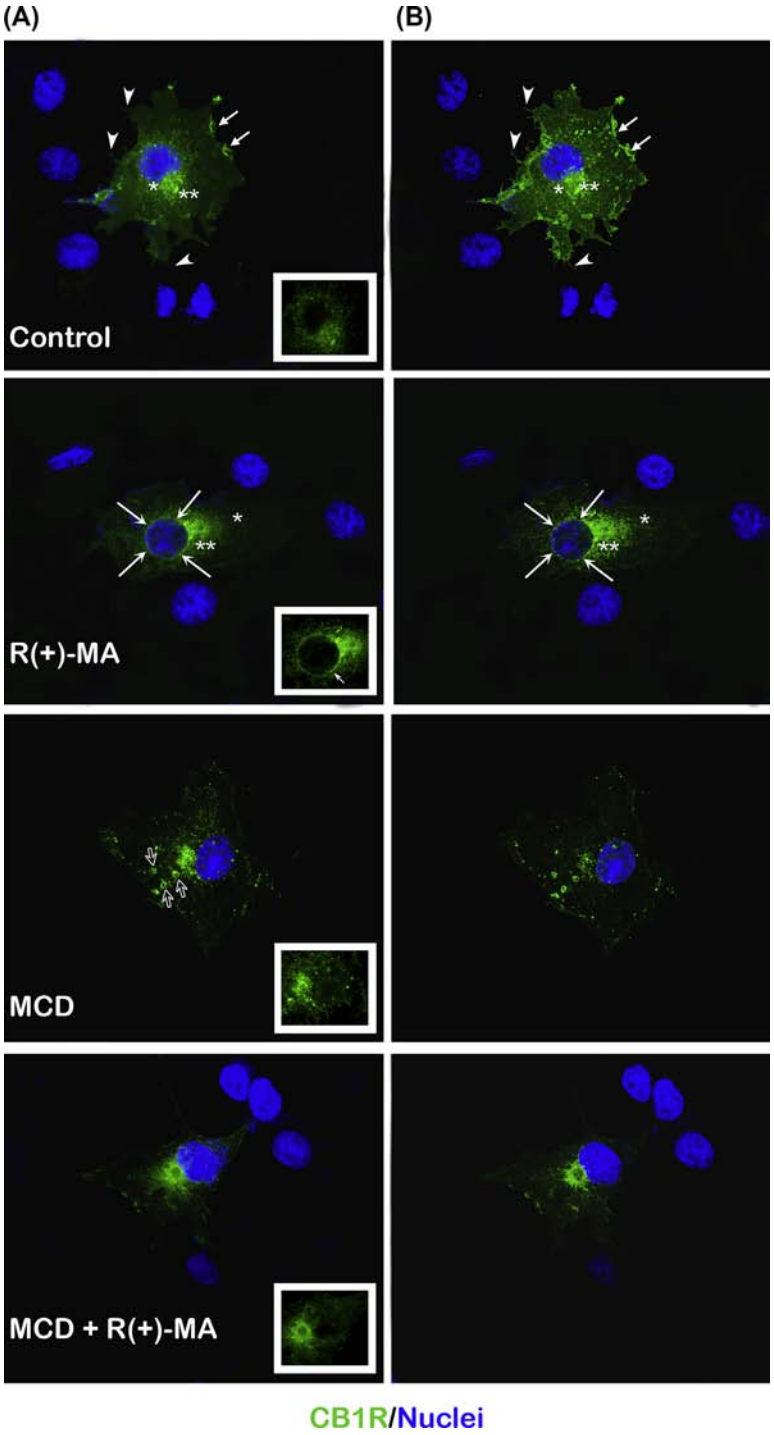

FIGURE 3 Subcellular distribution of CB1: effects of agonism and/or MCD. COS-7 cells were transfected with a CB1–GFP construct and were either left untreated (control) or treated with *R*(+)-MA (9 nM) for 15 min, with or without cholesterol extraction with 10 mM MCD, fixed with 4% paraformaldehyde, and stained with Hoechst 33258 to visualize chromatin (nuclei). Fluorescence was imaged using a Leica TCS SP5 inverted confocal microscope with a motorized stage, a 63× HC PL APO CS oil lens, and a tandem scanner (Biological Imaging Unit, Biomedical Research Foundation, Academy of Athens). Images are projections of four serial 0.25-μm-thick Z-stacks (~2 μm each panel) at the equatorial plane (left column) or at the adhering surface (right column). Detailed description is given in the text.

Effects of Microtubule Integrity

An integral functional partner of lipid rafts is the tubulins, resident proteins in raft microdomains (Palestini et al., 2000). Both α- and β-tubulin have been shown to physically interact with the lipid-raft markers flotillin 1, caveolin, and G-proteins (Dremina, Sharov, & Schoneich, 2005). Microtubules, the polymerized dimers of α- and β-tubulin, in the cell periphery are very dynamic and the rapid interchange between stable elongation and catastrophe is modulated by a number of molecules in or in close association with lipid rafts. Moreover, both internalizing endosomes and vesicles transported from the ER to the Golgi may utilize microtubules to translocate. Although the fixing conditions for visualizing receptors, in this case CB1–GFP, are not optimal for precisely preserving the architecture of microtubules and in particular peripheral tubulin dimers, a number of CB1 vesicles may be seen along the immunofluorescence of β-tubulin (Figure 5(A), arrows in inset), especially along thicker microtubule bundles

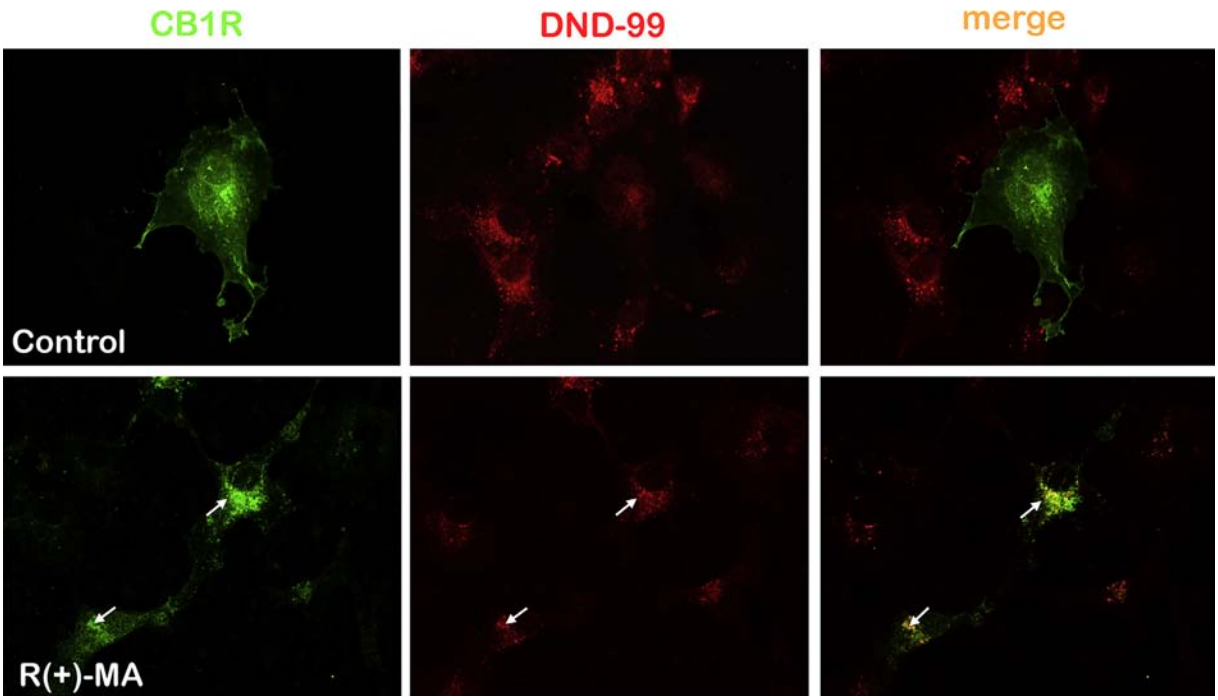

FIGURE 4 Limited localization of CB1 in lysosomes. CB1–GFP COS-7 cells were preincubated with LysoTracker DND-99 to detect acidic vesicular organelles (lysosomes) and then imaged under resting conditions (control) or after treatment with *R*(+)-MA. Intracellular patterns of CB1–GFP are quite distinct from those of DND-99 under either condition; arrows point to occasional colocalization upon receptor internalization. Images are projections of four serial 0.25-μm-thick Z-stacks (~2 μm each panel) at the equatorial plane.

(double arrows). This close association may also be seen in mitotic cells at telophase, in which internalization of the receptor after treatment with *R*(+)-MA may be readily seen (Figure 5(B)). The flow of CB1–GFP fluorescence appears to be in close association with microtubules emanating from the MTOC (yellow arrow), while there is an unexpected enrichment of small CB1 vesicles on the central spindle microtubules (double arrows) that promote ingression of the cleavage furrow and cytokinesis. When cells were mildly treated with nocodazole (660 nM × 1 h), a drug that binds to tubulins with a two-affinity Michaelis–Menten kinetics pattern and inhibits microtubule assembly, the cytoplasm collapses into few thick processes around the soma and the GFP-tagged receptors appear with a diffuse distribution in it, with no particular enrichments or PM presentation (Figure 6). Thus microtubules are essential for proper CB1 surface presentation and intracellular trafficking.

Effects of Filamentous-Actin Membrane Cytoskeleton Integrity

It is now adequately documented that lipid rafts coalesce within constraints applied by the underlying cortical filamentous-actin (F-actin) cytoskeleton and its associated proteins to form signaling platforms from where signaling cascades emanate. More specifically, constraints are imposed by the F-actin and cross-linking proteins that act as fences and by interacting proteins that act as pickets, namely by the cytosolic domains of transmembrane proteins or cytosolic proteins, tethered into the membrane through lipid posttranslational modifications (Kusumi & Suzuki, 2005; Morone et al., 2006). This extensive network of "fences" and "pickets" forms restraining "corrals" for the movement of lipid rafts through the entirety of the PM. F-actin-binding proteins are instantly modified by receptor-mediated signaling kinase activation and transiently assume new affinities and diffusion rates (Asimaki & Mangoura, 2011; Harder & Simons, 1999; Kusumi & Suzuki, 2005; Mangoura, 1997). Thus, a signal-dependent reorganization of the cortical actin cytoskeleton becomes important in the spatiotemporal activity of signaling pathways, because signaling complexes often internalize on pinched-off vesicles along cytoskeleton tracks, further propagating the signal intracellularly (del Pozo et al., 2005).

The importance of an intact F-actin cytoskeleton becomes apparent when imaging the subcellular localization of CB1 after the collapse of the F-actin cytoskeleton by cytochalasin D (10 μM × 1 h, at 37 °C) (Figure 7). More specifically, cytochalasin caused CB1–GFP fluorescence to appear as irregular, scattered foci, mostly throughout the cytoplasm (arrows), while some remained in the fine processes that are left as the cytoplasm retracts (arrowheads). Treatment with *R*(+)-MA resulted in further retraction of the fine processes, although cortical detection of the receptor was still evident (arrows, lower row), while some increase in the intracellular juxta- and perinuclear pools of CB1 was detectable, yet lower than with *R*(+)-MA alone (Figure 3, second row, vs Figure 7, lower row). It is thus possible that CB1 signaling and endocytosis may still take place when F-actin is disrupted, yet, the signal is modified as to (1) result in less intracellular trafficking of the receptor and (2) be "consumed" in the cell periphery, where more intense phosphorylation of F-actin-binding proteins, like MARCKS or p120 (Asimaki & Mangoura, 2011), leads to intense disengagement of the F-actin cytoskeleton from the PM and hence further collapsing of filopodia, as seen with CB1 agonism in hippocampal neurons (Roland et al., 2014).

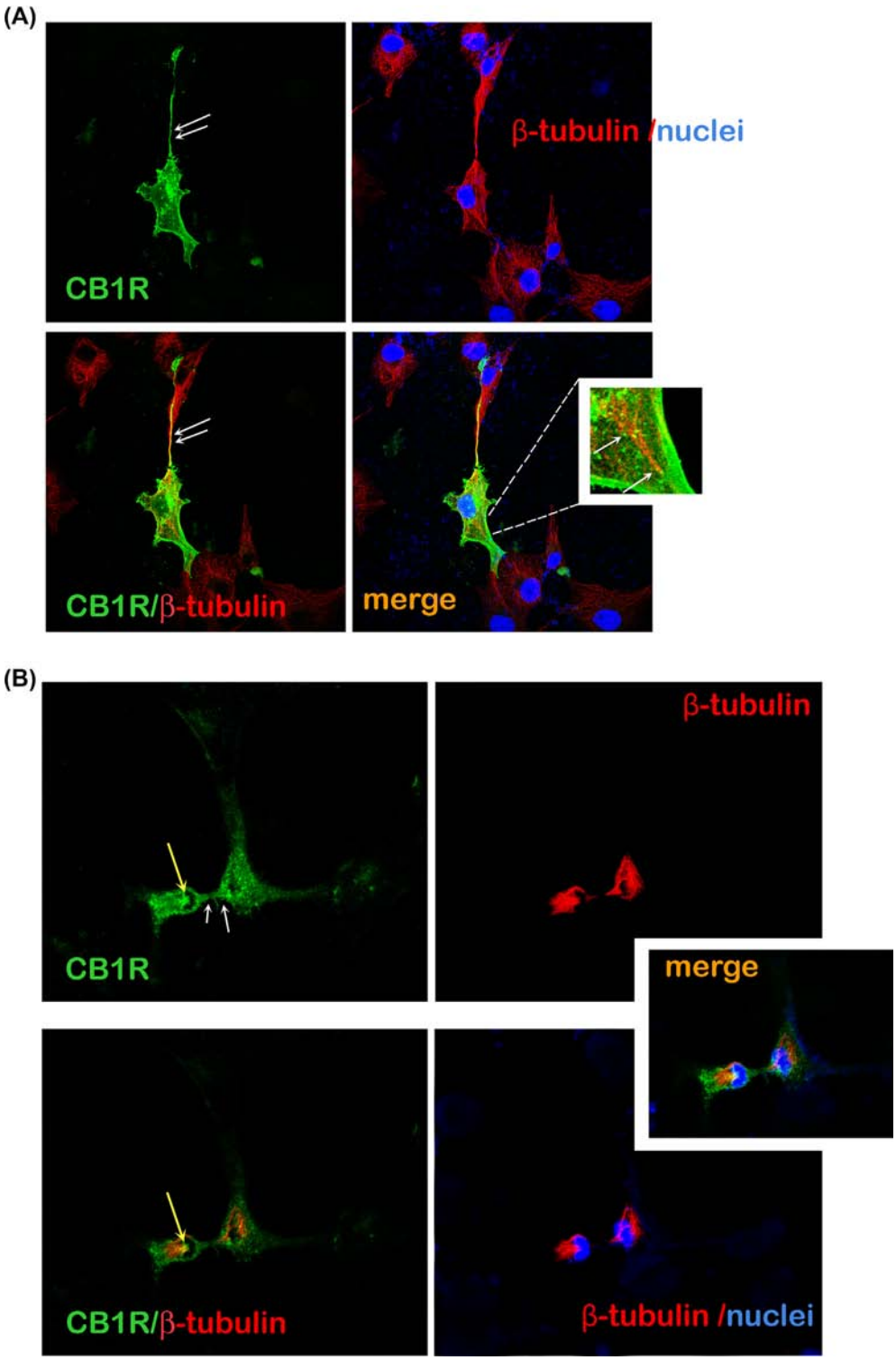

FIGURE 5 **CB1–GFP fluorescence partially localizes along microtubules.** CB1–GFP-expressing COS-7 cells were immunostained with β-tubulin, while nuclei are visualized with Hoechst 33258. (A) In control cells a number of CB1 vesicles may be seen along the microtubule tracks (arrows) detected with β-tubulin. (B) CB1–GFP internalization after treatment with *R*(+)-MA may be seen in cells at the last stage of mitosis, as deduced from chromatin (Hoechst 33258, blue) and β-tubulin patterns of staining. Yellow arrows point to a CB1 enrichment in the vicinity of the MTOC and double arrow to the central spindle microtubules. All images are projections of four serial 0.25-μm-thick Z-stacks (~2 μm each panel) at the equatorial plane.

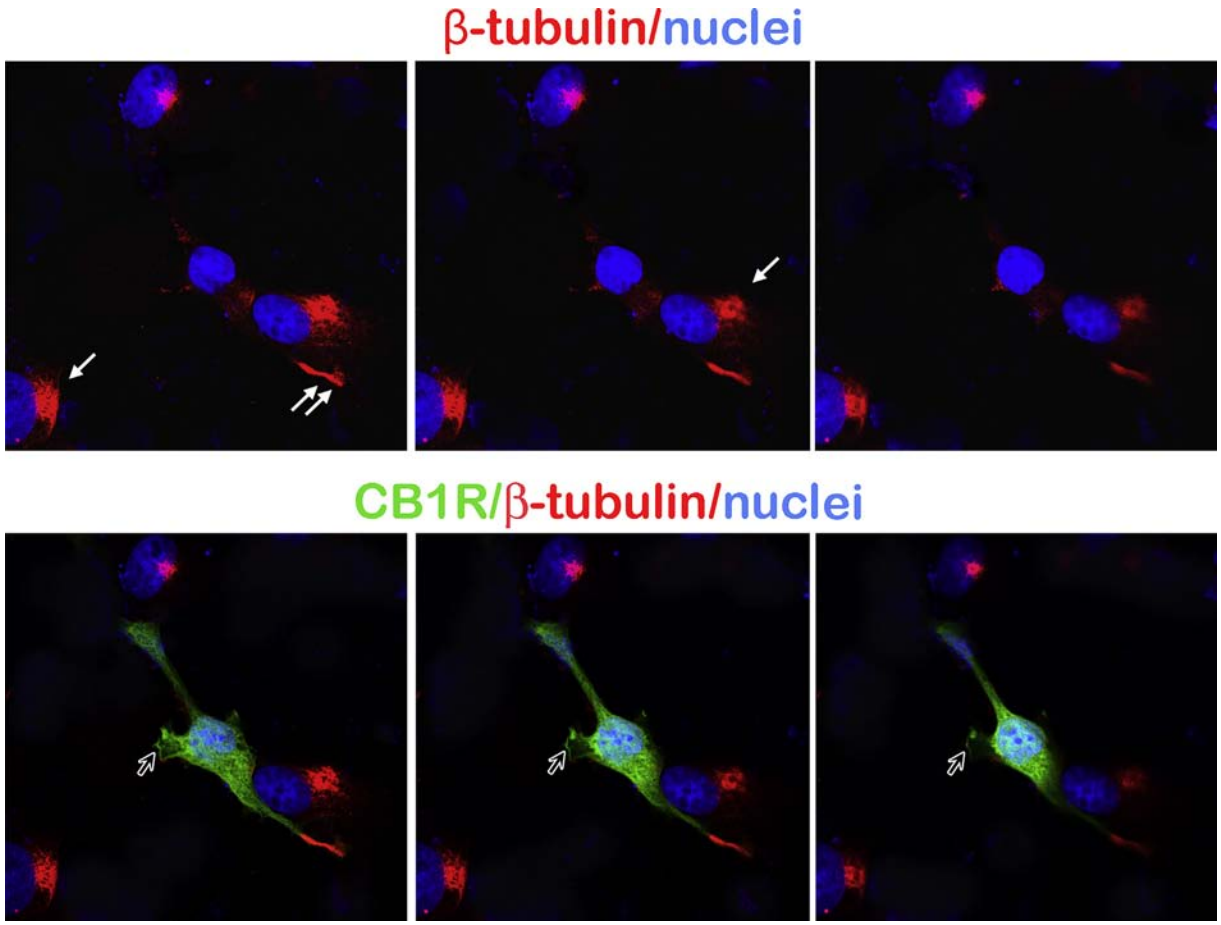

FIGURE 6 Microtubule integrity is essential for proper CB1 surface presentation and intracellular trafficking. CB1–GFP-expressing COS-7 cells were treated with the microtubule disrupter nocodazole (660 nM, 1 h, 37 °C) and immunostained with β-tubulin (red), while nuclei were visualized with Hoechst 33258. Nocodazole causes microtubule collapse toward the MTOC (single arrows) and in the leading process of a migrating cell (double arrow), and CB1–GFP fluorescence appears scattered throughout the cytoplasm with only limited membrane presentation (empty arrows). All images are projections of four serial 0.25-μm-thick Z-stacks (~2 μm each panel), starting at the equatorial plane (left column) and moving toward the free surface of the cell.

These data collectively indicate that CB1 targeting to and signaling/trafficking from the PM is regulated by lipid rafts and its functional partners, the F-actin membrane and the intracellular microtubule cytoskeleton.

FORMATION OF CB1 SIGNALING COMPLEXES AND TRANSACTIVATION OF TYROSINE KINASE RECEPTORS IN LIPID RAFTS

Interreceptor cross talk has long been recognized as an important mechanism that links GPCRs to ERK1/2 activation. CB1s may form homotypic dimers or even heterotypic dimers with other GPCRs for both signal amplification and receptor internalization (e.g., Ellis, Pediani, Canals, Milasta, & Milligan, 2006). The distinctly different heterotypic complexes of GPCRs and RTKs are, however, less understood and characterized, except in that they are known to require scaffolding proteins (Maudsley et al., 2000; Pyne, Waters, Moughal, Sambi, & Pyne, 2003). Cross talk between CB1 and RTKs was first described in CHO cells cotransfected with CB1 and insulin or insulin-like growth factor 1 receptors (Bouaboula et al., 1997), then EGFR was shown to integrate cannabinoid signaling to activate ERK1/2 in tumor cell lines (Hart, Fischer, & Ullrich, 2004; Rubovitch et al., 2004), while a significant level of cross talk between CB1 and FGFRs, also prominent receptors in promoting neuritic outgrowth, was shown to regulate the axonal growth of rat cerebellar granule neurons (Williams, Walsh, & Doherty, 2003) or neuritic outgrowth in chick embryo cortical neurons (Asimaki & Mangoura, 2011).

In canonical pathways, GPCR-dependent transactivation of RTKs requires tyrosine kinases of the Src family, which activate RTKs through intermolecular phosphorylation (e.g., Sandilands et al., 2007), while dependency on Ca^{2+}, G proteins, or PKC activity appears to be receptor and cell type specific (Ma, Huang, Ali, Lowry, & Huang, 2000; Mangoura, 1997; Mangoura & Dawson, 1993). Ligand-induced organization of GPCR–RTK multiprotein signaling complex provides amplification of the signal through the engagement of additional downstream effectors and may also contribute to the endocytosis and intracellular trafficking of the receptor. This highly complex regulation of membrane receptors, serving neurotransmission, growth, or polarity, is considered pivotal for CNS development and synapse function.

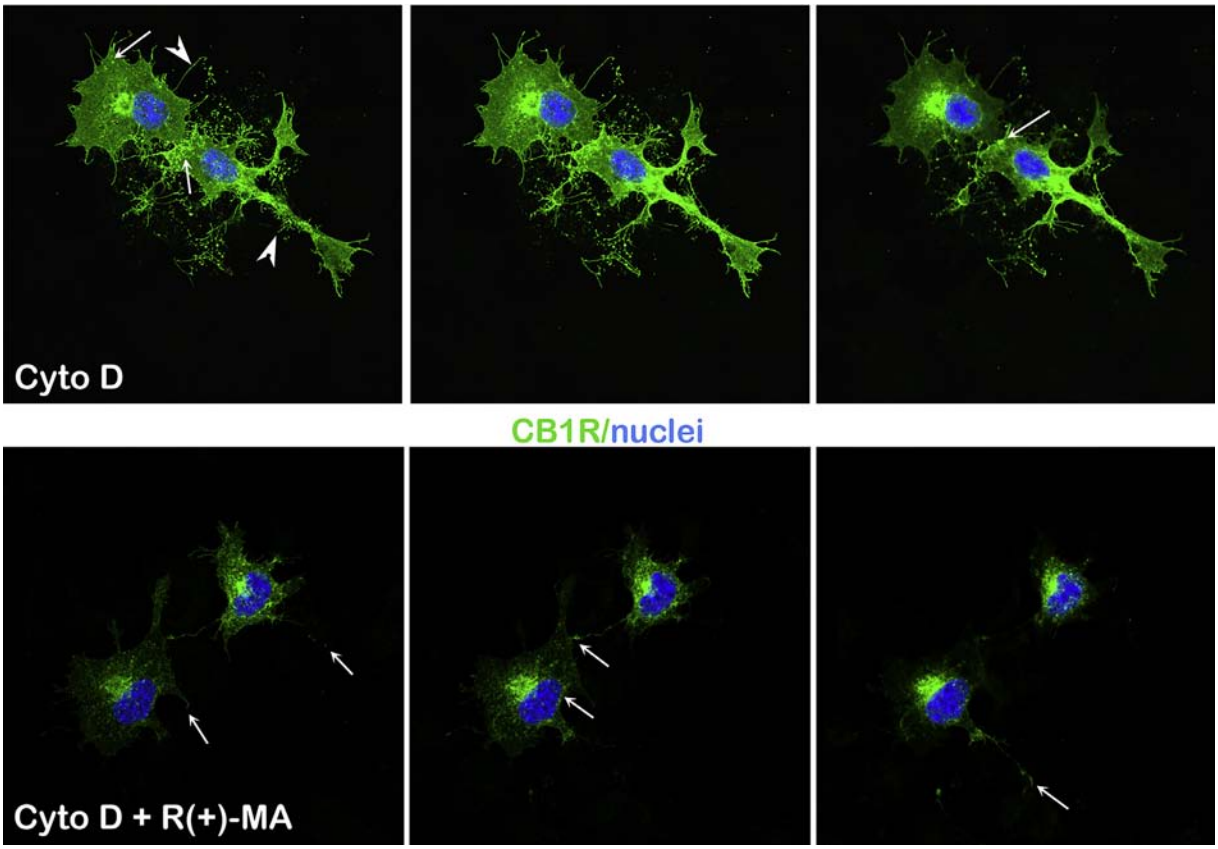

FIGURE 7 **Integrity of the F-actin cytoskeleton is essential for proper CB1 surface presentation, intracellular trafficking, and endocytosis.** CB1–GFP-expressing COS-7 cells were treated with 10 μM cytochalasin D (1 h at 37 °C) prior to addition of vehicle (upper row) or *R*(+)-MA for 15 min (lower row), fixed, stained with Hoechst 33258, and imaged. Detailed description is given in the text. All images are projections of four serial 0.25-μm-thick Z-stacks (~2 μm each panel), starting at the equatorial plane (left column) and moving toward the free surface of the cell.

Our investigations on CB1 proximal signaling in early cortical neurons in primary cultures have revealed that CB1 agonism with *R*(+)-MA induces a biphasic activation of ERK1/2 in a Src- and Fyn-dependent manner (Asimaki & Mangoura, 2011). Most interestingly this 90-min-long time course of ERK activation clearly exhibits two sequential increases: the first peak starts within seconds and reaches a plateau by 5 min, and the second, additional amplification at 15 min lasts for up to 30 min, decreasing slowly thereafter. This CB1-induced, sustained duration of ERK activation translates to induction of neuritic outgrowth in chick cortical neurons in the long term.

Among the earliest signaling events in this model of a CB1–ERK cascade is a $G_{q/11}$-mediated phospholipase C and PKC activation. With proper pharmacological analysis, we postulate that PKCε is the PKC isoform immediately activated. This isoform uniquely bears an actin-binding site, which allows it, upon activation, to bind to F-actin and modify F-actin-binding proteins, all leading to regulation of neurite outgrowth (Asimaki & Mangoura, 2011; Mangoura, 1997; Zeidman, Troller, Raghunath, Pahlman, & Larsson, 2002). Moreover, endogenous CB1 and PKCε physically associate and reciprocally coimmunoprecipitate, while stoichiometric cotransfections with appropriate constructs show that CB1 interacts directly with the regulatory domain of PKCε (Asimaki & Mangoura, 2011). Dissociation of the two molecules occurs probably owing to conformational changes in CB1 with ligand binding (Howlett et al., 2002) and to the phospholipase C (PLC)/diacyl glycerol-induced "opening" (activation) of PKCε; an open-conformation PKCε preferentially associates with Src-family kinases (Song et al., 2002). While activation of both Fyn and Src occurs upon *R*(+)-MA agonism in primary cortical neurons, their specific inhibition abolishes both peaks, suggesting that this CB1/G_q/PLC/PKCε/Src–Fyn/Ras/Raf/ERK pathway (Figure 8) induces a second, proximal signaling event that leads to intense amplification, possibly recruitment of an RTK.

Cross talk of CB1 and FGFR has been postulated in the axonal growth of rat cerebellar granule neurons (Williams et al., 2003) and, indeed, PD173074, an FGFR-specific inhibitor known to abolish neuritogenesis, significantly reduces the second phase of *R*(+)-MA-induced ERK1/2 activation, but not the first (Asimaki et al., 2011). As probed by immunoprecipitation, *R*(+)-MA-induced activation of CB1 indeed elicits tyrosine phosphorylation of FGFR (and therefore activation; Furdui, Lew, Schlessinger, & Anderson, 2006) in a time frame that correlates well with the second wave of ERK1/2 activation and constitutes an amplification of the first wave (Figure 8). Moreover, direct activation of PKCε (with the PKCε-specific activator peptide ψεRACK) increases tyrosine phosphorylation of FGFR (Asimaki et al., 2011). Further probing this transactivation, with analysis of CB1 immunoprecipitates during the second phase of ERK1/2 activation, shows that, upon CB1 agonism and $G_{i/o}$ stimulation, a complex containing CB1 and activated Src, Fyn, and FGFR is formed.

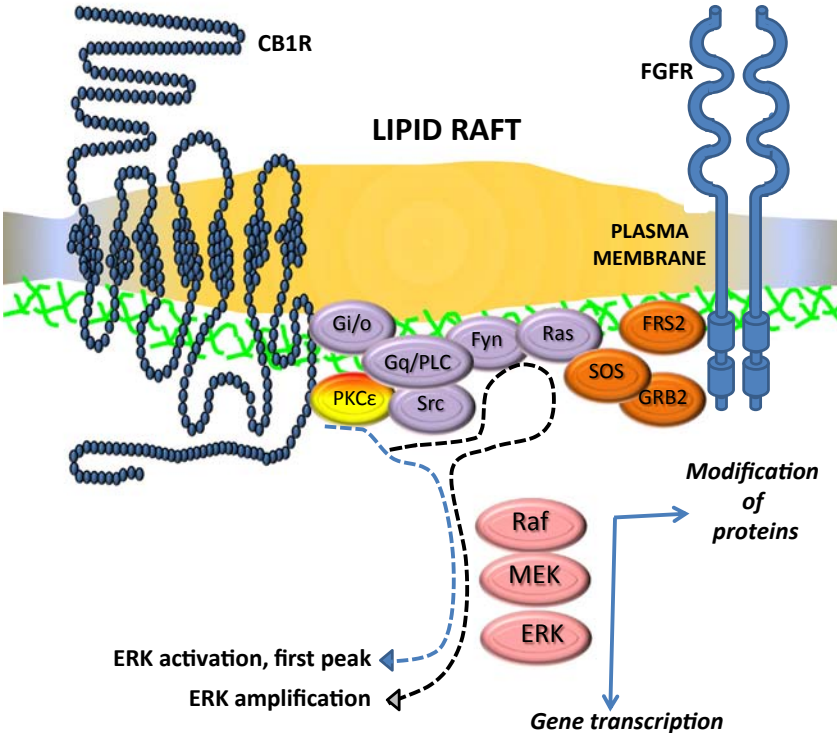

FIGURE 8 Schematic representation of CB1 signaling out of a lipid raft. CB1 causes a sustained and biphasic ERK activation in cortical neurons. A significant pool of CB1s preassociates with regulatory domains of PKCε, which upon ligand binding and sequential acute activation of $G_{q/11}$/PLC becomes activated and dissociates from CB1. The liganded receptor flows into lipid rafts, while the activated PKCε acutely forms transient signaling complexes with activated Src and Fyn kinases to elicit the first wave of ERK1/2 activation that lasts for 5 min (CB1/G_q/PLC/PKCε/Src–Fyn/Ras/Raf/ERK). Concurrently, several F-actin cytoskeleton proteins are modified by phosphorylation. A second pool of CB1s couples to activation of $G_{i/o}$ and, utilizing as effectors additional Src and Fyn molecules, transactivates FGFR to drive a second amplifying wave of ERK1/2 activation (CB1/$G_{i/o}$/Src–Fyn/FGFR/Ras/Raf/ERK).

Demonstrating specificity, specific CB1 antagonists, or potent inhibitors of PKCε and of Src or Fyn, abolish the cannabinoid-induced transactivation of FGFRs. This amplification of the magnitude and duration of the CB1-dependent activation of ERK through FGFR activation is of high functional significance, because inhibition of FGFR activation with PD173074 abolishes the CB1-dependent increase in the length of the major dendrites in primary cortical neurons.

More importantly, lipid-raft integrity is required for this CB1/G_i/PKCε/Src–Fyn/Ras/Raf/ERK pathway, because MCD diminishes the association of CB1 with Src, Fyn, and FGFR. Moreover, recruitment and activation of CB1 in lipid rafts constitutes an early event that precedes and is required for these functional CB1 multiprotein signaling complexes. As mentioned above, CB1 is widely distributed in raft and nonraft membranes of primary cortical neurons under basal conditions, when analyzed by a detergent-free lipid-raft preparation that provides sufficient resolution of genuine lipid rafts from nonraft PMs and intracellular ER and Golgi membranes (Figure 2). After 2 min of *R*(+)-MA, CB1 rapidly incorporates into the lipid-raft fractions and by 7 min it progressively traffics toward the nonraft membranes as well as the intracellular ER and Golgi membranes. After 15 min, the original pool of CB1 molecules in the lipid rafts has been restored. CB1 has evolved to bind lipid neurotransmitters, and at least for the prototypical agonist AEA, cholesterol is pivotal for insertion into the lipid bilayer (Di Pasquale, Chahinian, Sanchez, & Fantini, 2009). It is therefore possible that ligand binding induces conformational changes in CB1 that allow its flow in the lipid rafts, where it associates with other lipid-raft-prone signaling proteins. Indeed, Fyn kinase, a raft resident through its double lipid modification, is detected as activated in the lipid rafts within 2 min of treatment with *R*(+)-MA (Asimaki et al., 2011). Concurrently, that is by 2 min, *R*(+)-MA induces incorporation of an FGFR pool in the lipid rafts. FGFRs lack a GRB2 binding site and depend on recruiting the intermediate docking myristoylated phosphoprotein scaffolds FRS2 and FRS3, a function unique to vertebrates and available only to a limited repertoire of RTKs (Gotoh, 2008), in order to activate SOS and thus Ras, Raf, and ERK. Moreover, as ERK1/2 phosphorylates FRS2 to negatively affect FGF-induced tyrosine phosphorylation of FRS2 (Gotoh, 2008), the activation of FGFR provides another off switch in this signaling cascade. This is an additional mechanism to control the CB1-dependent ERK1/2 activation, the first being the PKC-dependent recruitment of off switches for the ERK pathway, such as neurofibromin (Mangoura et al., 2006). Taken together, FGFR1, known to signal mitogenic ERK1/2 signals from non-lipid-raft membranes, may also signal from lipid rafts in the case in which it is recruited as a tyrosine kinase that amplifies the CB1 signal in neurons.

CONCLUDING REMARKS

Collective knowledge has documented an important role for CB1 in a diverse array of physiological and pathophysiological processes ranging from normal development to control of appetite to pathogenesis of cancer and the development of addiction. Therefore understanding

the mechanistic details of CB1 signaling is of critical importance, as it holds great therapeutic promise. In this chapter we have reviewed experimental evidence that shows that lipid rafts constitute the PM platform on which both early CB1 signaling events and subsequent ordered complex formation with receptor tyrosine kinases, in particular with FGFR, are organized and primed toward the regulated propagation of ERK1/2 activation. These observations may provide new insights into the pharmacological effects and cellular functions modulated by the CB1-induced signaling events, which additionally underlie several of the less understood acute effects of cannabinoid drugs.

APPLICATIONS TO OTHER ADDICTIONS AND SUBSTANCE MISUSE

CB1 has evolved as a major regulatory molecule for almost all known aspects of the development and function of the CNS, while it is capable of establishing addiction. It is therefore very important to understand the precise mechanisms and intracellular signal transduction pathways that CB1 utilizes for its effects. As elaborated in this chapter, the highly complex intracellular signaling of CB1 is initiated and organized at specialized membrane microdomains, the lipid rafts.

Lipid rafts are also regulatory to a number of other receptors that CB1 interacts with and which may also induce addiction, such as the opioid receptors. Given that the composition of lipid rafts may be altered with various approaches, pharmacological or nutritional, understanding the role of lipid rafts in the signaling of membrane receptors opens new ways to target addiction.

Moreover, several of the intracellular signaling molecules that are utilized by both opioid receptors and CB1 are mediators of the effects of alcohol, such as PKCε, and understanding their function may lead to unraveling the molecular basis of addiction and eventually lead to its treatment.

DEFINITION OF TERMS

Plasma membrane This is a lipid bilayer (two leaflets), rich in proteins, that encloses each cell and separates it from its environment, thus generating the extracellular and the intracellular space; the latter includes all the cellular organelles and the nucleus with the genetic information.

The cannabinoid 1 receptor This is a protein interlaced in the PM in a specific manner, namely, its initial domain along with three other more centrally exposed to the extracellular space, while three other domains plus the terminal domain are exposed to the intracellular space. Exogenous (synthetic, plant-derived) or endogenous (produced by cells in the organism) cannabinoid substances (CB1 agonists) bind specifically to CB1; CB1 then changes its three-dimensional form and starts an intracellular signaling cascade.

Intracellular signaling cascades or signal transduction pathways or simply signaling These are a series of interactions among proteins that transmit the event or message that CB1 agonists have been bound to the CB1. Intracellular signaling cascades transmit this message (1) to proteins in the intracellular/cytoplasmic space and change transiently their function in physiological processes, e.g., shape of the cell, and (2) to the nucleus to modify gene expression.

Phosphorylation This is the addition of a phosphate group to certain amino acids, the building blocks of proteins. It is catalyzed by enzymes called *kinases*, and most kinases become activated during signaling. Phosphorylation is a reversible modification of proteins.

Lipid rafts These are specially organized microdomains with a diameter of 10–200 nm and slightly thicker than the rest of the PM; hence they appear as rafts in water. Lipid rafts have a particular composition of lipids and proteins (including receptors). This particular composition along with their ability to coalesce allows them to function as a regulatory platform for receptor signaling.

Lipid raft asymmetry This refers to the fact that the outer lipid layer or outer leaflet (which faces the extracellular space) and the inner leaflet (which faces the intracellular space) differ in protein and lipid composition, further regulating receptor signaling.

F-actin cytoskeleton or membrane skeleton or cortical cytoskeleton This is a special cytoskeleton structure composed of F-actin and proteins that bind to it. This fishnet-like structure underlies the PM and creates corrals that restrain the movement of lipid rafts. Several of its proteins interact with signaling cascades (act as scaffolds that facilitate propagation of the signal).

KEY FACTS OF CB1

- CB1 is expressed very early in embryo development and is involved in the mediation of important aspects of cell and tissue growth.
- CB1 is a prominent receptor in the CNS.
- CB1 is particularly expressed in the cerebral cortex, striatum, cerebellum, hippocampus, and hypothalamus, CNS areas that control cognition, memory, emotion, and movement, as well as appetite and metabolism.
- CB1 modulatory actions on cells are mediated by its intracellular signaling, which may transiently modify both preexisting proteins and protein dosage in general, via signaling to the nucleus for changes in gene expression (changes in gene transcription are translated to changes in protein content: the effects may be up- or downregulation of a given protein).
- Aberrant CB1 signaling and aberrations in protein dosage of proteins in CNS cells are the molecular basis of addiction.

SUMMARY POINTS

- Lipid rafts are important for CB1 plasma presentation, intracellular trafficking, and signaling.
- Integrity of F-actin and microtubule cytoskeletons is required for the same processes.
- CB1 flows to lipid rafts upon ligand binding.
- The CB1 signaling to ERK is served by two additive pathways:
  - CB1/G_q/PLC/PKCε/Src–Fyn/Ras/Raf
  - CB1/$G_{i/o}$/Src–Fyn/FGFR/Ras/Raf

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