

Themed Issue: Cannabinoids in Biology and Medicine, Part I

## REVIEW

# Cannabinoid receptor signalling in neurodegenerative diseases: a potential role for membrane fluidity disturbance

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Type-1 cannabinoid receptor (CB<sub>1</sub>) is the most abundant G-protein-coupled receptor (GPCR) in the brain. CB<sub>1</sub> and its endogenous agonists, the so-called 'endocannabinoids (eCBs)', belong to an ancient neurosignalling system that plays important functions in neurodegenerative and neuroinflammatory disorders like Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis and multiple sclerosis. For this reason, research on the therapeutic potential of drugs modulating the endogenous tone of eCBs is very intense. Several GPCRs reside within subdomains of the plasma membranes that contain high concentrations of cholesterol: the lipid rafts. Here, the hypothesis that changes in membrane fluidity alter function of the endocannabinoid system, as well as progression of particular neurodegenerative diseases, is described. To this end, the impact of membrane cholesterol on membrane properties and hence on neurodegenerative diseases, as well as on CB<sub>1</sub> signalling *in vitro* and on CB<sub>1</sub>-dependent neurotransmission within the striatum, is discussed. Overall, present evidence points to the membrane environment as a critical regulator of signal transduction triggered by CB<sub>1</sub>, and calls for further studies aimed at better clarifying the contribution of membrane lipids to eCBs signalling. The results of these investigations might be exploited also for the development of novel therapeutics able to combat disorders associated with abnormal activity of CB<sub>1</sub>.

**LINKED ARTICLES**

This article is part of a themed issue on Cannabinoids in Biology and Medicine. To view the other articles in this issue visit <http://dx.doi.org/10.1111/bph.2011.163.issue-7>

**Abbreviations**

2-AG, 2-arachidonoylglycerol; Aβ, amyloid β peptide; AD, Alzheimer's disease; AEA, N-arachidonylethanolamine or anandamide; ALS, amyotrophic lateral sclerosis; ApoE, apolipoprotein E; APP, amyloid precursor protein; BDNF, brain-derived neurotrophic factor; CB<sub>1</sub>, type-1 cannabinoid receptor; CB<sub>2</sub>, type-2 cannabinoid receptor; CNS, central nervous system; DAGL, diacylglycerol lipase; eCB, endocannabinoid; FAAH, fatty acid amide hydrolase; FMRP, Fragile X mental retardation protein; FXS, Fragile X syndrome; GPCR, G protein-coupled receptor; HD, Huntington's disease; IPSI, inhibitory postsynaptic currents; l<sub>c</sub>, liquid-disordered crystalline phase; l<sub>o</sub>, liquid-ordered phase; LR, lipid raft; MAGL, monoacylglycerol lipase; MCD, methyl-β-cyclodextrin; mGlu, metabotropic glutamate; MS, multiple sclerosis; NAPE-PLD, N-acyl-phosphatidylethanolamine (NAPE)-hydrolysing phospholipase D; NMDA, N-methyl-D-aspartate; PD, Parkinson's disease; PPAR, peroxisome proliferator-activated receptor; PrD, prion-related disorder; PrP, prion protein; TRPV1, type-1 transient receptor potential vanilloid

## Introduction

*N*-arachidonoylethanolamine [anandamide (AEA)] and 2-arachidonoylglycerol (2-AG) activate type-1 ( $\text{CB}_1$ ) (Howlett *et al.*, 2010; Pertwee, 2010) and type-2 ( $\text{CB}_2$ ) cannabinoid receptors (Patel *et al.*, 2010), and are the most active 'endocannabinoids' (eCBs) as yet characterized (Di Marzo, 2009; Maccarrone *et al.*, 2010a). AEA, but not 2-AG, also binds to and activates type-1 transient receptor potential vanilloid (TRPV1) channels, and hence is considered a true 'endovanilloid' (Di Marzo and De Petrocellis, 2010). The nomenclature of the major eCBs-binding receptors ( $\text{CB}_1$ ,  $\text{CB}_2$  and TRPV1) conforms to the *British Journal of Pharmacology's* Guide to Receptors and Channels (Alexander *et al.*, 2009). Here, the hypothesis that changes in membrane fluidity alter function of the eCBs signalling, as well as progression of particular neurodegenerative diseases, is described. To this end, the impact of membrane cholesterol on membrane properties and hence on neurodegenerative diseases, as well as on  $\text{CB}_1$  signalling *in vitro* and on  $\text{CB}_1$ -dependent neurotransmission within the striatum, is discussed.

$\text{CB}_1$  receptor is the most abundant G protein-coupled receptor (GPCR) in the brain (Howlett *et al.*, 2010). Together with its endogenous agonists,  $\text{CB}_1$  forms an ancient neuro-signalling system that plays important control functions within the central nervous system (CNS) (Katona and Freund, 2008). Alterations in eCBs signalling have been extensively investigated in a wide range of neurodegenerative and neuroinflammatory disorders, spanning from Alzheimer's disease (AD), Parkinson's disease (PD) and Huntington's disease (HD), to amyotrophic lateral sclerosis (ALS) and multiple sclerosis (MS) (Maccarrone *et al.*, 2007; Centonze *et al.*, 2008; Bisogno and Di Marzo, 2010). In addition, prion-related disorders (PrDs) seem to benefit from a nonpsychoactive phytocannabinoid like cannabidiol, that prevents prion accumulation and protects neurons against its toxicity (Dirikoc *et al.*, 2007; Iuvone *et al.*, 2009). Not surprisingly, research on the therapeutic potential of drugs modulating eCBs activity is very intense (Di Marzo, 2009). In recent years it has become increasingly evident the involvement of membrane lipids, especially cholesterol and glycosphingolipids, in regulating the function of GPCRs like  $\beta_2$ -adrenergic and serotonin<sub>1A</sub> receptors, as well as of several other membrane-associated proteins like caveolins (Pontier *et al.*, 2008; Prinetti *et al.*, 2009; Paila *et al.*, 2010; Shrivastava *et al.*, 2010).

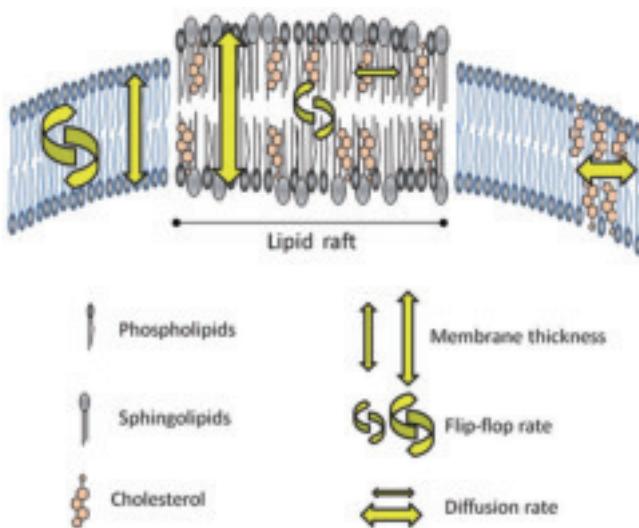
In the following sections the effect of cholesterol on membrane properties will be discussed, as well as its involvement in neurodegenerative diseases where eCBs signalling has been shown to play a role. Then, the effect of membrane cholesterol on  $\text{CB}_1$ -dependent signal transduction in *in vitro* models will be briefly reviewed, and the molecular determinants that underlie  $\text{CB}_1$ , but not  $\text{CB}_2$ , sensitivity to membrane cholesterol perturbation will be presented. Finally, recent evidence that demonstrates a role for membrane cholesterol on  $\text{CB}_1$ -dependent eCBs signalling in striatal neurotransmission will be summarized, in order to support the concept that membrane environment can indeed control eCBs-dependent neurotransmitter networks also *in vivo*. Incidentally, there is evidence that similar changes in plasma membrane fluidity occur in non-neuronal tissues where  $\text{CB}_1$  receptors are expressed, for instance within the immune system (Bari *et al.*,

2006). Yet, the effect of alterations of membrane cholesterol on peripheral  $\text{CB}_1$ -dependent signalling goes beyond the scopes of this review.

### *Cholesterol and membrane properties: the 'lipid raft' concept*

The classical 'fluid-mosaic' model of the biological membrane organization (Singer and Nicolson, 1972) has changed in recent years. Membrane proteins are no longer simply floating in a two-dimensional oriented solution, and the solvent is not only a simple viscous phospholipid bilayer. In fact, lateral compartmentalization and membrane asymmetry impose specific constraints to diffusion and partition between the two monolayers of specific hydrophobic molecules, and affect membrane-bound proteins (Forneris and Mattevi, 2008; Lindahl and Sansom, 2008). Some emerging concepts on membranes are that they are patchy structures, with segregated functional regions of various thickness and composition, and that crowding domains and ectodomains limit lipid exposure to the surrounding aqueous regions (Brown and London, 1998; Galbiati *et al.*, 2001). Therefore, it appears increasingly evident that changes in the physicochemical properties of the biological membrane directly affect the structure, and hence the function, of associated proteins. Rather than serving only as a medium through which membrane proteins diffuse, lipid bilayers have now been demonstrated to form compartmentalized subdomains with different biophysical properties.

Among the most interesting specialized microdomains of the plasma membrane are those enriched in cholesterol, sphingolipids, plasmenylethanolamine and arachidonic acid (Brown and London, 1998; Pike, 2005). These domains, characterized by a more tightly packed state responsible for their resistance to solubilization with non-ionic detergents (Brown and London, 1998), have been referred to as 'lipid rafts' (LRs). Interestingly, the physicochemical properties of LRs seem to be strictly related to their functional roles (Ostermeyer *et al.*, 1999). Phase separation between lipids in different physical states, most often the solid-like gel phase ( $\beta$ -phase) and the liquid-disordered crystalline phase ( $\alpha$ -phase,  $\text{l}_\alpha$ ), has been well characterized in model membranes. Sphingolipids differ from most biological phospholipids in that they contain long, largely saturated acyl chains. This allows them to pack tightly together. However, because of the high concentration of cholesterol in the plasma membrane and other membranes in which rafts form, sphingolipids in LRs do not exist in the gel phase. Different kinds of phase separation can occur in binary mixtures of individual phospholipids with cholesterol. In these mixtures, domains in a  $\text{l}_\alpha$ -like phase coexist with domains in a new state, the so-called 'liquid-ordered' ( $\text{l}_\text{o}$ ) phase. Interestingly, acyl chains of lipids in  $\text{l}_\text{o}$  phase are extended and tightly packed, as in the gel phase, but they have a high degree of lateral mobility (Brown and London, 1998). LRs obtained from detergent-resistant membranes isolated from cell lysates seem to exist in this  $\text{l}_\text{o}$  phase or in a state with similar properties (Ge *et al.*, 1999; Ostermeyer *et al.*, 1999). Thus, in the presence of cholesterol, lipid mixtures can undergo a  $\text{l}_\text{o}/\text{l}_\text{c}$  phase separation, instead of the  $\text{gel}/\text{l}_\text{c}$  distribution observed in the absence of cholesterol. Furthermore, cholesterol seems to promote phase separation, apparently because of favourable packing interactions between



**Figure 1**

Scheme of a biological membrane containing lipid rafts (LRs). The liquid-ordered regions of LRs are thicker than the liquid-disordered bulk of the membrane, and the cholesterol molecules within this region have a slower rate of lateral and interlayer diffusion (flip-flop). See text for details.

saturated lipids and cholesterol itself (Xu and London, 2000). This ‘cholesterol effect’ probably explains LRs formation also in cell membranes that contain low levels of sphingolipids, and clarifies why cholesterol depletion can induce LRs disruption, thus affecting raft function.

In the simplest case, LRs can be viewed as signalling platforms that serve to colocalize the required components, such as receptors, coupling factors, effectors and enzymes, facilitating their interaction and supporting signalling. The functions modulated by LRs include cholesterol transport (Fielding and Fielding, 2001; Uittenbogaard *et al.*, 2002; Smart *et al.*, 2004), organization of signalling protein complexes (Galbiati *et al.*, 2001), endocytosis (Pelkmans, 2005), potocytosis (Anderson *et al.*, 1992) and establishment of cell polarity (Ostrom and Insel, 2004). These lipid microdomains can serve also as specific sites of interaction for certain pathogens and toxins (Fivaz *et al.*, 2002), and they play an important role also in the organization of cell signalling machineries such as receptor tyrosine kinases and GPCRs (Barnett-Norris *et al.*, 2005). More generally, the liquid ordered regions of LRs are thicker than the liquid-disordered bulk of the membrane, and the cholesterol molecules within this region have a slower rate of lateral and interlayer diffusion (flip-flop) (Dainese *et al.*, 2010). These properties of LRs are schematically depicted in Figure 1.

### Cholesterol and neurodegenerative diseases

Compared with other tissues, mammalian brain contains the highest levels of cholesterol, that in humans represents ~25% of the amount in total body (~20 mg·g<sup>-1</sup>), although human brain accounts for ~2% only of total body weight (Dietschy and Turley, 2004; Vaya and Schipper, 2007). Brain cholesterol is mainly synthesized by oligodendrocytes and astrocytes,

without a significant contribution by blood circulation because cholesterol in lipoprotein particles [(most often apolipoprotein E (ApoE)] can not cross the blood–brain barrier (Liu *et al.*, 2010). While oligodendrocytes produce cholesterol to form the myelin sheaths, astrocytes supply cholesterol to neurons, by using ATP-binding cassette A1 and G1 transporters (Karten *et al.*, 2006). In addition, astrocytes are the major ApoE-producing cells within the CNS, and cholesterol/ApoE complexes may enter neurons by receptor-mediated endocytosis (Hayashi *et al.*, 2004), followed by transport of cholesterol to different intracellular organelles (e.g. lysosomes, endoplasmic reticulum, Golgi apparatus) and to the plasma membranes.

The relevance of membrane cholesterol in neurodegenerative processes is demonstrated by the number of research papers devoted to this issue: a PubMed search with ‘cholesterol’ and ‘neurodegenerative diseases’ scores more than 1500 entries, 675 of which were released in the last 5 years. Not surprisingly, the topic of cholesterol involvement in the pathogenesis of neurodegenerative diseases has been recently covered by several comprehensive review papers (Adibhatla and Hatcher, 2008; Schweitzer *et al.*, 2009; Liu *et al.*, 2010; Martin *et al.*, 2010; Schengrund, 2010; Zuccato *et al.*, 2010). Besides the effect on membrane properties outlined earlier, it is known that cholesterol in LRs modulates the binding and oligomerization of ‘amyloidogenic proteins’: these are a series of brain proteins with exceptional conformational plasticity and a high propensity for self-aggregation (Fantini and Yahi, 2010). By controlling the balance between unstructured monomers and  $\alpha$  or  $\beta$  conformers (the so-called ‘chaperone effect’). LRs can either inhibit or stimulate the oligomerization of amyloidogenic proteins. Cholesterol has a dual role in this process, because it regulates protein-sphingolipid interactions through a fine tuning of sphingolipid conformation (indirect effect), and/or facilitates pore (or channel) formation through direct binding to amyloidogenic proteins (Fantini and Yahi, 2010). Aberrantly folded proteins are hallmarks of amyloidogenic diseases, for instance AD and PrD that, although clinically different, have the same underlying pathogenetic mechanism, that is, an altered protein conformer with high  $\beta$ -sheet structure content: the amyloid  $\beta$  peptide ( $A\beta$ ) in the case of AD, and the aberrant prion protein,  $PrP^{Sc}$ , in PrD (Pani *et al.*, 2010). Growing evidence indicates the possible involvement of cholesterol in misfolded protein generation during AD and PrD (reviewed by Pani *et al.*, 2010). Furthermore,  $A\beta$  is a product of the amyloid precursor protein (APP) cleavage by secretase, and there is evidence that also the activity of the latter enzyme is modulated by membrane lipid environment (Eckert *et al.*, 2010). Conversely,  $A\beta$  disturbs the properties of artificial and isolated biological membranes, as well as those of plasma membranes in living cells. Thus, LRs may be the site where the neurotoxic cascade of  $A\beta$  is initiated (Eckert *et al.*, 2010). In addition, also glutamate receptors of the N-methyl-D-aspartate (NMDA) subtype show a very complex functional regulation, dependent on neuregulins and receptor tyrosine kinases localized in cholesterol-rich LRs (Schrattenholz and Soskic, 2006). It should be recalled that NMDA receptors are allosteric and ligand-gated calcium channels, with a pivotal role in memory-related signal transduction and other neurotransmission pathways (Chau, 2010). The brain pathologies

**Table 1**

Impact of membrane cholesterol on neurodegenerative diseases

Disease	Effect	Reference
Alzheimer's disease	LRs disruption by membrane cholesterol depletion (through MCD) leads to a lower rate of $\beta$ -amyloid accumulation, that impacts tyrosine kinase Fyn and hence tau phosphorylation. Instead, elevated levels of cholesterol enhance the activity of the amyloid precursor protein (APP)-cleaving enzyme BACE1, and that of $\gamma$ -secretase, also found in LRs.	Simons <i>et al.</i> (1998) Bhaskar <i>et al.</i> (2005) Kalvodova <i>et al.</i> (2005) Grimm <i>et al.</i> (2008)
Parkinson's disease	At least four proteins, which when mutated are associated with PD, reside in LRs: Parkin (an E3 ubiquitin-ligase); $\alpha$ -Synuclein; LRRK2 (leucine-rich repeat kinase 2); PINK1 (PTEN-induced kinase). Mutation of $\alpha$ -synuclein (A30P) leads to a reduced association with LRs.	Fallon <i>et al.</i> (2002) Fortin <i>et al.</i> (2004) Kubo <i>et al.</i> (2005) Silvestri <i>et al.</i> (2005) Hatano <i>et al.</i> (2007)
Huntington's disease	Mutant huntingtin associates with LRs more strongly than the wild-type protein, and inhibits the expression of several genes involved in cholesterol synthesis, vesicle synthesis and trafficking. In addition, mutant huntingtin increases raft-associated glycogen synthase kinase 3- $\beta$ , a marker of apoptotic stress.	Sipione <i>et al.</i> (2002) Valenza and Cattaneo (2006) Valencia <i>et al.</i> (2009) Zuccato <i>et al.</i> (2010)
Amyotrophic lateral sclerosis	LRs disruption by membrane cholesterol depletion (through MCD) protects motor neurons against brain-derived neurotrophic factor (BDNF)-induced excitotoxicity due to activation of TrkB receptors. Consistently, increased BDNF was found in muscles from ALS patients at an early stage of the disease.	Kust <i>et al.</i> (2002) Mojsilovic-Petrovic <i>et al.</i> (2006)
Multiple sclerosis	Lipid peroxidation that occurs in MS may be affected by cholesterol, that regulates membrane fluidity.	Carlson and Rose (2006)
Prion-related disorders	LRs are needed for the conversion of normal cellular prion protein ( $\text{PrP}^C$ ) into its toxic modified form ( $\text{PrP}^S$ ). Consistently, reduced cholesterol synthesis (by squalestatin) protects cells against prion neurotoxicity.	Johnson and Gibbs (1998) Baron <i>et al.</i> (2002) Bate <i>et al.</i> (2004)

where membrane cholesterol plays a critical role, and the main targets of cholesterol action, are summarized in Table 1. This table is restricted to neurodegenerative diseases where also eCBs signalling is known to play a major role, whereas other cholesterol-dependent disease conditions, like the Niemann-Pick diseases, were not covered. In fact, Niemann-Pick diseases encompass a heterogenous group of pathologies where lysosomal lipid storage is compromised, leading to cholesterol (and sphingolipids) accumulation in endosomes and lysosomes of neurons due to erroneous cholesterol trafficking (Vance *et al.*, 2006). Yet, for Niemann-Pick disorders a role for eCBs signalling has not yet been recognized, and only a preliminary study showing that sphingomyelin hydrolysis is stimulated by cannabidiol in fibroblasts from a Niemann-Pick patient has been reported (Burstein *et al.*, 1984).

### Cholesterol and $\text{CB}_1$ signalling in vitro

A role for membrane cholesterol in the functional regulation of  $\text{CB}_1$  has been well documented in neuronal and non-neuronal cells cultured *in vitro* (for an updated review see Dainese *et al.*, 2010). LRs disruption by acute cholesterol depletion with methyl- $\beta$ -cyclodextrin (MCD) has been shown to double  $\text{CB}_1$ -dependent signalling via adenylyl cyclase and mitogen-activated protein kinases in neuronal cells (Bari *et al.*, 2005a,b). Instead  $\text{CB}_2$  receptor, which is struc-

turally and functionally related to  $\text{CB}_1$ , and TRPV1 channel, which is also activated by eCBs like AEA (Di Marzo and De Petrocellis, 2010), are completely insensitive to the modulation of membrane cholesterol content (Bari *et al.*, 2006). Consistently, neither  $\text{CB}_2$  nor TRPV1 reside in cholesterol-rich LRs (Bari *et al.*, 2006; Maccarrone, 2008; Rimmerman *et al.*, 2008). In this context, it seems noteworthy that 2-AG has been shown to be entirely localized in LRs of dorsal root ganglion cells, where also part of AEA (~30%) can be detected (Rimmerman *et al.*, 2008). However, most of AEA (~70%) was found in non-LR fractions, and it remains to be clarified whether these eCBs are produced directly within LRs, or are transported to (or accumulated within) these microdomains. The different interaction of AEA and 2-AG with membrane microdomains might have significant implications for eCBs-dependent autocrine and/or retrograde-paracrine signalling pathways, and further studies are needed to clarify which structural determinants are responsible for a different localization of two apparently similar eCBs within lipid bilayers (Maccarrone, 2008). The effects of LRs disruption by cholesterol depletion on eCBs-binding receptors are summarized in Table 2. Recently, some details of the molecular basis for the different response of these two receptor subtypes to membrane cholesterol have been clarified, by showing that both  $\text{CB}_1$  and  $\text{CB}_2$  have a cholesterol-binding domain (CRAC,

**Table 2**

Effect of LRs disruption by membrane cholesterol depletion on CB<sub>1</sub>R, CB<sub>2</sub>R and TRPV1 binding and signalling

Receptor	Cholesterol depletion		[ <sup>35</sup> S]GTP <sub>γ</sub> S <sup>a</sup>	AC <sup>b</sup>	MAPK <sup>c</sup>
	Binding <i>K<sub>d</sub></i>	<i>B<sub>max</sub></i>			
CB <sub>1</sub> R	↑	↑	↑	↓	↑
CB <sub>2</sub> R	↔	↔	↔	↔	↔
TRPV1	↔	↔	nd	na	na

See text for reference to the original data.

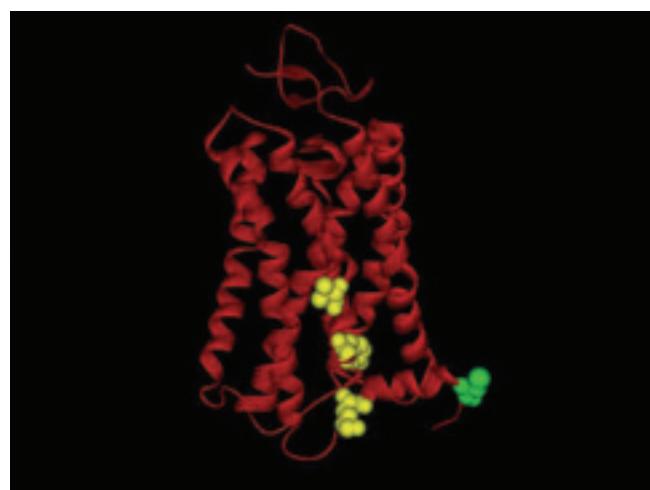
<sup>a</sup>Non-hydrolysable analogue of GTP: it is a measure of the extent of the interaction between receptor and associated G protein.

<sup>b</sup>Adenylyl cyclase.

<sup>c</sup>Mitogen-activated protein kinase.

na, not applicable; nd, not determined.

cholesterol recognition amino acid sequence consensus) in helix 7 (Oddi *et al.*, 2011). In this context, it should be recalled that as yet a unique conserved structural determinant for protein interaction with cholesterol has not been identified, however, CRAC [L/V-X<sub>(1–5)</sub>-Y-X<sub>(1–5)</sub>-R/K] is a well known motif that enables this interaction (Epand, 2006). For instance, CRAC has been demonstrated in caveolin-1, peripheral-type benzodiazepine receptor (Li and Papadopoulos, 1998; Jamin *et al.*, 2005), and in other proteins targeted to LRs (Xie *et al.*, 2010). Interestingly, by sequence alignment of human CB<sub>1</sub> and CB<sub>2</sub> we have demonstrated the presence of a CRAC-like motif in the last 11 amino acids of the transmembrane helix 7 of both CB<sub>1</sub> and CB<sub>2</sub>, yet with small but very important sequence differences between the two receptor subtypes (Oddi *et al.*, 2011). In particular, we found that in the highly conserved 'CRAC-like' region (82% amino acid identity), CB<sub>1</sub> differs from CB<sub>2</sub> for one residue only: lysine 402 of CB<sub>1</sub> corresponds to glycine 304 in CB<sub>2</sub> (Oddi *et al.*, 2011). Therefore, CB<sub>1</sub> has a true CRAC domain, whereas CB<sub>2</sub> has not. In keeping with this observation, we found that the CB<sub>1</sub>(K402G) mutant where the CRAC sequence of CB<sub>1</sub> was converted into that of CB<sub>2</sub> had a reduced propensity to reside in cholesterol-rich membrane regions, and lost its sensitivity to membrane cholesterol enrichment (Oddi *et al.*, 2011). Therefore, these data suggest that one residue in complex proteins like GPCRs can be enough to direct their interaction with membrane lipids, thus affecting their localization within LRs and signal transduction thereof. Additionally, we found that the C-terminal component of CB<sub>1</sub>, that is, the intracellular juxtamembrane helix 8, contains a cysteine residue (C415, in green) that could be constitutively palmitoylated (Dainese *et al.*, 2010). The latter reversible post-translational modification can be used by cells to regulate CB<sub>1</sub> targeting to LRs, thus influencing subsequent G protein-dependent signalling. Remarkably, a palmitoylation site corresponding to C415 is absent in CB<sub>2</sub> (Dainese *et al.*, 2010), and running experiments in our laboratory are aimed at ascertaining whether also this residue might contribute to LR localization of CB<sub>1</sub>. The positions of CRAC domain and C415 in the three-dimensional structure of CB<sub>1</sub> are depicted in Figure 2.

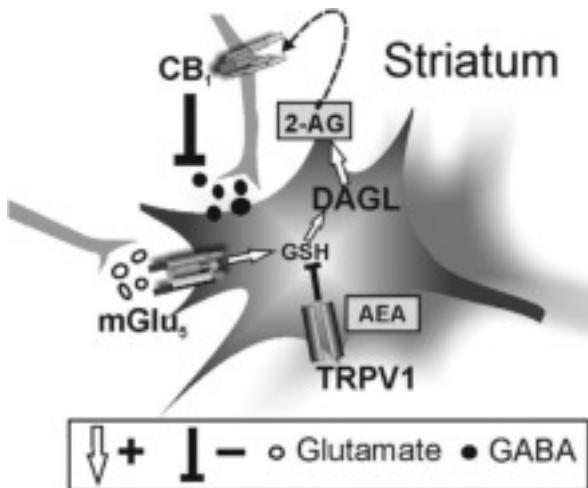
**Figure 2**

Three-dimensional model of type-1 cannabinoid receptor (CB<sub>1</sub>), based on sequence alignment with visual rhodopsin in the inactivated state (PDB code: 1F88). The model was obtained using the protein structure homology-modelling server SWISS-MODEL, integrated in the Deep-View program (Dainese *et al.*, 2010). The three residues (V392, Y397, K402) that form the CRAC sequence are represented as yellow spheres, sized to the Van der Waals radii; these residues belong to the transmembrane helix 7 of CB<sub>1</sub>. Additionally, the C-terminal component of CB<sub>1</sub>, that is, the intracellular juxtamembrane helix 8, contains a cysteine residue (C415, in green) that could be constitutively palmitoylated. The model was kindly provided by Dr. Enrico Dainese (University of Teramo, Italy). See text for further details.

Overall, we believe that the observation that even a single residue in CB receptors can regulate the activity of eCBs signalling might impact on the therapeutic exploitation of CB<sub>1</sub>-dependent versus CB<sub>2</sub>-dependent biological activity of these lipid signals. In the next section, evidence showing that within the striatum cholesterol regulation of eCBs signalling through CB<sub>1</sub> can indeed have major consequences on *in vivo* neurotransmission is presented.

### Impact of membrane cholesterol on CB<sub>1</sub>-dependent neurotransmission within the striatum

The nucleus striatum is a subcortical brain area involved in motor, cognitive and emotional processes (Packard, 2009; Rodriguez-Oroz *et al.*, 2009; Simpson *et al.*, 2010), and is highly enriched in CB<sub>1</sub> receptors controlling both glutamate and GABA transmission, mainly through presynaptic mechanisms (Ferré *et al.*, 2010; Rossi *et al.*, 2010a). Direct pharmacological agonists of CB<sub>1</sub> receptors, in fact, reduce the release of both transmitters, as demonstrated in several studies (Szabo *et al.*, 1998; Gerdeaman and Lovinger, 2001; Huang *et al.*, 2001; Köfalvi *et al.*, 2005; Narushima *et al.*, 2006; MacCarrone *et al.*, 2008). Evidence exists that the two sets of CB<sub>1</sub> receptors differ for the preferential eCB activating them, and also have different regulation mechanisms. CB<sub>1</sub> receptors controlling striatal glutamate synapses [CB<sub>1</sub>R(Glu)], but not those regulating GABA transmission [CB<sub>1</sub>R(GABA)], in fact,



**Figure 3**

Overall scheme of the interactions between *N*-arachidonoylethanolamine (AEA), type-1 transient receptor potential vanilloid (TRPV1), 2-arachidonoylglycerol (2-AG) and metabotropic glutamate 5 (mGlu<sub>5</sub>) receptors in the striatum. Stimulation of mGlu<sub>5</sub> receptors increases 2-AG synthesis by enhancing glutathione (GSH) production. 2-AG acts as retrograde signal to limit GABA release through the stimulation of presynaptic type-1 cannabinoid receptor (CB<sub>1</sub>) receptors. AEA, on the other hand, stimulates TRPV1 channels, presumably located in the somatodendritic region of striatal neurons, and modulates 2-AG metabolism and physiological effects by inhibiting GSH-stimulated diacylglycerol lipase (DAGL) activity (adapted from Maccarrone *et al.*, 2008).

seem to be targeted preferentially by AEoA, because we have found that genetic or pharmacological inhibition of the AEA degrading enzyme fatty acid amide hydrolase (FAAH), that selectively increases AEA levels, inhibits glutamate but not GABA transmission in a CB<sub>1</sub> receptor-dependent manner (Maccarrone *et al.*, 2008; Rossi *et al.*, 2010b). On the other hand, stimulation of endogenous 2-AG synthesis only affects GABA synapses by activating CB<sub>1</sub>R(GABA) (Maccarrone *et al.*, 2008).

Several neurotransmitters engage the eCB system in the striatum, and these include dopamine through D<sub>2</sub> receptors (Centonze *et al.*, 2004; Yin and Lovinger, 2006), neurotensin (Yin *et al.*, 2008), acetylcholine through M<sub>1</sub> receptors (Narushima *et al.*, 2007; Uchigashima *et al.*, 2007; Musella *et al.*, 2010) and glutamate through metabotropic glutamate 5 (mGlu<sub>5</sub>) receptors (Jung *et al.*, 2005; 2007; Maccarrone *et al.*, 2008). Stimulation of mGlu<sub>5</sub> receptor enhances the activity of the 2-AG synthesizing enzyme diacylglycerol lipase (DAGL) (Jung *et al.*, 2005; 2007), and the resulting 2-AG synthesis and physiological activity on CB<sub>1</sub> receptors inhibits GABAergic inhibitory postsynaptic currents (IPSCs) in the striatum (Maccarrone *et al.*, 2008). Of note, elevation of AEA content by pharmacological or genetic inhibition of FAAH regulates the mGlu<sub>5</sub> receptor/2-AG interaction, through the stimulation of TRPV1 channels and the resulting inhibition of glutathione metabolism (Maccarrone *et al.*, 2008) (Figure 3). Evidence exists that 2-AG is produced postsynaptically in striatal neurons in response to mGlu<sub>5</sub> receptor-dependent DAGL stimulation, and that it acts presynaptically on CB<sub>1</sub> receptors

to inhibit GABA release (Katona *et al.*, 2006). Accordingly, an ultrastructural study demonstrated that DAGL and mGlu<sub>5</sub> receptors are tightly associated on the somatodendritic surface of striatal projection neurons, whereas CB<sub>1</sub> receptors are particularly enriched on GABAergic axon terminals of striatal neurons (Uchigashima *et al.*, 2007).

Membrane cholesterol has been recently found to play a substantial role in mGlu<sub>5</sub> receptor/eCB coupling. Depletion of membrane cholesterol with MCD, in fact, failed to alter AEA metabolism in the striatum, because both the AEA synthesizing enzyme *N*-acyl-phosphatidylethanolamine-hydrolysing phospholipase D (NAPE-PLD) and FAAH were unaffected by the treatment. Instead, the activity of DAGL was significantly enhanced in MCD-treated striatal slices, whereas the activity of the 2-AG degrading enzyme monoacylglycerol lipase (MAGL) was not. As a result, 2-AG striatal contents were increased to levels that are able to stimulate CB<sub>1</sub> receptors in control slices (Maccarrone *et al.*, 2009). Surprisingly, however, the potentiated 2-AG synthesis observed after cholesterol depletion from striatal neuron membranes was not associated with increased activity of this eCB on CB<sub>1</sub> receptors, neither in basal conditions nor after the stimulation of mGlu<sub>5</sub> receptors with DHPG. The frequency of spontaneous and miniature GABA-mediated IPSCs was in fact normal in MCD-treated slices, while a frequency reduction of IPSCs normally accompanies the effect of 2-AG on CB<sub>1</sub>R(GABA) (Maccarrone *et al.*, 2008; 2009). Furthermore, blockade of CB<sub>1</sub> receptors did not increase IPSC frequency, again supporting the conclusion that MCD-mediated elevation of 2-AG did not result in enhanced activity of this eCB on CB<sub>1</sub> receptors. We did not observe any CB<sub>1</sub> receptor-mediated effect on GABA-mediated IPSCs even after the exacerbation of 2-AG metabolism with DHPG, an effect that was particularly surprising because both CB<sub>1</sub> receptor binding and activity were increased, and not down-regulated, in MCD-treated striatal slices. Furthermore, the sensitivity of CB<sub>1</sub> receptors was also addressed in physiological experiments, demonstrating that the ability of a synthetic agonist of CB<sub>1</sub> receptors to inhibit IPSCs was intact after cholesterol depletion with MCD (Maccarrone *et al.*, 2009).

MCD failed to affect mGlu<sub>5</sub> receptor binding, also ruling out that the sensitivity of this receptor was altered following cholesterol depletion (Maccarrone *et al.*, 2009). Instead, by means of double immunofluorescence, we demonstrated a relocalization of both CB<sub>1</sub> receptors and mGlu<sub>5</sub> receptors on striatal neurons after MCD treatment, while cellular fluorescence densitometry confirmed a significantly higher CB<sub>1</sub> receptor expression after MCD treatment (Maccarrone *et al.*, 2009). These results are therefore compatible with the idea that lipid rafts not only influence receptor expression and function in membranes but also their spatial orientation. Accordingly, MCD treatment of striatal slices did not alter the general staining of mGlu<sub>1</sub> receptors, which has many pharmacological, signal transduction and physiological homologies with the mGlu<sub>5</sub> receptors but is expressed outside lipid rafts in neuronal membranes (Becher *et al.*, 2001).

Given the localization of CB<sub>1</sub> receptors (Bari *et al.*, 2005b; 2008) and possibly of mGlu<sub>5</sub> receptors (Moffett *et al.*, 2000; Oh and Schnitzer, 2001; Fourgeaud *et al.*, 2003) in lipid rafts, and the substantial role of these cholesterol-enriched membrane subdomains in the limitation of movements of raft-

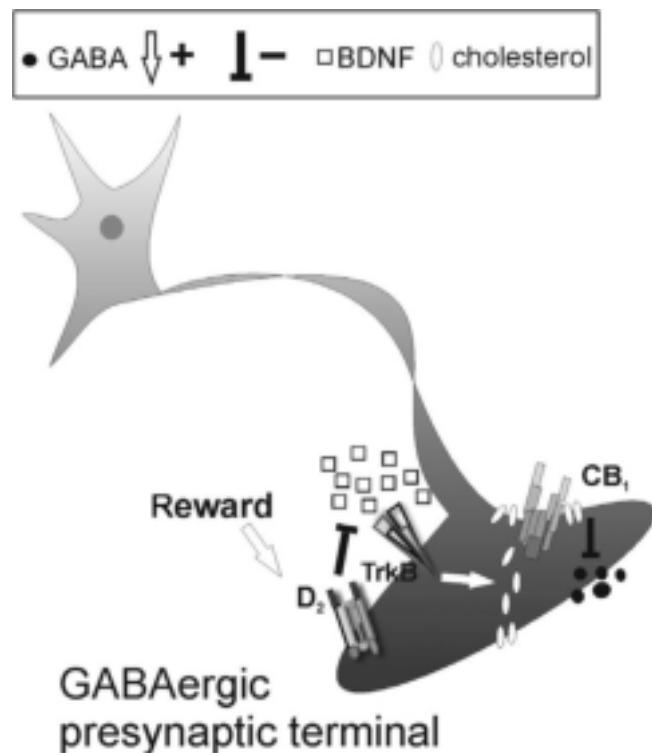
associated proteins (Lucero and Robbins, 2004; Hanzal-Bayer and Hancock, 2007), it is plausible that MCD disrupts 2-AG-mediated mGlu<sub>5</sub> receptor-CB<sub>1</sub> receptor interaction by altering CB<sub>1</sub> receptor and mGlu<sub>5</sub> receptor membrane localization (Maccarrone *et al.*, 2009).

### Involvement of striatal eCB signalling in physiological and pathological contexts

The data above assign to membrane cholesterol in lipid rafts a previously unexpected role in the regulation of synaptic transmission, with potentially very relevant consequences for the understanding of the cellular correlates of several physiological states and of pathological conditions. The pharmacological response of raft-associated striatal CB<sub>1</sub>Rs(GABA) and mGlu<sub>5</sub>R/2-AG coupling is in fact modulated by a variety of conditions, which include cocaine addiction, stress-induced anxiety, voluntary exercise, caffeine and palatable food assumption, as well as in models of neurological diseases such as HD, MS, ALS and Fragile X syndrome (FXS). It follows therefore that the modulation of cholesterol metabolism in membranes might be considered in the near future as a valuable alternative option for the treatment of frequent and severe neuropsychiatric conditions.

The maximal response of striatal CB<sub>1</sub>Rs(GABA) is significantly enhanced by chronic cocaine, when rodents develop overt addictive behaviours, but not by a single administration of the psychostimulant (Centonze *et al.*, 2007a; Rossi *et al.*, 2008; De Chiara *et al.*, 2010a,b). Similar potentiation of CB<sub>1</sub>R(GABA) signalling is seen in mice drinking chronically another psychoactive compound, caffeine (Rossi *et al.*, 2009), or exposed to running wheel or given access to a drinking solution containing sucrose (De Chiara *et al.*, 2010a). Of note, voluntary running activity has strong rewarding and reinforcing properties in rodents, and shares many neurochemical and behavioural characteristics with drug-induced reward situations, through the activation of dopamine signalling and the modulation of striatal neuron activity (Werme *et al.*, 2000; 2002; Lett *et al.*, 2001; De Visser *et al.*, 2007). Similarly, sweet foods and drinks also have intense rewarding properties (Lenoir *et al.*, 2007), and many commonalities exist between overconsumption of sugars and drug addiction, including the stimulation of the dopamine signalling in the striatum (Levine *et al.*, 2003; Kelley, 2004; Volkow and Wise, 2005). Thus, the evidence that chemical (cocaine) and natural rewards (running wheel and sucrose) share the common property of enhancing CB<sub>1</sub>R(GABA) responses in the striatum suggest that this receptor subtype is involved in the modulation of complex dopamine- and reward-based behaviours. Accordingly, blockade of dopamine D<sub>2</sub> receptors prevents the cocaine-, running- and sucrose-induced sensitization of CB<sub>1</sub>R(GABA) receptors (De Chiara *et al.*, 2010a,b).

Because of the ability of rewarding experiences to contrast the behavioural effects of stress, the data presented earlier might indicate that striatal CB<sub>1</sub>R(GABA) are involved in the mood disorders, such as anxiety and depression. Accordingly, we have demonstrated that stress-induced anxious-depressive behaviour is associated with the complete loss of the sensitivity of striatal CB<sub>1</sub>Rs(GABA), and that cocaine, caffeine, running wheel or sucrose are able to contrast these effects (Rossi *et al.*, 2008; 2009; De Chiara *et al.*, 2010a,b).



**Figure 4**

Schematic representation of the interaction between D<sub>2</sub> receptors, brain-derived neurotrophic factor (BDNF) and type-1 cannabinoid receptor (CB<sub>1</sub>) in striatal neurons. Stimulation of dopamine D<sub>2</sub> receptors through chemical (cocaine) and natural rewards (voluntary exercise or sucrose drinking) reduces striatal contents of BDNF, thus reducing cholesterol synthesis and incorporation within CB<sub>1</sub> receptor-containing lipid rafts mediated by the activation of the tyrosine receptor TrkB. This effect results in increased sensitivity of CB<sub>1</sub>Rs(GABA), and is limited to striatal GABAergic neuronal elements. The mechanism by which D<sub>2</sub> receptors reduce BDNF concentrations is conversely still unknown (adapted from De Chiara *et al.* 2010a,b).

Membrane cholesterol controls the pharmacological response of striatal CB<sub>1</sub>Rs(GABA) (Maccarrone *et al.*, 2009), and it can be hypothesized therefore that compounds able to interfere with lipid raft composition could be effective in the management of mood disorders. In agreement with this speculation, recent evidence showed that brain-derived neurotrophic factor (BDNF) levels in the striatum are regulated by dopamine D<sub>2</sub> receptors, and that BDNF is able to block CB<sub>1</sub>R(GABA) activity in the striatum. Importantly, this effect was dependent on increased synthesis and raft concentration of cholesterol mediated by BDNF (De Chiara *et al.*, 2010b) (Figure 4). Importantly, previous evidence showed that striatal infusion of BDNF elicits a depressive behaviour (Eisch *et al.*, 2003), and that the anxious-depressive behaviour induced by social stress is abolished by blockade of BDNF signalling in this brain area (Berton *et al.*, 2006). These observations therefore represent a first indication that raft-associated receptors, such as CB<sub>1</sub>Rs(GABA), are involved in mood disorders and that inhibition of cholesterol synthesis might be useful to treat anxious depressive symptoms.

Raft-associated CB<sub>1</sub>Rs(GABA) in the striatum are also altered in other pathological conditions, and namely neurodegenerative disorders. One of the earliest neurochemical alterations observed in HD patients, in fact, consists in the loss of CB<sub>1</sub> receptor binding in the striatum, an alteration that significantly precedes the development of identifiable striatal neuropathology, and that might play a critical role in the development of HD symptoms (Richfield and Herkenham, 1994; Glass *et al.*, 2000). In addition, down-regulation of CB<sub>1</sub> receptors also occurs in the striatum of transgenic mouse models of HD prior to the development of either neuropsychiatric symptoms or neuronal degeneration (Denovan-Wright and Robertson, 2000; Lastres-Becker *et al.*, 2002), and environmental enrichment, which delays the onset of HD symptoms in transgenic mice, is associated with a delayed loss of CB<sub>1</sub> receptors (Glass *et al.*, 2004). Together, these findings are compatible with the idea that CB<sub>1</sub> receptors are heavily implicated in HD pathophysiology and, in line with this idea, we have found in R6/2 HD mice that the sensitivity of CB<sub>1</sub>Rs(GABA) in the striatum was lost since the early phases of the disease, while the activity of CB<sub>1</sub>Rs(Glu) was intact (Centonze *et al.*, 2005).

Loss of CB<sub>1</sub>R(GABA) responses and preserved CB<sub>1</sub>R(Glu) function is also seen in experimental autoimmune encephalomyelitis (EAE), a model of MS in which the eCB system has been proposed to play a major role (Centonze *et al.*, 2007b; Rossi *et al.*, 2010a). In EAE, in fact, pharmacological stimulation of CB<sub>1</sub> receptors with HU210 fails to inhibit GABAergic IPSCs recorded from striatal neurons, while glutamate synapses are inhibited, as in control conditions, by HU210 (Centonze *et al.*, 2007b).

More complex alterations of the eCB system are observed in the striatum of experimental ALS, in which the maximal response of both CB<sub>1</sub>Rs(GABA) and CB<sub>1</sub>Rs(Glu) is enhanced (Rossi *et al.*, 2010c), and in the mouse model of FXTAS (Maccarrone *et al.*, 2009). In FXTAS mice, altered mGlu<sub>5</sub> receptor signalling has been demonstrated in several studies (Huber *et al.*, 2002; Bear *et al.*, 2004). Recently, we have provided evidence that also mGlu<sub>5</sub> receptor/2-AG coupling was altered in these mice. In the striatum of mice lacking fragile X mental retardation protein (FMRP), we found in fact enhanced activity of DAGL, associated with altered sensitivity of GABA synapses to the mobilization of 2-AG by mGlu<sub>5</sub> receptor stimulation (Maccarrone *et al.*, 2010b). Our data therefore indicate for the first time that mGlu<sub>5</sub> receptor-driven eCB signalling in the striatum is under the control of FMRP, and that abnormal mGlu<sub>5</sub> receptor/2-AG coupling might play a role in the synaptic defects of FXTAS.

## Conclusions

Modulation of CB<sub>1</sub> receptor activity is receiving increasing attention as a novel, promising strategy in the treatment of neuropsychiatric and neurodegenerative disorders. So far, however, *Cannabis sativa* extracts to activate these receptors (Howlett *et al.*, 2010; Pertwee, 2010), or synthetic antagonists to block them have been of limited clinical utility, because severe psychiatric symptoms have been associated with their use (Hill and Gorzalka, 2009). An alternative option intensely explored is the modulation of eCB activity at these receptors,

through the regulation of AEA or 2-AG metabolism (Petrosino and Di Marzo, 2010; Rossi *et al.*, 2010b). It is still fully unexplored whether the modulation of cholesterol/CB<sub>1</sub> receptor interaction could be a useful approach to modulate eCB signalling in the brain.

Here, we would like to comment that subtle, yet specific, differences might underpin the differential sensitivity of CB<sub>1</sub> and CB<sub>2</sub> to membrane cholesterol, possibly explaining the apparent redundancy of having two largely overlapping receptor subtypes that are activated by similar compounds (eCBs) and trigger similar transduction pathways (Di Marzo, 2009; Maccarrone *et al.*, 2010a).

In general, cholesterol may act on the conformation of a membrane receptor by indirectly altering the physicochemical properties of the bilayer, or by directly interacting with the receptor itself, for example, through the CRAC domain and/or palmitoylation sites. More generally, we believe that the comparison between CB<sub>1</sub> and CB<sub>2</sub> might represent an interesting paradigm that goes well beyond eCB signalling. In fact, the modulation of CB<sub>1</sub> by membrane cholesterol might disclose a novel ligand-receptor interaction, where a third player comes into the game: the membrane lipids. As a consequence, the membrane environment might play a role in receptor-dependent signalling, with a potential impact on several neurotransmission pathways, as well as several neurodegenerative/neuroinflammatory diseases where CB<sub>1</sub> is known to play a role. It should be recalled that CB<sub>1</sub>-dependent signalling impacts fundamental processes as different as immune response, energy homeostasis, reproduction and skin differentiation (Di Marzo, 2009; Maccarrone *et al.*, 2010a), thus it can be anticipated that cholesterol-dependent regulation of CB<sub>1</sub> can have a physiological relevance well beyond the CNS.

In conclusion, membrane environment seems to be critical for the regulation of signal transduction pathways triggered by G protein-coupled receptors like CB<sub>1</sub>. Despite the three dimensional complexity of these proteins, we learn from the comparison of CB<sub>1</sub> with CB<sub>2</sub> that just one amino acid residue can direct receptor functioning, calling for attention on the plasma membrane as a key-player in ligand recognition on the cell surface. Further studies aimed at clarifying the impact of these notions for the treatment of disorders associated with abnormal activity of CB<sub>1</sub> are desirable to come in the near future.

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## Conflict of interest

The authors have no conflict of interest to declare.

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