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REVIEW

Endocannabinoid tone versus constitutive activity of cannabinoid receptors

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This review evaluates the cellular mechanisms of constitutive activity of the cannabinoid (CB) receptors, its reversal by inverse agonists, and discusses the pitfalls and problems in the interpretation of the research data. The notion is presented that endogenously produced anandamide (AEA) and 2-arachidonoylglycerol (2-AG) serve as autocrine or paracrine stimulators of the CB receptors, giving the appearance of constitutive activity. It is proposed that one cannot interpret inverse agonist studies without inference to the receptors' environment vis-à-vis the endocannabinoid agonists which themselves are highly lipophilic compounds with a preference for membranes. The endocannabinoid tone is governed by a combination of synthetic pathways and inactivation involving transport and degradation. The synthesis and degradation of 2-AG is well characterized, and 2-AG has been strongly implicated in retrograde signalling in neurons. Data implicating endocannabinoids in paracrine regulation have been described. Endocannabinoid ligands can traverse the cell's interior and potentially be stored on fatty acid-binding proteins (FABPs). Molecular modelling predicts that the endocannabinoids derived from membrane phospholipids can laterally diffuse to enter the CB receptor from the lipid bilayer. Considering that endocannabinoid signalling to CB receptors is a much more likely scenario than is receptor activation in the absence of agonist ligands, researchers are advised to refrain from assuming constitutive activity except for experimental models known to be devoid of endocannabinoid ligands.

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; ABHD4,6 or 12, α/β hydrolase domain 4 (6 or 12); AEA, anandamide or *N*-arachidonylethanolamide; BRET, bioluminescence resonance energy transfer; CHO, Chinese hamster ovary cells; DAGL, diacylglycerol lipase; DSI or DSE, depolarization-induced suppression of inhibition or excitation; EPSP or IPSP, excitatory or inhibitory post-synaptic potential; ER, endoplasmic reticulum; FAAH, fatty acid amide hydrolase; FABP, fatty acid-binding protein; GPCR, G protein-coupled receptor; GP-NAE, glycerophospho-*N*-acylethanolamine; HEK293, human embryonic kidney cells clone 293; HFS, high-frequency stimulation; HSP, heat shock protein; IL3, intracellular loop 3; LPS, lipopolysaccharide; LTP, long-term potentiation; MAGL, monoacylglycerol lipase; MAPK, mitogen-activated protein kinase; NAE, *N*-acylethanolamine; NAPE, *N*-acyl phosphatidylethanolamine; NArPE, *N*-arachidonyl phosphatidylethanolamine NAT, *N*-acyl transferase; NMDA, *N*-methyl-D-aspartate; OEA, *N*-oleoylethanolamine; PEA, *N*-palmitoylethanolamine; PLC, phospholipase C; PLD, phospholipase D; POPC, palmitoyl, oleoyl-phosphatidylcholine; PTP, protein tyrosine phosphatase; TMH, transmembrane helix

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The endocannabinoid system

The endocannabinoid system in the body is comprised of the CB_1 and CB_2 receptors, the lipid mediators known as endocannabinoids [*N*-arachidonylethanolamine (AEA) and 2-arachidonoylglycerol (2-AG)] that serve as *orthosteric* agonists in their regulation, and the enzymes that produce and degrade the endocannabinoids. Recent reviews summarize the involvement of the endocannabinoid system in normal physiological and pathophysiological conditions (Banni and Di Marzo, 2010; Hill and McEwen, 2010; Izzo and Sharkey, 2010; Labar *et al*., 2010; Parolaro *et al*. 2010; Purohit *et al*., 2010). Our current understanding of the role of endocannabinoids in signalling to neighbouring cells comes from the study of synaptic retrograde signalling of the endocannabinoid system in the brain as summarized by Katona and Freund (2008). The activation of pre-synaptic CB_1 receptors by post-synaptic 2-AG results in a well-described feedback inhibition of neurotransmitter release via inhibition of voltage-activated Ca^{2+} channels and the enhancement of inwardly rectifying K^+ channels (Chevaleyre *et al*., 2006; Lovinger, 2008).

Considerable attention in recent years has been given to inverse agonist ligands that reduce the basal signal transduction responses of CB_1 receptors, studied most frequently in heterologous expressions systems like the human embryonic kidney (HEK293) cell (for review, see Reggio, 2003; Pertwee, 2005). Inverse agonists for the CB_2 receptor also have been characterized (Bouaboula *et al*., 1999; Cascio *et al*., 2010), although less research has been devoted to this receptor. Extrapolation beyond the cellular level of signal transduction to more complex responses in multicellular systems or even intact animal models can result in misinterpretation of pharmacological results. The present discussion evaluates the cellular mechanisms of constitutive activity of the CB_1 receptor and its reversal by inverse agonists using rimonabant as the prototype, and discusses pitfalls and problems in the interpretation of research data. The notion is presented that endogenously produced AEA or 2-AG can provide autocrine or paracrine stimulation of CB_1 receptors, giving the appearance of constitutive activity.

Definitions: agonist activation, constitutive activation, competitive and allosteric antagonism, and inverse agonism

Agonists stimulate G protein-coupled receptors (GPCRs) by hydrophobic or electrostatic interactions with multiple amino acid targets within their 'orthosteric' binding site, which initiates a series of microconformational changes in the receptor structure that ultimately leads to Gα activation by changes in the third intracellular loop (IL3) or the juxtamembrane C-terminal helix eight (H8). For the CB_1 receptor, recent reviews speculate on mechanisms of receptor activation and provide original references (Howlett, 2009; Howlett *et al*., 2009; Reggio, 2010). In experimental situations, the agonist is supplied exogenously, and the efficacy of that agonist is determined as the activation above basal of a signal transduction response, which, for the CB_1 receptor, includes stimulation of Gi/o protein activation, inhibition of adenylyl cyclase, activation of mitogen-activated protein kinase (MAPK) or Gβγ-mediated regulation of an ion channel. Competitive antagonists block the activities of GPCR agonists, as they compete for agonist binding sites, but fail to promote the conformational stimulus necessary to activate the associated G protein (i.e. they have no intrinsic efficacy to stimulate a response).

Constitutive activity of a GPCR is defined as the ability of the receptor to signal a response in the absence of agonist stimulation. For many cellular signals, the 'basal' activity may be the result of other, unrelated receptors that are stimulated by their endogenously produced agonists in the cell or tissue as a function of autocrine or paracrine regulation. For this reason, the means by which the constitutive activity of a GPCR can be distinguished above 'basal' noise from other receptor systems is by the ability of an 'inverse agonist' to reduce the activity below unstimulated levels.

Allosteric or non-competitive antagonists block activation of G proteins by binding to an 'allosteric' site on the receptor that is involved in the G protein activation process, thereby precluding the ability of the orthosteric agonist-mediated conformational stimulus to execute activation. Allosteric regulation of the CB_1 receptor has been recently reviewed (Ross, 2007a,b). Inverse agonists block the constitutive activation of G proteins in the absence of an agonist, presumably by binding to a site that ultimately blocks the ability to execute activation of G proteins. One can see that the possibility for overlap in functional definitions is inevitable. For example, the function of an inverse agonist may be comparable to that of the 'allosteric' site, with the only difference being whether or not an orthosteric agonist is actively promoting the stimulation of the G protein.

Constitutive activity of the CB_1 receptor and its reversal by inverse agonists

Rimonabant (also known as SR141716) and diarylpyrazole analogs act at the CB_1 receptor as competitive antagonists against agonists that are added exogenously, as well as endocannabinoids that are released endogenously. The present discussion will address the role of rimonabant as an inverse agonist to block constitutive activity of the CB_1 receptor. CB_1 receptor constitutive activity was first described by comparing 'basal' G protein activation and G protein-regulated signal transduction in cells expressing recombinant CB_1 receptors compared with CB_1-deficient host cells (Bouaboula *et al*., 1997; Pan *et al*., 1998; Vasquez and Lewis, 1999). Low concentrations of rimonabant (30 nM) reversed the increase in basal [^{35}S]GTPγS binding, the increase in MAPK activation, and the decrease in adenylyl cyclase activity that were promoted by expressing recombinant CB_1 receptors in CHO cells (Bouaboula *et al*., 1997; Landsman *et al*., 1997; MacLennan *et al*., 1998). Determination of constitutive activity in cells that endogenously express CB_1 receptors is based upon reduction of the basal response with rimonabant

or another inverse agonist. High concentrations of rimonabant reversed basal [^{35}S]GTPγS binding in rat brain membranes (Sim-Selley *et al.*, 2001) and neuronal cells endogenously expressing CB_1 receptors (Pan *et al.* 1998; Meschler *et al.*, 2000; Sim-Selley *et al.*, 2001). To explain the reduced basal responses by rimonabant, Bouaboula *et al.* (1997) proposed that the inverse agonist would induce an 'inactive' receptor conformational state, thereby preventing the spontaneous conversion to the activated state. This was the first suggestion that a class of inverse agonists could exert effects on the CB_1 receptor to promote a state of inactivity.

Inverse agonist regulation of the G protein cycle

As previously suggested (Sim-Selley *et al.*, 2001), assay of inverse agonist actions at the G protein level has some advantages compared to assay of signal transduction systems, including: (i) receptor-coupled G protein activity would be a direct measure of inverse agonists uncontaminated by regulatory processes that affect signal transduction systems; and (ii) assay of decreased basal G protein activity in different brain regions produced by inverse agonists might provide an effective measure of regional differences in constitutive CB_1 receptor activity.

Agonist-stimulated [^{35}S]GTPγS binding is a common measure of receptor-coupled G protein activation, and stimulation of [^{35}S]GTPγS binding by cannabinoid agonists is well established in both membranes (Selley *et al.* 1996; Breivogel *et al.*, 1998) and brain sections (Sim *et al.* 1996; Breivogel *et al.*, 1999). In the traditional agonist-dependent G protein activation cycle (Figure 1A), the classical agonist (Ag) can be regarded as a catalyst that accelerates the dissociation of the inactive receptor–G protein heterotrimer complex (*R-αβγ) into the free G protein subunits α + βγ, along with the low-affinity form of the receptor (*R*). The effector activation process occurs as Gα switches from a high affinity for GDP into a high affinity for GTP: the activated form of Gα (*α-GTP) is one of the heterotrimer components that produce effector activation. In the *in vitro* assay of [^{35}S]GTPγS binding, GTP is replaced by [^{35}S]GTPγS, and activation of G proteins by agonists is measured by a stimulation of [^{35}S]GTPγS binding. An inverse agonist (Inv Ag, Figure 1B) would produce the opposite effect of the agonist by stabilizing the inactive receptor–G protein heterotrimer, increasing the affinity of Gα for GDP and decreasing the affinity of Gα for GTP. In this version of the cycle, G protein activation occurs via constitutive activity of the receptor in the absence of agonist. Binding of the inverse agonist would reverse constitutive activity, while dissociation of the inverse agonist would allow constitutive activity to proceed in the absence of agonist. The result of inverse agonist effects would be a decrease in basal [^{35}S]GTPγS binding, and a reduction in the efficacy of agonist-stimulated [^{35}S]GTPγS binding. The actions of an inverse agonist at the level of receptor–G protein activation could be distinguished from the actions of a traditional competitive (or neutral) antagonist by the fact that the latter compound would have no effect on basal [^{35}S]GTPγS binding, and would decrease agonist potencies (i.e. increase agonist EC_{50} values)

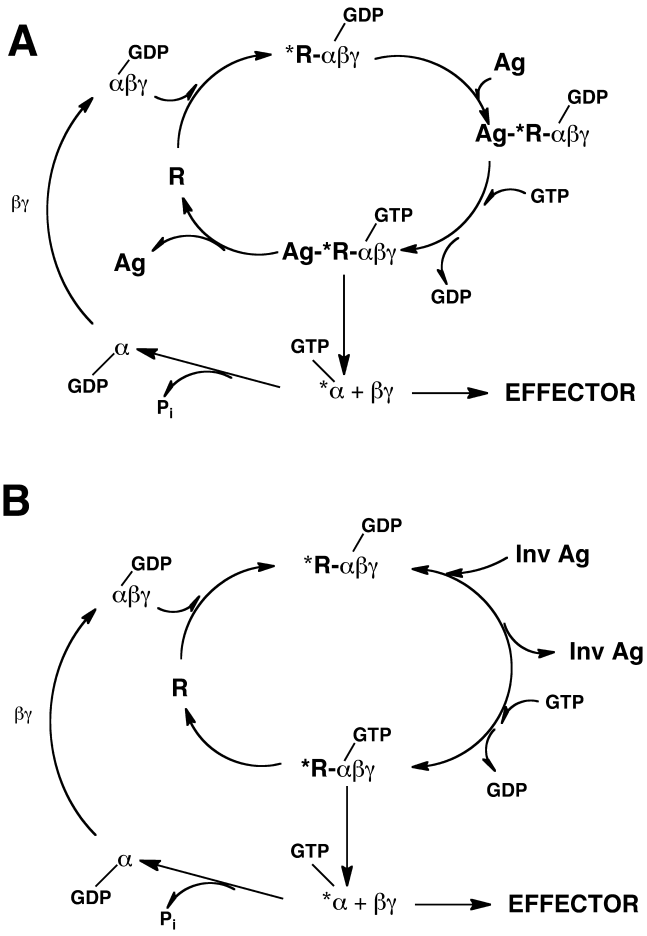

Figure 1

The G protein activation cycle, showing the opposite effects of a traditional agonist (Ag) and an inverse agonist (Inv Ag) on G protein activation and de-activation. (A) Agonist-dependent activation, by which the agonist serves as a catalyst that promotes the dissociation of receptor and G protein heterotrimer (high affinity for GDP) into the active components (Gα has a high affinity for GTP). (B) Constitutive activation, in which G protein activation occurs in the absence of agonist. In this scenario, an inverse agonist can stabilize the inactive receptor–G protein complex, further increasing its affinity for GDP and decreasing affinity for GTP, and prevents constitutive activation of G proteins.

with no effect on agonist efficacies. Moreover, a neutral antagonist may competitively block the actions of an inverse agonist by increasing the EC_{50} values of the inverse agonist, just as it does for traditional agonists.

The relative activity of inverse agonists is determined by the amount of constitutive receptors present. In *in vitro* studies, constitutive receptor activity can be increased by altering the concentrations of Na^+ and GDP (Sim-Selley *et al.*, 2001). These are some of the pharmacological criteria that should be used in the identification of novel CB_1 inverse agonists. On the other hand, the simple finding that a compound inhibits basal [^{35}S]GTPγS binding is not sufficient to establish the identity of an inverse agonist. This is especially problematic when high concentrations of cannabinoid compounds are used in *in vitro* assays. These are highly lipophilic

compounds, and when used at high micromolar concentrations, they may inhibit [^{35}S]GTPγS binding in non-specific ways.

In terms of an unambiguous identification of inverse agonist versus neutral antagonist actions of compounds at CB_1 receptors, most studies have utilized cell lines transfected with CB_1 receptors, in which the signal of [^{35}S]GTPγS binding is not complicated by the presence of other GPCRs, and the amount of CB_1 receptor protein is very high compared to native neuronal tissues. Early studies with rimonabant showed a significant decrease in basal [^{35}S]GTPγS binding in CB_1 receptor-transfected cell lines (Bouaboula *et al.*, 1995; Landsman *et al.*, 1997; MacLennan *et al.*, 1998). Rimonabant was potent as an inverse agonist in CB_1 receptor-transfected cells, with EC_{50} values between 1 and 10 nM, similar to its potency as a competitive antagonist. More recent studies have utilized inhibition of basal [^{35}S]GTPγS binding in transfected cells to screen for novel CB_1 inverse agonists (Thomas *et al.*, 2005; Wustrow *et al.*, 2008).

Evidence for agonist versus inverse agonist selectivity as a function of the Gi subtype

Stabilization of CB_1 receptor–G protein complexes by inverse agonist-bound CB_1 receptors was demonstrated in CHAPS extracts from N18TG2 cell membranes and brain (Mukhopadhyay and Howlett, 2005). In those studies, GTPγS promoted dissociation of Gαi proteins from CB_1 receptors, which was attenuated in the presence of rimonabant (Mukhopadhyay and Howlett, 2005), implicating an inverse agonist-induced CB_1 receptor conformation that precludes G protein dissociation. Similarly, desacetyllevonantradol behaved as an inverse agonist for CB_1-Gαi3, and (*R*)-methAEA behaved as an inverse agonist for CB_1-Gαi1/2 complexes. These findings suggest that these CB_1 ligands could serve as agonists to stimulate certain G proteins, but inverse agonists to block activation of others. It would appear that the inverse agonist binding could stabilize the receptor–G protein heterotrimer, thereby attenuating the potential for GTPγS to compete for GDP at the nucleotide binding site.

CB_1 inverse agonists affect other GPCR responses

Although putative CB_1 receptor inverse agonists decrease basal [^{35}S]GTPγS binding in brain membranes (Meschler *et al.*, 2000), studies of these effects are more difficult to interpret in brain compared to cell lines. One study (Sim-Selley *et al.*, 2001) has analysed the pharmacological properties of rimonabant on CB_1 receptor activation of G proteins in both brain membranes and sections, and found that although the competitive antagonist effects of rimonabant were extremely potent (EC_{50} value of 0.6 nM), the inverse agonist effect of rimonabant to inhibit basal [^{35}S]GTPγS binding was over a thousand times weaker (EC_{50} of 4 μM). It was concluded that either the apparent inverse agonist effects of rimonabant are not specific to CB_1 receptors, or that rimonabant was binding to different sites on the CB_1 receptor to produce inverse agonist and competitive antagonist effects. Support for the former conclusion was provided by the finding that rimonabant inhibited basal [^{35}S]GTPγS binding in brain membranes from CB_1 knock-out mice (Breivogel *et al.*, 2001). Similar differences in potencies of novel CB_1 antagonists as neutral antagonists versus inverse agonists have also been reported in more recent studies (Thomas *et al.*, 2005; Zhang *et al.*, 2008). Moreover, rimonabant displays a similarly low potency in producing inverse agonist effects on cAMP production in brain membranes (Mato *et al.*, 2002). In contrast, the novel nonapeptide hemopressin, which displays CB_1 receptor inverse agonist effects on adenylyl cyclase, was reported to inhibit [^{35}S]GTPγS binding in striatal membranes with a high potency, similar to its potency in blocking cannabinoid agonist effects (Heimann *et al.*, 2007). Finally, it is unlikely that inverse agonist activity at CB_2 receptors can be detected in brain, because the CB_2 inverse agonist SR144528 (Portier *et al.*, 1999) has no effect on [^{35}S]GTPγS binding in brain (Sim-Selley *et al.*, 2001).

Inverse agonism at the level of G protein stimulation has been used to determine the effects of constitutive CB_1 receptor activity on the functions of other GPCRs in cultured cells. In one study, the expression of exogenous CB_1 receptors in modified HEK293 cells reduced the activity of μ-opioid receptors (Canals and Milligan, 2008). The addition of rimonabant produced an inverse agonist effect at those exogenous CB_1 receptors, and thereby increased the apparent efficacy of a full agonist to stimulate G proteins through μ-opioid receptors (Canals and Milligan, 2008). Another study (Cinar and Szucs, 2009) showed that rimonabant had actions on μ-opioid receptors independent of its actions on CB_1 receptors. However, the high concentrations of rimonabant used in this study make it likely that non-specific effects were observed on basal [^{35}S]GTPγS binding with this highly lipophilic compound.

Endocannabinoids as autocrine or paracrine signals

Recent studies are providing greater evidence that endocannabinoids serve an autocrine or paracrine function to regulate the cannabinoid receptors. The production and removal of these endogenous agonists must be considered as intrinsic to the local responses directed by both CB_1 and CB_2 cannabinoid receptors. In many studies of cannabinoid receptor inverse agonist effects, the capability of local production of endocannabinoids was not investigated or even mentioned as a consideration. This is partly because of the paucity of selective inhibitors of synthetic and degradative enzymes for endocannabinoids, coupled to the complexities of determining endogenous levels of these and related lipid ligands. One cannot interpret inverse agonist studies without reference to the receptor's environment *vis-à-vis* the endocannabinoid agonists. The endocannabinoid levels are the results of a combination of synthetic pathways, storage and inactivation involving transport and degradation.

Regulation of the synthesis of endocannabinoids

2-AG Synthesis

2-AG is synthesized on demand from lipid in a two-step process in which phospholipase C-β hydrolyses phosphatidylinositol-4,5-bisphosphate to generate diacylglycerol, which is then hydrolysed by diacylglycerol lipase (DAGL-α) to yield 2-AG (Figure 2A, reaction 1) (Piomelli, 2003; Di Marzo, 2008). The biosynthetic enzymes for 2-AG are localized on post-synaptic neurons in dendritic spines and somatodendritic compartments (Katona, 2008). Released 2-AG controls the activity of the complementary pre-synaptic neuron, by binding to CB_1 receptors which are often expressed there. It is still unclear for retrograde signalling how newly synthesized 2-AG is induced to leave the post-synaptic cell plasma membrane to interact with the CB_1 receptor pre-synaptically. 2-AG may be secreted by simple diffusion; alternatively, passive (energy-independent) carrier proteins may be required to extrude 2-AG.

AEA synthesis

Early studies found that *N*-acylethanolamine (NAE) biosynthesis occurred by a two-step process by which: (i) a Ca^{2+}-dependent *N*-acyl transferase (NAT) transfers an sn-1 acyl chain from membrane phospholipids to the primary amine position of phosphoethanolamine resulting in a *N*-acyl phosphatidylethanolamine (NAPE); then (ii) a type-D phospholipase (PLD) hydrolyses NAPEs to NAEs [see review for original references (Schmid, 2000)]. The Ca^{2+}-dependent NAT has yet

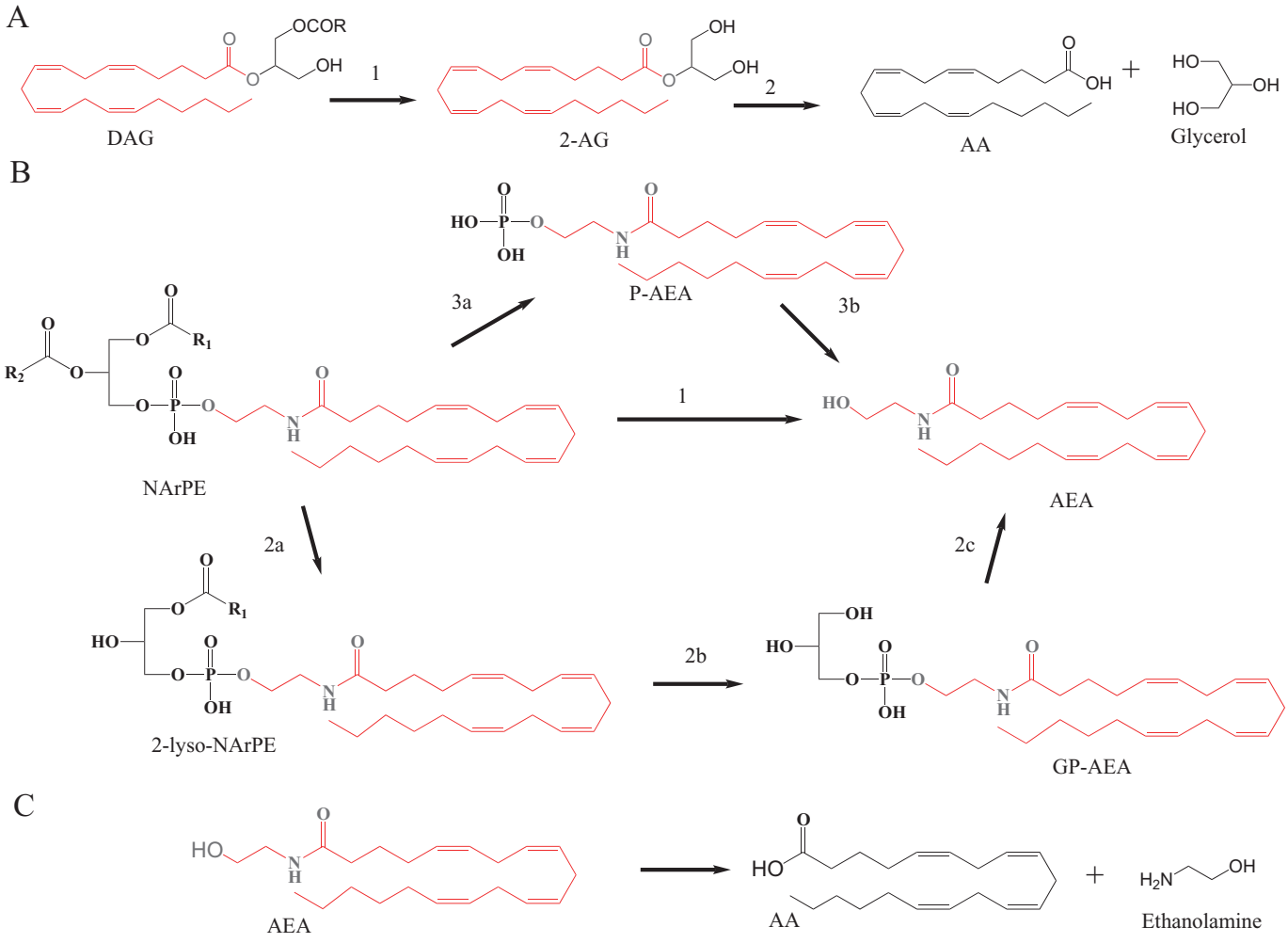

Figure 2

Pathways for 2-AG metabolism (2A), putative pathways for AEA synthesis (2B) and AEA breakdown (2C). (A) 2-AG is formed in step 1 by the action of DAG lipase upon DAG and 2-AG is metabolized in step 2 by MAG lipase, ABHD6 and ABHD12 to arachidonic acid and glycerol. (B) The metallo-β lactamase, NAPE–PLD, hydrolyses NArPE to form AEA via a one-step reaction (pathway 1). The serine hydrolase, ABHD4, sequentially removes acyl groups from NArPE to form lyso-NArPE and then GP-AEA (pathway 2a–b). The metal-dependant phosphodiesterase, GDE1, hydrolyses GP–AEA to form AEA (pathway 2c). A type-C phospholipase hydrolyses NArPE to pAEA (pathway 3a). PTPN22, SHIP1 or other uncharacterized phosphatases dephosphorylate pAEA to form AEA (pathway 3b). (C) Degradation of AEA to AA and ethanolamine by FAAH. The arachidonic acid moiety is highlighted in red.

to be cloned. A NAPE-specific phospholipase D (NAPE–PLD), a metallo-β lactamase sensitive to the sulfhydryl reagent *p*-chloromercuribenzoic acid, was cloned and shown to hydrolyse *N*-arachidonyl phosphatidylethanolamine (NArPE) to AEA *in vitro* (Figure 2B, pathway 1) (Okamoto *et al.*, 2004; Morishita *et al.*, 2005). Interestingly, knock-out of NAPE–PLD did not affect AEA levels in the mouse brain, but significantly decreased levels of congener saturated and monounsaturated NAEs such as *N*-palmitoylethanolamine (PEA, C16:0) and *N*-oleoylethanolamine (OEA, C18:1) (Leung *et al.*, 2006). It was proposed that perhaps NAPE–PLD primarily functioned to hydrolyse saturated and monounsaturated NAPEs, whereas a second enzyme was responsible for hydrolyzing polyunsaturated NAPEs such as NArPE. Indeed, subsequent studies revealed an α/β serine hydrolase, α/β hydrolase domain 4 (ABHD4), that possessed a Ca^{2+}-independent, NAPE-hydrolyzing activity (Simon and Cravatt, 2006). This enzyme would function *in vivo* to sequentially remove acyl groups from NArPE converting it first to lyso-NAPE and then glycerophospho-AEA (GP–AEA) (Figure 2B, pathway 2a–2b). The enzyme that then converts GP–AEA to AEA, a metal-dependent phosphodiesterase called GDE1, has also been characterized (Figure 2B, pathway 2c) (Simon and Cravatt, 2008). Brain homogenates pre-incubated with the serine hydrolase inhibitor methyl arachidonyl fluorophosphonate had decreased levels of lyso-NAPE. When treated with EDTA there were increased levels of endogenous GP–AEA compared to untreated controls, as well as a decrease in AEA synthesized from exogenously added NArPe (Liu *et al.*, 2008; Simon and Cravatt, 2008, 2010). *In vitro* studies showed that NAPE-to-NAE conversion in brain homogenates of mice lacking both NAPE–PLD and GDE1 genes is absent (Simon and Cravatt, 2010). However, these mice display no change in their total brain NAE levels when compared to wild-type mice. Neurons isolated from these double knock-out mice also retain their ability to convert NAPE to NAE (Simon and Cravatt, 2010). A third pathway for AEA synthesis was recently described where lipopolysaccharide (LPS)-induced synthesis of AEA in macrophages proceeds through a C-type phospholipase (PLC)/phosphatase pathway whereby NArPE is converted to phospho-AEA (pAEA) by a phospholipase C and then dephosphorylated to AEA (Figure 2B, pathway 3a–3b) (Liu *et al.*, 2006). No candidate PLC has been cloned, but a tyrosine phosphatase, PTPN22, and an inositol 5′ phosphatase, SHIP1, were found to dephosphorylate pAEA *in vitro*. Additionally, incubation of pAEA with brain homogenates from PTPN22 and SHIP1 knock-out mice reduced its conversion to AEA. The siRNA knockdown of PTPN22 enzymes also reduced the LPS-induced synthesis of endogenous AEA in macrophages (Liu *et al.*, 2008). Pre-incubation with the PLC inhibitor, neomycin, and the tyrosine phosphatase inhibitor, sodium metavanadate, reduced AEA synthesized post-LPS treatment. This indicates a role for the PLC/phosphatase pathway in this cell type. Although pAEA has been detected in the mouse brain, its dephosphorylation probably does not occur via PTPN22 as it is not highly expressed. There was no significant difference between endogenous AEA levels in PTPN22(–/–) and wild-type mice (Liu *et al.*, 2008). The expression pattern of SHIP1 in the brain has not been determined. However, it has been conjectured that this enzyme may be expressed in microglia (Liu *et al.*, 2008).

The NAPE–PLD pathway (pathway 1) is the only NAE synthetic pathway for which brain localization has been described (Egertova *et al.*, 2008; Nyilas *et al.* 2008). NAPE–PLD was most prominently expressed in the axons of granule cells (mossy fibers) of the dentate gyrus of the hippocampus. Although NAPE–PLD expression was detected in other brain regions (e.g. cortex, thalamus), the intensity of immunostaining was weaker than in mossy fibers (Egertova *et al.*, 2008). It was concluded that NAPE–PLD is targeted to axonal processes, and that NAEs generated by NAPE–PLD in axons may act as anterograde synaptic signalling molecules that regulate the activity of post-synaptic neurons (Egertova *et al.*, 2008). Nyilas *et al.* (2008) showed that NAPE–PLD is concentrated pre-synaptically in several types of hippocampal excitatory axon terminals and is associated with intracellular Ca^{2+} stores. These researchers concluded that the production of AEA of pre-synaptic origin may reflect the status of axon terminal [Ca^{2+}], in part following release from intracellular stores (Nyilas *et al.*, 2008). In cultured dorsal root ganglion cells, localization of AEA, 2-AG and their synthetic enzymes was observed in lipid raft domains, suggesting the scenario of intrinsic autocrine signalling (Rimmerman *et al.*, 2008). However, until the two enzymes for pathway 2 are immunolocalized, it is premature to speculate regarding AEA's site of synthesis and mode of action as a neuromodulator, although evidence exists for AEA localization to caveolin-rich membrane as recently reviewed (Placzek *et al.*, 2008). Although early reports implicated both AEA and 2-AG in retrograde signalling at the CB_1 receptor, recent evidence tends to favor 2-AG, based upon localization of synthetic and degrading enzymes and electrophysiological studies (Straiker *et al.*, 2009; Urbanski *et al.*, 2009).

Metabolic breakdown of endocannabinoids

2-AG metabolism

Degradation of 2-AG (Figure 2A, reaction 2) is accomplished principally by a membrane-associated, cytoplasm-facing soluble enzyme, monoacylglycerol lipase (MAGL) that appears pre-synaptically at axon terminals along with the CB_1 receptor targets for 2-AG (Dinh *et al.* 2002; Ghafouri *et al.*, 2004; Gulyas *et al.* 2004; Hohmann, 2007; Bisogno *et al.*, 2009; King *et al.* 2009; Long *et al.* 2009a,b; Pan *et al.*, 2009; Petrosino and Di Marzo, 2010). Approximately 15% of 2-AG in the mouse brain is hydrolysed by two novel enzymes, ABHD6 (4%) and ABHD12 (9%), with 1–2% of the 2-AG metabolism due to fatty acid amide hydrolase (FAAH) (Blankman *et al.*, 2007). Interestingly, both ABHD6 and ABHD12 are integral membrane proteins, but ABHD6 faces the cytosol and appears in the mitochondrial fraction, whereas ABHD12 faces the extracellular or intraluminal surface (Blankman *et al.*, 2007; Marrs *et al.* 2010). ABHD6 is localized post-synaptically on dendritic spines in neurons at sites of 2-AG production, and does not colocalize with axonal CB_1 receptors (Marrs *et al.*, 2010). It was suggested that the multiple enzymes for elimination might be directed at different pools of 2-AG (Blankman *et al.*, 2007). One distinct pool might be cytosolic versus synaptic, interstitial fluid or exogenously applied 2-AG (Marrs *et al.*, 2010).

AEA metabolism

Shortly after the discovery that AEA is an endogenous ligand for the CB_1 receptor (Devane *et al.*, 1992), it was shown that AEA was taken up into cells and readily broken down by an enzyme originally called AEA amidase (Deutsch and Chin, 1993), now referred to as FAAH (Cravatt *et al.*, 1996; McKinney and Cravatt, 2005). The AEA breakdown products were shown to be arachidonic acid and ethanolamine (Figure 2C). FAAH was shown to be present in the membrane fractions of the brain and other organs except muscle, as well as in a variety of cells (Deutsch and Chin, 1993). In basolateral amygdala, cerebellum and hippocampal principal neurons, FAAH appears post-synaptically in the soma and dendrites, primarily localized to intracellular organelles that serve as Ca^{2+} storage sites [e.g. mitochondria, smooth endoplasmic reticulum (ER)], with much less appearing at the plasma membrane (Gulyas *et al.*, 2004).

The uptake of AEA is coupled to its breakdown in cells in culture (Deutsch *et al.*, 2001). The driving force for the continued movement of AEA from the outside to the inside of the cell is its breakdown by FAAH, which results in low AEA concentrations inside the cell (Deutsch *et al.*, 2001). FAAH inhibitors would disrupt the establishment of equilibrium between AEA inside and outside the cell, leading to the build-up of AEA and increased binding at the cannabinoid receptors. In 1994, it was demonstrated that AEA levels were raised by treating cells with FAAH inhibitors, and this system was recognized as a therapeutic target: 'The development of inhibitors that block the breakdown of AEA may be significant therapeutically in any of the areas that Δ^9-tetrahydrocannabinol and AEA has been shown to play a role, including analgesia, mood, nausea, memory, appetite, sedation, locomotion, glaucoma, and immune function' (Koutek *et al.*, 1994). New generations of FAAH inhibitors have been synthesized that may eventually be employed therapeutically (Ahn *et al.*, 2009; Clapper, 2009; Fowler *et al.*, 2009).

Fatty acid-binding proteins (FABPs) as intracellular carriers for AEA

Recent work from the Deutsch laboratory (Kaczocha *et al.*, 2009) has shown that FABPs (FABP5 and FABP7) can shuttle AEA from the plasma membrane to the ER. Most neurotransmitters are hydrophilic and require protein transporters to traverse the cell membrane, but are freely diffusible once inside the cytosol. AEA, on the other hand, is an uncharged lipid that is insoluble in an aqueous environment, yet needs to traverse the cytosol in order to be metabolized by FAAH shown by immunohistochemistry to be localized to the ER (Arreaza and Deutsch, 1999; Gulyas *et al.* 2004). Accordingly, intracellular transporters are required to carry AEA through the cytoplasm. The FABPs serve this function and they are ubiquitous proteins expressed in all organs (Furuhashi and Hotamisligil, 2008). Three FABPs, expressed in brain (Owada *et al.*, 1996), were examined as possible intracellular AEA carriers. Recent molecular dynamics simulations of AEA in complex with FABP7 shows that the carboxamide oxygen of AEA can interact with FABP7 interior residues R126 and Y128, while the hydroxyl group of AEA can interact with FABP7 interior residues, T53 and R106 (see Figure 3) (Reggio *et al.*, 2009).

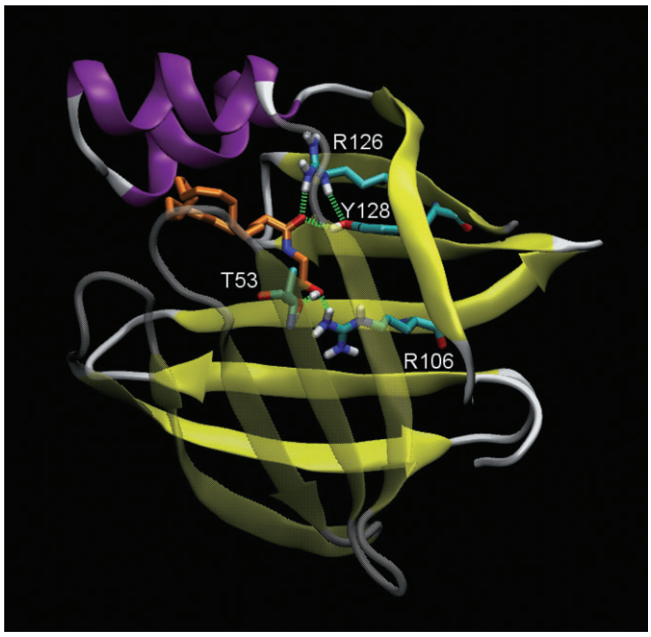

Figure 3

Depiction of AEA (orange) carried in the interior of FABP7. The carboxamide oxygen of AEA can interact with FABP7 interior residues R126 and Y128, while the hydroxyl group of AEA can interact with FABP7 interior residues, T53 and R106 (Reggio *et al.*, 2009).

It was found that AEA uptake and hydrolysis were significantly potentiated in N18TG2 neuroblastoma cells after overexpression of FABP5 or FABP7, but not FABP3 (Kaczocha *et al.*, 2009). Similar results were observed in COS-7 cells stably expressing FAAH. As expected, an FABP ligand, oleic acid, or the non-lipid FABP inhibitor BMS309403 decreased AEA uptake and hydrolysis in N18TG2 and the engineered COS-7 cells (Kaczocha *et al.*, 2009). Intracellular carriers, such as the FABPs, may account for the observation that endocannabinoids are accumulated inside the cell, and hence intracellular transporters may serve as a storage depot. In addition to the FABPs that transport AEA intracellularly from the plasma membrane to FAAH for inactivation, it has been reported that albumin and heat shock protein (Hsp)70 also function as AEA carriers (Oddi *et al.*, 2009). This topic has been recently reviewed (Maccarrone *et al.*, 2010). It remains to be shown how the endocannabinoids traverse the synapse and if they require any carriers. Interestingly, albumin has been shown to be synthesized and secreted from human microglial cells in culture (Ahn *et al.*, 2008), and it binds to the endocannabinoids, making it a candidate as a carrier, assuming that it is expressed in brain.

Evidence that autocrine or paracrine endocannabinoid signalling regulates CB_1 basal 'tone'

Given the evidence that endocannabinoid agonists can be synthesized and degraded by a number of enzymatic path-

ways, and carried intracellularly by FABPs and HSPs and extracellularly by albumins, what evidence exists for endocannabinoids to activate CB_1 receptors in an autocrine or paracrine fashion? Studies by Turu *et al*. (2007) identified CB_1 receptor 'tone' in receptor association with Gαo using a bioluminescence resonance energy transfer (BRET) signalling detection system when both proteins were exogenously expressed in CHO cells. This activation of G proteins was referred to as 'constitutive' because it was not stimulated by the addition of cannabinoid agonists and could be blocked by the CB_1 receptor inverse agonist AM251. However, the observation that AM251's response was completely attenuated by pretreatment with a DAGL inhibitor, tetrahydrolipstatin, provided compelling evidence that the production of 2-AG might underlie the increase in basal tone evoked by angiotensin II-stimulated AT_1 receptors. The 'constitutive internalization' that has been reported (Leterrier *et al*., 2004; 2006) was also attenuated by tetrahydrolipstatin (Turu *et al*., 2007), suggesting that the CB_1 receptor internalization occurred in response to endogenously produced 2-AG.

Production of 2-AG was demonstrated to result from stimulation of Gq/11-coupled AT_1 angiotensin receptors exogenously expressed in CHO cells leading to the activation of DAGL (Turu *et al*., 2009). When co-cultured, agonist-stimulation of AT_1 receptors in CHO cells was capable of generating sufficient 2-AG in the culture dish to stimulate exogenously expressed CB_1 receptors in a variety of host cells (CHO, COS7, HEK293), leading to activation Go interaction with the CB_1 receptors in a BRET signalling detection system (Turu *et al*., 2009). The AT_1-generated 2-AG was also able to regulate translocation of β-arrestin to the CB_1 receptors as an additional indicator of signal generation (Turu *et al*., 2009). To show how generalized this Gq/11-PLC-DAGL generation of 2-AG could be, agonist stimulation of exogenously expressed M1, M3 and M5 muscarinic; V1 vasopressin; α1a-adrenergic; and B_2-bradykinin receptors also activated a CB_1-Go BRET signal in co-cultured CHO cells (Turu *et al*., 2009). Cannabinoid receptor-mediated response to 'inverse agonists' that is influenced by basal tone set by adenosine receptors (Moore *et al*., 2000; Savinainen *et al*. 2003) might also have its origins in endogenously produced endocannabinoids.

Neurophysiological responses that can be attributed to tonic activation of CB_1 receptors

The most common neurophysiological demonstration of cannabinoid effects, indeed the field of greatest development in that past 10 years, regards the role of endocannabinoids in depolarization-induced suppression of inhibition (DSI) (Alger, 2002). As originally shown by Wilson and Nicoll (2001), rimonabant blocked a transient inhibition of GABAergic inhibitory post-synaptic potentials (IPSPs) in hippocampal principal cells. This phenomenon had been described by Pitler and Alger (1994) and proposed to incorporate a retrograde messenger to a pre-synaptic site of action. Wilson *et al*. (2001) demonstrated that the pre-synaptic receptor was the CB_1 receptor, and proposed that the retrograde messenger was an endocannabinoid. However, these and other studies (Maejima *et al*. 2001; Ohno-Shosaku *et al*., 2002; Hampson *et al*., 2003; Diana and Marty, 2004; Melis *et al*., 2004; Hashimotodani *et al*., 2008; Roux *et al*., 2009; Straiker and Mackie, 2009), demonstrating both DSI and the complementary depolarization-induced suppression of excitation (DSE) show only that CB_1 antagonists block the actions of exogenous or endogenous CB_1 ligands, and do not support inverse agonism manifested as facilitation of IPSPs or EPSPs by the antagonist alone.

Cannabinoid agonists have effects on long-term potentiation (LTP), the phenomenon by which trains of stimulation pulses either at continuous high frequency (100 Hz, HFS), or in bursts at the frequency of hippocampal theta rhythm (10 Hz, theta-burst), impart a long-lasting increase in synaptic activation of glutamate synapses in hippocampus and other brain areas. Exogenously applied cannabinoid agonists (WIN55212-2 and Δ^9-THC), as well as endocannabinoid degradation inhibitors, block the induction of LTP (Carlson *et al*., 2002; Slanina and Schweitzer, 2005; Hoffman *et al*., 2007; Abush and Akirav, 2010). Administration of AM251 (de Oliveira *et al*., 2006) or rimonabant (Sokal *et al*., 2008) also blocks the induction of LTP, interpreted as the blockade of endocannabinoid influence on GABAergic neurons (Abush and Akirav, 2010).

The direct actions of cannabinoid ligands can be observed on neural firing. In awake, behaving animals, hippocampal principal cell firing was suppressed by CB_1 agonists, and those effects were blocked by rimonabant (Hampson and Deadwyler, 2000; Deadwyler *et al*., 2007; Goonawardena *et al*., 2011). Only recently have any of these studies shown that rimonabant alone can enhance neural activity; however, this appears to more likely be the result of rimonabant blocking the endocannabinoids, rather than inverse agonism (Deadwyler *et al*., 2007; Deadwyler and Hampson, 2008). This hypothesis is confirmed by *in vitro* studies of hippocampal slices that measure changes in pyramidal cell intracellular Ca^{2+} concentration evoked by the application of the excitatory glutamatergic neurotransmitter *N*-methyl-D-aspartate (NMDA) (Hampson *et al*., 2009). NMDA application caused a transient 30–40% increase in intracellular Ca^{2+}, which was suppressed in the presence of cannabinoid agonists WIN55212-2 and Δ^9-THC. Rimonabant and AM251 not only blocked the agonist-induced suppression of intracellular Ca^{2+}, but also, *enhanced* the effects of NMDA, producing a 5–10% increase in NMDA-elicited Ca^{2+} concentration (Deadwyler and Hampson, 2008). Thus, when administered in the absence of CB_1 agonists, these antagonists do appear to have effects on basal tone. Recent results, however, suggest that the same effect can be obtained from blocking low levels of endocannabinoid activation of CB_1 receptors rather than by suppressing constitutive activity of the CB_1 receptor itself (Hampson *et al*., 2011).

What, then, is the electrophysiological evidence for such 'tonic' or 'background' release of endocannabinoids, which when blocked provide the same results as suppressing basal activity of CB_1 receptors? In hypothalamus, proopiomelanocortin neurons have been shown to continuously release endocannabinoids (Hentges *et al*., 2005), although curiously, the released endocannabinoids activated only the CB_1 receptors on inhibitory and not excitatory pre-synaptic neurons. Likewise, in hypothalamus, oxytocin-producing neurons tonically release both oxytocin and endocannabinoids, result-

ing in tonic inhibition of pre-synaptic GABA terminals as revealed by paired-pulse inhibition (Oliet *et al.*, 2007). In hippocampus, muscarinic or metabotropic glutamate receptor agonists produce DSI, while chelation of Ca^{2+} in the post-synaptic neuron has been shown to block DSI (Neu *et al.*, 2007). The fact that these same stimuli are implicated in the synthesis and release of endocannabinoids (Freund *et al.*, 2003) suggests that blockade of tonic CB_1 receptor activation by endocannabinoids, rather than suppression of constitutive CB_1 receptor activity, accounts for the 'inverse agonism' of rimonabant and AM251. A leading candidate for producing this release of endocannabinoids and tonic activation appears to be adenosine (Savinainen *et al*. 2003; Hoffman *et al*., 2010), with the possible involvement of oxytocin (Oliet *et al*., 2007) and angiotensin (Turu *et al*., 2007). Thus, in these neurophysiological preparations of CB_1 receptors, basal tone appears to be ligand dependent rather than constitutive activity.

Endocannabinoid interaction with cannabinoid receptors

Our understanding of agonist-stimulated GPCR activation generally presupposes that the agonist must diffuse to its binding site in aqueous solution from its origin in the blood or interstitial fluids. However, given the origin of the endocannabinoid ligands from membrane phospholipids, it is not unthinkable to suppose that 2-AG and AEA may reach the cannabinoid receptors by two-dimensional diffusion along the membrane. The orientation of both classical and non-classical cannabinoid, as well as endocannabinoid ligands in the lipid bilayer, has been established by small-angle X-ray diffraction/differential calorimetry experiments (Mavromoustakos *et al*., 1991), as well as by NMR (Tian *et al*., 2005; Kimura *et al*., 2009). These studies have shown that the C-3 side chain of classical and non-classical cannabinoids is aligned parallel with the membrane acyl chains (Mavromoustakos *et al*. 1991; Kimura *et al*., 2009), and that the fatty acid chain of anandamide orients parallel to membrane acyl chains with the terminal methyl near the centre of the bilayer (Tian *et al*., 2005).

The notion of lipid bilayer entry of endocannabinoids to the CB_1 receptor binding site is rendered more probable by simulations from the Reggio laboratory predicting that the initial contact of endocannabinoid agonists may be with the lipid face of the CB_1 seven transmembrane helix bundle (Lynch and Reggio, 2006; Hurst *et al*., 2010). Recent microsecond timescale molecular dynamics simulations of the CB_2 receptor in a palmitoyl, oleoyl-phosphatidylcholine (POPC) bilayer (Hurst *et al*., 2010) have suggested that: (i) 2-AG first partitions out of bulk lipid at the TMH6/7 interface of the CB_2 receptor; (ii) 2-AG then enters the CB_2 receptor binding pocket by passing between TMH6/7; (iii) the entrance of the 2-AG head group into the CB_2 binding pocket is sufficient to trigger breaking of the intracellular TMH3/TMH6 ionic lock and the movement of the TMH6 intracellular end away from TMH3; and (iv) subsequent to protonation at D3.49/D6.30, further 2-AG entry into the ligand-binding pocket results in both a W6.48 toggle switch change and large influx of water (see Figure 4).

Cannabinoid ligand entry at the TMH6/7 interface is supported by isothiocyanate labelling studies of CB_2 using the classical cannabinoid, AM841, functionalized at the C-3 dimethylheptyl side chain terminal carbon (Pei *et al*., 2008). Despite the fact that C7.42(288) faces into the CB_2 binding pocket and would be a likely covalent attachment site if the ligand entered the CB_2 binding pocket in the traditional way (from the extracellular aqueous space), AM841 was found to selectively label only one Cys residue, C6.47(257) (Pei *et al*., 2008). This residue is located in the TMH6/7 interface, facing lipid in a CB_2 model. This residue also faces lipid in rhodopsin (Palczewski *et al*., 2000; Okada *et al*., 2002; 2004; Li *et al*., 2004), and the β2-adrenergic (Olson *et al*., 2005; Cherezov *et al*., 2007; Rasmussen *et al*., 2007; Rosenbaum *et al*., 2007), β1-adrenergic (Warne *et al*., 2008) and adenosine A_{2A} (Jaakola *et al*., 2008) receptor crystal structures. Further, CB_2 receptor substituted cysteine accessibility method studies have indicated that C6.47(257) is not accessible from within the CB_2 ligand-binding pocket (Zhang *et al*., 2005). This suggests that AM841 may covalently label the outside facing C6.47(257) as it is gaining entrance to the binding domain. Interestingly, AM841 has also been shown to selectively label C6.47 in the CB_1 receptor (Picone *et al*., 2005), suggesting that a lipid pathway for ligand entry may also exist for the CB_1 receptor.

Conclusions

Research interest in separating 'inverse agonist' activity from 'neutral antagonist' activity has been accelerated in the last few years as the result of drug design goals to separate therapeutic effects from unwanted side effects for cannabinoid antagonists as medicinal compounds. As discussed herein, the inverse agonist actions of an antagonist are based solely on the ability to reverse constitutive activity. We have brought forward concerns that, for the endocannabinoid system, constitutive activity may be a misnomer. The use of the term 'constitutive activity' should be restricted to those experimental models in which it can be demonstrated that endocannabinoid agonists are not involved in the activation of the cannabinoid receptors. We recommend supplanting this term with phrases such as 'basal endocannabinoid system tone' or signal transduction 'in the absence of exogenously applied agonists' to be more accurate regarding the interpretation of experimental results. As noted, it is becoming more evident that endocannabinoid agonists signalling in nervous and other tissues occurs in an autocrine and paracrine manner. Given the diversity in pathways of synthesis and breakdown of 2-AG and AEA, it is likely that availability of endocannabinoid agonists will be governed by very different environmental stimuli in different tissues and cell types in the body. The ease with which endocannabinoid ligands can traverse the cell's interior and potentially be stored on FABPs suggests that endocannabinoids can be available for autocrine and paracrine signalling for extended periods of time. Furthermore, molecular modelling predicts that the endocannabinoids derived from membrane phospholipids have the capability of lateral diffusion in the lipid bilayer, giving these agonists a reasonable probability of interacting with their GPCRs without leaving their site of origin. These recently described insights into the workings of the endocan-

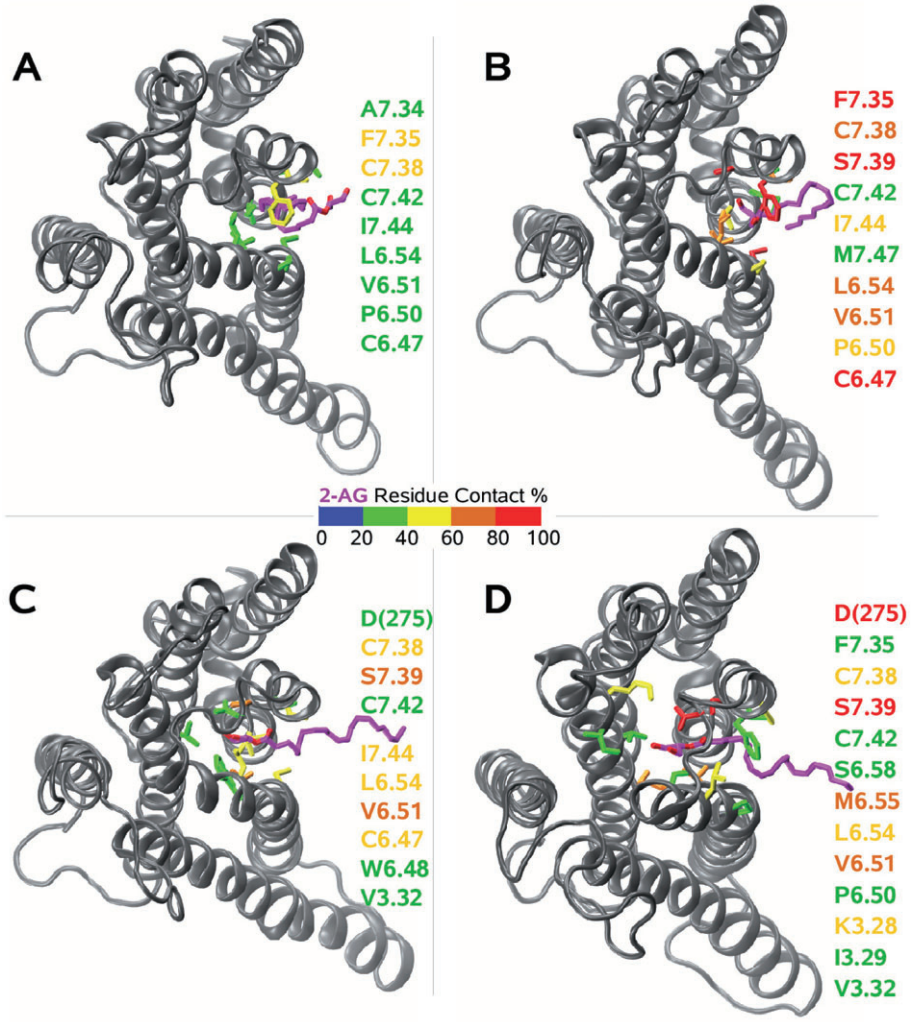

Figure 4

The results of microsecond timescale molecular dynamics simulations of 2-AG interacting with the CB_2 receptor embedded in a POPC bilayer. This figure illustrates the progress of 2-AG from the lipid bilayer into the CB_2 binding pocket as viewed from the extracellular surface of the receptor. 2-AG is located initially in the lipid bilayer surrounding CB_2. The lipid bilayer constituents are not displayed in order to simplify the view. The color scale represents the percentage of the trajectory in which any portion of 2-AG is within 4 Å of residues on CB_2 (defined here as within contact distance). Residues within contact distance are listed on the right and are color coded according to this scale. (A) The 2-AG has partitioned out of bulk lipid and contacts residues in or near the TMH6/7 interface. Highest contact is with F7.35(281) and C7.38(284). (B) 2-AG interaction with residues in the TMH6/7 interface increases with greater than 80% contact occurring with F7.35(281), S7.39(285) and C6.47(257). (C) After 2-AG entry into CB_2, 2-AG begins to contact binding pocket residues on TMH3 (V3.32(113)), TMH6 (W6.48(258)), TMH7 (C7.42(288)) and the EC-3 loop (D(275)). (D) Subsequent to protonation of D3.49 and D6.30, 2-AG contacts multiple residues on TMH3/6/7 and the EC-3 loop with formation of hydrogen bonds with D(275) in the EC-3 loop and to a lesser extent with S7.39(285) (Hurst *et al.*, 2010).

nabinoid system may serve as a prototype for other receptor systems that utilize lipid modulators as agonists.

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Conflict of interest

The authors have no conflict of interest to declare.

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