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REVIEW

The complications of promiscuity: endocannabinoid action and metabolism

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In this review, we present our understanding of the action and metabolism of endocannabinoids and related endogenous molecules. It is clear that the interactions between the multiple endocannabinoid-like molecules (ECLs) are highly complex, both at the level of signal transduction and metabolism. Thus, ECLs are a group of ligands active at 7-transmembrane and nuclear receptors, as well as transmitter-gated and ion channels. ECLs and their metabolites can converge on common endpoints (either metabolic or signalling) through contradictory or reinforcing pathways. We highlight the complexity of the endocannabinoid system, based on the promiscuous nature of ECLs and their metabolites, as well as the synthetic modulators of the endocannabinoid system.
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Keywords: cannabinoid receptors; endocannabinoid metabolism; TRPV1 vanilloid receptors; peroxisome proliferator-activated receptors

Abbreviations: 2AG, 2-arachidonoylglycerol; AEA, anandamide (*N*-arachidonoylethanolamine); AM404, *N*-(4-hydroxyphenyl)-5*z*,8*z*,11*z*,14*z*-eicosatetraenamide; ABC, ATP-binding cassette; CNS, central nervous system; CYP450, cytochrome *P*450; ECL, endocannabinoid-like molecules; ERK, extracellular signal-regulated kinase; FAAH, fatty acid amide hydrolase; GIRK, G protein-coupled inwardly rectifying potassium; GRK, G-protein-coupled receptor kinase; JNK, c-Jun N-terminal kinase; LOX, lipoxygenases; LY2183240, 5-biphenyl-4-ylmethyl-tetrazole-1-carboxylic acid dimethylamide; MAGL, monoacylglycerol lipase; MAP kinase, mitogen-activated protein kinase; NADA, *N*-arachidonoyldopamine; NAGly, *N*-arachidonoylglycine; NAPE, *N*-acylphosphatidylethanolamine; NAT, *N*-arachidonoyltaurine; ODA, oleamide (octadec(9,10*z*)enamide); OEA, *N*-oleoylethanolamine; PEA, *N*-palmitoylethanolamine; PLC, phospholipase C; PPAR, peroxisome proliferator-activated receptor; 7TM, 7-transmembrane; TRP, transient receptor potential; UCM707, *N*-(3-furanylmethyl)-5*z*,8*z*,11*z*,14*z*-eicosatetraenamide; URB597, cyclohexyl carbamic acid 3′-carbamoyl-biphenyl-3-yl ester; VDM11, *N*-(4-hydroxy-2-methylphenyl)-5*z*,8*z*,11*z*,14*z*-eicosatetraenamides

Introduction and scope of review

In this review, we highlight recent understanding of the mechanisms of signal transduction at cannabinoid and cannabinoid-like receptors, which, for this review, we consider to be CB_1, CB_2, GPR18, GPR55 and GPR119 7-transmembrane (7TM) receptors, TRPV1 (transient receptor potential) vanilloid transmitter-gated channels and peroxisome proliferator-activated receptor (PPAR) nuclear receptors (Table 1). Anandamide (AEA), the most intensively studied endocannabinoid, is the first endogenous agonist identified to be active at members of three of the four receptor superfamilies (there is, as yet, no evidence for activity at catalytic receptors), while the related endogenous molecule *N*-oleoylethanolamine (OEA) may prove to the second such agonist (Table 1). We describe the family of endocannabinoid-like molecules (ECLs), the majority of which have not been characterized at all the cannabinoid and cannabinoid-like receptors, or for activity as substrates or modulators of the associated enzymes. We highlight the limitations associated with the use of reported 'selective' metabolic inhibitors. We concentrate on signalling pathways identified for ECL action in native expression systems rather than in heterologous expression, since there are significant issues associated with the interpretation of studies on recombinant systems. Thus, it is likely that many signalling pathways are cell-specific, in that different contexts of receptor expression may favour one route of signalling over another. As such, therefore, heterologous expression of receptors (and enzymes) in naive cellular environments may provide a distorted picture of receptor signal transduction

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Table 1 Receptor targets of multiple endocannabinoid-like molecules (ECLs)

ECL	*CB_1*	*CB_2*	*GPR18*	*GPR55*	*GPR119*	*TRPV1*	*PPARα*	*PPARβ/δ*	*PPARγ*
ODA	+[1]								
PEA	+[2,3]	+[2,3]		++[4]	++[5]	+	++[6,7]		+[8]
OEA	+[9]	+[9]		++[4]	+++[5]	++[10]	++[11,7]	++[11]	+[11]
AEA	++[12,13,14]	++[12,13]		++[4]	+[5]	++[15]	++[7]		++[8]
NAGly	+[3]	+[16]	+++[17]			+[18]			
NADA	++[19]					+++[15]			
2AG	+++[14]	+++[20]		++[4]					
Noladin	++[14]	+[21]		++[4]			++[7]		
Virodhamine	−[22]	++[22]		+++[4]			++[7]		

Ligand activity at cannabinoid or cannabinoid-like receptors from three of the four superfamilies (7-transmembrane, transmitter-gated channels and nuclear receptors) of receptor: there is, as yet, no evidence for ECL activation of catalytic receptors. Scale within the target: − antagonist; + weak/no activity; ++ intermediate; +++ high relative activity; a blank space indicates the activity of the ligand has not been described. Indicated are five 7-transmembrane receptors (CB_1, CB2, GPR18, GPR55 and GPR119), one transmitter-gated channel (TRPV1) and three nuclear receptors (PPARα, PPARβ/δ and PPARγ).
[1]Leggett *et al.* (2004); [2]Vandevoorde *et al.* (2003); [3]Sheskin *et al.* (1997); [4]Drmota *et al.* (2004); [5]Overton *et al.* (2006); [6]Lo Verme *et al.* (2005); [7]Sun *et al.* (2006); [8]Bouaboula *et al.* (2005); [9]Lin *et al.* (1998); [10]Movahed *et al.* (2005); [11]Fu *et al.* (2003); [12]Felder *et al.* (1995); [13]Slipetz *et al.* (1995); [14]Sugiura *et al.* (1999); [15]Huang *et al.* (2002); [16]Sipe *et al.* (2005); [17]Kohno *et al.* (2006); [18]Huang *et al.* (2001b); [19]Bisogno *et al.* (2000); [20]Mechoulam *et al.*, 1995; [21]Hanus *et al.*, 2001; [22]Porter *et al.*, 2002.

pathways. For this reason, we have attempted to identify the cellular environment for signal transduction and, although the literature is limited, we have compared findings with *ex vivo*/*in vivo* tissues where possible.

Metabolic routes of ECL synthesis and catabolism are described, as these provide the potential for interactions between different components of the ECL system. Alongside traditional views of the signal transduction properties of ECL-activated receptors, we summarize the potential for convergence of signalling at these multiple receptors.

A number of most excellent reviews of cannabinoid receptors and endocannabinoids have been produced over recent years (aside from those in the current issue), and the reader is directed to these (Cota *et al.*, 2006; Demuth and Molleman, 2006; Di Marzo and De Petrocellis, 2006; Felder *et al.*, 2006; Jonsson *et al.*, 2006; Kogan and Mechoulam, 2006; Mackie, 2006; Pertwee, 2006; Sugiura *et al.*, 2006; Centonze *et al.*, 2007; Di Marzo and Petrosino, 2007; Di Marzo *et al.*, 2007; Fernandez-Ruiz *et al.*, 2007; Harkany *et al.*, 2007) for slightly differing perspectives on the field.

Multiple endocannabinoid-like molecules: synthesis and catabolism

Although we describe the compounds outlined in Figure 1 as endocannabinoids, one of the purposes of this review is to highlight the diversity of function within the group, with many of these entities inactive at the conventional cannabinoid receptors (Table 1); hence the appellation of endocannabinoid-like molecules (ECL, see below). To date, all of the endogenous ligands found to act at cannabinoid receptors are lipid-derived fatty acid congeners, although they can be structurally diverse within that group. Currently, endocannabinoids may be divided into N- or O-linked compounds (see Figure 1) ranging from the simplest generic structure of the amides (Figure 1a) to the more complicated glyceryl ethers (Figure 1f). There are questions about whether endogenous levels of some of these agents are sufficient to

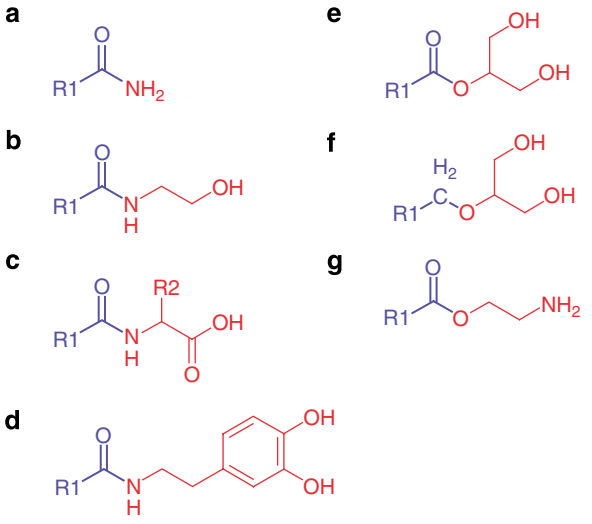

Figure 1 Endocannabinoid-like molecules (ECLs). In these structures, R1 is the hydrocarbon side chain, for example, $C_{19}H_{31}$ (generating arachidonyl/arachidonoyl), $C_{17}H_{33}$ (generating oleyl/oleoyl) and $C_{15}H_{31}$ (generating palmityl/palmitoyl). ECLs can be primary amides (**a:** for example, oleamide, ODA); *N*-acylethanolamides (**b:** for example, anandamide, AEA; *N*-oleoylethanolamide, OEA; *N*-palmitoylethanolamide, PEA); *N*-acylamino acids (**c:** for example, *N*-arachidonoylglycine, NAGly; *N*-arachidonoylGABA; *N*-arachidonoylalanine); *N*-acyldopamines (**d:** for example, *N*-arachidonoyldopamine, NADA; *N*-oleoyldopamine; *N*-stearoyldopamine; *N*-palmitoyldopamine), 2-acylglycerols (**e:** for example, 2-arachidonoylglycerol, 2AG), 2-alkylglycerols (**f:** for example, noladin ether-2-arachidonoylglyceryl ether) and *O*-acylethanolamines (**g:** for example, virodhamine).

allow them to be labelled as endocannabinoids (Oka *et al.*, 2003), although there is some variation in estimates of endogenous levels of these endocannabinoids, in various tissues, which may, at least in part, derive from differences in extraction methodologies (Kempe *et al.*, 1996). Overall, the picture we paint is of a complex spectrum of endogenous molecules, which have an array of activities at molecular targets, which may be claimed to be cannabinoid or cannabinoid-like receptors (Table 1).

Routes of N-linked endocannabinoid-like molecules synthesis

The simplest ECL molecules are the primary amides, such as oleamide (ODA) and arachidonamide (Figure 1a). A route for synthesis of primary amide ECLs has not been unequivocally demonstrated, but may involve metabolism of lipoamino acids (see below). An alternative is reversal of the enzymatic activity of fatty acid amid hydrolase (FAAH, see below), which has been suggested as a means of generating both primary amide ECLs, such as ODA (Sugiura *et al*., 1996b) as well as the ethanolamide ECLs (Devane and Axelrod, 1994; Kurahashi *et al*., 1997; Schmid *et al*., 1998), although the concentrations of substrates (ammonia and ethanolamine, respectively) required appear to be in excess of physiological levels and the use of enzyme inhibitors in intact cells seems to predicate against this route (Bisogno *et al*., 1997). Under extremely artificial conditions, non-mammalian lipases have been reported to hydrolyse lipids by ammonialysis to produce fatty acid amides such as ODA (Dezoete *et al*., 1996). Recently, cytochrome *c* has been proposed as a catalyst for ODA generation using oleoylCoA and ammonia as substrates (Driscoll *et al*., 2007).

The most intensively studied of the ECLs is anandamide (AEA, Figure 1b); in the chemistry laboratory, anandamide may be produced as a simple condensation product of arachidonic acid and ethanolamine. Generation of the ethanolamide ECLs *in vivo*, however, is thought to occur primarily as a result of hydrolysis of a minor membrane phospholipid, *N*-acylphosphatidylethanolamine (NAPE, (Di Marzo *et al*., 1994)). A novel phospholipase D (NAPE-PLD) has been described, which has the potential for generating the whole spectrum of endogenous fatty acid ethanolamides (Okamoto *et al*., 2004). Alternative indirect routes of synthesis of the acylethanolamines have also been described, however, including phospholipase C hydrolysis of NAPE and the consequent production of acylethanolamine-*O*-phosphates, which may be hydrolysed by a selective phosphatase, PTPN22 (Liu *et al*., 2006).

Conjugation of fatty acids with amino acids produces lipoamino acids (Figure 1c), which have recently been termed elmiric acids (Burstein *et al*., 2007). One proposed synthetic route of synthesis makes use of one of the mainstays of detoxification of xenobiotics, such as the conjugation of salicylic acid with glycine. In the case of carboxylic acid-containing molecules, this mechanism involves the formation of a CoA ester, followed by conjugation with glycine under the influence of the mitochondrial enzyme glycine *N*-acyltransferase (EC 2.3.1.13) or a soluble alternative bile acid-CoA: amino acid *N*-acyltransferase (O'Byrne *et al*., 2003). This has recently been observed for *N*-arachidonoyltaurine (NAT, Figure 1c) generation in rat liver fragments (Saghatelian *et al*., 2006). This is one possible route for *N*-arachidonoylglycine (NAGly, Figure 1c) synthesis, although an alternative route has been suggested through the stepwise actions of alcohol dehydrogenase and aldehyde dehydrogenase acting on AEA, via the intermediate formation of the aldehyde *N*-arachidonoylethanalamine (Burstein *et al*., 2000). *N*-Acylglycine may also be an intermediate in the synthesis of the primary amide ECLs, as it may be metabolized by a dual action of Golgi-located enzyme activity. The first step, peptidylglycine α-monooxygenase activity (EC 1.14.17.3), requires ascorbate and molecular oxygen as co-substrates and copper as a cofactor. The resulting 2-hydroxyglycine conjugate is unstable and dismutates to glyoxylate and the corresponding primary amide, a reaction catalysed by the second activity (peptidylamidoglycolate lyase, EC 4.3.2.5).

N-Arachidonoyldopamine (NADA, Figure 1d) is a condensation product of arachidonic acid and dopamine, which has been suggested to be generated in biological tissues by two alternative routes (Huang *et al*., 2002). A simpler alternative was the direct condensation of an arachidonoyl precursor (possibly arachidonoyl-CoA) with dopamine. A more intricate synthetic route for NADA involved production of an *N*-acylamino acid (Figure 1c), *N*-arachinodoyltyrosine, and then sequential hydroxylation (via tyrosine hydroxylase) and decarboxylation (presumably via aromatic amino acid decarboxylase) (Huang *et al*., 2002). Alternative routes of synthesis may yet be revealed.

Routes of O-linked endocannabinoid-like molecules synthesis

2-Arachidonoylglycerol (2AG, Figure 1e) has been suggested to be the primary endogenous agonist for CB_1 and CB_2 receptors, as it occurs in greater concentrations in tissues, and shows greater efficacy at these targets, than AEA (Sugiura *et al*., 1997, 1999, 2000; Sugiura and Waku, 2002) (see Table 1). 2AG synthesis is likely to come about from the sequential action of phospholipase C (PLC) and diacylglycerol lipase via the production of the intermediate diacylglycerol (Ben-Shabat *et al*., 1998; Parrish and Nichols, 2006), which is perhaps better known as a stimulus for protein kinase C activation. PLC-independent synthesis of 2AG has also been described in neuroblastoma (Bisogno *et al*., 1999), microglial cells (Carrier *et al*., 2004) and mouse ear *in vivo* (Oka *et al*., 2005), although the precise metabolic routes involved are unclear.

In contrast, the potential biosynthetic routes of production of the other O-linked ECLs noladin ether, the ether analogue of 2AG (Figure 1f) and virodhamine (Figure 1g) are much less clear (Fezza *et al*., 2002; Porter *et al*., 2002). In any event, there is doubt about whether these agents are present in brain to sufficient levels to be serious candidates as endogenous cannabinoid ligands (Oka *et al*., 2003), although extracellular levels of virodhamine measured in brain microdialysates are reported to be high (Porter *et al*., 2002).

Hydrolysis of N-linked endocannabinoid-like molecules

There are at least three enzymes described to have the capacity to hydrolyse fatty acid ethanolamides, although with distinct pH optima, subcellular localizations and substrate selectivities (Figure 2). A decade ago, an enzyme with the ability to hydrolyse both N- and O-linked ECLs, termed FAAH (EC 3.1.…), was cloned from mouse and

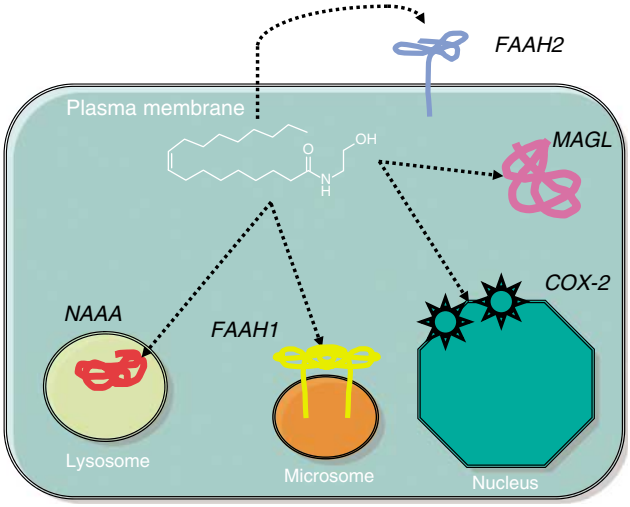

Figure 2 ECL catabolism: a schematic diagram of the enzymatic targets of endocannabinoids in a model cell. The chemical structure is of *N*-oleoylethanolamine, OEA, which may be hydrolysed at the intracellular face of FAAH1, the extracellular face of FAAH2 or within the lysosome by *N*-acylethanolamine acid amidase. Also indicated are COX-2 and MAGL, which are capable of metabolizing 2AG and AEA (rather than OEA).

human sources (Giang and Cravatt, 1997). However, more recently, a second enzyme with a more restricted species distribution in higher mammals (FAAH2) has been described with a similar inhibitor profile to FAAH1 (Wei *et al.*, 2006). The enzymes have distinct rank orders of substrate affinities, with FAAH1 hydrolysing AEA with a higher affinity than ODA, while the reverse is true for FAAH2. In comparison, the saturated fatty acid ethanolamines, such as *N*-palmitoylethanolamine (PEA) and *N*-stearoylethanolamine, are poor substrates for these enzymes. Of potentially greater interest is the observation that FAAH2 is likely to have an opposite membrane orientation to FAAH1 (Wei *et al.*, 2006). Thus, FAAH1 is a dimeric enzyme, which (based on the crystal structure of a complex with an inhibitor) has been proposed to be integrated into cell membranes, 'facing' the cytoplasm, while allowing direct access to the active site from the lipid bilayer (Bracey *et al.*, 2002). In heterologous expression studies, immunofluorescently imaged human and rat FAAH1 showed an intracellular location consistent with microtubular or Golgi/endoplasmic reticulum association, respectively (Giang and Cravatt, 1997). A similar intracellular pattern was observed for native expression of FAAH1 in mouse N18TG2 neuroblastoma cells (Deutsch *et al.*, 2001). In contrast, FAAH2 is predicted to have 'luminal' projection, which (if it proves to be a cell-surface enzyme) might have great significance in the regulation of levels of extracellular cannabinoids (Wei *et al.*, 2006).

A third enzyme activity hydrolysing fatty acid amides is located in the lysosomes of macrophages (Tsuboi *et al.*, 2007) and appears to be important for the regulation of ECL levels in macrophages, but not brain (Sun *et al.*, 2005). *N*-acylethanolamine acid amidase (Figure 2) has a preference for the saturated fatty acid ethanolamines, such as PEA and *N*-stearoylethanolamine (Ueda *et al.*, 2001), and exhibits a distinct inhibitor profile to the FAAH isoforms (Tsuboi *et al.*, 2004).

In comparison with hydrolysis of the fatty acid ethanolamines, metabolism of NADA may occur slowly via FAAH, but a more complicated scenario involves O-methylation (presumably via catechol *O*-methyltransferase) to a derivative much less potent at CB_1 and TRPV1 receptors (Huang *et al.*, 2002). NAGly was rapidly hydrolysed by rat brain membranes in a manner consistent with FAAH action ((Huang *et al.*, 2001b; Cascio *et al.*, 2004), while *N*-arachidonoylleucine and *N*-arachidonoylalanine exhibited cell/species variability in effectiveness as FAAH inhibitors/substrates (Cascio *et al.*, 2004). It is unclear, therefore, whether a common mechanism exists for all lipoamino acids.

Hydrolysis of O-linked endocannabinoid-like molecules

Monoacylglycerol lipase (MAGL, Figure 2) is a cytoplasmic enzyme, which hydrolyses 2-oleoyl- and 2-linoleoylglycerol at rates similar to 2AG (Ghafouri *et al.*, 2004). AEA was slightly less effective as an inhibitor/substrate, while PEA and NAGly were ineffective up to 100 μM. In the initial description of virodhamine levels in tissues from the rat, it was suggested that the esterase function of FAAH would also allow hydrolysis of virodhamine, analogous to the metabolism of 2AG (Porter *et al.*, 2002). In contrast, it has been suggested that noladin ether undergoes a distinct metabolic fate to the other endocannabinoids, such that it is incorporated into phospholipid (Fezza *et al.*, 2002).

Oxidative metabolism of endocannabinoid-like molecules

Of the two isoforms of cyclooxygenase activity, COX-2 (Figure 2) appears to be the more relevant for oxidative metabolism of endocannabinoids (see review from Fowler, this issue), with AEA (Yu *et al.*, 1997) and 2AG (Kozak *et al.*, 2000) reported to be metabolized to prostamides (prostaglandin ethanolamides) and glyceryl prostaglandins, respectively. Of the two endocannabinoids, endogenous levels of 2AG are more compatible with substrate affinities at COX-2, with an efficiency equivalent to arachidonic acid, leading to a suggestion that 2AG may be a (patho)physiological substrate of COX-2 action (Kozak and Marnett, 2002).

The prostamide analogues of PGD_2, PGE_2 and $PGF_{2\alpha}$ failed to interact significantly with human recombinant DP, EP, FP, IP or TP prostanoid receptors, human recombinant TRPV1 receptors or with FAAH preparations (Matias *et al.*, 2004), but instead appear to act upon a novel receptor target (Woodward *et al.*, 2007). Similarly, the glyceryl prostaglandins exhibit functional activity independent of cannabinoid receptors (Nirodi *et al.*, 2004; Sang *et al.*, 2007).

In addition to the 'mainstream' endocannabinoids, COX-2, but not COX-1, has also been reported to metabolize NAGly selectively to PGH_2 glycine and hydroxyeicosatetraenoyl glycine (Prusakiewicz *et al.*, 2002), the biological activity of which is obscure.

In contrast to cyclooxygenase, lipoxygenases (LOX) are theoretically able to produce eight distinct hydroperoxides from arachidonic acid. In practice, though, mammalian enzymes produce hydroperoxides in the 5*S*, 12*S*, 12*R* and 15*S* positions. AEA has been described to be hydroxylated in the 12 and 15 positions by distinct enzyme activities, of which the 12-hydroxy product was more CB_1 active (Ueda *et al*., 1995; Hampson *et al*., 1995a). In comparison, AEA was apparently inactive as a substrate for 5-LOX activity (Ueda *et al*., 1995). This spectrum of activity is mirrored for 2AG as substrate, in that 12-LOX and 15-LOX, but not 5-LOX, metabolize 2AG in cell-free and intact cell preparations (Moody *et al*., 2001; Kozak *et al*., 2002). Recently, 12-LOX and 15-LOX metabolism of NAGly, but much reduced activity against NADA, has been described (Prusakiewicz *et al*., 2007).

Oxidative metabolism of AEA and 2AG may lead to the production of ligands for PPARs (Kozak *et al*., 2002; Rockwell and Kaminski, 2004), TRPV1 receptors (Craib *et al*., 2001) and inhibitors of FAAH (Edgemond *et al*., 1998; Maccarrone *et al*., 2000).

Aside from metabolism by LOX/COX activities, arachidonic acid may be oxidized by cytochrome *P*450 (CYP450) oxygenase activities. Early studies using mouse liver microsomes suggested at least 20 metabolites of AEA, while brain metabolism appeared restricted to the production of two major metabolites (Bornheim *et al*., 1995). Using microsomes from human liver as a source of CYP450s, epoxyeicosatrienoyl ethanolamides with epoxides formed at all four potential positions (5,6-, 8,9-, 11,12- and 14,15-) have been observed (Snider *et al*., 2007). The corresponding dihydroxyeicosatrienoyl ethanolamides were also detected, following metabolism by microsomal epoxide hydrolase. Both human liver and kidney microsomes were able to produce a monooxygenated AEA, 20-hydroxyeicosatetraenoyl ethanolamine, through the action of CYP450 4F2 (Snider *et al*., 2007). CYP450 metabolism of 2AG has also been described recently using kidney-derived preparations (Chen *et al*., 2007). The epoxide metabolite, 2-(14,15-epoxyeicosatrienoyl)glycerol, stimulated cellular proliferation, apparently via activation of metalloproteinase activity, although a precise mechanism was not identified. The corresponding metabolite, 2-(14,15-dihydroxyeicosatrienoyl)glycerol, produced following epoxide hydrolysis is reported to be a PPARα agonist (Fang *et al*., 2006).

As mentioned above, the fatty acid moiety may not be the only site for oxidative metabolism as the ethanolamide portion of AEA may be sequentially oxidized by alcohol and aldehyde dehydrogenases to NAGly (Burstein *et al*., 2000).

Selectivity of inhibitors of endocannabinoid-like molecules turnover

A number of pharmacological inhibitors have been described for enzymes involved in the turnover of endocannabinoids. There are very few descriptions of inhibitors of NAPE-PLD described in the literature as a means of reducing levels of ethanolamide endocannabinoids. However, there are commercially (and clinically) available inhibitors of diacylglycerol hydrolysis to prevent 2AG formation: tetrahydrolipstatin and RHC80267 (Bisogno *et al*., 2003). Tetrahydrolipstatin, indicated for the treatment of clinical obesity under the name of Orlistat, is a pancreatic lipase inhibitor, thereby acting to reduce the metabolism and absorption of dietary fat. Presumably, the poor absorption of oral tetrahydrolipstatin militates against systemic effects on endocannabinoid production, although it is possible that tetrahydrolipstatin may have effects on ECL turnover within the gut. Further compounds based on methylfluorophosphonate analogues of 2AG have been described to be selective for diacylglycerol lipase over other related enzymes, including MAGL, NAPE-PLD and FAAH (Bisogno *et al*., 2006). Intriguingly, these compounds were ineffective in cultured cells (Bisogno *et al*., 2006), but elicited a response similar to tetrahydrolipstatin following injection into the rat periaqueductal grey matter *in vivo* (Maione *et al*., 2006).

One of the most widespread enzyme inhibitors utilized in endocannabinoid turnover studies is cyclohexyl carbamic acid 3′-carbamoyl-biphenyl-3-yl ester (URB597) (Kathuria *et al*., 2003; Mor *et al*., 2004). This inhibits both forms of FAAH with submicromolar potency with modest selectivity for FAAH2 (Wei *et al*., 2006). Although functional effects of URB597 administration *in vivo* are lost in FAAH-null mice (Fegley *et al*., 2005), there is controversy about its ability to inhibit other enzyme activities (Zhang *et al*., 2007), particularly triacylglycerol hydrolase (Lichtman *et al*., 2004; Clapper *et al*., 2006). Recently, high concentrations ($\geqslant 10\,\mu M$) of URB597 have been described to activate TRPA1, a cation channel expressed in sensory neurones (Niforatos *et al*., 2007) and to activate PPARγ in cells lacking native FAAH1 expression (Dionisi *et al*., 2007).

[1,1′-Biphenyl]-3-yl-carbamic acid, cyclohexyl ester (URB602) was initially characterized as a non-competitive inhibitor of MAGL, which elevated 2AG levels in the periaqueductal grey matter of rats *in vivo*, without altering AEA levels (Hohmann *et al*., 2005). Subsequent *in vitro* studies, however, suggested that this compound lacked selectivity, with significant inhibitory potency at FAAH (Vandevoorde *et al*., 2007). There are many situations, however, where mixed inhibition of FAAH and MAGL may prove useful and so this agent, or similar compounds, may have future therapeutic application.

6-Methyl-2-*p*-tolylaminobenzo[*d*]oxazin-4-one (URB754) was also initially described to be a selective MAGL inhibitor (Makara *et al*., 2005). Subsequently, the commercially available version has been described to be ineffective (Saario *et al*., 2006; Ho and Randall, 2007) or non-selective (Vandevoorde *et al*., 2007). A corrigendum to the original article identified that the custom-synthesized compound was contaminated with bis(dimethylthio)mercury (II), which proved an effective (albeit non-selective) MAGL inhibitor (Makara *et al*., 2007). These data together highlight the need for a selective MAGL inhibitor, which may allow the role of MAGL to be clarified in intact tissues and organisms.

There is a controversy over the existence of a selective transport mechanism for extracellular endocannabinoids, the putative anandamide transporter (Fowler *et al*., 2004; McFarland and Barker, 2004; Bojesen and Hansen, 2006;

Kaczocha *et al.*, 2006; Thors and Fowler, 2006). In the absence of a demonstrated molecular identity, the evidence for its existence relies on pharmacological data. Thus, AEA analogues, such as AM404, *N*-(4-hydroxy-2-methylphenyl)-5*z*,8*z*,11*z*,14*z*-eicosatetraenamide (VDM11) and *N*-(3-furanylmethyl)-5*z*,8*z*,11*z*,14*z*-eicosatetraenamide (UCM707), although reported as inhibitors of ECL accumulation, are also active at other sites. AM404 is an agonist at TRPV1 receptors (De Petrocellis *et al.*, 2000; Zygmunt *et al.*, 2000), and also inhibits FAAH (Jarrahian *et al.*, 2000), COX1 and COX2 (Hogestatt *et al.*, 2005) activities. VDM11 has been shown to act as a substrate for FAAH (although potency estimates appear to be highly dependent on enzyme source (Fowler *et al.*, 2004)) and to inhibit MAGL (Vandevoorde and Fowler, 2005). UCM707 shows significant occupancy of CB_2 receptors (Lopez-Rodriguez *et al.*, 2003). LY2183240 (5-biphenyl-4-ylmethyl-tetrazole-1-carboxylic acid dimethylamide) showed initial promise as a selective inhibitor of endocannabinoid transport, particularly as the structure was not fatty acid-based and a radiolabelled version showed saturable binding (Moore *et al.*, 2005). However, LY2183240 has been shown to be an inhibitor of multiple serine hydrolases including FAAH activity (Alexander and Cravatt, 2006). More recently, it has been argued that LY2183240 inhibits FAAH activity in intact RBL-2H3 cells as a consequence of a more potent effect on AEA accumulation (Ortar *et al.*, 2007).

Some inhibitors of other endocannabinoid-metabolizing enzymes show promiscuity of inhibition. For example, a number of COX inhibitors also inhibit FAAH activity (see review by Fowler, this issue). Thus, ibuprofen, ketorolac, flurbiprofen (Fowler *et al.*, 1999), indomethacin, but not nimesulide or SC58125 (Fowler *et al.*, 2003) exhibit FAAH inhibition, which is enhanced at acid pH. Particular LOX inhibitors are also inhibitors of FAAH (Patel and Alexander, 2007). Whether the combination of inhibition of COX and FAAH or LOX and FAAH (or indeed LOX, COX and FAAH) contributes to the therapeutic effects of any of these agents is yet to be determined.

Multiple targets for endocannabinoid-like molecules actions

There are multiple molecular targets for endocannabinoids, which can be grouped into 7TM receptors, transmitter-gated channels, nuclear receptors, ion channels, enzymes and transporters (Table 1, Figure 3). Cannabinoid receptors for a long time have been seen as a simple doublet of 7TM receptors, the predominantly neuronal CB_1 receptor and the predominantly immune system CB_2 receptor. While these receptors remain the most readily recognized and most deeply understood, recent years have seen the addition of three 7TM receptors from the stock of orphan receptors 'remaindered' on completion of the Human Genome Project, that is GPR18, GPR55 and GPR119. As there is a review in this themed issue on the topic of novel cannabinoid receptors (see review by Brown), only a cursory summary of the molecular pharmacology of these receptors will be presented, although it is apparent that much more is to be learned of ECL action at these recent additions (Table 1). Similarly, O'Sullivan describes activation of PPAR nuclear receptors by cannabinoids in this volume and so we discuss, only superficially, the signal transduction profile of these targets. The TRPV1 receptor is a transmitter-gated channel, which responds to AEA and other fatty acid ethanolamides, such as OEA (Table 1). Although all of these targets of cannabinoids have been identified at the molecular level, there is pharmacological evidence for further cannabinoid receptors (see below).

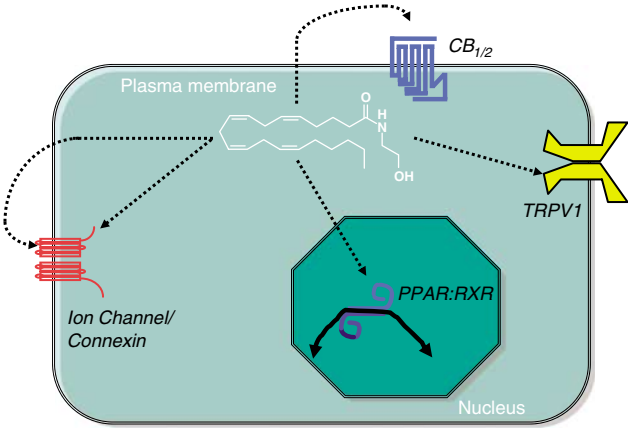

Figure 3 ECL action at receptors: a schematic diagram of the 'receptor' targets of endocannabinoids in a model cell. The chemical structure is of anandamide, which may act at the intracellular face of the TRPV1 receptor, the extracellular face of 7TM cannabinoid receptors, at undetermined site(s) of the ion channel or connexin gap junctions and at PPARs inside the nucleus.

7-Transmembrane receptors

Among the 7TM receptors, endocannabinoids act at the well-characterized CB_1 and CB_2 cannabinoid receptors (Showalter *et al.*, 1996; Bisogno *et al.*, 2000; Jonsson *et al.*, 2001; Leggett *et al.*, 2004). The 'best' candidates for endogenous agonists at these receptors are 2AG and AEA, with virodhamine acting as a putative endogenous CB_1 cannabinoid receptor antagonist (Table 1). In contrast, NAGly is ineffective at CB_1 or CB_2 receptors (Huang *et al.*, 2001b), but activates GPR18 at nanomolar concentrations (Kohno *et al.*, 2006). GPR55, recently described to be responsive to many endogenous and synthetic cannabinoids (Wise and Brown, 2001; Drmota *et al.*, 2004), awaits complete pharmacological, biochemical and physiological definition (see review by Brown, this issue). GPR119 has been proposed to be a receptor for OEA, while PEA is less potent, and AEA is almost ineffective (Overton *et al.*, 2006).

Agonist binding to the 7TM cannabinoid receptors leads to activation of heterotrimeric G-proteins of three of the four families of G-proteins (G_s, $G_{i/o}$ $G_{q/11}$ and $G_{12/13}$). Thus, GPR18, CB_1 and CB_2 receptors couple predominantly to $G_{i/o}$-proteins, while GPR119 couples to G_s (Overton *et al.*, 2006) and GPR55 couples to neither $G_{i/o}$ nor G_s (Drmota *et al.*, 2004), but likely couples to $G_{12/13}$. As a consequence of this coupling, CB_1 receptor signalling function in native

tissues is most commonly measured by the enhancement of [^{35}S]GTPγS binding and inhibition of adenylyl cyclase in preparations from brain tissue or cells and the inhibition of cyclic AMP accumulation in brain slice or cell preparations.

Immunoprecipitation of CB_1 receptors from mouse neuroblastoma N18TG2 cells allowed estimation of agonist-induced coupling to distinct isoforms of G_i, such that methanandamide (a stable anandamide analogue) promoted coupling selectively to $G_{\alpha i3}$, while the phytocannabinoid analogue desacetyllevonantradol promoted activation of $G_{\alpha i1}$ and $G_{\alpha i2}$, and WIN55212-2 allowed coupling to all three isoforms (Mukhopadhyay and Howlett, 2005). This agonist-selective activation of G proteins may underlie a recent observation of differences in coupling to tyrosine hydroxylase promoter activation in mouse N1E-115 neuroblastoma cells by the synthetic cannabinoid agonists HU210 and CP55940 (Bosier *et al*., 2007).

Similarly for CB_2 receptors, there appears to be agonist-dependent activation of distinct signalling pathways, at least in heterologous expression systems (Shoemaker *et al*., 2005). Intriguingly, there also appeared to be receptor-evoked responses that were differentially sensitive to the CB_2 cannabinoid receptor antagonists AM630 and SR144528 (Shoemaker *et al*., 2005).

CB_1 receptor-mediated enhancement of calcium transients was observed in neuroblastoma cells (Sugiura *et al*., 1996a, 1997, 1999). The mechanism suggested for this involved the enhancement of PLC activity via $G_{\beta\gamma}$ subunits. Although similar mechanisms are proposed to account for the modulation (both enhancement and inhibition) of inositol phospholipid turnover in rodent brain by other $G_{i/o}$-coupled receptors (a similar phenomenon in brain slices has been described for adenosine (Alexander *et al*., 1989) and Group II metabotropic glutamate receptors (Alexander *et al*., 1994; Mistry *et al*., 1998)), there appears to be no comparable literature for CB_1 receptor modulation of this pathway in *ex vivo* tissue preparations, although a recent study reports CB_1 receptor-mediated inhibition (potentially cross-desensitization) of bombesin-evoked calcium responses in mouse RINm5f insulinoma cells (De Petrocellis *et al*., 2007a).

There are well-established means of examining CB_1 receptor function in isolated tissues, primarily involving inhibition of autonomic function, including twitch responses of the mouse vas deferens and guinea-pig ileum preparations (Pertwee, 1997). In contrast, there is a paucity of methodologies for determining CB_2 cannabinoid receptor function in isolated tissues, with one example assessing the obligatory role of cAMP in the regulation of gene transcription and interleukin-2 production in murine T cells by CB_2 receptor activation (Condie *et al*., 1996).

Transmitter-gated channels

Although the TRPV1 receptor is the best candidate for a classical transmitter-gated channel site of ECL action (Table 1 and below), NMDA glutamate receptors have also been reported to be positively modulated by AEA (Hampson *et al*., 1998), while most other transmitter-gated channel are inhibited by endocannabinoids, including glycine receptors (Coyne *et al*., 2002), $GABA_A$ receptors (Yost *et al*., 1998; Coyne *et al*., 2002), α7-nicotinic receptors (Oz *et al*., 2003) and 5-HT_3 receptors (Fan, 1995; Barann *et al*., 2002). Additionally, calcium (Evans *et al*., 2004; Oz *et al*., 2005), potassium and sodium channels (Nicholson *et al*., 2001) have also been reported to be directly modulated by endocannabinoids (see below).

An interesting feature of cannabinoid receptor-independent AEA regulation of transmitter-gated and ion channels is the 'sidedness' of its action, acting on the intracellular face of the TRPV1 receptor (see below), but apparently on the extracellular face of the α7 nicotinic receptor (Spivak *et al*., 2007).

TRPV1 vanilloid receptors

A number of reports demonstrate direct interactions between ECLs and members of the transient receptor potential (TRP) receptor family, to the extent that some (particularly the non-selective cation-gating TRPV1 receptor) could be regarded as selective cannabinoid ion channel receptors (van der Stelt and Di Marzo, 2005; Starowicz *et al*., 2007). In the peripheral nervous system, TRPV1 receptors are widely expressed on small diameter primary afferent fibres (Caterina *et al*., 1997), where they act as a focal point for the summation of noxious stimuli such as high temperature and low pH. The receptors appear to be tonically active *in vivo* since antagonists of different structure cause hyperthermia via sites outside the central nervous system (Gavva *et al*., 2007). TRPV1 are also widely expressed in the brain (Toth *et al*., 2005) and TRPV1 deletion causes reduced anxiety, conditioned fear and hippocampal long-term potentiation (Marsch *et al*., 2007). In contrast to the cell-surface binding of ligands by CB_1 and CB_2 receptors, De Petrocellis *et al*. (2001a) demonstrated that AEA activates TRPV1 receptors via an intracellular binding site (Figure 3). AEA has been shown to be a full agonist at over-expressed TRPV1 receptors in model cells with a potency in the low micromolar range, but in comparable experiments using dorsal root ganglion neurones, its potency is approximately 10-fold lower and its efficacy possibly reduced (Jerman *et al*., 2002). Clearly, cell and experiment-specific factors such as expression levels of receptors and metabolic enzymes, pH and inclusion of interacting agents can all affect the measured affinity and efficacy of such a metabolically dynamic molecule as AEA and making generalizations concerning these parameters is inappropriate.

NADA and related congeners, including *N*-oleoyldopamine (Chu *et al*., 2003), are also reported to be agonists at TRPV1, with NADA being slightly more potent than AEA, with similar efficacy (Huang *et al*., 2002). Recently, it has been reported (Saghatelian *et al*., 2006) that, in addition to the *N*-acylethanolamines, another lipid family, the NAT, are able to activate multiple members of the TRP channel family including TRPV1 and TRPV4. These NATs are good FAAH substrates and peripheral blockade of the enzyme is reported to increase tissue levels of the NATs by more than 10-fold in 1 h. In addition to TRPV1, TRPV2 is widely distributed including in some sensory nerves, where it acts as a heat sensor, and in the cerebral cortex (Liapi and Wood, 2005).

The latter authors showed that TRPV1 and TRPV2 can hetero-multimerize to form channels with, as yet, uncharacterized properties. In contrast to their activating effects on TRPV1, the endocannabinoids/endovanilloids AEA and NADA potently inhibit TRPM8 receptors, which are also expressed in sensory nerves and which are gated by low ($<25\,^{\circ}C$) temperature (De Petrocellis *et al*., 2007b). Thus, the effects of a calcium-mobilizing stimulus in excitable cells, such as sensory nerves, will be subject to a complex array of modulating influences depending upon the expression of TRP family members.

Ion channels

One of the key physiological functions of the endocannabinoid signalling system is to regulate the degree of activation of excitable cells. This is largely achieved by modulation of cation channels either indirectly through the medium of G protein-coupled receptors or by more direct interactions with channel protein complexes. Currently, the evidence for regulation of anion channels seems to be limited to a CB_1 receptor-mediated, mitogen-activated protein (MAP) kinase-dependent activation of Cl^- currents in retinal pigmented epithelial cells (Shi *et al*., 2003).

Calcium channels

Cannabinoids, acting at CB_1 cannabinoid receptors, are widely recognized to reduce pre-synaptic neurotransmitter release via an inhibition of voltage-operated calcium channels (Sullivan, 1999; Hoffman and Lupica, 2000; Kreitzer and Regehr, 2001; Alger, 2002) at both excitatory and inhibitory synapses in the central nervous system. This action of CB_1 receptors via $G_{i/o}$ proteins seems to be effective at multiple forms of voltage-operated calcium channels; inhibition of N-type channels in NG108-15 cells (Mackie and Hille, 1992; Felder *et al*., 1993; Mackie *et al*., 1993) and in rat striatal neurones (Huang *et al*., 2001a); inhibition of P/Q-type Ca^{2+} fluxes in rat cortical and cerebellar brain slices (Hampson *et al*., 1998) and L-type channels in cat cerebral arterial smooth muscle cells (Gebremedhin *et al*., 1999).

In contrast, synthetic and endogenous cannabinoids have recently been shown to inhibit post-synaptic P-type currents in Purkinje neurones independently of CB_1 receptors (Fisyunov *et al*., 2006). This mirrors the findings regarding the ability of micromolar concentrations of AEA to inhibit T-type channels, either endogenously or heterologously expressed, in different cell types, which was not reproduced by 2AG or particular synthetic cannabinoid receptor agonists nor blocked by rimonabant, indicating a direct interaction with the channel (Chemin *et al*., 2001). No molecular modelling studies have been performed to clarify the nature of such direct inhibitory interactions between cannabinoids and voltage-operated calcium channels and there is no evidence that cannabinoids can positively modulate voltage-operated calcium channels function.

Potassium channels

Cannabinoids have been demonstrated to act on background, voltage-operated and G protein-coupled inwardly rectifying potassium (GIRK) channels. Maingret *et al*. (2001) reported a $CB_{1/2}$-independent blockade of the TASK-1 channel, which gates an acid- and anaesthetic-sensitive leak or background K^+ current, by submicromolar concentrations of AEA, CP55940 and WIN55212-2. As would be predicted, this induced a depolarization of the cerebellar granule cells expressing the channels and this was suggested by the authors to be involved in the effects of the endocannabinoid on motor behaviour.

Mackie *et al*. (1995) reported that WIN55212-2 activated an inward K^+ current following expression of CB_1 receptors in AtT-20 pituitary tumour cells. Similarly, in CB_1-transfected HEK-293 cells, WIN55212-2 and AEA activated, in a CB_1 antagonist-sensitive fashion, endogenously expressed inwardly rectifying K^+ channels (Vasquez *et al*., 2003). The Ba^{2+} sensitivity of cannabinoid agonist inhibited glutamate signalling in mouse nucleus accumbens suggested the involvement of a GIRK activation in the transmitter release process (Robbe *et al*., 2001). The mechanism underlying GIRK activation by cannabinoids is not entirely clear, but it has been suggested (Ho *et al*., 1999) to be mediated by $G_{\beta\gamma}$ rather than G_α subunits and that coupling to GIRK could be observed after expression of either CB_1 or CB_2 receptors in oocytes. GIRK coupling to CB receptors can be inhibited by stimulation of protein kinase C-mediated channel phosphorylation, as demonstrated by Garcia *et al*. (1998) in CB_1-transfected AtT-20 cells, but whether this occurs *in vivo* is not known.

Given the inhibitory effects of cannabinoids on neurotransmitter release, it is not surprising that they can enhance voltage-dependent A-type outward K^+ currents (Deadwyler *et al*., 1995) leading to hyperpolarization. This was shown to be mediated by CB_1 receptor-mediated reduction in cyclic AMP levels and protein kinase A activation (Deadwyler *et al*., 1995; Hampson *et al*., 1995b). Less predictable is the report of WIN55212-2 decreasing, via CB_1 receptors, M-type K^+ currents, leading to hyperexcitability in hippocampal CA1 neurones (Schweitzer, 2000). Whether this ability to enhance neuronal excitability directly, as opposed to disinhibition via reduction of inhibitory neurotransmitter release, is a widespread phenomenon remains to be seen.

Sodium channels

There is relatively little evidence of cannabinoid action at sodium channels, but an early report suggested that Δ^9-tetrahydrocannabinol (THC) was able to depress inward currents through voltage-operated sodium channels in neuroblastoma cells (Turkanis *et al*., 1991), although the mechanism and pharmacology of this phenomenon were not investigated. Kim *et al*. (2005a) reported that AEA inhibited both tetrodotoxin-sensitive and tetrodotoxin-resistant Na^+ currents in rat dorsal root ganglion neurones. This inhibition, which appeared to be on inactivated rather than on resting channels, was insensitive to antagonists of CB_1, CB_2 or TRPV1 receptors, suggesting a direct interaction of AEA with the channel. A similar direct inhibitory action on voltage-operated Na^+ channels was shown by Nicholson *et al*. (2003). These authors reported that AEA, AM404 and WIN55212-2 inhibited veratridine-dependent depolarization

of synaptoneurosomes and veratridine-dependent release of glutamate and GABA from purified synaptosomes with IC_{50} values in the micromolar range. The effects were all resistant to blockade with AM251 and the same group (Liao *et al*., 2004) demonstrated that AM251 ($IC_{50} = 9\ \mu M$) itself inhibited veratridine-dependent (tetrodotoxin-suppressible) release of glutamate and GABA from synaptosomes. The binding of the radioligand [^{3}H]batrachotoxinin A 20-α-benzoate to site 2 on sodium channels was displaced by AM251 with micromolar potency, as had previously been shown for AEA, AM404 and WIN55212-2.

Nuclear receptors

Among the nuclear hormone receptors, the PPARs have been reported to be a target of ECLs (see O'Sullivan, this issue). In brief, various members of the ECL family are able to activate PPARα (OEA, AEA, noladin) and/or PPARγ (AEA) to elicit gene transcription or suppression. Some of the synthetic cannabinoids, as well as some cannabinoid antagonists, are able to activate both PPARα and PPARγ. Activation of these receptors initiates or suppresses transcription of particular genes leading to the physiological responses of PPAR activation, including induction of enzymes of lipid metabolism and adipocyte differentiation.

Multiple enzyme regulation

There is evidence for receptor-mediated and direct regulation of a number of cellular enzymes by ECLs. Thus, AEA has been reported to inhibit Na^+/K^+-ATPase leading to a reduced synaptosomal accumulation of dopamine and 5-HT (Steffens and Feuerstein, 2004), while both AEA and OEA have been reported to activate extracellular signal-regulated kinase (ERK) directly (Berdyshev *et al*., 2001). One of the earliest actions (over 30 years ago) of a member of the ECL family was the description of the inhibitory actions of OEA on ceramidase (Sugita *et al*., 1975). AEA has also been reported to both inhibit and stimulate rat brain protein kinase C *in vitro*, dependent on the presence of calcium and phospholipid (De Petrocellis *et al*., 1995).

Protein kinases

Aside from the regulation of ion channels by 7TM receptors, the major alternative pathway of cell signalling is via protein kinases. The routes of 7TM receptor signalling to protein kinases is primarily via classical second messengers (for example, protein kinases A and C) through the medium of the G_α subunits, but can also be enacted via less conventional routes, including $G_{\beta\gamma}$ subunits.

The expected routes of $G_{i/o}$-coupled 7TM receptors via G_α subunits would be inhibition of Ca^{2+} channels and activation of K^+ channels via $G_{\alpha o}$ (see above) and inhibition of protein kinase A activity via $G_{\alpha i}$-mediated inhibition of adenylyl cyclase activity. Signalling via $G_{\beta\gamma}$ subunits is considered to be of much lower fidelity, in that the latter can regulate (activate or inhibit) multiple adenylyl cyclase isoforms, potentiate activity at particular isoforms of phospholipase Cβ, activate particular isoforms of phospholipase D, activate K^+ channels, activate members of the G-protein-coupled receptor kinase (GRK) family and lead to activation of members of the MAP kinase family.

GRKs: As the shortest cascade of activation following receptor stimulation, the GRKs are associated primarily with feedback inhibition of receptor signalling (Moore *et al*., 2007). In the *Xenopus* oocyte expression system, desensitization of the CB_1 receptor in the continuous presence of agonist is reported to depend upon the presence of GRK3 and β-arrestin 2 (Jin *et al*., 1999). Truncation of the receptor at residue 418 selectively interfered with the desensitization process, without affecting agonist activation. A distinct site appears to be the target for protein kinase C-mediated inhibition of CB_1 receptor coupling to K^+ channels in heterologous expression (Garcia *et al*., 1998).

In heterologous expression, the CB_2 receptor has been reported to be constitutively phosphorylated by a pertussis toxin-insensitive mechanism, possibly through a GRK-dependent mechanism (Bouaboula *et al*., 1999b).

MAP kinase: There is copious evidence for the coupling of both CB_1 and CB_2 receptors to activation of members of the MAP family, made up of principally isoforms of ERK, c-Jun N-terminal kinase (JNK; also known as stress-activated protein kinase) and p38. Thus, recombinant CB_1 cannabinoid receptors expressed in Chinese hamster ovary (CHO) cells couple to JNK and p38 MAP kinase, apparently dependent on tonic tyrosine kinase activity (Rueda *et al*., 2000). Coupling to ERK1/2 by CB_1 receptors expressed in CHO cells was blocked by pertussis toxin, implicating $G_{i/o}$ proteins in the signalling cascade (Bouaboula *et al*., 1995b). Coupling of natively expressed CB_1 receptors to ERK has been observed in both glial-derived (human astrocytoma U373MG) and neuronally derived (mouse Neuro-2a) cells (Bouaboula *et al*., 1995b; Graham *et al*., 2006).

In mouse and rat hippocampal slices *in vitro*, AEA and 2AG activation of CB_1 receptors led to enhanced activity of p38, but not JNK (Derkinderen *et al*., 2001), while in cortical neurones, CB_1 receptors couple to JNK (Downer *et al*., 2003). *In vivo*, in the mouse hippocampus, application of AEA and 2AG evoked CB_1 receptor activation enhanced ERK activation via a Fyn-dependent process, leading to gene transcription of c-fos, egr-1 (also known as krox-24, NGFI-A and zipf268) and brain-derived neurotrophic factor (BDNF) (Derkinderen *et al*., 2003).

An early observation in the characterization of CB_2 receptor signalling in heterologous expression was the involvement of ERK in gene transcription events (Bouaboula *et al*., 1996). Intriguingly, the same group reported later that, in HL-60 human promyelocytic cells, the CB_2 cannabinoid receptor antagonist SR144528 reduced ERK activation by endogenous CB_2, LPA and insulin, but not FGF, receptors (Bouaboula *et al*., 1999a) indicating an obligatory role for CB_2 receptor stimulation in ERK activation by multiple receptors. Other studies suggest, however, that cannabinoids can inhibit the activation of ERK/MAP kinases via CB_2 receptor activation (Faubert and Kaminski, 2000). It is possible that these differences in signalling route derive from cell-specific influences.

Phosphorylation of the CB_2 receptor overexpressed in HL-60 cells (Derocq *et al*., 2000) was stimulated by CP55940, simultaneously with ERK activation. CB_2 receptor activation in these cells lead to increased transcription of genes encoding cytokines (nonocyte chemoattractant protein-1; macrophage inflammatory protein-1, interleukin-8 and tumor necrosis factor-α), as well as regulators of cell cycling (Jun B and IκBα).

CB_1 receptors expressed in CHO cells were observed to activate protein kinase B following stimulation by AEA, in a pertussis toxin-sensitive manner (Gómez Del Pulgar *et al*., 2000). Activation of CB_1 receptors natively expressed in U373MG human astrocytoma cells also showed functional coupling to PKB activity, while HL-60 cells (expressing endogenous CB_2 receptors) did not (Gómez Del Pulgar *et al*., 2000).

AEA has been reported to elicit a rapid (<1 min), high potency ($pEC_{50} \sim 7.5$) increase in tyrosine phosphorylation in rat hippocampal slices, which was blocked by rimonabant (albeit at high concentrations—50 μM) and pertussis toxin, and reversed by stimulation of protein kinase A (Derkinderen *et al*., 1996).

Multiple transporters

Application of exogenous ECLs is reported to alter the function of glycine transporters, gap junctions and multi-drug resistance (ATP-binding cassette, ABC) transporters, although it is unclear whether endogenous ECLs are able to regulate these activities, and whether the action is on the intracellular or extracellular side of the plasma membrane. Thus, AEA, but not the synthetic cannabinoid WIN55212-2, has been reported to enhance GlyT1a glycine transport (Pearlman *et al*., 2003). In contrast, NAGly and *N*-arachidonoyl-L-alanine, but not AEA, have been shown to inhibit GlyT2a function (Wiles *et al*., 2006). Gap junctions are reported to be inhibited by both AEA (Venance *et al*., 1995) and ODA (Guan *et al*., 1997). In addition, high (>1 μM) concentrations of rimonabant are able to block gap junctions (Chaytor *et al*., 1999), which may help to explain some of its non-CB_1 receptor-mediated effects. Short-term (<1 h) exposure to AEA, but not 2AG or PEA, was found to block ABCB1 (MDR1, p-glycoprotein) function in HK2 human kidney cells (Nieri *et al*., 2006), while phytocannabinoids, particularly cannabidiol, inhibited ABCA1 function (<2 h) in Caco-2 human colon cancer cells (Zhu *et al*., 2006). In contrast, other authors have reported a lack of effect of short-term exposure (1 h) to cannabidiol and WIN5521-2 on ABCB1 activity, although longer-term (72 h) exposure evoked an inhibition (Holland *et al*., 2006). Intriguingly, activation of PPARα and PPARγ increased expression of ABCA1 transporter protein in human macrophage foam cells (Chinetti *et al*., 2001), while gene disruption of PPARγ reduced ABCA1 and ABCG1 expression in mouse macrophage foam cells (Akiyama *et al*., 2002).

Mechanisms awaiting molecular definition

There are further cannabinoid-like receptors with characteristics distinct from any of the targets described above (see also review by Brown, this issue), which are yet to be defined in a molecular sense. Thus, an abnormal-cannabidiol (*trans-p*-menthadien-[1, 8]-yl)-olivetol) receptor has been described on vascular endothelium (Jarai *et al*., 1999) and also on microglia (Walter *et al*., 2003). In the vasculature, this receptor is inhibited by pertussis toxin and a 'selective antagonist' O-1918 and evokes relaxation through activation of ERK1/2 (Offertaler *et al*., 2003). *N*-arachidonoylserine has been claimed to be the endogenous agonist at this receptor (Milman *et al*., 2006). Apparently, a distinct vascular cannabinoid-like receptor is present in rat small mesenteric arteries, activated by ODA and AEA and blocked by O-1918 and high concentrations of rimonabant. This receptor required an intact endothelium and sensory innervation (Hoi and Hiley, 2006). A further undefined vascular target of cannabinoids is present on sensory neurones, in which transmitter release is inhibited by noladin ether via a pertussis toxin-sensitive mechanism, independent of CB_1, CB_2 and TRPV1 receptors (Duncan *et al*., 2004). There is a poorly defined 'CBn receptor' on RBL-2H3 cells, which binds [^{3}H]WIN22512-2 and inhibits antigen-evoked 5HT release and which appears to be inhibited by AEA (Facci *et al*., 1995). Cannabidiol has also been reported to protect against cerebrovascular insult via a $5HT_{1A}$ receptor-mediated, but otherwise undefined, mechanism (Mishima *et al*., 2005).

Interactions between endocannabinoid signalling pathways

Regulation of canonical cannabinoid receptor function

Given that the locus of generation of the endocannabinoids is intracellular, one could readily conceive a temporal cascade of canonical/homologous receptor activation by ECLs, which would be, first, activation at the intracellular site of the plasma membrane TRPV1 receptor, followed by activation of nuclear PPARs and, third, activation at the extracellular binding site of the plasma membrane CB_1/CB_2 cannabinoid receptors following outwards transport. Since activation of TRPV1 receptors leads to elevation of cytoplasmic calcium levels and a consequent increase in endocannabinoid generation, this first step could be considered an autocatalytic amplification step (while extended activation of TRPV1 will also lead to calcium-mediated desensitization). Obviously, this sequence of activation depends very much on co-expression of these receptors, and would be subject to modification dependent on the cellular profile of metabolic enzymes. Co-expression of receptors responding to the same cognate agonist is not unusual, and is observed with (for example) adenosine, glutamate and 5-HT receptors. The rationale for co-expression often involves differences in agonist potency, divergence in signalling pathways, spatial divergence (particularly in neuronal expression) and differences in rates of activation or desensitization, to allow a greater control of the overall output from the cell.

TRPV1 receptors. TRP receptors are subject to a number of modulating influences, which increase their potential to act as focal points for cross-talk between intracellular signalling systems. The TRPV1 receptor is under the tonic inhibitory

control of phosphatidylinositol 4,5-bisphosphate (PIP_2), which can be relieved by G-protein receptor activation of PLC (Chuang *et al*., 2001). Indeed, there is evidence (based on immunoprecipitation studies) for a physical association between TRPV1 and PLCγ (Chuang *et al*., 2001). The release of diacylglycerol from PIP_2 can, via protein kinase C activation, lead to phosphorylation and sensitization of TRPV1 receptors (Premkumar and Ahern, 2000). Intriguingly, protein kinase C-mediated phosphorylation has been suggested to be an obligate step in OEA-evoked TRPV1 function (Ahern, 2003).

Acutely, phosphorylation-dependent sensitization can also be achieved via protein kinase A (De Petrocellis *et al*., 2001b; Bhave *et al*., 2002) and calmodulin-dependent protein kinase II (Tominaga *et al*., 2001). Desensitization of the TRPV1 channel has been reported to occur via dephosphorylation, which may be mediated by the protein phosphatase calcineurin (Jung *et al*., 2004). Whether there are differences in the levels, temporal or spatial location of the calcium-mediated activation of calcineurin compared to calmodulin-dependent protein kinase II remains to be identified.

Inhibition of p38 MAP kinase reduced the increase in TRPV1 immunoreactivity evoked by nerve growth factor (NGF) in rat dorsal root ganglion cells (DRG) *in vivo* (Ji *et al*., 2002), implying a role for p38 in TRPV1 expression. Additionally, a Ras/MEK/ERK pathway has been implicated in TRPV1 upregulation by NGF and glial-derived neurotrophic factor (GDNF) in cultured rat sensory neurones (Bron *et al*., 2003). Millns *et al*. (2001) showed that CB_1 receptor activation with HU210 inhibited capsaicin activation of TRPV1 channels in adult rat DRGs leading to the intriguing possibility that the action of ECLs, such as AEA, that have dual activity at inwardly facing TRPV1 and outwardly facing 7TM receptors could be under the control of membrane transporters.

In studies of heterologous co-expression of CB_1 and TRPV1 receptors in HEK-293 cells, forskolin-induced elevation of intracellular cyclic AMP potentiated TRPV1-evoked intracellular calcium responses (Hermann *et al*., 2003). In the absence of forskolin, CB_1 receptor activation enhanced TRPV1 responses (in a manner sensitive to inhibitors of PLC and phosphatidylinositol 3-kinase), while the presence of forskolin revealed a CB_1 receptor-mediated inhibition of TRPV1 activity. The latter inhibitory response was probably mediated via an inhibition of cyclic AMP accumulation and the reduced activity of protein kinase A (see Figure 4a). It is possible that the former, augmentatory response was an artefact of overexpression of CB_1 receptors, thereby allowing coupling to PLC activation. A subsequent report investigating rat mesencephalic cells in culture described TRPV1-mediated cell death, in part due to calcium influx (Kim *et al*., 2005b). In these cells, CB_1 receptor activation also led to cell death. The mechanisms of these actions is, however, obscured by the observation that the neurotoxic effects of CB_1 and TRPV1 receptor agonists could be blocked by antagonists of TRPV1 and CB_1 receptors, respectively (Kim *et al*., 2005b). The authors suggested that TRPV1 activation led to production of agents active at CB_1 receptors, exacerbating cell death (see the section Receptor-evoked modulation of ECL generation).

Phosphorylation-mediated activation of TRPV1 receptors appears likely to contribute to the heightened sensitivity to normal stimuli that is observed in inflammatory pain. At the cellular level of the primary afferent neurone expressing TRPV1 receptors, sensitizing agents such as bradykinin, ATP and histamine elicit an activation of PLC, thereby relieving a PIP_2 tonic inhibition, additionally sensitizing the receptor channel via protein kinase C and/or calmodulin-dependent protein kinase II. Additionally, the elevation of intracellular calcium is likely to lead to elevated ECL levels, thereby directly activating the TRPV1 receptor. Intriguingly, in rat dorsal root ganglion neurones *in vitro* or in TRPV1 receptor overexpressing cells, activation of the TRPV1 by protons (pH 5.5) required protein kinase C activation, and was additive or synergistic with AEA activation (Premkumar and Ahern, 2000; Vellani *et al*., 2001; Olah *et al*., 2002). A further complication arises from the report that WIN55212-2, a potent CB receptor agonist, has been reported to elicit a dephosphorylation of TRPV1 receptors in rat trigeminal neurones in culture (Jeske *et al*., 2006). Parallel investigation in a recombinant system suggested that WIN55212-2 directly activated TRPA1 channels, thereby inducing calcium influx, activation of calcineurin and, thus, dephosphorylation of TRPV1 receptors. Furthermore, small interfering RNA, directed against TRPA1 channels, proved an effective inhibitor of WIN55212-2-evoked dephosphorylation of TRPV1 receptors in sensory neurone culture.

Peroxisome proliferator-activated receptors. There is good evidence that PPAR activity is dependent on phosphorylation (Burns and Vanden Heuvel, 2007), which may be mediated through a variety of cellular protein kinases, including protein kinase A (Lazennec *et al*., 2000; Figure 4a) and AMP-dependent protein kinase. MAP kinases appear to have isoform- and tissue-selective effects on PPAR activity (Zhang *et al*., 1996; Juge-Aubry *et al*., 1999; Chen *et al*., 2003; Schild *et al*., 2006), which may be reciprocated. Thus, PPARα activation resulted in rapid ($\sim$20 min) activation of MEK-dependent ERK activity in mouse liver cells (Rokos and Ledwith, 1997), which led to enhanced expression of the immediate early genes *c-fos* and *egr-1*. Parallel investigations using central nervous system (CNS)-derived tissues indicated a CB_1 receptor-mediated, MEK-dependent activation of *egr-1* (Glass and Dragunow, 1995; Bouaboula *et al*., 1995a). Whether simultaneous activation of PPARα and CB_1 receptors converge in their effects on ERK and egr-1 mobilization remains to be identified. Egr-1 is described as a master regulator of gene transcription, which has been implicated in many disease-related phenomena, particularly in the vasculature (Khachigian, 2006), but whether endocannabinoid-evoked regulation of egr-1 is involved in these processes awaits further investigation. At this point, it is interesting to note a synergistic interaction between AEA and a selective PPARα agonist in the reduction of pain-associated responses in mice *in vivo* (Russo *et al*., 2007). The synergistic effect was entirely dependent on CB_1 receptor activation, as suggested by the inhibitory effect of rimonabant, and appeared to be mediated by activation of $K_{Ca}1.1$ potassium channels.

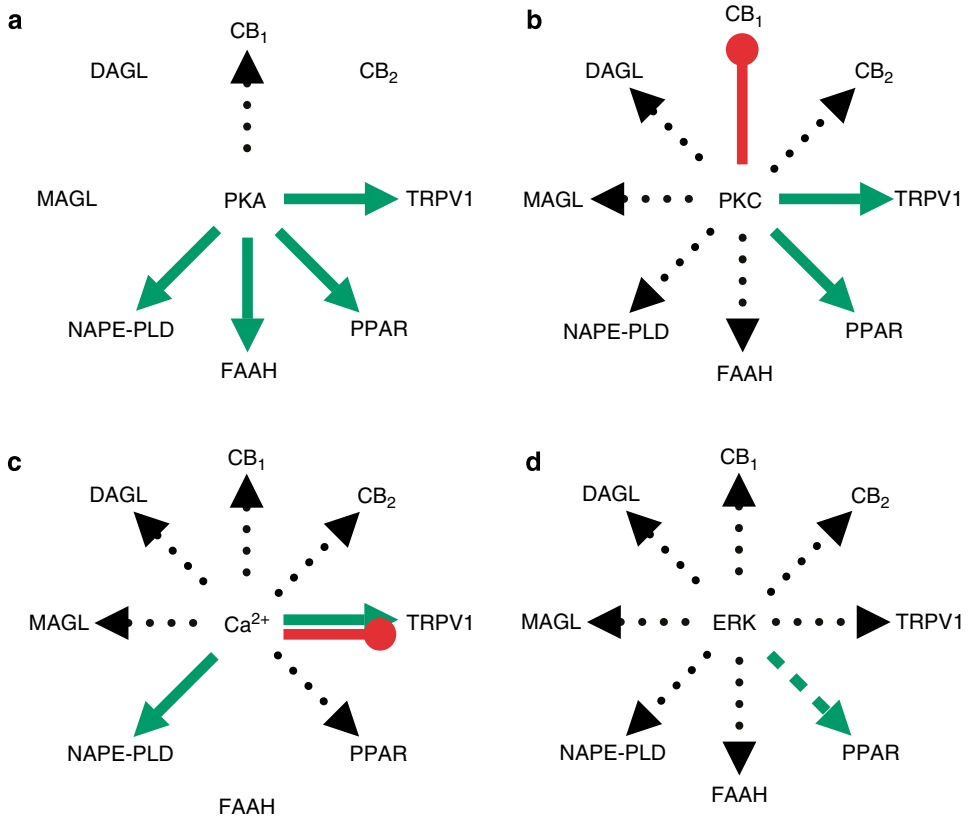

Figure 4 Compass points or the cannabinoid cartwheel of complicated cross-talk; green lines with arrow tips represent stimulation/enhancement of the indicated activity; red arrows with ball ends represent inhibition; dashed arrows represent unknown or variable relationships; no line represents no effect. For example, it appears that low levels of intracellular calcium enhance TRPV1, while prolonged/excessive calcium exposure leads to TRPV1 desensitization (**c**). ERK activity appears to have variable enhancing effects on PPAR activity that may be subtype- or tissue-dependent, but appears to have not been studied with respect to the majority of the endocannabinoid system, other than as a target of receptor activation (**d**). DAGL, diacylglycerol lipase, ERK, extracellular signal-regulated kinase; FAAH, fatty acid amide hydrolase; PKA, protein kinase A; PKC, protein kinase C; MAGL, monoacylglycerol lipase; NAPE-PLD, N-arylphosphatidylethanolamine-phospholipase D; PPAR, peroxisome proliferator-activated receptor.

CB receptors. Although being predominantly separately expressed, such that CB_1 and CB_2 receptors are often described as 'the CNS cannabinoid receptor' and 'the immune system cannabinoid receptor', respectively, there is evidence for co-expression of CB_1 and CB_2 receptors, chiefly on cells of the immune system. For example, in mouse bone marrow-derived dendritic cells, THC activation of both CB_1 and CB_2 receptors appeared to be required to see apoptosis (Do *et al.*, 2004). Recently, the presence of CB_2 receptors in the CNS has also been identified (Van Sickle *et al.*, 2005), and subsequently corroborated (Gong *et al.*, 2006), but it is unclear whether this expression is representative of co-expression. Given the similarity of signalling cascades activated by CB_1 and CB_2 receptors, it is likely that simultaneous activation of these receptors could conceivably result in additive or synergistic responses to extracellular ECLs.

Non-canonical receptors as loci of endocannabinoid influence
There is growing evidence that cannabinoid receptors, as with many other 7TM receptors, can form homomeric and heteromeric complexes. Using antibodies that preferentially recognize the dimerized form of the CB_1 receptor, it has been shown that this is apparently the preferred configuration of the receptors expressed in the brain (Hajos *et al.*, 2000; Katona *et al.*, 2001). CB_1 heterodimers also exist, the best described being complexes of dopamine D_2 and CB_1 receptors (Kearn *et al.*, 2005), although there is also evidence for dimerization with several of the opioid receptors (Rios *et al.*, 2006), OX_1 orexin receptors (Ellis *et al.*, 2006) and A_{2A} adenosine receptors (Carriba *et al.*, 2007), with many other combinations yet to be confirmed. It is probable that various 'flavours' of these multimers are expressed by different cells and this amplifies the possibilities for signal-transduction system coupling in different tissues. For example, heterodimers of dopamine D_2 and CB_1 receptors show dramatic increases in MAP kinase activation in response to CB_1 or D_2 agonists (Kearn *et al.*, 2005). It is also possible that heterodimers could have quite different coupling patterns and pharmacological profiles compared to each component receptor. For instance, given the preference of CB_1 receptors for $G_{i/o}$ proteins, the well-known mobilization of intracellular calcium by CB_1 agonists in NG108-15 neuroblastoma glioma cells (Sugiura *et al.*, 1999) was unexpected and it is possible that this requires the dimerization of CB_1 with

another, possibly $G_{q/11}$-linked, 7TM receptor. Similarly, the ability of ECLs to enhance 5-HT receptor function (Thomas *et al*., 1997; Boger *et al*., 1998; Kimura *et al*., 1998; Cheer *et al*., 1999) may well be mediated through plasma membrane-based interactions between molecular targets. Whether G protein-coupled CB receptors can form molecular complexes with receptors of other superfamilies is unknown, as is the potential for CB_2 receptor dimerization. Receptor complex formation might also explain the non-classical pharmacological profiles of some cannabinoid receptors (for example, microglial, abnormal-cannabidiol) without the need to invoke additional novel gene products.

Among the transmitter-gated channels, there is a considerable overlap in the activity that endocannabinoids and their analogues have at the TRPV1 receptor. Indeed, the activity is sufficient that many have also been termed endovanilloids (Di Marzo *et al*., 2001a), including AEA (Zygmunt *et al*., 1999; Smart *et al*., 2000, 2002), OEA (Ahern, 2003), *N*-oleoyldopamine (Chu *et al*., 2003) and NADA (De Petrocellis *et al*., 2004). In contrast, 2AG, PEA, noladin ether and virodhamine have been reported to be much less effective agonists than AEA at TRPV1 channels (Zygmunt *et al*., 1999; De Petrocellis *et al*., 2004; Duncan *et al*., 2004; Ho and Hiley, 2004).

Although, as shown above, cannabinoids almost invariably act in a way that results in inhibition of cell excitation there are a growing number of reports of positive modulation of neuronal activity. For example, Mendiguren and Pineda (2004) showed that micromolar concentrations of AEA or AM404 enhanced the NMDA-induced excitation of locus coeruleus neurones in rat brain slices. Similarly, the synthetic agonists WIN55212-2 and CP55940 enhanced the effect of NMDA. The AEA-induced enhancements were inhibited by the antagonists rimonabant and AM251, indicating CB_1 receptor involvement. Previously, Hampson *et al*. (1998) had demonstrated an intriguing dual effect of AEA, which was able to reduce NMDA-stimulated Ca^{2+} influx into rat brain slices in a manner sensitive to a CB_1 receptor antagonist, pertussis toxin treatment and agatoxin (a calcium channel inhibitor). However, in the presence of CB_1 blockade, AEA potentiated Ca^{2+} entry through NMDA channels in cortical, cerebellar and hippocampal slices. AEA (but not THC) also augmented NMDA-stimulated currents in *Xenopus* oocytes expressing cloned NMDA receptors, and enhanced neurotransmission across NMDA receptor-dependent synapses in hippocampus. Thus, the endocannabinoid seems to be able to enhance NMDA receptor function directly or to inhibit it via CB_1 receptor activation.

AEA and 2-AG have been shown to have receptor-independent inhibitory effects on nicotinic acetylcholine receptor channels (Oz *et al*., 2004). In submicromolar concentrations, the endocannabinoids and methanandamide reversibly inhibited currents generated via acetylcholine-stimulated homomeric α7-nicotinic acetylcholine receptors expressed in *Xenopus* oocytes. A functional relationship between CB_1 receptors and nicotinic channels is implied by the finding that the cognitive effects of nicotine and physostigmine were attenuated in CB_1 knockout mice (Bura *et al*., 2007), but the nature of this interaction and its physiological significance remains to be clarified.

Barann *et al*. (2002) demonstrated that a number of cannabinoids stereoselectively inhibited currents through recombinant human $5\text{-}HT_{3A}$ receptors overexpressed in HEK-293 cells, independently of cannabinoid receptors, and suggested that they might act allosterically at a modulatory site of the $5\text{-}HT_{3A}$ receptor.

Verdon *et al*. (2000) demonstrated that *cis*-ODA (but not *trans*-ODA) reversibly enhanced $GABA_A$ currents and depressed excitatory and inhibitory synaptic activity in cultured networks of embryonic rat neurones. The *cis* isomer stereoselectively blocked veratridine-induced [^{3}H]GABA release from mouse synaptosomes and, produced a marked inhibition of Na^+ channel-dependent increases in intrasynaptosomal Ca^{2+} concentrations. The data support the proposal that ODA is a stereoselective modulator of both postsynaptic $GABA_A$ receptors and presynaptic or somatic voltage-operated Na^+ channels.

Enzymes as loci of signalling convergence

It has been demonstrated that the effects of AEA on TRPV1 receptors in heterologous expression is limited by intracellular metabolism via FAAH and transport out of the cell (De Petrocellis *et al*., 2001a; Price *et al*., 2005). Thus, the effect of newly synthesized AEA in the plasma membrane will depend upon its local concentration in the region of the TRPV1 channels, which in turn will be determined by the rate of catabolism and export from the cell. It is conceivable that CB_1/CB_2 receptors occupied by exported AEA could influence TRPV1 activity and, in this regard, it has been shown that CB_1 activation with a synthetic agonist (HU210) inhibits TRPV1-mediated increases in intracellular Ca^{2+} concentrations in rat DRG neurones (Millns *et al*., 2001), suggesting a negative feedback circuit.

Fatty acid amid hydrolase. There is evidence that PEA downregulates FAAH expression in human breast cancer cells *in vitro* (Di Marzo *et al*., 2001b), possibly through a PPARα-mediated mechanism. Evaluation of the primary sequence of FAAH suggested a putative SH3-binding motif (Giang and Cravatt, 1997), although there is, as yet, no evidence for regulation by polyprolyl-professing protein partners. A lipase-sensitive messenger released from blastocysts has been suggested to activate FAAH activity in the uterus (Maccarrone *et al*., 2004) without altering NAPE-PLD activity, while bacterial lipopolysaccharide treatment of murine macrophage-like RAW 267 cells induced FAAH activity (as well as NAPE-PLD activity) (Liu *et al*., 2003). In these same cells, bacterial lipopolysaccharide and platelet-activating factor (as well as the phytocannabinoid Δ^9-THC) lead to an accumulation of AEA, simultaneous with arachidonate release (presumably reflecting phospholipase A_2 activation) (Pestonjamasp and Burstein, 1998). These two metabolic routes were dissociable, however, as nitric oxide stimulated arachidonate generation without AEA accumulation.

There is evidence for regulation of FAAH levels and activity by both sex and satiety hormones, progesterone and leptin (Gasperi *et al*., 2005), leading to a reduction in endocannabinoid levels in the responsive cells (U937 human lymphoma cells).

In mouse testicular Sertoli cells, follicle-stimulating hormone application evoked a 3- to 5-fold enhancement of FAAH activity over 24 h (Maccarrone *et al*., 2003), due to gene transcription/protein synthesis mechanisms, without any change in CB_2 receptor binding. Subsequent investigation suggested that these effects were mediated via cyclic AMP and protein kinase A (Rossi *et al*., 2007), and that the activity of the *N*-acyltransferase and NAPE-PLD enzymes responsible for AEA synthesis were unaltered (Figure 4a). Also unaffected were agonist binding to TRPV1 receptors, and the synthetic and degradative enzymes for 2AG, diacylglycerol lipase and MAGL (Rossi *et al*., 2007). Given that CB_2 receptors couple to inhibition of cAMP, it could be hypothesized that extracellular ECLs would lead to maintenance of a low level of FAAH activity and thus prolong ECL action.

In the same study, an aromatase-dependent pathway, allowing androgen conversion into estrogen was described, which was dependent on follicle-stimulating hormone receptor activation of phosphatidylinositol 3-kinase (Rossi *et al*., 2007). This pathway appeared to be independent of the cAMP/PKA pathway.

COX-2. OEA administration *in vivo* has been shown to reduce COX-2 expression in the cerebral cortex, presumably through a PPARα-dependent mechanism (Sun *et al*., 2007). In contrast, both AEA and 2AG (but not shorter chain analogues) were reported to increase both COX and 5-LOX activity in human neuroblastoma CHP100 cells (Maccarrone *et al*., 2000), generating agents (imprecisely defined) which inhibited FAAH activity within a timescale of minutes. Given that supposedly neither AEA nor 2AG are substrates of 5-LOX *in vitro* (see above), this phenomenon deserves closer scrutiny.

DAG kinase. PPARγ activation elevates DAG kinase activity (Verrier *et al*., 2004), which may lead to an inhibition of protein kinase C activation, and also reduce the substrate availability for DAG production.

Receptor-evoked modulation of endocannabinoid-like molecules generation

Since AEA synthesis via NAPE-PLD is apparently calcium-activated, van der Stelt *et al*. (2005) were led to hypothesize that AEA could act as a transducer and amplifier of Ca^{2+}-mobilising signals in particular cell types. Consequently, they demonstrated that carbachol- or ATP-generated Ca^{2+} increases in TRPV1 overexpressing HEK-293 cell and in primary cultured DRG neurones could be inhibited by TRPV1 blockade and enhanced by inhibitors of AEA catabolism or transport. The overall increases in Ca^{2+}-mediated AEA synthesis were relatively small in these studies and so this casts some doubt upon the sensitivity of AEA as an amplifier, given its low affinity for TRPV1. However, local concentration changes in the vicinity of the TRPV1 receptors might be much greater and it is possible that there are microdomains within the cell in which related component parts of the amplification mechanism are concentrated. One possible concentrating mechanism is the lipid raft, and McFarland and Barker (2005) have proposed these lipid raft/caveolae structures as microdomains for ECL synthesis. The cellular localization of CB receptors and TRPV channels is still incompletely characterized, however. Alternatively, it is possible that other ECLs or other endocannabinoid-sensitive Ca^{2+}-gated channels could play a role in Ca^{2+} amplification. OEA, for example, can activate TRPV1 under certain circumstances (Ahern, 2003). At submicromolar concentrations other unsaturated C_{18} *N*-acylethanolamines, *N*-linolenoylethanolamine, and *N*-linoleoylethanolamine, but not *N*-stearoylethanolamine and oleic acid, activate native rat TRPV1 on perivascular sensory nerves and with the exception of *N*-linolenoylethanolamine in rat sensory ganglia, the levels of C_{18} *N*-acylethanolamines are equal to or substantially exceed those of AEA (Movahed *et al*., 2005).

Given that stimulation of $G_{q/11}$-coupled $5HT_{2A}$ receptor evoked elevations of 2AG accumulation via PLC activity in a variety of cell types (Parrish and Nichols, 2006), it appears likely that endocannabinoid generation is a ubiquitous consequence of calcium-mobilizing agonists. It is tempting to speculate that this provides a global mechanism for either amplifying, via TRPV1, or inhibiting, via $CB_{1/2}$, Ca^{2+}-mobilizing receptor responses.

Integrating the regulation of the endocannabinoid-like molecules system

Figure 4 is an attempt to collate existing information about the role of four pivotal influences of cell signalling on the endocannabinoid system, as described in the previous section. The cyclic AMP/protein kinase A pathway appears not to affect 2AG metabolism, but enhances FAAH activity (at least in one study (Rossi *et al*., 2007)), which may have much wider implications for AEA (OEA, and so on) turnover (Figure 4a). cAMP/PKA enhances TRPV1 (in some studies, see above) and PPAR function, without altering CB_2 receptor activity. The impact of cAMP/PKA on CB_1 receptor function is unclear. Activation of the protein kinase C pathway enhances TRPV1 and PPAR function, while inhibiting CB_1 receptor activity (Figure 4b). Its influence on the remaining activities is unclear, but an absence of reporting probably reflects a lack of effect. While elevating Ca^{2+} appears to have no effect on FAAH activity and to enhance TRPV1 and (probably) NAPE-PLD function, it appears to have not been studied at the majority of the endocannabinoid system (Figure 4c). The regulation of the endocannabinoid system by ERK activity has been largely ignored, with the exception of subtype and tissue-selective effects on PPAR activity (Figure 4d).

Concluding remarks and implications for therapeutic exploitation of the endocannabinoid-like molecules system

In most signal-transduction systems, biological economy is served mainly by the provision of multiple receptors, often coupled to distinct molecular mechanisms. These receptors constitute the selectivity filters at which most modern

medicines have been aimed. As we have described above, ECLs and their metabolites are capable of acting upon members of three of the four superfamilies of receptor, potentially in the same cell (Table 1). How then is there any opportunity for pharmacological or therapeutic exploitation of the ECL system? Already, receptor ligands are in the clinic exploiting CB_1, TRPV1, PPARα and PPARγ receptors, so which other of these targets hold promise for future therapeutics? At the moment, the remaining receptors are largely unknown quantities in terms of patho-/physiological functions (see review by Brown, this issue). Given that ECLs are generated intracellularly, it is likely that transport and enzyme systems have the greatest influence on the site(s) of action of these entities. How ECLs are exported out of cells to act upon cell-surface 7TM receptors remains a mystery still and it is possible that specific mechanisms exist, which may be exploited pharmacologically in the future. Whether ECLs are targetted at TRPV1 and PPARs by other mechanisms is also obscure.

It is clear that the ECL system represents a major challenge both in our understanding of the complexity of signalling and in attempting to design drugs with selectivity of action; it does also provide an opportunity to develop novel therapeutic agents, probably not with 'magic bullet'-like specificity but more likely with multiple actions targeting different facets of the system. Perhaps it is time to embrace promiscuity!

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Conflict of interest

The authors state no conflict of interest.

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