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MiniReview

The Endocannabinoid System: Current Pharmacological Research and Therapeutic Possibilities

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Abstract: In the relatively short period of time since the discovery of cannabinoid receptors and their endogenous ligands, the endocannabinoids, an intensive research effort has resulted in the identification of agents that affect all aspects of the endocannabinoid system. The cannabinoid$_1$ receptor antagonist rimonabant is in phase III clinical trials for the treatment of obesity and as an aid to smoking cessation, and cannabinoid$_2$ receptor agonists are promising in animal models of inflammatory and neuropathic pain. In the present MiniReview, the endocannabinoid system is described from a pharmacological perspective. The main topics covered are: the mechanism of action of cannabinoid$_2$ receptor agonists; identification of the endocannabinoid(s) involved in retrograde signalling; the elusive mechanism(s) of endocannabinoid uptake; therapeutic possibilities for fatty acid amide hydrolase inhibitors; and the cyclooxygenase-2 and lipoxygenase-derived biologically active metabolites of the endocannabinoids.

The herb *Cannabis sativa* has been harvested for thousands of years because of its usefulness in production of hemp for ropes and textiles, but also for its psychotropic effects and the multitude of therapeutic indications ascribed to it. The use of the herb for medical purposes was described in Egyptian and Chinese sources as early as 2700 BC, revealing that the drug was used for rheumatic pains and other conditions (Adams & Martin 1996). In 1890, Dr J.R. Reynolds, then physician in ordinary to the household of the British Queen Victoria, wrote in his article 'Therapeutical uses and toxic effects of Cannabis indica' that "... Indian hemp, when pure and administered carefully, is one of the most valuable medicines we possess", and later in the article he reported that in his experiments "... it has relieved the lightning pains of the ataxic patient, and also the multiform miseries of tingling, formication, numbness, and other paræsthesiæ so common in gouty people" (Reynolds 1890). The area of "medical marijuana" remains of considerable public interest, albeit often fuelled by somewhat misleading articles in the mass media (Montane *et al.* 2005).

The major active constituent of cannabis, Δ^9-tetrahydrocannabinol (Δ^9-THC) was isolated in 1964 (Gaoni & Mechoulam 1964). Currently nabilone, a structural analogue of Δ^9-THC, is used in parts of the USA and the UK for treatment of chemotherapy-induced nausea and vomiting. Dronabinol, Δ^9-THC itself, is used as an appetite stimulator in AIDS patients in the USA (House of Lords 1998). In their hearing in 1998, the Science and Technology Committee of the UK House of Lords concluded that the large amount of anecdotal evidence as to the therapeutic efficacy of cannabis in multiple sclerosis and chronic pain conditions warranted "as a matter of urgency" investigation in proper clinical trials (House of Lords 1998). Clinical trials of standardised cannabis extracts are now being undertaken for these indications (Wade *et al.* 2003; Zajicek *et al.* 2003; Berman *et al.* 2004), and a new drug based on cannabis extracts (Sativex®) has recently been approved in Canada as adjunctive treatment for the symptomatic relief of neuropathic pain associated with multiple sclerosis. Nonetheless, the usefulness as therapeutic agents of such extracts, of Δ^9-THC itself, or of synthetic compounds with the same pharmacological actions as Δ^9-THC, is greatly hampered by their psychotropic effects (Pryce & Baker 2005). A major goal for many researchers is to find new approaches to harness the therapeutic potential of these cannabinoids without producing unwanted effects. Over the last fifteen years, huge advances in our knowledge of the physiology and pharmacology of the cannabinoid system of the body ("the endocannabinoid system") have taken place. In this MiniReview, we have focussed upon the life cycle of the endocannabinoids and the therapeutic opportunities afforded by agents preventing their breakdown. In addition, some recent developments in selected aspects of cannabinoid pharmacology have been described in Boxes for the reader more familiar with the endocannabinoid field.

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Cannabinoid receptors

In the 1970's and early 1980's, it was generally assumed that the psychotropic effects of cannabis and synthetic cannabinoids were rather unspecific on nerve cell membranes due to their high lipophilicity. However, by the mid 1980's, several groups had shown that cannabinoid activity was highly stereospecific (Razdan 1986) which led to the search for a specific receptor and its endogenous mediators. The first "hard" evidence for receptors was the finding that Δ^9-THC inhibited adenylyl cyclase activity in neuroblastoma cell membranes (Howlett 1984), followed by radioligand binding studies using the synthetic cannabinoid agonist CP55,940 ((-)-*cis*-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-trans-4-(3-hydroxypropyl)cyclohexanol) (Devane *et al.* 1988). Shortly after, the cannabinoid$_1$ receptor was cloned from rat brain and found to be a member of the seven transmembrane G-protein-coupled receptor superfamily (Matsuda *et al.* 1990). A peripherally located receptor was cloned from human promyelocytic leukaemia HL-60 cells and named the cannabinoid$_2$ receptor (Munro *et al.* 1993).

Cannabinoid$_1$ receptors are mainly neuronally located, and mediate the "high" produced by smoked cannabis (Huestis *et al.* 2001). These receptors couple to a variety of signalling pathways, including inhibition of adenylyl cyclase, inhibition of calcium channels and activation of potassium channels (Howlett *et al.* 2002). The ability of cannabinoid$_1$ receptors to inhibit neurotransmitter release, a key action of these receptors in the brain, may be related to actions upon these ion channels rather than upon inhibition of adenylyl cyclase (del Carmen Godino *et al.* 2005). Cannabinoid$_2$ receptors, on the other hand, are located mainly on peripheral immune cells and upon activated microglia, and also couple to the inhibition of adenylyl cyclase (Howlett *et al.* 2002). It is now clear that most of the actions of Δ^9-THC, the synthetic agonists CP55,940, HU-210 ((6aR)-*trans*-3-(1,1-dimethylheptyl)-6a,7,10,10a-tetrahydro-1-hydroxy-6,6-dimethyl-*6H*-dibenzo[b,d]pyran-9-methanol), WIN55,212-2 ((*R*)-(+)-[2,3-dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphthalenylmethanone mesylate), and the endocannabinoids (see below) are brought about via these two receptors, although there is evidence for interactions with other receptor systems, such as 5-HT$_{3A}$ receptors (Barann *et al.* 2002), with the GPR55 orphan receptor (Drmota *et al.* 2004; Sjögren *et al.* 2005; Brown *et al.* 2005), and with as yet unidentified receptors (Begg *et al.* 2005). Time will tell whether or not GPR55 is the long-awaited "cannabinoid$_3$" receptor.

The pharmacology of the cannabinoid$_1$ and cannabinoid$_2$ receptors is summarised in table 1. In terms of drug development, the cannabinoid$_1$ receptor antagonist rimonabant has progressed furthest and is in late phase III trials for the treatment of obesity and as an aid for smoking cessation (Cleland *et al.* 2004; Van Gaal *et al.* 2005). Non-selective and cannabinoid$_1$ receptor-selective agonists are likely to suffer from the same psychotropic side effects as Δ^9-THC, although therapeutic avenues for such compounds include their acute use in conditions where this is of secondary importance, such as following acute subdural haematoma (Mauler *et al.* 2002), and the use of cannabinoid$_1$

Table 1.

The pharmacology of the CB_1 and CB_2 receptors.

	CB_1 receptor	CB_2 receptor
Localisation	Mainly neurons in the CNS and periphery	Peripheral immune cells, activated microglia in spinal cord and CNS
Function in the CNS	↓ transmitter release	Role in neuroinflammation
Signal transduction	↓ cAMP, ↑ K^+ and ↓ Ca^{2+} conductances, MAPK activation	↓ cAMP, MAPK activation
Examples of selective agonists	ACEA	HU-308, GW405833, AM1241, JWH133
Examples of selective antagonists/inverse agonists	Rimonabant, AM251, LY320135	AM630, SR144528
Compounds in drug development	Selective antagonists/inverse agonists for treatment of obesity and an aid to smoking cessation	Selective agonists for the treatment of inflammatory and neuropathic pain
Possibilities for drug development (a selection)	Antagonists/inverse agonists for treatment of drug addiction and osteoporosis; peripheral agonists for treatment of pain; topical agonists for treatment of glaucoma	Agonists for treatment of: Alzheimer's disease, motility disorders secondary to intestinal inflammation, atherosclerosis
Selected recent references	Howlett *et al.* (2002); Freund *et al.* (2003); Di Marzo *et al.* (2004); Fride *et al.* (2004); Lange & Kruse (2004); Walter & Stella (2004); Idris *et al.* (2005); Lupica & Riegel (2005); Ramírez *et al.* (2005); Steffens *et al.* (2005); Valenzano *et al.* (2005); Van Gaal *et al.* (2005)	

Abbreviations: ACEA, *N*-(2-chloroethyl)-5*Z*,8 *Z*,11 *Z*,14 *Z*-eicosatetraenamide, AM251, *N*-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1 *H*-pyrazole-3-carboxamide; AM630, 6-iodo-2-methyl-1-[2-(4-morpholinyl)ethyl]-1H-indol-3-yl](4-methoxyphenyl) methanone; AM1241, 2-iodo-5-nitrophenyl)-(1-(1-methylpiperidin-2-ylmethyl)-1H-indol-3-yl)methanone; GW405833, 1-(2,3-dichlorobenzoyl)-5-methoxy-2-methyl-(2-(morpholin-4-yl)ethyl)-1H-indole; HU-308, 4-[4-(1,1-dimethylheptyl)-2,6-dimethoxyphenyl]-6,6-dimethyl-bicyclo [3.1.1]hept-2-ene-2-methanol; JWH133, (6a*R*,10a*R*)-3-(1,1-dimethylbutyl)-6a,7,10,10a-tetrahydro-6,6,9-trimethyl-6*H*-dibenzo[b,d]pyran; LY320135, 6-methoxy-2-(4-methoxyphenyl)benzo[*b*]thien-3-yl][4-cyanophenyl]methanone; rimonabant (SR141716A; N-piperidino-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-3-pyrazole-carboxamide), SR144528, N-[(1S)-endo-1,3,3-trimethyl bicyclo [2.2.1] heptan-2-yl]-5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)-pyrazole-3-carboxamide.

agonists that do not cross the blood brain barrier for the treatment of pain (Fride *et al.* 2004). Cannabinoid$_2$ receptor agonists have been more actively pursued, in particular for the treatment of pain conditions (see Box 1), although other possible indications include the treatment of atherosclerosis, certain types of glioma, glaucoma, and Alzheimer's disease (Velasco *et al.* 2004; Steffens *et al.* 2005; Ramírez *et al.* 2005; Zhong *et al.* 2005).

Box 1. Anti-nociceptive and anti-inflammatory effects of cannabinoid$_2$ receptor agonists – insights into mechanism(s) of action

It has long been known that cannabinoid receptor agonists have effects in a number of models of inflammatory and even neuropathic pain, and that these effects are brought about at supraspinal, spinal and peripheral levels (Pertwee 2001). The lack of psychotropic effects of cannabinoid$_2$ receptor agonists has made them an interesting group for development of new antinociceptive agents, and there are now a number of reports indicating that such compounds have efficacy in a variety of models of inflammation, inflammatory and neuropathic pain (Hanus *et al.* 1999; Malan *et al.* 2001, Clayton *et al.* 2002; Ibrahim *et al.* 2003; Hohmann *et al.* 2004; Valenzano *et al.* 2005, and references cited therein). The mechanism of action of these compounds is only beginning to be understood, but there is evidence that it may be indirect in nature:

- Cannabinoid$_2$ agonists reduce the oedema produced by skin mast cell degranulation *in vivo*, whereas the *in vitro* release of the granular protein β-hexosaminidase in skin slices is not affected by such compounds (Jonsson *et al.* 2006). This is consistent with an indirect mode of action, and there is immunochemical evidence that in the mouse skin the receptors are located in the epidermal, rather than the dermal layer (fig. 1).
- Activation of cannabinoid$_2$ receptors by the selective agonist AM1241 (structure given in table 1) results in the release of endogenous opioids from skin keratinocytes (Ibrahim *et al.* 2005). Furthermore, the efficacy of this compound in a test of thermal analgesia is blocked by the opioid receptor antagonist naloxone (Ibrahim *et al.* 2005). Whether such a mechanism of action is operative in inflammatory or neuropathic pain awaits elucidation. However, Wotherspoon *et al.* (2005) have reported that in the rat, L5 spinal nerve ligation or sciatic nerve section results in the appearance of cannabinoid$_2$ receptor immunoreactivity in the superficial laminae of the ipsilateral lumbar spinal cord. A similar result was seen in wild type mice, but not in cannabinoid$_2$ receptor knockout mice (Wotherspoon *et al.* 2005), which would suggest that the immunoreactivity seen was a true labelling of the cannabinoid$_2$ receptor (see legend to fig. 1 for a further discussion on cannabinoid$_2$ antibody specificity). The immunoreactivity, surprisingly, did not colocalize with microglia or astroglial cells, but rather with sensory neurone afferent terminals, leading the authors to conclude that "The direct demonstration ... of cannabinoid$_2$ expression on C fibers and an upregulation following nerve damage gives us cause to re-evaluate immune cells and keratinocytes as the main target of cannabinoid$_2$ agonists for attenuation of pain responses" (Wotherspoon *et al.* 2005).

Endocannabinoids

In 1992, the first endogenous compound to exert activity at cannabinoid$_1$ receptors, arachidonoylethanolamide, an *N*-acylethanolamine, was extracted from pig brain and named anandamide after the Sanskrit word for bliss, ananda (Devane *et al.* 1992; for structure, see fig. 2). Three years later, 2-arachidonoylglycerol, a monoacylglycerol involved as an intermediate in a variety of signalling pathways, was reported to interact with cannabinoid receptors (Mechoulam *et al.* 1995; Sugiura *et al.* 1995). This compound is present in the brain in higher concentrations than anandamide, but since it is also involved as an intermediate in a number of signalling pathways, the amount of 2-arachidonoylglycerol involved in endocannabinoid signalling may be similar to that of anandamide. It is believed that 2-arachidonoylglycerol has a lower affinity, but higher efficacy for cannabinoid receptors than anandamide (Howlett *et al.* 2002), although the observed efficacies and/or potencies may be dependent upon the G-protein subunit involved in the coupling (as has been shown for methanandamide, the stable analogue of anandamide, Mukhopadhyay & Howlett 2005) and/or the effector system measured (as has been shown for 2-arachidonoylglycerol, Shoemaker *et al.* 2005). In addition, anandamide is by no means a specific ligand for cannabinoid receptors, and can activate other targets such as TRPV1 receptors (Zygmunt *et al.* 1999) and PPARγ (Bouaboula *et al.* 2005).

Other endogenous compounds, including dihomo-γ-linolenoylethanolamide and docosatetraenoylethanolamide (Hanus *et al.* 1993), 2-arachidonoylglyceryl ether (noladin ether, Hanus *et al.* 2001), *O*-arachidonoyl-ethanolamine (virhodamine; Porter *et al.* 2002), *N*-arachidonoyl-dopamine (NADA; Bisogno *et al.* 2000) and oleamide (Leggett *et al.* 2004), have also been found to interact with cannabinoid receptors with differing potencies and efficacies, although it is not yet clear whether they act as endocannabinoids in the body (for a recent review on the pharmacology of these and related compounds, see Bradshaw & Walker 2005). Indeed, NADA is more likely to be an 'endovanilloid' than an endocannabinoid (Huang *et al.* 2002).

The synthesis of the endocannabinoids has been reviewed elsewhere (Freund *et al.* 2003), and so will only be dealt with briefly here. The main route for *N*-acylethanolamine synthesis from membrane phospholipids is by a two step reaction, where a fatty acyl chain is transferred in a Ca^{2+}-dependent manner from a phospholipid to a phosphatidyl ethanolamine, forming an *N*-acyl phosphatidyl ethanolamine (NAPE). The NAPE is then hydrolysed to its corresponding *N*-acylethanolamine by a phosphodiesterase of the phospholipase D-type (NAPE-PLD), an enzyme that has recently been cloned (Okamoto *et al.* 2004) or alternatively by phospholipase A_2 and a lysophospholipase D (Natarajan *et al.* 1984; Sun *et al.* 2004). In RAW264-7 macrophages, however, lipopolysaccharide-induced anandamide production appears mainly to involve a pathway whereby NAPE is hydrolysed to yield a phospho-AEA, which is then dephosphorylated (Liu *et al.* 2005). 2-Monoacylglycerols can be synthesised by several different pathways (Freund *et al.* 2003), but, as with the *N*-acylethanolamine synthetic pathway, synthesis can be regulated by calcium and other stimuli. The calcium dependency allows for regulation of

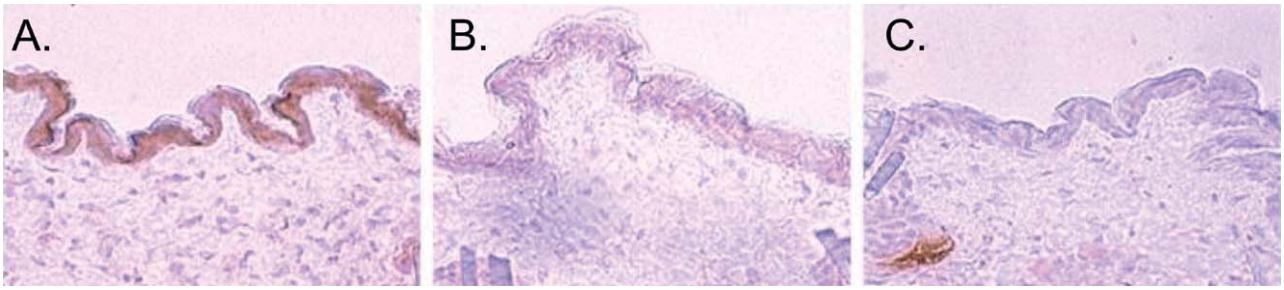

Fig. 1. Immunoreactivity of mouse skin samples seen using a commercially available antibody raised against a sequence (a.a. 20–33; NPMKDYMILSGPQK) between the N-terminus and the first transmembrane domain of the human cannabinoid$_2$ (CB_2) receptor (http://www.caymanchem.com/neptune/servlet/neptune/template/Product.vm/catalog/101550/a/z). Immunoreactivity was detected in the epidermis rather than in dermis, where skin mast cells are located (Panel A). The immunoreactivity could be blocked by preabsorption of the antibody with immunising peptide at 1:1 for 1 hr (Panel B) and was not seen for samples treated with the secondary antibody alone (Panel C). (Jonsson, Ny & Egelrud, unpublished data). A similar localisation has been seen by others using both this antibody (Casanova *et al.* 2003) and an antibody from a different source (Ibrahim *et al.* 2005). Of course, these data are dependent upon the specificity of the antibody used, and although Panel B is a useful indicator of specificity, it is by no means absolute proof. However, Steffens *et al.* (2005) have reported that in their hands, the antibody used here shows immunoreactivity in the spleen of wild type, but not $CB_2^{-/-}$ mice. Wotherspoon *et al.* (2005) were also able to demonstrate spleen immunoreactvity preventable by antibody preabsorbtion with peptide immunogen, as well as immunoreactivity in CHO cells expressing CB_2 receptors (but not the control cells). We, however, were unable to detect obvious immunoreactivity in our spleen samples from wild type mice (data not shown), so the sensitivity of the antibody appears to be highly dependent upon the conditions used.

anandamide and 2-arachidonoylglycerol synthesis, and there are several reports showing that their synthesis is increased both in physiological conditions (i.e. following depolarising stimuli and receptor-mediated activation of the inositol phospholipid signalling pathway (Stella & Piomelli 2001; Jung *et al.* 2005; van der Stelt *et al.* 2005)) and under conditions of cellular damage, such as is seen after neurotoxic insult, trauma or in models of multiple sclerosis or amyotrophic lateral sclerosis (Baker *et al.* 2001; Panikashvili *et al.* 2001; Berger *et al.* 2004; Witting *et al.* 2004). Anandamide is also produced in the periaqueductal gray region after nociceptive input (Walker *et al.* 1999). There is also data to suggest that inflammation also results in an increased rate of anandamide synthesis (Kondo *et al.* 1998; McVey *et al.* 2003; Dinis *et al.* 2004), but this may in some cases be a consequence of the accompanying cell damage rather than the inflammation per se (Holt *et al.* 2004).

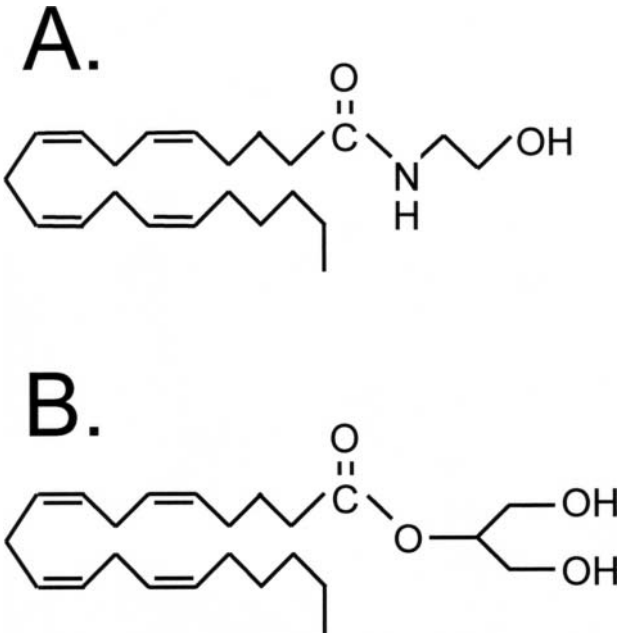

Fig. 2. Structures of A. anandamide (AEA) and B. 2-arachidonoylglycerol (2-AG).

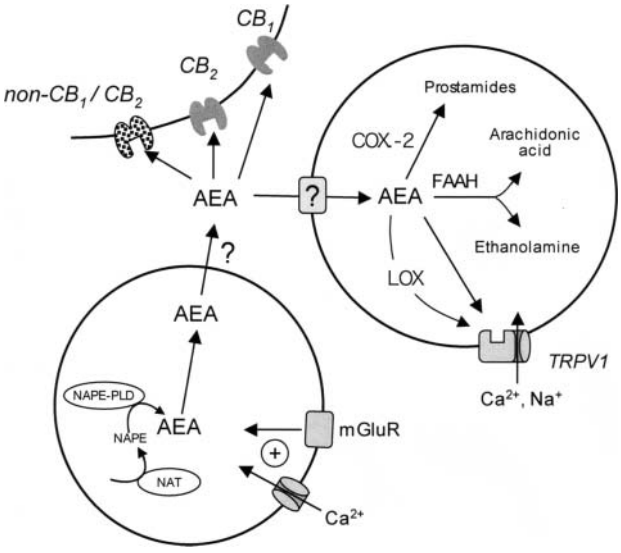

Fig. 3. The "life cycle" of anandamide (AEA) showing calcium and metabotropic glutamate receptor regulated synthesis, release and interaction with receptors, cellular reuptake and metabolism. AEA interacts with the intracellular side of the TRPV1 receptor. It should be stressed that the picture is an oversimplification of a somewhat complicated system: synthesis stimulated by other metabotropic receptors, actions of AEA upon TRPV1 receptors present in the same cell in which AEA is synthesised, and actions of AEA upon other biological targets have not been represented. Abbreviations. CB=cannabinoid, COX=cyclooxygenase, FAAH=fatty acid amide hydrolase, LOX=lipoxygenase.

Endocannabinoids are released by an as yet unclear mechanism. After interaction with the receptors, their action is terminated by cellular reuptake, followed by metabolism, mainly by the enzymes fatty acid amide hydrolase and monoacylglycerol lipase, but also by cyclooxygenase-2 and lipoxygenase enzymes (Kozak & Marnett 2002; Fowler 2004). The "life cycle" of anandamide is shown schematically in fig. 3. There are now pharmacological agents available that interfere with the synthesis and degradation of endocannabinoids, and these have provided useful information as to the physiological roles played by these mediators. As an example of this, the identity of the endocannabinoids involved in retrograde signalling in the brain is discussed in Box 2. The current status of the field with respect to the mechanism(s) governing anandamide uptake are summarised in Box 3, and the role of cyclooxygenase and lipoxygenase enzymes in endocannabinoid metabolism are discussed in Box 4.

Therapeutic opportunities for fatty acid amide hydrolase inhibitors

Fatty acid amide hydrolase is the most important enzyme involved in the removal of anandamide, and fatty acid amide hydrolase$^{-/-}$ mice show fifteen times higher brain levels of this endocannabinoid than their wild-type litter mates (Cravatt *et al.* 2001). In addition fatty acid amide hydrolase has a wide substrate specificity and can metabolise endogenous compounds such as palmitoylethanolamide and arachidonoylglycine, compounds which are known to have potentially beneficial effects upon pain and inflammation (Bradshaw & Walker 2005; Darmani *et al.* 2005). Fatty acid amide hydrolase$^{-/-}$ mice do not show overt signs of general central cannabinoid receptor activation, but show hypoalgesia in thermal nociceptive tests and in the formalin test of inflammatory pain, and have a reduced oedema and hyperalgesia following intraplantar carrageenan injection (Cravatt *et al.* 2001; Lichtman *et al.* 2004a). Fatty acid amide hydrolase$^{-/-}$ mice are also less sensitive to colitis produced by intrarectal administration of 2,4-dinitrobenzene sulfonic acid than their wild-type litter mates (Massa *et al.* 2004). In contrast, the fatty acid amide hydrolase$^{-/-}$ mice did not behave differently from their litter mates in the sciatic nerve chronic constriction injury model (Lichtman *et al.* 2004a). The relative involvement of central and peripheral fatty acid amide hydrolase in these effects was recently investigated using a transgenic mouse model (fatty acid amide hydrolase-NS) where fatty acid amide hydrolase expression was

Box 2. Unravelling the identity of endocannabinoid retrograde signalling molecules

It is now well established that an important function of the endocannabinoid signalling system in the brain is to regulate the release of neurotransmitters such as GABA and glutamate. Electrophysiologists studying the phenomena of depolarisation-induced suppression of inhibition and depolarisation-induced suppression of excitation demonstrated initially that endocannabinoids were involved. In the currently accepted model, the depolarising stimulus produces a synthesis and release of endocannabinoids from the postsynaptic cell. This then stimulates presynaptic cannabinoid$_1$ receptors which in turn acts to reduce the release of the presynaptic transmitter (Diana & Marty 2004). Whilst the phenomenon itself has been much studied, and there is evidence in hippocampal slices that phospholipase Cβ1 acts as a "coincidence detector" for production of endocannabinoid in response to depolarising- and $G_{q/11}$-mediated receptor-stimuli (Hashimotodani *et al.* 2005), it has not been until recently that the identity of the endocannabinoid involved has been clarified. Several separate approaches have suggested that 2-arachidonoylglycerol may be more important in this respect:

- Neural activity stimulates 2-arachidonoylglycerol production in hippocampal slices and exogenously added 2-arachidonoylglycerol modulates the electrophysiological properties of the hippocampal neurons (Stella *et al.* 1997; Hajos *et al.* 2004; Jung *et al.* 2005). In cerebellar slices, the combination of a depolarising stimulus (20 mM K^+) and a group I mGluR agonist, R,S-3,5-dihydroxyphenylglycine (10 μM) increased levels of 2-arachidonoylglycerol whereas either stimulus alone was without effect (Maejima *et al.* 2005).
- In hippocampal slices, exogenous 2-arachidonoylglycerol mimics the effect of WIN55212-2 in reducing paired-pulse depression, whereas anandamide produces a TRPV1-mediated increase over the concentration range tested (1–30 μM) (Al-Hayani *et al.* 2001).
- Monoacylglycerol lipase (the enzyme mainly responsible for 2-arachidonoylglycerol metabolism in the brain) is located presynaptically, whereas the anandamide metabolising enzyme fatty acid amide hydrolase is located postsynaptically (Gulyas *et al.* 2004). In addition there is a mismatch between the postnatal development of fatty acid amide hydrolase and cannabinoid$_1$ receptors in the hippocampus (Morozov *et al.* 2004). On the basis of phylogenetic and chemotaxonomic data, McPartland (2004) has argued that fatty acid amide hydrolase evolved at the time where TRPV1 receptors acquired its affinity for anandamide whereas monoacylglycerol lipase may be an older molecule. These data can be rationalised to suggest that the highly lipophilic endocannabinoid molecule is removed by uptake into the presynaptic component following association with the presynaptic cannabinoid$_1$ receptor rather than traversing back to the postsynaptic side.
- Inhibition of 2-arachidonoylglycerol synthesis results in a loss of suppression of excitation in dopaminergic neurones (Melis *et al.* 2004) and a reduction of depolarisation-induced suppression of inhibition in Purkinje cells from the cerebellum (Szabo *et al.* 2005). Conversely, inhibition of monoacylglycerol lipase potentiated depolarisation-induced suppression of inhibition in hippocampal pyramidal cells (Makara *et al.* 2005). The fatty acid amide hydrolase inhibitor URB597, on the other hand, did not affect either cerebellar nor hippocampal depolarisation-induced suppression of inhibition (Kim & Alger 2004; Makara *et al.* 2005; Szabo *et al.* 2005).

It would, however, be premature to suggest that 2-arachidonoylglycerol is the only endocannabinoid involved in retrograde signalling. In the striatum, for example, microdialysis experiments have found that a high K^+-pulse produces a large increase in anandamide release without a corresponding change in the release of 2-arachidonoylglycerol (Giuffrida *et al.*, 1999). In the amygdala, long-term depression of inhibitory GABAergic synaptic transmission is enhanced in mice lacking fatty acid amide hydrolase (Azad *et al.* 2004). A selective inhibitor of anandamide synthesis is sorely needed, since such a compound would greatly aid our understanding of the extent to which this endocannabinoid is involved in retrograde signalling in the brain.

Box 3. Cellular accumulation of anandamide – the current state of the art

Seldom have experiments that are at first sight relatively easy to undertake, caused such controversy as is the case for anandamide reuptake (see Glaser *et al.* 2005 for a thorough review of the potential pitfalls associated with assay of anandamide uptake). Following early suggestions that the uptake of anandamide was mediated by an energy and sodium-independent process of facilitated diffusion, much effort has been made toward revealing the mechanism(s) involved. There is today no clear consensus in this matter, although a number of different models have been proposed, including a) facilitated diffusion mediated by a membrane carrier protein (Di Marzo *et al.* 1994; Hillard *et al.* 1997); b) simple diffusion across the membrane driven by removal of the accumulated anandamide either by metabolism (Deutsch *et al.* 2001; Glaser *et al.* 2003) or by intracellular sequestration (Hillard & Jarrahian 2003); c) bidirectional carriers (that may or may not be specifically designated to the task) that shuttle newly synthesised anandamide from the endoplasmic reticulum to the plasma membrane and in the reverse direction for the removal of anandamide (Fowler *et al.* 2004; Ortega-Gutiérrez *et al.* 2004); and d) endocytotic internalisation of anandamide (McFarland *et al.* 2004). With respect to (c), exchange efflux measurements undertaken using resealed human red blood cells indicate that anandamide can traverse the red blood cell membrane very rapidly at 0° (Bojesen & Hansen 2005) and so incubation times of 5–10 min. commonly used in uptake experiments may be investigating events subsequent to plasma membrane translocation. On the other hand, evidence in favour of a plasma membrane transporter was provided by Oddi *et al.* (2005), who demonstrated that reconstituted plasma membrane vesicles were able to accumulate anandamide, whereas microsomal vesicles were not, and this pattern mirrored the levels of cholesterol and calveolin-1, but differed from that of fatty acid amide hydrolase (Oddi *et al.* 2005). In addition, the use of a novel radioligand with high potency as an inhibitor of uptake of anandamide was able to label a binding site in membranes prepared from RBL-2H3 cells in a manner that was inhibited by anandamide (Moore *et al.* 2005). However, as argued by Hillard & Jarrahian (2005), it is likely that different cells use different uptake mechanisms. These authors demonstrated that the pharmacological properties of cerebellar granule neurones and glioma cells differed with respect to inhibition of anandamide uptake, particularly by ethylarachidonoylamide and arachidonic acid. They suggested that mammalian cells are likely to have non-selective scavenging methods to conserve arachidonic acid (i.e. produced from anandamide by the action of fatty acid amide hydrolase) whereas cells using endocannabinoids specifically as signalling molecules would be expected to have designated processes for their reuptake (Hillard & Jarrahian 2005). Watch this space for further developments!

Despite the elusive nature of the transporter(s), there are now a number of compounds available that can prevent the accumulation of anandamide. These compounds potentiate the effects of anandamide *in vivo* (Fowler *et al.* 2005) and may be useful in conditions such as multiple sclerosis where they bolster up endocannabinoid tone that in itself is increased by the neuroinflammation (Baker *et al.* 2001). Most of these are arachidonoyl- or oleoyl-based compounds, although a few non-acyl based compounds have been reported (Hopkins & Wang 2004; Moore *et al.* 2005).

restricted to the central nervous system. The levels of anandamide (and the related *N*-acylethanolamine palmitoylethanolamide and oleoylethanolamide) were normal in the CNS and spinal cord but elevated in the periphery, in contrast to the fatty acid amide hydrolase$^{-/-}$ mice where a general increase was seen throughout the body (Cravatt *et al.* 2001 & 2004). The reduced oedema response to carrageenan was retained in the fatty acid amide hydrolase-NS mice, whereas the antihyperalgesic effects in tests of thermal nociception were lost (Cravatt *et al.* 2004).

Since the fortituitous discovery in 1993 that fatty acid amide hydrolase could be inhibited by phenylmethylsulfonyl fluoride (Deutsch & Chin 1993), a number of compounds have been identified as fatty acid amide hydrolase inhibitors (Fowler 2004). The most well characterized of these compounds, URB597 (cyclohexylcarbamic acid 3′ carbamoylbiphenyl-3-yl ester) and OL-135 (1-oxo-1[5-(2-pyridyl)-2-yl]-7-phenylheptane) have been found to produce effects that mirror the pattern seen with the fatty acid amide hydrolase$^{-/-}$ mice, namely increases in brain levels of anandamide, beneficial effects upon thermal and inflammatory pain and the oedema response to carrageenan without overt signs of general central CB_1 receptor activation (Kathuria *et al.* 2003; Lichtman *et al.* 2004b; Holt *et al.* 2005; Wilson *et al.* 2005). URB597 also enhances stress-induced analgesia in rats (Hohmann *et al.* 2005). In contrast, URB597 is less effective in the sciatic nerve chronic constriction injury model (Costa *et al.* 2005). When the knockout and inhibitor data are taken together, they suggest that fatty acid amide hydrolase inhibitors may be useful for the treatment of inflammation and inflammatory pain, but probably not neuropathic pain disorders. Other possible therapeutic areas include anxiety (Kathuria *et al.* 2003), multiple sclerosis (Baker *et al.* 2001), neuroprotection (Fowler 2003; Karanian *et al.* 2005) and certain forms of cancer (Bifulco *et al.* 2004), but as always, the transition from promising experimental data to positive results in clinical trials will never be as straightforward as preclinical pharmacologists such as the present authors like to believe!

Conclusions

In the relatively short time since the isolation of anandamide, the endocannabinoid field has literally exploded. As an example of this, a simple PubMed search with search word "cannabinoid" gave 112, 190, 360 and 593 hits each for the individual years 1992, 1996, 2000 and 2004, respectively. Pharmacological tools and in some cases genetically modified animals are available for many components of the cannabinoid system, but there are still important gaps in our knowledge. The mechanisms involved in the release and reuptake of endocannabinoids remain unclear, and selective compounds blocking the synthesis of anandamide have not been reported in the literature. In contrast to the situation for fatty acid amide hydrolase, the functional significance of monoacylglycerol lipase, the main enzyme for metabolism of 2-arachidonoylglycerol in the brain (Dinh *et al.* 2002), has not yet been probed by the use of knockout animals. The identity and structure of the additional "non-cannabinoid$_1$, non-cannabinoid$_2$" receptors awaits elucidation, although the identification of GPR55 as a receptor

Box 4. Cyclooxygenase-2 and lipoxygenase derived metabolites of anandamide and 2-arachidonoylglycerol

Whilst the main route of metabolism of anandamide and 2-arachidonoylglycerol is by hydrolysis to arachidonic acid, there is increasing evidence that cyclooxygenase-2 and lipoxygenase pathways may be important, not so much as for the removal of these endocannabinoids (although there is evidence in the hippocampus that COX-2 inhibition potentiates endocannabinoid retrograde signalling, Kim & Alger 2004), but in the production of biologically active compounds. In the case of the lipoxygenase pathway, relatively little is known, although there are reports that lipoxygenase derived metabolites of anandamide and 2-arachidonoylglycerol have actions at TRPV1 receptors and peroxisome proliferator activated receptor α, respectively (Craib *et al.* 2001; Kozak *et al.* 2002).

A number of effects of prostamides (prostaglandin ethanolamides, i.e. the compounds derived from cyclooxygenase-2 metabolism of anandamide) have been reported, including inhibition of contraction of the cat iris sphincter (Matias *et al.* 2004), and there is some evidence that this is mediated by a receptor different from those involved in the action of prostaglandins: both since prostamides interact weakly with these receptors (Ross *et al.* 2002; Matias *et al.* 2004), and since an antagonist of the actions of prostamide $F_{2\alpha}$ upon the cat iris sphincter has been discovered (Woodward *et al.* 2005). A cyclooxygenase product of anandamide also inhibits secretion of interleukin-2 from primary splenocytes (Rockwell & Kaminski 2004) and produces pulmonary hypertension in isolated rabbit lungs (Wahn *et al.* 2005). Although prostamide levels are difficult to detect, unless fatty acid amide hydrolase activity has been removed (Weber *et al.* 2004; Woodward *et al.* 2004), a recent study has reported that standard immuno-based assays of prostaglandins will also detect the corresponding prostamides and that the apparent production of prostaglandin E_2 following stimulation of a human amnion-derived cell line with anandamide in the presence of interleukin-1β was in fact due to production of the corresponding prostamide (Glass *et al.* 2005). Less is known about cyclooxygenase-2-derived metabolites of 2-arachidonoylglycerol, which can be produced from lipopolysaccharide-treated macrophages in response to zymosan phagocytosis (Rouzer & Marnett 2005), although the glyceryl ester of prostaglandin E_2 does affect the inositol phospholipid signalling pathway in RAW 264.7 macrophage cells (Nirodi *et al.* 2004) and the contraction of the cat iris sphincter (in a manner not blocked by the prostamide antagonist described above) *in vitro* (Woodward *et al.* 2005).

Given that the endocannabinoids can interact with cyclooxygenase-2, it is perhaps not surprising that compounds that inhibit cyclooxygenase enzymes also have effects upon the endocannabinoid system. Thus, the non-selective cyclooxygenase inhibitors indomethacin and flurbiprofen, when administered spinally, reduce the response to formalin injection in the mouse in a manner that can be blocked by cannabinoid$_1$ receptor antagonists or by genetic deletion of the cannabinoid$_1$ receptor (Gühring *et al.* 2002; Ates *et al.* 2003). The ability of systemically administered indomethacin to prevent the oedema response to injection of carrageenan into the paw of anaesthetised mice is blocked by the cannabinoid$_2$ receptor antagonist SR144528 (Holt *et al.* 2005). Ibuprofen and flurbiprofen inhibit the activity of fatty acid amide hydrolase in intact cells, particularly at low extracellular pH (i.e. at pH seen in inflamed tissue) (Holt & Fowler 2003), although it is not as yet established whether this contributes to the actions of these compounds *in vivo*.

A final note concerns the recent report that the analgesic and antipyretic agent acetaminophen is transformed via deacylation followed by fatty acid amide hydrolase-dependent arachidonic acid conjugation to give the anandamide transport inhibitor/TRPV1 agonist AM404 (*N*-(4-hydroxyphenyl)arachidonoylamide) (Högestätt *et al.* 2005). These authors showed further that AM404 could inhibit the activity of both cyclooxygenase-1 and cyclooxygenase-2 *in vivo*. Whether or not this pathway contributes to the actions of acetaminophen awaits elucidation. Nonetheless, the study further demonstrates the close link between the cyclooxygenase and endocannabinoid systems. In addition, the study is consistent with the suggestion that fatty acid amide hydrolase can act in the reverse direction as a synthase (both AM404 and the closely related uptake inhibitor VDM11 [*N*-(4-hydroxy-2-methylphenyl) arachidonoylamide] are hydrolysed by fatty acid amide hydrolase (Fegley *et al.* 2004; Vandevoorde & Fowler 2005) *in vivo*, an action that has previously been given little attention due to the high non-physiological substrate concentrations required to drive the reaction in that direction.

activated by cannabinoids may hopefully be a major breakthrough. In other words, it is likely that interest in the endocannabinoid system and compounds that modulate its function is likely to increase.

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