HEP (2005) 168:283–297

The Phylogenetic Distribution and Evolutionary Origins of Endocannabinoid Signalling

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Abstract The endocannabinoid signalling system in mammals comprises several molecular components, including cannabinoid receptors (e.g. CB_1, CB_2), putative endogenous ligands for these receptors [e.g. anandamide, 2-arachidonoylglycerol (2-AG)] and enzymes involved in the biosynthesis and inactivation of anandamide (e.g. NAPE-PLD, FAAH) and 2-AG (e.g. DAG lipase, MGL). In this review we examine the occurrence of these molecules in non-mammalian organisms (in particular, animals and plants) by surveying published data and by basic local alignment search tool (BLAST) analysis of the GenBank database and of genomic sequence data from several vertebrate and invertebrate species. We conclude that the ability of cells to synthesise molecules that are categorised as "endocannabinoids" in mammals is an evolutionarily ancient phenomenon that may date back to the unicellular common ancestor of animals and plants. However, exploitation of these molecules for intercellular signalling may have occurred independently in different lineages during the evolution of the eukaryotes. The CB_1- and CB_2-type receptors that mediate effects of endocannabinoids in mammals occur throughout the vertebrates, and an orthologue of vertebrate cannabinoid receptors was recently identified in the deuterostomian invertebrate *Ciona intestinalis* (CiCBR). However, orthologues of the vertebrate cannabinoid receptors are not found in

protostomian invertebrates (e.g. *Drosophila, Caenorhabditis elegans*). Therefore, it is likely that a CB_1/CB_2-type cannabinoid receptor originated in a deuterostomian invertebrate. This phylogenetic information provides a basis for exploitation of selected non-mammalian organisms as model systems for research on endocannabinoid signalling.

Keywords Cannabinoid · Anandamide · 2-Arachidonoylglycerol · Deuterostome · Protostome

1 Introduction

Cannabinoid receptors are activated by Δ^9-tetrahydrocannabinol, the main psychoactive constituent of the drug cannabis (Howlett et al. 2002). Two G protein-coupled cannabinoid receptors have been identified in humans and other mammals and are known as CB_1 and CB_2 (Matsuda et al. 1990; Munro et al. 1993). CB_1 is expressed by neurons and mediates effects of cannabis on the central nervous system (CNS) whereas CB_2 is associated with cells in the immune system. Following the discovery of CB_1 and CB_2, putative endogenous ligands for these receptors were isolated from mammalian tissues and identified as derivatives of arachidonic acid. The first "endocannabinoid" to be characterised was arachidonoylethanolamide ("anandamide"; Devane et al. 1992) followed by 2-arachidonoylglycerol (2-AG; Mechoulam et al. 1995; Sugiura et al. 1995). With these discoveries the concept of an endocannabinoid signalling system in mammals has emerged. Moreover, the physiological roles of the endocannabinoid signalling system in mammals are beginning to be elucidated. Recently it was established that endocannabinoids and the CB_1 receptor mediate retrograde signalling at synapses in the brain (Wilson and Nicoll 2002; Kreitzer and Regehr 2002), confirming a hypothesis first put forward by Egertová et al. (1998) and elaborated on by Elphick and Egertová (2001).

The purpose of this article is not to review research on endocannabinoid signalling in mammals, as this topic is covered in detail in other chapters of this volume and in other recent reviews (Freund et al. 2003; Piomelli 2003). The aim here is to examine the phylogenetic distribution and evolutionary origins of the molecular components that are recognised as constituents of the endocannabinoid signalling system in mammals. This is not the first article to discuss the evolution of endocannabinoid signalling; several reviews on comparative aspects of cannabinoid biology have been published in recent years, including: Salzet et al. (2000), Elphick and Egertová (2001), Salzet and Stefano (2002) and McPartland and Pruitt (2002). What then justifies writing another? First, important discoveries have been made since the last review appeared. Second, there are conflicting views on interpretation of some published data. For this review we will largely restrict our analysis to eukaryotes and in particular animals and plants, although in doing so we do not presume that some elements of the endocannabinoid signalling system in mammals might not have their origins in more ancient prokaryotic organisms.

To investigate the phylogenetic distribution of proteins that could mediate the biosynthesis, inactivation and physiological effects of endocannabinoids in non-mammalian organisms, in addition to surveying published papers, we have employed the basic local alignment search tool (BLAST; Altschul et al. 1990) to analyse the GenBank database and databases specifically associated with genome sequencing projects, using mammalian endocannabinoid-related proteins as search sequences. The primary focus for these searches were several non-mammalian animal species where complete or near complete genome sequence data are available. These include the vertebrate species *Fugu rubripes* (puffer fish; Aparicio et al. 2002), *Danio rerio* (zebrafish), *Xenopus laevis* (African clawed toad) and *Gallus gallus* (chicken), and the invertebrate species *Caenorhabditis elegans* (nematode worm; The *C. elegans* sequencing consortium 1998), *Drosophila elegans* (fruit fly; Adams et al. 2000), and *Ciona intestinalis* (sea-squirt; Dehal et al. 2002).

Interpretation of the significance of results obtained from BLAST analysis of genome sequence data from different species requires knowledge of animal phylogeny, and therefore a brief introduction is necessary here. Comparative analysis of extant animals based on both morphological and molecular data indicates that the animal kingdom comprises two main clades: (1) the deuterostomes, which include vertebrates, cephalochordates, urochordates (e.g. *Ciona*), hemichordates and echinoderms and (2) the protostomes, which are further sub-divided into two assemblages: (a) the ecdysozoa, which include nematodes (e.g. *C. elegans*) and arthropods (e.g. *Drosophila*) and (b) the lophotrochozoa, which include molluscs and annelids. Basal to the deuterostomes and protostomes are the cnidarians (e.g. *Hydra*), which are the most primitive animals with nervous systems (Adoutte et al. 2000).

2
The Phylogeny of Endocannabinoids

2.1
The Phylogenetic Distribution of Anandamide and Enzymes Involved in Anandamide Biosynthesis

Anandamide (arachidonoylethanolamide) is just one of a family of lipids known as *N*-acylethanolamines (NAEs), which are generated from membrane phospholipids via a common enzymatic pathway (see below). The occurrence of anandamide in an organism is dependent on: (1) the presence of the fatty acid arachidonic acid as a component of membrane phospholipids and (2) the presence of enzymes that can catalyse formation of NAEs from membrane phospholipids. Therefore, the phylogenetic distribution of anandamide is likely to reflect a combination of both the phylogenetic distribution of arachidonic acid as a fatty acid component of membrane lipids and the phylogenetic distribution of the enzymes that can catalyse formation of NAEs.

The presence of arachidonic acid in an organism is determined by diet and/or the presence of enzymes that catalyse formation of arachidonic acid from other

fatty acids. In mammals, arachidonic acid is synthesised from linoleic acid through the sequential activity of $\Delta 6$ fatty acid desaturase, $\Delta 6$ fatty acid elongase and $\Delta 5$ fatty acid desaturase (Nakamura and Nara 2003). Interestingly, zebrafish have a single gene encoding an enzyme with both $\Delta 5$ and $\Delta 6$ fatty acid desaturase activities, whereas the nematode *C. elegans* , like mammals, has two genes encoding a $\Delta 5$ fatty acid desaturase and a $\Delta 6$ fatty acid desaturase (Hastings et al. 2001; Napier and Michaelson 2001). These findings indicate that vertebrate and invertebrate species can generate arachidonic acid, although this fatty acid is not necessarily ubiquitous in animals. For example, arachidonic acid is not found as a component of phospholipids in *Drosophila* heads (Yoshioka et al. 1985), which may reflect lack of expression of genes encoding fatty acid desaturases and elongases or loss of genes encoding these enzymes. However, in animal species that lack genes encoding fatty acid desaturases and/or elongases, arachidonic acid may be a dietary constituent. Thus, determination of an organism's potential for generating anandamide from arachidonic acid, as a component of membrane phospholipids, may require assessment of both molecular genetic and dietary information. Consequently, there are unlikely to be discrete phylogenetic patterns in the distribution of arachidonic acid, and hence the potential to generate anandamide.

Anandamide and other NAEs are synthesised in mammalian tissues through the sequential action of two enzymes: (1) a *N*-acyltransferase that generates *N*-acylphosphatidylethanolamine (NAPE) from phosphatidylcholine and phosphatidylethanolamine and (2) a NAPE-phospholipase D (NAPE-PLD) that generates anandamide and other NAEs by cleavage of NAPE (Schmid et al. 1990; Di Marzo et al. 1994; Piomelli 2003). The presence of enzymes that catalyse these reactions has also been reported in invertebrate animals and in plant species (Bisogno et al. 1997; Chapman 2000), indicating that the enzymatic machinery for formation of NAEs may be evolutionarily ancient. Unfortunately, phylogenetic analysis of the distribution of these enzymes has been hindered by lack of sequence data for genes that encode these enzymes. An important breakthrough was reported recently, however, with the cloning and sequencing of cDNAs encoding NAPE-PLD in human, rat and mouse (Okamoto et al. 2004). Thus, it is now possible to investigate the occurrence of related proteins in non-mammalian species. Analysis of genome sequence data for the puffer fish *Fugu rubripes* reveals the presence of a gene encoding a protein that shares a high level of sequence identity (60%) with mammalian NAPE-PLDs. This protein is likely to be a fish orthologue of mammalian NAPE-PLDs, and therefore NAPE-PLDs probably occur throughout the vertebrates. However, genes encoding proteins resembling NAPE-PLD do not appear to be present in two of the invertebrate species for which there are complete genome sequence data available, the insect *Drosophila melanogaster* and the sea-squirt *Ciona intestinalis* . Proteins sharing approximately 40% sequence identity with mammalian NAPE-PLDs are present in the nematode worm *C. elegans* and in numerous bacterial species. However, experimental studies are required to determine if these proteins actually function as NAPE-PLDs.

2.2 The Phylogenetic Distribution of Fatty Acid Amide Hydrolase

The existence of an enzyme in mammalian tissues that catalyses hydrolysis of anandamide to arachidonic acid and ethanolamide was established soon after the identification of anandamide as an endogenous cannabinoid (Deutsch and Chin 1993; Di Marzo et al. 1994; Ueda et al. 1995). Molecular characterisation of this enzyme was accomplished by Cravatt et al. (1996) with the cloning and sequencing of a rat cDNA encoding a protein that is now known as fatty acid amide hydrolase (FAAH). Genes encoding orthologues of rat FAAH have been identified in human and mouse (Giang and Cravatt 1997), but relatively little is known about the occurrence of FAAH in non-mammalian animals. There are, however, several reports of FAAH activity in homogenates of tissues from a variety of invertebrate species. For example, FAAH-like activity has been detected in whole-animal homogenates of the cnidarian *Hydra viridis* (De Petrocellis et al. 1999), in the nervous system of the leech *Hirudo medicinalis* (Matias et al. 2001) and in the ovaries of the sea urchin *Paracentrotus lividus* (Bisogno et al. 1997). Moreover, FAAH-like activity has also been detected in plant tissues (Shrestha et al. 2002), indicating that FAAH may be an evolutionarily ancient enzyme.

An important recent discovery has been the identification of a FAAH gene in the plant species *Arabidopsis* (Shrestha et al. 2003). *Arabidopsis* FAAH is a 607 amino acid protein that shares only 18% overall sequence identity with rat FAAH, although this rises to 37%–60% in the catalytic domain, depending on the length of sequence compared. Analysis of the enzymatic properties of heterologously expressed *Arabidopsis* FAAH reveals that, like mammalian FAAHs, it catalyses hydrolysis of anandamide and other NAEs. Therefore, it appears that FAAH is an evolutionarily ancient enzyme whose ancestry dates back at least as far as the unicellular eukaryotic common ancestor of plants and animals. Moreover, the discovery of a plant gene encoding a protein that functions as a FAAH enzyme, but which shares relatively little sequence similarity with mammalian FAAHs, suggests that related genes in non-mammalian animal species may also encode enzymes that have FAAH activity. For example, genes encoding FAAH-like proteins that share much higher levels of sequence similarity with mammalian FAAHs than with *Arabidopsis* FAAH are present in the genomes of the bird *Gallus gallus* (chicken), the puffer fish *Fugu rubripes*, the urochordate *Ciona intestinalis* and the nematode *C. elegans*. Further studies are now required to characterise the properties of the enzymes encoded by these putative non-mammalian FAAH genes.

2.3 The Phylogenetic Distribution of 2-AG and Enzymes Involved in 2-AG Biosynthesis

2-AG was originally identified as a potential endogenous cannabinoid in mammals by Mechoulam et al. (1995) and Sugiura et al. (1995) and subsequent studies indicate that 2-AG is also present in several non-mammalian species, including the insect *Drosophila melanogaster* (McPartland et al. 2001) and the annelid *Hirudo*

medicinalis (Matias et al. 2001). These findings suggest that 2-AG may have a broad phylogenetic distribution. However, as with anandamide, the ability of organisms to generate 2-AG will depend on the presence of arachidonic acid as a component of phospholipids.

Two enzymatic pathways have been proposed as potential mechanisms for 2-AG biosynthesis in mammalian cells (Piomelli 2003). First, a pathway in which phosphatidylinositol is cleaved by phospholipase C (PLC) to generate 1,2-diacylglycerol (DAG), which is then converted to 2-AG through the action of DAG lipase. Second, a pathway in which phosphatidylinositol is cleaved by phospholipase A_1 to generate 2-arachidonoyl-lysophospholipid, which is then converted to 2-AG through the action of lyso-PLC. For the purposes of this review we will focus on the first pathway because: (1) PLC is a ubiquitous effector for G protein-coupled receptors throughout the animal kingdom and therefore a potentially important and evolutionarily ancient mediator of 2-AG formation and (2) genes encoding mammalian DAG lipases have recently been identified, opening up new opportunities for analysis of the molecular and cellular biology of 2-AG formation in cells.

Analysis of human genome sequence data revealed the presence of two genes that encode *sn*1-DAG lipases and which are now known as DAGLα and DAGLβ (Bisogno et al. 2003). Importantly, heterologous expression of DAGLα or DAGLβ conferred increased formation of 2-AG from *sn*-1-stearoyl-2-arachidonoyl-glycerol as a substrate, demonstrating that these enzymes can catalyse synthesis of 2-AG in cells. Therefore, expression of DAGLα or DAGLβ in cells and tissues may serve as molecular markers for cells that generate 2-AG in vivo. Consistent with this notion, DAGLα is expressed in the dendrites of cerebellar Purkinje cells, neurons which are sources of endocannabinoids that act as retrograde signalling molecules by activating presynaptic CB_1 receptors located on the axons of cerebellar granule cells (Kreitzer and Regehr 2002).

Genes encoding orthologues of DAGLα and DAGLβ are present in other mammals (e.g. mouse) and, more importantly for purposes of this review, in non-mammalian vertebrates that include the bird *Gallus gallus* (chicken) and the zebrafish *Danio rerio* (Bisogno et al. 2003). Moreover, a DAG lipase-like gene (CG33174) is also present in an invertebrate species, the insect *Drosophila melanogaster* (Adams et al. 2000).

2.4 The Phylogenetic Distribution of Monoglyceride Lipase

The inactivation of 2-AG in mammals is thought to be mediated by the enzyme monoglyceride lipase (MGL). However, molecular characterisation of MGL was not driven by an interest in 2-AG but by research directed at identification of the enzymes involved in the sequential hydrolysis of stored triglycerides. A mouse cDNA encoding this enzyme was cloned and sequenced by Karlsson et al. (1997) and found to encode a 302 amino acid protein that is expressed in a wide range of tissues, including brain. Subsequently, Dinh et al. (2002) demonstrated that rat MGL catalyses hydrolysis of 2-AG when expressed in cells. Interestingly, 2-AG is

also a substrate for the enzyme FAAH in vitro (Goparaju et al. 1998), but in mice lacking FAAH, the 2-AG content of the brain is not significantly different from that in wild-type mice (Lichtman et al. 2002). Therefore, it is thought that MGL is the primary physiological mediator of 2-AG inactivation in the mammalian brain (Dihn et al. 2002).

Analysis of the occurrence of MGL-like proteins in non-mammalian organisms by BLAST analysis reveals closely related proteins in the zebrafish *Danio rerio* and the chicken *Gallus gallus* . It is likely, therefore, that MGL occurs throughout the vertebrates. However, genes encoding proteins resembling MGL do not appear to be present in any of the invertebrate species for which complete genome sequence data are available (i.e. *Drosophila, C. elegans, Ciona*). Genes encoding related proteins are, however, present in the genomes of plant, bacterial and viral species. This is an unusual pattern of phylogenetic distribution that raises questions about the evolutionary origin of vertebrate MGL proteins. Relevant to this issue, it is interesting to note that a cowpox virus gene encodes a protein that shares 40% sequence identity with mammalian MGL proteins (Karlsson et al. 1997). Therefore, perhaps an ancestral MGL gene was introduced into the vertebrate genome by horizontal gene transfer mediated by a virus.

3
The Phylogeny of Cannabinoid Receptors and Other Endocannabinoid Receptors

What our survey of the phylogenetic distribution of endocannabinoids and associated enzymes indicates is that the ability of cells to produce and inactivate the molecules that we classify as endocannabinoids in mammals is an evolutionarily ancient phenomenon. Moreover, some components of the endocannabinoid system may date back as far as the common ancestor of all eukaryotic organisms. However, the ability of cells to produce these molecules does not necessarily imply that they function as signalling molecules in all eukaryotes. In assessing the evolution of endocannabinoid signalling, we should not assume that because endocannabinoids activate CB_1/CB_2-type G protein-coupled receptors in mammals that receptors of this type necessarily mediate effects of these molecules in other eukaryotes. Some organisms may have independently evolved their "own" endocannabinoid receptors unrelated to the mammalian cannabinoid receptors. Other organisms may be able to produce the chemicals that we, with our mammalian bias, refer to as "endocannabinoids" but lack receptors for these molecules.

3.1
Receptors Related to Mammalian CB_1 and CB_2 Cannabinoid Receptors

Genes encoding orthologues of the mammalian CB_1 and CB_2 receptors have been identified in the puffer fish *Fugu rubripes* (Yamaguchi et al. 1996; Elphick 2002). This indicates that the existence of CB_1 and CB_2 receptors in vertebrates can be traced back at least as far as the common ancestor of teleost fish like *Fugu* and

the amphibians, reptiles, birds and mammals. Accordingly, CB_1-type genes have also been identified in birds and amphibians (Soderstrom and Johnson 2000, 2001; Soderstrom et al. 2000). Thus far, the *Fugu* CB_2 gene is the only one reported for a non-mammalian vertebrate (Elphick 2002). However, BLAST analysis of genome sequence data for the bird *Gallus gallus* (chicken) reveals the presence of both CB_1-and CB_2-type genes in this species.

An interesting feature of the puffer fish *Fugu rubripes* is that it has one CB_2 gene (Elphick 2002) but two CB_1-type genes (CB_{1A} and CB_{1B}; Yamaguchi et al. 1996). The occurrence of duplicated genes, with respect to other vertebrates, is a feature of teleost fish that is thought to be a legacy of a whole-genome duplication event that occurred in an ancestral species (Taylor et al. 2001). However, duplicates of some genes will have been lost with the passage of evolutionary time, which probably explains the existence of only one CB_2 gene in *Fugu*.

Although both CB_1 and CB_2 genes have been found in the "higher" vertebrates, it remains to be established if CB_1 and CB_2 genes are also present in cartilaginous fish (e.g. sharks, rays) and in primitive agnathan vertebrates (e.g. hagfish, lamprey). However, progress has been made recently in investigating the occurrence of cannabinoid receptors in invertebrate chordates. The extant invertebrates that are most closely related to the vertebrates are the cephalochordates (e.g. Amphioxus), based on both morphological and molecular evidence (Adoutte et al. 2000). Unfortunately, relatively little is known about the physiology and biochemistry of these animals. However, because of the important phylogenetic position of these animals with respect to vertebrates, there are plans to sequence the genome of a cephalochordate species.

An invertebrate chordate species that has had its genome sequenced recently is the urochordate (sea-squirt) *Ciona intestinalis* (Dehal et al. 2002). As adults, these animals exhibit little similarity with other chordates (vertebrates and cephalochordates), but as larvae *Ciona* have several morphological characters that distinguish them as chordates. Moreover, urochordates are the most primitive of the extant chordates, and thus these animals are of particular interest for evolutionary studies. Analysis of the *Ciona* genome sequence has revealed the presence of a putative cannabinoid receptor gene (*CiCBR*) encoding a 423 amino acid protein that shares 28% and 24% sequence identity with the human CB_1 and CB_2 receptor, respectively (Elphick et al. 2003). These are relatively low levels of sequence similarity, but analysis of the relationship of CiCBR with cannabinoid receptors and other G protein-coupled receptors, by construction of a phylogenetic tree based on sequence alignments, demonstrated that CiCBR is an orthologue of the vertebrate cannabinoid receptors CB_1 and CB_2 (Elphick et al. 2003). Thus, CiCBR is the first putative cannabinoid receptor to be identified in an invertebrate species. Moreover, phylogenetic analysis indicates that the common ancestor of CiCBR and vertebrate CB_1 and CB_2 receptors predates a duplication event that gave rise to CB_1 and CB_2 in vertebrates. In this respect, cannabinoid receptor genes conform to a pattern seen in other gene families, where for each invertebrate gene there are often two or more related genes in vertebrates. This feature is thought to reflect a whole-genome duplication event that occurred in the invertebrate ancestor of the vertebrates (Furlong and Holland 2002).

The discovery of CiCBR indicates that the evolutionary history of cannabinoid receptors that are related to the vertebrate CB_1 and CB_2 receptors extends back at least as far as the common ancestor of vertebrates and the invertebrate chordates (urochordates, cephalochordates). What remains to be established is whether other, more distantly invertebrate animals also have orthologues of the vertebrate cannabinoid receptors. The invertebrate animals that are most closely related to the chordates are the hemichordates and echinoderms (Adoutte et al. 2000). Hemichordates are a relatively obscure group of animals (e.g. acorn worms) that have not been studied in great detail. There has, however, been a surge of interest in these animals recently with the advent of molecular techniques for research on developmental and evolutionary biology (e.g. Lowe et al. 2003). Moreover, there are plans to sequence the genome of a hemichordate species, the acorn worm *Saccoglossus kowalevskii* . Therefore, as with Amphioxus, there may be opportunities to investigate the occurrence of a cannabinoid receptor in hemichordates in the near future.

The echinoderms (e.g. sea urchins and starfish) are an invertebrate group that has been studied extensively, in particular for research on early stages of development. Moreover, at the time of writing, a genome sequencing project for the sea urchin species *Strongylocentrotus purpuratus* is ongoing (Cameron et al. 2000) and due to be completed during 2004. This is of special interest for research on cannabinoid receptors because this species has been the subject of a detailed study on the effects of cannabinoids. Herbert Schuel and colleagues demonstrated that cannabinoids block the acrosome reaction in sea urchin sperm, indicating that endocannabinoids may have a physiological role in preventing polyspermy (Schuel et al. 1991, 1994). Moreover, Chang et al. (1993) demonstrated that cannabinoid binding sites are present on sea urchin sperm. The molecular properties of these cannabinoid binding sites and their relationship to vertebrate cannabinoid receptors are currently unknown. However, analysis of sea urchin genome sequence data, when it is available, may provide new opportunities for further research on this issue.

Having considered the deuterostomian invertebrates, we will now turn our attention to the protostomian clade of the animal kingdom. First we will consider the ecdysozoa, which include two well-studied species for which complete genome sequence data are available—the insect *Drosophila melanogaster* and the nematode *C. elegans* . Analysis of the genome sequences of both of these species has revealed, however, that orthologues of cannabinoid receptors are not present (Elphick and Egertová 2001). Moreover, these species also do not have orthologues of the G protein-coupled receptors in vertebrates that are most closely related to CB_1 and CB_2—lysophospholipid receptors and melanocortin receptors (Elphick and Egertová 2001). These data indicate, therefore, that the group of G protein-coupled receptors that include cannabinoid receptors may have originated in the deuterostomian branch of the animal kingdom, after the deuterostomian-protostomian split. Consistent with these conclusions based on genome sequence data, biochemical analysis of insect species has not revealed the presence of cannabinoid binding sites (Egertová 1999; Elphick and Egertová 2001; McPartland et al. 2001).

Turning now to the lophotrochozoan phyla, here there have been a few studies that have reported detection of cannabinoid binding sites. Stefano et al. (1996) reported the presence of binding sites for anandamide on haemocytes from the bivalve mollusc *Mytilus edulis* , whilst Stefano et al. (1997) reported anandamide binding sites in the nervous system of the leech *Hirudo medicinalis* (Phylum Annelida). Interestingly, the latter study was accompanied by a partial leech cDNA sequence that shared sequence similarity with vertebrate CB_1 receptors. However, subsequent detailed analysis of this sequence revealed that it is chimeric with one region that shares 98% amino acid identity with the bovine adrenocorticotropic hormone receptor and two regions that share 68% and 65% amino acid identity with mammalian CB_1 receptors (Elphick 1998). It is unlikely, therefore, that this sequence represents part of a *bone fide* leech cannabinoid receptor cDNA. How, then, can the discovery of this unusual sequence be explained. One possibility is that leech cDNA was contaminated with bovine DNA derived from blood that leeches had fed on. Clearly, further work is required, but thus far there have been no follow-up studies to confirm the existence of a full-length cannabinoid receptor cDNA in the leech or in any other protostomian invertebrate.

The detection of cannabinoid binding sites in *Mytilus* and *Hirudo*, but not in insects, has been explained by some authors as a consequence of loss of cannabinoid receptor genes in the ecdysozoan lineage but not in the lophotrochozoan lineage (McPartland and Pruitt 2002). However, as highlighted above, both *Drosophila* and *C. elegans* also lack orthologues of the vertebrate G protein-coupled receptors that are most closely related to cannabinoid receptors (lysophospholipid and melanocortin receptors). Therefore, a more parsimonious explanation is that this group of receptors originated in the deuterostomian branch of the animal kingdom after the protostomian–deuterostomian split.

If orthologues of cannabinoid receptors are not present in protostomian invertebrates, as proposed above and in previous reports (Elphick and Egertová 2001; Elphick et al. 2003), how then can the existence of cannabinoid binding sites in *Mytilus* and *Hirudo* be explained? Detection of these binding sites may reflect interaction of cannabinoids with membrane proteins in these species that are unrelated to the vertebrate CB_1/CB_2-type cannabinoid receptors but which have evolved independently. However, demonstrating that these binding sites equate to functional receptors that mediate physiological effects of endocannabinoids in these organisms will require detailed molecular characterisation of the putative receptors, and thus far this has yet to be accomplished. The same applies to cannabinoid binding sites detected in the primitive cnidarian species *Hydra viridis* (De Petrocellis et al. 1999). If these putative receptors can be characterised then they may provide fascinating examples of convergent evolution in signalling mechanisms.

3.2 Other Endocannabinoid Receptors and Cannabinoid Receptors

Although the CB_1 and CB_2 cannabinoid receptors are by far the most well characterised receptors for endocannabinoids in vertebrates, it is important to recognise

that there are also other receptor types that may mediate physiological effects of anandamide and 2-AG. For example, there is evidence of a third G protein-coupled receptor in mammals that is activated by endocannabinoids (Breivogel et al. 2001). Without molecular characterisation of this putative receptor it is impossible to investigate its phylogenetic distribution. However, the possibility remains that this receptor may have more widespread phylogenetic distribution than CB_1/CB_2-related receptors and thereby account for cannabinoid binding sites that have been reported in some invertebrate species.

Another receptor that has been implicated as a mediator of physiological effects of the endocannabinoid anandamide in mammals is the vanilloid receptor VR1, more recently referred to as transient receptor potential vanilloid type 1 (TRPV1) (Zygmunt et al. 1999). However, VR1 is not activated by "classical" Δ^9-tetrahydrocannabinol-like cannabinoid agonists. Therefore, VR1 is an endocannabinoid receptor but not a cannabinoid receptor. Unlike CB_1 and CB_2, VR1 is not a G protein-coupled receptor but belongs to the TRP family of ligand-gated cation channels (Montell et al. 2002). Genes encoding proteins that are closely related to the mammalian VR1 receptor have been identified in *Drosophila* (nan) and in *C. elegans* (OSM-9) (Montell 2003). However, to the best of our knowledge, it is not known if these invertebrate VR1-like channels are activated by anandamide. Therefore, it remains to be determined if the ability of anandamide to activate TRP-type channels is an evolutionarily ancient phenomenon.

Another interesting member of the TRP channel family that has been characterised recently is ANKTM1, which is activated by Δ^9-tetrahydrocannabinol as well as being implicated in the detection of noxious cold (Jordt et al. 2004). However, the physiological relevance of the effect of Δ^9-tetrahydrocannabinol on ANKTM1 is unclear because the endocannabinoids anandamide and 2-AG do not activate this TRP channel (Jordt et al. 2004). Nevertheless, it is possible that other as-yet-unidentified endocannabinoids act as endogenous ligands for ANKTM1.

In conclusion, there is now an emerging concept of TRP-type ion channels that are receptors for cannabinoids and/or endocannabinoids, and an interesting area for future research will be to investigate the occurrence of invertebrate TRP-type channels that are also activated by cannabinoid-related molecules.

4
The Evolutionary Origins of Endocannabinoid Signalling

What can we conclude from our survey of the phylogenetic distribution of (1) endocannabinoids, (2) enzymes involved in endocannabinoid biosynthesis and inactivation and (3) cannabinoid/endocannabinoid receptors? It is clear that many of the components of the enzymatic machinery that are used for biosynthesis and inactivation of endocannabinoids in mammals are evolutionarily ancient. For example, there is evidence that enzymes involved in biosynthesis and inactivation of anandamide occur in animals and plants. Most notable in this respect has been the recent discovery and enzymatic characterisation of a FAAH-like enzyme in the plant *Arabidopsis* (Shrestha et al. 2003). Therefore, it appears that the ability

of organisms to synthesise endocannabinoids such as anandamide and 2-AG may date back at least as far as the unicellular eukaryotic common ancestor of plants and animals. However, exploitation of these molecules for intercellular signalling may have occurred independently in different lineages during the evolution of the eukaryotes. For example, there is evidence that plants may also have receptors for anandamide and/or related NAEs, because Tripathy et al. (2003) have detected binding sites for NAEs in cell membranes from the plant species *Nicotiana tabacum* (tabacco) and *Arabidopsis*. If molecular characterisation of a putative NAE receptor in plants can be accomplished, this may provide a fascinating example of how plants have independently exploited NAEs as signalling molecules.

So far, the best-characterised example of endocannabinoid signalling in eukaryotes is CB_1/CB_2-mediated processes in vertebrates. Moreover, our phylogenetic analysis of the occurrence of CB_1/CB_2 receptors in invertebrates indicates that the ancestor of these receptors originated in a deuterostomian invertebrate, and in accordance with this view receptors of this type have so far not been found in protostomian invertebrates. The CiCBR gene that was recently identified in the invertebrate chordate *Ciona intestinalis* (Elphick et al. 2003) is an example of a receptor in a deuterostomian invertebrate that may resemble the putative ancestor of the vertebrate CB_1 and CB_2 receptors. Therefore, analysis of CiCBR function in *Ciona* is now of particular interest.

Looking ahead, we hope that this review may stimulate scientists with an interest in endocannabinoid signalling to exploit not only the familiar mammalian model species (rats, mice) but also the rich diversity of non-mammalian animals where the existence of endocannabinoid receptors has been established.

References

Adams MD, Celniker SE, Holt RA, et al (2000) The genome sequence of Drosophila melanogaster. Science 287:2185–2195

Adoutte A, Balavoine G, Lartillot N, Lespinet O, Prud'homme B, De Rosa R (2000) The new animal phylogeny: reliability and implications. Proc Natl Acad Sci USA 97:4453–4456

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. J Mol Biol 215:403–410

Aparicio S, Chapman J, Stupka E, et al (2002) Whole-genome shotgun assembly and analysis of the genome of Fugu rubripes. Science 297:1301–1310

Bisogno T, Ventriglia M, Milone A, Mosca M, Cimino G, Di Marzo V (1997) Occurrence and metabolism of anandamide and related acyl-ethanolamides in ovaries of the sea urchin Paracentrotus lividus. Biochim Biophys Acta 1345:338–348

Bisogno T, Howell F, Williams G, Minassi A, Cascio MG, Ligresti A, Matias I, Schiano-Moriello A, Paul P, Williams EJ, Gangadharan U, Hobbs C, Di Marzo V, Doherty P (2003) Cloning of the first sn1-DAG lipases points to the spatial and temporal regulation of endocannabinoid signaling in the brain. J Cell Biol 163:463–468

Breivogel CS, Griffin G, Di Marzo V, Martin BR (2001) Evidence for a new G protein-coupled cannabinoid receptor in mouse brain. Mol Pharmacol 60:155–163

Cameron RA, Mahairas G, Rast JP, Martinez P, Biondi TR, Swartzell S, Wallace JC, Poustka AJ, Livingston BT, Wray GA, Ettensohn CA, Lehrach H, Britten RJ, Davidson EH, Hood L (2000) A sea urchin genome project: sequence scan, virtual map, and additional resources. Proc Natl Acad Sci USA 97:9514–9518

Chang MC, Berkery D, Schuel R, Laychock SG, Zimmerman AM, Zimmerman S, Schuel H (1993) Evidence for a cannabinoid receptor in sea urchin sperm and its role in blockade of the acrosome reaction. Mol Reprod Dev 36:507–516

Chapman KD (2000) Emerging physiological roles for N-acylphosphatidylethanolamine metabolism in plants: signal transduction and membrane protection. Chem Phys Lipids 108:221–229

Cravatt BF, Giang DK, Mayfield SP, Boger DL, Lerner RA, Guilula NB (1996) Molecular characterization of an enzyme that degrades neuromodulatory fatty-acid amides. Nature 384:83–87

De Petrocellis L, Melck D, Bisogno T, Milone A, Di Marzo V (1999) Finding of the endocannabinoid signalling system in Hydra, a very primitive organism: possible role in the feeding response. Neuroscience 92:377–387

Dehal P, Satou Y, Campbell RK, et al (2002) The draft genome of Ciona intestinalis: insights into chordate and vertebrate origins. Science 298:2157–2167

Deutsch DG, Chin SA (1993) Enzymatic synthesis and degradation of anandamide, a cannabinoid receptor agonist. Biochem Pharmacol 46:791–796

Devane WA, Dysarz FAI, Johnson MR, Melvin LS, Howlett AC (1988) Determination and characterization of a cannabinoid receptor in rat brain. Mol Pharmacol 34:605–613

Devane WA, Hanus L, Breuer A, Pertwee RG, Stevenson LA, Griffin G, Gibson D, Mandelbaum A, Etinger A, Mechoulam R (1992) Isolation and structure of a brain constituent that binds to the cannabinoid receptor. Science 258:1946–1948

Di Marzo V, Fontana A, Cadas H, Schinelli S, Cimino G, Schwartz J-C, Piomelli D (1994) Formation and inactivation of endogenous cannabinoid anandamide in central neurons. Nature 372:686–691

Dinh TP, Carpenter D, Leslie FM, Freund TF, Katona I, Sensi SL, Kathuria S, Piomelli D (2002) Brain monoglyceride lipase participating in endocannabinoid inactivation. Proc Natl Acad Sci USA 99:10819–10824

Egertová M (1999) Neuroanatomy and phylogeny of cannabinoid signalling. Ph.D. Thesis, University of London, UK

Egertová M, Giang DK, Cravatt BF, Elphick MR (1998) A new perspective on cannabinoid signalling: complementary localization of fatty acid amide hydrolase and the CB1 receptor in rat brain. Proc R Soc Lond B Biol Sci 265:2081–2085

Elphick MR (1998) An invertebrate G-protein coupled receptor is a chimeric cannabinoid/melanocortin receptor. Brain Res 780:170–173

Elphick MR (2002) Evolution of cannabinoid receptors in vertebrates: identification of a CB2 gene in the puffer fish Fugu rubripes. Biol Bull 202:104–107

Elphick MR, Egertová M (2001) The neurobiology and evolution of cannabinoid signalling. Philos Trans R Soc Lond B Biol Sci 356:381–408

Elphick MR, Satou Y, Satoh N (2003) The invertebrate ancestry of endocannabinoid signalling: an orthologue of vertebrate cannabinoid receptors in the urochordate Ciona intestinalis. Gene 302:95–101

Freund TF, Katona I, Piomelli D (2003) Role of endogenous cannabinoids in synaptic signaling. Physiol Rev 83:1017–1066

Furlong RF, Holland PWH (2002) Were vertebrates octoploid? Philos Trans R Soc Lond B Biol Sci 357:531–544

Giang DK, Cravatt BF (1997) Molecular characterization of human and mouse fatty acid amide hydrolases. Proc Natl Acad Sci USA 94:2238–2242

Goparaju SK, Ueda N, Yamaguchi H, Yamamoto S (1998) Anandamide amidohydrolase reacting with 2-arachidonoylglycerol, another cannabinoid receptor ligand. FEBS Lett 422:69–73

Hastings N, Agaba M, Tocher DR, Leaver MJ, Dick JR, Sargent JR, Teale AJ (2001) A vertebrate fatty acid desaturase with delta 5 and delta 6 activities. Proc Natl Acad Sci USA 98:14304–14309

Howlett AC, Barth F, Bonner TI, Cabral G, Casellas P, Devane WA, Felder CC, Herkenham M, Mackie K, Martin BR, Mechoulam R, Pertwee RG (2002) International Union of

Pharmacology. XXVII. Classification of cannabinoid receptors. Pharmacol Rev 54:161–202
Jordt SE, Bautista DM, Chuang HH, McKemy DD, Zygmunt PM, Hogestatt ED, Meng ID, Julius D (2004) Mustard oils and cannabinoids excite sensory nerve fibres through the TRP channel ANKTM1. Nature 427:260–265
Karlsson M, Contreras JA, Hellman U, Tornqvist H, Holm C (1997) cDNA cloning, tissue distribution, and identification of the catalytic triad of monoglyceride lipase. Evolutionary relationship to esterases, lysophospholipases, and haloperoxidases. J Biol Chem 27:27218–27223
Kreitzer AC, Regehr WG (2002) Retrograde signaling by endocannabinoids. Curr Opin Neurobiol 12:324–330
Lichtman AH, Hawkins EG, Griffin G, Cravatt BF (2002) Pharmacological activity of fatty acid amides is regulated, but not mediated, by fatty acid amide hydrolase in vivo. J Pharmacol Exp Ther 302:73–79
Lowe CJ, Wu M, Salic A, Evans L, Lander E, Stange-Thomann N, Gruber CE, Gerhart J, Kirschner M (2003) Anteroposterior patterning in hemichordates and the origins of the chordate nervous system. Cell 113:853–865
Matias I, Bisogno T, Melck D, Vandenbulcke F, Verger-Bocquet M, De Petrocellis L, Sergheraert C, Breton C, Di Marzo V, Salzet M (2001) Evidence for an endocannabinoid system in the central nervous system of the leech Hirudo medicinalis. Brain Res Mol Brain Res 87:145–159
Matsuda LA, Lolait SJ, Brownstein MJ, Young AC, Bonner TI (1990) Structure of a cannabinoid receptor and functional expression of the cloned cDNA. Nature 346:561–564
McPartland JM, Pruitt P (2002) Sourcing the code: searching for the evolutionary origins of cannabinoid receptors, vanilloid receptors, and anandamide. J Cannabis Ther 2:73–103
McPartland J, Di Marzo V, De Petrocellis L, Mercer A, Glass M (2001) Cannabinoid receptors are absent in insects. J Comp Neurol 436:423–429
Mechoulam R, Ben-Shabat S, Hanus L, Ligumsky M, Kaminski NE, Schatz AR, Gopher A, Almog S, Martin BR, Compton DR, Pertwee RG, Griffin G, Bayewitch M, Barg J, Vogel ZVI (1995) Identification of an endogenous 2-monoglyceride, present in canine gut that binds to cannabinoid receptors. Biochem Pharmacol 50:83–90
Montell C (2003) The venerable inveterate invertebrate TRP channels. Cell Calcium 33:409–417
Montell C, Birnbaumer L, Flockerzi V (2002) The TRP channels, a remarkably functional family. Cell 108:595–598
Munro S, Thomas KL, Abu-Shaar M (1993) Molecular characterization of a peripheral receptor for cannabinoids. Nature 365:61–65
Nakamura MT, Nara TY (2003) Essential fatty acid synthesis and its regulation in mammals. Prostaglandins Leukot Essent Fatty Acids 68:145–150
Napier J, Michaelson LV (2001) Genomic and functional characterisation of polyunsaturated fatty acid biosynthesis in Caenorhabditis elegans. Lipids 36:761–766
Okamoto Y, Morishita J, Tsuboi K, Tonai T, Ueda N (2004) Molecular characterization of a phospholipase D generating anandamide and its congeners. J Biol Chem 279:5298–5305
Piomelli D (2003) The molecular logic of endocannabinoid signalling. Nat Rev Neurosci 4:873–884
Salzet M, Stefano G (2002) The endocannabinoid system in invertebrates. Prostaglandins Leukot Essent Fatty Acids 66:353–361
Salzet M, Breton C, Bisogno T, Di Marzo V (2000) Comparative biology of the endocannabinoid system. Possible role in the immune response. Eur J Biochem 15:4917–4927
Schmid HH, Schmid PC, Natarajan V (1990) N-Acylated glycerophospholipids and their derivatives. Prog Lipid Res 29:1–43
Schuel H, Berkery D, Schuel R, Chang MC, Zimmerman AM, Zimmerman S (1991) Reduction of the fertilizing capacity of sea urchin sperm by cannabinoids derived from marihuana. I. Inhibition of the acrosome reaction induced by egg jelly. Mol Reprod Dev 29:51–59

Schuel H, Goldstein E, Mechoulam R, Zimmerman AM, Zimmerman S (1994) Anandamide (arachidonylethanolamide), a brain cannabinoid receptor agonist, reduces sperm fertilizing capacity in sea urchins by inhibiting the acrosome reaction. Proc Natl Acad Sci USA 91:7678–7682

Shrestha R, Noordermeer MA, van der Stelt M, Veldink GA, Chapman KD (2002) N-Acylethanolamines are metabolised by lipoxygenase and amidohydrolase in competing pathways during cottonseed imbibition. Plant Physiol 130:391–401

Shrestha R, Dixon RA, Chapman KD (2003) Molecular identification of a functional homologue of the mammalian fatty acid amide hydrolase in Arabidopsis thaliana. J Biol Chem 278:34990–34997

Soderstrom K, Johnson F (2000) CB1 cannabinoid receptor expression in brain regions associated with zebra finch song control. Brain Res 857:151–157

Soderstrom K, Johnson F (2001) Zebra finch CB1 cannabinoid receptor: pharmacology and in vivo and in vitro effects of activation. J Pharmacol Exp Ther 297:189–197

Soderstrom K, Leid M, Moore FL, Murray TF (2000) Behavioural, pharmacological and molecular characterization of an amphibian cannabinoid receptor. J Neurochem 75:413–423

Stefano GB, Liu Y, Goligorsky MS (1996) Cannabinoid receptors are coupled to nitric oxide release in invertebrate immunocytes, microglia and human monocytes. J Biol Chem 271:19238–19242

Stefano GB, Salzet B, Salzet M (1997) Identification and characterization of the leech CNS cannabinoid receptor: coupling to nitric oxide release. Brain Res 753:219–224

Sugiura T, Kondo S, Sukagawa A, Nakane S, Shinoda A, Itoh K, Yamashita A, Waku K (1995) 2-Arachidonoylglycerol: a possible endogenous cannabinoid receptor ligand in brain. Biochem Biophys Res Commun 215:89–97

Taylor JS, Van de Peer Y, Braasch I, Meyer A (2001) Comparative genomics provides evidence for an ancient genome duplication event in fish. Philos Trans R Soc Lond B Biol Sci 356:1661–1679

The C. elegans Sequencing Consortium (1998) Genome sequence of the nematode C. elegans: a platform for investigating biology. Science 282:2012–2018

Tripathy S, Kleppinger-Sparace K, Dixon RA, Chapman KD (2003) N-acylethanolamine signaling in tobacco is mediated by a membrane-associated, high-affinity binding protein. Plant Physiol 131:1781–1791

Ueda N, Kurahashi Y, Yamamoto S, Tokunaga T (1995) Partial purification and characterization of the porcine brain enzyme hydrolyzing and synthesizing anandamide. J Biol Chem 270:23823–23827

Wilson RI, Nicoll RA (2002) Endocannabinoid signaling in the brain. Science 296:678–682

Yamaguchi F, Macrae AD, Brenner S (1996) Molecular cloning of two cannabinoid type 1-like receptor genes from the puffer fish Fugu rubripes. Genomics 35:603–605

Yoshioka T, Inoue H, Kasama T, Seyama Y, Nakashima S, Nozawa Y, Hotta Y (1985) Evidence that arachidonic acid is deficient in phosphatidylinositol of Drosophila heads. J Biochem (Tokyo) 98:657–662

Zygmunt PM, Petersson J, Andersson DA, Chuang HH, Sorgard M, DiMarzo V, Julius D, Hogestatt ED (1999) Vanilloid receptors on sensory nerves mediate the vasodilator action of anandamide. Nature 400:452–457