The evolution and comparative neurobiology of endocannabinoid signalling

Maurice R. Elphick

Queen Mary University of London,
School of Biological & Chemical Sciences,
Mile End Road,
London, E1 4NS, UK

Correspondence to: Prof. M.R. Elphick,
Queen Mary University of London,
School of Biological & Chemical Sciences,
Mile End Road,
London, E1 4NS, UK

Tel: 44 207 882 5290
Fax: 44 208 983 0973
E-mail: M.R.Elphick@qmul.ac.uk

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Abstract

CB_1- and CB_2- type cannabinoid receptors mediate effects of the endocannabinoids 2-arachidonoylglycerol (2-AG) and anandamide in mammals. In canonical endocannabinoid-mediated synaptic plasticity, 2-AG is generated postsynaptically by diacylglycerol lipase alpha and acts via presynaptic CB_1-type cannabinoid receptors to inhibit neurotransmitter release. Electrophysiological studies on lampreys indicate that this retrograde signalling mechanism occurs throughout the vertebrates, whilst system level studies point to conserved roles for endocannabinoid signalling in neural mechanisms of learning and control of locomotor activity and feeding. CB_1/CB_2-type receptors originated in a common ancestor of extant chordates, and in the sea squirt *Ciona intestinalis* a CB_1/CB_2-type receptor (CiCBR) is targeted to axons, indicative of an ancient role for cannabinoid receptors as axonal regulators of neuronal signalling. Although CB_1/CB_2-type receptors are unique to chordates, enzymes involved in biosynthesis/inactivation of endocannabinoids occur throughout the animal kingdom. Accordingly, non-CB_1/CB_2-mediated mechanisms of endocannabinoid signalling have been postulated. For example, there is evidence that 2-AG mediates retrograde signalling at synapses in the nervous system of the leech *Hirudo medicinalis* by activating presynaptic TRPV-type ion channels. Thus, postsynaptic synthesis of 2-AG or anandamide may be a phylogenetically widespread phenomenon, and a variety of proteins may have evolved as presynaptic (or postsynaptic) receptors for endocannabinoids.

Key Words

Cannabinoid, anandamide, 2-AG, invertebrate, vertebrate, CRIP1a

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1. PREFACE

On March 29th 2001 a review titled "The Neurobiology and Evolution of Cannabinoid Signalling" was published in *Philosophical Transactions of the Royal Society B* (1). It was the first review on cannabinoid signalling to be published in this journal. Since then the field of research on cannabinoid signalling has grown exponentially. Accordingly, the present article is one of fourteen reviews that collectively form an entire journal issue devoted to "Endocannabinoids in nervous system health and disease". Writing of the 2001 review required a survey of approximately 3000 articles, which was challenging but feasible. Ten years later, by the end of 2011, the PubMed database had over 11,000 articles that could be found using the search term "cannabinoid*". Clearly, it is no longer feasible to comprehensively review this field of research in a journal article. Even a large book devoted to cannabinoid biology could not cover the range of papers on this topic. Therefore, it is necessary in a review such as this to focus on a specific aspect of cannabinoid biology and the theme here is "The Evolution and Comparative Neurobiology of Endocannabinoid Signalling", focusing largely on articles that have been published since 2001.

In discussing the evolution and comparative neurobiology of endocannabinoid signalling, it is necessary to first provide an overview of current understanding of mechanisms of endocannabinoid signalling in the group of animals in which this system was discovered – the mammals. It is fitting therefore that on March 29th 2001, three landmark experimental papers were also published that transformed our understanding of endocannabinoid signalling in the mammalian nervous system. Independently, three research groups obtained evidence that postsynaptic depolarisation of principal neurons in the hippocampus or cerebellar cortex triggers postsynaptic synthesis of endocannabinoids, which then act presynaptically to cause CB_1-mediated inhibition of neurotransmitter release (2-4) Thus, a mechanism of synaptic plasticity mediated by retrograde endocannabinoid signalling was discovered. The concept that endocannabinoids might act as retrograde synaptic signalling molecules had been proposed three years earlier based on our neuroanatomical observations (5) and a model of this putative signalling mechanism was presented

in the 2001 review article (1). But it was the three other papers published on the 29th March 2001 that converted a hypothesis into a textbook principle. Within a decade, the field of research on endocannabinoid signalling has moved from a marginal position to the centre stage of 21st century neuroscience. So looking back, the 29th March 2001 can be thought of as a turning point for cannabinoid research and indeed it has been referred to as a Dies Mirabilis for the field (6).

2. INTRODUCTION TO ENDOCANNABINOID SIGNALLING

(a) Discovery of CB_1 and CB_2 cannabinoid receptors

The existence of cannabinoid receptors in the brain was first inferred from the stereoselective pharmacological actions of Δ^9-tetrahydrocannabinol (Δ^9-THC), the psychoactive constituent of cannabis, and other cannabinoid-type compounds (7). However, demonstration of the existence of specific cannabinoid binding sites in the brain using the radiolabelled cannabinoid ^{3}H-CP-55,940 provided the first solid evidence that cannabinoid receptors exist in the brain (8). Molecular characterisation of a protein that confers cannabinoid binding-sites on rodent brain cell membranes provided the definitive proof of a receptor and revealed a 473-residue G-protein coupled receptor (9), which is now referred to as CB_1. This nomenclature distinguishes CB_1 from a related G-protein coupled receptor known as CB_2, which is predominantly associated with immune cells (10). Thus, in humans and other mammals there are two G-protein coupled cannabinoid receptors, CB_1 and CB_2, and analysis of CB_1-knockout mice and CB_2-knockout mice indicates that these two receptors are largely responsible for mediating the pharmacological effects of Δ^9-THC in mammals (11-13).

(b) Endocannabinoids and enzymes involved in endocannabinoid biosynthesis and inactivation

The discovery of CB_1 and CB_2 pointed to the existence of endogenous ligands for these receptors and two such "endocannabinoids" have been identified – *N*-arachidonoylethanolamide ("anandamide") and *sn*-2-arachidonoylglycerol (2-AG) (14-16).

2-AG is synthesized in the brain by the enzyme diacylglycerol lipase alpha (DAGLα), which catalyses cleavage of 2-AG from arachidonic acid containing diacylglycerols (DAGs) (17-19). A second diacylglycerol lipase that is related to DAGLα based on sequence similarity has been identified and is known as DAGLβ (17). However, whilst DAGLβ can catalyse formation of 2-AG *in vitro* (17), comparative analysis of the brain content of 2-AG in DAGLα- and DAGLβ-knockout mice indicates that the contribution of DAGLβ to 2-AG biosynthesis in adult brain is much less significant compared to DAGLα (18, 19). 2-AG is inactivated by the enzyme monoacylglycerol lipase (MAGL), which cleaves 2-AG into arachidonic acid and glycerol (20-22). Approximately 85% of brain 2-AG hydrolase activity is attributable to MAGL, whilst the remaining 15% is largely attributed to the α/β hydrolases ABH6 and ABH12 (23).

The mechanisms by which anandamide is synthesized in the brain are not yet fully characterised. *In vitro* studies suggested that anandamide may be synthesized by a two-step enzymatic pathway wherein a Ca^{2+}-activated *N*-acyltransferase transfers a *sn*-1 arachidonoyl acyl group of a phospholipid onto the amine of phosphatidylethanolamine (PE) to generate *N*-acyl PE (NAPE) and then NAPE is converted by a phospholipase D (NAPE-PLD) into anandamide and phosphatidic acid (24-27). However, the levels of anandamide in brains from NAPE-PLD-knockout mice are not significantly different from wild-type mice, arguing against a role for NAPE-PLD in anandamide biosynthesis in the brain. The levels of long-chain saturated *N*-acylethanolamines are substantially reduced in NAPE-PLD knockout mice though, indicating that the primary function of NAPE-PLD in the brain may be in biosynthesis of these molecules (28). The physiological roles of long-chain saturated *N*-acylethanolamines in the brain are unknown, but localisation of NAPE-PLD in the axons and axon terminals of sub-populations of neurons in the brain has provided a neuroanatomical framework for further investigation of this issue (29).

Other enzymatic pathways have also been implicated in biosynthesis of anandamide (30-35) but, as yet, definite proof that these are involved in *in vivo* production of anandamide in the brain has

not been forthcoming. It is possible that multiple and potentially interacting pathways are involved, which may make it difficult to pinpoint roles for particular enzymes.

Whilst our knowledge of mechanisms of anandamide biosynthesis in the brain remains incomplete, enzymes that catalyse inactivation of anandamide have been identified. In 1996 Cravatt *et al*. identified an enzyme known as fatty acid amide hydrolase (FAAH), which converts anandamide to arachidonic acid and ethanolamine (36) and subsequent analysis of FAAH knockout-mice and mice treated with selective FAAH inhibitors have demonstrated that FAAH has a major role in regulation of anandamide levels in the brain (37, 38). In humans, but not rodents, there is a second FAAH-like enzyme, which is known as FAAH-2 (39). Analysis of the biochemical properties of FAAH-2 reveals that it associated with lipid droplets in cells and hydrolyses anandamide at rates 30-40% of those of FAAH (40). Furthermore, cyclooxygenase-2 (COX-2) also contributes to the metabolism of anandamide in neurons and other cell types (41, 42).

Lastly, evidence for and against the existence of proteins involved in transport of endocannabinoids has been reported (43, 44) and recently it was proposed that a catalytically silent isoform of FAAH (FAAH-like anandamide transporter or FLAT) may drive anandamide transport into neurons (45).

(c) Putative regulators of cannabinoid receptor signalling

The existence of proteins that regulate the activity of G-protein coupled receptors (GPCRs) is well established. These include proteins such as G-protein coupled receptor kinases (GRKs), which phosphorylate serine and threonine residues in GPCR C-terminal of tails following G-protein dissociation, and arrestins, which bind to C-terminally phosphorylated GPCRs and then block interaction with G-proteins and mediate receptor internalisation (46). However, these are generic GPCR-interacting proteins that regulate the activity of many GPCRs. In addition to these generic GPCR-interacting proteins, other proteins that only interact with specific GPCRs have been

identified. For example, the melanocortin receptor accessory protein MRAP mediates targeting of MC2-type melanocortin receptors to the cell surface in adrenal cells (47-49).

The first report of candidate cannabinoid receptor interacting proteins (CRIPs) was published in 2007 (50). Deletion of the C-terminal region of the CB_1 receptor had been found to alter CB_1 signalling (51) and it was postulated that accessory proteins binding to this region of the receptor may modulate CB_1 activity. Using a polypeptide corresponding to the C-terminal 55 residues of the CB_1 receptor as bait, a yeast two-hybrid screen was employed to identify potential interacting partner proteins expressed in human brain. A 128 residue protein was identified as a positive hit and analysis of its sequence revealed that it is encoded by a gene containing four exons (1, 2, 3a and 3b) that is subject to alternative splicing, with exons 1, 2 and 3b encoding the 128 residue protein and exons 1, 2 and 3a encoding a 164 residue protein (50). Biochemical evidence that both the 164-residue protein and the 128-residue protein interact with the C-terminal tail of CB_1 was obtained and accordingly these two proteins were named cannabinoid receptor interacting protein 1a (CRIP1a) and cannabinoid receptor interacting protein 1b (CRIP1b), respectively. Furthermore, co-expression of CRIP1a or CRIP1b with CB_1 in superior cervical ganglion neurons revealed that CRIP1a, but not CRIP1b, suppresses CB_1-mediated tonic inhibition of voltage-gated Ca^{2+} channels, providing evidence of a role for CRIP1a in regulation of CB_1 signalling (50). More recently, it has been reported that co-expression of CRIP1a with CB_1 receptors in cultured cortical neurons alters the actions of cannabinoids in a neuroprotection assay, inhibiting the neuroprotective effect of a CB_1 agonist (WIN55,212-2) and conferring responsiveness to the CB_1 antagonist SR141716 as a neuroprotective agent (52). These data provide further evidence that CRIP1a may regulate CB_1 signalling. However, as yet, evidence that CRIP1a regulates CB_1 signalling *in vivo* has not been reported and for this we may have to await the characterisation of CRIP1a gene-knockout mice.

(d) Endocannabinoid signalling as a mediator of synaptic plasticity in the nervous system

Thus far, a catalogue of proteins that act as cannabinoid receptors or regulators of cannabinoid receptor signalling or catalyse biosynthesis/inactivation of endocannabinoids has been presented. However, from a neurobiological perspective our interest is in understanding how these proteins work together at the cellular level to enable neurophysiological mechanisms to operate. The term "cannabinoid or endocannabinoid signalling" first appears in the literature in 1998 (53, 54) but prior to this much was already known about the distribution of the CB_1 receptor in the brain and the effects of cannabinoids on neurotransmitter release. Based on an analysis of the distribution cannabinoid binding sites (using ^{3}H-CP-55,940 autoradiography) combined with lesion studies and analysis of patterns of CB_1 gene expression (using mRNA in situ hybridisation) it was concluded that the CB_1 receptor is targeted to the axons and axon terminals of neurons in the brain (55-58). This was then confirmed by a series of immunocytochemical studies published in 1998 (5, 59, 60). This presynaptic targeting of CB_1 receptors in neurons was consistent with electrophysiological studies demonstrating that cannabinoids cause inhibition of neurotransmitter release (61). Furthermore, evidence that endocannabinoids are released in response to neuronal stimulation was reported (62), which suggested that endocannabinoids act as intercellular (not intracellular) signalling molecules. A logical extrapolation of these anatomical and physiological observations was that endocannabinoids are synthesized postsynaptically and act as retrograde synaptic signalling molecules (5), which was subsequently proven to be correct.

Depolarisation of principal neurons in several brain regions causes CB_1-mediated inhibition of presynaptic release of the excitatory neurotransmitter glutamate (depolarisation-induced suppression of excitation or DSE) and/or CB_1-mediated inhibition of presynaptic release of the inhibitory neurotransmitter GABA (depolarisation-induced suppression of inhibition or DSI) (2-4). DSE and DSI are not observed in DAGLα-knockout mice, indicating that 2-AG mediates these mechanisms of synaptic plasticity (18, 19). DAGLα is concentrated postsynaptically in dendritic spines that are apposed to CB_1-expressing axon terminals (63), which is consistent with the notion that 2-AG is synthesized postsynaptically but acts presynaptically. The 2-AG degrading enzyme

MAGL is localised presynaptically in the axons of neurons, most notably in glutamatergic neurons (20, 64) and the duration of DSI and DSE in MAGL-knockout mice is prolonged when compared to wild-type mice, indicating that MAGL controls the temporal dynamics of 2-AG/CB_1-mediated retrograde synaptic signalling (65, 66). Accordingly, the MAGL inhibitor JZL184 also prolongs the duration of DSI and DSE in mice (67).

Endocannabinoid signalling also mediates long-term depression (LTD) of synaptic transmission. For example, stimulation of cortical glutamatergic input to the striatum causes activation of postsynaptic metabotropic glutamate receptors, leading to endocannabioid/CB_1-mediated long-term depression of transmission at excitatory cortico-striatal synapses (68). Endocannabioid/CB_1–mediated LTD has subsequently been reported in other brain regions and there is evidence that, as with DSE and DSI, it is postsynaptic formation of 2-AG that mediates this particular form of long-term synaptic plasticity (69).

The physiological roles of anandamide as an endogenous agonist for CB_1 receptors in the central nervous system are currently less well characterised when compared to 2-AG. Evidence that anandamide may also mediate retrograde signalling at synapses has also been reported (70) and it has been suggested that anandamide may mediate tonic endocannabinoid signalling, thereby performing a role that is distinct from the transient stimulated release of 2-AG (71). Furthermore, there is evidence that anandamide may mediate mechanisms of synaptic plasticity via CB_1-independent molecular pathways. Thus, postsynaptic elevation of intracellular anandamide levels is thought to cause LTD via a mechanism mediated by the cation channel TRPV1, which results in internalisation of postsynaptic AMPA-type glutamate receptors (6, 70, 72).

Whilst our knowledge and understanding of the roles of endocannabinoid signalling at the synaptic level have improved dramatically over the last decade, there is still much work to be done in linking processes at this level to the systems level. The CB_1 receptor is widely distributed in the brain but not all neurons express CB_1, so why do particular neural pathways in the brain utilise endocannabinoid signalling to regulate synaptic transmission, whilst others don't. Proximate answers

to this question will surely emerge as we learn more about the patterns of electrical activity that trigger synthesis of endocannabinoids in different regions of the brain and the net behavioural consequences of this. However, ultimate answers will only be obtained by comparative analysis of the physiological roles of the endocannabinoid system, which may shed light on how over evolutionary timescales the endocannabinoid system has been recruited as a regulator of neural processes in different lineages. Some roles of the endocannabinoid system in brain function may be ancient and highly conserved; other roles may have evolved more recently as neural adaptations that are unique to particular lineages. If we are to understand endocannabinoid signalling it will be necessary to explore the physiological roles of this system throughout the animal kingdom, and already important insights are beginning to emerge from comparative studies on non-mammalian animals, as discussed below.

3. THE PHYLOGENETIC DISTRIBUTION AND EVOLUTION OF ENDOCANNABINOID SIGNALLING

Canonical endocannabinoid signalling in the mammalian nervous system, as it is currently understood, could be characterised as a process in which postsynaptic formation of 2-AG by DAGLα in response to depolarisation-induced Ca^{2+} elevation or activation of metabotropic receptors coupled via G-proteins to PLC causes inhibition of neurotransmitter release when 2-AG binds to presynaptic CB_1 receptors, with the spatial and temporal dynamics of this signalling mechanism being controlled by presynaptic degradation of 2-AG by MAGL. Thus, in investigating the evolutionary origins of endocannabinoid signalling, one could specifically investigate the phylogenetic distribution DAGLα, MAGL and CB_1-type receptors. However, this would be a rather narrowly defined view of endocannabinoid signalling in the nervous system. It is true to say that at present our understanding of the physiological role of anandamide as an endogenous ligand for CB_1 receptors is incomplete by comparison with 2-AG. Nevertheless, the phylogenetic distribution of

enzymes involved or implicated in anandamide biosynthesis or inactivation is of interest. Likewise, it is important to investigate the phylogenetic distribution of proteins implicated as regulators of CB_1 signalling such as CRIP1a and CRIP1b, because this may inform understanding of their proposed functions. Whilst CB_1 is by far the most abundant cannabinoid receptor in the mammalian nervous system, there is evidence that CB_2 may have important roles in neural functions (73) and therefore the phylogenetic distribution of CB_2 receptors is also of interest from a neurobiological perspective.

(a) The phylogenetic distribution of* CB_1*/*CB_2*-type cannabinoid receptors

As mediators of the pharmacological effects of Δ^9-THC and the physiological actions of endocannbinoids, the G-protein coupled cannabinoid receptors CB_1 and CB_2 are the focal points for a phylogenetic survey of endocannabinoid signalling. CB_1 and CB_2 share more sequence similarity with each other (~44%) than with any other mammalian G-protein coupled receptors, indicating that they originated by duplication of a common ancestral gene (i.e. they are paralogs). Furthermore, the relatively low level of sequence similarity shared by CB_1 and CB_2 receptors in mammals is suggestive of an evolutionarily ancient gene duplication. Analysis of the phylogenetic distribution of CB_1 and CB_2 receptors indicates that the gene duplication that gave rise to these two receptors occurred in a common ancestor of extant vertebrates, probably concurrently with a whole-genome duplication event. Thus, CB_1 and CB_2 receptor genes can be found in the genomes of non-mammalian tetrapod vertebrates (amphibians e.g. *Xenopus tropicalis*; birds e.g. *Gallus gallus*) and in bony fish (e.g. the zebrafish *Danio rerio*) (74, 75). Interestingly, in teleosts duplicate copies of CB_1 or CB_2 genes are found, attributable to a genome-duplication in a common ancestor of teleosts followed by subsequent lineage-specific retention/loss of duplicate genes. Thus, in the zebrafish *Danio rerio* there is one CB_1 gene and two CB_2 genes, whereas in the puffer fish *Fugu rubripes* there are two CB_1 genes and one CB_2 gene. However, the functional significance of the differential retention of duplicate CB_1 or CB_2 genes in different teleost lineages is currently unknown (74, 75).

To date there are no published reports of CB_1 and CB_2 genes in the most basal of the extant vertebrate orders – the chondrichthyes (e.g. sharks and rays) and the agnathans (e.g. lampreys and hagfish). However, unpublished genome sequence data are available for the elephant shark *Callorhinchus milii* (http://esharkgenome.imcb.a-star.edu.sg/) and the sea lamprey *Petromyzon marinus* (http://genome.wustl.edu/genomes/view/petromyzon_marinus) and in both species a gene encoding a CB_1-type receptor can be found. Interestingly, a CB_2-type receptor gene is not evident in the currently available genome sequence data, which may simply reflect incomplete sequence data or perhaps more interestingly may reflect loss of CB_2 receptor genes in these basal vertebrates.

Genes encoding CB_1/CB_2-type receptors have been found in the invertebrate groups that are most closely related to the vertebrates (urochordates, e.g. CiCBR in *Ciona intestinalis*; cephalochordates, e.g. BfCBR in *Branchiostoma floridae*) but not in the non-chordate invertebrate phyla (74, 76-79). Thus, it appears that CB_1/CB_2-type receptors are unique to the phylum Chordata and as such they have a rather restricted phylogenetic distribution in the animal kingdom.

(b) The phylogenetic distribution of DAG lipases

The antiquity of DAG lipases is evident in the strategy that led to the discovery of the mammalian enzymes DAGLα and DAGLβ - the sequence of a DAG lipase originally identified in the bacterium *Penicillium* was used to identify related proteins in BLAST searches of the human genome sequence (17). This indicates that DAG lipases are an ancient enzyme family that originated in prokaryotes. Submission of human DAGLα and human DAGLβ as query sequences in BLAST searches of the GenBank protein database reveals orthologs of both isoforms in deuterostomian invertebrates and protostomian invertebrates. Thus, the gene duplication that gave rise to DAGLα or DAGLβ dates back at least as far as the common ancestor of extant bilaterian animals.

(c) The phylogenetic distribution of MAG lipase

MAG lipase (MAGL) was originally discovered on account of its role in fat metabolism (80) and subsequently it was proposed that MAGL may regulate 2-AG levels in the brain (20). Submission of human MAGL as a query sequence in BLAST searches of the GenBank protein database reveals orthologs in a wide range of animal species, including deuterostomian invertebrates, protostomian invertebrate and basal invertebrates such as cnidarians (*Nematostella vectensis*) and placozoans (*Trichoplax adhaerens*). Therefore, MAGL was present in the common ancestor of extant animals. However, there has been loss of MAGL in some lineages; for example, in *Drosophila* and other insects. Interestingly, MAGL is also found in poxviruses, which is probably a consequence of horizontal gene transfer from host species (81).

(d) The phylogenetic distribution of NAPE-PLD as an enzyme implicated in anandamide biosynthesis

Although analysis of NAPE-PLD knockout mice indicates that NAPE-PLD is not responsible for synthesis of the bulk of anandamide in the brain (28), this does not rule out the possibility that NAPE-PLD participates in anandamide biosynthesis in other organs and organisms. Therefore, it is of interest to determine the phylogenetic distribution of NAPE-PLD with respect to the evolution of endocannabinoid signalling. Orthologs of NAPE-PLD are found throughout the animal kingdom, in non-mammalian vertebrates, deuterostomian invertebrates (e.g. the sea urchin *Strongylocentrotus purpuratus*), protostomian invertebrates (e.g. the crustacean *Daphnia pulex* and the nematode *C. elegans*) and basal invertebrates such as the cnidarian *Nematostella vectensis* and the placozoan *Trichoplax adhaerens*. However, as with MAGL, there has been loss of NAPE-PLD in some lineages. For example, orthologs of NAPE-PLD are not present in *Drosophila* and other insects, the urochordate *Ciona intestinalis* and the cephalochordate *Branchiostoma floridae*. The functional significance of NAPE-PLD loss in some animal lineages is currently unknown. However, biochemical analysis of species that lack NAPE-PLD may provide useful new insights on NAPE-PLD-independent mechanisms of *N*-acylethanolamine biosynthesis.

(e) The phylogenetic distribution of FAAH and FAAH-2

Analysis of the phylogenetic distribution of FAAH and FAAH2 indicates that the gene duplication that gave rise to these related proteins probably predates the origins of the first animals with nervous systems. However, in addition to the loss of FAAH2 in rodents (see above), there are other examples of lineage-specific loss of FAAH or FAAH2. For example, only a FAAH2 ortholog is found in *Drosophila* and other insects.

(f) The phylogenetic distribution of CRIP1a and CRIP1b

Analysis of the phylogenetic distribution of CRIP1a and CRIP1b in mammals reveals that, whilst CRIP1a is found throughout the mammals, CRIP1b may be unique to catarrhine primates. For example, orthologs of human CRIP1b can be found in the chimpanzee *Pan troglodytes*, the gibbon *Nomascus leucogenys* and the rhesus monkey *Macaca mulatta*. Thus, it appears that exon 3b of the human CRIP1 gene, which is unique to CRIP1b, may have originated relatively recently in mammalian evolution. The functional significance of this is unknown and it will be interesting to investigate the roles of CRIP1b in brain function.

Unlike the restricted phylogenetic distribution of CRIP1b, CRIP1a has a much wider phylogenetic distribution that extends throughout much of the animal kingdom. Indeed orthologs of CRIP1a can be found in basal invertebrates such as the cnidarian *Nematostella vectenses*, indicating that CRIP1a is very ancient protein with origins dating back to the first animals with nervous systems. Accordingly, orthologs of human CRIP1a are found throughout the vertebrates and in deuterostomian invertebrates (e.g. in the cephalochordate *Branchiostoma floridae* and in the hemichordate *Saccoglossus kowalevski*) and protostomian invertebrates (e.g. in the insect *Bombus impatiens* and in the nematode *Caenorhabditis elegans*). This contrasts with the much more restricted phylogenetic distribution of CB_1/CB_2-type cannabinoid receptors, which, as highlighted above, are only found in vertebrates and invertebrate chordates. What this suggests is that CRIP1a is

evolutionarily much more ancient than the CB_1 receptor protein that it is thought to interact with. We can infer from this that CRIP1a must have other physiological roles in cells in addition to its proposed interaction with CB_1 receptors.

4. COMPARATIVE NEUROBIOLOGY OF ENDOCANNABINOID SIGNALLING

(a) Neurobiology of CB_1/CB_2-type endocannabinoid signalling in non-mammalian vertebrates

Given that a great deal is now known about the role of endocannabinoid-CB_1 signalling in mediating retrograde signalling at synapses in the mammalian brain, it is pertinent to pose the question: is there evidence that endocannabinoid-CB_1 mediated retrograde signalling operates at synapses in the central nervous systems of non-mammalian vertebrates? Addressing this question may shed light on the evolutionary origin of this particular mechanism of synaptic plasticity. Not surprisingly, direct evidence from electrophysiological studies comparable to those carried out on rodent brain slices is sparse. The strongest evidence can be found in an impressive series of studies investigating the roles of endocannabinoid signalling in the spinal neuronal network that controls swimming in the lamprey *Lampetra fluviatilis*. Collectively, the data obtained indicate that 2-AG is synthesized postsynaptically by neurons in the spinal locomotor network and acts presynaptically to inhibit both excitatory and inhibitory neurotransmission via CB_1-mediated mechanisms. Furthermore, nitric oxide and endocannabinoid signalling interact to regulate the frequency/amplitude of the locomotor rhythm by differentially modulating excitatory and inhibitory inputs to motoneurons (82-85). Thus, it appears that 2-AG/CB_1-mediated regulation of excitatory and inhibitory neurotransmission is a highly conserved mechanism throughout the vertebrates. Consistent with this hypothesis, recent electrophysiological studies have demonstrated that endocannabinoid-CB_1 signalling mediates DSE and metabotropic glutamate receptor - induced LTD in area X of the zebra finch brain (Thompson and Perkel, 2011). Furthermore, immunocytochemical

analysis of CB_1 expression in the nervous systems of non-mammalian vertebrates reveals patterns of expression consistent with axonal targeting and presynaptic sites of action (86-89).

Given the key role that DAGLα has in postsynaptic formation of 2-AG as a mediator of retrograde synaptic signalling in the mammalian CNS, it would be interesting to determine whether DAGLα is located in the somatodendritic compartment of neurons postsynaptic to CB_1-expressing axons in non-mammalian vertebrates. However, whilst the existence of DAGLα in non-mammalian vertebrates is confirmed by comparative analysis of genome sequence data (see above), detailed neuroanatomical analyses of DAGLα expression in the CNS of non-mammalian vertebrates have not yet been conducted.

It is perhaps not surprising that the physiological roles of 2-AG/CB_1-mediated endocannabinioid signalling at the sub-cellular/cellular level are conserved throughout the vertebrates. Are, however, the roles of endocannabinoid signalling also conserved at the system level e.g. with respect to the regions of the CNS where the CB_1 receptor is expressed and the physiological/behavioural processes that the endocannabinoid signalling system regulates? To address this question, we must look to a currently rather limited number of neuroanatomical and behavioural studies of the cannabinoid system in non-mammalian vertebrates.

Developmental analysis of the zebrafish *Danio rerio* reveals CB_1 mRNA expression in cells located in the presumptive preoptic area of the diencephalon at 24 hours post-fertilization (pf) and by 48 hours expression is observed in the telencephalon, the hypothalamus, the tegmentum and the hindbrain (ventral to cerebellum). In adult zebrafish, CB_1 mRNA expression is observed in the anterior region of the telencephalon and in the periventricular medial zone and central zone of the dorsal telencephalon. Expression is also evident in the hypothalamus and posterior tuberculum (diencephalon) and in the torus longitudinalis (mesencephalon) (90). Complementing the use of *in situ* hybridisation techniques by Lam et al. for analysis of CB_1 mRNA expression in *Danio rerio*, Cottone et al. have used immunocytochemical techniques to investigate the distribution of the CB_1 protein in the cichlid *Pelvicachromis pulcher* (86, 91). Immunostained neurons and/or fibres were

observed in several brain regions including the telencephalon, the preventricular preoptic nucleus, the lateral infundibular lobes of the hypothalamus, the pretectal central nucleus and the posterior tuberculum.

In amphibians, the distribution of CB_1 mRNA in the brain of the rough skinned newt *Taricha granulosa* has been examined using mRNA *in situ* hybridisation methods, revealing a widespread pattern of expression with CB_1 mRNA detected in the telencephalon (olfactory bulb, the pallium and amygdala), the diencephalon (preoptic area and thalamus), the mesencephalon (tegmentum and tectum) and the hindbrain (cerebellum and stratum griseum) (92). Complementing the use of *in situ* hybridisation techniques by Hollis et al. for analysis of CB_1 mRNA expression in *Taricha*, Cesa et al. have used immunocytochemical techniques to investigate the distribution of CB_1 in the brain of *Xenopus leavis*, revealing CB_1-immunoreactive cells and/or fibres in the olfactory bulbs, dorsal and medial pallium, striatum, amygdala, thalamus, hypothalamus, mesencephalic tegmentum and cerebellum (87). CB_1-immunoreactivity is also present in the dorsal and central fields of the *Xenopus* spinal cord, regions that correspond to laminae I-IV and X of the mammalian spinal cord (88).

In birds, CB_1 expression has been analysed in the brain of the chick *Gallus gallus* (93), the zebra finch *Taeniopygia guttata* (89) and the budgerigar *Melopsittacus undulates* (94), revealing some patterns of expression that are strikingly similar to findings in mammals (57, 95). For example, high levels of CB_1 expression are observed in the hippocampus and amygdala and, as in mammals, in the cerebellar cortex the CB_1 gene is expressed in granule cells and the receptor protein is targeted to parallel fibres in the molecular layer.

Detailed descriptions of the distribution of CB_1 receptor expression in the CNS provide valuable frameworks for further investigation of the roles of the endocannabinoid signalling system in non-mammalian vertebrates. However, the number of species analysed thus far are too few to enable any meaningful general conclusions on how the neuroarchitecture the cannabinoid signalling system has been shaped by lineage-specific changes in brain organisation over evolutionary time scales. Nevertheless, the expression of CB_1 in so many different brain regions suggests that

endocannabinoid signalling has been a fundamental and widely employed mechanism of synaptic plasticity throughout more than 400 million years of vertebrate brain evolution. Moreover, there is evidence that at least some of the physiological/behavioural roles of endocannabinoid signalling that have been discovered in mammals are also applicable to non-mammalian vertebrates, suggesting evolutionarily ancient origins.

Some of the most striking actions of CB_1 cannabinoid receptor agonists in mammals are dose-dependent modulatory effects on locomotor activity (96). These behavioural effects are consistent with abundant expression of the CB_1 receptor in brain regions involved in initiation (basal ganglia) and co-ordination (cerebellum) of movement (1). Furthermore, consistent with the notion that CB_1 has an evolutionarily ancient role in neural pathways that control movement, Valenti et al. have reported that the CB_1 receptor antagonist AM 251 (1μg/g body mass) causes a reduction in locomotor activity in the goldfish *Carassius auratus* (97). Behavioural effects of drugs that bind to the CB_1 receptor have also been investigated in an amphibian species, the rough skinned newt *Taricha granulosa*, revealing an inhibitory effect on spontaneous locomotor activity and courtship clasping behaviour (98). Likewise, the cannabinoid WIN 55,212-2 causes inhibition of locomotor activity in the zebra finch (99).

The brain endocannabinoid system is also involved in regulation of appetite and feeding in mammals (100), and again there is evidence that this role may be evolutionarily ancient. A study by Valenti et al. on the goldfish *Carassius auratus* found that food deprivation was accompanied by a significant increase in anandamide (but not 2-AG) in the telencephalic region of the brain and intraperitoneal injection of anandamide (1 pg/g body mass) caused an increase in food intake within 2 h of administration (97). Soderstrom et al. report that in the zebra finch a reduction in food availability causes elevation of 2-AG in the caudal telecephalon and a CB_1-mediated reduction in song-stimulated brain expression of the transcription factor ZENK and a CB_1-mediated reduction in singing (101). Thus, the endocannabinoid system may have a fundamental role in linking behavioural activity with food availability.

The endocannabinoid signalling system is also involved in mechanisms of learning and memory and studies on rodent models have, for example, provided evidence of roles in mechanisms of synaptic plasticity in brain regions critical for declarative memory (hippocampus) and in neural mechanisms underlying extinction of aversive memories (102). In this aspect of endocannabinoid signalling, research on a non-mammalian vertebrate, the zebra finch *Taeniopygia guttata*, has been particularly significant. The zebra finch is an attractive model system for research on neural mechanisms of learning because, in a manner analogous to human language acquisition, male zebra finches learn a song pattern during juvenile development (103). Ken Soderstrom and colleagues have found that cannabinoid exposure during sensorimotor stages of vocal development alters song patterns produced later during adulthood (104) with distinct sub-periods of sensitivity (105). Consistent with these findings, the CB_1 receptor is expressed in brain regions involved in song learning (89) and song production (106), with cannabinoid exposure during sensorimotor stages of vocal development leading to alterations in CB_1 expression and 2-AG levels in the adult brain (107). Further investigation of mechanisms of action have revealed that cannabinoid exposure during sensorimotor stages of vocal development leads to increased basal expression of the transcription factor FoxP2 in the striatum of adult birds, including the area X song region (108) and increased dendritic spine densities (109).

Analysis of the effects of cannabinoids on adult zebra finches reveals an inhibitory effect on song production (110) and an associated inhibition of expression of the transcription factor ZENK in a brain region that is involved in auditory perception (the caudomedial neostriatum) (111). Adult exposure to cannabinoids also causes dose-related inhibitory or stimulatory effects on neuronal activity (based on c-fos expression) in brain regions that control vocal motor output (112).

Thus far, the zebra finch cannabinoid studies have focused primarily on the effects of exogenous cannabinoids (in particular WIN 55,212-2) on song learning and song production. This has provided insights on how developmental exposure to cannabinoids can lead to permanent alterations in brain function and behaviour, which may be highly relevant to an understanding of the

risks associated with cannabis use in adolescents (113). With the recent development of drugs that selectively inhibit degradation of endocannabinoids (e.g. the MAGL inhibitor JZL184 and the FAAH inhibitor PF-3845), it may now be possible to obtain more insights on the physiological roles of the endocannabinoid signalling system in learning using the zebra finch as a model system.

(b) Neurobiology of* CB_1*/*CB_2*-type endocannabinoid signalling in invertebrate chordates

As highlighted above, the discovery of genes encoding co-orthologs of CB_1 and CB_2 in the urochordate *Ciona intestinalis* (CiCBR) (77) and in the cephalochordate *Branchiostoma floridae* (BfCBR) (76) revealed that the evolutionary origin of CB_1/CB_2-type cannabinoid receptors could be traced back beyond the vertebrates to the common ancestor of extant chordates. As of yet the pharmacological properties of CiCBR and BfCBR have not been determined and although these receptors are clearly CB_1/CB_2-type receptors based on sequence similarity, it should not be assumed that CiCBR and BfCBR are necessarily activated by the endocannabinoids 2-AG and anandamide *in vivo*. The G-protein coupled receptors in mammals that are most closely related CB_1 and CB_2 are activated by other lipid signalling molecules – the lysophosphoplipids (114). Therefore, whilst we cannot assume that CiCBR and BfCBR are activated by the endocannabinoids 2-AG and anandamide, it seems reasonable to assume that these receptors are activated *in vivo* by endocannabinoid/lysophospholipid-like lipid signalling molecules. Thus, determining the identity of endogenous ligands for CiCBR and BfCBR is of great interest because it may shed light on how and when CB_1/CB_2-type receptors acquired their property of binding 2-AG and anandamide.

Although the pharmacological properties of CiCBR and BfCBR are unknown, some insights into the physiological roles of CiCBR have been obtained by investigation of the distribution CiCBR expression in *Ciona intestinalis* using specific antibodies that bind to the C-terminal tail of the receptor. These immunocytochemical studies revealed that the ~46 kDa CiCBR protein is concentrated in the cerebral ganglion of *Ciona*, which is located between the inhalent and exhalent siphons that confer on this species and other sea squirts a filter-feeding lifestyle. Furthermore,

CiCBR-immunoreactivity is localised in a dense meshwork of neuronal processes in the neuropile of the cerebral ganglion. CiCBR-immunoreactivity is also present in the axons and axon terminals of neurons that project via peripheral nerves over and around the internal surfaces of the inhalent and exhalent siphons (115), a pattern of expression consistent with behavioural effects of cannabinoids on siphon activity in *Ciona* (116).

The axonal targeting of CiCBR in *Ciona* is intriguing because of its similarity to CB_1 receptor localisation in mammalian CB_1-expressing neurons. It suggests that CiCBR may have a similar role to CB_1 receptors by acting as an axonal regulator of neurotransmitter release. Furthermore, it implies that the role of CB_1 receptors as presynaptic regulators of neurotransmitter release may be very ancient, preceding the gene duplication that gave rise to CB_1 and CB_2 receptors and dating back at least as far as the common ancestor of vertebrates and urochordates. What isn't yet known is the molecular identity of neurotransmitter(s) or neurohormone(s) that are released by CiCBR-expressing neurons in *Ciona*. Is CiCBR expressed in GABAergic and/or glutamatergic neurons, as in mammals, or is CiCBR expressed in other types of neurons such as aminergic or peptidergic neurons? These are questions that need to be addressed if we are to gain an understanding of the physiological roles of CiCBR in *Ciona*. It would also be interesting to determine if BfCBR is expressed by neurons and targeted to axon terminals in *Branchiostoma floridae*. If it is, then this would indicate that the axonal targeting of CB_1-type receptors that is seen in vertebrates can be traced back to the common ancestor of all extant chordates.

It is important to note that because CiCBR and BfCBR are co-orthologs of CB_1-type and CB_2-type cannabinoid receptors, then these receptors in invertebrate chordates may have both CB_1-like and CB_2-like functional properties. It is of interest, therefore, that CiCBR is not only expressed in neurons but is also present in hemocytes in *Ciona* (117), which may be indicative of an ancient CB_2-like role in regulation of immunological processes. Thus, we can imagine a scenario where in the invertebrate chordate ancestor of vertebrates a CiCBR/BfCBR-like protein may have had both CB_1-type and CB_2-type functions and following duplication of the gene encoding a CiCBR/BfCBR-

like protein the duplicated receptors diverged and acquired their more specific CB_1-type and CB_2-type functions. Clearly this is speculative but it provides a rationale for further investigation of the physiological roles of CiCBR and BfCBR and the physiological roles of CB_1-type and CB_2-type cannabinoid receptors in non-mammalian vertebrates.

(c) Neurobiology of non-CB_1/CB_2 mediated endocannabinoid signalling in invertebrates

Whilst CB_1/CB_2-type receptors do not occur in the majority of invertebrates, as highlighted above, the biochemical pathways for biosynthesis/inactivation of 2-AG and anandamide occur throughout the animal kingdom. Therefore, it is of interest to review evidence of non-CB_1/CB_2 mediated endocannabinoid signalling in the nervous systems of invertebrates.

(i) Non-chordate deuterostomes - echinoderms and hemichordates

Effects of cannabinoids and endocannabinoids on fertilisation in the sea urchin *Strongylocentrotus purpuratus* (118) and the occurrence of an endocannabinoid-like signalling system in embryonic and larval sea urchins (Buznikov et al. 2010) have been reported. Furthermore, opportunities to investigate the existence and functions of endocannabinoid-like signalling systems in echinoderms and hemichordates have been facilitated recently by sequencing of the transcriptomes/genomes of the sea urchin *Strongylocentrotus purpuratus* and the hemichordate *Saccoglossus kowalevskii* (119-121).

(ii) Lophotrochozoan protostomian invertebrates – annelids

Investigation of a putative endocannabinoid-like signalling system in annelids has largely focussed on the medicinal leech *Hirudo medicinalis*, which is a well-established model system in neurobiology. In 1997, Stefano *et al*. reported the sequence of a putative leech cDNA encoding a partial (153 amino-acids) protein sequence sharing significant similarity with mammalian CB_1

cannabinoid receptors (122). However, subsequent analysis of the sequence revealed that it was chimeric, with a central region sharing 98% identity with the bovine adrenocorticotropic hormone (ACTH) receptor and outer regions sharing 65-68% identity with mammalian CB_1 receptors (123). Horizontal transfer of bovine DNA to leeches that feed on bovine blood was offered as a possible explanation for this unusual sequence (123) but perhaps a more likely explanation is that the sequence is an artefact (1). More recently, the genome of the leech *Hellobdella robusta* has been sequenced (http://genome.jgi-psf.org/Helro1) and analysis of the genomic sequence data does not reveal the presence of any CB_1-like genes, consistent with analysis of genomic sequence data from other protostomian invertebrates. However, there is evidence that an endocannabinoid-like system may exist in leeches and other annelids.

Detection of binding sites for ^{3}H-anandamide in cell membranes derived from the CNS of *Hirudo medicinalis* suggested the presence of putative receptors for this molecule (122), whilst binding sites for the cannabinoid ^{3}H-CP55,940 have been detected in the nervous system of another annelid species, the earthworm *Lumbricus terrestris* (79). Moreover, the detection of both anandamide and 2-AG and associated enzymatic activities in extracts of leech ganglia indicates that the biosynthetic machinery for synthesis of these molecules exists in annelids (124).

Building upon these biochemical studies are a recent series of papers by Brian Burrell and colleagues that have provided evidence that an endocannabinoid signalling system modulates synaptic transmission in the leech *Hirudo medicinalis*. Li and Burrell found that in the polysynaptic pathway from touch-sensitive mechanosensory neurons (T) to S interneurons in *Hirudo,* long-term depression (LTD) of synaptic transmission is observed following low frequency electrical stimulation (1 Hz) for 450 s or 900 s. LTD elicited by 450 s low frequency stimulation was blocked by NMDA receptor antagonists but LTD elicited by 900 s low frequency stimulation was unaffected by NMDA receptor antagonists. Interestingly, LTD elicited by 900 s low frequency stimulation was blocked by the cannabinoid receptor antagonist AM251 and by the DAG lipase inhibitor RHC80267, suggesting the involvement of an endocannabinoid-like signalling mechanism in this particular form

of synaptic plasticity. Importantly, application of 2-AG or the cannabinoid receptor agonist CP-55,940 induced LTD of the T-S synaptic pathway, providing further evidence of an endocannabinoid-like mechanism of synaptic plasticity in the leech (125).

Further characterisation of this system has revealed that LTD elicited by 900 s low frequency stimulation requires activation of metabotropic serotonin receptors and is dependent on Ca^{2+} elevation in the S interneuron, mediated by voltage-gated Ca^{2+} channels and intracellular inositol triphosphate receptors. Furthermore, this particular form of LTD also involves stimulation of nitric oxide synthase and a decrease in cAMP signalling (126). However, because synaptic plasticity is being examined here in the context of a polysynaptic pathway, mechanistic interpretation of these findings is complicated. Nevertheless, given that CB_1/CB_2-type cannabinoid receptors do not exist in annelids and other protostomian invertebrates, these findings raise intriguing questions concerning the molecular nature of the putative receptors that mediate effects of endogenous or exogenous 2-AG (and other related lipids) in the nervous system of the leech.

Research on mammalian models has provided evidence that transient receptor potential vanilloid (TRPV)-type receptors are activated by endocannabinoids *in vitro* and mediate *in vivo* effects of endocannabinoids (72, 127). Thus, Burrell and colleagues have investigated TRPV-type receptors as potential mediators of endocannabinoid-dependent LTD in the leech nervous system. In the leech there are three types of cutaneous mechanosensory neurons: low threshold touch (T), moderate threshold pressure (P) and high threshold nociceptive (N) neurons, all of which synapse onto the longitudinal motor neuron (L cell), which controls contraction during whole-body shortening. Low-frequency stimulation of the T neurons induces heterosynaptic LTD of glutamatergic transmission at the N-to-L synapse and, importantly, Yuan and Burrell found that this was blocked by DAG lipase inhibitors and the TRPV antagonists capsazepine and SB 366791. Furthermore, application of 2-AG and the TRPV agonist capsaicin mimicked LTD at the N-to-L synapses and these effects of 2-AG and capsaicin were blocked by capsazepine. Pre-treatment with 2-AG or capsaicin occluded subsequent expression of LTD induced by low-frequency stimulation.

Finally, presynaptic, but not postsynaptic, intracellular injection of capsazepine blocked both low-frequency stimulation-induced and 2-AG-induced LTD, indicating that presynaptic TRPV-type receptors mediate LTD at the N-to-L synapse. Collectively, these findings indicate that low-frequency stimulation of T neurons stimulates postsynaptic synthesis of 2-AG or a 2-AG-like molecule in L neurons, which then acts in a retrograde manner to inhibit heterosynaptic neurotransmitter release by N neurons via a TRPV-type receptor mediated mechanism (128).

Evidence that presynaptic TRPV-type receptor mediated LTD may be a widespread mechanism of synaptic plasticity in the leech nervous system has been obtained in a subsequent study using the leech T-S synaptic pathway as a model preparation (129). LTD is induced when a spike train is triggered in the S cell 1-10 s prior to stimulation of the T cell and this is blocked by perfusion of the preparation with the cannabinoid receptor antagonist AM251 or the DAG lipase inhibitor RHC80267 and by injection of the DAG lipase inhibitor tetrahydrolipstatin into the S cell. Perfusion with the TRPV anatagonist capsazepine also blocked LTD induced by a spike train in the S cell 1-10 s prior to stimulation of the T cell. This effect of capsazepine was observed when it was injected into the T cell but not when it was injected into the S cell. Thus, it appears that mechanisms of LTD involving postsynaptic synthesis of 2-AG or 2-AG-like molecules by DAG lipase and presynaptic activation of TRPV-type receptors occur widely in the leech nervous system. These findings raise interest in determination of the molecular identity of the putative TRPV-type receptors that mediate LTD in the leech nervous system. This would enable investigation of the cellular distribution of these receptors in the leech nervous system and comparison of their molecular properties with mammalian TRPV receptors. Likewise, it would be interesting to investigate the expression of DAG lipases in the leech nervous system at a cellular and sub-cellular level to assess DAG lipases as potential sources of 2-AG or 2-AG-like molecules that mediate LTD via retrograde synaptic signalling mechanisms.

The discovery of LTD mediated by 2-AG or 2-AG-like molecules and TRPV-type receptors in the leech nervous system suggest that endocannabinoid-mediated retrograde synaptic signalling is

an evolutionarily ancient mechanism that predates the origins of CB_1/CB_2-type cannabinoid receptors in chordates. If this is correct, these mechanisms of synaptic plasticity may also operate in the nervous systems of other invertebrates (see below). Thus, the findings of Burrell and colleagues have paved the way for further investigation of the function of endocannabinoid-type signalling mechanisms in the nervous systems of all animals, extending the scope for research on the comparative neurobiology of endocannabinoid signalling well beyond the phylum Chordata.

(iii) Lophotrochozoan protostomian invertebrates – molluscs

There has been relatively little investigation of endocannabinoid-like signalling systems in molluscs. This is perhaps surprising given the importance of molluscs as model systems in neurobiology, in particular the gastropod species *Aplysia californica* and *Lymnaea stagnalis* (130). The discovery that an endocannabinoid-type signalling system mediates synaptic plasticty in the leech *Hirudo medicinalis*, as highlighted above, may act as a stimulus for researchers to investigate if similar mechanisms operate in molluscan species.

Importantly, biochemical studies on bivalve molluscan species have revealed the presence of *N*-acylethanolamines, including anandamide, putative binding sites for anandamide and a FAAH-like enzymatic activity (131, 132). Furthermore, transcriptomic/genomic sequence data are available for molluscan species, including the gastropod *Aplysia californica* (133) and the bivalve *Crassostrea gigas* (134). Therefore, identification of genes encoding proteins implicated in endocannabinoid signalling (e.g. DAG lipases, MAGL, NAPE-PLD, FAAH) is now feasible for molluscan species, which will facilitate detailed investigation of endocannabinoid-like signalling systems in molluscan species.

(iv) Ecdysozoan protostomian invertebrates – nematodes

The nematode *Caenorhabditis elegans* was the first animal species to have its genome sequenced and analysis of this sequence provided the first evidence that CB_1/CB_2-type cannabinoid

receptors do not occur throughout animal kingdom (1). A gene encoding a G-protein coupled receptor (C02H7.2) that shares sequence similarity with CB_1/CB_2-type cannabinoid receptors is present in *Caenorhabditis elegans*, but analysis of its sequence indicates that it is not an ortholog (1, 78). Nevertheless, binding sites for the cannabinoid ^{3}H-CP-55,940 have been detected in the nematode *Panagrellus redivivus*, suggesting the presence of other non-CB_1/CB_2-type cannabinoid receptors in nematodes (79)

The presence of the endocannabinoids anandamide and 2-AG has been specifically investigated in nematodes by analysis of three species, *Caenorhabditis elegans*, *Caenorhabditis briggsae* and *Pelodera strongyloides* and both anandamide and 2-AG were detected in all three species. However, anandamide and 2-AG were not detected in a mutant strain of *Caenorhabditis elegans* (fat-3) that lacks functional activity of the delta-6 desaturase enzyme required for synthesis of long chain polyunsaturated fatty acids (including arachidonic acid) (135).

Importantly, the physiological roles of anandamide and other *N*-acylethanolamines (NAEs) in *C. elegans* have recently been investigated, exploiting the use of techniques to manipulate expression of genes encoding enzymes involved in NAE metabolism. Suppression of FAAH using RNA interference (RNAi) or FAAH inhibitors (URB597) caused an increase in the levels of anandamide and other NAEs and overexpression of the *faah-1* gene caused a decrease in levels of anandamide and other NAEs, demonstrating the importance of FAAH as a regulator NAEs in an invertebrate species (136). Furthermore, *faah-1* overexpression caused a developmental delay that was rescued by *faah-1* RNAi, indicating a role for NAEs in promotion of larval development in *C. elegans*. Peak levels of NAEs are detected during the second larval stage (L2) at which time animals are committed to reproductive growth, but NAE levels are reduced at L2 in animals committed to an alternative diapause stage (dauer) induced by dietary restriction. This suggested that NAEs may act as signals of an altered metabolic state and, consistent with this notion, exogenous application of the NAE eicosapentaenoyl ethanolamide (EPEA), and to a lesser extent anandamide, was found to rescue dauer formation. Worms overexpressing *faah-1* also exhibited resistance to thermal stress and

increased adult lifespan, whereas application of EPEA caused a reduction in thermotolerance and lifespan, most strikingly in animals under dietary restriction. Collectively, the data reported by Lucanic et al. indicate that the effect of dietary restriction on lifespan extension is mediated, at least in part, by a reduction in NAE signaling. However, the mechanisms by which NAEs exert effects on lifespan in *C. elegans* are as yet unknown and therefore identification of NAE receptors in nematodes now represents a fascinating objective for the future.

(v) Ecdysozoan protostomian invertebrates – arthropods

Sequencing of the genome of an arthropod species, the insect *Drosophila melanogaster*, provided key evidence that CB_1/CB_2-type cannabinoids receptors do not occur throughout the animal kingdom (1) and this has been supported by sequencing of other arthropod genomes. Nevertheless, binding studies using radiolabelled cannabinoids have revealed the presence of putative binding sites in a variety of arthropod species (79), although identification of membrane proteins that bind cannabinoids has yet to be accomplished for any arthropod species. Evidence that TRPV-type ion channels mediate effects of endocannabinoids in the nervous system of the leech *Hirudo medicinalis* (128) points to these proteins as potential endocannabinoid receptors in arthropods. It is noteworthy that the prototype for the TRP ion channel family was first discovered in *Drosophila* as a result of molecular analysis of the *transient receptor potential* (*trp*) mutant that has a defective phototransduction mechanism (137). Interestingly, analysis of phototransduction in *Drosophila* has revealed similarities with mechanisms of 2-AG biosynthesis. Thus, the photon-activated rhodopsin protein is coupled via G-proteins to stimulate activation of phospholipase C (PLC) and the diacylglycerols (DAGs) generated by PLC are substrates for a DAG lipase that is a homolog of the mammalian enzymes DAGLα and DAGLβ. The *Drosophila* DAG lipase mutant (*inaE*) is defective in photoreceptor responses to light and a DAG metabolite is thought to mediate phototransduction by activating TRP and/or TRP-like (TRPL) ion channels (138). However, the identity of the DAG metabolite that binds to and activates the TRP/TRPL phototransduction channels is not known,

although there is evidence that it may be a polyunsaturated fatty acid (PUFA) (137). This suggests the existence and activity of a MAG lipase in the phototransduction pathway, but one has yet to be identified. As highlighted above, *Drosophila* and other insects do not have an ortholog of the MAGL enzyme that has such a key role in 2-AG metabolism in mammals. Therefore other as yet uncharacterised enzymes may generate endogenous TRP/TRPL ligands from MAG substrates.

Interestingly, DAG lipase is not only expressed in *Drosophila* photoreceptors but is also expressed more widely in the brain (138). Therefore, investigation of the physiological roles of DAG lipase and its metabolites in the nervous system of *Drosophila* might be fruitful in revealing the existence of an endocannabinoid-like signalling system that regulates synaptic activity in insects and other arthropods.

(vi) Cnidarians

Cnidarians are considered to be the most primitive animals with nervous systems and evidence of an endocannabinoid-like system in the hydrozoan *Hydra viridis* has been reported (139). Sequencing of the genomes *Hydra magnipapillata* and the anthozoan *Nematostella vectensis* (140, 141) has presented new opportunities for molecular analysis of putative endocannabinoid signalling mechanisms in the simple nervous systems of these animals.

5. CONCLUDING COMMENTS

The value and importance of non-mammalian animals for cannabinoid research has been illustrated here with reference to studies on "model organisms" ranging from leeches to lampreys and from zebrafish to zebra finches. However, we should not look upon cannabinoid-related studies on non-mammalian animals solely from a utilitarian perspective. Research on the evolution and comparative neurobiology of endocannabinoid signalling is intrinsically interesting and there is great

potential to deepen our understanding of this aspect of nervous system function by revealing "the art of the possible" in the diverse branches of the tree of life.

ACKNOWLEDGEMENTS

I am grateful to Dr Bradley Alger (University of Maryland, USA), Dr Brian Burrell (University of South Dakota, USA) and Dr Michaela Egertová (Queen Mary, University of London, UK) for commenting on and suggesting improvements to the manuscript during its preparation.

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