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The neurobiology and evolution of cannabinoid signalling

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The plant *Cannabis sativa* has been used by humans for thousands of years because of its psychoactivity. The major psychoactive ingredient of cannabis is Δ^9-tetrahydrocannabinol, which exerts effects in the brain by binding to a G-protein-coupled receptor known as the CB_1 cannabinoid receptor. The discovery of this receptor indicated that endogenous cannabinoids may occur in the brain, which act as physiological ligands for CB_1. Two putative endocannabinoid ligands, arachidonylethanolamide ('anandamide') and 2-arachidonylglycerol, have been identified, giving rise to the concept of a cannabinoid signalling system. Little is known about how or where these compounds are synthesized in the brain and how this relates to CB_1 expression. However, detailed neuroanatomical and electrophysiological analysis of mammalian nervous systems has revealed that the CB_1 receptor is targeted to the presynaptic terminals of neurons where it acts to inhibit release of 'classical' neurotransmitters. Moreover, an enzyme that inactivates endocannabinoids, fatty acid amide hydrolase, appears to be preferentially targeted to the somatodendritic compartment of neurons that are postsynaptic to CB_1-expressing axon terminals. Based on these findings, we present here a model of cannabinoid signalling in which anandamide is synthesized by postsynaptic cells and acts as a retrograde messenger molecule to modulate neurotransmitter release from presynaptic terminals. Using this model as a framework, we discuss the role of cannabinoid signalling in different regions of the nervous system in relation to the characteristic physiological actions of cannabinoids in mammals, which include effects on movement, memory, pain and smooth muscle contractility.

The discovery of the cannabinoid signalling system in mammals has prompted investigation of the occurrence of this pathway in non-mammalian animals. Here we review the evidence for the existence of cannabinoid receptors in non-mammalian vertebrates and invertebrates and discuss the evolution of the cannabinoid signalling system. Genes encoding orthologues of the mammalian CB_1 receptor have been identified in a fish, an amphibian and a bird, indicating that CB_1 receptors may occur throughout the vertebrates. Pharmacological actions of cannabinoids and specific binding sites for cannabinoids have been reported in several invertebrate species, but the molecular basis for these effects is not known. Importantly, however, the genomes of the protostomian invertebrates *Drosophila melanogaster* and

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Caenorhabditis elegans do not contain CB_1 orthologues, indicating that CB_1-like cannabinoid receptors may have evolved after the divergence of deuterostomes (e.g. vertebrates and echinoderms) and protostomes. Phylogenetic analysis of the relationship of vertebrate CB_1 receptors with other G-protein-coupled receptors reveals that the paralogues that appear to share the most recent common evolutionary origin with CB_1 are lysophospholipid receptors, melanocortin receptors and adenosine receptors. Interestingly, as with CB_1, each of these receptor types does not appear to have *Drosophila* orthologues, indicating that this group of receptors may not occur in protostomian invertebrates. We conclude that the cannabinoid signalling system may be quite restricted in its phylogenetic distribution, probably occurring only in the deuterostomian clade of the animal kingdom and possibly only in vertebrates.

Keywords: CB_1; CB_2; anandamide; endocannabinoid; fatty acid amide hydrolase; G-protein-coupled receptor

1. INTRODUCTORY PERSPECTIVE AND AIMS

Located on human chromosome 6 is a gene encoding a protein known as the CB_1 cannabinoid receptor that makes us neurologically and psychologically responsive to drug preparations of the plant *Cannabis sativa* (Hoehe *et al*. 1991). The sequence of the human CB_1 gene was determined ten years ago (Matsuda *et al*. 1990; Gérard *et al*. 1990) and this discovery suggested that the brain may produce its own chemicals that interact with the CB_1 receptor as part of normal brain function. Thus, the concept of a *cannabinoid signalling* pathway in the brain has emerged and one aim of this review is to discuss the neurobiology of this system, focusing on experimental research using mammalian models. A second aim is to consider the evolution of cannabinoid signalling and, in particular, to assess the phylogenetic distribution and evolutionary origins of this pathway. In so doing, we hope to provide a useful framework for future research in this fascinating new field of neuroscience.

2. A HISTORICAL OVERVIEW OF CANNABINOID RESEARCH

The psychological and physiological effects of cannabis in humans are well known and include the feeling of euphoria, altered perception, sedation, impaired memory, effects on motor function, analgesia, anti-emesis and appetite stimulation (British Medical Association 1997). The search for the identity of the psychoactive ingredients of cannabis dates back to the 19th century (see Iversen (2000) for a detailed historical review). To date, over 60 chemical compounds derived from the *Cannabis* plant have been identified, but the majority of these compounds are not responsible for the psychoactivity of cannabis. The main pyschoactive *cannabinoid* in cannabis is Δ^9-tetrahydrocannabinol (Δ^9THC), which was isolated by Gaoni and Mechoulam in 1964. The discovery and chemical synthesis of Δ^9THC was the starting point for modern cannabis research because it enabled investigation of the effects and mode of action of cannabinoids in laboratory animals. Administration of Δ^9THC to laboratory animals gives rise to a number of now well-characterized effects such as static ataxia in dogs and catalepsy in mice (Howlett 1995).

The production of synthetic analogues of Δ^9THC enabled analysis of the structure–activity relationships of Δ^9THC, and a mouse behavioural assay was established in which a combination of catalepsy, reduced motility, analgesia and a reduction in body temperature could be correlated with the pyschoactivity of cannabinoids in humans (Martin *et al*. 1995*a*). An understanding of the structural basis for the activity of Δ^9THC in animals provided the foundation for investigation of the mode of action of this compound at the molecular level. In particular, the pharmacological data indicated that Δ^9THC might exert its effects by interacting with a specific receptor protein in the brain. The first indication of the molecular nature of this putative *cannabinoid receptor* was the discovery that Δ^9THC causes a decrease in the concentration of the second messenger molecule cyclic adenosine monophosphate (cAMP) in neuroblastoma cells (Howlett 1984). Moreover, analysis of the effects of other structurally related cannabinoids on neuroblastoma cells indicated that the ability to decrease cAMP correlated with activity in behavioural assays (Howlett & Fleming 1984). Thus, the effects of Δ^9THC at the behavioural level could be attributed to an interaction of this compound with a receptor protein in the brain that is negatively coupled to the enzyme that synthesizes cAMP, adenylyl cyclase. The interaction between activated membrane receptors and adenylyl cyclase was known by this time in the early 1980s to be mediated by a family of proteins known as G-proteins that bind GTP. Thus, the data obtained by Howlett (1984) and Howlett & Fleming (1984) provided the first indication that the putative cannabinoid receptor may be a G-protein-coupled receptor.

Further characterization of the putative cannabinoid receptor using conventional radioligand binding assay methods was hindered because the lipophilic nature of Δ^9THC gives rise to a high proportion of non-specific binding. The key breakthrough came with the development of novel, synthetic, more potent and more hydrophilic analogues of Δ^9THC by Pfizer, Inc., Groton, CT, USA. In particular, a compound known as CP-55,940 was developed and used as a radioligand in membrane-binding assays. A [^{3}H]CP-55,940 binding site in rat brain membranes was characterized and importantly the ability of Δ^9THC and other cannabinoids to displace [^{3}H]CP-55,940 correlated with their relative potencies in classical behavioural assays (Devane *et al*. 1988). Thus, the effects of Δ^9THC at the behavioural level could be attributed to an interaction of this compound with a specific cannabinoid receptor-binding site in the brain. Moreover, the inhibitory effects of a non-hydrolysable analogue of GTP on [^{3}H]CP-55,940 binding provided further evidence that the brain cannabinoid receptor is a G-protein-coupled receptor.

Molecular characterization of G-protein-coupled receptors was first accomplished in the mid-1980s with the

cloning and sequencing of cDNAs encoding a β-adrenergic receptor and a muscarinic acetylcholine receptor (Dixon *et al*. 1986; Kubo *et al*. 1986). These proteins share a common topological structure that is characteristic of all G-protein-coupled receptors and which comprises seven transmembrane domains, an extracellular N-terminal tail, three extracellular and three intracellular loops that link the transmembrane domains and an intracellular C-terminal tail. In 1987 the first sequence of a G-protein-coupled neuropeptide receptor was determined with the cloning and sequencing of a cDNA encoding the bovine receptor for substance-K (Masu *et al*. 1987). This discovery prompted scientists at the National Institute of Mental Health (NIMH) to search for related rat brain receptors that bind other substance-K-like peptides (Matsuda *et al*. 1990). A cDNA (SKR6) encoding a novel 473 amino-acid putative G-protein-coupled receptor was isolated from rat cerebral cortex but when expressed in cells none of the known neuropeptides caused activation of the receptor. Thus, SKR6 was a receptor without a known ligand (an 'orphan receptor'). Simultaneously at NIMH, however, Herkenham *et al*. (1990) were using autoradiography to map the distribution of [^{3}H]CP-55,940 binding sites in the rat brain. When the pattern of SKR6 mRNA expression in the rat brain was investigated using *in situ* hybridization methods, it was found that it corresponded to a large extent with the distribution of [^{3}H]CP-55,940 binding sites. This suggested that SKR6 might encode the brain cannabinoid receptor, and when cells were transfected with SKR6 this conferred responsiveness to the inhibitory effects of cannabinoids on adenylyl cyclase activity. Moreover, the potency of cannabinoids in inhibiting adenylyl cyclase activity in SKR6-transfected cells correlated with their relative potency in behavioural assays. These observations demonstrated that the G-protein-coupled receptor encoded by the SKR6 cDNA was indeed the rat brain cannabinoid receptor (Matsuda *et al*. 1990). Subsequently, a human orphan G-protein-coupled receptor was identified as an orthologue of the rat brain cannabinoid receptor on the basis of 98% amino-acid sequence identity (Gérard *et al*. 1990).

In 1993 a second G-protein-coupled cannabinoid receptor sequence (CX5) was identified amongst cDNAs from the human promyelocytic leukaemic cell line HL60 (Munro *et al*. 1993). CX5 shared 44% amino-acid sequence identity with the human brain cannabinoid receptor and when expressed in COS cells conferred binding sites for cannabinoids. The pattern of expression of a rat orthologue of CX5 was investigated and found to be expressed by macrophages in the marginal zone of the spleen but not in the brain. In order that the two cannabinoid receptors could be distinguished, Munro *et al*. (1993) suggested that the brain receptor be referred to as CB_1 and that the second receptor, which is expressed by cells of the immune system, be referred to as CB_2.

The discovery of CB_2 provided a molecular explanation for the effects that cannabis was known to have on the immune system, and Parolaro (1999) provide a detailed review of this aspect of cannabinoid biology. Moreover, it was clear that in order that the role of CB_1 or CB_2 in mediating pharmacological effects of cannabinoids could be determined, it would be necessary to develop selective agonists and/or antagonists for the two receptors. The first CB_1 antagonist was developed by Sanofi Recherche and is known as SR141716A. This compound displays nanomolar affinity for CB_1 but only micromolar affinity for CB_2 in ligand-binding assays (Rinaldi-Carmona *et al*. 1994). Subsequently, a CB_2 antagonist, SR144528, was developed by the same company and shown to have 700-fold lower affinity for CB_1 than CB_2 (Rinaldi-Carmona *et al*. 1998).

Probably the most powerful approach by which the involvement of either CB_1 or CB_2 in mediating the effects of cannabinoids in animals can be investigated has been the development of mice in which either the CB_1 gene or the CB_2 gene has been deleted. CB_1-knockout mice have been generated independently in two laboratories and shown to be unresponsive to cannabinoids in a standard set of behavioural assays (Ledent *et al*. 1999; Zimmer *et al*. 1999). Detailed characterization of the behavioural and physiological consequences of CB_1 gene inactivation is now ongoing and will be discussed in more detail below. Subsequently, CB_2-knockout mice have been generated and in these animals the immunomodulatory effects of cannabinoids are absent whilst behavioural effects mediated by CB_1 are normal (Buckley *et al*. 2000). Thus, it is now possible to distinguish effects of cannabinoids in animals that are mediated by either CB_1 or CB_2. The first part of this review (§ 3) discusses the neurobiology of cannabinoids and therefore focuses on CB_1 rather than CB_2. However, CB_2 will be considered again later with respect to cannabinoid receptor evolution.

The discovery of cannabinoid receptors that are activated by the plant-derived compound Δ^9THC was a major breakthrough for cannabis research, comparable in importance to the discovery of opioid receptors in the 1970s. Moreover, it suggested that endogenous cannabinoid ligands for the receptors or *endocannabinoids* may be present in the brain. Two endocannabinoids have so far been identified—arachidonylethanolamide (known as 'anandamide') and 2-arachidonylglycerol (2-AG) (Devane *et al*. 1992; Mechoulam *et al*. 1995; Sugiura *et al*. 1995). Importantly, both of these compounds display a profile of biological activities that is similar to 'classical' cannabinoids (Fride & Mechoulam 1993; Crawley *et al*. 1993; Mechoulam *et al*. 1995). Thus, molecular components of a putative cannabinoid signalling system in the brain have been identified and major aims of current cannabinoid research are to investigate how, where and when these molecules are produced and inactivated and their role in the physiological mechanisms of brain function. These issues will provide the main focus for Part 1 of this review.

3. PART 1: THE NEUROBIOLOGY OF CANNABINOID SIGNALLING

(a) *Functional neuroanatomy of the CB_1 cannabinoid receptor*

Analysis of the distribution of [^{3}H]CP-55,940 binding sites in the rat brain provides insight into the neuroanatomical basis for the behavioural effects of Δ^9THC in rodents (Herkenham *et al*. 1991). For example, the highest concentrations of cannabinoid binding sites are in the basal ganglia and cerebellum, regions of the brain that are involved in the initiation and coordination of

movement. This is consistent with the well-characterized effects of Δ^9THC on movement in rodents. Conversely, low level or no binding is present in the brainstem (medulla and pons), consistent with the lack of cannabinoid activity on vital respiratory and cardiovascular functions. The pattern of CB_1 mRNA expression in the rat brain is broadly similar to the distribution [^{3}H]CP-55,940 binding sites but with some discrepancies which can be largely accounted for by translocation of the CB_1 protein along axons to sites distal from the neuronal somata where the CB_1 gene is transcribed and translated (see Matsuda *et al*. 1993).

To assess whether CB_1 expression alone accounts for the distribution of cannabinoid binding sites as revealed by autoradiography, immunocytochemical methods can be used to localize the CB_1 protein in the brain. Antibodies to the rat CB_1 receptor that have then been used for such studies have been produced independently in at least three laboratories. Twitchell *et al*. (1997) raised antibodies to amino acids 1–77 that form a large part of the N-terminal tail of rat CB_1, whilst Pettit *et al*. (1998) raised antibodies to amino acids 83–98 located in the juxtamembrane region of the N-terminal tail of the receptor. Egertová *et al*. (1998*a*) targeted the C-terminal tail of CB_1 by raising antibodies to a sequence of 13 amino acids (461–473) that forms its most distal segment. Immunocytochemical studies using these different antibodies revealed a generally consistent pattern of staining that corresponded with the distribution of cannabinoid binding sites (figure 1; Egertová *et al*. 1998*a*; Egertová & Elphick 2000; Pettit *et al*. 1998; Tsou *et al*. 1998*a*). These studies provide important evidence that CB_1 is likely to be largely responsible for mediating the effects of cannabinoids in the brain. This is supported by autoradiographic analysis of CB_1-knockout mice in which no detectable [^{3}H]CP-55,940 binding sites were observed (Zimmer *et al*. 1999). However, more recent analysis of cannabinoid binding sites in CB_1-knockout mice indicates that an additional non-CB_1, non-CB_2 receptor may be present in the brain (Breivogel *et al*. 2000; Di Marzo *et al*. 2000). Nevertheless, based on comparison of data from CB_1 immunocytochemistry and [^{3}H]CP-55,940 autoradiography, this putative novel cannabinoid receptor is likely to represent a very small component of total cannabinoid binding sites in the rodent brain.

Determination of the neuroanatomical distribution of the CB_1 receptor in the brain has provided a framework for interpreting the behavioural effects of cannabinoids. In particular, it has identified regions of the brain, neural circuits and individual synapses in which effects of cannabinoids can be investigated using electrophysiological techniques. In this way, it has become possible to shed light on the role that putative endocannabinoid signalling molecules may have in normal brain function. Moreover, one of the advantages of CB_1 immunocytochemistry compared with autoradiographic localization of cannabinoid binding sites is that it enables resolution of the receptor in neurons at the cellular and subcellular level. In the following section, we will discuss some of the general principles that have emerged from localization of CB_1 at the cellular and subcellular level and, in particular, how this relates to the mechanisms of cannabinoid signalling in neurons.

(b) *Molecular mechanisms of cannabinoid signalling in neurons*

(i) *CB_1 receptor function*

In the context of classical concepts of synaptic physiology, it is important to establish whether CB_1 functions as a postsynaptic receptor or a presynaptic receptor or both. The first autoradiographic localization studies provided an insight into this issue: Herkenham *et al*. (1991, p. 577) state that the 'receptors are dense in the location of the striatal efferent pathway, suggesting that this entire array of dense labelling may represent receptors on axons and terminals of striatofugal neurons. Indeed, such "presynaptic" localisation of cannabinoid receptors was confirmed in studies showing loss of receptors in the striatum, pallidum, and nigra following destruction of striatal neurons with ibotenic acid.' Subsequent immunocytochemical studies have shown that the CB_1 receptor is targeted to the axons and terminals of neurons, not only in the basal ganglia, but probably in all neurons that express the CB_1 gene in the brain (Egertová *et al*. 1998*a*; Egertová & Elphick 2000). Indeed, it is pertinent to ask whether CB_1 is ever targeted to the somato-dendritic compartment of neurons to function as a 'postsynaptic' receptor. This is a controversial issue and contradictory data have been obtained in different immunocytochemical studies. Using antibodies to the C-terminal tail of CB_1, immunoreactivity was only observed in fibres and axon terminals and immunostaining was not observed in any neuronal somata in either the brain or spinal cord of rats under normal conditions (Egertová *et al*. 1998*a*; Egertová & Elphick 2000; Farquhar-Smith *et al*. 2000). The only occasion where CB_1-immunoreactivity has been observed in neuronal somata with C-terminal-directed antibodies was in hemisected spinal cord preparations close to the site of hemicordectomy, which may be due to interruption of receptor transport out of the cell bodies (Farquhar-Smith *et al*. 2000).

In contrast to the findings with C-terminal-directed antibodies, two studies using N-terminal-directed antibodies reported that, in addition to axonal staining like that seen with C-terminal-directed antibodies, CB_1-immunoreactivity was also seen in the somata of neurons known to express the CB_1 gene (Tsou *et al*. 1998*a*; Pettit *et al*. 1998). For example, CB_1-immunoreactivity was observed in the somata of GABAergic interneurons (basket cells) in the hippocampus (Tsou *et al*. 1998*a*). However, when these neurons were subsequently examined at the subcellular level using electron microscopic methods, the CB_1-immunoreactivity was found to be localized in the Golgi apparatus and not in the cell surface membrane (Katona *et al*. 1999). These findings indicate that the CB_1-immunoreactivity detected in hippocampal interneurons corresponds to newly synthesized receptors prior to translocation out of the somato-dendritic compartment into the axonal compartment. Therefore, the immunocytochemical data obtained so far indicate that CB_1 is typically, and possibly exclusively, a presynaptic receptor. This conclusion is generally supported by electrophysiological studies, which will be discussed below.

What is the role of the CB_1 receptor in the presynaptic terminals of neurons that express the CB_1 gene? Prior to the discovery of the CB_1 gene, it was already established that cannabinoids cause inhibition of neurotransmitter

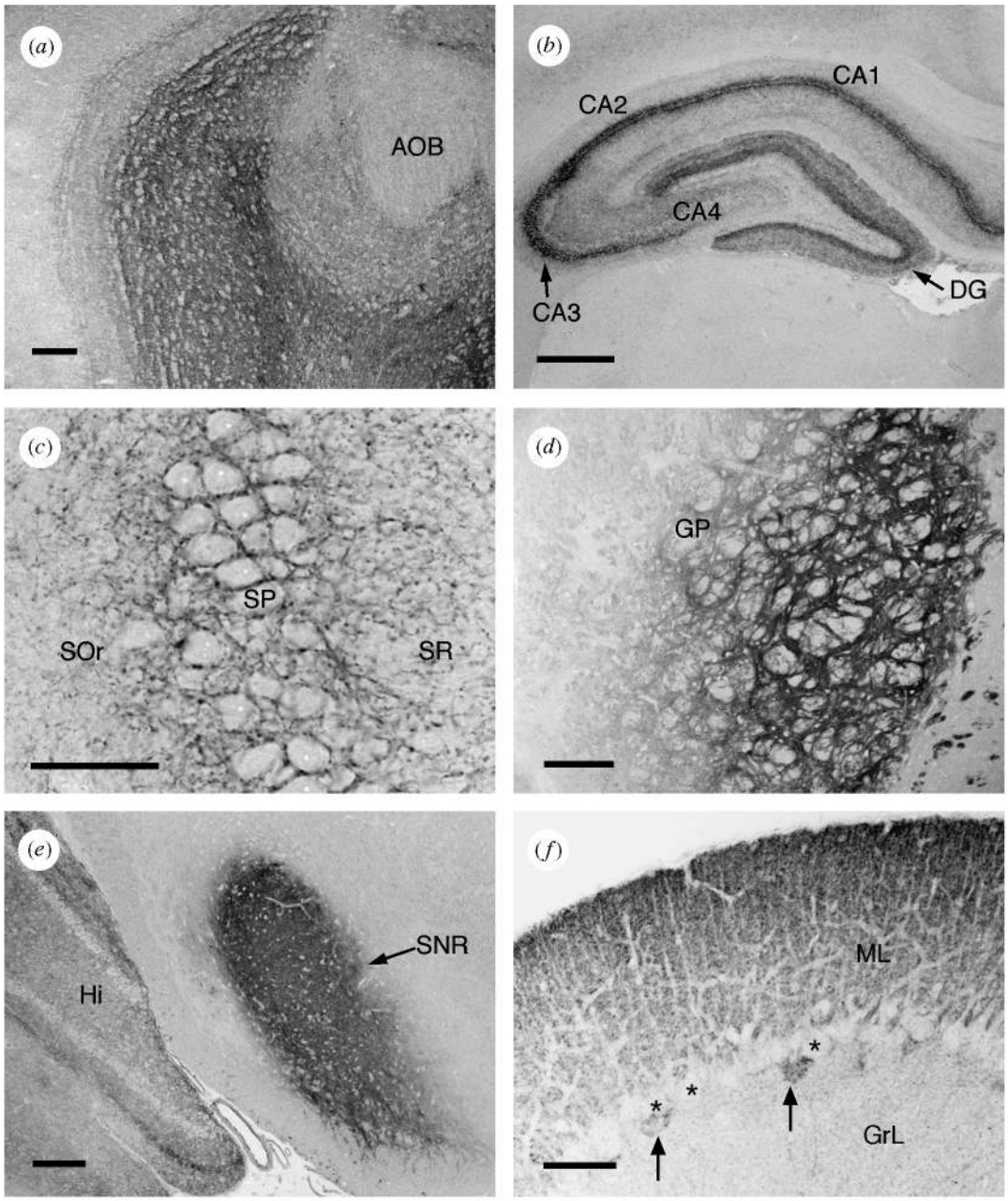

Figure 1. Immunocytochemical localization of the CB_1 cannabinoid receptor in rat brain. (*a*) Main olfactory bulb. (*b*,*c*) Hippocampal formation ((*c*) shows detail of CB_1-immunoreactive fibres located around the unstained cell bodies of pyramidal cells in the CA3 region of the hippocampus). (*d*) Globus pallidus. (*e*) Substantia nigra pars reticulata. (*f*) Cerebellar cortex with arrows indicating CB_1-immunoreactive terminals of basket cells and asterisks showing the positions of unstained Purkinje cells. Abbreviations: AOB, accessory olfactory bulb; CA1–CA4, fields 1–4 of the hippocampus; DG, dentate gyrus; SOr, stratum oriens; SP, stratum pyramidale; SR, stratum radiatum; GP, globus pallidus; Hi, hippocampus; SNR, substantia nigra pars reticulata; ML, molecular layer; GrL, granule cell layer. Scale bars: (*a*) 100 μm, (*b*) 500 μm, (*c*) 50 μm, (*d*) 100 μm, (*e*) 200 μm, (*f*) 50 μm. (Reproduced with permission of Wiley-Liss, Inc., from Egertová & Elphick 2000.)

release by neurons (Roth 1978) and subsequently numerous studies have confirmed this in several regions of the brain. How does activation of presynaptic CB_1 receptors cause inhibition of neurotransmitter release? The prototypical pathway for CB_1 signalling in neurons is that originally discovered by Howlett (1984) in which the activated receptor is coupled to inhibition of adenylyl cyclase with a consequent decrease in cAMP. How does this influence vesicular release of classical neurotransmitters from presynaptic terminals expressing CB_1? One potential mechanism would be to cause shortening of the duration of presynaptic action potentials due to activation of potassium currents. Deadwyler *et al.* (1993) have obtained experimental evidence for this mode of action: they demonstrated that cannabinoids enhance activation of A-type potassium currents in cultured hippocampal neurons. Moreover, the enhancement of A-type currents is due to decreased phosphorylation of the respective potassium channels by cAMP-dependent protein kinase A (PKA) (Deadwyler *et al.* 1995). Thus, activation of presynaptic cannabinoid receptors leads to inhibition of adenylyl cyclase, a reduction in cAMP and a decrease in

PKA-mediated phosphorylation of A-type potassium channels. The consequent enhancement of A-type currents could account for the inhibitory effects of cannabinoids on neurotransmitter release by shortening the duration of action potentials that invade the presynaptic terminals of neurons. A second potential mechanism by which cannabinoids could inhibit neurotransmitter release would be through inhibition of presynaptic calcium currents. In 1992, two groups demonstrated that N-type calcium currents are indeed inhibited by cannabinoids (Mackie & Hille 1992; Caulfield & Brown 1992); however, in this case the effect is not mediated by cAMP but through direct G-protein-mediated inhibition of calcium channel proteins. Thus, activation of presynaptic cannabinoid receptors can lead to inhibition of neurotransmitter release via two separate molecular pathways, although it remains to be demonstrated whether both mechanisms operate simultaneously in the presynaptic zone of an individual neuron.

(ii) *Endogenous cannabinoids*

The inhibitory effect of exogenously applied cannabinoids on neurotransmitter release from the terminals of brain neurons raises questions about the physiological relevance of this phenomenon. With the discovery of the endocannabinoids anandamide and 2-AG it may now be possible to identify the physiological circumstances in which these molecules are produced to modulate neurotransmitter release by CB_1-expressing neurons. However, before we can assess the physiological relevance of cannabinoid receptors in the brain, a number of basic questions relating to the properties of the putative endocannabinoid signalling molecules, anandamide and 2-AG, need to be addressed, in particular the following questions. How and where are these molecules synthesized in the brain? Are they released by neurons and if so what promotes their release? Are these putative endocannabinoids inactivated and if so by what mechanisms?

Measurement of anandamide and 2-AG concentrations in brain tissue provides an initial indication of their potential relative importance as endocannabinoids. Thus, for anandamide, concentrations typically ranging between 10 and 100 pmol g^{-1} (wet weight) have been reported, whilst 2-AG has been reported to occur at concentrations 170 times higher than anandamide (Stella *et al*. 1997). However, in terms of assessing the potential of these molecules to interact with CB_1 receptors, more useful information is provided by analysis of their concentrations in different regions of the brain. Felder *et al.* (1996) measured the concentration of anandamide in regions of both rat and human brain that contain a high density of CB_1 receptors (e.g. hippocampus, cerebellum and striatum) and in a region that is sparse in CB_1 receptors, the thalamus. In rat, the concentration of anandamide in all four regions was similar (*ca.* 20 pmol g^{-1} wet weight) whilst in human, the concentration of anandamide in the thalamus was approximately twice the concentration measured in the cerebellum. A more detailed analysis of rat brain was performed recently by Bisogno *et al.* (1999) in which the concentrations of anandamide and 2-AG were measured in nine regions of the brain and compared with the distribution of cannabinoid binding sites. The authors found higher levels of anandamide and 2-AG in the brainstem, hippocampus, striatum and medulla, while the cerebellum, diencephalon and cortex contained lower levels. In contrast to the findings of Stella *et al.* (1997), in all brain regions the concentration of anandamide was found to be higher than that of 2-AG (Bisogno *et al*. 1999).

It is clear from these data that for both anandamide and 2-AG, their relative regional abundance in the brain does not correlate with the distribution CB_1. The significance of this finding is not yet clear. However, what is immediately apparent is the considerable variability in the values obtained for brain endocannabinoid concentrations both between and within studies. Presumably this reflects variation between individual animals and/or methodological discrepancies. Clearly, more sophisticated methods for localizing endocannabinoids in the brain are required. One possibility would be to develop monoclonal antibodies that specifically recognize anandamide or 2-AG and which could be used for immunocytochemical localization studies. However, this approach is unlikely to be feasible because of the potential for cross-reactivity with numerous other brain lipids. Thus, characterization of the enzymes involved in endocannabinoid synthesis is required to facilitate more detailed analysis of the sites of anandamide and 2-AG production in the brain.

(iii) *Biosynthesis and release of endocannabinoids*

Soon after the isolation of anandamide from porcine brain, studies investigating how it is synthesized were initiated. Initially, it was thought that it may be formed by condensation of arachidonic acid and ethanolamine (Devane & Axelrod 1994), but this is now considered to be a manifestation of an inactivation mechanism (see below) acting in reverse with non-physiological concentrations of the relevant substrates. An alternative pathway was proposed in which anandamide is synthesized by phospholipase-D (PLD)-mediated hydrolytic cleavage of a phospholipid precursor, N-arachidonoylyl phosphatidylethanolamine (NAPE) in a calcium-dependent manner (Di Marzo *et al*. 1994; Cadas *et al*. 1996). Biosynthesis of NAPE is also calcium dependent and potentiated by cAMP, and it is thought that it is mediated by *N*-transacylation of phosphatidylethanolamine with arachidonic acid stereoselectively donated from the *sn*-1 position of di-arachidonylphosphatidylcholine (Cadas *et al*. 1996, 1997). Thus, a pathway for anandamide synthesis in neurons has been proposed in which membrane depolarization leads to calcium-dependent, *N*-acyltransferase-mediated formation of NAPE, which is then converted to anandamide by PLD (Cadas *et al*. 1997). The distribution of NAPE and *N*-acyltransferase activity in rat brain has been examined (Bisogno *et al*. 1999; Cadas *et al*. 1997) but as with anandamide, their distribution does not correlate in any obvious way with the distribution of CB_1. It is clear, therefore, that more detailed molecular characterization of enzymes involved in anandamide biosynthesis is now essential in order that their distribution can be compared neuroanatomically with CB_1. The same situation exists with respect to the mechanisms of 2-AG biosynthesis where potential pathways have been proposed but detailed molecular characterization has yet to be accomplished (Di Marzo & Deutsch 1998). This is essential if

we are to be able to assess the role of anandamide and 2-AG as potential components of a cannabinoid signalling system in the brain. The presence of these molecules in the brain by itself does not prove that they function as activating ligands for CB_1 under physiological conditions.

If anandamide and 2-AG function as endogenous ligands for cannabinoid receptors in the brain, then it is important to establish whether they are released and if so under what circumstances. This information is useful in assessing whether they may function as intercellular or intracellular signalling molecules. There is now evidence available from several studies indicating that anandamide is released by depolarized neurons *in vitro* (Di Marzo *et al.* 1994) and *in vivo* (Giuffrida *et al.* 1999), supporting the view that this molecule functions as an intercellular signal. However, 2-AG release was not observed when monitored under conditions in which anandamide release was detected in the striatum (Giuffrida *et al.* 1999). What might be the explanation for this observation? One possibility is that anandamide and 2-AG are used as intercellular endocannabinoid signals in a mutually exclusive fashion such that co-release of these molecules is not observed in any one region of the brain. However, to the best of our knowledge, there have so far been no reports of 2-AG release from neurons, whereas anandamide release has been observed in several regions of the brain (Giuffrida *et al.* 1999; Walker *et al.* 1999*b*). Therefore, whilst anandamide may function as an intercellular cannabinoid signalling molecule, perhaps 2-AG is not released by neurons in the brain and functions as an intracellular cannabinoid signalling molecule in neurons that express CB_1.

(iv) *Uptake and inactivation of endocannabinoids*

If, as the data indicate, anandamide is released by neurons and exerts physiological effects by binding to CB_1, then it is likely that there are physiological mechanisms for inactivation of this molecule. An enzyme that catalyses hydrolysis of anandamide, forming arachidonic acid and ethanolamine, was detected in rat brain tissue soon after the discovery of anandamide (Deutsch & Chin 1993) and is variously known as 'anandamide amidase' 'anandamide hydrolase' or 'anandamide amidohydrolase'. However, molecular characterization of this enzyme was accomplished via a different line of research. Cravatt *et al.* (1996) isolated an enzyme from rat liver that catalyses hydrolysis of the endogenous sleep-inducing lipid oleamide to oleic acid and ammonia (Cravatt *et al.* 1995). This enzyme was named 'oleamide hydrolase' and a cDNA encoding it was sequenced and found to encode a 579 amino acid protein with a predicted molecular mass of *ca.* 63 kDa. However, when the substrate selectivity of the cloned enzyme was analysed it was found that it also displayed 'anandamide amidohydrolase' activity. In fact, the rate of anandamide hydrolysis was higher than oleamide hydrolysis. This novel enzyme was therefore named fatty acid amide hydrolase (FAAH), in recognition of its ability to catalyse hydrolysis of two putative fatty acid amide signalling molecules, oleamide and anandamide (Cravatt *et al.* 1996). Subsequently, it has been found that FAAH also catalyses hydrolysis of 2-AG (Goparaju *et al.* 1998) and therefore it could participate in inactivation of both anandamide and 2-AG.

The existence of a membrane transporter that mediates uptake of anandamide has also been investigated. Di Marzo *et al.* (1994) and Hillard *et al.* (1997) demonstrated that anandamide is accumulated by cerebellar granule cells by a Na^+-independent, protein-mediated transport process that has characteristics of facilitated diffusion. Moreover, Beltramo *et al.* (1997) demonstrated that anandamide uptake by rat cortical neurons and astrocytes is selectively inhibited by the compound N-(4-hydroxyphenyl)arachidonylamide (AM404), invoking the existence of a specific transporter for anandamide. However, more recent studies have revealed that AM404 inhibits FAAH activity in a human neuroblastoma cell line (Verity *et al.* 2000). Thus, it is possible that the activity of FAAH alone may be sufficient to facilitate diffusion of anandamide across the cell membrane of FAAH-expressing cells (B. F. Cravatt, personal communication).

To assess the potential role of FAAH in cannabinoid signalling mechanisms of the brain, its distribution in the rat brain has been analysed in detail and compared with CB_1 receptor expression (Egertová *et al.* 1998*a*). Measurement of FAAH enzyme activity in eight regions of the rat brain revealed the highest concentrations in the hippocampus and cortex and the lowest concentrations in the brainstem and hypothalamus with intermediate concentrations in the cerebellum, olfactory bulb, striatum and thalamus (Thomas *et al.* 1997; Egertová *et al.* 1998*a*). Importantly, when the distribution of the FAAH protein was analysed using Western blotting methods, its relative abundance in rat brain regions correlated with the distribution of fatty acid amide hydrolysing activity, indicating that FAAH is probably the major enzyme in the brain responsible for inactivation of fatty acid amides (Egertová *et al.* 1998*a*). Moreover, comparison of the distribution of FAAH in rat brain regions based on enzyme assays and Western blotting data revealed a significant overlap with the expression of CB_1. For example, high concentrations of FAAH and CB_1 are found in both the hippocampus and neocortex whilst lower concentrations of FAAH and CB_1 are found in the brainstem and hypothalamus. To assess the potential role of FAAH in cannabinoid signalling mechanisms of the brain, the distribution of this enzyme was compared with CB_1 at the cellular and subcellular level using immunocytochemical methods. Three regions of the brain that are enriched with both FAAH and CB_1 were analysed in detail: the cerebellum, hippocampus and neocortex. Intriguingly, in all three regions a complementary pattern of FAAH and CB_1 expression was observed with FAAH occurring in the somato-dendritic compartment of neurons that are postsynaptic to CB_1-expressing axons. For example, in the cerebellum FAAH was detected only in the cell bodies and dendrites of Purkinje cells whilst CB_1 was localized only in the axons and terminals of two classes of neurons that are presynaptic to Purkinje cells: granule cells and basket cells. Similarly, in the hippocampus and neocortex FAAH is localized in pyramidal cells that are surrounded by the CB_1-containing terminals of neurons that are likely to be local GABAergic interneurons (Egertová *et al.* 1998*a*; Egertová & Elphick 2000). Thus, in three major regions of the brain there is a synaptic association of FAAH and CB_1, which supports the view that FAAH may participate in cannabinoid signalling mechanisms of the

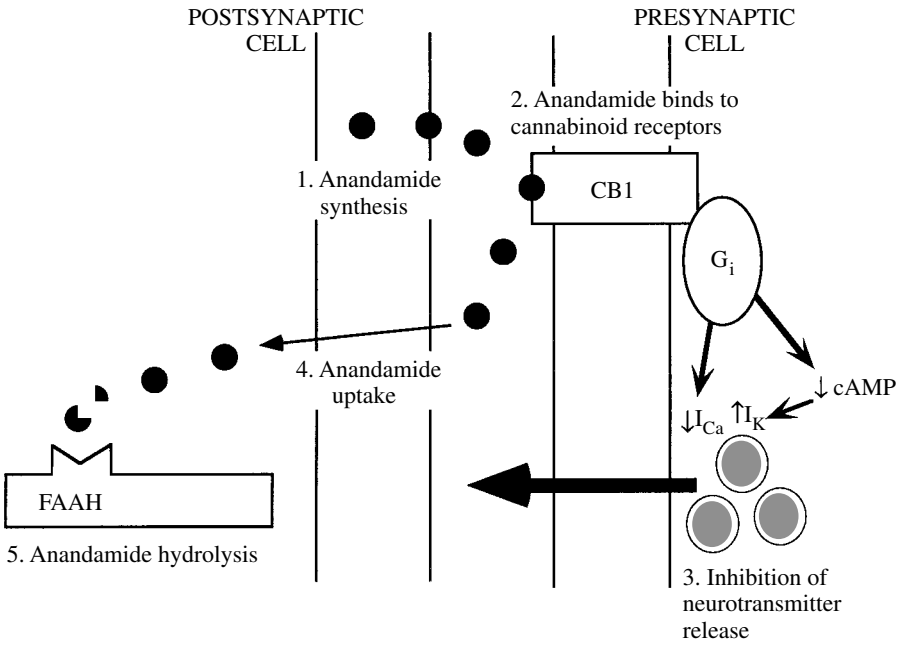

Figure 2. A model of synaptic cannabinoid signalling in which anandamide functions as a retrograde signalling molecule that modulates (inhibits) the release of 'classical' anterograde transmitters by presynaptic terminals. Anandamide is synthesized and released by the postsynaptic cell [1] and then diffuses into the synaptic cleft where it binds to and activates presynaptic CB_1-type cannabinoid receptors [2]. Activated CB_1 receptors cause inhibition of neurotransmitter release from presynaptic terminals via G-protein-mediated mechanisms involving activation of K^+ channels or inhibition of Ca^{2+} channels [3]. Anandamide dissociates from CB_1 receptors and then, following uptake into the postsynaptic cell [4], is hydrolysed intracellularly by FAAH [5].

brain by inactivating locally released endocannabinoids. However, it is unlikely that this is the only role of FAAH in the brain because FAAH is also expressed in regions of the brain that are largely void of CB_1 such as the thalamus and brainstem (Thomas *et al.* 1997; Tsou *et al.* 1998*b*; M. Egertová and M. R. Elphick, unpublished data). Perhaps in these regions of the brain other fatty-acid-related signalling molecules such as oleamide are regulated by FAAH. Moreover, FAAH is also expressed in non-neuronal cells of the brain including epithelial cells of the choroid plexus (Egertová *et al.* 2000). The physiological role of FAAH in these cells is unknown but the presence of FAAH at this critical site within the blood–brain barrier is likely to affect significantly the ability of anandamide, 2-AG or oleamide to reach the brain following experimental intraperitoneal or intracerebroventricular injection of these compounds. This may explain why the presence of non-selective FAAH inhibitors such as phenylmethylsulphonyl chloride (PMSF) is required for anandamide to mimic the behavioural effects of 'classical' cannabinoids at equivalent concentrations (Ueda & Yamamoto 2000).

(v) *A model of cannabinoid signalling*

A considerable amount of information has now been accumulated on the molecular mechanisms of cannabinoid receptor action in neurons and on the mechanisms of endocannabinoid synthesis, release and inactivation. There is still much that needs to be learnt, but with the available data it may be possible to formulate a model of how cannabinoid signalling operates at the synaptic level. In 1998, at the annual meeting of the International Cannabinoid Research Society in France, we proposed a model (Elphick *et al.* 1998) which we present here in the scientific literature for the first time (see figure 2) and which is based on our analysis of FAAH and CB_1 expression in the cerebellum, hippocampus and neocortex (Egertová *et al.* 1998*a*; Egertová 1999). In this model CB_1 is located presynaptically, consistent with overwhelming evidence from both electrophysiological and neuroanatomical data. Unknown at present is where a putative endocannabinoid such as anandamide is synthesized in order that it might gain access to binding sites on CB_1 receptors. One possibility is that anandamide is synthesized presynaptically within the same membranes as CB_1 and functions as a local, membrane-delimited, intraneuronal messenger. However, this runs contrary to the more widely assumed role of anandamide as an intercellular signalling molecule and the observation that anandamide is released into the extracellular medium by neurons (Di Marzo *et al.* 1994). Therefore, perhaps a more likely scenario is that presented in figure 2 in which anandamide is synthesized and released by neurons postsynaptic to CB_1-expressing axon terminals. In this model, endocannabinoids would function as retrograde synaptic messenger molecules that are produced by postsynaptic neurons to modulate (inhibit) the presynaptic vesicular release of 'classical' (anterograde) neurotransmitters, this effect being mediated by coupling of endocannabinoid-activated CB_1 receptors to inhibition of presynaptic calcium channels and/or activation of presynaptic potassium channels. At present, however, there is no direct experimental evidence that anandamide is preferentially synthesized and released by the somato-dentritic compartment of neurons that are postsynaptic to CB_1-expressing axon terminals. Supporting evidence, however,

can be found in the discovery that FAAH is targeted to the somato-dendritic compartment of neurons that are postsynaptic to CB_1-expressing axon terminals in the cerebellum, hippocampus and neocortex (Egertová *et al.* 1998*a*). The rationale for this part of the model is that the product of anandamide hydrolysis by FAAH (arachidonic acid) may then be reincorporated into membrane lipids that are used for subsequent synthesis of anandamide. As highlighted above, however, molecular characterization and neuroanatomical localization of the proteins involved in the synthesis of anandamide is now required to test this model experimentally. Nevertheless, the model provides a useful anatomical framework to interpret the growing number of electrophysiological studies that have examined the effects of cannabinoids on neural activity at the synaptic level and/or the circuit level and which are discussed in § 3(c).

(c) *The role of cannabinoid signalling in the nervous system*

(i) *The olfactory system*

CB_1 is expressed by a population of neurons that project from the anterior olfactory nucleus (AON) to the ipsilateral main olfactory bulb (MOB) and via the anterior commissure to the contralateral MOB where they form synapses with granule cells (figure 1*a*; Herkenham *et al.* 1991; Matsuda *et al.* 1993; Egertová & Elphick 2000). CB_1-immunoreactivity is present throughout the axons of these neurons, indicating that CB_1 receptors are targeted to their presynaptic terminals. To the best of our knowledge, the effects of cannabinoids on synaptic activity in the MOB have not been examined and therefore the functional significance of the presynaptic expression of CB_1 at the synapses between AON projection neurons and MOB granule cells is not yet known. However, the MOB has been studied in considerable detail with respect to synaptic connectivity (Shepherd & Greer 1990) and therefore it may represent an attractive system for analysis of cannabinoid function at the AON projection neuron–MOB granule cell synapse.

In addition to the MOB, CB_1 is also located in several regions of the olfactory cortex, including the AON itself where CB_1-immunoreactive fibres are evident surrounding unstained neuronal somata (Egertová & Elphick 2000). These fibres may be collateral branches of the neurons that project to the MOB or they may be derived from local interneurons. A similar pattern of CB_1-immunoreactive fibres surrounding unstained somata is evident in the piriform cortex and olfactory tubercle (Egertová & Elphick 2000). Therefore, there exists the potential for cannabinoids to exert effects at several levels in the olfactory system. Cannabis is not known to have effects on olfaction in humans but it may be worthwhile examining the effects of cannabinoids in olfactory-related behavioural assays in rodents where the sense of olfaction is of particular importance.

(ii) *The hippocampal formation and memory*

The effects of cannabinoids on hippocampal function has probably been investigated more extensively than in any other region of the brain. The basis for this is the well-established inhibitory effects of cannabis on memory in humans and the recognition that the hippocampus plays a critical role in memory. Moreover, the first investigations of cannabinoid receptor expression in rat and human brain clearly showed abundant expression throughout the hippocampal formation (Herkenham *et al.* 1990, 1991). Subsequently, the identity of neurons expressing CB_1 in the hippocampal formation has been determined by localization of CB_1 mRNA expression and CB_1-immunocytochemistry (figure 1*b*,*c*; Matsuda *et al.* 1993; Tsou *et al.* 1998*a*; Egertová & Elphick 2000). CB_1 mRNA transcripts are most abundant in GABAergic interneurons of the hippocampus and the CB_1 protein is targeted to their presynaptic terminals surrounding the neuronal somata of pyramidal cells in fields CA1–CA4 (figure 1*c*). GABAergic interneurons of the dentate gyrus also express CB_1, concentrated in a band of fibres at the boundary of the molecular layer and the granule cell layer (Tsou *et al.* 1998*a*; Egertová & Elphick 2000). In addition, however, the principal cells of the hippocampus (CA1–CA4 pyramidal cells) appear to express the CB_1 gene but at a lower level than the GABAergic interneurons (Matsuda *et al.* 1993). Furthermore, there is evidence from both anatomical and physiological studies that CB_1 receptors in the hippocampal formation are not only located on intrinsic neurons but also on axonal inputs from CB_1-expressing neuronal somata located in other regions such as the entorhinal cortex and the medial septal complex (Matsuda *et al.* 1993). Inputs from the septum release acetylcholine (ACh) on to granule cells of the dentate gyrus and CA3 pyramidal cells of the hippocampus (Breivogel & Childers 1998), and accordingly cannabinoids inhibit ACh release in hippocampal slices, an effect that is reversed by the CB_1 antagonist SR141716A (Gifford & Ashby 1996). For the purpose of this review, however, we will focus on the role of CB_1-expressing neurons that are intrinsic to the hippocampal formation. However, it should be recognized that modulation of hippocampal input may account for some of the effects of cannabinoids on hippocampal function and memory that are discussed below in relation to CB_1-expressing intrinsic neurons.

How does the pattern of CB_1 expression by intrinsic neurons of the hippocampal formation correlate with the effects of cannabinoids on hippocampal physiology and memory? The mechanisms of synaptic plasticity that are thought to be the neural correlates of learning and memory have been studied more extensively in the hippocampus than in any other region of the brain. In particular, the electrophysiological phenomena of *long-term potentiation* (LTP) and *long-term depression* (LTD) at glutamatergic synapses in the hippocampal formation are candidate mechanisms for memory formation in this part of the brain. Importantly, cannabinoids inhibit induction of LTP and LTD in the hippocampus (Collins *et al.* 1994, 1995; Terranova *et al.* 1995; Stella *et al.* 1997; Misner & Sullivan 1999). However, cannabinoid receptor activation does not directly inhibit the molecular mechanisms responsible for long-term synaptic plasticity, but impairs LTP and LTD by reducing presynaptic release of glutamate. This brings the level of glutamate release below that required to depolarize the postsynaptic membrane such that Mg^{2+} blockade of NMDA receptors is relieved (Shen *et al.* 1996; Misner & Sullivan 1999). This effect of cannabinoids is mediated by coupling of CB_1 to the inhibition of presynaptic N- and Q-type

calcium channels (Sullivan 1999). These electrophysiological data provide functional evidence that CB_1 receptors are targeted to the presynaptic terminals of glutamatergic pyramidal cells in the hippocampus, consistent with the expression of CB_1 mRNA in the somata of these cells. Moreover, these findings correlate with the inhibitory effects of cannabinoids on memory at the behavioural level and provide further evidence that LTP and LTD are neural correlates of memory acquisition in the hippocampus. Unfortunately, the brain is more complicated than we would like it to be and the cannabinoid system in the hippocampal formation is no exception because, in addition to CB_1 expression in pyramidal cells, we need to account functionally for the high level of expression in GABAergic interneurons.

Katona *et al.* (1999) demonstrated that CB_1 is expressed by cholecystokinin (CCK)-immunoreactive GABAergic basket cells of the hippocampus and that cannabinoids inhibit GABA release in the hippocampus. More recently, Hoffman & Lupica (2000) investigated the mechanisms of CB_1-mediated regulation of GABA release in the hippocampus and found that activation of CB_1 receptors reduces $GABA_A$ receptor-mediated (ionotropic, Cl^- currents) but not $GABA_B$ receptor-mediated (metabotropic, K^+ currents) synaptic inhibition of CA1 pyramidal neurons. Collectively, the data available indicate that cannabinoids inhibit GABA release from a specific population of hippocampal interneurons (CCK-immunoreactive) by activating CB_1 receptors located in their presynaptic terminals that are negatively coupled to voltage-sensitive calcium channels. What are the physiological consequences of this effect of cannabinoids? They are likely to be the exact opposite of the previously described inhibitory effects of cannabinoids on glutamate release, because inhibition of GABA release will cause disinhibition of post-synaptic pyramidal cells. So in theory the presynaptic effects of cannabinoids in the hippocampus on glutamatergic and GABAergic transmission could cancel each other out with no net effect postsynaptically. Yet it is clear that cannabinoids do affect memory and neural correlates of memory such as LTP and LTD. This suggests that the inhibitory effects of cannabinoids on excitatory glutamatergic transmission may override those on inhibitory GABAergic transmission under most experimental conditions.

Prior to studies that demonstrated that cannabinoids inhibit GABA release in the hippocampus (Katona *et al.* 1999; Hoffmann & Lupica 2000), Paton *et al.* (1998) investigated the relationship between the effects of cannabinoids on GABAergic transmission and on LTP. Their hypothesis was that cannabinoids *increase* GABA release in the hippocampus and that this may be the mechanism by which activation of CB_1 receptors blocks induction of LTP. In the light of more recent studies (Misner & Sullivan 1999; Hoffmann and Lupica 2000), it is not surprising that Paton *et al.* (1998) concluded that the blockade of LTP by cannabinoids is *not* via upregulation of GABAergic synaptic transmission. However, some of the experimental data reported by Paton *et al.* (1998) are interesting because they can now be reinterpreted with respect to Hoffmann & Lupica's finding that cannabinoids inhibit $GABA_A$-mediated synaptic transmission in the hippocampus. To assess the effects of cannabinoids on GABAergic transmission in the hippocampus, Paton *et al.* (1998) used an experimental phenomenon known as paired pulse depression (PPD) because this form of short-term plasticity is thought to be an indicator of the strength of GABAergic feedback inhibition (Schwartzkroin & Knowles 1983). The effects of paired pulse stimulation of the Schaffer-collateral excitatory inputs to CA1 pyramidal neurons were monitored by recording the population spike in the CA1 region. With low stimulus strengths and with interpulse intervals of less than 80 ms, the magnitude of the second spike was smaller than the first spike (PPD) whilst with interpulse intervals of 80–200 ms the second spike was larger than the first (paired pulse facilitation, PPF; see below). PPD is thought to be due, at least in part, to recurrent activation by Schaffer-collateral glutamatergic terminals of GABA release by hippocampal interneurons. These include CB_1-expressing GABAergic interneurons and therefore cannabinoids could modulate PPD. Consistent with what we now know about the mechanisms of cannabinoid inhibition of GABA transmission in the hippocampus (Hoffman & Lupica 2000), Paton *et al.* (1998) found that cannabinoids reduced PPD. Thus, the *in vitro* phenomenon of PPD provides an insight into the role CB_1 receptors in the regulation of GABA release in the hippocampus. However, the physiological significance of this effect of cannabinoids on PPD is not yet clear and the physiological role of CB_1-mediated inhibition of GABAergic interneurons remains unknown. Hoffman & Lupica (2000) have highlighted the role that GABAergic interneurons have in synchronization of pyramidal cell activity and their consequent role in the oscillatory activity of the hippocampus, which is thought to be important in the encoding of spatial and sensory information. Perhaps, therefore, the cannabinoid receptors in GABAergic interneurons are important for the ongoing basal oscillatory electrical activity of the hippocampus and it is only during phases of learning that the inhibitory effects of cannabinoids on glutamatergic activity are recruited and perhaps dominate modulation of GABAergic transmission. It is clear that we need to know much more about the endogenous cannabinoid signalling mechanisms of the hippocampus before it will be possible to address these issues experimentally. However, what has yet to be done is to measure the release of endocannabinoids in the hippocampus and to correlate this with the activity of neurons during learning and memory formation. The recent success in measuring endogenous cannabinoid release in the dorsal striatum and the periaqueductal gray of freely moving rats (Giuffrida *et al.* 1999; Walker *et al.* 1999*b*) suggests that application of such methods to the hippocampus may be feasible.

As in other regions of the brain, it will also be important to identify which neurons in the hippocampus are responsible for releasing endocannabinoids such as anandamide. Based on the localization of the FAAH in hippocampal pyramidal cells (Egertová *et al.* 1998*a*; Tsou *et al.* 1998*b*) and the model of cannabinoid signalling proposed in figure 2, we would predict that the postsynaptic pyramidal cell somata may be a source of anandamide. Entry of calcium through postsynaptic NMDA receptor channels may stimulate anandamide release, which may then act as a retrograde signalling

molecule to inhibit glutamate release presynaptically. Thus, anandamide would function as part of a system that prevents the strengthening of or weakens glutamatergic synaptic transmission in the hippocampus. As such, release of endocannabinoids in the hippocampus may be a neural correlate of the behavioural phenomenon of memory loss or 'forgetting'. Until recently, however, the only way to investigate the role of the cannabinoid system in hippocampal function was to test the effects of exogenously applied cannabinoid agonists. With the development of the CB_1 receptor antagonist SR141716A it has been possible to assess the physiological role of endocannabinoids in the hippocampus. Based on the inhibitory effects of cannabinoid agonists on LTP and memory, one might predict that an antagonist would enhance LTP and memory formation by preventing binding of endocannabinoids. However, whilst SR141716A blocks the inhibitory effect of cannabinoid agonists on LTP, by itself SR141716A has no effect on LTP (Terranova *et al.* 1995). With respect to effects on learning and memory, SR141716A blocks the inhibitory effects of cannabinoid agonists (Brodkin & Moerschbaecher 1997), as expected, but when tested alone, in some studies SR141716A improves memory (Terranova *et al.* 1996) whilst in other studies SR141716A has no effect (Mallet & Beninger 1998). Thus, the relative importance of tonic release of endocannabinoids in mechanisms of learning and memory is not yet clear.

It has become possible to assess the importance of the CB_1 receptor in mechanisms of learning and memory with the production of CB_1-knockout mice. To test the impact of CB_1 knockout on synaptic transmission in the hippocampus, Bohme *et al.* (2000) first compared short-term plasticity in wild-type and knockout mice using the PPF paradigm, referred to above. No difference in the extent of PPF was observed between both genotypes with interpulse intervals of 40–280 ms. However, LTP was enhanced by 45% in knockout mice compared with wild-type animals. Consistent with these *in vitro* observations, Reibaud *et al.* (1999) found that memory is also enhanced in mice lacking the CB_1 receptor. In a two-trial object recognition test, knockout mice were able to retain memory for at least 48 h after the first trial, whilst wild-type controls lost their capacity to retain memory after 24 h.

Whether it is meaningful to conclude from these findings that the cannabinoid signalling system of the brain is actively involved in physiological processes that underlie the behavioural phenomenon of memory loss or 'forgetting' is debatable. However, it is clear that the cannabinoid system has an important modulatory role in the mechanisms of learning and memory in the hippocampus. Because CB_1 receptors appear to be coupled to inhibition of *both* excitatory glutamatergic transmission and inhibitory GABAergic transmission they are ideally poised for such a role. Perhaps release of endocannabinoids and activation of presynaptic CB_1 receptors can lead to selective weakening of some synapses whilst others are enhanced. Moreover, there may be subtle, activity-dependent factors that determine whether the consequences of endocannabinoid release lead to strengthening or weakening of a particular synapse. Knocking out the CB_1 gene would disrupt these local control mechanisms and may simply and arbitrarily shift global hippocampal synaptic activity toward enhanced excitatory transmission as opposed to enhanced inhibitory transmission in a manner that is just as non-physiological as administering a high concentration of a CB_1 antagonist. A significant step toward a more detailed understanding of the role of cannabinoid receptors in the hippocampus would be to block CB_1 expression selectively in a specific population of hippocampal neurons (e.g. in GABAergic interneurons but not pyramidal cells or vice versa). Unfortunately, techniques to do this are not yet available. What we can look forward to, however, is the development of knockout mice for genes encoding other proteins that are thought to participate in cannabinoid signalling such as FAAH. However, interpretation of the phenotypes of these animals is likely to be even more complicated than CB_1-knockouts because FAAH may be involved in inactivation of other lipid signalling molecules (e.g. oleamide) in addition to the putative endocannabinoids, anandamide and 2-AG. Clearly, there is much still to be learnt about the role of cannabinoid signalling in the hippocampal formation. Nevertheless, it is remarkable how much we have learnt in the ten years since the discovery of the CB_1 gene (see also Hampson & Deadwyler 1998, 1999).

(iii) *The basal ganglia and control of movement*

The inhibitory effects of cannabinoids on movement are well characterized and, as highlighted above, provide two of the tetrad of behaviours that are used to assay cannabinoid action in mice: hypomobility and catalepsy. However, whilst cannabinoids most typically have inhibitory effects on movement, they can also increase movement in a time- or dose-dependent manner (Dewey 1986). Recently, Sañudo-Peña *et al.* (2000) investigated the effects of cannabinoids on movement in rats and discovered a triphasic pattern. At low concentrations ($0.2\ mg\ kg^{-1}$) Δ^9THC decreased locomotor activity while higher doses ($1–2\ mg\ kg^{-1}$) dose-dependently stimulated movement until catalepsy emerged at $2.5\ mg\ kg^{-1}$ accompanied by decreased activity. The physiological bases for these complex effects of cannabinoids on movement are not yet fully understood, but detailed analysis of CB_1 expression in the brain, combined with electrophysiological investigation of cannabinoid action in specific regions of the brain, has begun to provide some insights.

The regions of the brain that play a critical role in the initiation of movement are the basal ganglia, which comprise the caudate-putamen (striatum), external globus pallidus (GPe), internal globus pallidus (GPi) or entopeduncular nucleus (EP) as well as the associated substantia nigra (SN). It is beyond the scope of this review to discuss this system of interconnected nuclei in detail, but in order that the effects of cannabinoids can be explained it is necessary to have a rudimentary understanding of the neural circuitry of the basal ganglia. The striatum receives excitatory input from the neocortex and the major output from the striatum is via GABAergic medium-spiny neurons, which project to the GPe, GPi–EP and the substantia nigra pars reticulata (SNR). Feedback to the striatum is provided by dopaminergic neurons of the substantia nigra pars compacta (SNC). Feedback to the cortex is mediated indirectly by GABAergic neurons of the SNR and GPi–EP, which project to the thalamus and thereby influence movement by regulating the

activity of an excitatory thalamocortical pathway to the neocortex. Here in the thalamus, the basal ganglia input is integrated with input from the cerebellum (see below). In addition to this direct pathway from the neocortex to the striatum via the SNR, GPi–EP and thalamus and then back to the neocortex, there is also an indirect pathway in which GABAergic intrinsic neurons of the GPe project to the subthalamic nucleus (STN) where they influence the activity of glutamatergic excitatory neurons which themselves project back to the GPe as well as to the SNR and GPi–EP. These subthalamic excitatory inputs to the SNR and GPi–EP can thus influence the direct pathway to the thalamus.

How does cannabinoid receptor expression fit into this rather complex neural circuitry? This has been discussed in detail in a number of recent reviews (see Breivogel & Childers 1998; Rodríguez de Fonseca *et al.* 1998; Sañudo-Peña *et al.* 1999; Giuffrida *et al.* 2000). The CB_1 gene is expressed by striatal GABAergic medium-spiny projection neurons and by glutamatergic projection neurons of the STN (Matsuda *et al.* 1993). As in other regions of the brain, the CB_1 protein is targeted to the axons of these neurons, giving rise to a high concentration of cannabinoid binding sites in regions of the brain where they terminate. Thus, CB_1 receptors are particularly abundant in the GPe, GPi–EP and the SNR where the axons and axon terminals of striatal GABAergic medium-spiny projection neurons are located (figure 1*d*,*e*; Herkenham *et al.* 1991; Egertová & Elphick 2000). In addition, an inhibitory feedback pathway mediated by axon collaterals of the striatal GABAergic medium-spiny projection neurons may account for the high density of CB_1-immunoreactivity within the striatum itself. CB_1 receptors located on the axon terminals of the glutamatergic projection neurons of the STN are likely to contribute to the CB_1-immunoreactivity observed in the GPe, GPi–EP and the SNR. Based on this neuroanatomical data, combined with our knowledge of the molecular mechanisms of CB_1 receptor action, one could predict that activation of CB_1 receptors may inhibit GABA release in the striatum and both GABA and glutamate release in the GPe, GPi–EP and the SNR. However, when GABA release in the globus pallidus and substantia nigra was monitored, no change was observed after exposure to cannabinoids whilst in both cases a decrease in GABA uptake was observed (Maneuf *et al.* 1996; Romero *et al.* 1997).

What are functional consequences of CB_1-receptor activation in the basal ganglia? This issue has been investigated in detail by analysing the effects of cannabinoids on the electrophysiological activity of neurons in these regions of the brain. Szabo *et al.* (1998) demonstrated that cannabinoids inhibit GABA-mediated inhibitory postsynaptic currents in the somata of striatal medium-spiny neurons, probably by reducing presynaptic GABA release from the CB_1-expressing axon collaterals of these neurons. This disinhibitory effect of cannabinoids in the striatum will increase the firing rate of striatal medium-spiny neurons, which will complete a negative-feedback loop in the striatum whilst at the same time increasing GABAergic output in the GPe, GPi–EP and the SNR.

Miller & Walker (1995) examined the effects of intravenous administration of cannabinoids on electrical activity of neurons in the SNR and found that cannabinoids produced a modest but significant increase in their spontaneous activity and reversed striatal stimulation-evoked inhibition, also leading to a net increase in activity. These findings indicate that in the SNR, cannabinoid receptors located on striatonigral terminals may regulate movement by disinhibiting the activity of intrinsic neurons. This effect could be mediated by cannabinoids inhibiting GABA release from the striatonigral terminals, although this idea is not consistent with findings of Romero *et al.* (1997) who did not detect any change in basal or KCl-stimulated GABA release when the substantia nigra was incubated *in vitro* with cannabinoids.

Miller & Walker (1996) also examined the effects of intravenous administration of cannabinoids on electrical activity of neurons in the GPe and found that cannabinoids inhibited spontaneous activity and, as in the SNR, reversed striatal stimulation-evoked inhibition, leading to a net increase in activity. The reduction in spontaneous activity may be due to CB_1-mediated inhibition of glutamate release from the terminals of STN projection neurons because the subthalamic input to the GPe is known to be tonically active. In contrast, the GABAergic striatal input to the GPe is phasically active and therefore under basal conditions, effects of cannabinoids on this pathway could be overridden by effects on the tonically active STN–GPe pathway. However, when tonic activity of the GABAergic striatal–pallial pathway is induced experimentally, the disinhibitory effects of cannabinoids are observed. In a subsequent study, Miller & Walker (1998) locally injected cannabinoids into the GPe and, as with systemic injection, they found that spontaneous neuronal activity in the GPe was reduced but, in contrast to the findings with systemic injection, cannabinoids did not reverse striatal stimulation-evoked inhibition. Thus, the effects of systemic cannabinoid injection on striatal stimulation-evoked inhibition of pallial neurons and nigral neurons (see above) may be mediated indirectly by actions in other regions of the brain (e.g. the striatum). Clearly, further experimental analysis is required, but perhaps these antagonistic excitatory or inhibitory effects that cannabinoids can have on neuronal activity in the basal ganglia nuclei provide an explanation for the biphasic or triphasic nature of the effects of cannabinoids on movement at the whole-animal level.

One way in which the link between the effects of cannabinoids at the neuronal level and at the behavioural level has been investigated has been to examine the effects on movement of unilateral or bilateral injection of cannabinoids into specific nuclei of the basal ganglia. Cannabinoids activate movement when injected into the SNR, the direct pathway of the basal ganglia, but inhibit movement when injected into the GPe, the indirect pathway (Sañudo-Peña *et al.* 1999). The stimulatory effect of intranigral or intrapallial injection of cannabinoids can be explained as follows, in relation to effects of cannabinoids on the excitatory input to these structures from the STN. The tonic activity of intrinsic GABAergic SNR neurons is driven by stimulatory input from glutamatergic STN neurons and leads to inhibition of the thalamocortical pathway that drives movement. Intranigral injection of cannabinoids inhibits the release of glutamate by STN neurons leading to inhibition of GABAergic SNR

neurons, which relieves their inhibitory effect on the thalamocortical pathway that causes activation of movement. Conversely, cannabinoid-induced inhibition of glutamate release by STN neurons in the GPe reduces the inhibitory activity of GPe GABAergic interneurons, which causes disinhibition of the glutamatergic STN input to the SNR, leading to activation of the intrinsic GABAergic SNR neurons and consequent inhibition of the thalamocortical pathway that drives movement.

How do these effects of locally injected cannabinoids relate to the effects of systemic injection? Since movement can be either inhibited or stimulated, depending on which part of the brain receives cannabinoid injection, it is not surprising that biphasic or triphasic effects of cannabinoids on movement have been observed following systemic administration. Sañudo-Peña *et al.* (2000) have suggested that the primary role of cannabinoids, which is observed at lower doses, is in activation of movement via inhibition of tonic glutamate release by STN neurons in the SNR, as outlined above. These authors suggest that as the systemic cannabinoid dose is increased, the phasically active inhibitory striatal input to the SNR is affected, with cannabinoids causing reduced GABA release, which leads to disinhibition of the inhibitory SNR input to the thalamocortical pathway, resulting in inhibition of movement. Analysis of CB_1-knockout mice by Zimmer *et al.* (1999) supports this interpretation because they displayed reduced locomotor activity compared with wild-type controls, suggesting that basal activation of CB_1 receptors promotes movement in animals. However, in contrast to the findings of Zimmer *et al.* (1999), CB_1-knockout mice generated by Ledent *et al.* (1999) showed a similar level of locomotor activity as wild-type controls. Moreover, subsequent more detailed analysis of these mice by Valverde *et al.* (2000) has revealed that mutant mice exhibit a significant increase in horizontal locomotor activity compared with wild-type animals, indicating that basal activation of CB_1 receptors inhibits movement in animals.

Clearly, we do not yet have a complete understanding of the role of cannabinoids in the complex circuitry of the basal ganglia and, as in the hippocampus, the effects observed may be determined by the influence of other neurotransmitter systems. Although we now have a detailed body of data on the anatomical distribution of CB_1 in the basal ganglia and the effects of cannabinoids at the neuronal level and at the behavioural level, what we know little about is the physiological role of this system and the circumstances in which putative endocannabinoids such as anandamide are released in the basal ganglia. Recently, the first experiments directed at filling this gap in our knowledge were performed. Giuffrida *et al.* (1999) measured endocannabinoid release in the dorsal striatum of freely moving rats and under basal conditions anandamide, but not 2-AG, was detected. Stimulation of neural activity in the striatum with 60 mM KCl caused a 1.5–2-fold increase in anandamide release whilst the dopamine D_2 receptor agonist quinpirole caused an eight-fold increase in anandamide release. This is of physiological interest because dopamine is released by the nigrostriatal pathway and stimulates movement by activating postsynaptic receptors on striatal projection neurons. Giuffrida *et al.* (1999) investigated the role of dopamine-induced striatal anandamide release by systemic injection of the CB_1 antagonist SR141716A and found that this potentiated quinpirole-evoked hyperactivity. How can these observations be explained in the context of what we already know about cannabinoid action in the basal ganglia? If dopamine promotes anandamide release in the striatum, presynaptic CB_1 receptors located on the axon collaterals of the GABAergic striatal projection neurons could be activated leading to inhibition of GABA release, disinhibition of the striatal projection neurons and enhanced movement. If this were the case, then SR141716A would be expected to reduce quinpirole-evoked hyperactivity by blocking the interaction of anandamide with presynaptic CB_1 receptors in the striatum. Giuffrida *et al.* (1999) found the reverse of this, which suggests that systemically administered SR141716A may be exerting its effect in other regions of the brain (e.g. the SNR). This provides yet another example of the problem of interpreting the effect of cannabinoids on movement following systemic injection of drugs. Nevertheless, the work of Giuffrida *et al.* (1999) paves the way toward experiments where neuronal activity and endocannabinoid release are monitored in specific nuclei of the basal ganglia combined with local administration of drugs such as SR141716A and drugs that affect inactivation of endocannabinoids.

(iv) *The cerebellum and neocortex*

The role of cannabinoids in the regulation of movement is further complicated by the fact that, in addition to expression in the basal ganglia, CB_1 receptors are particularly abundant in another region of brain associated with the control of movement, the cerebellar cortex. The neuroanatomy of the cerebellar cortex is well characterized and is divided into three layers: the molecular layer (outer layer), the Purkinje cell layer (middle layer) and the granule cell layer (inner layer). The Purkinje cells are the principal cells of the cerebellar cortex, receiving direct input from climbing fibres (which originate in the inferior olivary complex) and producing the only output via axonal projections to the deep cerebellar nuclei. The granule cells, which receive input from mossy fibres, have an extensive network of axons in the molecular layer (parallel fibres) that form synapses with the dendrites of the Purkinje cells. In addition, there are a number of classes of local interneurons whose cell bodies are located in the molecular layer (e.g. basket cells, stellate cells). The Purkinje cells do not express the CB_1 gene but CB_1 receptors are particularly abundant in the molecular layer of the cerebellar cortex where they are located on the axons (parallel fibres) and axon terminals of glutamatergic granule cells (figure 1*f*; Herkenham *et al.* 1991; Matsuda *et al.* 1993; Egertová & Elphick 2000). In addition, GABAergic basket cells which form perisomatic synapses with Purkinje cells have CB_1 receptors located within their presynaptic terminals (figure 1*f*; Egertová *et al.* 1998*a*; Egertová & Elphick 2000; Tsou *et al.* 1998*a*). Thus, CB_1 receptors are located presynaptic to Purkinje cells on the excitatory glutamatergic (granule cells) and inhibitory GABAergic (basket cells) terminals of local interneurons. Therefore, CB_1 may modulate the electrical activity of Purkinje cells, and hence output from the cerebellar cortex, by regulating the release of glutamate from granule cell terminals and GABA from basket cell terminals.

To the best of our knowledge, the role of CB_1 receptors in GABAergic cerebellar basket cells has yet to be investigated, but the effects of cannabinoids on synaptic transmission between the parallel fibres of granule cells and Purkinje cells have been investigated by Lévénès *et al.* (1998). Cannabinoids depressed parallel-fibre-induced excitatory postsynaptic currents (EPSCs) in Purkinje cells but had no effect on EPSCs induced by iontophoretic application of glutamate, indicating that presynaptic CB_1 receptors inhibit glutamate release from parallel fibres. The effects of cannabinoids on long-term depression (LTD) of synaptic transmission at the parallel fibre–Purkinje cell synapse was also examined and, as in the hippocampus (see Misner & Sullivan 1999), cannabinoids impaired induction of LTD. Activation of postsynaptic ionotropic receptors (AMPA) and metabotropic glutamate receptors is required for induction of LTD, and therefore the most likely explanation for the effect of cannabinoids on cerebellar LTD is that they inhibit presynaptic release of glutamate by parallel fibres such that activation of these receptors is reduced.

It is not yet known to what extent the effects of cannabinoids on motor behaviour, as highlighted above in relation to the basal ganglia, may be attributable to actions on cerebellar neurons. It may, however, be possible to address this issue by local injection of cannabinoids and/or CB_1 antagonists into the cerebellar cortex of rats or mice. More importantly, if we are to understand the mechanisms of cannabinoid signalling in the cerebellum, it will be important to identify cells that may produce putative endocannabinoids such as anandamide and 2-AG. The enzyme FAAH is expressed in Purkinje cells, indicating that endocannabinoids are inactivated postsynaptically (Egertová *et al.* 1998*a*). Therefore, based on the model of cannabinoid signalling that we present in figure 2, we would predict that anandamide may be synthesized and released in a Ca^{2+}-dependent manner by Purkinje cells and function as a retrograde messenger molecule that acts via presynaptic CB_1 receptors to inhibit glutamate release from the parallel fibres of granule cells or to inhibit GABA release from the terminals of basket cells. Clearly, these two effects could cancel each other, leading to no net change in the activity of Purkinje cells. Thus, as in the hippocampus and the basal ganglia, the consequences of endocannabinoid release may depend critically on the relative activity of presynaptic excitatory and inhibitory neurons.

The distribution of CB_1 cannabinoid receptors in the neocortex has been described in detail by Herkenham *et al.* (1991), Matsuda *et al.* (1993) and Egertová & Elphick (2000) and, as in other regions of the brain, CB_1 expression appears to be particularly associated with local interneurons based on the observation that both CB_1 mRNA expression and CB_1-immunoreactivity are concentrated in layers II–III and layers V–VI, with layers I and IV displaying little or no expression (Matsuda *et al.* 1993; Egertová & Elphick 2000). Moreover, as in the hippocampus, the majority of cortical neurons expressing high levels of CB_1 are GABAergic neurons belonging to the cholecystokinin-positive and parvalbumin-negative type of interneurons (basket cells; Marsicano & Lutz 1999). However, despite the obvious importance of the neocortex in brain function and as a target for cannabinoids, it has so far been largely neglected with respect to electrophysiological analysis of the effects of cannabinoids on neural activity. This represents an important area for future experimental studies.

(v) *Supraspinal, spinal and peripheral mechanisms of analgesia*

The analgesic properties of cannabis and cannabinoids are widely documented but only recently have specific target sites in the central nervous system (CNS) been identified. Detailed reviews on the neurobiology of cannabinoid analgesia have been published recently (Martin & Lichtman 1998; Walker *et al.* 1999*a*; Fuentes *et al.* 1999) and therefore the aim here will be to provide a brief overview, incorporating some of the most recent findings. Several regions of the CNS are recognized as sites where both endogenous and exogenous analgesics exert their effects, including the periaqueductal gray (PAG) of the midbrain and the dorsal horn of the spinal cord, which receives the input from the central terminals of sensory neurons whose cell bodies are located in the dorsal root ganglia (DRG). In accordance with the analgesic properties of cannabinoids, there is evidence that cannabinoid receptors are expressed in both of these regions of the rat CNS, with moderate levels of specific [^{3}H]CP-55,940 binding sites detectable in the PAG (1.89 pmol mg^{-1} protein) and the dorsal horn of the spinal cord (0.95 pmol mg^{-1} protein) (Herkenham *et al.* 1991). Low level CB_1 mRNA expression is evident in the dorsal and lateral regions of the PAG (Matsuda *et al.* 1993) but, to the best of our knowledge, analysis of CB_1 mRNA expression in the spinal cord using anatomical *in situ* hybridization techniques has not yet been reported. CB_1 mRNA is, however, expressed in a subpopulation of medium- and large-size neuronal somata in the dorsal root ganglion (DRG), which are probably of the A∂- and Aβ-type (Hohmann & Herkenham 1999*a*). These sensory neurons project both centrally (to the dorsal horn of the spinal cord) and peripherally, but ligation studies indicate that CB_1 receptors are primarily targeted to their peripheral terminals (Hohmann & Herkenham 1999*b*).

Recently, CB_1 protein expression in the PAG and spinal cord has been investigated using immunocytochemical methods. Tsou *et al.* (1998*a*) describe fibres in the rat PAG that are immunoreactive with antibodies to the N-terminal tail of CB_1, whilst no immunoreactivity was evident in the PAG using antibodies to the C-terminal tail of CB_1 (Egertová & Elphick 2000). One possible explanation for this latter finding may be that the level of expression in the PAG is below the threshold of sensitivity of the immunocytochemical methods used with C-terminal CB_1 antibodies. However, comparison with other regions of the brain does not support this interpretation. For example, Herkenham *et al.* (1991) report 1.6 pmol mg^{-1} protein of [^{3}H]CP-55,940 binding in the olfactory tubercle, whilst in the PAG 1.89 pmol mg^{-1} protein of [^{3}H]CP-55,940 binding was detected. Based on these data and the failure to detect CB_1-immunoreactivity in the PAG, one would not expect to detect CB_1-immunoreactivity in the olfactory tubercle. However, by using C-terminal CB_1 antibodies, a fine meshwork of densely stained CB_1-immunoreactive fibres is evident in the olfactory

tubercle (Egertová & Elphick 2000). Therefore, alternative explanations for the absence of immunostaining in the PAG using C-terminal CB_1 antibodies must be sought. One possible explanation is that in the PAG there is a high level of phosphorylation of serine and/or threonine residues in the C-terminal tail of CB_1, which renders it non-immunoreactive with C-terminal-directed antibodies (see Egertová & Elphick (2000) for a detailed explanation of this hypothesis). An alternative and perhaps more controversial idea is that cannabinoid binding sites in the PAG (Herkenham *et al.* 1991) are largely due to a non-CB_1 cannabinoid receptor protein, the existence of which has been suggested from analysis of CB_1-knockout mice (Breivogel *et al.* 2000). Clearly, more detailed analysis of cannabinoid receptor expression in the PAG is now required to resolve this issue.

In the spinal cord, Herkenham *et al.* (1991) detected the highest levels of cannabinoid binding sites in layers 1 and 2 of the dorsal horn and in lamina 10 around the central canal. Lower levels of binding were detected in the ventral horn, which contains the cell bodies of motor neurons. Recently, a detailed analysis of CB_1 receptor localization in the rat spinal cord has been reported using C-terminal CB_1 antibodies (Farquhar-Smith *et al.* 2000). Consistent with the binding data, intense immunostaining was detected in the superficial layers of the dorsal horn and in lamina 10. However, the most intense staining was present lateral to the superficial dorsal horn in the dorsolateral funiculus (DLF). In retrospect, a high concentration of cannabinoid binding sites in the DLF can be seen in the autoradiographs shown in fig. 14a of Herkenham *et al.* (1991), although these authors did not draw attention to it.

The immunocytochemical analysis performed by Farquhar-Smith *et al.* (2000) has provided the first detailed neuroanatomical map of CB_1 expression in the spinal cord. Most importantly, by performing co-localization studies using antibodies to other spinal-cord-associated neuronal markers combined with lesion studies, it was established that CB_1-expressing spinal interneurons are likely to be responsible for the majority of cannabinoid binding sites in the superficial layers of the dorsal horn, where nociceptive processing takes place, and in the DLF. Prior to this study it was thought that CB_1 receptors may be located on the axonal terminals of primary afferents in the dorsal horn. However, it appears that the majority of CB_1 receptors in the spinal cord are located on local interneurons (Farquhar-Smith *et al.* 2000), whilst the CB_1-expressing DRG neurons seem to target the receptor to their peripheral terminals with little or no central targeting (Hohmann & Herkenham 1998, 1999*b*). It is with this neuroanatomical framework in mind that electrophysiological and behavioural analysis of cannabinoid-induced analgesia can be interpreted.

The ability of cannabinoids to block nociceptive stimuli (antinociception) has been observed in a wide range of behavioural studies (Martin & Lichtman 1998). Moreover, it has been established that cannabinoids may exert analgesic effects by interacting with cannabinoid receptors both at supraspinal sites and spinal sites as well as peripherally. By injecting cannabinoids into different regions of the PAG it has been established that this area is a likely target for the supraspinal analgesic effects of cannabinoids, although there is conflicting data with regard to which regions of the PAG are most responsive (Martin *et al.* 1995*b*; Lichtman *et al.* 1996). More recently, Vaughan *et al.* (2000) have examined the effects of cannabinoids on membrane properties and synaptic transmission in rat PAG neurons. Consistent with findings in other regions of the brain (see above), these authors found that cannabinoids inhibited electrically evoked inihibitory and excitatory postsynaptic currents in all PAG neurons tested, probably by presynaptic inhibition of GABA and glutamate release, respectively. As with other regions of the brain, however, little is known about the role of endocannabinoids in modulating the activity of PAG neurons. However, a significant breakthrough recently was the report of Walker *et al.* (1999*b*) in which anandamide release in the PAG was measured in freely moving rats. Electrical stimulation of the dorsal and lateral PAG produced CB_1-mediated analgesia accompanied by an increase in anandamide release in the PAG. Moreover, anandamide release in the PAG was also increased in response to pain-triggering stimuli. These observations provide important evidence that anandamide functions as an endogenous ligand for the CB_1 receptor and has an important role in physiological analgesic mechanisms.

In addition to the PAG, injection of cannabinoids into other brain regions including the rostral ventrolateral medulla (RVM) can also produce analgesia (Walker *et al.* 1999*a*). These findings are consistent with neural pathways that mediate analgesia because RVM neurons receive input from the PAG and project to the dorsal horn where they inhibit dorsal horn nociceptive neurons. Inactivation of the RVM prevents the analgesia produced by systemically administered cannabinoids, and cannabinoids produce analgesia by modulating RVM neuronal activity (Meng *et al.* 1998). In particular, cannabinoids cause disinhibition of RVM neurons by presynaptic inhibition of GABA release (Vaughan *et al.* 1999).

At the level of the spinal cord, cannabinoids suppress noxious-stimulus-evoked responses of neurons (wide dynamic range) in the dorsal horn (Hohmann *et al.* 1999). Moreover, Walker *et al.* (1999*a*) suggest that this effect is mediated by modulation of descending noradrenergic input from the brainstem. Evidence in support of this idea is provided by Lichtman & Martin (1991) who demonstrated that spinal administration of the adrenergic antagonist yohimbine reduced cannabinoid-induced analgesia. Therefore, the CB_1-expressing spinal interneurons reported by Farquhar-Smith *et al.* (2000) may mediate the analgesic effects of cannabinoids by modulating the release of noradrenaline from descending fibres. If, as in the brain, the CB_1-expressing interneurons in the spinal cord are primarily GABAergic, then cannabinoids may act to cause disinhibition of descending fibres, leading to increased noradrenaline release and attenuation of nociceptive transmission in the dorsal horn of the spinal cord.

In addition to the central actions of cannabinoids, there is now growing evidence for cannabinoid-induced analgesia at peripheral sites (Jaggar *et al.* 1998; Calignano *et al.* 1998). This is in accordance with the targeting of CB_1 to the peripheral terminals of a sub-population of DRG sensory neurons (Hohmann & Herkenham 1999*b*).

Thus, activation of CB_1 receptors located in the peripheral terminals of nociceptive DRG neurons may cause inhibition of voltage-activated calcium channels or activation of potassium channels, resulting in a reduction in the excitability of these neurons. However, it appears that the peripheral analgesic actions of cannabinoids are not solely mediated by CB_1-type cannabinoid receptors but also by CB_2 and/or CB_2-like receptors because the CB_2 antagonist SR144528 enhances nociception when administered peripherally (Jaggar *et al.* 1998; Calignano *et al.* 1998). CB_2 receptors are not expressed by nociceptive sensory neurons (Hohmann & Herkenham 1999*a*) and therefore CB_2-mediated analgesia is probably due to a peripheral action of cannabinoids on non-neuronal cells. Likely candidates are mast cells, which express the CB_2 receptor (Facci *et al.* 1995) and which have a critical role in pain because they release inflammatory mediators that activate the terminals of nociceptive sensory neurons. Activation of CB_2 receptors on mast cells may inhibit release of inflammatory mediators and thereby cause the CB_2-mediated antinociceptive effects of cannabinoids (Mazzari *et al.* 1996).

Analysis of the involvement CB_1 and CB_2 receptors in cannabinoid-induced analgesia has been facilitated with the development of CB_1- and CB_2-knockout mice. Importantly, Ledent *et al.* (1999) found that the analgesic effects of Δ^9THC were absent in CB_1-knockout mice. However, in a separate study, Zimmer *et al.* (1999) found some residual cannabinoid analgesia, which may be consistent with the occurrence of peripheral CB_2-mediated analgesia in CB_1-knockout mice. Assessment of the importance of endocannabinoids in the physiological mechanisms of analgesia is also possible using these knockout animals but this has produced conflicting evidence. Thus, in one behavioural assay (the tail-flick test) there was no difference in the response latency of CB_1-knockout and wild-type controls (Zimmer *et al.* 1999). However, in two other tests (hotplate assay and the formalin test) the CB_1-knockout animals were hypoalgesic compared with wild-type controls (Zimmer *et al.* 1999). This latter finding is the opposite of what would be expected based on the analgesic properties of cannabinoids. It is difficult to explain this finding but one possibility is that it reflects a physiological over-compensatory adaptation of another endogenous analgesic mechanism to the loss of a CB_1-mediated endocannabinoid analgesic system. Subsequently, Valverde *et al.* (2000) have compared the nociceptive thresholds of CB_1-knockout and wild-type mice in more detail using four behavioural assays and, consistent with the original findings of Ledent *et al.* (1999), they observed no differences. However, one significant difference was observed in that CB_1-knockout mice did not display the opioid-mediated antinociception that normally follows a forced swim at 34 °C in wild-type mice. Valverde *et al.* (2000) conclude from these findings that 'the presence of CB_1 receptors in the central nervous system does not play a major role in the endogenous control of pain'. However, it does appear that a physiological interaction between the opioid and CB_1-cannabinoid systems is required to allow the development of opioid-mediated responses to stress. The specific role of the CB_2 receptor in physiological mechanisms of analgesia has yet to be investigated in detail, but with the development of CB_2-knockout mice (Buckley *et al.* 2000) this should now be possible.

(vi) *The autonomic nervous system*

Having considered the peripheral expression of CB_1 and/or CB_2 receptors with respect to sensory processes (nociception), it is appropriate to consider at this point the role of cannabinoid receptors in the motor component of the peripheral nervous system (PNS). Although there have been occasional reports investigating the effects of cannabinoids on fast excitatory neuromuscular transmission (Van der Kloot 1994), there is no evidence that cannabinoid receptors are expressed by the motor neurons or the muscle cells that mediate voluntary control of skeletal muscles. However, there is a substantial body of pharmacological and biochemical evidence that the CB_1 receptor is expressed presynaptically by autonomic motor neurons that innervate visceral organs such as the vas deferens and bladder, as reviewed recently by Pertwee (1999). Cannabinoids inhibit electrically evoked contractions of *in vitro* preparations of these visceral organs by causing a reduction in noradrenaline release from sympathetic nerves (Pertwee *et al.* 1996; Ishac *et al.* 1996; Pertwee & Fernando 1996). Cannabinoids also cause inhibition of electrically evoked contractions of a myenteric plexus–longitudinal muscle preparation from the guinea-pig small intestine and here the effect is thought to be mediated by presynaptic inhibition of acetylcholine release from enteric neurons (Pertwee 1999). Thus, endocannabinoids may have a physiological role in the regulation of intestinal motility (Izzo *et al.* 2000).

In addition, to their effects on the innervation of smooth muscle associated with visceral organs of the digestive, urinary and reproductive systems, cannabinoids also cause inhibition of vascular smooth muscle. The main cardiovascular effects of cannabis on humans and of cannabinoids on laboratory animals are prolonged hypotension and bradycardia. Until recently it was thought that these effects were due to activation of cannabinoid receptors in the brain, but there is growing evidence for peripheral sites of action in the blood vessels and the heart. In particular, activation of CB_1 receptors located on the presynaptic terminals of sympathetic neurons causes inhibition of noradrenaline release, as highlighted above (Ishac *et al.* 1996). Noradrenaline exerts excitatory effects on the heart and on vascular smooth muscle tone, so cannabinoid-induced inhibition of sympathetic noradrenaline release could account for the bradycardic and hypotensive effects of cannabinoids, respectively (Kunos *et al.* 2000). Moreover, direct evidence for the involvement of the CB_1 receptor in the cardiovascular effects of cannabinoids has come from analysis of CB_1-knockout mice, which do not display cannabinoid-induced bradycardia and hypotension (Ledent *et al.* 1999).

Thus, the cardiovascular effects of 'classical' Δ^9THC-like cannabinoids are probably due to CB_1-mediated inhibition of sympathetic noradrenaline release. However, the cardiovascular effects of endocannabinoids such as anandamide appear to be more complex. In particular, it has been reported that anandamide may also cause vasodilation by binding to vanilloid (VR1) receptors located on the terminals of sensory neurons, thereby promoting Ca^{2+}-dependent release of the vasorelaxant neuropeptide calcitonin gene-related peptide (CGRP) (Zygmunt *et al.* 1999). This finding is of general interest because it highlights the fact that anandamide may have multiple

molecular targets, in addition to CB_1, which makes interpretation of the physiological effects of anandamide complicated. However, as in other parts of the body, nothing is known about the cellular source of endocannabinoids in the cardiovascular system. Nevertheless, based on the model that we present in figure 2 it is tempting to suggest that anandamide may be synthesized in a Ca^{2+}-dependent manner by postsynaptic smooth muscle cells. As such, anandamide could function as a retrograde neuromuscular signalling molecule that acts presynaptically as part of a negative feedback mechanism to reduce the magnitude of muscle contraction.

4. PART 2: THE EVOLUTION OF CANNABINOID SIGNALLING

(a) *Introduction*

The discovery of CB_1- and CB_2-cannabinoid receptors and putative endogenous ligands for these receptors has provided the foundation for the novel concept of cannabinoid signalling in the nervous and immune systems of mammals. With the discovery of any new signalling system, there arises the issue of its evolutionary origins. Investigating the occurrence of signalling pathways in non-mammalian vertebrates and invertebrates is of intrinsic scientific interest because it can identify those intercellular messenger systems, which by virtue of a widespread phylogenetic distribution can be considered to be evolutionarily ancient and therefore perhaps of fundamental importance to all animals. Moreover, it is of value to determine which of the 'mammalian' signalling systems are present in invertebrates because certain invertebrate species provide important model systems in animal physiology and genetics. The best examples of such 'invertebrate model organisms' are the insect *Drosophila melanogaster* and the nematode *Caenorhabditis elegans*, which by virtue of their small size and short generation times are important systems for the investigation of behavioural and developmental genetics. Moreover, increasingly the functions of genes are first defined in *Drosophila* or *C. elegans* and only subsequently are mammalian orthologues identified and characterized. In addition to genetic model organisms, non-genetic invertebrate model species are important systems in neurobiology for investigating the neural basis of behaviour by virtue of the relative simplicity of their nervous systems in which individual neurons can be repeatably identified from animal to animal. Examples of such organisms are the marine mollusc *Aplysia californica* (Pittenger & Kandel 1998) and the insect (locust) *Schistocerca gregaria* (Burrows 1996). A non-neurobiological example is the sea urchin *Strongylocentrotus purpuratus* (phylum Echinodermata), which is a model system for the study of the mechanisms of fertilization and embryonic development (Davidson *et al.* 1998; Cameron *et al.* 2000). Thus, in each of the major invertebrate phyla there is at least one species that has been used as a model system for the study of an important biological process.

(b) *Comparative pharmacology of cannabinoids*

The identification of Δ^9THC as the main psychoactive ingredient of cannabis by Gaoni and Mechoulam in 1964 provided comparative pharmacologists with a compound that could be tested for its effects on animal behaviour and on *in vitro* organ preparations. For example, McClean & Zimmerman (1976) reported the effects of Δ^9THC on cell division and macromolecular synthesis in the protozoan *Tetrahymena pyriformis*, whilst Waser (1979) reported the effects of Δ^9THC on behaviour in ants. Turkanis & Karler (1988) reported an example of a study in which the *in vitro* pharmacological effects of cannabinoids on an invertebrate species were examined. Here the effects of cannabinoids on neurotransmitter release at the neuromuscular junction of the lobster *Homarus americanus* were investigated. Importantly, in this study not only was Δ^9THC tested but also cannabidiol, a non-psychoactive component of cannabis. Δ^9THC increased the amplitude of the excitatory junctional potential whilst cannabidiol decreased the amplitude of the excitatory junctional potential; both compounds depressed the amplitude of miniature end-plate potentials. Thus, cannabinoids appear to exert both pre- and postsynaptic effects on neuromuscular transmission in a crustacean.

The most detailed and extensive set of studies investigating cannabinoid action in an invertebrate is those reported by Schuel *et al.* (1987, 1991) in which the effects of cannabinoids on sea urchin sperm were investigated. The effects of Δ^9THC, cannabinol and cannabidiol were examined and with all three compounds, pre-treatment of sperm inhibited fertilization of eggs by reducing sperm fertility (Schuel *et al.* 1987). However, pre-treatment of eggs had no effect on their receptivity to sperm. Subsequently, the effects of cannabinoids on sperm fertility were investigated in more detail and attributed to inhibition of the induction of the acrosome reaction by egg jelly (Schuel *et al.* 1991). Ultrastructural analysis of Δ^9THC-treated sperm revealed that the membrane fusion reaction between the sperm plasma membrane and the acrosomal membrane is completely blocked (Chang & Schuel 1991).

The questions that arise from these pharmacological studies concern the mode of action of cannabinoids in non-mammalian animals. How do cannabinoids exert physiological or behavioural effects in invertebrates and non-mammalian vertebrates? Do they bind to a specific membrane receptor protein and in so doing effect an endogenous signal transduction cascade? Alternatively, it is possible that the actions of cannabinoids observed in invertebrates are due to non-specific membrane disrupting effects of these lipophilic drugs. However, if cannabinoids do bind to specific membrane receptor proteins in non-mammalian animals, are they orthologues of the G-protein-coupled CB_1 and CB_2 cannabinoid receptors that have been identified in mammals, or do non-mammalian animals have other protein targets that cannabinoids interact with and which are molecularly quite distinct from the mammalian CB_1 and CB_2 receptors? These issues can only be addressed by detailed biochemical characterization of the respective cannabinoid binding sites and/or by cloning and sequencing of genes encoding non-mammalian orthologues of the mammalian CB_1 and CB_2 receptors.

(c) *The phylogenetic distribution of cannabinoid receptors*

(i) *Vertebrates*

Devane (1989) investigated the presence of specific cannabinoid binding sites in the nervous systems of a

variety of vertebrate species including chicken, turtle, frog, trout and lamprey. Specific binding sites for [^{3}H]CP-55,940 were detected on brain membranes from all of the animals tested except lamprey. These data indicate that brain cannabinoid receptors may occur in all of the major vertebrate classes. However, the detection of specific binding sites for [^{3}H]CP-55,940 on brain cell membranes of several vertebrate species does not in itself prove the existence of non-mammalian orthologues of the CB_1 receptor. To determine whether the [^{3}H]CP-55,940 binding sites in non-mammalian vertebrates are structural and functional equivalents of mammalian CB_1 receptors it is necessary to investigate the relative potency and stereoselectivity of cannabinoids in displacing [^{3}H]CP-55,940 from these sites, but this has yet to be done, with the exception of a recent study on an amphibian species (Soderstrom *et al.* 2000; see below). Alternatively, a molecular biological approach can be adopted in which the presence of genes in non-mammalian vertebrates that encode orthologues of the mammalian CB_1 or CB_2 receptors is investigated.

The first non-mammalian cannabinoid receptor genes were discovered in the puffer fish *Fugu rubripes* by Yamaguchi *et al.* (1996). Two genes that display sequence similarity with mammalian cannabinoid receptors were identified but both genes share more sequence similarity with mammalian CB_1 genes than with mammalian CB_2 genes and therefore the *Fugu* genes were named FCB_{1A} and FCB_{1B}. FCB_{1A} and FCB_{1B} share 66% sequence identity at the amino-acid level and share 72 and 59% sequence identity with the human CB_1 protein, respectively. Both FCB_{1A} and FCB_{1B} are highly expressed in the *Fugu* brain and to a lesser extent in testis, ovary and spleen, providing further evidence that these genes are both structural and functional orthologues of the mammalian CB_1 gene. An orthologue of the mammalian CB_2 gene was not detected in the *Fugu* genome, which is interesting because it suggests that the CB_2 gene may have evolved since the divergence of tetrapods and fish. The presence of two CB_1-like genes, both of which are expressed in the *Fugu* brain, is also of particular interest because it is likely to be a consequence of a whole-genome duplication event that is thought to have occurred after the divergence of ray-finned fish (subclass Actinopterygii) and lobe-finned fish (subclass Sarcopterygii) but before the radiation of the teleosts (which include *Fugu*) (Amores *et al.* 1998). Accordingly, comparative analysis of the genome of the zebrafish *Danio rerio* (also a teleost) has revealed pairs of genes that are orthologues of single mammalian genes (Gates *et al.* 1999). Thus, the existence of the FCB_{1A} and FCB_{1B} genes in *Fugu* that are orthologues of the mammalian CB_1 gene provides another example of this phenomenon. Moreover, the occurrence of two CB_1 genes in fish is interesting because it raises questions about their respective functions. The FCB_{1A} gene shares more sequence similarity with mammalian CB_1 genes than FCB_{1B}, and therefore it is the FCB_{1B} gene that has diverged most from the putative ancestral CB_1 gene that predates a genome duplication event in fish. Thus, it would be interesting to investigate the expression profile of FCB_{1A} and FCB_{1B} to establish whether they have acquired different functions during evolution or whether they are simply co-expressed and co-functional in a common population of cells in the *Fugu* brain. Unfortunately, nothing is known about the expression and functions of the CB_1 genes in *Fugu*. Nor is anything known about the properties of the protein products of the FCB_{1A} and FCB_{1B} genes or their relative capacity to bind and to be activated by 'classical' Δ^9THC-like cannabinoids or endocannabinoids such as anandamide.

The existence of CB_1 genes in a fish species (*Fugu*) indicates that CB_1 is likely to occur throughout the 'higher' non-mammalian vertebrate classes, which include amphibians, reptiles and birds, consistent with the detection of [^{3}H]CP-55,940 binding sites in the brains of species from these three classes (Devane 1989). Accordingly, the sequence of a CB_1 gene from an amphibian species, the roughskin newt *Taricha granulosa*, has been reported recently (Soderstrom *et al.* 2000). The *Taricha* CB_1 gene encodes a 473 amino acid protein that shares 84.2%, 75.5% and 61.6% sequence identity with the human CB_1, *Fugu* CB_{1A} and *Fugu* CB_{1B} receptor proteins, respectively. The *Taricha* CB_1 gene is highly expressed in the brain of this species and Soderstrom *et al.* (2000) characterized a [^{3}H]CP-55,940 binding site on brain membranes where the rank order for various cannabinoid ligands to displace [^{3}H]CP-55,940 was consistent with that reported in mammalian species. Moreover, cannabinoids cause inhibition of spontaneous locomotor activity and courtship clasping behaviour in *Taricha*, indicating that not only the biochemical properties of CB_1 may be similar in amphibians and mammals but also the role of this receptor in the neural mechanisms of motor control, presumably at the level of the basal ganglia (see § 3).

To the best of our knowledge, CB_1 genes have yet to be identified and characterized in any reptilian species. However, there is a recent report in which CB_1 expression and function in an avian species, the zebra finch *Taeniopygia guttata*, has been investigated (Soderstrom & Johnson 2000). A [^{3}H]CP-55,940 binding site was detected in the zebra finch brain and a 693 base pair cDNA fragment encoding part of a CB_1-like protein was amplified. Subsequently, the full-length sequence of a cDNA encoding a 473 amino acid zebra finch CB_1 receptor has been determined and submitted to GenBank (accession number AF255388). Soderstrom & Johnson (2000) investigated the expression of CB_1 in the zebra finch brain and obtained evidence that it is highly expressed in regions of the brain involved in song learning, indicative of a possible role for the cannabinoid signalling system in vocal development.

Collectively, the data discussed above indicate that cannabinoid receptors occur in the majority of extant vertebrates. Nevertheless, it would be interesting to investigate the presence of CB_1-like genes in fish such as sharks and rays because these cartilaginous fish are considered to be more primitive than other jawed vertebrates (Osteichthyes and Tetrapoda). The absence of binding sites in lamprey brain (Devane 1989) is of interest because lamprey is one of the few extant representatives of a primitive vertebrate class, the agnathans (jawless fish). It is possible that cannabinoid receptors may have been secondarily lost in agnathans or alternatively cannabinoid receptors may have evolved after the divergence of agnathans from the lineage that gave rise to the major

vertebrate classes. To address this issue further, more detailed experimental analysis of lampreys is required.

(ii) *Invertebrates*

If one were to trace the evolution of cannabinoid receptors beyond ancestral vertebrates then, based on our knowledge of the evolution of the animal kingdom, it is in extant representatives of deuterostomian invertebrates such as protochordates and echinoderms that one would first look. Protochordates (e.g. sea squirts) and echinoderms (e.g. starfish and sea urchins) share a common form of embryonic development with the vertebrates (deuterostomian) that distinguishes them from the other major invertebrate groups such as arthropods, molluscs and annelids, which have a protostomian mode of development. For this reason, the protochordates and echinoderms are recognized as sharing a more recent common ancestry with vertebrates than with the protostomian phyla. To the best of our knowledge, the existence of cannabinoid receptors in protochordates has yet to be investigated. However, as highlighted above, there has been a series of studies investigating the effects of cannabinoids on fertility in sea urchin sperm (Schuel *et al.* 1987, 1991; Chang & Schuel 1991). Moreover, Chang *et al.* (1993) have investigated the molecular basis for the effects of cannabinoids on sea urchin sperm by characterizing a cannabinoid binding site on live sea urchin sperm. Using a [^{3}H]CP-55,940 radioligand binding assay, Chang *et al.* (1993) demonstrated the presence of specific binding sites on sea urchin sperm. Moreover, the ability of stereoisomers of Δ^9THC to displace binding of [^{3}H]CP-55,940 was also analysed and importantly the K_i for (−)Δ^9THC (830 nM) was found to be lower than the K_i for (+)Δ^9THC (3700 nM), which is consistent with the stereoselectivity of mammalian CB_1 cannabinoid receptors. These data indicate that sea urchin sperm cells may express a cannabinoid receptor that is orthologous to vertebrate G-protein-coupled CB_1 receptors. Human sperm cells also express the CB_1 receptor (Gérard *et al.* 1991; Schuel *et al.* 1998) and therefore the expression of cannabinoid receptors by sperm cells may be an evolutionarily ancient association that occurs throughout the deuterostomian lineage of the animal kingdom. An important goal now will be to investigate the presence of a CB_1-like gene in the genome of an echinoderm (e.g. sea urchin) and to analyse expression of CB_1-like mRNA and protein in sea urchin sperm. This would establish for certain whether the origins of CB_1-like G-protein-coupled receptors can be traced beyond ancestral vertebrates to their invertebrate deuterostomian ancestors. Can cannabinoid receptors be traced even further back to the common ancestor of both the deuterostomian and the protostomian phyla? This question can be addressed by testing for the presence of cannabinoid receptors in the major protostomian phyla such as the arthropods (e.g. insects), molluscs and annelids.

Howlett *et al.* (1992) addressed this issue in insects by testing for [^{3}H]CP-55,940 binding sites in *D. melanogaster* and 'a low concentration of binding sites were discernible'. Subsequently, we have investigated the presence of cannabinoid receptors in insect tissue using the locust *Schistocerca gregaria* as a model system. No specific binding sites for [^{3}H]CP-55,940 were detected in locust brain membrane preparations, but a low level of specific binding appeared to be present in locust muscle (Egertová *et al.* 1997, 1998*b*; Egertová 1999). We have also tested for the presence of CB_1-like proteins using antibodies to the C-terminal tail of rat CB_1 and an immunoreactive protein of *ca.* 40 kDa was detected. However, more detailed analysis revealed that this CB_1-like immunoreactive locust protein is not a membrane-associated protein and therefore cannot be an orthologue of vertebrate cannabinoid receptors (Egertová *et al.* 1998*b*; Egertová 1999). Recently, Howlett *et al.* (2000) have characterized a cannabinoid binding site in *Drosophila* and detected specific binding sites for [^{3}H]CP-55,940 in heads at a threefold higher density than in bodies. Displacement of [^{3}H]CP-55,940 binding was observed with several cannabinoid ligands with the following rank order of potency: CP-55,940 > Δ^9THC > anandamide. However, [^{3}H]CP-55,940 binding was not displaced by aminoalkylindole cannabinoid agonist WIN55212-2 nor by the cannabinoid receptor antagonists SR141716A and SR144528. These data indicate that the cannabinoid-binding site in *Drosophila* may be structurally quite different to vertebrate CB_1/CB_2 cannabinoid receptors. Howlett *et al.* (2000) also report the presence of a *ca.* 105 kDa protein in the *Drosophila* head that is cross-reactive with antibodies to the N-terminal 14 amino acids of the *ca.* 53 kDa rat CB_1 protein. It remains to be determined, however, whether this CB_1-like immunoreactive protein is responsible for the binding sites in *Drosophila* heads and whether it is related to vertebrate CB_1 receptors. Since the molecular mass of the *ca.* 105 kDa protein is far in excess of that expected for a G-protein-coupled cannabinoid receptor, it is possible that this is an unrelated protein that just happens to cross-react with antibodies to the N-terminal tail of rat CB_1.

The definitive way of investigating the presence of a CB_1-related cannabinoid receptor in insects is to use a molecular biological approach and search for CB_1-related genes or proteins in *Drosophila*. With the completion of the *Drosophila* genome sequencing project (Adams *et al.* 2000), it has become possible to carry out such molecular biological 'experiments' *in silico* using computer programs such as the Basic Local Alignment Search Tool (BLAST; Altschul *et al.* 1990), and we have done this using the National Center for Biotechnological Information (NCBI) Web site at http://www.ncbi.nlm.nih.gov/. The *Drosophila* genes that display the highest level of sequence similarity with the rat CB_1 receptor at the amino-acid level are two genes codenamed CG6919 and CG12796. Reference to the flybase Web site (http://fly.ebi.ac.uk:7081/) in which the *Drosophila* genome annotation data are stored reveals, however, that whilst CG6919 encodes a putative G-protein-coupled receptor it is categorized as a serotonin or octopamine receptor. Meanwhile, BLAST analysis of the CG12796 gene product shows that the protein in the GenBank database that displays the highest sequence similarity with it is a chicken dopamine receptor. Thus, the G-protein-coupled receptors encoded in the *Drosophila* genome that display the highest level of sequence similarity with mammalian cannabinoid receptors are in fact insect catecholamine receptors. The *Drosophila* genome does not, therefore,

contain genes that are orthologues of mammalian cannabinoid receptors.

The only other invertebrate species in which a 'complete' genome sequence has been determined is the nematode worm *C. elegans*. Using the same *in silico* approach as described above for *Drosophila*, if the *C. elegans* genome is searched for CB_1-like sequences using the BLAST algorithm (http://genome.wustl.edu/gsc/blast/client.pl) the sequences that display the highest level of sequence similarity with rat CB_1 are several short fragments of clone Y54G2A. However, the level of sequence identity with CB_1 is very low and far less than the sequence identity shared by rat CB_1 and the putative *Drosophila* catecholamine receptors encoded by CG6919 and CG12796. Therefore, whilst this *C. elegans* gene sequence may encode a G-protein-coupled receptor, it is unlikely to be an orthologue of the mammalian cannabinoid receptors. If one searches GenBank for *C. elegans* genes that have been annotated as cannabinoid receptor-like, there is one gene that is described as having 'weak similarity to cannabinoid receptors'. However, BLAST analysis of the protein product of this gene (C02H7.2) reveals that whilst indeed it is the vertebrate cannabinoid receptors that display the highest sequence similarity with this protein, the level of sequence identity is very low (23% over a stretch of 311 amino acids). This level of sequence identity is in fact similar to that shared between rat CB_1 and the putative *Drosophila* catecholamine receptors CG6919 (24% over a stretch of 364 amino acids) and CG12796 (24% over a stretch of 314 amino acids). Therefore, whilst it is likely that C02H7.2 is a *C. elegans* G-protein-coupled receptor, it is unlikely that C02H7.2 represents an orthologue of vertebrate cannabinoid receptors.

The phylogenetic relationship of nematodes such as *C. elegans* with other invertebrates is controversial and some taxonomists have placed their evolutionary origin at a point that precedes the divergence of deuterostomian and protostomian animals. Recently, however, Aguinaldo *et al.* (1997) have presented evidence that nematodes belong to a protostomian clade known as the Ecdysozoa, which includes the arthropods (e.g. *Drosophila*). Therefore, the two invertebrate species for which we have 'complete' genome sequences appear to be more closely related than previously thought and may not necessarily be representative of other protostomian phyla. Therefore, it is important to assess the evidence for the occurrence of cannabinoid receptors in other protostomian invertebrates such as the molluscs and annelids.

Devane (1989) tested for the presence of [^{3}H]CP-55,940 binding sites in the marine mollusc *Aplysia californica* but no specific binding sites were detected in nervous tissue. Stefano *et al.* (1996) have reported the presence of a [^{3}H]anandamide binding site in blood cells from the bivalve mollusc *Mytilus edulis* and investigated the ability of cannabinoids to displace [^{3}H]anandamide. However, the methodology used for these radioligand-binding assays was non-standard and the K_i values for all cannabinoids tested were very similar. Subsequently, Stefano *et al.* (1997) investigated the presence of a specific [^{3}H]anandamide binding site in ganglia from the leeches *Hirudo medicinalis* and *Theromyzon tessulatum* (phylum Annelida). Again non-standard radioligand-binding assay methods were used and curiously the K_i values obtained for a variety of cannabinoids were virtually identical to those reported in the previous study on *Mytilus* blood cells. Anandamide is a very hydrophobic molecule and hence the binding sites detected in both *Mytilus* and *Hirudo* may simply reflect a non-selective but competitive interaction of cannabinoids with cell membranes as opposed to a specific interaction with a membrane protein. Nevertheless, Stefano *et al.* (1997) also report the isolation of a partial leech cDNA that when translated codes for a 153-amino-acid peptide that displays 61% sequence identity with the human CB_1 receptor. Subsequently, we have analysed this partial cDNA sequence in detail using the BLAST method and discovered that it is in fact chimeric (Elphick 1998). Two regions of the leech sequence display high levels of amino-acid identity with mammalian cannabinoid receptors, whilst a third region is 98% identical to part of the bovine adrenocorticotropic hormone receptor. We have offered a number of possible explanations for the isolation of this unusual cDNA sequence from leech (Elphick 1998), but one possibility that we did not suggest previously is that the leech sequence may be a PCR artefact arising from DNA contamination.

One other major branch of the animal kingdom that we have yet to consider is the diploblastic and radially symmetrical animals (coelenterates) that are exemplified by extant species belonging to the phylum Cnidaria (e.g. *Hydra*). These animals are of particular interest because they are recognized as being one of the most evolutionary ancient phyla, which probably evolved before the triploblastic and bilaterally symmetrical ancestor(s) of the protostomian and deuterostomian phyla. De Petrocellis *et al.* (1999) have investigated the presence of cannabinoid receptors in *Hydra vulgaris* and report the presence of specific binding sites for the CB_1 receptor antagonist SR141716A. Anandamide dose-dependently displaced binding of [^{3}H]SR141716A to these sites with a K_i value of 505 nM. The stereoselectivity of this binding site was not investigated and so therefore it is not possible to assess whether this binding site corresponds structurally and functionally with vertebrate cannabinoid receptors. However, against the overwhelming molecular evidence for the absence of cannabinoid receptor orthologues in other invertebrates such as *Drosophila* and *C. elegans*, as outlined above, it seems unlikely that the [^{3}H]SR141716A binding site in *Hydra* is a CB_1- or CB_2-like G-protein-coupled cannabinoid receptor. Throughout the animal kingdom there may be numerous proteins that are capable of specifically binding cannabinoids but which are structurally unrelated to the vertebrate CB_1 or CB_2 receptors. These proteins may be very interesting in their own right but it is important to recognize that not all cannabinoid-binding proteins are necessarily orthologues of vertebrate CB_1 and CB_2 receptors.

(d) *The evolution of cannabinoid receptors*

The determination of the sequences of genes encoding CB_1 genes in species belonging to three of the major non-mammalian vertebrate classes has provided an opportunity to compare the sequences of the proteins encoded by these genes and to investigate their relationship with mammalian CB_1 and CB_2 genes. In so doing it is possible to construct a phylogenetic tree that reflects the evolution of the cannabinoid receptor family in the vertebrates, as

illustrated in figure 3. There are two distinct clades in the tree, a CB_1 clade and a CB_2 clade. The CB_2 clade, based on the current state of knowledge, has only mammalian representatives. However, it is not known at what point in evolution the divergence of CB_1 and CB_2 receptors (node 1) occurred, although based on the absence of CB_2 genes in *Fugu*, the CB_2 gene may have evolved as a result of duplication of an ancestral CB_1-like gene since the divergence of tetrapods and fish. It is possible that this CB_1–CB_2 duplication event occurred more recently in early mammals, although this must then have been followed by a period of rapid sequence divergence to get to the point seen in extant mammals where CB_1 and CB_2 display a relatively low level of sequence identity. Regardless of the point in evolution that this duplication event occurred, with the existence of two cannabinoid receptor genes, it would have been possible for one of them to diverge from an ancestral type (CB_1-like?) and to acquire new functions associated with cells of the immune system (CB_2).

Analysis of the CB_1 clade of the phylogenetic tree reveals a pattern of relatedness that would be expected based on our knowledge of the sequence of events that gave rise to extant vertebrate classes. Thus, the set of four mammalian CB_1 receptors shares the highest level of sequence similarity, whilst the non-mammalian receptor that displays most similarity with the mammalian CB_1 receptors is the bird receptor. This reflects the fact that both mammals and birds evolved from reptilian ancestors (represent by node 5), and together with extant reptiles (which are not represented in the tree, at present) they form a monophyletic group of vertebrates known as the Amniota (Pough *et al*. 1999). The clade of amniotic vertebrates evolved from an amphibian-like ancestor (represented by node 4) and accordingly it is the amphibian CB_1 receptor (*Taricha*) along with Amniota that forms a clade of the CB_1 phylogenetic tree that corresponds with the tetrapod clade of the vertebrates. Finally, node 3 represents the common ancestry that tetrapods share with extant teleost fish (represented by *Fugu* CB_{1A}). The position of the *Fugu* CB_{1B} receptor in the tree is potentially confusing because one is given the impression that it occupies the position of a receptor that is ancestral to all the other CB_1 receptors in the tree. However, we know that FCB_{1B} is likely to have evolved due to a genome duplication event that occurred in the fish lineage, after the divergence of tetrapods. Because the sequence of FCB_{1B} has diverged from its ancestral form (FCB_{1A}-like), one is given the wrong impression that it arose from a duplication event that occurred before the divergence of fish and tetrapods. This reflects one of the limitations of trying to portray the molecular evolution of proteins in a two-dimensional diagram. Ideally phylogenetic trees that include both orthologues and paralogues need to be three dimensional and on a three-dimensional phylogenetic tree the *Fugu* CB_{1B} receptor would be positioned as a branch of the line linking node 3 to *Fugu* CB_{1A} but orientated at right-angles to the surface of the page. Similarly, the position of node 1 suggests that the CB_1–CB_2 duplication event occurred before the divergence of fish and tetrapods, which is not consistent with absence of a CB_2 gene in *Fugu*. These sorts of limitations in the presentation of molecular phylogenies occur when both paralogous and orthologous sequences are combined in a single tree, as in figure 3. Nevertheless, as more CB_1 and CB_2 receptor sequences are determined it will be interesting to expand the tree accordingly and in so doing portray when key events in the evolution of cannabinoid receptor family took place (e.g. the CB_1–CB_2 divergence).

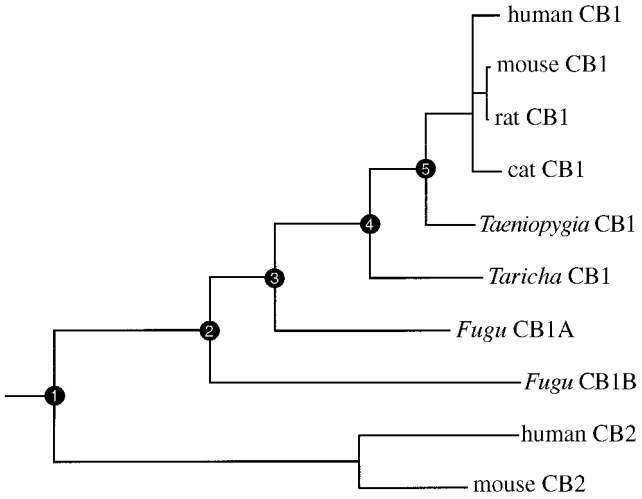

Figure 3. Phylogenetic analysis of vertebrate cannabinoid receptors. The amino-acid sequences of the receptors were aligned using the ClustalX (1.8) multiple alignment program (Thompson *et al*. 1997) and a phylogenetic tree was constructed using the neighbour-joining method (Saitou & Nei 1987) and viewed in NJ Plot. The position of the root for the phylogenetic tree was determined using human lysophospholipid receptors as an outgroup. The numbering of the nodes (1–5) is to facilitate discussion of the phylogenetic tree in the main text.

At what point in animal evolution did CB_1- and CB_2-related cannabinoid receptors appear? Collectively, the evidence for the existence of this family of cannabinoid receptors in the protostomian invertebrates is not compelling. In particular, the absence of genes in the genomes of *Drosophila* and *C. elegans* that display significant sequence similarity with vertebrate cannabinoid receptors indicates that orthologues of CB_1 and CB_2 do not exist in the protostomian phyla. Therefore, CB_1-like cannabinoid receptors probably did not exist before the divergence of protostomes and deuterostomes. The presence of a stereoselective binding site for [^{3}H]CP-55,940 in sea urchin sperm suggests that CB_1-like cannabinoid receptors may occur in echinoderms, in which case the family may have originated at the stem of the deuterostomian branch animal kingdom. However, without molecular characterization of the sea urchin protein(s) responsible for this binding site, this is speculation.

There is another way in which one can investigate the evolutionary origins of cannabinoid receptors and assess the likelihood that CB_1-like cannabinoid receptors could exist in invertebrate animals such as *Drosophila*. This approach involves the identification of vertebrate G-protein-coupled receptors that are most closely related to cannabinoid receptors and analysis of the occurrence of orthologues of these cannabinoid receptor paralogues in the *Drosophila* genome. BLAST analysis reveals that the mammalian G-protein-coupled receptors that are most closely related to CB_1 and CB_2 are

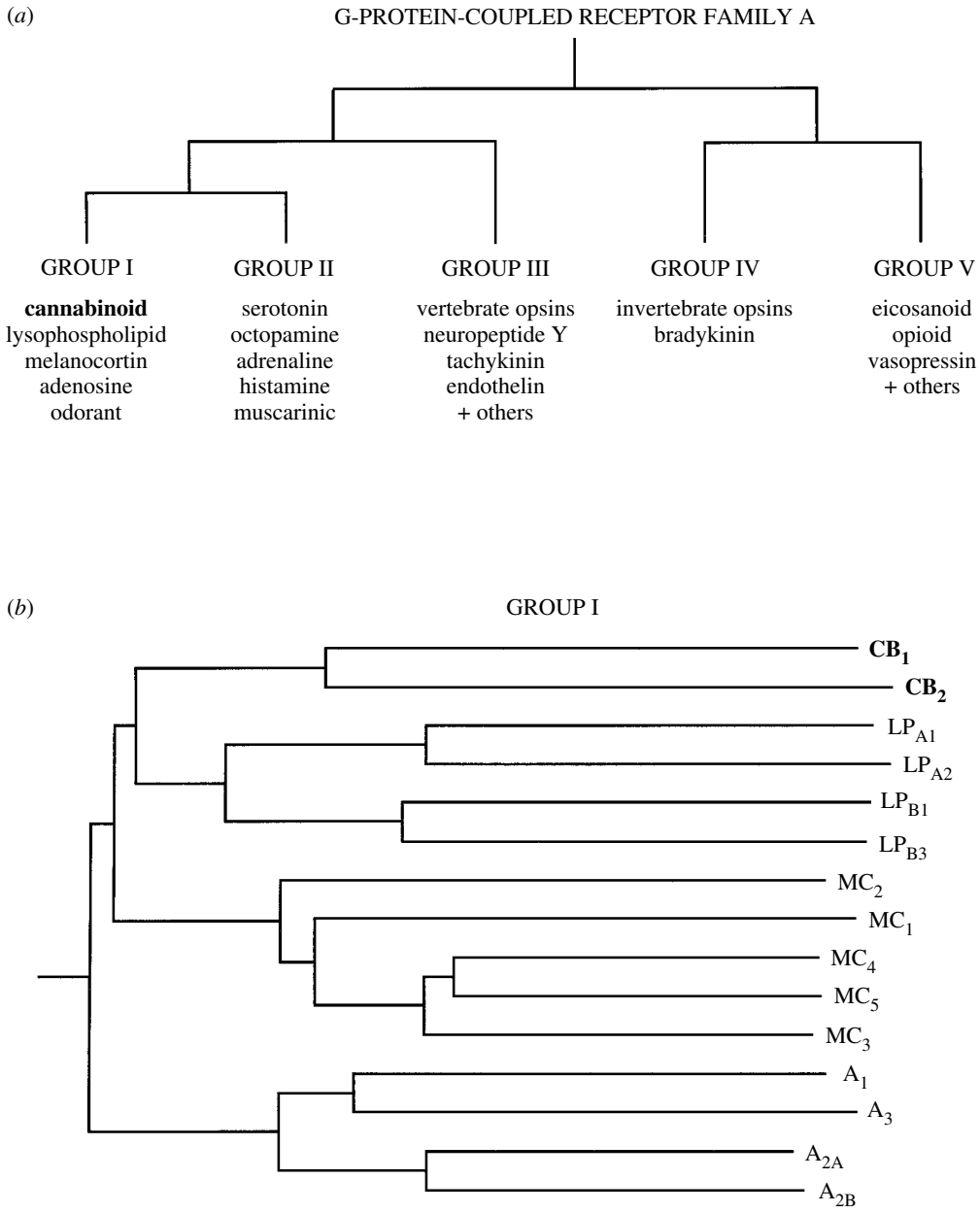

Figure 4. Phylogenetic analysis of the relationship of cannabinoid receptors (CB_1 and CB_2) with other G-protein-coupled receptors. (*a*) Classification of G-protein-coupled receptor family A into five groups (I–V) according to the phylogenetic analysis of Kolakowski (1994). The branch lengths are arbitrary, but the structure of the tree reflects the findings of Kolakowski (1994). (*b*) Phylogenetic analysis of human paralogues of the human CB_1 receptor that belong to group I-type receptors in Kolakowski's classification of G-protein-coupled receptors in family A. The amino-acid sequences of the receptors (CB, cannabinoid; LP, lysophospholipid; MC, melanocortin; A, adenosine) were aligned using the ClustalX (1.8) multiple alignment program (Thompson *et al*. 1997) and a phylogenetic tree was constructed using the neighbour-joining method (Saitou & Nei 1987) and viewed in NJ Plot. The position of the root for the phylogenetic tree was determined using a putative human odorant receptor (HSHGMP07J; Parmentier *et al*. 1992) as an outgroup.

members of a family of receptors that are activated by lysophospholipids (Chun 1999). Together with CB_1 and CB_2 cannabinoid receptors, these receptors form part of a distinct clade of the superfamily of G-protein-coupled receptors that are thought to have evolved from a common ancestral gene. By constructing phylogenetic trees, Kolakowski (1994; http://www.gcrdb.uthscsa.edu/) has subdivided the G-protein-coupled receptor superfamily into six families named A–F. The majority of known G-protein-coupled receptors falls within family A, including cannabinoid and lysophospholipid receptors. Moreover, family A can be subdivided further based on phylogenetic relatedness into five groups (I–V), as illustrated in figure 4. Cannabinoid receptors, along with lysophospholipid receptors, fall within group I along with melanocortin peptide receptors, adenosine receptors and a diverse set of putative odorant receptors. Thus, it is likely that the group I receptor types all evolved from a common ancestral gene and have arisen through a series of gene duplications over evolutionary time. What we do not know is over what time-span these duplication events, which ultimately

gave rise to vertebrate cannabinoid receptors, took place. One way to address this question is to investigate the presence of orthologues of the group I G-protein-coupled receptors in an invertebrate species such as *D. melanogaster*. We have already done this for the cannabinoid receptors, as discussed above, and we did not find *Drosophila* genes that share the high level of sequence similarity that one would expect for orthologues. What about the other members of group I? Using the BLAST method, we have searched the *Drosophila* genome for orthologues of several representative members of group I including a human melanocortin receptor, a human lysophosphatidic acid receptor and a human adenosine receptor, and in each case, as with cannabinoid receptors, the *Drosophila* gene products that displayed the highest amino-acid sequence identity with these group I receptors were the two putative catecholamine receptor genes CG6919 and CG12796. Thus, it appears that there are no *Drosophila* orthologues for any of the group I-type receptors. By way of comparison, orthologues of the group II receptors do occur in *Drosophila*. This is not surprising because the group II receptors include catecholamine receptors and muscarinic receptors, which are known to exist in insects based on decades of pharmacological data. However, this is interesting because, based on Kolakowski's phylogenetic tree, group II receptors share a more recent common ancestry with group I than groups III–V. Moreover, as highlighted above, the *Drosophila* receptors that display the highest level of sequence similarity with cannabinoid receptors and other group I receptors are putative catecholamine receptors. It appears, therefore, that members of the group I receptors may not exist in insects and the closest relatives of cannabinoid receptors that exist in insects are group II receptors. This suggests that the gene duplication event that gave rise to the group I receptors on the one hand and the group II receptors on the other probably occurred in the deuterostomian branch of the animal kingdom since its divergence from protostomes. Moreover, some of the group I receptors may be unique to vertebrates. Further insights into the evolutionary origin of cannabinoid receptors will be possible as more gene sequences are determined in invertebrate groups such as the echinoderms (Cameron *et al.* 2000), which occupy key positions in the evolutionary history of the animal kingdom. As the science of genomics develops we can look forward to a situation where complete genome sequences of species representing each of the major animal phyla are known. It may then be possible to construct evolutionary histories for all protein families, including cannabinoid receptors.

(e) *The evolution of cannabinoid signalling*

Based on the findings outlined above, it appears that the family of CB_1 or CB_2 G-protein-coupled cannabinoid receptors may not be widely distributed phylogenetically. Because G-protein-coupled receptors are essential molecular components of the cannabinoid signalling system, the evolution and phylogenetic distribution of cannabinoid signalling is likely to parallel that of the receptors. However, it is possible that other molecular components of the putative cannabinoid signalling system that have been characterized in mammals may be evolutionarily more ancient than the G-protein-coupled cannabinoid receptors with which they are associated. Therefore, it is of interest to investigate the phylogenetic distribution of cannabinoid-signalling-associated molecules such as anandamide, 2-AG and proteins involved in endocannabinoid synthesis and inactivation (e.g. FAAH).

Anandamide may occur throughout the animal kingdom because it has been detected in a variety of invertebrate species including the cnidarian *H. vulgaris* (De Petrocellis *et al.* 1998, 1999), the mollusc *Aplysia* (De Petrocellis *et al.* 1998) and the echinoderm *Paracentrotus lividus* (Bisogno *et al.* 1997). Moreover, it appears that 2-AG may be similarly widespread phylogenetically (De Petrocellis *et al.* 1998). However, the presence of these molecules should not in itself be interpreted as evidence that they necessarily function as part of an endocannabinoid signalling system in these animals. After all, anandamide has been found to present in chocolate (Di Tomaso *et al.* 1996), but nobody would suggest that this reflects the existence of a cannabinoid signalling pathway in the cocoa bean! Similarly, the amino acid glutamate occurs in all living organisms, but this is not considered evidence that it functions as an excitatory neurotransmitter in all living organisms.

Enzyme activities that catalyse hydrolysis of anandamide have also been detected in a variety of invertebrate species including *Hydra* and *Paracentrotus* (Bisogno *et al.* 1997; De Petrocellis *et al.* 1999). We have measured FAAH-like activity in the locust *Schistocerca gregaria* using [^{3}H]oleamide as a substrate and detected activity in a variety of tissues including brain, gut and muscle (Egertová *et al.* 1998*b*; Egertová 1999). However, as with anandamide itself, the presence of anandamide amidase-like or FAAH-like activity should not be necessarily interpreted as evidence for the existence of cannabinoid signalling in these wide-ranging animals or tissues. After all, FAAH was originally isolated from mammalian liver on account of its abundance in this tissue but the role of FAAH in liver may be unrelated to cannabinoid signalling. Moreover, FAAH may not be the only enzyme that catalyses the hydrolysis of anandamide and related lipid signalling molecules, and therefore FAAH-like activity is not necessarily indicative of the presence of an orthologue of FAAH. However, with the cloning and sequencing of mammalian FAAH genes (Cravatt *et al.* 1996; Giang & Cravatt 1997) it is now possible to search for related genes in non-mammalian animals. If GenBank is searched for proteins related to FAAH using the BLAST method, a number of related proteins are identified in both *C. elegans* and *Drosophila*. However, it remains to be determined whether these proteins are functional orthologues of the mammalian FAAHs. Nevertheless, based on the relatively low level of sequence similarity shared with mammalian FAAHs, it is possible that invertebrate FAAH-like proteins are simply related members of a family of enzymes that share an amidase signature sequence with FAAH.

Thus, in considering the evolution and phylogenetic distribution of cannabinoid signalling one must be cautious in drawing conclusions based solely on the presence of lipid molecules and enzymes that have been associated with this pathway in mammals. Recently, Salzet *et al.* (2000) reviewed the literature relating to

comparative aspects of cannabinoid biology and concluded that 'the endogenous cannabinoid system is conserved throughout evolution from coelenterates to man'. We would not make such a bold conclusion based on the evidence available. The molecules that are the key determinants for the existence of a cannabinoid signalling system are the CB_1 or CB_2 G-protein-coupled cannabinoid receptors and to date the existence of these receptors has only been firmly established in vertebrate species. Based on the data available, it seems likely that the cannabinoid signalling system may be quite restricted in its phylogenetic distribution, probably occurring only in the deuterostomian clade of the animal kingdom and possibly only in vertebrates.

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