THE ROYAL SOCIETY

A new perspective on cannabinoid signalling: complementary localization of fatty acid amide hydrolase and the CB1 receptor in rat brain

Michaela Egertová[1], Dan K. Giang[2], Benjamin F. Cravatt[2] and Maurice R. Elphick[1]*

[1]*School of Biological Sciences, Queen Mary and Westfield College, University of London, London E1 4NS, UK*
[2]*The Skaggs Institute for Chemical Biology and Department of Cell Biology, The Scripps Research Institute, La Jolla, CA 92037, USA*

CBl-type cannabinoid receptors in the brain mediate effects of the drug cannabis. Anandamide and *sn*-2 arachidonylglycerol (2-AG) are putative endogenous ligands for CBl receptors, but it is not known which cells in the brain produce these molecules. Recently, an enzyme which catalyses hydrolysis of anandamide and 2-AG, known as fatty acid amide hydrolase (FAAH), was identified in mammals. Here we have analysed the distribution of FAAH in rat brain and compared its cellular localization with CBl-type cannabinoid receptors using immunocytochemistry. High concentrations of FAAH activity were detected in the cerebellum, hippocampus and neocortex, regions of the rat brain which are enriched with cannabinoid receptors. Immunocytochemical analysis of these brain regions revealed a complementary pattern of FAAH and CBl expression with CBl immunoreactivity occurring in fibres surrounding FAAH-immunoreactive cell bodies and/or dendrites. In the cerebellum, FAAH was expressed in the cell bodies of Purkinje cells and CBl was expressed in the axons of granule cells and basket cells, neurons which are presynaptic to Purkinje cells. The close correspondence in the distribution of FAAH and CBl in rat brain and the complementary pattern of FAAH and CBl expression at the cellular level provides important new evidence that FAAH may participate in cannabinoid signalling mechanisms of the brain.

Keywords: cannabinoid receptor; fatty acid amide hydrolase; anandamide; arachidonylglycerol; cerebellum; rat

1. INTRODUCTION

The psychoactive ingredient of cannabis, Δ^9-tetrahydrocannabinol, exerts effects in the brain by binding to specific receptor proteins known as cannabinoid receptors (Devane *et al*. 1988; Pertwee 1995). Two types of cannabinoid receptor have been characterized in mammals and are known as CBl (Matsuda *et al*. 1990) and CB2 (Munro *et al*. 1993). The CBl cannabinoid receptor is expressed in the brain and testis, whereas the CB2 receptor is expressed by cells of the immune system (Felder & Glass 1998). The discovery of cannabinoid receptors in the brain heralded a search for endogenous ligands and to date two candidate molecules have been identified. The first putative endocannabinoid to be identified was arachidonylethanolamide (anandamide) which was isolated from porcine brain (Devane *et al*. 1992). More recently, another arachidonic acid derivative, *sn*-2 arachidonylglycerol (2-AG), has been identified as a possible ligand for brain cannabinoid receptors (Sugiura *et al*. 1995; Stella *et al*. 1997).

At present little is known about the mechanisms of anandamide and 2-AG biosynthesis in neurons although various models have been proposed (Cadas *et al*. 1996, 1997; Deutsch & Chin 1993; Devane & Axelrod 1994; Di Marzo *et al*. 1994; Felder & Glass 1998). Assessment of anandamide and 2-AG as potential ligands for CBl receptors in the brain will be facilitated when enzymes involved in their synthesis have been characterized. Progress has been made, however, in the characterization of an enzyme that hydrolyses anandamide and 2-AG. Anandamide amidohydrolase activity was detected in rat brain tissue soon after the discovery of anandamide (Deutsch & Chin 1993), but molecular characterization of this enzyme was accomplished via an independent line of research. Cravatt *et al*. (1996) isolated an enzyme from rat liver which hydrolyses a sleep-inducing fatty acid amide (oleamide) present in the cerebrospinal fluid of sleep-deprived cats (Cravatt *et al*. 1995). Characterization of the cloned 'oleamide hydrolase' revealed that it is a membrane protein of *ca*. 63 kDa which also displays 'anandamide hydrolase' activity (Cravatt *et al*. 1996). The new enzyme was therefore named fatty acid amide hydrolase (FAAH) in recognition of its ability to catalyse hydrolysis of two bioactive fatty acid amides, oleamide and anandamide.

The discovery of FAAH represents an important breakthrough in cannabinoid research because it provides new strategies for assessing anandamide as a potential ligand for cannabinoid receptors. Moreover, recently it has been shown that FAAH also catalyses hydrolysis of 2-AG, the second putative endogenous cannabinoid receptor ligand (Goparaju *et al*. 1998). It appears therefore that FAAH may represent an important component of cannabinoid

*Author for correspondence (m.r.elphick@qmw.ac.uk).

signalling mechanisms in the brain. In this paper we have addressed this issue by analysing the distribution of FAAH in the rat brain and comparing its expression pattern with CB1-type cannabinoid receptors at the cellular level.

2. MATERIALS AND METHODS

(a) *FAAH enzyme activity assay*

Male Sprague-Dawley rats were sacrificed by decapitation. Brains were removed immediately, dissected into regions and placed in ice-cold phosphate-buffered saline (PBS). Brain regions were homogenized in 1 mM $NaHCO_3$ and assayed for FAAH activity as follows: 4 µl of ^{14}C-oleamide (final concentration 100 µM, 50 µCi µmol^{-1}) was incubated at 37 °C for 1 h with 40 µl of protein (50 µg) and 156 µl of 125 mM Tris-HCl, pH 9.0. The reaction mixture was then partitioned between ethyl acetate (1 ml) and 0.07 M HCl (0.6 ml). The ethyl acetate layer was concentrated under a stream of N_2, and the remaining residue was resuspended in 15 µl of ethanol. Products were separated by thin-layer chromatography (60% ethyl acetate in hexanes), eluted from silica with scintillation fluid and quantified by scintillation counting. Each brain region was assayed in triplicate and mean activities with standard deviations were calculated. Furthermore, the experiment was repeated three times using brain tissue from different individuals, and a representative set of results from one of these experiments is illustrated in figure 1.

(b) *Western blot analysis*

Fifty micrograms of protein from homogenized whole rat brain and brain regions were run on a 10% polyacrylamide Tris-glycine gel. The gel was transferred to nitro-cellulose and probed with a 1:500 dilution of affinity-purified anti-FAAH rabbit polyclonal antibodies raised against a FAAH-GST fusion protein. The FAAH-GST fusion protein was generated by standard molecular biology procedures (Pharmacia) and included amino acids 38–579 of the rat FAAH protein. Affinity purification of anti-FAAH rabbit antibodies was conducted first by depleting rabbit antiserum of GST-cross-reactive antibodies and then isolating from this serum the FAAH-GST-reactive antibodies.

(c) *Immunocytochemistry*

A CB1 antiserum was generated by immunizing rabbits with a conjugate of thyroglobulin and a peptide comprising the C-terminal 13 amino acids of the rat CB1 receptor (KYTMSVSTDTSAEAL). An N-terminal lysine residue was incorporated into the peptide sequence to provide a reactive site for coupling to a carrier protein (thyroglobulin) by using glutaraldehyde. A tyrosine residue was also incorporated so that radioimmunoassay could be employed to monitor antibody production in rabbits using a ^{125}I-labelled peptide tracer. Male adult Wistar rats were asphyxiated with CO_2 and perfused through the heart with 50 ml of 4% formaldehyde in PBS. The brains were removed, post-fixed in Bouin's fixative and embedded in paraffin wax for sectioning (10 µm). Dewaxed sections were blocked with 3% normal goat serum/PBS +0.2% Triton X-100 (PBST) and then incubated with FAAH affinity-purified antibodies or CB1 antiserum diluted 1:50 and 1:1000 in PBST, respectively. Bound antibodies were revealed using the ABC peroxidase method (Vector Labs). The specificity of CB1 staining was established by performing pre-absorption experiments using the CB1-C-terminal peptide antigen (20 µM).

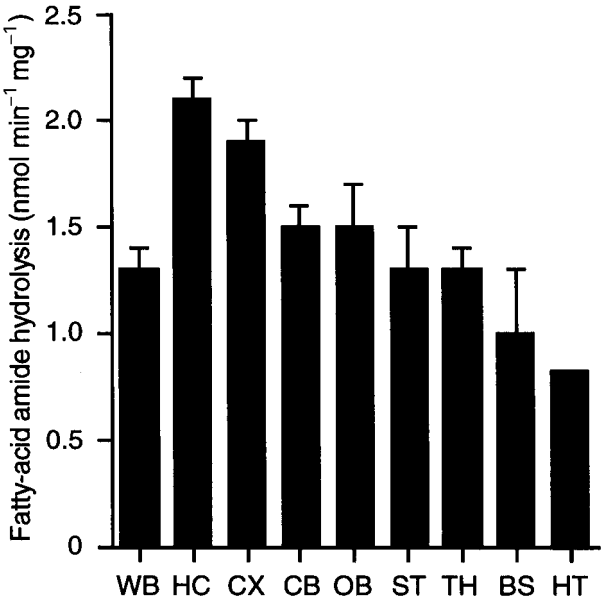

Figure 1. FAAH activity in homogenates (50 µg of protein) of whole rat brain compared with regions of the rat brain. WB, whole brain; HC, hippocampus; CX, neocortex; CB, cerebellum; OB, olfactory bulb; ST, striatum; TH, thalamus; BS, brain stem; HT, hypothalamus. Mean values from assays performed in triplicate with standard deviation error bars are shown. Similar findings were reported while this paper was in preparation (Thomas *et al*. 1997).

3. RESULTS AND DISCUSSION

FAAH enzyme activity was found to be concentrated in the hippocampus, neocortex, cerebellum and olfactory bulb (figure 1), regions of the rat brain which are also enriched with cannabinoid receptors (Herkenham *et al*. 1991). Lower levels of FAAH activity were detected in other brain regions analysed (figure 1). To establish whether FAAH activity correlated with FAAH protein distribution in the rat brain we applied Western blotting methods using affinity-purified rabbit polyclonal antibodies to an FAAH-GST fusion protein. Fifty micrograms of protein from each brain region were analysed, providing a semi-quantitative method for FAAH protein measurement (figure 2). The FAAH protein of *ca*. 63 kDa was most abundant in the hippocampus and neocortex, with lower levels of FAAH being detected in each of the other brain regions examined. These results generally matched well with measurements of FAAH activity in brain regions and are consistent with the distribution of FAAH mRNA in the rat brain (Thomas *et al*. 1997).

To identify which cells in the rat brain express FAAH we used immunocytochemical methods with the same FAAH antibodies that were used for Western blot analysis. FAAH immunoreactivity occurred in a distinct population of neurons in the brain but was not evident in glial cells. FAAH immunoreactive neurons were detected most predominantly in the cerebellum, hippocampus and neocortex (figure 3). FAAH-immunoreactive neurons were also observed in other regions of the brain (e.g. in the olfactory bulb; not shown) but not as clearly and strongly as in the cerebellum, hippocampus and neocortex. FAAH immunoreactivity was localized in the cytoplasmic compartment of the cell body and dendrites of

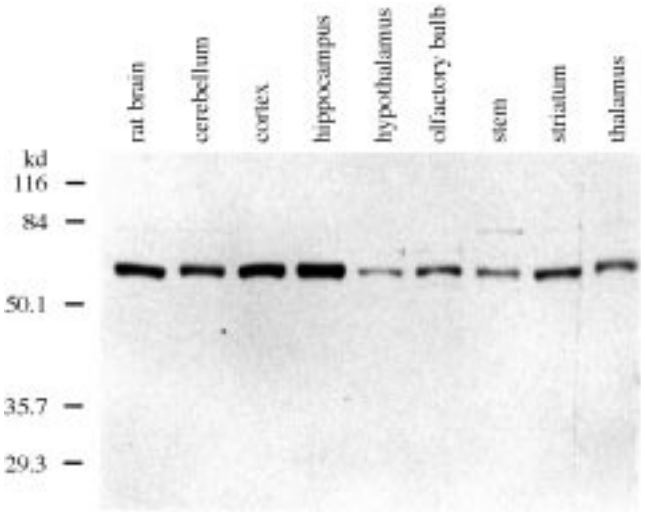

Figure 2. Western blot showing the FAAH protein of *ca*. 63 KDa in homogenates (50 µg protein) of whole rat brain and regions of the rat brain using affinity-purified rabbit antibodies to a GST-FAAH fusion protein. Note the higher concentrations of FAAH in the neocortex and hippocampus compared to other brain regions.

identified neurons in each brain region, including cerebellar Purkinje cells (figure 3*a*), hippocampal pyramidal cells (figure 3*b*) and neocortical pyramidal cells (figure 3*c*).

To evaluate the involvement of FAAH in cannabinoid signalling in the rat brain, we compared the anatomical distribution of FAAH with the CB1-type cannabinoid receptor at the cellular level. Antibodies to the C-terminal 13 amino acids of the rat CB1 protein were developed and used to map the distribution of cannabinoid receptors in the rat brain using immunocytochemistry. The gross distribution of CB1 immunoreactivity in the rat brain matched with previous descriptions based on cannabinoid receptor autoradiography (Herkenham *et al*. 1991). Moreover, Western blot analysis of rat brain homogenates using our CB1-C-terminal antisera (data not shown) revealed staining of a band similar in molecular mass to the 52.8 kDa protein predicted by Matsuda *et al*. (1990) from the cDNA sequence encoding the rat CB1 receptor. Also, while this paper was in preparation, Tsou *et al*. (1998) reported the distribution of CB1 in the rat brain as revealed using antibodies to the N-terminal region of the CB1 receptor. The pattern of staining revealed by N-terminal antibodies appears to be generally very similar to our findings using C-terminal antibodies. However, a striking novel observation of the present analysis was the absence of CB1-immunoreactivity in neuronal cell bodies. These differences in the pattern of staining observed with C-terminal and N-terminal antibodies are intriguing but it is not yet clear why such differences are observed. One possibility, however, is that whilst located in neuronal cell bodies the CB1 receptor is not immunoreactive with our C-terminal antibodies due to phosphorylation of serine and/or threonine residues in the C-terminal tail. Further work is required to investigate these potentially functionally important observations.

We focused our analysis of the cellular distribution of CB1 expression on the three regions of the brain in which FAAH-immunoreactive neurons were most abundant; the cerebellum, the hippocampus and the neocortex. Importantly, in all three regions, CB1 immunoreactivity was detected in nerve fibres located around the cell bodies and/or dendrites of neurons in which FAAH immunoreactivity was detected. The clearest example of this was seen in the cerebellum where identification of stained neurons is relatively easy (Palay & Chan-Palay 1974).

Purkinje cells of the cerebellum exhibited FAAH immunoreactivity in their cell body and dendrites (figure 3*a*) but were completely devoid of CB1-immunoreactivity (figure 4*a*). Surrounding the dendrites of Purkinje cells, however, there was CB1 immunoreactivity in fibres throughout the molecular layer of the cerebellum (figure 4*a*). This pattern of staining is consistent with expression of CB1 receptors on the axons of granule cells which form a network of parallel fibres in the molecular layer (Palay & Chan-Palay 1974). Moreover, this interpretation is supported by previous studies showing CB1 mRNA expression in granule cell somata (Matsuda *et al*. 1993). Granule cell axons are presynaptic to Purkinje cell dendrites (Palay & Chan-Palay 1974), and so in the cerebellum it appears that CB1-receptors are expressed in the axons of neurons (granule cells) that are presynaptic to neurons expressing FAAH (Purkinje cells). Another cerebellar cell type which is presynaptic to Purkinje cells are basket cells whose cell bodies are located in the molecular layer of the cerebellum with axons that make synaptic contact with the cell bodies of several Purkinje cells (Palay & Chan-Palay 1974). Basket cells are unique in forming a 'basket' of fibres around the cell bodies of Purkinje cells which makes their identification relatively easy. Analysis of CB1 immunoreactivity in the cerebellum clearly revealed stained structures around the base of Purkinje cells bodies (figure 4*a*) which match exactly with previous descriptions of basket cell axon terminals (Palay & Chan-Palay 1974). Moreover, as with granule cells, previous *in situ* hybridization studies have shown CB1 mRNA expression in the somata of basket cells (Matsuda *et al*. 1993). Colocalization of FAAH with CB1 was not observed in any part of the cerebellum; Purkinje cell bodies and dendrites did not exhibit CB1 immunoreactivity (figure 4*a*), and likewise granule cell axons and basket cell axons were void of FAAH immunoreactivity (figure 3*a*). These data indicate a complementary pattern of FAAH and CB1 expression, with FAAH being expressed in cell bodies and dendrites that are postsynaptic to CB1-expressing axon terminals.

A similar complementary localization of CB1 and FAAH appears to exist in the hippocampus and neocortex, although here the identification of individual neuronal types exhibiting CB1-immunoreactivity is more difficult than in the cerebellum. Pyramidal cells of both the neocortex and hippocampus were FAAH-immunoreactive (figure 3*b*,*c*) but were clearly void of CB1 immunoreactivity (figure 4*b*,*c*). However, surrounding the cell bodies of pyramidal cells in the hippocampus and neocortex was a network of beaded CB1-immunoreactive fibres (figure 4*b*,*c*). Although we are unable to identify the neurons which express CB1 immunoreactivity in the hippocampus and cortex, the pattern of pericellular stained fibres is not inconsistent with expression of CB1 in axons of neurons that are presynaptic to pyramidal cells.

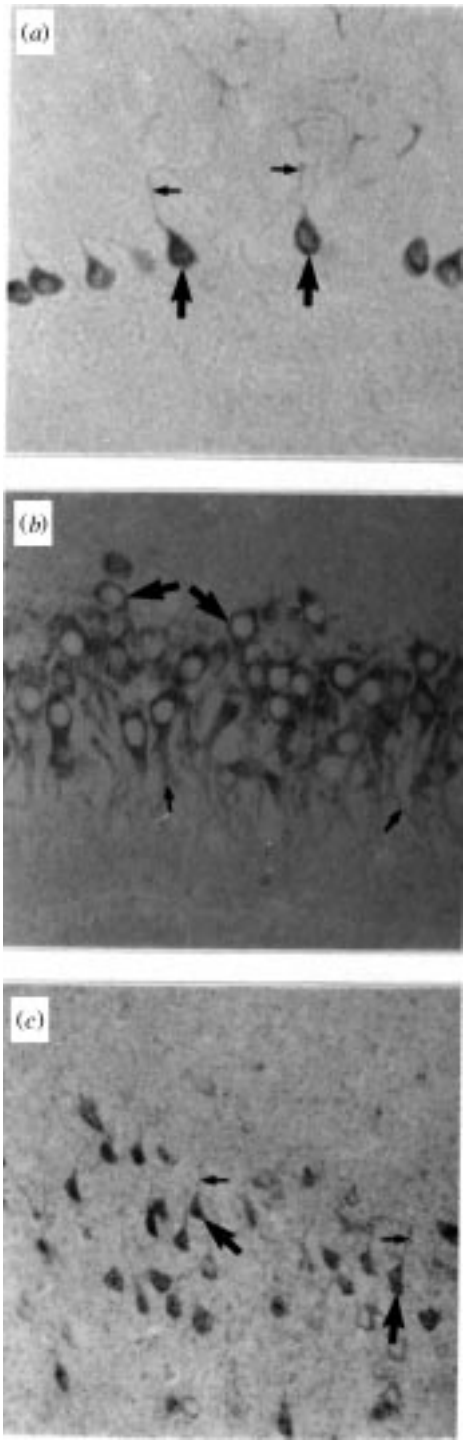

Figure 3. FAAH-immunoreactive neurons in rat brain. (*a*) Cerebellum: FAAH immunoreactivity in the cell bodies (large arrows) and dendrites (small arrows) of Purkinje cells. FAAH immunoreactivity is not evident in granule cells and basket cells (cf. figure 4*a*). (*b*) Hippocampus: FAAH immunoreactivity in the cell bodies (large arrows) and dendrites (small arrows) of pyramidal cells in the CA3 region of the hippocampus. (*c*) Neocortex: FAAH immunoreactivity in the cell bodies (large arrows) and dendrites (small arrows) of pyramidal cells in the frontal lobe of the neocortex. (Magnifications: (*a*), (*b*), × 295; (*c*), × 185.)

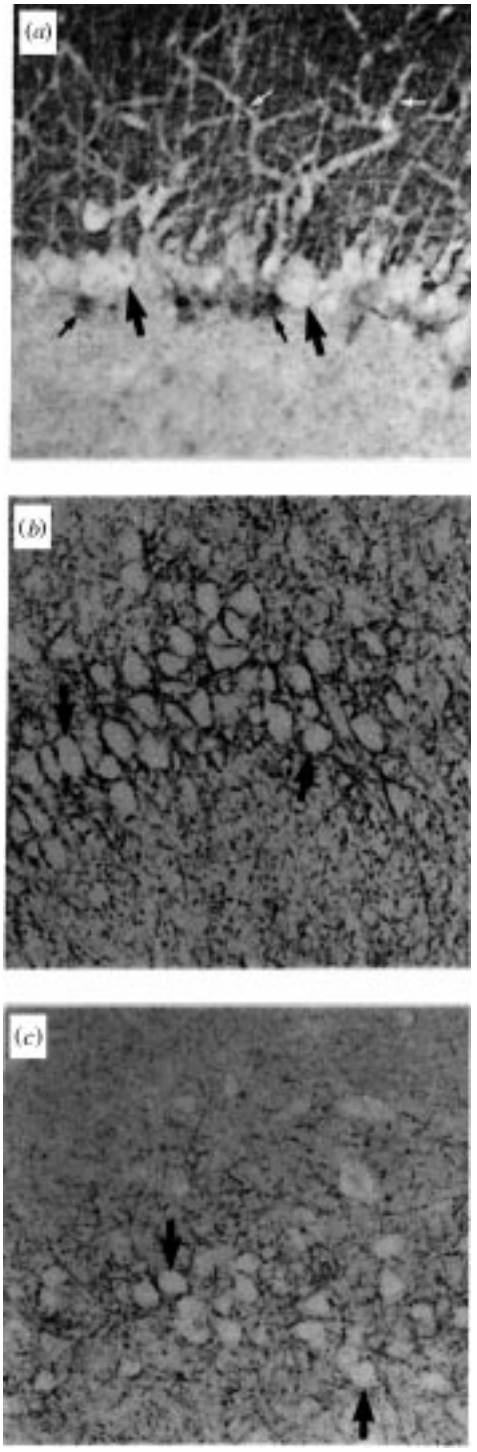

Figure 4. CB1 immunoreactivity in rat brain. (*a*) Cerebellum: dense CB1-immunoreactivity is present in the molecular layer where stained parallel fibres surround the unstained dendrites (white arrows) of Purkinje cells. The stained axon terminals of basket cells (small black arrows) can be seen around the unstained cell bodies (large black arrows) of Purkinje cells. (*b*) Hippocampus: CB1 immunoreactivity is present in beaded nerve fibres surrounding the unstained cell bodies of pyramidal cells (arrows) in the CA3 region of the hippocampus. (*c*) Neocortex: CB1 immunoreactivity is present in beaded nerve fibres surrounding the unstained cell bodies of pyramidal cells (arrows) in the frontal lobe of the neocortex. (Magnifications: (*a*), (*b*), × 295; (*c*), × 185.)

Our results indicate that there is a complementary pattern of FAAH and CBl expression in the cerebellum, hippocampus and neocortex with CBl immunoreactivity occurring in nerve fibres that surround the cell bodies and/or dendrites of neurons expressing FAAH. The striking correspondence in the distribution of FAAH and CBl in these three major regions of the brain provides important new evidence that FAAH participates in cannabinoid signalling mechanisms of the brain. Moreover, if, as the data indicate, FAAH is expressed in neurons that are postsynaptic to CBl-expressing axons, this provides a new perspective on cannabinoid signalling. It suggests that the endocannabinoids anandamide and 2-AG may be inactivated by FAAH in neurons that are postsynaptic to the CBl-expressing neurons where these molecules are presumed to exert effects. At present it is not known which neurons in the brain synthesize anandamide and 2-AG, and for this we must await the characterization of endocannabinoid synthases. One possibility, however, is that endocannabinoids are synthesized by neurons that are postsynaptic to CBl-expressing axon terminals. In this model, endocannabinoids could function as synaptic retrograde messenger molecules which modulate release of classical neurotransmitters by presynaptic axon terminals. This model is consistent with observed inhibitory effects of anandamide and other cannabinoids on synaptic transmission in the nervous system (Cadogan *et al*. 1997; Collins *et al*. 1995; Ishac *et al*. 1996; Schlicker *et al*. 1997; Terranova *et al*. 1995).

We are grateful to Richard Melarange for technical assistance. This work was supported by a grant (F476U) from the Leverhulme Trust to M.R.E. and a grant (MH58542) from the NIH to B.F.C.

REFERENCES

Cadas, H., Gaillet, S., Beltramo, M., Venance, L. & Piomelli, D. 1996 Biosynthesis of an endogenous cannabinoid precursor in neurons and its control by calcium and cAMP. *J. Neurosci*. **16**, 3934–3942.

Cadas, H., Di Tomaso, E. & Piomelli, D. 1997 Occurrence and biosynthesis of endogenous cannabinoid precursor, N-arachidonyl phosphatidylethanolamine, in rat brain. *J. Neurosci*. **17**, 1226–1242.

Cadogan, A. K., Alexander, S. P. H., Boyd, E. A. & Kendall, D. A. 1997 Influence of cannabinoids on electrically evoked dopamine release and cyclic AMP generation in the rat striatum. *J. Neurochem*. **69**, 1131–1137.

Collins, D. R., Pertwee, R. G. & Davies, S. N. 1995 Prevention by the cannabinoid antagonist, SR141716A, of cannabinoid blockade of long-term potentiation in the rat hippocampal slice. *Br. J. Pharmacol*. **115**, 869–870.

Cravatt, B. F., Prospero-Garcia, O., Siuzdak, G., Gilula, N. B., Henriksen, S. J., Boger, D. L. & Lerner, R. A. 1995 Chemical characterization of a family of brain lipids that induce sleep. *Science* **268**, 1506–1509.

Cravatt, B. F., Giang, D. K., Mayfield, S. P., Boger, D. L., Lerner, R. A. & Gilula, N. B. 1996 Molecular characterization of an enzyme that degrades neuromodulatory fatty-acid amides. *Nature* **384**, 83–87.

Deutsch, D. G. & Chin, S. A. 1993 Enzymatic-synthesis and degradation of anandamide, a cannabinoid receptor agonist. *Biochem. Pharmacol*. **46**, 791–796.

Devane, W. A. & Axelrod, J. 1994 Enzymatic-synthesis of anandamide, an endogenous ligand for the cannabinoid receptor, by brain membranes. *Proc. Natn. Acad. Sci. USA* **91**, 6698–6701.

Devane, W. A., Dysarz, F. A. I., Johnson, M. R., Melvin, L. S. & Howlett, A. C. 1988 Determination and characterization of a cannabinoid receptor in rat brain. *Molec. Pharmacol*. **34**, 605–613.

Devane, W. A., Hanus, L., Breuer, A., Pertwee, R. G., Stevenson, L. A., Griffin, G., Gibson, D., Mandelbaum, D., Etinger, A. & Mechoulam, R. 1992 Isolation and structure of a brain constituent that binds to the cannabinoid receptor. *Science* **258**, 1946–1949.

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

Felder, C. C. & Glass, M. 1998 Cannabinoid receptors and their endogenous agonists. *A. Rev. Pharmacol. Toxicol*. **38**, 179–200.

Goparaju, S. K., Ueda, N., Yamaguchi, H., & Yamamoto, S. 1998 Anandamide amidohydrolase reacting with 2-arachidonoylglycerol, another cannabinoid receptor ligand. *FEBS Lett*. **422**, 69–73.

Herkenham, M., Lynn, A. B., Johnson, M. R., Melvin, L. S., De Costa, B. R. & Rice, K. C. 1991 Characterization and localization of cannabinoid receptors in rat brain: a quantitative *in vitro* autoradiographic study. *J. Neurosci*. **11**, 563–583.

Ishac, E. J. N., Jiang, L., Lake, K. D., Varga, K., Abood, M. E. & Kunos, G. 1996 Inhibition of exocytotic noradrenaline release by presynaptic cannabinoid CBl receptors on peripheral sympathetic nerves. *Br. J. Pharmacol*. **118**, 2023–2028.

Matsuda, L. A., Lolait, S. J., Brownstein, M. J., Young, A. C. & Bonner, T. I. 1990 Structure of a cannabinoid receptor: functional expression of the cloned receptor. *Nature* **346**, 561–563.

Matsuda, L. A., Bonner, T. I. & Lolait, S. J. 1993 Localization of cannabinoid receptor mRNA in rat brain. *J. Comp. Neurol*. **327**, 535–550.

Munro, S., Thomas, K. L. & Abu-Shaar, M. 1993 Molecular characterization of a peripheral receptor for cannabinoids. *Nature* **365**, 61–65.

Palay, S. L. & Chan-Palay, V. 1974 *Cerebellar cortex: cytology and organization*. Berlin: Springer-Verlag.

Pertwee, R. G. (ed.) 1995 *Cannabinoid receptors*. London: Academic Press.

Schlicker, E., Timm, J., Zentner, J. & Gothert, M. 1997 Cannabinoid CBl receptor-mediated inhibition of noradrenaline release in the human and guinea-pig hippocampus. *Naunyn-Schmiedebergs Archives of Pharmacol*. **356**, 583–589.

Stella, N., Schweitzer, P. & Piomelli, D. 1997 A second endogenous cannabinoid that modulates long-term potentiation. *Nature* **388**, 773–778.

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

Terranova, J. P., Michaud, J. C., Lefur, G. & Soubrie, P. 1995 Inhibition of long-term potentiation in rat hippocampal slices by anandamide and WIN55212-2—reversal by SR141716-A, a selective antagonist of CBl cannabinoid receptors. *Naunyn-Schmiedebergs Archives of Pharmacol*. **352**, 576–579.

Thomas, E. A., Cravatt, B. F., Danielson, P. E., Gilula, N. B. & Sutcliffe, J. G. 1997 Fatty acid amide hydrolase, the degradative enzyme for anandamide and oleamide, has selective distribution in neurons within the rat central nervous system. *J. Neurosci. Res*. **50**, 1047–1052.

Tsou, K., Brown, S., Sañudo-Peña, M. C., Mackie, K. & Walker, J. M. 1998 Immunohistochemical distribution of cannabinoid CBl receptors in the rat central nervous system. *Neuroscience* **83**, 393–411.