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Cannabinoid receptors and their ligands

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Summary There are at least two types of cannabinoid receptors, CB_1 and CB_2, both coupled to G proteins. CB_1 receptors exist primarily on central and peripheral neurons, one of their functions being to modulate neurotransmitter release. CB_2 receptors are present mainly on immune cells. Their roles are proving more difficult to establish but seem to include the modulation of cytokine release. Endogenous agonists for cannabinoid receptors (endocannabinoids) have also been discovered, the most important being arachidonoyl ethanolamide (anandamide), 2-arachidonoyl glycerol and 2-arachidonyl glyceryl ether. Other endocannabinoids and cannabinoid receptor types may also exist. Although anandamide can act through CB_1 and CB_2 receptors, it is also a vanilloid receptor agonist and some of its metabolites may possess yet other important modes of action. The discovery of the system of cannabinoid receptors and endocannabinoids that constitutes the 'endocannabinoid system' has prompted the development of CB_1- and CB_2-selective agonists and antagonists/inverse agonists. CB_1/CB_2 agonists are already used clinically, as anti-emetics or to stimulate appetite. Potential therapeutic uses of cannabinoid receptor agonists include the management of multiple sclerosis/spinal cord injury, pain, inflammatory disorders, glaucoma, bronchial asthma, vasodilation that accompanies advanced cirrhosis, and cancer. Following their release onto cannabinoid receptors, endocannabinoids are removed from the extracellular space by membrane transport and then degraded by intracellular enzymic hydrolysis. Inhibitors of both these processes have been developed. Such inhibitors have therapeutic potential as animal data suggest that released endocannabinoids mediate reductions both in inflammatory pain and in the spasticity and tremor of multiple sclerosis. So too have CB_1 receptor antagonists, for example for the suppression of appetite and the management of cognitive dysfunction or schizophrenia.

THE ENDOCANNABINOID SYSTEM

Mammalian tissues contain at least two types of cannabinoid receptors. These are CB_1 receptors, cloned in 1990,[1] and CB_2 receptors, cloned in 1993.[2] Endogenous agonists for these receptors also exist: arachidonoyl ethanolamide (anandamide),[3] 2-arachidonoyl glycerol,[4–6] and 2-arachidonyl glyceryl ether (noladin ether).[7] Of these 'endocannabinoids', anandamide behaves as a partial cannabinoid receptor agonist with marginally greater CB_1 than CB_2 affinity but much less CB_2 than CB_1 efficacy.[8] The pharmacological properties of 2-arachidonoyl glycerol and 2-arachidonyl glyceryl ether have been less well characterized. The available data suggest that both are cannabinoid receptor agonists, that noladin ether has significantly higher affinity for CB_1 than CB_2 receptors whilst the affinity of 2-arachidonoyl glycerol for CB_1 and CB_2 receptors is similar to that of anandamide and that 2-arachidonoyl glycerol differs from anandamide in exhibiting higher-efficacy at CB_2 and probably also at CB_1 receptors.[7–9] Anandamide and 2-arachidonoyl glycerol may both serve as neurotransmitters or neuromodulators as there is evidence that they are synthesized by neurons 'on demand', that they can undergo depolarization-induced release from neurons and that once released they are rapidly removed from the extracellular space by a membrane transport process yet to be fully characterized.[5,10–13] Indeed, results from very recent experiments suggest that endocannabinoids

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function as retrograde synaptic messengers (see below). Once within the cell, anandamide is thought to be hydrolysed to arachidonic acid and ethanolamine by the microsomal enzyme, fatty acid amide hydrolase (FAAH)[5,10,12,14] 2-Arachidonoyl glycerol can also be hydrolysed enzymically, both by FAAH and by other hydrolases yet to be characterized.[5,12,15] Mechanisms underlying the release and fate of noladin ether remain to be identified. Cannabinoid receptors and their endogenous agonists constitute the 'endocannabinoid system'.

CB_1 receptors are present in the central nervous system and also in some peripheral tissues including pituitary gland, immune cells, reproductive tissues, gastrointestinal tissues, sympathetic ganglia, heart, lung, urinary bladder and adrenal gland.[16] CB_2 receptors, on the other hand, are expressed mainly by immune cells, particularly B-cells and natural killer cells.[16] Within the brain, the distribution of CB_1 receptors is heterogeneous, accounting for several well-documented pharmacological properties of CB_1 receptor agonists. For example, the cerebral cortex, hippocampus, lateral caudate-putamen, substantia nigra pars reticulata, globus pallidus, entopeduncular nucleus and the molecular layer of the cerebellum are all populated with particularly high concentrations of CB_1 receptors, a distribution pattern that is consistent with the well-established ability of cannabinoids and to alter locomotor activity and produce catalepsy, particularly in rodents, and to impair cognition and memory.[16,17] Additionally, CB_1 receptors are found on pain pathways in brain and spinal cord and probably also at the peripheral terminals of primary afferent neurons and these receptors presumably mediate cannabinoid-induced analgesia.[18]

As detailed elsewhere,[16,18,19] considerable information is now emerging about cannabinoid receptor signalling. More particularly, results obtained from experiments with tissues containing naturally expressed cannabinoid receptors and with cells that have been transfected with CB_1 or CB_2 receptors indicate that both these receptor types can couple through $G_{i/o}$ proteins, negatively to adenylate cyclase and positively to mitogen-activated protein kinase. CB_1 receptors are also coupled through $G_{i/o}$ proteins to ion channels, positively to A-type and inwardly rectifying potassium channels and negatively to N-type and P/Q type calcium channels and to D-type potassium channels.[16,18,19] In addition, there is evidence that CB_1 receptors are negatively coupled to postsynaptic M-type potassium channels in rat hippocampal CA1 pyramidal neurons and to voltage gated L-type calcium channels in cat cerebral arterial smooth muscle cells and in retinal bipolar cell axon terminals of larval tiger salamanders.[18,19] CB_1 receptors may also close $5HT_3$ ion channels, modulate nitric oxide production and mobilize arachidonic acid when activated.[16,19] There is evidence too that CB_1 receptors can mobilize intracellular calcium stores both in neurons[19–22] and in smooth muscle cells[23] and that CB_1 receptors on rat cortical astrocytes can activate sphingomyelin hydrolysis to ceramide through a non-$G_{i/o}$ mechanism.[24,25] Experiments with CB_1- and CB_2-transfected cells have revealed other signalling mechanisms for cannabinoid receptors. For example, CB_1 receptors have been reported to be positively coupled to phospholipase C through G protein in COS7 cells co-transfected with CB_1 receptors and G_α subunits of the $G_{\alpha q}$ family.[26] It is also possible for CB_2 receptors to couple to ion channels in transfected cells, there being reports that inwardly rectifying potassium channels provide a signalling mechanism for this receptor type in *Xenopus* oocytes transfected with potassium channels of this type together with CB_2 receptors.[26,27] However, the physiological significance of the signalling data obtained solely from transfected cell experiments remains to be established.

Experiments with tissues containing either naturally expressed or transfected CB_1 receptors have shown that under certain conditions, CB_1 receptors can couple to G_s proteins to activate adenylate cyclase and/or to reduce outward potassium K current, possibly through arachidonic acid-mediated stimulation of protein kinase C.[28–32] The questions of whether CB_1 receptor coupling to G_s proteins have physiological importance and of whether such coupling increases after $G_{i/o}$ protein sequestration by co-localized non-cannabinoid $G_{i/o}$ protein-coupled receptors are yet to be resolved.

CB_1 receptor signalling does not appear to be the same in all brain areas. Thus, whilst CB_1 receptors in rat hippocampal neurons seem to be negatively coupled to both N- and P/Q-type calcium channels,[33,34] in rat striatum they appear to be coupled only to N-type calcium channels[35] or possibly not to calcium channels at all.[36] There is also a report that CB_1 receptors are not coupled to N-, P/Q- or L-type calcium channels in mouse nucleus accumbens.[37] In addition, it has been found that cannabinoid receptor agonists inhibit adenylate cyclase in rat cerebellar and striatal membranes and, to a lesser extent, in rat frontal cortical membranes but not in membranes obtained from other brain regions.[38] Evidence also exists that cannabinoid receptor-G-protein coupling efficiency is not the same in all brain areas.[8]

CB_1- AND CB_2-SELECTIVE LIGANDS

The discovery of CB_1 and CB_2 receptors prompted a search for CB_1- and CB_2-selective antagonists. This led to the development of four agents of particular note: SR141716A, LY320135, SR144528 and 6-iodopravadoline (AM630).[8] Of these, SR141716A and LY320135 both exhibit significant CB_1-selectivity. As well as potently

preventing or reversing CB_1-mediated effects both in vitro and in vivo, SR141716A can by itself elicit responses in some CB_1 receptor-containing tissues that are opposite in direction from those produced by CB_1 receptor agonists. Whilst such 'inverse cannabimimetic effects' may in some instances be attributable to a direct antagonism of responses elicited at CB_1 receptors by released endocannabinoids, there is evidence that this is not the only mechanism and that SR141716A is in fact an inverse agonist.[39–43] Thus, SR141716A may produce inverse cannabimimetic effects in at least some tissues by somehow reducing the constitutive activity of CB_1 receptors (the coupling of CB_1 receptors to their effector mechanisms that it is thought can occur in the absence of exogenously added or endogenously produced CB_1 agonists). In some in vivo or in vitro investigations, SR141716A has been found to be more potent in blocking the actions of CB_1 receptor agonists than in eliciting inverse cannabimimetic responses by itself.[43–47] This may be because SR141716A binds with relatively low affinity to a site on the CB_1 receptor that is distinct from the agonist binding site for which it has higher affinity and that is the lower affinity site that is responsible for the inverse agonist properties of this agent.[43] LY320135 can also behave as an inverse CB_1 receptor agonist. However, it has less affinity for CB_1 receptors than SR141716A and, at concentrations in the low micromolar range, also binds to muscarinic and $5HT_2$ receptors.[8] The CB_2-selective agents, SR144528 and AM630, also both behave as inverse agonists rather than as 'silent' antagonists.[48,49] The inverse efficacy at CB_2 receptors but also the CB_2/CB_1 affinity ratio are less for AM630 (CB_2/CB_1 affinity = 165) than for SR144528 (CB_2/CB_1 affinity >700).[8,50] At CB_1 receptors, AM630 has been found to behave as a low-affinity partial agonist in some investigations but as a low-potency inverse agonist in another study.[8]

SR141716A and SR144528 are widely used as pharmacological tools for distinguishing between CB_1 and CB_2 receptor-mediated effects. Consequently, it is important to bear in mind that although these agents show considerable selectivity as CB_1 or CB_2 antagonists/inverse agonists, they lack absolute specificity.[8] Thus, whilst existing data suggest that it is safe to assume that in tissues containing both these receptor types, concentrations of SR141716A in the low or mid-nanomolar range will interact mainly with CB_1 receptors, this does not remain true for higher concentrations of SR141716A. Similarly, concentrations of SR144528 in the high nanomolar range and above can block CB_1 as well as CB_2 effects.[48] SR141716A, SR144528 and LY320135 are not commercially available. However, it is possible to purchase two structural analogues of SR141716A, AM251 and AM281, which have been found to be respectively three and eight times less potent than SR141716A in displacing [^{3}H]SR141716A from binding sites on mouse cerebellar membranes.[51] AM281 has also been found to bind more readily to CB_1 than CB_2 receptors and to behave as an inverse agonist when administered alone and both analogues share the ability of SR141716A to attenuate responses to established cannabinoid receptor agonists.[35,52–57]

Several agonists with CB_1- or CB_2-selectivity have also been developed. Of these, the CB_1-selective agents are all analogues of anandamide, for example *R*-(+)-methanandamide, arachidonyl-2′-chloroethylamide (ACEA) and arachidonylcyclopropylamide (ACPA).[58] *R*-(+)-methanandamide owes its CB_1-selectivity to the introduction of a methyl group on the 1′ carbon of anandamide, a structural change that also confers greater resistance to the hydrolytic action of FAAH. Neither ACEA nor ACPA show any sign of reduced susceptibility to enzymic hydrolysis. This is presumably because they lack a methyl substituent since the addition of a methyl group to the 1′ carbon of ACEA markedly decreases the susceptibility of this molecule to FAAH-mediated hydrolysis.[60] This structural change also reduces the affinity of ACEA for CB_1 receptors by about 14-fold. O-1812, which does possess a 1′ methyl substituent, appears to lack significant susceptibility to hydrolysis by FAAH.[59] It differs from anandamide not only in its higher affinity for CB_1 receptors, its rather high in vivo potency as a CB_1 receptor agonist and its high CB_1/CB_2 affinity ratio (1138) but also in the greater separation (573-fold) it exhibits between its CB_1 K_i value against [^{3}H]CP55940 (3.4 nM) and its EC_{50} for activating vanilloid receptors (1949 nM).[59] Notable CB_2-selective agonists to have been developed so far are L-759633, L-759656, JWH-133 and HU-308, all structural analogues of tetrahydrocannabinol.[58]

The cannabinoid receptor agonists that have been most widely used in pharmacological experiments bind equally well to CB_1 and CB_2 receptors or show only marginal CB_1- or CB_2-selectivity. These are the classical cannabinoids, (−)-Δ^9-tetrahydrocannabinol (Δ^9-THC) and HU-210, the non-classical cannabinoid, CP55940, and the aminoalkylindole, *R*-(+)-WIN55212.[8,58] CP55940 and *R*-(+)-WIN55212 have CB_1 and CB_2 affinities in the low nanomolar range and exhibit relatively high efficacy at both these receptor types. *R*-(+)-WIN55212 (WIN55212-2) has significantly higher affinity for CB_1 and CB_2 receptors than its enantiomer, *S*-(−)-WIN55212 (WIN55212-3).[16] Accordingly, the observation that a measured response is elicited much more readily by *R*-(+)-WIN55212 than by *S*-(−)-WIN55212 is often taken as an indication that the effect is cannabinoid-receptor mediated. HU-210 has efficacies at CB_1 and CB_2 receptors that match those of CP55940 and (+)-WIN55212 and affinities for CB_1 and CB_2 receptors that exceed those of these other cannabinoids.[8] It is, therefore, a particularly potent cannabinoid

receptor agonist. Its pharmacological effects in vivo are also exceptionally long-lasting. Δ^9-THC, which is also the main psychotropic constituent of cannabis, resembles anandamide in behaving as a partial agonist at CB_1 and CB_2 receptors and in exhibiting lower CB_2 than CB_1 efficacy.[8]

CANNABINOID RECEPTORS MODULATE THE RELEASE OF CHEMICAL MESSENGERS

A common property of CB_1 and CB_2 receptors appears to be the ability to modulate spontaneous or evoked release of chemical messengers. Thus neurotransmitter release from both central and peripheral neurons can be inhibited through presynaptic CB_1 receptors (see below) whilst cytokine release can be inhibited or facilitated by the activation of CB_2/CB_2-like receptors on immune cells.[61] Current knowledge of cannabinoid-receptor mediated effects on cytokine release was described only very recently by Berdyshev[62] and so this review will focus on the modulation of neurotransmitter release by CB_1 receptors.

There is evidence that the evoked release of a range of excitatory and inhibitory transmitters from neurons in several brain areas or from certain peripheral neurons can be inhibited by the activation of presynaptic CB_1 receptors (Table 1). For acetylcholine, noradrenaline, dopamine, 5-hydroxytryptamine, D-aspartate and cholecystokinin this evidence derives from experiments in which release has been directly monitored either in vivo or in vitro. In contrast, the evidence that cannabinoids can act through presynaptic CB_1 receptors to inhibit glutamate or glycine release from central neurons has so far come entirely from indirect electrophysiological data. Common electrophysiological findings relating to glutamate release have been demonstrations that a cannabinoid receptor agonist can act in an SR141716A-sensitive manner and sometimes also in a bicuculline- or picrotoxin-insensitive manner (a) to inhibit evoked excitatory postsynaptic currents (EPSCs) thought to be induced by neuronally released glutamate without affecting inward currents induced by local injection of a glutamate receptor agonist and/or (b) to reduce the frequency but not the amplitude of miniature EPSCs that are deemed to be postsynaptic responses to spontaneously released single glutamatergic synaptic vesicles (see Table 1 for references). Other electrophysiological indicators of presynaptic CB_1 receptor-mediated inhibition of glutamate release have been the production by a cannabinoid receptor agonist of an SR141716A-sensitive inhibition of spiking activity induced in putative glutamatergic neurons by low extracellular Mg^{2+} (Table 1) or of an increase in paired pulse facilitation at putative glutamatergic synapses.[34,35,100]

With two exceptions, the evidence that cannabinoids can act through presynaptic CB_1 receptors to inhibit GABA release from central GABAergic neurons also comes from experiments yielding indirect electrophysiological data (Table 1). Most of these experiments have shown that cannabinoid receptor agonists can inhibit evoked $GABA_A$ receptor-mediated inhibitory postsynaptic currents (IPSCs) in a manner that is sensitive to antagonism by SR141716A, AM251 or AM281.[36,52,85,86,88,90–95] Some investigations have also provided evidence that CB_1 receptor activation: (a) can increase the spontaneous firing of neurons in the pars reticulata of rat substantia nigra without affecting the response of these neurons to iontophoretically applied GABA,[65] (b) can reduce the frequency but not the amplitude of miniature IPSCs that are thought to be induced by spontaneous neuronal release of single GABAergic synaptic vesicles[85,87,91–94] or (c) does not affect decreased neuronal firing rates[65] or inward currents[89] induced by local administration of GABA. There is evidence to suggest that cannabinoid-induced suppression of the central neuronal release of glutamate or GABA depends on CB_1 receptor-mediated blockade of presynaptic voltage-dependent Ca^{2+} channels in rat hippocampus and substantia nigra pars reticulata[85,86,90] but not in rat striatum or mouse nucleus accumbens.[36,37] Presynaptic potassium channels may also be involved in at least some brain areas.[34,35,37] Although cannabinoids have been reported to inhibit GABA uptake in rat globus pallidus[109,110] and substantia nigra,[111] this action does not seem to contribute to their effect on GABAergic transmission in rat hippocampus.[85,112] It is noteworthy that whilst there are some electrophysiological data to support CB_1 receptor-mediated inhibition of GABA release in rat substantia nigra (Table 1), it has not proved possible to detect any cannabinoid-induced inhibition of spontaneous or evoked release of [^{3}H]GABA from fragments of rat substantia nigra or globus pallidus.[109,111]

The recent development of polyclonal antibodies to the *N*- and *C*-terminal amino acid tails of the CB_1 receptor has greatly facilitated the identification of neurons that express presynaptic CB_1 receptors. Immunocytochemical data obtained using CB_1 antibodies generally support and extend information about the distribution pattern of CB_1 receptors that has been obtained by applying the techniques of in situ hybridization or autoradiography.[16,113,114] In addition, dual immunostaining with CB_1 antibodies and antibodies for protein markers of nerve terminals or of particular types of neuron has so far greatly strengthened the evidence that presynaptic CB_1 receptors mediate cannabinoid-induced inhibition of GABA release in the hippocampus, cerebellum and substantia nigra, of glutamate release in the cerebellum, striatum and prefrontal cortex and of acetylcholine

Table 1 Inhibition of neurotransmitter release by cannabinoid receptor agonists through CB_1 receptors in mammalian tissues

Tissue preparation	Transmitter-releasing stimulus	Transmitter	References
In vivo			
Rat medial-prefrontal cortex	None	ACh#	46
Rat hippocampus	None	ACh#	44, 46, 63, 64
Rat striatum	None	GABA*	65
In vitro			
Rat hippocampal slices	ES	ACh	55, 66–68, 71
Rat hippocampal and frontal cortical synaptosomes	K^+ or Ca^{2+}	ACh	69
Mouse hippocampal or cerebrocortical slices	ES or Ca^{2+}	ACh	70–72
Guinea-pig intestinal tissue (MPLM)	ES	ACh	73, 74
Guinea-pig cerebrocortical slices	ES	NA	45
Human and guinea-pig hippocampal slices	ES or Ca^{2+}	NA	45
Guinea-pig hippocampal slices	NMDA or kainate	NA	75
Guinea-pig hypothalamic slices	ES	NA	45
Guinea-pig cerebellar slices	ES	NA	45
Guinea-pig retinal discs	ES or Ca^{2+}	NA	76
Human atrial appendage segments	ES	NA	77
Rat atria	ES	NA	78
Rat vas deferens	ES	NA	78
Mouse vas deferens	ES	NA	79
Mouse cultured sympathetic neurons	ES	NA	80
Rat striatal slices	NMDA	DA	75
Rat striatal slices	ES	DA	81
Guinea-pig retinal discs	ES	DA	76
Mouse cerebrocortical slices	ES or Ca^{2+}	5-HT	70
Human hippocampal slices	ES	GABA	83
Rat hippocampal slices	ES	GABA	84
Rat hippocampal slices	ES	GABA*	52, 85, 86
1^y cultures of neonatal rat hippocampal cells	None	GABA*	87
1^y cultures of neonatal rat hippocampal cells	ES	GABA*	88
Rat striatal slices	ES	GABA*	36
Rat midbrain slices (SNR)	ES	GABA*	89, 90
Rat brain slices (RVM)	ES	GABA*	91
Rat cerebellar slices	None	GABA*	92
Rat brain slices (PAG)	ES	GABA*	93
Rat brain slices (shell region of NAc)	ES	GABA*	94
Mouse brain slices (NAc)	ES	GABA*	95
Rat spinal trigeminal nucleus pars caudalis (SG)	ES	GABA*	82
Rat prefrontal cortical slices	ES	Glu*	96
Rat brain slices (PAG)	ES	Glu*	93
Mouse brain slices (NAc)	ES	Glu*	37
1^y cultures of rat hippocampal cells	Low $[Mg^{2+}]_o$	Glu*	33, 97–99
1^y cultures of rat hippocampal cells	ES	Glu*	34
Mouse hippocampal slices	ES	Glu*	100
Rat cerebellar slices	ES	Glu*	101,102
Rat striatal slices	ES	Glu*	35,103
Rat midbrain slices (SNR)	ES	Glu*	104
Rat spinal cord slices (SG)	ES	Glu*	105
1^y cultures of rat cerebellar granule cells	Low $[Mg^{2+}]_o$	Glu*	106
Rat spinal trigeminal nucleus pars caudalis (SG)	ES	Gly*	82
1^y cultures of rat cerebellar granule cells	K^+	D-Asp	107
Rat hippocampal slices	K^+	CCK	108

#ACh collected by microdialysis. *Indirect electrophysiological evidence for decreased transmitter release: in some of these investigations there was also evidence that cannabinoids inhibited spontaneous as well as evoked release of GABA or Glu. ES, electrical stimulation; NMDA, *N*-methyl-D-aspartate; NAc, nucleus accumbens; PAG, periaqueductal grey; RVM, rostral ventromedial medulla; SG, substantia gelatinosa; SNR, substantia nigra pars reticulata; MPLM, myenteric plexus-longitudinal muscle preparation; $[Mg^{2+}]_o$, extracellular magnesium concentration; ACh, acetylcholine, CCK, cholecystokinin; NA, noradrenaline; DA, dopamine; 5-HT, 5-hydroxytryptamine; GABA, γ-aminobutyric acid; Glu, glutamate; Gly, glycine; D-Asp, D-aspartate; 1^y, primary.

release in the myenteric plexus of the small intestine. More specifically, within the hippocampus, CB_1 immunostaining has been observed primarily on the terminals of hippocampal GABAergic interneurons (basket cells) that innervate pyramidal cells.[83,84,86,87,115–118] Thus virtually all hippocampal CB_1-immunoreactive neurons (95%) have so far proved to be GABAergic interneurons.[117] It remains likely, however, that other types of hippocampal neurons also express CB_1 receptors and that current techniques are insufficiently sensitive to detect CB_1 immunostaining on such neurons.[87,117] Indeed it is already known that CB_1 mRNA is present at low but

significant levels in many non-GABAergic neurons of mouse hippocampus and that these are likely to include the terminals of principal glutamatergic neurons.[113] It has also been reported that some CB_1 immunostaining[117] (or CB_1 mRNA[113]) is detectable both on the terminals of neurons that project to the hippocampus and on projecting principal hippocampal neurons. Many hippocampal GABAergic interneurons that show CB_1 immunoreactivity also contain cholecystokinin.[83,84,86,117] These are a subset of GABAergic interneurons that mainly innervate hippocampal pyramidal cell somata and send fibres largely to the molecular layer but also to the granule cell layer.[117] The immunohistochemical data are in line with the observation that cannabinoids inhibit evoked cholecystokinin release in rat hippocampal slices (Table 1), a finding that may explain why cannabinoids and cholecystokinin have some opposite effects, for example on feeding behaviour and nociception.[113]

In cerebellar tissue, CB_1 immunostaining has been detected on axon terminals of granule cells and basket cells that are presynaptic to Purkinje cells[116,118] and, more recently, at synaptic terminals identified as glutamatergic.[106] These findings are consistent with another report of intense CB_1 immunostaining in the molecular layer but not in the granular layer of rat cerebellum.[115] CB_1 immunostaining has also been detected on glutamatergic cortical pyramidal neurons.[115] As to the basal ganglia, CB_1 immunostaining has been detected in rat caudate nucleus and putamen,[118] on rat cortical-striatal glutamatergic terminals[119] and on rat striatal GABAergic spiny projection neurons,[119] including the terminals of medium spiny GABAergic neurons projecting from the striatum to rat substantia nigra pars reticulata.[115,118] Presumably, cannabinoids can inhibit GABA release in other basal ganglia as well since CB_1 immunostaining is also present on striatal nerve terminals in rat globus pallidus and entopeduncular nucleus.[115,118] The presence of CB_1 receptors on the terminals of striatal projection neurons is supported by results from in situ hybridization experiments in which CB_1 mRNA was used as a marker.[16,114] Finally, in myenteric neurons of porcine and guinea-pig small intestine, CB_1 immunostaining has been shown to be highly co-localized with immunoreactivity for choline acetyltransferase, a protein marker for cholinergic neurons.[120,121] The precise location of CB_1 receptors that mediate the inhibition of acetylcholine release that has been observed both in vivo and in brain slice experiments remains to be established. So too does the location of the CB_1 receptors that mediate the inhibition of noradrenaline, dopamine and 5-hydroxytryptamine release that has been observed in brain slice or peripheral tissue experiments.

There is evidence that the inhibitory effect of cannabinoids on evoked neurotransmitter release is not the same in all brain areas. For example, there are reports that cannabinoids inhibit acetylcholine release in slices of rat and mouse hippocampus and cerebral cortex but not in rat or mouse striatal slices[67,69,71,72,81] and that they inhibit cholecystokinin release in rat hippocampal slices but not in rat frontal cortical slices.[108] Apparent species differences have also been reported. Thus, signs of cannabinoid-induced inhibition of evoked noradrenaline release have been noted in human and guinea-pig hippocampal slices[45,75] but not in slices of rat or mouse hippocampus.[45,67,72]

Whilst most effects of cannabinoids on neurotransmitter release have been found to be inhibitory in nature, signs of a stimulatory effect of cannabinoids on the central release of dopamine, acetylcholine, glutamate and dynorphin have been noted in some experiments. With regard to dopamine, signs of increased release have been observed in cannabinoid experiments both with whole animals[122–129] and with brain slices.[130] Results from some of these experiments suggest that cannabinoids can act through CB_1 receptors to increase release of this transmitter in the nucleus accumbens by increasing the firing of dopaminergic neurons that project from this brain region to the nucleus accumbens (meso-accumbens neurons) and form part of a putative 'reward' circuit.[125,126,128–130] There is evidence that extrinsic glutamatergic afferents control the firing of GABAergic neurons that project from the nucleus accumbens to the ventral tegmental area and that these GABAergic neurons in turn exert an inhibitory influence in the ventral tegmental area on dopaminergic meso-accumbens neurons.[37] Consequently, since results from electrophysiological experiments suggest that CB_1 receptors mediate inhibition of glutamate release in the nucleus accumbens and since CB_1 immunostaining has been observed on large glutamatergic fibres that make synaptic-like contacts with GABA-containing perikarya or processes in this brain area, it is possible that increased dopamine release in the nucleus accumbens can be triggered indirectly by the activation of CB_1 receptors located at the terminals of the glutamatergic neurons in the nucleus accumbens.[37] Thus, by inhibiting glutamate release in the nucleus accumbens, CB_1 receptor agonists would be expected to reduce the firing of GABAergic neurons projecting to the ventral tegmental area and so disinhibit firing of dopaminergic meso-accumbens neurons.

Turning to the enhancement of acetylcholine release by cannabinoids, Acquas et al.[47,131] have found that low intravenous doses of the cannabinoid receptor agonists, *R*-(+)-WIN55212, HU-210 and Δ^9-THC increase concentrations of this neurotransmitter in dialysates obtained from rat prefrontal cortex or hippocampus and that these effects can be attenuated by SR141716A. Acetylcholine

release in the cortex is thought to be regulated by inhibitory GABAergic neurons that project from the nucleus accumbens, a brain area in which they receive synaptic inputs from dopaminergic meso-accumbens neurons.[47] Moreover, in the dose range in which they stimulate the central release of acetylcholine, cannabinoids also increase dopamine release in the nucleus accumbens.[47,131] Consequently, it is possible that cannabinoids augment acetylcholine release in the cortex indirectly by suppressing cortical GABA release through CB_1-mediated enhancement of dopamine release in the nucleus accumbens[47,131] and/or, more speculatively, through the activation of CB_1 receptors that may prove to be present on cortical GABAergic terminals. In experiments in which cannabinoids were found to inhibit in vivo acetylcholine release in rat prefrontal cortex or hippocampus (Table 1), injections were made intraperitoneally rather than intravenously as by Acquas et al.[47,131] Even so, it seems likely that cannabinoids have a dose-dependent biphasic effect on acetylcholine release in these brain regions: a stimulant effect at low doses and an inhibitory effect at higher doses.

Whilst there is electrophysiological evidence to suggest that cannabinoids inhibit glutamate release (Table 1), data obtained recently in experiments with unanaesthetized rats in which release was measured directly by microdialysis, suggest that *R*-(+)-WIN55212 can act through CB_1 receptors in the cerebral cortex to enhance calcium-dependent glutamate release.[132] The same investigation also provided evidence that *R*-(+)-WIN55212 can produce CB_1 receptor-mediated increases in spontaneous, calcium-dependent glutamate release in primary cultures of rat cerebral cortex. The reason for the apparent discrepancy between these data and those obtained previously in electrophysiological studies remains to be elucidated. As to the enhancement of dynorphin release by cannabinoids, this has been reported to occur in the spinal cord and may depend on CB_1-mediated inhibition of tonically active neurons that exert an inhibitory influence on dynorphinergic neurons.[18]

ENDOCANNABINOIDS MAY SERVE AS RETROGRADE SYNAPTIC MESSENGERS

Results from recent investigations suggest that as well as having pharmacological importance, the apparent ability of central presynaptic CB_1 receptors to mediate inhibition of neurotransmitter release when activated has physiological relevance. Thus, it seems likely that endocannabinoids may function as fast retrograde synaptic messengers. More specifically, recent electrophysiological experiments have yielded data that strongly support the hypothesis that increases in intracellular calcium caused by strong depolarization of postsynaptic hippocampal pyramidal cells or cerebellar Purkinje cells rapidly trigger the biosynthesis and non-vesicular release of endocannabinoid molecules. These are then thought to act through presynaptic CB_1 receptors to inhibit the presynaptic release of GABA from hippocampal neurons (depolarization-induced suppression of inhibition: DSI) or of glutamate from cerebellar climbing fibres that originate in the inferior olive or from parallel fibres of cerebellar granule cells (depolarization-induced suppression of excitation: DSE).[52,88,102] It is noteworthy that whilst DSE should provide a negative feedback mechanism for damping down high synaptic activity, DSI is expected to exacerbate intense synaptic activity. Some key observations supporting the hypothesis that endocannabinoid molecules mediate DSI and DSE are listed below.

- DSI and DSE are dependent both on rises in postsynaptic calcium levels and on a G protein-dependent suppression of presynaptic currents.[52,88,102]
- Neuronal synthesis and release of anandamide and 2-arachidonoyl glycerol is also calcium-dependent.[133,134]
- Cannabinoids appear to act through presynaptic CB_1 receptors to inhibit GABA release from hippocampal interneurons and glutamate from cerebellar basket cells (see above) and also to reduce calcium entry at parallel fibre synapses.[102]
- DSI and DSE are prevented by selective CB_1 receptor antagonists/inverse agonists.[52,88,102]
- The effect of DSI on evoked inhibitory postsynaptic currents can be mimicked by the endocannabinoid membrane transport inhibitor, AM404, in an SR141716A-sensitive manner.[52]
- GABA and glutamate do not appear to serve as the retrograde messengers responsible for DSI (in hippocampus) or DSE (in cerebellum); adenosine-mediated DSE production has also been ruled out.[52,88,102]

The retrograde inhibitory action of endocannabinoid molecules is most probably terminated after many seconds by uptake into the postsynaptic neurons for subsequent hydrolysis by FAAH.[102,135] This enzyme is present in the somato-dendritic compartments of neurons that are postsynaptic to CB_1-expressing axon terminals in the cerebellum, hippocampus and neocortex but absent from the presynaptic CB_1-expressing neurons in these pathways.[116,135,136] Indeed it was the localization of FAAH in neurons postsynaptic to CB_1-expressing neurons that led Elphick and colleagues to propose in 1998 that anandamide may function as a retrograde signalling molecule.[116,135] Given the widespread presynaptic distribution of CB_1 receptors in the central nervous system, it seems likely that fast retrograde modulation of neurotransmission by endocannabinoids serves as an important and widespread mechanism in the brain for modulating presynaptic input during periods of high

postsynaptic activity.[88,102] As direct evidence that endocannabinoids are released during DSI or DSE is currently lacking, further experiments directed at monitoring endocannabinoid release under these conditions are required. Additional research is also needed to determine the extent to which endocannabinoid molecules function as retrograde synaptic messengers in areas outside the hippocampus and cerebellum and to identify the endocannabinoids involved.

One obvious implication of the hypothesis that endocannabinoids function as synaptic messengers is that release of these molecules should be detectable in the brain under physiological and/or pathophysiological conditions. It is noteworthy, therefore, that increased concentrations of anandamide have been observed in rat whole brain in vivo after inhibition of FAAH by phenylmethylsulphonyl fluoride,[137] in rat plasma after administration of the anandamide membrane transport inhibitor, AM404,[138] in female rat pituitary and hypothalamus during phase changes in the ovarian cycle,[139] in rat periaqueductal gray in response to electrical stimulation of this brain area or after subcutaneous injection of a nociceptive/inflammatory dose of formalin into the hind paw[140] and in rat striatum after local perfusion with a depolarizing concentration of potassium chloride or with the D_2-like receptor agonist, quinpirole.[141] Some increases in anandamide concentration have been found not to be accompanied by any detectable increase in the concentration of 2-arachidonoyl glycerol.[139,141] However, there have also been experiments in which increases in 2-arachidonoyl glycerol concentration have been observed to be unaccompanied by any change in anandamide concentration. Thus, Stella et al.[134] found that high-frequency in vivo electrical stimulation of rat Schaffer collaterals (excitatory hippocampal CA1 afferents) provoked increased calcium-dependent hippocampal release of 2-arachidonoyl glycerol but not anandamide (or palmityl glycerol or stearyl glycerol) whilst Di Marzo et al.[142] found in vivo administration of reserpine to elevate 2-arachidonoyl glycerol but not anandamide levels in rat globus pallidus although not in other brain areas.

In some experiments, increases (or decreases) in the concentrations of both anandamide and 2-arachidonoyl glycerol have been observed in vivo. Baker et al.[143] detected elevated concentrations of anandamide and 2-arachidonoyl glycerol in the brain and spinal cord of spastic mice with chronic relapsing experimental allergic encephalomyelitis (CREAE), an autoimmune model of multiple sclerosis in which demyelination and axonal loss in the central nervous system are induced. These increases were not observed in CREAE mice that had not developed limb spasticity suggesting that endocannabinoid release is affected by spastic events in CREAE, possibly in response to abnormal neuronal signalling and/or neurodegenerative changes in damaged nerves. Di Marzo et al.[144] showed that in the hypothalami of normal rats, concentrations of both anandamide and 2-arachidonoyl glycerol were markedly depressed by intravenous leptin. They also found 2-arachidonoyl glycerol but not anandamide to be elevated in animals with defective leptin signalling (obese Zucker rats) or in animals which lack leptin (young *ob/ob* mice) and that both these fatty acid derivatives were elevated in animals with defective leptin receptors (young obese *db/db* mice). These findings raise the possibility that endocannabinoids may contribute to the hyperphagia that is responsible for obesity. More recently, in experiments with a rat model of Huntington's disease in which striatal efferent GABAergic neurons were lesioned by bilateral intrastriatal injection of 3-nitropropionic acid, Lastres-Becker et al.[145] found striatal concentrations of both anandamide and 2-arachidonoyl glycerol to be less in lesioned than in control animals. In contrast, in the ventral mesencephalon which contains the substantia nigra, levels of anandamide, although not of 2-arachidonoyl glycerol, increased after lesioning. The question of whether the release of noladin ether can be modulated in vivo has yet to be addressed.

The evidence that release of anandamide and 2-arachidonoyl glycerol can be independently modified, that these two endocannabinoids may be produced under different physiological circumstances and/or in distinct regions of the central nervous system and that anandamide and 2-arachidonoyl glycerol require different phospholipases for their biosynthesis (phospholipase D and phospholipase C, respectively) opens up the possibility of developing pharmacological agents that selectively modulate the production of a particular endocannabinoid.[134,141]

The observation that the D_2-like receptor agonist, quinpirole, elevates anandamide concentrations in rat striatum has prompted the hypothesis that anandamide release in the striatum and possibly also in other brain areas can be elicited physiologically or pathophysiologically through the occupation by dopamine of D_2-like receptors.[141] In support of this hypothesis are the findings that local perfusion with a depolarizing concentration of potassium chloride reversibly elevates striatal levels of both dopamine and anandamide with similar time courses, that the effect of potassium chloride on anandamide production is calcium-dependent and tetrodotoxin-sensitive, that quinpirole-induced increases in striatal anandamide are reversed by the D_2-like receptor antagonist, raclopride, and that striatal anandamide concentrations are unaffected by local administration of the D_1-like receptor agonist, SKF38393.[141] Since reserpine elevates 2-arachidonoyl glycerol in rat globus pallidus and since this effect is attenuated by quinpirole, it also seems likely that 2-arachidonoyl glycerol is released by striatopallidal

neurons when the firing of these neurons is disinhibited by reduced release of dopamine in the striatum.[142] Another finding, that quinpirole reduces anandamide levels in the globus pallidus of reserpinized rats, suggests that this endocannabinoid too can be released by striatopallidal neurons.[142] It is noteworthy that endocannabinoid levels in the globus pallidus of reserpinized rats have been found to be reduced by the D_1-like receptor agonist, Cl-APB, an observation which suggests that putative endocannabinoid release from striatopallidal neurons may also be regulated by a basal ganglia-thalamo-cortico-striatal loop.[142] One implication of these findings is that endocannabinoids participate in pathologies that may involve dysregulated dopaminergic neurotransmission, for example schizophrenia, Parkinson's disease, Huntington's disease and Tourette's syndrome.[141,142]

One consequence of increased endocannabinoid release in rats may be CB_1-mediated hypokinesia. Thus, both systemic administration of AM404 at a dose elevating plasma levels of anandamide and intracerebroventricular injection of AM404 have been found to induce SR141716A-reversible hypokinesia in rats.[138,146,147] In one of these investigations, it was also found that it was possible for AM404 to normalize motor activity in juvenile spontaneously hypertensive rats at a dose that did not affect motor activity in control animals (1 mg/kg s.c.).[147] In addition, intracerebroventricular AM404 pretreatment has been shown to enhance the initial phase of hypokinesia induced by quinpirole and to reduce the subsequent phase of hyperkinesia.[147] This finding supports the hypothesis that D_2-like receptor agonists stimulate anandamide outflow in vivo, because neurally released anandamide is expected to share the ability of quinpirole (acting on D_2 autoreceptors) to produce hypokinesia by inhibiting striatal dopamine release but to oppose the hyperkinesia resulting from quinpirole-induced activation of postsynaptic D_2 receptors.[147] The finding that quinpirole-induced hyperkinesia can be potentiated by SR141716A[141] is also in line with the hypothesis that anandamide is released onto presynaptic CB_1 receptors to suppress striatal dopamine release. It is also possible that AM404 may produce hypokinesia in rats by acting through vanilloid receptors as AM404 is a vanilloid receptor agonist (see next section) and there is evidence that vanilloid receptors can mediate a motor depressant effect in rats.[148]

Other likely consequences of increased endocannabinoid release in rats that have been revealed in experiments with AM404 are altered tyrosine hydroxylase activity in rat hypothalamus and substantia nigra and reduced plasma levels of prolactin.[146] In addition, there is evidence that released endocannabinoids may oppose full expression of limb spasticity in CREAE mice as such spasticity is ameliorated not only by direct cannabinoid receptor agonists but also by AM404, by a second inhibitor of endocannabinoid membrane transport (VDM11) and by the FAAH inhibitor, AM374.[143] Another probable consequence of endocannabinoid release in rats is antinociception. The evidence for this comes from experiments showing firstly, that electrical stimulation of the periaqueductal grey induces antinociception in the tail flick test, secondly, that this antinociceptive effect can be attenuated by intracerebroventricular SR141716A and thirdly, that it is possible to trigger anandamide release into the periaqueductal grey both by applying electrical stimuli to this part of the brain and by injecting formalin subcutaneously into the hind paw.[140] There is also a report that phenylmethylsulphonyl fluoride, an inhibitor of anandamide metabolism, shows antinociceptive activity in the mouse tail flick test.[149]

ANANDAMIDE IS A VANILLOID RECEPTOR AGONIST

The realization that anandamide is chemically similar to capsaicin and olvanil[150] prompted experiments that lead to the discovery that anandamide can serve as an agonist not only at cannabinoid receptors but also at vanilloid receptors. These experiments provided convincing evidence that anandamide can act on rat or human vanilloid receptors transfected into cultured cells to produce membrane currents or increase intracellular calcium.[151–153] Evidence was also obtained that anandamide can act on naturally expressed vanilloid receptors in neonatal rat dorsal root ganglia to produce membrane currents[154] and in rat or guinea-pig isolated arterial strips to produce release of calcitonin-gene-related peptide (CGRP) from perivascular sensory nerves and relaxation of precontracted tissues.[151] The ability of anandamide to stimulate vanilloid receptors appears to be governed by the state of activation of protein kinase A[155] and protein kinase C.[156] Methanandamide also activates vanilloid receptors, albeit less potently or effectively than anandamide[151,153,157] whilst 2-arachidonoyl glycerol lacks significant activity at these receptors altogether.[151] Interestingly, although HU-210 is not a vanilloid receptor agonist,[151] there is evidence that this potent cannabinoid receptor agonist can inhibit electrically evoked CGRP release from sensory nerves in rat precontracted mesenteric arteries by acting prejunctionally through a mechanism that is independent of both CB_1- and CB_2-like receptors.[158]

Because CB_1 receptors are negatively coupled to calcium channels whilst vanilloid receptors open cation channels, several investigations have been directed at exploring the consequences of simultaneous activation of these two receptor types in experiments with rat cultured dorsal root ganglion (DRG) neurons that are known to

co-express CB_1 and vanilloid receptors to a very high degree.[159] Millns et al.[160] found that capsaicin-induced increases in intracellular calcium in adult rat cultured DRG neurons could be inhibited by the cannabinoid receptor agonist, HU-210, in an SR141716A-sensitive manner. Tognetto et al.[154] have obtained evidence that at 10 nM, anandamide inhibits electrically evoked calcium mobilization from neonatal rat cultured DRG neurons by acting through CB_1 receptors. This concentration of anandamide also inhibited electrically evoked release of CGRP from slices of neonatal rat dorsal spinal cord. However, at higher concentrations, anandamide opposed its own CB_1-mediated inhibitory effect by acting on vanilloid receptors to mobilize intracellular calcium and so trigger CGRP release. These findings are consistent with evidence that anandamide acts more potently on CB_1 receptors than on vanilloid receptors.[153] CB_1 and vanilloid receptors are also both expressed in the mouse vas deferens. Inhibition of electrically evoked contractions of isolated strips of this tissue is mediated both by presynaptic CB_1 receptors through reduction of contractile transmitter release and by vanilloid receptors which trigger the release of neuropeptide molecules that then presumably inhibit contractile transmitter release.[16,153] Whilst *R*-(+)-WIN55212 appears to act solely through CB_1 receptors to inhibit electrically evoked contractions of this tissue preparation, the inhibitory effect of anandamide seems to be mediated by both CB_1 and vanilloid receptors.[153]

The finding that anandamide is an agonist for both cannabinoid and vanilloid receptors has prompted the development of anandamide analogues with a range of different relative potencies for these two receptor types. Particularly notable is arvanil, which has anandamide-like CB_1 affinity but less CB_1 efficacy than anandamide, and which shows greater potency than anandamide as a vanilloid receptor agonist.[161,162] Another anandamide analogue that activates vanilloid receptors is AM404.[153,163,164] This it does at concentrations no higher than those at which it inhibits anandamide membrane transport.[11,165] Somewhat greater separation between these two actions has been achieved through the development of the AM404 analogue, VDM11, which exhibits the same potency as AM404 for anandamide membrane transport inhibition but markedly less efficacy than AM404 at vanilloid receptors.[162]

NON-CB_1, NON-CB_2 CANNABINOID RECEPTORS

CB_2-like cannabinoid receptors

Calignano et al.[166] have proposed the existence of an SR144528-sensitive, non-CB_2 cannabinoid receptor ('CB_2-like' receptor). This hypothesis is based primarily on the finding that even though palmitoylethanolamide lacks significant affinity for CB_1 or CB_2 receptors,[3,167–170] it induces antinociceptive effects that can be attenuated by the CB_2-selective antagonist, SR144528, but not by the CB_1-selective antagonist, SR141716A. Antagonism of palmitoylethanolamide by SR144528 has been observed both in the mouse formalin paw test and in the mouse abdominal stretch test in which stretching behaviour was induced by intraperitoneal acetic acid, kaolin or $MgSO_4$.[166,171] Palmitoylethanolamide was injected directly into the paw for the formalin paw test and intraperitoneally for the abdominal stretch test. It was also found that in these bioassays, anandamide could be antagonized by SR141716A but not SR144528 and palmitoylethanolamide and anandamide acted synergistically. Unlike anandamide, palmitoylethanolamide did not show antinociceptive activity in the mouse hot plate test.[171] Nor did it share the ability of anandamide (and capsazepine) to suppress paw-licking behaviour when co-administered with capsaicin into mouse hind-paw, suggesting that the putative CB_2-like receptor is not a vanilloid receptor.[171] It seems likely, therefore, that unlike anandamide, palmitoylethanolamide may induce antinociception by acting through SR144528-sensitive, SR141716A-insensitive, non-vanilloid, CB_2-like receptors and that it does not interfere directly with neuronally mediated transmission of pain signals to the central nervous system. Evidence also exists for the presence of CB_2-like receptors in the mouse vas deferens.[172]

SR141716A-sensitive, non-CB_1, non-CB_2, non-vanilloid cannabinoid receptors in mesenteric vasculature

There is growing evidence for the presence of non-CB_1, non-CB_2, non-vanilloid receptors, at least in mesenteric vasculature, for which anandamide, methanandamide and certain analogues of cannabidiol are agonists and SR141716A is an antagonist with sub-CB_1 potency. This evidence, obtained from experiments with rat or mouse precontracted mesenteric arteries can be summarized as follows.[173–175]

- Both agonist and antagonist ligands for the putative SR141716A-sensitive, non-CB_1, non-CB_2, non-vanilloid anandamide receptor have been identified and these seem to show structure-activity relationships that are distinct from those of CB_1 and CB_2 receptors. More specifically, anandamide and methanandamide induce a concentration-related relaxation of rat precontracted mesenteric arteries, whereas other well-established cannabinoid receptor agonists, Δ^9-THC, HU-210, *R*-(+)-WIN55212 and 2-arachidonoyl glycerol, do not produce this effect.[173] In addition, at 10 μM, the plant cannabinoid, cannabidiol,

which lacks significant affinity for CB_1 or CB_2 receptors, attenuates anandamide-induced relaxation of rat precontracted mesenteric arteries without affecting vasorelaxation induced by acetylcholine, bradykinin or sodium nitroprusside.[174]

- Anandamide induces relaxation of precontracted mesenteric arteries both in CB_1 receptor knockout ($CB_1^{-/-}$) mice and in their homozygous controls ($CB_1^{+/+}$ mice).[174]
- Methanandamide also induces relaxation of precontracted mesenteric arteries in $CB_1^{-/-}/CB_2^{-/-}$ double-knockout mice, ruling out an involvement of CB_2 as well as CB_1 receptors.[174]
- At 1 or 5 μM, SR141716A attenuates anandamide-induced relaxation of precontracted mesenteric arteries in both $CB_1^{+/+}$ and $CB_1^{-/-}$ mice.[174]
- Experiments with rat precontracted mesenteric arteries have shown that anandamide-induced relaxation is attenuated by 0.5 μM SR141716A[173] whilst capsaicin-induced relaxation is unaffected by 1 μM SR141716A.[174] These findings suggest that anandamide acts independently of vanilloid receptors in this bioassay system and also that the interaction between SR141716A and anandamide possesses some degree of selectivity.

Anandamide-induced vasorelaxation is detectable both in endothelium-intact and in endothelium-denuded precontracted mesenteric arteries of rats.[173,175] However, SR141716A only attenuates this vasorelaxant effect of anandamide in the presence of endothelium, suggesting that there are at least two mechanisms by which anandamide relaxes precontracted mesenteric arteries and that SR141716A-sensitive, non-CB_1, non-CB_2, non-vanilloid receptors for anandamide are present on the endothelium but not on mesenteric smooth muscle. It is unlikely that the relaxant effect of anandamide in precontracted mesenteric arteries depends on its metabolic hydrolysis by FAAH. The vasorelaxant potency of anandamide is unaffected by the general protease inhibitor, phenylmethylsulphonyl fluoride, methanandamide also possesses vasorelaxant properties even though it is more resistant than anandamide to enzymic hydrolysis and the anandamide metabolite, arachidonic acid, lacks relaxant properties in rat precontracted mesenteric arteries.[173]

Two other agonists for the putative SR141716A-sensitive, non-CB_1, non-CB_2, non-vanilloid anandamide receptor are abnormal cannabidiol and its more potent analogue, O-1602, neither of which exhibit significant affinity for rat brain CB_1 receptors.[174] Abnormal cannabidiol shares the ability of anandamide or methanandamide to induce a concentration-related relaxation of precontracted mesenteric arteries obtained from rats, from $CB_1^{-/-}$ mice and from $CB_1^{-/-}/CB_2^{-/-}$ double-knockout mice. Moreover, in rats or $CB_1^{-/-}$ mice the vasorelaxant effect of abnormal cannabidiol, like that of anandamide, is attenuated both by 1 or 5 μM SR141716A and by 10 μM cannabidiol.[173,174] In rat precontracted mesenteric arteries, the relaxant effect of abnormal cannabidiol has been found to be unaffected by a concentration of capsazepine (5 μM) that can attenuate the relaxant effect of capsaicin, thereby ruling out any major involvement of vanilloid receptors in this effect of abnormal cannabidiol.[174] An observation that the vasorelaxant effect of abnormal cannabidiol is endothelium dependent[174] supports the hypothesis that the proposed mesenteric non-CB_1, non-CB_2, non-vanilloid anandamide receptors are endothelial and not present on mesenteric smooth muscle. Like the vasorelaxant effect of abnormal cannabidiol, that of O-1602 is both SR141716A-sensitive and largely endothelium-dependent.[174]

Interestingly, although anandamide and abnormal cannabidiol both induce hypotension in pentobarbitone-anaesthetized mice they seem to act through different mechanisms. The hypotensive effect of anandamide appears to be CB_1-receptor mediated as it is observed in $CB_1^{+/+}$ but not in $CB_1^{-/-}$ mice.[174] In contrast, abnormal cannabidiol may act through the putative SR141716A-sensitive, non-CB_1, non-CB_2, non-vanilloid anandamide receptor. Thus, its hypotensive effect has been observed in $CB_1^{-/-}$ and $CB_1^{+/+}$ mice as well as in $CB_1^{-/-}/CB_2^{-/-}$ double-knockout mice and this effect is antagonized by SR141716A in $CB_1^{+/+}$ and $CB_1^{-/-}$ mice and by CBD (20 μg/g i.v.) in $CB_1^{+/+}$ mice.[174] Hypotension induced in anaesthetized mice by anandamide or HU-210 has been shown not to be attenuated by CBD.[174]

Other mammalian cannabinoid receptors

Shire et al.[176] have isolated a spliced variant of CB_1 cDNA, from a human lung cDNA library. This CB_{1A} receptor differs from the CB_1 receptor in the predicted length and amino acid composition of its N-terminal tail and also in its binding properties, the CB_1 to spliced variant affinity ratio being 8.8 for SR141716A, 3–4 for Δ^9-THC, CP55940 and *R*-(+)-WIN55212 and 0.83 for anandamide.[177] The ratio of CB_{1A} to CB_1 mRNA in human brain tissue ranges from 0.25 to <0.005, indicating that the spliced variant exists only as a minor transcript.[176] Onaivi et al.[178] have reported the presence of three distinct CB_1 mRNAs in C57BL/6 mouse brain but just one CB_1 mRNA in ICR and DBA/2 mouse brain. C57BL/6 mice showed a reduced sensitivity to Δ^9-THC for the production of hypothermia and antinociception. Whether this reduced sensitivity to Δ^9-THC resulted from the presence of additional CB_1 mRNAs in C57BL/6 mouse brain remains to be established. Three orphan G protein-coupled receptors (GPRs)

which share about 35% identity with cannabinoid receptors have been detected in the central nervous system, one in mouse brain (GPR3), the second in human brain (GPR6) and the third in rat brain (GPR12).[179]

As discussed in greater detail elsewhere,[18] Sandra Welch's group has obtained pharmacological evidence suggesting that cannabinoid-induced antinociception in the mouse tail flick test is mediated by more than one type of receptor in the spinal cord. Briefly, this group has found the potency of intraperitoneal SR141716A against antinociception in the tail flick test induced by intrathecal administration of established cannabinoid receptor agonists to be agonist dependent.[180] SR141716A showed highest potency against CP55940, intermediate potency against Δ^9-THC, Δ^8-THC and deoxy-HU-210 and lowest potency against anandamide. In addition, this group has found that when the intrathecal route is used, morphine interacts synergistically with Δ^9-THC but not with anandamide or CP55940.[18] The same group has also obtained evidence that whilst intrathecal Δ^9-THC triggers spinal release of dynorphins A and B, intrathecal CP55940 increases the release of dynorphin B but not dynorphin A and intrathecal anandamide fails to affect the release of either peptide.[18,181] Signs of signalling differences between the mechanisms mediating the antinociceptive effects of intrathecal Δ^9-THC and anandamide in mice have also been observed.[18,182] Welch's group has also obtained some in vivo pharmacological evidence suggesting differences between cannabinoid receptor populations in mouse spinal cord and brain.[18]

Finally, evidence is emerging for the existence in mouse brain of a new receptor for which both anandamide and *R*-(+)-WIN55212 appear to be agonists.[183,184] In particular, it has been found that [^{35}S]GTPγS binding can be activated in brain membranes from $CB_1^{-/-}$ mice by anandamide (EC_{50} = 3.6 μM) and *R*-(+)-WIN55212 (EC_{50} = 1.8 μM), suggesting that the putative new receptor is coupled to G-proteins. Three other established cannabinoid receptor agonists, Δ^9-THC, HU-210 and CP55940, did not stimulate [^{35}S]GTPγS binding in $CB_1^{-/-}$ brain membranes. Membranes from $CB_1^{-/-}$ mice found to be responsive to anandamide and *R*-(+)-WIN55212 were obtained either from whole brain or from cerebral cortex, midbrain, hippocampus, diencephalon or brain stem. However, neither of these agonists activated [^{35}S]GTPγS binding to membranes from $CB_1^{-/-}$ caudate-putamen/globus pallidus or cerebellum, brain areas that are well-populated with CB_1 receptors in wild-type animals. Specific binding sites for [^{3}H]*R*-(+)-WIN55212 but not [^{3}H]CP55940 were detected in membranes from $CB_1^{-/-}$ cerebral cortex, hippocampus and brain stem. Neither tritiated ligand exhibited detectable specific binding in membranes from $CB_1^{-/-}$ diencephalon, midbrain, caudate-putamen/globus pallidus, cerebellum or spinal cord. Specific binding sites for [^{3}H]SR141716A were also detected in membranes from some $CB_1^{-/-}$ brain areas. An observation that near maximal concentrations of anandamide and *R*-(+)-WIN55212 were not fully additive is consistent with the hypothesis that these agents were acting through a common mechanism. The stimulatory effects of anandamide and *R*-(+)-WIN55212 on [^{35}S]GTPγS binding to $CB_1^{-/-}$ membranes were attenuated by SR141716A at micromolar concentrations. However, this attenuation was entirely attributable to the inverse inhibitory effect on [^{35}S]GTPγS binding that this agent produces, suggesting that neither anandamide nor *R*-(+)-WIN55212 was competitively antagonized by SR141716A. The apparent pharmacological properties of this possible new cannabinoid receptor distinguish it from the CB_2 receptor for which Δ^9-THC, HU-210 and CP55940 are all established agonists. They also distinguish it both from the SR141716A-sensitive, *R*-(+)-WIN55212-insensitive anandamide receptors that George Kunos' group has suggested may be present in mesenteric arteries[175] (see above) and from the vanilloid receptor which is not coupled to G-proteins and is unresponsive to *R*-(+)-WIN55212.[151]

PHARMACOLOGY OF ENDOCANNABINOID METABOLITES

There is no doubt that if it is protected from hydrolysis by FAAH, anandamide is a relatively potent CB_1 receptor agonist.[16] Recent evidence supporting this idea comes from experiments demonstrating that anandamide can produce hypokinetic, cataleptic, hypothermic and antinociceptive effects in FAAH knockout mice (at 12.5–50 mg/kg i.p.) or in control mice pretreated with the non-selective FAAH inhibitor, phenylmethylsulphonyl fluoride (PMSF) and that these effects can readily be reversed by SR141716A.[185, 186] When anandamide is not protected from hydrolysis by FAAH, it can still produce hypokinesia, catalepsy, hypothermia and antinociception in mice, albeit with reduced potency.[187] However, under these conditions there is no detectable antagonism by SR141716A,[187] making it likely that these effects are produced not by anandamide per se but rather by active metabolites that are formed as a result of rapid hydrolysis of the parent compound to arachidonic acid or other metabolites. In line with this hypothesis is the observation that reductions in motor activity and antinociception produced in mice by the metabolically stable anandamide analogue, 2-methyl-2′-fluoroethylanandamide, in the absence of any FAAH inhibitor can be blocked by SR141716A.[187] The formation of active metabolites from anandamide is also implicated by the finding that some pharmacological effects of this endocannabinoid remain after its levels in the brain have diminished.[188]

There is at least one in vivo effect of anandamide, its hypotensive effect in anaesthetized mice and rats, that does appear to be CB_1-receptor mediated even in the absence of any protection from the hydrolytic action of FAAH.[173–175,189] However, the hypotensive effect of 2-arachidonoyl glycerol is not CB_1 receptor mediated as it is not blocked by SR141716A and is detectable in $CB_1^{-/-}$ mice.[189] This effect of 2-arachidonoyl glycerol is attenuated by cyclooxygenase inhibitors,[189] indicating that it may be mediated by arachidonic acid or other metabolites of 2-arachidonoyl glycerol.

These observations have been paralleled by the emergence of evidence that anandamide and 2-arachidonoyl glycerol can undergo metabolism by cyclooxygenase, lipoxygenase and cytochrome P_{450} enzymes in a manner similar to the metabolism of arachidonic acid, leading to the generation of analogous prostaglandin and hydroxy-eicosatetraenoic acid (HETE) ethanolamide products.[190] In the next section, we discuss the pharmacology of some products of anandamide metabolism by cyclooxygenase and lipoxygenase.

Cyclooxygenase

There is evidence that anandamide is effectively oxygenated by human cyclooxygenase-2 (COX-2) but not by cyclooxygenase-1,[191] and that the COX-2 products are similar to those formed with arachidonic acid as the substrate. In some recent literature these novel prostaglandin products are referred to as 'prostamides'.[192] The major prostanoid product of COX-2 metabolism of anandamide, as determined by mass spectrometry was found to be PGE_2 ethanolamide (prostamide E_2).[191] In macrophages, it has been shown that PGE_2 ethanolamide is synthesized from anandamide and that pretreatment of the cells with lipopolysaccharide (LPS), which is an inducer of COX-2 expression, leads to a significant enhancement of the production of this metabolite.[190]

Berglund et al.[193] have shown that PGE_2 ethanolamide does not bind to CB_1 receptors (rat brain membranes), although it does have low affinity for CB_2 receptors (human tonsilar membranes). In the same study, PGE_2 ethanolamide was shown to activate G-proteins, in an SR141716A-independent manner and to stimulate cyclic AMP production. Because of the structural similarity between this compound and PGE_2 we have hypothesised that it may interact with the prostaglandin E_2 receptors (EP receptors). In the guinea-pig trachea PGE_2 has both a contractile (EP_1 receptor-mediated) and a relaxant action (EP_2 receptor-mediated).[194] PGE_2 ethanolamide-elicited contractions of this preparation were prevented by the EP_1 receptor antagonist SC-51089. However, in the presence of this antagonist, PGE_2 ethanolamide caused a concentration-related relaxation of histamine-induced contractions of this tissue, being only 10-fold less potent than PGE_2. EP_3 receptors mediate the inhibition of electrically evoked contractions of the guinea-pig vas deferens. We found that PGE_2 ethanolamide was around 40-fold less potent than PGE_2 in this preparation and that neither CB_1 nor vanilloid receptor antagonists blocked its action. EP_2 and EP_3 receptor antagonists are not available, thus it may be that this compound is interacting with other, as yet uncharacterized, receptors for prostaglandin ethanolamides in these tissues. Interestingly, the pharmacology of $PGF_{2\alpha}$ ethanolamide (prostamide $F_{2\alpha}$) suggests the existence of a novel 'prostamide' receptor. Thus, $PGF_{2\alpha}$ ethanolamide contracts the cat iris sphincter with potent activity that is not exhibited in other preparations that respond to $PGF_{2\alpha}$. Furthermore, this compound has little affinity for the recombinant cat or human $PGF_{2\alpha}$ receptor (FP receptor).[192] It remains to be established whether the pharmacological activity of the prostaglandin ethanolamides is due to their subsequent hydrolysis by FAAH to prostaglandins.

The physiological significance of the cyclooxygenase pathway of metabolism of anandamide has yet to be established. It may be that the prostaglandin ethanolamides are a new class of mediators that bind to 'prostamide' receptors. Alternatively, it could be speculated that increasing levels of anandamide competing with arachidonic acid might modulate production of prostanoids by COX-2. This, in turn, would result in less activation of EP receptors since the alternative product, PGE_2 ethanolamide, has lower potency at these receptors than PGE_2.

Kozak et al.[195] have demonstrated that 2-arachidonoyl glycerol is also a substrate for COX-2 and is metabolized by this enzyme as effectively as arachidonic acid. In the same study it was shown that cultured macrophages produce prostaglandin D_2 glycerol ester (PGD_2-G), but only in LPS/IFN-γ activated cells, in which COX-2 is induced. The pharmacology of this novel 2-arachidonoyl glycerol metabolite remains to be established.

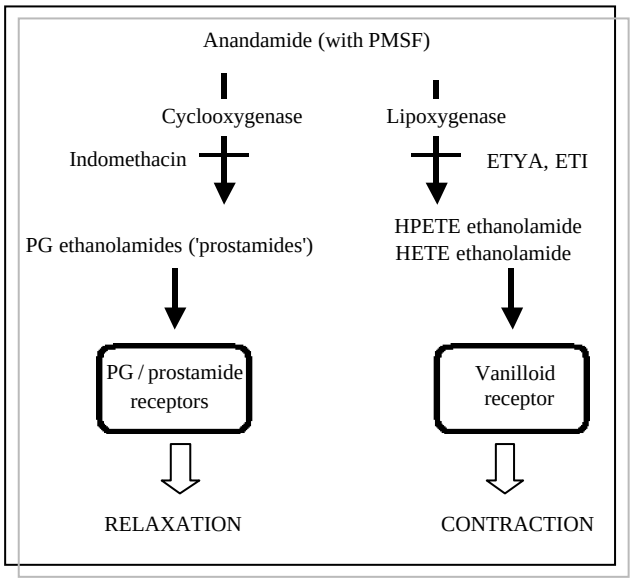
Lipoxygenase

Metabolism of anandamide by lipoxygenase enzymes purified from porcine brain (12-lipoxygenase) and rabbit reticulocytes (15-lipoxygenase) has been shown to produce 12(S) and 15(S) hydroxy-eicosatetraenoylethanolamide (HETEE), respectively, and the rates of conversion were similar to those for arachidonic acid.[196,197] In addition, it has been shown that human polymorphonuclear leukocytes produce 12(S) and 15(S) HETEE, while platelets produce 12(S) HETEE.[198] In this study it was found that these compounds were poor substrates for FAAH and did not bind to the anandamide membrane

transporter. Relatively little is known about the pharmacology and the possible physiological relevance of these novel metabolites. Radioligand binding studies suggest that 12(S) HETEE has affinity for the CB_1 and CB_2 receptors in a similar range to that of anandamide.[198] The 15(S) HETEE has negligible affinity for CB_2 and 4-fold lower affinity than anandamide for CB_1.[198] In addition, 12(S) HETEE has been shown to inhibit forskolin-stimulated cyclic AMP production, which is consistent with CB_1 receptor activity.[197]

There is evidence that lipoxygenase products of arachidonic acid may, in addition to anandamide, be candidates for endogenous vanilloid receptor agonists. 12(S) hydroperoxy-eicosatetraenoic acids (HPETE), 15(S) HPETE and leukotriene B4 (LTB4) have been shown to be more potent than anandamide as vanilloid receptor agonists.[199] It is possible that analogous anandamide lipoxygenase metabolites may have vanilloid receptor activity. Both 12(S) and 15(S) HETEE have been shown to inhibit electrically evoked contractions of the isolated mouse vas deferens,[196,197] the potencies being under 5-fold less than the potency of anandamide. It is important to note, however, that this measured response may be mediated by either CB_1 or vanilloid receptors.[153] More recently, it has been shown that 15(S) hydroperoxy-eicosatetraenoylethanolamide (HPETEE) stimulates an increase in cytosolic free Ca^{2+} in HEK cells transfected with vanilloid receptors, although it is significantly less potent than anandamide.[200] We have provided evidence for a possible role of lipoxygenase in the activation of vanilloid receptors by anandamide.[201] In the guinea-pig bronchus, we have demonstrated that in the absence of indomethacin, anandamide does not contract the tissue, but in the presence of indomethacin, anandamide elicits a contraction which is vanilloid receptor mediated. We also found that the vanilloid receptor-mediated contractile action of anandamide was attenuated by the lipoxygenase inhibitors, ETYA and ETI, suggesting that the action of anandamide may be due, at least in part, to lipoxygenase metabolites of this fatty acid amide that are vanilloid receptor agonists. As these experiments were carried out in the presence of PMSF, it seems unlikely that anandamide was acting via the arachidonic acid pathway in these experiments. A putative pathway for the action of anandamide in this tissue is represented in Box 1.

It is notable that in human cells, in culture and in brain, lipoxygenase derivatives of anandamide have been shown to be competitive inhibitors of FAAH.[10] Consequently, the formation of these derivatives may lead to an increase in the intracellular concentration of anandamide. There is also evidence that the receptor binding site for capsaicin and anandamide at the vanilloid receptor is intracellular,[200,202] suggesting that the formation of lipoxygenase products of anandamide may lead to an increase in the local concentration of anandamide available for vanilloid receptor activation.



BOX 1 Putative action of anandamide in the guinea-pig isolated bronchus.[201]

Finally, the production of 12(S) hydroxy-eicosatetraenoic acid glyceryl ester (HPETE-G) by leukocyte 12-lipoxygenase oxygenation of 2-arachidonoyl glycerol has been demonstrated.[203] The physiological relevance and pharmacology of the lipoxygenase products of 2-arachidonoyl glycerol have yet to be established.

The physiological role and significance of both the direct metabolism of anandamide and 2-arachidonoyl glycerol and their metabolism via arachidonic acid also remains to be established. It is noteworthy that the importance of these metabolites may change in disease states (e.g. inflammation, neuropathy, multiple sclerosis) where key enzymes may be activated.

THERAPEUTIC POTENTIAL OF CANNABINOID RECEPTOR LIGANDS

Several therapeutic targets have been suggested for CB_1 receptor antagonists/inverse agonists. These include appetite suppression, the reduction of l-Dopa-induced dyskinesia in patients with Parkinson's disease, the management of acute schizophrenia and the amelioration of cognitive/memory dysfunctions associated with disorders such as Alzheimer's disease.[58] As to CB_1 receptor agonists, there is growing evidence that in addition to their recognized uses in the clinic as appetite stimulants and anti-emetics, they may have therapeutic potential as neuroprotective agents through CB_1-mediated inhibition of glutamate release,[98] as anticancer agents,[204, 205] and

for the management of glaucoma,[206] pain,[18, 207] vasodilation that accompanies advanced cirrhosis[208] and various kinds of motor dysfunction that include the muscle spasticity/spasm/tremor associated with multiple sclerosis or spinal cord injury, the tics and psychiatric signs and symptoms of Tourette's syndrome and the dyskinesia that is produced by L-Dopa in patients with Parkinson's disease.[209–212] Of these potential therapeutic applications, pain and motor disorders associated with multiple sclerosis and spinal cord injury, are currently attracting particular attention.[209]

One challenge for future research is to develop strategies that maximize separation between the sought-after therapeutic effects of CB_1 receptor agonists and the unwanted effects of these drugs, particularly their psychotropic effects. One strategy could well be to use agents that activate the endogenous cannabinoid system indirectly by increasing extracellular levels of endocannabinoids through inhibition of their membrane transport or enzymic hydrolysis. Its success would depend on whether endogenous cannabinoids are released to a greater extent at sites at which they produce sought-after effects than at sites at which they provoke unwanted effects. It is, therefore, encouraging that signs of increased endocannabinoid release have already been observed in animal models of multiple sclerosis, obesity and inflammatory pain, and that inhibitors of the membrane transport or enzymic hydrolysis of endocannabinoids have been found to reduce limb spasticity in the multiple sclerosis model (see above).

CONCLUSIONS

Whilst a number of major advances have recently been made in the cannabinoid field many important questions still remain unanswered or incompletely addressed, prompting the need for more research. Particularly important at the pharmacological and physiological level is the need

- to gain a more complete understanding of the mode of action of known endocannabinoids
- to develop cannabinoid receptor antagonists that lack inverse agonist properties
- to achieve a more detailed understanding of the processes of endocannabinoid membrane transport and enzymic hydrolysis and to develop/characterize more fully agents that modulate either or both of these processes
- to establish the physiological importance and pharmacological properties of novel types of cannabinoid receptor that have already been proposed to exist
- to develop selective and potent agonists and antagonists for these novel receptors
- to seek out any additional endocannabinoids and types of cannabinoid receptor
- to obtain more detailed information about endocannabinoid release in both health and disease
- to comprehend more fully the role of endocannabinoids and their receptors both in modulating the release of neurotransmitters and other chemical messengers and in the aetiology and/or symptomatology of certain disorders, including multiple sclerosis and obesity.

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