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THE MOLECULAR LOGIC OF ENDOCANNABINOID SIGNALLING

Daniele Piomelli

The endocannabinoids are a family of lipid messengers that engage the cell surface receptors that are targeted by Δ^9 -tetrahydrocannabinol, the active principle in marijuana (*Cannabis*). They are made on demand through cleavage of membrane precursors and are involved in various short-range signalling processes. In the brain, they combine with CB_1 cannabinoid receptors on axon terminals to regulate ion channel activity and neurotransmitter release. Their ability to modulate synaptic efficacy has a wide range of functional consequences and provides unique therapeutic possibilities.

ARACHIDONIC ACID

Common name of 5,8,11,14-eicosatetraenoic acid, an essential (diet-derived) fatty acid that serves as precursor for eicosanoids and endocannabinoids.

The *Cannabis* plant has been used in Europe since antiquity, mostly to make cordage and fabric, but first attracted the attention of European scientists when Napoleon's troops brought back from Egypt intriguing accounts of its psychotropic activity. In 1810, a member of Napoleon's *Commission des Sciences et des Arts* wrote¹:

"For the Egyptians, hemp is the plant par excellence, not for the uses they make of it in Europe and many other countries, but for its peculiar effects. The hemp cultivated in Egypt is indeed intoxicating and narcotic."

Before long, detailed descriptions of the plant's properties began to appear^{2,3} and *Cannabis* extracts were introduced to the medical community. An 1848 commentary of the *British Pharmacopoeia* outlined quite accurately the psychotropic effects of *Cannabis* and pointed out its merit as an analgesic and antispasmodic⁴:

"Numerous observers have described the Indian hemp as producing in the natives of the East, who familiarly use it instead of intoxicating spirits, sometimes a heavy, lazy state of agreeable reverie, from which the individual may be easily roused to discharge any simple duty – sometimes a cheerful, active state of inebriation causing him to dance, sing and laugh, provoking the venereal appetite, and increasing the desire for food – and sometimes a quarrelsome drunkenness, leading to acts of violence. During this condition pain is assuaged and spasm arrested. [...] On the whole, it is a remedy which deserves a more extensive inquiry than any hitherto instituted."

The inquiry into the active chemical constituents of *Cannabis* turned out to be more time consuming than expected. Many other plant-derived compounds, such as morphine and atropine, had long been identified when the *Cannabis* plant finally yielded its active principle, the terpenoid derivative Δ^9 -tetrahydrocannabinol (THC)^{5,6} (FIG. 1).

The psychoactive properties of THC were recognized immediately, but the drug's unique chemical structure offered no hints as to its mechanism of action. To complicate matters further, the hydrophobic nature of THC delayed experimentation and indicated that the compound might act by influencing membrane fluidity, rather than by combining with a specific receptor. This impasse was resolved by the development of new classes of potent and selective THC analogue⁷ (FIG. 1), which led eventually to the pharmacological identification of cannabinoid-sensitive sites in the brain⁸.

The CB_1 cannabinoid receptor was molecularly cloned from rat brain in 1990 (REF. 9) and its immune-system counterpart, the CB_2 receptor, was identified by sequence homology three years later¹⁰. These discoveries not only established the mechanism of action of THC, thereby fuelling the development of subtype-selective agonists and antagonists (FIG. 1), but they also initiated a hunt for brain-derived cannabinoid ligands. Surprisingly, the first THC-like factor to be isolated was a lipid, rather than the peptide that had been expected on the basis of the precedent set by morphine and the enkephalins. It was identified as the amide of ARACHIDONIC ACID

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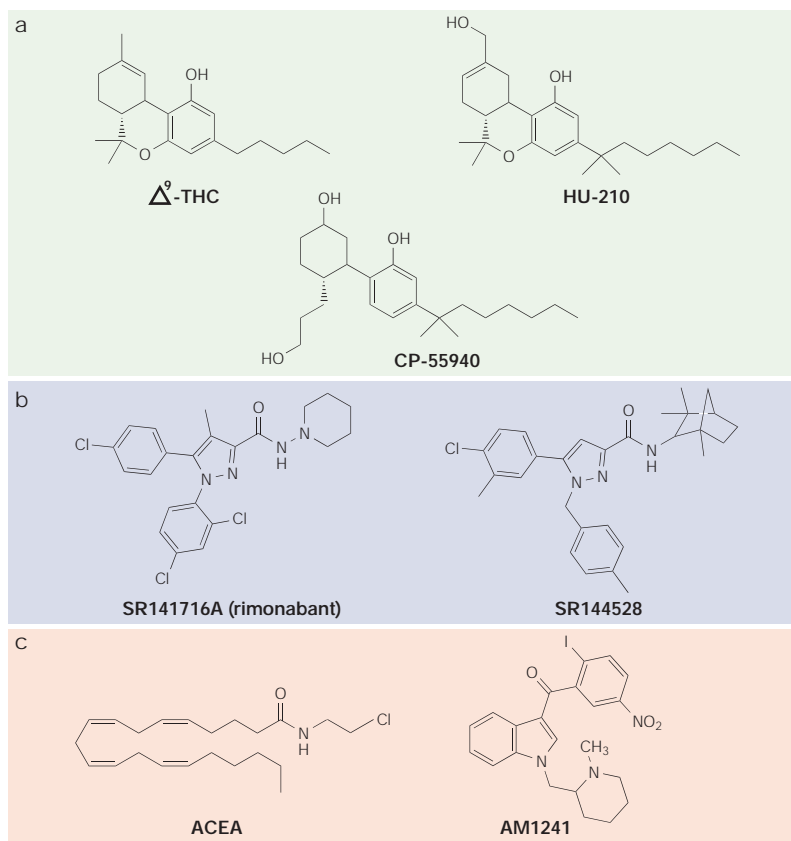


Figure 1 | Chemical structures of plant-derived and synthetic compounds that bind to cannabinoid receptors. **a** | Cannabinoid receptor agonists, which activate both CB₁ and CB₂ receptors. THC, tetrahydrocannabinol. **b** | Selective CB₁ antagonist (SR141716A, rimonabant) and CB₂ antagonist (SR144528). **c** | Selective CB₁ agonist (arachidonoyl-2'-chloroethanolamide, ACEA)¹⁴⁵ and CB₂ agonist (AM1241)¹⁴⁶.

with ethanolamine, and named anandamide after the Sanskrit word for bliss, *ananda*¹¹ (FIG. 2).

This small lipid molecule resembled no known neurotransmitter, but it did share structural features with the EICOSANOIDS, mediators of inflammation and pain with various functions in neural communication¹². Though initially controversial¹³, the signalling roles of anandamide were confirmed by the elucidation of the compound's unique metabolic pathways and the demonstration of its release in the live brain^{14–16}. As the search for THC-like compounds continued, other bioactive lipids were extracted from animal tissues. These include 2-arachidonoylglycerol (2-AG)^{17,18}, noladin ether¹⁹, virodhamine²⁰ and *N*-arachidonoyldopamine²¹ (FIG. 2).

In this article, I review the synthesis, release and deactivation of the endogenous cannabinoids (also called endocannabinoids). I then outline the properties and distribution of brain CB₁ receptors. Last, I describe the function of the endocannabinoids as local modulators of synaptic activity and their contribution to memory, anxiety, movement and pain.

Synthesis

Anandamide. The membranes of plant cells contain a family of unusual lipids that consist of a long-chain FATTY ACID tethered to the head group of

PHOSPHATIDYLETHANOLAMINE (PE) through an amide bond. When attacked by a PHOSPHOLIPASE D (PLD) enzyme, these membrane constituents generate a set of FATTY ACID ETHANOLAMIDES, which are used by plants as intercellular signalling molecules. They are released from cells in response to stress or infection, and stimulate the expression of genes engaged in systemic plant immunity²². This ancestral biochemical device is conserved in mammalian cells, which use the ethanolamide of arachidonic acid, anandamide, as a primary component of the endocannabinoid signalling system.

Anandamide formation in neurons is a two-step process, which parallels fatty acid ethanolamide production in plants^{14,23,24} (FIG. 3). The first step is the stimulus-dependent cleavage of the phospholipid precursor *N*-arachidonoyl-PE. This reaction is mediated by an uncharacterized PLD and produces anandamide and phosphatidic acid, a metabolic intermediate that is used by cells in the synthesis of other glycerol-derived phospholipids. Genes encoding two PLD isoforms have been cloned in mammals²⁵, but it is not known whether either of these enzymes is responsible for anandamide synthesis.

The brain contains tiny quantities of *N*-arachidonoyl-PE (20–40 pmol g⁻¹)^{23,24} — probably too little to sustain anandamide release for an extended time. The cellular stores of this precursor are replenished by the enzyme *N*-acyltransferase (NAT), which catalyses the intermolecular passage of an arachidonic acid group from the sn-1 position of PHOSPHATIDYLCHOLINE to the head group of PE^{14,23,24} (FIG. 3). In cultures of rat cortical neurons, two intracellular second messengers control NAT activity: Ca²⁺ and cyclic AMP. Ca²⁺ is required to engage NAT, which is inactive in its absence, whereas cAMP works through protein kinase A-dependent phosphorylation to enhance NAT activity²⁶. Although catalysed by separate enzymes, the syntheses of anandamide and its parent lipid are thought to proceed in parallel because Ca²⁺-stimulated anandamide production is generally accompanied by *de novo* formation of *N*-arachidonoyl-PE^{23,24}.

As expected of a Ca²⁺-activated process, anandamide formation can be elicited by Ca²⁺ ionophores, which carry Ca²⁺ ions across cell membranes. For example, in cultures of rat striatal neurons labelled by incubation with [³H]ethanolamine, the Ca²⁺ ionophore ionomycin stimulates accumulation of [³H]anandamide¹⁴. A similar stimulation is produced by kainate (a glutamate receptor agonist), 4-aminopyridine (a K⁺ channel blocker) or membrane-depolarizing concentrations of K⁺, and can be prevented by chelating extracellular Ca²⁺ (REFS 14,26). The Ca²⁺ dependence of anandamide synthesis was also demonstrated using MICRODIALYSIS. Administration of a high-K⁺ pulse in the rat striatum caused a reversible increase in interstitial anandamide concentrations, which was prevented by removal of Ca²⁺ from the perfusing solution¹⁵.

Although neural activity induces anandamide release in a Ca²⁺-dependent manner, Ca²⁺ entry into neurons is not the only determinant of anandamide generation: there is evidence that G-protein-coupled receptors can

EICOSANOIDS

A family of biologically active compounds produced through the enzymatic oxygenation of arachidonic acid. Examples are prostaglandins, leukotrienes and lipoxins.

FATTY ACID

An organic acid characterized by a non-branched carbon chain and an even number of carbon atoms. Examples of saturated fatty acids (without double bonds) are palmitic (16 carbons) and stearic (18 carbons). Examples of unsaturated and polyunsaturated fatty acids include oleic (18 carbons, one double bond) and arachidonic (20 carbons, 4 double bonds).

PHOSPHATIDYLETHANOLAMINE

An important class of membrane phospholipids comprising a glycerol skeleton linked to two fatty acid residues, phosphoric acid and ethanolamine.

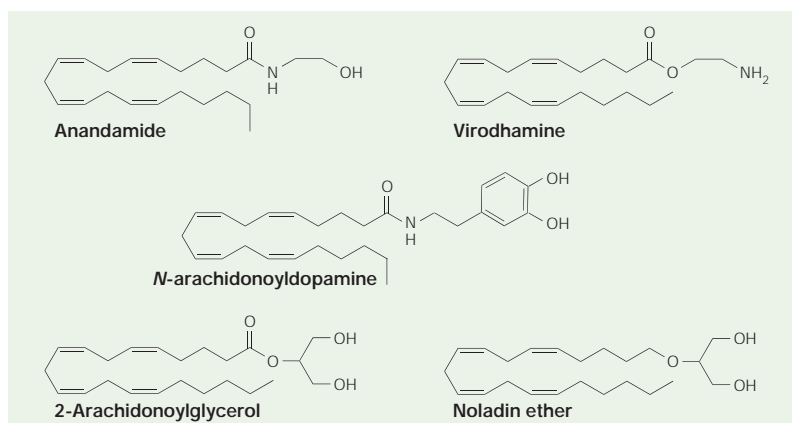


Figure 2 | Chemical structures of endogenous compounds that bind to cannabinoid receptors.

PHOSPHOLIPASE

(PL). A group of enzymes that catalyse the hydrolysis of phospholipids at their glycerol ester (PLA) or phosphodiester (PLC, PLD) bonds.

FATTY ACID ETHANOLAMIDE

A lipid-derived signalling molecule characterized by an ethanolamine residue linked to a long-chain fatty acid through an amide bond. Examples are anandamide (arachidonylethanolamide), oleoylethanolamide and palmitoylethanolamide.

SN: STEREOSPECIFIC NUMBERING

Defines a convention on how to designate the stereochemistry of glycerol-based lipids. When the glycerol moiety is drawn with the secondary hydroxyl to the left, the carbons are numbered 1,2,3 from top to bottom.

PHOSPHATIDYLCHOLINE

A major class of membrane phospholipids comprised of a glycerol skeleton linked to two fatty acid residues, phosphoric acid and choline. In the mammalian brain, the *sn*-2 position of phosphatidylcholine most often contains an arachidonic acid residue, but a small pool of this fatty acid is also stored in the *sn*-1 position.

MICRODIALYSIS

A technique that allows the sampling of neurochemicals in the brain of live animals.

also trigger this process. For example, application of the dopamine D_2 -receptor agonist quinpirole causes an eightfold increase in anandamide outflow in the rat striatum, which is prevented by the D_2 -receptor antagonist raclopride¹⁵. This response is accompanied by an elevation in tissue anandamide content, indicating that it might be due to a net increase in anandamide formation rather than to extracellular release of preformed anandamide²⁷. Muscarinic acetylcholine receptors and metabotropic glutamate receptors can also cause endocannabinoid release in hippocampal slices in a Ca^{2+} -independent manner, but the substance(s) involved have not been identified^{28,29}.

How does occupation of D_2 receptors initiate anandamide synthesis? Inhibition of cAMP formation, a hallmark of D_2 -receptor signalling, is unlikely to be responsible for this effect because cAMP positively regulates NAT activity²⁶. Alternatively, D_2 receptors could

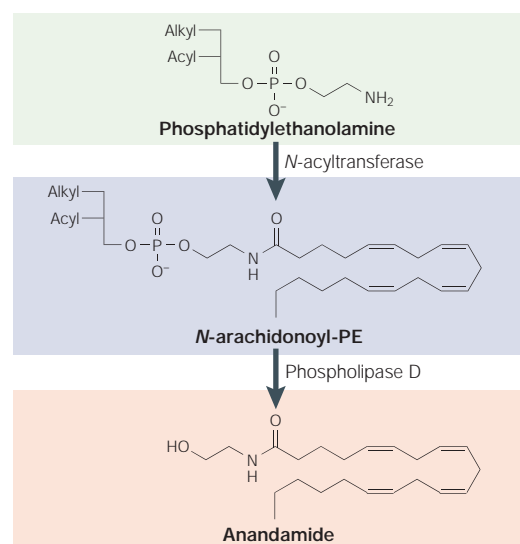


Figure 3 | Mechanism of anandamide formation in neurons. The sequence of reactions is thought to include: first, the synthesis of the anandamide precursor *N*-arachidonoyl-phosphatidylethanolamine (PE), catalysed by the enzyme *N*-acetyltransferase; second, the cleavage of *N*-arachidonoyl-PE to yield anandamide, catalysed by phospholipase D.

interact with the Rho family of small G proteins to stimulate PLD activity³⁰, or they might engage β - γ subunits of G proteins to activate phospholipase C_β (PLC $_\beta$)³¹. PLC $_\beta$ catalyses the cleavage of phosphatidylinositol-4,5-bisphosphate to produce inositol-1,4,5-trisphosphate, which might then recruit the NAT/PLD pathway by mobilizing Ca^{2+} from internal stores.

2-Arachidonoylglycerol. Like other MONOACYLGLYCEROLS, 2-AG is at the crossroads of multiple routes of lipid metabolism, where it can serve interchangeably as an end-product for one pathway and precursor for another. These diverse metabolic roles can explain its high concentration in brain tissue (about 200-fold greater than anandamide's)^{17,32}, and imply that a significant fraction of brain 2-AG is engaged in housekeeping functions rather than in signalling.

The place occupied by 2-AG at central intersections of lipid metabolism also complicates efforts to define the biochemical pathway(s) responsible for its physiological synthesis. There is, however, enough information to indicate two possible routes (FIG. 4). The first begins with the phospholipase-mediated formation of 1,2-diacylglycerol (DAG). This product regulates protein kinase C activity — an important second messenger function — and is a substrate for two enzymes: DAG kinase³³, which attenuates DAG signalling by catalysing its phosphorylation to phosphatidic acid; and DAG lipase (DGL), which hydrolyses DAG to monoacylglycerol³⁴. The fact that drug inhibitors of PLC and DGL block Ca^{2+} -dependent 2-AG accumulation in rat cortical neurons indicates primary involvement of this pathway in 2-AG formation³².

An alternative pathway of 2-AG synthesis begins with the production, mediated by phospholipase A1 (PLA1)^{35,36}, of a 2-arachidonoyl-LYSOPHOSPHOLIPID, which might be hydrolysed to 2-AG by lyso-PLC activity (FIG. 4). Although there is no direct evidence for this mechanism in 2-AG formation, the high level of PLA1 expression in brain tissue^{34,35} makes it an intriguing target for future investigation. In addition to the phospholipase-operated pathways outlined above, monoacylglycerols can be produced by hormone-sensitive lipase acting on triacylglycerols or by lipid phosphatases acting on lysophosphatidic acid. In general, however, these enzymes preferentially target lipids that are enriched in saturated or monounsaturated fatty acids, rather than the polyunsaturated species that would give rise to 2-AG.

Irrespective of its exact mechanism, neuronal 2-AG production can be initiated by an increase in the concentration of intracellular Ca^{2+} . In cultures of rat cortical neurons, the Ca^{2+} ionophore ionomycin and the glutamate receptor agonist NMDA (*N*-methyl-D-aspartate) stimulate 2-AG synthesis in a Ca^{2+} -dependent manner^{32,37}. Likewise, in freshly dissected hippocampal slices, high-frequency stimulation of the SCHAFER COLLATERALS produces a Ca^{2+} -dependent increase in tissue 2-AG content³². Importantly, this treatment has no effect on the concentrations of non-cannabinoid monoacylglycerols, such as 1(3)-palmitoylglycerol, which indicates

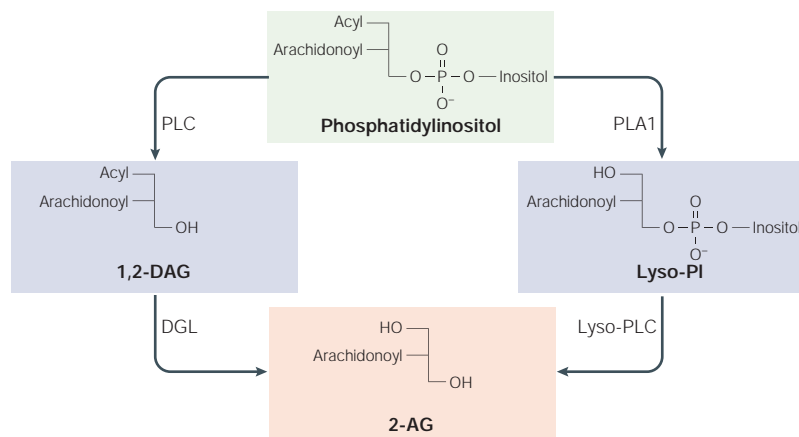


Figure 4 | Pathways of 2-arachidonoylglycerol (2-AG) formation in neurons. One possible sequence of reactions, shown on the left, includes the cleavage of phosphatidylinositol (PI) to yield 1,2-diaclyglycerol (DAG), catalysed by a phospholipase such as phospholipase C (PLC), and the subsequent conversion of DAG to 2-AG, catalysed by diacylglycerol lipase (DGL). An alternative route, shown on the right, comprises the formation of a 2-arachidonoyl-lysophospholipid such as lyso-PI, catalysed by phospholipase A1 (PLA1), followed by the hydrolysis of the lysophospholipid to 2-AG, catalysed by lyso-PLC.

that 2-AG formation is not due to a generalized increase in the rate of lipid turnover³². Furthermore, high-frequency stimulation does not alter hippocampal anandamide concentrations, indicating that the syntheses of 2-AG and anandamide can be independently regulated^{32,37}. In further support of this idea, activation of D₂ receptors — a potent stimulus for anandamide formation in the rat striatum — has no effect on striatal 2-AG concentrations^{15,27}.

Other putative endogenous ligands. Noladin ether is an ether-linked analogue of 2-AG that binds to and activates CB₁ receptors¹⁹ (FIG. 2). Its pathway of formation has not been characterized, and its occurrence in the normal brain has been questioned³⁸. Virodhamine, the ester of arachidonic acid and ethanolamine (FIG. 2), might act as an endogenous CB₁ antagonist²⁰. Its presence in brain tissue has been documented²⁰, but is intriguing because this chemically unstable molecule is rapidly converted to anandamide in aqueous environments. The mechanism of its synthesis is unknown, and its deactivation might share anandamide's pathways of uptake and intracellular hydrolysis²⁰. Finally, the endogenous vanilloid agonist, *N*-arachidonoyl-dopamine, also exhibits affinity for cannabinoid receptors *in vitro*²¹ (FIG. 2).

Release from neurons

How are endocannabinoids released from cells and how do they reach their targets? Classical transmitters and neuropeptides can diffuse through the water-filled space that surrounds neurons, but hydrophobic compounds such as anandamide and 2-AG tend to remain associated with lipid membranes. One possibility is that endocannabinoids might not leave the cell where they are produced; rather, they could move sideways within the plasmalemma until they collide with membrane-embedded CB₁ receptors. This hypothesis is supported

by the role of an intramembranous amino-acid residue (lysine-192) in the binding of anandamide to CB₁ (REF. 39), as well as by the finding that certain cannabinoid agonists can approach the receptor by lateral membrane diffusion⁴⁰. Nevertheless, it does not account for two pieces of evidence. First, anandamide is found in incubation media of cells and in brain interstitial fluid, implying that it can overcome its tendency to partition in membranes^{14–16}. Perhaps more importantly, physiological experiments have shown that an endocannabinoid substance does leave postsynaptic cells to activate CB₁ receptors on adjacent axon terminals^{41–46}. This unidentified compound might travel as far as 20 μm from its cell of origin before being eliminated⁴¹.

If endocannabinoids are released from neurons, what is the mechanism of their release? The fact that plasma membranes contain precursor molecules for both anandamide and 2-AG indicates that they could leave the cell as soon as they are formed. Extracellular lipid-binding proteins such as the lipocalins, which are expressed at high levels in the brain⁴⁷, might facilitate this step and help to deliver endocannabinoids to their cellular targets. Although this scenario awaits confirmation, it does mirror what happens in the bloodstream, where anandamide's movements are made possible by its reversible binding to serum albumin⁴⁸.

Deactivation

Two mechanisms cooperate in attenuating endocannabinoid signalling in the brain: carrier-mediated transport into cells and intracellular hydrolysis (FIG. 5).

Transport. Anandamide and 2-AG can diffuse passively through lipid membranes, but this process is accelerated by a rapid and selective carrier system that is present in both neurons and glial cells^{49,50}. Although it is superficially similar to other transmitter systems, endocannabinoid transport is not driven by transmembrane Na⁺ gradients, indicating that it might be mediated by a FACILITATED DIFFUSION mechanism^{49,50}. In this respect, neural cells seem to internalize anandamide and 2-AG in a manner similar to fatty acids, eicosanoids and other biologically relevant lipids, by using energy-independent carriers. Several lipid-carrier proteins have been molecularly cloned⁵¹, inspiring optimism that, despite current controversy (BOX 1), endocannabinoid transporter(s) will eventually be characterized.

Meanwhile, to gain insight into the role of transport in endocannabinoid inactivation, we can rely on an expanding series of pharmacological transport inhibitors. The prototype is AM404, which slows the elimination of both anandamide and 2-AG, magnifying their biological effects^{49,52,53} (FIG. 6). This inhibitor has helped to unmask important roles of the endocannabinoid system in the regulation of neurotransmission and synaptic plasticity, but suffers from various limitations, including an affinity for VANILLOID RECEPTORS and susceptibility to enzymatic attack by fatty acid amide hydrolase (FAAH). These limitations have prompted an ongoing search for more selective and stable analogues^{54,55} (FIG. 6).

MONOACYLGLYCEROL

A glycerol derivative in which one of the hydroxyl groups is linked to a fatty acid residue by an ester bond.

LYSOPHOSPHOLIPID

A phospholipid containing only one fatty acid chain. Examples include lysophosphatidic acid and lysophosphatidylethanolamine.

SCHAFFER COLLATERALS

Axons of the CA3 pyramidal cells of the hippocampus that form synapses with the apical dendrites of CA1 neurons.

FACILITATED DIFFUSION

A common mechanism of transmembrane transfer that involves a protein carrier, but does not require expenditure of cellular energy.

VANILLOID RECEPTORS

Membrane receptor-channels permeable to monovalent cations. They are activated by noxious heat and capsaicin, the active constituent of hot chili peppers.

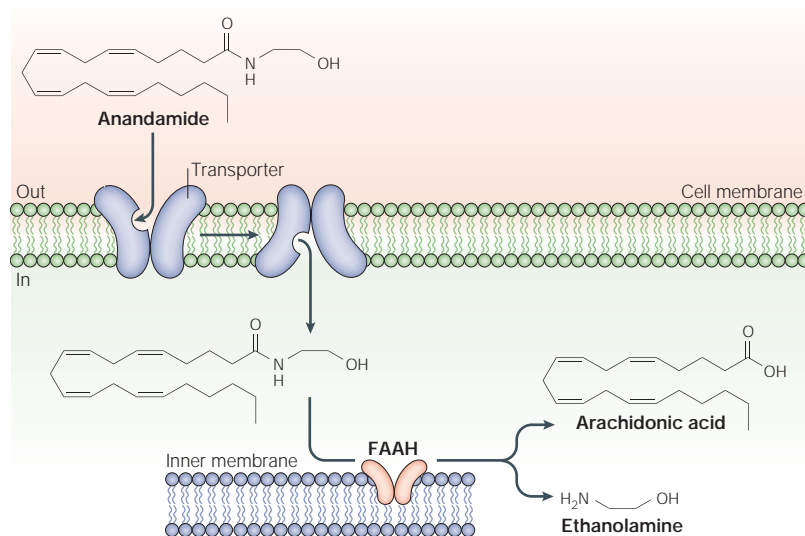


Figure 5 | Mechanisms of endocannabinoid deactivation in neurons. Anandamide and 2-arachidonoylglycerol (2-AG) can be internalized by neurons through a high-affinity transport mechanism, the 'endocannabinoid transporter'. Once inside cells, they can be hydrolysed by distinct serine hydrolases – anandamide by fatty acid amide hydrolase (FAAH) and 2-AG by monoglyceride lipase (MGL) (not shown) – to yield inactive breakdown products.

Fatty acid amide hydrolase. FAAH is an intracellular membrane-bound serine hydrolase that breaks down anandamide into arachidonic acid and ethanolamine^{56–59} (FIG. 5). It has been molecularly cloned and its catalytic mechanism, which allows it to recognize a broad spectrum of amide and ester substrates, has been elucidated in detail⁶⁰. Particularly notable is FAAH's ability to hydrolyse bioactive fatty amides, which do not bind to any of the known cannabinoid receptors: these include the satiety factor oleoylethanolamide⁶¹ and the anti-inflammatory/analgesic mediator palmitoylethanolamide^{62,63}. FAAH

tightly controls brain concentrations of these compounds^{64,65}, but the functional significance of this regulation is unknown.

FAAH is widely distributed in the rat brain, where it is expressed at high concentrations in cell bodies and dendrites of principal neurons^{66,67}. In the hippocampus, neocortex and cerebellum, FAAH-positive cell bodies are juxtaposed to axon terminals that contain CB₁ receptors, indicating not only that FAAH participates in the inactivation of neurally generated anandamide, but also that this process occurs postsynaptically. This idea can now be tested in FAAH-deficient mice⁶⁴ or using selective FAAH inhibitors with long-lasting systemic actions⁶⁵ (FIG. 6).

Monoacylglycerol lipase. The pig brain contains two chromatographically distinct 2-AG-hydrolysing activities⁶⁸, one of which is probably due to the enzyme monoacylglycerol lipase (MGL). The rat brain isoform of this cytosolic serine hydrolase has been characterized both molecularly and morphologically⁶⁹. It has a broad distribution in the central nervous system (CNS), which partially overlaps with that of FAAH; however, whereas FAAH is predominantly found in postsynaptic structures, MGL might be mostly associated with nerve endings⁶⁹. In the hippocampal CA1 field, MGL-positive axon terminals surround cell bodies of pyramidal neurons containing FAAH. This localization could reflect a functional role of presynaptic MGL in terminating RETROGRADE SIGNALLING events mediated by 2-AG (discussed later in this article).

CB₁ signalling

CB₁ is considered to be the most abundant G-protein-coupled receptor in the mammalian brain, and its presence in the neocortex, hippocampus, basal ganglia, cerebellum and brainstem accounts for most of the behavioural actions of cannabinoid drugs⁷⁰. The four symptoms that are often used to define cannabinoid intoxication in the rodent – hypothermia, rigid immobility, analgesia and decreased motor activity⁷¹ – are strikingly absent in mice in which the *cb1* gene has been deleted by targeted recombination^{72,73}.

Aside from its unusually high concentrations in the brain, CB₁ is a standard G_{i/o}-coupled receptor and can initiate signalling events typical of this class of transducing proteins. These include closure of Ca²⁺ channels, opening of K⁺ channels, inhibition of adenylyl cyclase activity (with its consequent decrease in cytosolic cAMP concentrations) and stimulation of kinases that phosphorylate tyrosine, serine and threonine residues in proteins. Each of these mechanisms seems to have distinct functions in translating CB₁-receptor occupation into biological responses.

Cannabinoid agonists inhibit N- and P/Q-type voltage-activated Ca²⁺ channels^{74–76}. This effect, which has been suggested to result from a direct interaction of G_{i/o}-protein β-γ subunits with the channels⁷⁷, might underlie CB₁-mediated depression of transmitter release at GABA (γ-aminobutyric acid) synapses in the CA1 field of the hippocampus⁷⁸ and at glutamatergic synapses in the dorsal striatum^{79,80} (FIG. 7a). Importantly,

Box 1 | To transport or not to transport?

Anandamide uptake in neural cells exhibits three identifying features of carrier-mediated transport. First, saturation kinetics: plots of the initial rate of [³H]anandamide accumulation against extracellular anandamide concentrations yield apparent Michaelis constants (K_M) of 0.32 μM in astrocytes and 1.2 μM in cortical neurons⁴⁹. These values are very similar to those obtained with the transporters for serotonin ($K_M = 0.3–0.5$ μM), dopamine ($K_M = 0.9–1.2$ μM) and noradrenaline ($K_M = 0.45$ μM)¹⁴⁸. Higher K_M values for anandamide transport were obtained, however, in cerebellar granule cells⁵⁰. Second, substrate specificity: rat brain neurons and other cells in culture internalize [³H]anandamide, but not closely related analogues^{14,49,50,52}. Third, selective inhibition: [³H]anandamide transport is blocked competitively by AM404 and other anandamide derivatives^{49,54,55}. Importantly, some chiral analogues of anandamide inhibit transport in a stereospecific manner, which is indicative of a macromolecular recognition site⁵².

Nevertheless, the finding that [³H]anandamide uptake does not require cellular energy^{49,50} has led to proposals that this process might be explained by passive membrane diffusion driven by intracellular fatty acid amide hydrolase (FAAH) activity¹⁴⁹. This hypothesis is readily testable, as it assumes that anandamide uptake inhibitors act by blocking FAAH, and that disruption of FAAH by either genetic or pharmacological means should eliminate anandamide transport. Although it is clearly possible to inhibit anandamide transport independently of FAAH^{54,55}, a stringent test of this hypothesis – for example, an examination of anandamide uptake in FAAH-knockout mice – has not yet been reported. Irrespective of the outcome of such a test, definitive proof of the transporter's existence will come only from its molecular characterization.

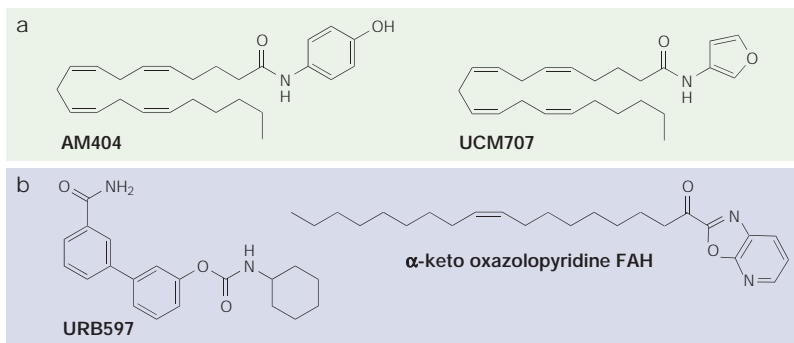


Figure 6 | Chemical structures of endocannabinoid deactivation inhibitors. **a** | Endocannabinoid transport inhibitors: AM404 (REF. 40) and UCM707 (REF. 54). **b** | Fatty acid amide hydrolase (FAAH) inhibitors: substituted carbamates (URB597)⁶⁵ and substituted α -keto oxazolopyridines¹⁴⁷.

however, endocannabinoid-mediated suppression of GABA release in hippocampal slices seems primarily to involve N-type Ca^{2+} channels⁸¹.

Cannabinoid regulation of voltage-gated K^+ currents⁸² is also implicated in presynaptic inhibition at GABA⁸³ and glutamate synapses. The latter include PARALLEL FIBRE–Purkinje cell synapses in the cerebellum, as well as synapses in the nucleus accumbens and lateral amygdala^{84–86} (FIG. 7b). The sensitivity of these responses to PERTUSSIS TOXIN implies that they are mediated by $G_{\text{i/o}}$ proteins, but it is still unclear whether transduction is direct (β - γ subunit-mediated) or indirect (second messenger-mediated). Inhibition of cAMP formation does not seem to be involved^{85,86}.

On the other hand, cAMP can contribute to the regulation of neuronal gene expression by CB_1 . This process, which is necessary to produce lasting changes in synaptic strength, depends on the recruitment of complex networks of intracellular protein kinases⁸⁷. Two components of these networks, extracellular signal-regulated kinase (ERK) and focal adhesion kinase (FAK), become activated when hippocampal slices are treated with cannabinoid agonists^{88,89}. This activation is mimicked by inhibitors of cAMP-dependent kinase and is lost when the slices are exposed to cell-permeant cAMP analogues, implying that it might result from a decrease in intracellular cAMP concentrations. The involvement of ERK and FAK in synaptic plasticity indicates that these protein kinases could participate in the changes in gene expression and the persistent neural adaptations that accompany cannabinoid administration⁹⁰.

CB_1 distribution

In the rodent and human cortices, CB_1 receptors are primarily found on axon terminals of cholecystokinin-8 (CCK-8)-positive GABA interneurons^{91–95}. This expression pattern dominates the neocortex, hippocampal formation and amygdala, where nerve terminals that form excitatory synapses are ostensibly devoid of CB_1 immunoreactivity^{91,94}. However, there is evidence that excitatory terminals in these regions do contain the receptor; for example, cannabinoid agonists reduce glutamatergic transmission in the amygdala of normal

mice, but fail to do so in CB_1 -deficient mutants⁸⁶. In addition, low concentrations of CB_1 messenger RNA have been found in many neurons of the cortex that do not contain GABA⁹².

CB_1 receptors are also expressed at very high levels throughout the basal ganglia. In the striatum they are localized to three distinct neuronal elements: glutamatergic terminals originating in the cortex^{79,80}, local-circuit GABA interneurons ('fast-spiking' interneurons that do not express CCK-8)⁹⁶ and axon terminals of GABA projection neurons ('medium spiny neurons')⁹⁷. Medium spiny neurons project to striatal outflow nuclei, where CB_1 receptors are especially abundant; for example, in the globus pallidus they outnumber dopamine D_1 receptors by a factor of 45 (REF. 97).

In the cerebellum, CB_1 is present on excitatory terminals of climbing and parallel fibres (but not on their postsynaptic partners, the Purkinje neurons) as well as on GABA interneurons^{70,93}. Smaller numbers of CB_1 receptors are also found in the thalamus (especially in the anterior dorsal nucleus and habenula), hypothalamus (ventromedial and anterior nuclei), midbrain (periaqueductal grey and superior colliculus), medulla (dorsal vagal complex and rostral ventromedial medulla) and spinal cord (dorsal horn)^{70,93}. Last, CB_1 is expressed in peripheral sensory neurons⁹⁸, where it is localized in cells that express N52, a protein marker of mechanosensitive A β fibres⁹⁹.

Another brain receptor?

A few cannabinoid effects persist in CB_1 -null mice, implying that this receptor might not act alone in mediating brain cannabinoid signalling^{73,100}. Although cannabinoid agonists lose their ability to inhibit GABA and glutamate transmission in some brain regions of adult CB_1 -knockout mice⁸⁶, they can still reduce excitatory transmission in the hippocampal CA1 field of these animals^{101,102}. This discrepancy is reinforced by the finding that GABA and glutamate synapses in CA1 respond in different ways to cannabinoid drugs. For example, cannabinoid depression of excitatory currents is blocked by CAPSAZEPINE, whereas depression of inhibitory currents is not¹⁰³. These results make a persuasive case for the existence of a hippocampal cannabinoid-sensitive site that is distinct from CB_1 (sometimes called ' CB_3 '), but other evidence appears to contradict them; for example, in newborn CB_1 -null mice, cannabinoid agonists affect neither GABA nor glutamate transmission¹⁰⁴. Although this difference could be due to the developmental stage of the preparation used – adult¹⁰¹ versus one-week-old mice¹⁰⁴ – more studies are needed to establish whether the CB_3 site is molecularly distinct from CB_1 . A novel cannabinoid site has also been identified in the vascular endothelium¹⁰⁵, but seems to be different from CB_3 because it is not antagonized by capsazepine¹⁰⁵ or activated by the CB_1/CB_2 agonist Win-55212-2 (REF. 106).

A local message

Outside the brain, the endocannabinoids are produced on demand and act on cells located near their site of synthesis. For example, they are formed by circulating

RETROGRADE SIGNALLING

The backward movement of signalling molecules from postsynaptic to presynaptic structures, which underlies a variety of short- and long-term changes in synaptic efficacy.

PARALLEL FIBRES

Axons of cerebellar granule cells. Parallel fibres emerge from the molecular layer of the cerebellar cortex towards the periphery, where they extend branches perpendicular to the main axis of the Purkinje neurons and form *en passant* synapses with this cell type.

PERTUSSIS TOXIN

The causative agent of whooping cough, pertussis toxin causes the persistent activation of G_i proteins by catalysing the ADP-ribosylation of the α -subunit.

CAPSAZEPINE

A synthetic drug that acts as a competitive antagonist of capsaicin at vanilloid receptors.

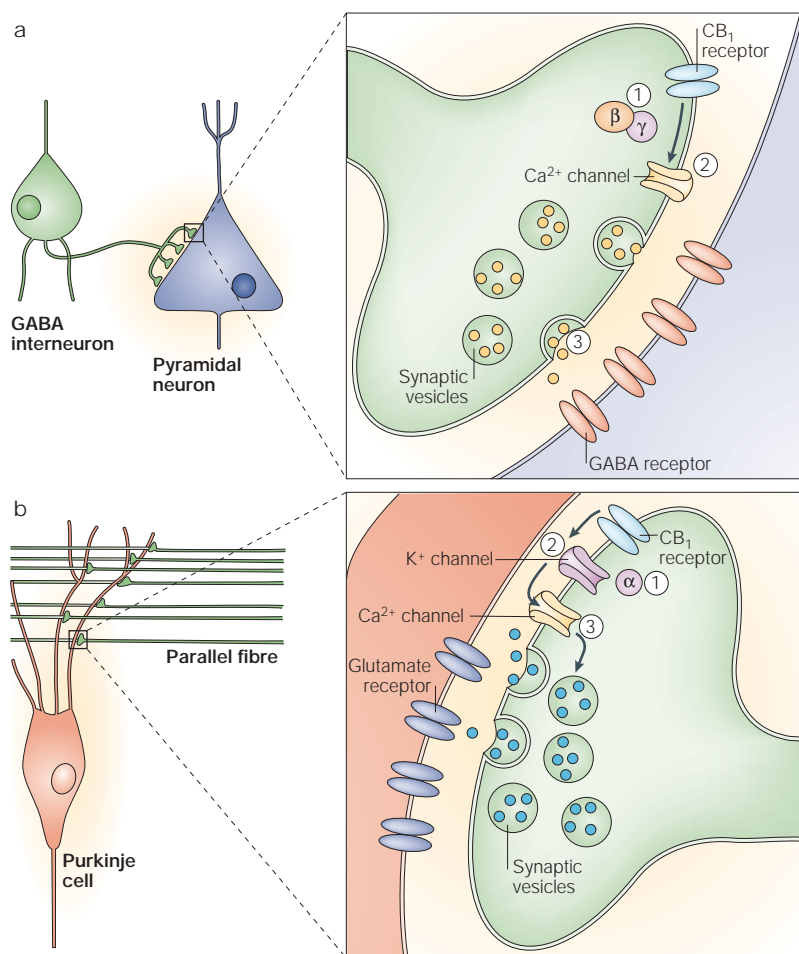


Figure 7 | Regulation of presynaptic ion channel activities by CB₁ cannabinoid receptors. **a** | At synapses between GABA (γ -aminobutyric acid) interneurons and pyramidal cells in the CA1 field of the hippocampus, activation of CB₁ receptors can initiate a series of intracellular events, which include (1) activation of G-protein β - γ subunits, (2) closure of voltage-gated Ca²⁺ channels and (3) inhibition of GABA release. **b** | At parallel fibre–Purkinje cell synapses in the cerebellum, CB₁ activation can (1) engage G-protein α -subunits that (2) cause the opening of K⁺ channels; the resulting membrane hyperpolarization can (3) reduce Ca²⁺ entry and inhibit glutamate release. Mechanisms similar to those illustrated above are thought to underlie cannabinoid-mediated inhibition of neurotransmitter release in other brain regions.

leukocytes and platelets, and induce vascular relaxation by interacting with cannabinoid receptors on the surface of neighbouring endothelial and smooth muscle cells¹⁰⁷. Similar PARACRINE actions are thought to occur in the CNS, where the endocannabinoids might mediate a localized signalling mechanism through which principal neurons modify the strength of incoming synaptic inputs.

Regulation of GABA transmission

Hippocampus: modulation of memory. When a pyramidal neuron in the CA1 field of the hippocampus is depolarized, the inhibitory GABA inputs received by that cell are transiently suppressed. This phenomenon, called depolarization-induced suppression of inhibition (DSI), is initiated postsynaptically by voltage-dependent influx of Ca²⁺ into the soma and dendrites of the neuron, but is expressed presynaptically through inhibition of transmitter release from axon terminals of GABA

interneurons¹⁰⁸. This indicates that a chemical messenger generated during depolarization of the pyramidal cell must travel backwards across the synapse to induce DSI (FIG. 8).

There is evidence that this retrograde signalling process involves an endocannabinoid substance, possibly 2-AG. First, CB₁ agonists mimic DSI, whereas CB₁ antagonists block it^{41–43}. Second, DSI is absent in CB₁-deficient mice^{81,109}. Third, the GABA interneurons that are implicated in DSI express high levels of CB₁ receptors, which are localized to their axon terminals⁹¹. Fourth, neural activity and Ca²⁺ entry stimulate the hippocampal synthesis of 2-AG, but have no effect on anandamide concentrations³². Nevertheless, we still don't know whether the endocannabinoid actually crosses back to the presynaptic nerve ending or is produced there by the action of another, unidentified retrograde signal (FIG. 8).

The fact that DSI is induced *in vitro*⁴³ by levels of neural activity that could also be encountered *in vivo* indicates that this process might have a role in normal brain function. Although this idea is still questioned¹¹⁰, various results link DSI to the regulation of hippocampal GAMMA OSCILLATIONS⁷⁷. These network oscillations are coordinated by CB₁-positive GABA interneurons and are influenced by cannabinoid agonists, raising the possibility that an endocannabinoid substance might modulate their expression¹¹¹ and be involved in the organization of hippocampal cell assemblies¹¹². Another function of DSI might relate to synaptic plasticity. By weakening GABA-mediated inhibition, DSI could facilitate the induction of long-term potentiation in individual CA1 pyramidal neurons; this might contribute in turn to the formation of 'place fields' or to other forms of hippocampus-dependent learning¹¹³. Such a cognitive-enhancing action would not contradict the well-known amnesic effects of cannabinoid drugs¹¹⁴ as the latter might result from a generalized, circuit-independent activation of CB₁ receptors in the hippocampus and other brain areas.

Outside the hippocampus, endocannabinoid-mediated DSI has been shown to occur at interneuron–principal cell synapses of the cerebellum^{115–117} and probably will soon be discovered elsewhere.

Amygdala: modulation of emotions. CB₁-bearing interneurons are selectively localized to a subdivision of the amygdala called the basolateral complex^{94,95}, a key station in the neural circuitry that processes emotions and a primary site of cannabinoid analgesia¹¹⁸. This localization, and the fact that CB₁ inactivation causes anxiety-like and aggressive responses in rodents^{119,120}, indicate that the endocannabinoid system might influence affective states through changes in the amygdala's efferent activity. This idea is further supported by two findings: first, presentation of anxiogenic stimuli increases anandamide and 2-AG concentrations in the mouse amygdala¹²¹; second, FAAH inhibitors exhibit marked anxiolytic-like properties in rats⁶⁵.

Locally formed endocannabinoids could modify the amygdala's output in two complementary ways.

PARACRINE

A bioactive substance formed in the body by the action of primary messengers (hormones, neurotransmitters) on their receptors, which produces its effects by acting on cells near its sites of synthesis.

GAMMA OSCILLATIONS

Fast (20–80 Hz) synchronous oscillations of brain activity, which are thought to contribute to cognition and movement.

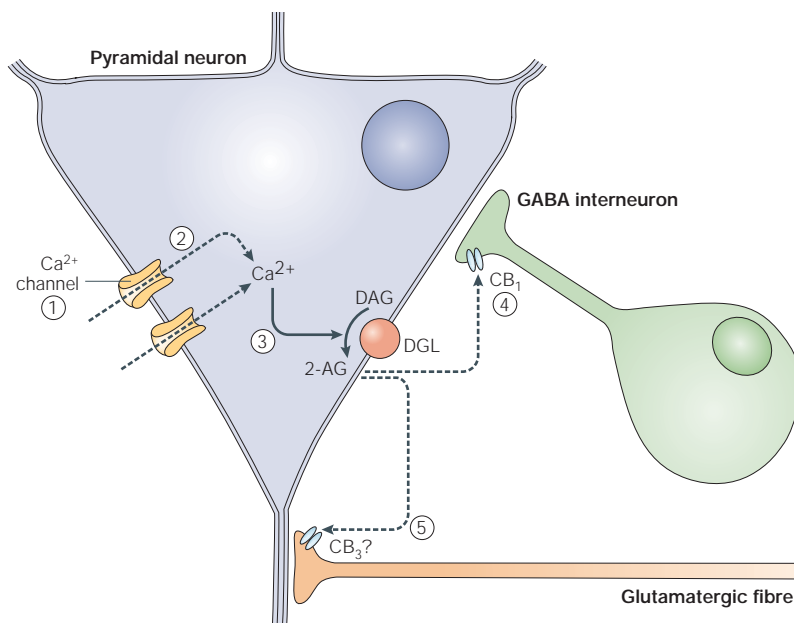


Figure 8 | Endocannabinoid-mediated synaptic signalling. In the CA1 field of the hippocampus, membrane depolarization (1) opens voltage-activated Ca^{2+} channels in pyramidal neurons, producing (2) an elevation of intracellular Ca^{2+} concentrations. Ca^{2+} can (3) stimulate the synthesis of 2-arachidonoylglycerol (2-AG) through the diacylglycerol lipase (DGL) pathway or the synthesis of anandamide through the phospholipase D pathway (not shown). The newly formed endocannabinoids might travel across the synapse to interact with (4) CB_1 receptors on axon terminals of GABA (γ -aminobutyric acid) interneurons, leading to depolarization-induced suppression of inhibition, or (5) ' CB_3 ' sites on glutamatergic terminals, leading to depolarization-induced suppression of excitation. Alternatively, the endocannabinoid might not actually cross back to the presynaptic nerve ending, but could be produced *in situ* by the action of another, unidentified retrograde signal.

They could depress glutamate release from axon terminals originating in the cortex and other brain regions⁸⁶. In addition, by reducing GABA release from basolateral interneurons, they might disinhibit GABA cells in the adjacent intercalated nuclei and consequently decrease the activity of their postsynaptic targets, the pyramidal neurons in the central nucleus of the amygdala, which constitute the structure's primary efferent pathway⁹⁴.

Basal ganglia: modulation of motor activity. The terminal fields of striatal projection neurons contain the highest densities of CB_1 receptors in the brain. Here, local administration of cannabinoid agonists inhibits GABA release and profoundly affects motor behaviours¹²². Membrane depolarization and dopamine D_2 -receptor activation stimulate striatal anandamide formation¹⁵, indicating that this endocannabinoid might contribute to the regulation of basal ganglia function. In agreement with this hypothesis, the CB_1 antagonist rimonabant enhances the stimulation of movement that is induced in rats by dopamine agonists¹²³, whereas the endocannabinoid transport inhibitor AM404 attenuates this stimulation in a CB_1 -dependent manner⁵³.

Anandamide might act at multiple sites in the basal ganglia, including GABA projection neurons, corticostriatal glutamatergic terminals and local-circuit interneurons^{79,96,97}. Local-circuit interneurons are particularly notable because of their functional resemblance

to CB_1 -positive interneurons in the hippocampus, with which they share not only a GABA-containing phenotype, but also the ability to discharge high-frequency bursts of action potentials that can inhibit firing in large assemblies of projection cells¹²⁴. Does locally released anandamide gain access to these interneurons? Or does it primarily act on medium spiny cells and their cortical afferents? We don't know yet. But these unanswered questions do not diminish the significance of striatal endocannabinoid signalling, which is further highlighted by the effectiveness of cannabinoid agonists in the symptomatic treatment of LEVODOPA-INDUCED DYSKINESIAS¹²⁵ and TOURETTE'S SYNDROME¹²⁶, two disorders with strong striatal underpinnings.

Hindbrain: central analgesia. Beside their actions in the amygdala, cannabinoid agonists can influence the central processing of pain by interacting with CB_1 receptors in the periaqueductal grey¹²⁷, rostral ventromedial medulla¹²⁸ and spinal trigeminal nucleus¹²⁹. At each of these sites, CB_1 activation depresses GABA release through a presynaptic mechanism, without causing significant changes in somatic membrane conductances^{129,130}. In the trigeminal nucleus, glycinergic transmission also is inhibited¹²⁹. Painful stimuli elicit anandamide release in the rat periaqueductal grey¹⁶, and systemic administration of CB_1 antagonists produces HYPERALGESIA in rats and mice^{63,131,132}. So, noxious stimuli can engage a central analgesic circuit operated by the endocannabinoids, which, working in combination with a parallel mechanism in the periphery, could underlie the analgesic properties of cannabinoid drugs¹³³.

Regulation of glutamate transmission
Principal neurons in the hippocampus and cerebellum use endocannabinoids to carry out a signalling process that is analogous in mechanism, but opposite in sign, to DSI, called depolarization-induced suppression of excitation (DSE)¹⁰⁸. Like DSI, DSE is induced by neuronal depolarization, it consists of a transient depression in neurotransmitter release, and it requires a retrograde endocannabinoid messenger. But unlike DSI, DSE targets glutamatergic rather than GABA axon terminals, and results therefore in reduced excitatory input to the affected cell¹⁰⁸ (FIG. 8).

Do DSI and DSE occur simultaneously in a single neuron and, if so, how are they coordinated? In cerebellar Purkinje cells, the two opposing phenomena can be elicited by similar stimulation protocols and so are likely to coexist⁴². Although they might be topographically segregated along the longitudinal axis of the neuron, the significance of their coexistence is not known. On the other hand, in the hippocampus, the induction of DSE requires longer periods of depolarization than does DSI, and its magnitude is smaller¹⁰⁴. This could be explained by the lower sensitivity of glutamatergic terminals to endocannabinoid activation¹⁰⁴, which would indicate that a switch from DSI to DSE might occur when endocannabinoid concentrations at hippocampal synapses attain a certain threshold value. Again, the role of such a switch, if any, is undefined.

LEVODOPA-INDUCED DYSKINESIAS
Unwanted movements that appear after prolonged use of the anti-Parkinsonian drug levodopa, β -(3,4-dihydroxyphenyl)-L-alanine.

TOURETTE'S SYNDROME
A psychiatric disorder of unknown aetiology, characterized by the presence of compulsive vocal and motor tics.

HYPERALGESIA
A state of enhanced sensitivity to painful stimuli.

Box 2 | Addicted to food

Although it is well established that marijuana smokers eat more marshmallows than they should, exactly why this occurs is still quite mysterious. However, a few facts seem to be clear¹⁵⁰. Cannabinoid agonists stimulate food intake in partially fed animals. This effect is probably due to the activation of CB₁ receptors and is accompanied by enhanced food palatability. Moreover, CB₁ antagonists such as rimonabant reduce food intake and body weight in animals. Last, feeding status and feeding-regulating hormones such as leptin can affect endocannabinoid synthesis in the hypothalamus¹⁵¹, as well as in intestinal tissue¹⁵². The neural substrates of these actions have not been elucidated, but their therapeutic potential is under study. The cannabinoid agonist THC (dronabinol, Marinol) has been approved by the US Food and Drug Administration for the treatment of anorexia associated with AIDS, and the antagonist rimonabant is in advanced clinical development for the treatment of obesity. In a proof-of-concept trial (Phase II), which was recently completed, the drug produced a marked reduction of body weight in obese patients; after four months of treatment, patients taking rimonabant lost 4.5 kg, whereas those taking placebo lost only 1 kg. Larger trials (Phase III) are now underway in the United States and Europe to compare the effects of rimonabant and placebo on obesity, hyperlipidaemia and diabetes. Importantly, rimonabant is also under consideration as a treatment for alcohol and tobacco abuse, a reminder of the common neural substrates that underlie drug and food rewards¹⁵³.

Inhibition of glutamatergic neurotransmission by cannabinoid agonists has been documented in a variety of brain structures besides the hippocampus and cerebellum. These include the prefrontal cortex¹³⁴, amygdala⁸⁶, nucleus accumbens⁸⁵, striatum⁷⁹ and substantia nigra pars reticulata¹³⁵. Whether such effects reflect the existence of regional DSE-like phenomena is an important question that remains to be addressed.

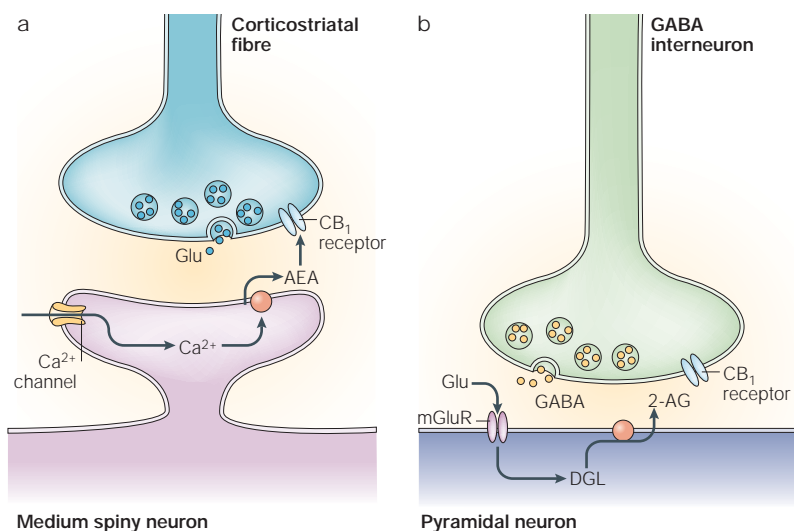


Figure 9 | Roles of the endocannabinoids in long-term synaptic plasticity. **a** | Repetitive activation of corticostriatal fibres causes a persistent reduction of glutamate release, called long-term depression (LTD), which might be mediated by anandamide. The elevated Ca²⁺ concentrations produced in postsynaptic spines of striatal medium spiny neurons after the stimulation could trigger anandamide (AEA) formation, which in turn might induce LTD by engaging CB₁ cannabinoid receptors on glutamatergic axon terminals. **b** | High-frequency stimulation of glutamatergic Schaffer collaterals in the hippocampus elicits a prolonged reduction of GABA (γ -aminobutyric acid) release that might be mediated by 2-arachidonoylglycerol (2-AG). This heterosynaptic form of plasticity, called inhibitory-LTD (I-LTD), is induced when glutamate activates metabotropic receptors (mGluR) on pyramidal neurons, eliciting 2-AG formation through the diacylglycerol lipase (DGL) pathway. 2-AG might then travel sideways to engage CB₁ receptors on contiguous terminals of GABA interneurons, producing I-LTD.

Other neurotransmitters

The ability of cannabinoid agonists to inhibit the release of neurotransmitters in the CNS is not restricted to glutamate and GABA¹³⁶. A particularly convincing case has been made for acetylcholine, the release of which is reduced by cannabinoids both *in vitro* and *in vivo*, and is enhanced by inactivation of CB₁ receptors^{136–138}. Acetylcholine release in the neocortex and hippocampus facilitates learning and memory, so disruption of this facilitatory process might contribute to the detrimental effects of cannabinoid drugs on cognition. Cannabinoids also reduce the release of the biogenic amines noradrenaline and serotonin¹³⁶, and the neuropeptide CCK-8 (REF. 139). Analogous, but as yet unknown, actions on peptide release in the hypothalamus might underlie the central involvement of the endocannabinoid system in the secretion of stress hormones and regulation of appetite (BOX 2).

Enduring inhibitions

Basal ganglia: a role in habit formation? High-frequency stimulation of cortical fibres that innervate the striatum leads to a form of persistent synaptic plasticity called long-term depression (LTD)¹⁴⁰. Like its hippocampal counterpart, striatal LTD is induced when Ca²⁺ enters the somatodendritic compartment of projection neurons, and is expressed as a decrease in glutamate release from axon terminals of corticostriatal fibres¹⁴¹. These analogies with DSI are suggestive of an endocannabinoid-dependent process, an idea that has been confirmed experimentally⁴⁴. Striatal LTD is absent in CB₁-deficient mice and is blocked by the CB₁ antagonist rimonabant; moreover, it is induced in a CB₁-dependent manner by anandamide or AM404 (REF. 44) (FIG. 9a).

A similar form of endocannabinoid-dependent LTD can be produced by low-frequency stimulation of cortical fibres that innervate the nucleus accumbens⁴⁵. Despite differences in induction protocols *in vitro* – one is produced by high-frequency⁴⁴, the other by low-frequency stimulation⁴⁵ – striatal and accumbal LTD could serve complementary functions. For example, they might both contribute to habit formation, a type of striatum-dependent learning that underlies the development of motor skills and is implicated in the pathogenesis of drug addiction¹⁴². Notably, cannabinoid drugs provoke in rats a relapse to drug-seeking behaviour after prolonged periods of abstinence, whereas CB₁ antagonists attenuate the relapse induced by drug-associated cues^{143,144}. These findings have provided the rationale for current clinical trials of rimonabant as a treatment for alcohol and tobacco addiction (BOX 2).

Hippocampus: a role in cognition? In the hippocampal CA1 field, stimulation protocols that cause long-term potentiation at excitatory synapses onto pyramidal neurons simultaneously produce LTD at adjacent inhibitory synapses (I-LTD)¹⁴⁶. Like striatal LTD, I-LTD might be endocannabinoid-mediated, but its molecular mechanism seems to be remarkably different. According to a current model, glutamate released from excitatory terminals activates metabotropic receptors on dendrites

of pyramidal neurons, which in turn stimulates 2-AG formation through the DGL pathway. The newly formed endocannabinoid can then depress GABA release by engaging CB₁ receptors on inhibitory nerve endings⁴⁶ (FIG. 9b). How this long-lasting disinhibitory process interacts with other forms of endocannabinoid-dependent plasticity and contributes to the overall effects of cannabinoids on hippocampus-dependent learning will surely be the object of future discussion and experiments.

Missing pieces

A decade of research in the biology of the endocannabinoid system has led to a series of exciting discoveries. We have learned that the brain contains multiple endocannabinoid lipids, and that neurons produce them using membrane constituents as starting material. We have also discovered that these lipids behave differently from traditional transmitters. Rather than being secreted

from vesicle stores, they are released in a non-synaptic manner and combine with cannabinoid receptors located near their sites of synthesis.

Despite this progress, many crucial pieces of the endocannabinoid puzzle are still missing. For example, we need to map the neuronal circuits that produce anandamide and 2-AG, and this requires in turn the molecular characterization of the synthetic enzymes involved. We also need to understand how classical neurotransmitters and drugs of abuse interact with these circuits, and to explore the functional consequences of such interactions. Last, but not least, we must continue to develop selective pharmacological tools that target not only the different cannabinoid receptor subtypes, but also the mechanisms of endocannabinoid synthesis and deactivation. Although these tasks are far from trivial, what is already known about the endocannabinoid system indicates that they are well worth pursuing.

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 **Online links**

DATABASES
The following terms in this article are linked online to: **LocusLink:** <http://www.ncbi.nlm.nih.gov/LocusLink/> CB₁ | CB₂ | D₂ receptor | FAAH | MGL

FURTHER INFORMATION
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International Association for Cannabis as Medicine: <http://www.acmed.org/>
National Institute on Drug Abuse: <http://www.drugabuse.gov/ResearchReports/Marijuana/Marijuana6.html>
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