# Role of Endogenous Cannabinoids in Synaptic Signaling

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Freund, Tamás F., István Katona, and Daniele Piomelli. Role of Endogenous Cannabinoids in Synaptic Signaling. Physiol Rev 83: 1017–1066, 2003; 10.1152/physrev.00004.2003.—Research of cannabinoid actions was boosted in the 1990s by remarkable discoveries including identification of endogenous compounds with cannabimimetic activity (endocannabinoids) and the cloning of their molecular targets, the  $CB_1$  and  $CB_2$  receptors. Although the existence of an endogenous cannabinoid signaling system has been established for a decade, its physiological roles have just begun to unfold. In addition, the behavioral effects of exogenous cannabinoids such as delta-9tetrahydrocannabinol, the major active compound of hashish and marijuana, await explanation at the cellular and network levels. Recent physiological, pharmacological, and high-resolution anatomical studies provided evidence that the major physiological effect of cannabinoids is the regulation of neurotransmitter release via activation of presynaptic CB<sub>1</sub> receptors located on distinct types of axon terminals throughout the brain. Subsequent discoveries shed light on the functional consequences of this localization by demonstrating the involvement of endocannabinoids in retrograde signaling at GABAergic and glutamatergic synapses. In this review, we aim to synthesize recent progress in our understanding of the physiological roles of endocannabinoids in the brain. First, the synthetic pathways of endocannabinoids are discussed, along with the putative mechanisms of their release, uptake, and degradation. The fine-grain anatomical distribution of the neuronal cannabinoid receptor CB<sub>1</sub> is described in most brain areas, emphasizing its general presynaptic localization and role in controlling neurotransmitter release. Finally, the possible functions of endocannabinoids as retrograde synaptic signal molecules are discussed in relation to synaptic plasticity and network activity patterns.

#### I. INTRODUCTION

Descriptions of the Cannabis sativa plant and its medicinal properties were already accessible to Greek and Roman physicians in the first century AD, when Dioscorides included the plant in his classic textbook of pharmacology, entitled Materia Medica ("The Materials of Medicine"). Ancient Indian and Chinese medical writers were even more accurate than their European colleagues in describing the remarkable physiological and psychological effects of this plant (for review, see Ref. 241). We know now that these effects, which in humans include a variable combination of euphoria, relaxation, reflex tachycardia, and hypothermia, are primarily produced by the dibenzopyrane derivative, delta-9-tetrahydrocannabinol (delta-9-THC), present in the yellow resin that covers the leaves and flower clusters of the ripe female plant. The chemical structure of delta-9-THC was elucidated by the pioneering studies of R. Adams (6) and Gaoni and Mechoulam (114). Unlike morphine, cocaine, and other alkaloids of plant origin, delta-9-THC is a highly hydrophobic compound, a property that, curiously enough, has slowed the progress on the mode of action of this compound for nearly three decades. The affinity of delta-9-THC for lipid membranes erroneously suggested, indeed, that the drug's main effect was to modify in a nonselective manner the fluidity of cell membranes rather than to activate a selective cell-surface receptor (157, 207).

Two series of events contributed to a radical change of this view. First, motivated by the potential therapeutic applications of cannabis-like ("cannabimimetic") molecules, laboratories in academia and the pharmaceutical industry began to develop families of synthetic analogs of delta-9-THC. These agents exerted pharmacological effects that were qualitatively similar to those of delta-9-THC but displayed both greater potency and stereoselectivity. The latter feature cannot be reconciled with nonspecific membrane interactions, providing the first evidence that delta-9-THC exerts its effects by combining with a selective receptor. Second, as a result of these synthetic efforts, it became possible to explore directly the existence of cannabinoid receptors by using standard radioligand binding techniques. In 1988, Howlett and her co-workers (84, 167) described the presence of high-affinity binding sites for cannabinoid agents in brain membranes and showed that these sites are coupled to inhibition of adenylyl cyclase activity. Conclusively supporting these findings, in 1990 Matsuda et al. (236) serendipitously came across a complementary DNA encoding for the first G protein-coupled cannabinoid receptor, now known as  $CB_1$ .

In heterologous expression systems,  $CB_1$  receptors were found to be functionally coupled to multiple intracellular signaling pathways, including inhibition of adenylyl cyclase activity, inhibition of voltage-activated calcium channels, and activation of potassium channels (56, 148, 221, 222, 236, 239). In situ hybridization and immunohistochemical studies have demonstrated that  $CB_1$  receptors are abundantly expressed in discrete regions and cell types of the central nervous system (CNS) (see also sect. III) but are also present at significant densities in a variety of peripheral organs and tissues (41, 225, 226, 235, 345). The selective distribution of  $CB_1$  receptors in the CNS provides a clear anatomical correlate for the cognitive, affective, and motor effects of cannabimimetic drugs.

The cloning and characterization of CB<sub>1</sub> receptors left several important problems unsolved. Since antiquity, it has been known that the actions of *Cannabis* and delta-9-THC are not restricted to the CNS, but include effects on nonneural tissues such as reduction of inflammation, lowering of intraocular pressure associated with glaucoma, and relief of muscle spasms. Are these peripheral effects all produced by activation of CB<sub>1</sub> receptors? An initial answer to this question was provided by the discovery of a second cannabinoid receptor exquisitely expressed in cells of immune origin (260). This receptor, called CB<sub>2</sub>, only shares  $\sim 44\%$  sequence identity with its brain counterpart, implying that the two subtypes diverged long ago in evolution. The intracellular coupling of the  $CB_2$  receptor resembles, however, that of the  $CB_1$ receptor; for example, in transfected cells, CB<sub>2</sub> receptor activation is linked to the inhibition of adenylyl cyclase activity (113).

The experience with opioid receptors and the enkephalins has accustomed scientists to the idea that whenever a receptor is present in the body, endogenous factor(s) that activate this receptor also exist. Not surprisingly, therefore, as soon as cannabinoid receptors were described, a search began to identify their naturally occurring ligand(s). One way to tackle this problem was based on the premise that, like other neurotransmitters and neuromodulators, an endogenous cannabinoid substance should be released from brain tissue in a calciumdependent manner. Taking this route, Howlett and coworkers incubated rat brain slices in the presence of a calcium ionophore and determined whether the media from these incubations contained a factor that displaced the binding of labeled CP-55940, a cannabinoid agonist, to brain membranes. These studies demonstrated that a cannabinoid-like activity was indeed released from stimulated slices, but the minute amounts of this factor did not allow the elucidation of its chemical structure (97, 98).

Devane, Mechoulam, and co-workers (85, 243), at the Hebrew University in Jerusalem, adopted a different strategy. Reasoning that endogenous cannabinoids may be as hydrophobic as delta-9-THC, they subjected porcine brains to organic solvent extraction and fractionated the lipid extract by chromatographic techniques while measuring cannabinoid binding activity. This approach turned out to be highly successful, and the researchers were able to isolate a lipid cannabinoid-like component, which they characterized by mass spectrometry and nuclear magnetic resonance spectroscopy as the ethanolamide of arachidonic acid. They named this novel compound "anandamide" after the sanskrit "ananda," inner bliss.

The chemical synthesis of anandamide confirmed this structural identification and allowed the characterization of its pharmacological properties (112). In vitro and in vivo tests showed a great similarity of actions between anandamide and cannabinoid drugs. Anandamide reduced the electrogenic contraction of mouse vas deferens and closely mimicked the behavioral responses produced by delta-9-THC in vivo; in the rat, the compound was found to produce analgesia, hypothermia, and hypomotility. However, these effects may not be exclusively due to cannabinoid receptor activation, as anandamide is readily metabolized to arachidonic acid, which can be converted in turn to a variety of biologically active eicosanoid compounds. Subsequent studies demonstrated that anandamide is released from brain neurons in an activity-dependent manner (89, 126) and elucidated the unique biochemical routes of anandamide formation and inactivation in the CNS (25, 44, 45, 69, 89). Thus anandamide fulfills all key criteria that define an endogenous cannabinoid (endocannabinoid) substance.

In their 1992 study, Devane, Mechoulam, and coworkers (242) reported that several lipid fractions from the rat brain contained cannabinoid-binding activity, in addition to anandamide's. In characterizing these fractions, they discovered that some of them were composed of polyunsaturated fatty acid ethanolamides similar to anandamide (e.g., eicosatrienoylethanolamide), but others were instead constituted of a distinct lipid component, sn-2-arachidonoyl-glycerol (2-AG) (242). Sugiura et al. (330) arrived independently to the same conclusion. That polyunsaturated fatty acid ethanolamides should mimic anandamide, to which they are structurally very similar, does not come as a great surprise. Moreover, the pharmacological properties of these fatty acid ethanolamides, essentially indistinguishable from those of anandamide, and their scarcity in brain relegate them, at least for the moment, to a position secondary to anandamide's. We cannot say the same for 2-AG. This lipid, considered until now a mere intermediate in glycerophospholipid turnover (see sect. II), is present in the brain at concentrations that are  $\sim$ 170-fold greater than those of an and amide and possesses two pharmacological properties that make it crucially different from the latter: it binds to both  $CB_1$  and CB<sub>2</sub> cannabinoid receptors with similar affinities, and it activates CB<sub>1</sub> receptors as a full agonist, whereas anandamide acts as a partial agonist.

Research of endocannabinoids begs for a conjunction of in situ biochemistry and physiology. We have learned much over the past 10 years on the behavioral effects of these molecules, on how these lipid mediators are produced physiologically, and on the functional roles that they may serve. A major step was the discovery that depolarization-induced suppression of inhibition (DSI; or excitation, DSE), a type of short-term synaptic plasticity originally discovered in the cerebellum and the hippocampus (214, 288), is mediated by endocannabinoids (199, 200, 271, 375). This discovery allowed the results of over a decade of research on retrograde synaptic signaling in these networks to be considered as functional characteristics of endocannabinoid signaling. The substrate of retrograde signaling and DSI is the predominantly presynaptic distribution of  $CB_1$  receptors on axon terminals in the hippocampus (188), as well as throughout the brain, where activation of  $CB_1$  by endocannabinoids can efficiently veto neurotransmitter release in many distinct types of synapses (see sect. IV). The conditions of synthesis, release, distance of diffusion, duration of effect, and site of action were all extensively characterized for the mediator of DSI (for review, see Ref. 10) that turned out to be an endocannabinoid (271, 375). The fact that neurons are able to control the efficacy of their own synaptic input in an activity-dependent manner (a phenomenon called retrograde synaptic signaling) is functionally very important, since this mechanism may subserve several functions in information processing by neuronal networks from temporal coding and oscillations to group selection and the fine tuning of signal-to-noise ratio. The crucial involvement of endocannabinoids in these functions just began to emerge from recent studies, which are reviewed in section v. Due to the exceptionally rapid expansion of this field in recent years (and to our special interest in neuronal signaling in complex integrative centres of the brain), we decided to focus the present review on questions related to the composition of the endocannabinoid system and its physiological roles in controlling brain activity at the regional and cellular levels as synaptic signal molecules. We did not aim to provide detailed accounts of studies dealing with other, similarly important, aspects of cannabinoid research, which have been dealt with in excellent recent reviews, e.g., about the relation of the endocannabinoid system to pain modulation (281, 366), the immune system (194), neuroprotection (136), and addiction (228).

The final message of the present review is that to understand the possible physiological functions of the endogenous cannabinoids, their roles in normal and pathological brain activity, pharmacological agents targeting the cascade of anandamide and 2-AG formation, release, uptake, and degradation will have to be developed. Such drugs, which undoubtedly will become invaluable research tools to study the potential functions listed above, may also provide novel therapeutic approaches to diseases whose clinical, biochemical, and pharmacological features suggest a link with the endogenous cannabinoid system.

# II. THE LIFE CYCLE OF THE ENDOCANNABINOIDS

### A. Introduction

A basic principle that has emerged from the last two decades of research on cellular signaling is that simple phospholipids such as phosphatidylcholine or phosphatidylinositol should be regarded not only as structural components of the cell membrane, but also as precursors for transmembrane signaling molecules. Intracellular second messengers like 1,2-diacylglycerol (DAG) and ceramide are familiar examples of this concept. Along with their intracellular roles, however, lipid compounds may also serve important functions in the exchange of information between cells. Indeed, biochemical mechanisms analogous to those involved in the generation of DAG or ceramide give rise to biologically active lipids that leave their cell of origin to activate G protein-coupled receptors located on the surface of neighboring cells. Traditionally overshadowed by amino acid, amine, and peptide transmitters, biologically active lipids are now emerging as essential mediators of cell-to-cell communication within the CNS, where G protein-coupled receptors for multiple families of such compounds, including lysophosphatidic acid and eicosanoids, have been identified (67, 285).

In this section, we discuss the biochemical properties of endogenous lipids that activate brain cannabinoid receptors. These compounds share two common structural motifs: a polyunsaturated fatty acid moiety (e.g., arachidonic acid) and a polar head group consisting of ethanolamine or glycerol (Fig. 1). Because of these features, endocannabinoid substances seemingly resemble the eicosanoids, ubiquitous bioactive lipids generated through the enzymatic oxygenation of arachidonic acid. However, the endocannabinoids are clearly distinguished from the eicosanoids by their different biosynthetic routes, which do not involve oxidative metabolism. The two best characterized endocannabinoids, anandamide (arachidonoylethanolamide) (85) and 2-AG (242, 330), may be produced instead through cleavage of phospholipid precursors present in the membranes of neurons, glia, and other cells. In the following sections, we will first focus on the biochemical pathways that lead to the formation of endocannabinoids in neurons and then turn to the mechanism by which these compounds are deactivated.

#### **B.** Biosynthetic Pathways

#### 1. Anandamide biosynthesis

Anandamide formation via energy-independent condensation of arachidonic acid and ethanolamine was described in brain tissue homogenates soon after the discovery of anandamide and was attributed to an enzymatic activity that was termed "anandamide synthase" (81, 83, 201). Subsequent work has demonstrated, however, that this reaction is in fact catalyzed by fatty acid amide hydrolase (FAAH), the primary enzyme of anandamide hydrolysis, acting in reverse (203). Since FAAH requires high concentrations of arachidonate and ethanolamine to synthesize anandamide, higher than those normally found in cells, this enzyme is unlikely to play a role in the physiological formation of anandamide (for further discussion, see sect.  $\Pi C6$ ).

Another model for anandamide biosynthesis is illustrated schematically in Figure 2. According to this model, anandamide may be produced via hydrolysis of the phospholipid precursor *N*-arachidonoyl phosphatidylethanolamine (PE), catalyzed by a phospholipase D (PLD)-type activity (89, 331, 332). The precursor consumed in this reaction may be resynthesized by a separate enzyme activity, *N*-acyltransferase (NAT), which may transfer an arachidonate group from the sn-1 glycerol ester position of phospholipids to the primary amino group of PE (89). The validity of this model was initially questioned, be-



Anandamide



Virodhamine



2-Arachidonoylglycerol

Noladin ether

FIG. 1. Molecular structure of endogenous lipids that activate brain cannabinoid receptors. These endocannabinoid compounds share two common structural motifs: a polyunsaturated fatty acid moiety (e.g., arachidonic acid) and a polar head group consisting of ethanolamine or glycerol. For details, see section II, *A* and *B*4.

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FIG. 2. Scheme illustrating the possible mechanism of anandamide formation. PE, phosphatidylethanolamine. For details, see section IIB1.

cause previous studies had failed to detect *N*-arachidonoyl PE in mammalian tissues (266, 267, 318). More recent chromatographic and mass spectrometric analyses have unambiguously shown, however, that *N*-arachidonyl PE is present in brain and other tissues, where it may serve as a physiological precursor for anandamide (44, 46, 89, 332).

Although biochemically distinct, anandamide formation and *N*-arachidonoyl PE synthesis are thought to proceed in parallel. Both reactions may be initiated by intracellular Ca<sup>2+</sup> rises (44, 45, 89, 315, 331, 332) and/or by activation of neurotransmitter receptors (125, 327). For example, administration of dopamine D<sub>2</sub>-receptor agonists to rats in vivo causes a profound stimulation of anandamide release in the striatum (125), which is likely mediated by de novo anandamide synthesis (A. Giuffrida and D. Piomelli, unpublished observations). Unfortunately, the two key enzyme activities responsible for these reactions, PLD and NAT, have only been partially characterized, and their molecular properties are still unknown (44, 45, 282, 283).

#### 2. 2-AG biosynthesis

There are two possible routes of 2-AG biosynthesis in neurons, which are illustrated in Figure 3. Phospholipase C (PLC)-mediated hydrolysis of membrane phospholipids may produce DAG, which may be subsequently converted to 2-AG by diacylglycerol lipase (DGL) activity. Alternatively, phospholipase  $A_1$  (PLA<sub>1</sub>) may generate a lysophos-



FIG. 3. Scheme illustrating the possible mechanism of 2-arachidonoylglycerol (2-AG) formation. DAG, 1,2-diacylglycerol; DGL, 1,2-diacylglycerol lipase; PI, phosphatidylinositol; PLC, phospholipase C; PLA<sub>1</sub>, phospholipase A<sub>1</sub>. For details, see section IIB2.

pholipid, which may be hydrolyzed to 2-AG by a lyso-PLC activity. In the intestine, where 2-AG was originally identified (242), this compound accumulates during the digestion of dietary triglycerides and phospholipids, catalyzed by pancreatic lipases (39). The fact that various, structurally distinct inhibitors of PLC and DGL activities prevent 2-AG formation in cultures of cortical neurons indicates that the PLC/DGL pathway may play a primary role in this process (328). The molecular identity of the enzymes involved remains undefined, although the purification of rat brain DGL has been reported (100, 101).

As first suggested by experiments with acutely dissected hippocampal slices, neural activity may evoke 2-AG biosynthesis in neurons by elevating intracellular  $Ca^{2+}$  levels (327, 328). In the hippocampal slice preparation, electrical stimulation of the Schaffer collaterals (a glutamatergic fiber tract that projects from CA3 to CA1 neurons) produces a fourfold increase in 2-AG formation, which is prevented by the Na<sup>+</sup> channel blocker tetrodotoxin or by removing  $Ca^{2+}$  from the medium. Noteworthy, the local concentrations reached by 2-AG after stimulation are in the low micromolar range (328), which should be sufficient to activate the dense population of CB<sub>1</sub> receptors present on axon terminals of hippocampal GABAergic interneurons (187, 188). The possible significance of this process for hippocampal network activity is discussed in sections v and vC.

In addition to neural activity, certain neurotransmitter receptors also may be linked to 2-AG formation. For example, in primary cultures of cortical neurons, glutamate stimulates 2-AG synthesis by allowing the entry of  $Ca^{2+}$  through activated *N*-methyl-*D*-aspartate (NMDA) receptor channels (327). Interestingly, this response is strongly enhanced by the cholinergic agonist carbachol, which has no effect on 2-AG formation when applied alone (327). The molecular basis of the synergistic interaction between NMDA and carbachol is unclear at present but deserves further investigation in light of the potential roles of 2-AG in hippocampal retrograde signaling (see sect. v*C*).

# 3. Fatty acid ethanolamides that do not interact with known cannabinoid receptors

The anandamide precursor *N*-arachidonoyl PE belongs to a family of *N*-acylated PE derivatives, which contain different saturated or unsaturated fatty acids linked to their ethanolamine moieties and give rise to the corresponding fatty acid ethanolamides (FAE). These compounds generally lack CB<sub>1</sub> receptor-binding activity but display a number of remarkable effects and possible biological functions. In this regard, two FAE have been studied in some detail, palmitoylethanolamide (PEA) and oleoylethanolamide (OEA). PEA exerts profound analgesic and anti-inflammatory effects in vivo, which have been attributed to its ability to interact with a putative receptor site sensitive to the CB<sub>2</sub>-preferring antagonist SR144528 (48, 99, 174, 238). The molecular identity of this site is unknown, although it is probably distinct from the CB<sub>2</sub> receptor whose gene has been cloned (260). PEA is present at high levels in skin and other tissues where, together with locally produced anandamide, may participate in the peripheral control of pain initiation (48).

Despite its chemical similarity with PEA, OEA shows weak analgesic properties (49) but exerts potent appetitesuppressing effects in the rat (303). Because these effects are prevented by sensory deafferentation, and intestinal OEA biosynthesis is linked to the feeding state (increasing in fed and decreasing in starved animals), it has been suggested that OEA may be involved in the peripheral regulation of feeding (303).

#### 4. Other endogenous agonists at cannabinoid receptors

A series of close structural analogs of anandamide with activity at cannabinoid receptors have been isolated from brain tissue. These compounds, which include eicosatrienoylethanolamide and docosatetraenoylethanolamide (144), may be generated through the same enzymatic route as anandamide, albeit in smaller quantities.

Distinct from these polyunsaturated ethanolamides as well as from 2-AG are two recently discovered brain lipids: 2-arachidonoyl glyceryl ether (noladin ether) (143) and O-arachidonoyl ethanolamine (virodhamine) (291) (Fig. 1). Noladin ether was isolated from porcine brain and identified by using a combination of mass spectrometry, nuclear magnetic resonance, and chemical synthesis. The compound binds to CB<sub>1</sub> receptors with high affinity in vitro [dissociation constant  $(K_{\rm D})$  21 nM] and produces cannabinoid-like effects in the mouse in vivo, including sedation, immobility, hypothermia, and antinociception (143). Virodhamine was identified in rat brain by mass spectrometry and chemical synthesis and shown to weakly activate CB<sub>1</sub> receptors in a <sup>35</sup>S-labeled guanosine 5'-O-(3-thiotriphosphate) (GTP $\gamma$ S) binding assay (halfmaximal effective concentration,  $1.9 \mu M$ ) in which the compound also displayed partial agonist activity (291). Moreover, virodhamine decreases body temperature in the mouse, although less effectively than anandamide, and inhibits anandamide transport in RBL-2H3 cells (291). A possible confounding factor in these studies is due, however, to the chemical instability of virodhamine, which in an aqueous environment is rapidly converted to anandamide. The formation and inactivation of these molecules, as well as their physiological significance, is the subject of ongoing investigations (105).

#### ENDOCANNABINOIDS IN SYNAPTIC SIGNALING

#### 5. Endocannabinoid release

Both anandamide and 2-AG may be generated by and released from neurons through a mechanism that does not require vesicular secretion. However, unlike classical or peptide neurotransmitters, which readily diffuse across the synaptic cleft, anandamide and 2-AG are hydrophobic molecules and, as such, are constrained in their movements through the aqueous environment surrounding cells. How may these compounds reach their receptors on neighboring neurons?

Experiments with bacterial PLD suggest that, in cortical neurons,  $\sim 40\%$  of the anandamide precursor *N*-arachidonoyl PE is localized to the cell surface (45), which also contains 2-AG precursors such as phosphoinositol phosphate and bisphosphate (341). This suggests that both endocannabinoids may be generated in the plasmalemma, where they are ideally poised to access the external medium. As with other lipid compounds, the actual release step may be mediated by passive diffusion and/or facilitated by the presence of lipid-binding proteins such as the lipocalins (9).

The existence of different routes for the synthesis of anandamide and 2-AG suggests that these two endocannabinoids could in principle operate independently of each other. This idea is supported by three main findings. First, electrical stimulation of hippocampal slices increases the levels of 2-AG, but not those of anandamide (328). Second, activation of dopamine  $D_2$  receptors in the striatum enhances the release of anandamide, but not that of 2-AG (125). Third, activation of NMDA receptors in cortical neurons in culture increases 2-AG levels but has no effect on an andamide formation, which requires instead the simultaneous activation of NMDA and  $\alpha$ -7 nicotinic receptors (327). It is unclear at present whether these differences reflect regional segregation of the PLC/ DGL and PLD/NAT pathways, the existence of receptoractivated mechanisms linked to the generation of specific endocannabinoids, or both.

# C. Termination of Endocannabinoid Effects: Transport and Degradation

#### 1. Anandamide transport

Carrier-mediated uptake into nerve endings and glia, probably the most frequent mechanism of neurotransmitter inactivation, is also involved in the clearance of lipid messengers. This idea may appear at first counterintuitive: why should a lipid molecule need a carrier protein to cross plasma membranes when it could do so by passive diffusion? A large body of evidence indicates, however, that even very simple lipids such as fatty acids are transported into cells by protein carriers, several families of which have now been molecularly characterized (2, 160, 316). Indeed, carrier-mediated transport may provide a rapid and selective means of delivering lipid molecules to specific cellular compartments (for example, enzyme complexes implicated in lipid metabolism). Thus it is not surprising that neural cells might adopt the same strategy to interrupt lipid-mediated signaling (Fig. 4).



FIG. 4. Scheme illustrating the possible mechanism of anadamide uptake and degradation by an as yet unidentified transporter and a hydrolytic enzyme, fatty acid amide hydrolase (FAAH), respectively. For details, see section II, C1 and C6.

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Anandamide transport meets four key criteria of a carrier-mediated process: saturability, fast rate, temperature dependence, and substrate selectivity (25, 89, 156). In rat cortical neurons in primary culture, the uptake of exogenous [<sup>3</sup>H]anandamide is saturable at 37°C, reaches 50% of its maximum within 4 min, and displays a Michaelis constant ( $K_{\rm m}$ ) of 1.2  $\mu$ M and a maximum accumulation rate ( $V_{\rm max}$ ) of 90.9 pmol  $\cdot$  min<sup>-1</sup>  $\cdot$  mg protein<sup>-1</sup> (25). Comparable kinetic values are observed in rat cortical astrocytes ( $K_{\rm m} = 0.32 \ \mu$ M;  $V_{\rm max} = 171 \ \text{pmol} \cdot \text{min}^{-1} \cdot \text{mg}$  protein<sup>-1</sup>) and human astrocytoma cells ( $K_{\rm m} = 0.6 \ \mu$ M;  $V_{\rm max} = 14.7 \ \text{pmol} \cdot \text{min}^{-1} \cdot \text{mg}$  protein<sup>-1</sup>) (25, 286), as well as in a variety of nonneural cells (for a review, see Ref. 108). For example, RBL-2H3 basophilic leukemia cells accumulate [<sup>3</sup>H]anandamide with a  $K_{\rm m}$  of 11.4  $\mu$ M and a  $V_{\rm max}$  of 17.5  $\times 10^{-17}$  mol/cell (293).

Anandamide transport differs from that of amine and amino acid transmitters in that it does not require cellular energy or external Na<sup>+</sup>, implying that it may be mediated through facilitated diffusion (25, 156, 286, 293). Because anandamide is rapidly hydrolyzed within cells (see sect. IIC6), it is reasonable to hypothesize that intracellular breakdown contributes to the rate of anandamide transport. Accordingly, HeLa cells that overexpress the anandamide-hydrolyzing enzyme FAAH also display higher than normal rates of [<sup>3</sup>H]anandamide accumulation (73). However, in primary cultures of rat neurons and astrocytes or in adult rat brain slices, FAAH inhibitors have no effect on [<sup>3</sup>H]anandamide transport at concentrations that completely abrogate [<sup>3</sup>H]anandamide hydrolysis (23, 25, 124). From these results it is reasonable to conclude that anandamide transport in the CNS is largely independent of intracellular hydrolysis. Whether persistent disruption of FAAH activity may eventually change the distribution of anandamide between intracellular and extracellular pools is an interesting question that warrants examination.

The substrate selectivity of anandamide transport has been investigated in rat cortical neurons and astrocytes (25, 89) and, more systematically, in human astrocytoma cells (286). In the latter model, [<sup>3</sup>H]anandamide uptake is not affected by a variety of lipids that bear close structural resemblance to anandamide, including arachidonic acid, PEA, ceramide, prostaglandins, leukotrienes, hydroxyeicosatetraenoic acids, and epoxyeicosatetraenoic acids. Furthermore, [<sup>3</sup>H]anandamide accumulation in these cells is insensitive to substrates or inhibitors of fatty acid transport (phloretin), organic anion transport (p-amino-hippurate and digoxin), and P-glycoproteins (verapamil, quinidine) (286). However, [<sup>3</sup>H]anandamide uptake is competitively blocked by nonradioactive anandamide (IC<sub>50</sub> = 15.1  $\mu$ M) and by the anandamide analog N-(4-hydroxyphenyl)-arachidonamide (AM404) (IC<sub>50</sub> =  $2.2 \ \mu\text{M}$ ) (24, 286). A similar sensitivity to AM404 has been reported for rat cortical and cerebellar neurons (25, 176),

rat cortical astrocytes (25), and rat brain slices (24). Inhibitory effects of AM404 on anandamide accumulation also have been observed in a number of nonneural cells, although the concentrations of AM404 needed to produce such effects are generally higher than in neurons (for a review, see Ref. 108). Together, these data are consistent with the view that anandamide is internalized by neurons and astrocytes through a selective process of facilitated diffusion. The molecular identity of the protein(s) responsible for this process is, however, unknown.

#### 2. 2-AG transport

Nonradioactive 2-AG prevents [<sup>3</sup>H]anandamide uptake in various cell types, suggesting that the two endocannabinoids may compete for the same transport system. Three observations support this hypothesis. First, in astrocytoma and other cells, [<sup>3</sup>H]anandamide and [<sup>3</sup>H]2-AG are accumulated with similar kinetic properties (26, 286). For example, in C6 glioma cells, [<sup>3</sup>H]2-AG uptake displays a  $K_{\rm m}$  of 1.7  $\mu$ M and a  $V_{\rm max}$  of 240 pmol  $\cdot$  min<sup>-1</sup>  $\cdot$ mg protein $^{-1}$ , values that are very close to those obtained with [<sup>3</sup>H]anandamide (26). Second, anandamide and 2-AG can prevent each other's transport (24, 26). Third, the accumulation of either endocannabinoid is blocked with similar potencies by the transport inhibitor AM404 (24, 26). Thus AM404 inhibits [<sup>14</sup>C]anandamide and [<sup>3</sup>H]2-AG accumulation in C6 glioma cells with  $IC_{50}$  values of 7.5 and 10.2  $\mu$ M, respectively (26).

Despite these similarities, differences in the properties of anandamide and 2-AG uptake also have been documented. For example, incubation with arachidonic acid causes a marked reduction in [<sup>3</sup>H]2-AG uptake by astrocytoma cells, but it has no effect on [3H]anandamide accumulation (24). Two alternative explanations may be offered for this discrepancy. Arachidonic acid may directly interfere with a 2-AG carrier distinct from anandamide's, or the fatty acid may indirectly prevent the facilitated diffusion of [<sup>3</sup>H]2-AG by inhibiting its conversion to arachidonic acid (possibly through product inhibition) in the intracellular compartment. If the latter explanation is correct, agents that interfere with the incorporation of arachidonic acid into phospholipids, such as triacsin C (an inhibitor of acyl-CoA synthesis), also should decrease <sup>[3</sup>H]2-AG uptake. Accordingly, triacsin C selectively prevents the uptake of [<sup>3</sup>H]2-AG by astrocytoma cells, but not that of [<sup>3</sup>H]anandamide (24). Thus, although anandamide and 2-AG may utilize similar transport mechanisms, or even share a common one, they may differ in how their intracellular degradation affects the rate of transport.

#### 3. Structure-activity relationship

Anandamide and 2-AG share three common structural features: 1) a highly hydrophobic fatty acid chain, 2) an amide (anandamide) or an ester (2-AG) moiety, and 3)

a polar head group (Fig. 1). Systematic modifications in the hydrophobic carbon chain indicate that the structural requisites for substrate recognition by the putative anandamide transporter may be different from those of substrate translocation. Substrate recognition may require the presence of at least one *cis* double bond in the middle of the fatty acid chain, indicating a preference for substrates (or competitive inhibitors) with a fatty acid chain that can adopt an extended U-shaped conformation. In contrast, a minimum of four cis nonconjugated double bonds may be required for translocation, suggesting that a closed "hairpin" conformation is required in order for substrates to be moved across the membrane (286). Molecular modeling studies show that transport substrates (such as anandamide and 2-AG) have both extended and hairpin low-energy conformers (286). In contrast, extended but not hairpin conformations may be thermodynamically favored in pseudo-substrates such as oleoylethanolamide, which displace [<sup>3</sup>H]anandamide from transport without being internalized (286, 295).

The effects of head group modifications on anandamide transport have also been investigated (176, 286). The results suggest that ligand recognition may be maintained when the head group is removed (as in arachidonamide), or replaced with substantially bulkier moieties (as in AM404), and when an ester bond substitutes the amide bond (as in 2-AG). Notably, ligand recognition appears to be favored by replacing the ethanolamine group with a substituted hydroxyphenyl group [as in AM404 and its derivative *N*-(2-methyl-4-hydroxy-phenyl)arachidonamide (25, 79) or a furane group (215)] (Fig. 1).

#### 4. Distribution of anandamide transport in the CNS

Biochemical experiments have demonstrated the existence of anandamide transport in primary cultures of rat cortical neurons and astrocytes (25), as well as rat cerebellar granule cells (156). But what brain regions express the transporter is still unclear, primarily due to a lack of molecular understanding of the transporter(s) involved in this process. In one study, the CNS distribution of anandamide transport was investigated by exposing metabolically active rat brain slices to [<sup>14</sup>C]anandamide and measuring the distribution of radioactivity by autoradiography. The  $CB_1$  antagonist SR141716A (rimonabant) was included in the incubation medium to prevent binding of <sup>14</sup>C]anandamide to CB<sub>1</sub> receptors, and AM404 was used to differentiate transport-mediated [<sup>14</sup>C]anandamide accumulation from nonspecific association with cell membranes and cell debris (124). These experiments suggest that the somatosensory, motor, and limbic areas of the cortex, as well as the striatum, contain substantial levels of AM404-sensitive [<sup>14</sup>C]anandamide uptake. Other brain regions showing detectable transport include the hippocampus, the amygdala, the septum, the thalamus, the substantia nigra, and the hypothalamus (124).

#### 5. Inhibitors of anandamide transport

Although a variety of compounds have been shown to inhibit anandamide transport, the anandamide analog AM404 remains a standard of reference, mainly because of its relatively high potency and its ability to block anandamide transport both in vitro and in vivo (24, 127, 156, 176, 286, 293).

AM404 inhibits [<sup>3</sup>H]anandamide uptake in rat brain neurons and astrocytes (25), human astrocytoma cells (286), rat brain slices (24), and a variety of nonneural cell types (see, for review, Ref. 108). The inhibitor also enhances several CB1 receptor-mediated effects of anandamide, without directly activating cannabinoid receptors (24, 25). For example, AM404 increases anandamideevoked inhibition of adenylyl cyclase activity in cortical neurons (25), augments the presynaptic inhibition of GABA release produced by anandamide in the midbrain periaqueductal gray (PAG) (358), and mimics the effects of cannabinoid agonists on hippocampal depolarizationinduced suppression of inhibition (375) (see sect. vC). The fact that the cannabinoid antagonist SR141716A prevents these effects suggests that AM404 may act by preventing anandamide inactivation and enhancing its interactions with cannabinoid receptors. Importantly, however, AM404 also can be transported inside cells (286), where it may reach levels that are sufficient to inhibit anandamide degradation by FAAH (176).

The target selectivity of AM404 has been investigated in some detail. Initial studies showed that AM404 has no affinity for a panel of 36 potential targets, including G protein-coupled receptors, ligand-gated channels, and voltage-dependent channels (22). Subsequent work suggested, however, that AM404 may activate the capsaicinsensitive VR1 vanilloid receptor in vitro (27, 325, but see Ref. 215 for opposing results). It is unlikely that this effect occurs in vivo, since AM404 does not display any of the pharmacological properties of a vanilloid agonist (see below). Yet, these findings underscore the need to design novel inhibitors of anandamide transport endowed with greater target selectivity. Ongoing research efforts in this direction have led to the development of several arachidonic acid derivatives that are equivalent or slightly superior to AM404 in inhibiting anandamide transport in vitro (79, 215) and in vivo, with effects similar to those of AM404 (77).

Consistent with its low affinity for  $CB_1$  receptors, AM404 does not act as a direct cannabinoid agonist when administered to live animals. The compound has no antinociceptive effects in the mouse hot-plate test (25) and does not reduce arterial blood pressure in the urethaneanesthetized guinea pig (47). In the same models, however, AM404 magnifies the responses elicited by exogenous anandamide, actions that are prevented by the  $CB_1$ antagonist SR141716A (25, 47). Furthermore, when administered alone, AM404 reduces motor activity (22), attenuates apomorphine-induced yawning (22), decreases the levels of circulating prolactin (132), and alleviates the motor hyperactivity induced in the rat by striatal 3-nitropropionic acid lesions (206). These actions resemble those of anandamide and are blocked by SR141716A (22, 127), suggesting that endogenous anandamide may be involved. In keeping with this notion, systemic administration of AM404 in the rat causes a time-dependent increase in circulating anandamide levels (127).

The participation of anandamide in the effects of AM404 in vivo has been questioned (108) based on the ability of this compound to interact with vanilloid receptors in vitro (27, 325; but see Ref. 215). Yet, the fact that SR141716A blocks the motor inhibitory actions of AM404 at doses that are selective for  $CB_1$  receptors strongly argues for a predominant, if not unique, role of the endocannabinoid system in the behavioral response to AM404 administration. Furthermore, the pharmacological properties of AM404 are very different, often opposite to those of capsaicin and other vanilloid agonists. For example, capsaicin produces pain and bronchial smooth muscle constriction (336), whereas AM404 has no such effect when administered alone, and in fact enhances anandamide's analgesic and bronchodilatory actions (22, 49). The ability of intraperitoneal capsaicin to inhibit movement, described by Di Marzo et al. (91), superficially mimics one property of AM404, but should be viewed with caution, as it most likely results from the strong visceral pain and subsequent "freezing response" elicited by capsaicin. In conclusion, current evidence suggests that AM404 may magnify the actions of anandamide primarily by inhibiting the clearance of this compound from its sites of action.

#### 6. Anandamide hydrolysis: role of FAAH

Almost a decade before anandamide was discovered, Schmid and collaborators (265) identified a hydrolase activity in rat liver that catalyzes the hydrolysis of fatty acid ethanolamides to free fatty acid and ethanolamine (265). That anandamide may be a substrate for such an activity was first suggested by biochemical experiments (80, 81, 89, 159, 351) and then demonstrated by molecular cloning, heterologous expression, and genetic disruption of the enzyme involved (68, 69).

FAAH (previously called anandamide amidohydrolase and oleamide hydrolase) is an intracellular membrane-bound protein whose primary structure displays significant homology with the "amidase signature family" of enzymes (69, 119). It acts as a hydrolytic enzyme for fatty acid ethanolamides such as anandamide, but also for esters such as 2-AG (134, 204) and primary amides such as oleamide (70). Site-directed mutagenesis experiments indicate that this unusually wide substrate preference may be due to a novel catalytic mechanism involving the amino acid residue lysine-142. This residue may act as a general acid catalyst, favoring the protonation and consequent detachment of reaction products from the enzyme's active site (279). This mechanism was recently confirmed by the solution of the crystal structure of FAAH complexed with the active site-directed inhibitor methoxy arachidonyl fluorophosphonate (34).

In addition to FAAH, other enzymes may participate in the breakdown of anandamide and its fatty acid ethanolamide analogs. A PEA-hydrolyzing activity distinct from FAAH was described in rat brain membranes (80) and human megakaryoblastic cells (352). This activity was purified to homogeneity from rat lung and shown to possess a marked substrate preference for PEA over anandamide (353). PEA does not bind to any of the known cannabinoid receptors but produces profound analgesic and anti-inflammatory effects (48, 238), which are prevented by the CB<sub>2</sub>-preferring antagonist SR144528 (48, 49). Future studies will undoubtedly address the relative roles of FAAH and this newly discovered enzyme in the biological disposition of PEA and anandamide.

The ability of FAAH to act in reverse (i.e., to synthesize anandamide from arachidonic acid and ethanolamine) has generated some confusion as to the mechanism of anandamide formation. Early reports of anandamide synthesis from free arachidonate and ethanolamine (81, 83) have now been unambiguously attributed to the reverse of the FAAH reaction (16, 181, 203). Because high concentrations of arachidonic acid and ethanolamine are needed to drive FAAH to work in reverse, it is unlikely that this reaction plays a physiological role in anandamide generation (see sect. IB1). One possible exception is represented by the rat uterus, where substrate concentrations in the micromolar range are required for the synthetic reaction to occur, implying that FAAH or a similar enzyme might contribute to anandamide biosynthesis in this tissue (319).

#### 7. Structure-activity relationship

Systematic structure-activity relationship investigations have identified several general requisites for substrate recognition by FAAH. First, FAAH accommodates a wide range of fatty acid amide substrates, but reducing the number of double bonds in the fatty acid chain generally results in a decrease in hydrolysis rate (29, 30, 80, 351). Second, replacing the ethanolamine moiety with a primary amide yields excellent substrates. For example, the rate of hydrolysis of arachidonamide is two to three times greater than anandamide's (29, 204). Third, anandamide congeners containing a tertiary nitrogen in the ethanolamine moiety are poor substrates (204). Fourth, introduction of a methyl group at the C2, C1', or C2' positions of anandamide yields analogs that are resistant to hydrolysis, probably due to increased steric hindrance around the carbonyl group (1, 204). Fifth, substrate recognition at the FAAH active site is stereoselective, at least with fatty acid ethanolamide congeners containing a methyl group in the C1' or C2' positions (1, 204). Finally, fatty acid esters such as 2-AG also are excellent substrates for FAAH activity in vitro (134, 279).

#### 8. FAAH distribution in the CNS

Early biochemical experiments showed that FAAH activity is abundant throughout the CNS, with particularly high levels in the neocortex, the hippocampus, and the basal ganglia (80, 159). Subsequent investigations have confirmed this wide distribution. Thus, in situ hybridization studies in the rat have found that FAAH mRNA expression is higher in the neocortex and hippocampus; intermediate in the cerebellum, thalamus, olfactory bulb, and striatum; and lower in the hypothalamus, brain stem, and pituitary gland (340). Immunohistochemical experiments suggest that large principal neurons in the cerebral cortex, hippocampus, cerebellum, and olfactory bulb have the highest levels of FAAH immunoreactivity (95, 347). For example, large pyramidal neurons in the neocortex are prominently stained together with their apical and basal dendrites in layer V (347). Moderate immunostaining is observed also in the amygdala, the basal ganglia, the ventral and posterior thalamus, the deep cerebellar nuclei, the superior colliculus, the red nucleus, and motor neurons of the spinal cord (347). A more recent study reported staining of principal cells and astrocytes in various regions of the human brain (307). However, the protein recognized by the antibody utilized in these experiments has an apparent molecular mass of  $\sim 50$  kDa (by SDS-polyacrylamide gel electrophoresis), which does not correspond to that of native FAAH ( $\sim 60$  kDa) (307).

Many FAAH-positive neurons throughout the brain are found in close proximity to axon terminals that contain  $CB_1$  cannabinoid receptors (see sect. III), providing important evidence for a role of FAAH in anandamide deactivation. Yet, there are multiple other regions of the brain where there is no such correlation, a discrepancy that likely reflects the participation of FAAH in the catabolism of other bioactive fatty acid ethanolamides, such as OEA (302) and PEA (48, 49).

#### 9. Inhibitors of FAAH activity

A number of inhibitors of anandamide hydrolysis have been described, including fatty acid trifluoromethylketones, fluorophosphonates,  $\alpha$ -keto esters and  $\alpha$ -keto amides (30, 82, 198), bromoenol lactones (23), and nonsteroidal anti-inflammatory drugs (109, 110). These compounds lack, in general, target selectivity and biological availability; thus attempts to use them in vivo (64) should be interpreted with caution.

An emerging second generation of FAAH inhibitors comprises three groups of molecules. The first are fatty acid sulfonyl fluorides, such as palmitylsulfonylfluoride (AM374). AM374 irreversibly inhibits FAAH activity with an IC<sub>50</sub> of 10 nM and displays a 50-fold preference for FAAH inhibition versus  $CB_1$  receptor binding (82). Systemic administration of AM374 enhances the operant lever-pressing response evoked by anandamide administration, but exerts no overt behavioral effect per se (310), raising the possibility that AM374 may protect anandamide from peripheral metabolism but may not have access to the brain. The second group of FAAH inhibitors is represented by a series of substituted  $\alpha$ -keto-oxazolopyridines, which are both reversible and extremely potent (30), but whose pharmacological selectivity and in vivo properties are not yet known. The third group is constituted by a class of aryl-substituted carbamate derivatives (185). The most potent member of this class, the compound URB597, inhibits FAAH activity with an  $IC_{50}$  value of 4 nM in brain membranes and an ID<sub>50</sub> value of 0.1 mg/kg in live rats. This compound has 25,000-fold greater selectivity for FAAH than cannabinoid receptors, which is matched by an apparent lack of cannabimimetic effects in vivo (185). The pharmacological profile of URB597, which is currently under investigation, includes profound antianxiety effects accompanied by modest analgesia (185).

#### 10. Physiological roles of FAAH

The generation of mutant mice in which the *faah* gene was disrupted by homologous recombination has shed much light on the role of FAAH in anandamide inactivation (68). FAAH -/- mice cannot metabolize anandamide and are therefore extremely sensitive to the pharmacological effects of this compound: doses of anandamide that are inactive in wild-type mice exert profound cannabimimetic effects in these mutants. FAAH -/- mice also have markedly elevated brain anandamide levels and reduced nociception (68). This finding is consistent with the roles of anandamide in the modulation of pain sensation (see, for review, Refs. 49, 173) and is supported by the analgesic activity of FAAH inhibitors (185).

Recently, a single nucleotide polymorphism in the human gene encoding for FAAH, which produces a proteolysis-sensitive variant of the enzyme, was found to be strongly associated with street drug and alcohol abuse (324). This important observation reinforces the central role played by the endocannabinoid system in the control of motivation and reward (228).

#### 11. 2-AG hydrolysis: the role of monoglyceride lipase

The fact that FAAH catalyzes the hydrolysis of 2-AG along with anandamide's has prompted the suggestion

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that this enzyme may be responsible for eliminating both endocannabinoids. There is, however, strong evidence against this hypothesis. First, inhibitors of FAAH activity have no effect on [<sup>3</sup>H]2-AG hydrolysis at concentrations that completely block anandamide degradation (24). Second, 2-AG hydrolysis is preserved in mutant FAAH -/-mice, which do not degrade either endogenous or exogenous anandamide (212).

In agreement with these results, a 2-AG hydrolase activity distinct from FAAH has been identified and partially purified from porcine brain (133). This activity likely corresponds to monoglyceride lipase (MGL), a cytosolic serine hydrolase that converts 2- and 1-monoglycerides to fatty acid and glycerol (180). Several findings support this conclusion (93). First, heterologous expression of rat brain MGL confers strong 2-AG-hydrolyzing activity and MGL immunoreactivity to HeLa cells. Second, adenovirusmediated transfer of the MGL gene in intact neurons increases MGL expression and shortens the life span of endogenously produced 2-AG, without any effect on either 2-AG synthesis or anandamide degradation. Third, MGL mRNA and protein are discretely distributed in the rat brain, with highest levels in regions where CB<sub>1</sub> receptors are also present (93).

The distribution of MGL in the rat hippocampus is particularly noteworthy. The high density of MGL immunoreactivity in the termination zones of the glutamatergic Schaffer collaterals suggests a presynaptic localization of this enzyme at CA3-CA1 synapses. 2-AG may be produced by CA1 pyramidal cells during Schaffer collateral stimulation (328), and the newly generated endocannabinoid may mediate depolarization-induced suppression of inhibition (271, 374, 375; see sect. v*C*), if able to diffuse to the nearby GABAergic boutons, or a suppression of excitation (273, but see sect. v*B2*). Thus MGL is exquisitely poised to terminate the actions of 2-AG at hippocampal synapses.

# III. REGIONAL AND CELLULAR DISTRIBUTION OF NEURONAL CB<sub>1</sub> CANNABINOID RECEPTORS

# A. Characteristic Differences in CB<sub>1</sub> Receptor Distribution in the Brain

In a landmark study published in 1990, Herkenham and co-workers took advantage of the newly developed cannabinoid agonist [<sup>3</sup>H]CP-55,940, the same highly selective ligand that had helped identify cannabinoid receptors two years earlier (84), to investigate for the first time the distribution of cannabinoid binding sites in the brain (152). Their results showed that these sites strikingly coincide with the neural substrates for cannabinoid actions predicted from behavioral experiments and started a season of intense research on the CNS distribution of cannabinoid receptors. In the following pages, we will summarize the current status of this research, highlighting the correspondence between cannabinoid receptor distribution and behavioral effects of cannabimimetic agents. In the next sections, we focus on the cellular and subcellular localization of cannabinoid receptors and on the consequences of their physiological or pharmacological activation.

Various radioactive ligands (both agonists and antagonists) have been used to identify the sites of action of cannabimimetic drugs at the regional and cellular level (129, 149–152, 225, 299, 370). One surprising observation stemming from these binding experiments, and confirmed later with other neuroanatomical techniques, is that cannabinoid receptors are much more densely expressed in the rat brain than are any other G protein-coupled receptors (Fig. 5, A and C) (152). Indeed, in several brain regions cannabinoid receptors are present in densities that are comparable to those of GABA or glutamate receptor channels, which, owing to their relatively low ligand affinities, are highly concentrated at synapses to allow fast neurotransmission to occur. This puzzling finding is still unexplained but can be conceptualized in the light of recent discoveries suggesting that the synaptic functions served by the endocannabinoid system may be much broader than previously suspected. These functions, which will be discussed in detail in section vC, appear to be primarily concerned with the short-range, activity-dependent regulation of synaptic strength and to extend to a diversity of CNS structures.

The broad regulatory roles of the endocannabinoids also may be surmised from the diverse effects of cannabimimetic drugs on physiology and behavior. In both animals and humans, these agents elicit a wide, but very distinctive spectrum of biological responses (166), which are epitomized by a tetrad comprising rigid immobility (catalepsy), decreased motor activity, analgesia, and hypothermia. This tetrad assay, developed by Billy R. Martin and his collaborators (see for example, Ref. 65), provides a convenient early screening to identify novel cannabimimetic drugs and highlights the role of the endocannabinoid system in motor behavior. Consistent with such a role, two brain regions that are intimately involved in movement regulation, the basal ganglia and the cerebellum, stand out among others for their very high densities of cannabinoid binding sites (Fig. 5, A and C). On the other hand, the marked binding capacities observed in limbic areas of the cerebral cortex, especially the cingulate and frontal cortices, as well as the amygdala, concord with the potent analgesic and antihyperalgesic properties of cannabinoid agonists and with their impact on emotional reactivity (Fig. 5, A and C) (115, 229). Although not as dense, significant cannabinoid binding is also found in other pain-processing areas of the CNS, including the



FIG. 5. Autoradiographic film images (A-C) show cannabinoid receptor localization in rat (A) and human brain (C) marked by the tritiated ligand CP-55,940 in an in vitro binding assay described by Herkenham et al. (152). Sagittal slide-mounted section of rat brain hybridized with a CB<sub>1</sub>-specific oligonucleotide probe (B) shows locations of neurons that express the mRNA at this level. In both rat and human, high levels of receptor protein are visible in the basal ganglia structures globus pallidus (GP), entopeduncular nucleus (Ep), and substantia nigra pars reticulata (SNR). High binding is also seen in the cerebellum; moderate binding levels are found in the hippocampus (Hipp), cortex, and caudate putamen (CPu); and low binding is seen in the brain stem and thalamus. Note that the GP, Ep, and SNR do not contain  $CB_1$  mRNA-expressing cells (B); this is because the receptors in these areas are on axons (large arrows in A and C) and terminals, and the mRNAexpressing cells of origin reside in the caudate and putamen. High-magnification photomicrographs  $(D-\tilde{G})$  of rat CPu show that CB1 mRNA-expressing neurons, marked by white dots (silver grains in the emulsion) are colocalized with enkephalin (Enk) and dynorphin (Dyn) mRNA-positive striatal projection neurons (D and E, respectively), but not with choline acetyltransferase (ChAT) or somatostatin (SOM) mRNA-positive striatal interneurons (F and G, respectively). Open arrows depict CB1-positive but dynorphin- or enkephalin-negative somata, whereas solid arrows indicate double-labeled cells. Scale bars: B, 1 mm; C, 2 mm; F, 20  $\mu$ m. [D-G from Hohmann and Herkenham (164); figure was kindly prepared by Miles Herkenham and Andrea Hohmann.]

PAG and the dorsal horn of the spinal cord. An important property of cannabimimetic agents, which is not modeled by the tetrad assay, relates to the ability of these compounds to influence cognitive functions, including short-term memory and attention (142). The high densities of cannabinoid binding sites in the hippocampus and other cortical structures provide a likely neural substrate for this property (Fig. 5, A and C).

Sheer density of CNS binding sites is not sufficient to precisely account for the spectrum of cannabinoid effects. Studies on the activation of G proteins by cannabinoid agonists in acutely dissected brain slices have revealed, indeed, the existence of an uneven coupling of cannabinoid receptors with G protein activation in different brain structures (37, 38, 304–306, 322). For example, receptors in structures such as the hypothalamus and the thalamus, although relatively low in number, display very tight G protein coupling, suggesting that they may be more efficacious than receptors found elsewhere in the brain. The molecular basis for these regional variations is unclear at present, but they may help reconcile the comparatively low density of cannabinoid receptors found in the hypothalamus with the profound neuroendocrine effects of cannabinoid drugs (261).

A quantitative summary of the distribution of cannabinoid binding sites in the rat brain has been provided (see Table 1 in Ref. 151). Similar distribution patterns have been found in other mammalian and nonmammalian species (see Fig. 5*C*), implying that the endocannabinoid system may play conserved roles in vertebrate phylogeny (57, 152).

# B. Selective Expression of CB<sub>1</sub> Cannabinoid Receptors by Identified Cell Types of Complex Networks

The mapping of brain cannabinoid binding sites by Herkenham et al. (152) preceded by a few months the molecular identification of the first cannabinoid receptor, the G protein-coupled receptor that is now called  $CB_1$ (236). A related gene encoding a second cannabinoidsensitive G protein-coupled receptor, the CB<sub>2</sub>, was identified soon afterward (260). The  $CB_1$  receptor is distributed throughout the body but is predominantly found in neurons of the central and peripheral nervous systems. In contrast, the CB<sub>2</sub> receptor is highly concentrated in immune cells and appears to be absent from CNS neurons (41, 113). Genetic deletion studies have confirmed that  $CB_1$  receptors contribute in a major way to the behavioral effects of cannabimimetic drugs. Thus mutant mice lacking functional CB<sub>1</sub> receptors do not exhibit the tetrad of behavioral responses evoked by cannabinoid agonists (208, 380). As mentioned above, the tetrad only partially illustrates the complexity of cannabinoid actions and ostensibly excludes those involving cognitive systems. It is conceivable therefore that certain responses to cannabimimetic agents may be preserved in mutant  $CB_1^{-/-}$ mice (36, 88, 253). This possibility is strongly supported by electrophysiological experiments, which show that  $CB_1^{-/-}$  mice, although impaired in their  $CB_1$ -mediated regulation of GABAergic transmission, retain an intact cannabinoid modulation of glutamate transmission (Fig. 12) (139). A parsimonious interpretation of these results, which is also consistent with current morphological data (Figs. 9A and 10, A and B) (138), is that glutamatergic axon terminals contain a cannabinoid-sensitive receptor that is molecularly distinct from  $CB_1$  (see sect. WB1B).

To understand the complex neurobiological effects of cannabinoid drugs and their endogenous counterparts, it is first necessary to precisely outline the neuronal cell types that express cannabinoid receptors. The molecular characterization of the CB<sub>1</sub> receptor opened the way to in situ hybridization studies on the CNS distribution of this receptor's mRNA (Fig. 5B) (236). The subsequent development of specific antibodies allowed the comparison of mRNA and protein expression, and investigators could now delve in greater detail into the cellular and subcellular localizations of this receptor (see Figs. 6-8) (138, 187, 188, 345). In this section, we synthesize the rapidly growing body of data from several laboratories about CB<sub>1</sub> cannabinoid receptor localization in particular cell types of given brain areas. Remarkably, these anatomical studies confirmed that, likewise to the patterned distribution of cannabinoid binding sites in certain brain regions, expression of the CB<sub>1</sub> receptor gene is restricted to specific cell types subserving distinct functional roles in certain



FIG. 6. Light micrographs of hippocampal sections (A and B from mouse, C from rat) immunostained for  $CB_1$  receptor using an antibody raised against a COOH-terminal intracellular epitope (A and B, showing the CA1 region), and another recognizing the NH2-terminal extracellular epitope (C, showing the CA3 region). The COOH-terminal antibody is more sensitive and provides somewhat stronger labeling, particularly in the dendritic layers, but the general staining pattern is similar. CB1positive axon terminals are seen in high density particularly in stratum pyramidale, where they surround the negative cell bodies of pyramidal cells in all subfields. Somatic staining appears only in interneurons mostly in strata radiatum and oriens, occasionally in stratum pyramidale (arrows). No immunostaining is visible in the CB<sub>1</sub> receptor knock-out mouse (B). s.o., Stratum oriens; s.p., stratum pyramidale; s.r., stratum radiatum;  $CB_1 + /+$ , wild-type mouse;  $CB_1 - /-$ ,  $CB_1$  receptor knock-out mouse. Scale bars, 50 µm. [Modified from Katona et al. (188) and Hájos et al. (138).]

neuronal networks, which may indeed account for the striking diversity of cannabinoid effects.

#### 1. Methodological considerations

Comprehensive in situ hybridization experiments have revealed three populations of brain cells that can be

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FIG. 7. A-C: immunostaining of the rat somatosensory cortex for CB1 reveals a dense axon labeling in all layers with variable densities. The highest density of labeled fibers can be seen in layers II and upper III, as well as in layers IV and VI. A much smaller amount of stained axons is visible in layer V and deep layer III, whereas layer I has only negligible labeling. Both synaptic varicosities (small arrows) and preterminal, thin axon segments show strong staining, as seen at higher power in B (layer II-III) and C(layer VI). A large number of varicosities surround the CB<sub>1</sub>-negative somata of pyramidal cells (asterisks). CB1-immunoreactive cell bodies show the characteristics of interneurons (large arrows). D: in the cerebellar cortex, Purkinje cells (P) are negative for CB<sub>1</sub>; their dendrites (arrowheads) appear as negative images in the otherwise strongly positive molecular layer. The dense terminal labeling in the molecular layer corresponds to both parallel and climbing fibers, and perhaps also includes stellate cell axon terminals. The somata and axon initial segments of Purkinje cells are surrounded by CB<sub>1</sub>positive axons of basket cells (arrows). Scale bars: A, 100 µm; B-D, 25 µm.

grouped according to their levels of  $CB_1$  mRNA (225, 231, 235). Cells with very high  $CB_1$  mRNA expression are found in many cortical regions, especially in the hippocampus, but also in the anterior olfactory nucleus, the neocortex, and the amygdala. Cells with moderate  $CB_1$  mRNA levels are characteristically present in the striatum and the cerebellum, whereas cells with very weak  $CB_1$  mRNA expression are widespread throughout the brain. Although this broad classification is generally accepted, more precise descriptions of  $CB_1$  mRNA distribution are still controversial. Discrepancies have been reported not

only in the intensity of labeling among brain regions, but even in the presence or absence of  $CB_1$  mRNA in certain cell types (even though the authors used the same oligonucleotides). Subsequent immunocytochemical studies have helped clarify some of these issues but have left others unsolved and, indeed, generated their own share of unexplained results. For example, recent reports of strong  $CB_1$  immunostaining in cerebellar Purkinje cells (252, 276) are in striking contrast to the lack of  $CB_1$ mRNA noted in these cells by many investigators (225, 235). Surely, these problems will be appropriately ad-



dressed and resolved in due time (e.g., by using  $\text{CB}_1^{-/-}$  mice for probe and antibody testing, see Fig. 6, *A* and *B*). Meanwhile, here we will primarily discuss those results, which are unequivocally supported by a combination of multiple neuroanatomical and functional approaches.

#### 2. Cortical areas

In situ hybridization and immunocytochemical studies consistently show that CB1 receptors are highly abundant in many forebrain areas, including the anterior olfactory nucleus, the hippocampal formation (Fig. 6), the neocortex (Fig. 7, A-C), and the basolateral as well as the cortical amygdaloid nuclei (Fig. 8) (138, 186-188, 225, 231, 235, 345).  $CB_1$ -positive cells in these areas display a scattered distribution pattern, represent only a small percent of the total cell population, and belong to the heterogeneous population of GABAergic interneurons (188, 231, 345). In the forebrain, GABAergic interneurons can be divided into various classes based on the cell typeselective expression of neurochemical markers, two prominent examples of which are the neuropeptide cholecystokinin (CCK) and the calcium-binding protein parvalbumin (111, 190). Double-labeling studies have revealed that only one subset of GABAergic interneurons contains  $CB_1$  receptors, those that also express and presumably release CCK. In contrast, other major interneuron types, such as those containing parvalbumin, lack CB<sub>1</sub> receptors. This pattern of expression is common to most forebrain areas, having been found in the anterior cortical nucleus (231), the basolateral amygdala (186, 231, 240), the cortical amygdaloid nuclei (186), the hippocampal formation (188, 231, 346), and the neocortex (28, 231). Moreover, an analogous pattern is also seen in the human hippocampal formation (187). This selective distribution implies that CB<sub>1</sub> receptor-dependent effects of cannabinoids on many of the physiological processes related to these forebrain areas (e.g., cognitive functions like learning and memory) might involve the modulation of a parFIG. 8. *A*: remarkable subregional selectivity of CB<sub>1</sub> receptor expression was found in the amygdala. The basolateral nucleus (BLA) is heavily positive for CB<sub>1</sub>, whereas the central nucleus (Ce) is devoid of any staining. The sharp border between them is visible at higher power in *B*. The CB<sub>1</sub>-positive fibers form a dense network in BLA and surround the negative principal cell bodies and dendrites. The white matter (ic, internal capsule) is dark due to the osmium treatment, not to immunostaining. Scale bars: *A*, 500  $\mu$ m; *B*, 100  $\mu$ m. [From Katona et al. (186), copyright 2001 by the Society for Neuroscience.]

ticular subpopulation of GABA ergic interneurons and predicts that this interneuron population may be closely connected with the participation of the endocannabinoids in the short-range modulation of synaptic activity, which will be further discussed in section vC.

Although the strong expression of CB<sub>1</sub> receptors in GABAergic interneurons of the cortex is now well established, the presence of CB<sub>1</sub> receptors in principal cells of the forebrain is still debated. Initial in situ hybridization studies reported a modest CB<sub>1</sub> mRNA expression in principal neurons of the neocortex (225, 235). Subsequent double-labeling experiments showed, however, that all CB<sub>1</sub>-expressing cells in this structure are also positive for the 65-kDa isoform of glutamic acid decarboxylase (GAD65), the GABA-synthesizing enzyme that marks GABAergic cells (231). Moreover, although several investigators have reported low CB<sub>1</sub> mRNA expression in principal neurons of the CA3 and CA1 subfields of the hippocampus (225, 231, 235), a more recent study suggested that  $CB_1$  labeling may be restricted to GABAergic interneurons (254). Even looking at the original figures claiming  $CB_1$  expression in pyramidal neurons (see, for example, Fig. 8B of Ref. 225), the density of labeling over the principal cells (5-10 silver grains) seems to be remarkably low compared with the interneuronal labeling (the cells are completely filled with a huge number of grains). This very low expression pattern within the principal neurons of cortical networks is similar in most other forebrain areas and was found in the human brain as well (231, 370).

This disagreement could not be settled by immunocytochemical localization of the  $CB_1$  protein. Experiments with antibodies raised against the  $NH_2$  terminus of the  $CB_1$  receptor found labeling of principal neurons in many forebrain areas (252, 276, 284). However, these studies also report  $CB_1$  immunoreactivity in cell populations from other brain areas, which were found to be

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negative for CB<sub>1</sub> mRNA in all in situ hybridization studies (i.e., Purkinje cells in the cerebellum, cells in the laterodorsal nucleus in the thalamus, in the substantia nigra, and in glial cells). Other investigators utilized different antibodies directed either against the NH<sub>2</sub> or the COOH terminus of the CB<sub>1</sub> protein, and unequivocally established antibody specificity with control tests on brains of mutant  $CB_1^{-/-}$  mice (Fig. 6, A and B). These carefully controlled studies found CB<sub>1</sub> immunostaining only in GABAergic interneurons of the cortex (138, 186-188, 345, 346). However, the fact that principal cells were not stained in these experiments does not rule out the possibility that a very low amount of CB<sub>1</sub> protein, undetectable by the antibodies, may be present in principal cells. Moreover, targeting of the receptor to axon terminals could further decrease antibody access to the antigen and account for the lack of cell body staining. Indeed, several laboratories have reported that glutamatergic synaptic currents in neurons of the prefrontal cortex and hippocampus are inhibited by cannabinoid agonists via a presynaptic mechanism (17, 139, 251, 323; see sect. IVB1B). Yet, the lack of CB<sub>1</sub> immunoreactivity on axon terminals forming asymmetrical synapses (which are typically excitatory) strongly argues against the presence of CB<sub>1</sub> receptors at these glutamatergic terminals (Figs. 9A and 10B) (138, 186–188). This clear morphological finding is also supported by work with CB<sup>-/-</sup> mice, which suggests that an additional receptor, pharmacologically related, but molecularly distinct from the CB<sub>1</sub>, may mediate the cannabinoid modulation of glutamatergic transmission in the hippocampus (Fig. 12) (139). We will return to this hypothesis in section IVB1B.

#### 3. Basal forebrain

 $CB_1$  receptors are also present in several subcortical nuclei of the basal forebrain. Cells expressing moderate levels of this receptor are located mainly in the tenia



FIG. 9. A–D: subcellular localization of CB1 receptors using an antibody raised against a COOH-terminal intracellular epitope, and the immunogold procedure in the hippocampal CA1 region of the rat. Silver-enhanced gold particles (small arrows) represent CB1-immunoreactive sites. Labeling was found to outline the inner surface of the membrane of axon terminals (b) that established symmetrical synaptic contacts (large arrows in A-D) characteristic of GABAergic synapses. Boutons (asterisk in A) forming asymmetrical synaptic specializations (open arrow in A) were always negative. CB1-receptor immunoreactivity was rarely seen on the plasma membrane of cell bodies or dendrites. Serial sections of the same boutons are shown in A and B, as well as in C and D, the former synapsing on a dendritic shaft (d) in stratum radiatum and the latter on a soma (s). E and F: colocalization of  $CB_1$  and cholecystokinin (CCK) in the same axon terminals using the diffusible (homogeneous electron dense) DAB end product to label CCK, and silver-gold to label the NH2-terminal extracellular epitope of  $CB_1$  (small arrows). The outer surface of the CCK-positive bouton, which forms a symmetrical synapse on a cell body (large arrow), is decorated with silver grains. G: no colocalization was found between parvalbumin (star), a marker of another basket cell type, and  $CB_1$  (small arrows) using the same technique. Both terminals form symmetrical synapses (large arrow) on the same proximal dendrite (d) of a pyramidal cell. Scale bars: A–G, 0.2  $\mu$ m. [Modified from Katona et al. (188) and Hájos et al. (138).]

tecta, the lateral and medial septum, and the nuclei of the vertical and horizontal limbs of the diagonal band (225, 231, 235, 345). Colocalization experiments show that  $CB_1$  receptors may be present in somatostatin-positive neurons of the lateral septum (164) and in cholinergic cells in the medial septum and the nucleus basalis of Meynert (216).

#### 4. Basal ganglia

In keeping with the profound impact of cannabimimetic drugs on motor activity (for a review, see Ref. 313), in situ hybridization studies have invariably reported strong expression of  $CB_1$  mRNA in the striatum (225, 235). Detailed analysis at the regional and cellular level uncovered a selective expression pattern in specific components of basal ganglia networks (164, 231). In rodents, the highest density of CB<sub>1</sub> mRNA is found in the dorsolateral portion of the striatum, where the transcript is primarily localized to GABAergic medium spiny cells, which constitute >90% of striatal neurons. In contrast, CB<sub>1</sub> mRNA expression is rather low in two key output structures of the basal ganglia in the globus pallidus and in the substantia nigra. This is also true for the human basal ganglia, which lack however the dorsoventral gradient of mRNA expression seen in rodents (225, 370).

Although the globus pallidus and the substantia nigra pars reticulata contain little CB<sub>1</sub> mRNA, cannabinoid binding is remarkably dense in these structures, implying that CB<sub>1</sub> receptors may be mainly localized to the axons of striatonigral and striatopallidal GABAergic neurons (150). Indeed, two colocalization studies have now established that CB<sub>1</sub> mRNA is expressed by neurons that also contain high levels of the enzyme GAD65 and low levels of its higher molecular mass isoform, GAD67 (164, 231). In a separate study, CB<sub>1</sub> mRNA was found to be coexpressed with both preproenkephalin (a marker of striatopallidal neurons) and prodynorphin (a marker of striatopallidal neurons), indicating that striatal projection neurons express CB<sub>1</sub> receptors irrespectively of their specific target region (Fig. 5, D-E) (164).

Interestingly, a small fraction of  $CB_1$ -positive neurons contain neither preproenkephalin nor prodynorphin (164) and express high levels of GAD67, which is typical of striatal interneurons. Thus, in addition to medium spiny projection cells, other neurons (presumably local-circuit interneurons) also may express  $CB_1$  mRNA. Because colocalization experiments revealed that  $CB_1$  is found neither in somatostatin-positive nor in cholinergic interneurons (Fig. 5, *F* and *G*), the presumptive candidates are the remaining parvalbumin-containing cells (164). Indeed, Marsicano and Lutz (231) demonstrated that  $\sim 15\%$  of the  $CB_1$ -expressing neurons are positive for parvalbumin, providing direct evidence that striatal local-circuit neurons express  $CB_1$  receptors. It is important to reiterate

that this expression pattern is opposite to the one found in cortical and amygdaloid structures, where parvalbumin-positive interneurons do not express  $CB_1$  receptors (186, 188, 231, 346). While the above results are based on the presence of  $CB_1$  mRNA in striatal projection neurons and local-circuit cells, the cellular expression pattern has not been confirmed yet at the protein level, although the presence of the  $CB_1$  protein in striatal neurons has already been demonstrated by immunostaining (345).

#### 5. Thalamus

In situ hybridization studies have reported very low levels of  $CB_1$  mRNA expression in the thalamus (225, 235). Subsequent work confirmed this finding both at the mRNA and at the protein level and extended it to the human brain (231, 345, 370). Neurons expressing moderate amounts of CB<sub>1</sub> mRNA were observed in the habenula and the anterior dorsal part of thalamus, while CB1-immunoreactive cells were found in the reticular nucleus and zona incerta (225, 231, 235, 284, 345). Further studies are needed, however, to unambiguously identify these cells and solve remaining inconsistencies in the literature regarding their exact location in different nuclei. This need is further underscored by the finding that anterior and dorsal nuclei of the thalamus may express high levels of monoacylglycerol lipase, an intracellular serine hydrolase implicated in terminating the biological effects of the endocannabinoid, 2-AG (93).

#### 6. Hypothalamus

There is a coherent body of evidence indicating that the endocannabinoid system participates in the hypothalamic regulation of feeding (90) and neuroendocrine function (261). Likewise, anatomical investigations agree in finding moderate levels of CB<sub>1</sub> receptor expression in the ventromedial and anterior nuclei of the hypothalamus (225, 231, 235), while pharmacological experiments suggest that these receptors may be particularly well coupled to G proteins (37, 38). Importantly, a double-labeling study showed that  $CB_1$  receptors are colocalized with calretinin, a marker for glutamatergic neurons in select hypothalamic nuclei (193), but not with GAD65 (231). This suggests that glutamatergic, but not GABAergic, cells may express  $CB_1$  receptors in these nuclei. Other hypothalamic nuclei display very low levels of CB<sub>1</sub> expression in a population of uniformly distributed cells. These nuclei include the medial and lateral preoptic nucleus, the magnocellular preoptic and hypothalamic nucleus, the premammilary nucleus and the lateral nucleus of the mammilary body, and the lateral hypothalamus (225, 231, 235). However, as elsewhere in the brain, there is still disagreement as to the precise identity and localization of hypothalamic CB<sub>1</sub>-expressing neurons, which will undoubtedly foster further scrutiny.

#### 7. Midbrain

The finding that noxious stimuli trigger anandamide release in the PAG, as assessed by in vivo microdialysis (365), implies that this midbrain structure may serve as a relay in the pain-processing circuit modulated by the endocannabinoids. Yet, a coherent description of the regional and cellular expression of  $CB_1$  receptors in the midbrain is still lacking. Although current data suggest that several midbrain nuclei may have very low to moderate expression of CB<sub>1</sub> mRNA, they are in conflict regarding the exact identity of these nuclei (225, 235). Immunostaining studies have shown that the superior colliculus contains CB<sub>1</sub>-positive neuronal cell bodies, but the identity of these cells was not determined (345). To be able to interpret the growing body of work on the analgesic and antihyperalgesic effects of cannabinoid agents, these morphological gaps need to be filled.

#### 8. Medulla and pons

Detailed morphological studies of the hindbrain are also rare. A notable exception is represented by the recent immunocytochemical demonstration of  $CB_1$  receptors in the dorsal vagal complex of the ferret, which may be relevant to the autonomic and antiemetic effects of cannabinoid agonists (355). The exclusive presence of these receptors in local GABAergic interneurons, but not in preganglionic motor neurons (355), shows how this intriguing morphological leitmotif may recurrently be found at most levels of the neuraxis.

#### 9. Cerebellum

 $CB_1$  receptor mRNA is highly abundant in the cerebellum (Fig. 5B) (225, 235). Owing to the well-determined circuitry of the cerebellar cortex, along with its laminar structure, the identification of neuronal elements expressing  $CB_1$  receptors in this region is relatively straightforward. Strong expression levels are found in glutamatergic granule cells, but not in the GABAergic Purkinje cells (Fig. 7D). In the molecular layer, several large cells were also reported to express  $CB_1$ , which might belong to the basket and stellate cells (345). However, it is not known whether all cerebellar interneurons express  $CB_1$  or a subtype selectivity exists among them.

#### 10. Spinal cord

One of the most important aspects of cannabinoids in terms of medicinal usefulness is their analgesic and antihyperalgesic effect at multiple stages of the pain-processing pathway, from high cognitive centers of the forebrain to the midbrain and down to peripheral tissues (48, 229, 234, 245, 297, 365). The spinal cord, where cells expressing CB<sub>1</sub> receptors have been extensively characterized, is obviously an integral component of this circuit. Most in situ hybridization and immunostaining studies agree that  $CB_1$  receptors are present in select neuronal populations of the spinal dorsal horn (7, 102, 165, 311). In lamina II, GABAergic neurons expressing  $CB_1$  also contain nitric oxide synthase (NOS), a marker for a subset of spinal interneurons called islet cells (311). In addition,  $CB_1$ -positive cells have also been found in lamina X, which surrounds the central canal of the spinal cord (311); however, by using a different antibody, these cells could only be visualized after spinal transection (102).

The presence of  $CB_1$  receptors in the dorsal root ganglia is now well established (for review, see Ref. 258). Primary sensory neurons in these ganglia are classified based on the selective expression of various neuropeptides [calcitonin gene-related peptide (CGRP), substance P, somatostatin], or the responsiveness to neurotrophic factors [nerve growth factor (NGF), glial-derived growth factor (GDNF), present in nociceptive neurons]. These cell-specific markers are rather heterogeneously colocalized with CB<sub>1</sub> receptors. In a small population of dorsal root ganglion cells, CB<sub>1</sub> receptors are present in CGRPand substance P-expressing neurons, but not in somatostatin-positive cells (165). This suggests that  $CB_1$  receptors may be expressed only by a subset of peptidergic nociceptive neurons, which represent  $\sim 25\%$  of all CB<sub>1</sub>positive cells, whereas the remaining CB<sub>1</sub>-expressing cells may belong to other subpopulations of nociceptive or nonnociceptive neurons. Work in dorsal root ganglion cultures suggests that CB<sub>1</sub> receptors colocalize with another nociceptor marker, the acid- and heat-sensitive vanilloid receptor 1 (VR1) (8). Further triple immunolabeling experiments confirmed this observation and suggested that  $\sim 25\%$  of CB<sub>1</sub>-bearing neurons are nonnociceptive and that distinct types of nociceptive neurons express the receptor as well (7, 258). This highly heterogeneous distribution may contribute to explain the unprecedented analgesic effectiveness of cannabinoid agents, particularly in animal models of persistent pain of neuropathic origin (154, 173).

# IV. ANATOMICAL, PHYSIOLOGICAL, AND PHARMACOLOGICAL EVIDENCE FOR THE PRESYNAPTIC LOCALIZATION OF CB<sub>1</sub> CANNABINOID RECEPTORS IN THE BRAIN

Based on the selective distribution of  $CB_1$  receptors in the CNS and their pervasive association with GABAergic interneurons, one would predict that the endocannabinoid system may play important and, possibly, unique roles in the local control of neuronal network activity. A growing body of functional evidence supports this prediction. For example, microdialysis experiments have found that anandamide is released in the striatum by activation of dopamine  $D_2$  receptor, where it may act as a shortrange mediator to counterbalance dopamine activity (22, 125). Furthermore, an endocannabinoid substance, which remains unfortunately uncharacterized, has been recently identified as a key component in two related forms of *trans*-synaptic communication, known as depolarization-induced suppression of inhibition (DSI) and depolarization-induced suppression of excitation (DSE) (200, 271, 375). In section v, we discuss how the endocannabinoid system may participate in these processes. But to do that, we first need to take a further step in the localization of CB<sub>1</sub> receptors, down to the subcellular level.

G protein-coupled receptors, such as the  $CB_1$ , are embedded within the lipid bilayer of the plasma membrane. The membrane surface of a nerve cell can be subdivided into two functionally distinct spatial domains. The dendritic tree and cell body are equipped to receive synaptic contacts at specialized structures called active zones, where receptors for fast-acting neurotransmitters such as glutamate or GABA are concentrated. G proteincoupled receptors are rarely associated with these structures; rather, a significant proportion of these receptors are found outside the synapse, within the so-called perisynaptic zone or even further away on the dendritic tree (see, for example, Ref. 19), where they can influence synaptic currents and neuronal excitability by triggering the formation of diffusible intracellular second messengers. Another group of G protein-coupled receptors is situated on axon terminals, where they are exquisitely poised to regulate the release of neurotransmitters, thereby controlling the final output of a neuron. Thus the question arises, in which neuronal surface domain are CB<sub>1</sub> receptors localized? The most direct way to approach this question consists, when a receptor-specific antibody is available, in analyzing the subcellular distribution of the receptor by using electron microscopy. This approach can also provide a wealth of information on the structure and function of the synapse, such as the complement of neurotransmitters and additional membrane receptors present. Evidence from anatomical studies such as these, as well as functional experiments, indicates that  $CB_1$  receptors are predominantly found in axon terminal membranes, where they may be involved in the presynaptic regulation of neurotransmitter release.

# A. Anatomical Evidence for Presynaptic Cannabinoid Receptors

Indirect anatomical evidence for the localization of  $CB_1$  receptors on axon terminals was first provided by in situ hybridization (236) and receptor binding experiments (152). These studies showed that, in the basal ganglia,  $CB_1$  receptor mRNA is almost exclusively localized to neurons within the striatum (236), whereas cannabinoid binding is

strongest in the globus pallidus and the substantia nigra pars reticulata (Fig. 5, A–C) (152, 236). This mismatch implies that CB<sub>1</sub> receptors synthesized in the cell bodies of striatal projection neurons are transported to axon terminal fields in the pallidum and substantia nigra. In keeping with this hypothesis, ibotenic acid lesion of the rat striatum produces a marked loss of cannabinoid binding in these two regions (150). A similar presynaptic localization also has been suggested for CB<sub>1</sub> receptors in dorsal root ganglion neurons, because resection of the dorsal root significantly decreases cannabinoid binding in the dorsal horn of the spinal cord (163).

An important achievement in cannabinoid research was the development of specific antibodies recognizing CB<sub>1</sub> receptors, which have become indispensable research tools (94, 138, 284, 345). Antibodies raised against either the NH<sub>2</sub> terminus or the COOH terminus of the CB<sub>1</sub> protein provided crucial information about the precise localization of  $CB_1$  receptors at the regional, cellular, and subcellular levels. However, immunohistochemical studies require careful investigation and well-designed controls, since it is rare that an antibody is absolutely specific for the desired target protein. Thus reports claiming immunoreactivity for  $CB_1$  receptors in cells (e.g., cerebellar Purkinje neurons), which do not produce the mRNA of  $CB_1$ , or immunolabeling of glial cells due to the antibody recognizing the antigen carrier protein should be viewed with caution (240, 252, 276, 302). Essential in this regard was the generation of mutant  $CB_1^{-/-}$  mice (208, 380), which were instrumental to demonstrate antibody specificity (Fig. 6, A and B) and limit the confusion resulting from staining artifacts (138, 186).

Initial light microscopy studies revealed the existence of numerous CB1-immunoreactive fibers throughout the brain (Figs. 6, A and C, 7, A–C, and 8B) (94, 95, 138, 186–188, 345). Based on their distinctive morphological appearance, thin and rich in varicosities, these fibers were tentatively identified as axons. This identification received its first subcellular confirmation from work conducted in the rat hippocampus (188). The varicosities observed at the light microscopy level were found to correspond to axon terminals packed with synaptic vesicles and to be densely covered by CB<sub>1</sub> receptors (Figs. 9, A-D, and 10). Notably, when an antibody against the extracellular NH<sub>2</sub> terminus of the CB<sub>1</sub> receptor was used in combination with silver-impregnated gold particles, the particles were exclusively found at the outer surface of the axonal plasma membrane (Fig. 10, C-E) (188). On the other hand, when the staining was carried out with a different antibody, specific for the COOH terminus, the gold particles only labeled the intracellular surface of the boutons (Figs. 9, A–D, and 10, A and B) (138).

 $CB_1$ -positive axons have a scattered pattern of distribution, which largely parallels that obtained with radioli-



FIG. 10. Subcellular localization of CB<sub>1</sub> receptors in the human hippocampal CA1 region using an antibody raised against a COOH-terminal intracellular epitope (*A* and *B*) and another recognizing an NH<sub>2</sub>-terminal extracellular epitope (*C*–*E*). Silver-enhanced gold particles (small arrows) represent CB<sub>1</sub>-immunoreactive sites on the inner (*A* and *B*) and outer (*C*–*E*) surface of axon terminal membranes, corresponding to the subcellular localization of the respective epitopes. Only boutons forming symmetrical synapses (large arrows) were labeled, as in the rat, which is characteristic of GABAergic, but not of glutamatergic (asterisk) axons. Scale bars: *A*–*E*, 0.2 µm. [From Katona et al. (187), copyright 2000 with permission from Elsevier Science.]

gand binding (94). An especially dense fiber meshwork is observed in the globus pallidus, the substantia nigra pars reticulata, and the entopeduncular nucleus, probably on axons deriving from the striatum. In many cortical areas, as well as in olfactory systems, CB<sub>1</sub>-immunoreactive axons are abundant and form pericellular baskets around CB<sub>1</sub>-negative cell bodies. Likewise, CB<sub>1</sub>-positive axons equipped with numerous boutons cover the somata of Purkinje cells in the cerebellum and shape the characteristic pinceaux (paint-brush) structures around the axon initial segments (Fig. 7D). In addition, the stratum moleculare of the cerebellum also exhibits strong CB<sub>1</sub> immunoreactivity, while leaving blank the dendritic tree of Purkinje cells (Fig. 7D).

The cell origin of these fibers can sometimes be inferred from the combination of cellular  $CB_1$  expression pattern and the distribution of  $CB_1$ -positive axons. For example, in the cerebellum, the dense staining seen in the stratum moleculare likely results from axons of  $CB_1$ -expressing granule cells, which constitute the so-called parallel fibers. In most cases, however, the cell origin and phenotype of  $CB_1$ carrying axons is still uncertain. Recent efforts have helped determine the precise subcellular distribution of  $CB_1$  receptors in the rodent somatosensory cortex, the hippocampus, and the amygdala, as well as in the human hippocampus (28, 138, 186–188). In these areas,  $CB_1$  receptors localize to specific types of axon terminals, and as a rule, boutons engaged in asymmetrical (excitatory) synapses do not carry CB<sub>1</sub> receptors, whereas boutons engaged in symmetrical (inhibitory) synapses do (see for example Fig. 10B). This indicates that GABAergic, but not glutamatergic, axon terminals contain the receptors. GABAergic interneurons are extremely heterogeneous, however, and not all of them express  $CB_1$  receptors. Indeed, only a subpopulation of GABAergic interneurons, those that utilize CCK as a peptide cotransmitter, was found to be  $CB_1$  positive (Fig. 9, *E* and *F*), whereas those marked by parvalbumin were not (Fig. 9G) (see sect. IIIB2). Because CCK- and parvalbumin-positive interneurons have distinct roles in the regulation of cortical activity, it is likely that endocannabinoid substances also have specific functions in the modulation of cortical network properties. This notion is strongly supported by the retrograde messenger role of endocannabinoids in DSI, which is clearly restricted to select inhibitory synapses within the hippocampus (233, 271, 374) (see sect. v).

Outside the cortex, detailed information on the subcellular distribution of  $CB_1$  receptors is only available for the peripheral nervous system, where  $CB_1$  receptors also appear to be concentrated at nerve endings. In the rat and guinea pig lung, sparse nerve fibers bearing  $CB_1$  receptors are found among bronchial smooth muscle cells (46, 363). Although such fibers rarely form true synapses, immunogold labeling reveals that  $CB_1$  receptors are located close to vesicle accumulations, where they may act to modulate neurotransmitter release. Importantly, neuropeptide Y, a neurochemical marker for noradrenergic sympathetic nerve fibers (18), was found to colocalize with  $CB_1$  in these axon terminals (46, 363). Accordingly, cannabinoids potently inhibit norepinephrine release in peripheral tissues and organs through a presynaptic mechanism (131, 172, 344, 363).



### **B.** Physiological and Pharmacological Evidence for Presynaptic Cannabinoid Receptors

Although anatomical studies may reveal the precise localization site of a particular receptor type, they may only provide predictions about its functional importance. In the last decade, two major approaches, electrophysiological recordings and neurochemical release studies, contributed fundamentally to our understanding of the physiological role of endocannabinoids and the conseguences of cannabinoid receptor activation. Most of these studies point to the same conclusion as anatomical studies, i.e., CB<sub>1</sub> receptors presynaptically regulate the release of certain types of neurotransmitters from axon terminals. The major goal of these studies is to establish which of the numerous types of neurotransmitters are influenced by cannabinoids at certain brain areas. Not surprisingly, the release of nearly all major neurotransmitter types was shown to be affected by cannabinoid agents.

Similarly to  $CB_1$ -specific antibodies in anatomical experiments, the development of pharmacological probes, such as selective  $CB_1$  receptor agonists and antagonists, was indispensable to advance the field (71, 158, 213, 298). However, as is the case with immunohistochemical experiments, the establishment of the role of  $CB_1$  receptors in many of the described processes requires careful evaluation. Recent studies using  $CB_1^{-/-}$  mice provided evidence that conventional cannabinoid receptor ligands, as well as the endocannabinoids, are not exclusively selective for  $CB_1$  receptors (36, 60, 88, 139, 175, 227, 253, 381).

In the following sections, we survey the various lines of pharmacological evidence for the existence of presynaptic cannabinoid receptors on many different types of axons in several brain areas and aim to evaluate in the light of anatomical data whether  $CB_1$  or another molecular target may underlie certain effects of cannabinoids.

#### 1. Cortical areas

A) CANNABINOID EFFECTS ON GABA RELEASE IN CORTICAL AR-EAS. In the hippocampus, electrophysiological and neurotransmitter release experiments concord in indicating that cannabimimetic agents modulate GABA release via a presynaptic mechanism. Whole cell patch-clamp experiments show that cannabinoid agonists decrease amplitude and frequency of GABA<sub>A</sub> receptor-mediated inhibitory postsynaptic currents (IPSCs) elicited by action potentials (Figs. 11, A-C, and 12B) (138, 161, 171). These effects are mediated by  $CB_1$  receptors, because they are blocked by the  $CB_1$  antagonist SR141716A (Fig. 11A) and are completely absent in  $CB^{-/-}$  mice (Fig. 12B) (138, 139). The presynaptic action of cannabinoids was suggested by the lack of effect on the amplitude of miniature IPSCs (Fig. 11D), as well as by a reduction in vesicle release probability (measured using the paired-pulse ratio). These data are in striking agreement with the anatomical studies showing the presynaptic localization of CB<sub>1</sub> receptors on GABAergic axon terminals. In the basolateral amygdala, which has a morphological architecture in many respects similar to the hippocampus, cannabinoid agonists produce comparable responses. The compounds inhibit synaptic GABA<sub>A</sub>-mediated currents in principal neurons of this region, but cause no such effect in the central nucleus, which does not contain  $CB_1$  receptors (186). The significance of these findings was also recently confirmed in vivo in the prefrontal cortex (104). In accordance with the exclusive expression of CB<sub>1</sub> by GABAergic neurons in the neocortex (231), the cannabinoid receptor agonist WIN 55,212-2 reduced cortical GABA levels, which was prevented by the cannabinoid receptor antagonist SR 141716A (104). Moreover, neurochemical release experiments extended the validity of this finding from the rat (188) to the human hippocampus (187). Taken together, these results indicate that GABAergic axon terminals are one of the major targets of cannabinoids in cortical networks, where they reduce the release of GABA in a  $CB_1$ receptor-mediated manner.

B) CANNABINOID EFFECTS ON GLUTAMATE RELEASE IN CORTICAL AREAS: INVOLVEMENT OF A NEW RECEPTOR? Results from a variety of cortical tissue preparations are consistent in indicating that cannabinoid agonists can reduce excitatory

FIG. 11. Synthetic cannabinoids suppress inhibitory postsynaptic currents (IPSC) in hippocampal pyramidal cells as revealed by whole cell patch-clamp recordings. A: plot of the IPSC amplitude shows an  $\sim$ 50% reduction of monosynaptic responses evoked by focal electrical stimulation after bath application of the cannabinoid receptor agonist WIN55,212-2 (WIN). Pretreatment with a cannabinoid receptor antagonist, SR141716A, and its coapplication with WIN55,212-2 prevents the suppression of the evoked IPSC amplitude. B: consistent with the anatomical results, only a subset of inhibitory axons is responsive to CB<sub>1</sub> receptor activation. Representative traces evoked by minimal stimulation from two different stimulus sites are shown. As the amplitude plot shows, one of the evoked IPSCs (left panel) was sensitive to another cannabinoid ligand, CP55,940, as indicated by the increased number of transmission failures. After washout, the synaptic responses returned to control levels. The IPSCs evoked by stimulation of a different site (right panel) were insensitive to the agonist application, since there was no obvious change in their failure rate during the CP55,940 treatment. C: activation of substance P receptors enhances the firing rate of predominantly those hippocampal interneurons (5), which express  $CB_1$  receptors (188). As a consequence of increased interneuron firing, both the conductance and frequency of IPSCs increased significantly, the increment of which could be reduced by 1  $\mu$ M WIN55,212-2 (bar graphs, n = 7). D: raw traces depicting mIPSCs in the presence of 0.5  $\mu$ M tetrodotoxin are shown before (top panel) and after (bottom panel) bath application of the synthetic cannabinoid CP55,940. The averaged mIPSC conductance or the averaged frequency did not differ significantly before or after applying CP55,940 (bar graphs, n = 8). [Modified from Hájos et al. (138); figure kindly prepared by Dr. Norbert Hájos.]



12. The cannabinoid receptor FIG. agonist WIN55,212-2 (WIN) inhibits glutamatergic but not GABAergic synaptic transmission in CB<sub>1</sub> receptor knock-out mice. A: in CA1 pyramidal neurons of both  $CB_1$  +/+ and  $CB_1$  -/- mice, the amplitudes of monosynaptically evoked excitatory postsynaptic currents (EPSCs) were reduced in a similar manner by bath application of 1  $\mu \mathrm{M}$  WIN, the effects of which could be readily reversed by 1  $\mu$ M SR141716A (SR), a cannabinoid receptor antagonist. B: 1 µM WIN decreased the amplitudes of evoked IPSCs in  $CB_1 + / +$ mice but had no effect in  $CB_1$  -/- animals. [Modified from Hájos et al. (139); figure kindly prepared by Dr. Nobert Hájos.]

synaptic neurotransmission (Fig. 12A) (15, 17, 251, 323, 333). These actions are probably exerted at a presynaptic locus, for three reasons: 1) cannabinoid agonists increase paired-pulse facilitation, 2) they do not change postsynaptic responses to glutamate or kainate applications, and 3) they cause a characteristic increase in response failures and coefficient of variation of excitatory postsynaptic currents (EPSCs). The ability of the  $CB_1$  antagonist SR141716A to prevent these inhibitory responses suggested early on that  $CB_1$  receptors might be involved. Nevertheless, the fact that careful anatomical analyses negated this hypothesis sent the field up an apparent cul-de-sac: how could cannabinoid agonists inhibit glutamate release if  $CB_1$  receptors are only weakly, if at all, expressed by glutamatergic neurons and are absent from glutamatergic terminals (138, 186-188, 231)? The use of  $CB_1^{-/-}$  mice offered a solution to this conundrum. Cannabimimetic agents reduce glutamatergic EPSCs in  $CB_1^{-/-}$  mice to the same degree as they do in wild-type ones, although they no longer affect GABAergic IPSCs (Fig. 12) (139). The most economical hypothesis compatible with this result is that glutamatergic axon terminals contain a novel cannabinoid-sensitive site, which is blocked by SR141716A, but is molecularly distinct from the cloned  $CB_1$  receptor.

Further pharmacological characterization revealed that the new cannabinoid-sensitive receptor has an order of magnitude lower affinity for WIN55,212–2 compared with CB<sub>1</sub> (137), as the EC<sub>50</sub> for the suppression of EPSCs was 2.01  $\mu$ M, whereas for IPSCs 0.24  $\mu$ M (161). In addition, cannabinoid effects on EPSCs could be antagonized by the vanilloid antagonist capsazepine, and mimicked by

the agonist capsaicin, whereas vanilloid compounds were without effect on GABAergic IPSCs (Fig. 13) (137). These data clearly indicate that cannabinoid receptors controlling IPSCs versus EPSCs are pharmacologically distinct. The latter type is unlikely to be the vanilloid receptor VR1, since WIN55,212-2 does not bind to VR1 on sensory nerves (381). Moreover, VR1 forms a nonselective cation channel (55), whereas cannabinoid effects on glutamatergic EPSCs are mediated via a pertussis toxin-sensitive G protein-coupled process (251, 329), which is in accordance with the ability of WIN 55,212-2 to stimulate  $[^{35}S]GTP\gamma S$  binding in several brain regions of CB<sub>1</sub> knockout mice (36). It is reasonable therefore to conclude that a cannabinoid-sensitive receptor other than CB<sub>1</sub> or VR1 is located on glutamatergic, but not on GABAergic, axons in the hippocampus and possibly other brain areas (though we do not know whether this site corresponds with the one identified by Breivogel and collaborators, Ref. 36).

C) CANNABINOID EFFECTS ON ACETYLCHOLINE RELEASE IN CORTICAL AREAS. The cannabinoid receptor agonist WIN 55,212–2 decreases acetylcholine release from electrically stimulated rat hippocampal slices (120). This effect is mimicked by other synthetic cannabinoid agonists, as well as by the endocannabinoid anandamide, and is prevented by the CB<sub>1</sub> antagonists SR141716A and AM281 (121–123, 182–184). Comparable inhibitory actions also have been demonstrated in the rodent neocortex (121, 183). The role of CB<sub>1</sub> receptors in these responses, suggested by the effects of CB<sub>1</sub> antagonists, is further supported by anatomical and genetic data. CB<sub>1</sub> receptors are expressed by neurons in the medial septum and ventral diagonal band, where cholinergic innervation of the hip-



FIG. 13. Vanilloid receptor ligands regulate glutamatergic, but not GABAergic, neurotransmission in the rat hippocampus. A: bath coapplication of the synthetic cannabinoid agonist WIN55,212-2 (1-5 µM, WIN), with the vanilloid receptor antagonist capsazepine (10  $\mu$ M; CZ), prevented the suppression of monosynaptically evoked EPSC amplitude, but not the amplitude of evoked IPSCs in CA1 pyramidal cells. B: 10 µM capsaicin, a vanilloid receptor agonist, suppressed the amplitude of evoked EPSCs, but not of evoked IPSCs. [Modified from Hájos and Freund (137); figure kindly prepared by Dr. Nobert Hájos.]

pocampus originates (225, 235). In the monkey forebrain, septal CB<sub>1</sub>-immunoreactive cells, along with other CB<sub>1</sub>positive neurons in the nucleus basalis of Meynert (where the cortical cholinergic pathway originates), express choline acetyltransferase (ChAT), the synthetic enzyme for acetylcholine (216). Furthermore, the cannabinoid modulation of acetylcholine release was reduced in "knockdown" experiments with antisense oligonucleotides (182) and abolished in the hippocampus and the neocortex of  $CB_1$  knock-out mice (184). Although unequivocal anatomical demonstration of CB<sub>1</sub> receptors on cholinergic axon terminals is still needed, physiological evidence also supports their existence. In hippocampal slices perfused with a Ca<sup>2+</sup>-free, K<sup>+</sup>-rich medium containing the Na<sup>+</sup> channel blocker tetrodotoxin, cannabinoid agonists attenuate Ca<sup>2+</sup>-evoked acetylcholine release, probably by inhibition of voltage-gated Ca<sup>2+</sup> channels (183). Importantly, a parallel result was obtained in cortical and hippocampal synaptosomes, again implying a presynaptic site of action (121).

What is the functional significance of these in vitro findings? Cholinergic innervation of cortical brain regions is thought to play an important role in cognitive processes, many of which are strongly impaired by cannabinoid treatment (35). An appealing causal link between these observations is strengthened by the finding that cannabinoid agonists reduce acetylcholine levels in rat cortical and hippocampal microdialysates, when administered at relatively high doses (mg/kg) (54, 118). However, recent experiments uncovered that lower doses of these drugs ( $\mu$ g/kg) cause an opposite effect, elevating acetylcholine level in the prefrontal cortex and the hippocampus (3, 4). Such an "inverted U" dose-response relationship warrants further investigation but may be explained by the activation of different cannabinoid receptor types, likely possessing distinct agonist sensitivity (see Refs. 36, 137), or by the dose-dependent engagement of excitatory or inhibitory afferent pathways of the basal forebrain, which may enhance or reduce the intrinsic activity of cholinergic neurons. In any case, the predominant effect of presynaptic  $CB_1$  receptors present on cholinergic axon terminals within the cortex is likely to be the inhibition of acetylcholine release, although such an effect alone may not entirely explain cannabinoid actions on cognition (268).

D) CANNABINOID EFFECTS ON NOREPINEPHRINE RELEASE IN CORTICAL AREAS. Along with cholinergic fibers, ascending noradrenergic pathways are also sensitive to cannabinoid modulation (182, 317). Norepinephrine release is inhibited by cannabinoid agonists, albeit in a species-specific manner, being reduced in human and guinea pig hippocampus and cortex, but not in rat hippocampus or mouse hippocampus, neocortex, and amygdala (122, 123, 153, 184, 317, 344). These species differences are intriguing, especially in light of the highly conserved distribution of  $CB_1$  receptors on the axon terminals of hippocampal GABAergic (138, 139, 187, 188) and septohippocampal cholinergic neurons (184, 216). In the rat locus coeruleus, where ascending noradrenergic pathways originate,  $CB_1$ mRNA expression is very low (225, 235). Thus it would be interesting to determine whether a diverging pattern of  $CB_1$  receptor expression might explain the greater sensitivity of human and guinea pig noradrenergic transmission to cannabinoid regulation.

E) CANNABINOID EFFECTS ON SEROTONIN RELEASE IN CORTICAL AREAS. The finding that cannabinoid agonists reduce both electrically and  $Ca^{2+}$ -evoked serotonin release in mouse brain cortical slices (264) accords with the prevailing

presynaptic localization of  $CB_1$  receptors. But whether serotonergic terminals do in fact contain such receptors is still unknown. Notably, the observed maximal reduction (~20%) in serotonin release is quite low compared with other transmitters like acetylcholine or GABA (50–80%) (120, 188, 264). Furthermore, in situ hybridization studies in the raphe nuclei have yielded inconsistent results (225, 235), and immunohistochemical investigations have not yet been reported.

F) CANNABINOID EFFECTS ON CCK RELEASE IN CORTICAL AREAS. As we have already pointed out, CB<sub>1</sub> receptors are located on the axon terminals of a specific GABAergic cell population in several cortical networks characterized by the expression of CCK (Fig. 9, E and F) (28, 186–188). Therefore, it is not unexpected that cannabinoid agonists inhibit potassium-evoked CCK release in rat hippocampal slices (21). More surprising, however, and still unexplained, is the observation that CCK release is unchanged in the frontal cortex (21). This discrepancy is surprising in light of the coexpression of  $CB_1$  and CCK in the entire neocortex and in the hippocampus (231). In addition, the observed maximal reduction of cholecystokinin was only  $\sim$ 40% in the hippocampus, which seems to be quite low considering the fact that nearly all CCK-containing axon terminals carry  $CB_1$  receptors in this brain region (188). Clarifying this point is particularly important in view of the possible interactions of CCK and anandamide in regulating anxiety and other emotional states (185).

#### 2. Basal ganglia

A) CANNABINOID EFFECTS ON GABA RELEASE IN THE BASAL GANGLIA. CB<sub>1</sub> cannabinoid receptors are expressed by at least three different GABAergic cell populations in the striatum (164, 231). Hence, cannabinoid effects on GABA release in different regions of the basal ganglia are well documented, and solid evidence for presynaptic cannabinoid receptors on GABAergic axon terminals derives from several different pharmacological approaches. Application of cannabinoid agonists to parasagittal slices of the rat midbrain causes a significant reduction of GABA<sub>A</sub> receptor-mediated currents recorded in substantia nigra pars reticulata neurons after stimulation of the internal capsule (367). Comparable results were obtained in coronal midbrain sections, although in this preparation GABAergic currents are more likely to derive from local GABAergic interneurons (58). The presynaptic nature of these responses is supported by the increased pairedpulse ratio of evoked IPSCs and by the lack of cannabinoid modulation on GABA<sub>A</sub> receptor-mediated currents elicited by bath application of GABA (59, 367).

In vivo experiments have provided additional insight on the roles of  $CB_1$  receptors on striatonigral GABAergic terminals (for review, see Ref. 313). Both systemically and locally applied cannabinoid agonists increase spontaneous activity of substantia nigra pars reticulata neurons, probably by removing an ongoing GABAergic inhibition (247, 339). Moreover, striatal stimulation inhibits the firing of nigral neurons, which is also alleviated by cannabinoids. Blocking of  $GABA_A$  receptors by bicuculline reverses this effect, indicating that cannabinoid treatment suppresses GABA release (247).

Striatal stimulation also results in reduced firing of pallidal neurons, and this effect is antagonized by systemic administration of a cannabinoid agonist (248). Surprisingly, local administration of the compound into the globus pallidus does not reverse this effect, raising doubts as to the role of striatopallidal GABAergic projections (249).

Although cannabinoid binding is lower in the striatum compared with its output structures, it is still quite abundant (152). In addition, endocannabinoid release and local cannabinoid receptors may participate in the modulation of striatal neuronal activity (125). Szabó et al. (334) provided electrophysiological evidence that cannabinoids inhibit the amplitude of IPSCs recorded from medium spiny neurons. One presumptive site for this action is the axon terminals of intrinsic inhibitory interneurons (parvalbumin positive; Ref. 231), which provide the major inhibitory control over the activity of striatal projection neurons (197). The contribution of recurrent axon collaterals of medium spiny neurons cannot be excluded at present.

In the shell of the nucleus accumbens, cannabimimetic agents decrease the amplitude of evoked IPSCs and increase the paired-pulse ratio, but do not alter the amplitude of miniature IPSCs, indicating a presynaptic inhibitory effect on GABA release (162, 230). In situ hybridization and immunostaining studies of this region report low CB<sub>1</sub> receptor levels (94, 225, 226, 235, 345), but this low signal may simply reflect a restricted distribution of the receptor to select interneuronal subtypes, as is the case elsewhere in the CNS. The important functions served by the nucleus accumbens in motivational and reward processes and the impact that cannabinoid drugs exert on such processes should encourage further studies aimed at establishing the precise localization of CB<sub>1</sub> receptors in this structure.

B) CANNABINOID EFFECTS ON GLUTAMATE RELEASE IN THE BASAL GANGLIA. The glutamatergic innervation of the basal ganglia derives from three main sources. Neurons in the striatum receive glutamatergic axon terminals from cortical and thalamic projection neurons, whereas neurons in the substantia nigra pars reticulata and globus pallidus receive glutamatergic input from the subthalamic nucleus. Both pathways can be modulated by cannabimimetic agents, which inhibit excitatory postsynaptic currents in the striatum as well as the substantia nigra pars reticulata (116, 168, 169, 335). The increased paired-pulse ratio and coefficient of variation, together with the lack of effect of cannabinoids on response to bath-applied glutamate, support a presynaptic site of action. In addition, currents evoked by direct glutamate application are not modulated by cannabinoids, demonstrating the lack of a postsynaptic component in these effects. A recent study by Gerdeman et al. (117) provided definitive evidence that the reduction of glutamate release in the striatum is mediated by CB<sub>1</sub> receptors, by showing that this effect is absent in CB<sub>1</sub><sup>-/-</sup> mice. Furthermore, these authors also demonstrated that the ability of cannabinoid agonists to acutely inhibit glutamate release is a crucial factor in the initiation of striatal long-term depression (117), a form of synaptic plasticity characterized by a persistent diminution in excitatory transmission.

The cannabinoid modulation of glutamatergic neurotransmission in the globus pallidus and the substantia nigra pars reticulata may be of considerable functional importance (313). Indeed, in contrast to striatal GABAergic projections to the output nuclei, which are usually quiescent, the subthalamic glutamatergic innervation to these two structures is tonically active. The administration of cannabinoid agonists produces changes in the firing of pallidal and nigral neurons, which are consistent with a decrease in this intrinsic activity (249, 314). It will be interesting to determine whether the endocannabinoid system plays a similar role and, if so, under which physiological circumstances.

In the nucleus accumbens, cannabinoid agonists reduce the amplitude of field excitatory postsynaptic potentials (EPSPs) as well as EPSCs recorded from medium spiny neurons in the core, but not the shell region of this nucleus (162, 300). The relatively high cannabinoid concentrations required to produce these effects, and the low expression of CB<sub>1</sub> receptors in the projection neurons of the prefrontal cortex, basolateral amygdala, and thalamus that innervate the nucleus accumbens, suggest that in this region, as in the hippocampus (139), cannabimimetic agents target a CB<sub>1</sub>-like receptor distinct from CB<sub>1</sub>. However, the possibility that the reduced cannabinoid sensitivity may reflect the very low expression level of CB<sub>1</sub> receptors in glutamatergic neurons cannot be excluded, and recent experiments demonstrating that evoked EPCS are not modulated by cannabinoids in CB<sub>1</sub> knock-out mice may also favor this explanation (301).

# 3. Cerebellum

A) CANNABINOID EFFECTS ON GABA RELEASE IN THE CEREBEL-LUM. The cerebellum contains one of the highest densities of CB<sub>1</sub> receptors in the brain. Expression of these receptors in local GABAergic interneurons (both basket and stellate cells) has been suggested by many studies, whereas Purkinje cells do not contain CB<sub>1</sub> mRNA (225, 235). Immunostainings revealed CB<sub>1</sub>-positive putative GABAergic axon terminals forming a pericellular matrix around the axon initial segment and cell body of Purkinje cells or impinging upon their dendritic tree (Fig. 7D) (87, 345).

In accordance, GABAergic synaptic currents recorded from Purkinje cells are strongly modulated by cannabinoids. Takahashi and Linden (337) provided the first evidence that spontaneous IPSCs are suppressed by cannabinoid agonists. They estimated that the amplitude of action potential-dependent IPSCs is reduced by  $\sim 75\%$ , whereas the amplitude of miniature IPSCs is not affected, suggesting a presynaptic mechanism of action. Subsequent experiments using paired recording and imaging of calcium transients in inhibitory axon terminals confirmed this observation (87) and extended it, by showing that endocannabinoids may also regulate afferent inhibitory inputs to Purkinje cells in a retrograde manner (see details in sect. v) (87, 199, 377). Verifying the role of  $CB_1$ receptors, this response is absent from  $CB_1^{-/-}$  mice (377).

B) CANNABINOID EFFECTS ON GLUTAMATE RELEASE IN THE CEREBELLUM. In the cerebellum, as in many other brain regions, cannabinoids can effectively modulate neurotransmission not only at inhibitory but also at excitatory synapses. Two pathways provide excitatory input to the cerebellum, the climbing fibers originating from the inferior olive and the parallel fibers deriving from local glutamatergic granule cells. Early anatomical studies reported a high density of cannabinoid binding sites in the molecular layer of the cerebellar cortex along with the expression of CB<sub>1</sub> mRNA in granule cells (152, 225, 235), indicating, but not proving, the presence of CB1 receptors on parallel fibers. Subsequent immunohistochemical experiments supported this notion by revealing a dense  $CB_1$ -positive axonal meshwork in the molecular layer (94, 345). Hence,  $CB_1$  receptors are situated in a central position to modulate the excitatory input of Purkinje cells. Indeed, whole cell patch-clamp studies revealed that cannabinoids effectively decrease parallel fiber EPSCs (211, 337). Experimental evidence supports a presynaptic effect, in which activation of cannabinoids results in a reduced probability of glutamate release. Neither the excitability of parallel fibers nor the response to locally applied glutamate was modified by cannabinoid receptor agonists (211, 337). Moreover, although the frequency of miniature EPSCs was decreased, the amplitude was also unchanged, indicating the presynaptic localization of cannabinoid receptors. An important consequence of this phenomenon is that cannabinoids may impair cerebellar long-term depression (211). In addition, recent experiments uncovered that endocannabinoids also serve as retrograde signaling molecules in DSE, a phenomenon discussed in detail in section v (200).

In contrast to the cannabinoid effects on parallel fibers, the localization of cannabinoid receptors on climbing fibers seems to be more modest. While a cannabinoid agonist strongly reduced the amplitude of parallel fiber EPSCs (~12% of baseline level), EPSCs deriving from putative climbing fibers were only slightly modulated (~74% of baseline level) (337). However, experimental evidence shows that modulation of glutamate release at climbing fibers is also under the control of endocannabinoids released by the postsynaptic Purkinje cells (200, 223). In this case, the role of CB<sub>1</sub> receptors is not clear yet, because only low levels of CB<sub>1</sub> mRNA were found in the inferior olive, where climbing fibers originate (235). Recent data also suggest the existence of additional cannabinoid binding sites in the cerebellum distinct from CB<sub>1</sub>, although the molecular identity and precise localization of these putative sites is still unknown (253).

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#### 4. Areas and pathways involved in pain perception

A) CANNABINOID EFFECTS ON GABA AND GLYCINE RELEASE IN SPINAL AND SUPRASPINAL NOCICEPTIVE AREAS. Cannabinoid agonists regulate pain sensation by acting at the supraspinal, spinal, and peripheral level (48, 154, 234, 245, 281, 297, 365). One common feature of the regulatory actions of these compounds is their ability to reduce inhibitory neurotransmission in the rostral ventromedial medulla, the PAG, and the trigeminal nucleus caudatus (177, 358, 359). Patch-clamp experiments revealed that in these three structures cannabinoid agonists reduce GABA release through a presynaptic mechanism. In the trigeminal nucleus, the release of another inhibitory transmitter, glycine, is also reduced (177). Although the presynaptic localization of cannabinoid receptors is confirmed by several experiments, the role of  $CB_1$  receptors remains equivocal. In addition, detailed studies clarifying the precise localization of CB<sub>1</sub> receptors at the subcellular level in these brain areas have not yet been conducted.

B) CANNABINOID EFFECTS ON GLUTAMATE RELEASE IN SPINAL AND SUPRASPINAL NOCICEPTIVE AREAS. The regulation of glutamatergic neurotransmission may also contribute to the antinociceptive activity of cannabinoid agonists. In the dorsal horn of the spinal cord, these agents suppress glutamate release from primary sensory afferents. Whole cell patch-clamp recordings in substantia gelatinosa neurons have indeed demonstrated that cannabinoid agonists reduce both the amplitude and frequency of spontaneous EPSCs (259). The frequency, but not the amplitude, of miniature EPSCs was diminished, indicating a presynaptic effect. The presumptive target sites of these effects are the axon terminals of afferent sensory fibers, since evoked EPSCs are also significantly decreased by cannabinoid agonists upon stimulation of the neighboring dorsal root ganglion. The stimulation protocol used in this study indicated that mainly Aδ- and C-fibers were affected, which was also confirmed by using the vanilloid agonist capsaicin (219, 259). These results parallel anatomical evidence that dorsal root ganglion neurons express  $CB_1$  receptors (165), and cannabinoid binding sites are reduced after dorsal rhizotomy or neonatal capsaicin treatment, although only 16% of the total  $CB_1$  receptor population was estimated to be located on C-fibers (163, 165).

Along with the spinal cord, glutamatergic neurotransmission is also affected in neurons of the PAG (358), which may also contribute to the role of cannabinoids in alleviating pain sensation. Interestingly, however, it seems that the inhibitory effect of cannabinoids on glutamate release cannot be extended to all regions involved in antinociceptive activity of cannabinoids. Remarkably, while GABAergic neurotransmission was massively inhibited by cannabinoids in the trigeminal nucleus caudalis, the evoked EPSCs upon stimulation of the trigeminal tract remained unaffected (177). However, the trigeminal tract contains a mixed population of glutamatergic axons with different conduction velocities and activation thresholds. The C-fibers exhibit a higher activation threshold, and thus the effect of cannabinoids on a selected small population of fibers (see above) may be masked by the use of different stimulation protocols (0.07–0.1 Hz in Ref. 177 and 10 Hz in Ref. 259).

C) CANNABINOID EFFECTS ON NEUROPEPTIDE RELEASE IN SPINAL AND SUPRASPINAL NOCICEPTIVE AREAS. As observed throughout the brain, CB<sub>1</sub>-bearing terminals in the spinal cord contain modulatory neuropeptides in addition to fast-acting amino acid neurotransmitters. Two neuropeptides, substance P (SP) and CGRP, are coexpressed with  $CB_1$  receptors in dorsal root ganglia (165). Accordingly, low doses of the endocannabinoid anandamide inhibit capsaicin-evoked CGRP release from both central and peripheral axon terminals of primary sensory neurons in the dorsal horn of the spinal cord, as well as in hindpaw skin (296, 297). In addition, at low concentrations, anandamide also inhibits SP and CGRP release elicited by electrical field stimulation (342), an effect that probably results from the activation of  $CB_1$  receptors and may contribute to the analgesic and anti-inflammatory properties of this lipid mediator (48, 297, 342). At high concentrations, which are unlikely to be attained in vivo, anandamide activates capsaicin-sensitive VR1 receptors, thereby stimulating SP and CGRP release (342). Similar concentrations of the compound also increase the frequency of miniature EPSCs in the spinal cord (258). The physiological significance of these findings, if any, is unknown at present.

#### C. Are There Postsynaptic CB<sub>1</sub> Receptors?

Cannabinoids can evoke physiological responses, which may not be mediated by presynaptic cannabinoid receptors. Recent reports indicate that both endocannabinoids and synthetic cannabinoid agonists modify the exENDOCANNABINOIDS IN SYNAPTIC SIGNALING

citability of neurons via regulation of distinct potassium conductances present on the extrasynaptic dendritic surface of neurons (74, 75, 148, 222, 227, 320). Within the synapse, the modulation of excitatory postsynaptic responses mediated by NMDA receptors was also reported (103, 141). In addition, cannabinoids are also able to induce or suppress gene expression patterns by activating signal transduction pathways likely to occur in the postsynaptic domain of neurons (32, 33, 128, 290, 354).

However, in most cases, the molecular substrates of these effects have not been unequivocally identified. Certain cannabinoid compounds were shown to activate ion channels and receptors other than CB<sub>1</sub> receptors (36, 60, 139, 227, 381). In addition, although many of the cannabinoid effects mentioned above were blocked by the CB<sub>1</sub> antagonist SR141716A, experiments with CB<sub>1</sub><sup>-/-</sup> mice demonstrate that this antagonist also recognizes other CB<sub>1</sub>-like receptors (Fig. 12) (139, 175). Further studies on genetically modified animals and novel, more selective pharmacological tools are thus needed to dissect all the molecular components of cannabinoid neuromodulation.

In contrast to the well-established evidence of presynaptic CB<sub>1</sub> receptors in various brain regions and on the axon terminals of a number of distinct cell types, the presence of functional CB1 receptors on the plasma membrane of the dendritic tree or somata of neurons requires more solid evidence than available at present. Although postsynaptic CB<sub>1</sub> receptors have been suggested to exist (276, 302, 311, 312), published data show a clear mismatch between the subcellular localization of the protein epitope used to generate the antibody and the distribution pattern of immunolabeling. The antibody used in these studies was generated against the NH<sub>2</sub> terminus of the  $CB_1$  receptor protein (345), which is expected to be situated on the outer surface of the plasma membrane. In contrast, in these studies dendritic  $CB_1$  labeling is invariably found inside cells and is often distant from the plasma membrane. This pattern might represent a labeling artifact, common in immunogold and immunoperoxidase staining, or may be biologically relevant. In some cases, dendritic CB<sub>1</sub> immunolabeling is clearly associated with intracellular organelles participating in the processing or degradation of proteins, such as the rough endoplasmic reticulum, the Golgi apparatus, or multivesicular bodies (302, 311, 312). Since these intracellular organelles usually intrude into the cytoplasm of proximal dendrites, in single electron microscopic sections they appear to be located within dendritic segments. Nevertheless, these segments belong functionally to the somatic (perinuclear) region, because they compose a continuous network within these structures. In our view, the most likely explanation for this labeling pattern is that the antibody also recognizes the freshly synthesized or degraded CB<sub>1</sub> protein. In support of this idea, correlated light and electron microscopy using high-resolution immunogold technique

provide clear-cut evidence that the CB<sub>1</sub> immunostaining visualizing cell bodies and proximal dendrites of interneurons at the light microscopical level is always associated with intracellular organelles, but never with the somatic or dendritic plasma membrane (186, 188). Moreover, the antibody recognizing the NH<sub>2</sub> terminus of the CB<sub>1</sub> receptor selectively labels the axon terminals of these interneurons, and the gold particles are found exclusively on the outer surface of the plasma membrane, demonstrating the availability of the NH<sub>2</sub>-terminal epitope for this antibody in conventional electron microscopic preparations (Fig. 10, *C–E*) (188). Thus, in contrast to presynaptic CB<sub>1</sub> receptors, establishing the presence of such receptors on the plasma membrane of the dendritic tree or somata of neurons will require further experimentation.

# V. PHYSIOLOGICAL ROLES OF ENDOCANNABINOIDS

In the year 2001, we witnessed the merger of two independent lines of research, namely, decades of investigations into the cellular and network effects of exogenous cannabinoids, and studies on the characteristics of DSI, a form of retrograde synaptic signaling. Wilson and Nicoll (375) and Ohno-Shosaku et al. (271) provided the missing link between these two lines by demonstrating that an as-yet-unidentified endocannabinoid substance mediates DSI. If we want to evaluate the studies that led to the present understanding of endocannabinoid functions, we should follow the milestones of research not only in the field of cannabinoid pharmacology, but also the sequence of discoveries that led to the establishment of the phenomenon, as well as the pharmacology and physiology of DSI, namely, the work that was initiated by the groups of Alger and Marty in the early 1990s (214, 288). Thus this section will synthesize the findings deriving from these two roots of research with the aim to better understand the functional roles of endocannabinoids at the synaptic and network levels.

# A. The Cannabinoid Root

It has been known for decades that cannabinoids have a profound influence on learning and memory (76, 155, 250). This may be related 1) to the impairment of long-term potentiation (LTP) that is generally believed to be linked to learning-associated synaptic plasticity of glutamatergic connections, 2) to a disturbance of fast and slow oscillations maintained by GABAergic interneurons that secure the necessary synchrony in the discharges of connected neurons, or 3) to alterations in the activity or release properties of monoaminergic and cholinergic subcortical pathways known to influence cortical plasticity and activity states. Thus the major questions here concern the brain region(s) and transmitter system(s) involved, as well as the network mechanisms underlying the cannabinoid effects.

In addition to showing an intense CB<sub>1</sub> receptor binding, the hippocampus is known to be a crucial area involved in learning and memory. LTP, as well as fast and slow oscillations, has been investigated most extensively and reproducibly in this brain region, and the underlying synaptic connectivity is relatively well understood. These features together provided sufficient reason to focus the majority of cannabinoid electrophysiology, and a large part of this section of the present review, on the hippocampus. On the other hand, the cerebellum and the basal ganglia are also extensively studied in cannabinoid physiology due to the well-known behavioral effects associated with these regions (for review, see Ref. 313), as well as to the very high density of cannabinoid binding sites (152). In addition, the cerebellum was one of the areas where DSI (and later DSE) was discovered (200, 214). Cannabinoid effects in these two brain regions have been discussed at the cellular level in sections III and IV; here we only focus on implications for DSI/DSE and, whenever data are available, possible network mechanisms.

Glutamate is the major mediator of intracellularly recorded as well as field EPSPs in the hippocampus, and it is the transmitter at synapses that are best known to show long-term plastic changes in strength. In addition, the laminar distribution of  $CB_1$  receptor binding in the hippocampus overlaps with glutamatergic pathways, which together explains why this transmitter has been investigated most extensively. On the other hand, both fast and slow oscillations rely on local GABAergic interneurons in the hippocampus (and neocortex), and in addition, GABA is by far the most dominant neurotransmitter in the cerebellum and basal ganglia as well, providing ample reason for focusing studies also on this transmitter. In addition to influencing learning and memory, cannabinoids have a profound effect on mood, emotions, and motivation, which are known to involve subcortical monoaminergic pathways. Therefore, effects of cannabinoids on dopaminergic, cholinergic, serotonergic, and noradrenergic transmission have also been extensively studied, mostly in the basal ganglia, and to some extent also in the hippocampus, amygdala, and neocortex (see sect. IV).

# 1. Effects on evoked potentials and long-term synaptic plasticity

In general, any drug actions on field EPSPs, population spikes, and paired-pulse (short term) synaptic plasticity are difficult to interpret, since several mechanisms may underlie any observed changes. These mechanisms should be studied by intracellular recordings from single cells or connected cell pairs, and parallel population data should be provided. Such combined studies are rather rare in the cannabinoid field; therefore, we chose to present the data from the literature without necessarily attempting to provide an explanation for the mechanism of cannabinoid actions, and for the conflicting data. Many of the conflicting results may be due to the dual cannabinoid actions on CB1 and on the new cannabinoid-sensitive receptor that is present on glutamatergic axons in the hippocampus (and possibly also in other areas), which can be influenced by several of the agonists and antagonists that are extensively used today as "selective" CB<sub>1</sub> ligands (36, 139; see sect.  $\mathbb{N}B1_{B}$ ). Some controversial interpretations of earlier studies may result from the shortage of data on the pre- or postsynaptic localization of the cannabinoid receptor(s). Our interpretation of these earlier results rests on the recent knowledge that these receptors are mostly, if not exclusively, presynaptic, as reviewed in section IV.

One of the earliest electrophyiological studies using cannabinoid agoinsts found that cannabinoids suppressed sensory-evoked or spontaneous firing of dentate granule cells and elicited characteristic changes in evoked potential waveform (50, 51). In the hippocampus, Wilkison and Pontzer (372) showed that  $CB_1$  agonists and antagonists had negligible effects on field EPSPs and population spikes, whereas in some other studies cannabinoids were shown to have a dose-dependent biphasic effect on evoked population spikes. At low doses, delta-9-THC augments evoked field EPSPs as well as orthodromically or antidromically evoked population spikes, whereas at higher doses the responses are depressed (202, 269, 356, 369). In recent studies, the endogenous ligand anandamide was shown to decrease the slope of Schaffer collateral-evoked field EPSPs, as well as the amplitude of population spikes in the CA1 region at relatively low  $(1 \ \mu M)$ and high (10  $\mu$ M) concentrations as well (15). The antagonist SR141716 prevented the effect of anandamide and when applied on its own induced a small increase in population spike amplitude. This suggests that endogenously released cannabinoids may be capable of inhibiting glutamate release. In contrast, another endocannabinoid, 2-AG, had no effect on the slope of evoked field EPSPs in CA1 (328), implying that the endogenous cannabinoid action observed by Ameri et al. (15) using SR141716A is likely exerted by anandamide alone. This effect is probably due to presynaptic inhibition of glutamate release, since population spike amplitudes evoked by antidromic stimulation (a reflection of excitability) did not change upon cannabinoid receptor activation (15). Higher doses of WIN55,212–2 also reduced paired pulse faciliation in the dentate gyrus, where the effect is again likely to be the inhibition of glutamate release from perforant path terminals (192).

Cannabinoid agonists were also found to decrease

paired-pulse depression of population spikes in the CA1 region (12, 13, 278). Paired-pulse depression of population spikes is thought to be due to recruitment of GABAergic inhibition, which normally decreases pyramidal cell excitability on the second stimulus; thus the interpretation of this result was that cannabinoids may be decreasing this paired-pulse effect by reducing feedback inhibition. Interestingly, a recent study from the same laboratory demonstrated that while 2-AG largely replicates the effect of WIN55,212-2 on paired-pulse inhibition, the other endocannabinoid, anandamide, had an opposite effect. It increased paired-pulse depression (PPD; Ref. 13), which was mimicked by the vanilloid agonist capsaicin, and antagonized by capsazepine. This may be interpreted as signifying an anadamide-induced reduction of glutamate release from axon terminals (including those that activate GABAergic feedback inhibition), which may result in a better activation of these interneurons by the second stimulus, and a concomittant increase in paired-pulse inhibition. These data, together with recent evidence that cannabinoid actions on GABAergic  $(CB_1)$  and glutamatergic transmission (new CBR), are mediated by distinct receptors (139, see also sect. WB1B and Fig. 12), and that their effect on glutamatergic EPSCs, but not those on GABAergic IPSCs, can be antagonized by capsazepine (Fig. 13A) (137), suggest that the two endocannabinoids may differentially act on the two receptors. Anandamide may selectively inhibit glutamate release, whereas 2-AG may preferentially act on GABAergic terminals, as also suggested by the data of Ameri et al. (15) and Stella et al. (328) discussed above.

One of the first experiments with delta-9-THC was to test its effects, and later those of anandamide, 2-AG, and synthetic cannabinoid ligands, on LTP. The first report suggesting a reduction of LTP in the hippocampus by delta-9-THC came from Nowicky et al. (269). Reduction of LTP was also observed by other laboratories using endogenous ligands, or various agonists and antagonists, which at that time were thought to act on  $CB_1$  receptors alone (62, 63, 251, 278, 328, 338). The mechanism of these cannabinoid actions is difficult to interpret knowing that the employed agonists (e.g., WIN55,212-2) and antagonists may act on both GABAergic and glutamatergic transmission (139). According to Terranova et al. (338), WIN55,212-2 had its maximal inhibitory effect on LTP at a concentration of 3  $\mu$ M, which is 50% over the EC<sub>50</sub> of this agonists on glutamatergic EPSCs, and it is more than 10 times the  $EC_{50}$  for GABAergic IPSC suppression (137, 161). Similarly, Paton et al. (278) observed a blockade of LTP by 5  $\mu$ M WIN55,212–2, whereas low doses (250 nM) decreased but did not block LTP. Both doses were effective in reducing paired-pulse inhibition in the same slices (278). The order of magnitude difference between the affinity of  $CB_1$  (located on GABAergic terminals) and the new CB receptor (located on glutamatergic terminals) for

WIN55,212–2 suggests that the blockade of LTP is due to a direct inhibitory action of the higher agonist dose on glutamate release. GABA release should be decreased by both of these concentrations of the agonist, which likely accounts for the reduced inhibition of the population spike evoked by the second pulse in the PPD paradigm. Similar cannabinoid effects on LTP were observed also in the presence of picrotoxin (251), which further confirms that the site of action is the glutamatergic axon terminal. Anandamide was also shown to have a concentrationdependent effect on LTP, although it did not block it completely (338). Bath application of 2-AG had a similar effect (328), which is difficult to explain knowing that in the same study 2-AG did not reduce field EPSPs and therefore is unlikely to inhibit glutamate release. It does inhibit GABA release, but that should rather enhance LTP. One possibility is that 2-AG might have a direct (non-CB receptor mediated) action on NMDA receptors, but in the opposite direction than an and amide (141), i.e., reducing Ca<sup>2+</sup> influx via NMDA receptors, or the inhibitory effect of 2-AG on glutamate release, if there is any, may become detectable only during high-frequency activation.

Another finding difficult to reconcile with the conclusions drawn so far is the enhanced LTP observed in CB<sub>1</sub> knock-out animals (31). It has been long known that suppression of inhibition (e.g., by pharmacological blockade of GABAergic neurotransmission or by induction of the DSI paradigm) facilitates the induction of LTP (53, 371). Thus a loss of endocannabinoid control of GABA release should increase inhibition, which likely counteracts LTP. An alternative explanation might involve a loss of CB<sub>1</sub> receptors from cholinergic or noradrenergic afferents to the hippocampus (see sect. w) resulting in an enhanced release of these transmitters, which may contribute to the facilitation of LTP (40, 189, 195, 326).

#### 2. Effects on population discharge patterns

The recent increase in cannabinoid research in the last 2–3 years brought about by the characterization of  $CB_1$  receptor-mediated actions on identified neurons and circuits has not as yet resulted in a similar boosting of in vivo research into the effects on network activity patterns. Most of the data that can be reviewed here are over 20 years old and were obtained without the currently available more selective and reliable drugs.

The septal driving of hippocampal theta rhythm was shown to be decreased by delta-9-THC, and the effect was attributed to a reduction of noradrenergic transmission (135), which normally acts in the medial septum as well as in the neocortex and hippocampus. This interpretation is consistent with recent evidence for a cannabinergic reduction of norepinephrine release (see sect. vB1D), but does not take into consideration direct cannabinoid effects on the GABAergic and glutamatergic components of the cortical/hippocampal circuitry. The peak-to-peak voltage of cortical electroencephalogram (EEG) recorded with chronic electrodes was found to decrease after acute delta-9-THC application. This reduction in spectral power subsided within 8 h, coincident with behavioral recovery (42). High-voltage EEG bursts have been reported to accompany the reduced low-voltage fast-frequency (desynchronized) activity both in rats (42) and monkey (237). EEG spike-bursts predominated over the temporal and frontal cortices in monkey. Interestingly, in the rat, the single theta peak ( $\sim 8$  Hz) in the power spectrum recorded during rapid-eye-movement sleep was broken up by cannabinoids into two peaks at 7 and 11 Hz, suggesting that different theta oscillator mechanisms may have been decoupled (309). The rat and monkey data are partly contradicted by EEG studies in the rabbit, where hippocampal theta and cortical EEG spike-bursts were found to be disrupted, and cortical voltage output was generally increased by delta-9-THC in a dose-dependent manner (66). Solid implants of delta-9-THC into the ventral hippocampus, however, induced epileptic activity that produced afterdischarges in the contralateral hippocampus and other distant brain areas (321). Systemic administration of delta-9-THC increased afterdischarge duration in the rat and facilitated transcallosal cortical evoked potentials (348). Taken together with our present knowledge of  $CB_1$ receptor distribution (see sects. III and IV), both studies suggest that disinhibition via  $CB_1$  receptors on basket cells may be the dominant effect in these cases.

Tests of cannabinoid effects using in vitro models of network oscillations are essentially limited to a single study, where Hájos et al. (138) demonstrated that the cannabinoid agonist, CP 55,940, reversibly reduces kainate-induced gamma oscillations in hippocampal slices. These data are consistent with the well-known interference of cannabinoid actions with basket cell function, which includes synchronization of pyramidal cell activity at both high and slow frequencies (61, 111).

# B. The DSI (DSE) Root: Control of GABAergic and Glutamatergic Synaptic Transmission via Retrograde Synaptic Signaling

Although modulation of synaptic transmission by retrograde messengers has been well established in the peripheral nervous system of vertebrates or invertebrates (72, 178), the potential importance and physiological role of this phenomenon in the vertebral CNS is still a debated question. Several classical and unconventional transmitters have been shown to be released from the postsynaptic neuron, and to influence synapse formation (107), as well as to modulate transmitter release from afferent boutons terminating on the same or an adjacent cell (10, 196, 373, 378, 379). Thus these signal molecules that include, e.g., amino acids, dopamine, neuropeptides, endocannabinoids, arachidonic acid, nitric oxide, or carbon monoxide, act in a retrograde fashion, whereby neurons may be able to regulate their own inputs and excitability in an activity-dependent manner.

### 1. DSI

A unique, slow,  $Ca^{2+}$ -dependent type of retrograde signaling was independently discovered a decade ago by two laboratories, one working in the cerebellum (214) and the other in the hippocampus (288). A train of postsynaptic action potentials, or a prolonged postsynaptic depolarization (0.1–2 s), was shown to induce a transient suppression of spontaneous or evoked GABAergic IPSP(C)s recorded in the postsynaptic neuron. This phenomenon was termed depolarization-induced suppression of inhibition, or DSI (10). Both in hippocampal pyramidal cells and cerebellar Purkinje cells evidence has been provided that DSI requires a large increase in intracellular Ca<sup>2+</sup> concentration on the postsynaptic side, which results in the release of a retrograde messenger that acts on the presynaptic terminals, reducing the probability of GABA release. DSI can be blocked by postsynaptic  $Ca^{2+}$  buffers or initiated by activity restricted to the postsynaptic side, and likely involves the opening of voltage-gated Ca<sup>2+</sup> channels (210, 214, 288, 289), or release from intracellular stores. Changes in postsynaptic GABAA receptor sensitivity have been excluded, since the response to iontophoretically applied GABA did not change, and DSI had no effect on the amplitude of miniature IPSCs. Despite the clearly postsynaptic site of initiation, numerous experiments demonstrated that DSI is expressed presynaptically, i.e., as a reduction in GABA release. With the use of minimal stimulation, DSI was found to increase failure rate, multiquantal components were also eliminated, and components of IPSCs were differentially influenced (11). In the cerebellum, axonal branch point conduction failure was shown to play a role (361). Furthermore, DSI was reduced by 4-aminopyridine and veratridine, both acting on the presynaptic terminal (11). Direct evidence for an inhibitory G protein-mediated presynaptic action has been provided by Pitler and Alger (289), as they showed that DSI was pertussis toxin sensitive.

Both laboratories hypothesized from the very beginning that they were dealing with a phenomenon that involves retrograde messengers. Llano et al. (214) stated that " $Ca^{2+}$  rise in the Purkinje cell leads to the production of a lipid-soluble second messenger." This was a remarkable prediction 10 years before the discovery that, indeed, the lipid-soluble endocannabinoids are these messengers (271, 375, for details, see below), although the earlier claim of a retrograde action of arachidonic acid in the presynaptic control of LTP (373) made this assumption rather plausible at that time.

The quest for identifying the chemical nature of this retrograde messenger began with the discovery of DSI. The slow onset ( $\sim 2-3$  s to a maximal effect), the requirement of a lasting  $Ca^{2+}$  rise, and the  $Ca^{2+}$  buffer effects (see below) were all consistent with a hormone or peptide rather than classical vesicular neurotransmitter. Yet the first substance suggested by direct experimental evidence was glutamate. In the cerebellum, metabotropic glutamate receptor (mGluR) agonists, acting on presynaptic group II mGluRs, were shown to mimic and occlude DSI, whereas antagonists reduced it (130). Activation of adenylate cyclase by forskolin reduced DSI, which is consistent with the proposed reduction of cAMP levels by mGluR2/3 activation that is known to lead to a reduction of GABA release (52). In contrast, in the hippocampus, forskolin and group II or III mGluR ligands were without effect on DSI; however, group I agonists occluded, and antagonists reduced it (257). Pharmacology and the anatomical distribution of the receptors suggested that mGluR5 is likely to be involved in the reduction of GABA release (257), but it appeared to be confined to the somadendritic compartment of the neurons perisynaptically around glutamatergic contacts (217, 218), which was difficult to reconcile with the hypothesis of glutamate being the retrograde signal molecule (but see sect. WC for the contribution of glutamate). The long duration of DSI is not due to the dynamics of the  $Ca^{2+}$  transient, as it was the same in EGTA and BAPTA (209), but probably to the slow disappearance of the retrograde messenger molecule from the site of action around the presynaptic terminal. This again is inconsistent with glutamate being the messenger (provided that it has no presynaptic mGluRmediated effect, see above), since this transmitter is known to be rapidly taken up.

The fast buffer BAPTA and the slow buffer EGTA reduced DSI to a similar degree, suggesting that the site of  $Ca^{2+}$  entry (for example, the voltage-dependent  $Ca^{2+}$ channels) and the site of calcium's action in DSI induction are relatively far from each other (209). One possibility is that the target of incoming  $Ca^{2+}$  may be an intracellular  $Ca^{2+}$  store that is able to produce large  $Ca^{2+}$  transients required for the release of the signal molecule. On the other hand, the selective N-type Ca<sup>2+</sup> channel blocker  $\omega$ -conotoxin was able to block DSI (209), which, according to recent evidence (374), turned out to be an action on the presynaptic terminals that are sensitive to DSI and selectively express the N-type Ca<sup>2+</sup> channel. These data suggest that Ca<sup>2+</sup> plays a dual role: it is involved in the initiation (priming) phase via Ca<sup>2+</sup>-induced Ca<sup>2+</sup> release from intracellular stores in the postsynaptic side as well as in the effector phase via N-type  $Ca^{2+}$  channels on presynaptic terminals (for details, see sect. vC).

Obviously, DSI-like phenomena can have a functional role in neuronal signaling only if they can be induced by physiologically occurring activity patterns. In cerebellar Purkinje cells, 100-ms depolarization (from -60 to +20mV) was required for a detectable reduction in IPSCs (214), which, under physiological conditions, may correspond to a few climbing fiber-induced complex spikes (30 ms each). Thus a short train of climbing fiber-induced spikes is expected to lead to an increased excitability of the innervated Purkinje cell for tens of seconds. Initiation by very few spikes, occasionally even two if closely spaced, has been reported in the hippocampus (288). With 100  $\mu$ M BAPTA in the pipette, detectable DSI could be evoked already by depolarization as short as 25 ms, and half-maximal effect was produced by 187 ms, or by 109-ms depolarization in the absence of BAPTA (209). This suggests a lower threshold, but also a smaller magnitude and shorter time course of DSI compared with the cerebellum. The behavior-dependent electrical activity patterns in the hippocampus that may lead to DSI (induced by endocannabinoid release, see below) are discussed in section vD.

#### 2. DSE

Recent studies by Kreitzer and Regehr (200) provided evidence that, at least in the cerebellum, excitatory synaptic transmission is also under the control of retrogradely acting signal molecules. Both parallel fiber and climbing fiber-evoked EPSCs were suppressed for tens of seconds by a 50- to 1,000-ms depolarization of the postsynaptic Purkinje cells from -60 to 0 mV. Due to the obvious similarity to DSI, this phenomenon has been termed depolarization-induced suppression of excitation (DSE). Paired-pulse experiments, showing that shortterm plasticity is affected by the depolarization paradigm for both parallel and climbing fiber responses, demonstrated that the site of expression of DSE is presynaptic and involves a reduction in the probability of transmitter release. BAPTA in the recording pipette completely abolishes DSE, providing evidence for the requirement of postsynaptic  $Ca^{2+}$  rise to trigger the event (see further details in sect. vC). Earlier reports are consistent with the lack of DSE in the hippocampus (364), but a recent study using excessive depolarization for 5-10 s (i.e., for much longer than required for DSI) argues for its existence also in this brain region (273). Whether the mechanisms of DSE are similar in the hippocampus and cerebellum is dicussed in the following section.

# C. Marriage of the Two Lines of Research Explains the Mechanism of DSI (and DSE) While Endowing Endocannabinoids With Function

The discovery by Wilson and Nicoll (375), Ohno-Shosaku et al. (271), and Kreitzer and Regehr (199, 200) that DSI/DSE are mediated by endocannabinoids revealed that investigations in both the cannabinoid and DSI/DSE

fields have been dealing accidentally with the same subject, i.e., the mechanism of retrograde synaptic signaling via endocannabinoids. Both receptor localization data and identification of the physiological actions of cannabinoids on synaptic transmission confirmed that cannabinoids act on presynaptic axons, reducing transmitter release (see sect. IV), whereas endocannabinoids are most likely released from the postsynaptic neuron upon strong stimuli that give rise to large Ca<sup>2+</sup> transients. Thus the signal molecules, which turned out to be endocannabinoids, travel from the post- to the presynaptic site and thus enable neurons to influence the strength of their own synaptic inputs in an activity-dependent manner. This may be considered as a short definition of retrograde synaptic signaling and perhaps, at the same time, summarizes the function of the endocannabinoid system. However, before trying to correlate the findings of cannabinoid and DSI (DSE) studies, one should be aware of the major limitations. There are numerous examples of mismatch in receptor/transmitter distribution in the brain; receptors can be found in locations where they hardly ever see their endogenous ligand. Nevertheless, these receptors readily participate in mediating the effects of its exogenous ligands, e.g., during pharmacotherapy. We are facing the same problems with the relative distribution of cannabinoid receptors versus endocannabinoid release sites both at the cellular and subcellular levels. In addition, the distance to which anandamide and 2-AG are able to diffuse (in the presence or absence of transporter blockers) is also an important question from the point of identifying the degree of mismatch. Thus correlation of the sites of action of cannabinoid drugs and the sites of expression of DSI (and DSE) should reveal the regional, cellular, and subcellular domains where receptor and endogenous ligand distributions match, i.e., where endocannabinoids are likely to have a functional role in synaptic signaling.

Several lines of evidence have been provided that endocannabinoids represent the retrograde signal molecules that mediate DSI both in the hippocampus and cerebellum, as well as DSE in the cerebellum. Antagonists of CB<sub>1</sub> receptors fully block (Fig. 14, A and B) and agonists occlude DSI and DSE, whereas DSI is absent in CB<sub>1</sub> receptor knock-out animals (Fig. 14, C and D) (87, 199, 200, 271, 374, 375, 377). In these experiments either singlecell or paired recording has been used, and retrograde synaptic signaling has been evoked by the same procedures as described in the original work of Alger's and Marty's groups (214, 288). In addition, Wilson and Nicoll (375) demonstrated that uncaging of Ca<sup>2+</sup> from a photo-



FIG. 14. A: in CA1 pyramidal neurons, a 5-s depolarizing step from -60 to 0 mV causes a transient suppression of GABAergic IPSCs. Depolarization-induced suppression of inhibition (DSI) is measured as a percentage depression of IPSC amplitude. Slices preincubated in the CB<sub>1</sub> antagonists AM251 (2  $\mu$ M) or SR141716 (2  $\mu$ M) show little or no DSI. Insets show average IPSCs in the 10 s before and 10 s just after the depolarizing step (overlaid). Glutamate receptor antagonists in the bath permit pharmacological isolation of IPSCs. B: average time course of eIPSC amplitudes after depolarization for control and AM251-treated slices. C: DSI is normal in  $CB_1 + / +$  and  $CB_1$ +/- mice, but completely absent in CB<sub>1</sub> -/- mice. Insets show eIPSCs for each genotype, with basal and depressed eIPSCs overlaid. D: average time course of eIPSC amplitudes after depolarization in  $CB_1 + /+,$  $CB_1 + /-$ , and  $CB_1 - /-$  mice. (Figure was kindly prepared by Rachel Wilson and Roger Nicoll.)

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labile chelator induces DSI that was indistinguishable from that evoked by depolarization. Thus a large intracellular Ca<sup>2+</sup> rise is a necessary and sufficient element in the induction of the release of endocannabinoids. As expected from the membrane-permeant endocannabinoids, their release does not require vesicle fusion, since botulinum toxin delivered via the intracellular recording pipette did not affect DSI. A further crucial question concerns the range to which the released endocannabinoids are able to diffuse. Recordings at room temperature from pyramidal cells at various distances from the depolarized neuron releasing the signal molecules revealed that it is only the adjacent cell, at a maximum distance of 20  $\mu$ m, to which endocannabinoids are able to diffuse in a sufficient concentration to evoke detectable DSI (360, 375). However, a considerably greater endocannabinoid uptake and metabolism should be expected at physiological temperatures, which likely results in a decreased spread and a more focused action.

Earlier data indicating the involvement of glutamate and mGluR receptors in DSI also needed clarification (256, 257). Varma et al. (357) demonstrated that enhancement of DSI by mGluR agonists could be blocked by antagonists of both group I mGluR and CB<sub>1</sub> receptors, whereas the same mGluR agonists were without effect in CB<sub>1</sub> receptor knock-out animals. This provides direct evidence that any mGluR effects on DSI published earlier were mediated by endocannabinoid signaling, and glutamate served here as a trigger for the release of endocannabinoids rather than as a retrograde signal molecule as thought earlier. These data were subsequently confirmed by paired recordings from cultured hippocampal neurons (272). In a recent paper, Maejima et al. (223) demonstrated that mGluR1 activation induces DSE in Purkinje cells even without changing the intracellular Ca<sup>2+</sup> concentration. This suggests that, at least in the case of cerebellar Purkinje cells, two independent mechanisms may trigger endocannabinoid synthesis (and release); one involves a transient elevation of intracellular  $[Ca^{2+}]$ , and the other is independent of intracellular [Ca<sup>2+</sup>] and involves mGluR1 signaling. This may imply that, under normal physiological conditions, different induction mechanisms may evoke the release of different endocannabinoids. With the growing number of potential endocannabinoids (see sect. IIB4), the question arises whether they are involved in distinct functions, i.e., by acting at different receptors and/or at specific types of synapses. This question represents one of the hot spots of current endocannabinoid research, and direct measurements of the different endocannabinoid compounds during retrograde signaling should provide an answer.

There are several mechanisms by which endocannabinoids may suppress transmitter release. They may induce branch-point failure, decrease action potential invasion of axon terminals, reduce  $Ca^{2+}$  influx into the

synaptic varicosities via N- or P/Q-type channels, or block the release machinery somewhere downstream from the  $Ca^{2+}$  signal. Using  $Ca^{2+}$  imaging of single climbing fibers (200) provided evidence that DSE involves a reduction of presynaptic  $Ca^{2+}$  influx, which has the same time course as the reduction of the EPSC. Branch-point failure was shown not to contribute to DSE, at least in the case of climbing fibers, as stimulation of the examined single axon evoked a uniform rise of Ca<sup>2+</sup> throughout its entire arbor. These findings are supported by the fact that cannabinoids are known to block N-type  $Ca^{2+}$  channels in neuroblastoma cells (221) and reduce synaptic transmission by inhibiting both N- and P/Q-type channels in neurons (349). Inhibition of the release machinery is unlikely to play a role, particularly in GABAergic transmission, since  $CB_1$  receptor activation has little if any effect on mIPSC frequency in the presence of tetrodotoxin and cadmium (138, 161, 186). Furthermore, CB<sub>1</sub> receptors tend to be localized away from the release sites, having a high density even on preterminal axon segments, which also argues against this possibility (138, 187, 188).

In the hippocampus, evidence has been provided that DSI likely involves a direct action of G proteins on voltage-dependent calcium channels. These included the demonstration that modulation of kinase and phosphatase activities or cAMP levels (257, 374) has no effect on DSI, while the relatively rapid onset (on average 1.2 s) of IPSC suppression makes a phosphorylation-mediated change in channel activity less likely, since that would typically require several seconds. They confirmed the findings of Lenz et al. (210) that  $\omega$ -conotoxin, but not  $\omega$ -aga-toxin is able to block DSI, which means that the G protein-mediated endocannabinoid actions target only the N-type but not the P/Q-type Ca<sup>2+</sup> channels in the hippocampus (Fig. 15). DSI or the selective  $Ca^{2+}$  channel inhibitors never block IPSCs completely, which may be due to a partial reduction of release from all terminals, or to the selective expression of CB<sub>1</sub> receptors together with the N-type channels only on a particular subset of interneurons. Wilson et al. (374) provided an elegant resolution to this dilemma using paired recordings, which revealed that interneurons producing IPSCs with distinct kinetics express different presynaptic Ca<sup>2+</sup> channels, and those that show DSI possess only N-type channels (see Fig. 15). This finding correlates well with the anatomical observations that CCK-containing basket cells selectively express  $CB_1$  receptors, whereas another basket cell type (that contains parvalbumin) lacks  $CB_1$  receptors (Fig. 9, E-G) (188). The differences in IPSC kinetics observed by Wilson et al. (374) may be due to CCK cells forming synapses that are enriched in  $\alpha_2$ -subunit-containing GABA<sub>A</sub> receptors (270), whereas parvalbumin-containing basket cells synapse onto GABA<sub>A</sub> receptors with five times less  $\alpha_2$ -subunits (likely having  $\alpha_1$  instead). Taken together, these data suggest that CCK-containing basket



FIG. 15. A: DSI was monitored by comparing eIPSC amplitudes just before (solid symbols) and just after (open symbols) depolarizing steps. After a stable baseline period, the N-type VDCC antagonist  $\omega$ -conotoxin GVIA ( $\omega$ -CTx-GVIA) was washed onto the slice, causing a depression of basal IPSC amplitude and a complete block of DSI. Subsequent wash-in of WIN55212-2 had no effect, indicating that N-type VDCCs are required for presynaptic inhibition by cannabinoids. Conversely, the P/Q-type VDCC antagonist ω-agatoxin TK (ω-Aga-TK) depressed basal IPSC amplitude but increased DSI magnitude. Subsequent wash-in of WIN55212-2 blocked most of the remaining IPSCs, indicating that the component of release mediated by N-type VDCCs is highly sensitive to cannabinoids. B: raw traces from unitary GABAergic connections, classified accordings to kinetics and DSI sensitivity. Three overlaid sweeps acquired just before depolarization are displayed next to three overlaid sweeps acquired just after depolarization. "Fast I" connections show both failures and smallamplitude successes after depolarization, whereas connections from the other two groups ("fast II", "slow") are not affected by depolarization. Average unitary IPSC amplitude is significantly larger for fast I connections compared with either of the other two groups. C: a representative experiment showing that ω-CTx-GVIA completely blocks synaptic transmission at a fast I synapse, whereas ω-Aga-TK has no effect. Inset shows averaged traces corresponding to baseline (1),  $\omega$ -Aga-TK (2), and  $\omega$ -CTx-GVIA (3). (Figure was kindly prepared by Rachel Wilson and Roger Nicoll.)

cell terminals selectively express N-type  $Ca^{2+}$  channels together with  $CB_1$  receptors predisposing them to DSI, whereas parvalbumin-containing interneurons may express only the P/Q-type  $Ca^{2+}$  channels, lack  $CB_1$  receptors, and are therefore unaffected by DSI.

This conclusion also suggests that the success of DSI induction in any hippocampal slice preparation depends on the relative contribution of the two basket cell types to the examined spontaneous or evoked IPSCs samples. Carbachol is known to enhance DSI, but the mechanism has

not been revealed to date (232). One possibility is that carbachol activates the inositol 1,4,5-trisphosphate (IP<sub>3</sub>) system via muscarinic receptors, thereby contributing to the large Ca<sup>2+</sup> transient required for endocannabinoid release (263, 292). This sounds unlikely as in most experimental paradigms massive depolarizations or uncaging of calcium has been used; thus it would be difficult to further enhance calcium levels by activation of IP<sub>3</sub> receptors on intracellular stores. Furthermore, a recent study showed in sympathetic neuronal cultures that muscarinic receptor-mediated activation of PLC- $\beta$  results in limited if any  $IP_3$ -mediated intracellular  $Ca^{2+}$  release (78); thus the major signaling pathway there is the production of DAG. However, under physiological conditions in the hippocampus, a cholinergic activation of PLC may well contribute to endocannabinoid release via both the IP<sub>3</sub> cascade and the DAG limb (see below). Another likely explanation for the experimental results with carbachol is that it may suppress IPSCs produced by parvalbumincontaining basket cells via presynaptic m2 receptors, which are selectively expressed by this interneuron type (140), whereas the spontaneous activity of CCK-containing interneurons may be increased via m<sub>1</sub> muscarinic, or perhaps even nicotinic actions of carbachol. The mutually exclusive distribution of CB1 and m2 receptors on two subsets of basket cell terminals is shown in Figure 16 (Katona and Freund, unpublished data). If this reasoning is correct, DSI could be facilitated via other receptors as well that are selectively (or preferentially) present on CCK cells but not on parvalbumin cells, e.g., substance P receptors (5) or 5-HT<sub>3</sub> receptors (255). Indeed, Hájos et al. (138) demonstrated that the increase in the amplitude and frequency of spontaneous IPSCs after bath application of substance P fragment was brought back to near control levels by the coapplication of the  $CB_1$  receptor agonist WIN55212-2.

Although endocannabinoid-mediated DSE has been convincingly demonstrated in the cerebellum, the existence of this phenomenon in the hippocampus could not be established with the same paradigm used for DSI (364) or DSE in the cerebellum (see sect.  $\mathbb{N}B$ ). Cannabinoids do reduce glutamatergic EPSCs in the hippocampus (139, 251, 323), but the receptor involved is unlikely to be  $CB_1$ (Fig. 12), since the effect was found to be the same in  $CB_1$ knock-out and wild-type animals (139; for details see sect. IV). However, in a recent study, prolonged (5–10 s) depolarization was found to readily induce DSE in hippocampal slices, which was absent in  $CB_1$  knock-out mice (273). This is in conflict with the data of Hájos et al. (139) and may be due to age or strain differences. Retrograde endocannabinoid signaling was shown to be responsible for another type of synaptic plasticity of glutamatergic transmission in the striatum. Long-term depression (LTD) of EPSCs induced by high-frequency stimulation of afferent fibers disappeared in CB<sub>1</sub> receptor knock-out animals



FIG. 16. Two nonoverlapping subsets of perisomatic axon terminals express CB<sub>1</sub> receptors (silver-gold particles labeled with small arrows) and muscarinic  $m_2$  receptors (diffuse DAB labeling, asterisks) in the rat hippocampus. A1-A3 show three adjacent ultrathin sections of the same boutons. The two axon terminals, one likely belonging to parvalbumin-containing ( $m_2$ -positive) and the other to CCK-containing (CB1-positive) basket cells, form symmetrical synapses (large arrows) on the same pyramidal cell body. Scale bars: 0.2  $\mu$ m.

(117, 301). Anatomical data to support or explain this phenomenon are still lacking (see sect. III). Interestingly, recent experiments uncovered that activation of postsynaptic type I mGluR receptors induce LTD in the hippocampus by decreasing glutamate release presynaptically (368). The striking similarity of induction parameters, as well as the potential role of type I mGluRs in

endocannabinoid synthesis (223, 357), suggests that retrograde signaling via postsynaptic release of endocannabinoids is likely to account for this phenomenon. Thus an important question for future research is to determine how DSE and mGluR-dependent LTD are related, along with the identification of how postsynaptic release of endocannabinoids may contribute to these phenomena.

The paragraphs above dealt with cannabinoid signaling phenomena that are, or could be, brought about by endogenously released cannabinoids. Some thought should be given also to those cannabinoid actions that are unlikely to be reproduced by endogenously released cannabinoids but may still be important for the interpretation of the mechanisms of action of delta-9-THC or synthetic ligands. For example, endogenously released cannabinoids are unlikely to act on LTP in the hippocampus, since 1) DSE could be evoked in this region only by prolonged (5–10 s) depolarization (273), 2) cannabinoids had no effect on LTP or LTD when Mg<sup>2+</sup>-free solution or pairing with strong postsynaptic depolarization was used (251), and 3) LTP induction under quasi-physiological conditions may be insufficient stimulation for a detectable endocannabinoid release (328). Single postsynaptic spikes are able to induce LTP if paired with presynaptic spikes or bursts (224, 280), and excess endocannabinoid release that would be capable of inhibiting glutamate release is unlikely to occur under these conditions. Thus whether endogenously released cannabinoids are able to influence the efficacy or plasticity of glutamatergic transmission in the hippocampus via a direct action on glutamate release is still to be shown. However, Carlson et al. (53) showed that a weak train of stimuli that normally does not induce LTP will induce NMDA-dependent LTP if given during the DSI period. The simultaneously recorded field EPSPs do not undergo LTP, showing that the weak stimulus train was indeed subthreshold for LTP induction except in disinhibited cells. The single-cell LTP was prevented by pretreatment with AM251, suggesting that locally released endocannabinoids can enhance LTP by causing disinhibition of a pyramidal cell.

# D. Electrical Activity Patterns Required for the Release of Endocannabinoids

As dicussed above, several lines of experimental evidence suggest that rather large increases in intracellular  $[Ca^{2+}]$  are required for the induction of DSI and DSE via the release of endocannabinoids (199, 200, 209, 288, 357, 375), and this elevation of  $Ca^{2+}$  is essential for the synthesis rather than the release of endocannabinoids (92, 223, 287, 375). Such profound  $Ca^{2+}$  transients may occur only under special physiological conditions, e.g., upon the release of  $Ca^{2+}$  from IP<sub>3</sub>- or ryanodine-sensitive intracellular stores via simultaneous activation of metabotropic receptors and voltage-gated  $Ca^{2+}$  channels (Fig. 17) (146, 147, 262, 263, 292, but see Ref. 210). Back-propagating action potentials are most likely responsible for the voltage-gated  $Ca^{2+}$  influx both in the proximal dendritic (perisomatic) and distal dendritic regions (spines), although in small cellular compartments like a spinehead, a single NMDA-mediated synaptic event may be sufficient to release  $Ca^{2+}$  from the local intracellular stores (96). In the perisomatic region (including the proximal main dendrites), type I mGluRs appear to supply IP<sub>3</sub> both in pyramidal and Purkinje cells (106, 262, 263), which may partly explain the apparent involvement of this receptor type in DSI (257). Indeed, recent papers (223, 272, 357) provide evidence that metabotropic glutamate effects on DSI are mediated by endocannabinoids, as described above. Pairing back-propagating action potentials with mGluR activation increases Ca<sup>2+</sup> release severalfold compared with spiking alone (262, 263). The largest amplitude  $Ca^{2+}$  transient was observed in the most proximal segment of the apical dendrite, an ideal location for endocannabinergic modulation of GABAergic axon terminals that innervate this region. Electron microscopic studies demonstrate the lack of glutamatergic synapses on the cell bodies and proximal apical shafts of pyramidal cells (244, 277), which suggests that intracellular Ca<sup>2+</sup> release in this region has to have a role other than conveying plasticity to glutamatergic synapses. One possibility is that this  $Ca^{2+}$  rise is sufficiently close to the nucleus to trigger transcriptional changes. Alternatively, it may be critically involved in the induction of endocannabinoid release, which results in the downregulation of perisomatic inhibition. Thereby action potentials could better back-propagate into the distal dendrites allowing associative LTP of distal glutamatergic synapses, or would enable the neuron to dissociate itself from the population oscillation maintained by basket cell-mediated inhibition (61, 246, 343, 376; for review, see Ref. 111). One problem with this hypothesis, and with the interpretation of the mGluR studies (223, 262, 263, 272, 357), is the source of glutamate required to activate mGluRs in the somatic/proximal dendritic region, since these parts of pyramidal cells do not receive glutamatergic synapses (244, 277). Thus, if mGluRs get activated at all in this region under physiological conditions, it either has to involve extrasynaptic mGluRs reached by diffusion of glutamate from distant synaptic sites, or mGluRs may be activated further away from the proximal apical dendrite (mostly on spines), and IP<sub>3</sub> would have to be able to diffuse very fast to its receptors located on the perisomatic or proximal dendritic endoplasmic reticulum. The latter alternative is possible, since IP<sub>3</sub> was calculated to be able to diffuse 50  $\mu$ m in 0.5 s, which is faster than  $Ca^{2+}$  diffusion in the cytosol containing  $Ca^{2+}$  buffers (14). Diffusion of synaptically released glutamate, however, is unlikely, since it is limited by the efficient glial and neuronal uptake machinery; a spillover even to the adjacent



FIG. 17. Schematic diagram of endocannabinoid-mediated retrograde synaptic signaling. The possible physiological mechanisms that may trigger endocannabinoid synthesis and release from hippocampal pyramidal neurons are outlined (similar mechanisms are likely to operate in most brain areas where endocannabinoid signaling takes place). The large  $Ca^{2+}$  transient required for endocannabinoid synthesis likely involves  $Ca^{2+}$  mobilization from intracellular stores upon activation of the inositol 1,4,5-trisphosphate (IP<sub>3</sub>) system (metabotropic receptors) and voltage-dependent  $Ca^{2+}$  channels (burst firing). Another root independent of intracellular  $Ca^{2+}$  transients is illustrated on the *left* of the schematized pyramidal cell body. Activation of phospholipase C (PLC) via group I metabotropic glutamate (mGluR) or muscarinic cholinergic receptors will produce, in addition to IP<sub>3</sub>, 1,2-diacylglycerol (DAG), which likely remains in the plasma membrane. This could then be converted to 2-arachidonoylglycerol (2-AG) by the enzyme 1,2-diacylglycerol lipase (DGL) still within the membrane, which may ensure a rapid diffusion into the extracellular space. The released endocannabinoid scat on CB<sub>1</sub> receptors located on axon terminals of GABAergic interneurons that contain CCK, or on a new cannabinoid receptor subtype (CB3?) expressed by glutamatergic axons. Activation of CB<sub>1</sub> reduces GABA release via a similar mechanism.

synapse is limited (86). An alternative trigger for  $IP_3$  synthesis is muscarinic activation. Indeed, Martin and Alger (232) demonstrated that DSI is enhanced by muscarinic  $m_1$  or  $m_3$  receptor stimulation. Varicose cholinergic fibers are abundant in all layers of the hippocampus and particularly enriched in stratum pyramidale and near the granule cell layer (220). Furthermore, principal cells are known to express muscarinic receptors on their perisomatic membrane (308). Activation of muscarinic receptors induces a profound  $Ca^{2+}$  rise in the soma, or  $Ca^{2+}$  waves that propagate into the soma, and increases the  $Ca^{2+}$  transients evoked by trains of action potentials (263, 292). Thus it is important to emphasize that, in addition to

group I mGluRs, cholinergic transmission may also contribute to the generation of sufficient IP<sub>3</sub> levels to trigger large Ca<sup>2+</sup> transients followed by endocannabinoid release when coinciding with trains of action potentials. However, muscarinic receptor-mediated activation of PLC- $\beta$  in sympathetic neuronal cultures results in limited if any IP<sub>3</sub>-mediated intracellular Ca<sup>2+</sup> release; thus the major signaling pathway there is the production of DAG (78), which, on the other hand, is the precursor of 2-AG synthesis (328). Whether muscarinic activation uses primarily the DAG limb in hippocampal endocannabinoid signaling remains to be established, although the lack of an antagonist (atropine) effect on DSI suggests that resting levels of acetylcholine are not involved in the generation of the required DAG pool (232). The same question arises also for the mechanism of mGluR-mediated endocannabinoid release, since in a recent study in hippocampal cultures, group I mGluR activation was shown to enhance DSI without increasing intracellular calcium signals (272). This raises the possibility that under some conditions, group I mGluR activation uses the alternative root; it may increase 2-AG synthesis via the DAG limb (328), and thereby could cooperate with depolarizationinduced Ca<sup>2+</sup> transients to enhance endocannabinoid release.

The physiologically most relevant question here is which are the behavior-dependent activity patterns that could ensure the coincidence of metabotropic receptor activation (IP3 and DAG synthesis) and bursts of action potentials that are able to induce sufficiently large Ca<sup>2+</sup> transients to release endocannabinoids in the hippocampus (Fig. 17). Spontaneous or low magnesium-evoked burst potentials that resemble physiological bursts were shown to induce DSI (20). Hippocampal pyramidal cells typically produce bursts of two to six action potentials at <6-ms intraburst intervals (294). These bursts were shown to invade parts of the dendritic tree quite efficiently, and therefore their pairing with presynaptic activity readily induces LTP (224, 280). Even much slower trains of action potentials (10-30 Hz) result in a buildup of Ca<sup>2+</sup> in pyramidal cell dendrites. This Ca<sup>2+</sup> level perfectly correlates with spike frequency (146); therefore, it may induce endocannabinoid release in an activity-dependent manner, if coupled to a coincident activation of the  $IP_3$  cascade and releases  $Ca^{2+}$  from intracellular stores. The probability of bursts was found to be highest at firing rates around theta frequency (145), and bursts at this frequency are particularly suitable for inducing LTP in hippocampal pyramidal cells (170, 205). Acetylcholine release in the hippocampus is large during theta activity, and it correlates with theta power (191), while muscarinic receptor activation induces Ca<sup>2+</sup> transients (and DAG synthesis). Therefore, theta is likely to be the behaviordependent EEG pattern that best couples burst-induced Ca<sup>2+</sup> influx with metabotropic activation of IP<sub>3</sub>/DAG synthesis. Endocannabinoid release that follows the resulting high Ca<sup>2+</sup> transients may reduce perisomatic inhibition of the burst-firing cells, which could facilitate LTP of distal dendritic synapses by allowing a more efficient backpropagation of action potentials. Interestingly, acetylcholine appears to use at least three different mechanisms to enhance communication between the soma and distal dendrites: 1) it is able to close transient  $K^+$  channels (I<sub>A</sub>) in the apical dendrites (179), 2) it reduces GABA release from parvalbumin-containing basket cell terminals via presynaptic  $m_2$  receptors (140), and 3) it may induce endocannabinoid release to reduce inhibition deriving from the other subset of perisomatic inhibitory cells, i.e.,

from those that contain CCK and express presynaptic  $CB_1$  receptors (188).

Another result of endocannabinoid-mediated downregulation of perisomatic inhibition may be that individual cells could dissociate the timing of their action potential firing from network oscillations during theta activity. During exploratory behavior and theta activity pyramidal cells tend to fire in specific areas of their environment, which are called place fields (274). When the animal enters the place field of the recorded neuron, it starts to fire at earlier phases of the theta waves relative to the population, which was called phase precession (275). Burst firing starts to occur preferentially at the periphery of the place field (145), creating, together with muscarinic receptor acivation, ideal conditions for endocannabinoid release. This would result in a gradual downregulation of basket cell-mediated inhibition, allowing the cell to fire at earlier and earlier phases of the theta cycle. The cell could still fire phase-locked to gamma oscillation, if the other basket cell population (the parvalbumin cells lacking CB<sub>1</sub> receptors) is able to convey this effect. The timing of the presumed endocannabinoid effect also seems optimal for this function, since the onset of DSI is  $\sim 1.2$  s after the somatic Ca<sup>2+</sup> rise, and lasts for a few seconds, or occasionally for >10 s (288, 374).

Another EEG pattern accompanied by synchronous pyramidal cell firing at relatively high frequencies are the sharp-wave bursts, which occur during non-theta behaviors (43). These events, however, are rather short (40-120)ms), and whether this is sufficient for endocannabinoid synthesis/release remains to be established. Nevertheless, the synchronous burst discharge of a large proportion of pyramidal cells may result in extensive Ca<sup>2+</sup> influx, activation of mGluR<sub>5</sub> receptors, and the synthesis of IP<sub>3</sub>/DAG (Fig. 17). The induced endocannabinoid action may start suppressing inhibition within a few hundred milliseconds, leading to downregulation of inhibition and the generation of the next sharp wave burst. The variable second messenger delay may account for the irregular occurrence of sharp waves and may explain why the same pyramidal cells initiate the subsequent bursts.

# VI. CONCLUSIONS

The aim of this review was to synthesize the currently available data about the life cycle of endocannabinoids; the conditions that result in their release in the brain; the precise sites of their action at the regional, cellular, and subcellular levels; and their physiological effects on neuronal networks. In addition, a major focus of this review was to generate testable hypotheses about the possible functional roles of endocannabinoids in complex integrative centers of the brain, such as the cerebral and cerebellar cortex and basal ganglia. A general view emerging from the synthesis of the available data is that endocannabinoids serve as mediators in neuronal communication that is distinct from synaptic and nonsynaptic (volume) transmission in its range and function. Endocannabinoids mediate retrograde synaptic signaling, Address

(volume) transmission in its range and function. Endocannabinoids mediate retrograde synaptic signaling, which has an intermediate range between synaptic and volume transmission. Synapses represent point-to-point connections where each of the contacts can be selectively activated and modified, whereas volume transmission employs mediators that can diffuse considerable distances (362) and are likely to be involved in the fine tuning of activity and plasticity in entire brain regions, subfields, or layers. In contrast, endocannabinoid diffusion is limited by uptake and metabolism basically to the axon terminals that form synapses on particular neurons that release them as retrograde signal molecules. Thus the generation of endocannabinoids by burst-firing and/or PLC activation via metabotropic receptors in a neuron will decrease the efficacy of the incoming inhibitory and/or excitatory synaptic signals primarily onto that neuron (Fig. 17). The functional importance of this mechanism is still under investigation. However, the available evidence suggests that endocannabinoids influence 1) transmitter release dynamics that play crucial roles in synaptic plasticity, 2) action potential back-propagation and timing relative to a phase-locked population activity in neuronal signaling, and 3) oscillations that are involved in higher cognitive functions such as feature binding during learning and memory processes. Remarkably, these processes may also represent the neurobiological substrate of the various behavioral effects of cannabis smoking.

The recent findings presented in this review on the function of endocannabinoids suggest that we are just at the beginning of a revolution in endocannabinoid research that may shed light not only on normal brain operations, but also on disease mechanisms that are so far poorly understood, like schizophrenia, anxiety, and other brain disorders. Future research should focus on 1) the molecular, physiological, and pharmacological characterization of missing key elements of the endocannabinoid system, such as new endocannabinoids and new cannabinoid receptors in the brain; 2) their precise cellular and subcellular localization; 3) the biochemical machinery involved in endocannabinoid synthesis, uptake, and degradation; 4) the physiological conditions necessary and sufficient for endocannabinoid release; and, last but not least, 5) the roles played by the endocannabinoid system in various neurological and psychiatric disorders.

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