Endocannabinoid Receptors: CNS Localization of the CB_1 Cannabinoid Receptor

István Katona

Contents

Abstract The evolution of plant metabolic pathways to invent compounds which distract predators, and the history of medicine to find treatments for diseases, often share a common logic. An attractive example to illustrate the rationale behind this is the *Cannabis sativa* plant, which was exploited for its widespread therapeutic effects for several thousand years, but historical "prescriptions" highlighted its distractive behavioral side-effects if abused. This chapter aims to explain the characteristically wide pharmacological and behavioral profile of the *Cannabis* plant by pointing to the ubiquitous anatomical distribution of CB_1 cannabinoid receptors, its predominant molecular target, throughout the nervous system. However, in contrast to their abundant regional and cellular localization, the subcellular arrangement of CB_1 receptors and the enzymes involved in the metabolism of its main endogenous ligand, 2-arachidonoylglycerol (2-AG), are strikingly polarized on the neuronal surface in the adult brain. Though there are still several unresolved issues,

I. Katona
Institute of Experimental Medicine, Hungarian Academy of Sciences, H-1083, Budapest, Szigony utca 43, Hungary
e-mail: katona@koki.hu

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the known pieces of the puzzle outline a picture in which the biosynthetic machinery for 2-AG is accumulated in the somatodendritic compartment of neurons, whereas its receptor and degrading enzyme are both found on axon terminals. This molecular architecture suggests that a main physiological role of endocannabinoid signaling is the retrograde regulation of synaptic transmission, and the present chapter aims to summarize compelling evidence that it is an ancient and fundamental component of several distinct types of synapses throughout the nervous system.

Keywords Synapse • Retrograde • DAGL • DGL-alpha • 2-AG • MGL • CB1 cannabinoid receptor

Abbreviations

2-AG	2-Arachidonoylglycerol
DGL	Diacylglycerol lipase
FAAH	Fatty acid amide hydrolase
MGL	Monoacylglycerol lipase
NAE	*N*-Acylethanolamine
RER	Rough endoplasmic reticulum
VGCC	Voltage-gated calcium channels

1 Introduction

The utility of molecular neuroanatomy lies in its ability to aid in correctly interpreting pharmacological, physiological and behavioral experiments by identifying the precise localization of molecular components involved in certain signaling pathways. Undoubtedly, this task requires investigations at several organizational levels of the nervous system with the main goal of finding certain patterns in the distribution of given molecular elements either at the regional, cellular or subcellular level. Compartmentalized localization of a given signaling pathway may predict its cell physiological role or may forecast its behavioral and therapeutic importance.

Molecular neuroanatomy has supported developments in the understanding of behavioral neurobiology of the endocannabinoid system for nearly two decades. Interestingly enough, the first neuroanatomical study describing the position of a putative cannabinoid (CB) receptor by Herkenham and colleagues served as a key trigger for the molecular identification of the receptor itself (Herkenham et al. 1990). This landmark study highlighted the ubiquitous distribution of cannabinoid binding sites throughout the brain, providing an explanation for the wide spectrum of cannabinoid behavioral effects. Furthermore, a pattern in the density of cannabinoid

binding clarified certain peculiar features of cannabinoid effects, e.g. the lack of lethal doses due to low density levels in lower brainstem areas that control cardiovascular and respiratory functions. Finally, the high abundance of cannabinoid binding sites was found to be comparable with the density of receptors for major neurotransmitters like glutamate or GABA, indicating well in advance that endogenous cannabinoid signaling would have a fundamental role in neuronal communication.

The subsequent molecular cloning of CB_1 receptors by Matsuda and colleagues paved the way for more detailed anatomical studies, which provided information, initially, on the cellular expression pattern at the mRNA level and – after the development of selective antibodies – on the subcellular localization pattern at the protein level (Matsuda et al. 1990; Tsou et al. 1998). These studies confirmed the widespread presence of CB_1 receptors throughout the central nervous system, but also highlighted characteristic cell-type-specific differences in its expression level. Further work on the subcellular distribution of CB_1 receptors uncovered the fact that the receptor protein is strikingly accumulated on the plasma membrane of axon terminals (Katona et al. 1999), which indicated that endocannabinoids may have a pivotal role in the regulation of synaptic neurotransmission. Indeed, this prediction has been confirmed by subsequent electrophysiological experiments (see the chapter "Endocannabinoid Signaling in Neural Plasticity" by Alger, this volume, for details). Finally, the molecular identification of metabolic enzymes involved in the synthesis or inactivation of endogenous cannabinoid molecules represented another milestone and opened the possibility of finding the start and finish of the endogenous signaling pathway mimicked by phytocannabinoids (Bisogno et al. 2003; Cravatt et al. 1996; Dinh et al. 2002; Okamoto et al. 2004; Simon and Cravatt 2008). Remarkably, follow-up molecular neuroanatomy studies soon confirmed that the biosynthetic and degrading enzymes for 2-arachidonoylglycerol (2-AG) are accumulated at synaptic junctions together with CB_1 receptors (Gulyas et al. 2004; Katona et al. 2006; Yoshida et al. 2006), in accordance with the emerging notion that 2-AG may be the predominant endogenous ligand of CB_1 receptors (Sugiura et al. 2006). While 2-AG is thought to be the key endocannabinoid molecule involved in the regulation of synaptic neurotransmission, the precise cell physiological role of anandamide, another endogenous cannabimimetic molecule, and its related bioactive congeners, the so-called *N*-acylethanolamines (NAEs), are still under intense investigation. These molecules have unique behavioral activity profiles and are suggested to activate various molecular targets; thus, molecular neuroanatomy studies in the future may facilitate understanding of the logic behind the existence of these distinct molecular signaling pathways. First attempts to localize some of the metabolic enzymes has already uncovered strikingly different subcellular localization (Egertova et al. 2008; Gulyas et al. 2004; Nyilas et al. 2008), indicating that the cell physiological function of NAEs is indeed distinct from the retrograde messenger role of 2-AG. Although the signaling pathways involving NAEs may certainly have a crucial role in behavioral neurobiology of the endocannabinoid system (see for example Maccarrone et al. 2008), current knowledge of the molecular architecture of these pathways is still very limited at the regional, cellular, and subcellular level. Therefore, the present chapter

will focus on the compartmentalized localization of molecular elements involved in 2-AG signaling and will highlight the presence of this signaling pathway as a common feature of several distinct types of synapses throughout the nervous system.

2 Methodological Considerations for Localization Studies on the Endocannabinoid System

2.1 Negative Controls for Positive Findings

The exponential growth in the accessibility of easily (and often freely, see Austin et al. 2004) available knockout mouse lines has transformed the view on molecular neuroanatomy findings in the last decade. As validation became a basic requirement for molecular neuroanatomy studies (see for example Saper and Sawchenko 2003), localization data double-checked in knockout controls is among the most solid information available for neuroscience research. This is strikingly different from physiological or behavioral studies in which interpretation of phenotypic differences in a certain experimental paradigm requires consideration of potential perturbations in developmental processes, though recent advancements in conditional gene deletion studies will hopefully soon circumvent this problem.

Knockout animals for quite a few molecular components of the endocannabinoid system are available from several sources (Buckley et al. 2000; Cravatt et al. 2001; Hasegawa et al. 2004; Johns et al. 2007; Ledent et al. 1999; Leung et al. 2006; Marsicano et al. 2002; Zimmer et al. 1999). Because all three major labeling methods may potentially result in non-specific staining patterns on brain sections (pharmacological compounds used in radioligand binding studies, riboprobes used to visualize mRNA expression, antibodies used to label the position of proteins), these knockout animals provide a unique opportunity to confirm any positive experimental findings regardless of the nature of the visualizing agent used in a given study. If knockout controls are not available, an alternative solution is the application of at least two independent visualizing agents, e.g. two riboprobes corresponding to two non-overlapping sequences or two antibodies raised against distinct non-overlapping epitopes (see for example Katona et al. 2006). If the two independent tools visualize identical staining patterns, then the probability of false positive staining is significantly reduced (but not entirely excluded). Careful targeted analysis of potential background caused by the tag in the fusion protein used to generate the antibody may also reveal that certain staining patterns are irrelevant, as has been elegantly demonstrated in the case of astrocytic labeling for CB_1 receptors in the amygdala (McDonald and Mascagni 2001). Three widely used control methods to rule out false positive stainings are not definitive enough. Omission of the primary agents (radioligand, riboprobe or antibody) from the staining procedure reveals that the procedure itself is specific, but does not rule

out the possibility that the agent binds to another molecular target in a non-specific manner. Competition experiments using excess "cold" (non-labeled) ligands and riboprobes confirm that the labeled and non-labeled agents recognize the same target or targets, but does not exclude that they compete for a wrong molecular identity. Finally, preabsorbtion tests with the immunizing protein for antibodies corroborate that a given antibody recognizes its epitope, but the disappearance of the staining pattern may simply be due to the fact that the antibody was titrated out by preabsorption and thereby has a reduced capability to bind to its non-specific target as well.

2.2 Positive Controls for Negative Findings

It is very difficult, if not impossible, to prove unequivocally the absence of a given molecule in certain brain regions, cell types, or subcellular compartments. Several factors may cause false negative findings. Most often, the quantity of a given molecule is not high enough at certain locations, so more sophisticated procedures or more sensitive agents are required to demonstrate its presence. This is especially true if the given molecule is also expressed at very high levels at another location. In this case, the quantity of the labeling agent may not be high enough to visualize smaller amounts of the molecule at another position as well. Research on the localization of CB_1 cannabinoid receptors followed the aforementioned trajectory, as first generation antibodies revealed its presence exclusively on GABAergic axon terminals (see for example Hajos et al. 2000; Katona et al. 1999); it took several years before a new generation of more sensitive antibodies and proper knockout controls could reveal unequivocally its presence also on glutamatergic axon terminals, where the number of CB_1 receptors is an order of magnitude lower compared with GABAergic axon terminals (Katona et al. 2006; Kawamura et al. 2006; Monory et al. 2006). Another explanation for false negative immunostainings may be the fact that a given molecule may have different binding partners at distinct subcellular compartments, and these binding partners may mask recognition by competing for an overlapping domain of the protein. Finally, expression levels of most genes are not constant in time and space; several factors including development stages, the internal state of the animal, or pathological processes during disease may result in lack of labeling. Certainly, in this case, absence of labeling may be physiologically relevant, but cannot be used to generalize a given distribution pattern. A noteworthy example is the developmental switch in the subcellular distribution of diacylglycerol lipase-α (DGL-α), a predominant biosynthetic enzyme of 2-AG, which is found in axons in the embryonic brain, but was shown to localize in dendrites after birth (Bisogno et al. 2003).

To circumvent the above difficulties, the best strategy to imply the absence of a given molecule is to study different experimental levels (e.g. absence of mRNA, protein, physiological effect) and paradigms; if all converge onto the same result, then the probability of reporting a false negative finding will be strongly reduced.

Nevertheless, the most appropriate way to discuss the absence of labeling is to use the term "the amount of the given molecule did not reach the detection threshold".

2.3 Quantification of Positive Findings

Quantitative molecular neuroanatomy is still a developing field because the same problems discussed above may also render precise quantification difficult. Nevertheless, quantification should always be a goal for neuroanatomists, because it may reveal or explain physiologically important differences, such as between brain regions, cell types or subcellular compartments; or during development and disease. Several approaches now help the investigator to quantify the amount of a given molecule, and these will be described in detail below along with the localization patterns of molecular players of endocannabinoid signaling.

3 Regional Distribution

The first two studies of Herkenham et al. (1990, 1991b) carried out radioligand binding on whole brain sections from several species using the tritiated CB_1 receptor agonist [^{3}H]-CP55940 (Fig. 1a). This compound is 45 times more potent on CB_1 receptors than Δ^9-THC, and, more importantly, it failed to stimulate [^{35}S]-GTPγS binding on brain membranes from CB_1 receptor knockout animals (Breivogel et al. 2001), suggesting that the regional localization pattern revealed by [^{3}H]-CP55940 binding must represent the presence of CB_1 receptors. Furthermore, immunostaining using a novel highly sensitive CB_1 antibody developed by the Watanabe group (Fukudome et al. 2004) reveals an almost entirely identical distribution pattern at the whole brain level (Fig. 1a, b), indicating that, indeed, the CB_1 protein may be responsible for the characteristic binding pattern of [^{3}H]-CP55940. The first studies on the regional distribution of CB_1 receptors established three conceptually important points, which are now – retrospectively – widely accepted as key features for explaining the physiological and pathophysiological importance of the endocannabinoid system. These features, summarized below, are the (a) high density (b) characteristic regional pattern, and (c) similarity across species (Herkenham et al. 1990, 1991b).

Although the brain utilizes a plethora of messenger molecules, the density of these molecules, their metabolic enzymes, and their receptors vary across a wide spectrum. Remarkably, the density of cannabinoid receptors in several brain areas was found to be in the range of whole-brain glutamate receptors, cortical GABA receptors, and striatal dopamine receptors (Herkenham et al. 1990), implying that endocannabinoid signaling requires the same amount of receptor proteins as classical neurotransmitters involved in basal synaptic neurotransmission, and highly exceeds the density of neuropeptide receptors, which are supposed to function

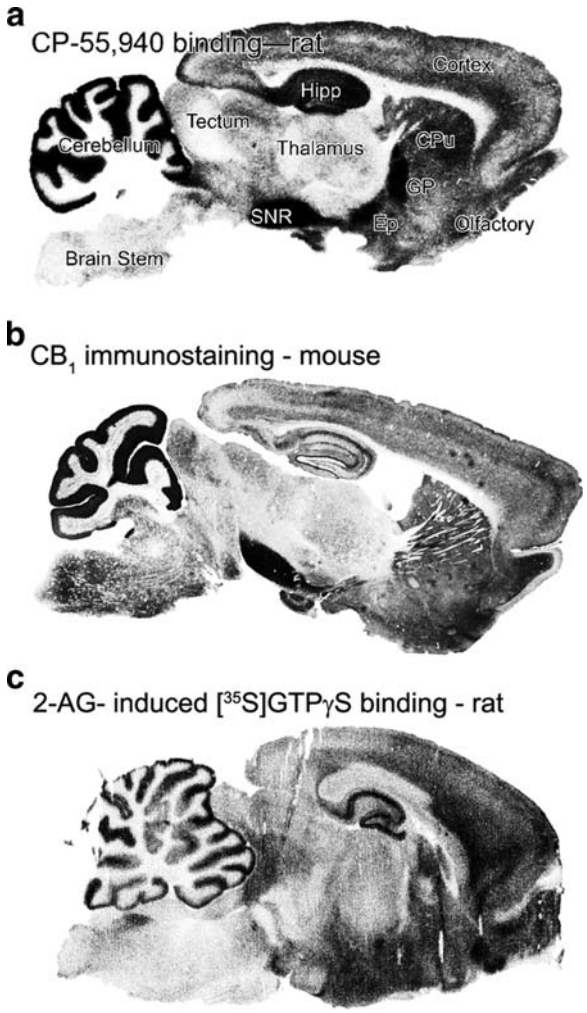

Fig. 1 Regional distribution of CB_1 cannabinoid receptors in the rodent brain. Three distinct experimental paradigms provide independent evidence that CB_1 cannabinoid receptors are widely distributed in the rodent brain. (**a**) Autoradiographic film image demonstrates that the CB_1 agonist CP55940 binds to CB_1 receptors in most brain areas, albeit with a different affinity. (**b**) A nearly identical pattern is visualized by immunostaining using the most sensitive antibody against CB_1 receptors. (**c**) Remarkably, the endogenous cannabinoid receptor ligand 2-AG also induces a similar pattern of CB_1 receptor activation, when its degrading enzyme is inhibited. Note that the three different experiments consistently show that high density of CB_1 is localized in the molecular layer of the cerebellum, in the basal ganglia (substantia nigra pars reticulata, SNR; caudate-putamen, CPU; globus pallidus, GP; hippocampus, Hipp) as well as in the neocortex. Interestingly, the thalamus and the brainstem contain only very few CB_1 receptors. Images in **a**, **b** and **c** were kindly provided by M. Herkenham (Herkenham et al. 1990); by M. Watanabe (Fukudome et al. 2004); and by J. Laitinen (Palomaki et al. 2007), respectively

instead as modulatory substances. In retrospect, this finding already suggested indirectly that endocannabinoid signaling may have a crucial role in the regulation of synaptic neurotransmission. On the other hand, its predominant endogenous ligand 2-AG was measured at a concentration of ~1–10 nmol g^{-1} in the brain (Stella et al. 1997), which is an order of magnitude lower in concentration than glutamate or GABA, two major synaptic neurotransmitters, and more closely resembles dopamine concentration. A potential explanation of this mismatch may be the peculiar synaptic physiological role of 2-AG. In contrast to glutamate or GABA, it is probably not involved in basal synaptic activity, but, rather, it is synthesized and released upon excess neuronal activity in an "on-demand" manner. Surprisingly, the brain concentration of anandamide is even lower (about ~10 pmol g^{-1}), which is in the range of neuromodulators (Cadas et al. 1996); thus, its physiological role may be even more specialized in time and space.

Brain areas are highly different in regard to the number of cells they contain, the number of synapses these cells receive, and the degree of plasticity their synapses express upon activity-dependent changes in neuronal function. Importantly, the general regional distribution of CB_1 receptors shows a peculiar pattern, which largely overlaps with other molecular elements implicated in synaptic neurotransmission and plasticity; furthermore, this pattern may explain several characteristic behavioral effects of phytocannabinoids. The highest density of CB_1 receptors was found in the cerebellum, especially in the molecular layer, where CB_1 was shown to be accumulated on the axon terminals of parallel fibers (Kawamura et al. 2006). In accordance with the striking cannabinoid effect on movement control, a very high density of CB_1 receptors was also found in the substantia nigra pars reticulata and in the globus pallidus; the underlying cellular localization of CB_1 was reported on the GABAergic axon terminals deriving from the striatum (Herkenham et al. 1991a). Finally, a very high density of CB_1 receptors was also found in the hippocampus, where the receptor protein is localized on both GABAergic and on glutamatergic axon terminals (Katona et al. 1999, 2006; Kawamura et al. 2006; Monory et al. 2006). Modest CB_1 receptor localization was reported in several other brain areas; for example, throughout the neocortex, in the amygdala, in the periaqueductal gray nucleus, in the medial hypothalamus, and in the superficial layers of the dorsal horn of the spinal cord (Herkenham et al. 1990). Remarkably, the brainstem contains only a very low density of CB_1 receptors, and several key medullary nuclei responsible for the organization of respiratory and cardiovascular functions seem to lack endocannabinoid signaling. This interesting paucity of a molecular pathway involved in synaptic plasticity may be explained by the well-known rigidity in the synaptic, neuronal, and network activity of these brain areas, which is indispensable for the rhythm generation in these key physiological processes. On a different note, this pattern may also explain why there is no history of fatal overdose due to cannabis consumption in contrast with other drugs of abuse (e.g. benzodiazepines or opiates).

Finally, a key observation of Herkenham et al. (1990) was the finding that CB_1 receptors show a largely similar regional distribution pattern across different mammalian species. This conservative localization pattern, which was later extended even to the subcellular level (namely CB_1 receptors are presynaptically localized

at least in vertebrates), in accordance with the surprisingly high sequence identity at the protein level suggested that endocannabinoid signaling may be ancient and may also have a basic physiological role in the regulation of neuronal activity. Nevertheless, subtle differences were uncovered between species, but this may well explain the different contributions of a given brain area to the general lifestyle of the given species. The two most striking examples are the particularly high density of CB_1 receptors in the molecular layer of the cerebellum in dogs, as compared with a relatively moderate labeling in this layer in humans, whereas our emotionally sophisticated species shows a characteristically higher density of CB_1 receptors in the basolateral amygdaloid complex not found in other mammalian species.

Importantly, the regional distribution pattern of CB_1 receptors established in the early studies of Herkenham et al. (1990, 1991b) was nicely confirmed by functional autoradiography experiments mapping the precise anatomical loci of the intrinsic endocannabinoid pathway itself (Palomaki et al. 2007). In their elegant paradigm, Palomäki and colleagues used potent inhibitors of monoacylglycerol lipase (MGL), the degrading enzyme of 2-AG, to increase the lifetime of this endogenous cannabinoid in brain tissue prepared for functional autoradiography (Palomaki et al. 2007). Remarkably, elevated intrinsic 2-AG level resulted in an almost entirely similar brain distribution of CB_1 receptor activation as obtained by [^{3}H]-CP55940, the exogenous CB_1 receptor agonist (Fig. 1a–c). Further pharmacological experiments using inhibitors of DGL uncovered the fact that "on-demand" activation of 2-AG biosynthesis was responsible for the characteristic regional pattern of endogenous 2-AG-mediated activation of CB_1 receptors. In contrast to 2-AG, which is a full efficacy agonist of CB_1 receptors, experimental increase in the tissue level of anandamide, a partial agonist of CB_1 receptors, by blocking its degrading enzyme FAAH, did not induce CB_1 receptor activation in this experimental paradigm. Taken together, these findings suggest that the operation of an endogenous chemical messenger, 2-AG, and its signaling pathway, the DGL-CB_1-MGL route, may explain the pattern in the specific regional distribution of cannabinoid receptors and may underlie the characteristic behavioral effects of cannabinoids.

Although the regional distribution of cannabinoid signaling in the brain is well established by the above studies in the *postmortem* brain, exciting new technologies are opening the way for in vivo approaches investigating this signaling system. Promising novel radioligands were recently developed to assess CB_1 receptors in live animals and even in humans using positron emission tomography (PET) (Burns et al. 2007; Yasuno et al. 2008). Notably, one of these radioligands, [^{11}C]-MePPeP, was also confirmed to be specific for CB_1 receptors in knockout animals (Terry et al. 2008). These exciting new tools provide ample opportunity to examine CB_1 receptor functioning in several behavioral or disease paradigms in the live brain. Most importantly, the first preliminary findings largely reproduced the regional distribution of CB_1 receptors in live human and monkey brains as revealed by the earlier autoradiography studies on postmortem brain tissue (Burns et al. 2007); thus, future work using these radioligands will hopefully facilitate our understanding of the physiological and pathophysiological importance of endocannabinoid signaling in the near future.

4 Cellular Distribution

The cellular expression pattern of CB_1 receptors in the central nervous system shows two important features. First and foremost, it is ubiquitous, i.e. nearly all neuronal cell types express this receptor, with some notable exceptions (see below). Secondly, the expression level in selected cell types varies greatly even under normal conditions; certain physiological stimuli or diseases can robustly change the expression level. Before going into details, one has to call attention to the fact that the most reliable technique of determining whether a given cell type is expressing CB_1 is still in situ hybridization. Because the majority of the functional CB_1 protein population is localized on axon terminals, the detection of proteins in the cell body may be ambiguous and a lack of labeling, again, does not exclude expression by the given cell type.

The expression pattern of CB_1 receptors in the brain was reported in the first landmark paper of Matsuda et al. (1990), which also included information about the molecular identification of CB_1 receptors. The authors used ^{35}S-labeled radioactive oligonucleotides on brain sections and demonstrated that certain cells express enormous amounts of CB_1 mRNA, especially in cortical areas. Although they reported that some of these cells are granule cells in the dentate gyrus, further studies revealed that granule cells do not express CB_1 mRNA or protein; instead, the neurons with high expression levels belong to a select subpopulation of GABAergic interneurons (Katona et al. 1999; Marsicano and Lutz 1999; Tsou et al. 1999). Intriguingly, similarly strong CB_1 expression was also found in scattered neurons throughout the neocortex (Bodor et al. 2005; Marsicano and Lutz 1999), as well as in the basolateral amygdala (Katona et al. 2001; Marsicano and Lutz 1999; McDonald and Mascagni 2001), and detailed analysis of their neurochemical markers revealed the presence of the neuropeptide cholecystokinin, a characteristic neurochemical marker of a special GABAergic cell population responsible for perisomatic and dendritic inhibition (Cope et al. 2002; Freund and Buzsaki 1996; Hajos et al. 2000; Klausberger et al. 2003; Pawelzik et al. 2002). Taken together, this suggests that the cortical type of neuronal networks may need specialized GABAergic inhibitory cells to regulate the activity of principal neurons, and principal cells may utilize 2-AG to escape from inhibition under certain circumstances by CB_1-mediated suppression of GABA release (Kim and Alger 2004; Makara et al. 2005; Ohno-Shosaku et al. 2001; Wilson and Nicoll 2001).

Matsuda and colleagues (Matsuda et al. 1990) also reported high CB_1 expression in the ventromedial hypothalamus, which was confirmed by follow-up studies investigating the whole-brain distribution of CB_1 receptors in rats and humans (Mailleux and Vanderhaeghen 1992; Matsuda et al. 1993; Westlake et al. 1994). These latter studies provide an important, comprehensive summary of the cellular expression pattern of CB_1 in nearly all brain regions and should be used as key references similar to the radioligand binding studies of Herkenham et al. (1990, 1991b). Without listing all brain regions, the key message derived from these studies is that the CB_1 receptor is indeed numerous and ubiquitous in the brain.

Most notably, the cerebellar cortex expressed the highest amount of CB_1 receptors outside the forebrain, particularly in the granule cell layer, which corresponded very well with the high density of radioligand binding in the molecular layer, where the axons of granule cells, the so-called parallel fibers, terminate. In addition, widely distributed GABAergic interneurons were also shown to synthesize CB_1 mRNA in a manner similar to the forebrain. A high intensity in situ hybridization signal was also observed in the dorsolateral part of the striatum, corresponding to GABAergic medium spiny neurons projecting to the substantia nigra (Mailleux and Vanderhaeghen 1992; Matsuda et al. 1993). Further detailed studies on selected brain regions largely confirmed these papers and provided the precise details of cellular expression patterns both in quality and quantity by investigating co-localization with cell-type-specific markers (see for example very comprehensive works by Marsicano and Lutz (1999) for cortical areas; or by Hohmann and Herkenham (2000) for the striatum).

Besides the high CB_1-expressing cell types found only in selected brain regions, most principal cell types also express a much lower amount of the receptor mRNA throughout the central nervous system. This rendered the appreciation of the functional significance of CB_1 receptors on these cells difficult for a long period of time; however, recent elegant cell-type-specific knockout models have provided convincing evidence that their presence serves important functions; for example, in protecting neurons from excitotoxicity (Marsicano et al. 2003; Monory et al. 2006). It is important to emphasize that, although these neurons express CB_1 at a lower magnitude, they outnumber high CB_1-expressing cell types by an order of magnitude. Therefore, the total CB_1 mRNA amount in most brain regions may be summed from these two populations in roughly equal levels (50–50%). Because these distinct types of neurons fulfill entirely different physiological roles at the network level in all brain regions, studies simply measuring expressional changes in the CB_1 level from homogenized tissue sources may not have the resolution to reveal cell-type-specific changes: this requires more tedious experimental analysis using cell-type-specific knockout animals or high resolution anatomical analysis (Ludanyi et al. 2008; Monory et al. 2006).

Cellular expression patterns can also be studied at the protein level using selective antibodies. However, one must remain very cautious when drawing conclusions from these experiments, especially in the case of negative findings. The author had earlier experience with antibodies raised against CB_1 receptors that were confirmed to be specific in knockout animals, which were excellent for visualizing CB_1 receptors in cell bodies but not axon terminals, and vice versa. This suggests that the CB_1 receptor protein may have different conformations in these two cellular domains, and the probability of a given antibody to recognize its epitope may, therefore, vary widely. In addition, a recent, careful analysis revealed that four widely used commercial antibodies against CB_1 failed to recognize CB_1 proteins, in contrast to other antibodies raised in academic research labs (Grimsey et al. 2008).

Two detailed whole-brain studies using these “home-made” antibodies for immunocytochemistry revealed a highly similar cellular distribution pattern for the CB_1 receptor protein, as compared to its mRNA (Pettit et al. 1998; Tsou et al.

1998). Intensely stained neurons were found in cortical structures, whereas weakly CB_1-positive cells were found throughout the brain, with a tendency for lower expression level cells to be located in the brainstem in comparison to midbrain and forebrain structures. Two years later, Egertova and Elphick (2000) extended these observations using another antibody to selectively label the axonal distribution pattern of CB_1 receptors but not the cell bodies producing the receptors. Briefly, CB_1-immunoreactive fibers cover several specific brain areas, and selected layers in these areas, in dense meshworks in agreement with the cellular expression pattern; whereas lower brain areas exhibited far fewer CB_1 receptors. Notable differences between mRNA and protein distributions revealed by the above studies can be explained by the presence of CB_1 receptors on the axons of projection neurons, e.g. high expression of CB_1 mRNA in neurons in the anterior olfactory nuclei may explain the high density of CB_1 protein in the internal granular layer of the olfactory bulb where otherwise CB_1 expression is under the detection threshold.

Finally, though with caution, one also has to emphasize that while CB_1 cannabinoid receptors appear to be a general feature of most cell types, we should note that there are a few neurons that are exceptions, in which neither the CB_1 mRNA levels nor the CB_1 protein levels in the cell bodies reach the detection threshold, and physiological studies also indicate a lack of cannabinoid receptors. These cell types include the midbrain dopaminergic neurons (Herkenham et al. 1991a), Purkinje cells of the cerebellum (Egertova et al. 1998), granule cells of the dentate gyrus (Katona et al. 2006; Marsicano and Lutz 1999), parvalbumin-positive interneurons of cortical areas (Bodor et al. 2005; Katona et al. 2000, 2001; Katona et al. 1999; Marsicano and Lutz 1999; McDonald and Mascagni 2001; Tsou et al. 1999) (but not in the basal ganglia, where parvalbumin-positive cells express CB_1 (Hohmann and Herkenham 2000; Uchigashima et al. 2007)), and cholinergic neurons of the striatum (Hohmann and Herkenham 2000; Uchigashima et al. 2007). Unfortunately, the reason for the absence of CB_1 on the boutons of these neurons is unknown. In addition, the presence of the related CB_2 receptors has also been demonstrated in the CNS in recent studies (Van Sickle et al. 2005); however, neither anatomical nor physiological evidence for the synaptic localization of CB_2 receptors is yet available.

5 Subcellular Distribution

The first indirect anatomical evidence that CB_1 receptors may be targeted to axonal processes is derived from the early work of Herkenham et al. (1991a). Selective ibotenic acid lesions of the CB_1-expressing striatal neurons resulted in a massive loss of cannabinoid binding sites in the substantia nigra and in the pallidum, indicating that striato-pallidal and striato-nigral projection neurons carry CB_1 receptors on their axonal processes (see also Matyas et al. 2006 for a more direct demonstration). Further immunostaining studies visualized dense meshworks of CB_1-positive fibers throughout the central nervous system (Egertova and Elphick

2000; Tsou et al. 1998). Higher resolution studies using electron microscopy confirmed in cortical areas that these immunostained profiles are indeed axons; more precisely, the vast majority of labeling was found on the plasma membrane of axon terminals, suggesting that CB_1 receptors may have a pivotal role in the regulation of synaptic transmission (Fig. 2e–h) (Katona et al. 1999, 2000, 2001;

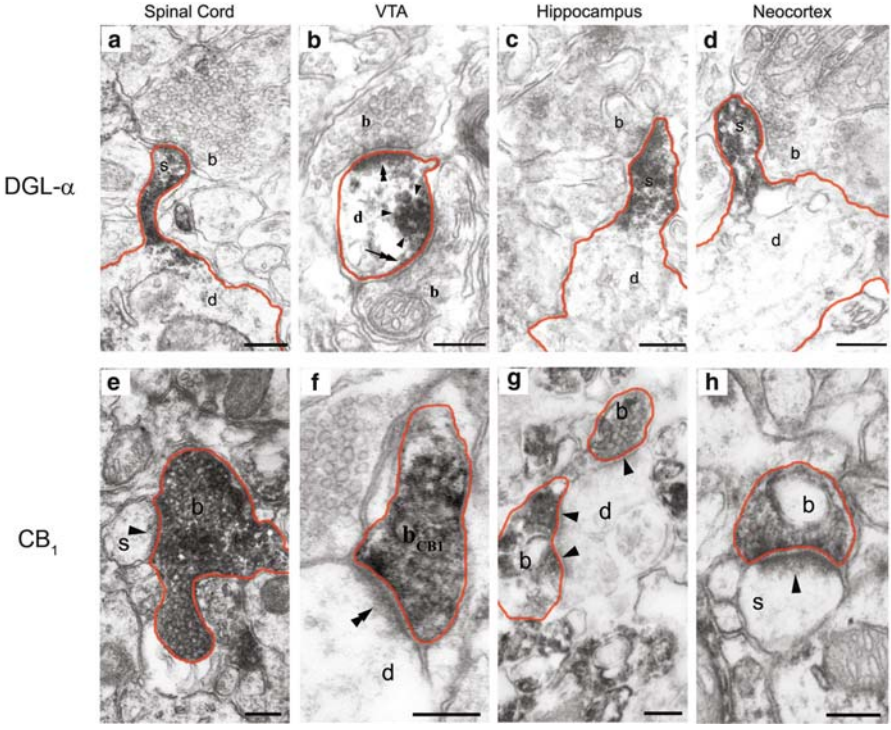

Fig. 2 Postsynaptic synthetic enzyme and presynaptic receptor for 2-AG are conserved features of excitatory synapses throughout the CNS. (**a**–**d**) Electron microscopic images demonstrate that DGL-α, the predominant biosynthetic enzyme of the endocannabinoid 2-AG, is accumulated at the postsynaptic side of excitatory synapses. The dense black end product of the immunoperoxidase reaction represents the position of DGL-α, and it shows a highly compartmentalized distribution limited to the spine head (s) in case of the spinal cord (**a**), hippocampus (**c**) or neocortex (**d**). Even in aspiny neurons like the dopaminergic cells of the ventral tegmental area (VTA), DGL-α is concentrated perisynaptically (**b**). Note that all of these DGL-α-containing postsynaptic profiles receive asymmetrical synapses from DGL-α-negative boutons (**b**). (**e**–**h**) High-resolution electron micrographs show that CB_1 cannabinoid receptors, the molecular targets of 2-AG, are located presynaptically on glutamatergic axon terminals (**b**) throughout the CNS. The dense end product of CB_1-immunostaining is restricted to boutons, whereas postsynaptic profiles, spines (s) or dendritic shafts (**d**) are always CB_1-immunonegative. The strongly asymmetrical postsynaptic density (*arrowheads*) demonstrates that these are excitatory, glutamatergic synapses. Red outlines assist the identification of the postsynaptic or presynaptic profiles (spines vs. axon terminals), which contain immunostaining for either DGL-α (**a**–**d**) or CB_1 (**e**–**h**), respectively. Scale bars: **a**–**h**, 0.2 μm. Images were modified from Katona et al. (2006) and Mátyás et al. (2008) or kindly provided by Rita Nyilas (**a**,**e**) and Barna Dudok (**d**,**h**)

Hajos et al. 2000). Further anatomical studies extended this finding to other brain areas or even to peripheral tissues (see for example Calignano et al. 2000; Vizi et al. 2001), and now it is very well established that most types of axon terminals bear presynaptic CB_1 receptors (but see the few exceptions above). While demonstration of co-localization of neurochemical markers with CB_1 receptors at the boutonal level was a very difficult task a few years ago, recent advances in confocal microscopy now provide ample opportunities to test various markers and to establish which axon terminals carry presynaptic CB_1 receptors (see for example two exemplary studies by Kawamura et al. 2006; Uchigashima et al. 2007). This widespread distribution of presynaptic CB_1 receptors was totally unexpected a few years ago; however, in light of the crucial physiological role of 2-AG in the negative feedback regulation of neurotransmitter release (see the chapter "Endocannabinoid Signaling in Neural Plasticity" by Alger, this volume, for details), it is conceivable retrospectively. For details regarding given types of axon terminals, a number of reviews provide a comprehensive summary of the anatomical, pharmacological and physiological evidence for presynaptic cannabinoid receptors and for their role in the regulation of synaptic plasticity (Chevaleyre et al. 2006; Freund et al. 2003; Schlicker and Kathmann 2001).

Axon terminals carry a conserved elaborate machinery mediating basal synaptic transmission, but are also equipped with various molecular signaling pathways, which help to adjust the efficacy of synaptic communication at a scale over an order of magnitude. Presynaptic CB_1 receptors seem to be involved in at least two pathways, both of which result in the attenuation of neurotransmitter release, albeit at a different time scale. Activation of presynaptic CB_1 receptors may result in the inhibition of voltage-gated calcium channels (VGCCs), of which N-type VGCCs seem to be particularly important (Wilson et al. 2001); engagement of this signaling pathway causes short-term synaptic depression. In addition, presynaptic CB_1 receptors may also down-regulate the activity of the adenylyl-cyclase-protein kinase A signaling pathway, which results in the long-term depression of synaptic neurotransmitter release (Chevaleyre et al. 2007). Whether these two molecular cascades evoking synaptic depression at a different time scale can be initiated by the same population of CB_1 receptors is unknown. On the other hand, high-resolution neuroanatomical studies suggest that there are two peaks of distribution of these receptors on given axon terminals, opening the possibility that there are two macromolecular signaling complexes involving CB_1 receptors. Interestingly, CB_1 receptors are positioned perisynaptically on hippocampal GABAergic axon terminals, but were not found intrasynaptically close to the vesicle docking sites (Nyiri et al. 2005). This distribution pattern fits well with the predicted subaxonal distribution of N-type VGCCs on the same type of axon terminals (Hefft and Jonas 2005) and is also in agreement with the notion that VGCC-mediated short-term depression is a homosynaptic phenomenon, i.e. the endocannabinoid ligand may arrive from the neighboring postsynaptic domains. Besides perisynaptic CB_1 receptors, a second population is also accumulated further away from the perisynaptic zone (Nyiri et al. 2005), which may be an ideal position in which to detect ligands coming from heterosynaptic sources, in harmony with the findings that

endocannabinoid-mediated long-term depression on GABAergic axon terminals is a heterosynaptic phenomenon (Chevaleyre and Castillo 2003). Importantly, identical dual peaks in the subaxonal distribution of CB_1 receptors were also reported on juvenile cerebellar parallel fibers; however the receptor localization becomes more concentrated perisynaptically in the adult cerebellum (Kawamura et al. 2006).

Besides presynaptically located CB_1 receptors, there is growing evidence that CB_1 receptors may also be found in the somatodendritic domain. While there is no unequivocal evidence that CB_1 is present on distal dendritic segments or close to postsynaptic specializations, complementary experimental approaches revealed its presence within the cell body or in thick proximal dendritic shafts of cortical GABAergic interneurons. Immunogold staining uncovered the fact that CB_1 receptor is present on the rough endoplasmic reticulum (RER), Golgi complex, multivesicular bodies and the endosome–lysosome system inside the cell bodies and proximal dendrites (Katona et al. 1999, 2001; Bodor et al. 2005). This suggests that the freshly synthesized CB_1 receptors are recognized by the antibodies, which may explain the labeling on the RER or Golgi complex. In addition, Leterrier and colleagues provided interesting data recently showing that EGFP-tagged CB_1 receptor proteins undergo a continuous recycling process between subcellular structures and somatic plasma membrane, which plays an important role in the subsequent axonal transport of CB_1 proteins (Leterrier et al. 2006) and may also explain the localization of CB_1 receptors on multivesicular bodies (Bodor et al. 2005; Katona et al. 2001). Finally, an exciting physiological paradigm discovered by Bacci and co-workers suggests that certain cortical interneuron types may undergo lasting self-inhibition via endocannabinoid signaling and intracellular CB_1 receptor activation (Bacci et al. 2004), though the anatomical substrates for this phenomenon require further investigations.

6 Distribution of Other Molecular Components Involved in 2-AG Signaling

Pharmacological and physiological studies converge on the notion that 2-AG may be the primary endogenous ligand of presynaptic CB_1 receptors (Kim and Alger 2004; Makara et al. 2005; Melis et al. 2004; Straiker and Mackie 2007). Recent breakthrough discoveries identified the enzymes responsible for the synthesis – two diacylglycerol lipases (DGL-α and DGL-β) (Bisogno et al. 2003) – and inactivation – MGL (Dinh et al. 2002) – of synaptic 2-AG (for details see the chapter "The Life Cycle of the Endocannabinoids: Formation and Inactivation" by Alexander and Kendall, this volume). In accordance with the retrograde transmitter function of 2-AG, subsequent high-resolution anatomical studies provided direct evidence that the synthetic enzyme of 2-AG is postsynaptic (Katona et al. 2006; Yoshida et al. 2006), whereas the degrading enzyme is presynaptic (Gulyas et al. 2004), implying that 2-AG must follow a retrograde route through the synaptic cleft. Interestingly, in

the case of excitatory synapses, DGL-α is not only found in postsynaptic dendritic spines, but it is also accumulated in a perisynaptic annulus around the postsynaptic density (Katona et al. 2006; Yoshida et al. 2006), where it is inserted into a perisynaptic signaling machinery together with $mGlu_5$ receptors and Homers (Jung et al. 2007). Remarkably, this specialized molecular architecture for retrograde regulation of neurotransmitter release is becoming widely appreciated as a common principle of excitatory synapses throughout the nervous system (Fig. 2). In addition to the various pharmacological and physiological evidence, anatomical studies revealed that the molecular components of this negative feedback pathway is conserved at glutamatergic synapses in the spinal cord (Fig. 2a, e) (Nyilas et al. 2009), midbrain (Fig. 2b, f) (Matyas et al. 2008), cerebellum (Yoshida et al. 2006), nucleus accumbens (Matyas et al. 2007), striatum (Uchigashima et al. 2007), hippocampus (Fig. 2c, g) (Katona et al. 2006; Yoshida et al. 2006) and in the prefrontal (Lafourcade et al. 2007) and somatosensory cortex (Fig. 2d, h) (Dudok et al. 2007). Regarding GABAergic synapses, there is experimental evidence for postsynaptic DGL-α in the ventral tegmental area (Matyas et al. 2008) and in the striatum (Uchigashima et al. 2007). Whether this biosynthetic enzyme of 2-AG is also present at other synapses is unknown, but the lack of labeling at cortical GABAergic synapses with the first generation of DGL-α antibodies indicate that its expression level is much lower compared to glutamatergic synapses. Nevertheless, this issue must be revisited in the future if more sensitive antibodies are available, because pharmacological evidence suggests that diacylglycerol lipases are involved in retrograde 2-AG signaling at cortical GABAergic synapses as well (Hashimotodani et al. 2008). In accordance with this, the presence of MGL in GABAergic axon terminals has already been demonstrated at the electron microscopic level (Gulyas et al. 2004).

While the evidence presented above demonstrates that the molecular architecture for retrograde 2-AG signaling is a conserved feature of synapses throughout the adult brain, the situation may be different in the embryonic brain. Bisogno and colleagues suggested that both DGLs are found in growing axonal tracts in the embryonic spinal cord and optic nerve (Bisogno et al. 2003), and this was confirmed in telencephalic axons at E14.5, although the expression of DGLs was shifted to the dendritic shafts already at E18.5 in the telencephalon (Berghuis et al. 2007). Further immunostaining revealed a high density of CB_1 receptors in the growth cones, where it has been shown to regulate maturation of both GABAergic and glutamatergic axons (Berghuis et al. 2007; Mulder et al. 2008).

7 Conclusions

Anatomical studies in the last decade have revealed the precise position of several molecular components of the endocannabinoid system at the regional, cellular and subcellular level. These studies contributed significantly to the notion that 2-AG is a retrograde messenger at most synapses of the nervous system. The underlying

molecular architecture involves (a) postsynaptic synthesis of 2-AG by DGL-α, (b) a presynaptic effector, the CB_1 cannabinoid receptor, and (c) presynaptic inactivation of 2-AG by MGL (Fig. 3). Ample anatomical evidence demonstrates that this molecular architecture is a general feature of synapses, and together with physiological and pharmacological data it is now clear that 2-AG signaling may be as common a principle of synaptic transmission as it is of glutamate or GABA signaling.

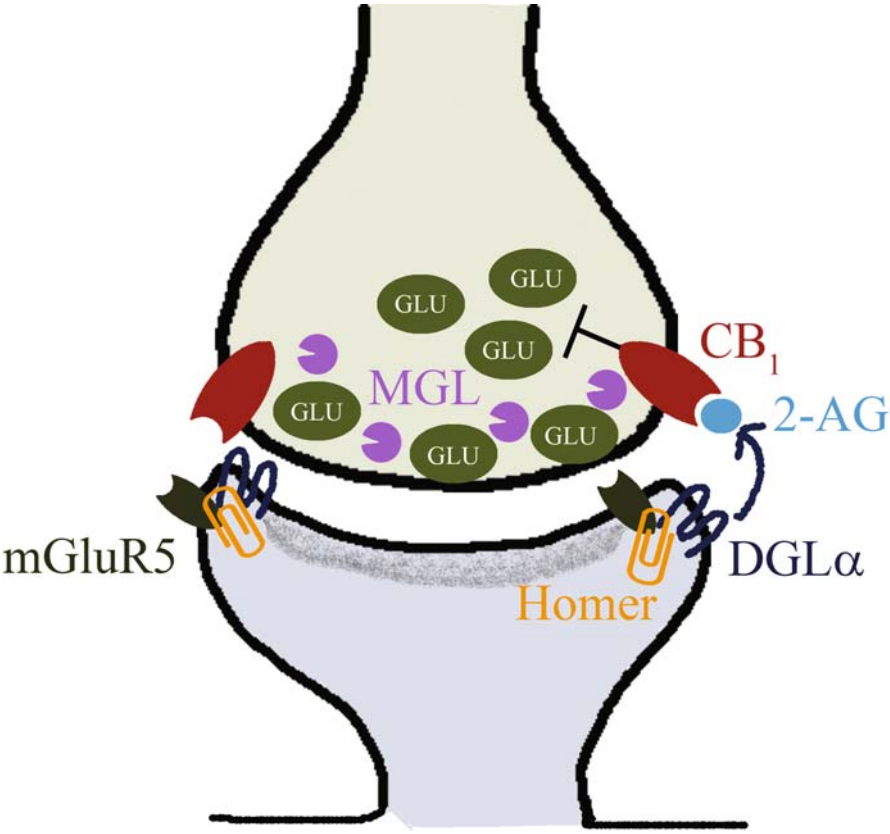

Fig. 3 Molecular architecture of synaptic 2-AG signaling pathway. The schematic diagram illustrates the synaptic position of molecular elements involved in retrograde 2-AG signaling. Spillover of glutamate from the synaptic cleft will activate perisynaptically located metabotropic glutamate receptors (predominantly $mGlu_5$ receptors), which are anchored together with 2-AG's synthetic enzyme diacylglycerol lipase (DGL-α) via the scaffolding protein Homer (note that other Homer-binding partners are not indicated for reasons of clarity). DGL-α produces 2-AG, which travels retrogradely through the synaptic cleft to activate presynaptic CB_1 cannabinoid receptors, which then suppress neurotransmitter release from the axon terminal. Finally, 2-AG is inactivated by the degrading enzyme monoacylglycerol (MGL), which is also localized in the axon terminal. This striking spatial organization of 2-AG signaling provides direct anatomical support for the view that 2-AG is a retrograde transmitter at glutamatergic synapses

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