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Novel Physiologic Functions of Endocannabinoids as Revealed through the Use of Mutant Mice*

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The presence in the mammalian brain of specific receptors for marijuana triggered a search for endogenous ligands, several of which have been recently identified. There has been growing interest in the possible physiological functions of endocannabinoids, and mutant mice that lack cannabinoid receptors have become an important tool in the search for such functions. To date, studies using CB1 knockout mice have supported the possible role of endocannabinoids in retrograde synaptic inhibition in the hippocampus, in long-term potentiation and memory, in the development of opiate dependence, and in the control of appetite and food intake. They also suggested the existence of as yet unidentified cannabinoid receptors in the cardiovascular and central nervous systems. The use of CB2 receptor knockout mice suggested a role for this receptor in macrophage-mediated helper T cell activation. Further studies will undoubtedly reveal many additional roles for this novel signaling system.

KEY WORDS: Anandamide; 2-arachidonoyl glycerol; cannabinoid receptors.

INTRODUCTION

The use of plant-derived substances to induce healing or pleasurable feelings is age-old. The unique specificity of such effects was well recognized in antiquity and had been frequently exploited by shamans and witch-doctors. With the rise of modern biology in the late 19th century, attention had first focused on isolating the bioactive principle in plants, which was followed by identifying their chemical structure. Extracts of hemp or 'cannabis sativa' have been used for their mood altering effects for millennia. Although the basic chemical structure of various constituents of the cannabis plant was revealed in the 1940's (1), it was not until 1964 that Gaoni and Mechoulam (2) correctly identified 9-tetrahydrocannabinol (THC) as its main bioactive constituent. This has opened the door for the synthesis of chemical analogs, and the use of such substances in pharmacological studies has revealed unique structure-activity relationships, the hallmark of cellular receptors. However, the notion of cannabinoid receptors present on mammalian neurons was only seriously entertained once the saturable, high affinity binding of radiolabeled cannabinoids to plasma membranes from the mammalian brain was documented in the 1980's (3). This was followed shortly by the molecular cloning of two cannabinoid receptors: the CB1 receptor (4), abundant in the brain (5) but also present in some peripheral tissues (6), and the CB2 receptor present almost exclusively in cells of the immune system (7).

The presence in the brain of specific receptors for a plant-derived substance rekindled memories of the discovery of endogenous opioid peptides following the identification of opiate receptor binding sites in the brain. Indeed, some early studies suggested the exis-

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tence of peptide-like substances in brain extracts that competed with labeled cannabinoid ligands for binding to the brain cannabinoid receptor (8). Mechoulam and coworkers started from a different premise, based on the highly lipophilic nature of THC. In 1992, they succeeded in identifying a brain-derived lipid, arachidonoyl ethanolamide or anandamide, as an endogenous ligand that binds to the brain (CB1) cannabinoid receptor and mimics the neurobehavioral effects of THC (9). Subsequent studies by others have revealed that anandamide is generated from a low abundance membrane lipid precursor, N-arachidonyl phosphatidyl ethanolamide (N-Ar-PE) by cleavage via phospholipase D (10). The rate limiting step in its synthesis, however, is more likely the generation of N-Ar-PE by the calcium-sensitive transacylation during which the arachidonate moiety of membrane phospholipids, such as phosphatidylcholine, is transferred to the primary amino group of PE (11). The enzyme originally thought to catalyze the synthesis of anandamide from arachidonic acid and ethanolamine (12) turned out to be the one responsible for its degradation by acting in the reverse direction as a fatty acid amidohydrolase (FAAH) (13). This membrane bound enzyme has a key role in the biodisposition of anandamide (14), together with a high affinity transporter that rapidly internalizes anandamide from the extracellular domain (15). In 1995, a second endogenous cannabinoid, 2-arachidonoyl glycerol (2-AG) was isolated from brain (16) and gut (17), which was found to interact with both CB1 and CB2 receptors (18). FAAH can also catalyze the degradation of 2-AG (19), although differences in the stability of the two substances under certain conditions (20) suggest that additional pathways, such as cleavage by a monoglyceride lipase, also contribute to the biodisposition of 2-AG. Finally, in 2001 a third endocannabinoid, a metabolically stable ether-linked analog of 2-AG, which binds to cannabinoid receptors, was isolated from the mammalian brain (21). Unlike classical neurotransmitters, endocannabinoids are not stored in nerve terminals and the mechanism of their release, which does not involve exocytosis, is unclear (22).

In the mid 1990's, selective antagonists for the CB1 and CB2 cannabinoid receptors have been introduced by scientists at the Sanofi company (23,24). These compounds and additional antagonists synthesized by others (25,26) have offered novel tools for exploring the physiological functions of endocannabinoids. The underlying assumption in such studies is that an effect produced by the antagonist alone, particularly if it is in the opposite direction form the effects of agonists, suggests 'tonic' activation of the receptor by an endogenous agonist. However, such an interpretation is complicated by the fact that both SR141716A, the prototypic CB1 antagonist, and its counterpart for CB2 receptors, SR144528, have inverse agonist activity in a number of test systems (27,28). Furthermore, SR141716A, the most widely used antagonist, has effects unrelated to blockade of CB_1 cannabinoid receptors when applied at doses or concentrations not too much higher than those required to produce significant blockade of CB1 receptors (29). The development of genetically altered mice that lack or overexpress a single gene product (knockout or transgenic mice, respectively) has offered powerful additional tools to explore the biological role of receptors and their endogenous ligands. The interpretation of results using such animals is not without problems, such as the possible compensatory changes in the expression of other genes or the role of the genetic background of the animals in their phenotype. However, their use circumvents the problems arising from non-specific effects of receptor antagonists or their possible intrinsic agonist or inverse agonist activity. Here we briefly review what has been learned about the physiological functions of endocannabinoids through the use of cannabinoid receptor knockout mice.

WHAT WE HAVE LEARNED FROM KNOCKING OUT THE CB1 RECEPTOR GENE

In 1999, two groups simultaneously and independently introduced CB1 receptor knockout mice. Ledent et al. developed their line by homologous recombination on a CD1 background (30), whereas Zimmer and colleagues using a similar approach backcrossed their chimeric animals to C57BL/6J mice (31). Not surprisingly, both studies clearly documented a major role of the CB1 receptor in mediating the neurobehavioral and some additional effects of cannabinoids, although there were some differences. In the first study, homozygous mice lacking the CB1 receptor were nearly completely unresponsive to THC in the classic tetrad of tests, including analgesia, hypomotility, catalepsy and hypothermia (30). The addictive properties of THC were absent in the knockout mice, as indicated by the lack of cannabinoid self-administration and precipitated withdrawal. The hypotensive and bradycardic effects of anandamide and the synthetic agonist WIN55,212-2, which had been attributed to CB1 receptor activation (32), were also absent in these animals (30).

Interestingly, CB1 receptor knockout mice displayed increased exploratory behavior under stressful conditions (30), which could indicate an impairment

of short-term memory and learning. Indeed, a recent study by Wilson and Nicoll demonstrated that the retrograde transmitter responsible for depolarization-induced synaptic inhibition (DSI) in the hippocampus, a process long thought to have an important role in learning and memory, is an endocannabinoid released from CA1 pyramidal cells which acts at presynaptic cannabinoid receptors at GABAergic interneurons (22). A subsequent study using CB1 receptor knockout mice developed by the Zimmer group provided definitive evidence for the involvement of CB1 receptors in DSI (66), which is in agreement with earlier findings of Hájos et al. (33) who demonstrated that cannabinoid-induced inhibition of inhibitory postsynaptic currents (IPSCs) in the hippocampus is absent in CB1 knockout mice. The role of endogenous cannabinoids in mediating DSI in the hippocampus suggests that they facilitate short-term memory and learning. In contrast, long-term potentiation was found enhanced in the Schaffer collateral-CA1 synapses of CB1 knockout mice (34), suggesting that endogenous cannabinoids acting at CB1 receptors may suppress long-term potentiation. Consistent with this possibility was another brief report that CB1 receptor knockout mice retained memory significantly longer than their wild-type controls, as assessed by a two-trial object recognition test (35).

The study by Ledent et al. has provided insight into another potential function of endocannabinoids and CB1 receptors. Morphine self-administration and naloxone-induced precipitated opiate withdrawal were significantly attenuated in CB1 knockout mice, suggesting that endocannabinoids acting at CB1 receptors may be involved in the development of physical dependence on opiates and in the somatic signs of opiate withdrawal (30). A possible underlying mechanism for this interaction is suggested by the finding that the dose-dependent increase in dopamine-release from the nucleus accumbens by morphine, which has been linked to the reinforcing properties of opiates (36), is absent in CB1 knockout mice (37). Although these findings raised the possibility that endocannabinoids and CB1 receptors may represent a common pathway for dependence on addictive drugs in general, in a subsequent study the failure of CB1 knockout mice to self-administer morphine was confirmed, but the animals did self-administer cocaine, d-amphetamine or nicotine (38). This highlights the specificity of the interaction between the opiate and cannabinoid systems in the brain.

The CB1 knockout mice developed by Zimmer and coworkers were also indifferent to most of the effects of THC (31). However, these animals displayed a significant residual analgesic response to THC in the tail-flick assay, and a similar but smaller residual response was also observed in the knockout mice developed by Ledent et al. (30). Although the mechanism underlying this effect was not explored, it may be mediated by CB2 receptors, as a significant role of CB2 receptors in cannabinoid-induced analgesia has been documented by others (39). An intriguing observation in the study of Zimmer and coworkers was the significant decrease in baseline locomotor activity, pain sensitivity and increased catalepsy, as compared to the wild-type controls (31). As these effects are similar in direction to the CB1 receptor-mediated effects of cannabinoids, it is unlikely that they reflect the loss of endocannabinoid tone. Instead, they may be the result of compensatory changes in the expression and activity of other neural regulatory mechanisms. Indeed, measurement of gene expression in these CB1 knockout mice by in situ hybridization histochemistry revealed major elevations in the mRNA levels of substance P, enkephalin, dynorphin and GAD 67 in striatal neurons projecting to the substantia nigra and globus pallidus, as compared to their control littermates (40).

The lack of cannabinoid induced hypotension and bradycardia in CB1 receptor knockout mice was subsequently confirmed in a study in which mice developed by the Zimmer group had been used (41). The complete absence of these in vivo cardiovascular responses to even very high doses of cannabinoids clearly illustrates the exclusive role of CB1 receptors in these effects (41), and indicates that a number of additional mechanisms recently identified as contributing to the in vitro vasodilator effect of anandamide in certain vascular beds (42) do not contribute to significant hypotension in vivo. Unexpected observations were made, however, when vascular responses to cannabinoids were examined in the isolated, buffer-perfused mesenteric arterial bed of rats and mice, including the CB1 receptor-knockout mice. These studies were prompted by earlier observations on the peculiar activity profile of abnormal cannabidiol (abn-cbd), a structurally modified analog of the plant derived substance, cannabidiol: in two relatively non-specific behavioral paradigms in dogs and mice, both compounds were found to be inactive, whereas abnormal cannabidiol, but not its parent compound cannabidiol, was found to cause profound hypotension in dogs (43). In retrospect, these findings could have suggested that the hypotensive effect of abn-cbd may be mediated by a receptor distinct from that involved in neurobehavioral effects. However, these observations were re-

ported in 1977, well before the existence of specific cannabinoid receptors has been proposed.

The possibility of an as yet unidentified cannabinoid receptor responsible for some of the cardiovascular effects of cannabinoids was further suggested by more recent findings of unusual structure-activity relationships in the rat isolated, buffer-perfused mesenteric vascular bed preparation. Bolus intraarterial injections of anandamide and its stable analog, methanandamide were found to elicit long lasting vasodilation sensitive to inhibition by SR141716A (44). However, potent synthetic CB1 receptor agonists or THC were completely without effect, suggesting that the receptor involved in the effect of anandamide is not CB1 (44). The results of this study further indicated that the effect of anandamide consisted of a major, endothelium-independent and a small but significant endothelium-dependent component, only the latter of which was sensitive to inhibition by SR141716A (44), which was subsequently confirmed by others (45).

The use of CB1 receptor knockout mice provided a way to unequivocally determine the role of CB1 receptors in these vascular effects (41). First, it was verified that abn-cbd was completely inactive in the behavioral tetrad at doses up to 60 mg/kg and that it did not bind to the brain CB1 receptors at concentrations up to 100 μM. At the same time, abn-cbd caused the same degree of SR141716A-sensitive hypotension and mesenteric vasodilation in wild type mice and in mice lacking either the CB1 receptor or both the CB1 and CB2 receptors (double knockouts). The SR141716A-sensitive vasodilator effect of anandamide also persisted in CB1 receptor knockout mice (41). However, unlike the effect of anandamide, which largely persisted after endothelial denudation, this procedure completely abolished the effect of abn-cbd. Furthermore, the vanilloid VR1 receptor antagonist capsazepine, which inhibited the effect of anandamide, had no influence over the vasodilation induced by abn-cbd, which was also resistant to inhibition of endothelial NO synthase and cyclooxygenase (41). In contrast, the effect of abn-cbd was inhibited by cannabidiol or by the combination of apamin and charybdotoxin (41). These latter two substances block certain subtypes of calcium-activated potassium channels, and their combination was found to inhibit vasodilation caused by the endothelium-derived hyperpolarizing factor (EDHF)(46).

These complex findings supported the following conclusions (41): 1. The endothelium-dependent, SR141716A-sensitive component of the effect of anandamide is mediated by an endothelial site distinct from CB1 or CB2 receptors or vanilloid VR1 receptors. 2. Abn-cbd appears to be a selective agonist and cannabidiol an antagonist of this site, activation of which causes vasodilation probably via the release of EDHF. 3. The endothelium-independent vasodilator effect of anandamide is probably mediated by vanilloid VR1 receptors located on sensory nerve terminals through a mechanism first proposed by Zygmunt and coworkers (47). The nature of the site responsible for the endothelium-dependent effect of anandamide and abn-cbd remains to be clarified. It may be a cell surface receptor, or it may be an intracellular site of action, such as the gap junctions between endothelial and vascular smooth muscle cells (45).

The results of another study using the same strains of CB1 knockout and wild-type mice suggested the existence of non-CB1/non-CB2 receptors in the CNS that could mediate some of the actions of anandamide, but not THC (48). In this study, anandamide at doses of 10–30 mg/kg was found to cause hypomotility, analgesia, and ring immobility in CB1 knockout mice, which correlated with earlier findings that these effects were not blocked by SR141716A in wild-type mice (49). Furthermore, anandamide-induced GTPγS labeling was reduced but not abolished in brain membranes from CB1 knockout compared to wild-type mice, and this residual activity was similarly resistant to inhibition of SR141716A. These findings could suggest that an SR141716A-insensitive G protein-coupled receptor mediates some of the effects of anandamide in the brain. In view of earlier findings that the behavioral effects of methanandamide, a metabolically stable analog of anandamide, could be blocked by SR141716A in the same animals (49), it will be important to test whether the actions of anandamide at this novel site in the brain can be potentiated by blocking its metabolism.

The use of CB1 receptor knockout mice was instrumental in the recent identification of a major role for endocannabinoids in the control of appetite (50). It has been known for a long time that smoking marijuana improves appetite (the ‘munchies’), and similar effects of cannabinoids have been reported in animal models of food intake (51,52). Reduction of food intake by SR141716A treatment has also been reported (53,54,55), but due to the inverse agonist property of this antagonist a possible role for endocannabinoids in the control of food intake could not be deduced from these findings. Such a conclusion was made possible, however, through the use of CB1 receptor knockout mice in a recent study (50). When subjected to brief food restriction to increase appetite, CB1 receptor

knockout mice ate significantly less than their controls. Furthermore, treatment with SR141716A reduced food intake in wild-type animals to levels seen in the knockouts, whereas SR141716A had no effect in these latter animals. Interestingly, the much lower level of food intake in animals with uninterrupted access to food was not different between the two strains and was unaffected by SR141716A. These findings clearly suggest that activation of CB1 receptors, most likely by an endocannabinoid, contributes to the hunger-induced increase in food intake (50).

Appetite and food intake are under the control of a complex neural circuitry located primarily in the hypothalamus, composed of a large number of orexigenic (appetite increasing) and anorexigenic (appetite decreasing) factors (56). A major regulator of appetite and energy balance is the adipocyte-derived hormone leptin, which acts on receptors in the hypothalamus to inform the brain of the state of the body's fat stores (57). Leptin decreases appetite and increases energy expenditure by increasing the expression of anorexigenic signals, such as alpha-melanocyte stimulating hormone (α-MSH)(58) and simultaneously decreasing the expression of orexigenic signals, such as neuropeptide Y (NPY) in the hypothalamus (59). Mutant rodents lacking leptin (*ob/ob* mice) or having a nonfunctional leptin receptors (*db/db* mice, *fa/fa* rats) are obese, and their symptoms of leptin deficiency, including hypomotility, hypothermia, overeating and immunosuppression, are remarkably similar to the effects of cannabinoids. This inverse relationship between the effects of leptin and cannabinoids, as well as examples of their opposite effects in other physiological systems (60), could suggest a regulatory link between these substances. Indeed, hypothalamic, but not cerebellar, levels of the endocannabinoids anandamide and 2-AG were found to be acutely reduced by leptin treatment in rats, and they were higher in the hypothalami of *ob/ob* and *db/db* mice and *fa/fa* rats than in their respective lean controls (50). These observations suggest that hypothalamic endocannabinoids are part of the leptin-regulated neural circuitry involved in the control of appetite and food intake, although they do not exclude the possible involvement of endocannabinoids at extrahypothalamic sites.

Whereas defects in anorexigenic signaling pathways almost always lead to obesity (61,62), loss of the major orexigenic signal NPY does not result in a lean phenotype (63). Redundancy in orexigenic signaling is not unexpected in view of the vital importance of feeding to survival. This has raised the issue of compensatory mechanisms that are apparently activated when NPY signaling is lost. Although endocannabinoids could be plausible candidates, SR141716A was found to suppress food intake to the same extent in NPY knockout mice and their wild-type controls (50). Thus, it appears that NPY and endocannabinoids promote food intake independently of one another.

As discussed above, in wild-type mice temporary food deprivation is required to unmask the orexigenic function of endocannabinoids. Genetically obese mice with defective leptin signaling undergo a period of hyperphagia early in life, which initiates obesity, whereas in later stages their food intake is normal or subnormal and obesity is maintained by reduced energy expenditure. When young *db/db* and *ob/ob* mice with ad libitum access to food were treated with SR141716A, their food intake was markedly reduced, and chronic treatment for a week resulted in significant retardation of weight gain when tested in *db/db* mice (50). These findings suggest that endocannabinoids acting at CB1 receptors contribute to overeating in obesity, and raises the potential therapeutic usefulness of CB1 receptor antagonism in obese humans.

LESSONS FROM A STUDY OF CB2 RECEPTOR KNOCKOUT MICE

Unlike the wide distribution of CB1 receptors in the brain and a number of peripheral tissues, the expression of CB2 receptors is limited to cells of the immune system, suggesting an involvement in the immunomodulatory effects of cannabinoids. Recently, a mouse strain deficient in CB2 receptors has been developed (64). These animals were healthy, fertile, had an essentially normal phenotype and their neurobehavioral response to THC was not different from responses of wild-type controls. Earlier studies have demonstrated that nanomolar concentrations of THC can inhibit helper T cell activation by macrophages (65), and this effect was found to be absent in in vitro T-cell costimulation assays when macrophages from CB2 receptor knockout mice were used (64). These findings confirm that the well-known immunosuppressive effects of marijuana are due, at least in part, to CB2 receptor activation. However, it remains unclear whether CB2 receptors on immune cells are the targets of endocannabinoids, and whether or not they play a role in the physiological or pathological regulation of the immune system.

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