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Analysis of the Endocannabinoid System by Using CB_1 Cannabinoid Receptor Knockout Mice

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Abstract The endocannabinoid system has been involved in the control of several neurophysiological and behavioural responses. To date, three lines of CB_1 knockout mice have been established independently in different laboratories. This chapter reviews the main results obtained with these lines of CB_1 knockout mice in several physiological responses that have been previously related to the activity of the endocannabinoid system. Studies using CB_1 knockout mice have demonstrated that this receptor participates in the control of several behavioural responses including locomotion, anxiety- and depressive-like states, cognitive functions such as memory and learning processes, cardiovascular responses and feeding. Furthermore,

the CB_1 cannabinoid receptor is involved in the control of pain by acting at peripheral, spinal and supraspinal levels. The involvement of the CB_1 cannabinoid receptor in the behavioural and biochemical processes underlying drug addiction has also been investigated. These CB_1 knockouts have provided new findings to clarify the interactions between cannabinoids and the other drugs of abuse such as opioids, psychostimulants, nicotine and ethanol. Recent studies have demonstrated that endocannabinoids can function as retrograde messengers, modulating the release of different neurotransmitters, including opioids, γ-aminobutyric acid (GABA), and cholecystokinin (CCK), which could explain some of the responses observed after the stimulation of the CB_1 cannabinoid receptor. This review provides an update of the apparently controversial data reported in the literature using the three different lines of CB_1 knockout mice, which seem to be mainly due to the use of different experimental procedures rather than any constitutive alteration in these lines of knockouts.

Keywords CB_1 knockout mice · Locomotion · Emotional-like behaviour · Cognitive functions · Cardiovascular responses · Nociception · Feeding behaviour · Drug addiction · Opioids · Psychostimulants · Nicotine · Ethanol · Retrograde neurotransmitter

In this chapter we will focus on the physiological functions of CB_1 cannabinoid receptors that have been reported in knockout mice, rather than review the general physiology of the CB_1 cannabinoid receptors.

1 Generation of CB_1 Knockout Mice

The murine CB_1 receptor is encoded by the *Cnr1* gene on chromosome 4. Like many other G protein-coupled receptors (GPCRs), the entire CB_1 receptor is encoded by a single large exon. To date three lines of CB_1 knockout mice have been established independently in three different laboratories. In the line generated by Ledent and her co-workers (1999), the first 233 codons were replaced by a phosphoglycerate kinase (PGK)-neo cassette. One of our laboratories (A.Z.) generated a knockout strain by replacing the region between amino acid 32 and 448 with PGK-neo (Zimmer et al. 1999). Both mutations constitutively invalidate the gene. The Ledent line has been crossed to an outbred CD1 genetic background, and thus individual mutant animals from this strain can be expected to have a heterogeneous genetic background. The initial results from the Zimmer line were also obtained with animals from a CD1 genetic background, but it has since been crossed for more than 10 generations to C57BL/6J mice, thus generating a congenic strain in which all animals are genetically homogeneous. Marsicano and colleagues (2002) generated a third line of mice that carries a CB_1 gene flanked by lox sites ("floxed"). These lox sites are recognized by the Cre enzyme, a DNA recombinase derived from P1 bacteriophages. When such mice are bred to a transgenic strain that express Cre, floxed genes will be deleted in all tissues in which the Cre enzyme is active. This

strategy is now frequently used for the tissue-specific inactivation of genes (Sauer 1998).

Mice develop apparently normally in the absence of the CB_1 receptor. They are fertile, care for their offspring, and do not show any behavioural abnormalities that would be obvious to the casual observer. However, CB_1-deficient animals have a much higher mortality rate than wild-type animals (Zimmer et al. 1999). Approximately 30% of the mutant animals die of natural causes during the first 6 months, in contrast to less than 5% of the heterozygous and wild-type control animals. The mortality rate in knockout mice is equally high in animals of different age, and death occurs suddenly without prior evidence of illness. Careful examination of dead animals has not yet revealed a cause of death. However, we have frequently observed epileptic seizures in mutant animals and believe that these may have contributed to the increased mortality rate.

2 Neurochemical and Biochemical Adaptive Changes Produced by the Lack of the CB_1 Cannabinoid Receptors

Genetic mutations or deletions can lead to molecular or cellular changes that have been interpreted as an attempt of the organism to compensate for the missing or malfunctioning gene product (Nelson and Young 1998; Pich and Epping-Jordan 1998). CB_1 receptor knockouts have been extensively studied to determine whether such compensatory changes occur in the absence of CB_1 receptors.

Binding of the CB_1-specific agonist CP55,940 was completely abolished in CB_1 knockout mice (Zimmer et al. 1999), and neither CP55,940 nor HU-210 [nor Δ^9-tetrahydrocannabinol (THC)] stimulated [^{35}S]GTP binding in brain tissues from these animals (Breivogel et al. 2001). These results indicated that the CB_1 receptor is the only target for these ligands. A 50% reduction of CB_1 sites was also observed in heterozygous mice when WIN55,212-2 was used. However, the maximal stimulation of [^{35}S]GTP binding was only reduced by 20%–25% in most brain regions, suggesting that there is a small receptor reserve in wild-type animals that was depleted in heterozygous mice (Breivogel et al. 2001). A notable exception was the striatum, where the decrease in stimulation was proportional to the receptor density. Interestingly, some stimulation of [^{35}S]GTP binding by WIN55,212-2 was still observed in homozygous mutant animals, strongly indicating that there is also a non-CB_1 target for this compound. Di Marzo and colleagues analysed anandamide levels in wild-type and CB_1-deficient animals (Di Marzo et al. 2000). They found that, in the absence of CB_1 receptors, anandamide levels were decreased in the hippocampus and to a lesser extent in the striatum. Because fatty acid amide hydrolase (FAAH) activity was unchanged in these animals, the authors argue that the CB_1 receptor may control anandamide biosynthesis. In contrast, Maccarone and co-workers reported that anandamide hydrolysis, mediated by FAAH, was age-dependently increased in CB_1-deficient, but not in wild-type, mice (Maccarrone et al. 2001). Old CB_1 knockouts also showed a significantly elevated enzyme activity (V_{max}), in the cerebral cortex. Although the reason for these disparate re-

sults are unclear, the different genetic backgrounds of the animals or, more likely, differences in holding conditions may have contributed.

3
CB_1 Cannabinoid Receptors Participate in the Control of Locomotion

Among the most striking behavioural effects of cannabinoids in rodents is a profound dose-dependent induction of catalepsy and reduction of locomotor activity (Rodriguez de Fonseca et al. 1998; Chaperon and Thiebot 1999). In contrast, even high doses of THC (up to 100 mg/kg) have no locomotor effects in CB_1-deficient animals, demonstrating that they are mediated by CB_1 receptors (Zimmer et al. 1999). An endocannabinoid tone in the regulation of locomotor activity has been suggested, because the CB_1 receptor antagonist SR141716A stimulates locomotor activity (Compton et al. 1996) and potentiates the locomotor stimulant effects of amphetamine and apomorphine (Masserano et al. 1999). This idea is supported by the observation of Ledent and co-workers (1999) that locomotor activity is slightly increased in mice without cannabinoid receptors. However, Steiner and colleagues (1999) found a decrease in open-field activity in the Zimmer CB_1 knockout strain. There are two explanations for these differences. First, because cannabinoids have biphasic effects (Chaperon and Thiebot 1999), it is conceivable that abolishing the endocannabinoid tone may lead to different outcomes, depending on the level of the endogenous tone. Secondly, because CB_1 knockout mice apparently have higher levels of anxiety (see below), the results may have been influenced by the experimental conditions. Indeed, Steiner et al. used a relatively large open field apparatus and regular laboratory illumination, whilst Ledent et al. conducted their open field test under low light conditions using a smaller device. The latter conditions are less anxiogenic in mice, thus resulting in a higher locomotor activity.

The locomotor effects of THC are thought to be mediated in part by CB_1 receptors in the basal ganglia (Rodriguez de Fonseca et al. 1998). In the striatum, CB_1 receptors display a distinct medial-to-lateral and dorsal-to-rostral distribution, with the highest receptor densities in the lateral part of the middle striatum (Steiner et al. 1999). The striatum has two distinct output pathways, one to the substantia nigra and one to the globus pallidus (Gerfen 1992, 1993). The primary neurotransmitter of both pathways is γ-aminobutyric acid (GABA), but they have different neuropeptide co-transmitters. Striato-pallidal neurons contain enkephalins, whilst striato-nigral neurons express substance P and dynorphin (Steiner and Gerfen 1998). Steiner and colleagues have shown that dynorphin and substance P mRNA levels were significantly elevated in the medio-lateral striatum of CB_1 knockout mice, which also contained the highest CB_1 receptor densities (Steiner et al. 1999). Enkephalin expression was also elevated in CB_1 knockout mice, but unrelated to CB_1 receptor densities. These results are consistent with a local CB_1 inhibition of striato-nigral neurons, whilst effects on striato-pallidal neurons probably involve network-level alterations.

4
CB_1 Cannabinoid Receptors and Emotional Behaviour

Different evidence suggests that the endocannabinoid system plays an important role in the regulation of emotional-like behaviour. Thus, the CB_1 cannabinoid receptor is widely distributed in limbic and cortical areas involved in the control of emotion. The administration of cannabinoid ligands produces emotional-like responses in different behavioural paradigms. Furthermore, cannabinoids also exert a modulatory role on the activity of the hypothalamic-pituitary adrenal axis (HPA), and these compounds modulate the release of several neurotransmitters involved in emotional behaviour, including CCK and GABA.

Studies using CB_1 knockout mice have supported and clarified the previous data reported by using different pharmacological approaches. Thus, it has been shown that CB_1 knockout animals (on a CD1 genetic background) displayed anxiogenic-like responses in different behavioural models, including the open-field, light-dark box and elevated plus maze (Haller et al. 2002; Maccarrone et al. 2002; Martin et al. 2002; Uriguen et al. 2004). Similar anxiogenic-like responses were exhibited in CB_1 knockout mice with an inbred genetic background (C57BL/6). Thus, an anxiogenic-like response in the elevated plus-maze and impairment in the extinction in auditory fear-conditioning test were revealed in these mice (Marsicano et al. 2002), supporting previous results obtained in the CB_1 knockout mice with a CD1 background. In agreement, the administration of SR141716A mimicked the phenotype of CB_1-deficient mice, supporting the role of the endocannabinoids in the control of emotional-like responses (Marsicano et al. 2002). Furthermore, the anxiogenic-like responses in the CB_1 knockout mice were accompanied by alterations in the HPA axis under basal conditions, as well as a hypersensitivity to stress and an impaired action of anxiolytic drugs (bromazepam and buspirone) in the light-dark box (Uriguen et al. 2004). Indeed, basal corticosterone concentrations in the plasma were lower in mutant CB_1 than in wild-type mice, whereas CB_1 knockout mice showed a greater increase in plasma corticosterone concentrations than wild-type littermates after the exposure to restraint stress, supporting the results obtained in the behavioural models (Uriguen et al. 2004). In addition to the anxiogenic-like profile observed in mice lacking CB_1 cannabinoid receptors, these animals also exhibited an increase in aggressive behaviour when exposed to the resident-intruder paradigm, and an enhanced sensitivity to develop a state of anhedonia (depressive-like state) during the exposure to the chronic unpredictable mild stress paradigm (Martin et al. 2002).

A strong impairment of short-term and long-term extinction in auditory fear-conditioning test has been also reported in CB_1 knockout mice (Marsicano et al. 2002). Thus, tone presentation during extinction trials resulted in elevated levels of endocannabinoids in the basolateral amygdala complex, a region known to control extinction of aversive memories, which indicates that endocannabinoids facilitate extinction of aversive memories through their selective blockade of local inhibitory networks in the amygdala (Marsicano et al. 2002). These authors proposed that the decrease of activity of local inhibitory networks within the basolateral amygdala induced by CB_1 activation leads to a disinhibition of principal neurons and finally

to extinction of the freezing response, this being a physiological function impaired in CB_1 knockout mice (Marsicano et al. 2002).

Studies using CB_1 knockout mice also suggest the existence of a novel cannabinoid receptor involved in the control of mood. A recent study has investigated the effects induced by SR141716A on CB_1 knockout mice and wild-type littermates in the elevated plus-maze, showing that surprisingly, the cannabinoid antagonist reduced anxiety in both wild-type and CB_1 knockout mice (Haller et al. 2002). This result shows a discrepancy between genetic and pharmacological blockade of the CB_1 receptor, supporting the hypothesis that a third cannabinoid receptor participates in the responses induced by SR141716A (Haller et al. 2002). Biochemical studies have supported this idea and provided evidence for putative "CB_3" or "CB_x" receptor binding sites in the brain that are sensitive to WIN55,212-2, anandamide and SR141716A (Di Marzo et al. 2000; Breivogel et al. 2001).

In conclusion, pharmacological studies show that cannabinoid agonists induce a broad spectrum of actions in different experimental models of anxiety. Data from knockout mice deficient in the CB_1 cannabinoid receptors demonstrate the existence of an endogenous cannabinoid tonus modulating mood through the stimulation of these CB_1 receptors and also support the possible existence of a third cannabinoid receptor, which seems to play an opposite role to the CB_1 receptor in emotional control. CB_1 cannabinoid receptors modulate the HPA axis activity and the release of several neurotransmitters such as CCK, GABA, serotonin and nicotine, providing a neurochemical substrate for this physiological role. The modulation of several neurotransmitter systems by CB_1 receptors would explain the different effects that cannabinoids can have on anxiety.

5
CB_1 Cannabinoid Receptors Participate in the Control of Cognitive Functions

Cannabinoid ligands produce clear effects on learning and memory that have been widely reported (Dewey 1986; Ameri 1999; Diana and Marty 2004). However, the precise role of the endocannabinoid system on these processes has not yet been completely clarified. In humans, THC administration induces the disruption of short-term recall, as well as disorienting effects (Miller and Branconnier 1983; Chait and Perry 1992). In animals, cannabinoid administration impairs memory and learning processes. In particular, there are reports that cannabinoids impair task acquisition and working memory in different animal species (Molina-Holgado et al. 1995; Lichtman and Martin 1996; Winsauer et al. 1999). The alterations are especially important for spatial memory (Molina-Holgado et al. 1995; Lichtman and Martin 1996) and short-term memory (Molina-Holgado et al. 1995). In rodents, endogenous cannabinoids have been reported to prevent the induction of long-term potentiation in the hippocampus (Stella et al. 1997), and to impair memory in different behavioural tasks, an effect attenuated by SR141716A administration (Mallet and Beninger 1998). On the other hand, the CB_1 antagonist SR141716A can induce an enhancement of memory in some experimental conditions (Hampson and Deadwyler 2000).

In agreement with these pharmacological data, mice lacking CB_1 cannabinoid receptors showed an improved performance in the active avoidance paradigm (Martin et al. 2002), and in the two-trial object recognition test (Reibaud et al. 1999; Bohme et al. 2000). A facilitation of long-term potentiation in the hippocampus was also reported in the same line of CB_1 knockout mice (Böhme et al. 2000). On the other hand, CB_1 knockout mice have been reported to exhibit similar acquisition rates in the Morris water maze as wild-type littermates, whilst CB_1 knockout animals demonstrated deficits in a reversal task in which the hidden platform was located in a different place, also suggesting that the endocannabinoid system has a role in facilitating extinction and/or forgetting processes (Varvel and Lichtman 2002). Indeed, CB_1 cannabinoid receptor-deficient mice exhibited strong impairments in short- and long-term extinction in the auditory fear-conditioning test, indicating that these animals have a prolonged aversive memory (Marsicano et al. 2002).

A recent study has shown that CB_1 knockout mice exhibited an increased acetylcholine release in the hippocampus (Kathmann et al. 2001). Inhibition of acetylcholine activity has been associated with cannabinoid-induced impairment of memory (Braida and Sala 2000). The hippocampus and the neocortex play a crucial role in the control of learning and memory. In both brain structures, CB_1 cannabinoid receptors are expressed in a well-defined subpopulation of GABAergic interneurons (Katona et al. 1999; Marsicano and Lutz 1999; Tsou et al. 1999). Moreover, CB_1 cannabinoid receptor-positive interneurons are distinctive in forming inhibitory synapses with particularly fast kinetics. These GABAergic interneurons seem to control plasticity at excitatory synapses, and thus the blockade of inhibition induced by cannabinoids generally promotes long-term potentiation at excitatory synapses (Wilson and Nicoll 2002; Diana and Marty 2004). This facilitation in the plasticity phenomenon seems to be mediated, at least in part, by extracellular-regulated kinases (ERK). THC has been reported to activate ERK and to induce expression of immediate early genes products in both hippocampal slices and in vivo in this brain structure (Derkinderen et al. 2003). In view of this facilitatory effect induced by cannabinoids in the hippocampal neurons, one may wonder if the endocannabinoid system facilitates learning. However, pharmacological and genetic studies have clearly demonstrated a cannabinoid-induced impairment of memory processes. A possible explanation for this apparent discrepancy has been proposed by Wilson and Nicoll (2002), who suggest that endocannabinoids modulate at a physiological level the activity of interneurons forming fast synapses in the hippocampus to orchestrate fast synchronous oscillations in the gamma range (Banks et al. 2000). The administration of marijuana derivatives might permit promiscuous plasticity, suppressing many hippocampal inhibitory synapses, and cause deficits in cognition and recall (Wilson and Nicoll 2002). Further studies are necessary in order to clarify the complex role of the endocannabinoid system on learning and memory processes and the nature of the changes promoted in the brain by the exogenous administration of cannabinoids.

6
CB_1 Cannabinoid Receptors Participate in the Control of Cardiovascular Responses

It is well known that the acute consumption of THC causes tachycardia in humans without any significant effect on blood pressure, whilst the chronic ingestion of cannabinoids leads to hypotension and bradycardia (Benowitz and Jones 1975). Pharmacological studies using selective CB_1 receptor antagonists (Varga et al. 1995; Lake et al. 1997) have suggested that some of these cardiovascular responses are mediated by CB_1 receptors.

Considering the pharmacological effects of cannabinoids, it was somewhat surprising to see that basal blood pressure and heart rate were normal in CB_1-deficient mice, thus suggesting that endogenous cannabinoids do not exert a tonic control on these cardiovascular parameters. However, when the CB_1 agonists anandamide or WIN55,212-2 were administered to CB_1 knockout animals, they failed to produce the sustained decrease in heart rate and blood pressure that was observed in control littermates (Ledent et al. 1999). A similar result was observed when CB_1-deficient and control mice were treated with 2-arachidonylglyceryl ether, a metabolically stable analogue of 2-arachidonoylglycerol (2-AG). In contrast, 2-AG, which is rapidly metabolized, still produced hypotension and tachycardia in the absence of CB_1 receptors, indicating that a metabolic product of 2-AG elicits cardiovascular effects that are not mediated by CB_1 receptors (Jarai et al. 2000).

Interestingly, "abnormal cannabidiol", a neurobiologically inactive cannabinoid, causes hypotension and mesenteric vasodilation in mice lacking CB_1 and CB_2 receptors that can be blocked by SR141716A (Jarai et al. 1999). These findings suggest the existence of a yet unidentified endothelial cannabinoid receptor. A further line of evidence was obtained when endotoxin lipopolysaccharide (LPS)-induced hypotension was studied in cannabinoid receptor-deficient animals. Intravenous injection of 100 μg/kg LPS caused a similar hypotension in phenobarbital anaesthetised wild-type animals and in mice deficient in CB_1 or both CB_1 and CB_2 receptors (Batkai et al. 2001). This hypotensive effect was also blocked by pretreatment with SR141716A (Batkai et al. 2004), again indicating that this compound exerts some of its effects through non-CB_1 receptors.

7
Participation of the CB_1 Cannabinoid Receptors in the Control of Pain

Cannabinoids produce antinociception through multiple mechanisms at peripheral, spinal and supraspinal levels through CB_1 and CB_2 cannabinoid receptors in several animal species, including mice, rats, rabbits, cats, dogs, monkeys and humans (Pertwee 2001). These responses were revealed in multiple acute nociceptive models using thermal (Buxbaum 1972; Hutcheson et al. 1998; Martin and Lichtman 1998), mechanical (Smith et al. 1998), chemical (Bicher and Mechoulam 1968; Welch et al. 1995) and electrical stimuli (Bicher and Mechoulam 1968; Weissman et al. 1982). Cannabinoid agonists also induce antinociception in inflammatory

models of pain, including hyperalgesia induced by carrageenan (Mazzari et al. 1996), capsaicin (Li et al. 1999), formalin (Calignano et al. 1998; Jaggar et al. 1998) or Freund's adjuvant (Martin et al. 1999). Cannabinoid agonists are also effective in visceral models of pain, such as inflammation of the bladder wall induced by turpentine administration (Jaggar et al. 1998), 2,4-dinitrobenzene sulphonic acid (DNBS)-induced colitis (Massa et al. 2004) and also in neuropathic pain models, such as the painful mononeuropathy induced by loose ligature of the sciatic nerve (Herzberg et al. 1997; Mao et al. 2000). Electrophysiological studies also provide evidence that cannabinoids attenuate nociceptive transmission in vivo (Pertwee 2001; Hohmann 2002). Thus, cannabinoids suppress noxious stimulus-evoked neuronal activity in nociceptive neurons in the spinal cord and thalamus (Hohmann et al. 1995; Martin et al. 1996; Tsou et al. 1996).

Several central structures involved in cannabinoid antinociception have been identified. Hence, the local microinjection of cannabinoid agonists in areas such as the periaqueductal grey matter (Martin and Lichtman 1998; Martin et al. 1999), the rostral ventromedial medulla (Martin et al. 1996), the submedius and lateroposterior nuclei of the thalamus (Mailleux and Vanderhaeghen 1992), the superior colliculus and the amygdaloid complex (Martin et al. 1996; Martin et al. 1999) was able to produce antinociceptive responses. All these neuroanatomical structures related to cannabinoid-induced antinociception are involved in pain transmission and constitute the descending system involved in the control of pain (Basbaum and Fields 1984; Fields et al. 1991). At the spinal level, CB_1 cannabinoid receptors are abundant in the dorsal horn responsible for pain transmission. Most primary afferent neurons that express CB_1 receptor mRNA are those with larger diameter fibres involved in the transmission of non-nociceptive-sensitive inputs (Hohmann and Herkenham 1998). However, CB_1 cannabinoid receptors also modulate the transmission of C fibre-evoked responses (Kelly and Chapman 2001), inhibiting the release of neurotransmitters responsible for pain transmission (Wilson and Nicoll 2002). CB_1 cannabinoid receptor mRNA was also highly expressed in dorsal root ganglion cells (Hohmann 2002; Bridges et al. 2003). At this level, CB_1 cannabinoid receptor stimulation seems to produce a presynaptic inhibition of Ca^{2+} channels, attenuating the release of neurotransmitters (Millns et al. 2001).

On peripheral terminals, the activation of CB_1 and CB_2 cannabinoid receptors was shown to inhibit nociceptive transmission, and both receptors seem to be implicated in mediating the existing endogenous cannabinoid tone (Calignano et al. 1998; Strangman et al. 1998; Hanus et al. 1999; Ko and Woods 1999). Thus, behavioural studies support a role for peripheral cannabinoid CB_2 receptors in animal models of persistent pain and the existence of a synergism between CB_1- and CB_2-mediated responses at this level (Malan et al. 2002). However, other studies do not support such a role of peripheral cannabinoid receptors (Di Marzo et al. 2000). CB_2 receptor activation can also inhibit oedema and plasma extravasations produced by inflammation at a peripheral level (Malan et al. 2002). Cannabinoid CB_2 receptors are likely located on non-neuronal cells in inflamed tissues, where they inhibit the release of inflammatory mediators that excite nociceptors (Mazzari et al. 1996).

Recent studies using knockout mice deficient in cannabinoid receptors have provided new and important information on the involvement of the cannabinoid system in nociception. Different results were reported on spontaneous nociceptive perception of CB_1 knockout mice, depending on the genetic construction of the knockout mice. In CB_1 knockout mice with an outbred CD1 genetic background, no changes in the nociceptive threshold were found after the application of thermal (tail-immersion and hot-plate tests), mechanical (tail-pressure) or chemical (writhing test) stimuli (Ledent et al. 1999; Valverde et al. 2000b). However, CB_1 knockout mice on an inbred C57BL/6J genetic background displayed hypoalgesia in the hot-plate and in the formalin test, whereas no difference in the tail-flick test was found (Zimmer et al. 1999). The hypoalgesic phenotype observed in this latter strain was surprising because CB_1 agonists produce similar behavioural effects in wild-type mice. Moreover, intrathecally administered SR141716A or antisense knockdown of spinal CB_1 receptors produced hyperalgesia in the hot-plate test (Richardson et al. 1998). The discrepancies between the two studies performed with knockout mice could be due to the different genetic background of the lines, but also to the different behavioural responses evaluated in the nociceptive test. Thus, Zimmer et al. (1999) measured the first discomfort response exhibited in the hot-plate test (paw lifting, paw shaking, paw licking or jumping), whereas Valverde et al. (2000b) have quantified jumping latency.

A recent study has demonstrated that the endogenous cannabinoid system mediates a protective role during visceral inflammation through the activation of the CB_1 cannabinoid receptors. Thus, CB_1 knockout mice exposed to an experimental colitis, induced by intrarectal DNBS, exhibited a higher sensibility to chemical-induced visceral inflammation. Pharmacological blockade of CB_1 receptors with the selective antagonist SR141716A led to a worsening of colitis similar to that observed in CB_1-deficient mice. Moreover, the cannabinoid agonist HU-210 reduced the severity of experimental colitis, and FAAH-deficient mice showed significant protection against DNBS treatment (Massa et al. 2004).

In mice lacking CB_1 cannabinoid receptors, the antinociceptive properties of THC were abolished in the hot-plate test, and were strongly reduced in the tail-immersion test. In this latter test, a slight antinociceptive response was still observed in mutant mice only at the highest dose of THC used (Ledent et al. 1999; Zimmer et al. 1999). In contrast, morphine-induced antinociception was preserved in these knockout mice in the tail immersion and the hot-plate tests. Furthermore, the antinociceptive effects induced by the selective δ-opioid agonists [D-penicillamine^{2,5}]enkephalin (DPDPE) and deltorphin II and by the selective κ-opioid agonist U-50,488H were unchanged (Valverde et al. 2000b). Therefore, CB_1 receptors do not seem to be involved in the antinociceptive responses induced by exogenous opioids. However, CB_1 receptors participate in the antinociceptive responses produced by non-steroidal anti-inflammatory drugs. Thus, the antinociceptive responses induced by the non-selective cyclooxygenase inhibitor indomethacin in the formalin test were abolished in CB_1 knockout mice (Guhring et al. 2002).

Several studies have shown tolerance to several behavioural responses induced by cannabinoids, including antinociception (Buxbaum 1972; Hutcheson et al. 1998;

Martin and Lichtman 1998; Pertwee 2001). The development of cannabinoid tolerance seems to be mainly due to pharmacodynamic events. Thus, a significant decrease in both CB_1 cannabinoid receptor binding sites and mRNA levels has been observed in different brain areas after a chronic treatment with cannabinoid agonists. Changes in G protein expression and functional activity were also observed in rats chronically treated with cannabinoids (Rodriguez de Fonseca et al. 1994; Rubino et al. 1994, 1998, 2000; Fan et al. 1996; Sim et al. 1996; Romero et al. 1998). Studies using knockout mice deficient in the different components of the endogenous opioid system provide new data concerning the possible mechanisms involved in the development of cannabinoid tolerance. Thus, knockout mice lacking the pre-proenkephalin gene showed a decrease in the development of tolerance to THC antinociceptive effects (Valverde et al. 2000a). A similar decrease in the development of cannabinoid tolerance was also observed in double mutant mice, lacking δ- and κ-opioid receptors (Castañe et al. 2003).

There is increasing evidence to support a role for peripheral CB_2 receptors in the analgesic effects of cannabinoids. Thus, chronic pain induced by peripheral nerve injury, but not that produced by peripheral inflammation, was associated with the enhancement of CB_2 cannabinoid receptor expression, specifically located in the lumbar spinal cord (Malan et al. 2002). Thus, a selective induction of spinal CB_2 expression presumably occurs on activated microglia in regions undergoing neuronal damage.

Taken together, these results show that the endocannabinoid system plays an important role in the physiological modulation of nociceptive transmission and in the development of inflammatory and neuropathic pain. Furthermore, the endocannabinoid system seems to participate in the antinociception induced by anti-inflammatory drugs, and displays an important synergic effect with opioid agonists. These data strongly support the therapeutic potential of cannabinoid receptor agonists for the treatment of chronic pain.

8
CB_1 Cannabinoid Receptors and Addiction

Behavioural and neurochemical studies have now clarified the controversy about the abuse liability of cannabinoids by demonstrating that such drugs fulfil most of the common features attributed to compounds with reinforcing properties. Cannabinoid rewarding properties have been identified using intracranial self-stimulation, conditioned place preference and intravenous self-administration paradigms. Furthermore, a cannabinoid withdrawal syndrome has also been characterized in different animal species (Lichtman and Martin 2002; Maldonado and Rodriguez de Fonseca 2002).

The administration of cannabinoid agonists can produce both rewarding and aversive/dysphoric effects in the place conditioning paradigm, depending on the dose and the experimental conditions. Thus, THC produced place preference in rats when administered at low doses and when animals were exposed to a 24-h washout period between the two THC conditioning sessions (Lepore et al. 1995). THC also

produces a clear place preference in mice when a long period of conditioning is used and the possible dysphoric consequences of the first drug exposure are avoided (Valjent and Maldonado 2000). Concerning intracranial self-stimulation, acute administration of THC has been reported to decrease the intracranial self-stimulation threshold in rats, suggesting the activation of central hedonic systems (Gardner et al. 1988; Lepore et al. 1996). In contrast, CP55,940 administration did not modify electrical brain stimulation, supporting the hypothesis that cannabinoids have a relatively modest influence on reward circuits (Arnold et al. 2001).

Different studies have reported that THC is unable to induce self-administration behaviour in any of the animal species studied (Corcoran and Amit 1974; Harris et al. 1974; Carney et al. 1977; Mansbach et al. 1996). However, one study has revealed THC intravenous operant self-administration behaviour in squirrel monkeys that have a previous history of cocaine self-administration (Tanda et al. 2000). Recently, Justinova et al. (2003) reported self-administration of THC by drug-naïve monkeys, demonstrating that THC can act as an effective reinforcer of drug-taking behaviour in monkeys with no history of exposure to other drugs (Justinova et al. 2003). The pharmacokinetic properties of THC seem to be crucial for the behavioural responses observed in the self-administration paradigm. Thus, the synthetic cannabinoid agonists WIN55,212-2 and CP55,940, which have a shorter half-life than THC, are intravenously self-administered by mice (Martellotta et al. 1998) and rats (Braida et al. 2001). A selective involvement of the CB_1 cannabinoid receptors is implicated in the reinforcing properties of all these cannabinoid compounds because the CB_1 receptor antagonist SR141716A completely blocked the self-administration induced by WIN55,212-2 (Martellotta et al. 1998), CP55,940 (Braida et al. 2001) and THC (Tanda et al. 2000). Furthermore, CB_1 knockout mice failed to self-administer WIN55,212-2 in contrast to wild-type animals (Fattore et al. 1999; Ledent et al. 1999).

Administration of the selective CB_1 cannabinoid receptor antagonist SR141716A to animals (mouse, rat and dog) chronically treated with THC has been shown to precipitate different somatic manifestations of cannabinoid withdrawal. In rodents, this cannabinoid withdrawal syndrome is characterized by the presence of a large number of somatic signs and the absence of vegetative manifestations (Lichtman and Martin 2002; Maldonado and Rodriguez de Fonseca 2002). However, the doses of THC required to induce physical dependence in rodents are extremely high, currently from 10 to 100 mg/kg of THC (i.p.), daily for 5 to 10 days (Tsou et al. 1995; Aceto et al. 1996; Cook et al. 1998; Hutcheson et al. 1998). CB_1 cannabinoid receptors are responsible for the somatic manifestations of cannabinoid withdrawal. Indeed, CB_1-deficient mice chronically treated with THC did not exhibit any manifestation of cannabinoid withdrawal (Ledent et al. 1999; Lichtman et al. 2001).

In conclusion, these data clearly demonstrate that the functional activity of the CB_1 cannabinoid receptor is necessary for the manifestation of the rewarding properties of cannabinoids and for the development of cannabinoid physical dependence and withdrawal.

9
Interaction Between Cannabinoid Receptors and Other Addictive Drugs

Different evidence supports the possible existence of functional interactions between cannabinoids and other drugs of abuse including opioids, psychostimulants, ethanol and nicotine. Findings in support of a link between cannabinoids and other drugs of abuse include: (1) the existence of common physiological and pharmacological properties (opioids, ethanol, nicotine); (2) the stimulation of dopamine release after their administration (psychostimulants, opioids, ethanol, nicotine); (3) the existence of interactions at a signal-transduction level (opioids, psychostimulants, ethanol and nicotine); and (4) the observation that many of these drugs are consumed together.

9.1
Interaction Between Cannabinoids and Opioids

The interaction between cannabinoids and opioids has been widely evaluated because of the diverse physiological effects shared by both types of compounds, including antinociception, hypothermia, and control of locomotion, rewarding properties and the ability to induce drug abuse. Interestingly, the interaction between these two systems seems to be bi-directional. Thus, morphine-induced intravenous self-administration (Ledent et al. 1999; Cossu et al. 2001) and conditioned place preference (Martin et al. 2002) was abolished in knockout mice lacking the CB_1 cannabinoid receptors. These studies underlie the relevance of CB_1 cannabinoid receptors for the manifestation of the reinforcing properties of morphine. The ability of cannabinoid agents to reinstate or prevent heroin-seeking behaviour after a period of extinction has been also evaluated. The cannabinoid agonists WIN55,212-2 and CP55,940, but not THC, restored heroin-seeking behaviour in rats, whereas the CB_1 cannabinoid antagonist SR141716A completely prevented the reinstatement of drug-seeking behaviour induced by a priming injection of heroin (Fattore et al. 2003), supporting the cooperation between opioid and cannabinoid systems in the modulation of addictive behaviour.

Different pharmacological and molecular approaches have been used to investigate the interaction between cannabinoids and opioids in physical dependence. For example, administration of the CB_1 cannabinoid antagonist SR141716A can precipitate behavioural and biochemical manifestations of withdrawal in morphine-dependent rats (Navarro et al. 2001). In contrast to these data, SR141716A did not precipitate any behavioural sign of withdrawal in morphine-dependent mice (Lichtman et al. 2001). These discrepancies could be due to the different animal species and/or differences in the experimental procedure. However, studies performed in CB_1 knockout mice clearly demonstrated the important role played by the CB_1 cannabinoid receptors in the physical manifestations of the morphine withdrawal syndrome. Thus, a robust decrease in the severity of naloxone-precipitated morphine withdrawal syndrome was reported in CB_1 knockout mice (Ledent et al. 1999). In agreement, the co-administration of SR141716A and morphine over

5 days produced an important attenuation in the incidence of the morphine withdrawal manifestations (Mas-Nieto et al. 2001). Early studies have also demonstrated that acute administration of cannabinoid agonists strongly attenuated the severity of morphine abstinence (Hine et al. 1975; Bhargava 1976a,b; Bhargava and Way 1976; Vela et al. 1995). Furthermore, a chronic pre-treatment with THC before starting chronic morphine administration reduced the somatic manifestations of naloxone-precipitated morphine withdrawal, without modifying the motivational responses of this opioid compound (Valverde et al. 2000b).

Reciprocally, the endogenous opioid system has been reported to be involved in the motivational responses and withdrawal manifestations induced by cannabinoids. Thus, the rewarding effects induced by THC were abolished in μ-opioid receptor knockout mice (Ghozland et al. 2002). Furthermore, the dysphoric effects induced by a high dose of THC (5 mg/kg) were slightly attenuated in μ-knockout mice and completely blocked in mice lacking κ-opioid receptors (Ghozland et al. 2002). The conditioned place aversion induced by a high dose of THC (5 mg/kg) was also abolished in prodynorphin knockout mice, also supporting the involvement of κ-opioid receptors in the motivational responses induced by cannabinoids (Zimmer et al. 2001). In addition, the rewarding responses induced by THC in the conditioned place paradigm were also abolished in double knockout mice lacking both μ- and δ-opioid receptors (Castañe et al. 2003). There is also evidence to suggest that the endogenous opioid system participates in the reinforcing properties of cannabinoids. Thus, the opioid antagonist naloxone partially blocked self-administration of the cannabinoid agonist CP55,940 (Braida et al. 2001). THC self-administration behaviour was also attenuated by a different opioid antagonist naltrexone (Justinova et al. 2004). Furthermore, naloxone precipitated some behavioural signs of abstinence in rats chronically treated with a cannabinoid agonist (Kaymakcalan et al. 1977; Navarro et al. 2001).

The role of the endogenous opioid peptides in cannabinoid dependence has also been investigated by using knockout mice. The expression of cannabinoid withdrawal was attenuated in THC-dependent knockout mice lacking the preproenkephalin gene (Valverde et al. 2000a). However, THC abstinence was not modified in μ-, δ- or κ-opioid receptor knockout mice (Ghozland et al. 2002). In contrast, another study reported a decrease in the severity of cannabinoid withdrawal syndrome in μ-opioid receptor knockout mice (Lichtman et al. 2001). The different genetic construction of knockout mice and the changes in the experimental conditions can explain these discrepancies. Finally, a significant decrease in the severity of cannabinoid withdrawal syndrome was observed in double μ-, δ-opioid receptor knockout mice (Castañe et al. 2003), suggesting that a cooperative action of μ- and δ-opioid receptors is required for the entire expression of THC dependence.

All these results indicate that the bi-directional interactions between the endogenous cannabinoid and opioid systems are crucial for the motivational properties and the development of physical dependence induced by these two kinds of drugs, and could provide new strategies for a more rational approach to the treatment of drug abuse.

9.2 Interaction Between Cannabinoids and Psychostimulants

The endogenous cannabinoid system has been reported to be involved in the addictive effects induced by other drugs of abuse, such as cocaine and other psychostimulants. Dopaminergic activity in the mesocorticolimbic system is considered a common feature mediating the primary reinforcing effects of most drugs of abuse (Di Chiara 1998). Psychostimulants facilitate this dopaminergic neurotransmission by different mechanisms, including the enhancement of extracellular dopamine concentrations, mainly through inhibition of the dopamine transporter. On the other hand, CB_1 cannabinoid receptors are important modulators of dopaminergic activity in the mesocorticolimbic system, suggesting that the endogenous cannabinoid system may contribute to the reinforcing properties of different drugs of abuse, including psychostimulants. However, the possible mechanisms involved in such an interaction remain controversial, because only a few studies have been performed on this topic and have frequently provided contradictory results.

Several studies suggest that CB_1 cannabinoid receptors do not participate in the acute rewarding properties of psychostimulants. Thus, cocaine-induced conditioned place preference and sensitization to the hyperlocomotor effects produced by chronic administration of the drug were preserved in CB_1 knockout mice (Martin et al. 2000). In addition, acute self-administration of cocaine, performed during a single session, was also maintained in mice lacking CB_1 receptors (Cossu et al. 2001). However, administration of the cannabinoid agonist WIN55,212-2 has been found to decrease the reinforcing actions of cocaine in a brain stimulation paradigm in mice (Vlachou et al. 2003), whereas the blockade of CB_1 receptors by SR141716A treatment decreased the reinforcing value of intracranial self-stimulation in rats (Deroche-Gamonet et al. 2001). These results suggest that the endogenous cannabinoid system could modulate cocaine reward. Other studies have also supported the existence of an interaction between cocaine and cannabinoids in reinforcing responses. Thus, pretreatment with WIN55,212-2 of rats self-administering cocaine reduces cocaine intake in a dose-dependent manner. The CB_1 antagonist SR141716A completely reversed these effects of WIN55,212-2, indicating that the reinforcing effects of CB_1-mediated and cocaine-induced reward mechanisms are additive (Fattore et al. 1999).

Furthermore, the endocannabinoid system plays an important role in the neuronal processes underlying cocaine-seeking behaviour. Thus, the cannabinoid agonist HU-210 induces relapse to cocaine seeking after prolonged withdrawal periods, and the antagonist SR141716A attenuates this response when it is induced by re-exposure to cocaine-associated cues or to cocaine itself (De Vries et al. 2001). It therefore seems necessary to perform further studies by using CB_1 knockout mice to evaluate the contribution of these receptors in processes related to the acquisition, maintenance and extinction of cocaine self-administration, and thus further clarify the nature of the interaction between cocaine and the endocannabinoid system.

Recent studies have also evaluated the interaction between cannabinoids and other psychostimulants such as amphetamine and MDMA (methylenedioxymethamphetamine; ecstasy) (Braida and Sala 2002; Parker et al. 2004). These studies showed that infusion of the cannabinoid agonist CP55,940 decreased intracerebroventricular MDMA self-administration in rats (Braida and Sala 2002). It remains to be determined, however, if cannabinoids modulate the addictive properties of psychostimulant drugs.

9.3 Interaction Between Cannabinoids and Nicotine

The consumption of cannabis is highly associated with tobacco, which contains nicotine, an important psychoactive compound (Nemeth-Coslett et al. 1986; McCambridge and Strang 2004). The administration of THC and nicotine in rodents produces multiple common pharmacological responses including analgesia, hypothermia, impairment of locomotor activity and addiction (Hildebrand et al. 1997; Ameri 1999; Maldonado and Rodriguez de Fonseca 2002). Nicotine responses are mediated by the activation of nicotinic acetylcholine receptors, which have a pentameric structure consisting of different receptor subunits (Grutter and Changeux 2001; Le Novere et al. 2002).

Several studies have suggested a possible functional interaction between cannabinoid and nicotinic systems. The specific behavioural and biochemical consequences of such an interaction are poorly documented in animal models in spite of the high frequency of association of these two substances in humans. Nicotine facilitated THC-induced acute pharmacological and biochemical responses in mice, including hypothermia, antinociception, hypolocomotion and anxiolytic-like responses. Furthermore, the co-administration of sub-threshold doses of THC and nicotine produced conditioned place preference (Valjent et al. 2002). Mice co-treated with nicotine and THC displayed attenuation in THC tolerance and an enhancement in the somatic expression of cannabinoid antagonist-precipitated THC withdrawal (Valjent et al. 2002). These findings showed that low doses of cannabinoids associated with nicotine could have a higher capability to induce behavioural responses related to addictive processes than THC administration alone, and could enhance the somatic consequences of chronic consumption of these drugs.

Some behavioural responses induced by nicotine were modified in mice lacking CB_1 cannabinoid receptors. Thus, whereas the severity of nicotine withdrawal syndrome was not affected in CB_1 knockout mice, the rewarding properties of nicotine, evaluated in the conditioned place preference assay, was abolished in these animals (Castañe et al. 2003). In contrast, the absence of CB_1 cannabinoid receptors did not modify acute self-administration induced by nicotine (Cossu et al. 2001). The effective doses in these two behavioural models (acute intravenous self-administration and conditioned place preference) are different, which makes it difficult to directly compare the results of these studies. However, the interaction between THC and nicotine previously reported by using pharmacological and bio-

chemical approaches (Valjent et al. 2002) are in agreement with the impairment of nicotine rewarding effects in CB_1 knockout mice (Castañe et al. 2002). In addition, the administration of SR141716A decreased nicotine self-administration in rats, and nicotine-induced dopamine release in the nucleus accumbens and the bed nucleus of the stria terminalis, supporting the role of the endocannabinoid system in nicotine rewarding effects (Cohen et al. 2002). SR141716A increased dopamine, noradrenaline and serotonin levels in the cortex and the nucleus accumbens (Tzavara et al. 2003), which could contribute to its ability to reverse nicotine-induced responses. SR141716A could have anti-smoking activity in humans, accordingly to promising findings obtained in a placebo-controlled phase III clinical trial using this compound (Fernandez and Allison 2004).

Studies into the addictive properties of cannabinoids using knockout mice lacking different protein subunits of nicotinic receptors could greatly extend our knowledge of the neurobiological mechanisms involved in the interaction between cannabinoids and nicotine.

9.4 Interaction Between Cannabinoids and Ethanol

There is now considerable evidence to suggest a possible involvement of the cannabinoid CB_1 receptor in the addiction-related effects of ethanol (Mechoulam and Parker 2003). Both, cannabinoids and ethanol produce some similar physiological and behavioural responses including euphoria, motor incoordination and hypothermia. CB_1 ligands are able to modulate ethanol preference and self-administration (Arnone et al. 1997; Freedland et al. 2001; Mechoulam and Parker 2003). Furthermore, chronic ethanol treatment increases the synthesis of endocannabinoids and down-regulates brain CB_1 receptors and their function (Basavarajappa and Hungund 2002), supporting the hypothesis of an interaction between these two drugs. Pharmacological studies reported that blocking the CB_1 receptor with SR141716A reduced ethanol consumption (Arnone et al. 1997; Freedland et al. 2001).

A recent study on a CD1 genetic background showed that ethanol consumption and preference were decreased in CB_1 knockout mice, whereas ethanol sensitivity and withdrawal severity were increased in these mice (Naassila et al. 2004). These observations are similar to those reported in a previous study showing decreased ethanol consumption and increased sensitivity to the acute effects of ethanol in CB_1 knockout mice on a C57BL/6J genetic background (Hungund et al. 2003). Furthermore, ethanol did not cause release of dopamine in the nucleus accumbens in CB_1 knockout mice, in contrast to the effects observed in wild-type littermates. In agreement, SR141716A completely abolished the enhancement of dopamine responses induced by acute ethanol in the nucleus accumbens of wild-type mice (Hungund et al. 2003). Similarly, a reduction in the effects of ethanol on extracellular levels of dopamine in the nucleus accumbens after SR141716A administration has been previously reported, suggesting that cannabinoids modulate the reinforcing properties of ethanol by decreasing the release of dopamine in limbic areas

(Cohen et al. 2002). Another study also supports the hypothesis that endocannabinoids acting on CB_1 receptors contribute to ethanol rewarding effects, albeit in an apparent age-dependent manner (Wang et al. 2003). Thus, a high ethanol preference was found in young (6–10 weeks) C57BL/6J mice that was reduced in CB_1 knockout mice. The administration of the antagonist SR141716A to young wild-type mice reduced ethanol preference to the level exhibited by CB_1 knockout mice. Ethanol preference declined in old wild-type mice (26–48 weeks), and this reached a level similar to that observed in CB_1 knockout mice (similar for young and old animals). Ethanol preference in old CB_1 knockout and wild-type littermates was unaffected by SR141716A (Wang et al. 2003). The age-dependent differences for ethanol preference reported in this study could probably explain some of the discrepancies between results that have been obtained from different studies with CB_1 knockout mice. Thus, Racz et al. (2003) reported that CB_1 knockout mice (on a C57BL/6J genetic background) showed initially an even higher preference for ethanol than wild-type littermates. After 1 week, the ethanol consumption was virtually identical in knockout and wild-type mice. Withdrawal symptoms after the cessation of chronic ethanol administration were completely absent in CB_1 knockout mice (Racz et al. 2003). Activation of the CB_1 receptor promotes alcohol craving and suggests a role of this receptor in excessive ethanol drinking behaviour and the development of alcoholism (Schmidt et al. 2002). Interestingly, this recent clinical study associated a CB_1 cannabinoid receptor gene polymorphism with the severity of withdrawal symptoms in humans (Schmidt et al. 2002).

Recently, a new CB_1 receptor antagonist, namely SR147778, has been developed. This compound is able to reduce both ethanol and sucrose consumption in mice and rats (Rinaldi-Carmona et al. 2004), supporting the involvement of the CB_1 cannabinoid receptor in ethanol consumption. Taken together, these results suggest an involvement of endocannabinoids in the rewarding effects, physical dependence and craving induced by ethanol. Further studies must to be performed in order to clarify the apparent discrepancies observed in the different studies performed with CB_1 knockout mice.

10
CB_1 Receptors in the Control of Feeding Behaviour

The appetite-stimulating effects of marijuana have been known for centuries and constitute one of the established medicinal uses of cannabis preparations. Today THC (dronabinol/Marinol) is clinically used for the treatment of cachexia-anorexia in human immunodeficiency virus (HIV) and palliative care patients. There have also been very promising advances in the development of a cannabinoid receptor antagonist (SR141716A, now named Rimonabant or Acomplia) for the treatment of obesity.

Pharmacological studies in animals are consistent with a role of the endogenous cannabinoid system in the regulation of feeding behaviours and food palatability (Williams and Kirkham 2002a,b; Higgs et al. 2003). Administration of THC to rats produced a significant hyperphagia that was reversed by SR141716A (Williams

et al. 1998; Williams and Kirkham 2002b). Since 2-AG is present in the milk of humans and animals, Fride and her collegues asked whether this endocannabinoid might promote appetite and suckling behaviour in newborn animals. Indeed, the administration of SR141716A to newborn mice, within the first 24 h after birth, had a devastating effect on milk ingestion and often led to the death of the treated animals. CB_1 receptor-deficient mice also failed to drink in the first 24 h after birth, but started to display milk bands from day 2. It seems that this delayed onset of milk intake affects the survival rate of CB_1 knockout pups, which was significantly lower than that of wild-type littermates in Fride's studies (Fride et al. 2001, 2003). Our (A.Z.) previous analysis of the distribution of genotypes among offspring of heterozygous matings indicated a small deviation from the expected Mendelian frequency at the time of weaning ($CB_1^{+/+}$, 29%; $CB_1^{+/-}$, 47,7%; $CB_1^{-/-}$, 23.3%; $n = 1{,}439$), thus also suggesting a somewhat reduced viability of homozygous and even heterozygous pups (Zimmer et al. 1999). These results suggest that endocannabinoids in the milk promote suckling behaviour during the early postnatal period.

The body weight of adult CB_1 receptor knockout mice was, however, similar to that of control animals, indicating that the endocannabinoid system is not critical for maintaining regular food intake under normal laboratory conditions (Zimmer et al. 1999). In contrast, when animals were food deprived for 18 h, wild-type mice consumed significantly more food at the end of the fasting period than CB_1-deficient animals (Di Marzo et al. 2001). Wild-type mice that were treated with 3 mg/kg SR141716A 10 min before the start of the testing period also showed a lower food intake, similar to that of CB_1 knockouts. The orexigenic effects of cannabinoids are thought to be mediated by hypothalamic CB_1 receptors, although the CB_1 receptor density in the hypothalamus is lower than in many other brain regions (Marsicano and Lutz 1999; Harrold and Williams 2003). The endocannabinoid system in the hypothalamus seems to be part of a leptin-sensitive regulatory pathway, as leptin decreases hypothalamic endocannabinoid synthesis, whilst defective leptin signalling in obese (ob/ob) or diabetic (db/db) mice is accompanied by elevated endocannabinoid levels (Di Marzo et al. 2001). Fasting also increased 2-AG levels in the hypothalamus and in the limbic forebrain, whilst hypothalamic 2-AG levels declined as animals ate (Kirkham et al. 2002). Together these results are consistent with a role of leptin-regulated endocannabinoids in the control of motivational aspects of feeding behaviour.

11
Endocannabinoid as Retrograde Neurotransmitter

Several recent studies have begun to elucidate the cellular and molecular mechanisms underlying the numerous and profound effects of cannabinoids on the brain. Indeed there is now compelling evidence that endocannabinoids act as activity-dependent retrograde inhibitors of synaptic transmission.

In the hippocampus, CB_1 receptors are localized presynaptically in GABA axon terminals, most of which originate from CCK-positive basket cells (Katona et al.

1999). Endocannabinoids are probably synthesized by Ca^{2+}-dependent postsynaptically localized enzymes (Bisogno et al. 2003). Activation of the presynaptic CB_1 receptors exerts diverse effects on synaptic functions, including the activation of inwardly rectifying K^+ channels, the inhibition of voltage-gated Ca^{2+} channels and the suppression of neurotransmitter release (Di Marzo et al. 1998; Freund et al. 2003). Because of the distribution and function of its various components, the endocannabinoid system seemed ideally suited to mediate a form of activity-dependent modulation of synaptic activity in the hippocampus that has been termed depolarization-induced suppression of inhibition (DSI). DSI describes a phenomenon in which a brief depolarization of a pyramidal neuron transiently suppresses the release of GABA from presynaptic terminals (Pitler and Alger 1992, 1994). A similar phenomenon affecting excitatory glutamatergic synapses has been described in the cerebellum and hippocampus, and is termed depolarization-induced suppression of excitation (DSE). Because DSI and DSE are initiated postsynaptically through an elevation of cytoplasmic Ca^{2+} and expressed presynaptically as an inhibition of neurotransmitter release, a retrograde signal that travels backwards across synapses had been postulated (Wilson and Nicoll 2002). Several studies have now conclusively demonstrated that the retrograde messengers responsible for this signalling are endocannabinoids. In the hippocampus, the CB_1-selective agonist WIN55,212-2 blocked GABA release and suppressed baseline inhibitory post-synaptic current (IPSC) amplitudes (Hajos et al. 2000; Wilson and Nicoll 2001). The CB_1 antagonists SR141716A and AM251 blocked DSI (Wilson and Nicoll 2001). Excitatory hippocampal synapses displayed an analogous reduction: WIN55,212-2 blocked excitatory post-synaptic currents (EPSC) and SR141716A blocked DSE. Importantly, DSI and DSE were completely absent in CB_1 knockout mice from the Zimmer laboratory in the hippocampus and in the cerebellum (Yoshida et al. 2002). However, Hajos and colleagues have pointed out that anatomical studies could not confirm the existence of CB_1 receptors on hippocampal glutamatergic terminals and have reported that CB_1-deficient mice generated by Ledent and co-workers still show a reduction of postsynaptic excitatory currents in hippocampal slices by WIN55,212-2 (Hajos et al. 2001). These authors speculate that the effect of cannabinoids on excitatory hippocampal neurons is mediated by a non-CB_1 receptor. Clearly, further studies are necessary to determine the reason for these contradictory findings.

12 Outlook

Knockout mice have revealed many novel and interesting aspects of the physiological functions of CB_1 receptors in locomotor activity, emotional behaviours, regulation of blood pressure, cognition, pain, reproduction and addiction. In addition, these animals have become invaluable tools for studying the interactions between cannabinoids and other drugs of abuse, i.e. opioids, nicotine, ethanol and cocaine. The multitude of phenotypes that have been observed in these an-

imals reflects the diversity of functions of the endogenous cannabinoid system. Undoubtedly, these results will further the potential medical uses of cannabinoid receptor agonist and antagonists.

Although the phenotype of the different knockout mice is very similar among the individual strains and laboratories involved, small differences do exist. It remains to be determined if these phenotypic differences are due to variations in the genetic background, different holding conditions, or both. Understanding the impact of these epigenetic factors may help us to appreciate the significance of the endocannabinoid system in environmentally and genetically more complex systems.

Whilst most of the research of the endocannabinoid system in the last decade has focussed on the CB_1 and CB_2 receptors, we have also made substantial advances in the identification of endocannabinoid degrading and synthesizing enzymes and the effects of endocannabinoids that are not mediated by these receptors. Future animal models will therefore increasingly address the relevance of non-CB_1 and non-CB_2 endocannabinoid binding sites and the regulation of endocannabinoid levels.

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