**Abstract:** Recently, there has been a rapidly growing interest in the role of cannabinoids in the regulation of skeletal remodeling and bone mass, addressed in basic, translational and clinical research. Since the first publications in 2005, there are more than 1000 publications addressing the skeletal endocannabinoid system. This review focuses on the roles of the endocannabinoid system in skeletal biology via the cannabinoid receptors CB1, CB2 and others. Endocannabinoids play important roles in bone formation, bone resorption and skeletal growth, and are sometimes age, gender, species and strain dependent. Controversies in the literature and potential therapeutic approaches targeting the endocannabinoid system in skeletal disorders are also discussed.

**Keywords:** cannabinoid receptors 1 and 2 (CB1, CB2); cannabinoids; osteoblasts; osteoclasts; osteoporosis.

**Introduction**

The cannabinoid receptor 1 (CB1) is considered the neuronal cannabinoid receptor, whereas CB2 is predominantly non-neuronal [1]. Both receptors are expressed in a wide array of tissues that regulate diverse processes. CB1 and CB2 are activated by endogenous fatty acid-derived ligands, arachidonoyl ethanolamide (anandamide) and 2-arachidonoylglycerol (2-AG) [1]. In the last decade, it was established that the skeleton harbors a local endocannabinoid (EC) regulatory system [1, 2]. The notion of the EC system that plays a role in skeletal biology originated from two important observations. One is that bone formation and bone mass, as well as the central production of at least one major EC, 2-AG, are subject to negative control by leptin [3]. The second observation is that traumatic brain injury (TBI) enhances both bone formation [4, 5] and central 2-AG production [6]. These findings prompted several follow-up studies that established the mechanism of action of the EC system in bones.

**The skeletal EC system**

The skeletal cannabinoid system is portrayed as part of the larger family of fatty acid amides, recently implicated in the regulation of skeletal remodeling and bone mass [7]. Several key components of the EC system have been identified in bone. The main ECs, anandamide and 2-AG, are present in this tissue in picomoles per gram and nanomoles per gram concentrations, respectively, levels similar to those found in the brain [8]. Because the blood EC levels are several orders of magnitude lower than those found in the bone, it is very likely that anandamide and 2-AG are synthesized locally in the skeleton [9]. Indeed, both ligands are produced by osteoblasts and osteoclasts in culture [10]. In addition, enzymes critically involved in 2-AG and anandamide biosynthesis are expressed in osteoblasts, osteocytes and bone lining cells [9]. Immunohistochemical analyses performed by Wasserman et al. [11] to assess the expression of EC and diacylglycerol lipases (DAGLs, critical biosynthetic enzymes of the main EC) in bone cells have unintentionally demonstrated the presence of these proteins not only in osteoblasts, osteocytes and osteoclasts, but also in the epiphyseal growth cartilage chondrocytes. In line with the occurrence of the EC in the bone, both CB1 and CB2 cannabinoid receptors are also present in the skeleton [10, 12]. CB1 is present in skeletal sympathetic nerve terminals, where it regulates norepinephrine production and/or release. CB2 is expressed in osteoblasts, osteocytes and osteoclasts [11]. Recent reports also show the expression of both cannabinoid receptors CB1 and CB2, and of 2-AG biosynthetic enzymes in hypertrophic chondrocytes in the growth plate [11].
Skeletal phenotypes of cannabinoid receptor-deficient mice

Studies investigating CB1 had reported different effects that are age and strain dependent [2, 10, 13–16]. The first study reporting on the skeletal effect of CB1 showed that young CB1–/– female mice on a CD1 background displayed a high bone mass (HBM) phenotype and were protected against ovariectomy (OVX)-induced bone loss [2]. The same group found that CB1–/– mice on the same CD1 background developed an increased age-related bone loss [14], attributing a bone protective role to CB1 during aging. This more recent observation is in line with other reports showing that CB1 in rats induced bone loss in young animals but prevented age-related osteoporosis later in life [15]. In C57Bl/6J mice, the role of CB1 seemed age independent as young CB1–/– animals also developed a low bone mass (LBM) phenotype [13]. Although it remains to be elucidated whether sex also affects the action of CB1 signaling, data from several groups support the idea that age and animal strain modulate the skeletal role of CB1.

The action of CB1 on bone formation vs. resorption is still controversial. In vivo, CB1 knockout either reduced or did not affect bone formation. In young CB1–/– mice on a CD1 background, bone formation was not affected, whereas it was reduced in both young C57Bl/6J CB1–/– mice and in all aged rodents independent of strain [2, 10, 14, 15, 17]. One important difference between past publications is the question of whether this effect on bone formation is mediated by direct CB1 signaling in osteoblasts. In these cells, CB1 is expressed at very low levels [2, 12, 13]. However, because these levels increase with age, it was suggested that CB1 up-regulation provides protection against age-related osteoporosis [14]. In line with this assumption, CB1 activation in isolated osteoblast precursors promoted osteoblast differentiation [14]. Surprisingly, this effect was observed in cultures from both young and aged animals, despite the paucity of CB1 expression in the young skeleton. An alternative mechanism is provided by other studies whose data support the notion that CB1 signaling does not have any direct effect on osteoblast proliferation [10, 18]. In a model of TBI, Tam et al. [10] demonstrated that CB1 mediates the increased bone formation induced by TBI. They showed that the TBI-induced increase in bone formation is preceded by an elevation of 2-AG and a decrease in noradrenalin levels in the bone tissue [10], TBI stimulation of bone formation is absent in CB1-deficient mice, but not in CB2-null mice. The skeletal effect of TBI can be mimicked in wild-type (WT) animals, including the suppression of noradrenalin levels, by exogenous 2-AG administration. Both the TBI- and 2-AG-induced stimulation of osteogenesis could be restrained by the β-adrenergic receptor (β2AR) agonist isoproterenol. These findings indicate that CB1 controls osteoblast function by negatively regulating noradrenalin release from sympathetic nerve terminals in the immediate vicinity of osteoblasts; noradrenalin suppresses bone formation by binding to osteoblastic β2AR, and this suppression is alleviated by the activation of sympathetic presynaptic CB1 [10] (Figure 1). Taken together, the relative contribution of osteoblastic, osteoclastic and sympathetic CB1 remains to be elucidated and further investigation on the mechanism of action of CB1 in bone formation is warranted.

The action of CB1 on bone resorption and osteoclasts is even more complex. In vivo, CB1–/– animals (on the CD1 background) displayed lower levels of osteoclastogenesis and bone resorption. The reduced osteoclastogenesis in CB1–/– animals may be mediated by CB1 signaling in osteoblasts. Idris et al. [14] showed that CB1-deficient osteoblasts have a reduced ability to support osteoclast formation owing to the lower expression of receptor activator of nuclear factor κ-B ligand. A direct effect via CB1 activation...
Recent studies examined the role of the EC system in skeletal elongation and showed that CB1 and CB2 cannabinoid receptors are expressed specifically in hypertrophic chondrocytes of the epiphyseal growth cartilage (EGC), which drives vertebrate growth [11]. These cells also express DAGLs and 2-AG, which are present at significant levels in the EGC. Results in vivo showed that the femora of CB1+/– and/or CB2+/– mice at the end of the rapid growth phase are longer than those of WT animals. It was found that Δ9-tetrahydrocannabinol (THC) slows skeletal elongation of female WT and CB2+/– mice, but not of CB1+/– mice, which is reflected in the femoral and lumbar vertebral body length. This, in turn, results in lower body weight, but unaltered fat content. THC inhibits EGC chondrocyte hypertrophy in ex vivo cultures and reduces the hypertrophic cell zone thickness of CB1+/– mice, but not of CB2+/– mice. These results demonstrate that (i) endochondral skeletal growth attenuation by THC is mediated by CB1 cannabinoid receptors and that (ii) the EGC displays a local growth-restraining EC system (Figure 2) [11].

Studies from Ofek et al. had shown that CB2–/– animals have a gender-independent skeletal phenotype [12]. During their first 2–3 months of life, CB2–/– mice accrue a normal peak trabecular bone mass, but later display a markedly enhanced age-related bone loss; their trabecular bone volume fraction at 1 year of age is approximately half that of WT controls (Figure 3) [12]. Reminiscent of human
postmenopausal osteoporosis [20], old male and female CB2+/− mice have a high bone turnover with increases in both bone resorption and formation, which are at a net negative balance [12, 14, 21]. In the cortical bone compartment, CB2 deficiency resulted in endosteal bone loss and cortical expansion [12]. Importantly, although many of the roles of CB2 in mediating various functions under stress or inflammation are already known, the age-related LBM was the first spontaneous phenotype reported in CB2−/− mice. The interaction between CB2 and sex hormones is less clear. Two separate studies from Idris et al. [17, 21] concluded that CB2 deficiency partially protects the skeleton against the catabolic effect of O VX in young females. This observation is in apparent contradiction with a prior report where attenuation of O VX-induced bone loss was observed following stimulation of CB2 signaling using the CB2 agonist HU-308 [12]. It should be noted that Idris et al. [17, 21] did not demonstrate that CB2+/− ovarioctomized mice have a higher bone mass than WT ovarioctomized animals. It is therefore possible that O VX in CB2−/− mice results in a slightly but not significantly higher bone mass than in WT mice, and in the control sham- ovarioctomized group, CB2 knockout animals had a slightly but not significantly lower bone mass than their WT counterparts, which could still lead to a statistically different effect of O VX in the two mouse strains. The discrepancy between the reports on the effect of CB2 signaling in O VX may also be due to a shared pathway between sex hormones and CB2 signaling in trabecular bone loss. Indeed, it has been shown that estrogens are able to modulate EC levels in rats as well as CB2 expression in both rats and humans [22, 23]. As discussed in the context of CB1, more recent studies indicate that the skeletal effect of CB2 is not only age but also strain dependent. Sophocleous et al. [24] reported that female CB2+/− mice on a CD1 background show a phenotype different from the C57Bl/6J mice, namely, HBM with low turnover at a young age, with no difference in aged mice. In this strain, males were not affected by CB2 knockout. In a subsequent study, transcriptomics data suggested that CB2 deficiency affects a large number of genes differentially in CD1 and C57Bl/6 mice, and these differences may explain the discrepancies between the strains in the CB2+/− skeletal phenotype [25]. Moreover, a recent study on an unspecified substrain of C57Bl/6 showed a HBM phenotype [26] in contrast to the original C57Bl/6 strain [24] but in line with the CD1 strain [24, 25]. There are many substra ins of C57Bl/6 mice that, due to random genetic drift, carry mutations that may affect the response to CB2 deletion. Such an example is the genetic drift that distinguishes C57Bl/6JolaHsd from C57Bl/6JrccHsd [27] or between the C57Bl/6J Kun and THE C57Bl/6J founder line [28]. Because the differences between the studies are not fully understood and the mice substrains are not mentioned in some of the studies, it is important in future studies to explicitly define the animal strain and use littermate controls rather than age-matched WT mice.

The exact mechanism of action of CB2 signaling on bone resorption and formation remains to be elucidated. The increased turnover rate observed in C57Bl/6J CB2+/− old mice indicates that CB2 signaling suppresses both bone resorption and formation in trabecular bone [12]. In the cortical bone, however, HU-308, a CB2 agonist, has been shown to stimulate endosteal bone formation [12], suggesting that CB2 has opposite roles in the trabecular and cortical bone compartments. This compartment-specific effect may be attributed to the contribution of NO release by non-skeletal cells responding to CB2 agonists in the trabecular bone (Figure 4). Over the years, many reports reached contradicting conclusions concerning the direct effect of CB2 signaling in osteoclasts in both murine and human-derived cultures. Although some of the studies suggest that CB2 stimulates osteoclast differentiation [2, 17], others showed evidence to support the notion that CB2 signaling inhibits osteoclastogenesis in both murine and human cells [12, 23, 29]. Although the jury is still out on this question, the results of these other studies are more in line with the consensual in vivo increased bone resorption in CB2-deficient mice.

The effect on trabecular bone formation in vivo seems to be mediated by non-osteoblastic cells as several studies demonstrated that CB2 signaling in isolated osteoblasts stimulates proliferation, differentiation and osteogenic activity [10, 12, 18, 21]. A plausible non-direct effect may be mediated via the increased/enhanced nitric oxide (NO) synthase activity and NO release from monocytes and mast cells in response to ECs [30]. NO inhibits bone formation and resorption [31], and this pathway therefore counteracts and supersedes the direct stimulation of osteoblasts by CB2 but further adds to the direct inhibition of osteoclasts by CB2. This may explain the high turnover bone loss observed in CB2−/− animals, in whom bone resorption increases to a greater extent than bone formation [12] (Figure 4).

Although the exact mechanism remains vague, it is becoming well established that CB2 in humans (CNR2) is strongly associated with bone mineral density in human populations [32–35]. The gene polymorphism that is significantly associated with the more severe form of osteoporosis is a non-conservative missense mutation that affects CNR2 expression and activity (Gln63Arg [36]). These human observations are in line with the mouse data demonstrating the bone protective actions of CB2 (discussed above).
It is interesting to note that the CB2-deficient animals had a significantly longer distal femoral metaphysis and also an increased length of vertebral bodies. In fact, the effect of CB2 deletion was much more pronounced than the effect of CB1 deletion (Figure 2) [11]. This suggests that the main physiologic involvement of CB2 is associated with a “CB2 tone,” regulating bone elongation in young growing animals and bone mass in the ageing skeleton [11, 12]. These two skeletal effects are the only reported phenotypes of naive CB2 mutant mice.

**Experimental inconsistencies related to the use of synthetic cannabinoids**

Cannabinoid research often employs synthetic cannabinoids that vary in their pharmacologic activity and are intended to mimic or antagonize the EC receptors in animals and cells. The use of such compound had often yielded contradictory results in different laboratories across the globe. For instance, AM630, defined as an antagonist/inverse agonist of CB2, has been shown to both stimulate [37] and inhibit [2, 17] osteoclastogenesis in vitro. HU308, a selective agonist of CB2, has also been shown to both stimulate [2] and inhibit [12] osteoclast formation. The reasons for these inconsistencies could be related to species differences, off target effects of CB receptor ligands at higher or lower concentrations [38], and/or endogenous cannabinoid activity in the serum used in cell cultures [39]. Another plausible reason for some of the inconsistencies is that some of the CB receptor ligands, thought to be specific for CB1 or/and CB2, are now reported to affect GPR55 (G protein-coupled receptor 55) receptor signaling in the bone [40–42]. Experimental results obtained with synthetic compound should therefore be considered with caution, and the exact pharmacologic activity, including...
the exact dose range, should be carefully defined before reaching biological conclusions. In this review, we based our conclusions on transgenic cells and animals, whenever possible.

**CB endogenous agonists**

Only a dearth of pharmacological studies dealt with the ECs and CB2, and the reported binding affinities are rather low, i.e. one order of magnitude lower than that of THC [43, 44]. Furthermore, published results from the laboratory of Tam et al. [10] and from other authors [45] suggest that anandamide and 2-AG, which are perceived as non-selective agonists of CB1 and CB2, may have opposing effects on CB2 signaling. Although 2-AG acts as a CB1 agonist in the sympathetic nerve terminals following a single or chronic administration to mice [10], it does not stimulate osteoblasts as HU308 (a selective CB2 agonist) does and may even act as a CB2 inverse agonist [9, 10]. Anandamide is considered a partial agonist for both CB1 and CB2, although it is a more potent agonist of CB1 than of CB2 [46].

Recent studies reported endogenous peptide ligands that bind to CB1 and activate or inhibit this receptor. These are segments of the hemoglobin protein and are present in the serum and brain, although the question of their physiologic function is still unclear [47]. These peptides are called pepcans, and because of their lipophobicity, the binding to the cannabinoid receptor is not likely to be the same as for the lipophilic agonists and is rather likely to occur via an extramembraneous allosteric domain. To date, the existence of an endogenous peptide that regulates CB2 activity has not been reported.

**Other receptors activated by cannabinoids in bone cells**

GPR55, a receptor that is known to be activated by ECs and synthetic cannabinoid ligands, is also expressed in osteoblasts and osteoclasts, although at lower levels as compared to the brain, adrenal and gut [40–42, 48]. Some reports show the stimulatory effect of GPR55 agonists on human and mice osteoclast function in vitro, consistent with in vivo evidence of decreased bone resorption in GPR55−/− mice [49]. This receptor may not be a specific cannabinoid receptor as it can also be activated by non-cannabinoid molecules such as l-α-lysophosphatidylinositol [50]. GPR55 is also involved in the stimulatory effect of cannabidiol on mesenchymal stem cell migration [51].

EC ligands can bind to other non-cannabinoid receptors such as calcium channels, nicotinic acetylcholine receptors, voltage-gated potassium channels and transient receptor potential vanilloid (TRPV) channels [38]. Although the physiological significance of TRPV channels in the skeleton is still unclear, studies show that genetic and pharmacological ablation of TRPV1 (transient receptor potential cation channel subfamily V member 1) signaling attenuates osteoclast activity in vitro and prevents O VX-induced bone loss in vivo [52]. Interestingly, CB2 expression on osteoclasts is increased in TRPV1−/− mice [52], which supports the aforementioned inhibitory effect of CB2 signaling in osteoclasts [12].

**Summary and therapeutic potential of cannabinoids in skeletal disorders and healing**

There are today no cannabinoid compounds approved for the treatment of bone disorders. However, the important skeletal actions of the EC system (Table 1) have prompted both preclinical researchers and industrial corporations to explore the clinical potential of therapies based on cannabinoids in the management of osteoporosis and other skeletal diseases. These approaches are targeting both CB1 and CB2 receptors, and are generally restricted to the peripheral nervous system and bone cells. This emphasizes the relevance of developing medicines that selectively modulate cannabinoid receptors located outside the blood-brain barrier, thus preventing any unwanted psychogenic side effects. Based on experimental observations in mice, agonist activation of either CB1 or CB2 or both may have bone protective functions via the receptors on peripheral nerve terminals, osteoblasts, osteoclasts and others. Although validation in humans is warranted, there is growing evidence that presynaptic CB1 activation can alleviate the tonic inhibition of the sympathetic nervous system on bone-forming osteoblasts via a receptor located outside the brain, on the sympathetic nerve terminals. CB1 expressed on bone cells may also play a role in the modulation of bone remodeling. CB2 activation, shown in mice to stimulate osteoblastic bone formation and inhibit bone resorption, has been shown in human studies to protect from osteoporosis. Taken together, pharmaceutical modulation of the EC system may be a valid approach in the prevention and treatment of age-related and steroid
deficiency-related osteoporosis. Preclinical studies also portray this system as a valid target for the therapeutic management of skeletal growth. Genetic tests aimed at detecting polymorphisms in CNR2 may serve as diagnostic measures to identify osteoporosis-susceptible individuals.

Synthetic agonists for CB2 such as HU-308 and HU-433 have shown to prevent osteoporosis in vivo and increase bone mass in sex hormone-deficient mice [12, 53]. These agonists are not psychoactive and therefore constitute a good therapeutic candidate in transitional studies that aim to validate the clinical use of CB2 agonists in osteoporosis treatment.

Similarly to CB endogenous agonists, oleoyl serine (OS) is an endogenous compound found in bones that belongs to the fatty acid amide family. Its role in bone remodeling consists of increasing bone mass by up-regulating bone formation and inhibiting bone resorption in mice [54]. However, the receptor of OS has not been identified and differences in cellular signaling indicate that CB2 is not the putative OS receptor [18, 54]. OS does not therefore belong to the skeletal EC system.

Cannabidiol (CBD) is another active component of cannabis (second to THC), with no psychoactive properties, and most reports agree that its actions are not mediated by the classical CB1 and CB2 due to its very low affinity to these receptors. Like OS, CBD is therefore not considered as an activator of the EC system. Notwithstanding, a recent article attributed a beneficial effect to CBD in bone fracture healing [55]. Interestingly, CBD did not increase the volume of mineral content of the fracture callus but still significantly improved its mechanical properties. This effect was associated with increased expression of the collagen cross-linking enzyme procollagen-lysine 2-oxoglutarate 5-dioxygenase 1 in osteoblasts and enhanced collagen maturation. Some of these effects were observed in THC-treated animals, although to a lesser extent than CBD. Whether only CBD or also the EC agonists stimulate bone healing remains to be elucidated.

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