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Mechanical and material properties of cortical and trabecular bone from cannabinoid receptor-1-null (*Cnr1*$^{-/-}$) mice

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ABSTRACT

The endocannabinoid system is known for its regulatory effects on bone metabolism through the cannabinoid receptors, *Cnr1* and *Cnr2*. In this study we analysed the mechanical and material properties of long bones from *Cnr1*$^{-/-}$ mice on a C57BL/6 background. Tibiae and femora from 5- and 12-week-old mice were subjected to three-point bending to measure bending stiffness and yield strength. Elastic modulus, density and mineral content were measured in the diaphysis. Second moment of area (MOA_2), inner and outer perimeters of the cortical shaft and trabecular fractional bone volume *(BV/TV)* were measured using micro-CT. In *Cnr1*$^{-/-}$ males and females at both ages the bending stiffness was reduced due to a smaller MOA_2. Bone from *Cnr1*$^{-/-}$ females had a greater modulus than wild-type controls, although no differences were observed in males. *BV/TV* of 12-week-old *Cnr1*$^{-/-}$ females was greater than controls, although no difference was seen at 5-weeks. On the contrary, *Cnr1*$^{-/-}$ males had the same *BV/TV* as controls at 12-weeks while they had significantly lower values at 5-weeks. This study shows that deleting *Cnr1* decreases the amount of cortical bone in both males and females at 12-weeks, but increases the amount of trabecular bone only in females.

1. Introduction

The strength of a bone is determined by the material properties of the matrix and the shape of the bone. These, in turn, are determined by a cellular process of modelling and remodelling involving matrix resorption and formation; osteoclasts resorb bone while osteoblasts lay down new bone [1]. The balance between formation and resorption determines the overall amount of bone, with osteoporosis resulting from resorption outweighing formation during remodelling [2] and high bone mass disorders when formation exceeds resorption, either because of increased anabolic activity [3] or defective osteoclastic resorption as in osteopetrosis [4,5] due to genetic abnormalities. These processes are governed centrally, e.g. via leptin and the hypothalamus [6], via the autonomic nervous system [7,8], as well as by local signals such as mechanical loads. Central and local regulation are coordinated to ensure that gross imbalances do not occur in bone deposition or resorption at different sites in the body.

Among many factors now identified, recent studies have uncovered a role for cannabinoid signalling in the regulation of bone [9–14]. The endogenous cannabinoid (endocannabinoid) system is widely studied for its regulatory effects on numerous physiological functions, including appetite, pain sensitivity and immune function [15–18]. In addition, it is being increasingly recognised as having a complex regulatory role in bone metabolism [10,19–21]. There are two classical cannabinoid receptors, *Cnr1* and *Cnr2* and these belong to the family of G-protein coupled receptors that, when activated, inhibit adenyl cyclase activity, and activate the MAPK signalling cascade [22]. *Cnr1* is expressed ubiquitously throughout the brain [23] and also on immune cells, in vascular tissue and adipocytes [16,24]. *Cnr2*, on the other hand, is predominately located in peripheral immune tissue such as macrophages [24,25]. Both *Cnr1* and *Cnr2* have been reported in osteoblasts and osteoclasts [11,13].

Several studies have indicated a role for the cannabinoid receptor *Cnr1* in bone metabolism [9–11,14,19,21,26] but, in trabecular bone, the phenotype resulting from deleting *Cnr1* in mice has been found to depend on mouse strain and sex. On a CD1 background, *Cnr1*$^{-/-}$ male mice exhibited a high trabecular bone mass, while the females had normal trabecular bone with slight cortical expansion [11]. Another group suggested that females also had a high bone mass and loss of *Cnr1* protected against ovariectomy-induced bone loss [10]. They later extended this to show that trabecular

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bone volume fraction, *BV/TV,* was significantly greater at 3 months of age in both male and female *Cnr1*$^{-/-}$ mice compared with wild-type animals, although it had fallen to become significantly lower by 12 months [26]. Different results were reported from mice on a C57BL/6 background; both male and female *Cnr1*$^{-/-}$ mice at 9–12 weeks of age exhibited a low bone mass phenotype, accompanied by an increase in osteoclast number and a reduction in bone formation rate [11].

The effects of *Cnr1* deletion on bone properties have mostly been investigated within the trabecular compartment. This is commonly done because the larger surface area of trabeculae results in a higher turnover rate and greater sensitivity to manipulation. It does not, however, reflect fully the range of bone properties. Bones can balance the quantity and quality of bone matrix and factors regulating bone properties could feasibly affect either or both of these; for instance, a weaker matrix may be compensated by increased geometrical properties. The purpose of this paper is to characterize cortical and trabecular bone from the tibia and femur of *Cnr1*$^{-/-}$ mice on a C57BL/6 background to address the discrepancies found in previous studies and extend our knowledge of bone regulation by *Cnr1*.

2. Materials and methods

2.1. Animals

C57BL/6 *Cnr1*$^{-/-}$ mice were available from a previous study in which they were generated by homologous recombination, as described previously [27]. For studying the effect of knocking out *Cnr1* on the mechanical, material and geometrical properties of bone, 5 and 12-week-old male (5 Wild-type (WT), 7 knock-outs (KO) and 5 WT, 4 KO) and female mice (5 WT, 6 KO and 5 WT, 9 KO) were euthanized and the hind limbs cleaned and stored in phosphate-buffered saline (PBS) at −20 °C until measurements were made. A series of mechanical and material measurements were made on all the bones, carefully ordered so that each bone was kept as intact as possible for as long as possible [28].

2.2. Mechanical properties

The lengths of the tibiae and femora were measured using a Mitutoyo digital micrometre (Mitutoyo, Kanagawa, Japan). The longest tibia and femur were selected from each animal and the mechanical properties measured by subjecting each bone to 3-point bending using an Instron 5564 materials testing machine (Instron, High Wycombe) fitted with a 2 kN load cell. The cross-head speed was set to descend at 1.00 mm min^{-1} and the span between the supports was adjusted to 9.93 mm to accommodate the shortest bone. The tibia was positioned with the fibula insertion pointing upwards and femur was loaded with the anterior surface upwards. The bending stiffness, failure load and work to fracture were calculated from the load-displacement graph as described previously [29,30] and summarized in the supplementary information. In brief, the bending stiffness was calculated from the maximum slope of the load-displacement curve, failure load as the maximum load supported by the bone and fracture as the point at which the load decreased suddenly; work to fracture being the area under the curve to this point.

2.3. Material properties

The modulus of the mineralized bone matrix was determined from the ultrasonic speed of sound and the Archimedean density. A Panametrics Pulser Receiver model 5052PR (Panametrics Inc, Waltham, MA, USA) with a V211BA piezoelectric transducer were used to generate pulses of 10 MHz ultrasound. A slice of cortical bone, ~1.5 mm thick, cut from the knee-end of the diaphysis of each bone, was placed on the wear-plate of the transducer, with a drop of conductivity gel to ensure acoustic coupling, and the transit time, t, of a pulse measured in pulse-echo mode using a dual beam oscilloscope (Hitachi V-665A, Tokyo, Japan) and the oscilloscope's internal callipers. The thickness of the slice, d, was measured using an electronic micrometre screw-gauge (Mitutoyo, Kanagawa, Japan). The longitudinal sonic plesio-velocity, v, was calculated from $2d/t$. The path length of sound in an inhomogeneous medium such as bone is unknown and will generally be longer than the specimen. Thus the calculated longitudinal velocity will be an underestimate and hence it has been termed the sonic plesio-velocity [31]. The density of the cortical bone was measured using Archimedes' principle by weighing the remaining diaphysis in distilled water, W_f, and in air, W_a [28]. Care was taken to ensure that water filled the medullary canal prior to weighing in water and all water was removed prior to weighing in air. Each measurement was performed three times and the density, ρ, calculated from $\rho_f W_a / (W_a - W_f)$, where ρ_f is the density of the water. The elastic modulus, E, was then calculated from $E = \rho v^2$. The mineral content of the bone matrix, expressed as a fraction of the dry weight, was determined by ashing the dried bones at 600 °C for 24 h [32].

2.4. Micro-computed tomography

A Skyscan 1072 x-ray Microtomograph (Skyscan, Aartselaar, Belgium) was used to obtain images of the proximal tibia and distal femur from each mouse. Voltage and current settings used were 50 kV and 197 μA and a 0.5 mm Al filter was inserted. Magnification was adjusted to x58 which gave a pixel size of 5 μm. The rotation step size between each image was 0.67 °. A 3D image was reconstructed from the image stack using NRecon version 1.4.4 (Skyscan) and the trabecular and cortical parameters measured using methods recommended by Skyscan [33]. A reference level was set at the level of the growth plate by scrolling down the image stack until the spots where the growth plate crossed the slice could be seen. Four spots were apparent in femora and two in tibiae. Following recommendations by Skyscan, an offset was determined as 50 slices from that reference level and the trabecular region was defined as the following 200 slices (Fig. 1) (using CTan version 1.7.0.2 software (Skyscan)). A region of interest (ROI) was drawn on the first slice to segment cortical and trabecular bone and 7–8 more drawn at regular intervals throughout the stack to ensure that in each slice this segmentation was maintained. Images were thresholded by adjusting the grey level to separate bone, shown by pixels with high grey levels, from non-bone until most of the image noise (speckle) was removed. The threshold grey level finally used in all images was 74. Measures of fractional bone volume (*BV/TV*), trabecular number (*Tb.N*), trabecular spacing (*Tb.Sp*) and trabecular thickness (*Tb.Th*) were calculated by the software. Volumetric Bone Mineral Density (BMD_v) values were calculated using calibration phantoms from primary micro-CT measurements from trabecular and cortical bone. The percentage porosity of cortical bone (5-week old mice) was calculated by measuring the difference between calculated bone volumes at high threshold values (160 for femora and 170 for tibiae) to enable identification of pores and low threshold values as above to identify total cortical bone area.

Micro-CT images were also used to measure the geometrical properties of each diaphysis from the lowest complete slice closest to the centre of the shaft of the bone (Fig. 1). A binary image representing this slice was imported to Image-J (version 1.42q) where a custom-written macro was used to compute the axial second moment of area, cross-sectional area, and the outer and inner

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