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Behavioural changes induced by a conditional disruption of bone formation

Introduction

Numerous epidemiological studies have revealed a significant comorbidity between major depression and osteoporosis. Yet, the interrelationship between these highly prevalent disorders has not been sufficiently clarified. A meta-analysis of 23 studies [1] comparing bone mineral density (BMD) in 2327 depressed and 21,141 non-depressed individuals revealed a strong correlation between reduced BMD and depression. This correlation has been interpreted as an indication that depression may be a risk factor for osteoporosis.

This hypothesis is in line with the notion that bone remodelling, a lifelong balanced process of bone resorption and formation, is modulated by the central nervous system through sympathetic and parasympathetic projections [2]. Norepinephrine, released from sympathetic nerve fibres in the skeleton, tonically restrains bone resorption and stimulates bone formation [3, 4]. Decreased skeletal sympathetic activity thus results in increased bone formation, a condition observed after traumatic brain injury [4], whereas increased skeletal sympathetic activity, which is observed in animal models of depression [5], leads to a low bone mass. Importantly, norepinephrine release from bone sympathetic terminals is inhibited by the activation of cannabinoid CB1 receptors through endocannabinoids produced by closely apposed osteoblasts [6, 7]. Parasympathetic nerve fibres also innervate bone. Acetylcholine released from these fibres activates nicotinic receptors on osteoclasts and thus stimulates bone mass accrual. Parasympathetic activity thus functionally antagonises the skeletal sympathetic tone.

It is less clear if and how bone affects brain functions. However, considering the important role of the skeleton in homeostasis and whole-organism physiology [8], this possibility deserves further exploration. Here we examine behavioural changes in a transgenic mouse model of osteoporosis, where osteoblasts express a herpes simplex virus thymidine kinase (TK). These cells can be selectively ablated with the antiviral compound aciclovir. This mouse model can thus be used to examine central nervous system effects caused by a disruption of bone formation.
Materials and methods

Animals

Experimental procedures complied with all regulations for animal experimentation in Germany and were approved by the Landesamt für Natur, Umwelt und Verbraucherschutz in Nordrhein-Westfalen, Germany (AZ: 84-02.04.2012.A146). Transgenic mice were generated by microinjection of a TK expression construct under control of the bone gamma-carboxyglutamate protein (Bglap, osteocalcin) promoter (Bglap-TK) into fertilised mouse eggs (C57BL/6 X DBA). The construct consisted of an 8 kb Bglap promoter fragment (amplified from a mouse BAC RF23-385K10 with the following primer sequences: GGACT TGCT TGTTT GCC ACC CTCCA GGTGC CAGTA GCATT TATAT CGCCT GCGGC CGGCC CTCCC TGTCC TGGAG AGGAC AAAT and GACAG TGGGA CAAA ATCTA AGAGA GAGAA GGGCT TTTTT TGGGG GTGGG GTGGA CTCAC AGGTT GCTCG TAGAA GTGAT GGCGC CGCGC TCTCC TGAGT AGGAC AAAT and GACAG TGGGA CAAA ATCTA AGAGA GAGAA GGGCT TTTTT TGGGG GTGGG GTGGA CTCAC AGGTT GCTCG TAGAA GTGAT GGCGC CGCGC TCTCC TGAGT AGGAC AAAT and GACAG TGGGA CAAA ATCTA AGAGA GAGAA GGGCT TTTTT TGGGG GTGGG GTGGA CTCAC AGGTT GCTCG TAGAA GTGAT). Hemizygous Bglap-TK transgenic mice and wild-type (Wt) litters were obtained from hemizygous breedings on a C57BL/6J genetic background. Transgenic animals and Wt controls were injected daily with 10 mg/kg aciclovir (9-(2-hydroxyethoxymethyl)-guanine) between postnatal days 29 and 100.

Micro-computed tomography (μCT)

The structure of whole femora fixed with 4% phosphate buffered formalin and stored in 70% ethanol was examined as described previously by a desktop μCT system (μCT 40, Scanco Medical AG, Bassersdorf, Switzerland) [5]. The specimen was scanned at a resolution of 20 μm in all three spatial dimensions. Two-dimensional CT images were reconstructed in 512 x 512 pixel matrices using a standard convolution-backprojection procedure with a Shepp and Logan filter. The mineralised tissues were differentially segmented by a global thresholding procedure. Morphometric parameters were determined using a direct 3D approach. Trabecular bone parameters were measured in the secondary spongiosa in a metaphyseal segment, 25% of the whole femoral length, extending proximally from the proximal tip of the primary spongiosa.

Histomorphometry and immunohistochemistry

After μCT image acquisition, the specimens were dehydrated in progressively increasing concentrations of ethanol, cleared in xylene, and embedded undecalcified in polymethylmethacrylate (Technovit 9100, Heraeus Kulzer, Wehrheim, Germany). Undecalcified, longitudinal 5-μm sections through the mid-frontal plane were left unstained for dynamic histomorphometric measurements. To identify osteoclasts, consecutive sections were deplasticised and stained for tartrate-resistant acid phosphatase and counterstained with Mayer’s haematoxylin. Histomorphometric analysis was carried out on digital photomicrographic images using Image-Pro Express Version 4.0.1 image analysis software (Media Cybernetics, Silver Spring, MD, USA). Dynamic histomorphometric parameters and osteoclast number (Oc.N/BS) were determined according to the convention of standardised nomenclature. Immunohistochemistry was performed using paraffin-embedded decalcified sections according to a standard protocol with a polyclonal first antibody (Cayman Chemical, Alexis, Nottingham, UK).

Behavioural studies

Home-cage activity was recorded using an infrared system (Mouse-E-Motion, Infra-e-motion, Henstedt-Ulzburg, Germany). Mice were housed single-caged under a reversed light/dark cycle (light phase for 12 h, light on at 9:00 pm), while movements were sampled every second and averaged over 30 min. In the Porsolt forced swim test, mice were placed individually into a glass cylinder (height 28 cm, diameter 20 cm) containing water (height 16 cm, 24–25 °C). Immobility time was recorded during the last 4 min of the 6 min testing period. For the social preference test, mice were first habituated to a transparent open-field box (44 cm x 44 cm) in three consecutive days for 5 min. On the testing day, an empty cage and a cage containing an unfamiliar mouse of the same gender and age were placed in the open field box. The test animals were introduced and their location was recorded for 10 min using an automatic video tracking system (EthoVision XT, Noldus, Wageningen, The Netherlands). The time spent investigating the partner mouse compared to the empty cage was calculated.

Results

Bglap-TK animals and Wt controls were treated with aciclovir daily over a 10-week period, starting 4 weeks after birth (Figure 1A). We did not observe any readily apparent adverse health effects or behavioural differences between the two genotypes during or after the treatment. Thus, the body weight, which was measured twice weekly, increased similarly during the aciclovir injection in both genotypes (Figure 1B). The activity of the animals in their home cage was also similar, and both genotypes displayed normal circadian activity patterns (Figure 1C). Together these data indicate that the treatment was well tolerated by the mice.

Analysis of the bone formation rate in the distal femoral metaphysis using vital fluorescent calcein staining after 4 weeks of acyclovir treatment revealed a 20% reduction in Bglap-TK animals compared to Wt mice (Figure 2A). This was not due to a decreased activity of osteoblasts, as the mineral appositional rate, a surrogate marker of osteoblast activity, was similar in both genotypes (Wt: 1.04±0.036 μm/day; Bglap-TK: 0.99±0.055 μm/day; p=0.452). However, the number of osteoblasts was significantly reduced in the Bglap-TK group as indicated by the lower mineralising perimeter, a surrogate marker of osteoblast number (Figure 2B). Interestingly, we also found an increased number of osteoclasts in Bglap-TK
Figure 1: Experimental design, body weight and home cage activity. (A) Schematic representation of the experimental design. Mice were treated daily with aciclovir from week 4 through week 14. Behavioural tests were performed at weeks 8, 9 and 10, followed by the analysis of bone structure and adrenal glands. (B) Bglap-TK and Wt mice showed a similar increase in body weight during the period of aciclovir injection. (C) Bglap-TK mice and Wt controls showed a similar home cage activity with typical circadian activity patterns.

Figure 2: Disruption of bone formation in Bglap-TK mice after aciclovir treatment. (A) Bone formation rate and (B) the mineralising perimeter were significantly reduced in Bglap-TK animals. (C) Bglap-TK mice showed an increased number of osteoclasts and (D) a significantly reduced bone volume density.

animals (Figure 2C), indicative of a concomitant increase in bone resorption. Accordingly, we observed a strikingly decreased trabecular bone volume density (BV/TV) (Figure 2D), with no change in cortical thickness (Wt: 0.184±0.004; Bglap-TK: 0.193±0.003). These results indicate that the aciclovir treatment disrupted bone formation through an ablation of osteoblasts in transgenic animals.

We next investigated behaviours that are relevant to the emotional condition of the animals. In the social preference test, both genotypes showed a clear preference for the conspecific over the empty cage and a similar overall exploratory activity (Figure 3A). However, in the Porsolt forced swim test, which has an excellent predictive validity for antidepressant drugs and is thought to assess a form of behavioural despair, Bglap-TK animals showed a clearly increased immobility time (Figure 3B). This increased immobility is often used as an indicator for an increased depression-like state. Interestingly, Bglap-TK animals also displayed an increased ratio of adrenal gland weight to body weight, indicative of a chronically elevated corticosterone production (Figure 3C).

Discussion

In this paper, we demonstrate that the disruption of bone formation through the ablation of osteoblasts resulted in behavioural and physiological changes that are relevant to depression. Thus, mice expressing an HSVTK gene in osteoblasts showed a striking loss of bone mass after 10 weeks of aciclovir administration in comparison to Wt controls treated identically. These animals showed a normal social behaviour and normal circadian activity,
but also increased despair behaviour. These findings indicate that a disruption of bone remodelling affects brain functions.

The conditional ablation of osteoblasts described here is a powerful method to selectively disrupt bone formation. A similar approach to disrupt bone physiology has previously been used in two different transgenic mouse strains, expressing the HSV-TK gene under the control of the Bglap2 and Col2.3 promotors, respectively [9, 10]. Bglap2-TK mice also showed a reduced bone density after 4 weeks of ganciclovir (2-amino-9-(1,3-dihydroxypropan-2-yloxymethyl)-guanine) treatment due to an ablation of osteoblasts, whereas osteoclasts were not affected. It should be pointed out that the effects of ganciclovir administration to Bglap2-TK seemed to be more severe than those reported here with aciclovir in Bglap-TK animals, because Bglap2-TK showed almost a complete absence of osteoblasts and bone formation, whereas we observed only a reduced bone formation rate. Also, Bglap2-TK mice showed an arrest of skeletal growth and a development of kyphosis after ganciclovir administration, which resulted in a striking growth difference to control animals. This was not the case in our study. Indeed, the Bglap-TK mice and Wt controls showed a similar increase in body weight during the aciclovir administration. Interestingly, we also found an increased number of osteoclasts in Bglap-TK mice, whereas osteoclasts function was not altered in Bglap2-TK mice. These differences are either related to differences in Bgalp and Baglap2 promotor activities, or to pharmacological differences between aciclovir and ganciclovir, or both. Common to both strains was a severe loss of bone mass.

The conditional disruption of bone formation in Bglap-TK mice did not interfere with basic behaviours such as circadian home cage activity, exploration of a novel environment or interactions with conspecifics. This further supports the notion that the health or general well-being of the animals was not severely compromised. Nevertheless, we observed increased adrenal gland weights, which is indicative of a chronic activation of the hypothalamic-pituitary-adrenal stress axis [11]. This is in agreement with the observed increased behavioural despair in the Porsolt forced swim test, which is widely used to investigate stress-related behavioural responses and antidepressant drug effects [12–15]. Such increased despair behaviour is often observed in mouse models of depression. Stress activates hypothalamic paraventricular neurons through synaptic inputs from central and peripheral stress-sensing neurons, thus triggering the release of corticotropin-releasing hormone and subsequently the release of adrenocorticotropic hormone from the pituitary and glucocorticoids from the adrenal cortex. Activation of this hypothalamic-pituitary-adrenal (HPA) axis is an important adaptation to stress, but its chronic activation can lead to severe clinical conditions, including psychiatric disorders. Thus, persistent elevation of cortisol levels through chronic activation HPA axis has been associated with major depression in humans.

Together these findings demonstrate that a disruption of bone formation can affect behaviours that are relevant to depression in the absence of any overt evidence of a general sickness. This finding warrants further studies to elucidate the precise endocrine and/or neuronal mechanisms by which the skeleton signals to the brain.

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