Brief Communication

MOLECULAR METABOLISM

Modulation of cognition and anxiety-like behavior by bone remodeling

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ABSTRACT

Objective: That the bone-derived hormone osteocalcin is necessary to promote normal brain development and function, along with its recently described sufficiency in reversing cognitive manifestations of aging, raises novel questions. One of these is to assess whether bone health, which deteriorates rapidly with aging, is a significant determinant of cognition and anxiety-like behavior.
Methods: To begin addressing this question, we used mice haploinsufficient for *Runx2*, the master gene of osteoblast differentiation and the main regulator of *Osteocalcin* expression. Control and *Runx2*+/− mice were evaluated for the expression of osteocalcin's target genes in the brain and for behavioral parameters, using two assays each for cognition and anxiety-like behavior.
Results: We found that adult *Runx2*+/− mice had defects in bone resorption, reduced circulating levels of bioactive osteocalcin, and reduced expression of osteocalcin's target genes in the brain. Consequently, they had significant impairment in cognitive function and increased anxiety-like behavior.
Conclusions: These results indicate that bone remodeling is a determinant of brain function.

1. INTRODUCTION

Osteocalcin is a bone-derived hormone that regulates a growing number of physiological functions. In particular, although *Osteocalcin* is not expressed in any part of the WT brain before or after birth [1], *Osteocalcin*−/− mice have increased anxiety-like behavior and depression, decreased exploratory behavior, and impaired learning and memory when compared to WT littermates [1]. Remarkably, these phenotypic abnormalities are already severe in young, i.e. 3-month-old, *Osteocalcin*−/− mice. Osteocalcin is secreted into the general circulation, crosses the blood brain barrier, and binds to discrete parts of the brain including neurons in the brainstem, ventral tegmental area (VTA), and hippocampus, where it signals through Gpr158, a recently-identified second receptor for this hormone [2]. In the brain, osteocalcin promotes the synthesis of monoamine neurotransmitters (i.e., serotonin, dopamine, and norepinephrine) and inhibits synthesis of GABA [1] by regulating the expression of key enzymes implicated in the synthesis of these various neurotransmitters. Together, these properties of osteocalcin contribute, at least in part, to its beneficial influence on cognition and anxiety.
The importance of osteocalcin's regulation of brain function was further underscored recently when it was shown that the presence of osteocalcin is necessary for the ability of plasma from young mice to improve cognition in older mice [2]. Accordingly, the systemic delivery of osteocalcin was shown to be sufficient to reverse age-related cognitive decline in 16-month-old mice [2]. In view of these findings and given that osteocalcin is produced only by osteoblasts, the cell type responsible for bone formation and osteoclast differentiation [3], we asked whether an impairment in osteoblast differentiation and function, as may occur in various skeletal dysplasias or with aging, could affect cognition or anxiety.
Remarkably, we note that cleidocranial dysplasia (CCD), a classical skeletal dysplasia in which cognitive functions are also affected, is an autosomal dominant disease caused by haploinsufficiency at the *Runx2* locus. The transcription factor Runx2 is the master regulator of osteoblast differentiation and the main regulator of *Osteocalcin* expression [4,5]. Since osteoblasts are responsible for the production of osteocalcin, this raises the question of whether decreased osteocalcin production could be the cause of the cognitive defects that were reported 60 years ago in certain, but not all, CCD patients [6]. To test this hypothesis, we used *Runx2*+/− mice, which phenocopy, at least in the skeleton, what is observed in *Runx2* haploinsufficient humans [4,7,8].

2. METHODS AND MATERIALS

2.1. Mice and treatment

Osteocalcin+/− and *Runx2*+/− mice have been previously described [4,9]. For all experiments, we used females and littermates as controls unless otherwise stated. *Osteocalcin*+/− mice were maintained on a pure 129-Sv/Ev genetic background and the *Runx2*+/− model was maintained on a C57BL/6J background. *Runx2*+/− mice were generated by breeding WT females with *Runx2*+/− male mice. Each group described is represented individually in each panel. Mice were housed two to five animals per cage (polycarbonate cages (35.5 × 18 × 12.5 cm)), under a 12 h light/dark cycle with ad libitum

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Received September 1, 2017 • Revision received September 28, 2017 • Accepted October 2, 2017 • Available online xxx

https://doi.org/10.1016/j.molmet.2017.10.001

access to food and water prior to experimentation, in a pathogen-free animal facility. All experiments involving animals were approved by the Institutional Animal Care and Use Committee of Columbia University Medical Center and with the European Communities Council Directive (2010/63/UE).

2.2. Behavioral studies

All animals of the same batch were born within an interval of 2 weeks and were kept in mixed genotype per group of 2–5 females in the same cage, at standard laboratory conditions (12 h dark/light cycle, constant room temperature and humidity, and standard lab chow and water ad libitum). For each test, the mice were transported a short distance from the holding mouse facility to the testing room in their home cages or in the transport boxes filled with bedding from their home cages. Behavioral testing of the mice was performed on a battery of functional tests between 3 and 5 months of age, and mouse weight was between 18 g and 28 g. The tests were performed by an experimentalist blind to the genotypes of the mice under study.

2.2.1. Elevated plus maze test (EPMT)

This test takes advantage of the aversion of rodents to open spaces. The EPM apparatus comprises two open and two enclosed arms, each with an open roof, elevated 60 cm from the floor [10,11]. Testing takes place in bright ambient light conditions. Animals are placed into the central area facing one closed arm and allowed to explore the EPM for 6 min. Time spent in open arms was recorded.

2.2.2. Light to dark transition test

This test is based on the innate aversion of rodents to brightly illuminated areas and on their spontaneous exploratory behavior in response to the stressor that light represents [12]. The test apparatus consists of a dark, safe compartment and an illuminated, aversive one. Mice were tested for 6 min, and time spent in the lit compartment was scored as an index of anxiety-related behavior as well as exploratory activity.

2.2.3. Morris water maze test

Animals are transported to the testing room in their home cages and left undisturbed for at least 30 min prior to the first trial. The maze is comprised of a large swimming pool (150 cm diameter) filled with water (23 C) made opaque with non-toxic white paint. The pool is located in a brightly lit room filled with visual cues, including geometric figures on the walls of the maze demarking the four fixed starting positions of the trials, at (12:00, 3:00, 6:00 and 9:00). A 15 cm round platform is hidden 1 cm beneath the surface of the water at a fixed position. Each daily trial block consisted of four swimming trials, with each mouse starting from the same randomly chosen starting position. The starting position is varied between days. On day 1, mice that fail to find the platform within 2 min are guided to the platform. They must remain on the platform for 15 s before they are returned to their home cage. Mice are not guided to the platform after day 1, and the time it takes them to reach the platform over repeated trials (4 trails/day for the next 10 days) is recorded as a measure of spatial learning.

2.2.4. Novel object recognition test (NOR)

The NOR paradigm assesses the rodent's ability to recognize a novel object in the environment. The NOR task is conducted, as previously described [13], in an opaque plastic box using 2 different objects: (1) a clear plastic funnel (diameter 8.5 cm, maximal height 8.5 cm) and (2) a black plastic box (9.5 cm^3). These objects elicit equal levels of exploration as determined in pilot experiments [1,14]. The NOR paradigm consists of 3 exposures over the course of 3 days. On day 1, the habituation phase, mice are given 5 min to explore the empty arena, without any objects. On day 2, the familiarization phase, mice are given 10 min to explore 2 identical objects, placed at opposite ends of the box. On day 3, the test phase, mice are given 15 min to explore 2 objects, one novel object and a copy of the object from the familiarization phase. The object that serves as the novel object (either the funnel or the box) as well as the left/right starting position of the objects is counterbalanced within each group. Mice are placed in the center of the arena at the start of each exposure. Between exposures, the objects and arenas are cleaned. Discrimination of the novel object is assessed by the following formula: ((time spent with novel object-time spent with old object/total exploration time)). Exploration of each object, as well as number of grid crossings, is scored from video recordings of each exposure and recorded using the Stopwatch program. Climbing or sitting adjancent to the object was not counted as exploration. An equal exploration time for the two objects, or a decreased percentage of time spent with the novel object compared to WT controls indicates impairment in hippocampal memory.

2.3. Hormonal measurement

Circulating levels of osteocalcin in mouse serum were determined with a specific ELISA previously described [15,16]. Total levels of osteocalcin and carboxylated osteocalcin were estimated using two different specific antibodies. Bioactive osteocalcin was determined by substracting the carboxylated osteocalcin levels from total osteocalcin levels [15,16].

2.4. Real-Time RNA transcript determination

All brain dissections were performed in ice-cold PBS 1× under a Leica MZ8 dissecting light microscope. The hypothalamus was removed from the midbrain during collection. All parts of the brain isolated were snap frozen in liquid nitrogen and kept at −80 C until use. No contusions were observed in any of the analyzed *Runx2*+/− brains. Tibia isolated for gene expression was centrifuged for 20 s at 16,100 × *g* to flush out the bone marrow. All isolated bones were snap frozen in liquid nitrogen and kept at −80 C until use.

RNA was isolated from brain tissue using TRIZOL (Invitrogen). cDNA synthesis was performed following standard protocol. Total RNA was first incubated with DNAse I for 30 min at room temperature to remove any genomic DNA. DNAse I-treated total RNA was converted to cDNA by using M-MLV reverse transcriptase (Thermo: 28025013) and random hexamers (Thermo: N8080127). qPCR was performed on a CFX96 Touch™ Real-Time PCR Detection System (BioRad), and analyses were done using specific quantitative PCR primers and expressed relative to *Gapdh* levels.

2.5. Biochemistry and molecular biology

For Western blotting, frozen hippocampi from adult mice were lysed and homogenized in 250 µl tissue lysis buffer (25 mM Tris HCl 7.5; 100 mM NaF; 10 mM $Na_4P_2O_7$; 10 mM EDTA; 1% NP 40). Proteins were transferred to nitrocellulose membranes, and blocked with TBST-5% BSA prior to overnight incubation with primary antibody in TBST–5%BSA. Antibodies: anti-Runx2: M-70 sc-10758, Santa Cruz, anti-BDNF: sc-546, Santa Cruz; anti-tubulin: T6199, Sigma. HRP-coupled secondary antibodies and ECL were used to visualize the signal.

2.6. Quantification and statistical analysis

All values are depicted as mean ± SEM. Statistical parameters including the exact value of n, post hoc test and statistical significance are reported in each figure and figure legend. The number of mice used for each experiment was estimated to be sufficient based on pilot

