Brain, bones and cognition: low-dose microCT in the search for treating Down syndrome

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Aims

Brain and bones: the integration of two developing systems. Brain malformations lead to cognitive impairment and are associated with malformation of other systems, such as the face. The brain and the face are integrated intimately, indeed, and this linked development has important implications for vertebrate and human evolution, as well as for clinical medicine[1-3]. Brain and craniofacial malformations co-occur in many congenital diseases, such as holoprosencephaly, cleft lip and palate, micro- and macrocephaly, Apert, Pfeiffer and Crouzon craniosynostosis syndromes and Down syndrome (DS). Although it is not known exactly how (mal-)formation of brain and face influence each other, it is clear that the interaction between common signaling pathways in neural and skeletal development underlie the tight link between the brain, the face and the skull[3,4]. It is likely that this close developmental relationship is not only restricted to these structures but also can be extended to other elements of the skeletal system, such as the bones of the appendicular skeleton. This integrated view of development is critical to understand diseases such as DS, with a complex and multisystemic phenotype involving cognitive, brain, craniofacial and skeletal malformations.

Down syndrome as a neuro-skeletal disorder. DS is a genetic condition caused by trisomy of chromosome 21, in which gene overexpression alters many systems and results in cognitive impairment, immunodeficiency, congenital heart diseases, skeletal and craniofacial dysmorphologies[5]. Cognitive impairment in DS is the result from altered neural development and can be directly related to brain malformations[6]. Reciprocally, the skull shape is significantly altered in DS, showing a typical shape with shorter, wider and rounder skulls and a flattened face[7]. Besides cranial and facial bones, long bones also show an altered pattern of skeletal development in individuals with DS, with reduced bone mineral density and abnormal bone remodeling and growth, which in adults results in osteopenia and osteoporosis[8]. Extensive research has identified genes and molecular mechanisms underlying the cognitive impairment associated with DS[6], but we know little about how trisomic genes affect bone maintenance and homeostasis. We still need to understand how the same signaling pathways are involved in both neurogenesis and skeletal development and how these interactions are altered in DS.

Integrated development, integrated treatment? Epigallocatechin-3-gallate (EGCG), a green tea flavonoid, is a strong candidate for pharmacotherapy in DS[8-13], possibly through normalization of the overexpression of *Dyrk1A*[9,11], a candidate gene for skeletal and brain co-development that lies in the DS critical region of chromosome 21[6,8,14-17]. Interestingly, the beneficial effects of EGCG may not be limited to neural and cognitive phenotypes, but may

extend to skeletal phenotypes[8,10,18-21] - although high doses of EGCG may have the contrary effect, suppressing bone formation and exacerbating skeletal defects[19,20].

Longitudinal evaluation of the integrated development of the skeleton, the skull and the brain. One of the main challenges to analyze the integration patterns between the skeleton and the brain is to follow their developmental trajectory on the same individuals, from birth to adulthood. Low-dose µCT is able to provide anatomical images from live, free-breathing animals in three dimensions with excellent resolution and contrast. The properties of µCT can be extended using diffusible iodine-based contrast-enhanced CT (diceCT), a staining technique that combines the assets of good soft tissue contrast for evaluation of brain anatomy with excellent visualization of bony structures versus soft and aerated tissues, and that is more cost-effective than MRI[22]. We propose non-invasive imaging to assess longitudinal changes in a preclinical study of the integration of skeletal, neural and cognitive development in DS and to explore the potential for EGCG treatment to ameliorate simultaneously neural and skeletal development from early in development into adulthood.

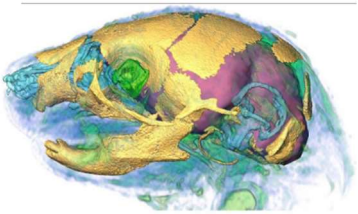

Figure 1: Superimposition of different scans to visualize the head of a P0 mouse and highlight the integration of the skull (yellow) and the brain (purple). From Martínez-Abadías et al (2013).

Method

We bred trisomic (Ts65Dn) mice and administered EGCG (30 mg/kg/ day) to half of the litters, starting when both the face and the brain start developing (embryonic day 9), and continued the treatment up to postnatal day 29. The mice were genotyped by quantitative PCR and distributed in four groups: euploid (WT) and trisomic (TS), treated and untreated. We followed the postnatal development of the skull and the brain by repeatedly scanning each mouse with both *in vivo* µCT (SkyScan1278) and MRI (9.4T, Bruker Biospin) at postnatal days (PD) 3 (infantile), 14 (juvenile) and 29 (early adult) with optimized scan settings per time point, and *ex vivo* diceCT after the last *in vivo* time point. We also assessed the psychomotor and neurobehavioral development of the mice. We segmented the MR images and estimated the volume of the whole brain, the four brain ventricles and the cerebellum. We also recorded 3D coordinates of landmarks located in these structures. From the µCT data, we estimated bone mineral density (BMD) in the humerus and performed volumetric and 3D morphometric analyses of the upper limb bones and skull to compare the skeletal development and skull morphometry among groups.

Results

EGCG treatment rescued the TS mice for some brain morphology and psychomotor measurements, such as normalized ventriculomegaly of the lateral ventricles and pivoting behavior. For the rest of measurements we analyzed, the EGCG treatment did not show any effect (i.e. volumes of the third and fourth ventricles, as well as blast response or tactile orientation) or even indicated a worsening of the DS phenotype, such as of walking behavior and bone development (Figure 2). The µCT scans showed that upon EGCG treatment, both euploid and trisomic mice presented a delay in bone growth, with overall lower BMD and obvious bone defects (Figure 2A-B). Statistical testing revealed that TS mice were smaller than WT and the EGCG significantly reduced skull size in both groups. The comparative analyses of skull shape revealed significant differences between trisomic and euploid mice that are evident from birth and increase with time (Figure 2C). In comparison to euploid mice, trisomic mice present flatter faces with more globular, brachycephalic skulls, which correspond

to the most common craniofacial traits in DS individuals. Unexpectedly, the EGCG treatment caused an exacerbation of the DS craniofacial traits, in both WT and TS mice. These severe effects suggest that over-inhibition of *Dyrk1A*-activity by EGCG treatment can have deleterious effects on bone development and growth. To look deeper into these effects, more volumetric and morphometric brain analyses, as well as systematic assessments of BMD on skull and long bones, are ongoing.

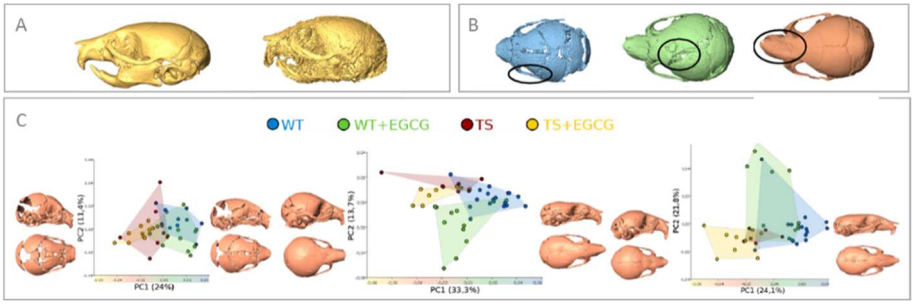

Figure 2: Skull shape and bone development assessment. A) Qualitative comparison showing lack of bone mineralization in EGCG treated mice. The same threshold was used to reconstruct the skulls, which reveals lower bone density in the EGCG treated case (right). B) Examples of abnormal skeletal development in EGCG treated mice. C) Skull morphometric analysis based on Principal Component analyses performed using the 3D coordinates of 27 anatomical landmarks recorded throughout the skulls of the same mice in the four groups of mice (WT, TS, WT+EGCG, TS+EGCG) at PD3 (left), PD14 (middle), PD29 (right). Convex hull shaded areas represent the range of variation of each group of mice, whereas 3D reconstructions depict the skull shape of groups lying at the extremes of variation associated with the first principal component, which explains the largest percentage of morphological variation.

Conclusion

Our results underscore the urgent need to test the effect of the EGCG at different concentrations with the aim to optimize the beneficial over the detrimental effects of this treatment. Our results clearly evidence that EGCG treatment can have pleiotropic effects and that at certain concentrations EGCG can have more negative than positive impact on bone, face and brain development. This is especially relevant when considering that in humans there is suggestive evidence that the EGCG treatment can have a positive impact on facial morphology and that many people are already taking green tea based treatments, which are freely commercially available. It is crucial to establish as soon as possible the efficiency and dose-effect of the treatment, aiming to identify the optimal EGCG dosage that provides maximal therapeutic effect while minimizing potential side effects.

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