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The Impact of Epigallocatechin-3-Gallate (EGCG) on Ts65Dn Down Syndrome Mouse Models

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ABSTRACT: Down syndrome (DS) is caused by the trisomy 21 genetic disorder, which produces a unique craniofacial phenotype. The purpose of this research is to better understand how Epigallocatechin-3-gallate (ECGC) influences the development of DS craniofacial phenotypes. Ts65Dn DS mouse models have been genetically modified to have 3 copies of numerous genes found on human chromosome 21, including *DYRK1A*, which plays a role in bone and brain development. EGCG is a known inhibitor of *Dyrk1a* activity. For this study, pregnant Ts65Dn mice were treated with 200 mg/kg of ECGC twice daily on days 7 and 8 of pregnancy. It was hypothesized that EGCG treatment will reduce *Dyrk1a* overexpression during development resulting in a measurable improvement in craniofacial morphology. To test this hypothesis, three mouse samples were analyzed: Ts65Dn, Ts65Dn + EGCG, and euploid. Skulls were imaged using high-resolution μCT at 6 weeks after birth. Anatomical landmark coordinates were measured from μCT images using Amira software. Euclidean Distance Matrix Analysis was used to assess craniofacial shape variation among samples. Results show that EGCG improves craniofacial morphology in treated Ts65Dn mice relative to untreated baselines, but improvements vary by region for the cranial vault, face, base, and mandible. Being able to understand how EGCG influences craniofacial development on a trisomic background is the first step in finding a way to improve phenotypic development to potentially avoid health issues of the craniofacial complex associated with DS. These results suggest that EGCG could be a useful therapeutic option.

KEYWORDS: EGCG; Dyrk1a; Ts65Dn; Down Syndrome; Trisomy 21

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INTRODUCTION

Trisomy 21 (Ts21), also known as Down syndrome (DS), is a genetic disorder where the body creates three functional copies of chromosomes 21 as opposed to the normal two. DS is associated with birth defects, intellectual disabilities, and characteristic facial features. Babies with DS are at increased risk of being born with heart defects, intestinal malformations, visual impairment, hearing loss, thyroid problems, and leukemia (Stanford Children's Health, 2018). Cognitive abilities such as recognitions, making specific connections, learning, memories, and reaction time are affected due to the gene-dosage imbalance resulting from Ts21.

DYRK1A (dual-specificity tyrosine-(Y)-phosphorylation-regulated kinase 1A) is a serine/threonine gene essential for brain development and function, located on chromosome 21 (Ogawa et al., 2010). This gene helps with signaling and correct protein function. Because a third chromosome is expressed, *DYRK1A* is overexpressed in individuals with DS, which leads to defective cortical pyramidal cell morphology, synaptic plasticity deficits, and altered excitation/inhibition balance (Ruiz-Mejias et al., 2016).

Dyrk1a has recently been connected to the regulation of bone development, homeostasis, and reabsorption (Blazek et al., 2010). Furthermore, *Dyrk1a* appears to have a crucial role during central nervous system development (CNS), via its regulation of multiple targets in both the nucleus and the cytoplasm (Stringer et al., 2017). The overexpression of *DYRK1A* is thought to strongly contribute to several of the facial characteristics and negative health problems that occur in individuals with DS.

EGCG (Epigallocatechin 3-gallate) is an antioxidant found in green tea and a natural inhibitor of *DYRK1A* activity (Ogawa et al., 2010). Previous trials with EGCG have shown improvement in the adaptive behavior and brain-related changes in young adults with DS because of EGCG's ability to cross the blood–brain barrier to inhibit *DYRK1A* (Wyganowska-Świątkowska et al., 2018).

Ts65Dn mice have been genetically modified to have 3 copies of numerous genes found on human chromosome 21, including *Dyrk1a*. As a model of human Ts21, Ts65Dn mice display a remarkably diverse array of DS-like phenotypes, including performance deficits in different behavioral tasks and alterations in synaptic plasticity and adult neurogenesis (Scott-McKean et al., 2010). Genetically, genes homologous to those found on human chromosome 21 are located on mouse chromosome 16 in Ts65Dn mice, with 55% of these genes being homologous to one another.

The objective of this investigation is to determine whether EGCG treatment produces an improvement in craniofacial development of Ts65Dn mouse models that overexpress *Dyrk1a* in addition to numerous other chromosome 21 genes. EGCG is administered during a critical window of craniofacial development to maximize potential therapeutic benefits. This research is a first step in finding a way to improve phenotypic development to potentially avoid health issues associated with DS.

METHODOLOGY

Pregnant Ts65Dn and euploid mice were force fed either 200 mg/kg of EGCG or H2O, twice daily on embryonic day 7 and 8. Treated and untreated Ts65Dn and euploid offspring were imaged at 6 weeks of age using micro-computed tomography (μCT; 35 mm resolution) to assess whether early EGCG treatment rescued skull morphology. In summary, μCT images were sent to the Starbuck lab at UCF for morphometric analysis, and μCT images were thresholded to visualize bone and viewed as 3D objects using Amira software, an extendable software system for scientific visualization, data analysis, and presentation of 3D and 4D data, to assess presence and absence of anatomical structures and to identify and measure anatomical landmarks located on bone surfaces.

Anatomical landmarks are biologically meaningful points that can be seen in every animal used to find forms and measure proportions. Here we find multiple skull and mandible landmarks as shown in Table 1. The number of landmarks measured from each craniofacial region includes 12 from the face, 13 from the vault, 7 from the base, and 10 from the mandible (Figure 1).

Eighty eight skulls and mandibles were landmarked and compared. Seven Ts65Dn offspring and euploid offspring from a Ts65Dn mother were treated with EGCG, 10 euploid offspring from a euploid mother were treated with EGCG, and 5 euploid offspring from a Ts65Dn mother and 11 euploid offspring from a euploid mother received no treatment.

A Euclidean Distance Matrix Analysis (EDMA)

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was employed to calculate all unique linear distance measurements between anatomical landmarks using the following formula:

d_((A,B))=√([[[(x]]_1-x_(2))]] ^2+([[y_1 [[- y]]_2)]] ^2+([[z_1- z_2)]] ^2)

Afterwards, an EDMA confidence interval testing procedure ($\alpha \leq 0.10$) was carried out on a region by region basis to identify local mean differences between samples, which resulted in the comparison of 66 linear distances for the face, 78 for the vault, 21 for the base, and 45 for the mandible.

RESULTS

Table 2 summarizes local test results with the first two-sample comparison, Euploid versus Ts65Dn (Figure 2), acting as a baseline for expected differences between genetically modified trisomic mice and disomic controls. The second two-sample contrast, Ts65Dn+EGCG versus Ts65Dn (Figure 3), compares trisomic mice treated with EGCG in utero and untreated trisomic mice to determine if and how EGCG influences craniofacial morphology. If EGCG had no effect on *Dryk1a* and craniofacial development, then no craniofacial differences would be expected between the treated and untreated trisomic mice. In reality, however, following EGCG treatment the percentages of significant morphological measures increased in the face, decreased in the base and vault, and stayed the same in the mandible (although specific local differences may vary despite having the same percentage). These results suggest that EGCG does alter typical Ts65Dn craniofacial morphology, likely by reducing *Dyrk1a* overexpression associated with trisomy. The last two-sample comparison, Euploid vs. Ts65Dn+EGCG (Figure 4), tells us how different disomic euploid controls are compared to trisomic genetically modified mice treated with EGCG. If EGCG is rescuing trisomic craniofacial morphology to euploid levels, then we should see fewer significant differences between these two samples relative to the first baseline comparison. Clear improvement in craniofacial morphology occurs across the craniofacial complex in the base, face, mandible, and vault, suggesting that EGCG has reduced the overexpression of *Dyrk1a* during development to ameliorate craniofacial morphogenesis and growth.

DISCUSSION

Ts65Dn mice models were specifically made in order to study the effects of DS, including the overexpression of *Dyrk1a*. EGCG was able to significantly change the morphological measurements of the Ts65Dn mice skulls and have a positive phenotypic effect, likely through the reduction of *Dyrk1a* overexpression. A low number of significant differences were also seen between the last two-sample comparison, Euploid vs. Ts65Dn+EGCG. This sample compares disomic mice to trisomic mice that were treated with EGCG. Fewer differences suggests that EGCG is helping to rescue the craniofacial phenotype relative to untreated Ts65Dn mice, which are very different from euploid controls.

Previous studies of EGCG have also recorded positive results on the cognitive deficits of the overexpression of *Dyrk1a* and improvement of behavior. For example, many Ts65Dn mice treated with EGCG for 1 month had visuospatial learning and memory improvement in addition to improvement of learning strategies and reference memory (De la Torre et al., 2013). Homocysteine (Hcy) is an amino acid that, when produced in high amounts, can cause brain atrophy, cognitive impairment, and dementia, among other things (Smith et al., 2010), Hcy decreases in mice when treated with EGCG (De la Torre et al., 2013). Having a higher amount of Hcy is common in individuals with DS because of Hcy's connection between *Dyrk1a* (De la Torre et al., 2013). In humans, improvement of episodic and working memory was shown after a three month treatment of capsules of EGCG as well as visual memory recognition, psychomotor speed, and social functioning (De la Torre et al., 2013).

The results from this experiment suggest that a high dose of EGCG treatment in utero improves craniofacial morphogenesis and growth. Additional research is necessary to determine if EGCG is a viable therapy option for individuals with DS to reduce health issues associated with the craniofacial complex as well as how long the treatment should be and what dosage should be used. Since EGCG is a kinase inhibitor, additional studies are also needed to determine if EGCG inhibits other genes, aside from *Dyrk1a*. In addition, other areas of the post-skeleton and brain should be studied to determine if EGCG affects these regions too. Moving into the future, a larger study of the effects of EGCG over a longer time period will be useful to assess side effects or complications. Differences between mice and

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humans also must be incorporated when evaluating different doses of EGCG, side effects, and differences in results both cross-sectionally and longitudinally.

CONCLUSIONS

This research was conducted to determine whether EGCG treatment improves craniofacial morphology in Ts65Dn DS mouse models. EGCG-treated mice showed improved craniofacial morphology in the face, vault, base, and mandible relative to untreated Ts65Dn mice and exhibited fewer differences with euploid mice, suggesting a positive effect from EGCG treatment overall. These results suggest that EGCG may be a useful therapeutic option for humans with DS, but additional studies are necessary to determine the best treatment timing and dosage.

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APPENDIX

Figure 1. Skull and mandible landmarks.

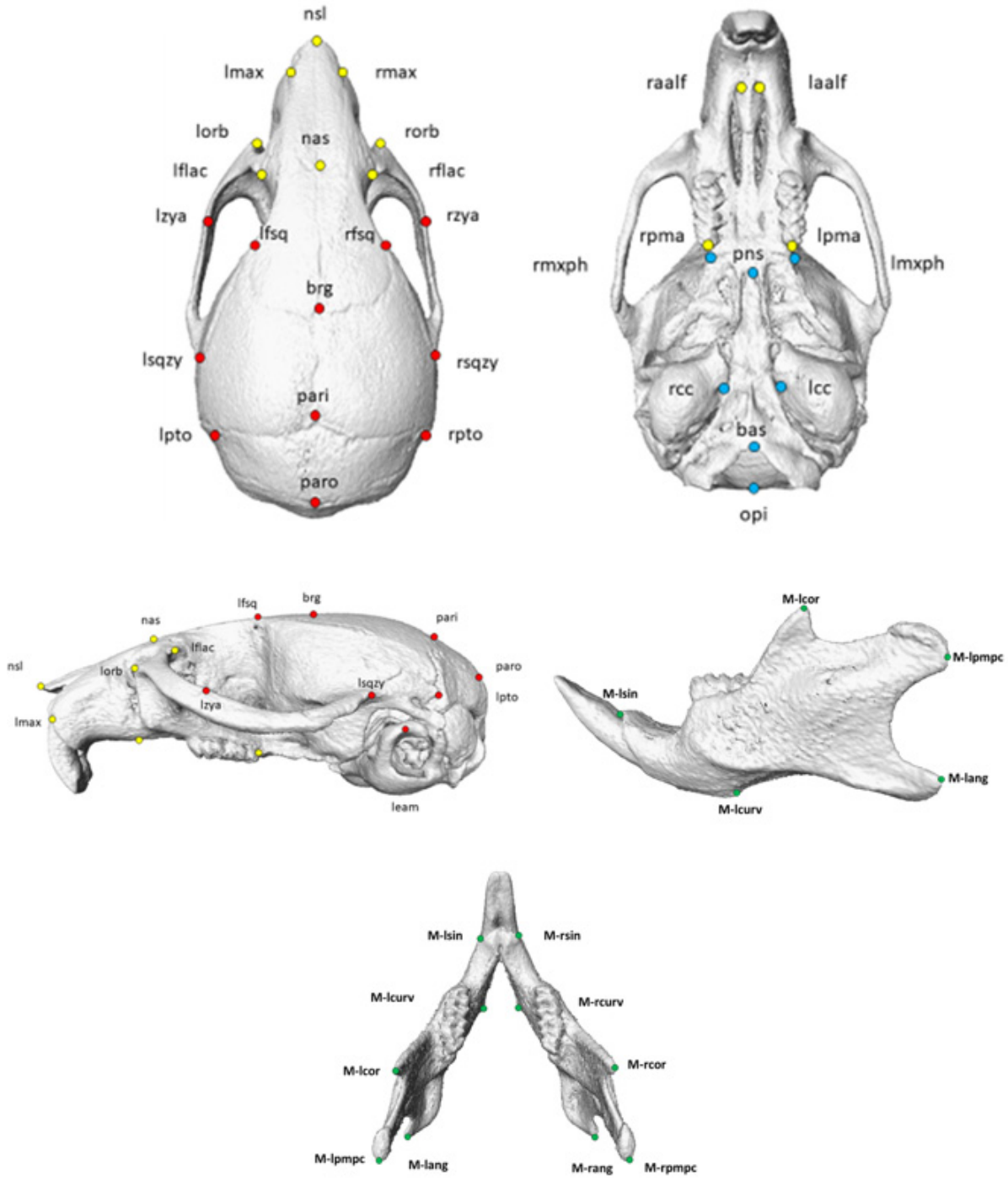

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Table 1. Skull and mandible anatomical landmark definitions.

Skull Landmarks
nsl (nasale) – the rostral intersection of the nasal bones, midline
nas (nasion) – the caudal intersection of the nasal bones, midline
brg (bregma) – the intersection of the frontal and parietal bones, midline
pari – the intersection of the parietal bones with the anterior aspect of the interparietal bone, midline
paro- the intersection of the interparietal bone with the squamous portion of the occipital bone, midline
opi (opisthion) – the midsagittal point on the posterior margin of the foramen magnum, midline
bas (basion) – the midsaggital point on the anterior margin of the foramen magnum, midline
pns (posterior nasal spine) – the most posterior point on the hard palate, midline
max– the center of the alveolar ridge over the maxillary incisor, bilateral
orb– the anterior notch on the frontal process, lateral to the infraorbital fissure, bilateral
flac – the intersection of the frontal process of the maxilla with the frontal and lacrimal bones, bilateral
zya – the intersection of the zygomatic process of the maxilla with the superior surface of the zygoma, bilateral
fsq – the fronto-squamosal intersection at the temporal crest, bilateral
sqzy– the posterior point at the joining of the squamosal body to the zygomatic process, bilateral
eam – the most posterioinferior point on the superior portion of the tympanic ring, bilateral
pto – the intersection of the parietal, temporal, and occipital bones, bilateral
aalf – the most anterior point on the anterior palatine foramen, bilateral
pma – the posterior-most point on the central anteriorposterior axis of the molaralveolus, bilateral
mxph– the intersection of the maxilla and sphenoid on the inferior alveolar ridge, bilateral
cc – the most anterior medial point on the carotid canal, bilateral

Mandible Landmarks
m-sin – Superior-most point on incisor alveolar rim at midline (at bone tooth-junction)
m-curv – Anterior edge of the coalescence of curve of masseteric ridge with post-symphyseal rugged area
m-ang – tip of mandibular angle
m-cor – apex of coronoid process
m-pmpc – posterior midline point on condyle

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Table 2. Comparison table of percentages and fractions of significant differences where 0% equals no differences and 100% equals all measurements were significantly different when compared.

Comparison (samples)	Cranial Base	Cranial Face	Mandible	Cranial Vault
Euploid vs. Ts65Dn	42% (9/21)	56% (37/66)	82% (37/45)	37% (27/78)
Ts65Dn+EGCG vs. Ts65Dn	33% (7/21)	68% (45/66)	82% (37/45)	14% (11/78)
Euploid vs. Ts65Dn+EGCG	14% (3/21)	18% (12/66)	9% (4/45)	9% (7/78)

Figure 2. Significant results from the comparison of Euploid and Ts65Dn mice.

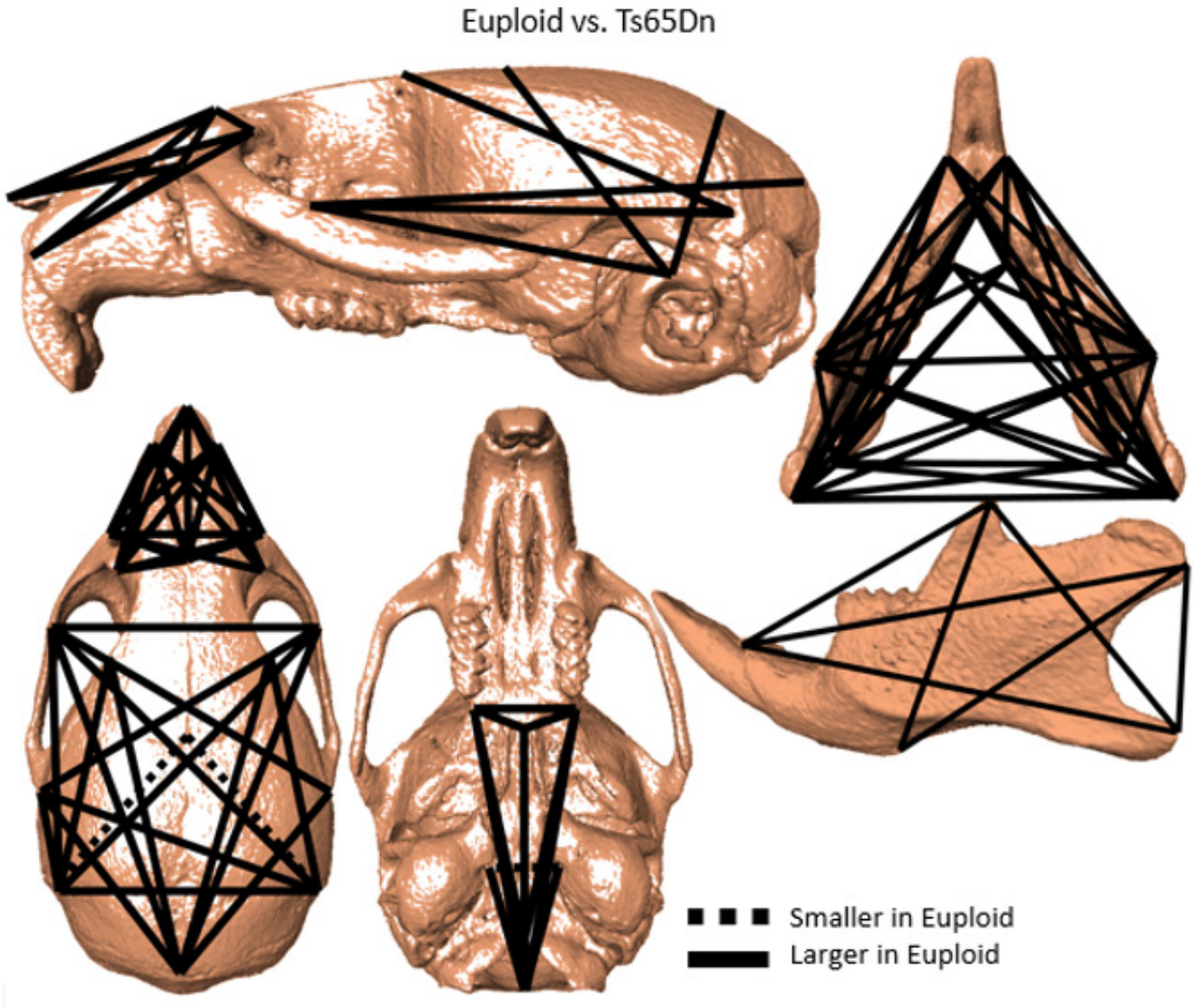

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Figure 3. Significant results from the comparison of Ts65Dn treated with EGCG and untreated Ts65Dn mice.

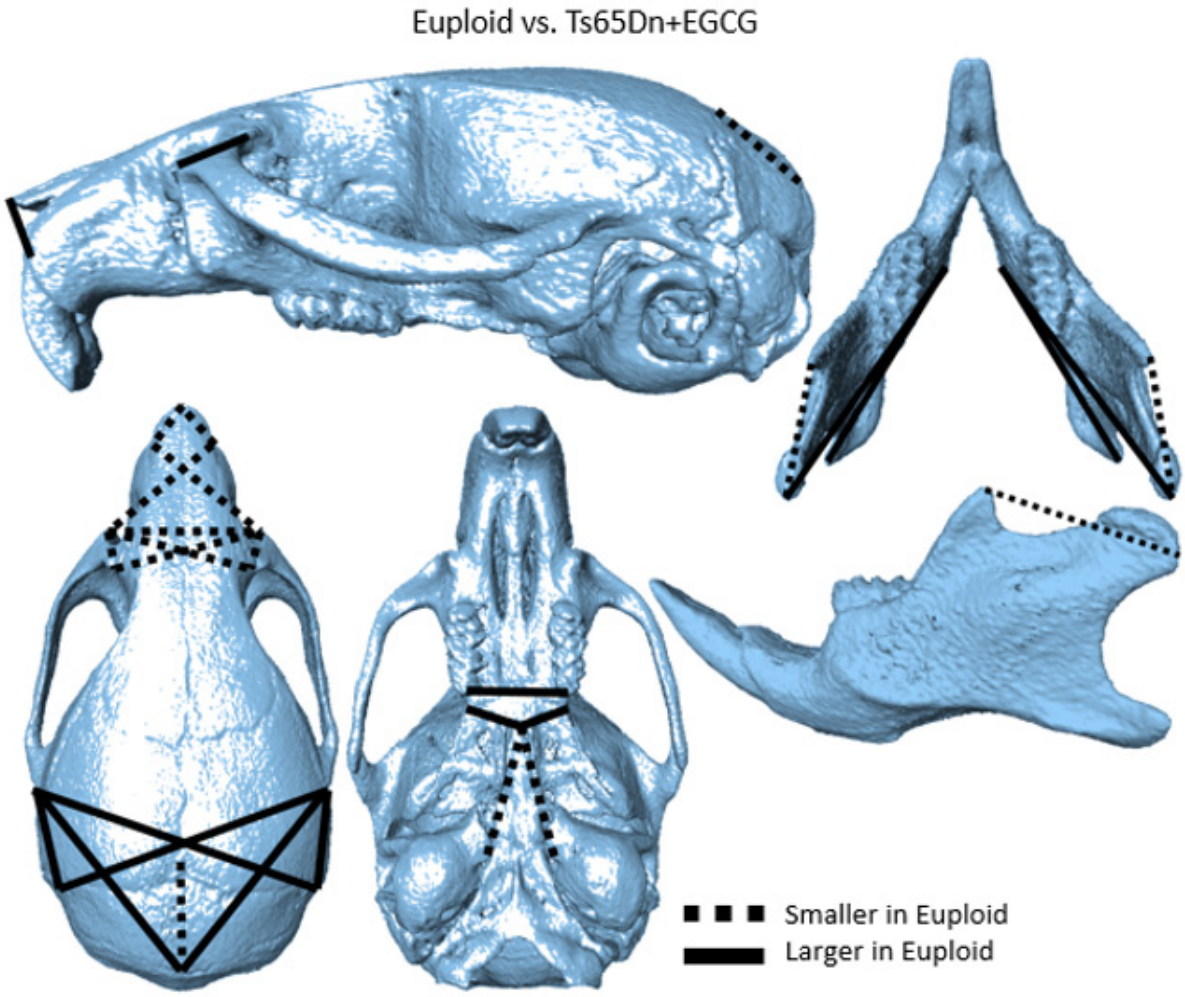

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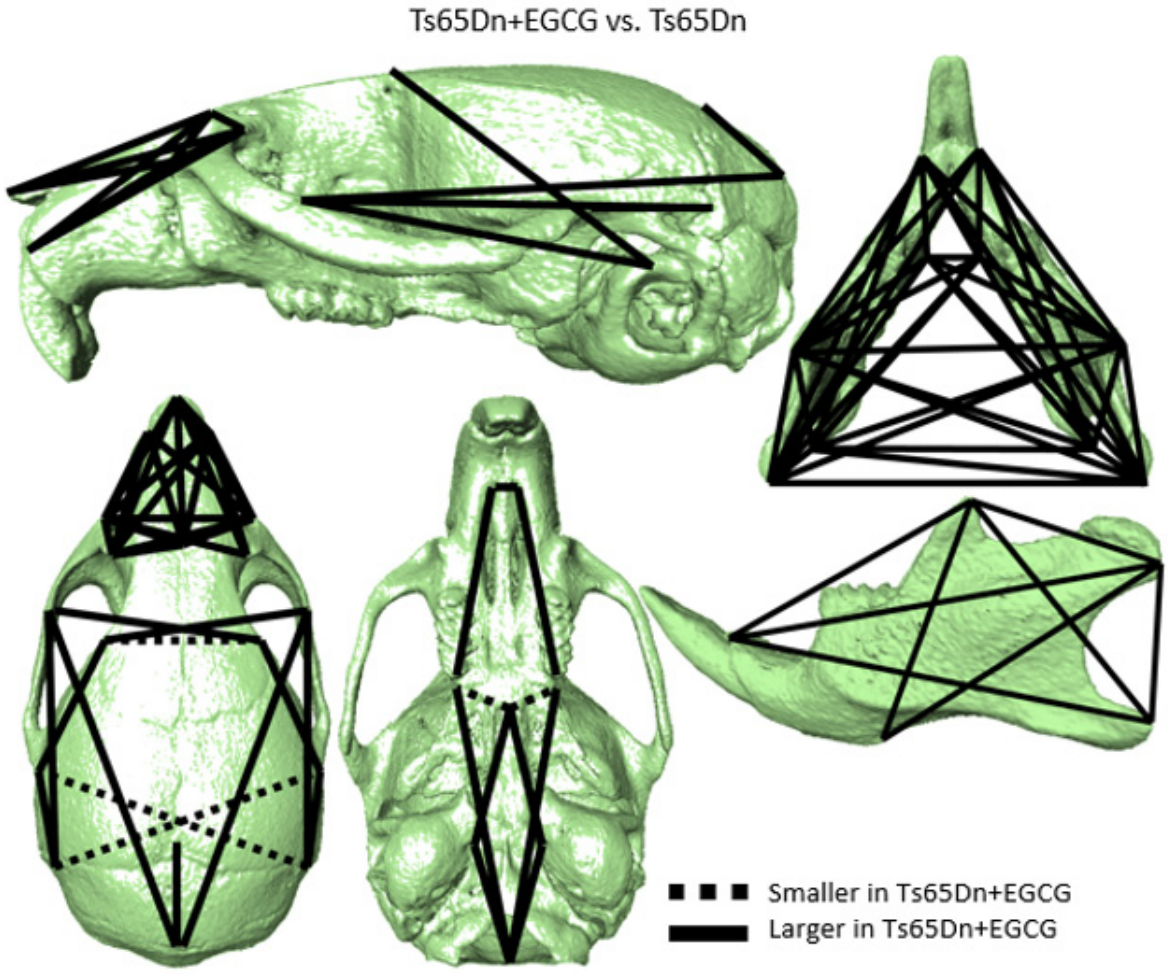
Figure 4. Significant results from the comparison of euploid mice and Ts65Dn mice that were treated with EGCG.



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