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Enamel alterations in serotonin 2B receptor knockout mice

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The role of the serotonin 2B receptor (5-HT_{2B}R) in enamel formation and mineralization was explored in adult 5HT_{2B}R knockout (KO) mice compared with wild-type (WT) mice. In the molar, quantitative data obtained by micro-computed tomography imaging showed that the overall volume of the enamel layer was firmly reduced in KO mice. Defective mineralization was ascertained by energy-dispersive X-ray microanalysis. We also observed, using scanning electron microscopy, that parazones in the KO mice included two or three helicoidally twisted rods within Hunter–Schreger bands, instead of a single rod, as found in the WT mice. Minor disturbances were also detected in the incisors of KO mice. Structural modifications, thinner enamel crystallites, and porosities observed in KO mice indicate that the 5-HT_{2B}R-mediated signaling pathways as part of the enamel formation process. These data provide a basis for evaluating the role of 5-HT_{2B}R in ameloblast functions. Defects observed in the mineralization and structure of enamel in KO mice highlight that the 5-HT_{2B}R interferes with the mechanisms directing amelogenesis.

Serotonin (5-hydroxytryptamine, 5-HT), a neurotransmitter in the central nervous system, modulates a wide variety of behavioral and physiological processes, such as appetite, sleep, anxiety, cognition, and memory. In the periphery, 5-HT is exclusively synthesized by enterochromaffin cells of the gut and stored by platelets. Platelet-stored 5-HT participates broadly in the homeostasis of various tissues and plays a role in cardiovascular functions, smooth muscle response, gastrointestinal contraction, regulation of the endocrine system [see for review (1)]. 5-Hydroxytryptamine also exerts a trophic function during animal development. Both 5-HT and the 5-HT transporter (SERT) have been detected in early development stages of mouse embryo (2, 3) and are involved in the migration of cells derived from the neural crest, and in neurogenesis, as well as in craniofacial and cardiovascular morphogenesis (4–6). Of note, embryos exposed to 5-HT_{2B} receptor (5-HT_{2B}R) antagonists or antidepressants, which block 5-HT uptake by the SERT, exhibit morphological defects in mesodermal and neural crest derivatives, including the craniofacial skeleton (4–8). Moreover, in adults, patients receiving serotonin reuptake inhibitor antidepressants appear to be at risk for osteoporosis (9–11).

The biological actions of 5-HT are mediated by numerous 5-HT receptors (5-HTRs). At present, 15, 5-HTR subtypes, divided into seven classes (5-$HT_{1–7}$R), have been characterized, based on their amino acid sequence, pharmacological profile, and transduction mechanisms (12). Such a diversity of 5-HTRs makes it difficult to elucidate their precise role in 5-HT-mediated functions (13). An alternative way to investigate the role of 5-HTRs is by the genetic inactivation of genes encoding these receptors.

Regarding mineralized tissues, the functional impact of 5-HT signaling in the skeleton is still debated because both enhancing and inhibitory effects on bone formation have been depicted in response to 5-HT (14). In previous studies, we identified the 5-HT_{2B}R as an important player in bone metabolism (15–17). We used an inducible mesoblastic cell line, C1, able to fully differentiate into osteocytes within 12 days, to assess the involvement of 5-HT_{2B}R during osteogenic differentiation. The 5-HT_{2B}R-mediated coupling to the nitric oxide synthase and the phospholipase A2 pathways is necessary for optimal bone matrix mineralization (15). Blockade of this receptor causes a 40% reduction in calcium incorporation within the matrix. Furthermore, we have identified the tissue non-specific alkaline phosphatase (TNAP) as a target whose activity is entirely controlled by the 5-HT_{2B}R (17). In agreement, primary calvarial osteoblasts obtained from 5-HT_{2B}R knockout (KO) mice suffering from osteopenia (6), exhibit defects in TNAP activity (16, 17).

The role of 5-HT in enamel and dentin formation is poorly documented, although the presence of 5-HT receptors (namely 5-$HT_{1A, 2A, 2B, 2C}$R), tryptophan hydroxylase 1 (TPH1; the key enzyme that synthesizes 5-HT), and SERT have been detected in the enamel organ of both molars and incisors (18).

As 5-HT contributes, during development, to the differentiation of neuroectodermal, neural crest, and mesodermal derivatives, and in view of the role of 5-HT_{2B}R in bone matrix mineralization, we hypothesize that this receptor may be crucial for proper enamel development and tooth homeostasis. We took advantage of 5-HT_{2B}R KO mice to investigate whether 5-HT_{2B}R depletion could be at the origin of defects in enamel formation. We compared the ultrastructure and mineralization of enamel in the wild type (WT) and the 5-HT_{2B}R KO mice, both in the continuously growing incisor and in the molar, a tooth of limited growth. The well-organized matrix framework for mineral deposition was altered in 5-HT_{2B}R KO mice, as was the mineral density of enamel. These data provide the first evidence that 5-HT_{2B}R-dependent signaling pathways act on enamel formation and introduce this receptor as a novel actor in tooth development.

Material and methods

This study was performed using adult 5-HT_{2B}R KO mice in which exon 2 of the 5-HT_{2B}R locus has been substituted for the selective bacterial neo cassette (6). The 5-HT_{2B}R KO mice have a 129sv/PAS background as WT (Charles River Laboratories, L'Arbresle, France). The mice were allowed free access to food and water, in full compliance with French Government and European Community Animal Welfare Policy.

One third of embryonic 5-HT_{2B}R KO mice die midgestation, and another third die at birth from cardiac failure. The mice that survive have a cardiac phenotype, but a normal lifespan (6, 16). In order to establish the potential indirect incidence of plasmatic calcemia and vascular endothelial growth factor (VEGF) on enamel formation, blood was collected by eye puncture. The mean values were determined for each group, which contained at least 10 mice.

Two groups of thirteen WT (eight male and five female) mice and 12, 5-HT_{2B}R KO (seven male and five female) mice were killed at 8 and 10 wk by cervical dislocation, and the mandibles were dissected out.

One hemimandible was fixed with formaldehyde, embedded in methylcetone peroxide-polymerized Stratyl, and cut with a diamond disk. To avoid variations in section angle, transverse sections were systematically performed in the middle of the first molar at right angles to the long axis of the mandible. The mandible slices had a thickness of 2 mm and were glued on an aluminum stub. After etching three times (for 10 s each time) with 1% nitric acid, they were rinsed with water and sputter coated with platinum for scanning electron microscopy examination (Zeiss Supra 40, Carl Zeiss SMT, Oberkochen, Germany). The ratio of calcium and phosphorus, from the enamel of mandible sections sputter-coated with carbon, was determined using energy-dispersive X-ray (EDX) microanalysis (Hitachi SU70, Hitatchi High Technologies Europa GmbH, Krefeld, Germany. SEM and INCA microanalysis system, Oxford Instruments, Oxford, UK).

The other mouse hemimandible was scanned by micro-computed tomography (micro-CT) for non-destructive studies (Viscom X8060, UsefulProgress, Saints Pères, Paris, France). The X-ray source was operated at 80 kV and 160 mA, with a 5 μm spatial resolution. Volume rendering, a technique for three-dimensional (3D) visualization of samples, was suitable for rendering images from mouse mandibles acquired by micro-CT and to identify the bone and different dental structures: pulp, dentin, and enamel (19). Volumetric measurements were carried out following the selection of a 3D volume of interest drawn around the first molar. Software was used to visualize and quantify the volume of the first molar enamel. A threshold image filter allowed the isolation of the enamel according to its mineral density. All samples were analyzed following the same threshold to obtain comparable volume measurements and visualization. By making the dentin, the pulp, and the bone translucent, the enamel cap was observed from various directions. The volume of the enamel was calculated by subtracting the less mineralized tissues in the 3D volume of interest.

Results

Our aim was to investigate whether the inactivation of 5-HT_{2B}R leads to disturbed enamel formation. We performed invasive and non-invasive techniques to analyze the composition, structure, and volume of enamel.

Table 1 shows the values found for plasmatic calcemia and VEGF in WT and 5-HT_{2B}R KO mice. The differences were not significant and demonstrate that the effects observed on amelogenesis are caused neither by hypocalcemia nor by variations of VEGF.

X-ray spectrophotometric analysis allowed comparison of the phosphorus and calcium levels in enamel of WT and 5-HT_{2B}R KO mice (Table 2). The phosphorus level was similar in both groups, whereas the calcium level was lower in the 5-HT_{2B}R KO group than in the control. Consequently, the calcium/phosphorus ratio

Table 1
Analysis of plasmatic calcemia and vascular endothelial growth factor (VEGF) in wild-type (WT) and serotonin 2B receptor (5-HT_{2B}R) knockout (KO) groups

	WT	5-HT_{2B}R KO
Plasmatic calcemia (mM)	1.43 ± 0.16	1.45 ± 0.11
Plasmatic VEGF (ng l^{-1})	15.7 ± 2.4	18.0 ± 1.9

Data are given as mean ± SD.

Table 2
Energy-dispersive X-ray microanalysis (EDX) of enamel in wild-type (WT) and serotonin 2B receptor (5-HT_{2B}R) knockout (KO) groups

EDX microanalysis	WT (atomic percentage)	5HT_{2B}R KO (atomic percentage)
Calcium	21.62 ± 4.26	20.43 ± 2.63*
Phosphorus	16.52 ± 1.92	16.16 ± 1.62*
Calcium/phosphorus ratio	1.33 ± 0.09	1.26 ± 0.09*

EDX for calcium and phosphorus showed the atomic percentage (mean ± SD) of K line elements. The calcium/phosphorus ratio from WT and 5-HT_{2B}R KO samples is presented as mean ± SD.
*$P < 0.05$ vs. WT.

was decreased in the 5-HT$_{2B}$R KO group, suggesting that the enamel was less calcified in this group.

Histological analyses of incisor and molar enamel structures were compared between 5-HT$_{2B}$R KO mice and WT mice. Depending on slight angle variations in the orientation of the small-sized samples, the enamel surface of the crown of the molar was more or less exposed, but included the buccal and lingual cusps, and presented an enamel-free occlusal surface. In the lower part of the mandible, the alveolar and basal bones surrounded transverse sections of the incisor, where enamel was always limited to a half-moon structure.

Incisor enamel

In the WT incisors, acid-etched transverse sections of enamel (total width about 120–130 μm) revealed a thin inner aprismatic enamel of about 4–5 μm located at the dentino–enamel junction (DEJ), followed by parallel rods at right angles to the DEJ (Fig. 1A). In the inner prismatic enamel, rows of prisms (or rods) were grouped within single-layered rows (lamellae) with a decussation pattern, and each alternate row was oriented in opposite directions. The lamellae were approximately parallel to the DEJ. In the outer enamel, the rods were parallel and at right angles to the enamel outer surface. The superficial border of the enamel, termed the outer aprismatic enamel, of about 10 μm thick, displayed a weaker acid-etched pattern, probably because enamel maturation was not complete, as shown also by small unfilled inter-rod spaces (Fig. 1A).

In the 5-HT$_{2B}$R KO mice (Fig. 1B), the inner aprismatic layer of the incisor enamel was thinner compared with that of the WT mice. The lamellae located in the inner prismatic enamel formed a 20–25° angle relative to the DEJ. It was obvious that the well-defined parallel pattern of enamel was disorganized. In particular, rods and inter-rods were more angulated and protruding mineral structures were formed, while numerous porosities could be visualized at the junction between the rods and inter-rods. The poorly etched outer layer was thicker in the 5-HT$_{2B}$R KO mice compared with the WT mice, suggesting that depletion of the 5-HT$_{2B}$R affects not only the angulation of mineral deposition but also the later stages of enamel maturation.

Molar enamel

As the organization of enamel differs according to its localization, enamel structure was compared between WT and 5-HT$_{2B}$R KO mice on molar sections performed in the cusps, and in the buccal and lingual areas.

In the outer buccal areas of WT mice (Fig. 2A), near the tip of the cusp, the rods and inter-rods displayed a feather-like structure in the inner half with some inclination to the DEJ. In the outer half, the rods became parallel to each other and at right angles to the surface. The outer aprismatic layer was almost undetectable.

In the 5-HT$_{2B}$R KO mice (Fig. 2B), the outer prismatic enamel appeared to be significantly thicker and the feather-like pattern was missing. Rods at right angles to the DEJ were first parallel and protruded, and then they curved in the inner half and finally expanded as long, thin, and straight parallel lines. In addition, a 10-μm-thick aprismatic outer layer was present. Thus, as observed for the incisor, depletion of the 5-HT$_{2B}$R

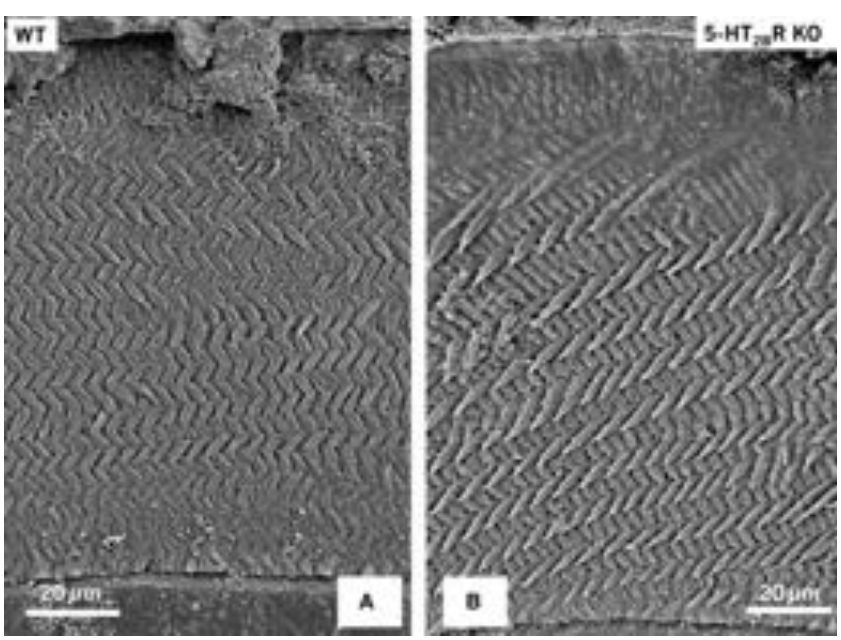

Fig. 1. Depletion of the serotonin 2B receptor (5-HT$_{2B}$R) alters formation of the mouse incisor enamel structure. Transverse sections of incisors were analyzed by scanning electron microscopy. In wild-type (WT) mouse incisor (A), decussating prisms formed rows (lamellae) parallel to the dentino–enamel junction (DEJ). Incisor enamel in 5-HT$_{2B}$R knockout (KO) mice (B) showed a thicker outer aprismatic zone than incisor enamel in WT mice, and lamellae displayed a 20–25° angle to the DEJ.

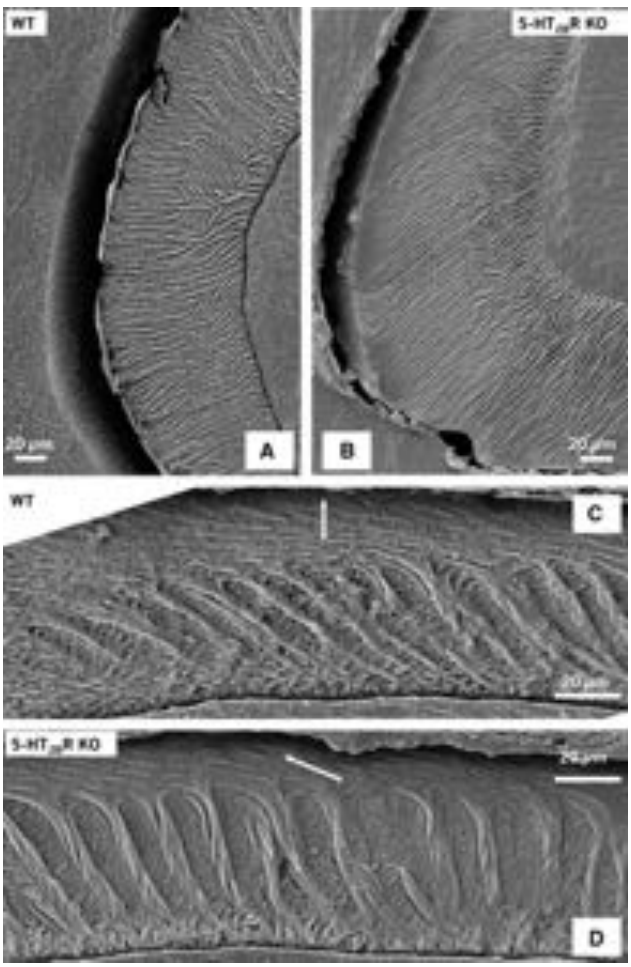

Fig. 2. Serotonin 2B receptor (5-HT_{2B}R) knockout (KO) mice display structural alterations in the buccal cusp of the molar enamel. In wild-type (WT) mice (A), rods and inter-rods seen in the inner and outer prismatic enamel are structural elements of the buccal tip of the cusp. The outer aprismatic border is thin. In the 5-HT_{2B}R KO mice (B), the thickness of the outer prismatic enamel appeared significantly increased. In WT mice (C), the inner prismatic enamel of the buccal enamel is formed by parazones and diazones of the Hunter–Schreger (HS) bands, which included one single rod. In the outer prismatic enamel layer, the rods were at right angles (arrow) to the enamel surface, Conversely, in 5-HT_{2B}R KO mice (D), the HS bands of the inner prismatic enamel included helicoidally twisted structures, formed by two or three rods. In the outer prismatic enamel layer, the rods were bent towards the tip of the cusp.

impacts on enamel spatial organization of the molar. This implies that the 5-HT_{2B}R contributes to the formation of the intricate matrix/mineral network elaborated by the ameloblasts.

In the WT mice, the aprismatic zone near the DEJ was 4–5 μm thick (Fig. 2C). Alternative Hunter–Schreger (HS) bands formed the inner prismatic enamel. Parazones included one single rod, and diazones encompassed a series of superposed rods. In the prismatic outer layer (Fig. 2C), rods perpendicular to the enamel surface crossed oblique lamellae before reaching the surface, where they formed ultimately a thin aprismatic outer layer.

In the 5-HT_{2B}R KO mice, the aprismatic enamel layer near the DEJ was enlarged, reaching about 10 μm (Fig. 2D). The inner prismatic enamel included helicoidally twisted structures, formed by two or three rods, which constitute thicker parazones, whereas diazones included piles of superposed rods. In the outer third, the rods fanned out and were bent towards the tip of the cusp direction forming an angle of 15–20° relative to the surface. Ultimately prisms merged and contributed to the formation of an aprismatic outer enamel (Fig. 2D).

At higher magnification (Fig. 3A–D), important changes in mineral density were pbserved between WT and 5-HT_{2B}R KO mice. In WT male mice, buccal enamel crystallites were densely packed in the molar (Fig. 3A). In contrast, crystallites were significantly thinner in 5-HT_{2B}R KO male mice, and enamel exhibited many porosities (Fig. 3B). Moreover, 5-HT_{2B}R KO female mice (Fig. 3D) displayed more pronounced defects than the WT female mice (Fig. 3C). The crystallites had a fibrillar appearance and were disassociated. Enamel crystallites of 5-HT_{2B}R KO male and female mice (82.80 ± 15.25 nm) was significantly thinner compared with those of WT male and female mice (118.25 ± 13.50 nm) ($P < 0.002$). In mutant mice, large

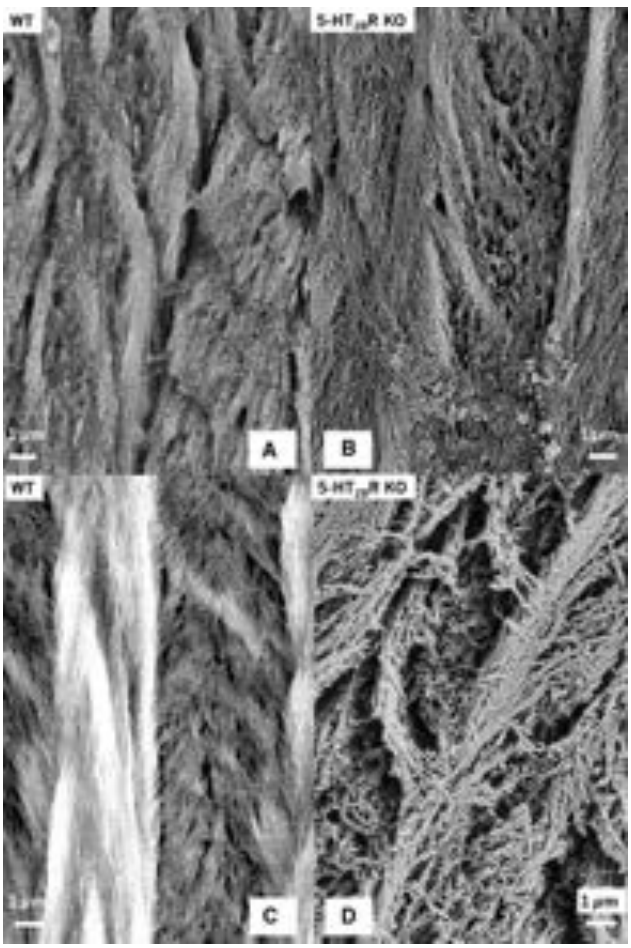

Fig. 3. Serotonin 2B receptor (5-HT$_{2B}$R) knockout (KO) mice display enamel porosities that are more pronounced in female mice. At higher magnification, the enamel of wild-type (WT) male mice (A) exhibited dense structures and large crystallites, whereas the enamel from 5-HT$_{2B}$R KO male mice (B) presented numerous porosities and thin crystallites. Compared with enamel from WT female mice (C), the enamel from 5-HT$_{2B}$R KO female mice (D) displayed increased porosities and thin fibril-like crystallites which formed a network at the hypomineralized enamel surface.

spaces increased the porous aspect of enamel at this magnification, indicating that the 5-HT$_{2B}$R may play a pivotal role in the mineralizing processes sustaining enamel formation.

The differences in enamel structure were less obvious in the lingual areas of WT and 5-HT$_{2B}$R KO mice (Fig. S1). Both mice displayed HS bands with similar appearance (Fig. S1A,B). The aprismatic outer enamel was denser in the WT mice compared with the 5-HT$_{2B}$R KO mice (Fig. S1C,D).

Shape and volume of enamel

We used micro-CT to analyze the shape, volume, and distribution of enamel in the molars of WT and 5-HT$_{2B}$R KO mice. The spatial resolution and the volume rendering isolated the enamel cap from the underlying structures and provided qualitative information on the enamel shape and thickness. As shown in Fig. 4A, volume analysis revealed that the 5-HT$_{2B}$R KO first molars display a statistically significant decrease in enamel volume (about 30%) compared with WT first molars ($P < 0.005$). The 3D reconstruction of the molar enamel was illustrated by volume rendering (Fig. 4B–E). The side view of the crown showed the cusps and fissures in WT mice (Fig. 4B). In the 5-HT$_{2B}$R KO mice, the enamel appeared rough and irregular, but hypoplasia was never seen (Fig. 4C). The volume-rendering method, allowing virtual and transverse sections of the enamel cap, also revealed a reduced enamel thickness in the 5-HT$_{2B}$R KO mice. Moreover, in the occlusal view, increased abrasion and exposure of a large area of dentin were observed in the 5-HT$_{2B}$R KO mice (Fig. 4E) compared with the WT mice (Fig. 4D).

Discussion

Identification of factors affecting enamel formation may have implications in tooth development and homeostasis, and in the fields of preventive and restorative dentistry. Serotonin reuptake inhibitors are widely used as antidepressants and for the treatment of anxiety disorders. These therapies may have clinical conse-

quences on mineralized tissue formation, namely on dentin and bone. Moreover, serotonin reuptake inhibitors also have adverse effects on enamel formation, especially during amelogenesis. The effects may be similar to those observed during congenital cardiac disease (CCD), with enamel hypoplasias observed in 52% of the children with CCD (20). As the 5-HT_{2B}R regulates cardiac embryonic development, discrete enamel hypomineralization may be associated with cardiomyopathy, as is the case for mutant mice (6, 7, 13). We are unaware whether it is a direct influence of the receptor (21), or an indirect action caused by changes in blood pressure and vascularization. However, using the mouse model it has been shown that blood pressure and lung remodeling associated with vascular proliferation are not detectable in normal and hypoxic mice (22). As no differences were found in the levels of calcium and in VEGF, a growth factor implicated in angiogenesis, a direct effect of 5-HT_{2B}R is privileged. However, we cannot rule out that blood pressure, changes in vascularization, and cardiac abnormalities have no influence on the enamel defects reported here. Identifying the actors that control the orientation of enamel rods is important in restorative dentistry because enamel with disordered microstructures is prone to fracture.

The present work provides, for the first time, evidence for involvement of the 5-HT_{2B}R in ameloblast function and enamel mineralization. The mineral density shown by EDX, the enamel volume evidenced by micro-CT, and the crystallite thickness and the 3D organization of enamel rods observed by scanning electron microscopy in the incisors and molars of 5-HT_{2B}R KO mice clearly differ from those of WT mice. In addition, as shown for bone (16), enamel defects are more pronounced in female mice than in male mice. Depletion of the 5-HT_{2B}R does not impair enamel formation but disturbs the intricate matrix/mineral network, leading to defects in the mineral repartition and in the orientation of the enamel rods. Thus, the 5-HT_{2B}R contributes to optimal structure formation and mineralization of enamel.

Of note, upon the depletion of 5-HT_{2B}R, defects could be observed in all layers of the DEJ up to outer aprismatic enamel, depending on the area observed. This implies that the 5-HT_{2B}R influences the backwards movement of secretory ameloblasts during amelogenesis and affects rod torsions and twisting that are probably postsecretory events linked to mechanical forces.

The oblique orientation of groups of rods, and a double-helix organization of prisms in mammalian teeth lead to the formation of HS bands (23). In the incisors of rodents, it has been proposed that each HS band includes one rod alone, involving one single rod displaying, respectively, an antiparallel orientation and consequently appearing either as diazones or as parazones (24). This organization is more complex in the molar and depends on the area observed (25, 26). In the buccal enamel of WT mice, HS bands are located only in the inner enamel, whereas near the tip of cusps and in grooves, rods display a parallel orientation at right angles to the surface. In the 5-HT_{2B}R KO mice, two major differences were detectable in the cervical part of the buccal enamel: (i) instead of one rod per HS band in the buccal enamel, two or more twisted rods were visible in each parazone; and (ii) in the outer part of the buccal cervical enamel, the rods were bent towards the outer surface, oriented in the direction of the tip of the cusps. The fact that such dif-

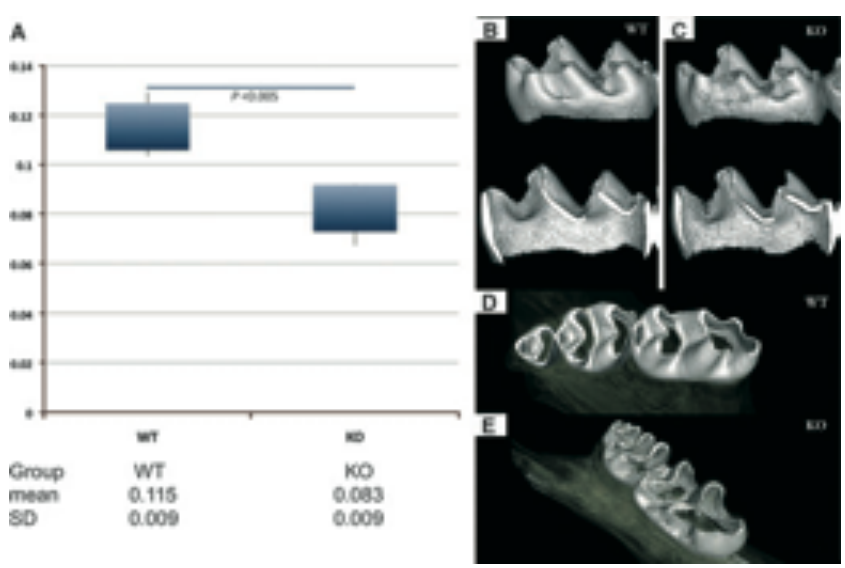

Fig. 4. Volume measurement and virtual reconstruction of mandibular first molar enamel in wild-type (WT) and serotonin 2B receptor (5-HT_{2B}R) knockout (KO) mice. Micro-computed tomography (micro-CT) analysis indicated that the enamel volume of the first mandibular molars was decreased in the 5-HT_{2B}R KO mice compared with the WT mice (A). Three-dimensional images and virtual sections of 5-HT_{2B}R KO mice (C) showed that the enamel surface appeared rough and thinner compared with the enamel surface of the WT mice (B). The enamel surface appeared rough and thinner compared to the enamel of the WT mice (D), while the molars from 5-HT_{2B}R KO mice presented increased exposure of dentin as a result of enamel abrasion (E).

ferences are not detectable in the lingual enamel suggests that these modifications are not dependent on cell movements but are under the control of pressure distribution; further investigations are needed to clarify the reasons for such differences.

Besides, mechanotransduction mechanisms are known to be involved in organogenesis and to mobilize diverse signaling pathways, such as nitric oxide (NO) and phospholipids, leading to changes in cytoskeletan, metabolism, cell adhesion, and gene expression. Still, very little is known about the signals and the mechanoreceptors that trigger cellular responses in numerous tissues. In the case of enamel, the hypomineralized porous enamel rods squeezed by occlusal pressures may receive a flux of forces expelling the residual matrix during rod formation and enamel maturation. The occlusal forces may produce unequal helicoidal pressures on a tooth, and consequently promote twisting of the prisms and their association into thicker HS bands. Unequal distribution of forces may merely affect the buccal part of the enamel cap, not the lingual enamel. Such a hypothesis cannot be excluded because of the impact of 5-HT$_{2B}$R on skeletal development and on bone turnover through diffusion of NO or eicosanoids (15). These data provide a basis to investigate a putative role of this receptor in mechanotransduction mechanisms, which might drive enamel structure formation.

In summary, this work, exploiting 5-HT$_{2B}$R KO mice, provides the first evidence for a functional relationship between 5-HT$_{2B}$R and enamel formation. These findings further substantiate the recent notion that the 5-HT system contributes to mineral tissue differentiation and skeletal development.

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Conflicts of interest – The authors declare no conflicts of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Lingual molar enamel.

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