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Serotonin and fluoxetine receptors are expressed in enamel organs and LS8 cells and modulate gene expression in LS8 cells

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Abstract

The selective serotonin re-uptake inhibitor (SSRI) fluoxetine is widely used in the treatment of depression in children and fertile women, but its effect on developing tissues has been sparsely investigated. The aim of this study was to investigate if enamel organs and ameloblast-derived cells express serotonin receptors that are affected by peripherally circulating serotonin or fluoxetine. Using RT-PCR and western blot analysis we found that enamel organs from 3-d-old mice and ameloblast-like cells (LS8 cells) express functional serotonin receptors, the rate-limiting enzyme in serotonin synthesis (Thp1), as well as the serotonin transporter (5HTT), indicating that enamel organs and ameloblasts are able to respond to serotonin and regulate serotonin availability. Fluoxetine and serotonin enhanced the alkaline phosphatase activity in the cell culture medium from cultured LS8 cells, whereas the expression of enamelin (*Enam*), amelogenin (*AmeI*), and matrix metalloproteinase-20 (*MMP-20*) were all significantly down-regulated. The secretion of vascular endothelial growth factor (VEGF), monocyte chemotactic protein 1 (MCP-1), and interferon-inducible protein 10 (IP-10) was also reduced compared with controls. In conclusion, enamel organs and ameloblast-like cells express functional serotonin receptors. Reduced transcription of enamel proteins and secretion of vascular factors may indicate possible adverse effects of fluoxetine on amelogenesis.

Keywords

ameloblasts; enamel; fluoxetine; serotonin

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Fluoxetine is a commonly used selective serotonin re-uptake inhibitor (SSRI), and because it has been extensively documented clinically, it is the preferred antidepressant when treatment is indicated in children and pregnant or breastfeeding women (1). A study on the developmental and behavioural consequences of prenatal exposure to fluoxetine concluded that this treatment might cause a transient delay in motor development, but does not adversely affect the postnatal behaviour (2). Functional serotonergic pathways are also present in bone, and, as shown by our group previously, fluoxetine modulates the function of bone cells *in vitro* (3). In accordance with this, an increased risk of fracture has been described in patients using SSRIs, which may be linked to a dose-dependent inhibition of the serotonin transporter system (4).

It has been reported that serotonin [or 5-hydroxytryptamine (5HT)] may regulate dental differentiation, act as a dose-dependent morphogenetic signal for craniofacial development (5), and regulate tooth-germ morphogenesis. Functional serotonin receptors and the rate-limiting enzyme in serotonin synthesis (Thp1) have been demonstrated in bone cells from mesenchymal (osteoblasts) and haematopoietic (osteoclasts) origins, and serotonin has been found to regulate bone metabolism in a direct manner (3, 6). The presence of serotonin has been demonstrated within dental mesenchyme and craniofacial epithelial structures (7), indicating a role in tooth development; however, it is not yet verified whether the effect is direct or indirect (8). The enamel of the developing tooth is composed of mineralized enamel matrix synthesized by ectodermal cells (i.e. ameloblasts). The presence of serotonin receptors has not yet been demonstrated in ameloblasts. The aim of this study was to examine whether 5HT receptors are expressed in murine enamel organs and in the ameloblast-like cell line, LS8, and if confirmed, to study the effects of serotonin and fluoxetine on protein secretion and gene expression.

Material and methods

Enamel organs

Enamel organs from incisors and from the first molars of 3 d postnatal BALB/c mice ($n = 18$) were isolated for serotonin receptor analysis, and brain-tissue samples from the same mice were used as a positive control. Aliquots from either six molars or three incisors were pooled before protein isolation. Frozen samples were homogenized using a knife disperser (T 50 basic ultra-turrax; IKA, Staufen, Germany) in Nonidet P-40 (NP-40) lysis buffer (Invitrogen, Paisley, UK), containing 1 mM phenylmethanesulphonyl fluoride (PMSF; Sigma-Aldrich, St Louis, MO, USA) and a protease inhibitor cocktail (Sigma-Aldrich), according to the manufacturer's protocol. The amount of total protein in each sample was measured according to the Bradford assay (9) using a Bradford dye reagent concentrate (Bio-Rad, Hercules, CA, USA). Samples were diluted in NP-40 lysis buffer until equal protein concentrations were achieved.

Western blotting

Thirty microlitres of each protein lysate containing either 50 ng of total protein (from lysed enamel organs) or 20 ng of total protein (from the brain tissue positive control) were separated on a 10% Bis-Tris SDS-polyacrylamide gel in 1× 3-morpholinopropane-1-

sulfonic acid (MOPS) running buffer (Invitrogen) before electrotransfer onto Hybond-P membranes (Amersham Pharmacia Biotech, Pittsburgh, PA, USA). The transfer was performed in 1× Transferbuffer (Invitrogen), supplemented with 20% (v/v) methanol, for 1 h at 175 mA. The membranes were dried, moistened quickly in 100% methanol, and incubated for 5 min in Trisbuffered saline containing Tween 20 (TTBS buffer: 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Tween 20) and placed in the SNAP i.d. Protein Detection System (Millipore, Molsheim, France). Then, the membranes were blocked by incubation in TTBS containing 0.5% non-fat dry milk (Nestle, Copenhagen, Denmark), and then incubated for 10 min with primary antibodies (listed later in this section) diluted in TTBS containing 1.0% bovine serum albumin (BSA). After washing three times with TTBS buffer, the membranes were incubated for 10 min with horseradish peroxidase-conjugated goat anti-rabbit IgG (Cell Signaling Technology, Danvers, MA, USA) diluted (1:5,000) in TTBS containing 1.0% BSA. After washing three times with TTBS buffer, the binding of secondary antibody (listed later in this section) was visualized using the SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, Rockford, IL, USA). Detection of the western blot bands was carried out using Kodak Image Station 2000R software (Kodak, Pittsburgh, PA, USA). The sizes of the detected bands were estimated by comparison with the Magic Mark protein standard (Invitrogen) loaded on the same gel.

The following antibodies were used: rabbit polyclonal 5HT1A receptor antibody (ab64994, 1.0 $\mu\text{g ml}^{-1}$; Abcam, Cambridge, MA, USA), rabbit polyclonal 5HT2A receptor antibody (ab16028, 1.0 $\mu\text{g ml}^{-1}$) (Abcam), rabbit polyclonal 5HT2B receptor antibody (ab77006, 1.0 $\mu\text{g ml}^{-1}$; Abcam), rabbit polyclonal 5HT2C receptor antibody (ab32172, 1.0 $\mu\text{g ml}^{-1}$; Abcam), rabbit polyclonal Tph1 antibody (ab78969, 1.0 $\mu\text{g ml}^{-1}$; Abcam), rabbit N-terminal 5HTT antibody (SAB4200039, 1.0 $\mu\text{g ml}^{-1}$; Sigma-Aldrich), mouse monoclonal glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (sc137179, 0.5 $\mu\text{g ml}^{-1}$; Santa Cruz Biotechnology, Santa Cruz, CA, USA), and horseradish peroxidase-conjugated goat anti-rabbit IgG (#7074, 1:2,000 dilution; Cell Signaling Technology).

Cell culture and treatment

The LS8 cell line was maintained in Dulbecco's modified Eagle's medium (Sigma-Aldrich) supplemented with penicillin (100 units ml^{-1}) and streptomycin (100 $\mu\text{g ml}^{-1}$). The cells were cultured until confluent and then incubated with serotonin and fluoxetine (5-hydroxytryptamine hydrochloride and fluoxetine hydrochloride, respectively, both from Sigma-Aldrich) at various final concentrations (0.1, 1.0, or 10 μM).

Both cell culture media and cells were harvested after 1, 3, and 7 d of incubation. Untreated cells were used as a control at each time-point studied. Data are presented as mean $\pm$ SD of three individual experiments calculated relative to each individual control of untreated cells at each time-point.

Quantification of serotonin in cell culture medium

The Serotonin-ELISA kit IBL (Immuno Biological Laboratories, Hamburg, Germany) was used to determine the serotonin levels in cell culture media. Determinations were made in

duplicate, and the procedures were performed according to the manufacturer's manual. The absorbance was measured at 405 nm with reference wavelength 600 nm.

Calcium atomic absorption spectrophotometry

The concentration of calcium ions, $[Ca^{2+}]$, in the cell medium was measured using atomic absorption spectrophotometry. The medium was added to a solution of 2.5% lanthanum oxide in 25% HCl. Analyses were performed using a PerkinElmer 2380 atomic absorption spectrophotometer (PerkinElmer, Norwalk, CT, USA) at 422.7 nm. The spectrometer was calibrated using a calcium concentration standard curve ranging from 2.5 to 2500 μM , created by the serial dilution of an atomic absorption calcium standard (Sigma Diagnostics, Sigma-Aldrich, St Louis, MO, USA).

Alkaline phosphatase activity

Alkaline phosphatase (ALP) activity was quantified by measuring the cleavage of p-nitrophenyl phosphate (pNPP) (Sigma, Sigma-Aldrich, St Louis, MO, USA) into a soluble yellow end-product that absorbs at 405 nm. In parallel to the samples, a standard curve was constructed with calf intestinal alkaline phosphatase (Promega, Madison, WI, USA). The amount of total protein in the medium was determined using the Sigma Microprotein PR assay kit with a Protein Standard Solution Calibrator (Sigma). Analyses were performed using a Cobas Mira chemistry analyzer (Roche Diagnostics, Mannheim, Germany). Intra-assay and interassay variations were < 2.4% and 3.2%, respectively. The detection range was 10–2000 $mg\ l^{-1}$. The ALP activity, expressed as $nmol\ of\ pNPP\ min^{-1}\ mg^{-1}$ of total protein in each sample, was expressed as a percentage of the control at the selected time-points.

Lactate dehydrogenase activity

Cell viability was monitored by measuring the lactate dehydrogenase (LDH) activity in the medium, using the Cytotoxicity Detection kit (LDH) (Boehringer, Mannheim, Germany). According to the manufacturers' protocol, triplets of 50 μl of sample were added to a 96-well plate together with 50 μl of mixture (catalyst and chromogenic solution), and incubated in the dark for 30 min before measuring the absorbance in an ELISA reader (Asys expert96; Asys Hitech, Eugendorf, Austria) at a wavelength of 450 nm.

Determination of cytokine secretion into the culture medium

Multi-analyte profiling was performed using the Luminex-100 system and the XY Platform (Luminex, Austin, TX, USA). Calibration microspheres for classification and reporter readings, as well as sheath fluid, were purchased from Luminex. Acquired fluorescence data were analyzed using the SStarStation software (Version 2.0; Applied Cytometry Systems, Sheffield, UK). Before analysis, the samples were concentrated $\times 10$ using Microsep Centrifugal tubes with a nominal 3 kDa cut-off (Pall Life Science, Ann Arbor, MI, USA).

The concentration of various cytokines [fibroblast growth factor (FGF), granulocyte-macrophage colony-stimulating factor (GM-CSF), interferon- γ (IFN- γ), interleukin(IL)-1 α , IL-1 β , IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p40/p70, IL-13, IL-17, interferon-inducible protein 10 (IP-10), keratinocyte-derived chemokine (KC), monocyte chemotactic protein 1 (MCP-1), macrophage-induced gene (MIG), macrophage inflammatory protein 1 α

(MIP-1 α), tumour necrosis factor- α (TNF- α), and vascular endothelial growth factor (VEGF)] in the cell culture medium was determined using the Murine Cytokine 20-plex (BioSource, Carmarillo, CA, USA), according to the manufacturer's instructions.

mRNA isolation

Cells were lysed in lysis/binding buffer [100 mM Tris-HCl, pH 8.0, 500 mM LiCl, 10 mM EDTA, pH 8.0, 0.5 mM dithiothreitol (DTT), and 1% SDS], and mRNA was isolated using magnetic beads [oligo (dT)₂₅], as described by the manufacturer (Dynal, Oslo, Norway). Beads containing mRNA were resuspended in 10 mM Tris-HCl, pH 8.0, and stored at -70°C until use. Ten microlitres of the mRNA-containing solution was used as the template to obtain a first-strand cDNA using the iScript cDNA Synthesis kit, which contains both oligo (dT) and random hexamer primers (Bio-Rad).

Real-time PCR quantification

Reactions were performed and monitored using Stratagene's MX3000P Real-time PCR system and the 2X iQ SYBR Green Supermix, based on iTaq DNA polymerase (Bio-Rad). cDNA samples were analyzed in triplicate both for the genes of interest and for reference (housekeeping) genes (β -actin and/or GAPDH). The amplification program consisted of a pre-incubation step for denaturation of the template cDNA (5 min, 95°C), followed by 40 cycles, each of which consisted of a denaturation step (30 s, 95°C), an annealing step (30 s, 60°C), and an extension step (30 s, 72°C). The Ct value, defined as the number of cycles required to produce a detectable product above background fluorescence, was measured for each sample, and arbitrary units were calculated using standard curves that consisted of serial dilutions of cDNA from a pool of samples or controls containing the highest amounts of the specific gene analyzed. Real-time RT-PCR amplifications of β -actin and GAPDH were run in triplicate as controls to monitor RNA integrity and for normalization. The specificity of each primer pair was confirmed by melting-curve analysis and agarose-gel electrophoresis. The PCR products were also sequenced to confirm amplification of the correct product. Table 1 shows the primer sequences, the expected PCR product sizes, and gene bank accession numbers used to design the primer pairs.

Statistical evaluation

Data obtained by real-time PCR (Ct values or relative mRNA expression, calculated using the standard curve method, for the serotonin receptors, and Tph1 and 5HTT) and protein analyses passed normality and equal variance tests. Statistical comparison between groups and treatments was performed using parametric one-way ANOVA and *post hoc* Holm-Sidak tests (SIGMASTAT software; Systat Software, San Jose, CA, USA). A probability of 0.05 was considered significant.

Results

Serotonin receptors, Tph1, and 5HTT in the murine enamel organ

Protein expression analyses of 3-d-old murine enamel organs confirmed the presence of the serotonin receptors 5HT1A, 5HT2A, 5HT2B and 5HT2C, and of Tph1 and 5HTT, in both molar and incisor teeth (Fig. 1). The concentration of receptors, as well as of Tph1, appeared

to be higher in molar samples compared with incisor tissue, probably because of the larger bulk of enamel organs present in the molar teeth at this stage.

Expression of mRNAs for serotonin receptors, Tph1, and 5HTT in cultured LS8 cells is regulated by fluoxetine

We found that the mRNAs for the serotonin receptors 5HT1A, 5HT2A, 5HT2B, and 5HT2C, and for Tph1 and 5HTT, are expressed in LS8 cells (Fig. 2). The expression of the receptors, as well as of 5HTT and Tph1, was regulated by fluoxetine. Fluoxetine (0.1 and 1.0 μM) enhanced the transcription of 5HT1A, 5HT2A, 5HT2B, 5HT2C, 5HTT, and Tph1 after 1 and 3 d of incubation compared with untreated cells. After prolonged incubation (day 7), and at a higher concentration (10 μM), no significant effects were found on the level of mRNA expressed for serotonin receptors or 5HTT; however, the expression of Tph1 mRNA was reduced (Table 2).

Serotonin (0.1, 1.0, and 10 μM) acutely enhanced the expression of 5HT2C (day 1), followed by a significant down-regulation of this receptor after 3 d. The expression of 5HT1A and 5HT2B was enhanced after 3 d, whereas the expression of 5HT2A was enhanced after 7 d of incubation with serotonin compared with untreated cells (Table 2).

Effect of fluoxetine and serotonin on expression of mRNA for enamel proteins in LS8 cells

At each time-point studied, the highest dose of fluoxetine (10 μM) significantly reduced the expression of amelogenin (*AmeI*) mRNA ($P < 0.001$, for days 1, 3, and 7), matrix metalloproteinase-20 (*MMP20*) ($P < 0.001$, for days 1, 3, and 7), and enamelin (*Enam*) ($P < 0.001$, for days 1, 3, and 7), but had no significant effect on the expression of ameloblastin (*Ambn*) mRNA compared with untreated cells (Table 3).

Serotonin had no significant effect on the expression of *AmeI* and *Ambn* mRNA, whereas the expression of *MMP20* ($P < 0.001$ and $P < 0.001$, respectively) and *Enam* ($P < 0.001$ and $P < 0.001$, respectively) mRNAs were reduced by all three serotonin concentrations after days 1 and 7 (Table 3).

Fluoxetine enhances the secretion of serotonin from LS8 cells

Fluoxetine induced a dose-dependent increase of serotonin release into the cell culture medium, of up to six-fold after 1 d ($P < 0.001$) and 3.5-fold after 7 d ($P < 0.001$) at the highest fluoxetine concentration (10 μM) (Fig. 3). We observed no significant effect of either fluoxetine or serotonin on LDH activity in the cell culture medium compared with untreated controls at any time-points (data not shown).

Effect of fluoxetine and serotonin on ALP activity and $[\text{Ca}^{2+}]$

Fluoxetine enhanced the ALP activity in the cell culture medium in a dose- and time-dependent manner. The ALP activity was enhanced threefold on day 1 ($P < 0.001$), fivefold on day 3 ($P < 0.001$), and fourfold on day 7 ($P < 0.001$) after fluoxetine (10 μM) administration (Fig. 4).

The effect of serotonin was similar, in that the highest dose of serotonin (10 μ M) induced a threefold increase in ALP on day 1 ($P < 0.001$) and an increase of more than fivefold on days 3 ($P < 0.001$) and 7 ($P < 0.001$) (data not shown).

The $[Ca^{2+}]$ in the culture medium was, however, not significantly altered by the treatments at any time-points (data not shown).

Fluoxetine reduces secretion of cytokines from LS8 cells into the culture medium

The secretion of VEGF, MCP-1, and IP-10 was enhanced in untreated LS8 cells during the test period, with levels ranging from 2,349–6,617, 2,302–3,561, and 688–1,419 pg ml⁻¹, respectively. Fluoxetine (10 μ M) reduced the secretion of VEGF ($P < 0.001$, for days 1, 3, and 7) (Fig. 5A), MCP-1 ($P < 0.001$, for days 1 and 7) (Fig. 5B), and IP-10 ($P < 0.001$, days 1 and 3, $P = 0.003$, day 7) (Fig. 5C) into the cell culture medium. The secretion of FGF, GM-CSF, IFN- γ , IL-1 α , IL-1 β , IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p40/p70, IL-13, IL-17, KC, MIG, MIP-1 α , and TNF- α was not significantly affected by either fluoxetine or serotonin in our study (data not shown).

Discussion

Differentiated ameloblasts enter a secretory phase where they are characterized by the presence of the Tomes' process, which displays the secretory surface responsible for enamel protein secretion. Once the maturation phase begins, the Tomes' processes are lost as enamel protein secretion slows to nearly zero. The mouse immortalized ameloblast-like cell-line, LS8, expresses amelogenin protein and secretes it into the medium, suggesting a quasi-Tomes' process. The LS8 cell line was first described by Chen *et al.* (10) in 1992 and has previously been used in studies to characterize both amelogenin (11) and ameloblastin (12) promoter effects. However, while the LS8 cells express many of the genes associated with amelogenesis, they do not organize a typical rodent extracellular enamel matrix (13–16). LS8 cells are not true ameloblasts but a cell line derived from ameloblasts.

We found that murine enamel organs, as well as cultured murine ameloblast-like cells (LS8), express serotonin receptors, Tph1, and 5HTT, and that the expression of the enamel protein gene was regulated by serotonin and the SSRI fluoxetine in cultured LS8 cells. Fluoxetine reduced the expression of enamel proteins as well as the secretion of vascular factors and cytokines. Taken together with their effect on tooth-germ morphogenesis (8), as well as the presence of serotonin uptake within cells of the dental mesenchyme and dental epithelium (5), this suggests that serotonin regulates LS8 function in a direct manner. Alterations in serotonin levels may thus directly affect enamel growth and mineralization during tooth development.

In addition to being a neurotransmitter in the central nervous system, serotonin also exerts key regulatory functions in the gastrointestinal and cardiovascular systems, and has been suggested to be involved in embryogenesis through a diversity of receptors (17) and rate-limiting feedback mechanisms. Tph1 has been demonstrated in nerves and in the odontoblast layer of human dental pulp (18). During tooth formation, epithelial signals from oral ectoderm induce odontogenic potential in the mesenchymal cells and initiate tooth formation

(19–21). The unanswered questions are thus whether neurotransmitters, such as serotonin, participate in the regulation of tooth formation through signalling to the dental epithelium and mesenchyme, and if serotonin is involved in the control of both enamel and dentin extracellular matrix protein secretion and mineral phase maturation. The presence of serotonin receptors in enamel organs suggests regulation via the blood circulation and/or paracrine and autocrine mechanisms, and not only indirectly via the central nervous system. Additionally, serotonin levels may fluctuate during the different stages of tooth formation and differentiation. In our *in vitro* study we found that the expression of serotonin receptors was up-regulated by low dose of fluoxetine and down-regulated by high dose of fluoxetine, as well as serotonin, in LS8 cells. Fluoxetine exerted a more potent effect on the expression of the receptors compared with serotonin, presumably because of the accumulation of serotonin when re-uptake by 5HTT is decreased. We found that fluoxetine also caused an immediate increase in the serotonin level in the cell medium.

The therapeutic range for fluoxetine in serum is 0.65–2.5 μM , while the concentration in tissues, such as the bone marrow, may be as high as 100 μM and detectable up to 3 months after termination of medication (22). The fact that fluoxetine may accumulate in the bone marrow, and perhaps also in the dental pulp, is a reason for extra caution when developing and growing individuals are treated with SSRIs. It has previously been demonstrated, in both *in vivo* and *in vitro* experiments, that fluoxetine has a negative effect on bone metabolism (23) and that growing children, when placed on SSRI treatment, may have an impaired general growth rate (24). This may indicate an adverse effect of SSRI medication on mineralizing tissue.

Both serotonin and fluoxetine reduced the expression of the enamel protein gene and enhanced the ALP activity in the cell medium in LS8 cells. The use of an *in vitro* system and a murine-derived ameloblast-like cell-line limits the interpretation of our results; nevertheless, the risk for similar negative effects in humans during treatment with fluoxetine must be considered. Reduced expression of the enamel matrix gene *in vivo* leads to the production of less enamel matrix, and the ameloblasts may prematurely switch from the secretory phase to a maturation phase, allowing teeth formed during this period to erupt with enamel that is hypomineralized or hypoplastic (25), with the associated complication of caries, material failure or aesthetic defects.

Fluoxetine reduced the amounts of IP-10, VEGF, and MCP-1 in LS8 cells by more than 95% compared with the control, indicating a cumulative negative effect on angiogenesis. The distribution of blood vessels in the initial stage of tooth development has been shown to be important for differentiation of the dental organ. Complexes of blood vessels are found around the tooth organ/primordia in the dental follicle, and they enter the dental papilla during the cap stage (26). Interferon-inducible protein 10 is an anti-angiogenic protein secreted by T helper 2 (Th2) cells (27) that selectively affects endothelial cells with respect to apoptosis and proliferation (28).

Vascular endothelial growth factor is an important signalling protein involved in both vasculogenesis (the *de novo* formation of the embryonic circulatory system) and angiogenesis (the growth of blood vessels from preexisting vasculature). The presence of

VEGF in enamel organs has previously been documented (29), and VEGF is closely associated with the differentiation of the inner enamel epithelium to ameloblasts (30). Monocyte chemotactic protein 1 and VEGF are expressed in the dental follicle and participate in tooth eruption by promoting osteoclastogenesis (31). Monocyte chemotactic protein 1 is synthesized and secreted by the dental follicle cells *in vitro* and seems to be a paracrine signalling molecule between the stellate reticulum and the dental follicle (32), and an *in vivo* study in rats concluded that it was likely that MCP-1 enhancement recruits the mono-nuclear cells to the dental follicle to initiate eruption (33). The cytokines IP-10, VEGF, and MCP-1 are vascular signalling factors and might affect the vascular supply that is fundamental for normal tooth development, including odontogenic cell proliferation and differentiation. The reduced production of these vascular signalling factors at high fluoxetine concentrations *in vitro* indicates that the blood supply may be affected by fluoxetine administered during tooth organogenesis.

The effects of SSRIs on dental tissue development are still inconclusive. Silva *et al.* (34) found no morphological or structural changes in the first molar of fetuses after fluoxetine administration to pregnant rats suggesting that, at the dosage used (10 mg/kg administered for 20 days), fluoxetine did not interfere with major development of the tooth. Shuey *et al.* (35) found that, similarly to our findings, different SSRIs, including fluoxetine, at 10 μ M induced a negative effect on epithelial–mesenchymal interactions in murine embryos. Shuey *et al.* (36) found also that the amount of serotonin taken up, and the level of expression of serotonin-binding protein are specific to the different stages during craniofacial development in mouse embryos.

In conclusion, enamel organs and LS8 cells derived from ameloblasts express functional serotonin receptors that are regulated and able to respond to serotonin availability.

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Fig. 1.

Expression of the serotonin receptors 5-hydroxytryptamine (5HT)1A, 2A, 2B, and 2C, tryptophan hydroxylase 1 (Tph1), and the serotonin transporter (5HTT) in enamel organs isolated from 3-d-old mice, as detected by western blot analyses. Fifty nanograms of total protein was loaded into lanes 1 and 2, and 20 ng of total protein was loaded into lane 3 (positive control). Lane 1, incisor; lane 2, first molar; lane 3, brain tissue (positive control). The images are from representative sets of analyses. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Fig. 2. Electrophoresis of the representative PCR products, obtained by one-step RT-PCR, for serotonin receptors [5-hydroxytryptamine (5HT)1A (244 bp), 5HT2A (420 bp), 5HT2B (209 bp), and 5HT2C (498 bp)] and for tryptophan hydroxylase 1 (Tph1) (192 bp) and the serotonin transporter (5HTT) (158 bp) in murine ameloblast cells (LS8). STD is a 100-bp DNA ladder. β -Actin is a housekeeping control.

Fig. 3. Effect of fluoxetine [0.1 (●), 1.0 (▽), and 10 (■) μM] on the serotonin level in the cell culture medium. The data are presented as mean $\pm$ SD in percent of untreated cells (=100%) at all three time-points (***) $P < 0.001$.

Fig. 4.

Effect of fluoxetine [0.1 (●), 1.0 (▽), and 10 (■) μM] on the alkaline phosphatase (ALP) activity in the cell culture medium. The ALP activity was calculated relative to the total amount of proteins in each sample, and the data are presented as mean $\pm$ SD in percent of untreated cells (=100%) at all three time-points (***) $P < 0.001$.

Fig. 5.

The effect of fluoxetine [0.1 (●), 1.0 (▽), and 10 (■) μM] on the concentrations of vascular endothelial growth factor (VEGF) (A), monocyte chemotactic protein 1 (MCP-1) (B), and interferon-inducible protein 10 (IP-10) (C) in the cell culture medium. The data are presented as mean $\pm$ SD and calculated in percent of untreated cells at each time-point (** $P < 0.01$, *** $P < 0.001$).

Table 1

Primers used in real-time PCR quantification

Gene	Primer sequence	Species	GenBank accession number
<i>5HT1A</i>	S 5'-GGCATTGCTCTTGTTACT-3' AS 5'-AGCATTGCCATTACTAAAGT-3'	Mouse	NM_008308.2.
<i>5HT2A</i>	S 5'-TTCAGAAAGAAGCCACCTTG-3' AS 5'-CCTTGACTGCGACTGAATG-3'	Mouse	NM_172812.1.
<i>5HT2B</i>	S 5'-GAAGGCCCTTGGAGTCGTGT-3' AS 5'-TGCCAAATGCTTCCCGAAAT-3'	Mouse	NM_008311.1.
<i>5HT2C</i>	S 5'-CTGAGGGACGAAAGCAAAG-3' AS 5'-CACATAGCCCAATCCAAACAAAC-3'	Mouse	NM_008312.2.
<i>5HTT</i>	S 5'-GCCCGGAATCTACTAGAACCCCT-3' AS 5'-GTAATGGGCCCGGAGTGT-3'	Mouse	NM_010484.1.
<i>Thp1</i>	S 5'-AGTTGCGGTATGACCTTGAT-3' AS 5'-AGGCGAGAGACATTGCTAA-3'	Mouse	NM_009414.2.
β -actin	S 5'-CTGGCTCCTAGCACCATGA-3' AS 5'-AGGCACCAATCCACACAGA-3'	Mouse/Rat	NM_031144.2.
<i>GAPDH</i>	S 5'-ACCCAGAAAGACTGTGGATGG-3' AS 5'-CACATTGGGGGTAGGAACAC-3'	Mouse	NM_008084.2.
<i>Anel</i>	S 5'-TGAGGTGCTTACCCCTTTGAAGTG-3' AS 5'-GGAACCTGGCATCATGGTTGC-3'	Mouse	NM_009666.1.
<i>Ambn</i>	S 5'-TTCTCCCAACGCATAACTCTTC-3' AS 5'-LTTTGTGTGTCATGGTCCCGG-3'	Mouse	NM_009664.1.
<i>Enam</i>	S 5'-TGGCAATGGACTTTACCCCTATC AS 5'-GCATCAGGCACAGTTGAGTTGTAG	Mouse	NM_017468.1.
<i>MMP20</i>	S 5'-TAAGAATGCTTGCTGCTCCA-3' AS 5'-AGCCACCAGAGGATCAGA-3'	Mouse	NM_013903.2.

AS, antisense; S, sense.

Table 2

Effect of fluoxetine and serotonin on the expression of mRNA for serotonin receptors [5-hydroxytryptamine (5HT) 1A, 2A, 2B, and 2C], tryptophan hydroxylase 1 (Tph1), and the serotonin transporter (5HTT) in LS8 cells

Treatment group	Treatment time-point and drug concentration (μ M)									
	Day 1			Day 3			Day 7			
	0.1	1.0	10	0.1	1.0	10	0.1	1.0	10	
Fluoxetine										
<i>5HT1A</i>	147 $\pm$ 1.5	279 $\pm$ 166	174 $\pm$ 28	963 $\pm$ 155 *	325 $\pm$ 21 **	144 $\pm$ 50	123 $\pm$ 17	170 $\pm$ 17	166 $\pm$ 14	
<i>5HT2A</i>	604 $\pm$ 148 **	1525 $\pm$ 320 *	375 $\pm$ 135	3978 $\pm$ 72 **	156 $\pm$ 40	153 $\pm$ 2.8	153 $\pm$ 48	251 $\pm$ 71	140 $\pm$ 47	
<i>5HT2B</i>	251 $\pm$ 49	462 $\pm$ 0 **	278 $\pm$ 47 *	1337 $\pm$ 26 **	374 $\pm$ 6.2 *	230 $\pm$ 25 *	99 $\pm$ 22	73 $\pm$ 14	101 $\pm$ 18	
<i>5HT2C</i>	471 $\pm$ 21 *	2220 $\pm$ 42 **	363 $\pm$ 61 *	6484 $\pm$ 204 **	147 $\pm$ 136	190 $\pm$ 93	166 $\pm$ 15	279 $\pm$ 35	62 $\pm$ 23	
<i>Tph1</i>	106 $\pm$ 10	186 $\pm$ 4.7	56 $\pm$ 10	2044 $\pm$ 272 **	215 $\pm$ 37 *	207 $\pm$ 135	45 $\pm$ 8.6 *	506 $\pm$ 52 **	11 $\pm$ 3.6 **	
<i>5HTT</i>	314 $\pm$ 46 *	662 $\pm$ 225	239 $\pm$ 51	1130 $\pm$ 87 **	419 $\pm$ 123	132 $\pm$ 36	118 $\pm$ 46	158 $\pm$ 69	80 $\pm$ 21	
Serotonin										
<i>5HT1A</i>	65 $\pm$ 24	134 $\pm$ 6.8	32 $\pm$ 13	311 $\pm$ 14 **	286 $\pm$ 42 *	175 $\pm$ 55	142 $\pm$ 30	203 $\pm$ 7.2 *	153 $\pm$ 6.2	
<i>5HT2A</i>	105 $\pm$ 23	181 $\pm$ 83	215 $\pm$ 56	59 $\pm$ 25	96 $\pm$ 3.5	60 $\pm$ 25	195 $\pm$ 3.5 *	64 $\pm$ 8.5	84 $\pm$ 33	
<i>5HT2B</i>	245 $\pm$ 85	165 $\pm$ 30	107 $\pm$ 17	305 $\pm$ 47 *	208 $\pm$ 16 *	87 $\pm$ 13	103 $\pm$ 16	100 $\pm$ 11	114 $\pm$ 16	
<i>5HT2C</i>	379 $\pm$ 21 **	516 $\pm$ 22 **	485 $\pm$ 30 **	37 $\pm$ 14 *	34 $\pm$ 7.7 **	28 $\pm$ 0.9 **	102 $\pm$ 13	232 $\pm$ 33	238 $\pm$ 16	
<i>Tph1</i>	123 $\pm$ 28	57 $\pm$ 46	120 $\pm$ 25	236 $\pm$ 72	115 $\pm$ 98	84 $\pm$ 54	60 $\pm$ 15	63 $\pm$ 0.4 *	89 $\pm$ 20	
<i>5HTT</i>	88 $\pm$ 2.7	139 $\pm$ 20	229 $\pm$ 37 *	34 $\pm$ 22	32 $\pm$ 28	111 $\pm$ 7.3	35 $\pm$ 49	171 $\pm$ 83	172 $\pm$ 55	

Data are given as mean $\pm$ SD. Fluoxetine and serotonin were each analyzed at three different concentrations (0.1, 1.0, and 10 μ M), and the results were determined at three time-points (days 1, 3, and 7). Expression of mRNA was calculated relative to the mean of two housekeeping genes, and the results are presented in percentage of untreated cells (=100%) at all three time-points.

* $P < 0.05$,

** $P < 0.001$.

Effect of fluoxetine and serotonin on the expression of mRNA for enamel proteins in LS8 cells

Table 3

Treatment group	Treatment time-point and drug concentration (μM)									
	Day 1			Day 3			Day 7			
	0.1	1	10	0.1	1	10	0.1	1	10	
Fluoxetine										
<i>Amel</i>	92.1 ± 1.6	94.4 ± 0.8	84.7 ± 1.8 *	94.9 ± 3.8	90.7 ± 0.2	72.7 ± 2.0 *	102 ± 1.7	70.7 ± 6.8	83.9 ± 0.7 *	
<i>MMP20</i>	72.4 ± 2.5	70.7 ± 1.7	67.1 ± 1.2 *	94.8 ± 0.7	98.7 ± 0.8	82.4 ± 3.3 *	72.0 ± 3.2	73.5 ± 2.1	65.4 ± 3.9 *	
<i>Enam</i>	87.5 ± 1.2	79.2 ± 1.9	78.0 ± 1.0 *	112.9 ± 1.7	103.5 ± 0.3	83.4 ± 3.8 *	93.2 ± 1.2	87.3 ± 1.6	78.2 ± 1.5 *	
<i>Ambn</i>	100.7 ± 1.8	89.9 ± 1.5	94.7 ± 1.1	102.6 ± 1.2	96.1 ± 3.1	85.5 ± 0.9	92.6 ± 1.2	91.1 ± 1.3	95.5 ± 1.6	
Serotonin										
<i>Amel</i>	107.3 ± 1.5	91.0 ± 2.5	92.8 ± 0.88	85.7 ± 1.3	95.6 ± 0.8	104.9 ± 2.3	92.5 ± 3.6	105.4 ± 1.1	95.9 ± 1.4	
<i>MMP20</i>	67.3 ± 0.3 *	72.0 ± 2.1 *	74.8 ± 1.4 *	98.4 ± 3.0	83.1 ± 3.7	87.9 ± 1.7	76.2 ± 3.3 *	68.1 ± 2.6 *	74.4 ± 2.4 *	
<i>Enam</i>	90.8 ± 2.9	86.9 ± 0.8 *	80.7 ± 1.4 *	116.5 ± 1.0	120.4 ± 0.7	111.5 ± 3.7	87.2 ± 1.0 *	84.6 ± 1.8 *	87.5 ± 2.0 *	
<i>Ambn</i>	105.7 ± 0.2	94.4 ± 0.6	83.5 ± 4.4	111.2 ± 2.1	111.2 ± 1.4	101 ± 1.1	84.6 ± 1.9	86.4 ± 3.1	89.6 ± 1.6	

Data are given as the mean $\pm$ SD of three biological replicates. Fluoxetine and serotonin were each analyzed at three different concentrations (0.1, 1.0, and 10 μ M), and the results were determined at three time-points (days 1, 3, and 7).

Expression of mRNA was calculated relative to the expression of two housekeeping genes [β -actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH)], and the results are presented in percentage of untreated cells at each time-point.

* $P < 0.05$.