Serotonergic 5-HT$_{2B}$ Receptor Controls Tissue-nonspecific Alkaline Phosphatase Activity in Osteoblasts via Eicosanoids and Phosphatidylinositol-specific Phospholipase C*†

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In previous studies, we observed that mice knocked out for the serotonin-2B receptor (5-HT$_{2B}$R) show defects in bone homeostasis. The present work focuses on the downstream targets relaying the anabolic function of this receptor in osteoblasts. A functional link between the 5-HT$_{2B}$R and the activity of the tissue-nonspecific alkaline phosphatase (TNAP) is established using the C1 osteoprogenitor cell line. During C1 osteogenic differentiation, both 5-HT$_{2B}$R and TNAP mRNA translations are delayed with respect to extracellular matrix deposition. Once the receptor is expressed, it constitutively controls TNAP activity at a post-translational level along the overall period of mineral deposition. Indeed, pharmacological inhibition of the 5-HT$_{2B}$R intrinsic activity or shRNA-mediated 5-HT$_{2B}$R knockdown prevents TNAP activation, but not its mRNA translation. In contrast, agonist stimulation of the receptor further increases TNAP activity during the initial mineralization phase. Building upon our previous observations that the 5-HT$_{2B}$R couples with the phospholipase A2 pathway and prostaglandin production at the beginning of mineral deposition, we show that the 5-HT$_{2B}$R controls leukotriene synthesis via phospholipase A2 at the terminal stages of C1 differentiation. These two 5-HT$_{2B}$R-dependent eicosanoid productions delineate distinct time windows of TNAP regulation during the osteogenic program. Finally, prostaglandins or leukotrienes are shown to relay the post-translational activation of TNAP via stimulation of the phosphatidylinositol-specific phospholipase C. In agreement with the above findings, primary calvarial osteoblasts from 5-HT$_{2B}$R-null mice exhibit defects in TNAP activity.

Imbalances in bone formation or resorption frequently result in pathological situations, such as osteoporosis, osteopenia, or osteomalacia. Proper skeletal development and bone homeostasis notably necessitate a tight regulation of mineralization. Over the past few years, growing attention has been paid to the involvement of serotonin (5-hydroxytryptamine, 5-HT) in bone biology (1). Indeed, beyond its role as a neurotransmitter in brain (2), platelet-stored 5-HT broadly participates to the homeostasis of various tissues. For instance, 5-HT regulates cardiovascular, smooth muscle, and endocrine functions (for review, see Ref. 3).

In the periphery, 5-HT is synthesized exclusively by enterochromaffin cells of the gut and stored by platelets. Thus, because they express the serotonin transporter and diverse serotonergic receptors (4–6), osteoblasts may be directly influenced by circulating 5-HT. In agreement with this idea, inhibition of the serotonin transporter reduces bone formation (7, 8). Moreover, patients receiving selective serotonin reuptake inhibitor antidepressants appear to be at risk for osteoporosis (9). Five types of serotonergic receptors have been depicted in osteoblast primary cultures or osteoblastic cell lines: 5-HT$_{1A}$, 5-HT$_{1B}$, 5-HT$_{1D}$, 5-HT$_{2A}$, and 5-HT$_{2B}$ (4, 6, 10, 11). For instance, focusing on the 5-HT$_{1B}$ subtype, Yadav et al. have recently reported that this receptor inhibits osteoblast proliferation and negatively controls bone mass (11). As in the central nervous system (12), the relative affinity of serotonergic receptors for 5-HT and the diversity of their couplings may allow a fine-tuned response of osteoblasts depending on the external 5-HT concentration.

In previous works, we identified the 5-HT$_{2A}$ receptor (5-HT$_{2AR}$) as an important player of bone metabolism. Our first series of observations was gained using the C1 osteoprogenitor cell line (13). These cells are endowed with the capacity to recapitulate osteogenic differentiation within 12–14 days in response to β-glycerophosphate and ascorbate, with a nearly 100% frequency of differentiation (13, 14). C1 osteogenic cells implement a functional 5-HT$_{2B}$R precisely when extracellular matrix (ECM) mineralization begins (10). No other 5-HT$_{1}$R subtype is expressed along C1 osteogenic differentiation (10). During the course of the program, the G protein-coupled 5-HT$_{2B}$R continuously displays constitutive activity toward both the nitric oxide (NO) and the phospholipase A2 (PLA2)

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§ The abbreviations used are: 5-HT, serotonin; 5-HT$_{2AR}$, serotonin-2A receptor; 5-HT$_{2B}$R, serotonin-2B receptor; AA, arachidonic acid; ALP, alkaline phosphatase; COX, cyclooxygenase; ECM, extracellular matrix; LOX, lipoxygenase; L-NNA, L-N monomethyl arginine; LTB4, leukotriene B4; PGE2, prostaglandin E2; PIPLC, phosphatidylinositol-specific phospholipase C; PLA2, phospholipase A2; TNAP, tissue-nonspecific alkaline phosphatase.
pathways. In response to 5-HT, the 5-HT₂⁄₂B receptor promotes further NO production and PLA2-dependent arachidonic acid (AA) release (10). In Ref. 10, we additionally reported that the 5-HT₂⁄₂B receptor does not recruit the inositol 1,4,5-trisphosphate pathway, thus excluding a coupling to the Gₛ/11 family of G proteins, as classically but not systematically described for receptors of the 5-HT₁ family (15). The 5-HT₂⁄₂B receptor contributes to mineralization in C1 cells because incorporation of calcium within the matrix is reduced by 40% upon inhibition of the receptor signaling activity. In addition, 5-HT₂⁄₂B knock-out mice were shown to display reduced bone density, thus confirming the involvement of the receptor in osteogenesis (6).

In the present study, we establish that the tissue-nonspecific alkaline phosphatase (TNAP), a glycosylphosphatidylinositol-anchored protein involved in ECM mineralization, is a target of the 5-HT₂⁄₂B signaling pathways in C1 cells. The 5-HT₂⁄₂B receptor does not interfere with the translation of TNAP mRNA. However, the 5-HT₂⁄₂B receptor controls the enzymatic activity of TNAP. The regulation of TNAP occurs at a post-translational level, downstream from the PLA2 coupling. During the initial mineralization phase, PLA2-mediated activation of TNAP is relayed by the cyclooxygenase (COX) pathway. Therefore, the COX pathway is switched off, and the 5-HT₂⁄₂B/PLA2-dependent control on TNAP activity is exerted by the lipoxygenase (LOX) pathway. Besides, we show that the positive action of the 5-HT₂⁄₂B receptor on TNAP downstream from eicosanoids requires the activity of the glycosylphosphatidylinositol-solubilizing enzyme phosphatidylinositol-specific phospholipase C (PIPLC). Finally, primary calvarial osteoblasts obtained from 5-HT₂⁄₂B knock-out mice, which suffer from osteopenia (6), exhibit defects in TNAP activity.

**EXPERIMENTAL PROCEDURES**

**Chemicals**—Ritanserin and BW723C86 were synthesized at Hoffmann-La Roche Ltd. All other chemicals were from Sigma. All tissue culture reagents were purchased from Invitrogen.

**Cell Culture and Differentiation**—The C1 cell line has been described in Ref. 13 and is available upon request, according to the ASBMB editorial policy. C1 cells were first seeded on untreated plastic Petri dishes at 6 × 10⁵ cells/10 ml of Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum (FCS) to form three-dimensional aggregates. After 10 days, C1 aggregates were transferred to DMEM supplemented with 1% FCS depleted for 5-HT (<1 nm) (10). Osteogenic differentiation was induced (day 0) by the addition of 0.25 mM ascorbic acid and 7 mM β-glycerophosphate. Because C1 cells do not synthesize 5-HT (tryptophan hydroxylase activity <0.5 pmol/10 min per mg of protein, 5-HT content <0.2 pmol/mg of protein) (10), the absence of 5-HT is guaranteed, thus ruling out any stimulation of 5-HT₂⁄₂B receptors. For thymidine incorporation, see supplemental “Experimental Procedures.”

**Properties of the Pharmacological Drugs Used**—Drugs used to target 5-HT receptors include ritanserin and BW723C86. Ritanserin is a nonselective inverse agonist of 5-HT₂ receptors (16, 17), which binds 5-HT₂⁄₂A, 5-HT₂⁄₂B, and 5-HT₂⁄₂C receptors. BW723C86 is a selective agonist of 5-HT₂⁄₂B receptors (16, 17). Drugs used to discriminate TNAP from other alkaline phosphatase (ALP) activities include levamisole and vanadate. Levamisole is a noncompetitive inhibitor of TNAP (18). Vanadate is an inhibitor of ALP enzymes with lower affinity toward TNAP than other phosphatases (19). Other drugs used include the nonselective COX-1/COX-2 inhibitor indomethacin (20), the LOX inhibitor MK-886 (21), the NO synthase inhibitor L-NAME (1-NMA), the PIPLC inhibitor U73122 and its neutral congener U73343 (22).

**RNA Isolation and Reverse Transcription-PCR Analysis**—Total RNA isolation and semiquantitative reverse transcription-PCR were performed as previously described (10). The following specific primers were used: TNAP, forward 5′-GGAGTTGGACCGGACACTATG-3′ and reverse 5′-TTCTT-GCTCATGACGGCGGTGAAGC-3′ (432 bp); 5-HT₂⁄₂B, forward 5′-AGGAATCGAGACTGATGTGAT-3′ and reverse 5′-CTTAGGAAAACGTTGGGCACA-3′ (230 bp); glyceraldehyde-3-phosphate dehydrogenase (GAPDH), forward 5′-TGAGTTGCGTGTAACGGATTGCGC-3′ and reverse 5′-CATGTAGGCCATGAGGTCACAC-3′ (982 bp), used as internal standard.

**5-HT₂⁄₂B Receptor Knockdown**—Oligonucleotides coding for short hairpin RNA (shRNA) specifically against 5-HT₂⁄₂B were cloned into pLenti6 (a lentiviral RNAi expression system; Invitrogen). Lentivirus production and cell infection were done according to the manufacturer’s instructions. The following target region of 5-HT₂⁄₂B was chosen: GACACACTTCTGGAGCACATTT. Stable transduced clones were established by selection with blasticidin (Invitrogen). It was verified in all experiments that a scramble shRNA (sequence GCAACACCTC-CATGACTTGT) yielded values similar to controls (data not shown).

**ALP and Eicosanoid Determinations**—C1 cells were washed twice with ice-cold PBS and scraped in 10 mM Tris-HCl containing 2 mM MgCl₂ and 0.05% Triton X-100, pH 8.2. The cell suspension was treated or not with the indicated drugs before homogenization (MM301 homogenizer; Retsch AG, Haan, Germany) at 4 °C. Aliquots of supernatants were subjected to ALP activity measurement using an Elecsys automat (Roche Diagnostics) with CSPD as substrate. Cell extracts were incubated in the presence of levamisole or vanadate for 10 min prior to the measurement of phosphatase activity. TNAP protein amounts were quantitatively measured using the immunonephelometric assay Tandem-R Ostase (Beckman-Coulter). The accumulations of PGE₂ and LTB₄ in the C1 cell culture supernatants were determined using high sensitivity PGE₂ and LTB₄ enzyme immunoassay kits (Cayman Chemicals).

**Measurement of PIPLC Activity**—PIPLC activity was measured by monitoring the conversion of [³H]phosphoinositides to [³H]glycerophosphoinositol. Briefly, C1 cells were labeled with [³H]phosphatidylinositol 4,5-bisphosphate (5 μCi, 200 μCi) for 24 h at 37 °C. After labeling, cells were washed three times in HBSS containing calcium and magnesium and supplemented with 20 mM HEPES and 0.1% BSA (experimental medium). Following exposure to the indicated drugs in experimental medium for 24 h, C1 cells were washed and scraped, and analysis of PIPLC products was carried out as in Ref. 23. Specific PIPLC activity was calculated as the phosphatidylinositol 4,5-
bisphosphate-sensitive formation of [3H]glycerophosphoinositol/min per mg of protein.

Calvarial Osteoblast Primary Culture—Primary osteoblasts were enzymatically isolated from calvaria of neonatal (2–3-day old) wild-type (WT) and 5HT2BR−/− mice as described in Ref. 6. Briefly, dissected calvaria were sequentially digested in a PBS collagenase solution containing 0.2% collagenase IV and 0.01% deoxyribonuclease for 70 min at 37 °C. After centrifugation, calvaria cells were collected and expanded for 5 days in α-MEM supplemented with 10% FCS depleted of 5-HT, 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cells were then plated at 10⁴ cells/cm² in the differentiation medium containing 50 μg/ml ascorbic acid and 10 mM β-glycerophosphate. ALP activity was measured after 7 days of culture.

Data Analysis and Statistics—The nonparametric Kolmogorov-Smirnov test was used for statistical analysis of small groups. All values are given as means ± S.E.

RESULTS

5-HT2B Rs Control TNAP Activity via PLA2 and PIPLC

5-HT2B Rs Intrinsic Signaling Controls the Peak in ALP Activity Associated with Osteogenic Differentiation—In 5-HT2B Rs knock-out mice, the age-dependent decrease in bone formation was shown to correlate with low levels of plasma ALP activity (6). Our goal was to delineate the relationship between the 5-HT2B Rs and ALP further. In a first set of experiments, we exploited the kinetics of C1 osteogenic differentiation to evaluate the dynamics of ALP activity in relation to other steps of the program, and notably the onset of 5-HT2B Rs prior to mineral deposition. Total ALP activity was measured in homogenates of C1 nodules during the kinetics of differentiation (Fig. 1A). Before the onset of the receptor, at day 5 of the program, ALP activity was faintly detectable. At day 7, ALP activity became measurable, reaching its maximal value at day 10. Between day 10 and day 12, when C1 cells converted into osteocytes and ceased dividing (supplemental Fig. 1), ALP activity remained stable.

To assess a functional link between 5-HT2B Rs and ALP, the receptor constitutive activity was quenched with ritanserin (30 nm) from day 0 (or day 5, not shown) of the osteogenic program. Ritanserin is a nonselective inverse agonist of 5-HT2 receptors, i.e. 5-HT2A, 5-HT2B, and 5-HT2C (16, 17). The absence of 5-HT2A and 5-HT2C receptors along C1 osteogenic differentiation warrants the use of ritanserin to quench the constitutive activity of the 5-HT2B Rs (10). Although ritanserin does not hinder the conversion of C1 cells into osteocytes, it reduces ⁴⁵Ca²⁺ incorporation within the ECM by 40% (10). Upon addition of ritanserin, ALP activity was still turned on at day 7. However, the intensity of phosphatase activity was reduced by 45% and remained constant from day 7 up to day 12 (Fig. 1A). In parallel experiments, we analyzed the impact of shRNA-mediated knockdown of the 5-HT2B Rs on total ALP activity during the kinetics of C1 differentiation. As shown in Fig. 1A, 5-HT2B Rs silencing interfered with the raise in ALP activity from day 7 and yielded ALP activity values comparable with those obtained with ritanserin. We therefore conclude that the 5-HT2B Rs controls a component of ALP activity that normally peaks during matrix mineralization.

To account for this observation, we may assume that at least two distinct ALP enzymatic activities superimpose during differentiation. A first set of phosphatase activity would sustain the basal level of ALP, independently from the onset of 5-HT2B Rs signaling. A second phosphatase activity would yield the peak of ALP activity that is switched off by ritanserin.

We may also note that the above experiments were performed in the absence of 5-HT (see “Experimental Procedures” and Ref. 10). Thus, the control of ALP activity by the 5-HT2B Rs between days 7 and 12 has to be ascribed to the intrinsic activity of the receptor.

TNAP Is a Downstream Target of 5-HT2B Rs Intrinsic Signaling—Although it is not osteoblast-specific, TNAP is considered as a phenotypic marker of osteogenic cells. Its activity is necessary to achieve optimal bone mineralization (24). We therefore evaluated whether the 5-HT2B Rs-dependent ALP activity identified above could be assigned to TNAP. To this purpose, C1 cell extracts were incubated with levamisole (4.5 mM), an inhibitor of TNAP (18). Under this treatment, ALP activity in samples collected at days 7, 10, and 12 of differentiation remained at the value measured with extracts from ritanserin-treated cells (Fig.
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5-HT2B Rs Control TNAP Activity through Post-translational Mechanisms—The question then arose as to whether, from day 7, TNAP molecules were rendered functional by de novo mRNA transcription or translation or through post-translational maturation. The levels of TNAP transcripts and proteins were followed in C1 cells throughout differentiation (Fig. 1, B and C). TNAP mRNAs were present as soon as day 0, thus indicating that, before their recruitment toward the osteogenic program, C1 mesoblastic cells already transcribe the TNAP gene. The amount of 5-HT2B R and TNAP mRNAs did not vary during the 12 days of differentiation (Fig. 1B). Thus, the 5-HT2B Rs-mediated TNAP regulation necessarily involves post-transcriptional events.

Anti-TNAP antibodies were then used to follow TNAP synthesis (Fig. 1C). In radioimmunoassays using C1 cell homogenates, the TNAP protein was faintly detectable up to day 5. At day 6, i.e. 1 day before the increase in levamisole-sensitive phosphatase activity, TNAP concentration increased and reached a plateau from day 8 onward (Fig. 1C). Interestingly, TNAP protein expression was not changed upon exposure of C1 cells to ritanserin. This indicates that the 5-HT2B R does not interfere with the translation of TNAP mRNA. The amounts of immunoreactive TNAP molecules and total ALP activity during differentiation are plotted on Fig. 1C. Clearly, de novo TNAP synthesis starts 1 day before ALP activity becomes detectable. Moreover, although it cancels TNAP activity, ritanserin does not change the capacity of C1 cells to synthesize TNAP. Our data thus favor the view that in osteogenic cells, preexisting TNAP molecules are turned on from an inactive to an active state by post-translational controls driven by functional 5-HT2B Rs.

Agonist-dependent Coupling of 5-HT2B R to TNAP Is Restricted to the Beginning of Matrix Mineralization—We next evaluated the impact of a 5-HT2B R stimulation on ALP function. Total ALP activity was measured after exposure of C1 cells to BW723C86, a selective agonist of the 5-HT2B R (25). Addition of 1 μM BW723C86 at day 5, i.e. when the receptor is implemented, did not elicit any additional ALP activity. However, upon stimulation at day 6, a 2-fold increase in ALP activity was recorded at day 7 (Fig. 2, inset). Fig. 2 shows the values of ALP activity in homogenates of cells exposed or not to BW723C86 at days 6, 9, or 11. All of the values were measured 1 day after agonist addition. Up to day 9, BW723C86 promoted a significant stimulation of ALP activity. From day 10 onward, ALP values remained at the level measured in the absence of agonist.

We further observed that, upon exposure of BW723C86-treated cell extracts to levamisole, ALP activity systematically decreased to values equal to those obtained after inhibition of the 5-HT2B R by ritanserin (Fig. 2). Thus, the agonist-dependent rise in ALP activity may unambiguously be assigned to TNAP.

In a more physiological paradigm, we examined the impact of 5-HT stimulation (10 nM, 24 h) on total ALP activity along the osteogenic differentiation of C1 cells. The value obtained at day 7 was similar to that obtained following the BW723C86 treatment (Fig. 2). At day 10, 5-HT still triggered an increase in ALP activity, although less important than that registered with BW723C86. In contrast, at day 12, 5-HT promoted a reduction of ALP activity to a value similar to that measured in levamisole-treated extracts, which corresponds to non-TNAP phosphatase activity (Fig. 2).

Altogether, the data show that, from day 7 to day 10 of the osteogenic program, agonist- or 5-HT-mediated activation of the 5-HT2B R promotes further recruitment of TNAP. This time window coincides precisely with the very beginning of bone matrix mineralization. When C1 cells convert into osteocyte-like cells (supplemental Fig. 1) (10), i.e. from day 10 onward, the 5-HT2B R loses its ability to stimulate TNAP activity in an agonist-dependent manner. At this stage, the induction of another yet to be identified 5-HT receptor subtype must be postulated to account for the negative action exerted by 5-HT on the 5-HT2B R-mediated control of TNAP activity.

5-HT2B R Turns on TNAP Activity via PLA2—In C1 cells, from day 5 of differentiation onward, the 5-HT2B R is coupled to NO production and PLA2-mediated AA release (10). As shown in Fig. 3, the increase in TNAP enzymatic activity was not
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FIGURE 3. PLA2/AA signaling pathway mediates activation of TNAP by 5-HT<sub>2B</sub>R during the overall period of mineral deposition. In C1 osteogenic cells, from day 5 until terminal differentiation, 5-HT<sub>2B</sub>Rs couple to NO synthases and PLA2 in both agonist-dependent and -independent manners. Exposure of C1 cells to L-NMA (100 μM, 24 h), a selective inhibitor of NO synthases, in the presence or absence of 1 μM BW273C86, has no significant impact on the increase in ALP activity accompanying osteogenic differentiation. L-NMA does not affect the basal level of phosphatase/ATPases either. In contrast, exposure of C1 cells to mepacrine (100 nm, 24 h), a specific inhibitor of PLA2, alone or in combination with BW273C86, abrogates the 5-HT<sub>2B</sub>R-dependent TNAP activation. Values recorded along differentiation coincide with those measured after inactivation of the receptor by ritanserin. Values are the means ± S.E. (error bars) of four independent experiments.

affected by L-NMA addition (100 μM) to the culture medium throughout differentiation. Thus, we may exclude an involvement of NO in the 5-HT<sub>2B</sub>R-mediated TNAP activation. In contrast, addition at day 6 of the PLA2 inhibitor mepacrine (100 nm) abolished the 5-HT<sub>2B</sub>R-dependent TNAP activation normally observed from day 7 (Fig. 3). Values recorded on days 7, 10, and 13 coincided with those measured after direct inhibition of the 5-HT<sub>2B</sub>R signaling activity by ritanserin (Fig. 1A). Addition of BW273C86 (1 μM) in combination with mepacrine did not restore any TNAP activity (Fig. 3). We therefore conclude that (i) the autocrine activation of TNAP observed during the overall period of matrix mineralization is elicited by the constitutive PLA2 coupling to the receptor; and (ii) the rise in TNAP activity upon agonist stimulation of the receptor, as observed between day 7 and day 10, is also relayed by the PLA2 pathway.

5-HT<sub>2B</sub>R/PLA2 Coupling Contributes to Osteogenic Differentiation of C1 Cells by Controlling the COX and LOX Pathways in a Stepwise Manner—In C1 osteoblasts, the 5-HT<sub>2B</sub>R is coupled to AA release from day 5 until terminal differentiation. From day 5 to day 10, AA release is followed by a COX-dependent PG2 synthesis (Fig. 4A) (10). PG2 production depends on the 5-HT<sub>2B</sub>R because it is canceled by ritanserin (Fig. 4A) (10) or upon shRNA-mediated down-regulation of the receptor (Fig. 4A). From day 10, we observed a shunt from the COX to the LOX pathway, downstream from the 5-HT<sub>2B</sub>R/PLA2/AA coupling. Indeed, at this stage, PG2 production was quenched to the benefit of LTb4 synthesis (Fig. 4A). Again, ritanserin- or 5-HT<sub>2B</sub>R-specific shRNA canceled LTb4 synthesis, thus demonstrating that the 5-HT<sub>2B</sub>R intrinsic activity controls the basal production of eicosanoids.

We next evaluated the contribution of COX/LOX signaling on 5-HT<sub>2B</sub>R-dependent TNAP regulation. First, C1 cells were exposed for 24 h to 5 μM indomethacin, a COX inhibitor, in combination or not with 1 μM BW723C86. As shown in Fig. 4B, blockade of the COX pathway switched off the 5-HT<sub>2B</sub>R-related TNAP activation observed between day 7 and day 10 of differentiation. As anticipated, ALP activity was insensitive to indomethacin at later stages (Fig. 4B). ALP activity was further measured in C1 cells exposed for 24 h to the LOX inhibitor MK-886 (10 μM). Again, as expected, ALP activity was insensitive to MK-886 up to day 10 (Fig. 4B). Added at day 12, MK-886 abolished the 5-HT<sub>2B</sub>R-dependent TNAP activity (Fig. 4B).

Altogether, these data highlight that the switch from PG2 production to leukotrienes synthesis delineates specific stages in the regulation of TNAP activity. The shunt from COX-derived prostaglandins synthesis to LOX-derived leukotrienes production coincides with the conversion of C1 osteoblasts into osteocytes.

Control Exerted by 5-HT<sub>2B</sub>R on TNAP Is Relayed by PIPLC Activity—The above findings show that the 5-HT<sub>2B</sub>R turns on TNAP activity by post-translational mechanisms. The report by Ciancaglini et al. that the activity of PIPLC-solubilized TNAP is largely increased (>50-fold) compared with the membrane-bound enzyme (26) prompted us to assess whether PIPLC may contribute to TNAP activation downstream from the 5-HT<sub>2B</sub>R. First, total ALP activity was measured along differentiation after exposure of C1 cells to 50 nm U73122 (24 h), a selective inhibitor of PIPLC (22), or its neutral congener U73343 (22). With U73122, but not U73343, total ALP activity was reduced to values similar to those measured in cell extracts treated with the TNAP inhibitor levamisole (Fig. 5A). Hence, blockade of PIPLC activity switches off the peak in ALP activity that corresponds to TNAP. We further quantified PIPLC activity along C1 osteogenic differentiation. As shown in Fig. 5B, PIPLC activity was turned on from day 7 and increased further.
at day 10. Similarly to TNAP activity, PIPLC activity remained stable between days 10 and 13. Of note, whatever the differentiation stage, inhibition of the 5-HT<sub>2B</sub>R intrinsic activity with ritanserin drastically reduced PIPLC activity to a basal level corresponding to <13% of the maximal value, observed at day 10 (Fig. 5B). On the contrary, agonist stimulation of the 5-HT<sub>2B</sub>R by addition of BW723C86 up-regulated PIPLC activity between days 7 and 10. If added at day 12, BW723C86 failed to induce any PIPLC activity. Finally, PIPLC activity remained at its basal value, as measured with ritanserin, when C1 cells were exposed to the COX inhibitor indomethacin (5 μM, 24 h) or MK-886 (10 μM, 24 h) also reduces PIPLC activity, thus indicating that the control exerted by the 5-HT<sub>2B</sub>R intrinsic activity on PIPLC is relayed by the downstream eicosanoid effectors. With the agonist BW723C86 (1 μM, 24 h), further PIPLC activity is measured up to day 10, which is sensitive to ritanserin or indomethacin. Values are the means ± S.E. of four independent experiments.

5-HT<sub>2B</sub>R Controls TNAP Activity in Primary Calvarial Cells—
We next took advantage of 5-HT<sub>2B</sub>R knock-out mice to extend our results to ex vivo cultures. Primary osteoblasts were isolated from calvaria of neonatal WT and 5-HT<sub>2B</sub>R knock-out mice. As shown in Fig. 6, in a 5-HT<sub>2B</sub>R-null context, total ALP activity as measured at day 7 of the culture was 50% of that in WT cells. No change in ALP activity was observed upon exposure of WT or 5-HT<sub>2B</sub>R<sup>−/−</sup> calvarial cells to 5-HT (10 nM, 24 h) (Fig. 6). This likely relates to the presence of 5-HT in the cultures, despite the use of 5-HT-depleted serum (6). The presence of 5-HT in the medium may thus impair the detection of biochemical effects in response to an addition of extra serotonin. Treatment of WT osteoblasts with the inverse agonist ritanserin (30 nM) from day 0 yielded a 70% reduction in total ALP activity at day 7. This stronger effect upon pharmacological inhibition compared with genetic inactivation of the 5-HT<sub>2B</sub>R may be accounted for by the expression of 5-HT<sub>2B</sub>R in calvarial cultures (6). These receptors are absent in C1 osteogenic cells (10). These 5-HT<sub>2B</sub>Rs are also targets of ritanserin (16, 17), and their expression is up-regulated in a 5-HT<sub>2B</sub>R-null context (6). In agreement with the above idea, we observed that addition of ritanserin to 5-HT<sub>2B</sub>R<sup>−/−</sup> mice-derived osteoblasts promoted a 40% reduction in the level of total ALP activity (Fig. 6). This resulting ALP activity compared with that measured in WT calvarial cultures exposed to ritanserin.
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**FIGURE 7. Dynamic regulation of TNAP activity by 5-HT_{2B}R signaling to PLA2 and PIPLC during osteogenic differentiation.** Dormant mRNAs encoding 5-HT_{2B}R and TNAP are present from the mesoblastic stage. Translation occurs once C1 cells are embedded in a type 1 collagen matrix (day 5). The 5-HT_{2B}R is rendered functional on day 5, prior to mineral deposition. At this stage, TNAP is present but in an inactive form. The 5-HT_{2B}R turns on TNAP to an active state through PLA2 signaling from day 7 until terminal differentiation. The osteogenic program is associated with a switch in signaling downstream from the 5-HT_{2B}R-related PLA2 coupling. The shunt from COX-dependent PGE2 to LOX-dependent LTB4 production at day 10 of the osteogenic program delineates two distinct stages of TNAP regulation. Both eicosanoids recruit PIPLC to relay the 5-HT_{2B}R-mediated activation of TNAP.

**DISCUSSION**

Despite considerable effort to dissect the molecular pathways sustaining osteogenesis, the mechanisms allowing proper mineralization and bone formation are not fully understood. Critical players of bone biology include the tissue-nonspecific ALP, whose expression with type 1 collagen is currently assumed to be necessary and sufficient to account for the spatial restriction of mineralization to bone and teeth (27). In osteoblasts, TNAP is involved in the control of the P_{i}/P_{i} ions ratio. It thereby governs hydroxyapatite crystals formation within the type 1 collagen bone ECM (28–30). Such a role is exemplified by the severe decreases in ECM mineralization observed in TNAP-deficient mice or in human patients with hereditary hypophosphatasia (24, 31, 32). In addition, ectopic TNAP expression in collagen-expressing cells induces pathological mineralization (27).

To gain insight into the mechanisms involved in the implementation and regulation of TNAP activity during mineralization, we took advantage of the C1 mesoblastic cell line, which recapitulates osteogenic differentiation in vitro in a homogeneous and synchronous manner. The data show that the 5-HT_{2B}R signaling plays a role in the control of TNAP activity.

At day 0, condensing C1 mesoblastic cells instruct permissive signals that turn on the production of mRNAs related to osteogenic differentiation, in agreement with in vivo observations (33). TNAP and 5-HT_{2B}R mRNAs are among such transcripts. Their levels remain roughly constant from the stem cell state up to terminal differentiation. However, these transcripts are dormant during the initial stages of differentiation, thus pointing to yet-to-be elucidated signals repressing their maturation and/or translation.

Between day 2 and day 4, C1 cells build up a profuse type 1 collagen matrix (14). The translation of both TNAP and 5-HT_{2B}R mRNAs begins at day 5. Possibly, common signal(s) turn on the translation of the two transcripts. At this stage, C1 cells have elaborated a widespread type 1 collagen matrix. As suggested by the recent work of Hennessy et al. (34), we may propose that activation of collagen-selective integrins recruits downstream signaling cascades that switch on the translation of TNAP and 5-HT_{2B}R mRNAs.

At day 6, 5-HT_{2B}Rs functionally couple with NO and PLA2/AA. At variance with the most classic schemes of 5-HT_{2} receptor signaling, the 5-HT_{2B}R does not function through the G_{i/11} family of G proteins in C1 cells (10). Instead, we may consider an involvement of Go13 (35) or Goz (36) in the recruitment of the NO and PLA2 pathways, respectively, as observed in neuronal cells. Another possibility would be G protein-independent signaling (35). At this stage, the TNAP protein becomes overtly expressed but still lacks catalytic activity. A post-translational induction of TNAP activity thus appears to be one limiting step during the program. It may account for the delay between ECM formation and mineral deposition, despite the presence of P_{i} (β-glycerophosphate) in the culture medium from the earliest stages of the program.

Day 7 marks the beginning of mineralization. It coincides with the onset of TNAP activity from preexisting TNAP molecules and that of other yet-unidentified ALP activities. The latter phosphatase(s) sustain(s) an unvariant basal level of activity from day 7 up to the end stage of differentiation. Although these phosphatases are not controlled by the 5-HT_{2B}R, the receptor switches on TNAP activity by post-translational mechanisms. TNAP activation involves PIPLC, whose activity is stimulated from day 7 and up to day 10 (i.e. during the initial period of mineralization) by the PLA2/AA/COX/PGE2 pathway. The regulation of TNAP activity is under the control of the receptor intrinsic activity, as shown by either pharmacological or shRNA knock-down approaches. However, additional up-regulation of TNAP activity can be obtained in response to agonist stimulation.

From day 10 onward, the 5-HT_{2B}R continues to control TNAP activity in a cell-autonomous manner. However, it loses its ability to elicit further TNAP activation upon agonist exposure. This time window coincides with a switch in signaling intermediates downstream from the 5-HT_{2B}R-PLA2 coupling.
with AA being now metabolized into LTB4 through LOX instead of prostaglandins. leukotoienes continue to mediate the intrinsic action of the 5-HT_{2B}R on TNAP activity. They also act upstream from PIPLC in the pathway.

The functional link between the 5-HT_{2B}R and TNAP activity in C1 cells is summarized in Fig. 7. The control exerted by the 5-HT_{2B}R on TNAP activity is imparted by eicosanoids, which in turn activate PIPLC. Eicosanoids are known to be local regulators of bone metabolism (37). Some pathways sustaining the anabolic action of prostaglandin EP2 and EP4 receptors have been recently uncovered (38). Our unpublished results indicate that EP4 receptors are expressed at later stages will deserve further investigation. Further work will also be necessary to solve the mechanisms through which eicosanoids control PIPLC activity.

Our study also shows defects in TNAP activity in primary calvarial osteoblasts derived from 5-HT_{2B}R knock-out mice. Moreover, as observed in C1 cells, the activity of TNAP in WT calvaria is controlled by PIPLC.

In conclusion, our data establish a positive involvement of the 5-HT_{2B}R in mineralization and allow us to relate the osteopenic phenotype of 5-HT_{2B}R knock-out mice (6) to a deficit in TNAP activity. The functional impact of 5-HT signaling in the skeleton is still very much debated because both enhancing and inhibitory effects on bone formation have been depicted in response to serotonin (1). Such diverse effects may be explained by the concurrent expression of several 5-HT receptor subtypes (4, 6, 10, 11) and downstream couplings, which may vary according to the differentiation state of bone-forming cells. For instance, the negative regulation exerted by 5-HT on TNAP activity when C1 cells have reached the osteocyte stage, at variance with the positive effect at terminal differentiation state of bone-forming cells. For instance, the negative regulation exerted by 5-HT on TNAP activity when C1 cells have reached the osteocyte stage, at variance with the positive effect at terminal differentiation state of bone-forming cells. For instance, the negative regulation exerted by 5-HT on TNAP activity when C1 cells have reached the osteocyte stage, at variance with the positive effect at terminal differentiation state of bone-forming cells. For instance, the negative regulation exerted by 5-HT on TNAP activity when C1 cells have reached the osteocyte stage, at variance with the positive effect at terminal differentiation state of bone-forming cells. For instance, the negative regulation exerted by 5-HT on TNAP activity when C1 cells have reached the osteocyte stage, at variance with the positive effect at terminal differentiation state of bone-forming cells. For instance, the negative regulation exerted by 5-HT on TNAP activity when C1 cells have reached the osteocyte stage, at variance with the positive effect at terminal differentiation state of bone-forming cells.

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