Pathway of Maternal Serotonin to the Human Embryo and Fetus

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Serotonin [5-hydroxytryptamine (5-HT)] is essential to intrauterine development, but its source is debated. We used immunocytochemistry to gauge 5-HT, its biosynthetic enzyme tryptophan hydroxylase 1 (TPH1); an importer (serotonin transporter, 5-HTT/SERT/SLC6A); other transporters [P-glycoprotein 1 (P-gp/ABCB1), OCT3/SLC22A3, and gap junction connexin-43]; and the 5-HT degradative enzyme monoamine oxidase A (MAOA) in sections of placentas. In humans, 5-HT was faintly stained only in first-trimester trophoblasts, whereas TPH1 was not seen at any stage. SERT was expressed in syncytiotrophoblasts and, more strongly, in cytotrophoblasts. MAOA was prominent in syncytiotrophoblasts, OCT3 and gap junctions were stained in cytotrophoblasts, and P-gp was present at the apical surfaces of both epithelia. 5-HT added to cultured placental explants accumulated in the trophoblast epithelium and reached the villus core vessels. Trophoblast uptake was blocked by the SERT inhibitor escitalopram. Inhibition of gap junctions with heptanol prevented the accumulation of 5-HT in cytotrophoblasts, whereas blocking OCT3 with decynium-22 and P-gp with mitotane led to its accumulation in cytotrophoblasts. Reducing 5-HT destruction by inhibiting MAOA with clorgyline increased the accumulation of 5-HT throughout the villus. In the mouse fetus, intravascular platelets stained prominently for 5-HT at day 13.5, whereas the placenta and yolk sac endoderm were both negative. TPH1 was not detected, but SERT was prominent in these mouse tissues. We conclude that serotonin is conveyed from the maternal blood stream through syncytiotrophoblasts, cytotrophoblasts and the villus core to the fetus through a physiological pathway that involves at least SERT, gap junctions, P-gp, OCT3, and MAOA. (***Endocrinology*** **159: 1609–1629, 2018)**

Serotonin [5-hydroxytryptamine (5-HT)] is a multifunctional intercellular signal molecule (1–4). It plays an important role in intrauterine development (5–11). The involvement of 5-HT in the development of the fetal brain and in autism spectrum disorder is of particular interest (12–20).

Our focus here is on the source of the 5-HT provided to the conceptus. In principle, it could be supplied by the embryo and/or the fetus itself, the placenta, and/or the maternal bloodstream (21). Evidence for the production of 5-HT in the conceptus is limited (8, 21, 22). Rather, recent reports have concluded that embryonic and fetal 5-HT is produced by placental tissues (18, 19, 23–27). In contrast, our study found no evidence for placental 5-HT synthesis in either mice or humans. Consistent with this observation, other researchers suggest that some of the copious 5-HT in the platelets in the mother's blood that bathes the placenta could be transferred to the embryo and fetus in a regulated fashion (6, 8, 28). That a physiologic pathway might convey 5-HT from the maternal blood stream to the embryo and fetus is the premise we have now investigated.

Materials and Methods

Specimens

First- and second-trimester specimens from the elective termination of pregnancy, ranging from 7 to 15 weeks of gestation (based on last menstrual period), were collected from

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Abbreviations: 5-HT, 5-hydroxytryptamine; CAS, Chemical Abstracts Service; DMSO, dimethyl sulfoxide; IgG, immunoglobulin G; IHC, immunohistochemistry; MAOA, monoamine oxidase A; NBF, neutral buffered formalin; OCT3, organic cation transporter 3; P-gp, P-glycoprotein; TPH1, tryptophan hydroxylase 1.

otherwise healthy women with no known genetic or other abnormalities, as previously described (29). All women who provided first- and second-trimester placentas signed an informed consent (protocol no. 021-06-972) approved by the ethical committee of the Bnai Zion Medical Center, Haifa, Israel, under Helsinki convention guidelines. Samples from term placentas were collected anonymously from normal healthy patients undergoing elective, repeated cesarean sections. All women who provided term placental samples signed an informed consent (Yale Institutional Review Board protocol no. 1208010742). Specimens were fixed in 10% neutral buffered formalin (NBF) for at least 1 day and embedded in paraffin, after which 5-μm sections were placed on coated glass slides designed for immunohistochemistry (IHC) processing.

C57BL/6J wild-type mice were purchased from Jackson Laboratories (Bar Harbor, ME). For timed pregnancy experiments, female mice, each 8 weeks old, were mated with male mice, 10 to 14 weeks old and of proven fertility, with the morning of vaginal plug detection being considered as embryonic day 0.5. Mice were maintained in the Animal Facility of Yale School of Medicine and were treated under an approved Yale University Institutional Animal Care and Use Committee protocol. They were euthanized by cervical dislocation and gravid mouse uteri at 13.5 days' gestation, along with samples of internal organs from the same mothers, were placed in NBF, fixed overnight, and processed as described. All tissues from the same mouse were analyzed in the same IHC run to avoid staining variability among experiments.

Explant culture

Pieces of placenta, approximately 5 × 3 × 3 mm, were excised from the soft parenchymal center of placentas collected no more than 20 minutes after cesarean section. Approximately 1-cm thin slices were cut across the width of the still-warm placentas, laid on their sides immersed in warmed Dulbecco's modified Eagle medium /F12 media (to minimize clotting of the maternal blood) on a Teflon cutting board and then cut into thin strips. These were cut—avoiding the maternal and fetal surfaces—into the final explants that were placed in Dulbecco's modified Eagle medium /F12 in a sterile Petri dish and incubated on a gyrating platform in a 37°C chamber for 10 to 90 minutes with or without 5-HT and/or inhibitors. Serotonin hydrochloride [H9523; Chemical Abstracts Service (CAS) no. 153-98-0; Sigma-Aldrich, St. Louis, MO] was dissolved in sterile medium. Escitalopram oxalate (PHR1733; CAS no. 219861-08-2; Sigma-Aldrich), a competitive inhibitor (K_i = 2.5 nM) of SERT, was dissolved in sterile medium and added to the tissue pieces 10 minutes before the 5-HT. *N*-Methyl-*N*-propargyl-3-(2,4-dichlorophenoxy)propylamine hydrochloride (clorgyline; M3778; CAS no. 17780-75-5; Sigma-Aldrich), a selective irreversible inhibitor of monoamine oxidase A (MAOA), was dissolved in water and added to the tissue pieces 30 minutes before the 5-HT. 1-Heptanol (H2805; CAS no. 111-70-6; Sigma-Aldrich), a competitive inhibitor of gap junction connexins, was dissolved in dimethyl sulfoxide (DMSO; D4540; CAS no. 67-68-5; Sigma-Aldrich) and added to the tissue pieces 20 minutes before the 5-HT. 1,1′-Diethyl-2,2′-cyanine iodide (D22; 323764; CAS no. 977-96-8; Sigma-Aldrich), a competitive inhibitor of organic cation transporter 3 (OCT3), was dissolved in DMSO and added to the tissue pieces 20 minutes before the 5-HT. Mitotane (25925; CAS no. 53-19-0; Sigma-Aldrich), an inhibitor of P-glycoprotein (P-gp), was dissolved in DMSO and added to the tissue pieces 20 minutes before the 5-HT. After incubations, the tissue pieces were fixed overnight in NBF and processed as described to generate sections for IHC. After IHC (described in detail later), the explants were assessed for 5-HT staining in the outer (*i.e.*, most peripheral) one to 10 villus cross-sections of each explant.

Antibodies and IHC

The following rabbit polyclonal antibodies were used: anti–5-HT [S5545, used at 1.6 μg immunoglobulin G (IgG)/mL; Sigma-Aldrich]; anti-MAOA (sc-20156, used at 0.063 μg IgG/mL; Santa Cruz Biotechnology, Santa Cruz, CA); anti–tryptophan hydroxylase 1 (TPH1; HPA022483, used at 0.5 μg IgG/mL; Sigma-Aldrich); anti-SERT (LS-C179236, used at 0.5 μg IgG/mL; LifeSpan BioSciences, Seattle, WA); anti-ABCB1 (P-gp 1; HPA002199, used at 0.1 μg IgG/mL; Sigma-Aldrich); anti–connexin-43 (gap junctions; C6219, used at 0.18 μg IgG/mL; Sigma-Aldrich); anti-SLC22A3 (OCT-3; HPA029750, used at 1 μg IgG/mL; Sigma-Aldrich); and, as a negative control, normal rabbit serum (R9133, used at 1 μg IgG/mL; Sigma-Aldrich). Antibody concentrations were chosen to produce strong staining in the positive cellular structures without background staining.

Serial sections (5 microns each) were immunohistochemically stained using EnVision+ horseradish peroxidase Rabbit DAB+ (K4011; Dako Agilent, Santa Clara, CA) to mark the sites of antibody binding with a brown deposit [Supplemental Materials and Methods; and see Kliman *et al.* (29)]. To minimize run-to-run variability, replicate samples were stained simultaneously with one antibody. Positive control sections from a de-identified normal human appendix or mouse colon were analyzed concurrently with each batch of slides. The stained sections were counterstained with hematoxylin.

Figure 1 presents positive controls for the key immunochemical reactions. Figure 1A shows the intense staining of 5-HT in the enterochromaffin cells of the appendix and their characteristic thin apical snout making contact with the gland lumen (arrow). Extracellular 5-HT is seen in spaces at the base of some positive cells (arrowheads) and in neighboring stromal nuclei. Figure 1B shows intense staining of TPH1 confined to enterochromaffin cells, often filling their thin snout as it contacts the gland lumen (arrow). MAOA immunoreactivity (Fig. 1C) is seen as a punctate pattern throughout the cytoplasm of appendix glandular cells, consistent with a mitochondrial disposition (arrowheads). Figure 1D shows no SERT staining in appendix cells; however, platelets within the vascular spaces (arrows and arrowheads) are clearly positive. Normal rabbit serum served as our primary antibody negative control and revealed no DAB staining (Fig. 1D inset).

For quantification of immunoreactivity, normal-term placentas were each randomly sampled four times, processed for IHC, and inspected microscopically using a raster pattern to ensure the entire histologic section was examined. The percentage of cells stained in the four samples was scored and averaged. Pixel sampling was performed using the Photoshop eyedropper tool in Adobe's Creative Cloud Suite (Adobe Systems, San Jose, CA) for Apple Macintosh computers (Cupertino, CA).

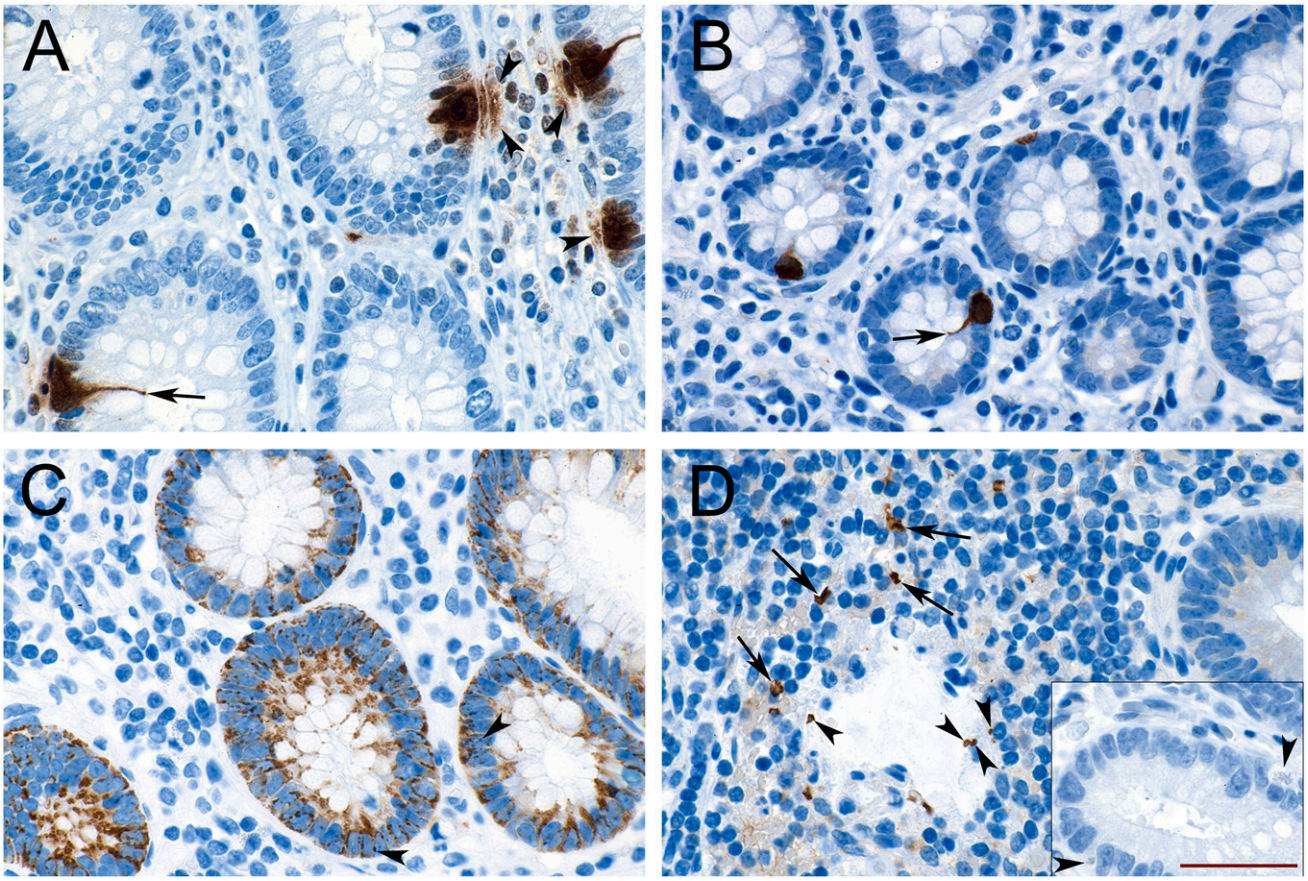

Figure 1. Sections of human appendix stained immunohistochemically. (A) 5-HT in enterochromaffin cells (arrow) and the basolateral stroma beneath these cells (arrowheads). (B) TPH1 in enterochromaffin cells (arrow). (C) MAOA in glandular cells; some mitochondria are visible (arrowheads). (D) SERT in individual platelets (arrowheads) and platelet aggregates (arrows). (D, inset) Appendix reacted with normal rabbit serum. No staining was seen in this control, including in the basal granules of enterochromaffin cells (arrowheads). Red scale bar = 50 μm. All panels are at the same magnification.

Results

Placental 5-HT, MAOA, TPH1, SERT, and other transporters

Do trophoblasts contain 5-HT?

The chorionic villus blood vessels in first- and second-trimester human placentas were strongly stained for 5-HT, presumably reflecting its presence in the platelets in the embryonic and fetal circulation (Fig. 2). In contrast with the 5-HT reactivity in these platelets and in the enterochromaffin cells in samples of appendix stained at the same time (Fig. 1A), only traces of 5-HT were seen in the cytoplasm of trophoblast cells in the first and second trimesters of gestation (Fig. 2A–2D). No staining of either the syncytiotrophoblast or cytotrophoblast layers was detected in 13 normal-term placentas, as verified by a quantitative analysis (Fig. 2E–2F), whereas the platelets in the maternal intervillous space were clearly stained for 5-HT (Fig. 2F, black arrowheads).

We surmised that 5-HT was either not produced in detectable amounts in human trophoblasts or was rapidly removed from productive cells. Consistent with the latter possibility, MAOA was prominent in the syncytiotrophoblasts between 8 and 40 weeks of gestation (Fig. 3). The nucleated fetal erythrocytes also showed MAOA reactivity at 8 weeks (marked with a V in Fig. 3A), presumably within their mitochondria (30).

Do trophoblasts make 5-HT?

TPH1 (enzyme entry EC 1.14.16.4; https://enzyme.expasy.org) is the rate-limiting enzyme for 5-HT biosynthesis (31). Appendiceal enterochromaffin cells used in our study as a positive control showed the strong IHC reactivity of this enzyme (Fig. 1B). In contrast, TPH1 was not detected in placentas between 7 and 15 weeks (Fig. 4A–4D). Thirteen term placentas showed rare TPH1 reactivity not seen with nonimmune rabbit serum (Fig. 4E and Fig. 4F; red arrowhead inset in Fig. 4F). In contrast, more than half of the syncytiotrophoblasts of the same 13 placentas were strongly stained for MAOA (Fig. 3).

Can trophoblasts take up 5-HT?

Cytotrophoblasts were rich in the 5-HT transporter SERT/SLC6A (Fig. 5). Staining was seen on their apical surfaces (Fig. 5C, black arrowheads; Fig. 5D, inset, black

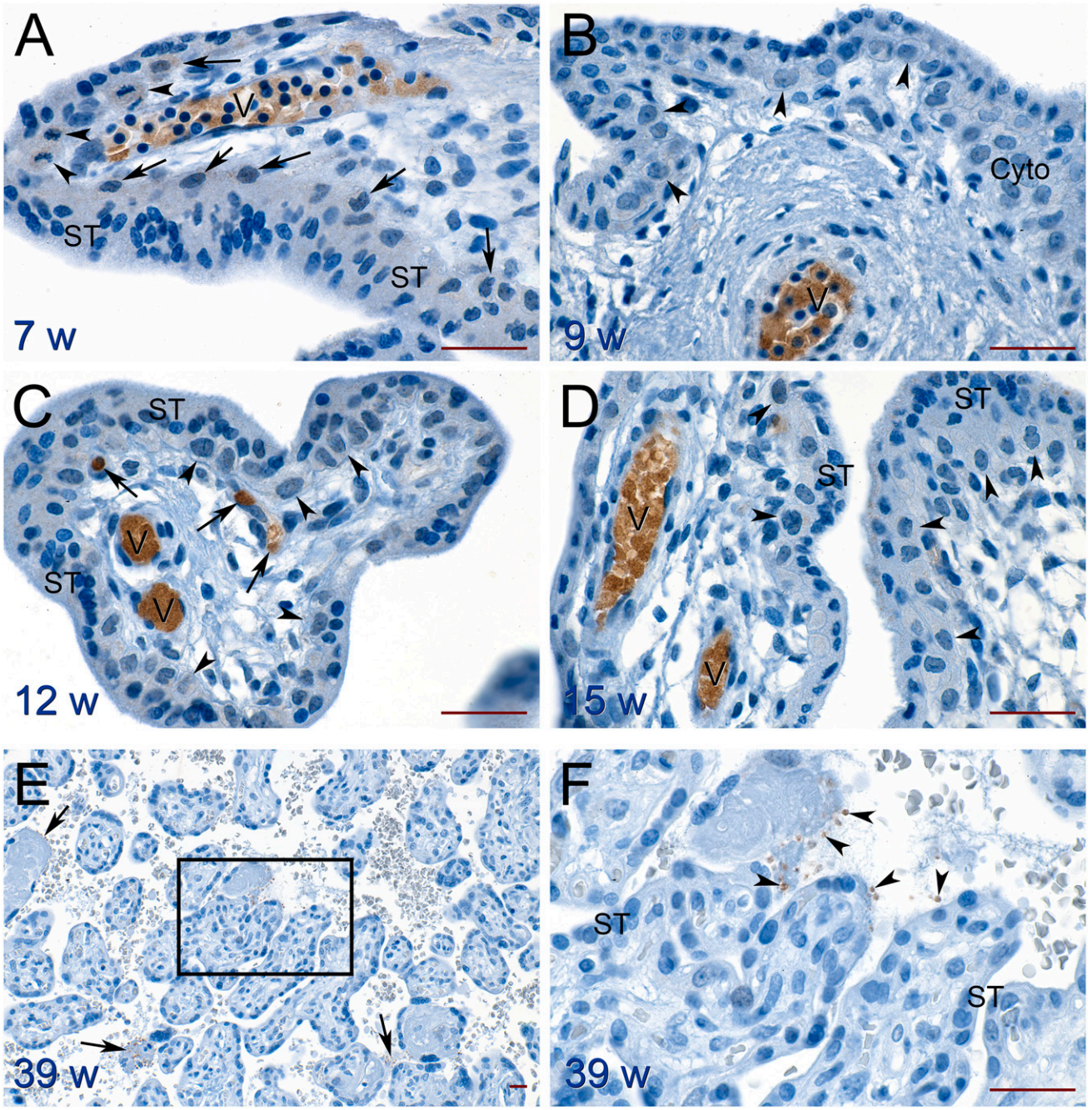

Figure 2. 5-HT immunoreactivity in 7- to 39-week-old human placentas. (A) A 7-week placenta. Fetal vessels were intensely stained. The cytoplasm of cytotrophoblasts with large, round nuclei (arrows) and syncytiotrophoblasts with smaller oval nuclei (ST) was lightly stained. This was most clearly seen in cytotrophoblasts undergoing mitosis (arrowheads). (B) A 9-week placenta. 5-HT reactivity was barely detectable in the cytotrophoblasts (arrowheads and Cyto), while the lumen of a fetal vessel was intensely stained. (C) A 12-week placenta. Very light 5-HT reactivity was seen in the cytotrophoblasts (arrowheads) and in the syncytiotrophoblast layer. The vessels were intensely stained for 5-HT, as were the capillary lumens (arrows). (D) A 15-week placenta. Virtually no 5-HT reactivity was visible in the trophoblast layers (arrowheads and ST), while the vascular lumens were strongly stained. (E) A 39-week placenta. No staining of 5-HT was seen in the trophoblasts. Clumps of 5-HT positive platelets were prominent in the intervillus space (arrows). (F) A magnified field from the box in (E). Platelets in maternal intervillous blood were positive (arrowheads), whereas all trophoblasts were negative. Red scale bars = 25 μm. Cyto, cytotrophoblast; ST, syncytiotrophoblast; V, fetal vessel.

arrowheads; Fig. 5F, red arrowheads; and Fig. 5F inset, red arrowheads) and, more extensively, in their cytoplasm (Fig. 5). Less SERT was associated with the syncytiotrophoblasts, and it mostly appeared in their cytoplasm (marked with an ST in Fig. 5A and Fig. 5D) rather than on their apical plasma membranes (Fig. 5E and inset, red arrowheads). SERT-positive platelets were identified near and occasionally adherent to the apical surfaces of the syncytiotrophoblasts (Fig. 5B and Fig. 5C, red arrowheads). Very fine linear staining for SERT was

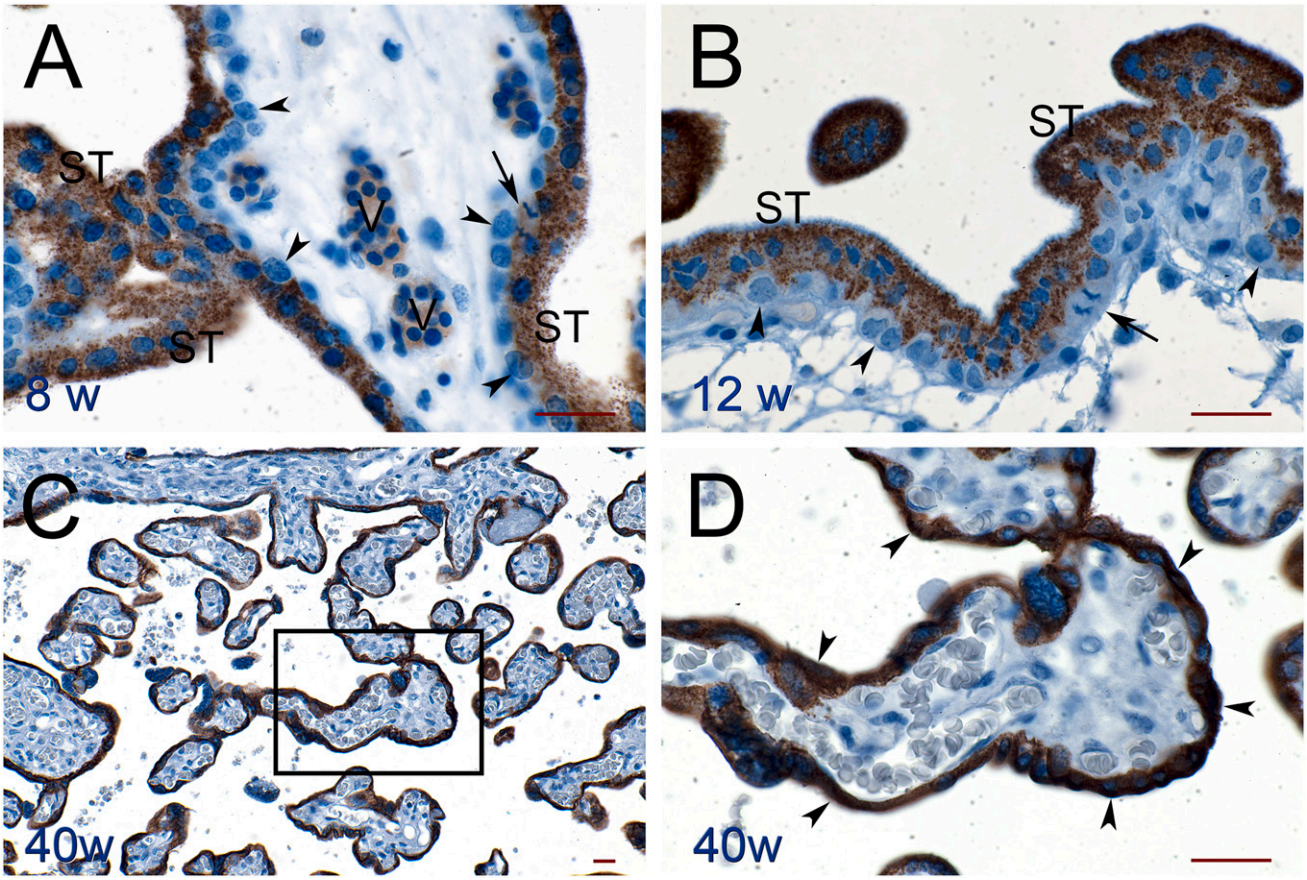

Figure 3. MAOA immunoreactivity in 8- to 40-week human placentas. (A) An 8-week placenta. The syncytiotrophoblast layer was intensely stained in a finely punctate (mitochondrial) pattern, whereas the cytotrophoblast cells were negative (arrowheads). Nucleated fetal erythrocytes also showed MAOA reactivity (V). A cytotrophoblast undergoing mitosis is marked with an arrow. (B) A 12-week placenta. Intense MAOA reactivity was seen in the syncytiotrophoblast layer, whereas the cytotrophoblasts were negative (arrowheads). A cytotrophoblast undergoing mitosis is marked with an arrow. (C) A term placenta. Diffuse reactivity was observed in the syncytiotrophoblasts. (D) A higher-power image from the box in (C) showing the intense staining of syncytiotrophoblasts (arrowheads). Red scale bars = 25 μm. ST, syncytiotrophoblast; V, fetal vessel.

occasionally observed on fetal capillary endothelial cell surfaces (32).

Can trophoblasts export 5-HT?

The gap junctions in trophoblasts channel the inter-epithelial transfer of small solutes, presumably including 5-HT (33, 34). The major gap junction subunit, connexin-43, was stained primarily in the cytoplasm of cytotrophoblasts with decreasing reactivity between 7 and 39 weeks and was significantly diminished in term placentas (Fig 6A–6F, black arrows). The cytoplasmic extensions of the cytotrophoblasts were sometimes also positive (Fig. 6A, red arrows). Connexin-43 was sometimes seen at the cytotrophoblast–syncytiotrophoblast interface (Fig. 6B, red arrowheads). No reaction was seen in the syncytiotrophoblasts themselves (black arrowheads).

Another putative 5-HT transporter is the bidirectional, electrogenic, OCT3 (OCT3/SLC22A3) (35–38). Staining for OCT3 was prominent in the cytoplasm of cytotrophoblasts in all three trimesters (Fig 7A–7F, black arrows) but not in the syncytiotrophoblasts (black arrowheads). OCT3 was also observed at the basolateral surface of the cytotrophoblasts (Fig. 7E and Fig. 7F, black arrows; Fig. 7E inset) and in their cytoplasmic extensions (Fig. 7E and Fig. 7F, red arrowheads). The transporter was seen as well in the cytoplasm of villus core mesenchymal cells (red arrows) and fetal vessel endothelial cells (marked with a V in Fig. 7F). The presence of OCT3 in villus mesenchymal cells is of unknown significance, but that this expression was seen most prominently at term suggests specific placental programming.

Another trophoblast transporter is the adenosine triphosphate–driven solute export pump, called ATP-binding cassette, subfamily B member 1 (also known as multidrug resistance protein and P-gp) (39–43). P-gp immunoreactivity was strong in the apical brush border of the human syncytiotrophoblasts (Fig. 8A–8F, black arrowheads). The interface between the two trophoblast epithelial layers was also well stained. A close inspection of many fields assigned this reactivity to the apical surface of the cytotrophoblasts (Fig. 8A–8D and Fig. 8F, and insets,

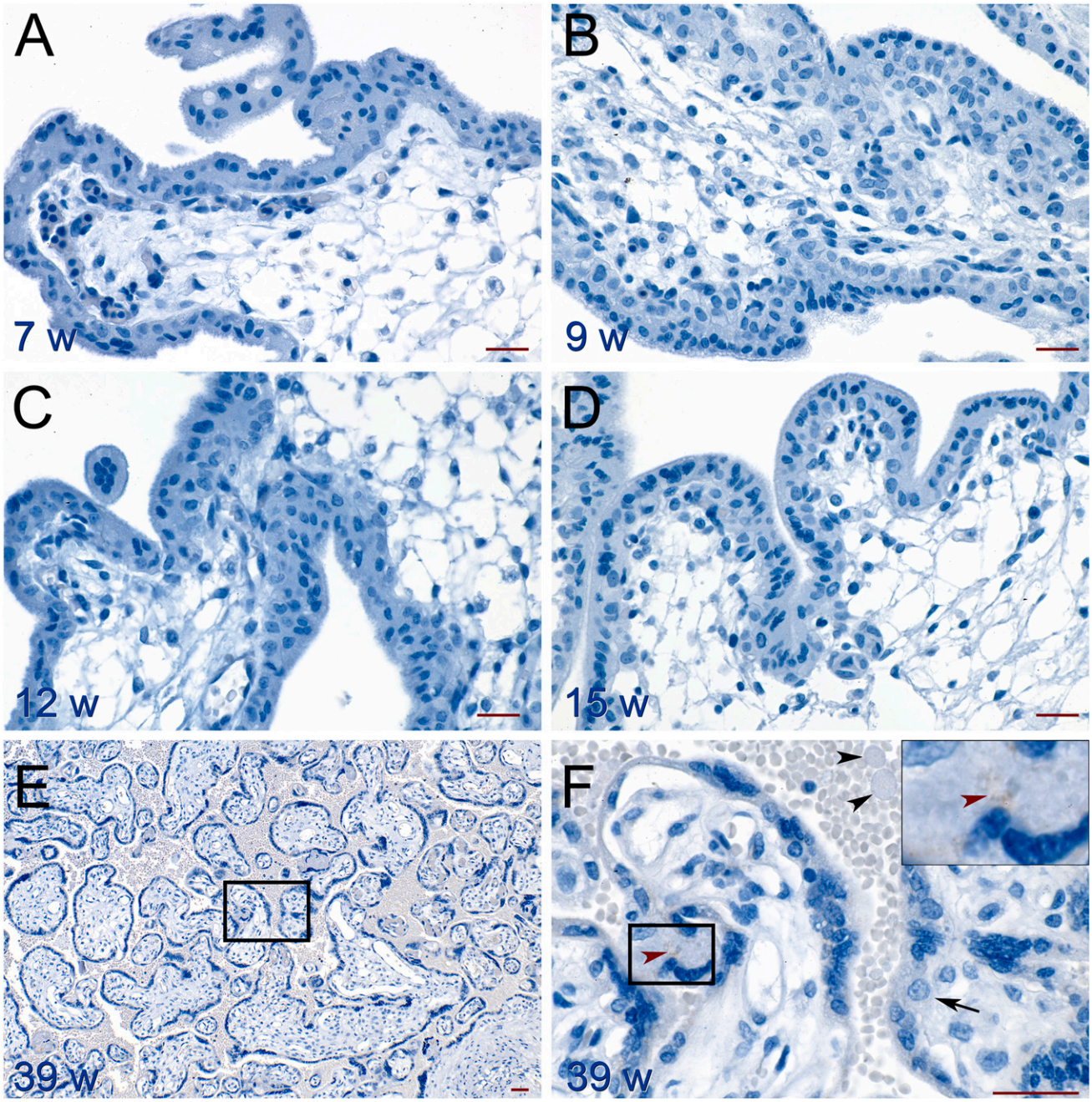

Figure 4. TPH1 immunoreactivity in 7- to 39-week human placentas. No TPH1 was seen in placentas at (A) 7, (B) 9, (C) 12, or (D) 15 weeks of gestation. (E) A 39-week placenta showed negligible TPH1 staining at low power. (F) A higher-power view of the syncytiotrophoblast layer from the box in (E). The arrow marks a negative cytotrophoblast. Platelet aggregates in the maternal blood were also negative (black arrowheads). The inset has been magnified an additional ×2.4 and shows a rare lightly stained region (red arrowhead). Red scale bars = 25 μm.

red arrowheads). Subsyncytiotrophoblast extensions of the cytotrophoblast cytoplasm were sometimes stained as well (Fig. 8E, red arrowheads).

Mouse placental 5-HT, TPH1, and SERT

The antibodies used for human tissues reacted strongly, specifically, and appropriately with mouse placental tissues, as can be seen in a comparison of Figs.1 and 9. Figure 9A shows that murine maternal colonic enterochromaffin cells stained intensely for 5-HT. These are recognized by their characteristic cytoplasmic snouts extending to the lumens of the glands (arrowheads), just as in the human appendix (Fig. 1A). Figure 9B shows these enterochromaffin cells also stained strongly for TPH1 (Fig. 9B, arrows). Here, too, characteristic snout-like extensions extend toward the glandular lumens (Fig. 9B, arrowheads). Figure 9C documents SERT reactivity in platelets (arrowheads) in a vessel within the murine pericolonic adipose tissue.

We then examined the 13.5-day placentas from the same gravid mouse used for reagent controls in Fig. 9A–9C

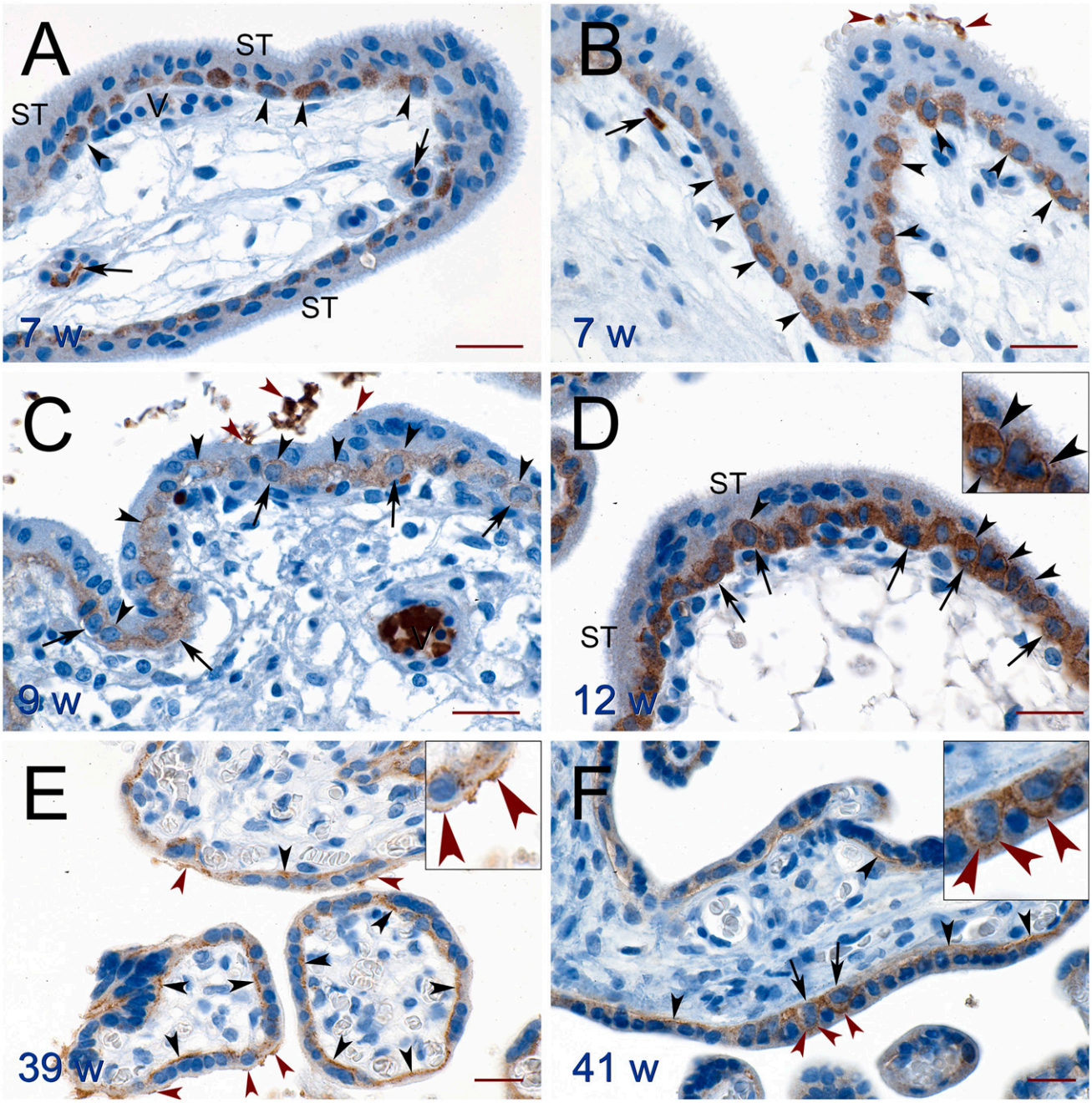

Figure 5. SERT immunoreactivity in 7- to 41-week human placentas. (A) A 7-week placenta. The cytoplasm of the cytotrophoblasts was strongly SERT positive (arrowheads), whereas the syncytiotrophoblast layer showed only light staining. The platelets in fetal vessels were also stained (arrows). Vessels contained nucleated erythrocytes. (B) A different 7-week placenta. A continuous row of SERT-positive cytotrophoblasts is marked with arrowheads. A fetal capillary with SERT-positive platelets is marked with an arrow. A clump of maternal erythrocytes and SERT-positive platelets was adherent to the apical surface of the syncytiotrophoblast layer (red arrowheads). (C) A 9-week placenta. A continuous row of SERT-positive cytotrophoblasts is marked with black arrows. Some cytotrophoblasts had strong linear cytoplasmic staining at the contact zone with syncytiotrophoblasts (black arrowheads). Some aggregated platelets were adherent to the syncytiotrophoblast microvillus border (red arrowheads). A blood vessel had strongly positive fetal platelets. (D) A 12-week placenta. The cytoplasm of the syncytiotrophoblast layer was lightly stained. The continuous row of SERT-positive cytotrophoblasts (black arrows) had strong linear cytoplasmic staining at the syncytiotrophoblast contact zone (black arrowheads). This is magnified 1.6-fold in the inset. (E) A 39-week placenta. The thin cytotrophoblast layer is stained for SERT (black arrowheads). In addition, a few SERT-positive linear areas were present on the apical surface of the syncytiotrophoblasts (red arrowheads), magnified twofold in the inset. (F) A 41-week placenta. Two cytotrophoblasts (arrows) and multiple areas of the thin cytotrophoblast layer (black arrowheads) were positive. As in (D), intense reactivity was observed within the cytotrophoblast cytoplasm at their interface with syncytiotrophoblasts (red arrowheads and magnified 1.8-fold in the inset). Red scale bars = 25 μm. ST, syncytiotrophoblast; V, fetal vessel.

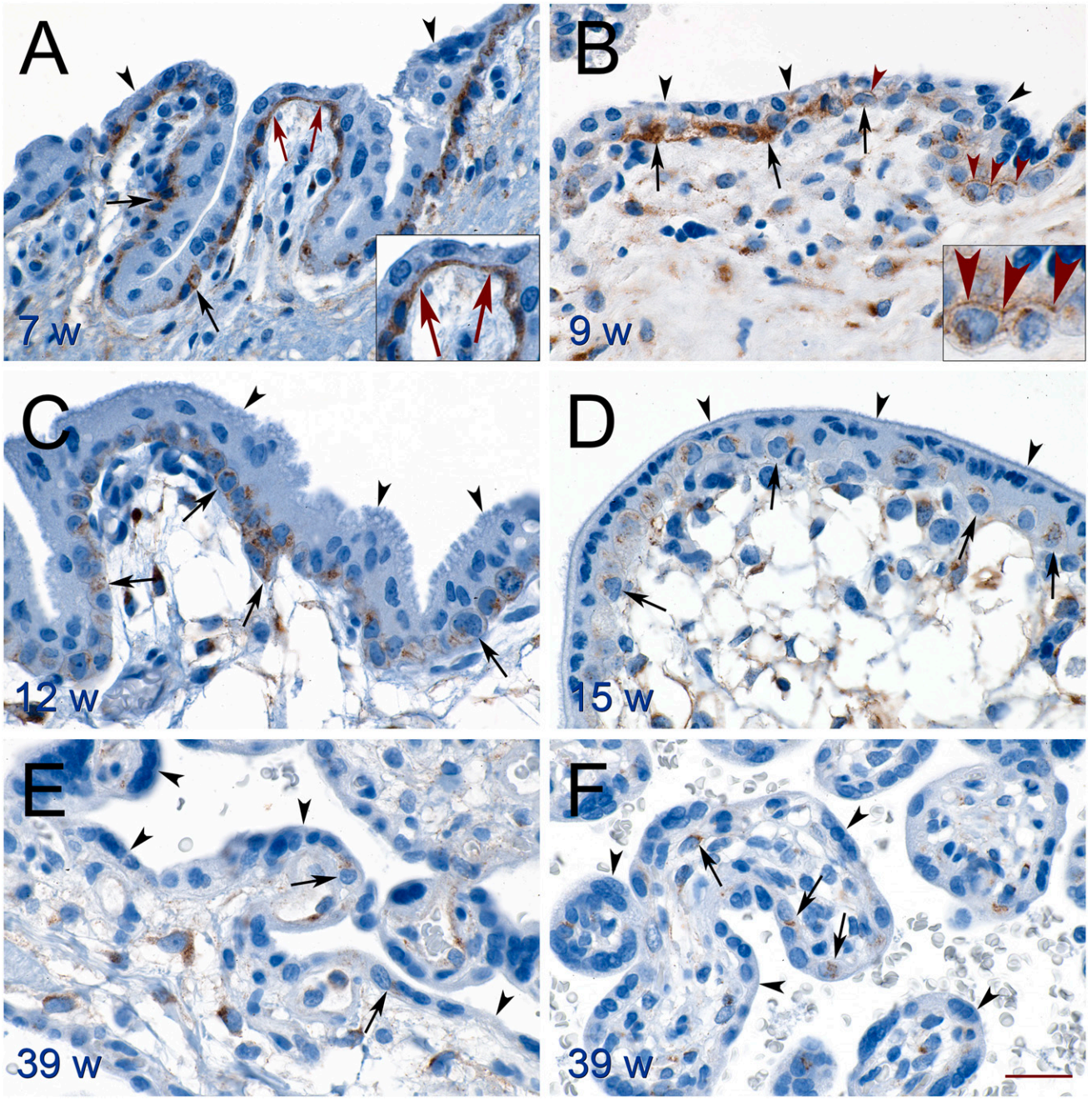

Figure 6. Connexin-43 immunoreactivity in 7- to 39-week human placentas. (A) At 7 weeks; (B) 9 weeks; (C) 12 weeks; (D) 15 weeks; (E, F) 39 weeks. Staining was confined to the cytoplasm of cytotrophoblasts (black arrows) and, occasionally, to their (A) cytoplasmic extensions (red arrows; magnified 1.5-fold in the inset). No reactivity was seen in syncytiotrophoblasts (black arrowheads). (B) Connexin-43 was sometimes identified at the interface between the apical surface of the cytotrophoblasts and syncytial basal membrane (red arrowheads; magnified twofold in the inset). Cytotrophoblast immunoreactivity decreased after 12 weeks of gestation. All images are at the same magnification. Red scale bar = 25 μm.

No 5-HT reactivity was detected in their trophoblasts (Fig. 9D) or in their yolk sac epithelium (Fig. 9G). In contrast, the platelets in maternal blood vessels were well stained for 5-HT (Fig. 9D and Fig. 9G, arrowheads; Fig. 9D, inset). TPH1 reactivity was not observed in serial sections of these same tissues (Fig. 9E and Fig. 9H). Figure 9F shows diffuse, light SERT reactivity in trophoblasts, as well as in maternal (arrowhead) and embryonic (asterisk) platelets identified by their proximity to nucleated erythrocytes (Fig. 9F, arrow). There was also diffuse SERT staining of the yolk sac epithelium (Fig. 9I, arrows) and in individual platelets (arrowheads) and clumps of platelets (asterisk) in the vascular spaces of the adjacent placenta.

5-HT transit in cultured placental explants

The immunohistochemical identification of various 5-HT transporters does not assure that they function in

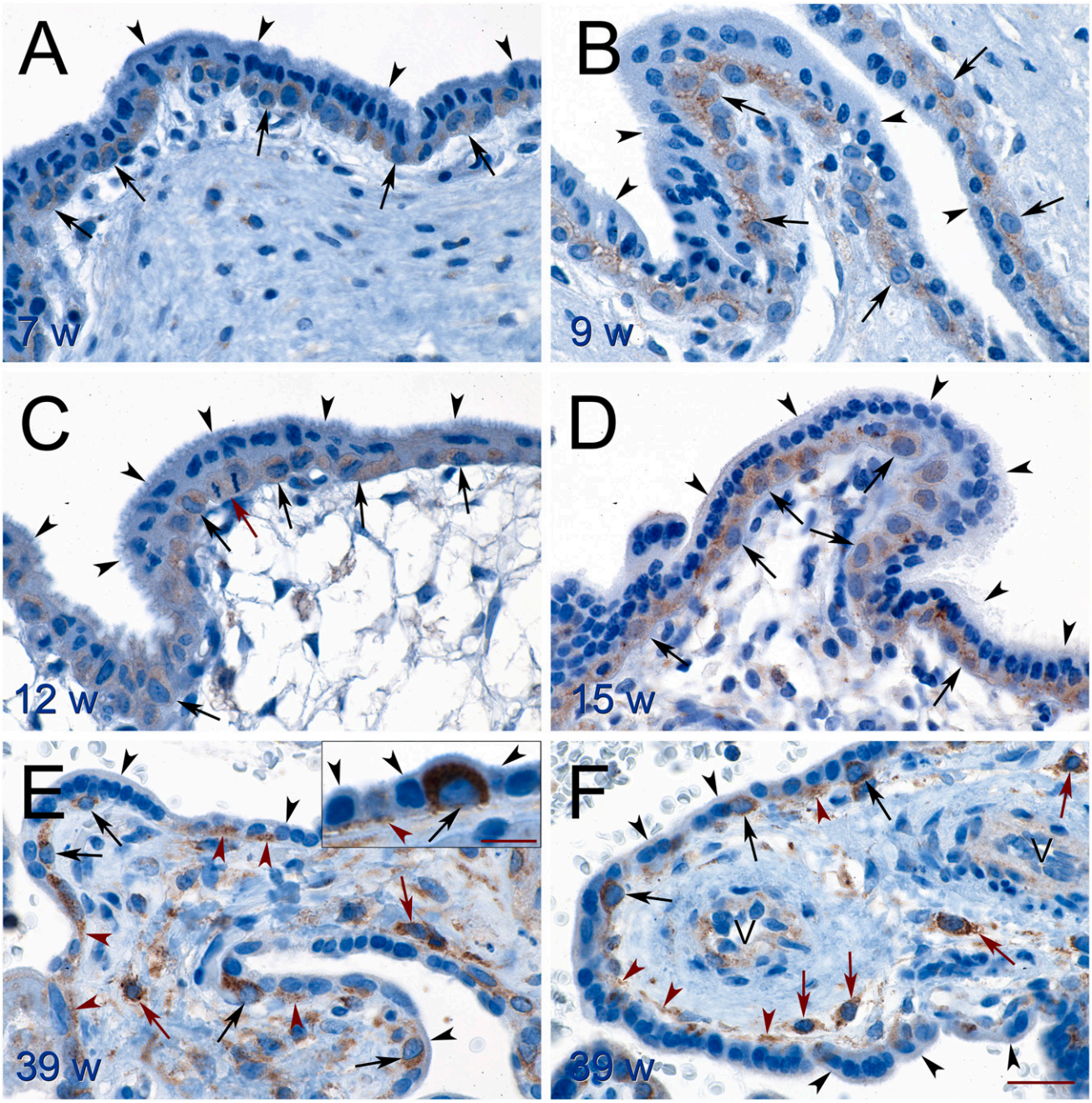

Figure 7. OCT3 immunoreactivity in 7-39 week human placentas. (A) At 7 weeks; (B) 9 weeks; (C) 12 weeks; (D) 15 weeks; (E, F) 39 weeks. Staining was prominent in cytotrophoblasts (black arrows) but not syncytiotrophoblasts (black arrowheads). (C) Mitotic figure (red arrow). (E, F) OCT3 was stained in the cytoplasm of the sparse cytotrophoblasts at 39 weeks (black arrows) as well as in the apparent extensions of the cytotrophoblast cytoplasm under the syncytium (red arrowheads). The inset in (E) shows a clear demarcation between the OCT3-positive cytotrophoblast (black arrow) cytoplasm and the nonreactive syncytiotrophoblast cytoplasm (black arrowheads). Red scale bar in the inset = 12.5 μm. Villus core mesenchymal cells (red arrows) and the endothelial cells in fetal vessels were sometimes OCT3 positive. All full-sized images are at the same magnification. (F) Red scale bar = 25 μm. V, fetal vessel.

the placenta, especially when their IHC localization is not confined to the plasma membrane. Therefore, we tracked the movement of exogenous 5-HT through pieces of intact placenta. Explants of human placental tissue were first incubated with 200 μM escitalopram (a competitive inhibitor of SERT) for 10 minutes, or 10 μM clorgyline (an irreversible inhibitor of MAOA) for 30 minutes, then with 100 μM 5-HT for 60 minutes, followed by IHC.

No 5-HT was observed in the untreated control trophoblast epithelium, blood vessel endothelium, or macrophages, but fetal vessels were sometimes positive, presumably because of their endogenous platelets (Fig. 10A, black arrows). Exogenous 5-HT was rapidly taken up by the trophoblasts (Fig. 10B). Unstained syncytiotrophoblast nuclei were blue (Fig. 10B inset, green arrows), whereas those that took up 5-HT were stained

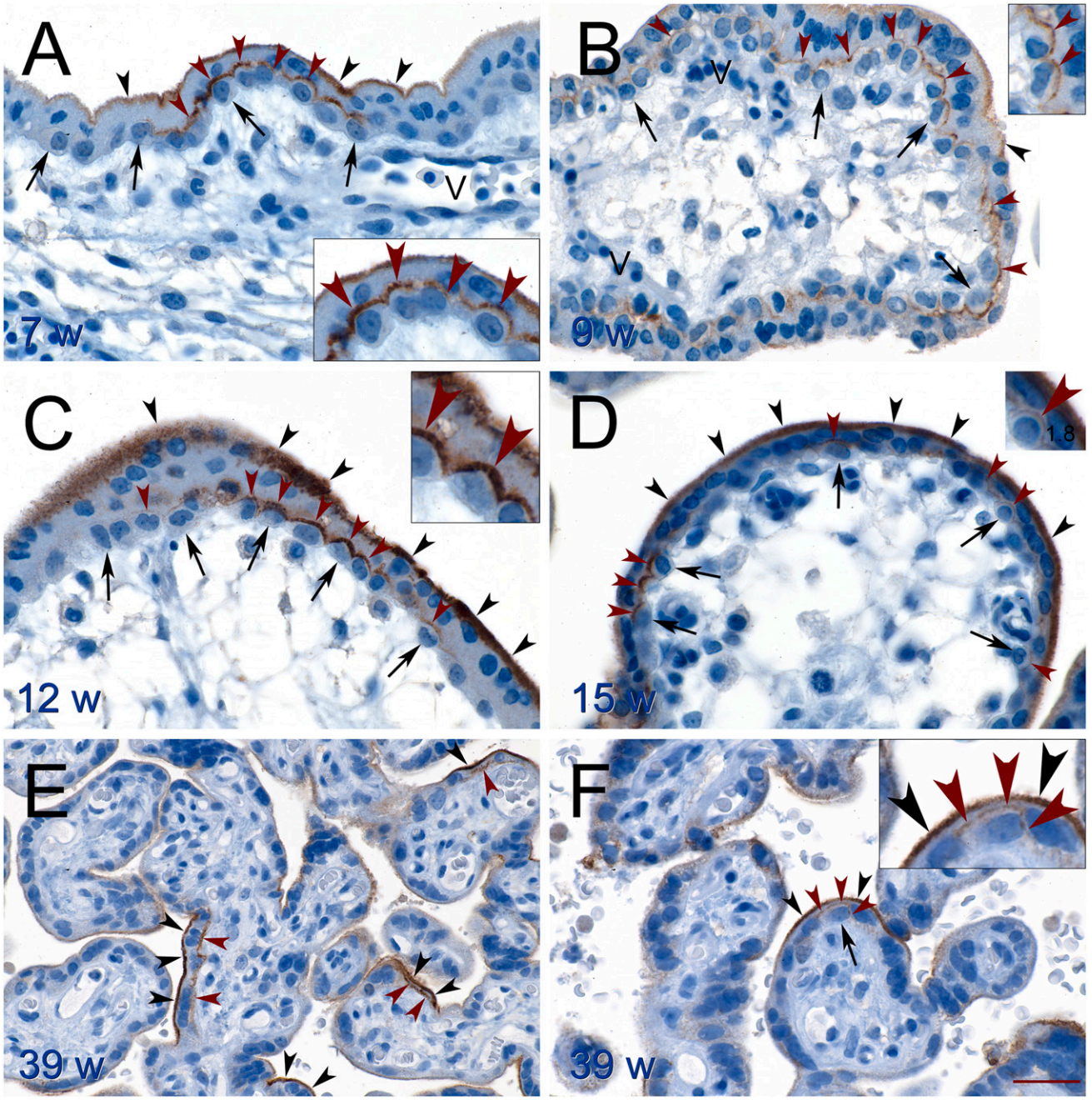

Figure 8. P-gp immunoreactivity in 7- to 39-week human placentas. Intense staining was observed on most of the syncytiotrophoblast apical surfaces (black arrowheads) and less often at the syncytiotrophoblast–cytotrophoblast interface [(A), (B), (D), and (F), red arrowheads; and insets in (A–F), red arrowheads]. The insets in (A) and (C) illustrate the intense granular reactivity in the interepithelial space (red arrowheads). [(A–D) and (F), black arrows] No staining was apparent at the basal surface of the cytotrophoblasts. (E) Extensions of cytotrophoblasts were P-gp immunoreactive (red arrowheads). (A and B) Villus vessels contained nucleated erythrocytes. All full-sized panels are at the same magnification; insets are magnified as follows: (A, B) 1.5-fold; (C, F) twofold; and (D) 1.8-fold. Red scale bar = 25 μm. V, fetal vessel.

brown-black (Fig. 10B inset, red arrows). The cytoplasm and nuclei of the cytotrophoblasts were stained more strongly than those of the syncytiotrophoblasts (Fig. 10B and inset, red arrowheads). These cells often had long 5-HT–positive cytoplasmic extensions running beneath the syncytiotrophoblast layer (Fig. 10B inset, green arrowheads). 5-HT was also detected in villus macrophages, the nuclei of the endothelial cells lining blood vessels (black arrowheads), and the lumens of the fetal vasculature, presumably in platelets (Fig. 10B, black arrow).

Figure 10C shows that escitalopram markedly reduced 5-HT uptake throughout. However, light, endogenous platelet staining similar to that seen in the control condition (Fig. 10A) is still present (black arrows). The minor amount of staining of a few macrophages (Fig. 10C, red arrows) and a rare cytotrophoblast nucleus (red arrowhead) is attributed to

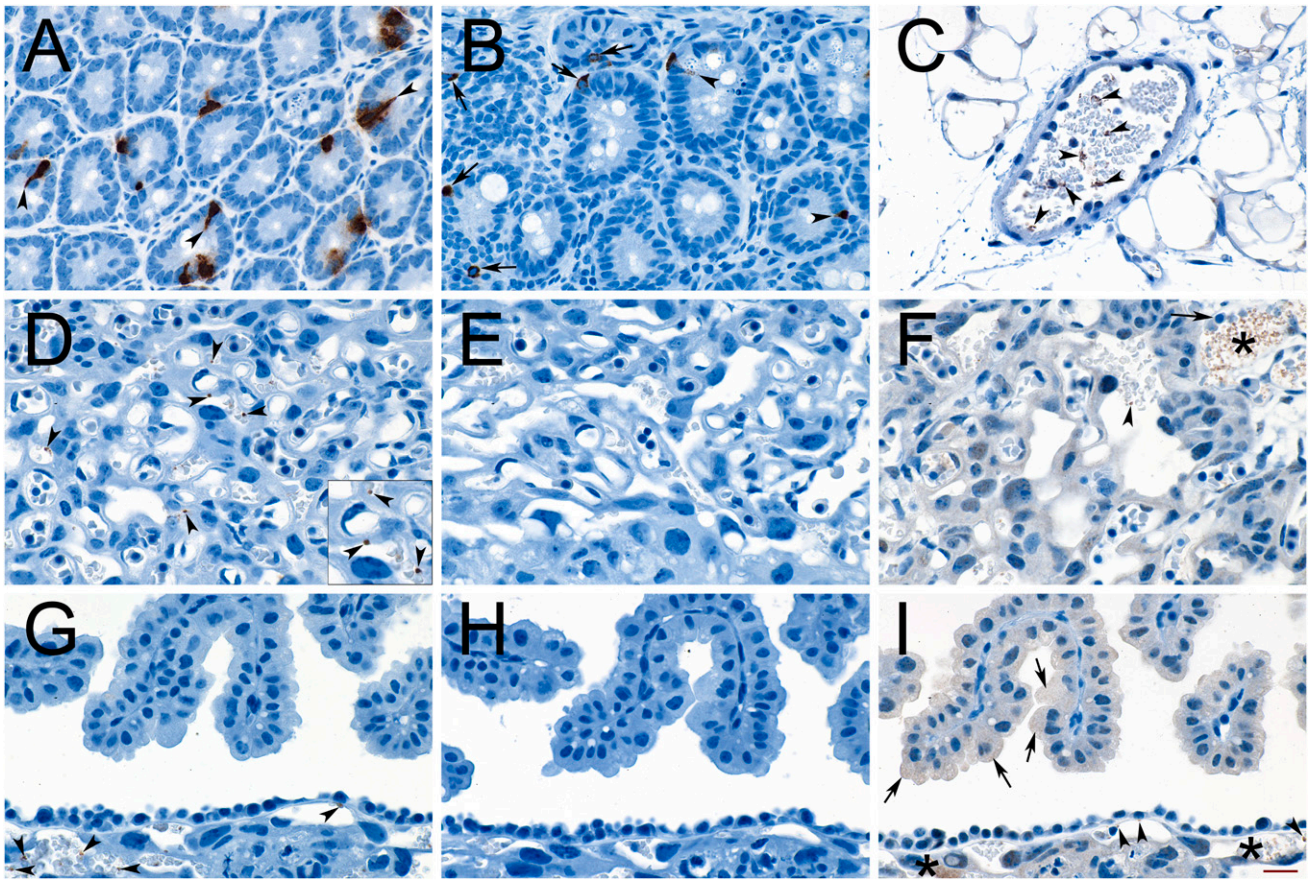

Figure 9. Immunoreactivity of 5-HT, TPH1, and SERT in the (A–C) colon of a gravid mouse at gestation day 13.5 and in the (D–F) trophoblasts and (G–I) yolk sac from a placenta of that mouse. (A) The enterochromaffin cells of the maternal colon were positive for 5-HT (arrowheads). (B) This maternal colon also had TPH1 positive enterochromaffin cells (arrows) with characteristic snout-like apical extensions (arrowheads). (C) The maternal colon showed SERT-positive intravascular platelets (arrowheads). (D) Placental tissue from the same mouse showed no 5-HT reactivity, but 5-HT positive platelets (arrowheads) were demonstrated in the vascular spaces, seen more clearly in the inset magnified 1.5-fold. (E) The same placenta was negative for TPH1. (F) That placenta showed diffuse light SERT reactivity. Maternal platelets (arrowhead) and embryonic platelets (*) near a nucleated erythrocyte (arrow) were also stained for SERT. (G) The yolk sac epithelium in the same placenta showed no 5-HT staining, but intravascular platelets in the adjacent placenta were positive (arrowheads). (H) A serial section of the yolk sac tissue shown in (G) showed no TPH1 reactivity. (I) A serial section of the yolk sac tissue shown in (G) was diffusely and lightly stained for SERT (arrows). Placental intravascular platelets (arrowheads), some in clumps (*), were positive for SERT. All full-sized panels are at the same magnification. Red scale bar = 25 μm.

the high level of 5-HT used here, because total inhibition by escitalopram was achieved with a lower concentration of the monoamine (not shown).

By inhibiting its destruction by MAOA, clorgyline markedly enhanced 5-HT staining (Fig. 10D), especially in the nuclei of syncytiotrophoblasts (red arrows), fetal vessel endothelial cell nuclei (black arrowheads), and vessel lumens (black arrows). Some syncytiotrophoblast nuclei were not stained (Fig. 10D inset, green arrows). The nuclei (Fig. 10D inset, red arrowheads) and cytoplasm (green arrowheads) of the cytotrophoblasts were strongly 5-HT positive.

Inhibitors of 5-HT transport in cultured placental explants

The photomicrographs in Fig. 10 suggest that 5-HT can move from the maternal blood space to the blood vessel lumens in the villus core in a SERT-dependent pathway. Therefore, we sought other participants in this pathway. For this, placental explants were pretreated with relevant inhibitors, then incubated with a low level of 5-HT, and its distribution traced by IHC. In the absence of an inhibitor (Fig. 11A), the added 5-HT was found in syncytiotrophoblast nuclei (red arrows), cytotrophoblast nuclei and cytoplasm (red arrowheads), fetal vascular endothelial cell nuclei (black arrowhead), and vessel lumens (black arrows), much as seen in Fig. 10B using a high concentration of 5-HT. The analysis of many such fields is summarized diagrammatically in Fig. 11a. The brown shading in the diagrams in Fig. 11a–11d was determined using quantitative pixel sampling of the images in Fig. 11A –11D; the intensity of the brown color in the diagrams thus conveys the location and abundance of the 5-HT under each condition.

The presence of the gap junction inhibitor heptanol increased the staining of syncytiotrophoblast nuclei and cytoplasm (red arrows) and, concomitantly, markedly reduced the staining of the cytotrophoblasts (Fig. 11B, red

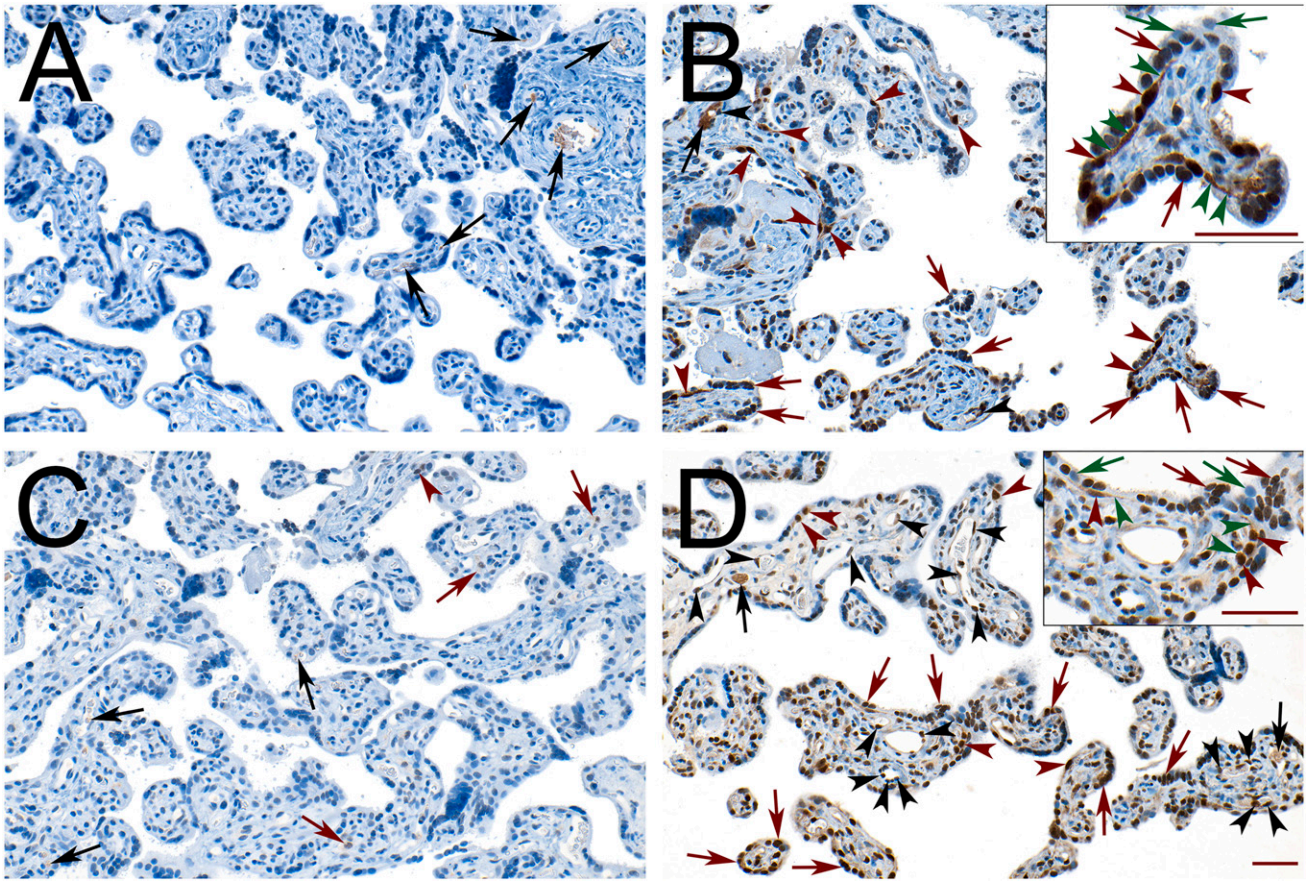

Figure 10. Disposition of exogenous 5-HT in cultured tissue explants from 39-week human placentas in the presence of escitalopram and clorgyline. (A) Untreated control tissues were incubated for 1 hour at 37°C then stained immunohistochemically for 5-HT. The modest reactivity in some vessels is ascribed to their endogenous platelets (black arrows). (B) As in (A), but the 1-hour incubation included 100 μM 5-HT. Staining was strong in syncytiotrophoblast nuclei (red arrows), cytotrophoblast nuclei (red arrowheads), and fetal vessel endothelial cell nuclei (black arrowheads); it was increased in the platelets in the vascular lumens (black arrow). The inset demonstrates that some syncytiotrophoblast nuclei were blue (hence, negative; green arrows), whereas others were intensely brown (signifying marked 5-HT uptake; red arrows). Virtually all the cytotrophoblast nuclei contained 5-HT (red arrowheads) and exhibited strongly 5-HT–positive cytoplasmic extensions (green arrowheads). (C) As in (B) but preceded by a 10-minute incubation with 200 μM escitalopram. This treatment blocked most of the 5-HT uptake into all compartments except for scattered villus core macrophages (red arrows) and a rare cytotrophoblast nucleus (red arrowhead). The staining of platelets in villus vessels was reduced to control levels (black arrows). (D) As in (B) but preceded by a 30-minute incubation with 10 μM clorgyline. 5-HT staining was markedly increased in all compartments of the villus (as manifested by the pervasive light brown coloration); these compartments included the syncytiotrophoblast nuclei and cytoplasm (red arrows), cytotrophoblast nuclei (red arrowheads) and cytoplasm (inset, green arrowheads), fetal vascular endothelial cell nuclei (black arrowheads), and the presumptive platelets in vascular lumens (black arrows). Scattered syncytiotrophoblast nuclei were unstained (inset, green arrows marking blue nuclei). All of the main panels are at the same magnification. Red scale bars = 50 μm.

arrowhead, and Fig. 11b). The endothelial cells in the villus core vessels remained positive in the presence of heptanol (black arrowheads), suggesting a heptanol-resistant 5-HT transfer path to the villus core. We denoted this hypothetical path in the syncytiotrophoblasts in Fig. 11a as orange cones labeled with a question mark (?) and the 5-HT efflux therefrom by the orange arrows in Fig. 11b.

D22, an inhibitor of OCT3, caused the marked accumulation of exogenous 5-HT in cytotrophoblast nuclei and cytoplasm (Fig. 11C, red arrowheads). In contrast, the syncytiotrophoblasts were no longer stained (Fig. 11C, red arrows). D22 did not block 5-HT flux to the lumens of fetal vessels (Fig. 11C, black arrow) nor to the sparse villus stromal cells (Fig. 11C, white arrow), suggesting that 5-HT was able to exit the cytotrophoblasts via P-gp (Fig. 11c, green curved arrows) and/or move directly to the villus core from syncytiotrophoblasts.

Blocking P-gp with mitotane similarly caused 5-HT to accumulate in the cytotrophoblasts (Fig. 11D, red arrowheads). No 5-HT was detected in the syncytium (red arrows) nor in the villus stromal cells. However, there was weak staining within the fetal vessel lumens (black arrow), suggesting that 5-HT was exported from the cytotrophoblasts via OCT3 (Fig. 11d, blue arrow) and/or directly from the syncytiotrophoblasts. A model summarizing all these findings is presented in Fig. 12.

Discussion

Figure 12 conveys the implications of our findings. We suggest that 5-HT is released from maternal platelets and

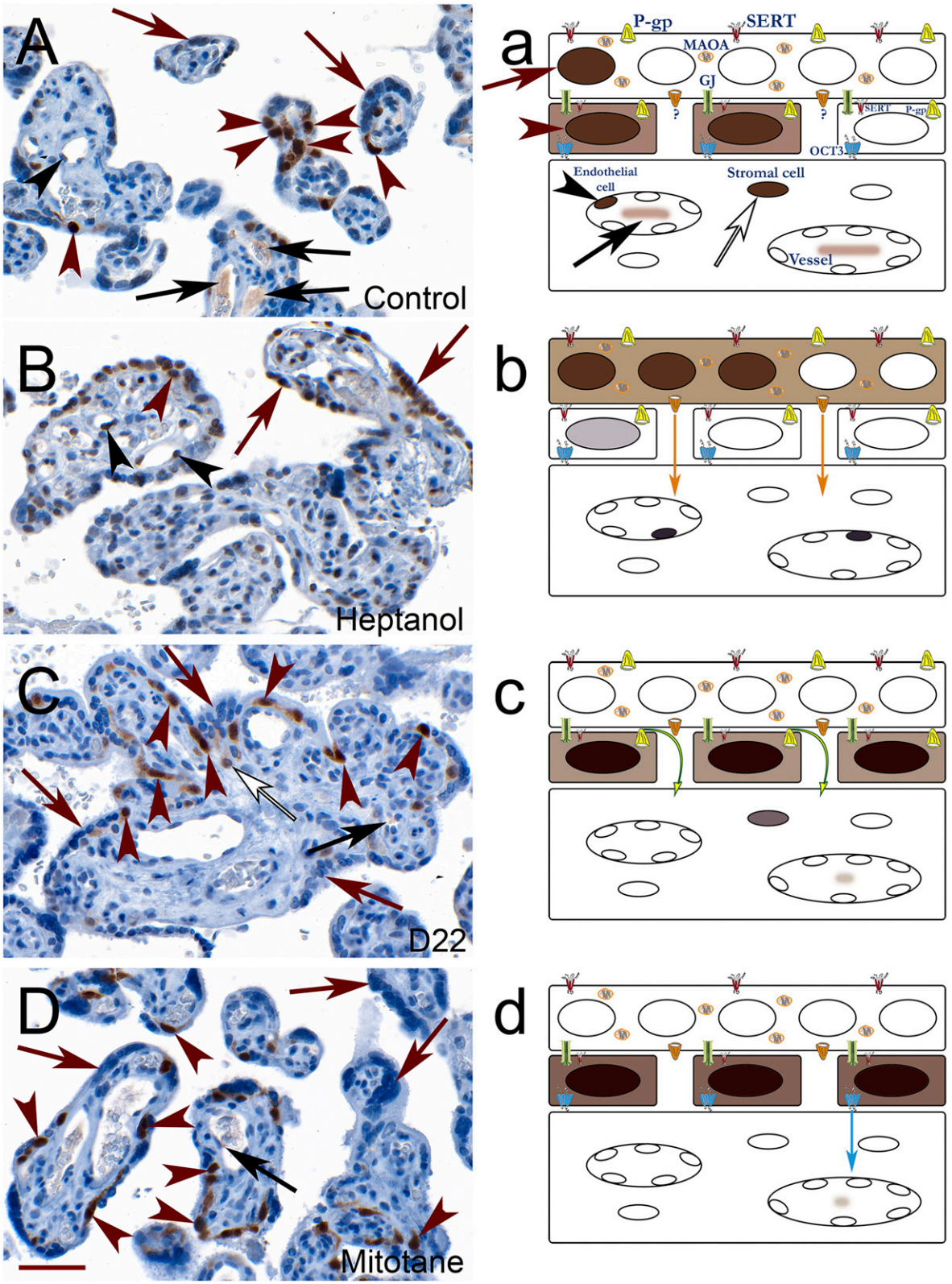

Figure 11. Effect of transport inhibitors on the distribution of exogenous 5-HT. (A–D) Tissue explants from 39-week placentas were preincubated with control media, 5 mM heptanol, 100 μM D22, or 200 μM mitotane for 20 minutes, followed by incubation with 0.3 μM 5-HT for 60 minutes, then stained for 5-HT by IHC. (a–d) Summary interpretation of the 5-HT disposition and its transporters. Brown [reflecting pixel quantitation of the representative images shown in (A–D)] denotes the abundance and location of the 5-HT. All the transporters are designated in (a), but only the uninhibited transporters are shown in (b–d) to highlight the 5-HT pathway. (A) Control: no inhibitors. The added 5-HT reached the syncytiotrophoblast nuclei (red arrows), cytotrophoblast nuclei (red arrowheads) and cytoplasm, the nuclei of fetal vessel endothelial cells (black arrowhead), and the fetal vascular lumens (black arrows). (B) Heptanol, an inhibitor of gap junctions [GJ, green cylinder in (a)], increased the staining of syncytiotrophoblast nuclei and cytoplasm (red arrows) and blocked the staining of the cytotrophoblast cytoplasm. The cytotrophoblasts had rare 5-HT–positive nuclei (red

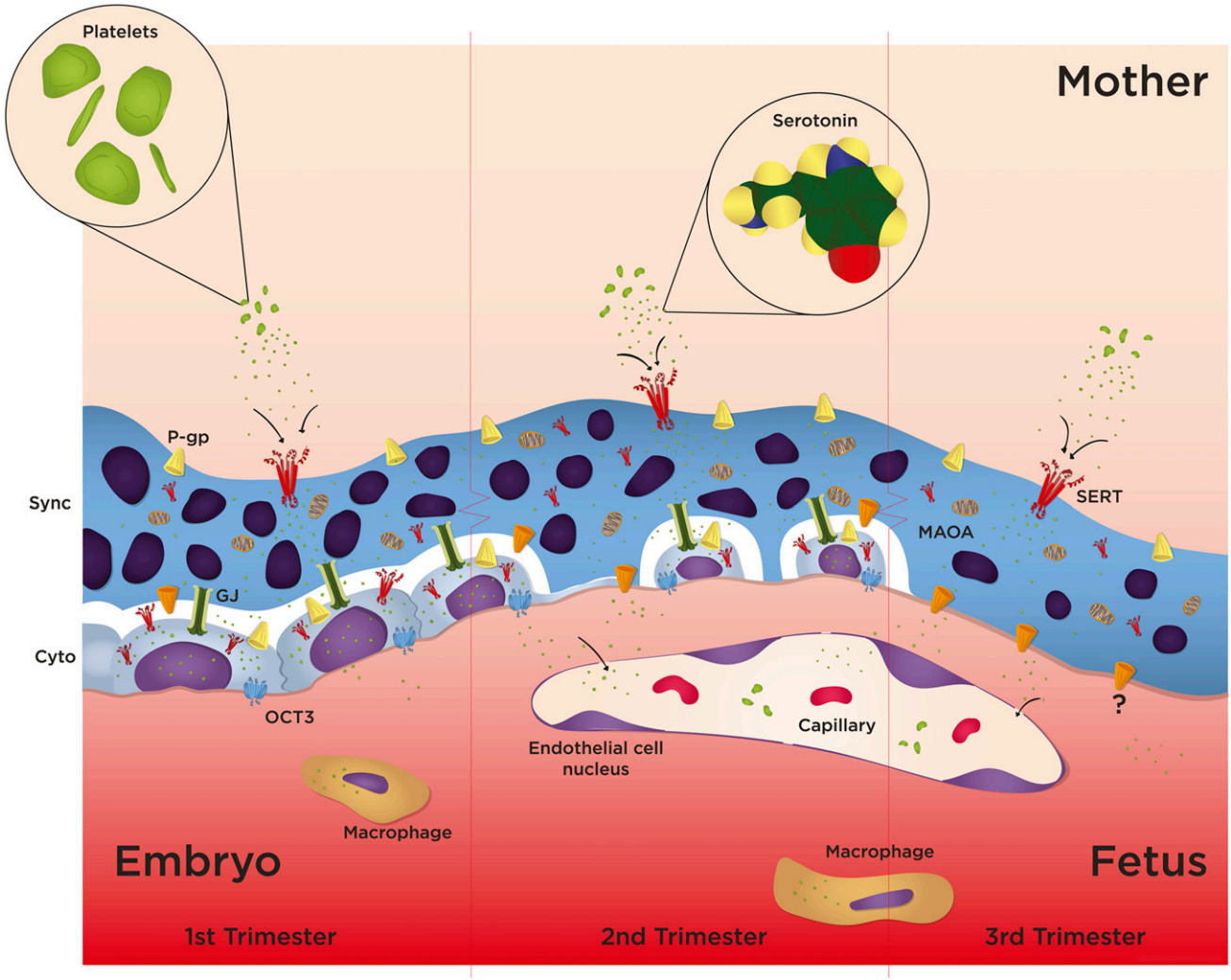

Figure 12. Proposed pathway for maternal 5-HT delivery to the human placenta, embryo, and fetus in each trimester. Maternal platelets (upper left, light green) degranulate in the intervillus space of the placenta. The released 5-HT (green dots) is taken up by syncytiotrophoblasts via SERT (dark red polytopic apical plasma membrane protein). The syncytiotrophoblast 5-HT has multiple fates: oxidation by MAOA (in brown mitochondria); return to the intervillus space by apical P-gp (yellow cones); transport to cytotrophoblasts via gap junctions (green cylinders); and transfer from its basal membrane to the interepithelial space by a hypothetical mechanism [orange cones (**?**)]. Interepithelial 5-HT is taken up by the cytotrophoblasts via their SERT (dark red polytopic apical plasma membrane protein). Cytotrophoblast 5-HT can then be transported distally by OCT3 (blue basal plasma membrane proteins). In addition, cytotrophoblast P-gp (yellow cones in the apical plasma membrane) can transport its 5-HT to the interepithelial space. Especially after the continuous cytotrophoblast barrier becomes fenestrated late in gestation, the interepithelial 5-HT exported from both the syncytiotrophoblasts and cytotrophoblasts diffuses to the villus core. This 5-HT can then be taken up by fetal vessels [in particular, pumped by SERT into platelets (light green)] and taken to the embryo and fetus. The light brown line represents the basement membrane between the trophoblast epithelium and the mesenchymal villus core. Cyto, cytotrophoblast; GJ, gap junction; Sync, syncytiotrophoblast.

taken up by syncytiotrophoblasts by SERT at their maternal surfaces. Some syncytiotrophoblast 5-HT could be returned to the maternal blood by P-gp, some destroyed by their mitochondrial MAOA, and some moved through gap junctions to the underlying cytotrophoblasts. SERT in the apical plasma membranes of cytotrophoblasts would take up the 5-HT released to the interepithelial space by the hypothetical syncytiotrophoblast mechanism proposed earlier in this article. Cytotrophoblast 5-HT is then delivered to the interstitium by its apical P-gp

Figure 11. (Continued). arrowhead). The staining of the nuclei in the endothelial cells of fetal blood vessels (black arrowheads) was mostly unaffected by the heptanol. This suggests a direct pathway for 5-HT from the syncytiotrophoblasts to the villus core via an unknown transporter [indicated by the orange cones labeled with a question mark (?) in (a) and by the orange arrows in (b)]. (C) D22 inhibition of OCT3 (the blue transporter) caused 5-HT accumulation in cytotrophoblast nuclei and cytoplasm (red arrowheads), and eliminated the reaction in syncytiotrophoblasts (red arrows). 5-HT was detected in villus core stromal cells (white arrow) and fetal blood vessels (black arrow), presumably having exited from cytotrophoblasts via P-gp [curved green arrows in (c)] and/or from syncytiotrophoblasts by the hypothetical pathway [orange arrow in (b)]. (D) Mitotane inhibition of P-gp (yellow cones) caused 5-HT to accumulate in the nuclei and cytoplasm of cytotrophoblasts (red arrowheads) and voided the syncytiotrophoblasts (red arrows). A small amount of 5-HT staining was seen in the villus core blood vessel lumens (black arrow), perhaps exported from cytotrophoblasts via OCT3 transporters [blue arrow in (d)] or by the hypothetical pathway [orange arrow in (b)]. All photomicrographs are at the same magnification. Red scale bar = 50 μm.

and its basal OCT3. Interstitial 5-HT in the villus core is transferred to the conceptus by the villus fetal blood vessels.

5-HT plays an essential role in intrauterine development, with the brain being the most intensively studied. The level of 5-HT in the conceptus increases during its development (28). However, there has been uncertainty as to the source of this effector (8, 19, 21, 22). In adults, the vast preponderance of 5-HT is made in and secreted by intestinal chromaffin cells, then taken up by and carried throughout the body by platelet granules for use in hemostasis (44, 45). However, enterochromaffin cells are not the main source of embryonic 5-HT in the mouse embryo, given that TPH1 does not appear in the intestine until day 15.5 of a 20-day gestation (6). 5-HT is also synthesized in neurons by the isoenzyme TPH2, but the 5-HT critical to brain development is not of neuronal origin early on (19). In particular, neither the serotoninergic neurons nor the pineal gland in the embryonic rodent brain express TPH1 or TPH2 until after day 9.5 (*i.e.,* midgestation) (6, 46).

Our findings support the hypothesis that maternal 5-HT is delivered to the embryo and fetus of the mouse and human, as was strongly suggested by two early studies (6, 8). We applied transport inhibitors to dissect this putative pathway. Using placental explants, we showed that escitalopram, heptanol, D22, and mitotane each inhibited the accumulation of exogenous 5-HT in the villus core, suggesting the involvement of multiple transporters in diverse locations. These results are summarized in Fig. 12, and our conclusions are discussed in the following points:

1. 5-HT is delivered from mother's blood. TPH1 was not detected in either human or mouse trophoblast tissues (Fig. 4 and Fig. 9E), providing no evidence for the biosynthesis of this monoamine therein. Rather, 5-HT is one of a myriad of solutes brought to the conceptus by the maternal circulation (47, 48). The delivery of this potent effector is presumably highly controlled but is not understood. Plasma 5-HT is avidly sequestered by platelets, leaving only traces free in the plasma (49, 50). It is possible that maternal platelets release their contents into the intervillus space by controlled degranulation [Fig. 5B and 5C; (51, 52)]. Furthermore, traces of 5-HT released to the plasma can stimulate further platelet exocytosis through a positive feedback loop (53). Also, the conceptus might clear incoming 5-HT so efficiently that the small fraction free in the mother's plasma would suffice (19).
2. Maternal 5-HT is taken up by trophoblasts. The syncytiotrophoblast layer provides a continuous barrier to the passive (hence, uncontrolled) diffusion of solutes from the maternal plasma to the chorionic villi (47, 48). Rather, maternal 5-HT is retrieved from the intervillus space by syncytiotrophoblast SERT [Fig. 5E; (32, 54–57)]. Although SERT was only weakly stained in the apical plasma membranes of syncytiotrophoblasts (Fig. 5), its role is made clear by the strong inhibition by escitalopram of the accumulation of exogenous 5-HT in these cells (Fig. 10C). That escitalopram greatly reduced the appearance of added 5-HT all the way to the villus core highlights the role of syncytiotrophoblast SERT as the entry point in the pathway. We know of no direct demonstration that maternal 5-HT traverses the placenta *in vivo*. However, there is clear evidence that the SERT-initiated pathway for 5-HT delivers closely related drugs to the conceptus. That is, at least two competitive inhibitors (namely, amphetamine and fluoxetine) are transported by SERT and pass from mother to offspring (56, 58–61). These findings support the premise that 5-HT is similarly physiologically conveyed.
3. 5-HT is removed from the syncytiotrophoblasts by several dissipation pathways. Little or no 5-HT was found in untreated human and mouse syncytiotrophoblasts compared with its abundance in enterochromaffin cells (Figs. 1, 2, and 9). Presumably, its level in the syncytium is tightly controlled. One mechanism is its destruction by MAOA [Figs. 3 and 10; (62, 63)]. This enzyme is regulated at the level of gene expression and would appear to modulate placental 5-HT (24). Although exogenously added 5-HT filled both the cytotrophoblast cytoplasmic and nuclear compartments (Fig. 10B, inset), only the nuclei in the syncytiotrophoblasts were stained for 5-HT, suggesting that the 5-HT in the syncytiotrophoblast cytoplasmic compartment was rapidly degraded by MAOA. The robust catalytic capacity of this oxidizer was demonstrated by the enhancement of villus 5-HT staining by the MAOA inhibitor clorgyline (Fig. 10D).

   Unlike neurons and platelets, syncytiotrophoblasts do not accumulate and release 5-HT through a regulated vesicle storage system (64). The export of 5-HT from the syncytium, therefore, is not quantal. Rather, P-gp is abundant in the apical brush border of the syncytium [Fig. 8; (41, 43, 65, 66)]. Thus, P-gp could pump 5-HT back to the intervillus space in opposition to SERT; however, this raises the possibility of a futile transport cycle unless some form of coordinated regulation supervenes.

   Gap junctions provide a conduit from syncytiotrophoblasts to cytotrophoblasts for small cytoplasmic

solutes [Fig. 6; (34, 67, 68)]. Their role in the export of syncytiotrophoblast 5-HT was made clear by the elimination of its accumulation in cytotrophoblasts by the gap junction inhibitor, heptanol [Fig. 11B and Fig. 11b; (69)]. But why did blocking the transfer of 5-HT from the syncytium to the cytotrophoblasts through the gap junctions not block its flux to the villus core (Fig. 11B)? Indeed, none of the inhibitors entirely eliminated the transfer of exogenous 5-HT to the villus core. It could be that these inhibitors were simply not completely effective, as is often the case. However, the evidence supports a more interesting alternative: the existence of a parallel route that transports 5-HT from the syncytium to the interepithelial space and thence to the villus core, bypassing the cytotrophoblasts. We have pictured this activity as an unknown basolateral syncytiotrophoblast transporter in Figs. 11 and 12 [the orange cones marked with a question mark (?)] in the basal plasma membranes of syncytiotrophoblasts.

4. 5-HT is taken up by cytotrophoblasts. As mentioned, 5-HT was transferred from syncytiotrophoblasts to cytotrophoblasts through their gap junctions (Fig. 6 and Fig. 11B). In addition, SERT is present on the apical surface of the cytotrophoblasts near the basal surface of the syncytiotrophoblasts [Fig. 5D and Fig. 5F insets; (32)]. Cytotrophoblast SERT is thus positioned to take up 5-HT exiting the syncytiotrophoblasts to the interepithelial space by way of the hypothetical pathway just mentioned. Indeed, as depicted in Fig. 12, no other source of 5-HT or function for cytotrophoblast SERT is apparent.
   Why was SERT more prominent in the cytotrophoblast interior than in its plasma membrane, as seen in Fig. 5 and noted by Viau *et al.* (32)? This integral plasma membrane protein has been widely reported to be internalized in cytoplasmic vesicles as part of an elaborate system of regulation involving endocytosis, interprotein interactions, and other molecular mechanisms (10, 70–76). Indeed, 5-HT is known to stimulate the rapid internalization of SERT (77). Perhaps our handling of the placentas *ex vivo* caused ambient maternal 5-HT to induce such internalization.
5. 5-HT is exported from cytotrophoblasts. OCT3, a bidirectional organic cation exchanger, is situated at the basal surface of cytotrophoblasts where it can transport 5-HT down its concentration gradient [Fig. 7; (35, 37, 78–80)]. That OCT3 performs this function was shown by the ability of its specific inhibitor, D22, to cause 5-HT accumulation in cytotrophoblasts (Fig. 11C and Fig. 11c). Furthermore, *in vivo* evidence suggests that OCT3 participates in the *trans*-placental transport of 5-HT; that is, it mediates the movement of another monoamine substrate of OCT3, metformin, from mother to fetus (81). OCT3 staining was also observed in the cytoplasm of cytotrophoblasts (Fig. 7). The dynamics of this protein in the cell interior have been described previously but are of unknown functional significance (36, 82). Finally, we note that OCT3 is absent from syncytiotrophoblasts, even though they are derived entirely from OCT3-rich cytotrophoblasts. Presumably, it is important for this transporter to be removed promptly as the syncytium forms.
   P-gp expression and function have been observed in human cytotrophoblasts *in situ* and in culture (40, 65, 83–86). That its inhibitor, mitotane, caused 5-HT accumulation in the cytotrophoblasts of placental explants confirms that P-gp works in tandem with OCT3 to remove their 5-HT (Fig. 11D and Fig. 11d). 5-HT is transported by this P-gp from the apical surface of the cytotrophoblasts to the interepithelial space (Fig. 8 insets; Fig. 12). There is no apparent pathway for this 5-HT to move retrograde to the syncytium or the maternal blood stream, but it could diffuse past the cytotrophoblasts to the villus core along with the aforementioned 5-HT exported from the syncytium by the hypothetical basal transporter (Fig. 12).
6. 5-HT moved to the interstitial spaces in the chorionic villus core. Exogenous 5-HT was transported to the trophoblast villus cores of placental explants, as manifested by the staining of the macrophages, the nuclei of the endothelial cells lining fetal capillaries, and the platelets within those vessels (Fig. 10B and Fig. 10D). We used a high concentration of 5-HT in those studies to make sure we could visualize the relevant compartments. However, similar results were obtained with a more physiologic 5-HT concentration (Fig. 11A and Fig. 11a). The syncytiotrophoblast epithelium forms an impermeable barrier in the human placenta (47, 48). Consequently, the 5-HT released by the P-gp at the apical surface of cytotrophoblasts would not be returned to the maternal blood space but, rather, would head distally. But how would this interepithelial 5-HT reach the villus core as we propose? One route would be its reclamation by cytotrophoblast SERT followed by its release via basolateral OCT3. A more direct pathway would be the diffusion of interepithelial 5-HT through the intercellular junctions between the cytotrophoblasts, given that these zones seem to be permeable to small solutes (33, 68, 87). Furthermore, the

cytotrophoblast layer becomes fenestrated and then increasingly sparse in the second and third trimesters (88–90). This patency would allow the monoamine released from the syncytiotrophoblasts into the interepithelial space to bypass the cytotrophoblasts and pass directly into the villus core. This anatomical transition means that, over time, the cytotrophoblasts would play a diminishing role in transferring maternal 5-HT to the villus core, while the direct delivery of the 5-HT released by the syncytiotrophoblasts would become increasingly important. Supporting this premise is the observed decline in the abundance of interepithelial gap junctions after 12 weeks of gestation (Fig. 6). It thus appears that 5-HT can be sent to the villus core from the basal surface of the syncytium as well as from both the apical and basolateral surfaces of the cytotrophoblasts—with the role of the cytotrophoblasts diminishing during gestation.

7. Implications for regulation: The proposed pathway would serve to provide the villus interior with a physiological amount of 5-HT to supply the fetal blood stream appropriately. This hypothesis suggests an interplay among the various transporters. Furthermore, these transporters are known to be modulated with gestational age, presumably to tune their homeostatic functions (24, 36, 42, 79, 91).

   In addition to their role in delivering 5-HT to the conceptus, cytotrophoblasts themselves may be subject to the influence of 5-HT as a morphogen. Indeed, it is their proliferation and organization that elaborate the overlying syncytiotrophoblast layer and shape the villus architecture (92–97). In this regard, we report that 5-HT accumulates in the nuclei (in addition to the cytoplasm) of different cell types (Fig. 1A, Fig. 2A, and Figs. 10 and 11]. Similar observations have been made previously (8, 98, 99). A nuclear 5-HT binding protein is suggested by these data. Furthermore, 5-HT receptors have been observed in nuclei, and G protein-coupled receptors may be associated with the inner nuclear membrane (100, 101). This issue is clearly worthy of investigation.
8. 5-HT is delivered from the villi to fetal tissues. There are small clefts between the endothelial cells of the villus capillaries that would permit interstitial 5-HT to diffuse into the fetal blood stream (102). Although the delivered 5-HT would have to pass the blood-brain barrier to reach the nervous system of the conceptus, that checkpoint is probably patent to small solutes at least until midpregnancy (103–105). Consistent with this hypothesis, serotonergic neurons do not synthesize their own 5-HT in the early embryo; rather, they express SERT for the uptake of exogenous 5-HT (16, 22).
9. Some unresolved issues:
   - This initial foray did not provide a comprehensive analysis of the multiple functions involved in the management of trophoblast 5-HT in the several stages of gestation. Indeed, the snapshots we provide of various cellular compartments at different times do not reveal the physiological activities of the different transporters, the magnitudes of the various 5-HT fluxes, or their changes over time; nor do we have a measure of 5-HT in the maternal space or that leaving the villi. More detailed studies are warranted.
   - A physiological pathway for 5-HT transit from mother to fetus has not been shown. Rather, we have visualized some trophoblast transport events *in vitro* and speculated on their functions. On the other hand, as mentioned, amphetamine, fluoxetine, and metformin are SERT and OCT3 substrates that reach the fetus from the mother, plausibly by the route proposed in Fig. 12 (56, 81). In fact, these *in vivo* findings provide a proof of principle for our model.
   - It is puzzling that blocking 5-HT efflux from cytotrophoblasts with either D22 or mitotane eliminates the staining of the syncytiotrophoblasts, because 5-HT could presumably remain in or reflux to the syncytiotrophoblasts through their gap junctions. Possibly, 5-HT was removed from the syncytiotrophoblasts by the combined actions of MAOA, their apical brush border P-gp, and the putative basal exporter (Fig. 12). But the phenomenon could be more complicated than that.
   - Other known trophoblast 5-HT transporters might also affect the proposed pathway (37, 43, 65, 106–108). In particular, MATE1 is thought to transfer 5-HT from rat syncytiotrophoblasts to the mother's blood (36, 78). It, and others, might have evolved to limit the flood of maternal 5-HT from reaching the fetus, given that its excess can be injurious (109, 110).
   - Conflicting models for mouse and human embryogenesis. Our results suggest a pathway through the human placenta in which syncytiotrophoblast SERT initiates the delivery of 5-HT from mother's blood to the conceptus (Fig. 12). However, the mouse placenta expresses modest levels of SERT on day 13.5 (Fig. 9F), only becoming abundant on day 18 of a 20-day gestation (111). Thus, SERT may not be important

in the early development of the mouse embryo (24, 63). This supposition is strengthened by the demonstration that SERT knock-out mouse embryos mature relatively normally (112).

The findings just discussed do not support a maternal source model. Rather, they favor a placental source model, in which the placental TPH1 synthesizes 5-HT for delivery to the conceptus. This premise has received considerable support in both mice and humans (18, 19, 24–27). However, we did not observe appreciable TPH1 staining in either human or mouse placentas, in contrast to those positive reports. It should be noted that we optimized our IHC method to produce strong staining of known positive enterchromafin cells while maintaining a clean, negative background, so that traces of TPH1 may not have been visualized in the placenta. In any case, *tph*$^{-/-}$ mouse embryos develop normally, whereas the phenotype of the offspring of mothers lacking this gene is drastically affected (6, 113). Other findings also suggest a role for maternal 5-HT in the development of the mouse conceptus (114).

Confounding this analysis are the significant anatomical and functional differences between mouse and human concepti (48, 115). Furthermore, the mouse placenta deploys the vesicular monoamine transporter 2, presumably to store nascent 5-HT (24), whereas human trophoblasts do not (64). As delineated in this discussion, SERT seems not to be important in early mouse embryogenesis but is prominent in human trophoblasts throughout gestation (Fig. 5) (32, 54–57). Therefore, it is not clear how well mouse studies bear on human embryogenesis.

- Are there two sources of 5-HT? These findings lead to a unifying hypothesis: that both maternal and placental 5-HT contribute to intrauterine development to varying degrees and at different times in different species. There may be functional redundancy—and perhaps coordination—between these two sources of 5-HT. In any case, the pathway we have proposed from syncytiotrophoblasts to fetal blood vessels would be the same for both maternal and placental sources of serotonin (Fig. 12; Fig. 6 in Wu *et al.* (24)].

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