

Effects of Constitutive Depletion or Excess of Serotonin during Development on the Maturation of Prefrontal Monoaminergic Circuits

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Marta C. F. Samina, Maria Stanciu, Thibault Merckelijn, Lidiane P. Garcia, Sabrina Hanswijk, Josefine S. Witteveen, Natalia Alenina, Michael Bader, Judith R. Homberg, and Sharon M. Kolk*



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ABSTRACT: Serotonin (5-hydroxytryptamine; 5-HT) is a critical neuromodulatory morphogen-like signal that governs prenatal cortical circuit assembly, and altered 5-HT signaling is implicated in a wide range of psychiatric disorders. Yet the structural consequences of lifelong 5-HT imbalance during the initial blueprinting phase remain poorly defined. Here, we used two genetic rat models representing the extremes of 5-HT homeostasis, *Tph2* knockout (*Tph2*^{−/−}; chronic depletion of 5-HT) and *5-HTT* knockout (*5-HTT*^{−/−}; chronic excess of 5-HT), to investigate sub-region-specific alterations in the embryonic medial prefrontal cortex (mPFC) and in prefrontal monoaminergic circuit maturation. Our results demonstrate that the developing mPFC is not uniformly vulnerable to 5-HT dysregulation. Instead, the prelimbic (PL) cortex emerged as a region of heightened sensitivity to low 5-HT levels, exhibiting localized hyperinnervation of catecholaminergic fibers and an increase in deep-layer *Tbr1*⁺ neurons. In contrast, within the infralimbic (IL) cortex, 5-HT deficiency induced a selective loss of reelin (*Reln*)-producing Cajal-Retzius (CR) cells, indicating a sub-region-specific disruption of the cortical scaffold. Transcriptomic analyses identified selective upregulation of the low-affinity monoamine transporter *Slc29a4* and the monoamine oxidase A (*Maoa*) in *5-HTT*^{−/−} embryos, suggesting activation of compensatory extracellular 5-HT clearance pathways during embryogenesis. Together, these findings establish prenatal 5-HT levels as an essential determinant of afferent innervation and cortical scaffold integrity, and suggest that late-gestational developmental blueprinting errors may provide a neurodevelopmental substrate for later monoaminergic circuit dysfunction linked to social and cognitive phenotypes.

KEYWORDS: neurodevelopment, medial prefrontal cortex (mPFC), serotonin (5-HT), serotonin transporter (5-HTT), tryptophan hydroxylase 2 (TPH2), Cajal-Retzius cells

INTRODUCTION

The mPFC undergoes prolonged developmental maturation and is particularly vulnerable to early-life perturbations that disrupt executive, emotional, and social behavior.^{1–3} Its assembly, including neuronal proliferation, migration, and differentiation of cortical progenitors, is a tightly coordinated process governed by intrinsic genetic programs and extrinsic factors, such as morphogens, guidance cues, afferent projections, and interneuron integration.^{4,5} Among these extrinsic signals, the 5-HT system is one of the earliest neuromodulatory inputs to the developing forebrain.^{6,7} In the rat, 5-HT axons from the dorsal and median raphe nuclei innervate the mPFC as early as embryonic day (E)17, well before synaptic connectivity is established, and they play an instructive role in corticogenesis.^{8,9} During this early developmental window, 5-HT functions as a morphogen-like signal, regulating key processes such as progenitor proliferation, neuronal migration, and dendritic

maturation.^{10–16} By interacting with specific 5-HT receptors on migrating neurons and radial glia scaffolds, 5-HT fine-tunes radial and tangential cortical organization, thereby contributing to the precise laminar positioning of cortical neurons.^{17,18}

The assembly of the mPFC is further shaped by coordinated interactions between serotonergic and catecholaminergic systems, including dopamine (DA) and norepinephrine (NA). During corticogenesis, 5-HT and catecholaminergic axons, originating from the hindbrain and midbrain, respectively,

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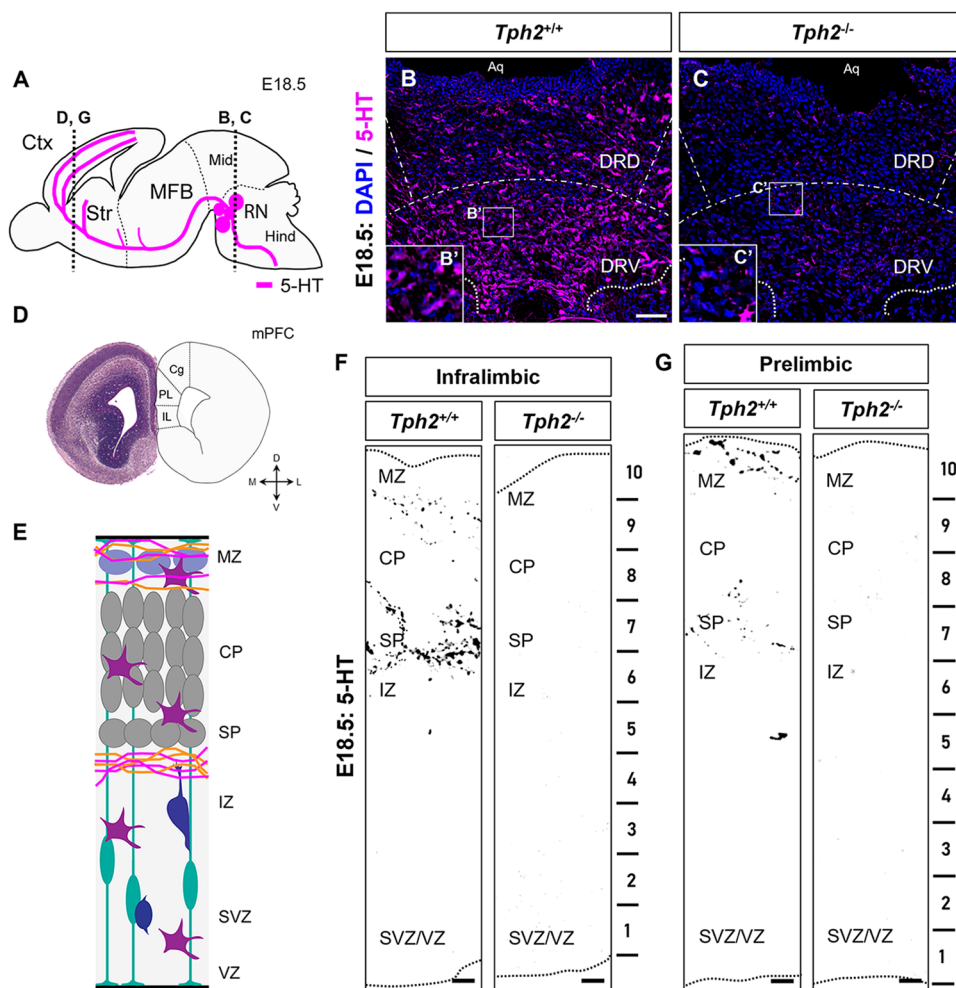


Figure 1. The developing serotonergic system is disrupted in *Tph2*^{−/−} embryos. (A) Schematic representation of a sagittal view of the rat brain at embryonic day (E)18.5. The 5-HT rhombo-prefrontal topographic projection (pink) is shown targeting the medial prefrontal cortex (mPFC). (B, C) Representative coronal prefrontal cryosections of E18.5 *Tph2*^{+/+} and *Tph2*^{−/−} rat brains immunostained for 5-HT (magenta) and counterstained with DAPI (blue), illustrating the raphe nuclei (RN). Aq: Aqueduct; DRD: dorsal raphe nucleus, dorsal part; DRV: dorsal raphe nucleus, ventral part. Scale bar: 50 μ m. (D) Schematic representation of a coronal view at E18.5. A 0.1 mm-wide rectangular region of interest (ROI) was positioned over the center of each mPFC subarea, spanning the entire cortical thickness. (E) Schematic representation of the developing cortical wall in the mPFC at E18.5. Serotonergic (5-HT) and dopaminergic (DA) fibers reach mPFC subregions in two bundles, one within the marginal zone (MZ) and the other beneath the subplate (SP), and make contact with Cajal-Retzius (CR) cells, interneurons, and proliferating neurons. Adapted from.^{11,23} Light blue cells: CR cells; cyan cells: radial glia; dark blue cells: migrating neurons; purple cells: migrating interneurons; magenta tracings: 5-HT fibers; orange tracings: DA fibers. (F, G) Enlargements of E18.5 *Tph2*^{+/+} and *Tph2*^{−/−} prefrontal cryosections showing the infralimbic (IL) and prelimbic (PL) cortices immunostained for 5-HT (black). Scale bar: 20 μ m. Cg: cingulate cortex; CP: cortical plate; Ctx: cortex; Hind: hindbrain; IZ: intermediate zone; MFB: median forebrain bundle; Mid: midbrain; RN: raphe nucleus; Str: striatum; SVZ/VZ: subventricular zone/ventricular zone. *Tph2*: Tryptophan hydroxylase 2.

innervate frontal cortical targets within overlapping embryonic time windows and often share migratory routes and molecular guidance cues.^{19–23} This temporal and spatial convergence suggests that monoaminergic systems interact to shape the developing prefrontal cortex. A critical cellular substrate for this monoaminergic regulation is the CR cell population, a transient neuronal population in the marginal zone (MZ; presumptive layer I) that secretes the glycoprotein Reelin.^{24,25} Reelin serves as an essential detachment signal for radially migrating neurons, ensuring proper inside-out cortical lamination.^{26–28} Importantly, CR cells express multiple monoaminergic receptors, making them direct targets of 5-HT, DA, and NA afferents.^{29,30} Experimental evidence indicates that 5-HT signaling supports the maintenance and survival of CR cells, as disruption of the high-affinity 5-HT transporter (5-HTT) alters Reelin⁺ cell density and causes laminar abnormalities during early postnatal life.³¹

These findings suggest that monoaminergic signaling can directly modulate the cortical structural scaffold.

Disruptions in 5-HT homeostasis during corticogenesis are strongly implicated in the etiology of multiple neuropsychiatric disorders, including autism spectrum disorder (ASD),^{32–34} depression,³⁵ and anxiety-related disorders.³⁶ Experimental investigation of these developmental perturbations has relied extensively on genetic models that bidirectionally alter 5-HT availability, including tryptophan hydroxylase 2 (TPH2)-null (*Tph2*^{−/−}) animals, which model lifelong central 5-HT depletion, and 5-HTT^{−/−} animals, which exhibit elevated extracellular 5-HT tone.^{37,38} In *Tph2*^{−/−} rodents, the absence of brain 5-HT results in profound 5-HT denervation and widespread alterations in cortical and subcortical architecture during postnatal maturation.³⁹ By contrast, 5-HTT-deficient models exhibit constitutively elevated 5-HT signaling, dis-

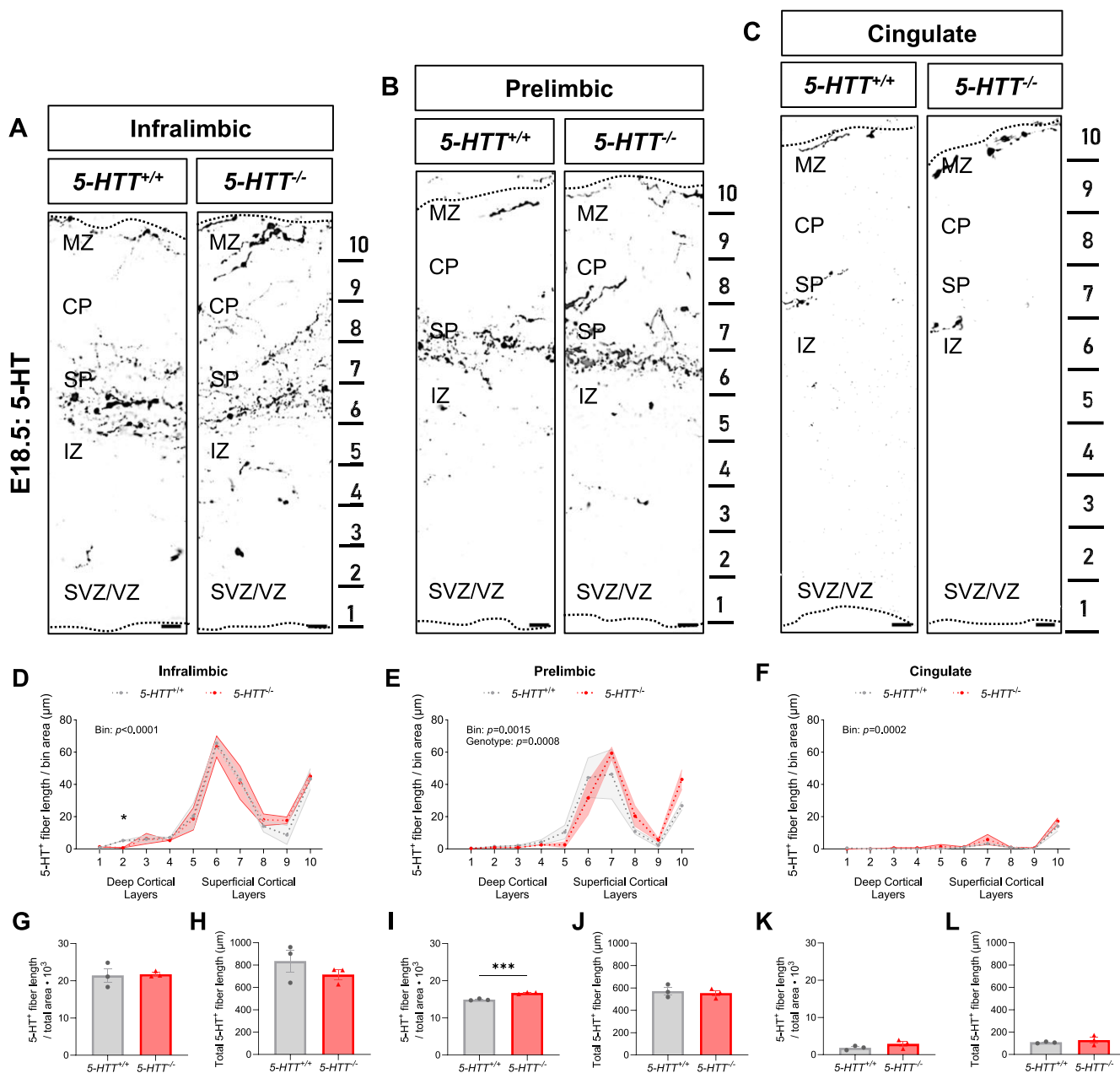


Figure 2. Serotonergic innervation of the developing prelimbic cortex is altered in $5\text{-HTT}^{-/-}$ embryos. (A–C) Enlargements of E18.5 $5\text{-HTT}^{+/+}$ and $5\text{-HTT}^{-/-}$ coronal prefrontal cryosections displaying the infralimbic (IL), prelimbic (PL), and cingulate (Cg) cortices immunostained for 5-HT (black). CP: cortical plate; IZ: intermediate zone; MZ: marginal zone; SP: subplate; SVZ/VZ: subventricular zone/ventricular zone. Scale bar: 20 μm . (D–F) Quantification of 5-HT⁺ fiber length (μm) across cortical bins in the IL, PL, and Cg cortices of $5\text{-HTT}^{-/-}$ compared to $5\text{-HTT}^{+/+}$ embryos. (G–L) Quantification of total 5-HT⁺ fiber length (μm), presented as both raw values and normalized to the total cortical area in the IL, PL, and Cg cortices. Sample size: $n = 6$ males (3 $5\text{-HTT}^{+/+}$, 3 $5\text{-HTT}^{-/-}$). Each data point represents one animal. Data are presented as the mean $\pm$ standard error of the mean (SEM). Panels (D–F): Two-way ANOVA with genotype and bin as factors. Panels (G–L): Unpaired two-tailed Student's t -test. Statistical significance: * $p < 0.05$ for $5\text{-HTT}^{+/+}$ compared to $5\text{-HTT}^{-/-}$. 5-HTT: serotonin transporter.

ruption of cortical organization, and progressive structural abnormalities.^{22,31,40}

While the long-term behavioral consequences of early 5-HT dysregulation have been extensively examined in both models, encompassing deficits in social interaction, cognitive flexibility, and emotional regulation,^{37,39,41–48} far less is known about how altered 5-HT levels affect the initial structural assembly of the mPFC. Although postnatal abnormalities in $5\text{-HTT}^{-/-}$ animals have been well documented,³¹ the state of the mPFC during late gestation remains poorly characterized. In particular, it is unclear

whether embryonic mPFC subregions show uniform vulnerability to 5-HT imbalance and whether compensatory mechanisms operate prenatally to preserve regional structural integrity.

Here, we hypothesized that lifelong 5-HT imbalance would differentially affect subregional mPFC assembly during late gestation. By studying cortical integrity in both $Tph2^{-/-}$ and $5\text{-HTT}^{-/-}$ rat models at E18.5, we aimed to identify early developmental blueprinting defects that precede postnatal circuit maturation. Through a sub-region-specific analysis, we

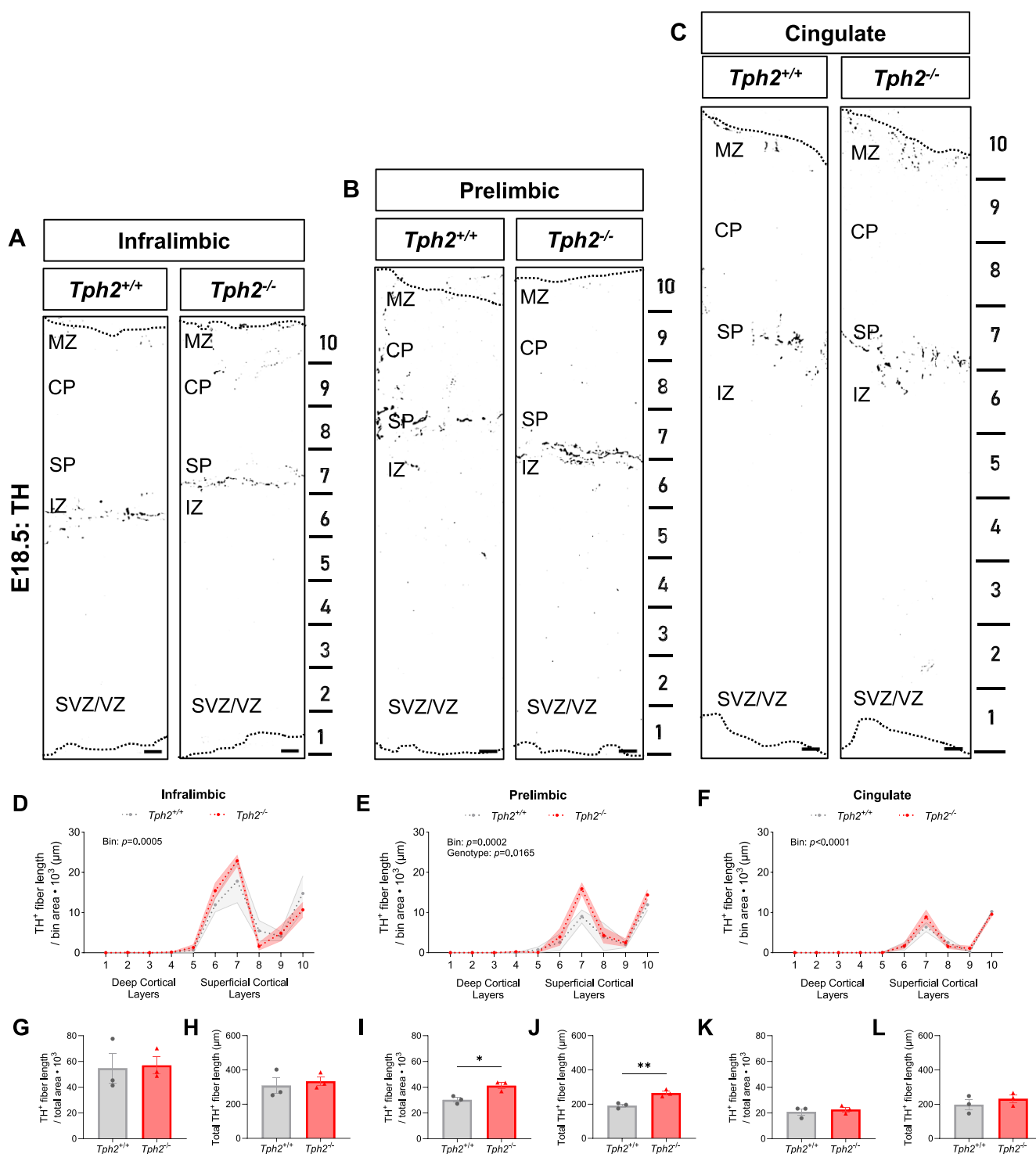


Figure 3. Catecholaminergic prefrontal innervation is disrupted in *Tph2*^{-/-} embryos. (A–C) Enlargements of E18.5 *Tph2*^{+/+} and *Tph2*^{-/-} coronal prefrontal cryosections showing the infralimbic (IL), prelimbic (PL), and cingulate (Cg) cortices immunostained for tyrosine hydroxylase (TH; black). CP: cortical plate; IZ: intermediate zone; MZ: marginal zone; SP: subplate; SVZ/VZ: subventricular zone/ventricular zone. Scale bar: 20 μ m. (D–F) Quantification of TH⁺ fiber length (μ m) across cortical bins in the IL, PL, and Cg cortices of *Tph2*^{-/-} compared to *Tph2*^{+/+} embryos. (G–L) Quantification of total TH⁺ fiber length (μ m), presented as both raw values and normalized to the total cortical area within the IL, PL, and Cg cortices. Sample size: $n = 6$ males (3 *Tph2*^{+/+}, 3 *Tph2*^{-/-}). Each data point represents one animal. Data are presented as the mean $\pm$ standard error of the mean (SEM). Panels (D–F): Two-way ANOVA with genotype and bin as factors. Panels (G–L): Unpaired two-tailed Student's *t*-test. Statistical significance: * $p < 0.05$ for *Tph2*^{+/+} compared to *Tph2*^{-/-}. *Tph2*: Tryptophan hydroxylase 2.

examined the spatial patterning of 5-HT and catecholaminergic fibers, the integrity of ReIn-producing cells, and the organization of deep-layer glutamatergic neurons. In parallel, we assessed

embryonic transcriptomic changes in the fore-, mid-, and hindbrain to uncover molecular mechanisms contributing to embryonic structural vulnerability. Together, these approaches

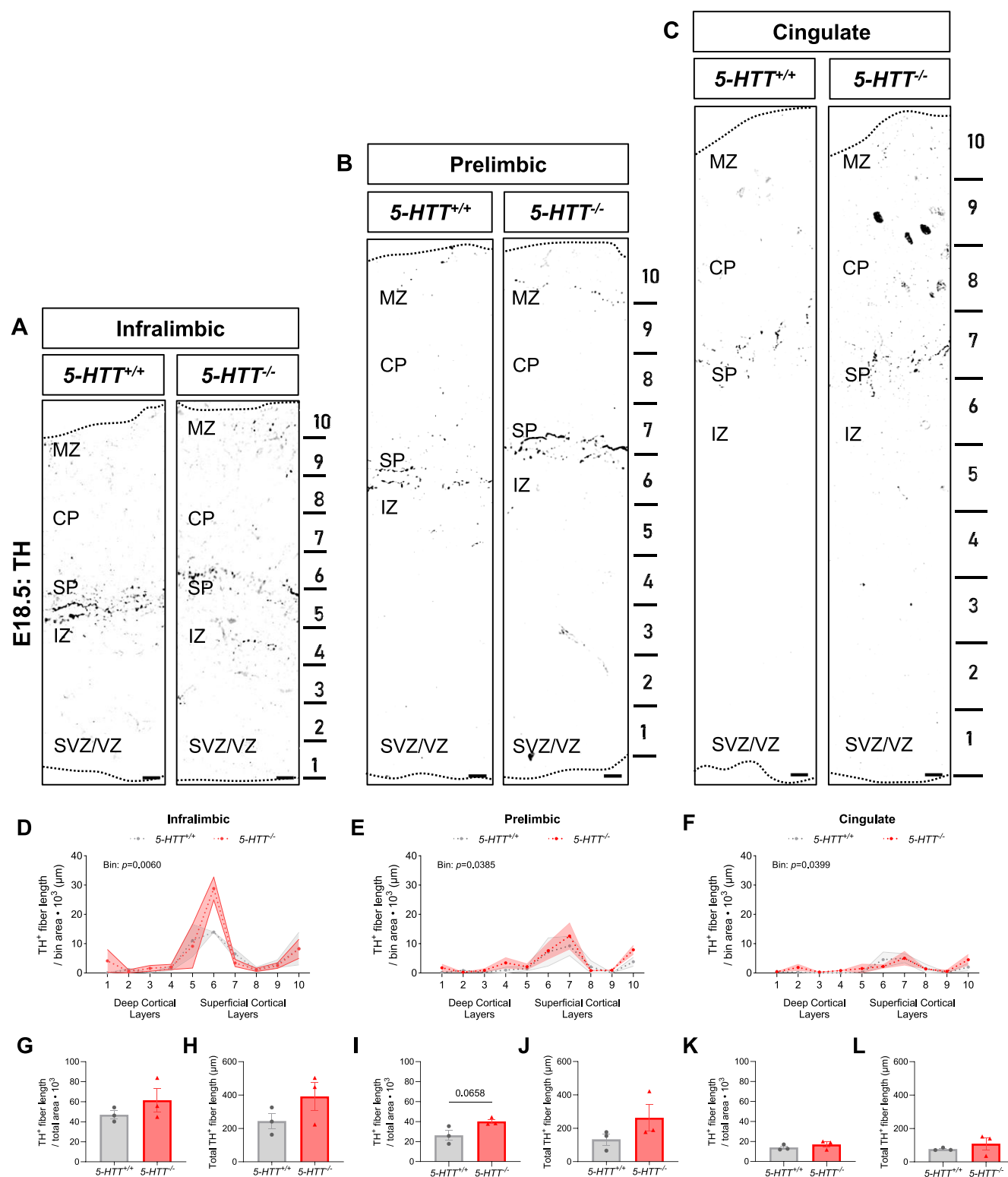


Figure 4. Alterations in catecholaminergic innervation of prefrontal regions in *5-HTT*^{-/-} embryos. (A–C) Enlargements of E18.5 *5-HTT*^{+/+} and *5-HTT*^{-/-} coronal prefrontal cryosections displaying the infralimbic (IL), prelimbic (PL), and cingulate (Cg) cortices immunostained for tyrosine hydroxylase (TH; black). CP: cortical plate; IZ: intermediate zone; MZ: marginal zone; SP: subplate; SVZ/VZ: subventricular zone/ventricular zone. Scale bar: 20 μm. (D–F) Quantification of TH⁺ fiber length (μm) across cortical bins in the IL, PL, and Cg cortices of *5-HTT*^{-/-} compared to *5-HTT*^{+/+} embryos. (G–L) Quantification of total TH⁺ fiber length (μm), presented as both raw values and normalized to the total cortical area within the IL, PL, and Cg cortices. Sample size: $n = 6$ males (3 *5-HTT*^{+/+}, 3 *5-HTT*^{-/-}). Each data point represents one animal. Data are presented as the mean ± standard error of the mean (SEM). Panels (D–F): Two-way ANOVA with genotype and bin as factors. Panels (G–L): Unpaired two-tailed Student's *t*-test. *5-HTT*: serotonin transporter.

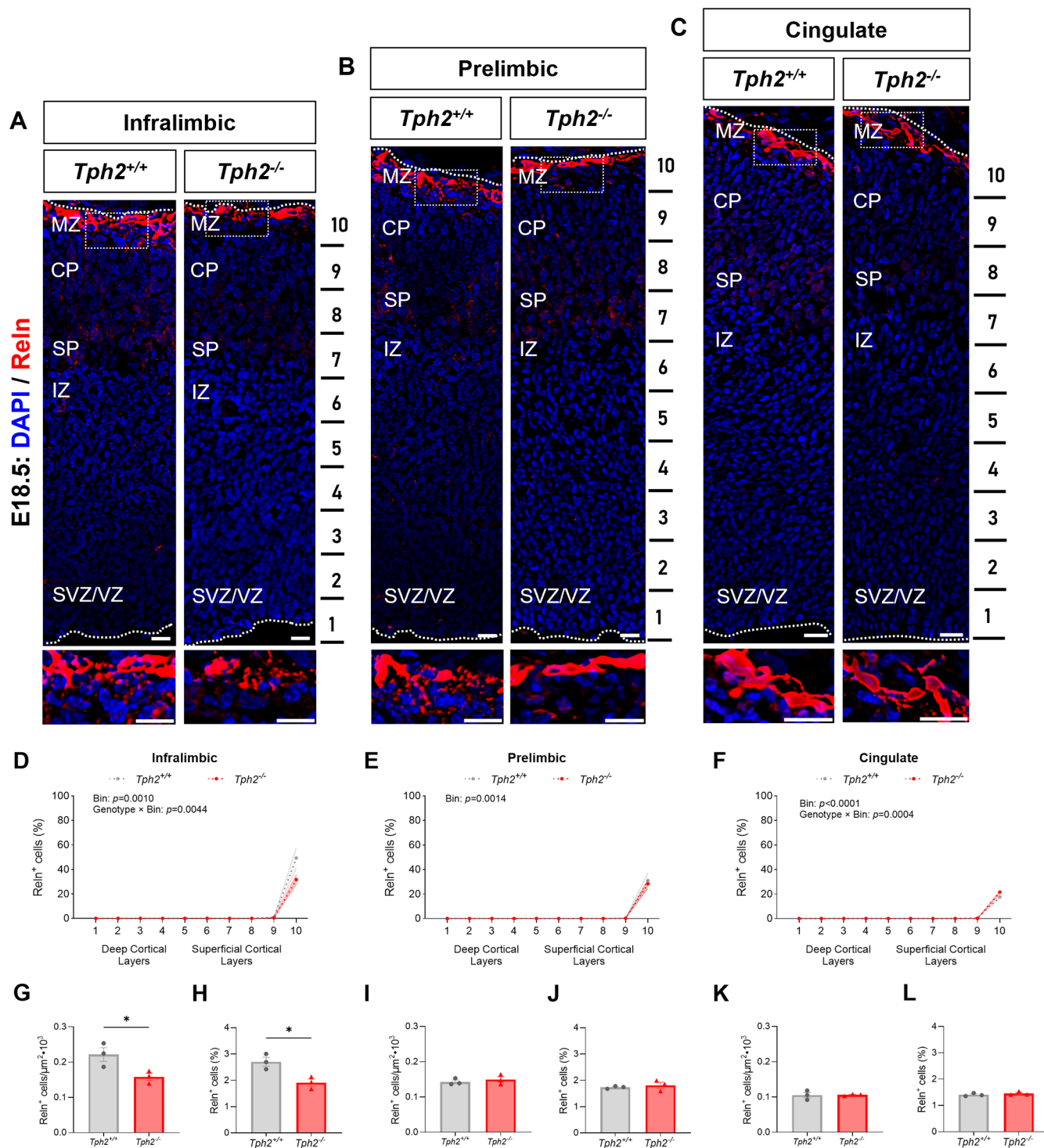


Figure 5. The number of Reelin-positive cells is reduced in *Tph2*^{-/-} embryos. (A–C) Enlargements of E18.5 *Tph2*^{+/+} and *Tph2*^{-/-} coronal prefrontal cryosections showing the infralimbic (IL), prelimbic (PL), and cingulate (Cg) cortices immunostained for reelin (Reln; red). CP: cortical plate; IZ: intermediate zone; MZ: marginal zone; SP: subplate; SVZ/VZ: subventricular zone/ventricular zone. Scale bar: 20 μm. (D–F) Quantification of Reelin⁺ cell density across cortical bins in the IL, PL, and Cg cortices of *Tph2*^{-/-} compared to *Tph2*^{+/+} embryos. (G–L) Quantification of total Reelin⁺ cells, presented as both raw counts and normalized to the total cortical area within the IL, PL, and Cg cortices. Sample size: *n* = 6 males (3 *Tph2*^{+/+}, 3 *Tph2*^{-/-}). Each data point represents one animal. Data are presented as the mean ± standard error of the mean (SEM). Panels (D–F): Two-way ANOVA with genotype and bin as factors. Panels (G–L): Unpaired two-tailed Student's *t*-test. Statistical significance: **p* < 0.05 for *Tph2*^{+/+} compared to *Tph2*^{-/-}. *Tph2*: Tryptophan hydroxylase 2.

aim to identify the earliest developmental perturbations that

underlie monoamine-related neurodevelopmental disorders.

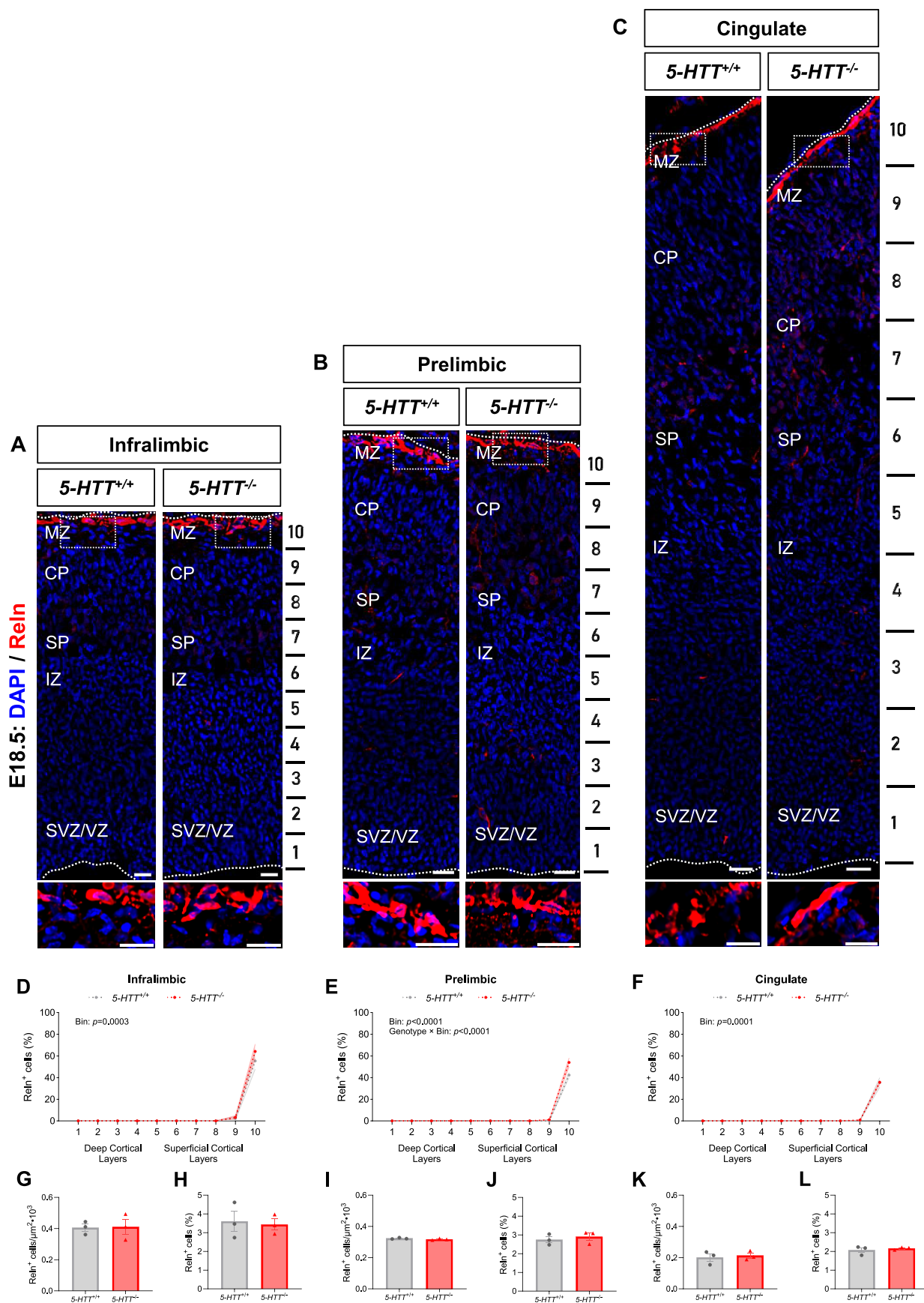


Figure 6. Reelin-positive cell distribution and number are preserved in $5\text{-HTT}^{-/-}$ embryos. (A–C) Enlargements of E18.5 $5\text{-HTT}^{+/+}$ and $5\text{-HTT}^{-/-}$ coronal prefrontal cryosections displaying the infralimbic (IL), prelimbic (PL), and cingulate (Cg) cortices immunostained for reelin (Reln; red). CP: cortical plate; IZ: intermediate zone; MZ: marginal zone; SP: subplate; SVZ/VZ: subventricular zone/ventricular zone. Scale bar: 20 μm . (D–F)

Figure 6. continued

Quantification of ReIn^+ cell density across cortical bins in the IL, PL, and Cg cortices of $S\text{-HTT}^{-/-}$ compared to $S\text{-HTT}^{+/+}$ embryos. (G–L) Quantification of total ReIn^+ cells, presented as both raw counts and normalized to the total cortical area within the IL, PL, and Cg cortices. Sample size: $n = 6$ males (3 $S\text{-HTT}^{+/+}$, 3 $S\text{-HTT}^{-/-}$). Each data point represents one animal. Data are presented as the mean $\pm$ standard error of the mean (SEM). Panels (D–F): Two-way ANOVA with genotype and bin as factors. Panels (G–L): Unpaired two-tailed Student's t -test. $S\text{-HTT}$: serotonin transporter.

RESULTS

Disrupted 5-HT and Catecholaminergic Fiber Innervation in 5-HT rat Models

5-HT acts as a pivotal neurotrophic signal during embryonic development.^{10–15} The 5-HT system develops in close spatiotemporal coordination with catecholaminergic projections,^{23,31} and both systems co-innervate common targets, including the mPFC. Given that the absence of 5-HTT leads to a paradoxical increase in both 5-HT- and tyrosine hydroxylase (TH)-positive fiber density in the IL and PL cortices by P6,^{22,31} we sought to determine whether these alterations are established prenatally. To this end, we performed immunohistochemical (IHC) analysis of coronal sections at E18.5 in two distinct 5-HT models (Figures 1–4).

In the $Tph2^{-/-}$ model, 5-HT⁺ immunoreactivity in the raphe nuclei (RN) was markedly reduced compared with $Tph2^{+/+}$ controls (Figure 1B,C). Consequently, 5-HT⁺ fibers were virtually absent from the IL, PL (Figure 1F,G), and Cg cortices (data not shown). In $S\text{-HTT}^{-/-}$ rats, however, the impact on 5-HT innervation was more nuanced and sub-region-specific (Figure 2). Laminar analysis revealed a significant bin-dependent distribution of 5-HT⁺ fibers across all mPFC subregions (IL: $F_{(2.145, 8.581)} = 46.07$, $p < 0.0001$; PL: $F_{(1.707, 6.830)} = 20.90$, $p = 0.0015$; Cg: $F_{(1.905, 7.620)} = 34.73$, $p = 0.0002$; Figure 2D–F). A genotype effect was observed in the PL ($F_{(1, 4)} = 81.34$, $p = 0.0008$). Detailed analysis of laminar distribution revealed a significant, localized reduction in 5-HT⁺ fibers in Bin 2 of the IL of $S\text{-HTT}^{-/-}$ embryos ($p = 0.0355$; Figure 2D). However, total cumulative fiber length in the IL, both raw and normalized to total cortical area, remained unchanged (Figure 2G,H). Within the PL, although the overall laminar pattern appeared similar across genotypes, the overall innervation density (total cumulative fiber length normalized by area) was significantly higher in $S\text{-HTT}^{-/-}$ compared to $S\text{-HTT}^{+/+}$ rats ($p = 0.0009$; Figure 2E,I,J). No significant changes were observed in the Cg cortex in either bin distribution or total cumulative 5-HT⁺ innervation (Figure 2F,K,L).

Given the absence of 5-HT in the $Tph2^{-/-}$ model, we next investigated whether catecholaminergic projections to the mPFC were affected by the loss of 5-HT fibers targeting the mPFC (Figure 3). Similar to our previous observations, analysis of TH⁺ fiber distribution revealed a significant main effect of bin across all subregions (IL: $F_{(1.588, 6.351)} = 35.01$, $p = 0.0005$; PL: $F_{(2.135, 8.541)} = 27.73$, $p = 0.0002$; Cg: $F_{(1.857, 7.426)} = 68.55$, $p < 0.0001$; Figure 3D–F). A genotype effect was observed in the PL ($F_{(1, 4)} = 15.80$, $p = 0.0165$). In addition to the complete absence of 5-HT⁺ fibers, overall catecholaminergic innervation density normalized by area was significantly increased in the PL of $Tph2^{-/-}$ embryos compared to $Tph2^{+/+}$ rats ($p = 0.0165$; Figure 3I). This was further supported by a significant increase in total cumulative TH⁺ fiber length ($p = 0.0090$; Figure 3J). No significant differences in TH⁺ innervation density or cumulative length were observed in the IL and Cg cortices (Figure 3D,F–H,K,L).

Parallel analysis of the 5-HTT strain revealed a similar main effect of bin on TH⁺ fiber distribution across all subregions (IL: $F_{(2.105, 8.422)} = 9.770$, $p = 0.0060$; PL: $F_{(1.542, 6.170)} = 6.155$, $p = 0.0385$; Cg: $F_{(2.779, 11.12)} = 3.978$, $p = 0.0399$; Figure 4D–F). Although no significant genotype differences were observed in individual bins, the overall TH⁺ innervation density in the PL, normalized for area, showed a nonsignificant tendency toward an increase in $S\text{-HTT}^{-/-}$ compared to $S\text{-HTT}^{+/+}$ rats ($p = 0.0658$; Figure 4I).

Taken together, these results demonstrate that 5-HT dysregulation, whether driven by loss of endogenous synthesis or by the absence of the presynaptic transporter, triggers an early onset reorganization of monoaminergic prefrontal afferents, with the PL cortex showing the greatest susceptibility to altered innervation patterns at E18.5.

5-HT Dysregulation Selectively Disrupts Laminar Distribution in the Embryonic Prefrontal Cortex

To determine whether changes in embryonic 5-HT levels affect the structural organization of the mPFC, we examined the expression of key markers of cortical lamination and neuronal laminar identity.

CR cells in the MZ secrete ReIn ,^{24,31} a critical glycoprotein that orchestrates the inside-out migration of glutamatergic neurons and establishes proper cortical layering.²⁹ We hypothesized that the altered 5-HT environment in $Tph2^{-/-}$ and $S\text{-HTT}^{-/-}$ embryos might disrupt ReIn expression (Figures 5 and 6), potentially leading to downstream defects in the positioning of deep-layer neurons (Figures 7 and 8).

Analysis of ReIn^+ cells in the $Tph2^{-/-}$ IL cortex revealed a significant main effect of bin ($F_{(1.011, 4.043)} = 71.34$, $p = 0.0010$) and a genotype $\times$ bin interaction ($F_{(9, 36)} = 3.366$, $p = 0.0044$; Figure 5D). This was reflected in the overall population density, with a significant reduction in ReIn^+ cell density normalized by area ($p = 0.0451$; Figure 5G) and a corresponding decrease in the cumulative percentage across the IL ($p = 0.0205$; Figure 5H). In contrast, the PL and Cg cortices were less affected. Although a bin effect was detected in both regions (PL: $F_{(1.002, 4.007)} = 62.73$, $p = 0.0014$; Cg: $F_{(1.048, 4.193)} = 439.20$, $p < 0.0001$; Figure 5E,F), no significant genotype differences were observed in the PL (Figure 5E,I,J). In the Cg, however, a significant genotype $\times$ bin interaction was detected ($F_{(9, 36)} = 4.688$, $p = 0.0004$; Figure 5F), although no differences were observed in the cumulative ReIn^+ cell density (Figure 5K,L). In $S\text{-HTT}^{-/-}$ embryos, ReIn^+ cell density remained largely unaffected. A significant main effect of bin was observed across all subregions (IL: $F_{(1.027, 4.108)} = 133.90$, $p = 0.0003$; PL: $F_{(1.103, 4.054)} = 383.90$, $p < 0.0001$; Cg: $F_{(1.023, 4.090)} = 204.2$, $p = 0.0001$; Figure 6D–F). Although a significant genotype $\times$ bin interaction was detected in the PL ($F_{(9, 36)} = 5.537$, $p < 0.0001$), post hoc analyses did not reveal significant genotype differences within individual bins, and cumulative ReIn^+ cell density was unaltered (Figure 6G–L). These findings suggest that although endogenous 5-HT synthesis is required for the proper

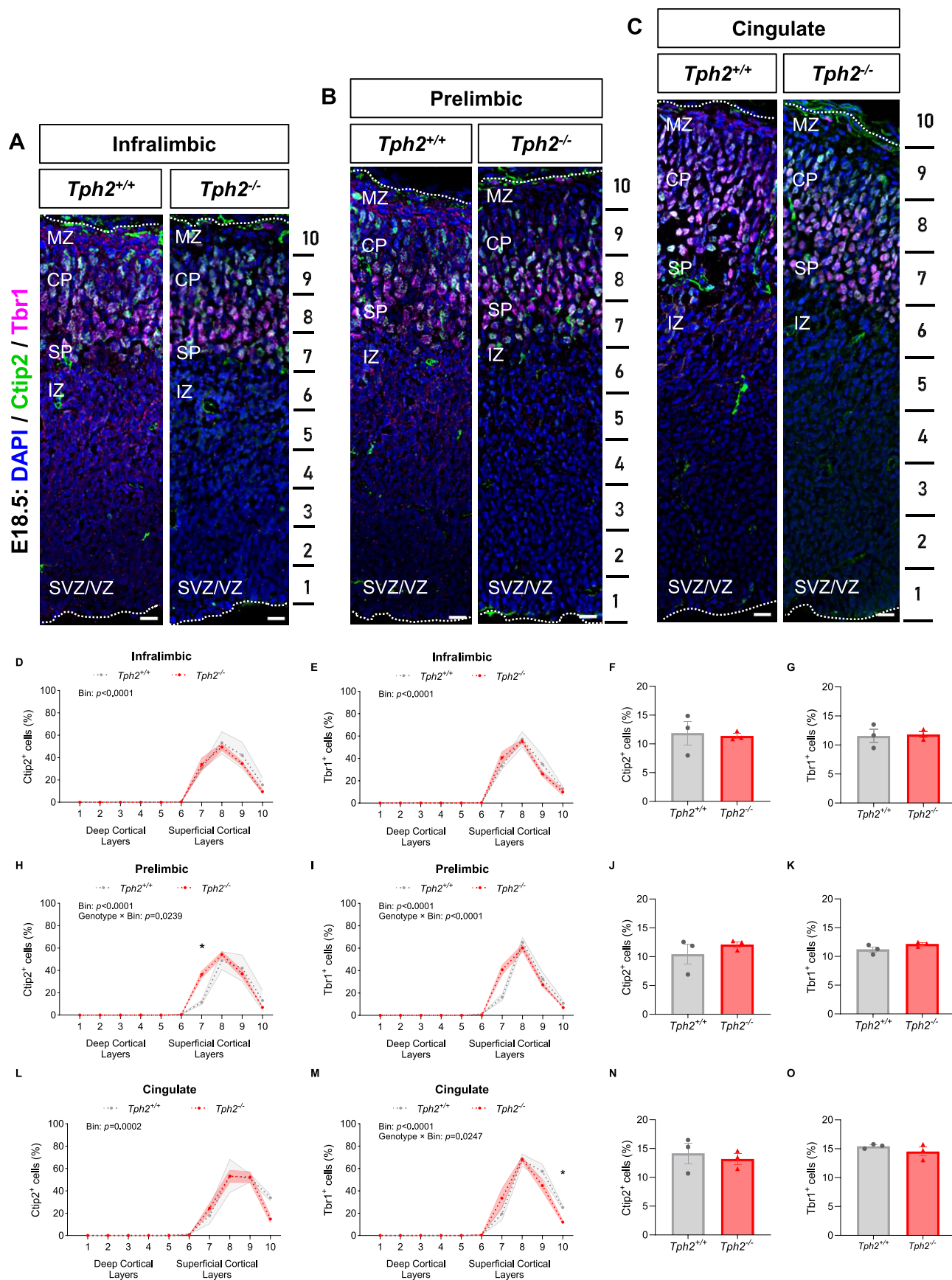


Figure 7. Prefrontal cytoarchitecture is altered in the prelimbic cortex of *Tph2*^{-/-} embryos. (A–C) Enlargements of E18.5 *Tph2*^{+/+} and *Tph2*^{-/-} coronal prefrontal cryosections showing the infralimbic (IL), prelimbic (PL), and cingulate (Cg) cortices immunostained for CtIP2 (layers V–VI; green) and Tbr1 (layer VI; magenta). CP: cortical plate; IZ: intermediate zone; MZ: marginal zone; SP: subplate; SVZ/VZ: subventricular zone/

Figure 7. continued

ventricular zone. Scale bar: 20 μm . (D, E, H, I, L, M) Quantification of Ctip2⁺ and Tbr1⁺ cell density across cortical bins in the IL, PL, and Cg cortices of *Tph2*^{-/-} compared to *Tph2*^{+/-} embryos. (F, G, J, K, N, O) Quantification of the percentage of total Ctip2⁺ and Tbr1⁺ cells within the IL, PL, and Cg cortices. Sample size: $n = 6$ males (3 *Tph2*^{+/-}, 3 *Tph2*^{-/-}). Each data point represents one animal. Data are presented as the mean $\pm$ standard error of the mean (SEM). Panels (D–F): Two-way ANOVA with genotype and bin as factors. Panels (G–L): Unpaired two-tailed Student's *t*-test. *Tph2*: Tryptophan hydroxylase 2.

maintenance or recruitment of CR cells in the IL, an excess of extracellular 5-HT does not acutely disrupt this scaffold at E18.5.

To further characterize the impact of altered 5-HT homeostasis on cortical cytoarchitecture, we assessed the distributions of subcerebral projection neurons (Ctip2⁺, layers V–VI) and corticothalamic neurons (Tbr1⁺, layer VI). In the *Tph2*^{-/-} model, consistent with cortical lamination, a significant main effect of bin was observed on both Ctip2⁺ and Tbr1⁺ cell percentages across all subregions: IL (Ctip2: $F_{(2.258, 9.031)} = 52.46$, $p < 0.0001$; Tbr1: $F_{(1.954, 7.816)} = 100.80$, $p < 0.0001$), PL (Ctip2: $F_{(1.905, 7.621)} = 48.61$, $p < 0.0001$; Tbr1: $F_{(1.546, 6.184)} = 170.00$, $p < 0.0001$), and Cg (Ctip2: $F_{(1.489, 5.956)} = 53.69$, $p = 0.0002$; Tbr1: $F_{(1.425, 5.700)} = 107.50$, $p < 0.0001$; Figure 7D,E,H,I,L,M). Detailed post hoc analysis revealed localized laminar disruptions. In the PL, significant genotype $\times$ bin interactions were detected for both Ctip2⁺ and Tbr1⁺ cell distributions (Ctip2: $F_{(9, 36)} = 2.514$, $p = 0.0239$; Tbr1: $F_{(9, 36)} = 6.937$, $p < 0.0001$; Figure 7H,I). *Tph2*^{-/-} rats showed a localized increase in Ctip2⁺ cells in Bin 7 of the PL ($p = 0.0475$; Figure 7H). Furthermore, the absolute number of Tbr1⁺ neurons was significantly higher in *Tph2*^{-/-} embryos than in *Tph2*^{+/-} rats ($p = 0.0008$, Figure S1D). In the Cg cortex, a significant genotype $\times$ bin interaction was observed ($F_{(9, 36)} = 2.499$, $p = 0.0247$; Figure 7M). Post hoc analyses revealed a significant reduction in Tbr1⁺ cells in the MZ of *Tph2*^{-/-} embryos ($p = 0.0415$), although overall cell numbers remained unchanged. No significant genotype-driven differences were observed in the IL (Figures 7D–G and S1A,B). In *5-HTT*^{-/-} embryos, the cellular architecture of the mPFC remained largely preserved. Two-way ANOVA of Ctip2⁺ and Tbr1⁺ cell percentages revealed main effects of bin across all subregions: IL (Ctip2: $F_{(1.916, 7.666)} = 36.98$, $p = 0.0001$; Tbr1: $F_{(1.425, 5.700)} = 21.61$, $p = 0.0029$), PL (Ctip2: $F_{(1.582, 6.326)} = 60.53$, $p < 0.0001$; Tbr1: $F_{(1.602, 6.410)} = 36.45$, $p = 0.0010$), and Cg (Ctip2: $F_{(2.037, 8.146)} = 106.70$, $p < 0.0001$; Tbr1: $F_{(2.122, 8.489)} = 58.26$, $p < 0.0001$; Figure 8D,E,H,I,L,M). In the PL, significant genotype $\times$ bin interactions were detected for both Ctip2⁺ and Tbr1⁺ cell distributions (Ctip2: $F_{(9, 36)} = 2.989$, $p = 0.0092$; Tbr1: $F_{(9, 36)} = 4.154$, $p = 0.0010$; Figure 8H,I). In the Cg, a significant genotype $\times$ bin interaction was detected only for the Tbr1⁺ cell distribution ($F_{(9, 36)} = 3.251$, $p = 0.0055$; Figure 8M). Although no significant differences were observed in the spatial distribution (Figure 8F,G,J,K,N,O), absolute cell counts showed a nonsignificant increase in density in *5-HTT*^{-/-} embryos. Specifically, the total number of Ctip2⁺ cells in the IL approached statistical significance ($p = 0.0549$; Figure S2A), as did the total number of Tbr1⁺ cells within the PL ($p = 0.0556$; Figure S2D).

Finally, we evaluated whether these alterations were associated with gross morphological changes. Analysis of DAPI⁺ cell density and cortical thickness revealed no significant differences between genotypes in either model across any subregion (Figure S3). These data indicate that 5-HT-mediated effects on Reln distribution and laminar identity occur

independently of changes in the overall physical dimensions or the total cellular population of the embryonic mPFC.

In summary, 5-HT dysregulation triggers distinct, sub-region-specific structural phenotypes at E18.5. Loss of 5-HT synthesis disrupts the IL MZ, whereas the absence of 5-HT reuptake largely preserves the cellular scaffold with only trends toward increased cell populations. Importantly, these cellular shifts occur without gross morphological defects, suggesting that 5-HT homeostasis is specifically required for the fine-tuning of prefrontal cytoarchitecture during late gestation.

Temporal Dynamics of Monoaminergic Gene Expression in the Developing Rat Brain

Having established the structural and cellular abnormalities present at E18.5, we aimed to contextualize our findings within the broader temporal landscape of monoaminergic gene expression. To achieve this, we first analyzed the expression trajectories of key genes involved in monoamine transport, receptor signaling, and metabolism, including *Slc6a3*, *Drd2*, *Ddc*, *Slc22a3*, *Comt*, and *Maob* (Figure S4A) using the EMBL-EBI Expression Atlas. *In silico* profiling from E14 to postnatal day (P)0 revealed distinct spatial and temporal patterns across the developing forebrain and hindbrain (Figure S4B–G).

Among the markers investigated, *Slc6a3* exhibited the most robust overall expression throughout this developmental window (Figure S4B). Notably, *Drd2* and *Maob* showed consistent increases in expression across gestation, with both transcripts reaching nearly 30 transcripts per million (TPMs) by P0 (Figure S4C,G). In contrast, the organic cation transporter *Slc22a3* remained low during early gestation, with a late-stage increase only after E19 (Figure S4E). Spatial differences were also evident, particularly for *Ddc*, which was markedly enriched in the hindbrain relative to forebrain regions (Figure S4D). Meanwhile, the metabolic regulator *Comt* exhibited a relatively stable and uniform expression pattern (Figure S4F). These database-derived expression profiles confirm that the prenatal period is marked by a dynamic, region-specific monoaminergic transcriptional environment, supporting our rationale for targeted RT-qPCR analysis of the fore-, mid-, and hindbrains at E20.5.

Late-Prenatal Transcriptional Dysregulation of Monoaminergic Signaling in *5-HTT*^{-/-} Rats

To assess the molecular consequences of 5-HTT deficiency, we performed RT-qPCR analysis at E20.5 in the fore-, mid-, and hindbrain of *5-HTT*^{-/-} embryos and their *5-HTT*^{+/-} littermates. This developmental stage follows the peak of prefrontal fiber organization observed at E18.5, allowing us to capture the transcriptional state of the monoaminergic system just before birth.

Two-way ANOVA analysis of *Slc6a3* revealed a significant main effect of brain region ($F_{(2,20)} = 5.987$, $p = 0.0092$; Figure S5B), characterized by a decrease in midbrain expression in *5-HTT*^{-/-} embryos compared to *5-HTT*^{+/-} rats ($p = 0.0286$). Furthermore, in *5-HTT*^{-/-} animals, *Slc6a3* levels in the

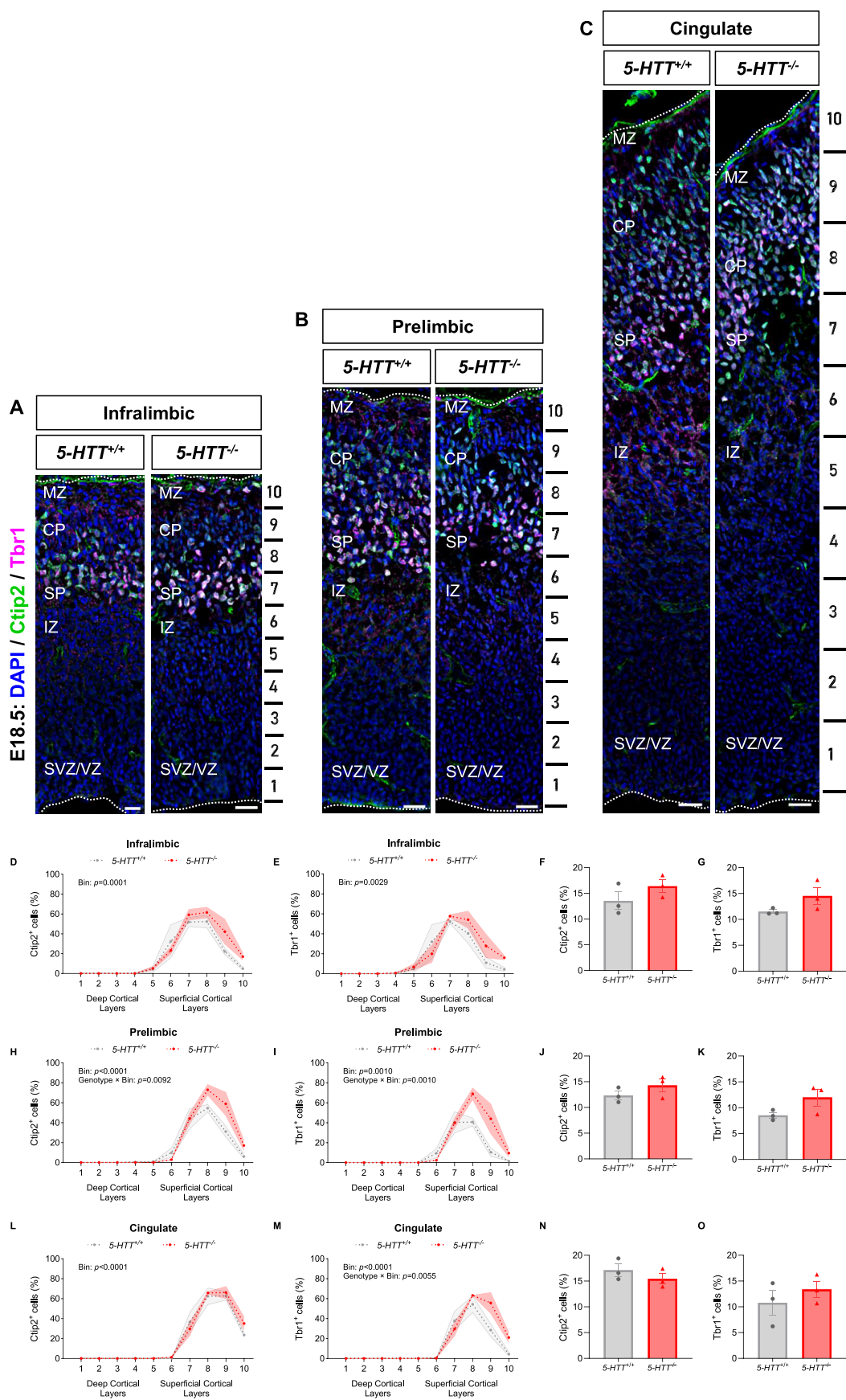


Figure 8. Prefrontal cortical organization is preserved in 5-HTT^{-/-} embryos. (A–C) Enlargements of E18.5 5-HTT^{+/+} and 5-HTT^{-/-} coronal prefrontal cryosections displaying the infralimbic (IL), prelimbic (PL), and cingulate (Cg) cortices immunostained for Ctbp2 (layers V–VI; green) and Tbr1 (layer VI; magenta). CP: cortical plate; IZ: intermediate zone; MZ: marginal zone; SP: subplate; SVZ/VZ: subventricular zone/ventricular zone.

Figure 8. continued

Scale bar: 20 μm . (D, E, H, I, L, M) Quantification of Ctip2⁺ and Tbr1⁺ cell density across cortical bins in the IL, PL, and Cg cortices of *5-HTT*^{−/−} compared to *5-HTT*^{+/+} embryos. (F, G, J, K, N, O) Quantification of the percentage of total Ctip2⁺ and Tbr1⁺ cells within the IL, PL, and Cg cortices. Sample size: $n = 6$ males (3 *5-HTT*^{+/+}, 3 *5-HTT*^{−/−}). Each data point represents one animal. Data are presented as the mean $\pm$ standard error of the mean (SEM). Panels (D–F): Two-way ANOVA with genotype and bin as factors. Panels (G–L): Unpaired two-tailed Student's *t*-test. *5-HTT*: serotonin transporter.

midbrain were markedly lower than those in the forebrain ($p = 0.0042$).

Beyond the high-affinity reuptake system, we identified significant genotype effects for the low-affinity plasma membrane monoamine transporter (*Slc29a4*: $F_{(1,24)} = 6.764$, $p = 0.0157$; Figure S5F) and the metabolic enzyme *Maoa* ($F_{(1,24)} = 4.391$, $p = 0.0469$; Figure S5I). Both transcripts were elevated in the *5-HTT*^{−/−} model, suggesting a targeted compensatory response to increased extracellular 5-HT during late gestation. In contrast, the organic cation transporter *Slc22a3* showed no significant differences across regions or genotypes (Figure S5G), indicating that this “uptake 2” component does not contribute to the observed molecular adaptations at this stage.

Finally, other dopaminergic markers (*Drd2*, *Ddc*, *Slc18a2*; Figure S5C–E) and metabolic regulators (*Comt*, *Maob*; Figure S5H,J) remained transcriptionally stable. Consistent with our immunohistochemical findings, *Reln* mRNA levels were unchanged (Figure S5K), confirming that the *Reln*-mediated scaffold remains resilient to *5-HTT* deficiency at the transcript level.

Altogether, our results indicate that *5-HTT* deficiency at E20.5 does not cause a global dysregulation of monoaminergic signaling but instead induces selective upregulation of alternative transport (*Slc29a4*) and degradative (*Maoa*) machinery. Moreover, the localized reduction in midbrain *Slc6a3* suggests a specific interaction between 5-HT homeostasis and dopamine system development. This suggests that while the *Reln* cortical scaffold remains transcriptionally stable, the embryonic brain undergoes targeted molecular shifts in response to the lifelong loss of *5-HTT*.

DISCUSSION

This study evaluated whether lifelong low (*Tph2*^{−/−}) and high (*5-HTT*^{−/−}) 5-HT levels influence the development of 5-HT and catecholaminergic prefrontal innervation, as well as the structural organization of the mPFC during late prenatal development in rats. In *Tph2*-deficient rats, we observed a marked reduction in 5-HT⁺ fiber density and robust hyperinnervation of TH⁺ fibers, particularly within the PL cortex. These changes were accompanied by a localized disruption of the *Reln*-positive scaffold in the IL and alterations in deep-layer lamination. In contrast, in the *5-HTT*^{−/−} model, we observed similar trends toward increased TH⁺ innervation and cellular density, but overall embryonic cortical architecture was largely preserved at E18.5.

Despite the genetic deletion of *Tph2*, a sparse population of 5-HT⁺ neurons was detected in the embryonic RN. The presence of 5-HT in the absence of its rate-limiting enzyme suggests uptake of exogenous 5-HT from maternal or placental sources, which are known to provide a critical 5-HT supply to the fetal brain prior to maturation of the blood-brain barrier.^{49,50} However, a potential contribution of transient TPH1 expression cannot be excluded.^{51,52} Future assessment of hindbrain lineage markers such as *Lmx1b* and *Pet1*^{53,54} is necessary to determine

whether these residual 5-HT⁺ neurons retain 5-HT identity and mature properly despite impaired endogenous synthesis.

A key outcome of this study is the regional segregation of afferent defects within the mPFC, with the PL cortex identified as a primary hotspot for catecholaminergic fiber reorganization. The reduction in 5-HT⁺ fiber density observed in *Tph2*^{−/−} embryos is consistent with previous reports of widespread 5-HT denervation following *Tph2* deletion.³⁹ Loss of 5-HT input allows compensatory ingrowth of other monoaminergic systems. Hyperinnervation of TH⁺ fibers in *Tph2*^{−/−} embryos and similar trends in *5-HTT*^{−/−} rats support the idea that 5-HT may act as a developmental gatekeeper that constrains the timing and innervation of catecholaminergic afferents. During corticogenesis, 5-HT and DA axons share migratory routes and target overlapping cortical layers,²³ so disruption of 5-HT homeostasis may lead to a compensatory response by DA fibers. Notably, these alterations are already evident by E18.5 and persist postnatally. In *5-HTT*^{−/−} rats, both 5-HT and catecholaminergic hyperinnervation remain prominent features of the mPFC at P6,^{22,31} indicating that the prenatal blueprinting errors have long-term consequences for prefrontal circuit wiring. Such early structural disturbances may contribute to the altered prefrontal connectivity observed across neurodevelopmental disorders linked to 5-HT dysfunction. Altered 5-HT signaling has been implicated in several neurodevelopmental and psychiatric conditions,^{10,55,56} many of which are associated with disrupted maturation and connectivity of prefrontal circuits. Although these disorders are multifactorial and cannot be attributed to 5-HT imbalance alone, our findings support the concept that perturbation of 5-HT homeostasis during critical embryonic developmental windows may alter the establishment of prefrontal circuits, potentially contributing to later functional impairments. Further elucidation of the mechanisms underlying cortical reorganization requires examination of alterations in monoaminergic source nuclei. In *5-HTT*^{−/−} rats, assessment of the RN at P6 revealed a reduced number of 5-HT⁺ neurons in both the dorsal and median raphe, accompanied by increased nuclear surface area.³¹ It remains to be determined whether cortical hyperinnervation results from altered neuronal proliferation at the source or dysregulation of axonal guidance and terminal branching.

Another important finding of this study is that *Reln* expression within the MZ is selectively reduced in the absence of 5-HT (*Tph2*^{−/−}), while it remains intact in *5-HTT*^{−/−} embryos at E18.5. These results indicate that 5-HT signaling is necessary for the maintenance or survival of *Reln*-producing CR cells rather than for their initial specification or positioning. 5-HT fibers are in proximity to CR cells in the MZ, where they can modulate CR cell activity and survival via 5-HT_{1A} or 5-HT₃ receptors.^{24,29,55,57,58} CR cells originate primarily from the cortical hem and migrate tangentially through to the MZ to populate distinct cortical subregions.^{59–61}

The IL cortex was particularly vulnerable to *Reln* disruption. As one of the earliest mPFC subregions to receive 5-HT input during gestation,^{6,62} the IL may have a narrower window of

plastic resilience, making its early scaffold more dependent on 5-HT-mediated support. Although *Reln* expression was preserved in *5-HTT*^{−/−} rats at E18.5, previous analyses at P6 revealed significant reductions in *Reln*⁺ cells across the PL and Cg cortices.³¹ This temporal divergence indicates that lifelong 5-HT excess does not acutely disrupt the embryonic scaffold but instead accelerates CR cell loss or scaffold degradation during early postnatal development. Further studies incorporating apoptotic and neuronal markers will be necessary to determine whether this reduction reflects the selective loss of *Reln*-producing cells or a broader decrease in neuronal density. Reduced *Reln* expression in *Tph2*-deficient models indicates an alteration of the *Reln*⁺ cortical scaffold during embryogenesis. In contrast, the subsequent loss of *Reln*⁺ cells in *5-HTT*^{−/−} models³¹ suggests that the effects of increased 5-HT availability on *Reln* expression arise during postnatal stages, a period characterized by active synaptic pruning and circuit refinement.⁶³

Beyond the structural scaffold, our findings demonstrate that 5-HT homeostasis is critical for the lamination of deep-layer glutamatergic neurons. In the PL cortex of *Tph2*^{−/−} embryos, we observed a significant increase in the absolute number of *Tbr1*⁺ neurons, suggesting altered timing of neurogenesis. Given that 5-HT regulates cortical progenitor proliferation,⁵⁷ its absence may disrupt the temporal regulation of neurogenesis, leading to altered numbers or distribution of early-born neuronal populations. In parallel, *Tph2*^{−/−} embryos exhibited a reduction of *Tbr1*⁺ cells within the MZ. As 5-HT functions as a guidance cue during neuronal migration,^{17,18,22} this reduction likely reflects redistribution of the *Tbr1*⁺ population, with neurons less prone to over-migrate into the MZ. Notably, although we observed increased *Tbr1*⁺ cell numbers at embryonic stages, analyses at P6 and P25 in the *5-HTT*^{−/−} model reveal a reduction in *Tbr1*⁺ cells throughout the PL.³¹ This inversion suggests that early-born neurons may be overproduced or mispositioned prenatally, which could affect their incorporation into postnatal prefrontal circuits and contribute to altered circuit maturation.

Although the present prenatal analysis focused on deep-layer populations, future studies should extend these observations to superficial cortical layers (II–III) at embryonic stages. This is particularly relevant given that both *Tph2*^{−/−} and *5-HTT*^{−/−} models exhibit significant postnatal alterations in upper-layer organization during early postnatal stages, including reduced layer II–IV thickness and decreased numbers of *Cux1*⁺ neurons at P7 and P6, respectively.^{31,44}

An important question raised by our findings is why the *5-HTT*^{−/−} model retains preserved cortical architecture at E18.5 despite clear trends toward fiber hyperinnervation in the PL cortex. Our mRNA expression data suggest that this resilience may result from activation of a molecular buffering mechanism. Specifically, upregulation of *Slc29a4* and *Maoa* suggests recruitment of low-affinity “uptake 2” transporters and metabolic enzymes to compensate for the loss of high-affinity 5-HT reuptake.^{64,65} However, this buffering may be limited; as 5-HT⁺ fiber density increases postnatally, compensatory mechanisms may become insufficient, resulting in the scaffold disruption observed at P6.³¹ Additionally, reduced midbrain *Slc6a3* expression in *5-HTT*^{−/−} embryos suggests that altered prenatal 5-HT homeostasis also impacts dopaminergic system identity. Previous studies using *5-HTT*^{−/−} models have shown that altered 5-HT availability during early development can influence postnatal prefrontal synaptic organization and laminar

maturation,^{22,31,66,67} supporting the long-lasting impact of developmental 5-HT imbalance on cortical circuit refinement. It remains to be determined whether the embryonic alterations observed in this study arise primarily from disrupted 5-HT input from raphe-derived projections or are also influenced by transient developmental 5-HTT expression within the cortex.⁶⁶ Unpublished observations from our group indicate that 5-HTT-positive fibers are present in the developing mPFC prior to the establishment of mature 5-HT innervation and are located in close proximity to both superficial and deep-layer neuronal populations. These findings suggest that transient cortical 5-HTT expression may contribute to the local regulation of 5-HT availability during early cortical assembly, although the relative contributions of cortical and raphe-derived 5-HTT expression require further investigation. Overall, these findings indicate that the *5-HTT*^{−/−} brain is not merely a state of high 5-HT levels but a dynamic system that actively maintains homeostasis through alternative pathways to delay structural damage.⁶⁸ However, protein-level validation and functional transporter assays will be required to determine whether these changes translate into an effective compensatory clearance mechanism *in vivo*.

This study provides a comprehensive characterization of prenatal mPFC organization. A major strength is the parallel examination of loss-of-function (*Tph2*^{−/−}) and gain-of-function (*5-HTT*^{−/−}) models, enabling direct assessment of how 5-HT homeostasis, rather than 5-HT tone alone, regulates cortical assembly. Analysis of the IL, PL, and Cg cortices as distinct anatomical and functional units revealed region-specific vulnerability profiles, such as IL-selective *Reln* disruption and PL-specific fiber reorganization, which would have been obscured by bulk mPFC analysis. Furthermore, integration of hindbrain, midbrain, and cortical transcriptomic data with protein expression and cellular distributions established a robust framework for identifying the molecular compensatory mechanisms underlying *5-HTT*^{−/−} embryonic resilience.

Several limitations warrant consideration. The modest sample size and inclusion of only male offspring reduce the sensitivity to subtle effect sizes and limit generalization to female developmental trajectories. In addition, analyses were restricted to late gestation (E18.5–E20.5), a period characterized by highly dynamic monoaminergic signaling and rapidly fluctuating transcript levels. Furthermore, 5-HT afferents were assessed by using 5-HT immunoreactivity. While this approach allows visualization and quantification of 5-HT-containing projections, additional analyses using 5-HT-specific markers, such as 5-HTT, are needed to determine whether the observed changes reflect differences in the density of 5-HT fibers reaching the mPFC or in the amount of 5-HT within those projections. In addition, although this study focused on neuronal populations and monoaminergic projections, the possibility that alterations in glial populations contribute to the observed developmental phenotypes cannot be excluded. Similarly, alterations in the development or maturation of cortical interneuron populations, which are also regulated by developmental 5-HT signaling, were not investigated in the present study. Consequently, these findings primarily describe the structural blueprinting phase of mPFC development. Future longitudinal studies are needed to clarify how early structural alterations identified in this work influence postnatal circuit maturation, given evidence that developmental alterations in 5-HT signaling affect prefrontal circuit assembly, synaptic maturation, and connectivity.^{66,67,69}

Table 1. List of Antibodies Used for Immunostaining

targeted protein	catalogue number	host	clonality	dilution
5-hydroxytryptamine (5-HT)	S5545	rabbit	polyclonal	1:500
tyrosine hydroxylase (TH)	P40101	rabbit	polyclonal	1:1000
Reelin (Reln)	MAB5364	mouse	monoclonal	1:200
COUP-TF-interacting protein 2 (Ctip2)	Ab18465	rat	monoclonal	1:500
T-box brain transcription factor 1 (Tbr1)	Ab31940	rabbit	monoclonal	1:500

CONCLUSIONS

In summary, our findings identify 5-HT as a critical spatiotemporal regulator of mPFC assembly, essential for maintaining the Reln-producing scaffold and guiding the patterned ingrowth of catecholaminergic afferents. Disruption of 5-HT homeostasis does not uniformly affect the mPFC; instead, the IL and PL cortices show distinct, region-specific vulnerabilities that likely reflect their developmental timing. The prenatal “blueprinting” abnormalities described here represent early structural perturbations that precede the emergence of social and cognitive deficits in models of 5-HT imbalance. Together, these results highlight the embryonic period as a critical window in which monoaminergic homeostasis establishes the structural framework necessary for proper maturation of the mPFC circuit.

MATERIALS AND METHODS

Animals and Experimental Procedures

Generation of *5-HTT*^{−/−} and *Tph2*^{−/−} rats by ENU-driven target-selected mutagenesis and zinc-finger nuclease technology, respectively, has been previously described.^{38,70} For each strain, heterozygous (*Tph2*^{+/-} or *5-HTT*^{+/-}) breeder pairs were set up to generate homozygous (*Tph2*^{−/−}; *5-HTT*^{−/−}) offspring, heterozygous (*Tph2*^{+/-}; *5-HTT*^{+/-}), and wild-type (*Tph2*^{+/+}; *5-HTT*^{+/+}) littermates. The day a copulatory plug was observed was defined as embryonic (E) day 0.5. All animals were bred and housed in the Central Animal Laboratory of Radboud University Nijmegen, the Netherlands, under controlled conditions (21 ± 1 °C, 50–60% humidity, 12-h light-dark cycle) with ad libitum access to food (sniff, Germany) and water. Timed-pregnant dams were euthanized using CO₂/O₂. For IHC studies, brains were harvested at E18.5, a developmental stage shortly after which 5-HT⁺ fibers establish innervation of the mPFC, allowing assessment of early alterations in monoaminergic circuit maturation. For molecular analyses, tissue from the *5-HTT* strain was collected at E20.5, after the peak of prefrontal 5-HT fiber organization observed at E18.5, to capture the transcriptional state of the monoaminergic system immediately before birth.

Genotyping of the offspring was performed *post-mortem* using tail tissue by high-resolution melting (HRM) curve analysis (for a detailed description, see below). Wild-type (*Tph2*^{+/+}; *5-HTT*^{+/+}) and homozygous (*Tph2*^{−/−}; *5-HTT*^{−/−}) male rat embryos were included in all experiments. All procedures were performed under project license AVD10300 2020 11424, issued by the Central Committee on Animal Experiments (Central Commissie Dierproeven, The Hague, the Netherlands), in full compliance with Dutch legislation on the use and protection of laboratory animals (Animal Testing Act).

Genotyping

Genomic DNA was isolated from embryonic tail tissue. Tissue samples were incubated overnight at 55 °C in a lysis buffer (1 M Tris-HCl, pH 8; 0.5 M EDTA; 10% SDS; 5 M NaCl) containing 20 mg/mL Proteinase K (Thermo Fisher Scientific, Waltham, MA). After protein digestion, DNA was precipitated with isopropanol, washed with 70% ethanol, and resuspended in Tris-EDTA (TE) buffer (pH 7.4). Genotyping was performed using HRM analysis on a CFX96 Touch Real-Time Detection System (Bio-Rad, Hercules, CA). The following primer sequences were used: *5-HTT*, forward, 5'-CTGGACTAGCTGCAC-

GAATC-3'; reverse, 5'-GCGTCCAGGTGATGTTGTC-3'; *TPH2*, forward, 5'-CACGCAGCTTAGCTCTGACA-3'; reverse, 5'-GGCCTACAGAGTATCCACTGC-3'. PCR consisted of an initial denaturation step at 95 °C (5 min), followed by 40 cycles of 95 °C (10 s), 60 °C (30 s), and 72 °C (10 s). A pre-melt conditioning step (90 °C, 65 s) and a heteroduplex formation step (95 °C, 60 s) preceded the HRM acquisition, which was performed from 65 °C to 95 °C in 0.1 °C increments. Data were analyzed using the uAnalyzeSM web tool⁷¹ (v2.1), and genotypes were assigned based on distinct melting curve profiles. Sex was determined by PCR for the SRY gene (forward primer: 5'-GCTCAACAGAATCCCAGCAT-3', reverse primer: 5'-CCATTG-CAGCAGGTTGTACA-3'), with MYOG serving as an internal control (forward primer: 5'-AGAGAAGCACCCTGCTCAAC-3', reverse primer: 5'-GTCTGACACCAACTCAGGGG-3').

Tissue Section Preparation, Immunohistochemistry, and Image Acquisition

Brains were rapidly dissected in ice-cold Dulbecco's Modified Eagle Medium (DMEM) from E18.5 male rat embryos, washed in phosphate-buffered saline (PBS), and fixed by immersion for 2 h in 4% paraformaldehyde (PFA) in PBS, pH 7.4, at 4 °C. After fixation, brains were cryoprotected by incubation in 30% sucrose in PBS at 4 °C until they sank (approximately 24 h). Embryonic brains were frozen in M-1 embedding matrix (Thermo Fisher Scientific, Waltham, MA), frozen on dry ice, and stored at −80 °C. Coronal cryosections were cut at 16 μm, collected as a series of eight on Superfrost Plus slides (Thermo Fisher Scientific), air-dried, and stored desiccated at −20 °C.

For IHC, sections were left to acclimatize at room temperature (RT) for 1 h, rehydrated in 1× PBS for 30 min, and incubated for 30 min in blocking solution consisting of 1.67% normal donkey serum, 1.67% normal goat serum, 1.67% normal horse serum, 1% bovine serum albumin, 1% glycine, 0.1% D-lysine, and 0.4% Triton X-100 in PBS. Sections were subsequently incubated overnight at 4 °C with the primary antibodies listed in Table 1, diluted in blocking solution. The following day, slides were left to acclimatize to RT for 30 min and washed three times in 1× PBS (10 min each) before incubation for 1 h at RT with species-specific fluorophore-conjugated secondary antibodies (Alexa Fluor Antibodies, Thermo Fisher Scientific). Following three washes in 1× PBS (10 min each), sections were counterstained with DAPI (0.5 μg/mL in 1× PBS; Merck, D9542), washed extensively again in 1× PBS, dried for 5 min, and mounted with Aqua-Poly/Mount (Polysciences Inc., 18606-100) under 1.5 mm coverslips.

Fluorescent signals were acquired on a Leica DMI8 inverted wide-field epifluorescence microscope equipped with a DMC4500 digital camera and LASX software (Leica Microsystems). Acquisition parameters, including the z-step size and optical section thickness, were optimized for each sample and kept consistent across experiments.

To ensure high spatial resolution, wide-field z-stack images were deconvolved using Huygens Professional (v19.10; Scientific Volume Imaging, The Netherlands; <http://svi.nl>). Deconvolution was performed using standard expression profile settings (acuity on) with channel-specific parameters. Image processing and analysis, including z-stack projections and reconstruction, were performed using ImageJ (v2.16; NIH, Bethesda).

Data Analysis

All data analysis was performed in a double-blind manner. The lengths of 5-HT⁺ and TH⁺ fibers, the densities of DAPI⁺, Reelin⁺, Ctip2⁺, and Tbr1⁺ cells, and cortical thickness were quantified within specific mPFC subregions: the IL, PL, and Cg cortices. Quantification was performed using ImageJ with the NeuroJ plugin (NIH, Bethesda). Brain

Table 2. Intron-Spanning Primers Used in RT-qPCR^a

gene	mRNA sequence ID	forward primer (5'–3')	reverse primer (5'–3')	product length (bp)
<i>Slc6a3/Dat</i>	NM_012694.2	CTCCTTCTGGGGTACATGG	TGTCGATACCCAGAGTGAGC	170
<i>Drd2</i>	NM_012547.2	CCAGTGAACAGGCGGAGAA	AGGACTGTCAGGGTTGCTAT	177
<i>Ddc</i>	NM_012545.4	TGCCATGTGGGTGAAGAAGA	ACCACATTTTCAGGGAGCGA	155
<i>Slc18a2/Vmat2</i>	NM_013031.2	CTATGGACTCATCGCTCCCA	CCCATACAAAAGGCCACGTC	153
<i>Slc29a4/Pmat</i>	NM_001105911.1	ATCCAGGCATCAGAGTCAGG	GGTTCCTGGGTACTTGTGGT	182
<i>Slc22a8/Oct3</i>	NM_031332.1	AGTGTCTCCGCTTCGTACAT	AGGTCCTCCAACCAGTATGC	200
<i>Comt</i>	NM_012531.2	AAGACCGCTACCTTCCAGAC	CCTTCTCCAAGCCGTCTACA	198
<i>Maoa</i>	NM_033653.1	ATGGGAAAAGAGATCCCCGT	CCACAGGGCAGATACCTCAT	180
<i>Maob</i>	NM_013198.1	AATGCGATGTGATCGTGGTG	TTGTGTAAGTCCTGCCTCCC	126
<i>Reln</i>	NM_080394.3	CCTGGAAGCACGGATGAAAG	CGCCTTCGGTTGTAGAAGTG	180
<i>Gapdh</i>	NM_017008.4	ATGGTGAAGGTCGGTGTGAA	CAGCTTCCCATTCTCAGCCT	195

^aRelative gene expression of target genes in the fore-, mid-, and hindbrain of E20.5 *5-HTT*^{+/+} and *5-HTT*^{−/−} embryos was calculated using the 2^{−ΔCt} method, with *Gapdh* as the reference gene. bp: base pairs.

cryosections from *Tph2*^{+/+}, *Tph2*^{−/−}, *5-HTT*^{+/+}, and *5-HTT*^{−/−} rats at E18.5 were analyzed. Three male embryos per genotype were analyzed as biological replicates, with each replicate defined as a single embryo. Embryos from the TPH2 rat line were collected from five independent litters, each generated by five breeding pairs. For the *5-HTT* rat line, embryos were collected from four independent litters generated by four breeding pairs. To reduce potential litter-specific effects, biological replicates were obtained from multiple independent litters, and wild-type (*Tph2*^{+/+}; *5-HTT*^{+/+}) and homozygous (*Tph2*^{−/−}; *5-HTT*^{−/−}) littermates were included when feasible. For each biological replicate, three to five sections (spaced 128 μm apart) were selected at matching neuroanatomical levels along the rostral-to-caudal axis. To standardize quantification, a 0.1 mm-wide rectangular region of interest (ROI) was placed over the center of each mPFC subarea, spanning the entire cortical wall. Each ROI was divided into 10 equal bins, with bin 10 representing the superficial presumptive layer I and bin 1 representing the deepest cortical layer. Data were normalized to total fiber length per bin per area or to a percentage of the total cell number and averaged for each embryonic replicate. Cortical thickness for each ROI was calculated as the average of three measurements taken at the left, center, and right margins of the swath.

In Silico Gene Expression Profiling

Temporal expression profiles of *5-HT*⁺ and catecholaminergic-related genes during rat brain development were obtained from the EMBL-EBI Expression Atlas using the publicly available RNA-seq developmental atlas 'Rat RNA-seq time-series of the development of seven major organs'⁷² (accession E-MTAB-6811). This dataset, generated as part of a comparative developmental transcriptome resource, contains bulk RNA-seq data from multiple rat organs across embryonic and postnatal development. For the present study, expression levels, quantified as TPM, were extracted exclusively from forebrain and hindbrain samples across developmental stages from E14 to P0. Based on data availability and relevance to monoaminergic signaling, a subset of six representative monoaminergic genes (*Slc6a3*, *Drd2*, *Ddc*, *Slc22a3*, *Comt*, and *Maob*) was selected for analysis.

RNA Extraction, cDNA Synthesis, and Real-Time Quantitative Polymerase Chain Reaction (RT-qPCR)

Forebrain, midbrain, and hindbrain tissues from E20.5 *5-HTT*^{+/+} and *5-HTT*^{−/−} rat embryos were dissected under a stereomicroscope using gross anatomical landmarks. The forebrain was isolated with a transverse cut caudal to the frontal lobes. The remaining tissue was separated along visible surface grooves at the midbrain-hindbrain junction (isthmus region) to isolate the midbrain and hindbrain. Dissected tissues were immediately collected, snap-frozen in liquid nitrogen, and stored at −80 °C until further processing. Total RNA from each brain region was isolated using TRIzol (Thermo Scientific). Briefly, embryonic brain samples were macerated in 800 μL of TRIzol and incubated at RT for 5 min to dissociate nucleoprotein complexes. Chloroform (160 μL; Thermo Scientific, 99.8%) was then added, mixed, and vortexed briefly. The homogenate was centrifuged at 12,000

rpm at 4 °C for 15 min, and the aqueous (upper) phase was transferred to a new 1.5 mL tube. RNA was precipitated with 0.7 volumes of isopropyl alcohol, incubated at RT for 10 min, and then centrifuged at 12000 rpm at 4 °C for 10 min. The RNA pellet was washed twice with ice-cold 75% ethanol, centrifuged (750 rpm, 4 °C, 5 min), air-dried, resuspended in RNase-free water, and incubated at 50 °C for 10 min. Total RNA was quantified using a NanoDrop ND-2000 UV/Vis Spectrometer (Thermo Scientific), and integrity was assessed by electrophoresis on 1% agarose gels under RNase-free conditions. Residual genomic DNA was removed by treatment with RNase-free DNase I (EN0521, Thermo Scientific) at 37 °C for 30 min. DNase I was inactivated with 50 mM EDTA at 75 °C for 10 min. cDNA was synthesized using the SensiFAST cDNA synthesis kit (Bioline, Taunton). Briefly, 1 μg of DNase-treated RNA from each brain region was heated at 70 °C for 5 min to disrupt secondary structures. Reverse transcription was performed according to the manufacturer's instructions. A no-RT control was included to assess residual genomic DNA contamination. Primers were designed using Primer-BLAST, and their sequences are provided in Table 2. Primer specificity was verified by melting curve analysis and amplicon size confirmation via agarose gel electrophoresis. qPCR reactions were performed using SensiFAST SYBR No-ROX Mix (Bioline) on a CFX96 Touch Real-Time PCR Detection System (Bio-Rad). The cycling protocol consisted of an initial denaturation at 95 °C for 2 min, followed by 40 cycles of 95 °C for 5 s and 60 °C for 20 s. A melt-curve analysis (65–95 °C, 0.5 °C increments) was performed at the end of each run to verify amplicon specificity. All reactions were run in technical triplicate.

Statistical Analysis

Data are presented as mean ± standard error of the mean (SEM). Statistical analysis was performed using GraphPad Prism (v10.2.1; San Diego, CA). Immunohistochemical comparisons between *Tph2*^{−/−} and *Tph2*^{+/+} animals, as well as between *5-HTT*^{−/−} and *5-HTT*^{+/+} rats, regarding total *5-HT*⁺ and TH⁺ fiber lengths, the total number of DAPI⁺, Reelin⁺, Ctip2⁺, and Tbr1⁺ cells, and cortical thickness were analyzed using an unpaired Student's *t*-test. To analyze the spatial distribution of fibers and cells across the cortical wall (10 bins) and the relative gene expression across brain regions (fore-, mid-, and hindbrain), two-way ANOVAs were conducted with bin or brain region as a within-subjects factor and genotype as a between-subjects factor. For RT-qPCR analysis, relative expression values were log₂-transformed before statistical testing to meet the assumptions of normality. Šidák's and Tukey's multiple comparison tests were used for post hoc analysis when significant main effects or interactions were detected. All statistical tests were performed two-tailed, and the level of significance was set at *p* < 0.05. Comprehensive statistical summaries, including test statistics, post hoc comparisons, and corresponding *p*-values, are provided in the Supporting Information (Tables S1–S8).

■ ASSOCIATED CONTENT

SI Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acscchemneuro.6c00382>.

Prefrontal cytoarchitecture is selectively altered in the prelimbic cortex in the absence of *Tph2* (Figure S1); deep-layer neuronal density is largely preserved in *5-HTT*^{−/−} embryos (Figure S2); total DAPI⁺ cell density and cortical thickness are preserved across models of 5-HT imbalance (Figure S3); transcriptional expression profiles of monoaminergic genes during prenatal development (Figure S4); molecular characterization of monoaminergic markers in *5-HTT*^{−/−} embryos (Figure S5); statistical analysis of the spatial distribution of monoaminergic innervation and cortical cellular populations across bins of the infralimbic (IL) cortex within the medial prefrontal cortex of *Tph2*^{−/−} rats (Table S1A); statistical analysis of the spatial distribution of monoaminergic innervation and cortical cellular populations across bins of the prelimbic (PL) cortex within the medial prefrontal cortex of *Tph2*^{−/−} rats (Table S1B); statistical analysis of the spatial distribution of monoaminergic innervation and cortical cellular populations across bins of the cingulate (Cg) cortex within the medial prefrontal cortex of *Tph2*^{−/−} rats (Table S1C); significant Šidák's-corrected post hoc comparisons following spatial distribution analyses of monoaminergic innervation and cortical cellular populations across bins within the medial prefrontal cortex of *Tph2*^{−/−} rats (Table S2); overall quantification of monoaminergic innervation and cortical cellular populations in the infralimbic (IL) cortex of *Tph2*^{−/−} rats (Table S3A); overall quantification of monoaminergic innervation and cortical cellular populations in the prelimbic (PL) cortex of *Tph2*^{−/−} rats (Table S3B); overall quantification of monoaminergic innervation and cortical cellular populations in the cingulate (Cg) cortex of *Tph2*^{−/−} rats (Table S3C); statistical analysis of the spatial distribution of monoaminergic innervation and cortical cellular populations across bins of the infralimbic (IL) cortex within the medial prefrontal cortex of *5-HTT*^{−/−} rats (Table S4A); statistical analysis of the spatial distribution of monoaminergic innervation and cortical cellular populations across bins of the prelimbic (PL) cortex within the medial prefrontal cortex of *5-HTT*^{−/−} rats (Table S4B); statistical analysis of the spatial distribution of monoaminergic innervation and cortical cellular populations across bins of the cingulate (Cg) cortex within the medial prefrontal cortex of *5-HTT*^{−/−} rats (Table S4C); significant Šidák's-corrected post hoc comparisons following spatial distribution analyses of monoaminergic innervation and cortical cellular populations across bins within the medial prefrontal cortex of *5-HTT*^{−/−} rats (Table S5); overall quantification of monoaminergic innervation and cortical cellular populations in the infralimbic (IL) cortex of *Tph2*^{−/−} rats (Table S6A); overall quantification of monoaminergic innervation and cortical cellular populations in the prelimbic (PL) cortex of *Tph2*^{−/−} rats (Table S6B); overall quantification of monoaminergic innervation and cortical cellular populations in the cingulate (Cg) cortex of *Tph2*^{−/−} rats (Table S6C); statistical analysis of relative monoaminergic gene

expression in *5-HTT*^{−/−} rats (Table S7A); RT-qPCR analysis of gene expression in the forebrain of *5-HTT*^{−/−} rats (Table S8A); RT-qPCR analysis of gene expression in the midbrain of *5-HTT*^{−/−} rats (Table S8B); and RT-qPCR analysis of gene expression in the hindbrain of *5-HTT*^{−/−} rats (Table S8C) (PDF)

■ AUTHOR INFORMATION

Corresponding Author

Sharon M. Kolk – Section Neurobiology, Donders Centre for Neuroscience, Donders Institute for Brain, Cognition and Behavior, Radboud University, NL-6525 AJ Nijmegen, The Netherlands; Email: s.kolk@donders.ru.nl

Authors

Marta C. F. Samina – Section Neurobiology, Donders Centre for Neuroscience, Donders Institute for Brain, Cognition and Behavior, Radboud University, NL-6525 AJ Nijmegen, The Netherlands; orcid.org/0009-0002-9766-1333

Maria Stanciu – Section Neurobiology, Donders Centre for Neuroscience, Donders Institute for Brain, Cognition and Behavior, Radboud University, NL-6525 AJ Nijmegen, The Netherlands

Thibault Merkelijn – Department of Molecular Biochemistry and Biophysics, Karolinska Institute, SW-17177 Stockholm, Sweden; orcid.org/0009-0003-7053-7188

Lidiane P. Garcia – Section Neurobiology, Donders Centre for Neuroscience, Donders Institute for Brain, Cognition and Behavior, Radboud University, NL-6525 AJ Nijmegen, The Netherlands

Sabrina Hanswijk – Department of Medical Neuroscience, Donders Institute for Brain, Cognition and Behavior, Radboud University Medical Centre, NL-6525 EN Nijmegen, The Netherlands

Josefine S. Witteveen – Section Neurobiology, Donders Centre for Neuroscience, Donders Institute for Brain, Cognition and Behavior, Radboud University, NL-6525 AJ Nijmegen, The Netherlands

Natalia Alenina – Molecular Biology of Peptide Hormones, Max-Delbrück-Centrum for Molecular Medicine in the Helmholtz Association (MDC), D-13125 Berlin, Germany; German Center for Cardiovascular Research (DZHK), D-10785 Berlin, Germany; orcid.org/0000-0002-6071-5433

Michael Bader – Molecular Biology of Peptide Hormones, Max-Delbrück-Centrum for Molecular Medicine in the Helmholtz Association (MDC), D-13125 Berlin, Germany; German Center for Cardiovascular Research (DZHK), D-10785 Berlin, Germany; Charité University Medicine Berlin, D-10117 Berlin, Germany; Institute for Biology, University of Lübeck, D-23562 Lübeck, Germany; orcid.org/0000-0003-4780-4164

Judith R. Homberg – Department of Medical Neuroscience, Donders Institute for Brain, Cognition and Behavior, Radboud University Medical Centre, NL-6525 EN Nijmegen, The Netherlands

Complete contact information is available at:

<https://pubs.acs.org/doi/10.1021/acscchemneuro.6c00382>

Author Contributions

M.C.F.S. acquired the data, performed statistical analysis, designed and executed figures and tables, wrote the original draft, and edited and reviewed the manuscript. M.S. and T.M.

supported data acquisition and statistical analysis. L.P.G. and S.H. helped with data acquisition. J.R.H. and S.M.K. designed the study, acquired funding, supervised the work, and supported manuscript writing. T.M., M.S., L.P.G., S.H., J.S.W., N.A., M.B., J.R.H., and S.M.K. read, critically revised, and approved the final manuscript.

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Notes

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■ ABBREVIATIONS

5-HT	5-hydroxytryptamine, serotonin
5-HTT	serotonin transporter
ASD	autism spectrum disorder
Cg	cingulate cortex
CP	cortical plate
CR	Cajal–Retzius
DA	dopamine
IL	infralimbic cortex
IZ	intermediate zone
MFB	median forebrain bundle
mPFC	medial prefrontal cortex
MZ	marginal zone
NA	norepinephrine
PL	prelimbic cortex
Reln	reelin
RN	raphe nucleus
SP	subplate
SVZ	subventricular zone
TPH2	tryptophan hydroxylase 2
VZ	ventricular zone

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