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Abnormal neurogenesis and cortical growth in congenital heart disease

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Abstract

Long-term neurological deficits due to immature cortical development are emerging as a major challenge in congenital heart disease (CHD). However, cellular mechanisms underlying dysregulation of perinatal corticogenesis in CHD remain elusive. The subventricular zone (SVZ) represents the largest postnatal niche of neural stem/progenitor cells (NSPCs). We show that the piglet SVZ resembles its human counterpart and displays robust postnatal neurogenesis. We present evidence that SVZ-NSPCs migrate to the frontal cortex and differentiate into interneurons

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Supplementary Materials:

Materials and methods

Author Contributions:

P.D.M., N.I., V.G., and R.A.J. designed the experiments. P.D.M. carried out neurosphere assays, immunocytochemistry, immunohistochemistry with the assistance of L.K., MRI analyses, nanoparticle analyses, iDISCO and 3D sementation and rendering, human tissue studies, and cell fate analysis. S.B. contributed to multiphoton image acquisition of optically cleared brain tissue. N.I. conceived the project and performed surgeries on piglets with the assistance of L.K. L.K. and S.D.R. processed human and piglet brain tissue. J.Z. and B.K.L. performed MRI scans. J.A.F., J.Z., S.M., and B.K.L. contributed to MRI data processing and analysis. P.D.M. and N.I. analyzed the experimental data. D.Z. contributed to statistical analysis. P.D.M. prepared the figures and wrote the manuscript with N.I., V.G., and R.A.J.

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in a region-specific manner. Hypoxic exposure of the gyrencephalic piglet brain recapitulates CHD-induced impaired cortical development. Hypoxia reduces proliferation and neurogenesis in the SVZ, which is accompanied by reduced cortical growth. We demonstrate a similar reduction in neuroblasts within the SVZ of human infants born with CHD. Our findings demonstrate that SVZ-NSPCs contribute to perinatal corticogenesis and suggest that restoration of SVZ-NSPCs' neurogenic potential is a candidate therapeutic target for improving cortical growth in CHD.

Introduction

Within the postnatal and adult mammalian brain, the subventricular/subependymal zone (SVZ/SEZ) represents the largest source of neural stem/progenitor cells (NSPCs)(1). Under normal physiological conditions, adult NSPCs in the rodent continuously generate interneurons, which migrate through the rostral migratory stream (RMS) to the olfactory bulb where they integrate into the existing circuitry(2, 3). However, in various pathologies of the developing brain including traumatic brain injury, ischemic stroke, and seizures, NSPCs can exit the rostral migratory stream and migrate to the site of injury to replace damaged or lost neurons(4, 5). To functionally replace damaged or lost neurons, SVZ-derived NSPCs must proliferate, migrate to the correct site, differentiate into the appropriate type of neuron, and integrate into the local circuitry(5). Because these cells retain their regenerative capacity throughout life, a more complete understanding of endogenous SVZ NSPCs may foster regenerative strategies for the treatment of neurological disorders(3, 6).

Congenital heart disease (CHD) is the most common major birth defect and often results in an array of neurological deficits throughout life including behavioral, cognitive, social, motor, and attention disorders(7–10). Genetic, environmental, pre-, intra-, peri-, and post-operative factors are associated with poor neurological outcomes in CHD(11). Severe delays in cortical gray and white matter development have been identified in fetuses and neonates with complex CHD(12–15); one converging etiology of these impairments includes a reduced supply of cerebral oxygen associated with cardiac anomalies(11, 16, 17). Advanced fetal brain imaging has revealed reduced cortical expansion and gyrification during the third trimester, a period when the brain undergoes robust synapse formation, requiring high oxygen demands(18, 19). However, the effects of CHD-induced brain injury at the cellular level, specifically during early life when the brain is rapidly expanding and vulnerable to the manifestation of neurological impairments, are unknown. Furthermore, it is unknown how CHD affects NSPCs within the SVZ, a highly vascularized niche for brain development and regeneration(20–23).

Analyses of postmortem tissue have provided insights into the cellular composition of the developing human brain, though seminal findings from these studies often require further validation in rodent models where cell dynamic studies, genetic manipulations, and mechanistic investigations can be performed(24). However, there are strong dissimilarities between the human and rodent brain: (i) humans have a highly evolved, gyrencephalic neocortex; (ii) 50% of human brain volume is white matter; (iii) the human SVZ has laminar organization; and (iv) humans have an expansive outer SVZ densely populated with radial glia(25–33). In addition, a limited window of neurogenesis is present in the human infant

SVZ, and these newborn neurons migrate not only to the olfactory bulb but also to the prefrontal cortex(34). Although the importance of NSPCs in the SVZ during normal development as well as their response to several pathological conditions has been extensively studied in rodent models, there is a need for additional animal models suitable for dynamic cellular and physiological studies.

Though non-human primates share some key structural characteristics with the human SVZ, direct investigations of non-human primates are often limited in number due to challenging technical and ethical scrutiny(35). Piglets are powerful model organisms in which to study human brain development because they possess a highly evolved, gyrencephalic neocortex. Unlike rodents, the progression of porcine white matter development parallels its human counterpart(36, 37). In the present study, we show that the cytoarchitecture of the porcine SVZ is similar to the human SVZ. Through multidisciplinary approaches, we demonstrate that the SVZ plays a role in gyrencephalic neocortical growth during postnatal life. Using a chronic hypoxia model, we challenge the growth of this key structure and confirm our findings in SVZ specimens from human infants born with complex CHD to reveal insights into the cellular mechanisms contributing to CHD-induced brain injury and dysmaturation.

Results

The anterior-dorsolateral region is the most active neurogenic niche in the postnatal porcine SVZ

In the human brain, specific differences have been identified in the structural and cellular properties of subregions of the SVZ(27, 34). To account for regional differences in the porcine SVZ, we first divided this region into four rostrocaudal domains as previously described in the human brain(27) (Fig. 1A–E). Within the anterior-end (AE)-, anterior (A)-, and middle (M)-SVZ, the SVZ was further subdivided into lateral and dorsolateral regions based on studies in rodents(38) (Fig. 1F). Immunohistochemical analysis revealed that the A-lateral-SVZ had a laminar organization composed of four distinct layers (Fig. 1G–I). The presence of neuroblasts in layer II and an astrocyte ribbon in layer III have been reported as unique features of the infant and adult human SVZ, respectively(28, 31). As seen in human neonates, the ventral region of the piglet AE-lateral-SVZ lacked laminar organization and an astrocyte ribbon(34) (fig. S1A–D). Within the A-SVZ, glial fibrillary acidic protein positive (GFAP⁺) processes were immuno-positive for the neuroepithelial marker vimentin (Vim) (fig. S1E–H). Furthermore, the thickness of the astrocyte ribbon varied as seen in humans(27) (fig. S1I–L), demonstrating that the early postnatal porcine SVZ shares structural features with the postnatal human SVZ.

During development, two features of the SVZ are evident in many gyrencephalic species: the outer-radial glia (oRG), and an expansive outer region termed outer-SVZ(29, 30). Both features were present within the porcine posterior (P)-SVZ (Fig. 1J–M). In addition, oRG exhibited Vim⁺GFAP⁺ processes that lined the lateral ventricle and extended radially (figs. S1M–P and S2A–G); this mesh-like distribution has also been reported in human fetuses(39).

We next assessed the proliferative capacity of NSPCs in distinct SVZ regions during postnatal development. In the P-SVZ, NSPCs and proliferating NSPCs were less abundant when compared to other regions (Fig. 1N,O and fig. S3A–K). The outer-SVZ is an important cell source during early human fetal life(30). Within the P-SVZ, NSPCs of the outer-SVZ were significantly less abundant and prolific compared with the inner-SVZ (Sox2⁺: $P=0.0115$; Sox2⁺Ki67⁺: $P=0.0009$) (Fig. 1P,Q), suggesting that, in contrast to early human fetal development, the outer-SVZ is not the predominant proliferating region in the postnatal piglet brain.

Because the A-SVZ displayed the most proliferating NSPCs and resembles the human SVZ (Fig. 1 F–I,O and fig. S3K), we next determined the density and proliferative capacity of NSPCs in different SVZ subregions throughout postnatal development. NSPCs were more abundant and proliferative in the dorsolateral-SVZ as compared with the lateral-SVZ at early developmental stages (Fig. 1, R and S). However, the number and proliferative activity of NSPCs significantly declined with age (Fig. 1R,S and fig. S3L). We found an interaction between age and sub-region (Fig. 1, R and S), and there were more NSPCs in the A-dorsolateral-SVZ than the A-lateral-SVZ at all ages (Fig. 1R). When a neurosphere assay was performed to further characterize the intrinsic developmental potential of porcine SVZ NSPCs, cells isolated at postnatal day 2 (p2) generated more spheres than at p14 (Fig. 1, T and U). The neurospheres generated from the A-SVZ at p2 achieved larger sizes than those from p14 (Fig. 1V and fig. S4). Altogether, these findings indicated that the porcine SVZ resembles its human counterpart and the A-dorsolateral-SVZ is the most prolific NSPC pool during early postnatal porcine development.

The SVZ supplies the frontal cortex with GABAergic interneurons during postnatal porcine development

In the developing piglet brain, immunohistochemical analysis revealed several clusters of doublecortin (Dcx⁺) neuroblasts coexpressing the neuronal cell migration marker polysialylated-neural cell adhesion molecule (PSA-NCAM) surrounding the SVZ (fig. S5). In addition, we identified many elongated Dcx⁺PSA-NCAM⁺ cells extending laterally, streaming from the SVZ to the cortex (Fig. 2A–C and fig. S6). To better visualize these abundant populations of neuroblasts, we developed a protocol to optically clear thick (3 mm) postnatal porcine brain slices. Three-dimensional (3D) segmentation and rendering of immunolabeled cells revealed complex patterns of Dcx⁺ neuroblasts (Fig. 2D–H): (i) streaming medially, adjacent to the lateral ventricle (Fig. 2E); (ii) clustered in the SVZ and projecting/streaming rostrally (Fig. 2F); (iii) throughout the subcortical white matter (Fig. 2G); and (iv) fanning laterally to the upper layers of the frontal cortex (Fig. 2H).

To determine the destination and fate of SVZ-derived cells, we used focal tracers. Cell-Tracker Green (CTG) was injected into the A-dorsolateral-SVZ (Fig. 3A) during the observed peak of NSPCs generation (Fig. 1R). One week post-injection (1 wpi), CTG⁺ cells were seen tangentially and rostrally through the SVZ (Fig. 3B–D). Further analysis near the injection site revealed that the majority of CTG⁺ cells were present in the SVZ and co-labeled with the neuroblast marker Dcx (Fig. 3E–I). A minimal percentage of CTG⁺ cells co-expressed ganglionic eminence markers within the AE-SVZ (Table S1). We also

observed CTG⁺ cells in the corpus callosum, periventricular white matter, and caudate on the plane of the injection site (A-SVZ) (Fig. 3F). On the plane of the AE-SVZ, rostral to the injection site (Fig. 3, A and D), CTG⁺Dcx⁺ cells were seen in the rostral migratory stream (Fig. 3J), periventricular white matter (Fig. 3K), and CTG⁺ cells were seen in the subcortical white matter (Fig. 3L) underlying the frontal cortex. In addition, SVZ-derived cells expressing the postmitotic neuronal marker NeuN were observed in the frontal cortex (Fig. 3M), demonstrating that the postnatal SVZ supplied the developing cortex with newly generated neurons.

We analyzed long-term migration and cell fate using superparamagnetic-iron-oxide (SPIO) nanoparticles that are visualized using magnetic resonance imaging (MRI) and microscopy(40) (Fig. 4A and fig. S7A). We focused our analyses on SVZ-derived cells populating the frontal cortex. SPIO⁺Dcx⁺ neuroblasts were detected not only in the SVZ and white matter, but also in layers II/III of the frontal cortex at 2 wpi (fig. S7, B and C). Long chains of SPIO⁺Dcx⁺ cells were also present in the white matter underlying the prefrontal cortex (Fig. S7, D and E). T2* weighted MRI scans indicated that SPIO nanoparticles, seen as hypointense voxels, were present in several frontal cortices at 4 wpi (Fig. 4A and fig. S8). The majority of SVZ-derived cells were seen in the prefrontal (67%) and somatosensory (22%) cortices during early postnatal development (Fig. 4B). Several SPIO signals were also seen in the piglet brain within the anatomical equivalent of the human ventromedial prefrontal cortex (Fig. 4A), a known destination for migrating immature neurons during human infancy(34). Together, our results suggest that newly generated neurons migrate from the SVZ to specific cortices - primarily the prefrontal cortex.

Based on these findings, we next analyzed the cell fate of SVZ-derived cells within the upper and lower layers of the prefrontal, primary somatosensory, and insular cortex. Quantification of SPIO⁺ cells within the upper and lower layers and subcortical white matter of the prefrontal cortex revealed that the majority of SPIO⁺ cells were located in the upper layers (Fig. 4C). Because our findings using CTG labeling demonstrated that SVZ-derived cells were primarily neuroblasts and GFAP⁺ cells - as opposed to oligodendrocytes or microglia (Fig. 3F–I) - we restricted our cell fate analyses to neurons, interneurons, and astrocytes. We found that somatostatin (SST⁺) and vasoactive intestinal polypeptide-expressing (VIP⁺) interneurons were scarce compared to calretinin (Calr) and parvalbumin (PPV)-positive interneurons (fig. S9); therefore, SPIO⁺ cells co-labeled with NeuN, Calr, PPV, and GFAP were quantified throughout the frontal cortex. SPIO⁺Calr⁺NeuN⁺ gamma-Aminobutyric acid (GABAergic) interneurons and SPIO⁺Calr⁺NeuN⁺ neurons were seen throughout the prefrontal cortex and SPIO⁺GFAP⁺ cells were less common (Fig 4, C–F). SPIO⁺Calr⁺NeuN⁺ GABAergic interneurons were also visible in the primary somatosensory and insular cortex (Fig. 4, C and G). Quantification revealed that the majority of SPIO⁺ cells were within the upper layers of the prefrontal cortex (Fig. 4, C and H). Calr⁺NeuN⁺ GABAergic interneurons within the upper layers of the prefrontal cortex were most abundant, with the upper layers of the somatosensory cortex showing the second highest concentration of these cells (Fig. 4C and table S2). Calr⁺NeuN⁺ neurons within the upper layers of the prefrontal cortex were the second most prominent type of SPIO⁺ cells identified (Fig. 4I and table S2). We did not find any SPIO⁺PPV⁺ interneurons in our analyses.

To determine multipotent differentiation potential and assess regional heterogeneity of the intrinsic cell fate of NSPCs, we analyzed non-dissociated neurospheres from different SVZ subregions. Spheres generated from the A-SVZ primarily differentiated into neurons, when compared to the P-SVZ (Fig. 4, J and K), indicating that the rostral SVZ is more neurogenic than the caudal SVZ during early postnatal development. Consistent with our analysis of the proliferative capacity of SVZ NSPCs (Fig. 1V), neuroblasts were most abundant at 1 week of age within the A-dorsolateral-SVZ and abundance declined with age (Fig. 4L and fig. S10A–F). Altogether, these findings indicate that the A-SVZ is a source of newborn neurons destined to populate the upper layers of the cortex.

Chronic hypoxia in piglets recapitulates impaired cortical development in human CHD and reduces NPC proliferation and neurogenesis in the SVZ

To assess the effects of chronic hypoxia on the developing gyrencephalic cortex, we used a porcine model of hypoxia injury (fig. S11A). Brains from hypoxic animals were smaller, weighed significantly less than controls, and exhibited a significant reduction in cortical gray matter volume (weight: $P < 0.0001$; volume: $P = 0.0248$) (Fig. 5A–F). Animals exposed to hypoxia showed a reduced gyrification index (GI) compared to controls (Fig. 5G–I). The GIs of control and hypoxic animals in our experimental paradigm were nearly identical to those of normal and CHD human fetuses near the end of the 3rd trimester, respectively(18) .

Similar to CHD brains, the lateral ventricles of the piglet brain underlying the A-dorsolateral-SVZ were enlarged after hypoxia compared to controls (Fig. 5, J and K). Hypoxia reduced the width of the SVZ, the size of the NSPC pool, and the length of GFAP⁺ processes in the A-dorsolateral-SVZ (Fig. 5L–O and fig. S11B,C). We found a significant reduction in proliferation in the A-dorsolateral-SVZ ($P = 0.0294$) (fig. S11D), but no differences in cell death within the SVZ in hypoxic brains (fig. S11, E and F). Consistent with this finding, we also observed a significant decrease in the number of Sox2⁺ ($P = 0.0284$) and Sox2⁺Ki67⁺ NSPCs in the same region after hypoxia (Sox2⁺: $P = 0.0284$; Sox2⁺Ki67⁺: $P = 0.0122$) (Fig. 5P–U). Similar effects of prolonged hypoxia on the NSPC niche were also found in the AE-SVZ (fig. S12A–H), but these alterations were not displayed in the A-lateral-SVZ (Fig. 5, R and U), suggesting a region-specific effect. Finally, we observed a significant reduction in the number of Dcx⁺ cells after hypoxia, specifically in the A-dorsolateral-SVZ ($P = 0.0125$) (Fig. 5V–X). Taken together, these data demonstrated that hypoxia reduced neurogenesis and progenitor numbers, particularly within the largest NSPC pool (A-dorsolateral-SVZ) of the postnatal porcine brain.

Congenital heart disease disrupts the cytoarchitecture and reduces neuroblast numbers in the SVZ of human infants

Immature cortical development is common in CHD patients, yet the state of the neuroblast supply within the SVZ in this population remains unknown. In parallel with our investigation of the hypoxic piglet brain, we analyzed brain specimens from human infants who experienced a reduction of cerebral oxygen supply during gestation due to complex CHD (table S3). These CHD specimens were compared to specimens from infants born with normal fetal cerebral circulation (no CHD) (table S3).

We analyzed the SVZ underlying the frontal cortex and found a global reduction in the average length of GFAP⁺ processes spanning layer II, a measure of laminar thickness, in the dorsolateral (DL-) and lateral (L-) SVZ in CHD infants (n=4) compared to no CHD (n=5) (Fig. 6A–K). In addition, we found a reduction in the number of Dcx⁺ neuroblasts within the L-SVZ in CHD infants compared with no CHD (Fig. 6L–T). Infants without CHD (n=4) had an abundance of Dcx⁺ neuroblasts within the DL- and L-SVZ and the density of this cell population was inversely proportional to age, specifically in the DL-SVZ (Fig. 6U–V). CHD infants (n=3) displayed a reduction in Dcx⁺ neuroblasts within the SVZ independent of age (Fig. 6U–V).

Chronic hypoxia impairs SVZ-derived cellular generation and migration in piglets

Based on our finding that SVZ-derived cells populate the frontal cortex in neonatal piglets during normal development, we hypothesized that a reduction in neuroblast numbers in the SVZ could play a role in the immature cortical expansion seen in human CHD patients. To determine if reduced cerebral oxygenation altered the cortical contribution of SVZ-derived cells, we performed cell fate analysis on piglets undergoing chronic hypoxic exposure (Fig. 7). We assessed the effects of hypoxia on cell migration from the A-dorsolateral-SVZ within the AE-SVZ (Fig. 7A–D). After hypoxia, nearly all CTG⁺ cells originating from the site of injection were of the neuronal lineage (Fig. 7E–J and fig. S12I–P), similar to normoxic conditions; however, a significant decrease in the number of CTG⁺ cells and CTG⁺ neuroblasts rostral (AE-SVZ) to the site of injection was noted in hypoxia (CTG⁺: $P=0.0253$; CTG⁺Dcx⁺: $P=0.0051$) (Fig. 7K,L). Nearly all CTG⁺Dcx⁺ cells co-expressed the cell migration-associated marker PSA-NCAM (Fig. 7, G and J), indicating that these cells are likely migrating immature neurons. When the effects of hypoxia on the regional distribution of SVZ-derived neuroblasts were determined with MRI, SPIO signals were seen throughout the forebrain, primarily in the prefrontal and somatosensory cortices in hypoxic and normoxic groups (Fig. 7M). A slight decrease in the percentage of SPIO⁺ SVZ-derived cells was observed in the insular cortex, and there were no differences in the overall regional distribution of nanoparticles within the cortex after hypoxia (Fig. 7M).

Chronic hypoxia alters interneuron and excitatory neuronal cell populations in the cortex and limits cortical expansion in a region-specific manner

To further understand the relationship between immature cortical development and the depletion of neuroblasts, we analyzed immature and mature neuronal populations within the cortex. We found a significant reduction in the number of Dcx⁺ immature neurons in layers II/III of the frontal cortex after hypoxia, independent of apoptotic cell death ($P=0.0191$) (Fig. 8A–G and fig. S13A–C), indicating that apoptotic cell death is not a major underlying mechanism in the reduction of cortical neuroblasts. When hypoxia-induced changes of immature neuronal numbers were analyzed between distinct cortical areas, a significant decrease in neuroblasts in layers II/III was seen specifically in the prefrontal and insular cortices (prefrontal: $P=0.0345$; insular: $P=0.0243$) (Fig. 8D). In addition, a significant reduction in white matter neuroblasts was displayed in the prefrontal cortex ($P=0.0412$) (Fig. 8H). Together, these findings suggested that impaired neurogenesis within the SVZ represents a cellular mechanism underlying hypoxia-induced, region-specific reduction in

immature neurons in the cortex and that the prefrontal cortex is most affected by this pathological insult.

We next analyzed the effects of chronic hypoxia on mature excitatory and inhibitory neuronal populations throughout the upper (II/III) and lower layers of the cortex. In contrast with the reduced density of immature neurons (Fig. 8D,H and fig. S13A), there were no differences in NeuN⁺ cell density in layers II/III of the cortex between normoxic and hypoxic conditions (tables S4 and S5). However, we found a significant reduction in the number of mature neurons throughout layers II/III of the frontal cortices after hypoxia, paired with a significant reduction in cortical volume (NeuN⁺: $P = 0.0065$; volume: $P = 0.0050$) (Fig. 8I,J and tables S4,5). Within individual cortical regions, the prefrontal and insular cortices had significantly fewer NeuN⁺ neurons and less volume after hypoxia (prefrontal: $P = 0.0017$; insular: $P = 0.0145$) (Fig. 8K,L and tables S4,5).

Consistent with our aforementioned findings, chronic hypoxia reduced the number of Calr⁺ and Calr⁺NeuN⁺ GABAergic interneurons in layers II/III of the prefrontal cortex (Fig. 8M and tables S4,5). There was also a significant reduction in the percentage of Calr⁺ interneurons (Calr⁺/NeuN⁺) in the upper layers of the prefrontal cortex after hypoxia ($P = 0.0234$) (tables S4 and S5). We found no difference in the number of T-box, brain 1 (Tbr1)⁺NeuN⁺ excitatory neurons throughout the frontal cortex (tables S4 and S5). However, there was an increase in excitatory neuron cell density in the upper layers of the cortex, specifically the somatosensory cortex (Fig. 8N and tables S4,5).

We found fewer alterations in the lower layers of the cortex after hypoxic exposure, including a significant decrease in the lower layer volume of the prefrontal cortex ($P = 0.0093$) (Fig. 8O and tables S4,5). We also found a significant reduction in the number and density of NeuN⁺ cells within the prefrontal cortex after hypoxia (cell number: $P = 0.0004$; cell density: $P = 0.0205$) (Fig. 8P and tables S4,5). On the other hand, there were no differences in Calr⁺ interneurons or Tbr1⁺ excitatory neurons throughout the lower layers of the cortex (Fig. 8Q and tables S4,5).

Discussion

This study addressed the relationship between the SVZ and cortical growth in CHD. We demonstrated that newly generated neurons in the postnatal SVZ migrated to frontal cortices and differentiated into interneurons in a gyrencephalic brain. We also challenged this process through chronic hypoxic exposure, which severely impaired neurogenesis within the SVZ, resulting in a depletion of a source of interneurons destined to populate and potentially fine-tune the postnatal frontal cortex. This pathology limited cortical expansion and gyrencephaly and mirrored the brain signatures seen in CHD patients. Consistent with these findings, we demonstrated that an even more drastic depletion of neuroblasts within the SVZ – particularly the A-lateral-SVZ - of infants born with complex CHD, associated with abnormal blood flow essential for cerebral oxygen and nutrient delivery, experienced a depletion of neuroblasts within the SVZ – particularly the A-lateral-SVZ. We identified the A-dorsolateral-SVZ as a contributor to postnatal cortical development in piglets – at an age

similar to human infancy - and demonstrated that NSPCs within the A-dorsolateral-SVZ region were vulnerable to the hypoxic conditions seen in CHD patients.

Cortical development is a dynamic and complex process and proper cognitive function requires a balance of excitation from pyramidal neurons and inhibition from GABAergic interneurons(41–43). Little is known regarding the contribution of the SVZ to cortical growth in gyrencephalic species during the early postnatal stages of life(44, 45). Here, we have shown that the A-dorsolateral-SVZ is a source of Calr⁺ interneurons during postnatal development in neonatal piglets. This study revealed the region-specific contributions of SVZ-derived cells to cortical growth during early postnatal life in a gyrencephalic mammal under normal as well as pathological conditions. We demonstrated that neuroblasts in the SVZ migrated postnatally to frontal cortices, where they differentiated primarily into Calr⁺ interneurons. There is growing evidence to support the presence of neuroblasts in the postnatal and adult cortex of the human and non-human primate(46–49). These studies commonly report neuroblasts in the upper layers of the frontal cortex, a phenomenon also seen in our study. Our analysis performed in piglets supports the possibility that newly born neurons seen in the human frontal cortex are generated within the postnatal SVZ, as observed in the porcine brain.

Our findings supported the notion that exploration of porcine SVZ development is relevant to elucidating the cellular and molecular mechanisms underlying SVZ development in the perinatal human brain. In this study, we were unable to define the embryonic origin of Calr⁺ interneurons, as only a small percentage (<2%) of CTG⁺ cells expressed ganglionic eminence markers within the AE-SVZ at this developmental time window. To define the fetal origin of interneurons, new approaches will need to be developed in the porcine model, such as viral delivery to label specific cell populations within the ganglionic eminences in utero and subsequent postnatal analyses. Future advances of such innovative techniques in the porcine developmental model should provide a more complete picture of the complexities of brain development and pathologies in gyrencephalic species.

There are many spatiotemporal similarities between human and porcine brain development; for example, both achieve a maximal phase of brain growth and peak myelination near birth (50, 51). The gyrification index of a 2 week old piglet is similar to that of a human fetus in the late 3rd trimester(18). The reduction in the gyrification index seen in piglets after hypoxic exposure at 2 weeks of age corresponds to the decline in gyrencephaly seen in human CHD fetuses in the late 3rd trimester(18). We show that the SVZ of a 7 week old piglet shares similarities to the human fetus, infant, neonate, and adult (27–29, 31, 34, 49). In the context of neuroblast populations, both the 1 week old piglet and the human neonate have an abundance of neuroblasts in layer II of the SVZ. The onset of hypoxia at 3 days of age in piglets corresponds to the late 3rd trimester human fetus, and our findings regarding the influence of hypoxia at 2 weeks of age reflect the developmental equivalence of a human neonate.

Genetic links between abnormal heart and brain development have recently been brought into focus(52); cardiac anomalies also reduce the supply of blood flow and cerebral oxygen throughout the postnatal period (11, 16, 17). Underdevelopment of the frontal cortex is a

distinct structural phenotype of the developing brain that is seen in many neonates with complex CHD(53). Our study indicates that delayed cortical expansion in a porcine model of CHD was due, in part, to depletion of NSPCs within the SVZ. We showed that depletion of NSPCs was specific to certain SVZ subregions in piglets (DL- vs. L-) and was associated with reduced interneuron populations within particular frontal cortices, primarily the prefrontal cortex. In addition our data in human tissue indicated that complex CHD is associated with a near depletion of neuroblasts within the SVZ. Altogether, these findings suggest that diminished SVZ neurogenesis caused by chronic hypoxia may represent a cellular mechanism underlying immature cortical development in the CHD population.

We cannot conclude that the reduction in cortical growth seen after hypoxic conditions was solely due to depletion of NSPCs within the A-SVZ and we do not rule out the possibility that other factors and sources of NSPCs could play roles in this pathology. Future studies focused on NSPCs within select subregions of the postnatal SVZ under normal conditions and after different pathological insults will be invaluable in understanding the regional heterogeneity of the regenerative capacity of NSPCs. In the present study, we showed that NSPCs within the DL-SVZ in piglets are particularly vulnerable at an age corresponding to human infancy.

Impairments in executive functions, a set of higher order cognitive functions involving the prefrontal cortex, are commonly associated with CHD(54). Our multilevel cortical analyses demonstrated that cellular alterations were most common in the prefrontal cortex after hypoxia. Hypoxic exposure resulted in region-specific imbalances in excitatory and inhibitory neurons throughout the cortex. An increase in excitatory neuronal density in the absence of changes in cell numbers in the somatosensory cortex after hypoxia was also seen in an ovine prenatal cerebral hypoxia-ischemia model of preterm birth(55). Imbalances between excitatory and inhibitory neurons in the frontal cortex are associated with behavioral and intellectual disabilities including attention deficits, hyperactivity, as well as learning and working memory impairments(56, 57). Many CHD children are affected by such behavioral and intellectual alterations(8–10). Our findings suggest it is plausible that developmental defects in interneurons and excitatory neurons play an important role in behavioral and intellectual deficits seen in CHD.

Our preliminary findings demonstrated alterations in the SVZ cytoarchitecture and neuroblast numbers associated with CHD. However, we cannot exclude the possibility that complicated surgical procedures contributed to the brain injury noted in human specimens. Postmortem human CHD and age-matched control samples are rare, which precludes extensive cellular analyses. The recent developments and expansion of banking and indexing human infant and neonatal brain tissue across hospitals will enable these analyses and greatly enhance our understanding of CHD-induced brain injury. Additional human cases and future studies comparing preoperative and postoperative CHD brain tissues will be necessary to conclude that these aberrations in the SVZ are specific to fetal and early postnatal cerebral hypoxia.

Our piglet model enabled us to reproduce several pathological signatures of the fetal/neonatal human brain with CHD: reduced brain weight, cortical volume, and

gyrencephaly(15, 18, 58). However, our model does not completely reflect the clinical state of abnormal physiology in fetal CHD, such as altered cerebral blood flow and cerebrovascular resistance. The bilateral carotid occlusion fetal sheep hypoxia-ischemia model of preterm brain injury does include cerebral hypoperfusion(55, 59, 60): however, this model also requires maternal anesthesia and the surgery induces inflammation. Both animal models reproduce brain alterations, such as a reduction in cortical volume, that are similar to those seen in CHD patients (55). To our knowledge, there are no well-established, large-animal, genetic models of severe or complex CHD. Such models will be essential in reproducing the fetal cerebral circulation defects seen in CHD. Because alterations of the developing piglet brain due to hypoxia are very similar to those observed in newborns with CHD, we are confident that many cellular events in the human fetal brain with CHD can be reproduced in the proposed animal models. With the growing availability of genetic tools such as CRISPR, future studies aimed at generating large animal genetic models are achievable and will aid in understanding brain injuries associated with abnormal fetal circulation.

Current knowledge of the cellular processes governing unfavorable neurological outcomes in humans is primarily associative and indirect. Therefore, elucidating the cellular and molecular mechanisms contributing to CHD-induced immature cortical growth is not only a fundamental research endeavor, but also is vital for the healthcare of this growing community of patients; complimentary animal models and integrative approaches that recapitulate clinical findings will be necessary to accomplish this goal. Our porcine model allows us to gain insight into the effects of CHD on immature brain development at the cellular level, and demonstrates that the analysis of hypoxia-induced brain injury in the piglet can help fill current gaps in knowledge. Future studies addressing different lengths and severity of hypoxic exposure in animals at different ages will be invaluable in determining regional and temporal vulnerability and therapeutic windows. There are no treatments for immature cortical development caused by CHD or for prolonged neurological impairments in this population, which represent substantial socioeconomic and management challenges for patients, families, and society(8–10, 61). Our findings indicate that treatments designed to protect or restore the neurogenic potential of NSPCs within the SVZ offer a promising avenue to improve neurological deficits in individuals with CHD by preventing or reversing immature or delayed brain development.

Materials and Methods

Study design

This study was designed to determine the potential deleterious cellular outcomes of subnormal cerebral oxygenation commonly associated with complex CHD. Because the porcine species shares many similarities to human brain structure and development - as well as physiology - we utilized a chronic hypoxia model in the piglet to mimic cerebral hypoxia in CHD. We utilized several in vivo approaches to assess the outcome of perinatal chronic hypoxia at the macrostructural and cellular level; in addition, we examined human tissue from postmortem CHD patients and controls to compare and validate our salient cellular findings in the piglet SVZ. Sample sizes were chosen based on the standard used in the field

and on previous experience with analyzing similar data sets. Animals were randomly housed in normoxic or hypoxic conditions. Inclusion criteria for human postmortem tissue was: (i) human infants who suffered a reduction of cerebral oxygen supply during gestation due to congenital heart disease; and (ii) human infants who did not suffer a reduction of cerebral oxygen supply; for immunohistochemistry analysis of Dcx⁺ cells, two samples (1 CHD, 1 No CHD) were excluded because of a lack of antibody penetrance. All animals were included in this study; there were no inclusion/exclusion criteria for animals. For neurosphere analysis: contaminated tissue culture samples were not analyzed; random images were acquired and quantified; for differentiation potential, unbiased random sampling was performed with a Stereo Investigator (MicroBrightField, Inc.) system with the optical fractionator probe. Investigators were blinded in MRI volumetric analysis, SPIO analysis, human tissue analysis, and in animal cellular analysis. Full details for the methods used for sample preparation, hypoxia studies, and image analysis can be found in the supplementary materials.

Statistical analysis

A two-tailed, unpaired Student's *t*-test was performed for single comparisons; data were considered significantly different if $P < 0.05$. For multiple comparisons, a one-way or two-way analysis of variance (ANOVA) was applied with Bonferroni post-hoc test; $p < 0.05$ determined significance. GraphPad Prism software was used to run Student's *t*-tests and ANOVAs. For neurosphere diameter frequencies, an ordinal logistic regression model with a likelihood ratio test was performed using International Business Machines Corporation SPSS Statistics software, version 23.0 (IBM). *P* values not listed in the main text are listed in table S6; *P* values less than 5 significant digits are listed as $P < 0.0001$.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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One Sentence Summary

Congenital heart disease depletes SVZ neural stem/progenitor cell pools critical for normal cortical growth.

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Fig. 1. The piglet SVZ is structurally similar to its human counterpart and displays robust postnatal neurogenesis

(A) 3D model of the pig brain at 7 weeks (w) of age. (B–E) Photographic images on the coronal plane of pig brain tissue at 7 weeks of age. The SVZ is divided into four rostrocaudal domains: (B) anterior end (AE), located at the rostral extremity of the anterior horn; (C) anterior horn (A); (D) middle (M), spanning the body of the ventricle; and (E) posterior (P), near the atrium of the lateral ventricle (LV). (F) Magnified image from the location marked by inset in (C). DAPI stain; L-, lateral; DL-, dorsolateral; scale bar, 1 mm.

(**G-I**) GFAP expression in the four distinct layers of L-SVZ: (I) ependymal layer; (II) Dcx⁺ neuroblasts; (III) GFAP⁺ astrocyte ribbon; (IV) transitional zone. Magnified images from the location marked by inset in (F). (**G**) GFAP expression; (**H**) GFAP expression and DAPI stain; (**I**) GFAP, DAPI, and Dcx immunostains; scale bars, 50 μ m. (**J-M**) Immunostains at 7 weeks of age. (**J**) Magnified image from the location marked by inset in **E** Vimentin (Vim) immunostain; I-, inner; O-, outer; WM, white matter; scale bar, 100 μ m. (**K-M**) Magnified images from the location marked by the inset in (J) showing: (**K**) Vim and Pax6 expression; (**L**) Vim and Sox2 expression; (**M**) Vim and Ki67 expression; scale bars, 50 μ m. Quantification of (**N**) Sox2⁺ and (**O**) Sox2⁺Ki67⁺ cells in the A- and P-SVZ. Quantification of (**P**) Sox2⁺ and (**Q**) Sox2⁺Ki67⁺ cells in I- and O-SVZ (n=6 animals). Quantification of (**R**) Sox2⁺ and (**S**) Sox2⁺Ki67⁺ cells at 1w, 7w, and 15w (n=5). (**T**) Neurospheres isolated from the A- and P-SVZ at p2 and p14 and cultured for 1w; scale bars, 100 μ m. (**U**) Number of neurospheres generated (n=3 animals, p2; n=5–6 animals, p14) and (**V**) distribution of neurosphere diameter. Data expressed as the mean $\pm$ SEM. *p < 0.05, **p < 0.01, ***p < 0.001, unpaired Student's t-test (**N-Q**); #p < 0.05, analysis of variance (ANOVA) with Bonferroni post-hoc test (**R,S,U**); ordinal logistic with likelihood ratio test (LRT) (**V**).

Fig. 2. Diverse patterns of neuroblast cell populations are present throughout the developing piglet brain

(A) MRI of piglet brain at 1 week (w) of age. (B-C) Magnified images of insets corresponding to (A). Immunofluorescence analysis of Dcx and PSA-NCAM expression in the (B) cortex and (C) subventricular zone (SVZ). Dcx⁺PSA-NCAM⁺ cells extend (magnified in inset) from the SVZ (C) to the cortex (B,C); scale bars, 50 µm (B), 200 µm (C). (D) 3D reconstruction of a tiled immunostain for Dcx in 3 mm thick, optically cleared brain tissue encompassing the SVZ and prefrontal cortex; curved dashed line marks the boundaries of the surface of the lateral ventricle (LV) and the SVZ. Reconstruction is a magnification of the inset denoted in (A). (E-H) 3D segmentation of Dcx⁺ cells within select

regions of interest demarcated in panel D illustrating multiple patterns of neuroblast cell distribution (**E**) adjacent to the LV, (**F**) within and near the SVZ, (**G**) in the subcortical white matter (WM), and (**H**) in the cortex (ctx); individual and groups of cells are assigned random colors. Note: segmentation is throughout the entire depth (2.4 mm after optical clearing) of the tissue and therefore not all segments are visible in the 3D reconstruction shown in D, particularly segments from panel F near the SVZ where Dcx⁺ cells are highly dense.

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Fig. 3. Focally labeled SVZ-derived cells are primarily of the neuronal lineage in piglets
(A) Schematic of CTG injection (green) into the A-DL-SVZ of the 1 week old piglet brain.
(B) Schematic corresponding to the AE-SVZ noted in (A) denoting regions of interest identified in higher magnification in (D,J,L,M). CTG⁺ cells in the **(C)** A-SVZ (region noted in (A) and **(D)** AE-SVZ (region noted in A), 1 week post injection (wpi); scale bars, 1 mm **(C)**, 500 μm **(D)**, 50 μm (inset in **C**). **(E)** Higher magnification of CTG⁺Dcx⁺ in the AE-SVZ denoted in (D), 1 wpi; scale bar, 50 μm. **(F)** Quantification of the number of CTG⁺ cells near the injection site at 2 weeks of age (n = 3 animals). **(G-I)** Quantification of the cell fate of CTG⁺ cells in the **(G)** DL-Med-SVZ, **(H)** DL-Mid-SVZ, and **(I)** L-SVZ (n = 3 animals); GFAP represents astrocytes and radial glia, Olig2 represents oligodendrocytes, and Iba1 represents microglia. CTG⁺Dcx⁺ cells in the **(J)** rostral migratory stream, **(K)** AE-SVZ and periventricular white matter (PVWM), 1 wpi. **(L)** CTG⁺ cells within the subcortical white matter (SCWM) and **(M)** CTG⁺NeuN⁺ neurons in the prefrontal cortex, 1 wpi; scale bars, 50 μm **(E,J-M)**. DAPI counterstain (blue) is shown in **(C,E,J,K,L,M)**. Panels on the

right in (K) and (M) identify single channels and merge images for insets. CTG, cell tracker green; A, anterior; AE, anterior end. LV, lateral ventricle; DL, dorsolateral; L, lateral; Med, medial; Mid, middle; CC, corpus callosum. Data expressed as the mean $\pm$ SEM. * $p<0.05$, ** $p<0.01$ compared with CC-Mid, CC-L, PVWM, and Caudate (**F**) by one-way ANOVA with Bonferroni post-hoc comparisons. *** $p<0.0001$, compared with GFAP, Olig2, and Iba1 (**G-I**) by 1-way ANOVA with Bonferroni post-hoc comparisons. # $p<0.05$, GFAP compared with Dcx, Olig2, and Iba1 (**H**) by one-way ANOVA with Bonferroni post-hoc comparisons.

Fig. 4. SVZ-derived cells migrate to the frontal cortex and differentiate into neurons in piglets (A) T2* weighted MRI scans displaying hypointense voxels illustrating the presence of SPIO⁺ nanoparticles (magenta arrowheads in insets), 4 weeks post injection (wpi) in the piglet brain. Cortices are color coded as follow: blue, prefrontal; pink, primary (P) somatosensory; green, insular. (B) Quantification of the distribution of hypointense voxels generated by SPIO nanoparticles as measured by T2* weighted MRI, 4 wpi (n=3); Ctx, cortex. (C) Quantification of SPIO⁺ co-labeled cells within the upper (UL) and lower (LL) layers, and subcortical white matter (WM), of the cortices where the majority of hypointense voxels were located in (B), 4wpi (n=3). (D-G) Examples of SPIO⁺ cells in the cortex, 4 wpi.

(**D**) SPIO⁺Calr⁺NeuN⁺ interneurons and (**E**) SPIO⁺Calr⁻NeuN⁺ neurons in the upper layers (II/III) of the prefrontal cortex. (**F**) SPIO⁺GFAP⁻Iba⁻ cells in layer I of the prefrontal cortex. (**G**) SPIO⁺Calr⁺NeuN⁺ interneurons in layers (II/III) of the primary somatosensory cortex. (**D-G**) DAPI⁺ nuclei are seen in the blue channel; dashed white lines separates layer I from II/III of the cortex; insets represent higher magnification and different combinations of channels to better visualize nanoparticles within specific cell types; Scale bars, 100 μ m. (**H**) Quantification of the distribution of SPIO⁺ co-labeled cells throughout the prefrontal cortex, where the majority of the nanoparticles were found in (**B**). (**I**) Quantification of the cell fate of co-labeled cells in the upper layers of the prefrontal cortex, where the majority of the nanoparticles were located (**H**). (**J,K**) Neurosphere differentiation potential, isolated from p14 SVZ (n=6, anterior; n=5 animals/group, posterior); scale bars, 100 μ m; Tuj1 (Neuron-specific class III beta-tubulin) labels neurons, O4 labels oligodendrocytes. (**L**) Quantification of Dcx⁺ cells at 1, 7, and 15w (n=4 each). Data expressed as the mean $\pm$ SEM. ***p < 0.001, prefrontal ctx and p somatosensory cortex vs. all other regions (**B**); **p<0.01, UL vs. LL and SCWM (**H**); **p<0.01, Calr⁺NeuN⁺ vs. Calr⁻NeuN⁺ and GFAP⁺ (**I**); **p<0.01, anterior Tuj1 vs. all markers in anterior and posterior (**J**); one-way ANOVA with Bonferroni post-hoc comparisons (**B**, **H-K**). More data are presented in table S2.

Fig. 5. Chronic hypoxia impairs cortical development and reduces neurogenesis in the SVZ in piglets

(A,B) Reconstruction of MRI scans at p14. (C) Quantification of average brain weight (n=15 animals/group). (D,E) Cortical gray matter volume (green). (F) Quantification of cortical volume (n=4–5 animals/group) in (D,E). (G,H) Perimeter of the total pial surface (red lines, inner) and total perimeter of the brain (yellow lines, outer); scale bars, 1 cm (D,E,G,H). (I) Quantification of cortical folding determined by the gyrification index (n=5 animals/group) expressed as a ratio of the inner vs. outer perimeter traces in (G,H). (J,K) DAPI stains of A-SVZ at p14; scale bars, 500 μm. (L) Quantification of the average width/thickness of the A-SVZ (n=4 animals/group). (M,N) Immunostains of GFAP. (O) Quantification of the average length of GFAP⁺ processes. (P,Q) Immunostains of Sox2⁺ cells. (R) Quantification of the average number of Sox2⁺ cells. (S,T) Immunostains of Sox2⁺Ki67⁺ cells. (U) Quantification of the average number of Sox2⁺Ki67⁺ cells. (V,W) Immunostains of Dcx⁺ cells. (X) Quantification of the average number of Dcx⁺ cells. Scale bars, 50 μm; n=3–4 animals/group (M,N,P,Q,S,T,V,W). LV, lateral ventricle; Nx, normoxia; Hx, hypoxia. Data expressed as the mean ± SEM. *p<0.05, **p<0.0001, Hx vs. Nx, unpaired Student's t-test (C,F,I,L,O,R,U,X).

Fig. 6. Structural and cellular alterations/abnormalities of the SVZ are associated with CHD in humans

(A,B) GFAP⁺ cells within the SVZ underlying the frontal cortex of human infants or neonates born (A) without or (B) with severe or complex CHD; insets represent areas where images were acquired at higher magnification, corresponding to C,D,F,G; dashed lines illustrate the boundary between the lateral ventricle and SVZ; scale bars, 1 mm. (C-H) Immunostains illuminating GFAP⁺ cell processes spanning layer II of the (C-E) DL- and (F-H) L-SVZ; insets in D,G represent higher magnification shown in E,H. (C,F) without CHD

and **(D,E,G,H)** with severe or complex CHD; scale bars, 50 μm (**C,D,F,G**), 20 μm (**E**), and 10 μm (**H**). Quantification of the average length of GFAP⁺ processes within **(I)** the SVZ, **(J)** the DL-SVZ, and **(K)** the L-SVZ (n=5 with no CHD and n=4 with CHD). **(L,M)** Immunostains illustrating Dcx⁺ cells within the SVZ for a human infant **(L)** without and **(M)** with CHD; insets represent areas where images were acquired at higher magnification corresponding to N-S; dashed lines illustrate the boundary between the lateral ventricle and SVZ; Scale bars, 1 mm. Images in panels P and S were acquired in the same regions marked by the insets in M from a different CHD specimen. Dcx⁺ cells within the DL- and L-SVZ of a human infant born **(N,Q)** without CHD and two different infants born **(O,P,R,S)** with severe or complex CHD; scale bars, 50 μm . Quantification of the average number of Dcx⁺ cells within **(T)** the SVZ, **(U)** the DL-SVZ, and **(V)** the L-SVZ (n=4 with no CHD and 3 with CHD). CHD, congenital heart disease; LV, lateral ventricle; DL, dorsolateral; L, Lateral; N, normal. Data are expressed as the mean $\pm$ SEM. *p < 0.05, **p < 0.01, No CHD vs. CHD, unpaired Student's t-test (**I,T**). Two-way ANOVA with Bonferroni comparisons (**I,T**). Pearson correlation (**U**).

Fig. 7. Chronic hypoxia impedes the contribution of SVZ-derived neurons to cortical development in piglets

(A,B) CTG⁺ cells within the AE-SVZ; insets represent higher magnification shown in (C,D); (A,C) normoxia, (B,D) hypoxia. Scale bars, 500 μm. A Schematic of CTG injection (green) into the A-DL-SVZ is shown at the top of (B). Dashed lines in D outline the SVZ. (E, F, H, I) CTG⁺Dcx⁺ and (G,J) CTG⁺Dcx⁺PSA-NCAM⁺ cells, originating from the A-SVZ, within the AE-SVZ. Quantification of the number of (K) CTG⁺ and (L) CTG⁺Dcx⁺ cells within the AE SVZ at p14, 11 days after hypoxic exposure (n=3 animals/group). Insets

show higher magnification labeled cells in **(E-J)**. **(M)** Quantification of the regional distribution of hypointense voxels from SPIO⁺ labeled cells present in the cortex (n=6 animals/group), expressed as a percentage of the total number of hypointense voxels. LV, lateral ventricle; Nx, normoxia; Hx, hypoxia; dpi, days post injection; Ctx, cortex. Data expressed as the mean $\pm$ SEM. *p<0.05, **p < 0.01, unpaired Student's t-test.

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Fig. 8. Chronic hypoxia alters immature and mature neuronal populations in a region-specific manner in piglets

Immunostains for (A) Dcx, (B) Casp3, and (C) Dcx/Casp3 within layers II/III of the prefrontal cortex at p14, normoxia (Nx); scale bars, 50 μm. (D) Quantification of Dcx⁺ cell density within the upper layers (UL) of distinct cortices. Immunostains for (E) Dcx, (F) Casp3, and (G) Dcx/Casp3 within layers II/III of the prefrontal cortex at p14, hypoxia (Hx); scale bars, 50 μm. (H) Quantification of Dcx⁺ cell density within the subcortical white matter at p14. Immunostains of NeuN within the prefrontal cortex under (I) normoxia and

(J) after hypoxia at p14; dashed lines border layers II/III of the cortex; scale bar, 500 μ m. Quantification of (K) the number of NeuN⁺ cells, (L) layer volume, (M) number of Calr⁺ cells, and (N) Tbr1⁺NeuN⁺ cell density within the upper layers of the cortex, p14. Quantification of (O) layer volume, (P) the number of NeuN⁺ cells, and (Q) the number of Calr⁺ in the lower layers (LL) of the cortex, p14. WM, white mater; Ctx, cortex; PF, prefrontal cortex; SS, primary somatosensory cortex; I, insular cortex; C, cingulate cortex; P, prepyriform area; PV, periventricular white matter. Data expressed as the mean $\pm$ SEM. n=6 animals per group. *p < 0.05, **p < 0.01, ***p<0.001, Hx vs. Nx, unpaired Student's t-test (D,H, K-Q). More data are presented in Supplemental Tables 4,5.