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Prenatal Cerebral Ischemia Disrupts MRI-Defined Cortical Microstructure Through Disturbances in Neuronal Arborization

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Abstract

Children who survive preterm birth exhibit persistent unexplained disturbances in cerebral cortical growth with associated cognitive and learning disabilities. The mechanisms underlying these deficits remain elusive. We used ex vivo diffusion magnetic resonance imaging to demonstrate in a preterm large-animal model that cerebral ischemia impairs cortical growth and the normal maturational decline in cortical fractional anisotropy (FA). Analysis of pyramidal neurons revealed that cortical deficits were associated with impaired expansion of the dendritic arbor and reduced synaptic density. Together, these findings suggest a link between abnormal cortical FA and disturbances of neuronal morphological development. To experimentally investigate this possibility, we measured the orientation distribution of dendritic branches and observed that it corresponds with the theoretically predicted pattern of increased anisotropy within cases that exhibited elevated cortical FA after ischemia. We conclude that cortical growth impairments are associated with diffuse disturbances in the dendritic arbor and synapse formation of cortical neurons, which may underlie the cognitive and learning disabilities in survivors of preterm birth.

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SUPPLEMENTARY MATERIALS

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Further, measurement of cortical FA may be useful for noninvasively detecting neurological disorders affecting cortical development.

INTRODUCTION

The leading causes of chronic neurological impairment in survivors of premature birth include a spectrum of cerebral palsy and intellectual disabilities (1, 2). Although preterm infants most commonly sustain white matter injury (WMI), there has been a recent decline in its overall severity (3). Nevertheless, up to 25 to 50% of preterm survivors continue to display a wide range of unexplained cognitive and learning disabilities, attentional deficits, and impaired social interactions (4), which suggests a role for gray matter pathology. Indeed, several large magnetic resonance imaging (MRI) studies have identified significant reductions in cerebral cortical and subcortical growth in survivors of preterm birth (5–10). This impairment in cortical growth correlates with cognitive, but not motor, outcomes in early childhood (11).

The underlying cellular mechanisms related to impaired cortical growth in contemporary cohorts of preterm survivors continue to be controversial. Loss of cortical neurons and axonal degeneration were reported in preterm human autopsy cases with significant necrotic WMI (12, 13). However, no neuronal loss was observed in cases with nonnecrotic diffuse WMI (12), which is the predominant type of WMI seen with modern medical management of premature infants (3, 14). Because a mechanism of cortical neuronal degeneration appears inconsistent with the patterns of WMI currently observed in preterm survivors, we considered an alternative mechanism in which cerebral ischemia disrupts cortical development via disturbances in neuronal maturation.

During the last trimester of fetal development, before the onset of myelination, a marked expansion of the cerebral cortex occurs (15) that is associated with prolific axonal growth, dendritic sprouting, and synapse formation (16, 17). This highly active period of neuronal elaboration may be particularly sensitive to insults that disrupt normal cortical maturation. There is, however, no defined role for reduced neuronal complexity as a cause of cortical volume loss and cognitive deficits in survivors of preterm birth. Diffusion tensor imaging (DTI) studies in humans (18, 19) and other species (20–22) demonstrated that normal cortical maturation is associated with a decline in MRI measurements of fractional anisotropy (FA), which is hypothesized to arise from increasing morphological complexity of cortical neurons (18, 21, 23). Recently, we demonstrated that water diffusion anisotropy is directly related to characteristics of neuronal morphology, including the orientation and maturation of the arbor of neuronal cell processes (24), and that FA measurements have sufficient sensitivity to discriminate between normal and abnormal cortical differentiation (25). Thus, measurement of cortical FA may be useful for detecting neurological disorders affecting cortical development.

Hence, we tested the hypothesis that cerebral ischemia disrupts cortical maturation by disturbing dendrite and spine formation, but not by causing neuronal loss, and that these changes disrupt cortical micro-structure as defined by MRI measurements of FA. We used a well-established preterm fetal sheep cerebral ischemia model (26–29) that generates a spectrum of moderate WMI, which closely reproduces the patterns seen in contemporary cohorts of human preterm survivors (3). We used high-field *ex vivo* MRI diffusion-weighted data combined with a Golgi impregnation analysis of neuronal complexity to provide an explanation for cognitive disturbances in preterm survivors that involves diffuse abnormalities in the dendritic arbor and synapse formation of cortical neurons. By applying a recently developed mathematical model, we demonstrate that disturbances in MRI-defined

FA were replicated by calculated estimates of FA derived from the neuronal dendritic arbor of the growth-impaired cortex.

RESULTS

Preterm ischemia disrupts long-term cortical growth

To define mechanisms of impaired growth of the preterm cerebral cortex, we determined the effect of in utero global cerebral ischemia induced by bilateral carotid artery occlusion at 0.65 gestation (equivalent to ~26 to 28 weeks of human gestation) on subsequent cortical development in our fetal sheep model of WMI (26, 27). Ex vivo high-field MRI studies were performed at 1, 2, and 4 weeks after the ischemic event to classify cortical voxels based on maps of FA and the apparent diffusion coefficient (ADC) (Fig. 1A). A progressive expansion in cortical volume was observed between 1 and 4 weeks after surgery in both the ischemia group and their twin controls, who had been subjected to the same general surgical procedure without subsequent ischemia [Fig. 1D; analysis of variance (ANOVA), $P < 0.001$]. After 1 week of recovery, there was no difference in cortical volume between the control and ischemia groups. However, at 2 and 4 weeks, cortical volumes were significantly lower in the ischemia group compared to age-matched controls (percentage decrease: ~14% at 2 weeks and ~18% at 4 weeks). Thus, ischemia disrupted the normal maturational increase in cortical volume, with a blunting of cortical growth that began between 1 and 2 weeks after the insult.

Diffusion MRI identifies alterations in cortical microstructure

We next hypothesized that disturbances in cortical growth would be accompanied by abnormalities in cortical microstructure defined by diffusion MRI. A decline in FA occurs with normal cortical development in humans (18, 19) and other species (20–22), but it has not been described after fetal cerebral ischemia. Consistent with these studies, both the control and ischemia groups exhibited a progressive decline in cortical FA with increasing gestational age (Fig. 1E; ANOVA, $P < 0.005$). However, by 4 weeks, the cortical FA was significantly higher in the ischemia group compared to age-matched controls, at values similar to the 2 week group. ADC also progressively declined with gestation time in both the control and ischemia groups (ANOVA, $P < 0.0001$) but showed no effect of ischemia (Fig. 1F). Thus, preterm ischemia inhibited the normal maturation-dependent decrease in cortical FA, consistent with a progressive disturbance in cortical microstructure and disrupted maturation of the cerebral cortex. In both the control and ischemia groups, the water diffusion principal eigenvector orientations for each cortical voxel were perpendicular to the cortical surface (fig. S1) and were aligned parallel to the apical dendrites of pyramidal neurons, indicating that the changes in cortical FA after ischemia were not due to alterations in the primary direction of water diffusion.

Impaired cortical growth is not caused by a loss of cortical neurons

We determined whether the disturbances in cortical growth and microstructure were related to a loss of cortical neurons. We used unbiased stereology to quantify the total number of neurons in the cortical mantle (Fig. 2, A to C). The control group demonstrated no significant change in the number of cortical neurons between 1 and 2 weeks after the ischemic episode (Fig. 2D), which supported the idea that addition of new neurons to the cortex was largely complete by this time. Moreover, compared to age-matched controls, there was no effect of ischemia on the number of cortical neurons at 1 or 2 weeks. A reduction in the density of neurons in controls by 2 weeks (Fig. 2E) reflected the normal pronounced growth of the cortex at this time. Cortical neuronal density at 2 weeks after ischemia appeared to increase compared to age-matched controls, but the difference was not

statistically significant (Fig. 2E; $P = 0.07$); this finding likely reflected the ischemia-induced reduction in cortical volumes at this time (see Fig. 1D).

Because previous human autopsy studies have reported neuronal loss in cases with significant white matter necrosis (12, 13), we also determined whether preterm ischemia triggered delayed neuronal degeneration in our model of diffuse but more mild nonnecrotic WMI (26, 27). Neuronal death in the cerebral cortex was analyzed by staining for activated caspase-3 (AC3) after 1, 2, and 4 weeks of recovery. At all ages, there were very low numbers of cortical AC3-positive cells in the control and ischemia groups, with a random distribution and without any predilection for a particular cortical gyrus or layer (fig. S2). Further, we previously reported no acute elevation of AC3 in the cortex at 24 hours and 3 days after ischemia in this model (26, 27). Thus, these data suggested that a reduction in neuronal number did not contribute to cortical volume loss. However, the paradoxical decrease in cortical volume was associated with an increase in neuronal density. These unexpected findings supported an alternative mechanism for cortical volume loss that was related to neuronal dysmaturation.

Normal cortical growth is accompanied by a marked increase in dendritic arborization

A marked growth of the cerebral cortex during the latter third of human gestation is associated with a rapid progressive increase in neuronal complexity (30). To determine whether increased neuronal complexity also contributes to growth of the fetal ovine cerebral cortex during the third trimester, we reconstructed Golgi-impregnated neurons at 0.65 gestation (at the time of ischemia, defined here as 0 weeks) and 4 weeks later. As an index of normal neuronal maturation, we analyzed the complexity of the basal dendritic arbor of pyramidal neurons because they are the most common population of excitatory cells in the cortex. At 0 weeks (at the time of surgery), control pyramidal neurons exhibited a simple morphology (Fig. 3A), which became markedly more complex after 4 weeks (Fig. 3B), with a significant increase in the number of basilar dendritic branches (by ~250%; Fig. 3C), basilar dendritic length (by ~390%; Fig. 3D), and number of basilar branch points (nodes) (by ~330%; Fig. 3E). There was no difference in the number of primary basal dendrites [0 weeks (5.7 ± 0.4) versus 4 weeks (6.2 ± 0.2); fig. S3, B to D] or neuronal soma size [0 weeks ($347 \pm 13 \mu\text{m}$) versus 4 weeks ($370 \pm 10 \mu\text{m}$); fig. S3, A and F] between these ages.

To define the dendritic branching properties of individual neurons, we next performed a Sholl analysis to quantify the differences in morphological complexity of the pyramidal neurons in the 0 week and 4 week groups. There was a marked increase in the number of dendritic intersections (a measure of the complexity of the dendritic arborization) by 4 weeks (Fig. 3F; ANOVA, $P < 0.0001$), with the most significant differences observed 25 to 235 μm from the cell soma. Further analysis according to centrifugal nomenclature suggested that this increase in dendritic complexity at 4 weeks was a response to an increase in total branch number (Fig. 3G) and dendritic length (Fig. 3H) at branch orders 2 to 5. This was supported by the marked increase in numbers of nodes observed at branch orders 1 to 3 at this time (Fig. 3I).

Impaired cortical growth is related to deficits in dendritic arbor maturation

Because we observed a marked increase in dendritic arborization during the phase of rapid cortical growth, we hypothesized that impairment in cortical growth in response to ischemia might be related to disruption of this critical phase of neuronal maturation. To test this, we reconstructed Golgi-impregnated neurons at 4 weeks after ischemia and determined whether the reduction in cortical volume at this time was associated with disturbances in maturation of the basal dendritic arbor of pyramidal neurons (Fig. 4, A and B). Compared to age-matched controls, at 4 weeks of recovery from cerebral ischemia, pyramidal neurons

exhibited a significant reduction in the total number of basilar dendritic branches (by ~12%; Fig. 4C), total basilar dendritic length (by ~18%; Fig. 4D), and the total number of basilar nodes (by ~24%; Fig. 4E). There was no change in the number of primary basilar dendrites [control (6.2 ± 0.2) versus ischemia (5.9 ± 0.2); fig. S3, B to D] or in neuronal soma size [control ($370 \pm 10 \mu\text{m}$) versus ischemia ($351 \pm 12 \mu\text{m}$); fig. S3, A and F]. Secondary analysis of dendritic complexity based on neuronal location in the supragranular or infragranular cortical layers indicated no layer-specific effects (fig. S3).

We next performed a Sholl analysis to quantify the differences in morphological complexity of the pyramidal neurons studied in the control and ischemia groups. The ischemia group displayed an overall reduction in the number of dendritic intersections (Fig. 4F; ANOVA, $P < 0.0001$), with the most significant differences observed at 25 to 75 μm from the cell soma, the region of highest dendritic complexity in both groups. Further analysis according to centrifugal nomenclature suggested that this decrease in dendritic complexity was a response to a decrease in total branch number (Fig. 4G) and dendritic length (Fig. 4H) at branch orders 2 to 4. This was supported by the decrease in the numbers of nodes observed at branch orders 1 to 3 in the ischemia group (Fig. 4I). Overall, these data were consistent with diffuse global impairment of the normal maturation of basal dendrites of cortical pyramidal neurons as a result of ischemia during a critical window when rapid expansion of the cortex normally occurs.

Impaired dendritic arbor maturation is accompanied by reduced dendritic spine density

To further define the effect of ischemia on neuronal maturation, we analyzed the density of spines on basal dendrites of pyramidal neurons in the control animals compared to those subjected to ischemia. Dendritic spines were visualized on the Golgi-impregnated neurons (Fig. 5, B to D). To analyze equivalent dendritic branches between the more complex neurons of the control group and the simplified neurons of the ischemia group, we quantified spine density on second-order terminal dendritic branches (Fig. 5A), which were commonly identified for both groups of neurons. Compared to age-matched controls, the ischemia group showed a significant reduction in spine density at 4 weeks of recovery (by ~10%; Fig. 5E). These data indicate that ischemia resulted in a diffuse global reduction in neuronal connectivity in the cortex.

Deficits in dendritic arbor maturation underlie the abnormal cortical FA after ischemia

Another morphological analysis was performed to directly determine whether properties of neuron morphology underlie the differences in cerebral cortical FA between control and ischemia animals at 4 weeks after surgery (Fig. 1E). We have recently defined the FA of the dendritic arbor orientation distribution, FAn, and derived expressions that lead to the prediction that FA is proportional to FAn (24). By applying this mathematical model, we calculated FAn derived from the neuronal dendritic arbor of the growth-impaired cortex. To assess whether differences in FA observed by DTI between control and ischemia animals correspond with differences in FAn, we used neuron tracings performed by NeuroLucida software to determine the orientation distribution of dendritic elements of the basal arbors. The three-dimensional (3D) reconstructions of Golgi-stained pyramidal neurons (Fig. 6, A and B) were used to identify 3D line segments parallel to the dendritic local symmetry axis over each sequential 10- μm stretch of each neuronal process (Fig. 6C) (24), and for each neuron, FAn was determined. At 4 weeks of recovery, we observed a significantly higher FAn of reconstructed cortical pyramidal neurons in the ischemia group versus the control group (Fig. 6D), which was consistent with the higher cortical FA observed by MRI for ischemia animals at this time (Fig. 1E). These data provide direct evidence that the impaired decline in cortical FA after ischemia is associated with impaired maturation of the basal dendritic arbor of cortical neurons and changes in water diffusion anisotropy.

DISCUSSION

To investigate the mechanisms that underlie the persistent disturbances in cortical growth and cognition in survivors of pre-term birth, we used a combination of high-field MRI and Golgi impregnation of neurons to analyze microstructural development of the cerebral cortex in a preclinical model of ischemia in preterm-equivalent fetal sheep. Unlike rodents, fetal sheep display cerebral hemodynamics and brain maturation similar to humans, and develop similar patterns of diffuse WMI (28). This model also reproduces the deficits in cortical growth observed clinically in preterm survivors with WMI. We provide evidence that these cortical growth disturbances may be related to a delayed maturation of cortical microstructure defined by FA, diffuse impaired development of the dendritic arbor of cortical neurons, and reduced dendritic spine density. Because neuronal connectivity is critically dependent upon the fine structure of neurons, these disturbances in the dendritic arbor and associated spines could explain the disturbances in cortical growth and cognition that are now the major cause of disability in survivors of premature birth.

Deficits in dendritic branching and spine formation are a feature of several human neurodevelopmental disorders associated with mental retardation, including Down and Rett syndromes [for review, see (31)]. Further, infants with extensive necrotic WMI and axonopathy or with direct cortical lesions exhibit marked alterations in neuronal dendritic development (32, 33). Currently, there is no direct evidence for a similar impairment of cortical circuitry in preterm survivors with cognitive disabilities. Nevertheless, preterm infants can exhibit dysmature electroencephalogram (EEG) patterns at 6 weeks of life (34) and late adolescence (35), which could be a result of abnormal cortical circuitry. As observed here for fetal ovine cerebral development, human cortical neurons display a marked increase in dendritic arborization and synapse formation during the latter half of gestation (16, 17) that parallels a marked expansion in cortical volume (15, 36). During this period in development, ischemia may disrupt cortical growth and connectivity. Unexpectedly, we found that moderate cerebral ischemia in sheep was not associated with early or delayed neuronal loss or apoptosis in the cortex, but did trigger significant disruptions in dendritic maturation and connectivity. By contrast, in the same animals, moderate cerebral ischemia was sufficient to cause diffuse but largely nonnecrotic WMI with selective degeneration of pre-oligodendrocytes (27). Thus, an ischemia-reperfusion insult that we have shown to be similar in magnitude in the cortex and white matter (29) appears to trigger very different neuronal versus glial responses. Cortical neurons were markedly more resistant to degeneration but were susceptible to global disturbances in dendritic maturation independent of neuronal supragranular or infragranular location. Similarly, neurons in the pre-term human cerebral cortex were markedly more resistant to damage in cases where the cerebral white matter sustained significant nonnecrotic WMI and glial degeneration (37). Cortical growth is a strong predictor of later neurocognitive outcome in preterm survivors because neurocognitive testing at 2 and 6 years of age correlated with the rate of cerebral cortical growth between 24 and 44 weeks postmenstrual age (11). Widespread disturbances in maturation of the dendritic arbor may provide a structural explanation for the decrease in cortical growth observed in survivors of premature birth.

The normal maturational decrease in cortical FA that occurs in several species including human (18–22) is hypothesized to relate to morphological differentiation of the cortical neuropil (18, 21). We found that the fetal ovine cortex also demonstrated a progressive developmental decrease in cortical FA, which was disrupted at 4 weeks after preterm ischemia. Consistent with the notion that normal developmental changes in FA are related to increasing structural complexity of neurons, we observed a progressive increase in cortical volume and dendritic arbor complexity during the 4-week period of our study. It has been proposed that in the immature cortex, water diffusion is highly anisotropic because of the

predominant alignment of cellular processes perpendicular to the pial surface (18, 38), thus restricting water diffusion in a direction parallel to the pial surface. With neuronal differentiation and elaboration of the dendritic arbor, the distribution of orientations of phospholipid bilayer structures that restrict water diffusion is believed to become more isotropic, causing cortical FA to progressively decrease (21). In support, we recently quantified the orientation distributions of processes of Golgi-impregnated cortical neurons in a model of bilateral enucleation in neonatal ferrets (25) and found that impairment in the developmental decline in cortical FA was related to a reduced complexity of dendritic arbors of cortical neurons. Here, we demonstrated that the impaired decline in cortical FA after preterm ischemia was a direct result of the reduced elaboration of basal dendritic arbors of pyramidal neurons and the associated changes in water diffusion anisotropy. In a related study in this issue, Vinall et al. performed serial DTI scans in preterm babies and found that impaired postnatal growth was independently associated with higher cortical FA at ~40 weeks postmenstrual age compared to appropriate weight infants, even after adjusting for gestational age, birth weight, sex, postmortem interval, severity of brain injury, and systemic illness (39). This delayed maturation of cortical microstructure was related to altered diffusion perpendicular to the pial surface (direction of basal dendrites). Thus, our findings provide an explanation for delayed cortical maturation in this cohort of growth-impaired preterm infants, namely, impaired maturation of the basal dendritic arbor of cortical neurons.

Our findings question current assumptions that the cognitive and learning disabilities in preterm survivors arise principally from irreversible brain injury resulting from neuronal degeneration (40). Whereas secondary neuronal loss has been observed in preterm human autopsy cases that displayed significant necrotic WMI and axonal degeneration (12, 13), cases with moderate diffuse nonnecrotic WMI do not exhibit evidence of early or delayed neuronal degeneration (12, 37). Similarly, despite a reduction in cortical volumes at 2 and 4 weeks of recovery from preterm ischemia, we found no loss of cortical neurons or evidence of neuronal degeneration. Moreover, in recent quantitative ultrastructural studies of high-field MRI-defined WMI, diffuse nonnecrotic WMI was not accompanied by primary axonal loss or degeneration (41). In addition, contemporary cohorts of preterm survivors display a spectrum of milder nonnecrotic WMI (3), which appears unrelated to neuron-axonal degeneration. Nevertheless, a direct role for diffuse nonnecrotic WMI in regulating cortical dysmaturation requires further investigation because these lesions display extensive astrogliosis and microgliosis (3, 12) that may potentially disrupt the function of intact axons in the white matter. Diffusion MRI studies also suggest microstructural and functional changes in white matter connectivity in preterm survivors that correlate with cognitive outcomes (42), although whether these effects are secondary to altered cortical development is unclear. Retrograde axonal signaling is one regulator of dendritic growth and synapse formation (43). In addition, cortical ischemia-reperfusion may directly alter the complex intrinsic programs required for normal dendritic development (44) or alter the function of subplate neurons (45), which are critical in establishing cortical networks during early development (46).

Our study supports the idea that DTI measurement at early developmental stages can be used to detect abnormalities in neuronal maturation in the cortex and to evaluate potential therapeutic strategies. Given the paucity of neuronal death, preventive therapies might consider agents that promote dendritic growth and spine formation, such as rehabilitative training and environmental enrichment (36, 47, 48). In a randomized control trial of socially deprived young children, those children that received foster care experienced a significant improvement in cognitive function compared to the marked intellectual deficits in children reared in institutions (49).

Although we focused on the maturation state of pyramidal neurons, other neuronal populations may be similarly affected. Imaging studies of human preterm survivors have revealed reduced volumes of other gray matter structures, including the thalamus, basal ganglia, and hippocampus (5–7, 9). The mechanisms underlying these volume changes are unknown and require further study, although an impairment of neuronal maturation similar to that seen in cortical pyramidal neurons may occur. Our studies do, however, suggest a mechanism of dysmaturation of cortical neurons that may account for the pronounced and varied disturbances in cognition, learning, attention, and socialization that are common in survivors of preterm birth.

MATERIALS AND METHODS

All studies were performed in a core facility of the Oregon Health & Science University (OHSU) Department of Comparative Medicine strictly according to protocols for which approval was obtained through the OHSU Institutional Animal Care and Use Committee.

Animal surgery and cerebral hypoperfusion studies

This instrumented fetal cerebral hypoperfusion preparation and the general surgical procedures were described previously (26–28). Time-bred preterm fetal sheep (~26 to 28 weeks human gestation-equivalent) of mixed Western breed between 88 and 91 days of gestation (term 145 days) were studied. For cerebral hypoperfusion studies, on postoperative day 2 or 3, sustained cerebral hypoperfusion (37-min duration) was initiated by bilateral carotid artery occlusion after inflation and reestablished by deflation of the carotid occluders (27).

Physiological monitoring

On the second or third postoperative day, at least 30 min before the start of the experiment, pressure transducers and a chart recorder (PowerLab 16/30; ADInstruments) were used to record pressure in the fetal artery relative to amniotic fluid (mean arterial blood pressure). Fetal heart rate was calculated from triplicate measurements of the arterial pressure pulse intervals over a continuous recording of no less than 20 s.

Blood analysis

Blood samples (1 ml) taken anaerobically from the fetal axillary artery (28) at 15 min before and at 10 min after occlusion were analyzed for arterial pH, P_{O_2} , and P_{CO_2} corrected to 39°C, hemoglobin content, oxygen saturation (SO_2), and hematocrit (ABL725 blood gas analyzer; Radiometer Medical A/S). After a 24-hour recovery from surgery, fetuses were entered into the study if they demonstrated normal fetal oxygenation (26, 50). Data from 4 week recovery animals are summarized in table S1. Data from 1 and 2 week recovery animals are as previously reported (26).

Tissue collection and processing

The ewe and fetuses were sacrificed (barbiturate overdose, Euthasol) at 1 week (control, $n = 9$; ischemia, $n = 7$), 2 weeks (control, $n = 5$; ischemia, $n = 6$), or 4 weeks (control, $n = 6$; ischemia, $n = 5$) after completion of the occlusion protocol. An additional group of four unoperated fetuses was collected at 95 to 96 days of gestation (age at ischemia; 0 week group). Fetal brains were hemisected and cut into five equivalent coronal blocks in proportion to the distance between the frontal and the occipital poles. Tissue blocks from the left hemisphere were immersion-fixed at 4°C in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 3 to 5 days and then stored in phosphate-buffered saline (PBS) at 4°C. Tissue blocks from the right hemisphere were processed for Golgi impregnation, as

described below. All analyses used region-matched frontal blocks that spanned from the genu of the corpus callosum to the optic chiasm, and were specific to all gyri and cortical locations of the dorsal cerebral cortex.

Ex vivo MRI

Experimental tissue was embedded alongside a twin control tissue block from the same level in 0.5% agarose and immersed in PBS within a 4-cm-diameter Plexiglass tube (custom-manufactured). A custom single-turn solenoidal coil (5-cm diameter, 5-cm length) was used for radio-frequency transmission and reception. Experiments were performed with an 11.7-T magnet interfaced with a magnetic field gradient coil of 9-cm inner diameter (Bruker). Procedures generally followed the previously published strategy that used DTI to characterize postmortem tissue from sheep and other species (21, 26, 51). A Stejskal-Tanner multislice spin-echo pulse sequence ($\delta = 12$ ms, $\Delta = 21$ ms, and $G = 11.6$ G/cm; resulting in $b = 2.5$ ms/mm²) was used for DTI measurements. The b value for this study was selected to provide an approximate match in diffusion sensitization to a typical in vivo measurement in which $b = 1$ ms/mm² (the water ADC is ~2.5-fold smaller in postmortem tissue than in vivo) (52). Diffusion anisotropy measurements were made with a 25-direction, icosahedral sampling scheme in combination with two measurements in which $b = 0$ (53). Other pulse sequence settings were as follows: repetition time = 10 s, echo time = 42 ms, and number of averaged transients = 1. The image resolution was isotropic, with voxel dimensions of 0.3 mm and a 128 voxel (phase-encode) by 256 voxel (readout) by 70 voxel (slice-select) field of view. Standard procedures were followed to determine eigenvalues of the diffusion tensor and the signal amplitude in the absence of diffusion weighting for each voxel from the set of 27 3D images (53). The ADC and FA, defined in (54), were calculated from the eigenvalues for each voxel.

MRI segmentation

ADC and FA images, herein defined as an image in which the intensity is $S(0) * \exp(-ADC)$, were used to classify all tissue and agarose voxels with Matlab 2008b software (MathWorks). Voxels for each tissue block were manually classified by an individual who was blinded to neuropathologic classification as either cortex or white matter with the standard functionalities of the ITK-snap program (<http://www.itksnap.org>) (26, 55). Mean values for MRI-derived parameters within the cortex for a given case were computed as means over the set of voxels classified as cortex.

Immunohistochemistry studies

After MRI, left hemisphere frontal blocks from each animal were cryo-protected by sequential equilibration in 15 and 30% sucrose solutions over 3 days at 4°C. Tissue was rapidly frozen with a liquid nitrogen interface. For each tissue block, a series of evenly spaced coronal sections (50- μ m thick, section interval = 12) were collected with a CM 1950 cryostat (Leica Microsystems Inc.). Rabbit anti-AC3 antibody (9661B; 1:500; Cell Signaling Technology) was used to identify apoptotic cells. A fluorescent goat anti-rabbit Alexa Fluor secondary antibody was used for AC3 visualization (1:500; Invitrogen/Life Technologies), and tissue sections were counterstained with Hoechst 33342 (Invitrogen/Life Technologies). Neurons were identified with a mouse anti-NeuN antibody (1:500; MAB377; Merck Millipore) and visualized with the peroxidaseimmunoperoxidase reaction (Vectastain ABC Kit; PK4000; Vector Laboratories Inc.) and 3,3'-diaminobenzidine with nickel enhancement (SK4100; Vector Laboratories Inc.).

Quantification of AC3

All AC3-positive cells throughout the cerebral cortex of control and ischemia cases were counted with a 20× objective on a minimum of two adjacent sections by an observer blinded to the groups (K.C.). The cortical/white matter boundaries in each section were defined with Hoechst 33342.

Unbiased quantification of cortical neurons

The numbers of cortical neurons were assessed by high-precision design-based unbiased stereology with a stereology workstation (Stereo Investigator; MicroBrightField Inc.) connected to a Leica DMREII inverted microscope (Leica Microsystems Inc.) with a motorized stage equipped with xyz-axis transducers. An investigator (K.H.) was blinded to the treatment groups of the animals, and all counts were performed by the same investigator. Contours outlining the entire dorsal cerebral cortex were traced on NeuN-stained sections with a 5× objective. Cortical neurons were counted by light microscopy [63× oil, numerical aperture (NA) = 1.40; Leica Microsystems Inc.]. With the optical fractionator probe, a digital sampling grid of 1500 × 1000 μm was laid over the entire cortical region of interest. Cells were counted with a 15 × 15-μm counting frame within each counting grid rectangle. The distance from the top of the section to the unbiased virtual counting zone was fixed at 4 μm (guard zone), and the height of the unbiased virtual counting zone (optical dissector) was set at 10 μm. Section thickness was measured at every sampling site. NeuN-positive neurons were counted in every 12th section. The inclusion criteria were a distinct and complete NeuN-positive nucleus in which the maximal nuclear diameter was in sharp focus within the optical dissector. These parameters were established to allow for coefficient of error values of NeuN cell counts to be <0.1. This counting scheme resulted on average in counting 220 neurons in 315 optical dissectors per animal.

Golgi-Cox staining

Right hemisphere frontal blocks of fresh tissue from the 0 week (95 to 96 days of gestation) and 4 week recovery animals were processed for Golgi-Cox impregnation with the FD Rapid Golgi Stain Kit (FD Neurotechnologies Inc.). Each block was serially sectioned (200 μm thick) in the coronal plane with a vibrating microtome (VT1200S; Leica Microsystems Inc.). The sections were mounted onto coverslips, processed for Golgi visualization, dehydrated in a graded alcohol series, and coverslipped. A region-matched section at the level of the frontal periventricular white matter was chosen from each case for further analysis.

Pyramidal neuron sampling criteria and dendritic reconstruction

Region-matched Golgi-stained tissue sections were used to assess dendritic morphology of cortical pyramidal neurons with Neurolucida software (MicroBrightField Inc.). All the tissue sections were coded, and pyramidal neuron selection and tracing was performed by an individual blinded to the study groups (E.M.). Details of the sampling work-flow used for unbiased and blinded selection of comparable populations of cortical pyramidal neurons are shown in fig. S4. For each section, a region of interest for the entire dorsal cerebral cortex was outlined under a 5× objective. Next, under 20× objective, all cortical fields were viewed with the meander scan function, and all pyramidal neurons were selected by placing a digital marker when they met the following criteria that have been applied in similar morphometric studies (56, 57): (i) triangular-shaped soma and apical dendrite perpendicular to pial surface; (ii) complete impregnation of the cell with Golgi-stained material that permitted visualization of the entire arbor of dendrites and spines; (iii) neuronal soma and processes not obscured by other neurons, glia, or vasculature; and (iv) neurons exhibiting largely

complete basilar dendritic tree with few truncated or cut processes. No distinction was made between subtypes of pyramidal neurons.

With this initial selection procedure, more than 500 cortical pyramidal neurons were selected per section per case (fig. S4B). Next, every 15th of these preselected pyramidal neurons was used for dendritic reconstruction (fig. S4C). Thus, this sampling procedure provided randomized and unbiased selection of pyramidal neurons throughout the cerebral cortex, which was independent of cortical gyrus or layer. Under a 63 $\times$ air objective (NA = 0.70; Leica Microsystems Inc.), the outline of the cell soma and the entire apical and basilar dendritic tree structure for every 15th preselected pyramidal neuron was traced in the x , y , and z coordinates. The outline of the soma was traced at its widest point in the 2D plane to provide an estimate of its cross-sectional area. Dendritic processes were not followed into adjacent sections, and the dendritic diameter was not examined. Broken tips and unclear terminations were identified as incomplete endings. In the 0 week (95 to 96 days of gestation) group, a total of 48 cortical pyramidal neurons were reconstructed (animal 1, $n = 10$; animal 2, $n = 11$; animal 3, $n = 11$; animal 4, $n = 16$). In the 4 week recovery animals, a total of 100 cortical pyramidal neurons per group (control: animal 1, $n = 27$; animal 2, $n = 33$; animal 3, $n = 21$; animal 4, $n = 19$; ischemia: animal 1, $n = 26$; animal 2, $n = 25$; animal 3, $n = 21$; animal 4, $n = 28$) were reconstructed. There was a similar distribution of pyramidal neuron soma size between the 0 week, 4 week control, and 4 week ischemia group (fig. S3A), providing support for the validity of our sampling scheme.

Dependent dendritic measures

A morphometric analysis of total dendritic length (summed lengths of all basal dendritic branches per cell) and dendritic complexity, including numbers of branches, branch points (nodes), and terminal tips (endings), for basal dendrites of all reconstructed neurons was performed with Neuroexplorer software (MicroBrightField Inc.). Branch order analysis for all these parameters was also performed according to a centrifugal nomenclature (56, 58), where dendritic branches arising from the soma are considered first-order segments until they branch into second-order segments, which branch into third-order segments, etc. Apical dendrites were excluded from analysis because of their high rates of truncation after tissue sectioning. To further assess dendritic complexity, we performed Sholl analysis (59) on all reconstructed neurons to calculate the number of intersections of dendrites per each Sholl ring (5- μ m interval concentric spheres centered on the soma).

Secondary classification of pyramidal neuron location in the cerebral cortex

Functionally, the layers of the neocortex can be divided into supragranular (laminae II to III) and infragranular (laminae V to VI) layers. To assess for supragranular/infragranular layer-specific effects of ischemia, we performed secondary analysis of dendritic complexity based on neuronal location in the supragranular or infragranular cortical layers in the 4 week groups (fig. S4, D to F). A brain section immediately adjacent to the Golgi-stained section from each case was stained with Nissl to define the cortical location. A low-magnification (5 $\times$ objective) 2D montage of each Nissl-stained section was created with Neurolucida software and a motorized xy stage mounted on an optical microscope (fig. S4D). Next, each Nissl montage was overlaid and aligned with the appropriate digital cortical region of interest initially created for sampling of pyramidal neurons (see "Pyramidal neuron sampling criteria and dendritic reconstruction") (fig. S4E). During this procedure, the previously acquired pyramidal neuron tracings were masked from view. On the basis of the Nissl montage, a line was drawn through layer IV (line tool in Neurolucida software), thus dividing the cortical digital region of interest into upper layers I to III and lower layers V to VI (fig. S4F). Finally, the tracings of the pyramidal neurons were unmasked, and the cells were classified into supragranular or infragranular locations for secondary analysis of

dependent dendritic measures with existing data sets. There was a near-equal distribution of pyramidal neurons in the supragranular or infragranular locations (supragranular: control, $n = 49$ cells; ischemia, $n = 52$ cells; infragranular: control, $n = 51$ cells; ischemia, $n = 48$ cells), providing further support for the validity of our sampling scheme.

Analysis of dendritic spine density

With Neurolucida software, the density of the dendritic spines was assessed on the same population of cortical pyramidal neurons sampled for dendritic morphology. All the tissue sections were coded, and spine counts were performed by an individual blinded to the study groups (E.M.). With light microscopy (100 $\times$ oil, NA = 1.30; Leica Microsystems Inc.), spine density was determined by counting all visible spines on all terminal second-order branches of basal dendrites that were at least 20 μm long. About 25% of neurons in both groups did not have dendrites that met these criteria. Other neuronal exclusion criteria included dendrites that were incompletely filled or that were obscured by other processes. No distinction was made between subtypes of spines. In the 4 week recovery animals, spine densities were assessed on a total of 72 neurons in the control group (animal 1, $n = 18$; animal 2, $n = 24$; animal 3, $n = 17$; animal 4, $n = 13$) and 73 neurons in the ischemia group (animal 1, $n = 19$; animal 2, $n = 20$; animal 3, $n = 15$; animal 4, $n = 19$). Spine data were calculated on a per-neuron basis by averaging the spine densities from all assessed dendrites for each neuron. Data are presented as number of spines per micrometer.

Quantification of the orientation distributions of neuronal processes of Golgi-impregnated cortical pyramidal neurons

The 3D orientation distributions of basal dendritic processes of cortical pyramidal neurons were characterized for the same population of pyramidal neuron tracings collected for dendritic morphology. First, Neurolucida was used to generate an ASCII file in which each basal dendrite was encoded as a list of 3D coordinates. In the Neurolucida format, the sets of coordinates defining a dendritic segment are not uniformly spaced. However, characterization of the dendritic arbor orientation distribution requires the orientations of each consecutive 10- μm segment of each dendrite to be determined (24). Thus, the lists of coordinates generated by Neurolucida were interpolated in terms of uniform 1- μm steps with scripts written in Matlab. The orientation of each 10- μm segment was then determined by computing the 3D orthogonal distance regression line from the interpolated coordinates (24). The fiber orientation tensor of neuronal processes was measured from this distribution, and the anisotropy of the neuronal processes (termed FAn) was calculated for the basal arbor of each neuron, as previously described (24, 60).

Statistical analysis

All data analysis was performed with Prism 4 statistical software (GraphPad Software Inc.). Data are expressed as means $\pm$ 1 SEM. Analyses of dendritic morphology parameters and Sholl analyses of ring intersections were performed for all reconstructed cells. Comparisons of group means for cell counts, dendritic morphology parameters, spine densities, and MRI-defined volume and diffusion data between age-matched control and ischemia animals were performed with two-tailed t tests. Sholl (radius $\times$ treatment group) and branch order (branch order $\times$ treatment group) analyses of dendritic morphology parameters between age-matched control and ischemia animals were performed by two-way ANOVA followed by Bonferroni post tests. Changes in MRI-defined volume and diffusion data, dendritic morphology parameters, and spine densities over gestation were performed with one-way ANOVA. A value of $P < 0.05$ was considered statistically significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. High-field MRI studies demonstrate progressive disturbances in cortical growth and dysmaturation of cortical microstructure. Coronal MRI images of one hemisphere at the level of the frontal periventricular white matter are presented at 4 weeks. (A to C) Representative cortical segmentation (A) (light gray shading) used to analyze cortical volume, (B) FA, and (C) ADC. Orientation marker indicates superior (S), inferior (I), medial (M), and lateral (L). (D to F) Changes in (D) cortical volume, (E) FA, and (F) ADC at 1, 2, and 4 weeks of recovery in control (Con; black bars) and ischemia (HI; white bars) groups: 1 week (control, $n = 9$; ischemia, $n = 7$); 2 weeks (control, $n = 5$; ischemia, $n = 6$); 4 weeks (control, $n = 6$; ischemia, $n = 5$). Data are shown as 25th, 50th, and 75th percentiles, and minimum and maximum data points. * $P < 0.05$, ** $P < 0.01$ versus age-matched controls.

Fig. 2.

Cerebral ischemia did not cause a loss of cortical neurons. (**A to C**) Representative NeuN-stained brain tissue showing (**A**) delineation of cortical boundaries (dotted line), (**B**) laminar distribution of neurons, and (**C**) nuclear labeling (white arrows). Scale bar, 5 μm . (**D** and **E**) Unbiased stereological estimates of neuron number (**D**) and neuron density in the cerebral cortex (**E**) of control (Con; black bars) and ischemia (HI; white bars) groups: 1 week (control, $n = 6$; ischemia, $n = 6$); 2 weeks (control, $n = 5$; ischemia, $n = 6$). Data are means $\pm$ SEM.

Fig. 3.

Pyramidal neuron complexity increases with gestation in the fetal cerebral cortex. (**A** and **B**) Golgi-stained pyramidal neurons in the cerebral cortex at (**A**) 0.65 gestation (0 weeks) and (**B**) 4 weeks later. Scale bars, 20 μ m. (**C** to **E**) Total number of basal dendritic branches (**C**), total basal dendritic length (**D**), and total number of nodes in control animals (**E**) at 0 weeks (black bars) and 4 weeks (white bars). (**F**) Sholl analysis of the number of basal dendritic intersections at 0 weeks (closed circles) and 4 weeks (open circles). (**G** to **I**) Branch order analysis of (**G**) total number of basal dendritic branches, (**H**) total basal dendritic length, and (**I**) total number of nodes at 0 weeks (black bars) and 4 weeks (white bars). Analyses were performed independent of cortical location: 0 weeks, $n = 48$ cells; 4 weeks, $n = 100$ cells. Data are means $\pm$ SEM. * $P < 0.05$, ** $P < 0.001$, *** $P < 0.0001$.

Fig. 4.

Abnormal development of basal dendritic arborization of cortical pyramidal neurons was seen in the cerebral cortex at 4 weeks after ischemia. **(A)** Example of Golgi-stained pyramidal neurons in the control cortex. Scale bar, 20 μm. **(B)** Example of computer-assisted reconstructions of representative neurons in the control (Con) and ischemia (HI) groups. **(C to E)** Total number of basal dendritic branches (C), total basal dendritic length (D), and total number of nodes (E) in control (black bars) and ischemia (white bars) groups. **(F)** Sholl analysis of the number of basal dendritic intersections in control (closed circles) and ischemia (open circles) groups. **(G to I)** Branch order analysis of total number of basal dendritic branches (G), total basal dendritic length (H), and total number of nodes (I) in control (black bars) and ischemia (white bars) groups. Analyses were performed independent of cortical location. $n = 100$ cells per group. Data are means $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Fig. 5.

Abnormal spine density on cortical pyramidal neurons was noted at 4 weeks after ischemia. For the same population of pyramidal neurons that were sampled for dendritic morphology, spine density was quantified on second-order terminal dendritic branches, which were commonly identified for both groups of neurons. (A to C) Examples of second-order terminal branches (arrows) on (A) Neurolucida tracing (white lines) and (B and C) the corresponding Golgi-impregnated neuron. In (A), the yellow, white, pink, green, and blue lines represent first-, second-, third-, fourth-, and fifth-order branches, respectively. (D) Dendritic spines (arrowheads) visualized on a Golgi-impregnated neuron. Scale bars, 10 μm (B), 5 μm (C), and 2 μm (D). (E) Spine density in control (Con; black bars) and ischemia (HI; white bars) groups at 4 weeks of recovery. Analyses were performed independent of cortical location. Control, $n = 72$ cells; ischemia, $n = 73$ cells. Data are means $\pm$ SEM. $*P < 0.05$.

Fig. 6.

Anisotropy of the basal dendrites of cortical pyramidal neurons in the cerebral cortex was increased at 4 weeks after ischemia. (**A to D**) NeuroLucida (A and A') and Matlab 3D reconstructions (B and B') of a Golgi-impregnated cortical neuron were used to generate a set of orthogonal distance regression line segments (C and C') of the basal dendrites, from which (D) the anisotropy of the dendritic processes (FAn) was calculated. Images in A', B', and C' are rotated 90° relative to the corresponding images in A, B, and C. (D) FAn in control (Con; black bars) and ischemia (HI; white bars) groups at 4 weeks of recovery. $n = 100$ cells per group. Data are means $\pm$ SEM. $*P < 0.01$.