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14. ABSTRACT The proposed studies will provide cellular/molecular insights into caspase-induced non-apoptotic neuronal degeneration and will establish a highly translational treatment aimed at reducing neurotoxicity due to neonatal cardiac surgery and improving neurological outcome in children with CHD. The proposed study is of broad relevance for not only for CHD but other populations who need to undergo major surgery early in life.					
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TABLE OF CONTENTS

	<u>Page</u>
1. Introduction	1
2. Keywords	2
3. Accomplishments	3
4. Impact	12
5. Changes/Problems	13
6. Products	14
7. Participants & Other Collaborating Organizations	15
8. Special Reporting Requirements	18
9. Appendices	19

Introduction: Significant neurological impairment is emerging as one the most important challenges for survivors of complex congenital heart disease (CHD) repair. Prolonged anesthesia for CHD surgery early in life can damage the developing brain. Cardiopulmonary bypass (CPB) causes systemic inflammation: in addition both deep hypothermic circulatory arrest (HCA) and low flow cerebral perfusion cause relative or complete cerebral ischemia under hypothermia and expose neonatal brains to reperfusion/reoxygenation. Prolonged anesthesia and CPB are indispensable for the care of children with complex CHD: however the cellular and molecular mechanisms whereby anesthesia and cardiac surgery impact cortical development are poorly understood, thereby inhibiting development of effective treatment to minimize surgery-induced neurotoxicity.

Various operative factors such as anesthetic agents and inflammation activate caspases in the developing brain. Although caspase activation has been used widely as the standard measure for cell apoptosis, it does not always lead to cell death but rather alters cellular status at the levels of epigenetic marking on the cellular genome, organelle dynamics and cytoskeletal rearrangement. Indeed there has been little evidence for a reduction of cell numbers associated with neonatal anesthesia neurotoxicity. Consistently our studies have shown no reduction of cortical neurons after activation of caspases. We have also observed increases of markers for caspase-mediated non-apoptotic neurite degeneration in anesthesia-exposed cortices. The most common neurologic deficit seen in children after CHD repair is impairment of fine and gross motor skill development. Our data show that non-apoptotic caspase activation in the neonatal mouse brain impairs fine and gross motor skill learning. Consistent with these findings, we have found that extensive caspase activation after CPB did not change the number of neurons but did alter cortical microstructures. These results have led to our primary hypothesis that: **extensive caspase activation due to the global impact of cardiac surgery leads to non-apoptotic neuronal degeneration thereby contributing to neurological deficits in CHD.** Given that caspase is a ubiquitous housekeeping enzyme during development, long-term caspase inhibition potentially has undesirable side effects. Our proposed studies therefore will test a potent pan-caspase inhibitor, Q-VD-OPh, in a limited time-frame that specifically targets caspase activation. Notably inhibition of caspase in this crucial period improves impairments of fine and gross motor skill learning in mice. **To test our central hypothesis and design new neuroprotective treatment in children with CHD,** the proposed studies will use a cross-species approach in our unique piglet model as well as a well-established mouse model.

Aim 1: Determine the effects of surgery on caspase-induced non-apoptotic neuronal degeneration by prolonged anesthesia. Obvious difficulties prohibit distinguishing anesthesia neurotoxicity from the overall impact of surgery in children. We hypothesize that systemic inflammation and reoxygenation resulting from CPB/DHCA exacerbate caspase-induced non-apoptotic neuronal degeneration due to prolonged anesthesia. As observed in mice, our study in piglets shows extensive caspase activation after anesthesia. In the piglet model we will test whether CPB exacerbates the effects of anesthesia at the cellular level. Advanced diffusion MRI will estimate the complexity of neuronal dendrites/axons and determine structural cortical maturation. Finally, we will determine differences in long-term epigenetic changes between anesthesia and surgery.

Aim 2: Determine the effects of brief caspase inhibition on neuronal degeneration and cortical dysmaturation after cardiac surgery. We will test the hypothesis that brief inhibition of caspase activation is a potential treatment to limit the adverse impact of neonatal cardiac surgery. Q-VD-OPh will be introduced in the piglet CPB model to determine the effects on caspase-induced neuronal degeneration and cortical dysmaturation. The same methodologies in Aim 1 will be applied to define cellular and structural changes. Finally we will assess clinically-relevant physiological biomarkers to define the possible positive/negative effects of brief pan-caspase inhibition on multi-organ function after surgery.

Aim 3: Determine the contribution of caspase-induced non-apoptotic neuronal degeneration to fine and gross motor deficits. Leveraging sophisticated genetic tools and behavioral assays in the mouse model, we will examine poorly understood non-apoptotic caspase functions in developmental neurotoxicity. We will use anesthetics to induce extensive caspase activation in the neonatal mouse cortex. We will first determine cellular/molecular events in cortical neurons. Long-term epigenetic effects of non-apoptotic caspases will also be determined. Given that fine/gross motor deficits are commonly seen in children after complex CHD surgery as well as in our mouse model of anesthesia, the effects on neuronal plasticity will be analyzed in the primary motor cortex during motor skill learning. The pharmacological inhibition of caspases will be performed to determine the contribution on behavioral deficits.

The proposed studies will provide cellular/molecular insights into caspase-induced non-apoptotic neuronal degeneration and will establish a highly translational treatment aimed at reducing neurotoxicity due to neonatal cardiac surgery and improving neurological outcome in children with CHD.

KEYWORDS:

congenital heart disease

neurologic injury

neuroprotection

surgery

anesthesia

neurotoxicity

caspase

ACCOMPLISHMENTS:

Major goals of the project:

The major goals of the project are to **provide cellular/molecular insights into caspase-induced non-apoptotic neuronal degeneration and establish a highly translational treatment aimed at reducing neurotoxicity due to neonatal cardiac surgery and improving neurological outcome in children with CHD.** The proposed specific goals and major tasks with timeline are listed below as stated in the approved SOW.

Specific Goal 1: Determine the effects of surgery on caspase-induced non-apoptotic neuronal degeneration by prolonged anesthesia

Major Task 1: Define effects of cardiac surgery on caspase activation and neural degeneration due to prolonged anesthesia. (Proposed timeline: 1-36 months)

Major Task 2: Define effects of anesthesia and cardiac surgery on structural cortical maturation. (Proposed timeline: 1-36 months)

Major Task 3: Define effects of anesthesia and cardiac surgery on epigenetic changes. (Proposed timeline: 1-36 months)

Specific Goal 2: Determine the effects of brief caspase inhibition on neuronal degeneration and cortical dysmaturation after cardiac surgery

Major Task 4: Define the effects of brief caspase inhibition on neuronal degeneration after neonatal cardiac surgery. (Proposed timeline: 23-36 months)

Major Task 5: Define the effects of brief caspase inhibition on cortical dysmaturation after neonatal cardiac surgery. (Proposed timeline: 23-36 months)

Major Task 6: Determine the possible positive/negative effects of brief pan-caspase inhibition on multi-organ function after neonatal cardiac surgery. (Proposed timeline: 23-36 months)

Specific Goal 3: Determine the contribution of caspase-induced non-apoptotic neuronal degeneration to fine and gross motor deficits.

Major Task 7: Determine the contribution of caspase-mediated non-apoptotic cellular degeneration caused by anesthesia. (Proposed timeline: 1-12 months)

Major Task 8: Determine caspase-mediated long-term effects on neuronal plasticity. (Proposed timeline: 13-24 months)

Major Task 9: Determine the effect of extensive caspase activation and the inhibition on neuronal plasticity during motor skill learning. (Proposed timeline: 25-36 months)

Accomplishment under these goals:

Our goal in Year 2 remained to test our principal hypothesis, namely that: **extensive caspase activation due to the global impact of cardiac surgery leads to non-apoptotic neuronal degeneration thereby contributing to neurological deficits in CHD.** As proposed and discussed below, our activities during the Year 2 period focused on Specific Goal 1 and 3 including major task 1, 2, 3, and 8.

Major activities:

To accomplish the goal, we utilized a neonatal piglet model **to determine the effects of surgery on caspase-induced non-apoptotic neuronal degeneration by prolonged anesthesia (Specific Goal 1).** As observed in mice, our study in piglets showed extensive caspase activation after anesthesia. The hypothesis to be tested in Goal 1 was “*systemic inflammation and reoxygenation resulting from CPB and HCA exacerbate caspase-induced non-apoptotic neuronal degeneration due to prolonged anesthesia*”. In year 1 we determined that the effects of cardiac surgery on caspase-induced non-apoptotic degeneration of cortical neurons. Caspase activation has been increasingly associated with pathophysiological outcomes that impact cellular morphology and function, such as cell proliferation and differentiation¹. Thus, our year 2 studies further assessed the effects of cardiac surgery on caspase-induced non-apoptotic functions in proliferation and maturation of neural stem/progenitor cells (NSPCs) at cellular (Major Task 1), structural (Major Task 2), molecular (Major Task 3) levels using our well-established piglet CPB model²⁻⁴ (see details in our accomplishment under specific Goal 1). In many institutions in the US that perform complex neonatal cardiac surgery, anesthesia was maintained using the synthetic opioid fentanyl in combination with inhaled Isoflurane. We used a similar anesthesia protocol which

we had previously utilized in a porcine CPB model.^{2, 3, 5} A hematocrit level of 30% was maintained and the pH-stat strategy was performed as is our standard technique^{6, 7} for the approved Goal 1 studies.

One of the significant aspects of our project is we examine poorly understood non-apoptotic functions of caspases. Leveraging sophisticated genetic tools, our Year 2 studies continued to use the mouse model of neonatal anesthesia **to determine the contribution of caspase-induced non-apoptotic neuronal degeneration to fine and gross motor deficits (Specific Goal 3)**. Recent clinical studies identified a number of de novo mutations in the patients with CHD^{8, 9}. At least 50% of these genes are associated with primary cilia. Similarly, a large scale screen in the mouse model of CHD found the enrichment of primary cilia-related genes¹⁰. It has been well recognized that the primary (immotile) cilium acts as a sensory organelle that alters signal transductions thereby regulates cellular physiology. Indeed, our previous studies supported by this grant found that primary cilia play an integral role in mitigating adverse impacts of environmental stressors such as anesthetics on perinatal brain development¹¹. In order to establish a highly translational treatment aimed at reducing neurotoxicity and improving neurological outcome in children with CHD, our Year 2 studies further assessed the caspase-mediated long-term effects on neuronal plasticity (Major Task 8) using *Emx1cre::lft88ff* mice, in which primary cilia are lost specifically in cortical excitatory neurons (see details in our accomplishment under Specific Goal 2).

Accomplishment under Specific Goal 1: The subventricular zone (SVZ) is a key source of neurons generated from NSPCs. Furthermore young neurons in the SVZ migrate postnatally along the lateral ventricles to populate cortical gray matter, contributing to its further cortical maturation¹⁴. In the previous study period, therefore, we examined the impact of CPB on neurogenic activity of the SVZ as well as growth and maturation of the frontal lobes. We first assessed the NSPC pool within the whole SVZ 3 days post-surgery. Although there were no differences in the density of Sox2⁺ NSPCs, detailed examination of the SVZ revealed a trend toward reduced NSPC proliferative capacity in the HCA group compared to control (Figure 1A-D). On the other hand, differences between CPB and HCA were not observed (Figure 1D,E). Neurogenic activity was further assessed by quantifying the average length of GFAP⁺ processes spanning tier 1 and expressed within radial-glia like cells (Figure 1F-I). Both CPB and HCA groups displayed reduced GFAP⁺ process length. When we analyzed neuroblasts within the SVZ, decreased density of DCX⁺ neuroblasts was observed in the peripheral region (tiers 2-3) in both CPB and HCA groups compared to control (Figure 1K-O). A previous study in the human infant brain identified a chain-like structure of actively migrating DCX⁺ neuroblasts with adherent junctions¹⁴. These chains principally located in the peripheral-SVZ (i.e., Tier 3) have been associated with a tangential migration oriented toward the developing frontal cortex. To determine whether CPB-induced brain insults also alter neuroblast migratory chains, we analyzed DCX⁺ clusters - coronal view of the migratory stream - within the SVZ (Figure 1P-R). Our analysis revealed that the overall occupancy of the clusters was significantly reduced in both CPB and HCA groups (Figure 1T). Together our results indicate that systemic inflammation and reoxygenation resulting from CPB and HCA alters neurogenesis in the piglet SVZ and disrupts neuroblast migrating chains toward the frontal lobe (Task 1).

To determine the long-term effects on SVZ neurogenic activity and the neuroblast population, we next examined the SVZ 4 weeks postoperatively. NSPC proliferation in the SVZ was significantly decreased for both CPB and HCA groups (Figure 2A-D). Notably changes in NSPC proliferation from day 3 to week 4 significantly differed according to the type of CPB-induced insult (Figure 2E), indicating the critical role of systemic inflammation on the prolonged decline of proliferative ability after pediatric cardiac surgery. These observations were coupled to a marked reduction of GFAP⁺ process length after both CPB and HCA insults (Figure 2F-J). When we assessed the long-term effect of cardiac

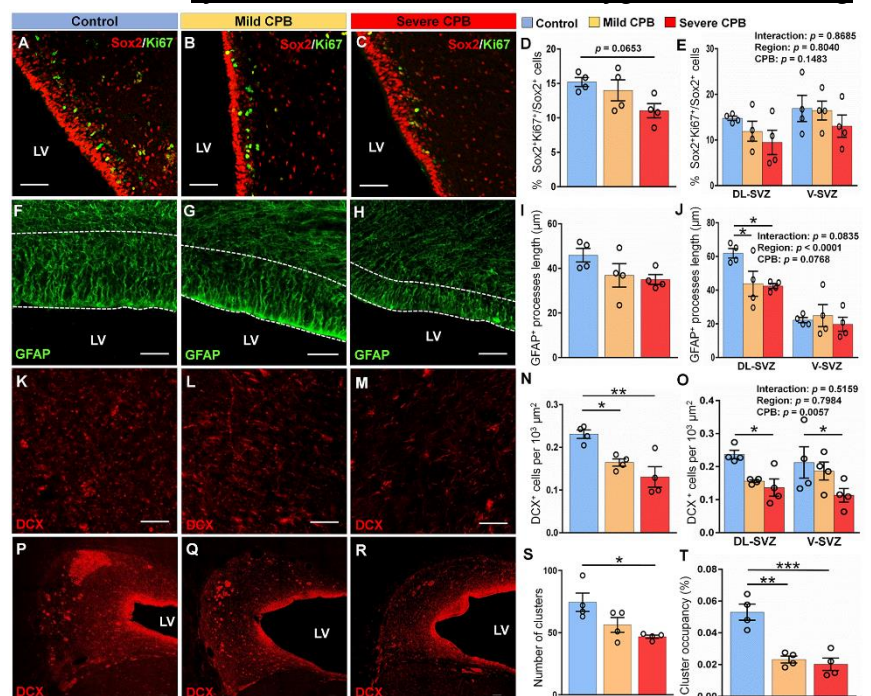


Figure 1

surgery on SVZ neuroblasts, reductions in cell density were displayed in both CPB and HCA groups (Figure 2K-N). Consistent with our findings for NSPCs, there was no recovery in neuroblast density over time (Figure 2O), indicating prolonged inhibition of SVZ neurogenesis due to CPB. Finally, a marked reduction of cluster number and a trend in cluster occupancy was shown 4 weeks after HCA (Figure. 2P-T), demonstrating that systemic inflammation and reoxygenation resulting from CPB and HCA cause prolonged alterations in SVZ neurogenic properties and neuroblast migrating chains (Task 1).

Based on our previous finding that SVZ-derived cells populate the frontal cortex in neonatal piglets during normal development¹⁵, we further assessed structural maturation of the developing cortex using a clinically-relevant neuroimaging approach 4 weeks after surgery. When the volumes of the entire frontal lobe and frontal cortex were quantified, however, both measurements in the CPB and HCA groups were decreased compared to control (Figure 3A-E). Consistent with previous findings in gyrencephalic brain development^{23,24}, these observations were paired with a significant reduction in gyrification (Figure 3F-I), demonstrating structural brain immaturity due to systemic inflammation and reoxygenation resulting from CPB and HCA (Task 2). Moreover, when we further compared diffusion MRI metrics between the three experimental groups, we found that animals exposed to HCA insult showed a significant increase in fractional anisotropy (FA)(Figure 3J). FA values in the HCA group (i.e. Severe CPB) were higher compared to CPB FA values. As cortical microstructural maturation is associated with FA decrease²⁶, these observations indicate that CPB-induced systemic inflammation can cause aberrations in macrostructural cortical development, whereas HCA-induced injury during CPB induces microstructural changes (Task 2). To further understand the relationship between immature cortical development and depletion of neuroblasts in the SVZ, mature neuronal populations were analyzed in frontal cortices (Figure 3K-M). Despite no statistical differences in the average density of neurons between the three groups (Figure 3N,O), together with decreased cortical volume in the CPB and HCA groups (Figure 3E) the results indicate an overall reduction in the total number of mature neurons in the frontal cortex after CPB. Together, *our porcine model has given us insights into the cellular alterations that explain cortical dysmaturation due to systemic inflammation and reoxygenation resulting from CPB and HCA (see more details in Appendices).*

The transcriptome analysis of single cells allows for molecular profiling of individual cells within the SVZ responsible for cortical maturation. Several steps are required before performing single-nuclei RNA sequencing: 1) successful nuclei isolation, 2) single-nuclei capture on the Chromium Controller, and 3) generation of barcoded cDNA followed by library preparation. Our studies during Year 2 have confirmed that these experiments are feasible on the piglet SVZ (Task 3). Cellular composition of the piglet SVZ was visualized using t-distributed stochastic

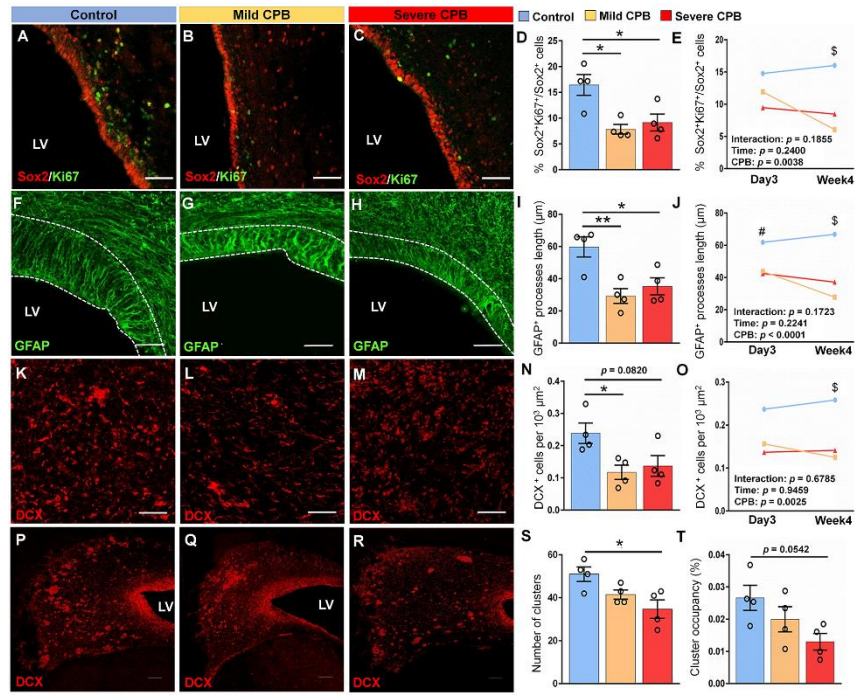


Figure 2

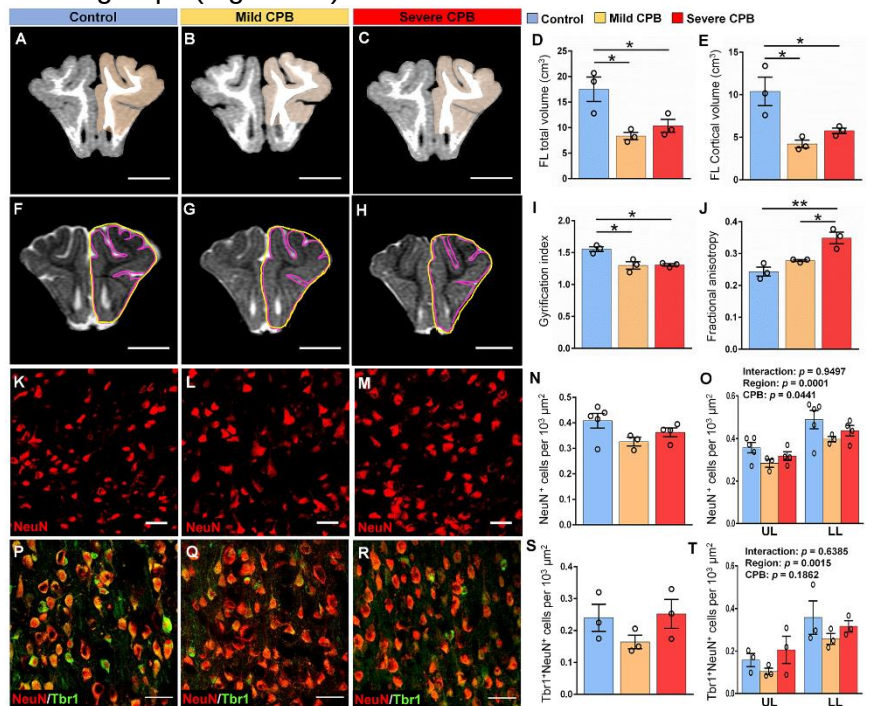


Figure 3

neighbor embedding (t-SNE). Individual single-cell transcriptomes were automatically coded according to cluster identity. Notable our data has uncovered cell populations with molecular profiles of immature excitatory neurons within the piglet postnatal SVZ that are not found in rodents (Figure 4A box). We have confirmed the immature excitatory neurons in the outer-SVZ of postnatal piglets *in vivo* (Figure 4B). This unique population is recently recognized in the human amygdala between 4–15 years of age¹². Cell clusters will be compared, and differentially expressed genes between the experimental groups will be assessed for each cell subtype to determine molecular events underlying CPB and anesthesia-induced alterations in neurogenic capacities of the SVZ NSPC population.

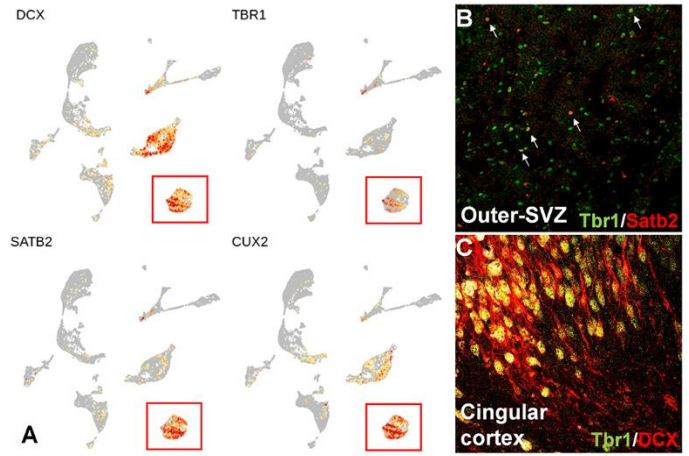


Figure 4

Accomplishment under Specific

Goal 3: As proposed, the mouse model of neonatal anesthesia have been used for Specific Goal 3. Pups were weighed and intraperitoneally injected twice with 2.5 g/kg of EtOH (or PBS control) at 2-hour intervals, or once with 20 mg/kg ketamine (or PBS control) at postnatal day (P) 7. Using *Emx1cre::lft88/f/f* mice, we examined markers of cytoskeletal

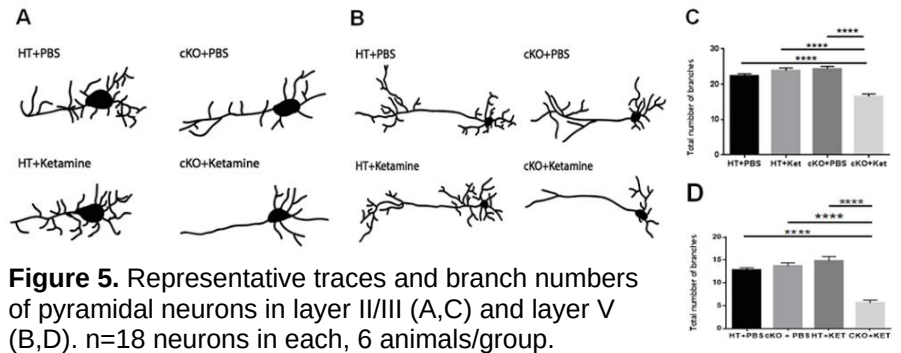


Figure 5. Representative traces and branch numbers of pyramidal neurons in layer II/III (A,C) and layer V (B,D). *n*=18 neurons in each, 6 animals/group.

degeneration in the medial prefrontal cortex as well as behavioral testing to assess their motor functions. When the expression of cleaved caspase-3 and cleaved tubulin was analyzed in layers II/III and V of the frontal cortex P8, we found that the homozygous (cKO) mutant mice, but not heterozygous (cHET), treated with Ketamine displayed a significant increase in both cleaved caspase-3 and cleaved tubulin expression in both layers of the cortex demonstrating that loss of primary cilia results in non-apoptotic caspase-mediated cytoskeletal degeneration following acute ketamine exposure (not shown). Our studies also found that ketamine exposed *lft88* cKO mice display decreased dendritic arborization at P30 following mild ketamine exposure (20mg)(Figure 5). The results indicated that enhanced caspase-3 activation due to anesthesia is associated with decreased long-term dendritic arborization in both layer II/III and V neurons (Task 8). The most common neurological deficits seen in infants with CHD after cardiac surgery are motor deficits^{13, 14}. Modifications of chromatin accessibility due to caspase-involved alterations in nucleosome architecture likely cause long-term epigenetic changes and alter neuronal plasticity.¹⁵ The formation and elimination of synaptic structures happen rapidly in cortical neurons during various sensorimotor learning experiences^{16, 17}. During this approved period, we assessed the effect of caspase activation on neuronal plasticity by *in vivo* imaging of spine dynamics in the primary motor cortex (M1) during motor learning (Figure 6A). Before imaging, an accelerated rotarod test was performed for prescreening problems in locomotor activity as these factors critically affect motor learning (Figure 6B). Our studies displayed that the alteration in dendritic spine adaptation and motor learning capabilities in mice lacking primary cilia following

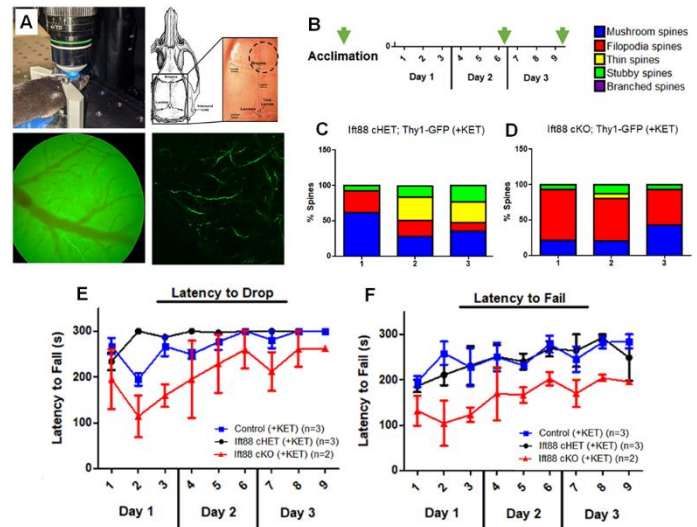


Figure 6. (A) Representation demonstrating the method used to visualize dendritic spines in a live mouse. (B) Schematic of the rotarod timeline. Dendritic spines were categorized in *lft88* cHETs (C) and cKOs (D) based on structure and identified as either mushroom (blue), filopodia (red), thin (yellow), stubby (green), or branched (purple). In the accelerated rotarod paradigm, cKO mice treated with ketamine demonstrate a trend towards impaired motor capability in both latency to drop (E) as well as latency to fail (F) compared to control and cHET mice treated with Ketamine.

ketamine treatment (Figure 6), indicating that extensive caspase activation due to neonatal anesthesia in primary cilia-deficient mice impairs neuronal plasticity during motor skill learning (Task 8).

Additional accomplishment: The proposed project addresses a following FY19 PRMRP Area of Encouragement: **Research both on the risk of neurologic injury and on enhanced neuroprotection before, during, and after surgery for CHD.** As proposed and approved, caspases are known to have phylogenetically conserved roles in the sculpting of neuronal dendritic arbors through cytoskeletal remodeling and have been demonstrated to contribute to synaptic plasticity¹⁸. In addition, caspase signaling has been linked to excitotoxic injuries in prior work¹⁸. Programmed cell death and neurodegeneration has been frequently reported in developmental brain injury scenarios such as anesthesia exposure¹⁹⁻²¹. Importantly, the neurotoxic potential of excessive non-apoptotic caspase signaling has not been well characterized in the developing brain. Given the capacity for caspase activation by commonly used agents such as volatile anesthetics and opioids, addressing the possibility of non-apoptotic, sub-lethal neurotoxicity is particularly pertinent. To disseminate the issue to academia and accelerate research on the risk of neurologic injury during surgery for CHD, our recent publication discussed key molecular characteristics and roles of the noncanonical caspase pathway and how its dysregulation may adversely affect brain development (Figure 7 and 8). We highlighted both genetic and environmental factors that regulate apoptotic and sublethal caspase responses and discussed potential interventions that target the noncanonical caspase pathway for developmental brain injuries (see more details in Appendices).

Figure 7. Overview of extrinsic and intrinsic apoptosis and caspase activation modifiers.

The flowchart presents a summary of the extrinsic and intrinsic arms of the apoptotic pathway, while highlighting known and hypothetical molecular modifiers of initiator and effector caspase activation. Extrinsic, death receptor-dependent signaling crosstalks with the mitochondrial arm through BCL-2 family proapoptotic proteins such as BID, which when translocated to the mitochondria (translocated BID/tBID)

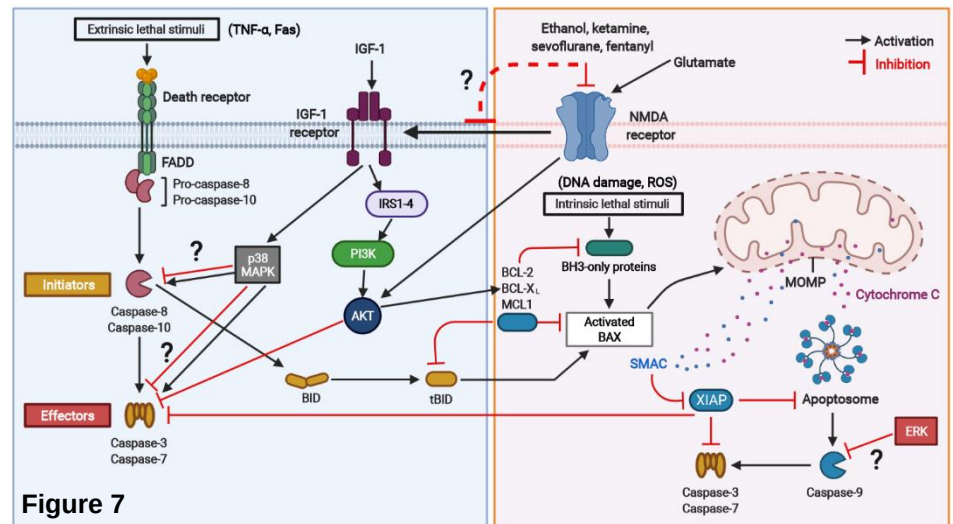


Figure 7

results in activation of the protein BAX, mitochondrial membrane permeabilization, and release of cytochrome C. Cytochrome C forms structures known as apoptosomes, which initiate caspase activation. Effector caspase activation is inhibited by X-linked inhibitor of apoptosis proteins (XIAPs), which are ubiquitin ligases. SMAC acts as a potentiator of apoptosis by inhibiting XIAP function. BCL-2 family antiapoptotic proteins BCL-XL and MCL1 suppress the intrinsic apoptotic cascade, acting to maintain homeostasis. Other signaling pathways such as PI3K/AKT, p38 MAPK, and ERK act as modulators of caspase signaling through their phosphorylation activity. Chemical agents with both neurophysiological and neurotoxic properties, such as glutamate and ketamine, function as agonists and antagonists of NMDA receptors, which interface with intracellular signaling regulators of caspase activation. Differential recruitment of these signaling pathways and their variable interaction with environmental stimuli may underlie functional differences between apoptotic and nonapoptotic caspase activation.

Figure 8. Caspase activation mode and corresponding outcomes in physiological and injury scenarios. The schematic offers a simplified overview of variable, caspase activation level-dependent neurotoxic outcomes (with caspase cleavage and/or phosphorylation levels being key modulators of activation). In pyramidal neurons, dendritic processes contact and receive synaptic inputs from other neurons and enable crosstalk with glial cells. Often, dendrites are covered in dendritic spines, postsynaptic sites of neurotransmission that are dynamically turned over and either stabilized or lost, processes that contribute to synaptic plasticity. These structures are supported and maintained by networks of filament proteins, including actin and tubulin. During normal physiological neuronal development and maturation, a low level of caspase pathway activation is required for dendritic branching and shifts in plasticity required during learning. However, upon exposure to environmental neurotoxins, such as anesthetics, enhanced activation of executioner caspases leads to the induction of

apoptosis via mitochondria (intrinsic pathway) and extensive cleaving of cytoskeletal proteins. A more complex scenario is increasingly being recognized, whereby a moderate level of caspase activity postexposure may lead to cytoskeletal remodeling, without full cell death. How this level of activation is achieved and modulated is unclear, but may involve variable availability of X-linked inhibitor of apoptosis proteins (XIAPs), genetic susceptibility combined with low-dose neurotoxin exposures, and altered neuronal–glial crosstalk.

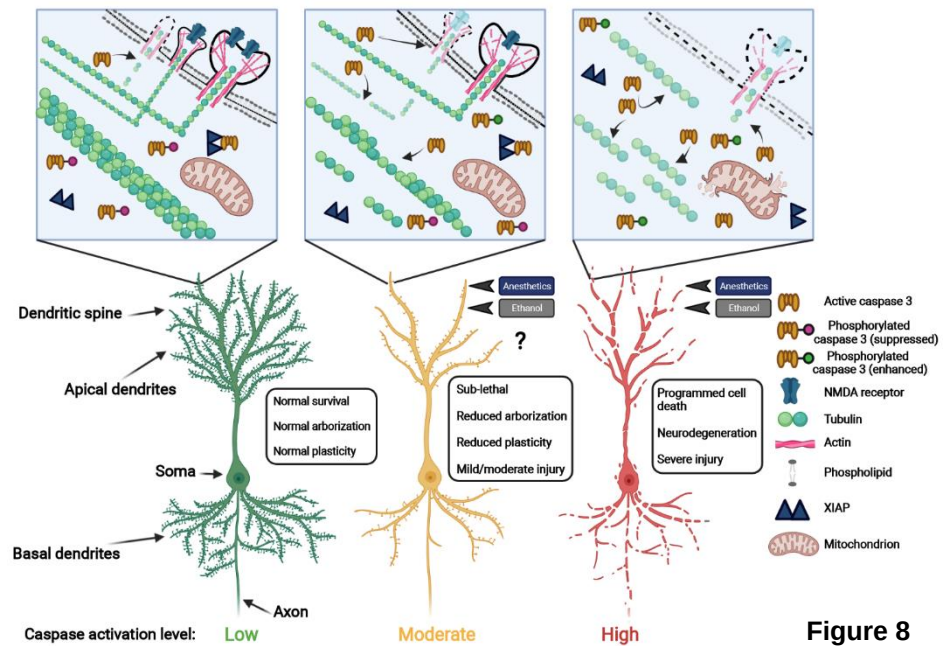


Figure 8

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Opportunities for training and professional development:

Nothing to Report

Dissemination to communities of interest:

Our four key audiences are: 1) Patients, patient families, and the public; 2) Cardiologists, cardiac surgeons, neuroscientists, and health care professionals working in the field of neuroprotection in the CHD population; 3) Academia; 4) Industry (who might be interested in possible therapeutic approach from this project). To ensure

that this work is disseminated, we have developed the following dissemination plan based on the four key audiences.

Patients, Patient Families, and the Public: To educate patients, their families, and the public we worked with our Public Relations and Marketing team (see the section of PRODUCT).

Health care professionals working in the field of neuroprotection in the CHD population: To educate physicians about our findings we presented and discussed results from this project at regional and national conferences during this reporting period (Details are shown in the section of PRODUCT).

Academia: The focus for academia is on publication of study results in a high-impact journal to support dissemination of this work. Our work from the Goal 1 studies was successfully published at Annals of Neurology highly respected journal in the field, in December 2021. In addition, two review articles were published at Trends in Neuroscience and Children for dissemination of our work. (Details are shown in the section of PRODUCT)

Industry: The focus was to identify industry partners with whom we will partner to support a pathway to regulatory approval and to potentially develop product therapeutically. For this aim, we worked with our Public Relations and Marketing team (see the section of PRODUCT).

Plan during the next reporting period to accomplish the goals:

Our goal in Year 3 is to continue testing our principal hypothesis, namely that: **extensive caspase activation due to the global impact of cardiac surgery leads to non-apoptotic neuronal degeneration thereby contributing to neurological deficits in CHD.**

To accomplish the goal, we will continue to utilize a neonatal piglet CPB model to **determine the effects of surgery on caspase-induced non-apoptotic neuronal degeneration by prolonged anesthesia (Specific Goal 1)**. As stated in the approved SOW, our Year 3 studies will continue to pursue the following major tasks: 1) Define effects of cardiac surgery on caspase activation and neural degeneration due to prolonged anesthesia (Major task 1); 2) Define effects of anesthesia and cardiac surgery on structural cortical maturation (Major Task 2); and 3) Define effects of anesthesia and cardiac surgery on long-term epigenetic changes (Major Task 3).

Major Task 1: We will obtain numbers of Caspase3+ neurons in upper/lower layers and Caspase3+ NSPCs in SVZ from analyzed cortical regions between tested groups including anesthesia group. The expression level of fractin and c-tubulin in layer II/III and V will also be determined. A stereological counting system will determine numbers of Iba1+ microglia cells. At 4 weeks, the number of neurons and the morphometric parameters of dendrites will also be compared. The analyses will determine effects of anesthesia and CPB on the dendritic complexity of cortical neurons and neuronal maturation delivered from the SVZ NSPCs.

Major Task 2: In addition to DTI induces (FA), we will obtain measures for NODDI parameters such as orientation dispersion index (ODI), intracellular volume fraction (ICVF), and isotropic volume fraction (ISOVF). By comparing between Control and Anesthesia, we will first determine the effect of anesthesia on microstructural changes. Then the effect of CPB on these outcomes will be determined using data generated in Year 1 and 2. Notably results during this reporting period indicated that systemic inflammation and reoxygenation resulting from CPB and HCA reduce NSPC proliferation and neurogenesis from the SVZ thereby impairing structural cortical maturation²² (See ACCOMPLISHMENT). In addition, logistic regression analysis will determine the relationship between morphological changes of cortical neurons and image biomarkers.

Major Task 3: Results from scRNA seqs during this reporting period indicated that these experiments are feasible on the piglet SVZ (See ACCOMPLISHMENT). During the next reporting period, we will rank the potential contribution of the gene expression changes based on the conservation of the defined loci in mRNA seqs between control and anesthesia and between anesthesia and anesthesia with CPB in cell each cluster.

In Year 3, we will also start testing our hypothesis in the approved Goal 2 studies that “brief inhibition of non-apoptotic caspase activation is a potential treatment to limit the adverse effect of cardiac surgery”. Specific goal for this study will be to **determine the effects of brief caspase inhibition on neuronal degeneration and cortical dysmaturation after cardiac surgery (Specific Goal 2)**. Q-VD-OPh will be introduced in the piglet model to determine dose-related effects on caspase-induced neuronal degeneration and cortical dysmaturation. Q-VD-OPh will be introduced at 30min before the onset of anesthesia. Because of its short half-life, Q-VD-OPh dosing will be repeated every 8hrs following initial administration during caspase activation. In our Year 1 studies

we have defined the time course of CPB-induced caspase activation. The effects of Q-VD-OPh will be compared with animals with CPB after administration of vehicle in same amount. The results will also be compared with naïve control animals used in the Goal 1 studies to define the effects of brief caspase inhibition on: 1) neuronal degeneration (Major Task 4); and 2) cortical dysmaturation (Major Task 5) and to determine the possible positive/negative effects of brief pan-caspase inhibition on multi-organ function (Major Task 6).

Major Task 4: At day 3, we will obtain numbers of: i) Caspase3+NeuN+; ii) Caspase6+NeuN+; and iii) Caspase7+NeuN+ neurons in layer II and V between tested groups. The expression level of fractin and c-tubulin in layer II and V will be determined. A stereological counting system will determine numbers of Iba1+ microglia cells. At 4 weeks after CPB, the number of neurons and morphometric parameters of dendrites (see Figure 6) will be compared. We will define dose-effects of Q-VD-OPh on: 1) acute caspase activation; 2) non-apoptotic neurite degeneration; and 3) long-term neuronal degeneration.

Major Task 5: We will obtain measures for: i) cortical volume; ii) FA; iii) MD; iv) ODI; v) ICVF; and vi) ISOVF in defined regions among the proposed and approved dose groups. Using two-way ANOVA with dose and region as the factors, we will define the dose-effects of Q-VD-OPh on altered cortical microstructures determined by dMRI-derived markers. The Logistic regression analysis will determine the relation between morphological changes of cortical neurons and image biomarkers.

Major Task 6: To determine the broad systemic effects of Q-VD-OPh we will analyze the pathophysiological biomarkers. We will use the blood analysis system (Epoc) to measure metabolites/electrolytes of arterial blood^{2, 5, 23}. The values will be used to determine alveolar-arterial gradient (AaDO₂) to analyze respiratory function. Additionally, biochemical tests and blood counting will be performed as we have previously in the model^{2, 5, 24}. Cerebral and somatic oxygenation status will be continuously monitored by Near-Infrared spectroscopy (NIRO-300)^{5, 25}. Finally, neurological and behavioral evaluations will be performed at 24hr intervals beginning on post-CPB day 1, as we have previously performed regularly^{2, 5, 23}. A score of 100 is assigned to each of 4 general components^{2, 3, 5}. Overall Performance Category assesses outcome in five categories^{2, 3, 5}. The score will be accessed in a blinded fashion^{2, 5, 23}. Finally open field testing will be conducted. Behaviors will be automatically tracked during each minute-long epoch and will be recorded using EthoVision XT platform (Noldus). Aforementioned biomarkers will be compared by two-way ANOVA with time and dose as factors to define dose-effect of Q-VD-OPh on overall physiological function. Longitudinal data analysis will also be applied using GEE strategy to incorporate nesting and multiple measurements at varying time points.

Leveraging sophisticated genetic tools, our Year 3 studies will continue to use the mouse model to **determine the contribution of caspase-induced non-apoptotic neuronal degeneration to fine and gross motor deficits (Specific Goal 3)**. In Year 2, we successfully accomplished Major Task 8 (see details in the section of ACCUMPLISHMENT). Thus, our studies during the next reporting period will focus on determining the effect of extensive caspase activation and the inhibition on neuronal plasticity during motor skill learning (Major Task 9). As previously described²⁶ Q-VD-OPh will be dissolved in 10% DMSO in PBS and introduced via subcutaneous injection at 30mins before the onset of anesthesia at 5 mg/kg per injection. As activation of caspase disappears by 24hrs post anesthesia in mice, we repeat the same volume once at 2.5hrs post anesthesia. The vehicle (10% DMSO in PBS) will be injected as control. The effects of Q-VD-OPh will also be tested in normal development. The primary motor cortex will be selected, as previous and our ongoing studies showed significant contributions of this region to learning using motor skills^{27, 28}. The plan is consistent with the approved SOW.

Major Task 9: In the accelerated rotarod test we will obtain the time (seconds) and terminal speed (rpm) before each mouse falls in 6 trials between tested groups including Q-VD-OPh treatment. The single-pellet reaching test will measure: 1) the success rate of obtaining food pellets in total trials (%); 2) the learning rate per day; and 3) the number of successful attempts per minute. Using *in vivo* imaging, spine formation and elimination (see Figure 6) will be determined in layer II and V neurons in the primary motor cortices. The relation between fine/gross motor function and neuronal plasticity will be assessed to define the effects of caspase activation and inhibition on neuronal plasticity in fine/gross motor skill learning.

IMPACT:

Impact on the development of the principal discipline of the project:

Anesthetics are an indispensable component of complex pediatric surgeries, for instance, corrective heart surgery in patients with CHD. Our study published during this reporting period is the first to reveal the impact of cardiac surgery on postnatal SVZ and cortical development in a porcine model which is anatomically and physiologically similar to humans. We demonstrated over a 4-week recovery period that there are significant alterations of the NSPC pool and neuroblast populations in the SVZ resulting in a depletion of a source of interneurons destined to populate and potentially fine-tune development of the postnatal frontal cortex. Furthermore, we noted decreased gyrencephaly and impairment of cortical expansion and maturation, which are pathological signatures observed commonly in survivors of neonatal cardiac surgery for complex CHD. Finally, our study highlights CPB-induced excitatory and inhibitory imbalance throughout the frontal cortex and the distinct role of CPB-induced ischemia-reperfusion injury in cortical dysmaturation. *Our findings published at Annals of Neurology provide new cellular insights into surgery-induced neonatal brain injury and guide us towards improving management of pediatric cardiac surgery to minimize neurological and behavioral consequences in the CHD population.* As proposed, our Year 3 studies will determine the effects of brief caspase inhibition on neuronal degeneration and cortical dysmaturation after cardiac surgery using the piglet model of CPB.

Significant progress has been made over the last decades in understanding the molecular mechanisms behind programmed cell death in the nervous system and its effects on cell morphology, physiology and organismal behavior. Detailed characterization of caspases, their substrates and the outcomes of their activity has informed the design of new classes of caspase inhibitors with demonstrated efficacy. A primary driving force behind this work was to understand canonical apoptotic signaling with the hope of either suppressing it in the context of neurodegenerative insults or promoting it during carcinogenesis. In parallel, the non-lethal mode of caspase activation and signaling has been gaining growing interest. Progress has been made in clarifying the important functions this signaling pathway plays in neuronal dendritic extensions and arborization, experience-induced plasticity and learning, as well as how it might be co-opted in injury scenarios. To disseminate the issue to academia and accelerate research on the risk of neurologic injury during surgery for CHD, *our article published at Trends in Neuroscience during this reporting period discussed key molecular characteristics and roles of the noncanonical caspase pathway and how its dysregulation may adversely affect brain development.* Our advances in super-resolution imaging together with established injury models are positioning research to dissect the conditions under which sub-lethal caspase activation takes precedence over caspase-driven apoptosis. These prospective studies will be especially important for deciphering the fine details behind developmental anesthesia neurotoxicity.

Impact on other disciplines:

As addressed a following FY19 PRMRP Area of Encouragement “Research both on the risk of neurologic injury and on enhanced neuroprotection before, during, and after surgery for CHD”, neurological deficits are a serious and common sequelae of CHD. While their underlying mechanisms have not been fully characterized, their manifestations are well-known and understood to persist through adulthood. Development of therapies to address or prevent these deficits are critical to attenuate future morbidity and improve quality of life including our ongoing DoD-approved project. In our second review article published at *Children* during this reporting period, we summarized the current status of neuroprotective therapy in CHD. Through an exploration of present research in the pre-operative, intra-operative, and post-operative phases of patient management, we described existing clinical and bench efforts as well as current endeavors underway within this research area.

Impact on technology transfer:

Nothing to report

Impact on society beyond science and technology:

Nothing to report

CHANGES/PROBLEMS:

Due to COVID, several key meetings were held virtually in Year 2 resulting in a reduced number of attendees. Although our works were presented at national and regional meetings, it is likely that dissemination to health care professionals working in the field of neuroprotection in the CHD population was still limited during this reporting period. Our goals during the next study period remain same and are to test our principal hypothesis, namely that: extensive caspase activation due to the global impact of cardiac surgery leads to non-apoptotic neuronal degeneration thereby contributing to neurological deficits in CHD. However, we will follow all guidelines in local government, Children's National, and Howard University and will decide our activities in Year 3 based on their guidance.

PRODUCTS

Publications, conference papers, and presentations:

Journal publications.

Authors: Dhari Z, Leonetti C, Lin S, Prince A, Howick J, Zurakowski D, Wang PC, Jonas RA, Ishibashi N.

Title: Impact of Cardiopulmonary Bypass on Neurogenesis and Cortical Maturation.

Journal: Ann Neurol. 2021 Dec;90(6):913-926. (PMID: 34590341)

Status: published

Acknowledgement of federal support: yes

Authors: Sarić N, Hashimoto-Torii K, Jevtovic-Todorovic V, Ishibashi N.

Title: Non-apoptotic caspases in neural development and in anesthesia-induced neurotoxicity.

Journal: Trends Neurosci. 2022 Apr 21:S0166-2236(22)00058-3. (PMID: 35491256)

Status: published

Acknowledgement of federal support: yes

Authors: Kobayashi K, Liu C, Jonas RA, Ishibashi N.

Title: The Current Status of Neuroprotection in Congenital Heart Disease.

Journal: Children 2021;8(12):1116. (PMID: 34943311)

Status: published

Acknowledgement of federal support: yes

Books or other non-periodical, one-time publications.

Nothing to report

Other publications, conference papers, and presentations.

Authors: Sarić N, Somaa F, Strauss M, Foster C, Bejugama S, Rivero Ballon GN, Wang L, Hashimoto-Torii K, Ishibashi N.

Title: Primary cilia loss renders pyramidal neurons susceptible to perinatal ketamine-induced dendritic degeneration and learning deficits.

Presentation: 50th Annual Meeting of the Society for Neuroscience, November 13-16, 2021, Chicago, IL.

Authors: Strauss M., Sarić N., Rivero Ballón G.N., Foster C., Ishibashi N.

Title: Primary cilia deficiency exacerbates neonatal anesthesia neurotoxicity.

Meeting: Research Education & Innovation Week 2022, April 4-8, 2022, Washington, DC.

Authors: Foster CS, Kobayashi K, Shepard CT, Saric N, Ayodeji M, Tu SC, Ishibashi N.

Anesthesia neurotoxicity in a piglet model.

Meeting: Research Education & Innovation Week 2022, April 4-8, 2022, Washington, DC.

Website or other Internet site:

To educate patients, their families, and the public and to identify potential industry partners we worked with our Public Relations and Marketing team. Our research activities as well as the results have been published through our website "innovation district".

<https://innovationdistrict.childrensnational.org/caspases-may-link-brain-cell-degeneration-and-cardiac-surgery/>

Technologies or techniques:

Nothing to report

Inventions, patent applications, and/or licenses:

Nothing to report

Other Products:

Nothing to report

PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

Individuals on the project:

Name	Nobuyuki Ishibashi
Project Role	PD/PI
Researcher Identifier	0000-0002-7910-4050
Months worked	1.9
Contribution to Project	Dr. Ishibashi has supervised all aspects of the experiments and contributed to analyze the data as well as prepare manuscripts. He is responsible for communication with DoD.
Funding support	National Institutes of Health, Children's Research Institute (All changes in active other support listed do not impact the effort on the project).
Name	Kazue Hashimoto-Torii
Project Role	Co-investigator
Researcher Identifier	0000-0003-4001-8259
Month worked	1.2
Contribution to Project	Dr. Hashimoto-Torii has designed experiments and analyses including RNA profiling of mouse and pig. She has completed report of the data and wrote a manuscript.
Funding support	National Institutes of Health, Children's Research Institute (All changes in active other support listed do not impact the effort on the project)
Name	Courtney Shepard
Project Role	Postdoc fellow
Researcher Identifier	0000-0002-1975-6188
Month worked	4.0
Contribution to Project	After departure of Dr. Leonetti, Dr. Shepard has carried out the experiments and data collection in the piglet studies.
Funding support	None
Name	Van K. Lam
Project Role	Postdoc fellow
Researcher Identifier	0000-0002-2571-7599
Month worked	3.0
Contribution to Project	After departure of Dr. Li, Dr. Lam has carried out image data collection and analysis.
Funding support	None
Name	Nemanja Saric
Project Role	Postdoc fellow
Researcher Identifier	0000-0001-5336-4180
Month worked	1.0
Contribution to Project	After departure of Dr. Maeda, Dr. Saric has carried out data collection and analysis in the Aim 1 model with assistance of Dr. Shepard.
Funding support	None
Name	Shahid Mohammad
Project Role	Postdoc fellow
Researcher Identifier	0000-0001-5239-7861
Month worked	5.0
Contribution to Project	Dr. Mohammad has carried out the experiments and data collection in the proposed Aim 3 studies.
Funding support	None
Name	Ankush Bansal
Project Role	Postdoc fellow
Researcher Identifier	0000-0002-5491-1243
Month worked	4.2

Contribution to Project	Dr. Bansal has carried out analyses of RNA sequencing.
Funding support	None

Each person has worked at least one month per year on the project during the reporting period.

Change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period:

The change of active support for the PD/PI and key personnel are described below. **All changes in active other support listed do not impact the effort on the project.**

Nobuyuki Ishibashi, M.D., Principal Investigator (PD/PI):

Closed grant:

Nothing to report

New active grant:

R21NS127051 (Ishibashi, PI)

National Institutes of Health

The developmental origins and fate of neurons in the gyrencephalic neocortex

R01HD108839 (Posnack, PI)

National Institutes of Health

Off-label drugs in cardiology: evaluating age- and disease-appropriate therapies

Kazue Hashimoto-Torii Ph.D., Co-investigator:

Closed grant:

Nothing to report

New active grant:

Nothing to report

Richard A. Jonas, M.D., Co-investigator:

Closed grant:

Nothing to report

New active grant:

Nothing to report

Paul Wang, Ph.D., Consultant:

Closed grant:

Nothing to report

New active grant:

Nothing to report

David Zurakowski, Ph.D., Consultant:

Closed grant:

Nothing to report

New active grant:

Nothing to report

Nina Deutsch, M.D., Other Significant Contributor:

Closed grant:

Nothing to report

New active grant:

Nothing to report

Vesna Jevtovic-Todorovic, MD, PhD, MBA, Other Significant Contributor:

Closed grant:

R01NS091353 (Jevtovic-Todorovic, PI)

National Institutes of Health

Molecular mechanisms of glycosylation of Cav3.2 channels in pain pathway

R01GM102525 (Todorovic, PI)

National Institutes of Health

Effects of anesthetics on thalamic excitability

New active grant:

T32GM135169 (Jevtovic-Todorovic, PI)

National Institutes of Health

Anesthesiology Mentored Research Training

Silicon Valley Community Foundation (Jevtovic-Todorovic, PI)

DNA epigenetic modification in human sperm – the role of general anesthesia exposure

Other organizations as partners:

Organization Name: Howard University

Location of Organization: 2041 Georgia Ave, NW, Washington, DC, 20059

Partner's contribution to the project: Facilities

Animal imaging has been performed at Molecular Imaging Laboratory Facility at Howard University which functions as preclinical neuroimaging core of the District of Columbia Intellectual and Developmental Disabilities Research Center (DC-IDDRC) at Children's National Hospital.

Organization Name: Boston Children's Hospital

Location of Organization: 300 Longwood Avenue, Boston, MA 02115

Partner's contribution to the project: Collaboration

Dr. Zurakowski, Director of Biostatistics at Boston Children's Hospital, serve as Biostatistician in the project.

Organization Name: University of Colorado School of Medicine

Location of Organization: 12401 East 17th Avenue, Aurora, CO 80045

Partner's contribution to the project: Collaboration

Dr. Jevtovic-Todorovic, a Professor of Anesthesiology at University of Colorado School of Medicine, is Other Significant Contributor and guides the preclinical studies of neonatal anesthesia.

SPECIAL REPORTING REQUIREMENTS:

Nothing to report

APPENDICES:

Original copy of our articles published on *Annals of Neurology* and *Trends in Neuroscience* has been attached as APPENDICES.

Review

Nonapoptotic caspases in neural development and in anesthesia-induced neurotoxicity

Nemanja Sarić,¹ Kazue Hashimoto-Torii,^{1,2} Vesna Jevtović-Todorović,³ and Nobuyuki Ishibashi ^{1,2,4,*}

Apoptosis, classically initiated by caspase pathway activation, plays a prominent role during normal brain development as well as in neurodegeneration. The non-canonical, nonlethal arm of the caspase pathway is evolutionarily conserved and has also been implicated in both processes, yet is relatively understudied. Dysregulated pathway activation during critical periods of neurodevelopment due to environmental neurotoxins or exposure to compounds such as anesthetics can have detrimental consequences for brain maturation and long-term effects on behavior. In this review, we discuss key molecular characteristics and roles of the noncanonical caspase pathway and how its dysregulation may adversely affect brain development. We highlight both genetic and environmental factors that regulate apoptotic and sublethal caspase responses and discuss potential interventions that target the noncanonical caspase pathway for developmental brain injuries.

Caspases: more than cell death mediators

Cell death is currently understood to occur in one of the four morphologically defined manners: **apoptosis** (see [Glossary](#)), autophagy, necrosis, and entosis [1]. Apoptotic cell death as an intrinsically programmed phenomenon was originally described in the roundworm *Caenorhabditis elegans*. Of the 1090 somatic cells generated during *C. elegans* development, 302 are genetically predetermined to become neuronal cells. Additional 131 cells are produced, however, and these cells undergo programmed cell death with high temporal fidelity during the development of each worm [2]. Unlike in *C. elegans*, mammalian development relies on a more stochastic process of cell elimination [3]. The apoptotic mechanism is orchestrated by **caspases**, cysteine protease enzymes that utilize both other caspase proteins and diverse cellular peptides as their substrates [4].

Caspases are classified into initiator caspases, such as caspase 2, 8, 9, and 10, which are the first to become catalytically active during apoptosis, and effector caspases 3, 6, and 7, which rely on initiator activation and mediate the final steps of programmed cell death [5]. The end result of this sequential protease activity is DNA fragmentation and, ultimately, cell death [4,6]. Apoptosis proceeds either through a death receptor **extrinsic apoptotic pathway** or an intracellular, mitochondria-based intrinsic pathway [7]. The triggers for extrinsic caspase activation involve binding of tumor necrosis factor- α (TNF- α) and Fas to death receptors, while intrinsic, mitochondrial mechanisms are initiated by DNA damage and/or excess reactive oxygen species production [7] ([Figure 1](#)).

Over the course of several decades, important neurodevelopmental and injury response roles have been described for the canonical apoptotic pathway [7–9]. More recently, caspase activation has increasingly been associated with both physiological and pathological nonapoptotic outcomes that do not result in neuronal cell death, yet have significant impacts on cell morphology and function. Caspase activation status and catalytic efficiency is known to be influenced by phosphorylation of the enzymes themselves, as well as their target substrates, by kinases [10–12]. Whether

Highlights

Caspase activity is necessary for normal neuronal development and maturation. Studies are increasingly showing important caspase roles in axonal pathfinding, dendritic arborization, and synaptic plasticity.

There is growing evidence for nonlethal, yet damaging caspase responses to commonly used chemical agents such as ethanol and anesthetics.

Nonlethal, excess caspase activation may result in impaired neuronal arborization and synaptic transmission, leading to long-term behavioral deficits.

Children undergoing surgeries, such as those with congenital heart defects, may be at increased risks for nonapoptotic caspase injuries due to exposure to anesthetics and accompanying genetic susceptibility.

Understanding the molecular and cellular differences between apoptotic and nonlethal caspase activity is crucial for more targeted neuroprotection.

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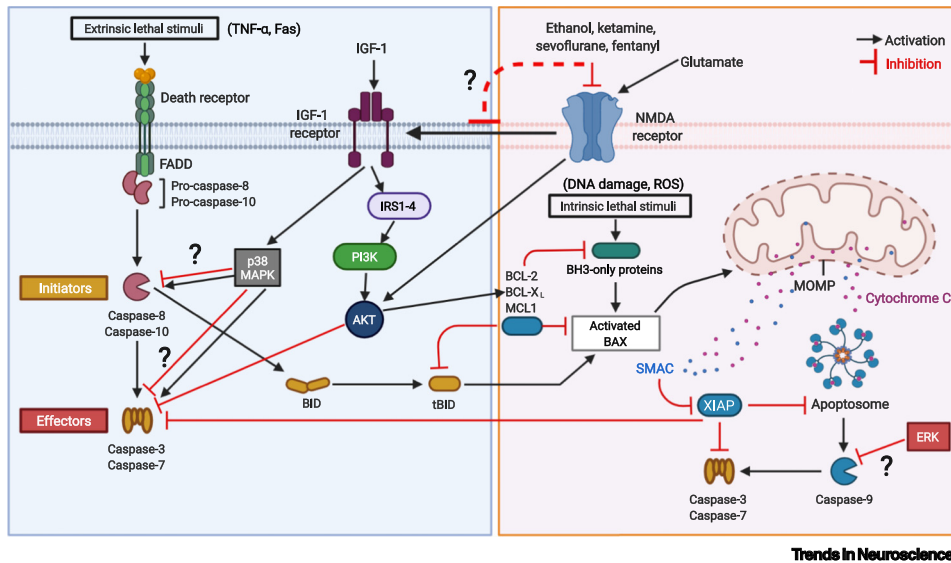


Figure 1. Overview of extrinsic and intrinsic apoptosis and caspase activation modifiers. The flowchart presents a summary of the extrinsic and intrinsic arms of the apoptotic pathway, while highlighting known and hypothetical molecular modifiers of initiator and effector caspase activation. Extrinsic, death receptor-dependent signaling crosstalks with the mitochondrial arm through BCL-2 family proapoptotic proteins such as BID, which when translocated to the mitochondria (translocated BID/tBID) results in activation of the protein BAX, mitochondrial membrane permeabilization, and release of cytochrome C. Cytochrome C forms structures known as apoptosomes, which initiate caspase activation. Effector caspase activation is inhibited by X-linked inhibitor of apoptosis proteins (XIAPs), which are ubiquitin ligases. SMAC acts as a potentiator of apoptosis by inhibiting XIAP function. BCL-2 family antiapoptotic proteins BCL-X_L and MCL1 suppress the intrinsic apoptotic cascade, acting to maintain homeostasis. Other signaling pathways such as PI3K/AKT, p38 MAPK, and ERK act as modulators of caspase signaling through their phosphorylation activity. Chemical agents with both neurophysiological and neurotoxic properties, such as glutamate and ketamine, function as agonists and antagonists of NMDA receptors, which interface with intracellular signaling regulators of caspase activation. Differential recruitment of these signaling pathways and their variable interaction with environmental stimuli may underlie functional differences between apoptotic and nonapoptotic caspase activation. The figure was created with [BioRender.com](https://www.biorender.com). Abbreviations: AKT, protein kinase B; BAX, BCL2-associated X protein; BCL-2, B-cell lymphoma 2; Bcl-X_L, B-cell lymphoma-extra large; BH3, Bcl-2 homology 3; BID, BH3 interacting-domain death agonist; ERK, extracellular-regulated protein kinase; FADD, Fas-associated death domain-containing protein; FAS, Fas cell surface death ligand; IGF-1, insulin-like growth factor 1; IRS1–4, insulin receptor substrate 1–4; MAPK, mitogen-activated protein kinase; MCL1, myeloid cell leukemia 1; MOMP, mitochondrial outer membrane permeabilization; PI3K, phosphoinositide 3-kinase; ROS, reactive oxygen species; SMAC, second mitochondria-derived activator of caspases; TNF-α, tumor necrosis factor-α.

caspase activation and the switch to nonapoptotic signaling predominantly depends on levels of caspase phosphorylation and/or cleavage (Figures 1 and 2) or on modulation by other signaling pathways (Figure 1) is unclear. It also remains unknown how precisely this shift in caspase activity diverts the neuron from proceeding with canonical apoptosis to instead undergoing structural changes (see Outstanding questions).

Neuronal structure is of critical importance to the assembly of functional brain circuits [13]. Disruptions of virtually any of the aspects of neuron structure have been implicated in disorders of neurodevelopment, age-related neurodegeneration, as well as various brain injury scenarios. Caspases are known to have phylogenetically conserved roles in the sculpting of neuronal dendritic arbors through cytoskeletal remodeling and have been demonstrated to contribute to synaptic plasticity [4]. In addition, caspase signaling has been linked to excitotoxic injuries in prior work [4]. Programmed cell death and neurodegeneration has been frequently reported in developmental brain injury scenarios such as anesthesia exposure and alcohol-induced neurotoxicity [9,14,15]. Importantly, the neurotoxic potential of excessive nonapoptotic caspase

Glossary

Apoptosis: a series of intrinsically programmed molecular steps leading to cell death.

Canonical apoptotic signaling: the most well-studied set of reactions leading to cell death, consisting of an extrinsic and intrinsic pathway/arm. Noncanonical apoptotic signaling describes caspase-dependent reactions that do not lead to cell death, yet are important for synaptic pruning, neuritogenesis, and synaptic plasticity.

Caspase: a protease enzyme involved in the execution of apoptosis as well as other nonapoptotic cellular processes.

Extrinsic apoptotic pathway: also termed death receptor pathway, involves binding of an extracellular death ligand (e.g., TNF-α) to a plasma membrane death receptor (e.g., TNF receptor) to initiate apoptosis.

Intrinsic apoptotic pathway: also termed the mitochondrial apoptotic pathway, it is initiated intracellularly, due to DNA damage, ischemia, and oxidative stress.

Sublethal caspase activation: caspase signaling that involves cleaving and activation of effector caspases, without subsequently leading to cell death (as assessed by cell number, nuclear fragmentation via TUNEL staining, and markers of mitochondrial integrity).

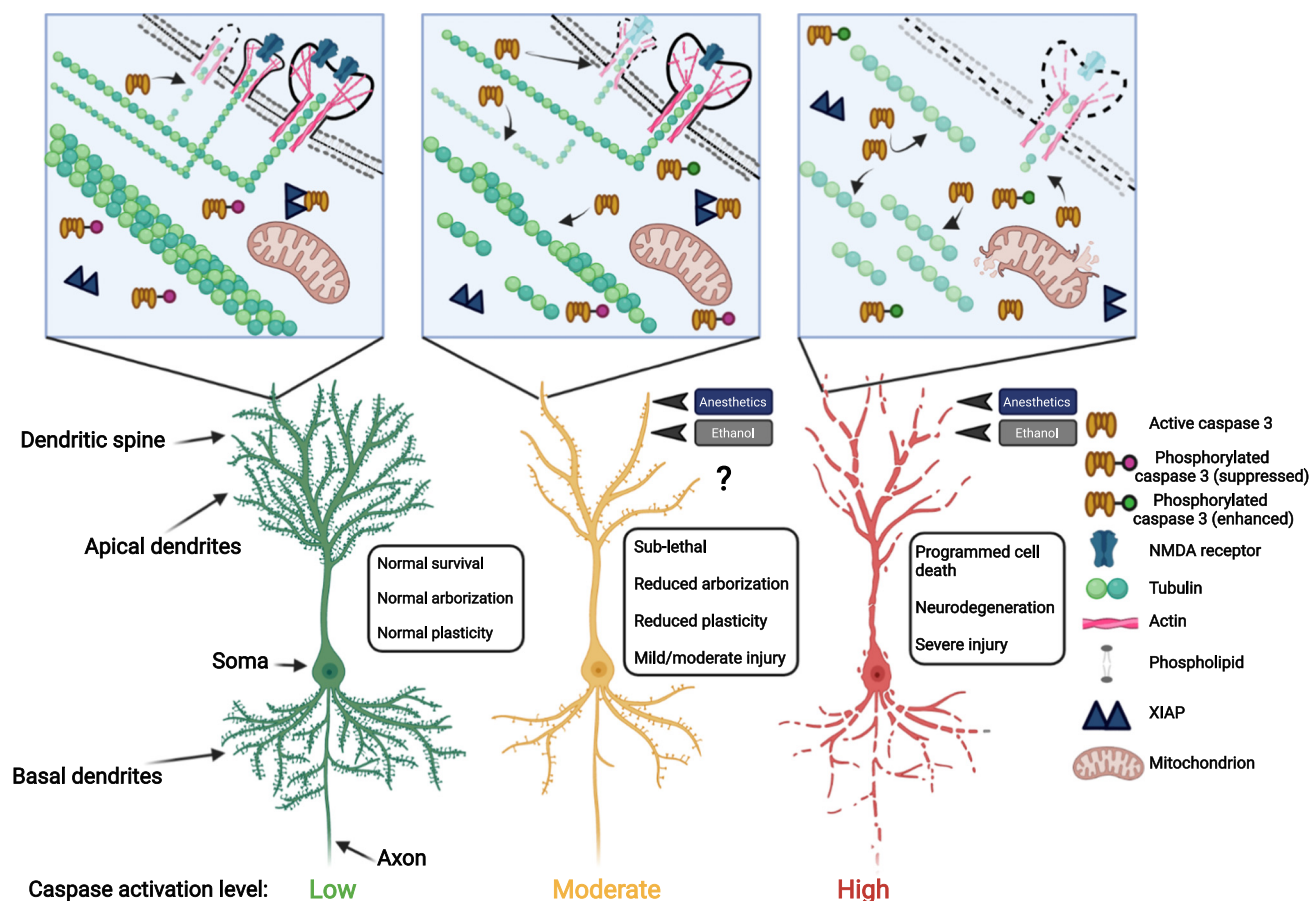


Figure 2. Caspase activation mode and corresponding outcomes in physiological and injury scenarios. The schematic offers a simplified overview of variable, caspase activation level-dependent neurotoxic outcomes (with caspase cleavage and/or phosphorylation levels being key modulators of activation). In pyramidal neurons, dendritic processes contact and receive synaptic inputs from other neurons and enable crosstalk with glial cells. Often, dendrites are covered in dendritic spines, postsynaptic sites of neurotransmission that are dynamically turned over and either stabilized or lost, processes that contribute to synaptic plasticity. These structures are supported and maintained by networks of filament proteins, including actin and tubulin. During normal physiological neuronal development and maturation, a low level of caspase pathway activation is required for dendritic branching and shifts in plasticity required during learning. However, upon exposure to environmental neurotoxins, such as anesthetics and ethanol, enhanced activation of executioner caspases leads to the induction of apoptosis via mitochondria (intrinsic pathway) and extensive cleaving of cytoskeletal proteins. A more complex scenario is increasingly being recognized, whereby a moderate level of caspase activity postexposure may lead to cytoskeletal remodeling, without full cell death. How this level of activation is achieved and modulated is unclear, but may involve variable availability of X-linked inhibitor of apoptosis proteins (XIAPs), genetic susceptibility combined with low-dose neurotoxin exposures, and altered neuronal–glial crosstalk. The figure was created with [BioRender.com](https://www.biorender.com).

signaling has not been well characterized in the developing brain. Given the capacity for caspase activation by commonly used environmental agents, such as volatile anesthetics, ethanol, and opioids, addressing the possibility of nonapoptotic, sublethal neurotoxicity is particularly pertinent.

In this review, we highlight key aspects of nonapoptotic caspase functions during neuronal development and maturation, as well as unaddressed questions concerning their contribution to pathological processes triggered by developmental brain injury. We then summarize recent findings on how the nonapoptotic caspase pathway contributes to long-term neurological deficits and discuss potential therapeutic avenues.

Diverse roles of the nonapoptotic pathway arm

Caspases during neurogenesis

Besides the canonical apoptotic pathway, caspase activity that triggers nonapoptotic events in the cell is involved in a diverse repertoire of roles, including proper immune function, cell proliferation and differentiation during development, cell fate specification, and cytoskeletal function in both vertebrates and invertebrates [16]. In *Drosophila melanogaster*, the fruit fly ortholog of mammalian initiator caspase 9/2, called *dronc*, restricts the expansion of enteroblast cells, a type of intestinal precursor cell, through a nonlethal regulation of the proliferation-differentiation transition [17,18]. In the *D. melanogaster* CNS, *dronc* plays a similar role, suppressing the production of excess sensory neural precursors in a nonapoptotic manner, thereby contributing to a system-level fine-tuning during cell differentiation [19]. Proper differentiation of mammalian neural stem cells also requires nonapoptotic caspase signaling [20]. For instance, the differentiation of cerebellar granule neurons and Bergmann glia is precisely controlled by nonapoptotic activity of caspase 3 [20–22]. Given the extensive caspase-dependent apoptosis in the rodent cerebellar cortex during the first postnatal week [23,24], a key question that remains to be addressed is how the two disparate functions of caspase 3 are spatiotemporally coordinated. A possible explanation is dosage dependence on caspase 3 cleavage levels (Figures 1 and 2). To test this hypothesis, expression constructs encoding catalytically active caspase 3 subunits under the control of variable strength synthetic promoters may be introduced in different neuronal cell lines [25,26]. This strategy could also be used at different stages of neuronal differentiation and maturation *in vivo*, using tissue- and cell-specific promoters, to clarify if levels of active caspase 3 determine the switch from nonapoptotic to apoptotic pathways.

Caspase-mediated cytoskeletal remodeling

One of the major targets of nonapoptotic caspase signaling is the cytoskeleton. Some of the earliest studies of caspase function, focused on understanding canonical cell death signaling, implicated caspases in the destabilization and cleaving of cytoskeletal proteins such as actin and tau [27,28]. Cytoskeletal remodeling and reorganization underlie cellular morphological changes, such as during cell division, movement, and acquisition of a mature morphology. During neural development, the process of axon guidance is necessary for the establishment of correct early wiring patterns and subsequent synaptic maturation. Inhibition of localized caspase 3 and 8 activity in mouse hippocampal neurons leads to a loss of neurite outgrowth [29]. This effect seems to be dependent on caspase-mediated cleaving of spectrin, a plasma membrane-associated cytoskeletal protein necessary for axonal bending and stretching [30,31]. The aforementioned *D. melanogaster* caspase ortholog *dronc* has been shown to cleave F-actin filaments in the developing salivary glands in nonapoptotic fashion and subsequently prune dendritic arbors in a class of sensory neurons [32]. Similarly, axonal misrouting and impaired synaptic contact establishment occurs in olfactory sensory neurons of mice mutant for caspase 9, an initiator caspase [33]. Furthermore, overexpression of a dominant negative mutant caspase 3 precursor in developing chick embryos, specifically in midbrain presynaptic neurons, led to significantly reduced dendritic complexity, as seen through fewer neuronal branch points and higher order branches [34]. Experiments from these studies show a conserved caspase function in neuronal arbor remodeling across different model species that is strongly dependent on cytoskeletal modulation. Cytoskeletal substrate preference seems to vary to some extent between different caspases, as caspase 3 has been shown to cleave both beta-actin and alpha-tubulin, whereas caspase 6 only targets tubulin [35].

How this substrate specificity is established and regulated in a nonapoptotic context remains an open question. One possibility is the involvement and variable availability of X-linked inhibitor of apoptosis proteins (XIAPs), such as E3 ubiquitin ligase, throughout the neuron [36] (Figures 1

and 2). Given the system-level need for nonapoptotic caspase function in regulating neuronal cytoskeletal architecture, distinguishing apoptotic from sublethal caspase impacts on cytoskeletal proteins at high resolution is an important yet overlooked avenue. Super-resolution microscopy techniques coupled with single-molecule fluorescence tagging of microtubule and actin bundles could provide insights into these distinct caspase-mediated outcomes [37–39].

Caspase signaling in synapses

Apart from being important in the process of neuronal process outgrowth and arborization, caspases have increasingly been linked to synaptic plasticity modulation and normal synaptic physiology (Figure 2). The process of long-term depression (LTD), by which neuronal synapses progressively weaken functionally, accompanied by a reduction in dendritic spine size, depends on a low level of caspase 3/9 activation within dendrites [40]. Curiously, the converse process of long-term potentiation, by which neuronal synapses strengthen over time, seems to be unaffected by suppressing caspase 3/9 activation genetically or pharmacologically. Furthermore, the LTD process dependent on NMDA receptor (NMDAR) function seems to be uniquely dampened by reduced expression of BAD and BAX proteins, both of which are classically associated with promoting intrinsic apoptosis via mitochondria (Figure 1) [41]. More recently, the BAD-BAX-caspase 3 signaling cascade was demonstrated to regulate synaptic vesicle pools by autophagy, with the process being tightly coordinated during associative fear learning in mice [42]. BAX-caspase 3 activation plays a prominent role in the activity-dependent reorganization of corticospinal projections necessary for the acquisition of fine motor task skills [43]. Caspase 3 also directly regulates the levels of AMPA receptor subunit GluA1 in response to chronic stimuli *in vivo* [44]. In zebra finch songbirds, cleaved caspase 3 levels are strongly increased in postsynaptic compartments of the auditory cortex following exposure to novel sounds [45].

Collectively, these findings provide evidence for the involvement of caspase activity during normal physiological processes underlying learning and memory. Better characterization of **sublethal caspase activation** and localization within dendrites and dendritic spines during various learning paradigms will help clarify how caspase activity impacts spine turnover and ion channel expression in experience-dependent plasticity. Are caspases differentially recruited depending on the degree of experience-induced plasticity? Important questions also remain regarding how endogenous caspase activity in these contexts interacts with extrinsic environmental factors known to modify neuronal activity in both physiological and pathological scenarios.

Environmental influences and injury mechanisms

Caspase signaling in excitotoxicity

Intrinsically activated caspases do not operate in isolation, but rather can be influenced by a variety of environmental factors. In neurons, nonapoptotic signaling engagement likely requires a lower activation threshold compared with full programmed cell death. Accordingly, the presence of one or several environmental agents might easily compound the likelihood of pathway activation. Glutamate-induced neurotoxicity, also termed excitotoxicity, is a well-characterized neuronal injury paradigm, particularly in the context of canonical apoptosis [46,47]. Glutamate-induced neurotoxicity occurs due to the presence of supra-physiological levels of the neurotransmitter glutamate, leading to excessive stimulation of ligand-gated ion channel receptors such as NMDA, AMPA, and kainic acid receptors, which culminates in a neurotoxic influx of calcium ions [48,49]. This toxicity is typically manifested through mitochondrial dysfunction, increased generation of reactive oxygen and nitrogen species, a drop in mitochondrial membrane potential, and, ultimately, mitochondrial pore opening followed by apoptosis [49]. The calcium ion influx is known to activate calcium-dependent proteases and phosphatases such as calpains and calcineurin, respectively, which in turn are capable of inducing executioner caspase activity as well, forming a feedback loop [50]. Calpain-10 has been shown to

control actin filament dynamics [51], providing a secondary caspase-independent avenue to excitotoxic dysregulation of the cytoskeleton. More recently, calcineurin signaling downstream of calcium influxes into hippocampal CA1 neurons was found to mediate damage to Ankyrin G, a cytoskeletal protein crucial to the organization of the axon initial segments in said cells [52]. In this study, the injury was ischemic, derived from medial cerebral artery occlusion, which, despite leading to major cytoskeletal reorganization and impaired action potential generation, did not result in significantly increased cell death. Given the known interplay between caspases and calcium-dependent proteases/phosphatases [52–56], and their respective capacities for altering cytoskeletal dynamics, it will be important to dissect their relative contributions to sublethal excitotoxic outcomes in different neuronal populations.

Role of neuronal–glial crosstalk in caspase activation

Neuronal cells do not undergo neurotoxic responses in isolation. The environmental agent causing the insult also acts on glial cells as well as endothelial cells of the vasculature. These non-cell autonomous responses are multi-pronged, with non-neuronal cells experiencing both shared and cell-specific insult effects, which then feed back onto neuronal cells in their proximity. Under physiological conditions, neurons and glial cells engage in extensive crosstalk, due to both physical proximity-based cell–cell contacts, cytoskeletal protrusions known as tunneling nanotubes, extracellular vesicles, as well as paracrine signals [57–59]. In microglial cells, exposure to inflammatory agents such as lipopolysaccharide triggers activation of initiator caspase 8 as well as caspases 3 and 7, which in turn drive the microglia to a proinflammatory phenotype via protein kinase C (PKC)- δ signaling [60]. The caspase activation in this paradigm is not lethal to microglia, yet indirectly leads to neurotoxic outcomes due to microglial activation. In neuronal and astrocyte cocultures, exposure to ketamine, which is known as an NMDAR antagonist, enhances lethal neurotoxicity compared with treatment of neurons alone [61]. Extracellular vesicles, and in particular the long noncoding RNA targeting BDNF contained within them, isolated from the ketamine-treated astrocytes seem to be partially responsible for this effect. Modulatory effects on caspase-dependent apoptosis in neurons by non-neuronal cells may play an even more pertinent role in sublethal caspase activity contexts, with comparatively lower caspase activation thresholds [62].

Anesthetics and ethanol in developmental neurotoxicity

Anesthetics and ethanol are among the best-studied environmental agents implicated in developmental neurotoxicity (Figures 1 and 2 and Box 1) [9,15]. Early work showed that both acute and

Box 1. Clinical findings in anesthesia toxicity and neurodevelopment

Approximately 100 clinical studies have been published evaluating neurodevelopmental outcomes after childhood surgery and anesthesia [110]. Despite concerning evidence from animal studies, clinical results are mixed, likely due to various challenges and limitations inherent to the design of optimal clinical studies (Box 3) [111]. Exposure to general anesthesia is not consistently associated with deficits in academic achievement, general intelligence, or language [88,111,112]. However, a growing number of studies has been reporting associations between general anesthesia exposure during infancy and increased risks for adverse behavioral outcomes, including deficits in executive function, social communication, motor function, and diagnoses of attention deficit hyperactivity disorder (ADHD) [112–114]. These deficits have been reported even after single exposures to anesthesia [115]. Anesthesia length, repeated exposure, dosage regime, and younger age all contribute to enhanced risk for such neurobehavioral impairments [110,112]. Multiple infant anesthetic exposures have been found to more than double the hazard ratio for learning disabilities [116]. The same study found a 30% increase in the hazard ratio for ADHD with a single anesthetic exposure, which, although not statistically significant, is suggestive of the cumulative nature of the damaging effect. It is plausible that lower dose exposures or specific anesthetics in isolation may preferentially trigger nonapoptotic caspase responses, resulting in outcomes different from those of classical apoptosis (see Figure 2 in main text).

The link between anesthesia and canonical caspase-mediated cell death and neurodegeneration in animal models is well established. Whether anesthetics can elicit brain injury via nonapoptotic caspase activation is a pertinent yet largely unexplored avenue of research.

chronic ethanol exposure was associated with caspase 3 activation, neuronal cell death, and neurodegeneration in mouse and rat models, with the response heavily dependent on BAX protein activity [9,63–65]. More recent work intriguingly found that mice lacking primary cilia in fore-brain excitatory neurons exhibited sublethal caspase 3 activation after acute ethanol treatment [66]. Dampened PI3K/AKT signaling caused by cilia loss seems to be a key causal factor behind the enhanced caspase response. The same effect on caspase 3 activation was observed with a single dose of ketamine. Curiously, the same ethanol dosage regime used elicited marked neurodegeneration at similar end points in prior studies, with evidence for neuronal cell loss at later times. This discrepancy may be partially explained by the dampening of the caspase 3 response over time, although questions remain over the inconsistency regarding cell loss. In line with prior developmental evidence, the sublethal caspase 3 activation [66] was punctate in presentation and was associated with actin and tubulin degeneration, followed by loss of pyramidal neuron dendritic complexity in ciliopathic mice. Neuronal safeguarding mechanisms, such as PI3K/AKT signaling via primary cilia, may be important modifiers of the degree of caspase activation and determinants of whether a cell commits to apoptosis or undergoes sublethal neurotoxic changes (Figure 1).

Anesthetics as a key risk factor for caspase-induced injury after major neonatal surgeries

Anesthetics in caspase-dependent injury

Anesthetics such as sevoflurane and isoflurane are an indispensable component of complex pediatric surgeries, for instance, corrective heart surgery in patients with congenital heart disease (CHD) (Box 2). Both volatile and injectable anesthetics have been extensively linked to canonical, cell death-associated caspase activity in various animal models of exposure. Originally described in rat models, combinations of midazolam, nitrous oxide, and isoflurane exposure were associated with extensive caspase activation, neurodegenerative changes, and subsequent learning impairments [8,15]. Many of the anesthetics used for major pediatric surgery function as either NMDAR blockers or GABA receptor agonists [8], suggestive of a converging mode of neurotoxicity. Isoflurane and sevoflurane have both been demonstrated to mediate their caspase-dependent neurotoxic effects through disrupting calcium homeostasis and activation of the intrinsic mitochondrial apoptotic cascade [67–70]. In rodent anesthesia models, perinatal exposures to sevoflurane and isoflurane induced long-term behavioral impairments, including memory deficits, increased anxiety, decreased sociability, and impaired motor coordination [14,71]. In infant rhesus macaques, repeated doses, but not a single dose, of isoflurane were associated with deficits

Box 2. Etiologies of neurological deficits observed in congenital heart disease (CHD)

CHD is one of the most common birth defects. Neonatal surgical interventions are critical for patients with complex CHD, leading to significantly reduced case mortality. Given its high prevalence, accounting for around 1% of live births, CHD has huge societal impacts. Despite improvements in mortality prevention, a substantial fraction of CHD patients (estimated at about 1 in 150) go on to exhibit various neurobehavioral abnormalities, including attention deficit, social difficulties, motor issues, and intellectual disability [117]. The Pediatric Heart Network and National Heart, Lung, and Blood Institute working group has reported that one of the most important challenges for neonates undergoing surgery for CHD is long-term sequelae, such as neurodevelopmental deficits [118].

Known and postulated causative factors range from genetic, including *de novo* mutations in chromatin modifier genes [90], to perioperative factors such as hypoxia/ischemia and exposure to anesthetics [119]. Neonates undergoing complex surgeries present a uniquely challenging scenario due to the temporal overlap between surgical factors, such as anesthesia, and ongoing development of several organ systems, including the brain. Past studies indicate that anesthetic use during the neonatal time window is associated with neurotoxicity and an increased risk of behavioral deficits in later life [120]. These include sensory issues, motor deficits, as well as cognitive delays. The neonatal period is characterized by crucial early brain maturation processes, such as early cortical circuit establishment, synaptogenesis, and cerebellar neurogenesis. Disruptions in any of these developmental steps carry a potential risk of physiological dysfunction and resultant behavioral disturbances.

in motor reflexes and increased social anxiety [72]. The dose-dependent effect points to a cumulative model of anesthetic neurotoxicity, with greater exposure frequency leading to greater neuroapoptosis and neurodegenerative changes. Genetic susceptibility or involvement of a second environmental factor (a dual-hit scenario) could therefore plausibly decrease the threshold for caspase activation as well as modify the nature of the response, rerouting it from cell death to structural and physiological remodeling of the cell. Ketamine administration is also commonplace in pediatric cardiac surgery as premedication. Recent reports indicate that multiple courses of ketamine in developing rat models elicit caspase 3-associated neurodegenerative changes in the prefrontal cortex and hippocampus as well as neurobehavioral alterations such as impairments in spatial memory, decreased anxiety, and enhanced novelty-induced behaviors [73–75].

Anesthetic exposure neurotoxicity has also been characterized in the context of age-related cognitive decline and neurodegeneration. Alzheimer's disease has been postulated to result primarily from abnormal accumulation and reduced clearance of β -amyloid (A β) peptides in the aging brain [76]. Isoflurane-exposed neonatal mice as well as human neuroglioma cells exhibited increased oligomerization of A β 42 peptides, which was accompanied by enhanced caspase 3 activation [77] and is known to be upstream of caspase signaling [78], leading to synaptic injuries [79]. Unexpectedly, the use of another anesthetic, propofol, attenuated caspase activation in this paradigm, likely through suppressing A β 42 oligomerization [77]. The apparently contradicting effects on caspase activation point to the possibility of complex interactions between different anesthetics, when used in combination. The caspase activation signature resulting from their combinatorial use may therefore fall in a sublethal range, where apoptotic and autophagic cell death are avoided, but cytoskeletal and synaptic damage persist.

Potential for sublethal insults

While the majority of anesthetic neurotoxicity research has focused on the canonical, cell death-inducing context, sublethal mechanisms have also been implicated. *In vitro* evidence in neural precursor cells (NPCs) derived from human embryonic stem cells suggests short-term (2 h) exposures to clinically relevant sevoflurane concentrations do not induce cell death, with higher, supra-clinical concentrations transiently inhibiting differentiation [80]. In concordance with these findings, earlier research in cultured hippocampal NPCs found that exposures to isoflurane [81] and clinically relevant concentrations of propofol [82,83] did not induce cell death, but did promote neuronal differentiation. Ketamine treatment of *ex vivo* rat cortical NPCs showed an identical trend, with no effect on apoptosis and enhanced neuronal differentiation [84]. These studies collectively suggest a greater resistance to anesthetic-induced cell death in immature neuronal precursors and potentially a more prominent role for nonapoptotic mechanisms in neurogenic regions of the brain during the neonatal time window. In a separate study, isoflurane-treated hippocampal NPCs showed robust increases in cleaved caspase 3 levels without accompanying cell death, as assessed by TUNEL staining [85]. Interestingly, at least part of the mechanistic explanation for the enhanced neuronal differentiation also observed in this study is increased caspase 3 activation, as treatment with Z-DEVD-fmk, a caspase 3 inhibitor, rescued caspase activation and neuronal differentiation levels. If caspase activation in neurogenic regions such as the hippocampus can be sublethally modulated by anesthetics, the neonatal period should be a particularly vulnerable time window, due to extensive neurogenesis taking place.

Detrimental anesthetic effects on dendritic arborization and synaptic ultrastructure have also been demonstrated. Acute exposures of *ex vivo* primary rat cortical neurons for 1 h to sevoflurane or desflurane did not result in overt cell death in the case of sevoflurane; however, both anesthetics led to reduced neurite lengths and dampened spontaneous synaptic currents [86]. Intriguingly, the damaging effects of both anesthetics were mediated through increased

mitochondrial fragmentation, as treatment with an inhibitor of mitochondrial division, Mdivi-1, rescued mitochondrial morphology as well as the synaptic and electrophysiological defects. Mitochondrial fragmentation is a key step during activation of the **intrinsic apoptotic pathway** and is upstream of caspase 3 cleaving and activation (Figure 1). Given the differential level of mitochondrial fragmentation observed between sevoflurane and desflurane [86], which was correlated to cell death, one could speculate that an ‘intermediate’ level of mitochondrial ‘leakiness’ leads to a corresponding sublethal caspase activation, which works to suppress neuritogenesis and synaptic maturation while avoiding triggering cell death (as postulated in Figure 2). The findings from this study point to the importance of careful characterization of neuronal phenotypes in anesthetic exposure paradigms where overt neurodegeneration and cell death are not observed. These more subtle phenotypes may be otherwise missed in studies focused on detecting signs of canonical caspase signaling. A major caveat of the aforementioned studies is that they were performed in cultured cells, raising the question of whether these unique, sublethal scenarios can be replicated *in vivo*.

Recently, a large-scale randomized control trial found no difference in neurodevelopmental milestones in 2-year-old children with a follow-up at 5 years, between a group exposed to 1 h of general anesthesia and a control group receiving awake-regional anesthesia [87,88]. While the trial design reflected commonly used regimes during pediatric surgery, it could not assess the effects of repeat exposures nor could it account for potential gene–environment interactions and combinatorial mechanisms (Box 3). Children with CHD often carry polymorphisms in genetic loci that are associated with regulation of apoptosis and cell survival. Among these are the *Akt1* and *Gdf1* genes [89,90], which have known roles in modulating caspase signaling and cell survival [91–94]. This suggests a scenario where genetically susceptible populations, such as certain CHD patients, may be at an increased risk for anesthesia-induced neurotoxicity due to an enhanced likelihood of caspase activation following exposure and caspase-mediated neurodegeneration.

How nonlethal yet damaging caspase responses may be implicated in these cases is unknown and warrants further study. Understanding the switch from cell death-promoting caspase activity to sublethal outcomes would potentially allow for better stratification and more informed treatment of patients suffering from caspase-elicited neurotoxicity.

Therapeutic potential and outlook

Caspase-dependent neurotoxicity is a complex, multistep process involving both autonomous and non-cell autonomous functions and is heavily influenced by timing, dosage, and other underlying

Box 3. Current controversies in anesthesia neurotoxicity

Studies on developmental anesthesia neurotoxicity have mostly focused on canonical, apoptotic caspase signaling and preclinical animal models, primarily rodents and monkeys. Repeated anesthetic exposures in animals lead to robust neurotoxic effects on brain development and maturation [71,74]. A recent clinical trial found no significant effects of a single, 1-h exposure to anesthesia on developmental milestones and neurobehavioral outcomes at 3 and 5 years of age [88]. The difficulty in assessing comprehensive outcomes in humans, as well as the small sample size and fact that trial participants were not screened for genetic status, presents difficulties for fully excluding anesthetics as neurodevelopmental risk factors. In addition, obvious technical and ethical difficulties prohibit distinguishing anesthesia neurotoxicity from the global impact of neonatal surgery such as systemic inflammation. Measuring apoptotic outcomes may also miss more subtle, yet potentially important cellular alterations due to nonlethal caspase-dependent mechanisms.

The cellular and molecular mechanisms behind nonapoptotic neuronal injury and their influence on behavior are largely unexplored, primarily due to historical focus on the canonical caspase pathway. Given the clear importance of this pathway as a source of potential long-term neurological injury, there is an urgent need for in-depth mechanistic characterization in both basic and translational animal models, as well as design of new targeted therapeutics.

covariates. Assuming sublethal neurodegenerative caspase activation is mostly a constrained version of caspase-dependent cell death, the increasing repertoire of caspase inhibitors would provide candidate therapeutics for neuroprotection. Among these, Z-VAD-FMK and Boc-D-FMK show potent anticaspase properties, albeit without inhibiting activity of all caspases and showing non-overlapping specificity [95–97]. Both inhibitors are commercially available; however, they have been shown to induce adverse effects in the past [98,99]. A viable alternative is the pan-caspase inhibitor Q-VD-OPh, with higher efficacy, demonstrable diffusion through the blood–brain barrier, and a better toxicity profile [100,101]. Q-VD-OPh has shown promise in preventing apoptosis in models of perinatal stroke [102], spinal cord injury [103], and Alzheimer’s disease [104].

Apart from broad-spectrum caspase enzyme inhibition, therapeutic strategies may be aimed at indirectly dampening caspase activation and subsequent neuronal damage. Mitochondrial fission and fragmentation is a known inducer of intrinsic caspase 3 activation, and inhibitors such as the quinazolinone Mdivi-1 have been shown to protect neuronal stem cells from oxidative damage and apoptosis [105]. The PI3K/AKT pathway has long been known to play a prominent neuroprotective role under various injury paradigms [92,106], partly through restricting caspase activation. Employment of the PI3K/AKT small molecule activator, SC79, may be beneficial in both apoptotic and sublethal injury scenarios, as it has been demonstrated to protect neurons from oxidative stress [107,108].

Given the variety of nonapoptotic roles caspases play during normal neural development and maturation processes, using broad-spectrum caspase inhibitors at critical developmental time points may pose risks. Since many of the sublethal effects of caspase activation take place at lower activation thresholds, it will be important to assess for remnant caspase activity and cytoskeletal integrity in paradigms using caspase inhibitors to suppress cell death. Interference with physiological caspase functioning is of particular concern in scenarios likely requiring continuous inhibitor treatments, such as in progressive neurodegenerative disorders like Alzheimer’s, Parkinson’s, and Huntington’s diseases. Future research on developmental anesthesia neurotoxicity will need to more carefully address the potential sublethal, caspase-dependent adverse outcomes in terms of cell morphology, establishment of functional neural circuitry, and behavior. Assessing caspase activation thresholds in preclinical models alongside thorough titration of therapeutics targeting caspase activation, intracellular signaling such as PI3K/AKT and mitochondrial stability [109] will be necessary to fully characterize and target the sublethal response.

Concluding remarks

Significant progress has been made over the past decades in understanding the molecular mechanisms behind programmed cell death in the nervous system and its effects on cell morphology, physiology, and organismal behavior. Detailed characterization of caspases, their substrates, and the outcomes of their activity has informed the design of new classes of caspase inhibitors with demonstrated efficacy. A primary driving force behind this work was to understand **canonical apoptotic signaling** with the hope of either suppressing it in the context of neurodegenerative insults or promoting it during carcinogenesis. In parallel, the nonlethal mode of caspase activation and signaling has been gaining growing interest. Progress has been made in clarifying the important functions this signaling pathway plays in neuronal dendritic extensions and arborization, experience-induced plasticity, and learning, as well as how it might be co-opted in injury scenarios. Advances in super-resolution imaging, *in vitro* neuronal culture, CRISPR-Cas9 genome editing, and next-generation sequencing methodologies, together with established injury models, are positioning research to dissect the conditions under which sublethal caspase

Outstanding questions

What is the main driver behind the shift from apoptotic to nonapoptotic caspase functions during neuronal development and maturation?

Is the functional divergence in neuronal caspase activity primarily dependent on quantitative, caspase activation threshold-associated changes in signaling, or do other indirect factors play a greater role?

Are neuronal cells more sensitive to nonapoptotic insults at particular times during their morphological and physiological maturation? Are different classes and types of neurons differentially sensitive to caspase signaling?

Are cytoskeletal proteins the primary targets of nonapoptotic caspase activity? How is this substrate specificity governed in neuronal cells? Are different caspases recruited to different substrates in a nonapoptotic context?

To what extent and by what mechanism are environmental neurotoxins implicated in nonlethal caspase signaling? How is the damage constrained and apoptosis avoided?

What role do gene–environment interactions in neurons play in nonapoptotic caspase signaling in physiological and injury contexts? Is there potential for harmful interactions between low-dose anesthetic exposures and genetic susceptibility at the level of nonapoptotic caspases?

If risks for particular pediatric populations (such as those with CHD) are identified, what caspase-targeting therapeutic options are optimal for neuroprotection?

activation takes precedence over caspase-driven apoptosis (see Outstanding questions). These prospective studies will be especially important for deciphering the fine details behind the neurotoxicity of clinically relevant agents such as anesthetics, which remain indispensable for surgical procedures like those in the pediatric CHD population.

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Declaration of interests

The authors declare no competing interests in relation to this work.

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Impact of Cardiopulmonary Bypass on Neurogenesis and Cortical Maturation

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Objective: Neurodevelopmental delays and frontal lobe cortical dysmaturation are widespread among children with congenital heart disease (CHD). The subventricular zone (SVZ) is the largest pool of neural stem/progenitor cells in the postnatal brain. Our aim is to determine the effects of cardiopulmonary bypass (CPB) on neurogenesis and cortical maturation in piglets whose SVZ development is similar to human infants.

Methods: Three-week-old piglets ($n = 29$) were randomly assigned to control (no surgery), mild-CPB (34°C full flow for 60 minutes) and severe-CPB groups (25°C circulatory-arrest for 60 minutes). The SVZ and frontal lobe were analyzed with immunohistochemistry 3 days and 4 weeks postoperatively. MRI of the frontal lobe was used to assess cortical development.

Results: SVZ neurogenic activity was reduced up to 4 weeks after both mild and severe CPB-induced insults. CPB also induced decreased migration of young neurons to the frontal lobe, demonstrating that CPB impairs postnatal neurogenesis. MRI 4 weeks after CPB displayed a decrease in gyrification index and cortical volume of the frontal lobe. Cortical fractional anisotropy was increased after severe CPB injury, indicating a prolonged deleterious impact of CPB on cortical maturation. Both CPB-induced insults displayed a significant change in densities of three major inhibitory neurons, suggesting excitatory-inhibitory imbalance in the frontal cortex. In addition, different CPB insults altered different subpopulations of inhibitory neurons.

Interpretation: Our results provide novel insights into cellular mechanisms contributing to CHD-induced neurological impairments. Further refinement of CPB hardware and techniques is necessary to improve long-term frontal cortical dysmaturation observed in children with CHD.

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Children with complex congenital heart diseases (CHD) are at risk of developing a wide spectrum of neurobehavioral problems.^{1,2} Neuroimaging studies have shown that altered cortical morphology particularly affecting the frontal lobe, including reduced folding and volume, is present in children with severe and complex CHD.^{3,4} Many of these changes are similar to the reductions in both cortical gray and white matter volume seen in children born prematurely.⁵ Studies using diffusion tensor imaging (DTI) have demonstrated a high incidence of white matter dysmaturation

and injury in CHD patients, including adolescents who underwent surgery in infancy.^{6,7}

Considerable previous work including our own has focused on the mechanisms of abnormal white matter development observed in the individual with CHD.⁸ Exposure to the inflammatory effects of cardiopulmonary bypass (CPB) and possible hypoxia/ischemia associated with surgical repair in early infancy has been documented to interfere with white matter development.^{8–11} The generation, migration, and maturation of oligodendrocytes

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responsible for myelin/white matter synthesis may be delayed by hypoxia due to CHD in the fetus.^{12,13}

Oligodendrocytes are glial cells generated by the sub-ventricular zone (SVZ) particularly during the third trimester of human pregnancy and the first 2 or 3 years of life. However, in addition to generating glial cells responsible for white matter development, the SVZ is a key source of neurons generated from neuronal stem precursor cells (NSPCs). Furthermore, young neurons in the SVZ migrate postnatally along the lateral ventricles to populate cortical gray matter, contributing to its further maturation.¹⁴ Using a piglet model of fetal and neonatal hypoxia, we have previously documented reduced proliferation and neurogenesis in the SVZ accompanied by reduced cortical growth. We showed a similar reduction in neuroblasts within the SVZ of human infants born with CHD.¹⁵

In the current study we examine the impact of cardiopulmonary bypass on neurogenic activity of the SVZ as well as growth and maturation of the frontal lobes.

Methods

Animals

A total of 49 Yorkshire pigs were studied. CPB results in SIRS due to various factors including the repeated passage of blood through non-endothelial surfaces.¹⁶ In addition, CHD patients are at risk of global cerebral ischemia during CPB resulting from a steal of blood by aortopulmonary collateral vessels and/or co-existing cerebral vessel abnormalities.^{17,18} Emboli from solid particles or gaseous origin due to CPB are also an important cause of ischemia/reperfusion (I/R) injury.¹⁷ Finally, the circulatory arrest technique involves risk of I/R-injury such as the “no-reflow phenomenon”.¹⁹ To investigate the effects of CPB on postnatal neurogenesis in the SVZ and frontal lobe development, CPB-induced brain insults were designed for this study to be primarily a result of SIRS alone or both SIRS and I/R-injury (Fig 1A). A total of 29 female piglets at 3 weeks of age were randomly assigned to one of three groups with different CPB-induced brain insults that involved I/R injury and SIRS (1) No surgery (control, no insult, $n = 9$); (2) 34°C full-flow bypass for 60 min (mild-CPB insult, CPB-induced SIRS, $n = 10$); and (3) 25°C hypothermic circulatory arrest (HCA) for 60 minutes (severe-CPB insult, CPB-induced SIRS with I/R injury, $n = 10$) (Fig 1A). Animals were euthanized at either day 3 ($n = 12$) or week 4 ($n = 17$) post CPB to determine time-related changes in postnatal SVZ neurogenesis and frontal lobe development after cardiac surgery. Heterologous blood was obtained from a donor pig ($n = 20$). We performed all experiments in compliance with the National Institutes of Health’s

“Guide for the Care and Use of Laboratory Animals” and the study was approved by the Animal Care and Use Committee at Children’s National Hospital.

Juvenile Porcine CPB Model

A thoracotomy was performed, and an arterial cannula and a venous cannula were inserted after heparinization. Fresh whole blood from a donor pig was transfused into the CPB circuit. The pump prime included normal saline, methylprednisolone, furosemide, sodium bicarbonate and cephazolin. The animals were perfused for 10 minutes at 37°C and then cooled to 25°C or 34°C (Table 1). After cooling, HCA or maintenance of full flow was chosen (Table 1). After 40 minutes of rewarming, animals were weaned from CPB. The hematocrit was maintained at 30% and pH-stat strategy was applied (Table 1).^{17,20} Blood pressure and temperature were monitored continuously. Arterial PO₂ and PCO₂, arterial pH, hematocrit value, and arterial lactate were measured. We used tissue oxygenation index (TOI) with Near-Infrared spectroscopy (NIRO-300) to assess cerebral ischemic status. Systemic leukocyte counts were performed to identify systemic inflammation. Neurological and behavioral evaluations were performed at 24 hour intervals beginning on post-operative day 1. A total score of 400 indicates brain death while a score of 0 is considered normal. The score was accessed in a blinded fashion.^{9,10} The details were described previously.^{8–10}

Cellular Assessment

On post-operative day 3 or week 4 brains were resected and further post-fixed. Sections at 20 µm thickness were incubated in primary and secondary antibodies as described previously.^{9,15} The following antibodies were used for immunohistochemistry: anti-GFAP (1:1000; DAKO), Sox2 (1:500; Millipore), anti-ki67 (1:1000; BD Biosciences), anti-Doublecortin (1:1000; Millipore), anti-NeuN (1:500; Millipore), anti-Cleaved Caspase-3 (1:1000; Cell Signaling), anti-Calretinin (1:1000; Millipore), anti-Somatostatin (1:100; Millipore), anti-Parvalbumin (1:500; Millipore) and anti-Tbr1 (1:200; Abcam). Analysis of the SVZ was performed on coronal sections of the brain, adjacent to the wall of the lateral ventricles. We previously demonstrated that the anterior SVZ displays the most proliferating NSPCs and resembles its human counterpart.¹⁵ Therefore we focused our analysis on the anterior SVZ (Fig 1B, C). An imaginary line was drawn to divide the anterior SVZ into two regions: dorsolateral and ventral (Fig 1D). The SVZ was further subdivided into three tiers based on the density and distribution of DCX⁺ cells as previously described in human brain.¹⁴ All images were acquired with a Leica SP5

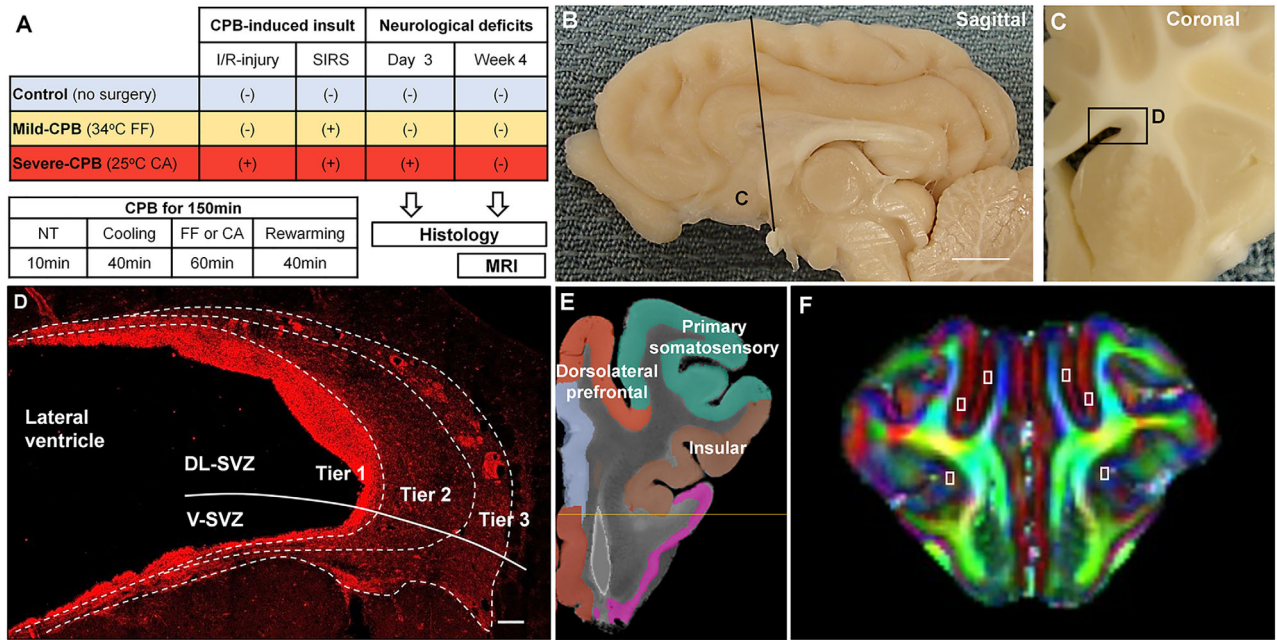


FIGURE 1: Methods. (A) Overall study design. (B) Sagittal plane of the porcine brain. Scale bar, 1 cm. (C) Coronal section and the SVZ (box D). (D) Magnified image from the boxed region in (C) and subdivision of the SVZ with doublecortin stain. Scale bar, 100 μ m. (E) Anatomical location of cortical ROIs (Prefrontal, Primary Somatosensory, and Insular cortices). (F) DTI FA image of pig brain showing ROI of assessed cortical regions. CA = circulatory arrest; CPB = cardiopulmonary bypass; DL-SVZ = dorsolateral-SVZ; FF = full-flow perfusion; I/R = ischemia/reperfusion; NT = normothermia; SIRS = systemic inflammatory response syndrome; SVZ = subventricular zone; V-SVZ = ventral-SVZ.

confocal laser-scanning microscope and counted in a blinded fashion. Given high cell density and homogeneous cell distribution, DCX⁺ cell density was quantified by acquiring a total of three images for each region from separate microscopic fields at $\times 40$ magnification. For other stainings, the whole quantified area was scanned at $20\times$ and tiled to avoid biased counting due to heterogeneous distribution in the large surface area. Regarding DCX⁺ cluster analysis, only clusters with a surface larger than $140\ \mu\text{m}^2$ were counted in the analysis. The cluster occupancy was calculated by dividing the sum of all clusters surface area by the SVZ total surface area. The length of GFAP⁺ processes lining the SVZ was perpendicularly measured every $50\ \mu\text{m}$ along the surface of the ventricle in a tile scan. For cellular analysis within the cortex, the following cortices were scanned separately based on the anatomical atlas and functional cortical map of the porcine brain: prefrontal cortex, somatosensory cortex, and insular cortex (Fig 1E,F).

MRI and DTI

We performed a total of 9 MRI studies (control; $n = 3$, mild-CPB; $n = 3$, severe-CPB; $n = 3$) at week 4 after CPB (Fig 1A). The images were transferred offline and reviewed by an MR physicist and a neuroradiologist. Conventional anatomical T1 and T2 MRI and diffusion tensor imaging (DTI) images were acquired on a GE Sigma

HDxt 3.0 T scanner. A minimum of 19 serial, coronal MRI images per piglet were analyzed for volumetric assessment and the gyrification index (GI).¹⁵ For DTI analysis: nine serials of coronal images per piglet were used to analyze fractional anisotropy (FA) value in cortical grey matter. We applied a region-of-interest (ROI) approach to test specific structures throughout the brain selected. All ROIs were drawn and quantified manually by a blinded experimenter as we performed previously.⁸

Statistics

One-way analysis of variance (ANOVA) with Tukey's post-hoc comparisons was used to evaluate cellular and imaging outcome between the three treatment groups. Two-way ANOVA was used to look at CPB insult effects within SVZ and cortical regions, cortical layers, cell types or over time. Group-by-time and group-by-region interaction terms were evaluated in the ANOVA models. Statistical analysis was performed with PRISM software (GraphPad Software, Inc., La Jolla, CA). Significance was determined as two-tailed $p < 0.05$ using Tukey post-hoc adjustment to control the experiment-wise error due to multiple comparisons.²¹ Sample sizes of piglets for treatment and exposure groups provided 80% power to detect moderate to large effect sizes regarding neuronal cell measures at 3 days and 4 weeks post-surgery and group

TABLE 1. Experimental Conditions

	Mild CPB (n = 10)		Severe CPB (n = 10)		<i>p</i> value
	Mean	SD	Mean	SD	
Temperature (°C)					
Pre CPB	36.2	1.0	36.2	0.8	0.9999
Cooling	33.8	0.3	26.6	0.7	<0.0001 ^a
FF or CA	33.1	0.3	24.7	0.8	<0.0001 ^a
Rewarming	36.4	0.9	35.6	0.9	0.2399
3 h	37.0	0.8	37.0	0.5	0.9999
MBP (mmHg)					
Pre CPB	83.9	19.6	80.7	15.0	0.9999
Cooling	99.7	14.1	95.5	12.9	0.9999
FF or CA	113.5	17.1	5.4	1.9	<0.0001 ^a
Rewarming	130.4	18.3	114.9	17.9	0.3526
3 h	92.8	14.9	87.1	13.0	0.9999
pH					
Pre CPB	7.585	0.098	7.582	0.082	0.9999
Cooling	7.489	0.036	7.471	0.042	0.9999
Rewarming	7.546	0.033	7.458	0.034	<0.0001 ^a
3 h	7.605	0.047	7.570	0.053	0.5423
PaCO ₂ (mmHg)					
Pre CPB	32.1	6.7	33.3	6.5	0.9999
Cooling	42.8	3.1	41.8	2.2	0.9999
Rewarming	41.5	3.4	39.3	2.7	0.5092
3 h	32.4	3.8	30.4	3.3	0.8892
Hematocrit (%)					
Pre CPB	35.5	3.6	36.9	2.3	0.9999
Cooling	34.1	1.5	33.9	1.6	0.9999
Rewarming	32.4	1.8	33.7	1.8	0.4967
3 h	36.9	3.7	33.7	1.9	0.1200
TOI					
Pre CPB	47.7	5.3	47.2	2.4	0.9999
Cooling	58.1	3.4	64.7	5.4	0.0236 ^a
FF or CA	57.6	2.6	34.7	4.4	<0.0001 ^a
Rewarming	53.7	2.8	53.2	1.6	0.9999
3 h	48.9	1.9	47.3	4.1	0.9999
Lactate (mmol/l)					
Pre CPB	1.4	0.4	1.4	0.4	0.9999
Cooling	2.4	0.8	2.5	0.5	0.9999
Rewarming	1.8	0.9	6.9	1.3	<0.0001 ^a
3 h	2.0	0.9	5.4	1.4	<0.0001 ^a
Leukocyte (10 ³ /μl)					
Pre CPB	5.4	1.4	6.9	1.2	0.0787
Cooling	5.7	1.8	5.3	1.4	0.9999
Rewarming	5.9	1.9	7.8	3.3	0.5948
3 h	11.8	4.5	14.0	3.7	0.9560

^aStatistically significant.

CPB = cardiopulmonary bypass; MAP = mean arterial pressure; TOI = tissue oxygen index.

differences in cortical impairment.²² All statistical analyses were reviewed by a biostatistics expert (D.Z.).

Results

Cardiopulmonary Bypass Alters Neurogenesis in the Piglet SVZ and Disrupts Neuroblast Migrating Chains toward the Frontal Lobe

There were no differences among CPB groups in preoperative conditions (Tables 1 and 2). The TOI value during HCA was significantly lower than full-flow CPB (Table 1). The index was then normalized during the rewarming period (Table 1) demonstrating I/R injury in the severe-CPB insult (Fig 1A). Lactate levels during cooling and at 3 hours post CPB were significantly higher in the severe-CPB group compared to the mild-CPB group (Table 1). Both mild- and severe-CPB insults caused a significant increase in leukocyte numbers 3 hours post CPB, but no difference was detected between the two CPB groups (Time $p < 0.0001$, CPB $p = 0.1413$) suggesting SIRS in the mild- and severe-CPB groups (Fig 1A). Severe-CPB insult caused acute neurological symptoms, including seizures, whereas mild-CPB insult did not (Table 2). Neurological symptoms improved with time and were not present on postoperative day 7 (Table 2).

The SVZ retains its highly mitotic potential throughout postnatal life. We first examined the NSPC pool within the whole SVZ 3 days post-surgery. Although there were no differences in the density of Sox2⁺ NSPCs at postoperative day 3 (control vs mild $p = 0.0605$, control vs severe $p = 0.0916$, mild vs severe $p = 0.9743$), detailed examination of the SVZ revealed a trend toward reduced NSPC proliferative capacity in the severe group compared to control (Fig 2A–D). On the other hand, differences between CPB groups were not observed (Fig 2D, E). There were no differences in the proliferative capacity between dorsolateral- and ventral-SVZ (Fig 2E) as well as between analyzed tiers ($p = 0.2198$), indicating no regional effects. Neurogenic activity was further assessed by quantifying the average length of GFAP⁺ processes spanning tier 1 and expressed within radial-glia like cells (Fig 2F–I). Both mild and severe groups displayed reduced GFAP⁺ process length. This effect was confined to the dorsolateral part of the SVZ, as no differences were observed in the ventral part (Fig 2J). These results are consistent with our previous studies showing that the dorsolateral SVZ is a more active neurogenic region than the ventral SVZ.¹⁵

When we analyzed neuroblasts within the SVZ, decreased density of DCX⁺ neuroblasts was observed in

TABLE 2. Changes in Body Weight and Neurological Outcome

	Control (n = 4–10)	Mild CPB (n = 4–10)		Severe CPB (n = 4–10)						Control vs mild	Control vs severe	Mild vs severe
	Mean	SD	Mean	SD	Mean	SD	<i>F</i> value	<i>p</i> value				
Weight (kg)												
Pre	5.1	0.7	5.0	0.8	5.0	0.4	0.072	0.930				
Day 3	5.4	0.8	5.0	0.9	4.9	0.6	1.280	0.293				
Week 4	10.5	1.7	9.1	1.8	9.1	2.2	1.161	0.338				
NDS												
Day 1	0.0	0.0	29.0	26.0	150.0	35.2	99.140	<0.0001 ^a	0.041 ^a	<0.0001 ^a	<0.0001 ^a	<0.0001 ^a
Day 2	0.0	0.0	0.0	0.0	89.0	52.0	29.350	<0.0001 ^a	0.9999	<0.0001 ^a	<0.0001 ^a	<0.0001 ^a
Day 3	0.0	0.0	0.0	0.0	61.5	32.5	35.820	<0.0001 ^a	0.9999	<0.0001 ^a	<0.0001 ^a	<0.0001 ^a
Day 4	0.0	0.0	0.0	0.0	26.7	35.5	3.395	0.061	0.9999	0.094	0.094	0.094
Day 5	0.0	0.0	0.0	0.0	12.5	14.8	4.310	0.033 ^a	0.9999	0.055	0.055	0.055
Day 6	0.0	0.0	0.0	0.0	6.7	10.3	2.500	0.116				
Day 7	0.0	0.0	0.0	0.0	0.0	0.0						

^aStatistically significant.

CPB = cardiopulmonary bypass; NDS = neurological deficit score.

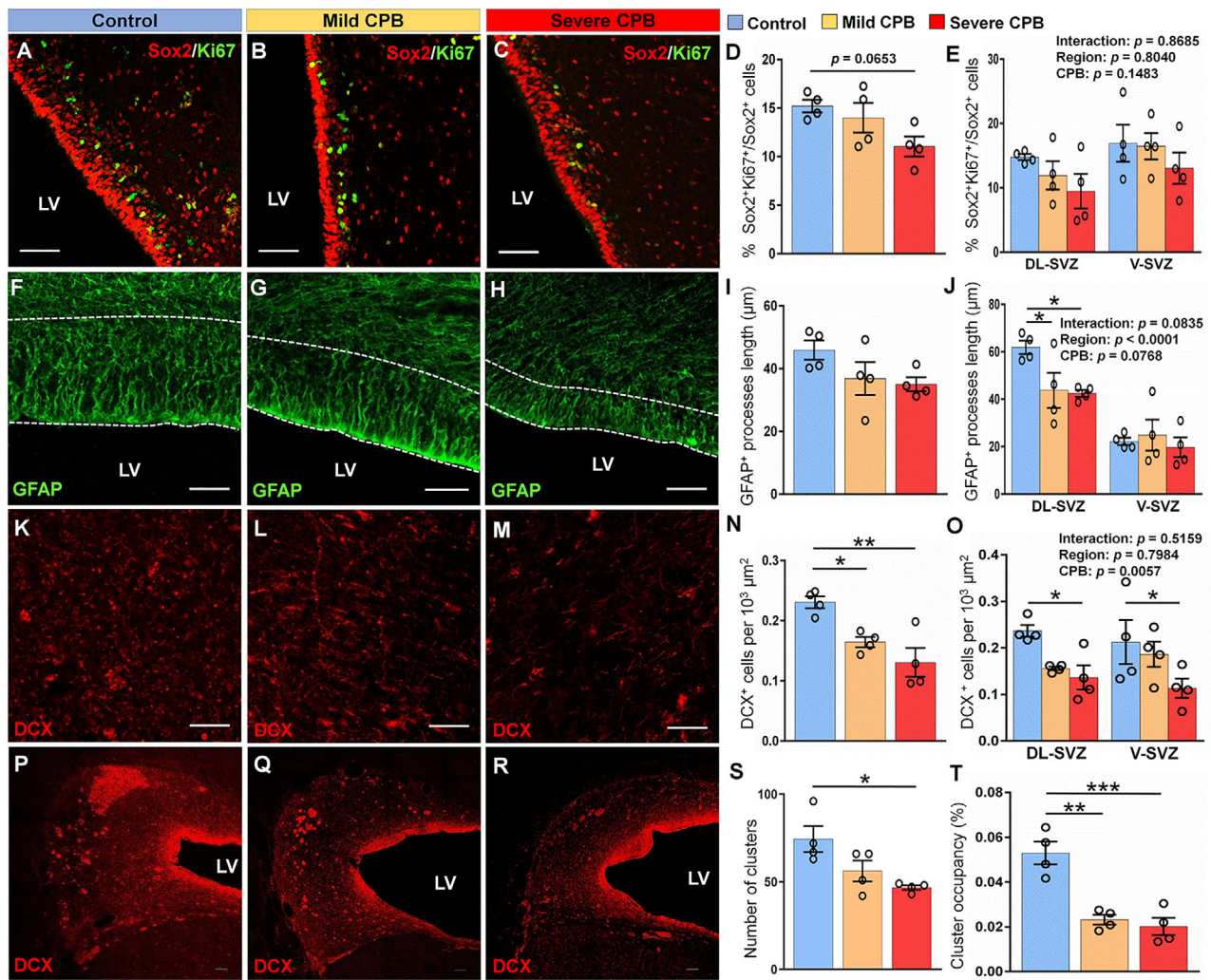


FIGURE 2: CPB impairs postnatal neurogenesis in the piglet SVZ 3 days post-surgery. (A–C) Immunostains of Sox2⁺Ki67⁺ cells in the DL-SVZ. (D, E) Quantification of the percentage of proliferative Sox2⁺ neural stem/progenitor cells. (F–H) Immunostains of GFAP in the DL-SVZ. (I, J) Quantification of the average length of GFAP⁺ processes. (K–M) Immunostains of DCX⁺ cells in tiers 2–3 of the DL-SVZ. (N, O) Quantification of the average density of DCX⁺ cells. (P–R) Coronal sections of the SVZ showing immunostains of DCX⁺ cell clusters. (S) Quantification of the average number of DCX⁺ cell clusters. (T) Quantification of the average cluster occupancy of the SVZ. Scale bars, 50 μm (A, B, C, F, G, H, K, L, M) and 100 μm (P, Q, R). $n = 4$ animals per group. Data are expressed as mean ± SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, one-way ANOVA with Tukey's post-hoc test (D, I, N, S, T) and two-way ANOVA with Tukey's post-hoc test (E, J, O). CPB = cardiopulmonary bypass; DL-SVZ = dorsolateral-SVZ; DCX = doublecortin; GFAP = glial fibrillary acidic protein; LV = lateral ventricle; SVZ = subventricular zone; V-SVZ = ventral-SVZ.

the peripheral region (tiers 2–3) in both mild and severe-CPB groups compared to control (Fig 2K–O). While the effect of CPB on radial-glia like cells (ie, neural stem cells) was restricted to the dorsolateral region (Fig 2J), the density of neuroblasts was decreased in both dorsolateral and ventral parts of the SVZ after CPB (Fig 2O). Although our previous studies found a unique role of HCA (ie, severe-CPB insult) on developing white matter,⁹ we did not observe differences between mild and severe-CPB groups in NSPC proliferation, morphology of radial-glia like cells, or neuroblast density during the acute period after surgery. Together these data demonstrate that CPB

alone can limit SVZ neurogenesis and reduce neuroblast density within the anterior dorsolateral-SVZ, the largest progenitor pool of the postnatal porcine brain.

A previous study in the human infant brain identified a chain-like structure of actively migrating DCX⁺ neuroblasts with adherent junctions.¹⁴ These chains principally located in the peripheral-SVZ (ie, Tier 3) have been associated with a tangential migration oriented toward the developing frontal cortex. To determine whether CPB-induced brain insults also alter neuroblast migratory chains, we analyzed DCX⁺ clusters - coronal view of the migratory stream - within the SVZ (Fig 2P–R). Although the average size of

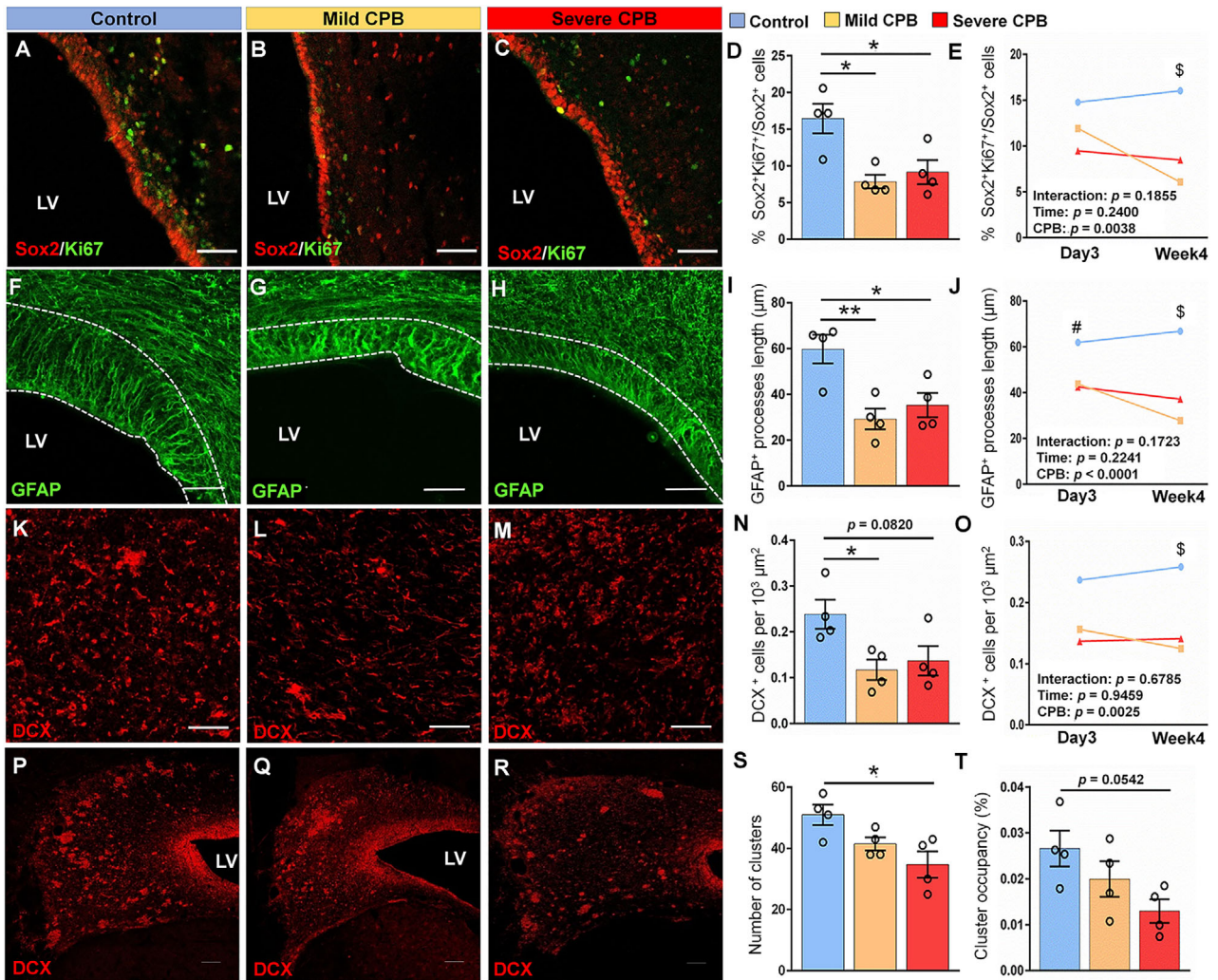


FIGURE 3: CPB causes a long-term disruption of SVZ neurogenesis 4 weeks post-surgery. (A–C) Immunostains of Sox2⁺Ki67⁺ cells in the DL-SVZ. (D) Quantification of the percentage of proliferative Sox2⁺ cells in the whole anterior SVZ. (E) Differences in the percentage of proliferative Sox2⁺ cells in the DL-SVZ between day 3 and week 4 post-surgery. (F–H) Immunostains of GFAP in the DL-SVZ. (I) Quantification of the average length of GFAP⁺ processes in anterior SVZ. (J) Differences in the average length of GFAP⁺ processes in the DL-SVZ between day 3 and week 4 post-surgery. (K–M) Immunostains of DCX⁺ cells in tiers 2–3 of the DL-SVZ. (N) Quantification of the average density of DCX⁺ cells. (O) Differences in the average density of DCX⁺ cells in the DL-SVZ between day 3 and week 4 post-surgery. (P–R) Coronal sections of the SVZ showing immunostains of DCX⁺ cell clusters. (S) Quantification of the average number of DCX⁺ cell clusters. (T) Quantification of the average cluster occupancy of the SVZ. Scale bars, 50 μm (A, B, C, F, G, H, K, L, M) and 100 μm (P, Q, R). n = 4 animals per group. Data are expressed as mean ± SEM. **p* < 0.05, ***p* < 0.01, #*p* < 0.05 control compared to severe group, \$*p* < 0.05 control compared to severe and mild groups. One-way ANOVA with Tukey's post-hoc test (D, I, N, S, T). CPB = cardiopulmonary bypass; DL-SVZ = dorsolateral-SVZ; DCX = doublecortin; LV = lateral ventricle; SVZ = subventricular zone; V-SVZ = ventral-SVZ; GFAP = glial fibrillary acidic protein.

clusters was not different between the three groups tested, the number of clusters within the SVZ was reduced after severe-CPB insults (Fig 2S). Our analysis also revealed that the overall occupancy of the clusters was significantly reduced in both mild and severe-CPB groups (Fig 2T). In addition to reduction in SVZ neurogenesis, these data suggest that cardiac surgery during the early postnatal period alters neuroblast colonization into the frontal lobe of the neonatal and infant brain.

Cardiopulmonary Bypass Causes Prolonged Alterations in SVZ Neurogenic Properties and Neuroblast Migrating Chains

To determine the long-term effects of cardiac surgery on SVZ neurogenic activity and the neuroblast population, we next examined the SVZ 4 weeks postoperatively. NSPC proliferation in the SVZ was significantly decreased for both mild- and severe-CPB groups (Fig 3A–D). The extensive neurogenic activity of these regions has been

described during the postnatal period.¹⁵ Consistent with our previous findings, this prolonged decrease of proliferative capacity was specifically observed in the dorsolateral-SVZ (Fig 3E). Our results also indicate that CPB insults significantly altered the number of Sox2⁺ NSPCs ($p = 0.0452$). Interestingly changes in NSPC proliferation from day 3 to week 4 significantly differed according to the type of CPB-induced insult (Fig 3E). In the naïve SVZ, approximately 15% of NSPCs are able to maintain their proliferative capacity during this early postnatal period (Fig 3E). In severe-CPB group, the proliferation of

SVZ NSPCs was significantly inhibited as soon as 3 days after surgery and remained consistently low over time (Fig 3E). On the other hand, we found that only 6% of Sox2⁺ NSPCs on average can proliferate 4 weeks after CPB insult (Fig 3E), indicating the critical role of systemic inflammation on the prolonged decline of proliferative ability after pediatric cardiac surgery. These observations were coupled to a marked reduction of GFAP⁺ process length after both mild- and severe-CPB insults (Fig 3F–I). Similar to the effects on NSPC proliferation, the impact of CPB on radial glia-like cells was especially deleterious

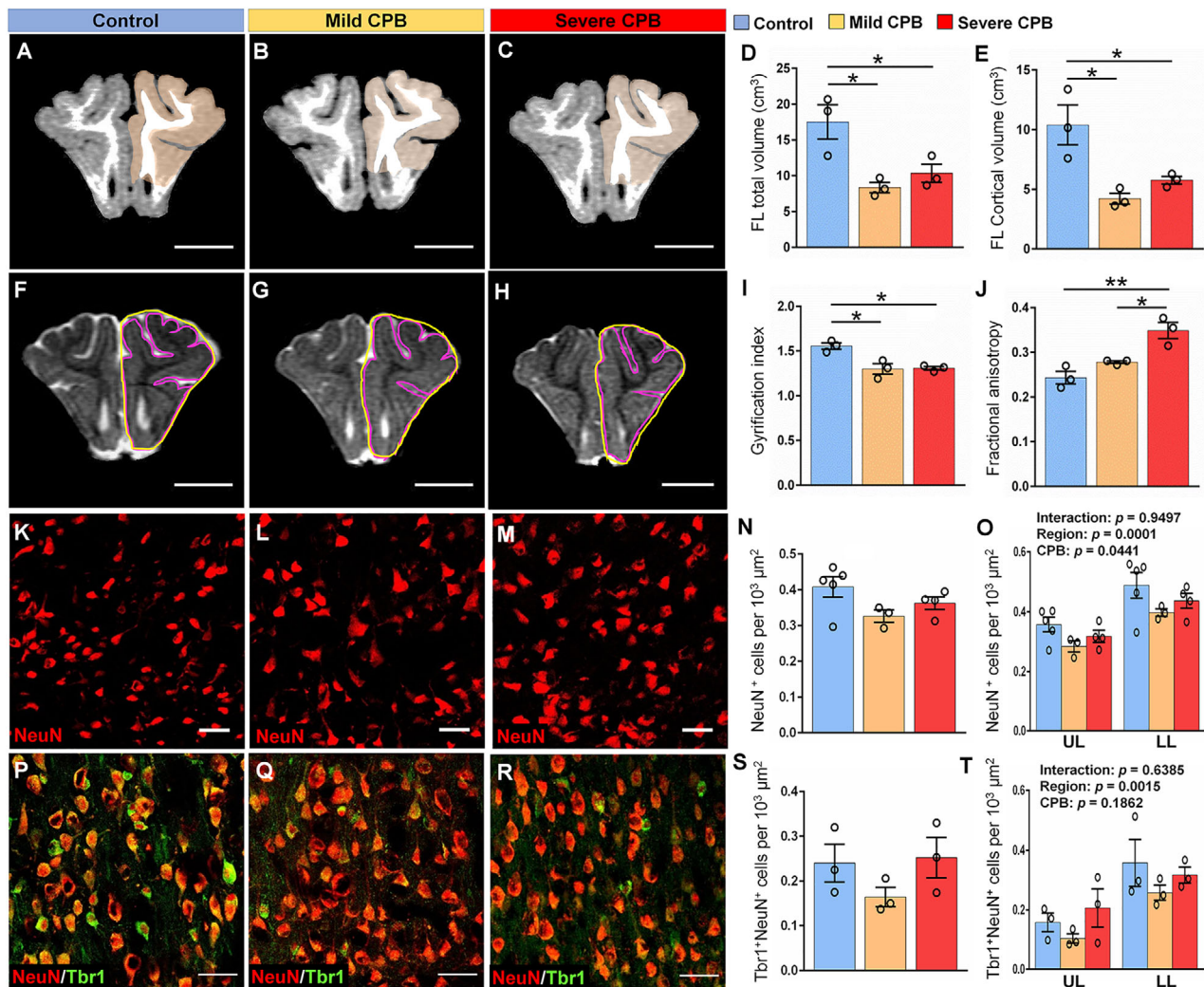


FIGURE 4: CPB impairs cortical development and limits cortical expansion. (A–C) T1 MRI scans showing cortical gray matter of frontal lobe. Scale bars, 1 cm. (D, E) Quantification of frontal lobe total and cortical volumes. (F–H) Perimeter of the total pial surface (purple lines, inner) and total perimeter of the brain (yellow lines, outer) on T2 MRI scans. Scale bars, 1 cm. (I) Quantification of cortical folding determined by the GI expressed as a ratio of the inner versus outer perimeter traces in (F, G, H). (J) Quantification of fractional anisotropy in the cortex of frontal lobe. (K–M) Immunostains of NeuN within the lower cortical layers of the frontal lobe. Scale bars, 50 μm. (N, O) Quantification of the average density of NeuN⁺ cells in the frontal lobe. (P–R) Immunostains of NeuN and Tbr1 within the lower cortical layers of the frontal lobe. (S, T) Quantification of the average density of NeuN⁺Tbr1⁺ cells in the frontal lobe. Scale bars, 50 μm. Data are expressed as mean ± SEM ($n = 3$ –4 animals per group). * $p < 0.05$, ** $p < 0.01$. One-way ANOVA with Tukey's post-hoc test (D, E, I, J, N, S) and two-way ANOVA with Tukey's post-hoc test (O, T). CPB = cardiopulmonary bypass; FL = frontal lobe; LL = lower layers; NeuN = neuronal nuclear protein; Tbr1 = T-box brain 1; UL = upper layers.

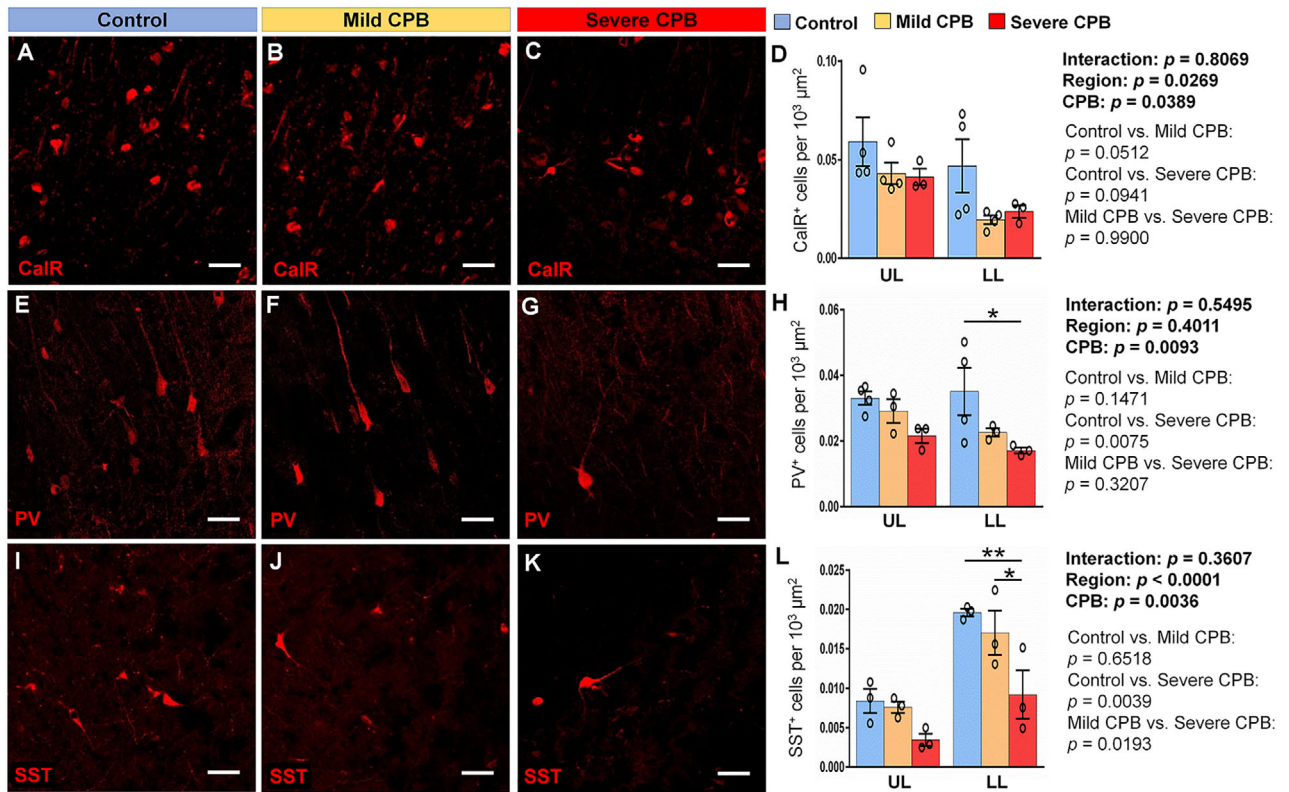


FIGURE 5: CPB alters interneuron cell populations in the cortex 4 weeks post-surgery. (A–C) Immunostains of Calretinin within the lower cortical layers of the frontal lobe. (D) Quantification of the average density of CalR⁺ cells. (E–G) Immunostains of Parvalbumin within the lower cortical layers of the frontal lobe. (H) Quantification of the average density of PV⁺ cells. (I–K) Immunostains of Somatostatin within the lower cortical layers of the frontal lobe. (L) Quantification of the average density of SST⁺ cells. Scale bars, 50 μm. Data are expressed as mean ± SEM (n = 3 to 4 animals per group). * $p < 0.05$, ** $p < 0.01$, two-way ANOVA with Tukey's post-hoc test (D, H, L). CalR = calretinin; CPB = cardiopulmonary bypass; FL = frontal lobe; LL = lower layers; PV = parvalbumin; SST = somatostatin; UL = upper layers.

in the dorsolateral-SVZ where GFAP⁺ processes of radial glia-like cells remained shorter over time in both CPB groups compared to control (Fig 3J). Altogether these results demonstrate the significant and prolonged deleterious impact of cardiac surgery on the largest NSPC pool in the neonatal and infant brain.

When we assessed the long-term effect of cardiac surgery on SVZ neuroblasts, reductions in cell density in the whole SVZ were displayed in both CPB groups (Fig 3K–N). Consistent with our findings for NSPCs, the neuroblast population was reduced specifically in the dorsolateral-SVZ 4 weeks post CPB in both mild and severe insult groups (Fig 3O), whereas no differences were observed in the ventral-SVZ (control vs mild $p = 0.1316$, control vs severe $p = 0.3868$, mild vs severe $p = 0.7261$). Moreover, similar to NSPCs, there was no recovery in neuroblast density over time (Fig 3O), indicating prolonged inhibition of SVZ neurogenesis due to CPB. Finally, a marked reduction of cluster number and a trend in cluster occupancy was shown 4 weeks after severe-CPB (Fig 3P–T). This latter observation specifically found in the group suggests a high susceptibility of migratory

processes in neuroblasts to HCA-induced injury (Fig 1A). Overall, our cellular analysis of the SVZ up to 4 weeks post-operatively provides evidence that a CPB-induced insult not only reduces postnatal SVZ neurogenesis and neuroblast migration in the short-term, but also prevents recovery of the neurogenic properties of the largest progenitor pool for a prolonged period.

Cardiopulmonary Bypass Impairs Cortical Development and Limits Cortical Expansion

Based on our previous finding that SVZ-derived cells populate the frontal cortex in neonatal piglets during normal development,¹⁵ we hypothesized that a reduction in the neuroblast number in the SVZ and structural alteration of neuroblast clusters could play an important role in cortical maturation after cardiac surgery. To test this hypothesis, we first assessed structural maturation of the developing cortex using a clinically-relevant neuroimaging approach 4 weeks after surgery. We did not observe any evidence of focal and/or multifocal injury post CPB. When the volumes of the entire frontal lobe and frontal cortex were quantified, however, both measurements in the mild and

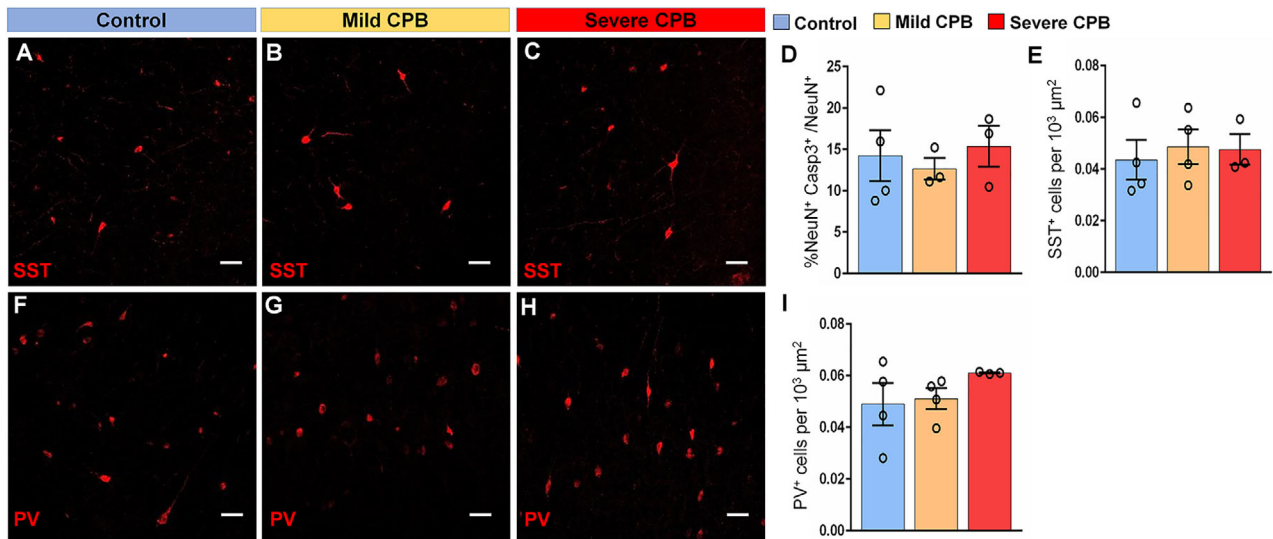


FIGURE 6: CPB does not alter interneuron densities in the piglet cortex 3 days after surgery. (A–C) Immunostains of Somatostatin within the lower cortical layers of the frontal lobe. (D) Quantification of the average percentage of Casp3⁺ NeuN⁺ cells within the piglet frontal lobe. (E) Quantification of the average density of SST⁺ cells. (F–H) Immunostains of Parvalbumin within the lower cortical layers of the frontal lobe. (I) Quantification of the average density of PV⁺ cells. $n = 3$ to 4 animals per group. Two-way ANOVA with Tukey's post-hoc test. Scale bars, 50 μm . Casp3 = cleaved-caspase 3; NeuN = neuronal nuclear protein; PV = parvalbumin; SST = somatostatin.

severe insult groups were significantly decreased compared to control (Fig 4A–E). There were no differences in volumes between right and left hemispheres (total volume; $p = 0.7211$, cortical volume; $p = 0.9887$) indicating a global impact. Consistent with previous findings in gyrencephalic brain development,^{23,24} these observations were paired with a significant reduction in GI (Fig 4F–I), demonstrating structural brain immaturity. Reduced cortical volume and impaired gyrification as observed in this animal model have been well documented in survivors after pediatric cardiac surgery.²⁵ Moreover, when we further compared DTI metrics between the three experimental groups, we found that animals exposed to a severe-CPB insult showed a significant increase in FA (Fig 4J). FA values in the severe group were higher compared to mild-CPB FA values. As cortical microstructural maturation is associated with FA decrease,²⁶ these observations indicate that CPB-induced systemic inflammation can cause aberrations in macrostructural cortical development, whereas HCA-induced injury during CPB induces microstructural changes.

To further understand the relationship between immature cortical development and depletion of neuroblasts in the SVZ, mature neuronal populations were analyzed within upper and lower layers of frontal cortices (Fig 4K–M). We found that the density of NeuN⁺ mature neurons varies between both cortical layers and experimental groups (Fig 4O). Despite no statistical differences in the average density of neurons between the three groups (Fig 4N), together

with decreased cortical volume in the mild and severe groups (Fig 4E) the results indicate an overall reduction in the total number of mature neurons in the frontal cortex after CPB.

Quantification of Tbr1⁺NeuN⁺ excitatory neurons showed a significant difference between layers as for NeuN⁺ cells (Fig 4O–T). Consistent with our previous findings,¹⁵ higher densities of excitatory neurons were found in the cortical lower layers compared to upper layers (Fig 4T). On the other hand, no differences were observed in the density of excitatory neurons between CPB groups throughout the frontal lobe (Fig 4P–T). Similarly, we did not observe differences in the densities of NeuN⁺ mature neurons ($F = 0.72$, $p = 0.49$) and Tbr1⁺NeuN⁺ excitatory neurons ($F = 0.89$, $p = 0.43$) between prefrontal, somatosensory and insular cortices. Altogether our data demonstrate that CPB impairs cortical expansion and microstructural maturation in an insult-dependent manner and limits the number of mature neurons in the frontal cortex of the infant brain.

Cardiopulmonary Bypass Alters Specific Subpopulations of Interneurons in the Frontal Cortex

Given previous findings demonstrating that postnatal migrating neuroblasts differentiate into inhibitory neurons,^{14,15} we next examined the effects of CPB on GABAergic interneuron populations (ie, inhibitory neurons) throughout the frontal cortex. Both experimental groups ($F = 4.58$, $p = 0.0224$) and distinguished cell types ($F = 27.31$, $p < 0.0001$) significantly affected the

cell population 4 weeks post CPB. We found that CPB induced a significant change in the densities of three major GABAergic cell subpopulations: CalR⁺, PV⁺ and SST⁺ interneurons (Fig 5A–L, CPB: $p < 0.05$). Moreover, a severe-CPB insult induced a significant decrease in densities of both PV⁺ and SST⁺ interneurons compared to control, specifically in lower cortical layers (Fig 5E–L). There were no regional differences in the CPB-induced reduction of three interneuron populations (CalR⁺ cells; $p = 0.1709$, PV⁺ cells; $p = 0.0860$, SST⁺ cells; $p = 0.7555$). Notably, SST⁺ interneuron density was also statistically lower in the severe insult group compared to the mild group (Fig 5L). Together with our observations of an unchanged excitatory neuron population (Fig 4S, T), this reduction of interneuron subpopulations 4 weeks after surgery indicates that CPB likely results in excitatory/inhibitory imbalance throughout the frontal cortex.

To assess whether the reduction in interneuron subpopulations was due to CPB-induced cell death or impaired integration of interneurons into the frontal cortex, we examined the effect of CPB on these cortical cell populations 3 days post-surgery. There was no difference in neuronal apoptosis, quantified as percentage of Casp3⁺NeuN⁺ apoptotic neurons per total NeuN⁺ cells (Fig 6D). Moreover, SST⁺ and PV⁺ cell densities were not affected 3 days after CPB (Fig 6A–I), suggesting that cell apoptosis due to CPB is not a major underlying mechanism in the reduction of cortical interneurons observed following CPB. Since severe-CPB specifically decreases cluster number and occupancy in the SVZ for a prolonged period (Fig 3P–T), our results suggest that HCA causes prolonged impairment in neuroblast migration from the SVZ and interferes with the integration of SST⁺ and PV⁺ interneurons into the frontal lobe, thereby causing microstructural alterations of the frontal cortex.

Discussion

This study is the first to reveal the impact of CPB on postnatal SVZ and cortical development in a porcine model which is anatomically and physiologically similar to humans. We demonstrated over a 4-week recovery period that there are significant alterations of the NSPC pool and neuroblast populations in the SVZ resulting in a depletion of a source of interneurons destined to populate and potentially fine-tune development of the postnatal frontal cortex. Furthermore, we noted decreased gyrencephaly and impairment of cortical expansion and maturation, which are pathological signatures observed commonly in survivors of neonatal cardiac surgery for complex CHD. Finally, our study highlights CPB-induced excitatory/inhibitory imbalance throughout the frontal cortex and

the distinct role of CPB-induced I/R injury in cortical dysmaturation. Our findings provide new cellular insights into surgery-induced neonatal brain injury and guide us towards improving management of pediatric cardiac surgery to minimize neurological and behavioral consequences in the CHD population.

In the present study, the mild-CPB group was designed to determine the effects of common stressors in cardiac surgery including SIRS and oxidative stress, while the severe insult group allowed assessment of the additional impact of HCA-induced I/R injury. Our study demonstrated that both mild and severe-CPB insults induced early and late deleterious effects on the largest NSPC pool of postnatal brain and SVZ neurogenesis. It has been well documented previously that NSPCs are highly sensitive to inflammation^{27,28} consistent with our finding of the impact of CPB-induced SIRS on neurogenesis in the neonatal brain. It is encouraging to note that stem cell proliferation can be restored by anti-inflammatory treatments.²⁸

The SVZ generates many neurons endogenously after birth.²⁹ It was recently found that neuroblasts continue to migrate postnatally along the lateral ventricles to populate the frontal lobe in the human infant, contributing to its further maturation.¹⁴ We have previously demonstrated that reductions in NSPC proliferation and SVZ neurogenesis result in reduced cortical growth of the postnatal brain. We also showed that hypoxia affects CalR⁺ interneurons and causes imbalance between excitatory and inhibitory neurons.¹⁵ Consistent with these previous findings, our current data demonstrate reduced cortical folding (GI) and gray matter volume of the frontal lobe as well as excitatory/inhibitory imbalance after both mild- and severe-CPB insults indicating multifactorial etiology of impaired cortical maturation in children with CHD. Interestingly, reduction in these macrostructural MRI indices has been linked to delayed cognitive function.^{30,31} Furthermore, excitatory/inhibitory imbalance in the prefrontal cortex has been strongly associated with intellectual and academic impairment.³² Children with CHD frequently face delays in cognitive milestones that can be attributed to such imbalances.^{3,33} In addition, changes in gamma oscillations, which are a product of the interplay between interneurons and excitatory principal cells,³⁴ are commonly reported in psychiatric conditions.^{35,36} To explore possible therapeutic approaches for improvement of cortical development in children with CHD, it will be important to define molecular targets that can prevent depletion of NSPC neurogenic potential and accelerate endogenous recovery through postnatal SVZ neurogenesis.

Our study demonstrates that I/R stress, only present in the severe group, is critical for the cortical dysmaturation of the frontal lobe determined by DTI.

Similar with our findings, an increase in FA values is observed in the immature cortex of preterm children.^{37,38} Current literature suggests that maturation of the neonatal cortex is associated with increased dendritic complexity which is correlated to decreased FA. Our detailed analysis of neuronal subpopulations revealed that severe-CPB decreases PV⁺ and SST⁺ interneuron populations. SST⁺ interneurons influence other interneuron classes and also target the dendritic spines of pyramidal neurons. It has been demonstrated that this sub-population controls dendritic calcium spikes in pyramidal neurons, thus affecting learning tasks.³⁹ Fast-spiking PV⁺ interneurons regulate local circuit dynamics,⁴⁰ and PV⁺ structural and physiological abnormalities have been strongly linked to psychiatric disorders.⁴¹ Thus, a lack of these inhibitory interneurons could provide some insight into the underlying mechanisms behind microstructural alterations such as reduced dendritic complexity observed after severe-CPB insults. Each interneuron subpopulation has a specific spatial origin and birthdate, and migrates during different developmental time windows.⁴² In the present study, both mild and severe-CPB insults resulted in reduced cluster number and occupancy of neuroblast migratory chains on postoperative day 3, (ie, 3 weeks of age) and affected the CalR⁺ interneuron population. Similarly, in our previous study, chronic hypoxia from postnatal day 3 to 14 reduced CalR⁺ interneurons.¹⁵ On the other hand, we observed reduced migratory chains at 4 weeks post CPB (7 weeks of age) in the severe-CPB group together with decreases in PV⁺ and SST⁺ interneurons. The critical timing of damage in migrating neuroblasts may contribute to the distinct disruptions of this interneuron subpopulation that we noted between the mild and severe CPB groups.

We previously identified that the FA value from diffusion tensor imaging in the same piglet white matter structures at postnatal 3 weeks was equivalent to those of human post-conceptual 41–53 weeks.⁸ On the other hand, GI of 7-week-old control piglet cortex in this study was approximately 1.5, which is similar to that of a human cortex at 37–38 week gestation in previous clinical studies. It is challenging to define the epochs in human cortical maturation that correspond to this piglet model because obvious technical/ethical difficulties prevent cellular investigations in the human brain during the late fetal and postnatal periods. However, our imaging studies indicate that the developmental time windows in the piglet model at this age correspond with the late gestation and early neonatal period in the human brain.

Higher mammals like pigs avoid many of the translational limitations encountered when studying rodents. The porcine brain has a gyrencephalic cortex and a multi-layered SVZ similar to the human SVZ.¹⁵ The pig model

has been widely used to investigate adverse effects of cardiac surgery and CPB.^{17,43} Piglets undergoing CPB display white matter injury similar to that seen in human patients.^{8,9} On the other hand, there are limitations in using juvenile pigs. Compared to studies with rodents, a small number of animals was assessed in each group. Although the deviation of physiological parameters in our studies were limited because of a well-controlled procedure, it is possible that the small animal number caused the lack of difference between the mild and severe groups in NSPC proliferation, morphology of radial-glia like cells, or neuroblast density during the acute period after surgery. In addition, behavior tests used in this study showed that neurological deficits were resolved 1 day after mild-CPB and 7 days after severe-CPB injury, suggesting the limited sensitivity to assess long-term neurological impairments. Compared to rodents, region-specific neurobehavioral tests have not been well established in a porcine animal. Establishment of sophisticated behavior assays will be required for further improvement of this unique model. Our studies found increased cortical fractional anisotropy after severe CPB injury. However, the relationships between DTI signals and neuropathology in the cortex is more complex compared to white matter. For instance, previous studies indicated that the increased FA in the cortex following traumatic brain injury is associated with reactive astrocytes.⁴⁴ In addition, the presence of inflammation with microglia and macrophage activation significantly alters DTI indices.⁴⁵ More detailed analyses using the gyrencephalic piglet cortex will provide important data needed to better interpret clinical human imaging studies. Our findings reflect the response of a normally developed brain to CPB because the animals used in this study did not experience fetal cerebral hypoxia. Therefore, it is possible that infants with complex CHD may experience these stresses differently based on their brain maturation status. Finally, in our studies two CPB injury groups were compared to naïve control, but not surgical control. Therefore, common surgery-induced insults such as anesthesia may affect some of our findings. Brain injury associated with pediatric cardiac surgery are multi-etiological. Although this study distinguished major CPB-induced insults between SIRS and I/R, future studies will be necessary to distinguish each factor individually from the global impact of cardiac surgery. On the other hand, our study provides a unique view of the roles of CPB and I/R on the largest NSPC niche and on cortical development.

In summary, our porcine model has given us insights into the cellular alterations that explain cortical dysmaturation occurring after CPB. Unraveling cellular/molecular events during surgery will enable us to design therapeutic approaches that can be restorative or reparative to the neurogenic potential

of SVZ NSPCs and may reduce the undesirable impact of cardiac surgery. Further studies using this model will likely contribute to continuing refinement of pediatric cardiac surgery thereby improving the quality of life for children with complex CHD.

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Author Contributions

For this manuscript, C.L. and N.I. were responsible for study concept and design. Z.D., C.L., S.L., A.P., J.H., D.Z., and P.C.W. contributed to data acquisition and analysis. Z.D. and C.L. drafted the manuscript and figures with R.A.J. and N.I. Z.D. and C.L. share the first author position. The authorship order among co-first authors was decided by volume of data acquisition used in the manuscript. All authors reviewed the manuscript before submission.

Potential Conflicts of Interest

Nothing to report.

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Review

Nonapoptotic caspases in neural development and in anesthesia-induced neurotoxicity

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Apoptosis, classically initiated by caspase pathway activation, plays a prominent role during normal brain development as well as in neurodegeneration. The non-canonical, nonlethal arm of the caspase pathway is evolutionarily conserved and has also been implicated in both processes, yet is relatively understudied. Dysregulated pathway activation during critical periods of neurodevelopment due to environmental neurotoxins or exposure to compounds such as anesthetics can have detrimental consequences for brain maturation and long-term effects on behavior. In this review, we discuss key molecular characteristics and roles of the noncanonical caspase pathway and how its dysregulation may adversely affect brain development. We highlight both genetic and environmental factors that regulate apoptotic and sublethal caspase responses and discuss potential interventions that target the noncanonical caspase pathway for developmental brain injuries.

Caspases: more than cell death mediators

Cell death is currently understood to occur in one of the four morphologically defined manners: **apoptosis** (see [Glossary](#)), autophagy, necrosis, and entosis [1]. Apoptotic cell death as an intrinsically programmed phenomenon was originally described in the roundworm *Caenorhabditis elegans*. Of the 1090 somatic cells generated during *C. elegans* development, 302 are genetically predetermined to become neuronal cells. Additional 131 cells are produced, however, and these cells undergo programmed cell death with high temporal fidelity during the development of each worm [2]. Unlike in *C. elegans*, mammalian development relies on a more stochastic process of cell elimination [3]. The apoptotic mechanism is orchestrated by **caspases**, cysteine protease enzymes that utilize both other caspase proteins and diverse cellular peptides as their substrates [4].

Caspases are classified into initiator caspases, such as caspase 2, 8, 9, and 10, which are the first to become catalytically active during apoptosis, and effector caspases 3, 6, and 7, which rely on initiator activation and mediate the final steps of programmed cell death [5]. The end result of this sequential protease activity is DNA fragmentation and, ultimately, cell death [4,6]. Apoptosis proceeds either through a death receptor **extrinsic apoptotic pathway** or an intracellular, mitochondria-based intrinsic pathway [7]. The triggers for extrinsic caspase activation involve binding of tumor necrosis factor- α (TNF- α) and Fas to death receptors, while intrinsic, mitochondrial mechanisms are initiated by DNA damage and/or excess reactive oxygen species production [7] ([Figure 1](#)).

Over the course of several decades, important neurodevelopmental and injury response roles have been described for the canonical apoptotic pathway [7–9]. More recently, caspase activation has increasingly been associated with both physiological and pathological nonapoptotic outcomes that do not result in neuronal cell death, yet have significant impacts on cell morphology and function. Caspase activation status and catalytic efficiency is known to be influenced by phosphorylation of the enzymes themselves, as well as their target substrates, by kinases [10–12]. Whether

Highlights

Caspase activity is necessary for normal neuronal development and maturation. Studies are increasingly showing important caspase roles in axonal pathfinding, dendritic arborization, and synaptic plasticity.

There is growing evidence for nonlethal, yet damaging caspase responses to commonly used chemical agents such as ethanol and anesthetics.

Nonlethal, excess caspase activation may result in impaired neuronal arborization and synaptic transmission, leading to long-term behavioral deficits.

Children undergoing surgeries, such as those with congenital heart defects, may be at increased risks for nonapoptotic caspase injuries due to exposure to anesthetics and accompanying genetic susceptibility.

Understanding the molecular and cellular differences between apoptotic and nonlethal caspase activity is crucial for more targeted neuroprotection.

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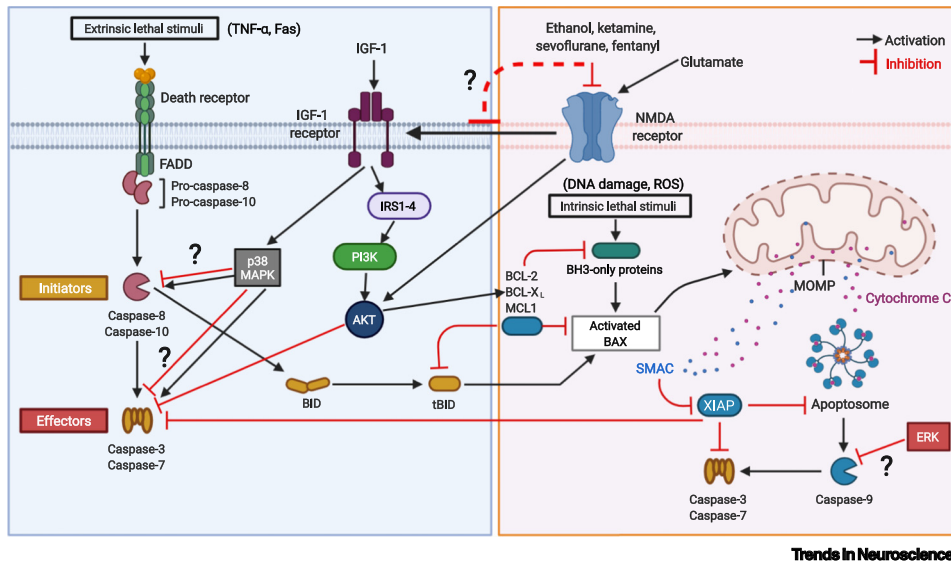


Figure 1. Overview of extrinsic and intrinsic apoptosis and caspase activation modifiers. The flowchart presents a summary of the extrinsic and intrinsic arms of the apoptotic pathway, while highlighting known and hypothetical molecular modifiers of initiator and effector caspase activation. Extrinsic, death receptor-dependent signaling crosstalks with the mitochondrial arm through BCL-2 family proapoptotic proteins such as BID, which when translocated to the mitochondria (translocated BID/tBID) results in activation of the protein BAX, mitochondrial membrane permeabilization, and release of cytochrome C. Cytochrome C forms structures known as apoptosomes, which initiate caspase activation. Effector caspase activation is inhibited by X-linked inhibitor of apoptosis proteins (XIAPs), which are ubiquitin ligases. SMAC acts as a potentiator of apoptosis by inhibiting XIAP function. BCL-2 family antiapoptotic proteins BCL-2 and MCL1 suppress the intrinsic apoptotic cascade, acting to maintain homeostasis. Other signaling pathways such as PI3K/AKT, p38 MAPK, and ERK act as modulators of caspase signaling through their phosphorylation activity. Chemical agents with both neurophysiological and neurotoxic properties, such as glutamate and ketamine, function as agonists and antagonists of NMDA receptors, which interface with intracellular signaling regulators of caspase activation. Differential recruitment of these signaling pathways and their variable interaction with environmental stimuli may underlie functional differences between apoptotic and nonapoptotic caspase activation. The figure was created with [BioRender.com](https://www.biorender.com). Abbreviations: AKT, protein kinase B; BAX, BCL2-associated X protein; BCL-2, B-cell lymphoma 2; Bcl-X_L, B-cell lymphoma-extra large; BH3, Bcl-2 homology 3; BID, BH3 interacting-domain death agonist; ERK, extracellular-regulated protein kinase; FADD, Fas-associated death domain-containing protein; FAS, Fas cell surface death ligand; IGF-1, insulin-like growth factor 1; IRS1–4, insulin receptor substrate 1–4; MAPK, mitogen-activated protein kinase; MCL1, myeloid cell leukemia 1; MOMP, mitochondrial outer membrane permeabilization; PI3K, phosphoinositide 3-kinase; ROS, reactive oxygen species; SMAC, second mitochondria-derived activator of caspases; TNF- α , tumor necrosis factor- α .

Glossary

Apoptosis: a series of intrinsically programmed molecular steps leading to cell death.

Canonical apoptotic signaling: the most well-studied set of reactions leading to cell death, consisting of an extrinsic and intrinsic pathway/arm. Noncanonical apoptotic signaling describes caspase-dependent reactions that do not lead to cell death, yet are important for synaptic pruning, neuritogenesis, and synaptic plasticity.

Caspase: a protease enzyme involved in the execution of apoptosis as well as other nonapoptotic cellular processes.

Extrinsic apoptotic pathway: also termed death receptor pathway, involves binding of an extracellular death ligand (e.g., TNF- α) to a plasma membrane death receptor (e.g., TNF receptor) to initiate apoptosis.

Intrinsic apoptotic pathway: also termed the mitochondrial apoptotic pathway, it is initiated intracellularly, due to DNA damage, ischemia, and oxidative stress.

Sublethal caspase activation: caspase signaling that involves cleaving and activation of effector caspases, without subsequently leading to cell death (as assessed by cell number, nuclear fragmentation via TUNEL staining, and markers of mitochondrial integrity).

caspase activation and the switch to nonapoptotic signaling predominantly depends on levels of caspase phosphorylation and/or cleavage (Figures 1 and 2) or on modulation by other signaling pathways (Figure 1) is unclear. It also remains unknown how precisely this shift in caspase activity diverts the neuron from proceeding with canonical apoptosis to instead undergoing structural changes (see Outstanding questions).

Neuronal structure is of critical importance to the assembly of functional brain circuits [13]. Disruptions of virtually any of the aspects of neuron structure have been implicated in disorders of neurodevelopment, age-related neurodegeneration, as well as various brain injury scenarios. Caspases are known to have phylogenetically conserved roles in the sculpting of neuronal dendritic arbors through cytoskeletal remodeling and have been demonstrated to contribute to synaptic plasticity [4]. In addition, caspase signaling has been linked to excitotoxic injuries in prior work [4]. Programmed cell death and neurodegeneration has been frequently reported in developmental brain injury scenarios such as anesthesia exposure and alcohol-induced neurotoxicity [9,14,15]. Importantly, the neurotoxic potential of excessive nonapoptotic caspase

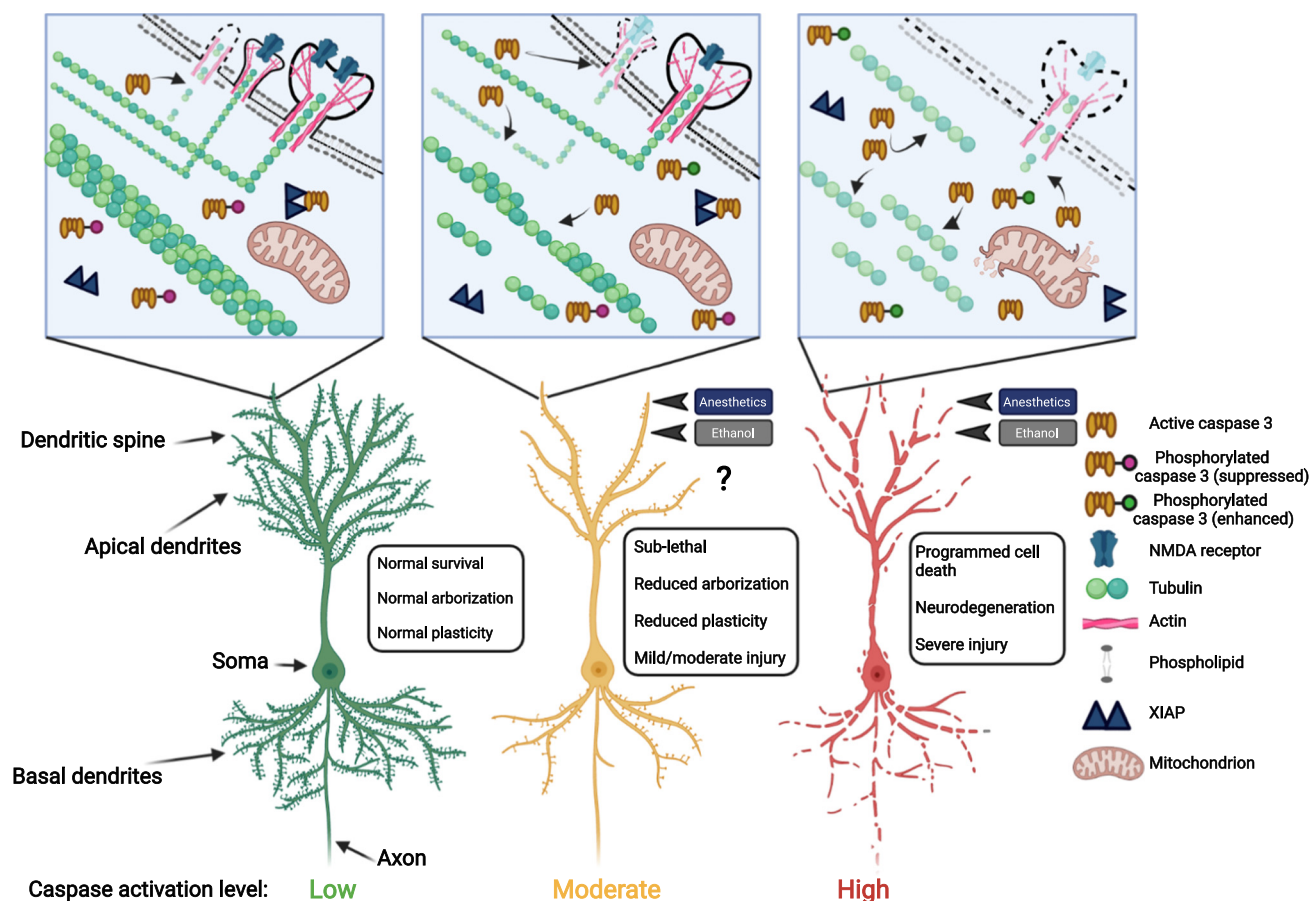


Figure 2. Caspase activation mode and corresponding outcomes in physiological and injury scenarios. The schematic offers a simplified overview of variable, caspase activation level-dependent neurotoxic outcomes (with caspase cleavage and/or phosphorylation levels being key modulators of activation). In pyramidal neurons, dendritic processes contact and receive synaptic inputs from other neurons and enable crosstalk with glial cells. Often, dendrites are covered in dendritic spines, postsynaptic sites of neurotransmission that are dynamically turned over and either stabilized or lost, processes that contribute to synaptic plasticity. These structures are supported and maintained by networks of filament proteins, including actin and tubulin. During normal physiological neuronal development and maturation, a low level of caspase pathway activation is required for dendritic branching and shifts in plasticity required during learning. However, upon exposure to environmental neurotoxins, such as anesthetics and ethanol, enhanced activation of executioner caspases leads to the induction of apoptosis via mitochondria (intrinsic pathway) and extensive cleaving of cytoskeletal proteins. A more complex scenario is increasingly being recognized, whereby a moderate level of caspase activity postexposure may lead to cytoskeletal remodeling, without full cell death. How this level of activation is achieved and modulated is unclear, but may involve variable availability of X-linked inhibitor of apoptosis proteins (XIAPs), genetic susceptibility combined with low-dose neurotoxin exposures, and altered neuronal–glial crosstalk. The figure was created with [BioRender.com](https://www.biorender.com).

signaling has not been well characterized in the developing brain. Given the capacity for caspase activation by commonly used environmental agents, such as volatile anesthetics, ethanol, and opioids, addressing the possibility of nonapoptotic, sublethal neurotoxicity is particularly pertinent.

In this review, we highlight key aspects of nonapoptotic caspase functions during neuronal development and maturation, as well as unaddressed questions concerning their contribution to pathological processes triggered by developmental brain injury. We then summarize recent findings on how the nonapoptotic caspase pathway contributes to long-term neurological deficits and discuss potential therapeutic avenues.

Diverse roles of the nonapoptotic pathway arm

Caspases during neurogenesis

Besides the canonical apoptotic pathway, caspase activity that triggers nonapoptotic events in the cell is involved in a diverse repertoire of roles, including proper immune function, cell proliferation and differentiation during development, cell fate specification, and cytoskeletal function in both vertebrates and invertebrates [16]. In *Drosophila melanogaster*, the fruit fly ortholog of mammalian initiator caspase 9/2, called *dronc*, restricts the expansion of enteroblast cells, a type of intestinal precursor cell, through a nonlethal regulation of the proliferation-differentiation transition [17,18]. In the *D. melanogaster* CNS, *dronc* plays a similar role, suppressing the production of excess sensory neural precursors in a nonapoptotic manner, thereby contributing to a system-level fine-tuning during cell differentiation [19]. Proper differentiation of mammalian neural stem cells also requires nonapoptotic caspase signaling [20]. For instance, the differentiation of cerebellar granule neurons and Bergmann glia is precisely controlled by nonapoptotic activity of caspase 3 [20–22]. Given the extensive caspase-dependent apoptosis in the rodent cerebellar cortex during the first postnatal week [23,24], a key question that remains to be addressed is how the two disparate functions of caspase 3 are spatiotemporally coordinated. A possible explanation is dosage dependence on caspase 3 cleavage levels (Figures 1 and 2). To test this hypothesis, expression constructs encoding catalytically active caspase 3 subunits under the control of variable strength synthetic promoters may be introduced in different neuronal cell lines [25,26]. This strategy could also be used at different stages of neuronal differentiation and maturation *in vivo*, using tissue- and cell-specific promoters, to clarify if levels of active caspase 3 determine the switch from nonapoptotic to apoptotic pathways.

Caspase-mediated cytoskeletal remodeling

One of the major targets of nonapoptotic caspase signaling is the cytoskeleton. Some of the earliest studies of caspase function, focused on understanding canonical cell death signaling, implicated caspases in the destabilization and cleaving of cytoskeletal proteins such as actin and tau [27,28]. Cytoskeletal remodeling and reorganization underlie cellular morphological changes, such as during cell division, movement, and acquisition of a mature morphology. During neural development, the process of axon guidance is necessary for the establishment of correct early wiring patterns and subsequent synaptic maturation. Inhibition of localized caspase 3 and 8 activity in mouse hippocampal neurons leads to a loss of neurite outgrowth [29]. This effect seems to be dependent on caspase-mediated cleaving of spectrin, a plasma membrane-associated cytoskeletal protein necessary for axonal bending and stretching [30,31]. The aforementioned *D. melanogaster* caspase ortholog *dronc* has been shown to cleave F-actin filaments in the developing salivary glands in nonapoptotic fashion and subsequently prune dendritic arbors in a class of sensory neurons [32]. Similarly, axonal misrouting and impaired synaptic contact establishment occurs in olfactory sensory neurons of mice mutant for caspase 9, an initiator caspase [33]. Furthermore, overexpression of a dominant negative mutant caspase 3 precursor in developing chick embryos, specifically in midbrain presynaptic neurons, led to significantly reduced dendritic complexity, as seen through fewer neuronal branch points and higher order branches [34]. Experiments from these studies show a conserved caspase function in neuronal arbor remodeling across different model species that is strongly dependent on cytoskeletal modulation. Cytoskeletal substrate preference seems to vary to some extent between different caspases, as caspase 3 has been shown to cleave both beta-actin and alpha-tubulin, whereas caspase 6 only targets tubulin [35].

How this substrate specificity is established and regulated in a nonapoptotic context remains an open question. One possibility is the involvement and variable availability of X-linked inhibitor of apoptosis proteins (XIAPs), such as E3 ubiquitin ligase, throughout the neuron [36] (Figures 1

and 2). Given the system-level need for nonapoptotic caspase function in regulating neuronal cytoskeletal architecture, distinguishing apoptotic from sublethal caspase impacts on cytoskeletal proteins at high resolution is an important yet overlooked avenue. Super-resolution microscopy techniques coupled with single-molecule fluorescence tagging of microtubule and actin bundles could provide insights into these distinct caspase-mediated outcomes [37–39].

Caspase signaling in synapses

Apart from being important in the process of neuronal process outgrowth and arborization, caspases have increasingly been linked to synaptic plasticity modulation and normal synaptic physiology (Figure 2). The process of long-term depression (LTD), by which neuronal synapses progressively weaken functionally, accompanied by a reduction in dendritic spine size, depends on a low level of caspase 3/9 activation within dendrites [40]. Curiously, the converse process of long-term potentiation, by which neuronal synapses strengthen over time, seems to be unaffected by suppressing caspase 3/9 activation genetically or pharmacologically. Furthermore, the LTD process dependent on NMDA receptor (NMDAR) function seems to be uniquely dampened by reduced expression of BAD and BAX proteins, both of which are classically associated with promoting intrinsic apoptosis via mitochondria (Figure 1) [41]. More recently, the BAD-BAX-caspase 3 signaling cascade was demonstrated to regulate synaptic vesicle pools by autophagy, with the process being tightly coordinated during associative fear learning in mice [42]. BAX-caspase 3 activation plays a prominent role in the activity-dependent reorganization of corticospinal projections necessary for the acquisition of fine motor task skills [43]. Caspase 3 also directly regulates the levels of AMPA receptor subunit GluA1 in response to chronic stimuli *in vivo* [44]. In zebra finch songbirds, cleaved caspase 3 levels are strongly increased in postsynaptic compartments of the auditory cortex following exposure to novel sounds [45].

Collectively, these findings provide evidence for the involvement of caspase activity during normal physiological processes underlying learning and memory. Better characterization of **sublethal caspase activation** and localization within dendrites and dendritic spines during various learning paradigms will help clarify how caspase activity impacts spine turnover and ion channel expression in experience-dependent plasticity. Are caspases differentially recruited depending on the degree of experience-induced plasticity? Important questions also remain regarding how endogenous caspase activity in these contexts interacts with extrinsic environmental factors known to modify neuronal activity in both physiological and pathological scenarios.

Environmental influences and injury mechanisms

Caspase signaling in excitotoxicity

Intrinsically activated caspases do not operate in isolation, but rather can be influenced by a variety of environmental factors. In neurons, nonapoptotic signaling engagement likely requires a lower activation threshold compared with full programmed cell death. Accordingly, the presence of one or several environmental agents might easily compound the likelihood of pathway activation. Glutamate-induced neurotoxicity, also termed excitotoxicity, is a well-characterized neuronal injury paradigm, particularly in the context of canonical apoptosis [46,47]. Glutamate-induced neurotoxicity occurs due to the presence of supra-physiological levels of the neurotransmitter glutamate, leading to excessive stimulation of ligand-gated ion channel receptors such as NMDA, AMPA, and kainic acid receptors, which culminates in a neurotoxic influx of calcium ions [48,49]. This toxicity is typically manifested through mitochondrial dysfunction, increased generation of reactive oxygen and nitrogen species, a drop in mitochondrial membrane potential, and, ultimately, mitochondrial pore opening followed by apoptosis [49]. The calcium ion influx is known to activate calcium-dependent proteases and phosphatases such as calpains and calcineurin, respectively, which in turn are capable of inducing executioner caspase activity as well, forming a feedback loop [50]. Calpain-10 has been shown to

control actin filament dynamics [51], providing a secondary caspase-independent avenue to excitotoxic dysregulation of the cytoskeleton. More recently, calcineurin signaling downstream of calcium influxes into hippocampal CA1 neurons was found to mediate damage to Ankyrin G, a cytoskeletal protein crucial to the organization of the axon initial segments in said cells [52]. In this study, the injury was ischemic, derived from medial cerebral artery occlusion, which, despite leading to major cytoskeletal reorganization and impaired action potential generation, did not result in significantly increased cell death. Given the known interplay between caspases and calcium-dependent proteases/phosphatases [52–56], and their respective capacities for altering cytoskeletal dynamics, it will be important to dissect their relative contributions to sublethal excitotoxic outcomes in different neuronal populations.

Role of neuronal–glial crosstalk in caspase activation

Neuronal cells do not undergo neurotoxic responses in isolation. The environmental agent causing the insult also acts on glial cells as well as endothelial cells of the vasculature. These non-cell autonomous responses are multi-pronged, with non-neuronal cells experiencing both shared and cell-specific insult effects, which then feed back onto neuronal cells in their proximity. Under physiological conditions, neurons and glial cells engage in extensive crosstalk, due to both physical proximity-based cell–cell contacts, cytoskeletal protrusions known as tunneling nanotubes, extracellular vesicles, as well as paracrine signals [57–59]. In microglial cells, exposure to inflammatory agents such as lipopolysaccharide triggers activation of initiator caspase 8 as well as caspases 3 and 7, which in turn drive the microglia to a proinflammatory phenotype via protein kinase C (PKC)- δ signaling [60]. The caspase activation in this paradigm is not lethal to microglia, yet indirectly leads to neurotoxic outcomes due to microglial activation. In neuronal and astrocyte cocultures, exposure to ketamine, which is known as an NMDAR antagonist, enhances lethal neurotoxicity compared with treatment of neurons alone [61]. Extracellular vesicles, and in particular the long noncoding RNA targeting BDNF contained within them, isolated from the ketamine-treated astrocytes seem to be partially responsible for this effect. Modulatory effects on caspase-dependent apoptosis in neurons by non-neuronal cells may play an even more pertinent role in sublethal caspase activity contexts, with comparatively lower caspase activation thresholds [62].

Anesthetics and ethanol in developmental neurotoxicity

Anesthetics and ethanol are among the best-studied environmental agents implicated in developmental neurotoxicity (Figures 1 and 2 and Box 1) [9,15]. Early work showed that both acute and

Box 1. Clinical findings in anesthesia toxicity and neurodevelopment

Approximately 100 clinical studies have been published evaluating neurodevelopmental outcomes after childhood surgery and anesthesia [110]. Despite concerning evidence from animal studies, clinical results are mixed, likely due to various challenges and limitations inherent to the design of optimal clinical studies (Box 3) [111]. Exposure to general anesthesia is not consistently associated with deficits in academic achievement, general intelligence, or language [88,111,112]. However, a growing number of studies has been reporting associations between general anesthesia exposure during infancy and increased risks for adverse behavioral outcomes, including deficits in executive function, social communication, motor function, and diagnoses of attention deficit hyperactivity disorder (ADHD) [112–114]. These deficits have been reported even after single exposures to anesthesia [115]. Anesthesia length, repeated exposure, dosage regime, and younger age all contribute to enhanced risk for such neurobehavioral impairments [110,112]. Multiple infant anesthetic exposures have been found to more than double the hazard ratio for learning disabilities [116]. The same study found a 30% increase in the hazard ratio for ADHD with a single anesthetic exposure, which, although not statistically significant, is suggestive of the cumulative nature of the damaging effect. It is plausible that lower dose exposures or specific anesthetics in isolation may preferentially trigger nonapoptotic caspase responses, resulting in outcomes different from those of classical apoptosis (see Figure 2 in main text).

The link between anesthesia and canonical caspase-mediated cell death and neurodegeneration in animal models is well established. Whether anesthetics can elicit brain injury via nonapoptotic caspase activation is a pertinent yet largely unexplored avenue of research.

chronic ethanol exposure was associated with caspase 3 activation, neuronal cell death, and neurodegeneration in mouse and rat models, with the response heavily dependent on BAX protein activity [9,63–65]. More recent work intriguingly found that mice lacking primary cilia in fore-brain excitatory neurons exhibited sublethal caspase 3 activation after acute ethanol treatment [66]. Dampened PI3K/AKT signaling caused by cilia loss seems to be a key causal factor behind the enhanced caspase response. The same effect on caspase 3 activation was observed with a single dose of ketamine. Curiously, the same ethanol dosage regime used elicited marked neurodegeneration at similar end points in prior studies, with evidence for neuronal cell loss at later times. This discrepancy may be partially explained by the dampening of the caspase 3 response over time, although questions remain over the inconsistency regarding cell loss. In line with prior developmental evidence, the sublethal caspase 3 activation [66] was punctate in presentation and was associated with actin and tubulin degeneration, followed by loss of pyramidal neuron dendritic complexity in ciliopathic mice. Neuronal safeguarding mechanisms, such as PI3K/AKT signaling via primary cilia, may be important modifiers of the degree of caspase activation and determinants of whether a cell commits to apoptosis or undergoes sublethal neurotoxic changes (Figure 1).

Anesthetics as a key risk factor for caspase-induced injury after major neonatal surgeries

Anesthetics in caspase-dependent injury

Anesthetics such as sevoflurane and isoflurane are an indispensable component of complex pediatric surgeries, for instance, corrective heart surgery in patients with congenital heart disease (CHD) (Box 2). Both volatile and injectable anesthetics have been extensively linked to canonical, cell death-associated caspase activity in various animal models of exposure. Originally described in rat models, combinations of midazolam, nitrous oxide, and isoflurane exposure were associated with extensive caspase activation, neurodegenerative changes, and subsequent learning impairments [8,15]. Many of the anesthetics used for major pediatric surgery function as either NMDAR blockers or GABA receptor agonists [8], suggestive of a converging mode of neurotoxicity. Isoflurane and sevoflurane have both been demonstrated to mediate their caspase-dependent neurotoxic effects through disrupting calcium homeostasis and activation of the intrinsic mitochondrial apoptotic cascade [67–70]. In rodent anesthesia models, perinatal exposures to sevoflurane and isoflurane induced long-term behavioral impairments, including memory deficits, increased anxiety, decreased sociability, and impaired motor coordination [14,71]. In infant rhesus macaques, repeated doses, but not a single dose, of isoflurane were associated with deficits

Box 2. Etiologies of neurological deficits observed in congenital heart disease (CHD)

CHD is one of the most common birth defects. Neonatal surgical interventions are critical for patients with complex CHD, leading to significantly reduced case mortality. Given its high prevalence, accounting for around 1% of live births, CHD has huge societal impacts. Despite improvements in mortality prevention, a substantial fraction of CHD patients (estimated at about 1 in 150) go on to exhibit various neurobehavioral abnormalities, including attention deficit, social difficulties, motor issues, and intellectual disability [117]. The Pediatric Heart Network and National Heart, Lung, and Blood Institute working group has reported that one of the most important challenges for neonates undergoing surgery for CHD is long-term sequelae, such as neurodevelopmental deficits [118].

Known and postulated causative factors range from genetic, including *de novo* mutations in chromatin modifier genes [90], to perioperative factors such as hypoxia/ischemia and exposure to anesthetics [119]. Neonates undergoing complex surgeries present a uniquely challenging scenario due to the temporal overlap between surgical factors, such as anesthesia, and ongoing development of several organ systems, including the brain. Past studies indicate that anesthetic use during the neonatal time window is associated with neurotoxicity and an increased risk of behavioral deficits in later life [120]. These include sensory issues, motor deficits, as well as cognitive delays. The neonatal period is characterized by crucial early brain maturation processes, such as early cortical circuit establishment, synaptogenesis, and cerebellar neurogenesis. Disruptions in any of these developmental steps carry a potential risk of physiological dysfunction and resultant behavioral disturbances.

in motor reflexes and increased social anxiety [72]. The dose-dependent effect points to a cumulative model of anesthetic neurotoxicity, with greater exposure frequency leading to greater neuroapoptosis and neurodegenerative changes. Genetic susceptibility or involvement of a second environmental factor (a dual-hit scenario) could therefore plausibly decrease the threshold for caspase activation as well as modify the nature of the response, rerouting it from cell death to structural and physiological remodeling of the cell. Ketamine administration is also commonplace in pediatric cardiac surgery as premedication. Recent reports indicate that multiple courses of ketamine in developing rat models elicit caspase 3-associated neurodegenerative changes in the prefrontal cortex and hippocampus as well as neurobehavioral alterations such as impairments in spatial memory, decreased anxiety, and enhanced novelty-induced behaviors [73–75].

Anesthetic exposure neurotoxicity has also been characterized in the context of age-related cognitive decline and neurodegeneration. Alzheimer's disease has been postulated to result primarily from abnormal accumulation and reduced clearance of β -amyloid (A β) peptides in the aging brain [76]. Isoflurane-exposed neonatal mice as well as human neuroglioma cells exhibited increased oligomerization of A β 42 peptides, which was accompanied by enhanced caspase 3 activation [77] and is known to be upstream of caspase signaling [78], leading to synaptic injuries [79]. Unexpectedly, the use of another anesthetic, propofol, attenuated caspase activation in this paradigm, likely through suppressing A β 42 oligomerization [77]. The apparently contradicting effects on caspase activation point to the possibility of complex interactions between different anesthetics, when used in combination. The caspase activation signature resulting from their combinatorial use may therefore fall in a sublethal range, where apoptotic and autophagic cell death are avoided, but cytoskeletal and synaptic damage persist.

Potential for sublethal insults

While the majority of anesthetic neurotoxicity research has focused on the canonical, cell death-inducing context, sublethal mechanisms have also been implicated. *In vitro* evidence in neural precursor cells (NPCs) derived from human embryonic stem cells suggests short-term (2 h) exposures to clinically relevant sevoflurane concentrations do not induce cell death, with higher, supra-clinical concentrations transiently inhibiting differentiation [80]. In concordance with these findings, earlier research in cultured hippocampal NPCs found that exposures to isoflurane [81] and clinically relevant concentrations of propofol [82,83] did not induce cell death, but did promote neuronal differentiation. Ketamine treatment of *ex vivo* rat cortical NPCs showed an identical trend, with no effect on apoptosis and enhanced neuronal differentiation [84]. These studies collectively suggest a greater resistance to anesthetic-induced cell death in immature neuronal precursors and potentially a more prominent role for nonapoptotic mechanisms in neurogenic regions of the brain during the neonatal time window. In a separate study, isoflurane-treated hippocampal NPCs showed robust increases in cleaved caspase 3 levels without accompanying cell death, as assessed by TUNEL staining [85]. Interestingly, at least part of the mechanistic explanation for the enhanced neuronal differentiation also observed in this study is increased caspase 3 activation, as treatment with Z-DEVD-fmk, a caspase 3 inhibitor, rescued caspase activation and neuronal differentiation levels. If caspase activation in neurogenic regions such as the hippocampus can be sublethally modulated by anesthetics, the neonatal period should be a particularly vulnerable time window, due to extensive neurogenesis taking place.

Detrimental anesthetic effects on dendritic arborization and synaptic ultrastructure have also been demonstrated. Acute exposures of *ex vivo* primary rat cortical neurons for 1 h to sevoflurane or desflurane did not result in overt cell death in the case of sevoflurane; however, both anesthetics led to reduced neurite lengths and dampened spontaneous synaptic currents [86]. Intriguingly, the damaging effects of both anesthetics were mediated through increased

mitochondrial fragmentation, as treatment with an inhibitor of mitochondrial division, Mdivi-1, rescued mitochondrial morphology as well as the synaptic and electrophysiological defects. Mitochondrial fragmentation is a key step during activation of the **intrinsic apoptotic pathway** and is upstream of caspase 3 cleaving and activation (Figure 1). Given the differential level of mitochondrial fragmentation observed between sevoflurane and desflurane [86], which was correlated to cell death, one could speculate that an ‘intermediate’ level of mitochondrial ‘leakiness’ leads to a corresponding sublethal caspase activation, which works to suppress neuritogenesis and synaptic maturation while avoiding triggering cell death (as postulated in Figure 2). The findings from this study point to the importance of careful characterization of neuronal phenotypes in anesthetic exposure paradigms where overt neurodegeneration and cell death are not observed. These more subtle phenotypes may be otherwise missed in studies focused on detecting signs of canonical caspase signaling. A major caveat of the aforementioned studies is that they were performed in cultured cells, raising the question of whether these unique, sublethal scenarios can be replicated *in vivo*.

Recently, a large-scale randomized control trial found no difference in neurodevelopmental milestones in 2-year-old children with a follow-up at 5 years, between a group exposed to 1 h of general anesthesia and a control group receiving awake-regional anesthesia [87,88]. While the trial design reflected commonly used regimes during pediatric surgery, it could not assess the effects of repeat exposures nor could it account for potential gene–environment interactions and combinatorial mechanisms (Box 3). Children with CHD often carry polymorphisms in genetic loci that are associated with regulation of apoptosis and cell survival. Among these are the *Akt1* and *Gdf1* genes [89,90], which have known roles in modulating caspase signaling and cell survival [91–94]. This suggests a scenario where genetically susceptible populations, such as certain CHD patients, may be at an increased risk for anesthesia-induced neurotoxicity due to an enhanced likelihood of caspase activation following exposure and caspase-mediated neurodegeneration.

How nonlethal yet damaging caspase responses may be implicated in these cases is unknown and warrants further study. Understanding the switch from cell death-promoting caspase activity to sublethal outcomes would potentially allow for better stratification and more informed treatment of patients suffering from caspase-elicited neurotoxicity.

Therapeutic potential and outlook

Caspase-dependent neurotoxicity is a complex, multistep process involving both autonomous and non-cell autonomous functions and is heavily influenced by timing, dosage, and other underlying

Box 3. Current controversies in anesthesia neurotoxicity

Studies on developmental anesthesia neurotoxicity have mostly focused on canonical, apoptotic caspase signaling and preclinical animal models, primarily rodents and monkeys. Repeated anesthetic exposures in animals lead to robust neurotoxic effects on brain development and maturation [71,74]. A recent clinical trial found no significant effects of a single, 1-h exposure to anesthesia on developmental milestones and neurobehavioral outcomes at 3 and 5 years of age [88]. The difficulty in assessing comprehensive outcomes in humans, as well as the small sample size and fact that trial participants were not screened for genetic status, presents difficulties for fully excluding anesthetics as neurodevelopmental risk factors. In addition, obvious technical and ethical difficulties prohibit distinguishing anesthesia neurotoxicity from the global impact of neonatal surgery such as systemic inflammation. Measuring apoptotic outcomes may also miss more subtle, yet potentially important cellular alterations due to nonlethal caspase-dependent mechanisms.

The cellular and molecular mechanisms behind nonapoptotic neuronal injury and their influence on behavior are largely unexplored, primarily due to historical focus on the canonical caspase pathway. Given the clear importance of this pathway as a source of potential long-term neurological injury, there is an urgent need for in-depth mechanistic characterization in both basic and translational animal models, as well as design of new targeted therapeutics.

covariates. Assuming sublethal neurodegenerative caspase activation is mostly a constrained version of caspase-dependent cell death, the increasing repertoire of caspase inhibitors would provide candidate therapeutics for neuroprotection. Among these, Z-VAD-FMK and Boc-D-FMK show potent anticaspase properties, albeit without inhibiting activity of all caspases and showing non-overlapping specificity [95–97]. Both inhibitors are commercially available; however, they have been shown to induce adverse effects in the past [98,99]. A viable alternative is the pan-caspase inhibitor Q-VD-OPh, with higher efficacy, demonstrable diffusion through the blood–brain barrier, and a better toxicity profile [100,101]. Q-VD-OPh has shown promise in preventing apoptosis in models of perinatal stroke [102], spinal cord injury [103], and Alzheimer’s disease [104].

Apart from broad-spectrum caspase enzyme inhibition, therapeutic strategies may be aimed at indirectly dampening caspase activation and subsequent neuronal damage. Mitochondrial fission and fragmentation is a known inducer of intrinsic caspase 3 activation, and inhibitors such as the quinazolinone Mdivi-1 have been shown to protect neuronal stem cells from oxidative damage and apoptosis [105]. The PI3K/AKT pathway has long been known to play a prominent neuroprotective role under various injury paradigms [92,106], partly through restricting caspase activation. Employment of the PI3K/AKT small molecule activator, SC79, may be beneficial in both apoptotic and sublethal injury scenarios, as it has been demonstrated to protect neurons from oxidative stress [107,108].

Given the variety of nonapoptotic roles caspases play during normal neural development and maturation processes, using broad-spectrum caspase inhibitors at critical developmental time points may pose risks. Since many of the sublethal effects of caspase activation take place at lower activation thresholds, it will be important to assess for remnant caspase activity and cytoskeletal integrity in paradigms using caspase inhibitors to suppress cell death. Interference with physiological caspase functioning is of particular concern in scenarios likely requiring continuous inhibitor treatments, such as in progressive neurodegenerative disorders like Alzheimer’s, Parkinson’s, and Huntington’s diseases. Future research on developmental anesthesia neurotoxicity will need to more carefully address the potential sublethal, caspase-dependent adverse outcomes in terms of cell morphology, establishment of functional neural circuitry, and behavior. Assessing caspase activation thresholds in preclinical models alongside thorough titration of therapeutics targeting caspase activation, intracellular signaling such as PI3K/AKT and mitochondrial stability [109] will be necessary to fully characterize and target the sublethal response.

Concluding remarks

Significant progress has been made over the past decades in understanding the molecular mechanisms behind programmed cell death in the nervous system and its effects on cell morphology, physiology, and organismal behavior. Detailed characterization of caspases, their substrates, and the outcomes of their activity has informed the design of new classes of caspase inhibitors with demonstrated efficacy. A primary driving force behind this work was to understand **canonical apoptotic signaling** with the hope of either suppressing it in the context of neurodegenerative insults or promoting it during carcinogenesis. In parallel, the nonlethal mode of caspase activation and signaling has been gaining growing interest. Progress has been made in clarifying the important functions this signaling pathway plays in neuronal dendritic extensions and arborization, experience-induced plasticity, and learning, as well as how it might be co-opted in injury scenarios. Advances in super-resolution imaging, *in vitro* neuronal culture, CRISPR-Cas9 genome editing, and next-generation sequencing methodologies, together with established injury models, are positioning research to dissect the conditions under which sublethal caspase

Outstanding questions

What is the main driver behind the shift from apoptotic to nonapoptotic caspase functions during neuronal development and maturation?

Is the functional divergence in neuronal caspase activity primarily dependent on quantitative, caspase activation threshold-associated changes in signaling, or do other indirect factors play a greater role?

Are neuronal cells more sensitive to nonapoptotic insults at particular times during their morphological and physiological maturation? Are different classes and types of neurons differentially sensitive to caspase signaling?

Are cytoskeletal proteins the primary targets of nonapoptotic caspase activity? How is this substrate specificity governed in neuronal cells? Are different caspases recruited to different substrates in a nonapoptotic context?

To what extent and by what mechanism are environmental neurotoxins implicated in nonlethal caspase signaling? How is the damage constrained and apoptosis avoided?

What role do gene–environment interactions in neurons play in nonapoptotic caspase signaling in physiological and injury contexts? Is there potential for harmful interactions between low-dose anesthetic exposures and genetic susceptibility at the level of nonapoptotic caspases?

If risks for particular pediatric populations (such as those with CHD) are identified, what caspase-targeting therapeutic options are optimal for neuroprotection?

activation takes precedence over caspase-driven apoptosis (see Outstanding questions). These prospective studies will be especially important for deciphering the fine details behind the neurotoxicity of clinically relevant agents such as anesthetics, which remain indispensable for surgical procedures like those in the pediatric CHD population.

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Declaration of interests

The authors declare no competing interests in relation to this work.

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Impact of Cardiopulmonary Bypass on Neurogenesis and Cortical Maturation

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Objective: Neurodevelopmental delays and frontal lobe cortical dysmaturation are widespread among children with congenital heart disease (CHD). The subventricular zone (SVZ) is the largest pool of neural stem/progenitor cells in the postnatal brain. Our aim is to determine the effects of cardiopulmonary bypass (CPB) on neurogenesis and cortical maturation in piglets whose SVZ development is similar to human infants.

Methods: Three-week-old piglets ($n = 29$) were randomly assigned to control (no surgery), mild-CPB (34°C full flow for 60 minutes) and severe-CPB groups (25°C circulatory-arrest for 60 minutes). The SVZ and frontal lobe were analyzed with immunohistochemistry 3 days and 4 weeks postoperatively. MRI of the frontal lobe was used to assess cortical development.

Results: SVZ neurogenic activity was reduced up to 4 weeks after both mild and severe CPB-induced insults. CPB also induced decreased migration of young neurons to the frontal lobe, demonstrating that CPB impairs postnatal neurogenesis. MRI 4 weeks after CPB displayed a decrease in gyrification index and cortical volume of the frontal lobe. Cortical fractional anisotropy was increased after severe CPB injury, indicating a prolonged deleterious impact of CPB on cortical maturation. Both CPB-induced insults displayed a significant change in densities of three major inhibitory neurons, suggesting excitatory-inhibitory imbalance in the frontal cortex. In addition, different CPB insults altered different subpopulations of inhibitory neurons.

Interpretation: Our results provide novel insights into cellular mechanisms contributing to CHD-induced neurological impairments. Further refinement of CPB hardware and techniques is necessary to improve long-term frontal cortical dysmaturation observed in children with CHD.

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Children with complex congenital heart diseases (CHD) are at risk of developing a wide spectrum of neurobehavioral problems.^{1,2} Neuroimaging studies have shown that altered cortical morphology particularly affecting the frontal lobe, including reduced folding and volume, is present in children with severe and complex CHD.^{3,4} Many of these changes are similar to the reductions in both cortical gray and white matter volume seen in children born prematurely.⁵ Studies using diffusion tensor imaging (DTI) have demonstrated a high incidence of white matter dysmaturation

and injury in CHD patients, including adolescents who underwent surgery in infancy.^{6,7}

Considerable previous work including our own has focused on the mechanisms of abnormal white matter development observed in the individual with CHD.⁸ Exposure to the inflammatory effects of cardiopulmonary bypass (CPB) and possible hypoxia/ischemia associated with surgical repair in early infancy has been documented to interfere with white matter development.^{8–11} The generation, migration, and maturation of oligodendrocytes

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responsible for myelin/white matter synthesis may be delayed by hypoxia due to CHD in the fetus.^{12,13}

Oligodendrocytes are glial cells generated by the sub-ventricular zone (SVZ) particularly during the third trimester of human pregnancy and the first 2 or 3 years of life. However, in addition to generating glial cells responsible for white matter development, the SVZ is a key source of neurons generated from neuronal stem precursor cells (NSPCs). Furthermore, young neurons in the SVZ migrate postnatally along the lateral ventricles to populate cortical gray matter, contributing to its further maturation.¹⁴ Using a piglet model of fetal and neonatal hypoxia, we have previously documented reduced proliferation and neurogenesis in the SVZ accompanied by reduced cortical growth. We showed a similar reduction in neuroblasts within the SVZ of human infants born with CHD.¹⁵

In the current study we examine the impact of cardiopulmonary bypass on neurogenic activity of the SVZ as well as growth and maturation of the frontal lobes.

Methods

Animals

A total of 49 Yorkshire pigs were studied. CPB results in SIRS due to various factors including the repeated passage of blood through non-endothelial surfaces.¹⁶ In addition, CHD patients are at risk of global cerebral ischemia during CPB resulting from a steal of blood by aortopulmonary collateral vessels and/or co-existing cerebral vessel abnormalities.^{17,18} Emboli from solid particles or gaseous origin due to CPB are also an important cause of ischemia/reperfusion (I/R) injury.¹⁷ Finally, the circulatory arrest technique involves risk of I/R-injury such as the “no-reflow phenomenon”.¹⁹ To investigate the effects of CPB on postnatal neurogenesis in the SVZ and frontal lobe development, CPB-induced brain insults were designed for this study to be primarily a result of SIRS alone or both SIRS and I/R-injury (Fig 1A). A total of 29 female piglets at 3 weeks of age were randomly assigned to one of three groups with different CPB-induced brain insults that involved I/R injury and SIRS (1) No surgery (control, no insult, $n = 9$); (2) 34°C full-flow bypass for 60 min (mild-CPB insult, CPB-induced SIRS, $n = 10$); and (3) 25°C hypothermic circulatory arrest (HCA) for 60 minutes (severe-CPB insult, CPB-induced SIRS with I/R injury, $n = 10$) (Fig 1A). Animals were euthanized at either day 3 ($n = 12$) or week 4 ($n = 17$) post CPB to determine time-related changes in postnatal SVZ neurogenesis and frontal lobe development after cardiac surgery. Heterologous blood was obtained from a donor pig ($n = 20$). We performed all experiments in compliance with the National Institutes of Health’s

“Guide for the Care and Use of Laboratory Animals” and the study was approved by the Animal Care and Use Committee at Children’s National Hospital.

Juvenile Porcine CPB Model

A thoracotomy was performed, and an arterial cannula and a venous cannula were inserted after heparinization. Fresh whole blood from a donor pig was transfused into the CPB circuit. The pump prime included normal saline, methylprednisolone, furosemide, sodium bicarbonate and cephazolin. The animals were perfused for 10 minutes at 37°C and then cooled to 25°C or 34°C (Table 1). After cooling, HCA or maintenance of full flow was chosen (Table 1). After 40 minutes of rewarming, animals were weaned from CPB. The hematocrit was maintained at 30% and pH-stat strategy was applied (Table 1).^{17,20} Blood pressure and temperature were monitored continuously. Arterial PO₂ and PCO₂, arterial pH, hematocrit value, and arterial lactate were measured. We used tissue oxygenation index (TOI) with Near-Infrared spectroscopy (NIRO-300) to assess cerebral ischemic status. Systemic leukocyte counts were performed to identify systemic inflammation. Neurological and behavioral evaluations were performed at 24 hour intervals beginning on post-operative day 1. A total score of 400 indicates brain death while a score of 0 is considered normal. The score was accessed in a blinded fashion.^{9,10} The details were described previously.^{8–10}

Cellular Assessment

On post-operative day 3 or week 4 brains were resected and further post-fixed. Sections at 20 µm thickness were incubated in primary and secondary antibodies as described previously.^{9,15} The following antibodies were used for immunohistochemistry: anti-GFAP (1:1000; DAKO), Sox2 (1:500; Millipore), anti-ki67 (1:1000; BD Biosciences), anti-Doublecortin (1:1000; Millipore), anti-NeuN (1:500; Millipore), anti-Cleaved Caspase-3 (1:1000; Cell Signaling), anti-Calretinin (1:1000; Millipore), anti-Somatostatin (1:100; Millipore), anti-Parvalbumin (1:500; Millipore) and anti-Tbr1 (1:200; Abcam). Analysis of the SVZ was performed on coronal sections of the brain, adjacent to the wall of the lateral ventricles. We previously demonstrated that the anterior SVZ displays the most proliferating NSPCs and resembles its human counterpart.¹⁵ Therefore we focused our analysis on the anterior SVZ (Fig 1B, C). An imaginary line was drawn to divide the anterior SVZ into two regions: dorsolateral and ventral (Fig 1D). The SVZ was further subdivided into three tiers based on the density and distribution of DCX⁺ cells as previously described in human brain.¹⁴ All images were acquired with a Leica SP5

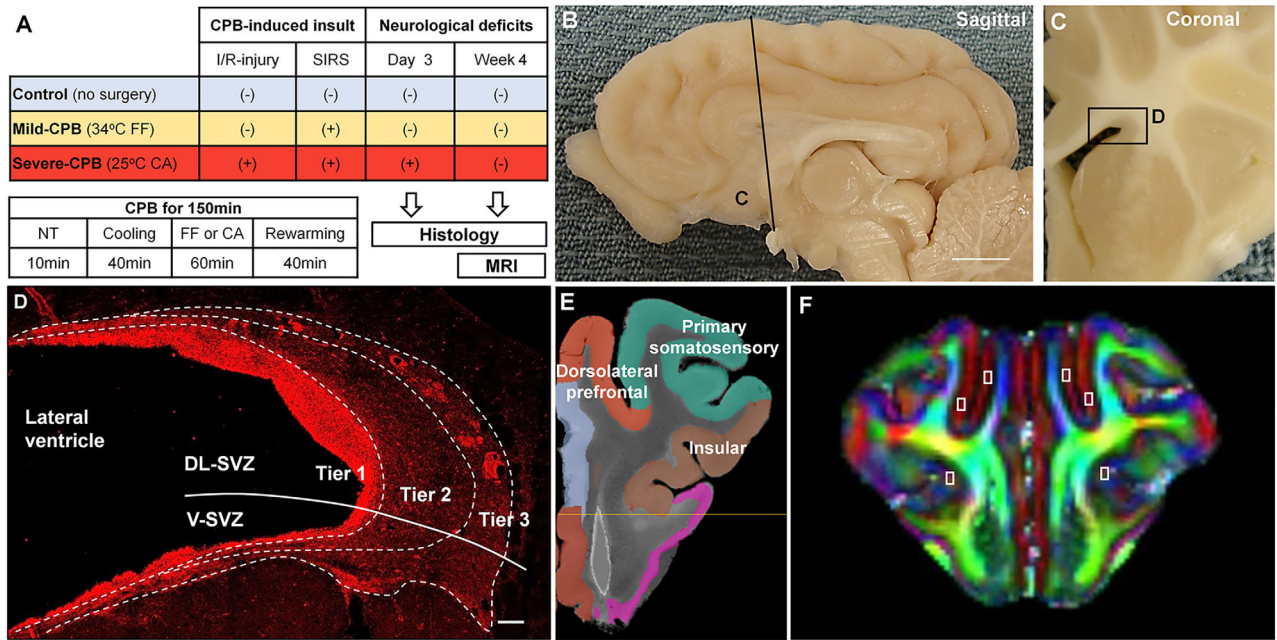


FIGURE 1: Methods. (A) Overall study design. (B) Sagittal plane of the porcine brain. Scale bar, 1 cm. (C) Coronal section and the SVZ (box D). (D) Magnified image from the boxed region in (C) and subdivision of the SVZ with doublecortin stain. Scale bar, 100 μ m. (E) Anatomical location of cortical ROIs (Prefrontal, Primary Somatosensory, and Insular cortices). (F) DTI FA image of pig brain showing ROI of assessed cortical regions. CA = circulatory arrest; CPB = cardiopulmonary bypass; DL-SVZ = dorsolateral-SVZ; FF = full-flow perfusion; I/R = ischemia/reperfusion; NT = normothermia; SIRS = systemic inflammatory response syndrome; SVZ = subventricular zone; V-SVZ = ventral-SVZ.

confocal laser-scanning microscope and counted in a blinded fashion. Given high cell density and homogeneous cell distribution, DCX⁺ cell density was quantified by acquiring a total of three images for each region from separate microscopic fields at $\times 40$ magnification. For other stainings, the whole quantified area was scanned at $20\times$ and tiled to avoid biased counting due to heterogeneous distribution in the large surface area. Regarding DCX⁺ cluster analysis, only clusters with a surface larger than $140\ \mu\text{m}^2$ were counted in the analysis. The cluster occupancy was calculated by dividing the sum of all clusters surface area by the SVZ total surface area. The length of GFAP⁺ processes lining the SVZ was perpendicularly measured every 50 μ m along the surface of the ventricle in a tile scan. For cellular analysis within the cortex, the following cortices were scanned separately based on the anatomical atlas and functional cortical map of the porcine brain: prefrontal cortex, somatosensory cortex, and insular cortex (Fig 1E,F).

MRI and DTI

We performed a total of 9 MRI studies (control; $n = 3$, mild-CPB; $n = 3$, severe-CPB; $n = 3$) at week 4 after CPB (Fig 1A). The images were transferred offline and reviewed by an MR physicist and a neuroradiologist. Conventional anatomical T1 and T2 MRI and diffusion tensor imaging (DTI) images were acquired on a GE Sigma

HDxt 3.0 T scanner. A minimum of 19 serial, coronal MRI images per piglet were analyzed for volumetric assessment and the gyrification index (GI).¹⁵ For DTI analysis: nine serials of coronal images per piglet were used to analyze fractional anisotropy (FA) value in cortical grey matter. We applied a region-of-interest (ROI) approach to test specific structures throughout the brain selected. All ROIs were drawn and quantified manually by a blinded experimenter as we performed previously.⁸

Statistics

One-way analysis of variance (ANOVA) with Tukey's post-hoc comparisons was used to evaluate cellular and imaging outcome between the three treatment groups. Two-way ANOVA was used to look at CPB insult effects within SVZ and cortical regions, cortical layers, cell types or over time. Group-by-time and group-by-region interaction terms were evaluated in the ANOVA models. Statistical analysis was performed with PRISM software (GraphPad Software, Inc., La Jolla, CA). Significance was determined as two-tailed $p < 0.05$ using Tukey post-hoc adjustment to control the experiment-wise error due to multiple comparisons.²¹ Sample sizes of piglets for treatment and exposure groups provided 80% power to detect moderate to large effect sizes regarding neuronal cell measures at 3 days and 4 weeks post-surgery and group

TABLE 1. Experimental Conditions

	Mild CPB (n = 10)		Severe CPB (n = 10)		<i>p</i> value
	Mean	SD	Mean	SD	
Temperature (°C)					
Pre CPB	36.2	1.0	36.2	0.8	0.9999
Cooling	33.8	0.3	26.6	0.7	<0.0001 ^a
FF or CA	33.1	0.3	24.7	0.8	<0.0001 ^a
Rewarming	36.4	0.9	35.6	0.9	0.2399
3 h	37.0	0.8	37.0	0.5	0.9999
MBP (mmHg)					
Pre CPB	83.9	19.6	80.7	15.0	0.9999
Cooling	99.7	14.1	95.5	12.9	0.9999
FF or CA	113.5	17.1	5.4	1.9	<0.0001 ^a
Rewarming	130.4	18.3	114.9	17.9	0.3526
3 h	92.8	14.9	87.1	13.0	0.9999
pH					
Pre CPB	7.585	0.098	7.582	0.082	0.9999
Cooling	7.489	0.036	7.471	0.042	0.9999
Rewarming	7.546	0.033	7.458	0.034	<0.0001 ^a
3 h	7.605	0.047	7.570	0.053	0.5423
PaCO ₂ (mmHg)					
Pre CPB	32.1	6.7	33.3	6.5	0.9999
Cooling	42.8	3.1	41.8	2.2	0.9999
Rewarming	41.5	3.4	39.3	2.7	0.5092
3 h	32.4	3.8	30.4	3.3	0.8892
Hematocrit (%)					
Pre CPB	35.5	3.6	36.9	2.3	0.9999
Cooling	34.1	1.5	33.9	1.6	0.9999
Rewarming	32.4	1.8	33.7	1.8	0.4967
3 h	36.9	3.7	33.7	1.9	0.1200
TOI					
Pre CPB	47.7	5.3	47.2	2.4	0.9999
Cooling	58.1	3.4	64.7	5.4	0.0236 ^a
FF or CA	57.6	2.6	34.7	4.4	<0.0001 ^a
Rewarming	53.7	2.8	53.2	1.6	0.9999
3 h	48.9	1.9	47.3	4.1	0.9999
Lactate (mmol/l)					
Pre CPB	1.4	0.4	1.4	0.4	0.9999
Cooling	2.4	0.8	2.5	0.5	0.9999
Rewarming	1.8	0.9	6.9	1.3	<0.0001 ^a
3 h	2.0	0.9	5.4	1.4	<0.0001 ^a
Leukocyte (10 ³ /μl)					
Pre CPB	5.4	1.4	6.9	1.2	0.0787
Cooling	5.7	1.8	5.3	1.4	0.9999
Rewarming	5.9	1.9	7.8	3.3	0.5948
3 h	11.8	4.5	14.0	3.7	0.9560

^aStatistically significant.

CPB = cardiopulmonary bypass; MAP = mean arterial pressure; TOI = tissue oxygen index.

differences in cortical impairment.²² All statistical analyses were reviewed by a biostatistics expert (D.Z.).

Results

Cardiopulmonary Bypass Alters Neurogenesis in the Piglet SVZ and Disrupts Neuroblast Migrating Chains toward the Frontal Lobe

There were no differences among CPB groups in preoperative conditions (Tables 1 and 2). The TOI value during HCA was significantly lower than full-flow CPB (Table 1). The index was then normalized during the rewarming period (Table 1) demonstrating I/R injury in the severe-CPB insult (Fig 1A). Lactate levels during cooling and at 3 hours post CPB were significantly higher in the severe-CPB group compared to the mild-CPB group (Table 1). Both mild- and severe-CPB insults caused a significant increase in leukocyte numbers 3 hours post CPB, but no difference was detected between the two CPB groups (Time $p < 0.0001$, CPB $p = 0.1413$) suggesting SIRS in the mild- and severe-CPB groups (Fig 1A). Severe-CPB insult caused acute neurological symptoms, including seizures, whereas mild-CPB insult did not (Table 2). Neurological symptoms improved with time and were not present on postoperative day 7 (Table 2).

The SVZ retains its highly mitotic potential throughout postnatal life. We first examined the NSPC pool within the whole SVZ 3 days post-surgery. Although there were no differences in the density of Sox2⁺ NSPCs at postoperative day 3 (control vs mild $p = 0.0605$, control vs severe $p = 0.0916$, mild vs severe $p = 0.9743$), detailed examination of the SVZ revealed a trend toward reduced NSPC proliferative capacity in the severe group compared to control (Fig 2A–D). On the other hand, differences between CPB groups were not observed (Fig 2D, E). There were no differences in the proliferative capacity between dorsolateral- and ventral-SVZ (Fig 2E) as well as between analyzed tiers ($p = 0.2198$), indicating no regional effects. Neurogenic activity was further assessed by quantifying the average length of GFAP⁺ processes spanning tier 1 and expressed within radial-glia like cells (Fig 2F–I). Both mild and severe groups displayed reduced GFAP⁺ process length. This effect was confined to the dorsolateral part of the SVZ, as no differences were observed in the ventral part (Fig 2J). These results are consistent with our previous studies showing that the dorsolateral SVZ is a more active neurogenic region than the ventral SVZ.¹⁵

When we analyzed neuroblasts within the SVZ, decreased density of DCX⁺ neuroblasts was observed in

TABLE 2. Changes in Body Weight and Neurological Outcome

	Control (n = 4–10)	Mild CPB (n = 4–10)		Severe CPB (n = 4–10)						Control vs mild	Control vs severe	Mild vs severe
	Mean	SD	Mean	SD	Mean	SD	<i>F</i> value	<i>p</i> value				
Weight (kg)												
Pre	5.1	0.7	5.0	0.8	5.0	0.4	0.072	0.930				
Day 3	5.4	0.8	5.0	0.9	4.9	0.6	1.280	0.293				
Week 4	10.5	1.7	9.1	1.8	9.1	2.2	1.161	0.338				
NDS												
Day 1	0.0	0.0	29.0	26.0	150.0	35.2	99.140	<0.0001 ^a	0.041 ^a	<0.0001 ^a	<0.0001 ^a	<0.0001 ^a
Day 2	0.0	0.0	0.0	0.0	89.0	52.0	29.350	<0.0001 ^a	0.9999	<0.0001 ^a	<0.0001 ^a	<0.0001 ^a
Day 3	0.0	0.0	0.0	0.0	61.5	32.5	35.820	<0.0001 ^a	0.9999	<0.0001 ^a	<0.0001 ^a	<0.0001 ^a
Day 4	0.0	0.0	0.0	0.0	26.7	35.5	3.395	0.061	0.9999	0.094	0.094	0.094
Day 5	0.0	0.0	0.0	0.0	12.5	14.8	4.310	0.033 ^a	0.9999	0.055	0.055	0.055
Day 6	0.0	0.0	0.0	0.0	6.7	10.3	2.500	0.116				
Day 7	0.0	0.0	0.0	0.0	0.0	0.0						

^aStatistically significant.

CPB = cardiopulmonary bypass; NDS = neurological deficit score.

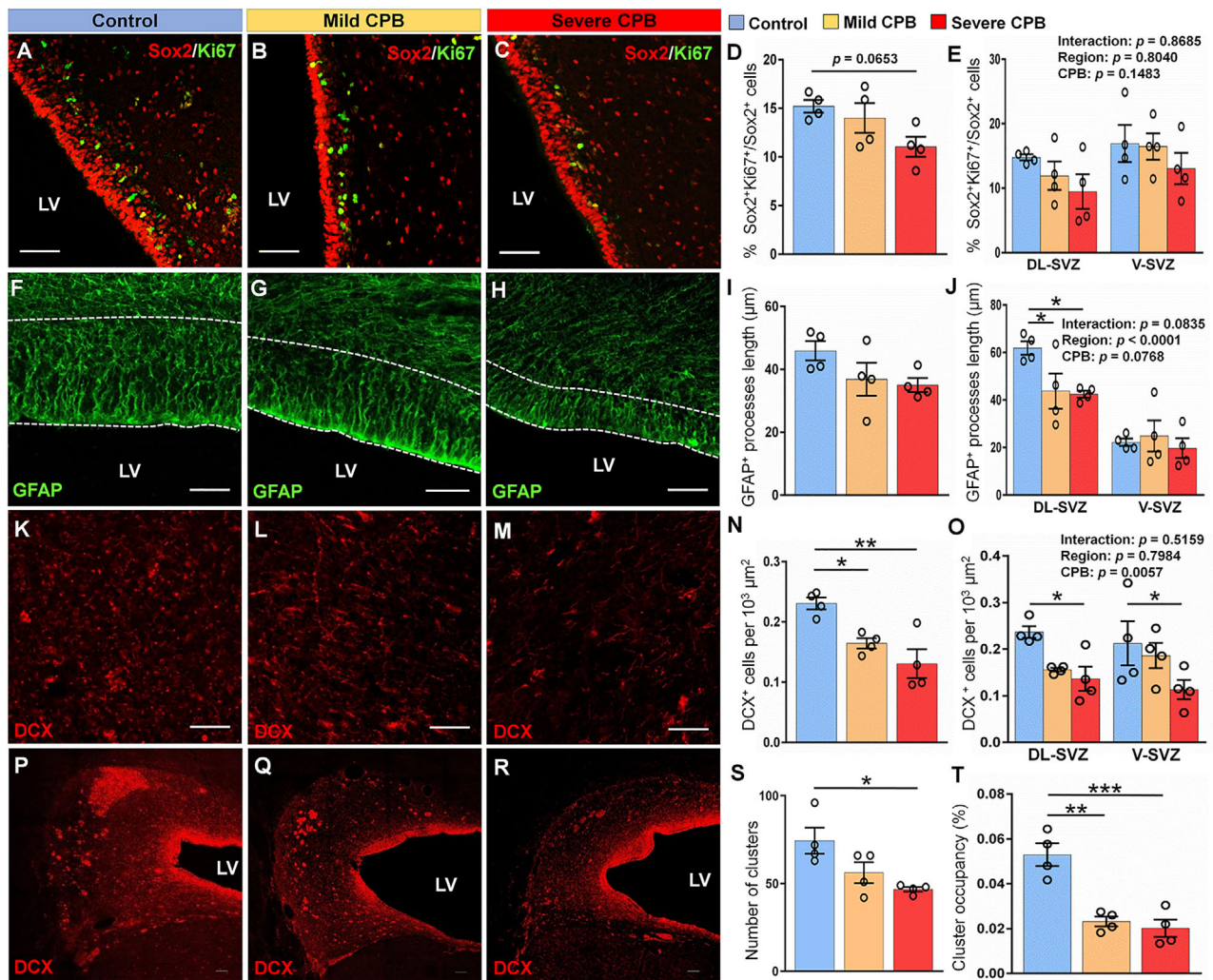


FIGURE 2: CPB impairs postnatal neurogenesis in the piglet SVZ 3 days post-surgery. (A–C) Immunostains of Sox2⁺Ki67⁺ cells in the DL-SVZ. (D, E) Quantification of the percentage of proliferative Sox2⁺ neural stem/progenitor cells. (F–H) Immunostains of GFAP in the DL-SVZ. (I, J) Quantification of the average length of GFAP⁺ processes. (K–M) Immunostains of DCX⁺ cells in tiers 2–3 of the DL-SVZ. (N, O) Quantification of the average density of DCX⁺ cells. (P–R) Coronal sections of the SVZ showing immunostains of DCX⁺ cell clusters. (S) Quantification of the average number of DCX⁺ cell clusters. (T) Quantification of the average cluster occupancy of the SVZ. Scale bars, 50 μ m (A, B, C, F, G, H, K, L, M) and 100 μ m (P, Q, R). $n = 4$ animals per group. Data are expressed as mean $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, one-way ANOVA with Tukey's post-hoc test (D, I, N, S, T) and two-way ANOVA with Tukey's post-hoc test (E, J, O). CPB = cardiopulmonary bypass; DL-SVZ = dorsolateral-SVZ; DCX = doublecortin; GFAP = glial fibrillary acidic protein; LV = lateral ventricle; SVZ = subventricular zone; V-SVZ = ventral-SVZ.

the peripheral region (tiers 2–3) in both mild and severe-CPB groups compared to control (Fig 2K–O). While the effect of CPB on radial-glia like cells (ie, neural stem cells) was restricted to the dorsolateral region (Fig 2J), the density of neuroblasts was decreased in both dorsolateral and ventral parts of the SVZ after CPB (Fig 2O). Although our previous studies found a unique role of HCA (ie, severe-CPB insult) on developing white matter,⁹ we did not observe differences between mild and severe-CPB groups in NSPC proliferation, morphology of radial-glia like cells, or neuroblast density during the acute period after surgery. Together these data demonstrate that CPB

alone can limit SVZ neurogenesis and reduce neuroblast density within the anterior dorsolateral-SVZ, the largest progenitor pool of the postnatal porcine brain.

A previous study in the human infant brain identified a chain-like structure of actively migrating DCX⁺ neuroblasts with adherent junctions.¹⁴ These chains principally located in the peripheral-SVZ (ie, Tier 3) have been associated with a tangential migration oriented toward the developing frontal cortex. To determine whether CPB-induced brain insults also alter neuroblast migratory chains, we analyzed DCX⁺ clusters - coronal view of the migratory stream - within the SVZ (Fig 2P–R). Although the average size of

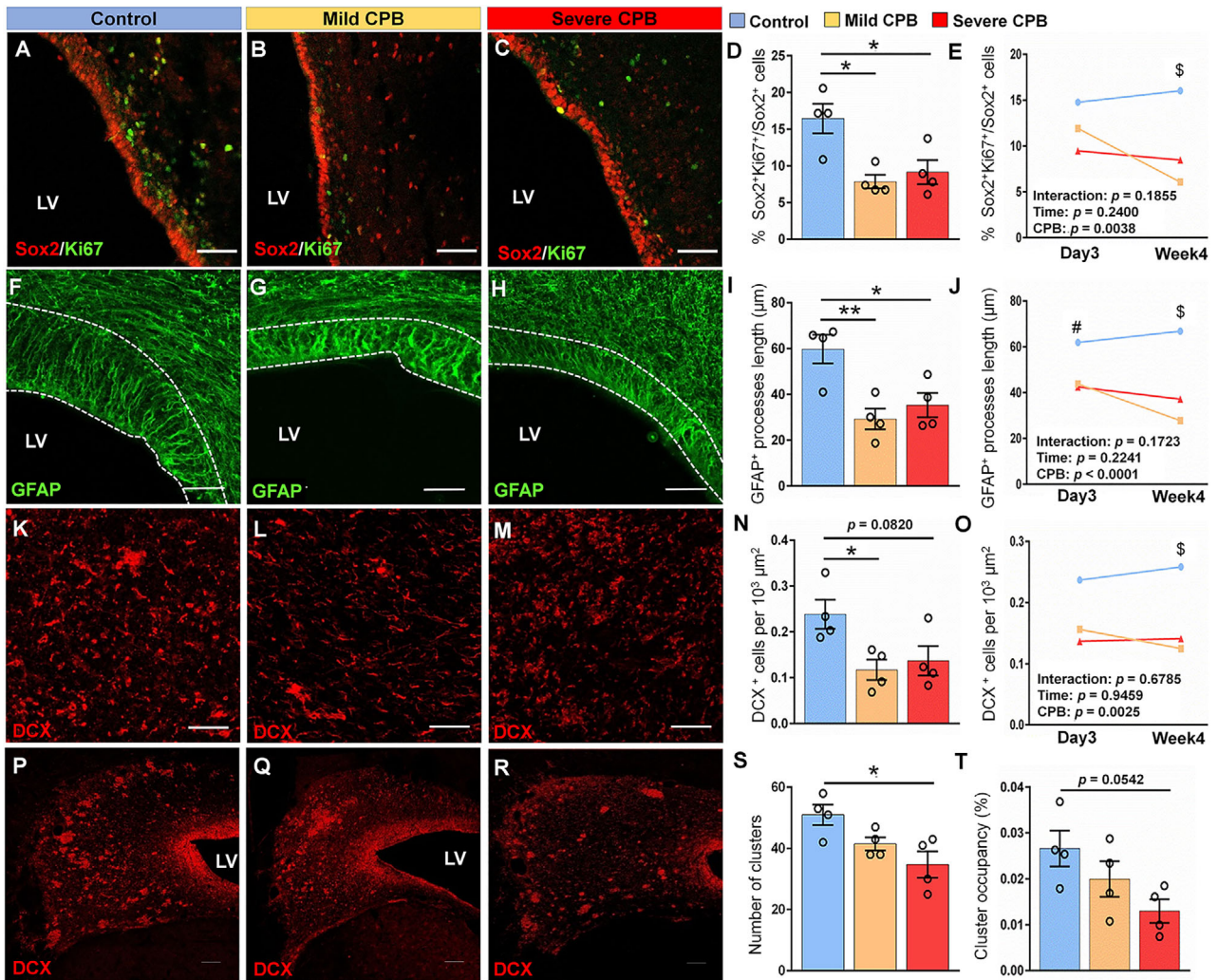


FIGURE 3: CPB causes a long-term disruption of SVZ neurogenesis 4 weeks post-surgery. (A–C) Immunostains of Sox2⁺Ki67⁺ cells in the DL-SVZ. (D) Quantification of the percentage of proliferative Sox2⁺ cells in the whole anterior SVZ. (E) Differences in the percentage of proliferative Sox2⁺ cells in the DL-SVZ between day 3 and week 4 post-surgery. (F–H) Immunostains of GFAP in the DL-SVZ. (I) Quantification of the average length of GFAP⁺ processes in anterior SVZ. (J) Differences in the average length of GFAP⁺ processes in the DL-SVZ between day 3 and week 4 post-surgery. (K–M) Immunostains of DCX⁺ cells in tiers 2–3 of the DL-SVZ. (N) Quantification of the average density of DCX⁺ cells. (O) Differences in the average density of DCX⁺ cells in the DL-SVZ between day 3 and week 4 post-surgery. (P–R) Coronal sections of the SVZ showing immunostains of DCX⁺ cell clusters. (S) Quantification of the average number of DCX⁺ cell clusters. (T) Quantification of the average cluster occupancy of the SVZ. Scale bars, 50 μ m (A, B, C, F, G, H, K, L, M) and 100 μ m (P, Q, R). n = 4 animals per group. Data are expressed as mean $\pm$ SEM. * p < 0.05, ** p < 0.01, # p < 0.05 control compared to severe group, \$ p < 0.05 control compared to severe and mild groups. One-way ANOVA with Tukey's post-hoc test (D, I, N, S, T). CPB = cardiopulmonary bypass; DL-SVZ = dorsolateral-SVZ; DCX = doublecortin; LV = lateral ventricle; SVZ = subventricular zone; V-SVZ = ventral-SVZ; GFAP = glial fibrillary acidic protein.

clusters was not different between the three groups tested, the number of clusters within the SVZ was reduced after severe-CPB insults (Fig 2S). Our analysis also revealed that the overall occupancy of the clusters was significantly reduced in both mild and severe-CPB groups (Fig 2T). In addition to reduction in SVZ neurogenesis, these data suggest that cardiac surgery during the early postnatal period alters neuroblast colonization into the frontal lobe of the neonatal and infant brain.

Cardiopulmonary Bypass Causes Prolonged Alterations in SVZ Neurogenic Properties and Neuroblast Migrating Chains

To determine the long-term effects of cardiac surgery on SVZ neurogenic activity and the neuroblast population, we next examined the SVZ 4 weeks postoperatively. NSPC proliferation in the SVZ was significantly decreased for both mild- and severe-CPB groups (Fig 3A–D). The extensive neurogenic activity of these regions has been

described during the postnatal period.¹⁵ Consistent with our previous findings, this prolonged decrease of proliferative capacity was specifically observed in the dorsolateral-SVZ (Fig 3E). Our results also indicate that CPB insults significantly altered the number of Sox2⁺ NSPCs ($p = 0.0452$). Interestingly changes in NSPC proliferation from day 3 to week 4 significantly differed according to the type of CPB-induced insult (Fig 3E). In the naïve SVZ, approximately 15% of NSPCs are able to maintain their proliferative capacity during this early postnatal period (Fig 3E). In severe-CPB group, the proliferation of

SVZ NSPCs was significantly inhibited as soon as 3 days after surgery and remained consistently low over time (Fig 3E). On the other hand, we found that only 6% of Sox2⁺ NSPCs on average can proliferate 4 weeks after CPB insult (Fig 3E), indicating the critical role of systemic inflammation on the prolonged decline of proliferative ability after pediatric cardiac surgery. These observations were coupled to a marked reduction of GFAP⁺ process length after both mild- and severe-CPB insults (Fig 3F–I). Similar to the effects on NSPC proliferation, the impact of CPB on radial glia-like cells was especially deleterious

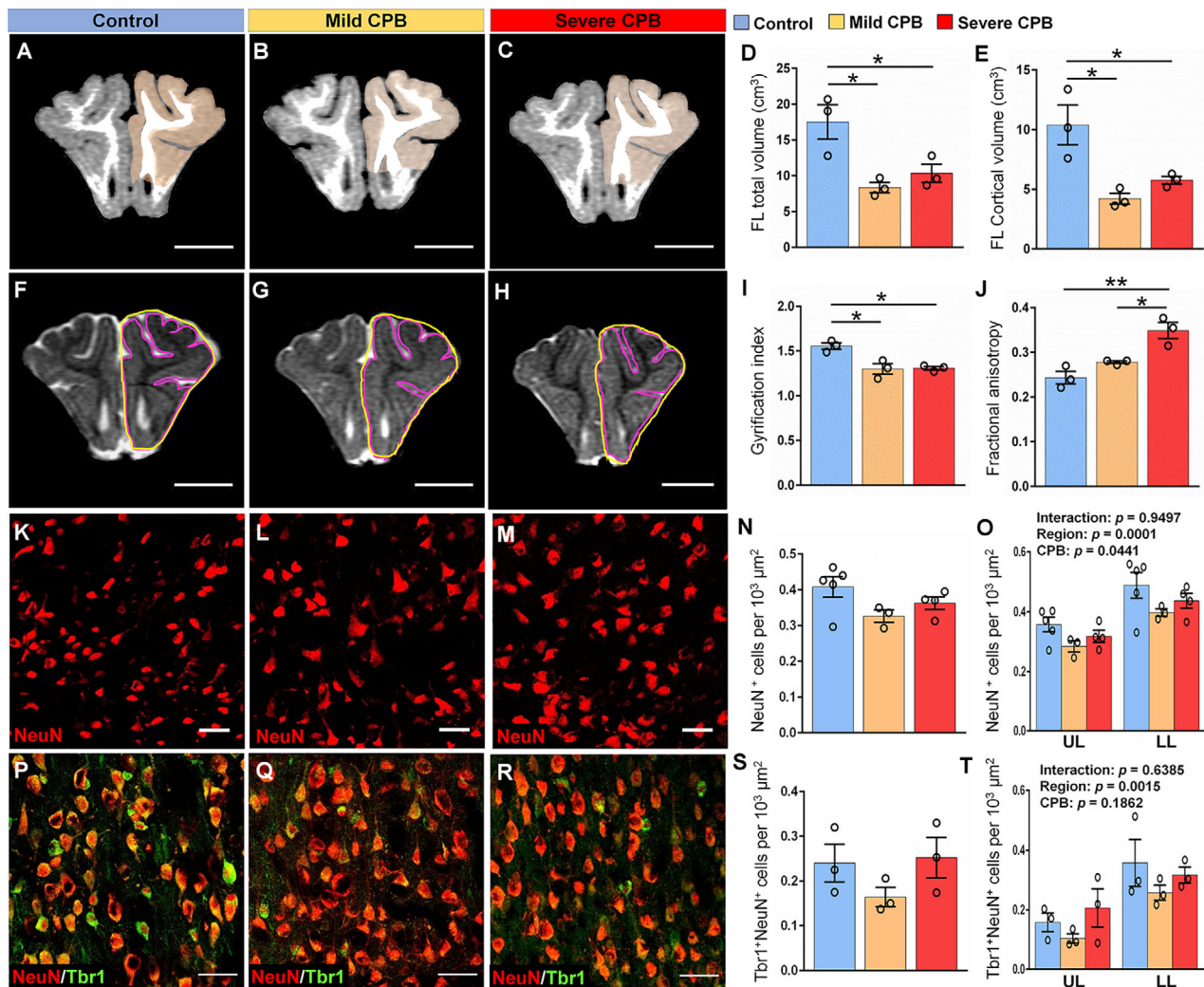


FIGURE 4: CPB impairs cortical development and limits cortical expansion. (A–C) T1 MRI scans showing cortical gray matter of frontal lobe. Scale bars, 1 cm. (D, E) Quantification of frontal lobe total and cortical volumes. (F–H) Perimeter of the total pial surface (purple lines, inner) and total perimeter of the brain (yellow lines, outer) on T2 MRI scans. Scale bars, 1 cm. (I) Quantification of cortical folding determined by the GI expressed as a ratio of the inner versus outer perimeter traces in (F, G, H). (J) Quantification of fractional anisotropy in the cortex of frontal lobe. (K–M) Immunostains of NeuN within the lower cortical layers of the frontal lobe. Scale bars, 50 μm. (N, O) Quantification of the average density of NeuN⁺ cells in the frontal lobe. (P–R) Immunostains of NeuN and Tbr1 within the lower cortical layers of the frontal lobe. (S, T) Quantification of the average density of NeuN⁺Tbr1⁺ cells in the frontal lobe. Scale bars, 50 μm. Data are expressed as mean ± SEM (n = 3–4 animals per group). * $p < 0.05$, ** $p < 0.01$. One-way ANOVA with Tukey's post-hoc test (D, E, I, J, N, S) and two-way ANOVA with Tukey's post-hoc test (O, T). CPB = cardiopulmonary bypass; FL = frontal lobe; LL = lower layers; NeuN = neuronal nuclear protein; Tbr1 = T-box brain 1; UL = upper layers.

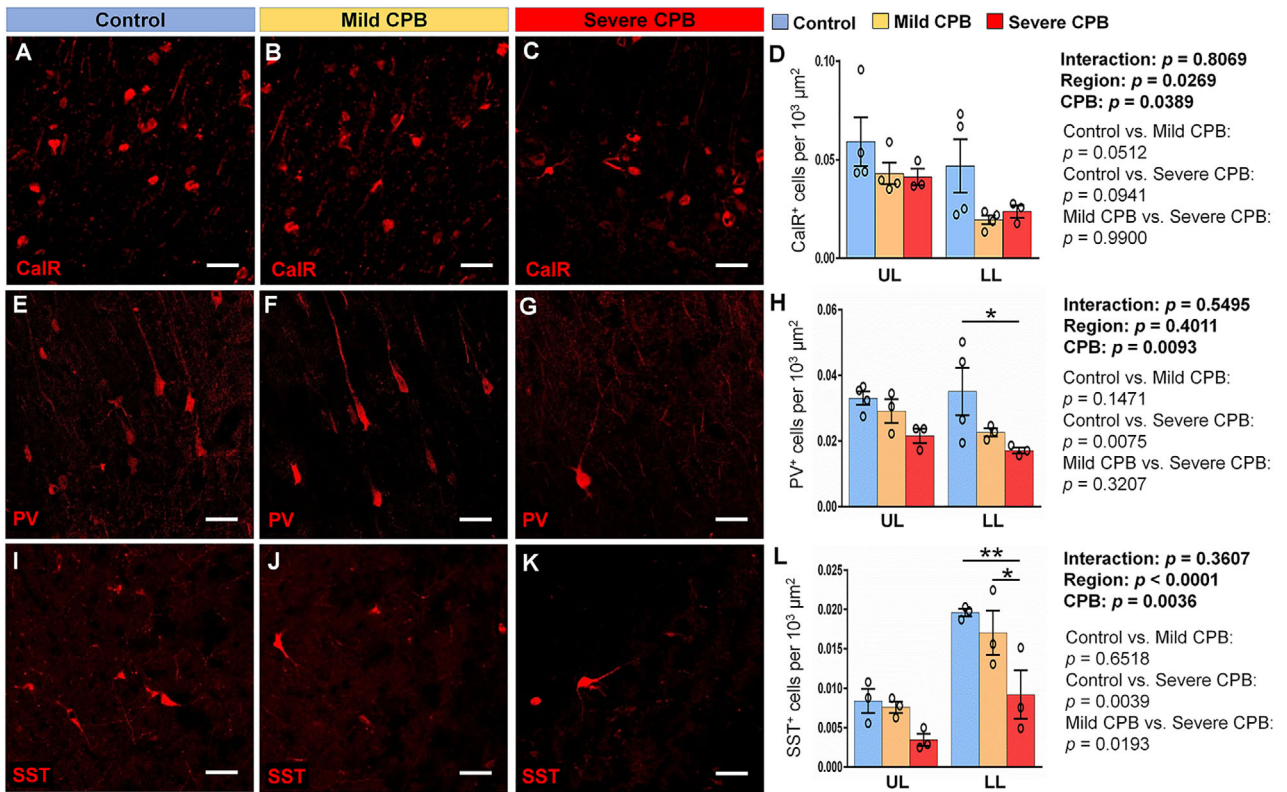


FIGURE 5: CPB alters interneuron cell populations in the cortex 4 weeks post-surgery. (A–C) Immunostains of Calretinin within the lower cortical layers of the frontal lobe. (D) Quantification of the average density of CalR⁺ cells. (E–G) Immunostains of Parvalbumin within the lower cortical layers of the frontal lobe. (H) Quantification of the average density of PV⁺ cells. (I–K) Immunostains of Somatostatin within the lower cortical layers of the frontal lobe. (L) Quantification of the average density of SST⁺ cells. Scale bars, 50 μm. Data are expressed as mean ± SEM (n = 3 to 4 animals per group). * $p < 0.05$, ** $p < 0.01$, two-way ANOVA with Tukey's post-hoc test (D, H, L). CalR = calretinin; CPB = cardiopulmonary bypass; FL = frontal lobe; LL = lower layers; PV = parvalbumin; SST = somatostatin; UL = upper layers.

in the dorsolateral-SVZ where GFAP⁺ processes of radial glia-like cells remained shorter over time in both CPB groups compared to control (Fig 3J). Altogether these results demonstrate the significant and prolonged deleterious impact of cardiac surgery on the largest NSPC pool in the neonatal and infant brain.

When we assessed the long-term effect of cardiac surgery on SVZ neuroblasts, reductions in cell density in the whole SVZ were displayed in both CPB groups (Fig 3K–N). Consistent with our findings for NSPCs, the neuroblast population was reduced specifically in the dorsolateral-SVZ 4 weeks post CPB in both mild and severe insult groups (Fig 3O), whereas no differences were observed in the ventral-SVZ (control vs mild $p = 0.1316$, control vs severe $p = 0.3868$, mild vs severe $p = 0.7261$). Moreover, similar to NSPCs, there was no recovery in neuroblast density over time (Fig 3O), indicating prolonged inhibition of SVZ neurogenesis due to CPB. Finally, a marked reduction of cluster number and a trend in cluster occupancy was shown 4 weeks after severe-CPB (Fig 3P–T). This latter observation specifically found in the group suggests a high susceptibility of migratory

processes in neuroblasts to HCA-induced injury (Fig 1A). Overall, our cellular analysis of the SVZ up to 4 weeks post-operatively provides evidence that a CPB-induced insult not only reduces postnatal SVZ neurogenesis and neuroblast migration in the short-term, but also prevents recovery of the neurogenic properties of the largest progenitor pool for a prolonged period.

Cardiopulmonary Bypass Impairs Cortical Development and Limits Cortical Expansion

Based on our previous finding that SVZ-derived cells populate the frontal cortex in neonatal piglets during normal development,¹⁵ we hypothesized that a reduction in the neuroblast number in the SVZ and structural alteration of neuroblast clusters could play an important role in cortical maturation after cardiac surgery. To test this hypothesis, we first assessed structural maturation of the developing cortex using a clinically-relevant neuroimaging approach 4 weeks after surgery. We did not observe any evidence of focal and/or multifocal injury post CPB. When the volumes of the entire frontal lobe and frontal cortex were quantified, however, both measurements in the mild and

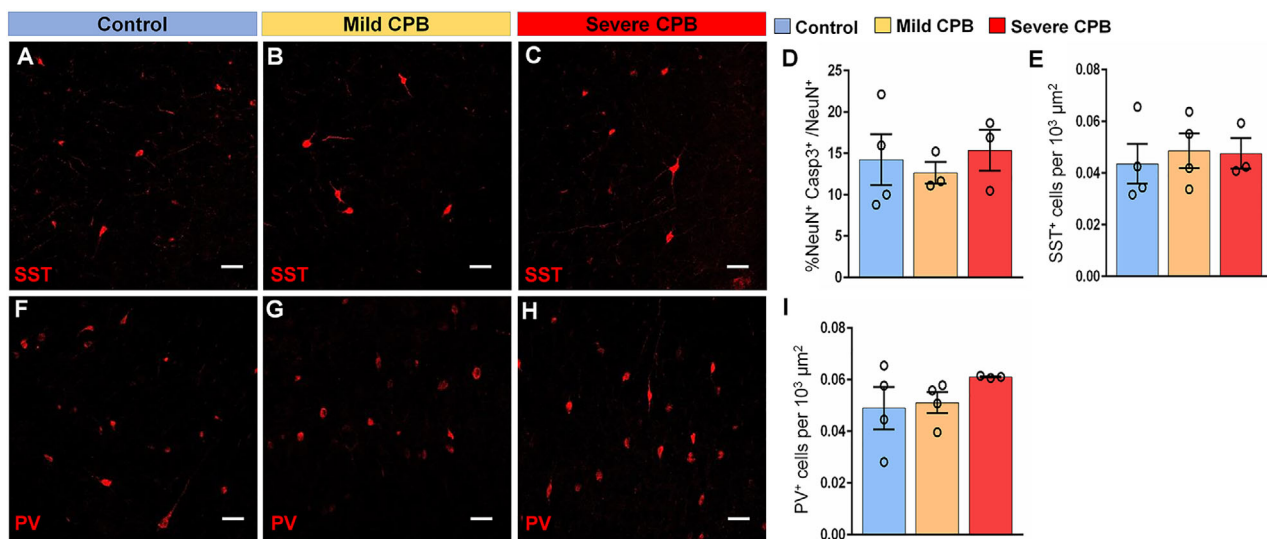


FIGURE 6: CPB does not alter interneuron densities in the piglet cortex 3 days after surgery. (A–C) Immunostains of Somatostatin within the lower cortical layers of the frontal lobe. (D) Quantification of the average percentage of Casp3⁺ NeuN⁺ cells within the piglet frontal lobe. (E) Quantification of the average density of SST⁺ cells. (F–H) Immunostains of Parvalbumin within the lower cortical layers of the frontal lobe. (I) Quantification of the average density of PV⁺ cells. *n* = 3 to 4 animals per group. Two-way ANOVA with Tukey's post-hoc test. Scale bars, 50 μm. Casp3 = cleaved-caspase 3; NeuN = neuronal nuclear protein; PV = parvalbumin; SST = somatostatin.

severe insult groups were significantly decreased compared to control (Fig 4A–E). There were no differences in volumes between right and left hemispheres (total volume; $p = 0.7211$, cortical volume; $p = 0.9887$) indicating a global impact. Consistent with previous findings in gyrencephalic brain development,^{23,24} these observations were paired with a significant reduction in GI (Fig 4F–I), demonstrating structural brain immaturity. Reduced cortical volume and impaired gyrification as observed in this animal model have been well documented in survivors after pediatric cardiac surgery.²⁵ Moreover, when we further compared DTI metrics between the three experimental groups, we found that animals exposed to a severe-CPB insult showed a significant increase in FA (Fig 4J). FA values in the severe group were higher compared to mild-CPB FA values. As cortical microstructural maturation is associated with FA decrease,²⁶ these observations indicate that CPB-induced systemic inflammation can cause aberrations in macrostructural cortical development, whereas HCA-induced injury during CPB induces microstructural changes.

To further understand the relationship between immature cortical development and depletion of neuroblasts in the SVZ, mature neuronal populations were analyzed within upper and lower layers of frontal cortices (Fig 4K–M). We found that the density of NeuN⁺ mature neurons varies between both cortical layers and experimental groups (Fig 4O). Despite no statistical differences in the average density of neurons between the three groups (Fig 4N), together

with decreased cortical volume in the mild and severe groups (Fig 4E) the results indicate an overall reduction in the total number of mature neurons in the frontal cortex after CPB.

Quantification of Tbr1⁺NeuN⁺ excitatory neurons showed a significant difference between layers as for NeuN⁺ cells (Fig 4O–T). Consistent with our previous findings,¹⁵ higher densities of excitatory neurons were found in the cortical lower layers compared to upper layers (Fig 4T). On the other hand, no differences were observed in the density of excitatory neurons between CPB groups throughout the frontal lobe (Fig 4P–T). Similarly, we did not observe differences in the densities of NeuN⁺ mature neurons ($F = 0.72$, $p = 0.49$) and Tbr1⁺NeuN⁺ excitatory neurons ($F = 0.89$, $p = 0.43$) between prefrontal, somatosensory and insular cortices. Altogether our data demonstrate that CPB impairs cortical expansion and microstructural maturation in an insult-dependent manner and limits the number of mature neurons in the frontal cortex of the infant brain.

Cardiopulmonary Bypass Alters Specific Subpopulations of Interneurons in the Frontal Cortex

Given previous findings demonstrating that postnatal migrating neuroblasts differentiate into inhibitory neurons,^{14,15} we next examined the effects of CPB on GABAergic interneuron populations (ie, inhibitory neurons) throughout the frontal cortex. Both experimental groups ($F = 4.58$, $p = 0.0224$) and distinguished cell types ($F = 27.31$, $p < 0.0001$) significantly affected the

cell population 4 weeks post CPB. We found that CPB induced a significant change in the densities of three major GABAergic cell subpopulations: CalR⁺, PV⁺ and SST⁺ interneurons (Fig 5A–L, CPB: $p < 0.05$). Moreover, a severe-CPB insult induced a significant decrease in densities of both PV⁺ and SST⁺ interneurons compared to control, specifically in lower cortical layers (Fig 5E–L). There were no regional differences in the CPB-induced reduction of three interneuron populations (CalR⁺ cells; $p = 0.1709$, PV⁺ cells; $p = 0.0860$, SST⁺ cells; $p = 0.7555$). Notably, SST⁺ interneuron density was also statistically lower in the severe insult group compared to the mild group (Fig 5L). Together with our observations of an unchanged excitatory neuron population (Fig 4S, T), this reduction of interneuron subpopulations 4 weeks after surgery indicates that CPB likely results in excitatory/inhibitory imbalance throughout the frontal cortex.

To assess whether the reduction in interneuron subpopulations was due to CPB-induced cell death or impaired integration of interneurons into the frontal cortex, we examined the effect of CPB on these cortical cell populations 3 days post-surgery. There was no difference in neuronal apoptosis, quantified as percentage of Casp3⁺NeuN⁺ apoptotic neurons per total NeuN⁺ cells (Fig 6D). Moreover, SST⁺ and PV⁺ cell densities were not affected 3 days after CPB (Fig 6A–I), suggesting that cell apoptosis due to CPB is not a major underlying mechanism in the reduction of cortical interneurons observed following CPB. Since severe-CPB specifically decreases cluster number and occupancy in the SVZ for a prolonged period (Fig 3P–T), our results suggest that HCA causes prolonged impairment in neuroblast migration from the SVZ and interferes with the integration of SST⁺ and PV⁺ interneurons into the frontal lobe, thereby causing microstructural alterations of the frontal cortex.

Discussion

This study is the first to reveal the impact of CPB on postnatal SVZ and cortical development in a porcine model which is anatomically and physiologically similar to humans. We demonstrated over a 4-week recovery period that there are significant alterations of the NSPC pool and neuroblast populations in the SVZ resulting in a depletion of a source of interneurons destined to populate and potentially fine-tune development of the postnatal frontal cortex. Furthermore, we noted decreased gyrencephaly and impairment of cortical expansion and maturation, which are pathological signatures observed commonly in survivors of neonatal cardiac surgery for complex CHD. Finally, our study highlights CPB-induced excitatory/inhibitory imbalance throughout the frontal cortex and

the distinct role of CPB-induced I/R injury in cortical dysmaturation. Our findings provide new cellular insights into surgery-induced neonatal brain injury and guide us towards improving management of pediatric cardiac surgery to minimize neurological and behavioral consequences in the CHD population.

In the present study, the mild-CPB group was designed to determine the effects of common stressors in cardiac surgery including SIRS and oxidative stress, while the severe insult group allowed assessment of the additional impact of HCA-induced I/R injury. Our study demonstrated that both mild and severe-CPB insults induced early and late deleterious effects on the largest NSPC pool of postnatal brain and SVZ neurogenesis. It has been well documented previously that NSPCs are highly sensitive to inflammation^{27,28} consistent with our finding of the impact of CPB-induced SIRS on neurogenesis in the neonatal brain. It is encouraging to note that stem cell proliferation can be restored by anti-inflammatory treatments.²⁸

The SVZ generates many neurons endogenously after birth.²⁹ It was recently found that neuroblasts continue to migrate postnatally along the lateral ventricles to populate the frontal lobe in the human infant, contributing to its further maturation.¹⁴ We have previously demonstrated that reductions in NSPC proliferation and SVZ neurogenesis result in reduced cortical growth of the postnatal brain. We also showed that hypoxia affects CalR⁺ interneurons and causes imbalance between excitatory and inhibitory neurons.¹⁵ Consistent with these previous findings, our current data demonstrate reduced cortical folding (GI) and gray matter volume of the frontal lobe as well as excitatory/inhibitory imbalance after both mild- and severe-CPB insults indicating multifactorial etiology of impaired cortical maturation in children with CHD. Interestingly, reduction in these macrostructural MRI indices has been linked to delayed cognitive function.^{30,31} Furthermore, excitatory/inhibitory imbalance in the prefrontal cortex has been strongly associated with intellectual and academic impairment.³² Children with CHD frequently face delays in cognitive milestones that can be attributed to such imbalances.^{3,33} In addition, changes in gamma oscillations, which are a product of the interplay between interneurons and excitatory principal cells,³⁴ are commonly reported in psychiatric conditions.^{35,36} To explore possible therapeutic approaches for improvement of cortical development in children with CHD, it will be important to define molecular targets that can prevent depletion of NSPC neurogenic potential and accelerate endogenous recovery through postnatal SVZ neurogenesis.

Our study demonstrates that I/R stress, only present in the severe group, is critical for the cortical dysmaturation of the frontal lobe determined by DTI.

Similar with our findings, an increase in FA values is observed in the immature cortex of preterm children.^{37,38} Current literature suggests that maturation of the neonatal cortex is associated with increased dendritic complexity which is correlated to decreased FA. Our detailed analysis of neuronal subpopulations revealed that severe-CPB decreases PV⁺ and SST⁺ interneuron populations. SST⁺ interneurons influence other interneuron classes and also target the dendritic spines of pyramidal neurons. It has been demonstrated that this sub-population controls dendritic calcium spikes in pyramidal neurons, thus affecting learning tasks.³⁹ Fast-spiking PV⁺ interneurons regulate local circuit dynamics,⁴⁰ and PV⁺ structural and physiological abnormalities have been strongly linked to psychiatric disorders.⁴¹ Thus, a lack of these inhibitory interneurons could provide some insight into the underlying mechanisms behind microstructural alterations such as reduced dendritic complexity observed after severe-CPB insults. Each interneuron subpopulation has a specific spatial origin and birthdate, and migrates during different developmental time windows.⁴² In the present study, both mild and severe-CPB insults resulted in reduced cluster number and occupancy of neuroblast migratory chains on postoperative day 3, (ie, 3 weeks of age) and affected the CalR⁺ interneuron population. Similarly, in our previous study, chronic hypoxia from postnatal day 3 to 14 reduced CalR⁺ interneurons.¹⁵ On the other hand, we observed reduced migratory chains at 4 weeks post CPB (7 weeks of age) in the severe-CPB group together with decreases in PV⁺ and SST⁺ interneurons. The critical timing of damage in migrating neuroblasts may contribute to the distinct disruptions of this interneuron subpopulation that we noted between the mild and severe CPB groups.

We previously identified that the FA value from diffusion tensor imaging in the same piglet white matter structures at postnatal 3 weeks was equivalent to those of human post-conceptual 41–53 weeks.⁸ On the other hand, GI of 7-week-old control piglet cortex in this study was approximately 1.5, which is similar to that of a human cortex at 37–38 week gestation in previous clinical studies. It is challenging to define the epochs in human cortical maturation that correspond to this piglet model because obvious technical/ethical difficulties prevent cellular investigations in the human brain during the late fetal and postnatal periods. However, our imaging studies indicate that the developmental time windows in the piglet model at this age correspond with the late gestation and early neonatal period in the human brain.

Higher mammals like pigs avoid many of the translational limitations encountered when studying rodents. The porcine brain has a gyrencephalic cortex and a multi-layered SVZ similar to the human SVZ.¹⁵ The pig model

has been widely used to investigate adverse effects of cardiac surgery and CPB.^{17,43} Piglets undergoing CPB display white matter injury similar to that seen in human patients.^{8,9} On the other hand, there are limitations in using juvenile pigs. Compared to studies with rodents, a small number of animals was assessed in each group. Although the deviation of physiological parameters in our studies were limited because of a well-controlled procedure, it is possible that the small animal number caused the lack of difference between the mild and severe groups in NSPC proliferation, morphology of radial-glia like cells, or neuroblast density during the acute period after surgery. In addition, behavior tests used in this study showed that neurological deficits were resolved 1 day after mild-CPB and 7 days after severe-CPB injury, suggesting the limited sensitivity to assess long-term neurological impairments. Compared to rodents, region-specific neurobehavioral tests have not been well established in a porcine animal. Establishment of sophisticated behavior assays will be required for further improvement of this unique model. Our studies found increased cortical fractional anisotropy after severe CPB injury. However, the relationships between DTI signals and neuropathology in the cortex is more complex compared to white matter. For instance, previous studies indicated that the increased FA in the cortex following traumatic brain injury is associated with reactive astrocytes.⁴⁴ In addition, the presence of inflammation with microglia and macrophage activation significantly alters DTI indices.⁴⁵ More detailed analyses using the gyrencephalic piglet cortex will provide important data needed to better interpret clinical human imaging studies. Our findings reflect the response of a normally developed brain to CPB because the animals used in this study did not experience fetal cerebral hypoxia. Therefore, it is possible that infants with complex CHD may experience these stresses differently based on their brain maturation status. Finally, in our studies two CPB injury groups were compared to naïve control, but not surgical control. Therefore, common surgery-induced insults such as anesthesia may affect some of our findings. Brain injury associated with pediatric cardiac surgery are multi-etiological. Although this study distinguished major CPB-induced insults between SIRS and I/R, future studies will be necessary to distinguish each factor individually from the global impact of cardiac surgery. On the other hand, our study provides a unique view of the roles of CPB and I/R on the largest NSPC niche and on cortical development.

In summary, our porcine model has given us insights into the cellular alterations that explain cortical dysmaturation occurring after CPB. Unraveling cellular/molecular events during surgery will enable us to design therapeutic approaches that can be restorative or reparative to the neurogenic potential

of SVZ NSPCs and may reduce the undesirable impact of cardiac surgery. Further studies using this model will likely contribute to continuing refinement of pediatric cardiac surgery thereby improving the quality of life for children with complex CHD.

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Author Contributions

For this manuscript, C.L. and N.I. were responsible for study concept and design. Z.D., C.L., S.L., A.P., J.H., D.Z., and P.C.W. contributed to data acquisition and analysis. Z.D. and C.L. drafted the manuscript and figures with R.A.J. and N.I. Z.D. and C.L. share the first author position. The authorship order among co-first authors was decided by volume of data acquisition used in the manuscript. All authors reviewed the manuscript before submission.

Potential Conflicts of Interest

Nothing to report.

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