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Neurodevelopment: Parental influences, in utero exposures, and genetics

Edited by
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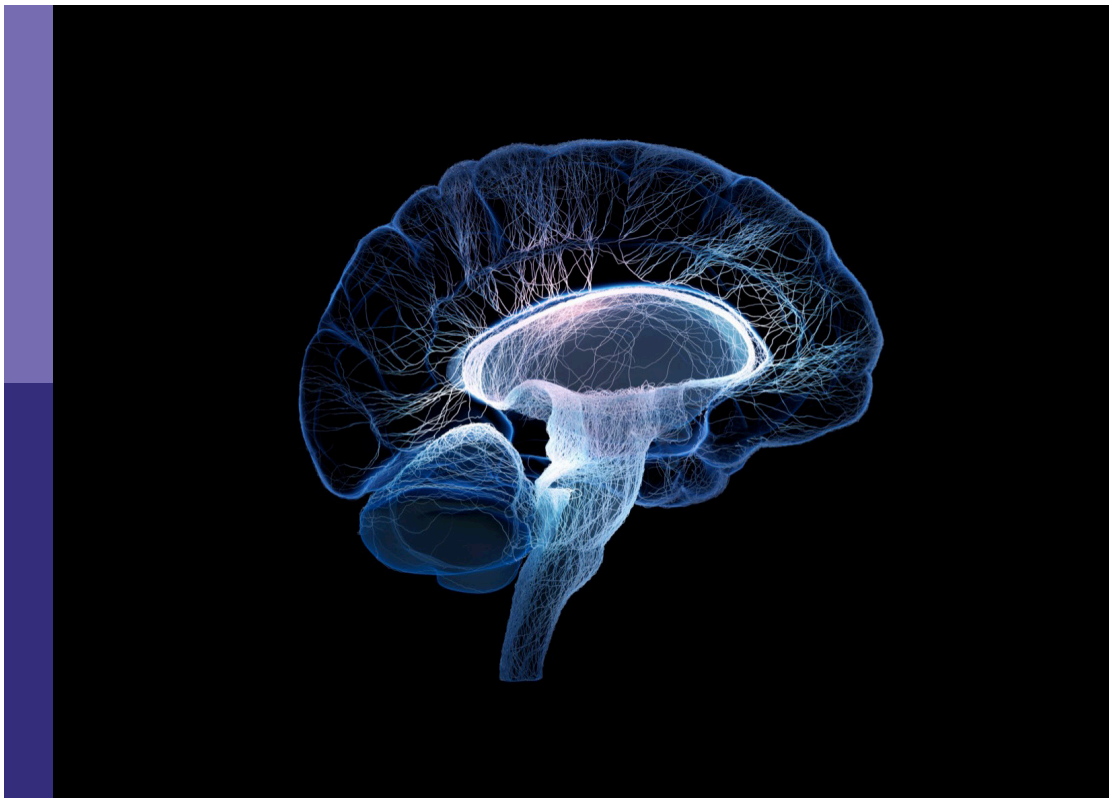

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Neurodevelopment: Parental influences, in utero exposures, and genetics

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Editorial: Neurodevelopment: parental influences, *in utero* exposures, and genetics

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Editorial on the Research Topic
Neurodevelopment: parental influences, *in utero* exposures, and genetics

The development of the central nervous system is subject to a broad range of disruptions both environmental and genetic in nature. These disruptions to neurodevelopmental trajectories can result in significant changes to brain structure, connections, and eventually alterations of behavioral outcomes in the short and long-term. Neurodevelopmental disorders and conditions remain a global concern, however, emerging reports indicate that low and middle-income countries bear a more heavy burden related to these conditions (Bitta et al., 2018). While rates of prevalence of neurodevelopmental disorders and related conditions vary between countries, it is reasonable to assume that current estimates are undercounting actual global prevalence of these conditions based on the most updated clinical criteria for diagnoses (Francés et al., 2022). Importantly, pharmacological and other therapeutic interventions have not kept a pace of neurodevelopmental conditions, likely due to a relative lack of basic biomedical research into these conditions.

Although much work remains to be done in the field of neurodevelopmental disorders, over the last half-century there is a greater appreciation of the multifactorial etiology of these conditions. While genetics has long been understood as a critical determinant of these conditions, the developmental environment has received greater scientific and research scrutiny. Importantly, the role of both the prenatal and postnatal environment on cellular and molecular mechanisms affecting neurodevelopment has become a topic of surging interest. This includes important research interfaces with neurodevelopment including the role of the gut-brain axis, substance abuse, viral infections, and signal transduction pathway analysis for diagnostics and therapeutics.

This Research Topic includes nine exciting manuscripts covering important questions across the field of neurodevelopment and the disorders that can result due to specific environmental and genetic disruptions to brain development.

In their impactful research study, Perez et al. tackle the problem of maternal alcohol consumption and its effect on offspring. While a number of studies have investigated the effect of maternal alcohol consumption

during the prenatal period, only a handful if any have attempted to define the potential role of maternal alcohol consumption during the lactational period in causing developmental brain deficits in offspring. Using a combination of behavioral, anatomical and cellular-morphological quantification in a novel lactational ethanol exposure mouse model, the authors identify the effects of maternal alcohol consumption during this key postnatal developmental period.

Along a related theme, Crawford et al. in their research manuscript address the important and challenging issue of fentanyl use during pregnancy and neonatal development. Using a rat model of fentanyl use, the authors assess potential effects of this exposure using a battery of behavioral tests at late adolescence. This study lays the groundwork for future preclinical studies on the lasting consequences of fentanyl on neurodevelopment.

Rodent models of neurodevelopmental disorders with complex etiologies often capture different aspects of symptomology; hence it is important to assess potential differences between models using standardized behavioral tests. Carbajal et al. in their research manuscript define the amount of impulsivity in two rat models of attention deficit hyperactivity disorder (ADHD) including spontaneously hypertensive rats and a transgenic rat model in which the ADHD-linked gene Lphn3 has been knocked out. The authors use the delay-discounting task to quantify impulsive choice in both mouse models with interesting differences between the models.

Increasingly, the recognition that neurodevelopmental trajectories can be significantly altered due to infection, neuroimmune responses, and inflammatory processes has come to the fore. Three provocative and insightful review manuscripts cover different angles of the intersection between neurodevelopmental disorders and infectious disease. In their review, Hall et al. take an epidemiological view of neurodevelopmental disorders with an emphasis on maternal immune activation—maternal immune responses that somehow disrupt fetal neural development. Helpfully, the authors also survey important factors that are involved in the onset of neurodevelopmental disorders and how translatable these factors are from animal models to human disorders.

In their review, Recaioglu and Kolk focus on viral infections and how these could potentially alter neurodevelopment. The authors cover more recent viral infections affecting many countries particularly many in the global south including Zika, Chikungunya, and SARS CoV-2. The authors pay special attention to potential routes of entry of these viruses to the developing fetal brain and the cellular populations that are particularly vulnerable to virus-mediated damage.

Zooming in on SARS-CoV-2, Dubey et al. perform a deep dive on the potential ways by which this pandemic-causing virus could affect the prenatal brain, especially considering that early studies indicated that pregnant women were especially vulnerable to COVID-19. The authors cover a lot of ground, looking at clinical evidence from published reports and point to a broad range of cellular, genetic, epigenetic, and brain pathways that future biomedical studies could follow-up on. These studies could then shed light on a disease for which the potential to disrupt neurodevelopmental trajectories appears to be very significant.

The interaction between the maternal microbiome and the developing fetus has been increasingly implicated in affecting long-term neurodevelopmental outcomes of offspring via the maternal-gut-fetal-brain axis. In their important study, Castillo-Ruiz et al. determine if the effects of germ-free gestation—fetal brain development in a maternal environment that is devoid of microorganisms—are more due to *in utero* cellular events or postnatal programming. The authors perform a careful analysis using cellular-morphological techniques as well as gene sequencing analysis to answer this question.

Finally, addressing the molecular and mechanistic bases of neurodevelopmental disorders and conditions we have two review manuscripts covering different signal transduction pathways, both of which have important insights in their particular biological contexts. Ca^{2+} is a critical second messenger in the cell that regulates a host of different cellular processes. Klocke et al. in their manuscript shine a spotlight on available evidence linking Ca^{2+} activity and homeostasis to neurodevelopmental disorders including autism spectrum disorder, ADHD, and schizophrenia.

Neonatal intensive care units (NICUs) have grown significantly efficient over the past 25 years, so much so that neonatal mortality rates have drastically reduced (Driscoll and Ely, 2020). However, certain perinatal complications still cause significant neonatal morbidity. A major cause of neonatal morbidity across the globe is Hypoxic-ischemic encephalopathy (HIE), however, few effective treatment options have been put forward for neonates affected by HIE. Christidis et al. perform a systematic review on a particular class of treatment option offered to HIE-affected neonates—drugs that target the Src kinase signaling pathway. The authors identify evidence on targeting this pathway in preclinical animal models with the hope that this would clarify how effective exactly it is to target the Src kinase pathway in HIE.

In summary, the collection of manuscripts we have edited in this Research Topic represents cutting-edge research papers and reviews across the field of neurodevelopmental disorders and conditions. It is our hope that this Research Topic would help answer important research questions and lead to many more important questions in this field.

Author contributions

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Conflict of interest

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Neuroprotective effect of Src kinase in hypoxia-ischemia: A systematic review

Panagiotis Christidis[1], Abhya Vij[2], Stamatios Petousis[3], Javid Ghaemmaghami[4], Bhairav V. Shah[5], Ioannis Koutroulis[6] and Panagiotis Kratimenos[4,7]*

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Background: Hypoxic-ischemic encephalopathy (HIE) is a major cause of neonatal morbidity and mortality worldwide. While the application of therapeutic hypothermia has improved neurodevelopmental outcomes for some survivors of HIE, this lone treatment option is only available to a subset of affected neonates. Src kinase, an enzyme central to the apoptotic cascade, is a potential pharmacologic target to preserve typical brain development after HIE. Here, we present evidence of the neuroprotective effects of targeting Src kinase in preclinical models of HIE.

Methods: We performed a comprehensive literature search using the National Library of Medicine's MEDLINE database to compile studies examining the impact of Src kinase regulation on neurodevelopment in animal models. Each eligible study was assessed for bias.

Results: Twenty studies met the inclusion criteria, and most studies had an intermediate risk for bias. Together, these studies showed that targeting Src kinase resulted in a neuroprotective effect as assessed by neuropathology, enzymatic activity, and neurobehavioral outcomes.

Conclusion: Src kinase is an effective neuroprotective target in the setting of acute hypoxic injury. Src kinase inhibition triggers multiple signaling pathways of the sub-membranous focal adhesions and the nucleus, resulting in modulation of calcium signaling and prevention of cell death. Despite the significant heterogeneity of the research studies that we examined, the available evidence can serve as proof-of-concept for further studies on this promising therapeutic strategy.

KEYWORDS

Src, hypoxic-ischemic encephalopathy (HIE), hypoxia, neonatal brain, neuroprotection

Introduction

One in four perinatal deaths is attributed to hypoxic-ischemic (HI) brain injury, a term that describes acute interruptions in oxygenated blood flow to the brain (Lawn et al., 2005; Pauliah et al., 2013). Following the perinatal asphyxiation insult, HI involves a cascade of biochemical events that cause cerebral edema, inflammation, neuronal cell injury, and ultimately, neuronal cell death over a period of hours to days (Gunn and Thoresen, 2006; Cilio and Ferriero, 2010; Juul and Ferriero, 2014; Hagberg et al., 2015; Van Bel and Groenendaal, 2016; Delivoria-Papadopoulos et al., 2018). While there have been improvements in survival rates after neonatal HI, many of these patients suffer ongoing neurological impairments that both lessen quality of life and incur burdensome healthcare costs (Blencowe et al., 2013; Eunson, 2015).

The only evidence-based treatment currently available for neonatal HI is therapeutic hypothermia (TH). This approach is targeted at modulating the deleterious cytotoxic and inflammatory processes that occur during HI by tightly regulating temperature (Wyatt et al., 2007), blood pressure, ventilation, and glucose metabolism; for some patients, application of TH has resulted in improved neurological outcomes (Filan et al., 2006; Tam et al., 2012; Wong et al., 2013).

While conceptually promising, the application of TH has several limitations. First, it is mainly available in high-resource countries. Moreover, the protocol requires that total body cooling be initiated within 6 h of birth, leaving clinicians with a narrow window to establish the diagnosis, assess the severity of HI and implement treatment with TH. This time is further compressed for centers that do not have the necessary advanced equipment, staffing, monitoring and experience to provide neonatal TH, as attempts are made to transfer the patient to a suitable facility (Olsen et al., 2013). Finally, despite the use of TH, both the overall mortality rates and the disability rates following application of TH after neonatal HI in published trials remain high (Shankaran et al., 2012).

Most strikingly, even when TH is timely applied in an advanced neonatal intensive care unit, only a subset of neonates with HI have been shown to benefit (Gunn and Thoresen, 2006). Thus, there is a critical and urgent need to develop additional therapeutic strategies that address both morbidity and mortality following neonatal HI. To this end, multiple pre-clinical *in vivo* studies have focused primarily on (a) elucidating the molecular biology underlying HI, (b) identifying potential molecular targets in pathways integral to cerebral injury, (c) optimizing cooling strategies, and (d) recognizing adjuvant therapies that could augment the neuroprotective effects of TH (Jacobs et al., 2013). These studies implicate potential targets of the apoptotic cascade that may, when modulated by pharmacological intervention, offer additional or alternative therapies for HI, critical in cases where TH is not available, particularly in low-resource countries (Robertson et al., 2008; Pauliah et al., 2013; Montaldo et al., 2015), and in treatment of patients who do not respond or respond insufficiently to TH.

In particular, these studies have shown that Src kinase is involved in numerous activated intracellular pathways during HI (Paul et al., 2001; Mishra et al., 2009; Haass and Mandelkow, 2010; Ittner et al., 2010; Delivoria-Papadopoulos et al., 2011; Liu and Sharp, 2011; Hossain et al., 2012; Angelis and Delivoria-Papadopoulos, 2017a,b; Kratimenos et al., 2017). However, there is conflicting evidence regarding its regulatory role, which may differ depending upon brain maturation. Hossain et al. demonstrated that Src kinase activation improves neuronal survival in primary cortical cell cultures (Paul et al., 2001; Haass and Mandelkow, 2010; Ittner et al., 2010; Liu and Sharp, 2011; Hossain et al., 2012), whereas several other studies have shown that Src kinase phosphorylation causes neuronal damage in ischemic stroke, intracerebral hemorrhage, and Alzheimer's disease (Haass and Mandelkow, 2010; Ittner et al., 2010; Liu and Sharp, 2011; Hossain et al., 2012). Porcine experimental models have been used to examine the deleterious effects of Src kinase in neonatal HI and have shown that it can induce the production of free radicals, causing secondary inflammation and excitotoxicity (Kratimenos et al., 2017, 2018, 2022).

Selective Src inhibitors (Src-i) exhibit effectiveness against neuronal cell injury in neonatal and developing animal models and offered neuroprotection as demonstrated by histologic, biochemical, and neurobehavioral assessments (Mishra et al., 2009; Delivoria-Papadopoulos et al., 2011; Angelis and Delivoria-Papadopoulos, 2017a,b; Kratimenos et al., 2017). Contrary to those results, experiments in adult mice showed that Src kinase inhibition worsens cerebral injury (Wang et al., 2004; Guo et al., 2006; Wu et al., 2008; Hu et al., 2009; Tian et al., 2009). In addition, several studies have highlighted the role of Src kinase in neuronal survival after ischemia/reperfusion (I/R) through interactions with the extracellular signal-regulated kinase (ERK) (Wang et al., 2004; Guo et al., 2006; Wu et al., 2008; Hu et al., 2009; Tian et al., 2009).

This systematic review aims to examine the current knowledge regarding the role of Src kinase in neonatal HI and the potential neuroprotective effects of selective Src manipulation.

Materials and methods

Protocol

A review of relevant preclinical studies was performed to summarize the current knowledge regarding the role of Src kinase inhibition and its potential benefits on the neonatal hypoxic-ischemic brain. We utilized the CAMARADES (Collaborative Approach to Meta-Analysis and Review of

Animal Data from Experimental Studies) guidelines in the methodology (De Vries et al., 2015).

Literature search

A literature search for the Medline electronic database for all studies up to July 01, 2022. Below, the search strategy for the database is presented: *((("hypoxia"[MeSH Terms] OR "hypoxia"[All Fields]) OR ("ischaemia"[All Fields] OR "ischemia"[MeSH Terms] OR "ischemia"[All Fields])) OR (("cerebrum"[MeSH Terms] OR "cerebrum"[All Fields] OR "cerebral"[All Fields] OR "brain"[MeSH Terms] OR "brain"[All Fields]) OR ("brain"[MeSH Terms] OR "brain"[All Fields])))) AND Src kinase[tiab]*. The reference lists of the retrieved articles were subsequently manually reviewed to identify any additional studies that would be considered for inclusion.

Inclusion criteria

Preclinical studies were included. For inclusion, experimental study protocols were required to involve animals treated with any kind of Src kinase inhibitor before or after the induction of HI. The outcome measures of the studies analyzed were required to include "neuroprotection" defined by histologic, biochemical, and/or neurobehavioral findings.

The current review defines HI as an acute interruption of blood flow and oxygen to the brain. In preclinical animal models, HI is typically induced *via* ligation or occlusion of the common carotid artery or by decreasing oxygen concentration in mechanically ventilated animals. Given the bilaterality of neonatal HI, we focused on experimental protocols that induced global brain hypoxia rather than unilateral hypoxia. Global transient hypoxia could be induced by four-vessel occlusion (4VO) of both vertebral arteries and common carotid arteries, by bilateral occlusion of common carotid arteries (2VO), or by titration of the FiO_2 below 0.21 (drop of FiO2 to 0.05–0.006 within 5 min, maintained for the 60 min period and titrated to achieve a 40 % reduction in systolic BP from baseline) in a controlled environment for a period of time (Traystman, 2003).

Exclusion criteria

We excluded all studies that were based on cell lines and *in vitro* experiments. Articles written in languages other than English were also excluded. We also excluded studies that treated animals with unilateral ligation of one of the carotid arteries or with occlusion of fewer than four vessels since these techniques are commonly used in stroke models. Studies that did not use selective Src kinase inhibitors or studies in which the intervention did not directly result in Src kinase modulation were not eligible for inclusion.

Risk of bias assessment

Assessment of risk for bias was based on the Systematic Review Center for Laboratory Animal Experimentation (SYRCLE) Risk of Bias (RoB) tool (Hooijmans et al., 2014), which was derived by the Cochrane Risk of Bias tool. SYRCLE RoB tool consists of nine questions adjusted for the specific characteristics of bias contributing to the results of interventional preclinical studies. Each question was marked as "Yes," "No," or "Unclear."

Data extraction

From each study, the following data were extracted: authors' names, year of publication, sample size, type of animal model and age, type of Src kinase inhibitor and timing of administration, method for HI induction, Src kinase inhibition outcomes, role of Src kinases (protective/damaging on neuron's survival) and whether reperfusion took place.

Results

Eligible studies

Results from the Medline database were combined by a citation manager software (Mendeley Desktop, Version 1.19.8), and duplicate entries were removed. Our search yielded 443 studies that were then screened by title and abstract to meet our criteria. Eighty-nine studies were found relevant to this review and required further analysis. Access to full-text articles, despite repeated efforts, was not possible for six studies, thus these were excluded. The remaining eighty-three studies were screened using our pre-defined inclusion and exclusion criteria. Sixty-three studies did not meet our inclusion criteria. Most studies (twenty-one) were excluded due to the use of cell cultures. Other common reasons for exclusion were the lack of an Src kinase inhibitor in the protocol (16 studies) or of a hypoxic event (nine studies). Six studies described unilateral brain hypoxia (mimicking stroke), seven studies were literature reviews and, finally, four articles were published in a non-English language. Table 1 summarizes the reasoning used for exclusion of the sixty-three studies. Ultimately, twenty studies were considered eligible for this analysis. Due to the variability in experimental methods and outcomes, it was impossible to perform a meta-analysis on the effect of Src kinase inhibition on the neonatal brain after hypoxia, so a qualitative approach was taken instead. An illustration of our methodology and approach is demonstrated in Figure 1.

TABLE 1 Studies not eligible based upon the exclusion criteria.

Exclusion criteria	No of studies
No hypoxic-ischemic brain injury	9
No global (unilateral) ischemia	6
No Src kinase inhibitor	16
Other types of cells/cell lines	21
Non-English	4
Review	7
Overall	63

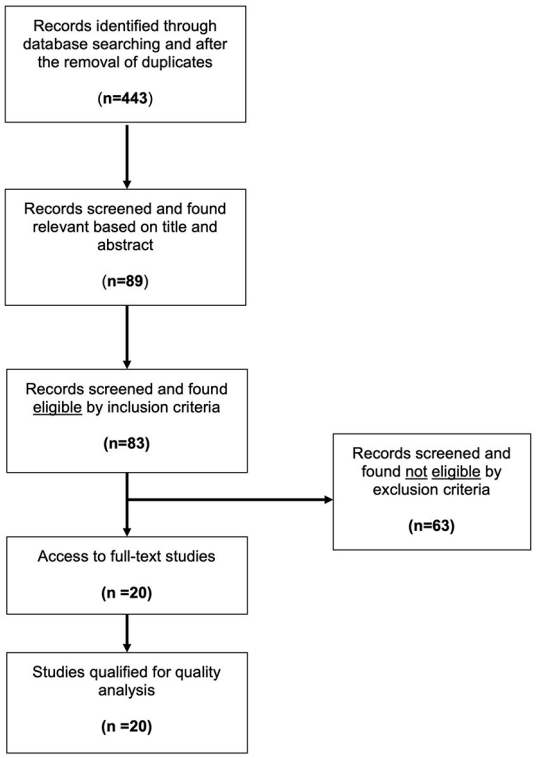

FIGURE 1
Flow diagram of the literature search. Modified from the preferred reporting items for systematic review and meta-analysis protocols (PRISMA-P) and modified to accurately depict the literature research (Moher et al., 2009).

Risk for bias assessment

As previously described, the evaluation of risk for bias was based on the SYRCLE RoB tool (Hooijmans et al., 2014). The twenty studies that were included in this review were evaluated for selection, detection, performance, and attrition bias. All but six studies (14/20, 70%) were prone to selection bias because the authors did not adequately describe the methodology used for assigning the animals to the experimental groups. In all twenty studies (20/20, 100%), the investigators were not blinded to the intervention (performance bias), the animals were not housed randomly (performance bias), or the animals were not randomized for outcome assessment (attrition bias). Only two studies (2/20, 10%) had two independent assessors review their results (neuropathology scores) and only two studies (2/20, 10%) specifically described the allocation concealment process. All the studies had animals that were comparable at baseline prior to group assignment, did not selectively present their outcomes, and appropriately addressed incomplete data. The results from the risk for bias analysis are shown in Table 2.

Study characteristics

Four different species of animals were used in the included studies: Sprague-Dawley rats ($n_{studies}$ = 7, 35%), Yorkshire newborn piglets ($n_{studies}$ = 10, 50%), Swiss albino mice ($n_{studies}$ = 2, 10%), and mice CD1 strain ($n_{studies}$ = 1, 5%). Twelve studies ($n_{studies}$ = 12, 60%) specified the total number of animals used; the remaining studies ($n_{studies}$ = 8, 40%) did not document the number of animals and the size of the assigned groups.

In all studies, a single dose of Src inhibitor was administered. In eighteen studies ($n_{studies}$ = 18, 90%) the investigators administered a selective Src kinase inhibitor (PP1, PP2, PP3, or SU6656). The remaining two studies ($n_{studies}$ = 2, 10%) included two different non-selective inhibitors of Src kinase, the neuronal nitric oxide synthase inhibitor (nNOSi) and (*RS*)-2-amino-3-(3-hydroxy-5-*tert*-butylisoxazol-4-yl) propanoic acid (ATPA), which also modulated the activity of the Src kinase after the hypoxic insult. In addition, one study ($n_{studies}$ = , 5%) combined therapeutic hypothermia with Src-i. The Src-i and their characteristics are presented in Supplementary Table 1.

Different models of HI were used in the included studies, primarily depending on the animal species involved. Eleven studies ($n_{studies}$ = 11, 55%) used FiO2 titration to establish transient cerebral hypoxia; most of these studies (10/11, 91%) utilized piglets. In two studies ($n_{studies}$ = 2, 10%), investigators performed bilateral carotid occlusion (2VO) in Swiss albino mice and in seven of them ($n_{studies}$ = 7, 35%) they used the method of 4VO in Sprangue-Dawley rats. According to the data that were extracted, reperfusion ensued after HI in every study. Analyzed studies, the species and number of animals, method of HI and type of Src-i used are summarized in Table 3.

Concerning the timing of intervention, the experimental compound was administered after the onset of the HI event in only three of the twenty studies ($n_{studies}$ = 3, 15%), mimicking the actual sequence of events in clinical practice. In the remaining seventeen studies ($n_{studies}$ = 17, 85%), the compound was given prior to the induction of the hypoxic insult. Two studies ($n_{studies}$ = 2, 10%) described conditioning which entails several brief repetitive cycles of ischemia with intermittent reperfusion prior to

TABLE 2 Assessment of risk of bias based upon SYRCLE RoB tool.

Risk of bias	Type of bias	Selected Studies																				
		Wang et al. (2004)	Guo et al. (2006)	Zhang et al. (2007)	Wu et al. (2008)	Xu et al. (2008)	Jiang et al. (2008)	Mishra et al. (2009)	Hu et al. (2009)	Tian et al. (2009)	Rehni et al. (2011)	Delivoria-Papadopoulos et al. (2011)	Delivoria-Papadopoulos (2012)	Kumar et al. (2014)	Angelis et al. (2014)	Angelis et al. (2015)	Angelis and Delivoria-Papadopoulos (2017a,b)	Angelis and Delivoria-Papadopoulos (2017a,b)	Kratimenos et al. (2017)	Kratimenos et al. (2018)	Kratimenos et al. (2022)
1 Was the allocation sequence adequately generated and applied?	Selection bias	1	1	1	1	1	1	2	1	1	1	2	1	2	1	1	1	1	2	2	2
2 Were the groups similar at baseline or were they adjusted for confounders in the analysis?	Selection bias	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
3 Was the allocation adequately concealed?	Selection bias	1	1	1	1	1	2	1	1	1	1	1	1	1	1	1	1	1	1	1	2
4 Were the animals randomly housed during the experiment?	Performance bias	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
5 Were the caregivers and /or investigators blinded from knowledge which intervention each animal received during the experiment?	Performance bias	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
6 Were animals selected at random for outcome assessment?	Detection bias	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7 Was the outcome assessor blinded?	Detection bias	1	1	1	1	1	1	1	1	1	1	1	1	2	1	1	1	1	1	2	1
8 Were incomplete outcome data adequately addressed?	Attrition bias	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
9 Are reports of the study free of selective outcome reporting?	Reporting bias	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2

(Continued)

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TABLE 2 (Continued)

Risk of bias	Type of bias	Selected Studies																						
		Wang et al. (2004)	Guo et al. (2006)	Zhang et al. (2007)	Wu et al. (2008)	Xu et al. (2008)	Jiang et al. (2008)	Mishra et al. (2009)	Hu et al. (2009)	Tian et al. (2009)	Rehni et al. (2011)	Delivoria-Papadopoulos et al. (2011)	Delivoria-Papadopoulos (2012)	Kumar et al. (2014)	Angelis et al. (2014)	Angelis et al. (2015)	Angelis and Delivoria-Papadopoulos (2017a,b)	Angelis and Delivoria-Papadopoulos (2017a,b)	Kratimenos et al. (2017)	Kratimenos et al. (2018)	Kratimenos et al. (2022)
Overall assessment		IR	IR	IR	IR	IR	IR	IR	IR	IR	IR	IR	IR	LR	IR	IR	IR	IR	IR	IR	IR	IR	IR	IR
		11	11	11	11	11	12	12	11	11	11	12	11	13	11	11	11	11	12	13	13			
YES (2)																								
UNCLEAR/NOT MENTIONED (1)																								
NO (0)																								
Low Risk (13-18)	LR																							
Intermediate Risk (7-12)	IR																							
High Risk (1-6)	HR																							

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TABLE 3 The included studies and their descriptive statistics.

No	Year	Authors	Type of animals	No of animals	Interventions	Method of HI
1	2004	Wang et al., 2004	Adult Sprague-Dawley rats	-	10 μl PP2 (icv)	4VO
2	2006	Guo et al., 2006	Adult Sprague-Dawley rats	-	5 μg/μl PP2 (icv), 5 μg/μl PP3 (icv), 5 μg/μl locostatin (icv)	4VO
3	2007	Zhang et al., 2007	Adult Sprague-Dawley rats	-	1 mg/kg muscimol (ip), 20 mg/kg baclofen (ip), 15 μg PP2 (ip), 15 μg PP3 (ip), 3 mg/kg MK-801 (ip)	4VO
4	2008	Wu et al., 2008	Adult Sprague-Dawley rats	-	25 μg PP2 (icv)	4VO
5	2008	Xu et al., 2008	Adult Sprague-Dawley rats	-	2 nmol ATPA in 5 μl of 0.9% NaCl (icv)	4VO
6	2008	Jiang et al., 2008	Newborn mice CD1 strain	-	1 μg/mg PP2 (ip), 1 μg/mg PP3 (ip)	Titration of FiO2
7	2009	Mishra et al., 2009	Newborn Yorkshire piglets	5	0.4 mg/Kg nNOSi (iv)	Titration of FiO2
8	2009	Hu et al., 2009	Adult sprague-Dawley rats	-	5 μM SU-6656 (icv)	4VO
9	2009	Tian et al., 2009	Adult sprague-Dawley rats	-	100 pmol/animal SU-6656 (icv),	4VO
10	2011	Rehni et al., 2011	Adult swiss albino mice	28	0.1 mg/kg and 0.2 mg/kg PP1 (ip), 2 mg/kg and 4 mg/kg SU-6656 (ip)	2VO
11	2011	Delivoria-Papadopoulos et al., 2011	Newborn Yorkshire piglets	5	1 mg/kg PP2 (iv)	Titration of FiO2
12	2012	Delivoria-Papadopoulos, 2012	Newborn Yorkshire piglets	5	0.4 mg/kg PP2 (iv)	Titration of FiO2
13	2014	Kumar et al., 2014	Adult swiss albino mice	48	0.1 mg/kg and 0.2 mg/kg PP1 (ip), 2 mg/kg and 4 mg/kg SU-6656 (ip)	2VO
14	2014	Angelis et al., 2014	Newborn Yorkshire piglets	5	1 mg/kg PP2 (iv)	Titration of FiO2
15	2015	Angelis et al., 2015	Newborn Yorkshire piglets	4	1 mg/kg PP2 (iv)	Titration of FiO2
16	2017	Angelis and Delivoria-Papadopoulos, 2017a	Newborn Yorkshire piglets	5	1 mg/kg PP2 (iv)	Titration of FiO2
17	2017	Angelis and Delivoria-Papadopoulos, 2017b	Newborn Yorkshire piglets	5	1 mg/kg PP2 (iv)	Titration of FiO2
18	2017	Kratimenos et al., 2017	Newborn Yorkshire piglets	5	1 mg/kg PP2 (iv)	Titration of FiO2
19	2018	Kratimenos et al., 2018	Newborn Yorkshire piglets	5	PP2 and hypothermia	Titration of FiO2
20	2022	Kratimenos et al., 2022	Newborn Yorkshire piglets	5	1 mg/kg PP2 (iv)	Titration of FiO2

iv, intravenous; icv, into the cerebral ventricle; ip, intra-peritoneal; mg, milligram; kg, kilogram.

or subsequently to prolonged ischemia (Rehni et al., 2011; Kumar et al., 2014). Both studies demonstrated the neuroprotective properties of the Src kinase under HI conditions. It is worth mentioning that studies ($n_{studies}$ = 12, 60%) that focused exclusively on biochemical analyses did not include a sham or control group for direct comparisons (treated vs. non-treated) of the effectiveness of the therapy with Src-i.

The inclusion of eligible studies led to the assessment of histologic, biochemical, and neurobehavioral outcomes. Histologic analyses included cortical and striatal lesions. Moreover, biochemical parameters, such as the enzymatic expression or activity related to neurological damage, and the cerebral energy status as quantified by ATP and phosphocreatine (PCr) concentrations, were evaluated. In ten out of twenty studies ($n_{studies}$ = 10, 50%) sufficient cerebral hypoxia was

induced for both the treatment and control groups as confirmed by energy production levels. Neurobehavioral indicators were examined with neurobehavioral tests designed to measure cognitive function (memory) and motor coordination. Two studies ($n_{studies}$ = 2, 10%) included both histologic and neurobehavioral outcomes, eleven studies ($n_{studies}$ = 11, 55%) only biochemical outcomes and six ($n_{studies}$ = 6, 30%) reported both biochemical and histologic outcomes. It is worth mentioning that one study ($n_{studies}$ = 1, 5%) utilized experimental data to create and validate a computational model of the critical intracellular signaling components of HI in neonatal brain (Kratimenos et al., 2022).

Of the studies included in our analysis, six ($n_{studies}$ = 6, 30%) demonstrated that Src kinase phosphorylation was neuroprotective, whereas 14 studies ($n_{studies}$ = 14, 70%) provided evidence that the effects of Src kinase can be deleterious while its inhibition provides neuroprotection. Six out of twenty studies ($n_{studies}$ = 6, 30%), where Src kinase activity had a beneficial impact, were conducted on adult animal models. The studies in which Src kinase activity led to worse outcomes were conducted on either newborn animals ($n_{studies}$ = 9, 45%) or adult animals ($n_{studies}$ = 5, 25%). The time of intervention, type of outcome measures, and the role of Src kinase in selected studies are presented in Table 4.

Discussion

This paper highlights the current evidence on neuroprotective effects of Src kinase modulation as demonstrated by histologic, biochemical, and neurobehavioral outcomes in twenty eligible studies. Notably, all the included studies were based on a single dose regimen, but only 15% of the studies administered Src-i post-HI. This treatment timing is a key consideration, as in actual clinical practice, treatment with Src-i would likewise occur post-HI.

Although various animal models were utilized in the studies included, all inhibitors used were selective for Src kinase. Each preclinical study that examined models of the neonatal age group reported neuroprotective effects of treatment with Src-i, whereas studies in adult rats showed the opposite effect. This discrepancy may be attributed to the pathophysiologic differences between neonatal and adult brains as it pertains to susceptibility to injury, plasticity and cell death pathway activation (Sands et al., 1979; Clancy et al., 2007; Pressler and Auvin, 2013).

Methodological quality assessment using the SYRCLE's RoB tool yielded an intermediate risk for bias scores for the evaluated studies in this review. Due to the variability of experimental methods and outcomes used, it was not possible to perform a meta-analysis on the effect of Src kinase inhibition on the neonatal brain after hypoxia.

Pathophysiology of HI in neonatal brain

There are three major mechanisms of neuronal cell death during global ischemia: generation of free radicals, excitotoxicity, and inflammation. Each mechanism is mediated through inflammatory cascades that require phosphorylation of enzymatic regulatory sites by Src kinases (Mishra et al., 2009; Delivoria-Papadopoulos et al., 2011; Delivoria-Papadopoulos, 2012; Angelis et al., 2014, 2015; Angelis and Delivoria-Papadopoulos, 2017a,b; Kratimenos et al., 2017, 2018). Aligned with these findings, experiments on newborn piglets demonstrated that inhibition of Src kinase phosphorylation after HI by a selective antagonist (Src-i) is a novel mechanism of neuroprotection (Mishra et al., 2009; Delivoria-Papadopoulos et al., 2011; Delivoria-Papadopoulos, 2012; Angelis et al., 2014, 2015; Angelis and Delivoria-Papadopoulos, 2017a; Kratimenos et al., 2017, 2018). The proposed mechanism of the apoptosis-induced cell death in a developing neuron is illustrated in Figure 2.

The activation of Src kinases in HI is dependent, in part, upon formation of free radicals, which are known to develop during HI in the setting of decreased oxidative phosphorylation given decreased O2. Nitric oxide (NO) free radicals, which are produced by neuronal nitric oxide synthase (nNOS), react with superoxide to form peroxynitrate. Peroxynitrate then inactivates protein tyrosine phosphatases, SH-PTP-1 and SH-PTP-2 *via* reduction mechanisms on cysteine residues (Lee et al., 1998; Barrett et al., 1999; Takakura et al., 1999), facilitating the activation of Src kinases (Mishra et al., 2009).

In addition to the generation of free radicals as oxidative phosphorylation fails, this metabolic alteration also induces excitotoxicity through the depolarization and activation of voltage-gated Ca^{2+}-channels with a subsequent rise in intracellular Ca^{2+} (White et al., 2000). Depolarization of those channels results in the release of excitatory neurotransmitters such as glutamate into the synaptic cleft (Fujimoto et al., 2004) and inability of the glutamate reuptake mechanisms to clear glutamate from the cleft. This cascade of events leads to the upregulation of gated NMDARs, further increasing intracellular Ca^{2+} and activating calcium-dependent apoptotic pathways (excitotoxicity) (Arundine and Tymianski, 2004; Delivoria-Papadopoulos et al., 2011). Additionally, excitotoxicity is augmented by the concurrent phosphorylation of certain subunits of NMDAR by Src kinase (Chen et al., 2003; Salter and Kalia, 2004).

Calcium itself plays a critical role in HI-mediated neuronal cell injury (Delivoria-Papadopoulos et al., 2007). During hypoxia, nuclear Ca^{2+} forms a complex with calmodulin (CaM), which activates an apoptotic cascade (Delivoria-Papadopoulos et al., 2011). This cascade involves Ca^{2+}-dependent kinases such as the CaM kinase-dependent kinase (CaMKK) and CaM kinase IV (CaMKIV), primarily located in the neuronal cell nucleus. CaMKK directly activates CaMKIV *via* phosphorylation of

TABLE 4 The included studies and their descriptive statistics.

No	Year	Authors	Time of intervention	Type of outcomes	Outcomes with Src kinase inhibition	Role of Src
1	2004	Wang et al., 2004	Pre HI	Biochemical	Reduced ERK5	Protective
2	2006	Guo et al., 2006	Pre HI	Biochemical	Reduced Ras/Raf-1/ERK	Protective
3	2007	Zhang et al., 2007	Pre HI	Biochemical	Reduced NR2A, PSD-95, Src, increased GABA	Deleterious
				Histologic	Reduced neuronal injury	
4	2008	Wu et al., 2008	Pre HI	Biochemical	Reduced Spry2	Deleterious
5	2008	Xu et al., 2008	Pre HI	Biochemical	Reduced NR2A, PSD-95, Src	Deleterious
				Histologic	Reduced neuronal injury	
6	2008	Jiang et al., 2008	Post HI	Biochemical	Reduced Src, NR2A, NR2B, unchanged PDS95	Deleterious
				Histologic	Reduced neuronal injury	
7	2009	Mishra et al., 2009	Pre HI	Biochemical	Increased ATP, PCr	deleterious
					Reduced Src kinase	
8	2009	Hu et al., 2009	Pre HI	Biochemical	Reduced ERK, Era, CREB	Protective
					Increased PP2A	
9	2009	Tian et al., 2009	Pre HI	Biochemical	Reduced ERK	Protective
				Histologic	Reduced neuronal injury	
10	2011	Rehni et al., 2011	Pre HI	Histologic	Increased infarct size	Protective
				Neurobehavioral	Impaired memory (elevated plus maze test)	
					Motor incoordination (rota-rod test)	
11	2011	Delivoria-Papadopoulos et al., 2011	Pre HI	Biochemical	Increased ATP, PCr	Deleterious
					Reduced CaM, CaM kinase IV, CREB	
12	2012	Delivoria-Papadopoulos, 2012	Pre HI	Biochemical	Increased ATP, PCr	Deleterious
					Reduced caspase-3/-9	
13	2014	Kumar et al., 2014	Pre HI	Histologic	Increased infarct size	Protective
				Neurobehavioral	Impaired memory (Morris-water-maze test)	
					Motor incoordination (rota-rod test)	
					Motor incoordination (inclined beam walking)	
					Motor incoordination (lateral push test)	
14	2014	Angelis et al., 2014	Pre HI	Biochemical	Increased ATP, PCr	Deleterious
					Reduced both caspase-1, IL-1β	
15	2015	Angelis et al., 2015	Pre HI	Biochemical	Increased ATP, PCr	Deleterious
					Reduced caspase-1/-8	
16	2017	Angelis and Delivoria-Papadopoulos, 2017a	Pre HI	Biochemical	Increased ATP, PCr	Deleterious
					Reduced PTP-1B	
17	2017	Angelis and Delivoria-Papadopoulos, 2017b	Pre HI	Biochemical	Increased ATP, PCr	Deleterious
					Reduced caspase-2	
18	2017	Kratimenos et al., 2018	Post HI	Biochemical	Increased ATP, PCr	Deleterious
					CaM kinase IV, additive effect of hypothermia	
				Histologic	Reduced neuronal injury	
19	2018	Kratimenos et al., 2017	Pre HI	Biochemical	Increased ATP, PCr	Deleterious
					Reduced caspase-3, cytochrome c, smac/diablo, AIF	
				Histologic	Reduced neuronal injury	
20	2022	Kratimenos et al., 2022	Post-HI	Biochemical	Increased ATP, PCr	Deleterious
					Increased Ca^{2+} influx, CaMKK2	
				Computational model	Ca^{2+} influx and Bax expression are dissociable	
					Reduced Bax expression by altering NMDAR – Src kinase interaction	

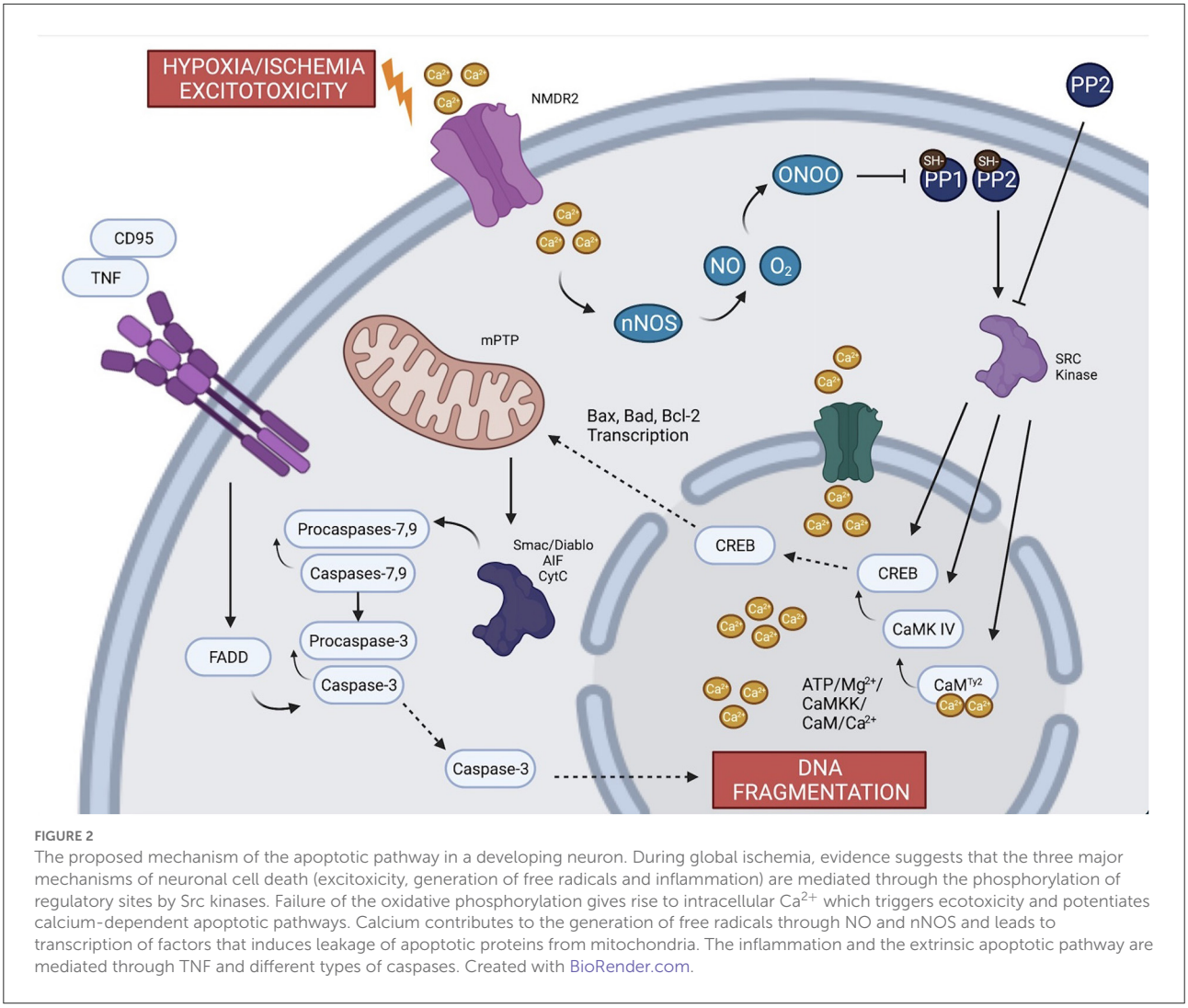

FIGURE 2
The proposed mechanism of the apoptotic pathway in a developing neuron. During global ischemia, evidence suggests that the three major mechanisms of neuronal cell death (excitoxicity, generation of free radicals and inflammation) are mediated through the phosphorylation of regulatory sites by Src kinases. Failure of the oxidative phosphorylation gives rise to intracellular Ca^{2+} which triggers ecotoxicity and potentiates calcium-dependent apoptotic pathways. Calcium contributes to the generation of free radicals through NO and nNOS and leads to transcription of factors that induces leakage of apoptotic proteins from mitochondria. The inflammation and the extrinsic apoptotic pathway are mediated through TNF and different types of caspases. Created with BioRender.com.

threonine 200 (Thr200) or threonine 196 (Thr196) in a process that requires Ca^{2+}/CaM and ATP/Mg^{2+}. Activated CaMKIV mediates DNA transcription by phosphorylating cyclic AMP response binding protein (CREB) (Mishra et al., 2006; Delivoria-Papadopoulos et al., 2007, 2011; Hornick et al., 2007). During HI, Src kinase phosphorylates CaM at tyrosine 99 (Tyr99), CaMKIV at Thr200 or Thr196 and CREB protein at Ser133, promoting the expression of pro-apoptotic proteins (Delivoria-Papadopoulos et al., 2011). As discussed, inhibition of Src kinase by a potent selective inhibitor has been shown to ameliorate the impact of HI in the cerebral cortex of newborn piglets (Delivoria-Papadopoulos et al., 2011).

Programmed cell death occurs through two pathways: intrinsic and extrinsic. The intrinsic pathway is mediated by the generation of free radicals (oxidative stress) and caspases, whereas the extrinsic pathway is mediated by inflammation and tumor necrosis factor (TNF) (Bleicken et al., 2013; Lukyanova and Kirova, 2015). The Bcl-2 family protein includes several anti-apoptotic (Bcl-2, Bcl-xL, and Bcl-w) and pro-apoptotic (Bax, BAD, Bak, or Bok) proteins, whose transcription is induced by HI (Bleicken et al., 2013; Lukyanova and Kirova, 2015). Activation of the CREB protein leads to apoptosis by the transcription of Bax and suppression of Bcl-2 (Kratimenos et al., 2017). Porcine animal models have shown that activated Bax forms pores in the mitochondrial membrane, allowing the leakage of apoptosis-inducing factor (AIF), Smac/Diablo and cytochrome c in the cytosol (Kratimenos et al., 2017). The release of these molecules is further facilitated by the action of Src kinases that expand the opening of the mitochondrial permeability transition pore (mPTP) (Kratimenos et al., 2017). The translocation of apoptotic factors activates caspases −3, −7, and −9, which results in DNA fragmentation and neuronal cell death (Kratimenos et al., 2017). These caspases mediate the activation of the intrinsic apoptotic pathway, whereas the extrinsic pathway is activated by TNF through the binding of Fas ligand (CD95L) to the CD95 receptor. This process results

in the formation of the complex FAS-associated death domain (FADD) which then activates caspase-8 (Boatright et al., 2003). Caspase-8 induces apoptosis *via* caspase-3 activity. Additionally, *in vivo* studies have demonstrated the inhibitory effect of Protein Phosphatase 2 (PP2) on caspase-8, which would in this case ameliorate the observed apoptosis (Angelis et al., 2015).

Research indicates that activity of both caspase-8 and caspase-1 acutely increases in the newborn piglet brain following hypoxia (Angelis et al., 2015). Capsase-1 activation is directly related to neuro-inflammation and contributes to the production of IL-1β through the formation of inflammasomes (Angelis et al., 2015). Furthermore, Src kinase inhibition protects cortical neurons from the deleterious sequelae of inflammation caused by HI (Angelis et al., 2015). Additionally, there is evidence that Src kinase is involved in apoptotic cascade activation and pro-neuroinflammatory pathways, ultimately leading to neuronal cell death (Angelis et al., 2015).

N-methyl-D-aspartate receptors (NMDARs) in HI

The balance between neuronal inhibition and excitation is an essential homeostatic mechanism for typical brain function and development. HI disrupts this homeostasis by upregulating excitation, resulting in apoptosis and neuronal cell death (Seeburg, 1993; Hollmann and Heinemann, 1994). The excitatory component of this mechanism is mediated by glutamate and the NMDARs (Kumari and Ticku, 2000). NMDARs and their associated signaling pathways are located at the electron-dense matrix beneath the postsynaptic membrane of excitatory synapses, called postsynaptic density (PSD) (Kennedy, 1997; Martone et al., 1999). HI-induced activation of Src kinases upregulates NMDARs, increasing excitation by phosphorylating tyrosine residues on the NR2A and NR2B subunits. In addition, transient ischemia causes changes in the structure and protein composition of the PSD, enhancing their association with certain proteins (Kennedy, 1997; Martone et al., 1999).

The interaction between NR2A, Src kinase and post-synaptic density protein 95 (PSD-95) has been implicated in HI-induced neuronal injury. Liu et al. suggest that this mechanism also includes the activation proline-rich kinase 2 (Pyk2). After ischemia-reperfusion (I/R), activated Pyk2 binds to Src kinase, promoting phosphorylation of NR2A and calcium overload via PSD-95 (Liu et al., 2005). PP2 can block the NR2A-PSD95-Src signaling pathway and alleviate neuronal cell injury (Zhang et al., 2007; Jiang et al., 2008). Other than PP2, MK-801, a selective antagonist of NMDARs, can reverse Src kinase's activation and its effect on NR2A during HI. A novel approach using a computational model also predicted that Src-i can modulate the interaction between the NMDARs and Src and can significantly reduce Bax expression (39).

In the setting of HI-induced excitotoxicity, targeting gamma-aminobutyric acid (GABA) signaling is a potentially effective therapeutic strategy. GABA is the primary inhibitory neurotransmitter in the CNS that balances the excitatory effects of glutamate (Oja et al., 1990; Rosenbaum et al., 1990; Johansen and Diemer, 1991; Sivilotti and Nistri, 1991). Several researchers have proposed that enhancing GABAergic activity could potentially alleviate the excitotoxic effects of ischemic brain injury (Oja et al., 1990; Rosenbaum et al., 1990; Johansen and Diemer, 1991; Sivilotti and Nistri, 1991). The effects of GABA on ischemia are mediated by the activation of $GABA_A$, which increases Cl^- permeability and hyperpolarizes cells. Hyperpolarized cells demonstrate reduced excitability due to decreased glutamate concentrations and calcium influx (Oja et al., 1990; Rosenbaum et al., 1990; Johansen and Diemer, 1991; Sivilotti and Nistri, 1991). Furthermore, Zhang et al. demonstrated that muscimol and baclofen, both GABA receptor agonists, prevent hippocampal CA1 neurons' death during cerebral I/R via suppression of the phosphorylation of excitatory NMDA receptor subunit NR2A (Zhang et al., 2007). Interestingly, muscimol's and baclofen's neuroprotective properties are linked to the downregulation of the phosphorylation of Src kinase and NMDARs. In addition, administration of (RS)-alpha-amino-3-hydroxy-5-tert-butyl-4-isoxazolepropionic acid (ATPA), an agonist of GluR5 (glutamate receptor 5)-containing kainate receptor, also demonstrated neuroprotective effects (Zhang et al., 2007). Xu et al. hypothesized that this was secondary to increased GABA release and inhibition of the NR2A-PSD95-Src signaling pathway (Xu et al., 2008). However, some researchers have also reported conflicting results, indicating that increased GABA signaling after HI may accelerate neuronal cell loss (Rosenbaum et al., 1990; Stokes et al., 2001).

The dual role of Src kinase following ischemia-reperfusion (I/R)

Several studies have highlighted Src kinase's role in neuronal survival after I/R through interactions with the extracellular signal-regulated kinase (ERK) (Wang et al., 2004; Guo et al., 2006; Wen et al., 2008; Wu et al., 2008; Hu et al., 2009; Tian et al., 2009). Following I/R, Src kinase and NMDARs upregulate ERK, increasing neuronal survival (Wang et al., 2004; Guo et al., 2006). HI-induced activation of Src kinase leads to the phosphorylation of Raf at the Tyr340/341 position. Raf-1, an upstream molecule of the ERK pathway, subsequently induces the phosphorylation of estrogen receptor a (ERa) and CREB at Ser133 position, promoting neuronal cell survival (Wang et al., 2004; Guo et al., 2006; Wu et al., 2008; Hu et al., 2009). Despite

some reports of Src kinase-related increases in neuronal survival, several studies showed that Src kinase inhibition by PP2A was neuroprotective following I/R. Wu et al. demonstrated that Src kinase's induction of neuronal apoptosis following I/R, is mediated by the phosphorylation of Spry2, a down-regulator of Raf/ERK pathway (Wu et al., 2008). Administration of PP2 or SU6656 shows an attenuation of Src kinase's negative effect on cellular death in rat hippocampi (Wang et al., 2004; Guo et al., 2006; Hu et al., 2009; Tian et al., 2009). The results of the aforementioned studies cannot be translated to clinical neonatology practice because of the use of adult mice and their differences in pathophysiology, mainly on the mechanism of injury and recovery when compared to neonatal mice (Sands et al., 1979; Clancy et al., 2007; Pressler and Auvin, 2013). Moreover, intact Src kinase is correlated with improved neuronal survival, whereas in conditions of excitotoxicity, calpain cleavage of Src kinase generates a neurotoxic truncated Src fragment (Hossain et al., 2015).

Pre-/post-conditioning

As mentioned previously, Src kinase plays a pivotal role in neuronal health among multiple disease models, including conditioning. Conditioning involves the intermittent reperfusion that precedes or follows prolonged ischemia. These are termed ischemic preconditioning (IPrCo) and ischemic post-conditioning (IPoCo), respectively (Kumar et al., 2014). Studies have shown that both IPrCo and IPoCo prevent cerebral infarct formation by ischemia-reperfusion and prevent neurobehavioral impairment in Swiss albino mice (Rehni et al., 2008a, 2011; Kumar et al., 2014). IPrCo's neuroprotective effect on the brain is likely attributable to amelioration of the ischemia and reperfusion sequela through the activation of the Akt/p38-mitogen/ERK pathway (Bochelen et al., 1999; Rehni et al., 2008b, 2009; Kumar et al., 2014). However, Rehni et al. demonstrated that the effect of IPrCo is exerted through phosphorylation of Src kinase, even though the exact activation transduction pathway is not yet well understood (Rehni et al., 2008a, 2011). Neuronal cell injury was also ameliorated by IPoCo through the activation of Src kinase (Kumar et al., 2014). Although the exact mechanism remains unknown, Kumar et al. suggested that it also involves the activation of Akt/p38-mitogen/ERK (Kumar et al., 2014). In a clinical setting, IPrCo is not feasible due to the inability to predict the onset of ischemia. IPoCo, on the other hand, is clinically relevant.

Why are findings for Src-i not yet translatable?

Although Src kinase inhibition exhibits a neuroprotective effect on neonatal animal models, this has not yet been confirmed in clinical trials. The variability of experimental results and animal models used has prevented the introduction of Src kinase inhibition as a therapeutic approach in clinical trials. Neurobehavioral experiments could provide additional data to support the use of Src-i, however they require a longer follow-up period with highly trained personnel, as well as validated scoring systems for accuracy and consistency. In the present analysis, only two studies provided a complete set of outcomes that were evaluated with multiple different approaches (Rehni et al., 2011; Kumar et al., 2014). The use of histologic and biochemical markers has been proven to be a cost-effective alternative approach. Further investigations are required to elucidate the precise mechanism by which Src kinase affect cortical neurons. Many Src kinase inhibitors like dasatinib are currently being tested as adjuvant therapies in cancer. Pharmacokinetic data of such inhibitors including PP2, a more selective Src kinase inhibitor used in thirteen out of the 20 included studies, are still lacking. Finally, the potential addition of therapeutic hypothermia to Src-i has not been sufficiently explored.

Future directions

To date, Src-i have only been examined in clinical trials for cancer and neurodegenerative conditions such as Alzheimer's and Parkinson's disease (ClinicalTrials.gov Identifier: NCT00779389, NCT02167256, and NCT03661125). We anticipate that further research will involve large animals and primates. Large animal studies, although expensive, offer the greatest potential of translation to humans, due to the similarities in brain size, gray/white matter ratio, developmental ages and morphology, as well as the localization of injury after HI (Odden et al., 1989; Thoresen et al., 1996; Haaland et al., 1997; Björkman et al., 2006). Additionally, larger sample sizes can help to decrease bias and improve study validity. Standard reporting methods for preclinical studies focused on Src-i are also necessary to minimize reporting bias. Future work needs to focus on HI pathophysiologic mechanisms and Src-i dosage, timing, route of administration, and potential adverse events. Moreover, as therapeutic hypothermia is considered standard of care for HI in neonates, the additive effects of a combined hypothermia/Src kinase inhibition protocol should be further investigated (Kratimenos et al., 2018). Recently, our team validated a computational model with experimental measurements of critical intracellular signaling components and captured key molecular trends in this pathway (Kratimenos et al., 2022). Our computational model indicated that Src-i disassociates Ca^{2+} influx from Bax expression and modulates the interaction between the NMDAR and Src reducing Bax expression (Kratimenos et al., 2022). This model could provide a translational platform to design and

screen drugs in neonatal hypoxic brain (Kratimenos et al., 2022).

Strengths and limitations of the study

To our knowledge, this is the first attempt to systematically evaluate the current literature on preclinical evidence supporting the use of Src kinase inhibitors in models of HI. Notably, we included ten studies in which large animals, including Yorkshire newborn piglets, were treated with Src-i following HI. Yorkshire newborn piglets' brains share many characteristics with the human brain (Odden et al., 1989; Thoresen et al., 1996; Haaland et al., 1997; Björkman et al., 2006). Human neonates suffer from somatosensory cortical and basal ganglia damage after perinatal asphyxia, which has many similarities to findings in term piglets aged 1–5 days after similar insults (Thoresen et al., 1996).

Although every effort was made for a thorough literature search, it is possible that some relevant studies were missed. It was not feasible to perform a quantitative analysis (meta-analysis) of studies with a focus on Src-i in neonatal HI as there was a significant variability in experimental animals used, sample size and reported outcomes (histologic, biochemical, neurobehavioral). Most of those studies were using inhibitors of the Src kinase to investigate mechanistic questions rather than examining its role as a therapeutic target. The lack of neurobehavioral assessments did not allow for the study of HI-induced visual, motor, and cognitive impairments. Moreover, we are unable to examine the clinical safety of Src-i due to the lack of long term follow-up. Most of the studies were characterized as intermediate when assessed for risk for bias, which can be attributed to the insufficient description of experimental methods, protocols and interventions as evaluated by the SYRCLE's RoB tool. Following the ARRIVE (Animal Research: Reporting of *in vivo* Experiments) guidelines could have improved the reporting of results by minimizing publication bias (Hooijmans et al., 2014). However, these guidelines were not published prior to June 2010, and many studies that were included in our review were conducted before that time. Not all studies explicitly added control groups to compare effectiveness against the treatment group again likely because the studies were not designed to examine therapeutic effects. Despite the moderate quality assigned to the examined studies by the SYRCLE RoB tool, the evidence presented still indicates the potential benefits of Src kinase inhibition in neonates suffering perinatal asphyxia.

Conclusions

This systematic review demonstrates that inhibition of Src during hypoxia-ischemia results in neuroprotection. However, these protective properties were assessed based on varying animal models, study designs, and intervention characteristics. Further preclinical studies on large animals and specific experimental models are required to examine the pharmacokinetics of Src-i and its exact role in programmed neuronal death. While heterogeneity and risk for bias were limiting factors, the overall results indicate that Src-i neuroprotective properties could be a promising therapeutic strategy to neonates after hypoxic events.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding author/s.

Author contributions

PK and PC conceptualized the manuscript. PC wrote the manuscript with contribution from IK, AV, JG, and SP. PK, BS, and IK edited the manuscript. All authors have read and approved the final manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

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SARS-CoV-2 (COVID-19) as a possible risk factor for neurodevelopmental disorders

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Pregnant women constitute one of the most vulnerable populations to be affected by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection, the cause of coronavirus disease 2019. SARS-CoV-2 infection during pregnancy could negatively impact fetal brain development via multiple mechanisms. Accumulating evidence indicates that mother to fetus transmission of SARS-CoV-2 does occur, albeit rarely. When it does occur, there is a potential for neuroinvasion via immune cells, retrograde axonal transport, and olfactory bulb and lymphatic pathways. In the absence of maternal to fetal transmission, there is still the potential for negative neurodevelopmental outcomes as a consequence of disrupted placental development and function leading to preeclampsia, preterm birth, and intrauterine growth restriction. In addition, maternal immune activation may lead to hypomyelination, microglial activation, white matter damage, and reduced neurogenesis in the developing fetus. Moreover, maternal immune activation can disrupt the maternal or fetal hypothalamic-pituitary-adrenal (HPA) axis leading to altered neurodevelopment. Finally, pro-inflammatory cytokines can potentially alter epigenetic processes within the developing brain. In this review, we address each of these potential mechanisms. We propose that SARS-CoV-2 could lead to neurodevelopmental disorders in a subset of pregnant women and that long-term studies are warranted.

KEYWORDS

SARS-CoV-2, COVID-19, HPA axis, preeclampsia, brain development, inflammation, pregnancy

Introduction

In December of 2019 a novel virus, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), caused a pneumonia outbreak in Wuhan City, Hubei Province in China. The disease caused by the virus was designated coronavirus disease 19 (COVID-19). SARS-CoV-2 expanded rapidly across the globe, and on March 11, 2020 the World Health Organization [WHO] (2022) declared COVID-19 a pandemic and global

public health emergency. As of November 22, 2022 [WHO Coronavirus (COVID-19) Dashboard | WHO Coronavirus (COVID-19) Dashboard with Vaccination Data], 634 million cases have been detected worldwide. SARS-CoV-2 has already posed a great threat not only to the health of the people but also to the economy and healthcare system.

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is an enveloped, positive stranded ribonucleic acid (RNA) virus of the family of Coronaviridae that causes respiratory and gastrointestinal infections ranging from mild, self-limiting conditions to more serious disorders such as viral pneumonia with systemic impairment (di Mascio et al., 2020). Unfortunately, pregnant women constitute one of the most vulnerable groups to be affected by this viral infection, due to anatomical, reproductive, endocrine, and immune changes (Zhao et al., 2020). With specific regard to the latter, immunological changes in pregnancy result in suppressed cell mediated immunity which would increase susceptibility to SARS-CoV-2 (Liu et al., 2020). In addition, pregnant women are more likely to have severe disease and ICU admissions compared to their non-pregnant counterparts after adjusting for age, underlying medical conditions, race, and ethnicity (Ellington et al., 2020; Sutton et al., 2020). As per a systematic review of over 11,000 pregnant women with suspected or confirmed COVID-19, the disease commonly manifests as fever (40%), cough (39%), dyspnea (19%), loss of taste (15%), myalgia (10%) and diarrhea (7%) (Allotey et al., 2020).

The novel SARS-CoV-2 infection could negatively impact fetal brain development in both direct and indirect ways (**Figure 1**) (Ellul et al., 2020). Regarding the direct route, an increasing number of case studies provide evidence for transplacental transmission of SARS-CoV-2, which could invade the central nervous system and disrupt brain development. Regarding indirect routes, SARS-CoV-2 could produce placental dysfunction, preeclampsia, and preterm birth, and trigger immune responses in the mother, which could, in turn affect the developing fetus. Interestingly, many of these routes involve the action of pro-inflammatory cytokines. Preclinical studies have revealed that inducing inflammation during the perinatal period produces long-term alterations in brain structure and function and a wealth of epidemiological studies have documented associations between infection-induced immune activation and offspring neuropsychiatric risk. In this review, we address each of these potential mechanisms and propose that SARS-CoV-2 could lead to neurodevelopmental disorders in a subset of pregnant women. We also review emerging empirical evidence supporting this hypothesis. This manuscript builds upon prior review articles on this topic such as (Shook et al., 2022), and (Figueiredo et al., 2021), which focused primarily on emerging evidence for transplacental transmission and the role of maternal immune activation (MIA), and (Kleeman et al., 2022), which focused primarily on epigenetic mechanisms in MIA. The current manuscript aims to be both comprehensive and concise. It provides a more detailed discussion of how altered levels of glucocorticoids in the context of SARS-CoV-2 could affect fetal neurodevelopment, and is the first, to our knowledge, to raise the possibility that SARS-CoV-2 induced hypocortisolism could be a risk factor for adverse neurodevelopmental outcomes. Finally, this review includes the most recent empirical studies on neurodevelopmental consequences of *in utero* exposure to SARS-CoV-2 including (Aldrete-Cortez et al., 2022; Hessami et al., 2022; Shuffrey et al., 2022).

The direct route: Evidence of maternal transmission of SARS-CoV-2 infection to fetuses/neonates

Several viruses are known to be transmitted from pregnant women to their children and subsequently disrupt neurodevelopment. These are the TORCH pathogens. TORCH is an acronym standing for *Toxoplasma gondii*, Other infections, Rubella, human Cytomegalovirus (HCMV), and Herpes simplex viruses 1 and 2 (HSV-1 and HSV-2, respectively). "Other infections" include human immunodeficiency virus (HIV), syphilis, parvovirus B19 (fifth disease), varicella (chickenpox) and Zika (reviewed in Schwartz and Hyg, 2017). HCMV and HSV infections are the most common causes of neonatal morbidity worldwide (Looker et al., 2017; Marsico and Kimberlin, 2017) and in recent years Zika virus remains a threat for pregnant women (reviewed in Spitz, 2019). These TORCH pathogens can induce brain calcifications, major brain malformations including microcephaly, and neurodevelopmental disorders (Chen et al., 2021; Krenn et al., 2021). Consequently, one of the first questions we ought to ask when considering the adverse neurodevelopmental potential of SARS-CoV-2 is whether there is evidence of maternal transmission to the fetus or neonate.

Transmission of SARS-CoV-2 from mother to child could occur transplacentally, during labor and delivery, or in the early post-partum period. The possibility of vertical transmission of SARS-CoV-2 from mother to fetus *in utero* is currently a topic of widespread debate. The published literature is both sparse and contradictory, with some reports supporting direct *in utero* transmission (Bahadur et al., 2020; Dong et al., 2020; Patanè et al., 2020), and others suggesting little or no vertical transmission (Dashraath et al., 2020; Khan et al., 2020; Xiong et al., 2020; Zhu et al., 2020). The presence of SARS-CoV-2 either in amniotic fluid, placental samples, or infant nasopharyngeal swabs collected shortly after birth,

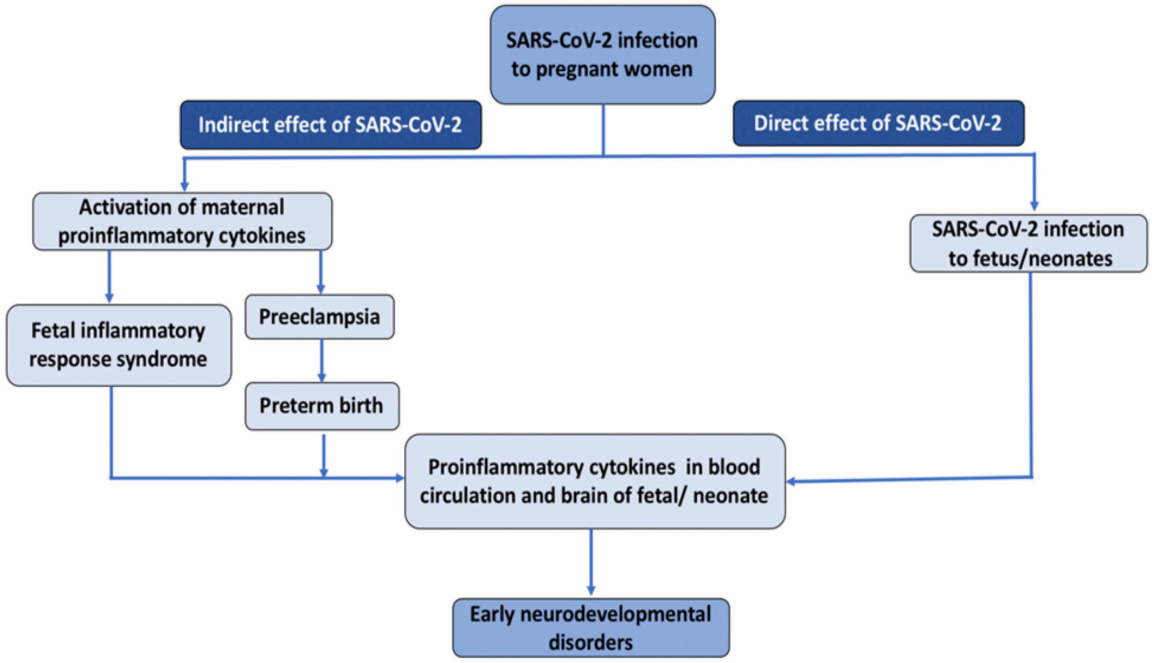

FIGURE 1
Direct and indirect effect of SARS-CoV-2 infection on fetal/neonatal brain development.

represents compelling evidence for an *in utero* infection. Several case studies have been published documenting such evidence. Sisman et al. (2020) reported a case of congenital SARS-CoV-2 in an infant born through vaginal delivery to a COVID-19 positive mother. This report confirmed the intrauterine transmission of SARS-CoV-2 *via* presence of SARS-CoV-2 nucleocapsid protein and viral particles in placental syncytiotrophoblastic cells and nasopharyngeal samples of the infant (Sisman et al., 2020). Similarly, Patanè et al. (2020), demonstrated vertical transmission of SARS-CoV-2 from mother to fetus *in utero* as evidenced by the presence of SARS-CoV-2 RNA on the fetal side of placental tissues (Patanè et al., 2020). Transplacental transmission of SARS-CoV-2 is also supported by reports of caesarean delivery where strict neonatal isolation was implemented immediately after birth without delayed cord clamping or skin to skin contact. In one such case study, neonatal nasopharyngeal swabs were positive for SARS-CoV-2 RT-PCR test within 16 h of birth despite these precautions (Alzamora et al., 2020) and within 24 h in the other (Kirtsman et al., 2020). Perhaps the strongest early evidence supporting congenital infection was reported by Kirtsman et al. (2020). In this case, a woman with active SARS-CoV-2 delivered via caesarean delivery. The neonate had no contact with vaginal secretions or maternal skin. Artificial rupture of membranes was performed at operation, which was conducted with airborne, droplet, and contact precautions. The infant was immediately removed from the operative field, in a sterile fashion, to a resuscitator 2 m away in the same room. Never-the-less, neonatal nasopharyngeal swabs were SARS-CoV-2 positive by RT-PCR test on the day of birth as well as day 2 and day 7. Furthermore, placental micrographs revealed multiple areas of infiltration by inflammatory cells and extensive early infarction (Kirtsman et al., 2020). While, a number of researchers cautioned against treating early data as conclusive (Kimberlin and Stagno, 2020), evidence for transplacental transmission has continued to accumulate, though it appears to be a very rare event. In a study of 427 pregnant women from the UK admitted to hospital with SARS-CoV-2 infection, 12 of 265 infants tested positive, a rate of 5%, though only 6 of those did so within the first 12 h after birth (Knight et al., 2020). A recent systematic review and meta-analysis including data up to 3 August 2021 and including over 14,000 babies born to mothers with SARS-CoV-2 infection found about 2% of babies tested positive with 14 confirmed mother-to-child vertical transmission, seven of which occurred *in utero* (Allotey et al., 2022). A slightly smaller systematic review of 47 studies and over 900 neonates reported that slightly less than 1% had a confirmed or probable vertical transmission of infection (Jeganathan and Paul, 2022). Similarly, a recent "systemic review of systematic reviews" suggested that mother to child transmission was relatively rare with

about 70% of cases attributable to environmental exposure and about 20% related to potential vertical transmission (Musa et al., 2021). Overall, it appears that transplacental transmission is possible, but rare.

The direct route: The neuroinvasive potential of SARS-CoV-2

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is similar in many ways to SARS-CoV, a virus identified in 2003, which is known have neuroinvasive potential (Ding et al., 2004; Xu et al., 2005), as evidenced by its presence in neural tissue from SARS autopsies (Gu et al., 2005). Studies indicate that the genomic sequence is similar between SARS-CoV-2 and SARS-CoV (Lu et al., 2020; Yu et al., 2020). It is particularly notable that the receptor-binding domain of SARS-CoV is structurally similar to SARS-CoV-2 (Lu et al., 2020). Hence, it is possible that SARS-CoV-2 follows the same path of neuroinvasiveness as SARS-CoV using the ACE2 receptor for cellular entry into the human brain. Several hypotheses have been put forth regarding possible mechanisms of SARS-CoV-2 mediated neural invasion including: (1) Transplacental transmission could induce viremia which would promote viral binding to the endothelial ACE2 receptors of the blood brain barrier (BBB) and subsequently entry into the central nervous system (CNS). Electron micrography on post-mortem brain biopsies revealed viral particles in the frontal cortex of a SARS-CoV-2 infected adult. The presence of particles in brain capillary endothelium and blebbing of viral-like particles coming in/out of the endothelial wall strongly suggested neuroinvasion through the BBB (Paniz-Mondolfi et al., 2020). (2) Cells of the immune system (macrophages and monocytes), which may express the ACE2 receptor, could act as a reservoir for dissemination into the CNS (Desforges et al., 2014). Further, infected immune cells (monocytes neutrophils and T cells) may disseminate into brain via various entry points including meninges, vasculatures, and the choroid plexus (Iadecola et al., 2020). (3) Neurons in the gut could carry the virus into the CNS via retrograde axonal transport (Esposito et al., 2020). (4) The virus could enter the CNS through the olfactory bulb. This possibility is strengthened by SARS-CoV-2 induced anosmia being a notable symptom during viral infection. Studies have shown expression of ACE2 receptors and other receptors that can facilitate SARS-CoV-2 binding in the olfactory epithelium (Fodoulian et al., 2020). This could play a role in neonatal infection during delivery through contact with vaginal secretions or soon after delivery through other means (physical or airborne). (5) Finally, the lymphatic pathway represents another possible route for neuroinvasion by the SARS-CoV-2 virus. The virus may directly enter the brain via olfactory/cervical lymphatic vessels (Bostancıklıoğlu, 2020).

One key question when considering the neuroinvasive potential of SARS-CoV-2 *in utero* is whether the fetal brain expresses cellular components that interact with the spike protein of SARS-CoV-2. Using publicly available RNA sequencing datasets, Varma et al. (2021) revealed that while *ACE2* mRNA is expressed at relatively low levels in the fetal brain, other spike protein interactors including *FURIN*, *ZDHHC5*, *GOLGA7*, and *ATP1A1* are highly expressed, especially in neurons. These proteins may play key roles in SARS-CoV-2 fetal brain pathogenesis, especially during the 2nd and 3rd trimesters of pregnancy (Varma et al., 2021).

The indirect route: SARS-CoV-2 effects on the placenta and ensuing complications

Even in the absence of direct transmission of a pathogen from mother to child, infections can disrupt neurodevelopment in indirect ways. For example, the H1N1 influenza virus is not teratogenic, but severe infections were associated with elevated risks for adverse infant outcomes, such as preterm birth, which have neurodevelopmental consequences [Maternal and Infant Outcomes Among Severely Ill Pregnant and Postpartum Women with 2009 Pandemic Influenza A (H1N1) — United States, April 2009–August 2010; Newsome et al., 2019]. In this section we discuss emerging evidence that SARS-CoV2 impacts placental functioning and how this could lead to altered neurodevelopment.

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) binding ACE2 receptors are highly expressed in placental tissues. SARS-CoV-2 infected pregnant women have placental inflammatory signs along with systemic maternal inflammation (Vivanti et al., 2020), which can lead to placental microvascular dysfunction. This may present clinically as preeclampsia or preeclampsia-like features, fetal distress, intrauterine growth restriction, and/or or preterm labor depending on gestational age at time of SARS-CoV-2 infection (Mulvey et al., 2020).

To understand the impact of COVID-19 infection on the placenta, a brief review of the salient aspects of the renin–angiotensin system (RAS) axis in the formation of a well perfused placental vascular bed may be helpful. In the maternal portion of the human placenta, which is derived from the maternal stromal cells, ACE2 is highly expressed in the invading and intravascular trophoblast and in decidual cells. ACE2 is also found in arterial and venous endothelium and smooth muscle of the umbilical cord (Valdés et al., 2006). Levels of ACE2 vary temporally depending on gestational age in both humans and rodents

(Valdés et al., 2006; Ghadhanfar et al., 2017). The various components of RAS-Ang II, ACE2, and Ang-(1-7) function mainly to regulate blood pressure and fetal development. Ang II stimulates trophoblast invasion in rat and human cells (Hering et al., 2010). Ang-(1-7) and ACE2 may act as local autocrine/paracrine regulators in the early (angiogenesis, apoptosis, and growth) and late (uteroplacental blood flow) events of pregnancy (Neves et al., 2008). ACE2 hydrolyzes Ang II into Ang-(1-7), and Ang I into Ang-(1-9), which is quickly converted to Ang-(1-7), thereby controlling the blood pressure and hydro-salinity balance of pregnant women (Pringle et al., 2011).

Preeclampsia is a serious pregnancy complication which typically begins after the 20th week of gestation and manifests as high blood pressure and proteinuria. The exact cause of preeclampsia is not known, but it is generally believed to arise due to improper functioning of the placenta. Placental vascular anomalies and inflammation are often observed in women affected by preeclampsia (Harmon et al., 2016; Ramos et al., 2017). Brosnihan et al. (2004), reported that pre-eclamptic women presented with suppressed plasma Ang-(1-7) levels when compared with normal pregnancy subjects (Brosnihan et al., 2004). Furthermore, high expression of Ang II in the placental villus during preeclampsia can cause decreased blood flow and nutrition supply to the fetus (Shibata et al., 2006; Anton and Brosnihan, 2008; Anton et al., 2009). An observational study published early in the pandemic suggested that prevalence of preeclampsia is remarkably higher in SARS-CoV-2 infected pregnant women, with five out of eight infected pregnant women admitted to the intensive care unit having preeclampsia like syndrome (Mendoza et al., 2020), which may lead to higher occurrence of preterm birth (Yan et al., 2020). A case report by Hosier and colleagues might be an example of placental infection with SARS-CoV-2 or manifestation of SARS-CoV-2 induced cytokine release or both presenting as severe early-onset preeclampsia. The patient, who had a history of gestational hypertension in a prior pregnancy, but normal blood pressure during early pregnancy and normal baseline preeclampsia evaluation, presented acutely at 22-weeks' gestation with features mimicking preeclampsia with Disseminated Intravascular Coagulation (DIC) and fever. SARS-CoV-2 RNA was positive in a nasopharyngeal swab. Patient's thrombocytopenia and hypofibrinogenemia were more severe than what would have been expected from SARS-CoV-2 alone. Placental pathology post termination of the pregnancy revealed SARS-CoV-2 localized to the syncytiotrophoblast layer and the intervillous invasion of macrophages (intervillositis) (Hosier et al., 2020). A study by Mulvey et al. (2020), on placenta (after term delivery) of COVID-19 infected pregnant women found fetal vascular malperfusion due to focal avascular villi and thrombi in large fetal vessels (Mulvey et al., 2020). Vivanti et al. (2020) also reported a case of delivery at 35 weeks through cesarean section following fetal distress indicated by category 3 fetal heart rate tracing, and RT-PCR in the placenta was positive for SARS-CoV-2. The mother who was having an uneventful pregnancy until diagnosis of COVID-19, without any severe or critical presentation of the infection, had thrombocytopenia, lymphopenia, elevated acute phase reactants, and abnormalities in coagulation cascade on admission. Three days after hospitalization, without any deterioration of maternal status, a category 3 fetal heart tracing was observed, representing fetal compromise likely due to uteroplacental insufficiency for which delivery through cesarean section was performed (Vivanti et al., 2020). This strongly indicates that SARS-CoV-2 can cause uteroplacental dysfunction and can induce a preeclampsia like picture either due to direct placental invasion or through induction of excess cytokine release in the mother or both. However, one must acknowledge that much of the empirical evidence for this hypothesis comes from case studies, which may not generalize to all SARS-CoV-2 infected mothers. Case studies may also be subject to researcher bias and do not allow the production of quantifiable risk estimates. A recent systematic review of cardiovascular complications among pregnant women with COVID-19 found substantial variance in estimates across studies with some reporting rates of preeclampsia as high as 69% and others as low as 0.5% (Yaghoobpoor et al., 2022).

A potential mechanism by which SARS-CoV-2 could induce placental dysfunction and preeclampsia is illustrated in **Figure 2**. The viral spike protein of SARS-CoV-2 facilitates binding to the ACE2 receptor. When viremia occurs in the mother during severe SARS-CoV-2 infection, the virus may invade the placenta. The viral spike protein of SARS-CoV-2 after binding with ACE2 receptor, enters the placental trophoblast with the help of protease-mediated cleavage (TMPRSS2 or cathepsin L) of the S protein subunit- S2. This internalization of the virus along with the ACE2 receptor into the placental trophoblast may increase the activity of ADAM17 (a matrix metalloproteinase) (reviewed in Schreiber et al., 2021; Jackson et al., 2022), as seen in previous SARS-CoV infection (Haga et al., 2008). ADAM17 up-regulation leads to proteolytic cleavage of the ACE2 ectodomain (Lambert et al., 2005; Heurich et al., 2014), which results in reduced membrane ACE2. The resulting imbalance in AngII/ACE2 interaction may result in hypertension of pregnancy, pre-eclampsia, or eclampsia in susceptible mothers. Of note, systemic inflammation and oxidative stress induced by SARS-CoV-2 infection may also lead to increased ADAM17 expression in the placental trophoblasts promoting the development of preeclampsia in susceptible women even in the absence of direct placental infection by SARS-CoV-2 (Gooz, 2010) (**Figure 2**).

Even in the absence of preeclampsia, SARS-CoV-2 infection in the early stages of pregnancy can cause fetal growth restriction (FGR)/intrauterine growth restriction (IUGR)

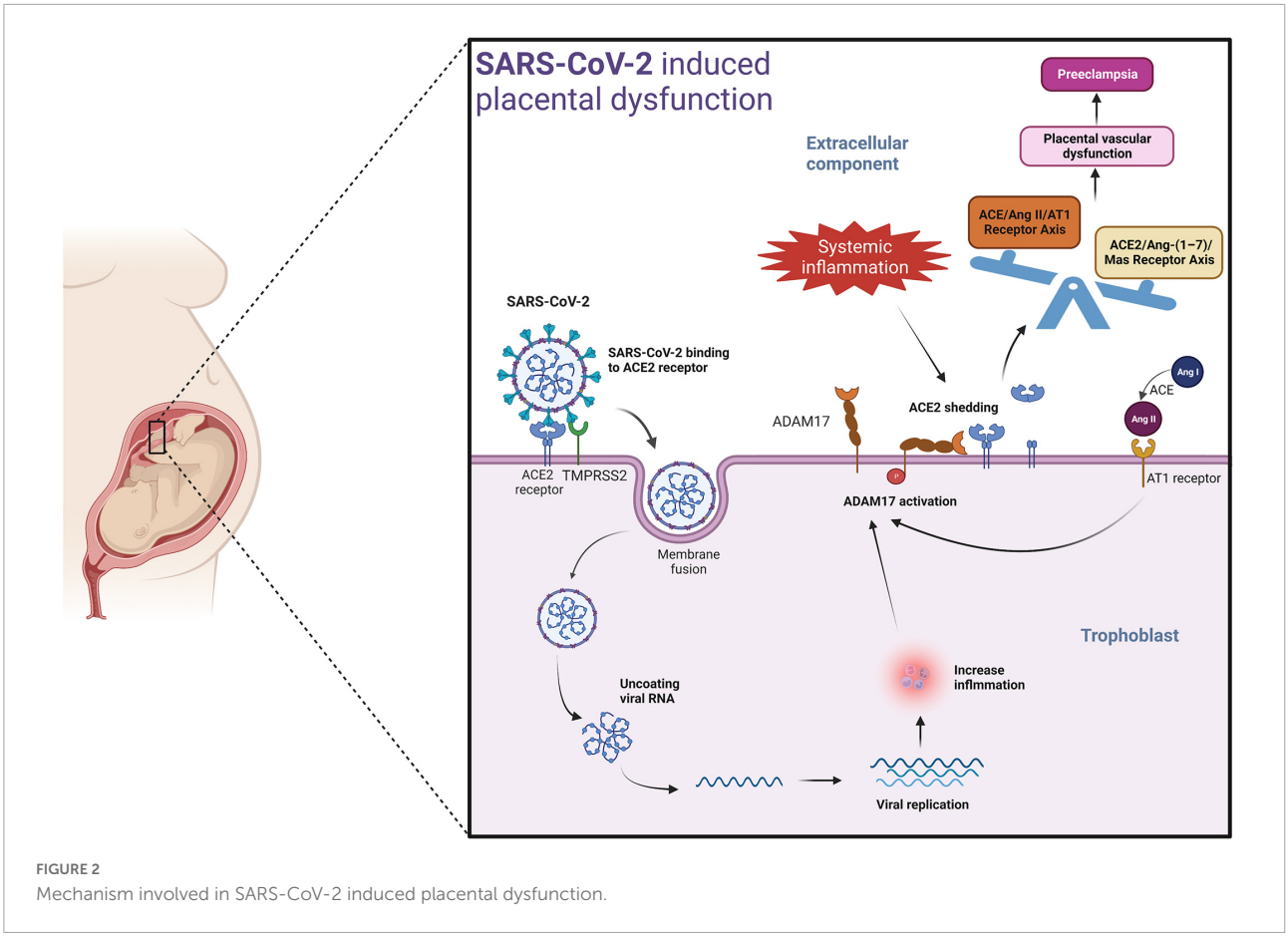

FIGURE 2
Mechanism involved in SARS-CoV-2 induced placental dysfunction.

(Allotey et al., 2020), which is itself a risk factor for abnormal postnatal neurodevelopment in babies (Adams Waldorf and McAdams, 2013; Dang et al., 2020). IUGR influences the overall growth of fetus and is accompanied by reduced total brain volume, which may cause cognitive (Hartkopf et al., 2018; reviewed in Miller et al., 2016) and motor regulation deficits (Dubois et al., 2008; Miller et al., 2016; Hartkopf et al., 2018; reviewed in Miller et al., 2016), and as well as some neurodevelopmental disorders. More specifically, reduced brain volumes have been observed in schizophrenia and bipolar disorder (Wright et al., 2000; McDonald et al., 2004; Arnone et al., 2009; Ellison-Wright and Bullmore, 2010; Haijma et al., 2013; Hibar et al., 2016, 2018; van Erp et al., 2016, 2018) and in ADHD (Boedhoe et al., 2020).

The indirect route: SARS-CoV-2 induced inflammation may disrupt offspring neurodevelopment

Epidemiological studies indicate a strong correlation between maternal viral infection and neuropsychiatric disorders in offspring, especially for ASD and schizophrenia (reviewed in Estes and McAllister, 2016). For ASD, this includes a large-scale registry-based study in Denmark which revealed that severe viral infections (requiring hospitalization) during the first trimester of pregnancy are associated with increased risk of ASDs in offspring (Atladóttir et al., 2010) and a cohort study conducted in Finland which suggested that early stage increases in gestational CRP due to prenatal infection increases risk of ASD in children by 43% (Brown et al., 2014). A meta-analysis of 15 studies, conducted in 2016 and including over 40,000 ASD cases, reported an OR of 1.13 for the association between maternal infection during pregnancy and increased risk of ASD in offspring [95% confidence interval (CI): 1.03–1.23], with risk being moderated by the severity of infection, type of infectious agent, time of infectious exposure, and site of infection. Greater severity of infection (indexed by hospitalization) was associated with higher risk. Bacterial infections appeared to confer greater risk than viral infections and genitourinary and skin infections appeared to confer higher risk than gastrointestinal or respiratory infections. With specific regard to infection timing, second trimester exposures conferred the greatest risk, followed by first trimester exposures, and third trimester exposures had minimal effects (Jiang et al., 2016). This differs from a recent meta-analysis

evaluating the impact of maternal fever on neurodevelopmental disorders in general (includes ASD, ADHD, Developmental Delay, and Developmental Coordination Disorder) which suggested first trimester exposures were most detrimental. Regarding schizophrenia, since Mednick et al. (1988) published their seminal, ecological study revealing increased risk of schizophrenia in pregnant women exposed to the 1957 influenza A epidemic in Helsinki, evidence linking schizophrenia to maternal infection has accumulated and includes (Mednick et al., 1988; Barr et al., 1990; Susser et al., 2000; Brown et al., 2001, 2004a,b, 2005; Buka et al., 2001; Mortensen et al., 2007). A recent meta-analysis of seven cohort studies reported that maternal infection during gestation increased the risk of non-affective psychosis with a relative risk (RR) of 1.28 (95% CI:1.05-1.57) (Saatci et al., 2021). Relative risk was even high for schizophrenia (1.63) with the strongest effects observed in the second trimester. Several studies have highlighted the second trimester as the time of greatest risk (Brown et al., 2000; Nielsen et al., 2013), which may indicate an impact of infection on gestational neurogenesis, which peaks during this period (Stiles and Jernigan, 2010). However, another study reported that the risk of schizophrenia was increased 7-fold for influenza exposure during the first trimester with no increased risk of schizophrenia for exposure during the second or third trimester (Brown et al., 2004a). Another study indicating that first trimester prenatal exposures may increase risk for schizophrenia in offspring is (Clarke et al., 2009). Despite these inconsistencies regarding timing of exposure, the general idea that gestational infection predisposes individuals to schizophrenia is widely accepted. This is not to say there are no controversies in the literature. For example, Selten et al. (2010) published a meta-analysis challenging earlier studies linking the 1957 influenza pandemic to schizophrenia (Selten et al., 2010). In addition, even large population-based studies can suffer from methodological problems such as misclassification of exposure and genetic confounding. Karlsson and Dalman (2020) argue that infections in general appear to have a much smaller effect on schizophrenia risk compared to specific exposures such as *Toxoplasma gondii* (Karlsson and Dalman, 2020). Of particular relevance to SARS-CoV-2, research suggests that second trimester respiratory infections are a risk factor for schizophrenia spectrum disorders (Brown et al., 2000). There are also epidemiological studies linking maternal infections to ADHD (Pineda et al., 2007; Mann and McDermott, 2011; Silva et al., 2014) and mood disorders (see Simanek and Meier, 2015 for review), but as the evidence is more limited (for the former) and ambiguous (for the latter), we do not provide additional details here. Activation of the pregnant mother's immune system in response to infection is thought to be the primary mechanism responsible for these associations, a hypothesis that is supported by a substantial body of preclinical research (reviewed in Boksa, 2010; Careaga et al., 2017).

Like other viral infections, SARS-CoV-2 can trigger systemic inflammation during pregnancy in both mother and fetus. ACE2 receptors are expressed widely in the mouth, tongue, respiratory tract, lung, heart, kidney, gut, endothelium, and in other tissues like placental tissues (Deverman and Patterson, 2009). Binding of ACE2 located on the surface of the target cells with the receptor-binding domain of SARS-CoV-2 results in endocytosis and translocation of both viruses and ACE2 into the endosomes located in the cell. Inside the cell, it replicates and induces cytotoxicity. The damaged host cell undergoes pyroptosis and releases damage-associated molecular patterns resulting in the initial inflammatory response.

As noted in the introduction, pregnant women are more likely to experience severe SARS-CoV-2 infection and ICU admissions compared to their non-pregnant counterparts, and COVID-19 can induce a systemic inflammatory disorder. With increasing severity of the infection, higher levels of circulating cytokines and other inflammatory biomarkers like IL6, IL-1β, TNFα, C-reactive protein (CRP) and D-dimer occurs (Smith et al., 2007; Deverman and Patterson, 2009; Wu et al., 2017; Zupan et al., 2017). These proteins attract monocytes, macrophages, and T-cells to the site of infection, promoting further inflammation and establishing a pro-inflammatory feedback loop. In addition, non-neutralizing antibodies produced by B-cells may enhance SARS-CoV-2 infection through antibody-dependent enhancement, further exacerbating organ damage (Deverman and Patterson, 2009). The resulting cytokine storm circulates to other organs, leading to multi-organ damage.

Even in the absence of a cytokine storm, maternal immune activation during COVID-19 along with proinflammatory changes in the placental vascular bed could potentially activate interleukin (IL-6) signaling in the syncitiotrophoblast layer. Furthermore, increased cytokines and complement factors in the maternal environment can bleed over into the fetus, altering neurodevelopment. Pro-inflammatory cytokines including IL-6, IL-1β, and TNF-α have a molecular mass of about 50kDa and can easily cross the placental barrier, passing from mother to fetus (Zaretsky et al., 2004; Aaltonen et al., 2005; Smith et al., 2007; Deverman and Patterson, 2009; Ratnayake et al., 2013; Wu et al., 2017; Zupan et al., 2017). All neural and non-neural cell types within the developing CNS use cytokines for paracrine and autocrine signaling. Thus, maternal immune activation secondary to maternal infection can disrupt brain development in multiple ways, which we review briefly in the following paragraphs and summarize in **Figure 3**. Most of the studies discussed in the ensuing paragraphs used rodent models of maternal immune infection (MIA). Multiple models of MIA exist including prenatal administration of immunogenic liposaccharides (LPS), transmembrane protein toll-like receptor (TLR) 4, or polyriboinosinic–polyribocytidilic acid [Poly(I:C)]. The specific models used are noted throughout.

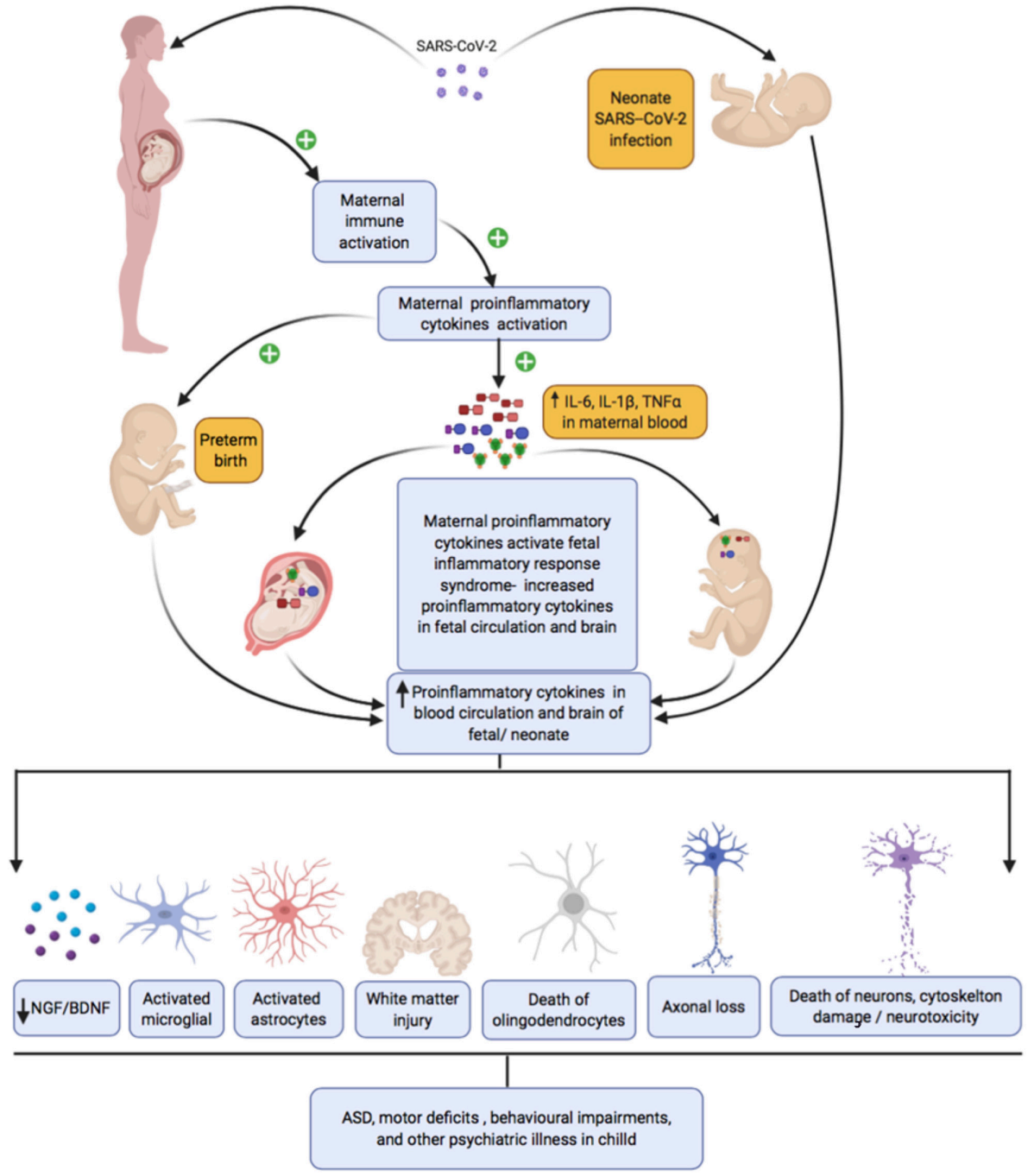

FIGURE 3
Maternal SARS-CoV-2 infection, inflammation, and subsequent fetal/neonatal brain development.

First, maternal immune activation can trigger periventricular white matter damage. White matter damage has been observed in the context of many different prenatal infections in human newborns and in animal models [Yoon et al., 1997 (rabbit, E. coli infection); Dammann et al., 1999; reviewed in Malaeb and Dammann, 2009], and in a newborn baby following transplacental transmission of SARS-CoV2 infection (Vivanti et al., 2020). These observations partly reflect associations between prenatal infection and preterm delivery, which is a well-established risk factor for intraventricular hemorrhage, neonatal white matter damage, and subsequent cerebral palsy. However, rodent studies confirm that white matter injury in offspring can be induced by intrauterine maternal infection in the absence of preterm birth

[Wang et al., 2007; Zhan et al., 2021 (LPS)]. Mechanisms of injury may include both direct effects of pro-inflammatory cytokines on oligodendrocytes and axons and indirect effects via activation of microglia (reviewed in Robinson, 2005; Burd et al., 2012). Microglial cells enter the human brain as early as 4 weeks of gestation and accumulate in the prospective white matter of the corona radiata between weeks 19 and 24 (Monier et al., 2007). When activated, microglia cause localized neuroinflammation and injury [Monier et al., 2007; reviewed in Burd et al., 2012]. Once activated, the microglia may continue to remain activated into infancy or early childhood, resulting in sustained production of pro-inflammatory cytokines, oxidative and nitrosative products, and excitotoxic metabolites such as glutamate and quinolinic acid, all of which can injure oligodendrocytes. Specific pro-inflammatory cytokines that have been linked to oligodendrocyte injury include IL-1β which impairs myelination by reducing the number of developing oligodendrocytes when injected into the cerebrum of rat pups (Cai et al., 2004) and TNF-α which induces death of human oligodendrocyte cells by activation of apoptosis-inducing factor (Yoon et al., 1997; Saliba and Henrot, 2001), With regard to axonal development, Makinodan et al. (2008) report that juvenile mice had reduced axonal diameters in the hippocampus following maternal immune activation [poly(I:C)], a phenotype that normalized by adulthood.

Second, maternal immune activation can influence developmental neurogenesis and neurodifferentiation. In general, hyperactivation of the immune response is thought to impair survival and differentiation of neural progenitors (Borsini et al., 2015; Kim et al., 2016) by attenuating the production of neurotrophic factors including brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), platelet-derived growth factors (PDGF), and neurotrophins (NT-3,4). Much of this evidence is based on *in vitro* models, which are though to best model human midgestational neurogenesis. However, *in vitro* models cannot fully capture the impact of infection on a pregnant mother. Furthermore, different cytokines induce different effects in *in vitro* models – some positive and some negative. For example, IL-1β and TNF-α reduce neurogenesis of fetal hippocampal neural progenitor cells (NPCs) (Johansson et al., 2008; Zunszain et al., 2012; Chen et al., 2013), while IL-6 was reported to increase neurogenesis in human hippocampal NPCs (Johansson et al., 2008). Very few animal studies have directly assessed the impact of MIA on developmental neurogenesis. One recent study examined Ki67 + /Nestin + and Tbr2 + neural progenitor cells in the subventricular zone (SVZ) of neonatal mice following mid-gestation MIA (LPS) and reported robust increases (Loayza et al., 2022). In a similar manner, significant increases in the proportion of Pax6-positive neural progenitor cells and Pax6/Tbr2 double-positive cells have been observed in mouse fetal brains 24 h after poly(I:C) injection (Tsukada et al., 2021). This contrasts with the findings of Canales et al. (2021) who reported evidence of overall decreased neurogenesis by E17.5, following poly(I:C) injection at E12.5 (Canales et al., 2021) and with Tsukada et al. (2015) who reported that mid-gestatational MI (Poly:I:C) impairs neurogenesis in the cerebellum. There is a rich body of literature demonstrating that pro-inflammatory cytokines disrupt neurogenesis in the adult rodent hippocampus [see (Kim et al., 2016) for review] and several MIA studies examined adult hippocampal neurogenesis. Mid-gestation MIA (LPS) suppressed hippocampal neurogenesis in adult rat (Okano et al., 2022), as did late gestation treatment with Poly(I:C) (Zhao et al., 2019), while suppression of maternal IL-6 enhanced it (Mouihate and Kalakh, 2021). Finally, defective neurogenesis in the subventricular zone (SVZ)-olfactory bulb (OB) pathway has been reported following early gestational exposure to Poly:I:C in mouse (Liu et al., 2013). The mechanisms by which MIA primes dysfunction in the unique hippocampal pool of neural stem/progenitor cells in adulthood remains to be fully elucidated (Couch et al., 2021). Furthermore, effects of specific cytokines on neurogenesis and differentiation may vary based on brain region, species, and developmental stage.

Finally, pro-inflammatory cytokines can promote cytoskeletal damage and neural apoptosis. Astrocytes may play a key role in this process. MIA (Poly:I:C) induces a hypertrophied morphology and intense GFAP immunoreactivity in astrocytes in the hippocampus that persist at least until weaning (Patro et al., 2013), with upregulation of GFAP detectable in adulthood following LPS (Berkiks et al., 2019). Hypertrophied morphology and upregulation of GFAP indicate astrocytic activation and astrocytes produce reactive oxygen species (ROS) including nitric oxide (NO), which are neurotoxic. Activated microglia also produce ROS, as discussed previously, which could damage neurons as well as glia. Increased oxidative stress has been observed in the hippocampus and cerebral cortex of adult rats exposed to LPS MIA (Cieślik et al., 2020, 2021). In Cieślik et al. (2021), oxidative stress did not appear to result from activated microglia but was accompanied by evidence of mitochondrial dysfunction (Cieślik et al., 2021), which has also been linked to oxidative damage in mouse models of autism spectrum disorder (Yui et al., 2015). Cieślik et al. (2021) also observed abnormal phosphorylation and dysfunction of MAPT, which is involved in assembling and stabilizing microtubules, which make up the cytoskeleton (Cieślik et al., 2021). Increased neural apoptosis appears to be linked to late-gestational MIA, rather than mid-gestation MIA (Meyer et al., 2006) [poly(I:C), mice] and appears to arise due to the interactive effect of multiple cytokines (Matelski et al., 2021) (*in vitro* model).

We end this section by noting that animal models of MIA can also help address questions about how timing of infection relates to neurodevelopmental sequelae. For example, Nakamura et al. (2022) recently reported that early gestational exposure to MIA [poly(I:C)] disrupted working memory and reduced perseverative behavior in female offspring while late gestational exposure induced male-specific deficits in working

memory and reversal learning (Nakamura et al., 2022). Guma et al. (2022) have reported that early gestational exposure to MIA [poly(I:C)] induces profound reductions in certain regions of the embryonic brain, likely through increased apoptosis, while late gestational exposure induced volume expansions, possibly due to acute inflammatory responses (Guma et al., 2022). The same group has also presented timing of exposure specific effects on neonatal mice brain volumes in regions of the amygdala, hippocampus, entorhinal cortex, striatum, and periaqueductal gray matter and reported that neonatal communication abilities, indexed by ultrasonic vocalizations, are reduced following early, but not late exposure (Guma et al., 2021b). Early exposure also appears to produce more profound effects on anxiety-like, stereotypic, and sensorimotor gating behaviors, measured in adolescence, than late exposure, changes that are accompanied by transcriptional alteration in genes linked to inflammation and autistic behaviors (Guma et al., 2021a).

A key role for IL-6 in abnormal neurodevelopment following maternal immune activation

Maternal immune activation (MIA) is accompanied by increased levels of multiple pro-inflammatory cytokines. However, IL-6 appears to play an especially important role in mediating the impact of maternal infection on offspring neurodevelopment (Smith et al., 2007). This was demonstrated by Smith et al. (2007) via an elegant series of rodent experiments. First, they showed that administration of IL-6 during pregnancy was sufficient to induce prepulse inhibition (PPI) and latent inhibition (LI) deficits in adult offspring, while administration of IFNγ was not. Second, they demonstrated that administration of an IL-6-neutralizing antibody during MIA [poly(I:C)] rescued deficits in PPI and IL and normalized exploratory and social behavior. Next, they showed that IL-6 knock-out mice failed to exhibit deficit in PPI, social interaction, or exploratory behavior following MIA. Finally, they demonstrated that administration of an IL-6-neutralizing antibody during MIA also normalized gene expression differences in the brains of offspring (Smith et al., 2007). Hence, IL-6 emerged as the main driving factor through which MIA causes long term behavioral changes in offspring (Smith et al., 2007).

The importance of IL-6 in human brain development has been demonstrated via neuroimaging studies of infants and children. Specifically, Rudolph et al. (2018) reported associations between maternal IL-6 levels and neonatal functional brain connectivity, with the salience, dorsal attention, and subcortical networks being most extensively involved. Furthermore, these associations may explain associations between maternal IL-6 and offspring working memory performance at 2 years of age in the same sample (Rudolph et al., 2018). Effects of IL-6 on the salience network were also reported by Spann et al. (2018). Also, MRI data of infants (n = 30) shows that higher levels of IL-6 during pregnancy may lead to disruption in frontolimbic white matter and cognitive development (Rasmussen et al., 2019). Furthermore, children born to women (n = 86) with high IL-6 levels during early pregnancy showed larger right amygdala volumes and stronger bilateral amygdala connectivity to other parts of brain including fusiform, somatosensory cortex and thalamus (for sensory processing and integration), anterior insula (for salience detection), caudate and parahippocampal gyrus (for learning and memory) at 24 months age. Moreover, volume of the right amygdala and stronger left amygdala connectivity mediated associations between maternal IL-6 and compromised impulse control in offspring (Graham et al., 2018). While these studies are relevant to the issue at hand, it is important to note that the sample sizes are relatively small. A recent study by Marek et al. (2022), suggests that rigorous and reproducible associations between brain structure or function and complex cognitive or behavioral data may require thousands of individuals (Marek et al., 2022). In addition to being insufficiently powered, small sample sizes are vulnerable to sampling variability, inflated effect sizes, high statistical error rates, and poor reproducibility.

Various mechanisms have been proposed to explain how IL-6 induces abnormal neurodevelopment (Boulanger-Bertolus et al., 2018). Many of these mechanisms highlight the placenta as a key organ in this pathophysiological process. Knockout of the trophoblastic IL-6 receptor in mice prevents cerebellar neuropathology and behavioral impairments following MIA [poly(I:C)] and attenuates immune responses in the fetal brain (Wu et al., 2017). This suggests that placental IL-6 signaling, specifically in the trophoblast, is required for MIA-induced acute immune activation in the fetal brain and subsequent detrimental effects on offspring neurodevelopment, at least in rodents. The authors of this study proposed three different ways placental IL-6 signaling might impact the fetal brain. First, they proposed that the placenta may initiate a feed-forward cycle of IL-6 induction in the embryo. Second, they proposed effects of placental IL-6 signaling on the fetal brain might be mediated by changes in placental hormones including prolactin and corticotrophin-releasing factor (CRF). Finally, they suggested that IL-6 might induce trophoblasts to produce factors that increase vascular permeability in the placenta, thereby altering the metabolic and nutritional environment of the fetus. An earlier study by the same group revealed another placental hormone system disrupted by poly(I:C) MIA – the growth hormone-insulin-like growth factor (GH-IGF) axis. Levels of growth hormone (GH), insulin like growth factor 1 (IGF1), and insulin like growth factor binding protein 3 (IGFBP3) levels were all reduced following MIA (Hsiao and Patterson, 2011). More recently, Monteiro et al. (2022) reported that mid-pregnancy MIA [poly(I:C)] alters expression of placental ATP-Binding Cassette (ABC) efflux transporters, which transport a variety of substances including cholesterol, drugs, xenobiotics,

and cytokines across the placental barrier (Monteiro et al., 2022). While this data is clearly of interest, it should be noted that there are substantial differences between the most frequently used rodent models and human placentas, which make it difficult to extrapolate directly from mice and rats to human. Schmidt et al. (2015) and Carter (2020) review several key differences in anatomy including (1) in human placenta maternal blood perfuses the intervillous space, while in mice and rats exchange of material is between fetal and maternal capillaries, (2) humans do not have an inverted yolk sac placenta in addition to the chorioallantoic placenta, while mice and rats do, (3) mice and rats have trichorial placentas while humans have monochorial placentas, and (4) in humans there is deep interstitial and endovascular invasion of trophoblast cells into the inner third of the human myometrium, while in mice the invasion is restricted to the decidua basalis (Schmidt et al., 2015; Carter, 2020). The latter difference is particularly problematic when studying preeclampsia. There are also important species differences in placental endocrinology, molecular features, and immune responses. For example, the human placenta can actively transport protective immunoglobulin IgG antibodies to the fetus during gestation, while rodents do not transport IgG as efficiently and mice acquire maternal IgG antibodies via yolk sac–derived cells and after birth via suckling (reviewed in Ander et al., 2019). In addition, the human placenta secrets primate-specific antiviral microRNAs (miRNAs) from a cluster on chromosome 19 (C19MC) from syncytiotrophoblast layer that broadly restrict viral infections while mouse placenta uses interferons to restrict viral infections (Ander et al., 2019). To overcome this translational challenge, additional studies using *in vitro* models and alternative animal models are needed (Schmidt et al., 2015; Ander et al., 2019; Carter, 2020).

Maternal infection and fetal hypothalamic-pituitary-adrenal axis modulation

It is well established that viral infection increases the production of proinflammatory cytokines which in turn activate the HPA axis, resulting in increased glucocorticoid production (reviewed in Silverman et al., 2005; Raony et al., 2020). When the maternal HPA axis is activated, levels of glucocorticoids in maternal blood increase. Glucocorticoids can cross the placental barrier thereby increasing fetal glucocorticoid levels. Also, maternal cytokines can cross the placenta and activate the fetal HPA axis and stimulate the release of corticotrophin releasing hormone (CRH). This subsequently would stimulate secretion of adrenocorticotrophic hormone (ACTH) from the fetal anterior pituitary and glucocorticoids from the fetal adrenals (reviewed in Seckl, 2004; Ratnayake et al., 2013). Glucocorticoids play a central role in the fetal programming of HPA function (reviewed in Kapoor et al., 2006). Exposure to high levels of glucocorticoids *in utero* can manifest as disrupted HPA axis reactivity in later life and may underlie cognitive deficits and addictive behaviors in childhood and adulthood (reviewed in Waffarn and Davis, 2012; Moisiadis and Matthews, 2014; Granja et al., 2021).

In a recent review article, Granja et al. (2021) proposed a possible mechanism by which SARS-CoV-2 infection in a pregnant woman may disrupt fetal brain development via interference with the HPA axis. They explain that during a normal pregnancy, levels of 11β-HSD2 (glucocorticoid inactivating hormone) increase to ensure the appropriate exposure of glucocorticoids to the fetus. At the same time progesterone levels are also increasing to counter the cytokine balance toward an anti-inflammatory profile at the maternal-fetal interface (Granja et al., 2021). They hypothesize that viral infection (e.g., SARS-CoV-2 infection) may disrupt placental 11β-HSD2 expression resulting in increased exposure of the fetus to glucocorticoids (Granja et al., 2021). We are unaware of any studies directly testing Granja et al's hypothesis, but there is a growing body of literature on the immune environment of the human placenta during COVID-19 infection. Lu-Culligan et al. (2021) reported robust inflammatory responses in placenta tissue from third trimester COVID-19 infections including increased expression of pro-inflammatory genes and chemokines, revealed by single-cell transcriptomic profiling (Lu-Culligan et al., 2021). In contrast, Juttukonda et al. (2022) have reported that while decidual tissue from individuals with third trimester COVID-19 infections have increased macrophages, NK cells, and T cells, levels of IL-8 are reduced compared to controls and levels of IFN-γ, IL-1β, IL-6, IL-10, and TNF do not differ (Juttukonda et al., 2022). In the same study, decidual tissue from individuals with second trimester infections showed a significant decrease in IL-6, IL-8, IL-10, and TNF-α and no change in abundance for IL-1β or IFN-γ (Juttukonda et al., 2022). Bordt et al. (2021) reported increased levels of IFN-α, IFN-γ, and IL-10 in placentas from individuals with third trimester infections, but only in males (Bordt et al., 2021). Thus, there is still much to be done in terms of understanding how maternal COVID-19 impacts inflammatory profiles of the placenta and a dearth of studies on how this might impact glucocorticoids.

Maternal glucocorticoids can reach fetal brain and bind with glucocorticoid receptors (GR) to exert detrimental effects (reviewed in Miranda and Sousa, 2018). In fact, disrupted placental 11β-HSD2 expression, may lead to abnormal glucocorticoid receptor (GR) expression in hippocampus and amygdala, leading to a hyperreactive HPA axis and increased anxiety-like behaviors in adult rat offspring (Welberg et al., 2000). Furthermore, higher glucocorticoid exposure to the fetus increases inducible nerve growth factor A and activates transcriptional activity thereby disrupting fetal brain development (Andrews et al., 2004) (guinea pig). In addition to these molecular changes, fetal exposure to high levels of

glucocorticoids also impacts neurogenesis in both rodent and human *in vitro* models and changes hippocampal structure (Gould et al., 1992; Lemaire et al., 2000; Provençal et al., 2020). Similarly, higher levels of glucocorticoid may alter microglial function (reviewed in Walker et al., 2013), disrupting synaptogenesis, neurogenesis, synaptic pruning, axonal growth, myelination and astrocyte maturation (reviewed in Harry and Kraft, 2012; Schafer and Stevens, 2015).

It is reasonable to hypothesize that MIA in the context of SARS-CoV-2 would produce similar effects on the HPA axis which may increase risk for behavioral problems in offspring as shown in **Figure 4A**. Indeed, SARS-CoV-2 infected patients may experience a 'cytokine storm' leading to excessive glucocorticoids that may have deleterious effects on the host (**Figure 4A**) (Silverman et al., 2005; Raony et al., 2020). On the other hand, there is also evidence to suggest that SARS-associated coronaviruses can produce hypocortisolism (**Figure 4B**) (Leow et al., 2005). In a prospective cohort study of SARS-CoV survivors, 24 of 61 patients developed HPA axis dysfunction resulting in reduced blood cortisol levels during the 3-month follow-up period (Leow et al., 2005). A published case report suggests that SARS-CoV-2 infection can also produce hypocortisolism; a 69-year-old Iranian man, admitted to the ICU with SARS-CoV-2, developed adrenal insufficiency and reduced serum cortisol level (Heidarpour et al., 2020). Two mechanisms have been proposed for the association of SARS-associated coronaviruses with hypocortisolism: (1) destruction of ACTH due to infection and (2) damage to the hypothalamus. Regarding the first potential mechanism, in 2004, Wheatland proposed the molecular mimicry theory of ACTH in SARS. This theory is based on the observation that SARS-CoV expresses certain amino acid sequences that mimic the adrenocorticotropic hormone (ACTH). Thus, antibodies produced by the host in response to SARS-CoV may also destroy host ACTH thereby reducing the patient's cortisol level (Wheatland, 2004). Regarding the second potential mechanism, the authors of the prospective cohort study of SARS-CoV survivors discussed above proposed in their discussion that SARS-CoV-2 infection could damage the hypothalamus and/or pituitary via the ACE2 receptor or CD209L/L-SIGN, a C-type lectin surface glycoprotein implicated in viral pathogenesis, leading to HPA axis dysfunction and hypocortisolism (Leow et al., 2005). Support for this mechanism has recently been provided by a French group, who reported that ACE2 and the transmembrane proteinase, serine 2 (TMPRSS2), which cleaves the SARS-CoV-2 spike protein, are expressed in the adult human hypothalamus, with the paraventricular nucleus showing the highest expression among hypothalamic nuclei. Interestingly, a KEGG pathway enrichment analysis suggested that both ACE2 and TMPRSS2 play important roles in the "neuroactive ligand-receptor interaction" pathway supporting an impact of SARS-CoV-2 on neuroendocrine function, including interactions between corticotropin releasing hormone and its receptor. Furthermore, they report that viral markers for SARS-CoV-2 were abundant in the hypothalamus of a 63-year-old male patient who died of COVID, but absent from the hypothalamus of controls (Nampoothiri et al., 2020). Very little work has been done on the possible neurodevelopmental consequences of low cortisol levels during pregnancy, but pregnancy is typically accompanied by substantial increases in cortisol (Mastorakos and Ilias, 2003; Jensen et al., 2011; Guardino et al., 2016). Several studies, conducted in sheep, indicate that lowering maternal cortisol during pregnancy alters placental morphology and reduces placental and uterine blood flow, which could result in restricted fetal growth (Jensen et al., 2005, 2007) and altered neurodevelopment, as previously discussed. Overall, depending on the timing of SARS-CoV-2 infection, the developing fetus may be exposed to both abnormally high and abnormally low levels of cortisol with potential consequences for neurodevelopment.

Maternal infection and epigenetic modulation

Accumulating evidence suggests that maternal infection during gestation may affect intergenerational and transgenerational offspring neurodevelopmental process via epigenetic modifications (reviewed in Kleeman et al., 2022). Epigenetic processes produce long term and heritable modifications in gene expression without changing the DNA sequence (reviewed in Bale, 2015). These processes include DNA methylation, histone modification, and expression of microRNA (miRNA) (reviewed in Bale, 2015; Szyf, 2015; Weber-Stadlbauer, 2017). Epigenetic processes play a critical role in linking early environmental experiences to long-term changes in brains structure and function and are likely to play a key role in explaining the impact of maternal infection on brain development as described in subsequent paragraphs (reviewed in Bale, 2015; Dubey et al., 2018; Bergdolt and Dunaevsky, 2019).

DNA methylation involves the attachment of a methyl (CH3) group to cytosines within the DNA sequence, a reaction that requires both methyltransferases, such as DNA cytosine-5-methyltransferase 1 (DNMT1), and methyl donors, which are derived from nutrients such as folate. Methylated DNA attracts methyl binding proteins, such as methyl CpG binding protein 2 (MeCP2), that condense the structure of the nucleosome, thereby preventing transcription. A growing body of research suggests that infection during pregnancy alters DNA methylation in the offspring brain in ways that are both complex and region-specific (Richetto et al., 2017b). Richetto and colleagues observed that MIA following treatment with the viral mimetic Poly(I:C) altered DNA methylation in the medial prefrontal cortex of adult offspring. Adult offspring of immune activated mothers showed hyper or hypomethylation

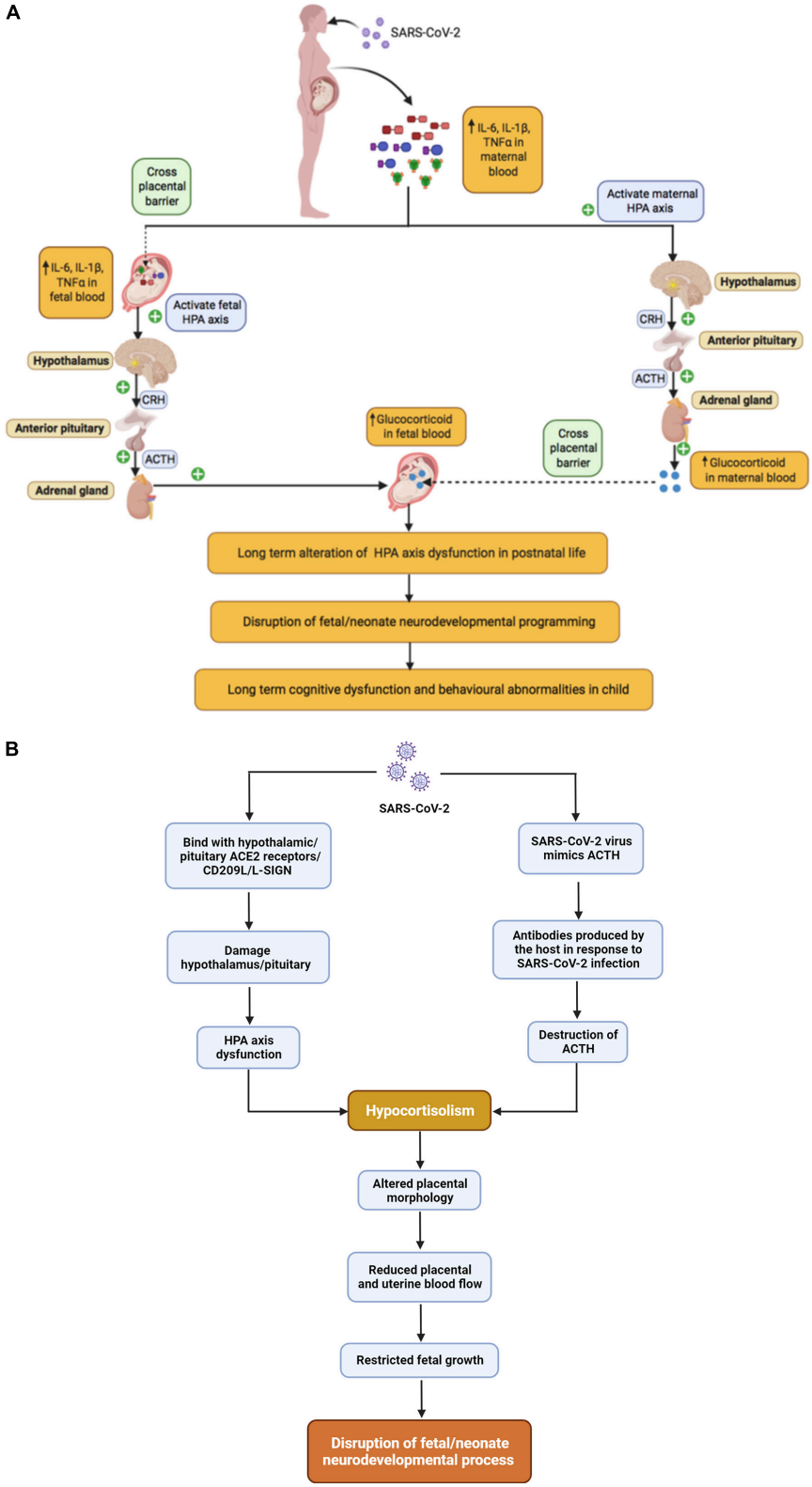

FIGURE 4
The relationship between maternal SARS-CoV-2 infection and Fetal HPA axis modulation, **(A)** SARS-CoV-2 infection and hypercortisolism, **(B)** SARS-CoV-2 infection and hypocortisolism.

of CpGs at various loci including loci influencing GABAergic differentiation and signaling (*Dlx1, Lhx5, Lhx8*), Wnt signaling (*Wnt3, Wnt7b, Wnt8a*), neural development (*Efnb3, Mid1, Nlgn1, Nrxn2*) (Richetto et al., 2017b), and myelination related gene α-myelin-associated oligodendrocytic basic protein (*mobp*) in prefrontal cortex and nucleus accumbens of adult offspring (Richetto et al., 2017a), suggesting that epigenetic modification might mediate the impact of prenatal infections on offspring behavior (Richetto et al., 2017a,b). Labouesse and colleagues have also investigated Poly(I:C) induced MIA impacts on prefrontal cortex in mice and found increased methylation of promotors for GAD1 and GAD2, key enzymes in GABA synthesis (Labouesse et al., 2015). Poly(I:C) induced MIA also induces significant global DNA hypomethylation in the hippocampus, including in the promotor of *Mecp2*, but not in the mice striatum (Basil et al., 2014), as well as increased expression of DNase I hypersensitivity sites (DHSs) and MECP2 binding sites genes, namely *Abat* and *Gnas9* in mice hypothalamus (Basil et al., 2018). In another study, poly(I:C) induced MIA increased methylation of the promoter region of tyrosine hydroxylase (*Th*) gene in the dopaminergic neurons of ventral midbrain in adult mice (Weber-Stadlbauer et al., 2021). All the above-mentioned studies used Poly (I:C) induced MIA model, because it is widely applied to study neurodevelopment (reviewed in Bao et al., 2022). Further, Poly (I:C) induced MIA are mainly driven via IL-6 activity (reviewed in Bao et al., 2022). IL-6 may provide a mechanistic link between infection and altered DNA methylation as it promotes nuclear translocation of DNMT1, the major enzyme responsible for maintaining methylation patterns following DNA replication (Hodge et al., 2007). Further, alterated or defective DNA replication may lead to impaired neuronal development (Kalogeropoulou et al., 2019).

Histone modifications are post translational modifications of histone protein (Szyf, 2015). There can be hundreds of modifications on a given histone producing a cumulative effect on how DNA around that histone is packaged. The addition or removal of acetyl groups is one biochemical process known to be important for transcriptional regulation and is accomplished by two types of enzymes: histone acetyltransferases (HATs) and histone deacetylases (HDACs). HATs promote DNA histone acetylation, which loosens the chromatin and facilitates gene transcription, while HDACs remove acetyl groups, which condenses the chromatin and reduces gene transcription (reviewed in Dubey et al., 2018). LPS-induced inflammation increases expression of HDAC2 and HDAC5 in the brain, an effect that can be attenuated by treating with an HDAC inhibitor prior to administering LPS. Moreover, pre-treatment with an HDAC inhibitor diminished LPS-induced anhedonia, anorexia, and microglia activation, supporting histone deacetylation as a key mechanism linking systemic inflammation to cognitive dysfunction in adult and juvenile animals (Hsing et al., 2015). Another study in mice suggests that MIA [Poly(I:C)] regulates hippocampal serotonin transporter (SERT) levels via modulation of histone acetylation which results in anhedonic behavior in offspring (Reisinger et al., 2016). Specific cytokines linked to histone modification include TNF-alpha, which increases histone acetylation in human alveolar epithelial cells (Rahman et al., 2002) and IL-17a, which reduces HDAC2 activity via the PI3K pathway in human bronchial epithelial cells (Zijlstra et al., 2012). Whether these cytokines have similar effects in the developing brain is currently unknown. In one study, MIA [Poly(I:C)] during pregnancy did not appear to alter histone modification in cerebral cortex of adult offspring, although activation of cytokine signaling in primary cultures from fetal forebrain influenced trimethylated histone H3-lysine 4 (H3K4me3) marks in a limited set of genes (Connor et al., 2012). In contrast, Tang et al. (2013), observed that MIA [Poly(I:C)] leads to global hypoacetylation of histone H3 at H3K9K14 and H4K8 in the cortex of juvenile mice. This was accompanied by reduced expression of genes involved in neuronal development, synaptic transmission, and immune signaling. Specific genes exhibiting hypoacetylation included *Robo1*, which is involved in axon guidance and neuronal precursor cell migration, *arhgap18*, and *Ntrk3*, which is likely involved with cell survival and differentiation in the nervous system. Hyperacetylation was also observed at specific loci in the hippocampus of juvenile mice including *Disc1*, which is involved in many aspects of nervous system development, *Nr2f1*, *Ntrk3*, which is a transcriptional regulator, and *Gria1* and *Gria2*, which are both subunits of AMPA-type ionotropic glutamate receptors (Tang et al., 2013). Further, glutamate activities may modulate the early brain developmental process (Tanaka, 2013). Another, mouse study suggested that prenatal poly (I:C) exposure at late gestation (embryonic day 17) leads to deficits in working memory of the adult animal due to altered histone H3K4me3 methylation in approx. 30 genes, including Disc1 (Connor et al., 2012).

MicroRNA (miRNA) are small endogenous non-coding RNAs involved in post-transcriptional gene regulation (Ha and Kim, 2014; Weber-Stadlbauer, 2017). They target most protein coding transcription and prevent the production of specific proteins by binding to and destroying messenger RNA (mRNA). miRNA are highly expressed in brain and essential for brain development and neuronal function (Petri et al., 2014). Several recent studies demonstrate an impact of MIA on miRNA expression in offspring brain. Sunwoo et al. (2018) have reported that MIA [Poly(I:C)] alters brain miRNA expression in offspring at 3 weeks of age, a time when both synaptogenesis and myelination are ongoing. 8 miRNA were upregulated and 21 were downregulated. Furthermore, target genes of 18 downregulated and 3 upregulated miRNA were found to be significantly enriched among differentially expressed genes, confirming that MIA induced alterations in miRNA have functional consequences, at least at the level

of gene expression. Offspring exhibited behavioral changes typical of MIA including lack of a preference for social novelty and reduced prepulse inhibition, but the study design did not address whether miRNA alterations played a causal role in the behavioral abnormalities (Sunwoo et al., 2018). Berger et al. examined how MIA [Poly(I:C)] affected 13 specific miRNA in offspring hippocampus and observed increased levels of miR-15b-2, miR-98-1, miR-103-2, and miR-124-1. None of these overlap with the differentially expressed miRNA in Sunwoo et al., indicating that additional studies are needed to clarify which miRNA are most robustly associated with MIA. Interestingly, MIA also influenced MiRNA expression in F2 generations of offspring, along the paternal line. The specific miRNA involved differed from observations in the F1 generation. This and the modest magnitude of changes led the authors to conclude that miRNA did not play a substantial role in the behavioral impacts of MIA (Berger et al., 2018).

With specific regard to SARS-CoV-2, no studies have examined miRNA levels in the brains of exposed fetuses. However, a recently published cohort study indicated that various miRNAs are upregulated in both plasma and placental tissues of pregnant mothers infected with SARS-CoV-2 (Saulle et al., 2021). 35 miRNA were differentially expressed in human plasma including seven antiviral miRNAs (miR-21, miR-23b, miR-28, miR-29a, miR-29c, miR-98 and miR-326) and six immunomodulatory miRNAs (miR-17, miR-92, miR-146, miR-150, miR-155, miR-223), all upregulated in infected mothers. In placenta, eight miRNA were upregulated in the context of maternal infection including ones with direct effects on viral replication (miR-21b, miR-29c, miR-98) and ones influencing viral replication by indirect mechanisms (miR-146, miR-155, miR-190, miR-346, and miR-326) (Saulle et al., 2021). Further, an *in silico* study showed that miR-21, miR-16 and miR-146a have high affinity to the SARS-CoV-2 virus (Jafarinejad-Farsangi et al., 2020).

Interestingly, several of these miRNAs have also been implicated in neurodevelopment. miR-146a is one of the most commonly dysregulated miRNAs in neurodevelopmental disorders (Fregeac et al., 2016; Nguyen et al., 2016, 2018; Schepici et al., 2019). In H9 human neural stem cells, miR-146a overexpression enhances neurite outgrowth and branching and favors differentiation into neuronal like cells (Nguyen et al., 2018). In mouse primary cell cultures, miR-146a overexpression leads to impaired neuronal dendritic arborization and increased astrocyte glutamate uptake capacities (Nguyen et al., 2016). miR-146a is highly expressed in mouse hippocampus, amygdala and entorhinal cortex, areas with important roles in social cognition, memory, spatial navigation, and the perception of time, and targets genes with known roles in neurodevelopment including *MAP1B, FMR1*, and *KCNK2* (Nguyen et al., 2016). Furthermore, miR-146a overexpression promotes oligodendrocyte differentiation and myelination in the context of neurological injury (Santra et al., 2014; Liu et al., 2017; Zhang et al., 2017, 2019), raising the possibility that it also effects these processes during neurodevelopment. In addition, downregulation of miRNA-146a expression in mice leads to impaired neurogenesis, abnormal brain anatomy, along with deficits in working and spatial memory (Fregeac et al., 2020). Apart from miR-146a, miR-21, miR-146b, miR-23a, miR-23b, miR-92(a1-a2) and miR-23a-3p have been reported to differentiate between controls and individuals with ASD in peripheral tissues, e.g., lymphoblastoid cell lines and saliva (Talebizadeh et al., 2008; Sehovic et al., 2020; Frye et al., 2021), while miR-21-3p is overexpressed in the cortex of post-mortem ASD patients (Wu et al., 2016). These miRNAs have been less thoroughly studied than MiR-146a in terms of their roles in neurodevelopment, but miR-21 expression in the placenta is associated with fetal growth (Maccani et al., 2011), which could explain the association of maternal SARS-CoV-2 infection with intrauterine growth restriction, a known risk factor for altered neurodevelopment. miR-23 regulates progenitor fate decisions by inhibiting cyclin D1 mRNA. Inhibition of miR-23 increases cyclin D1 protein in mouse progenitor cells leading to reduced neuronal differentiation during cortical neurogenesis (Ghosh et al., 2014). miR-21-3p downregulates multiple genes in a specific gene co-expression module enriched for ASD risk genes in post-mortem human brain (Wu et al., 2016). This module is upregulated in early cortical development and is enriched for genes implicated in neural development and synaptic function (Parikshak et al., 2013). In addition, miR-21-3p over-expression led to a pronounced decrease in the *PCDH19* gene (Wu et al., 2016), which encodes a cell-adhesion protein primarily expressed in the brain. Mutations in *PCDH19* are associated with both epilepsy and ASD (Redies et al., 2012).

Overall, maternal immune activation can affect multiple epigenetic processes in the developing brain leading to long-lasting behavioral changes in offspring as summarized in **Figure 5**. Readers interested in additional details may find the following reviews of interest: (Woods et al., 2021; Kleeman et al., 2022). The latter provides a systematic review of MIA-induced changes in gene expression and epigenetic features. Additional research is needed to study these relationships in the context of maternal SARS-CoV-2.

Emerging evidence linking gestational SARS-CoV-2 infection to altered neurodevelopment

The information presented thus far strongly suggests that gestational SARS-CoV-2 infection could alter fetal neurodevelopment. Empirical evidence supporting this hypothesis is currently sparse and somewhat inconsistent, but intriguing. A preliminary study conducted in 2020 (N = 57) reported that a substantial proportion of children born to

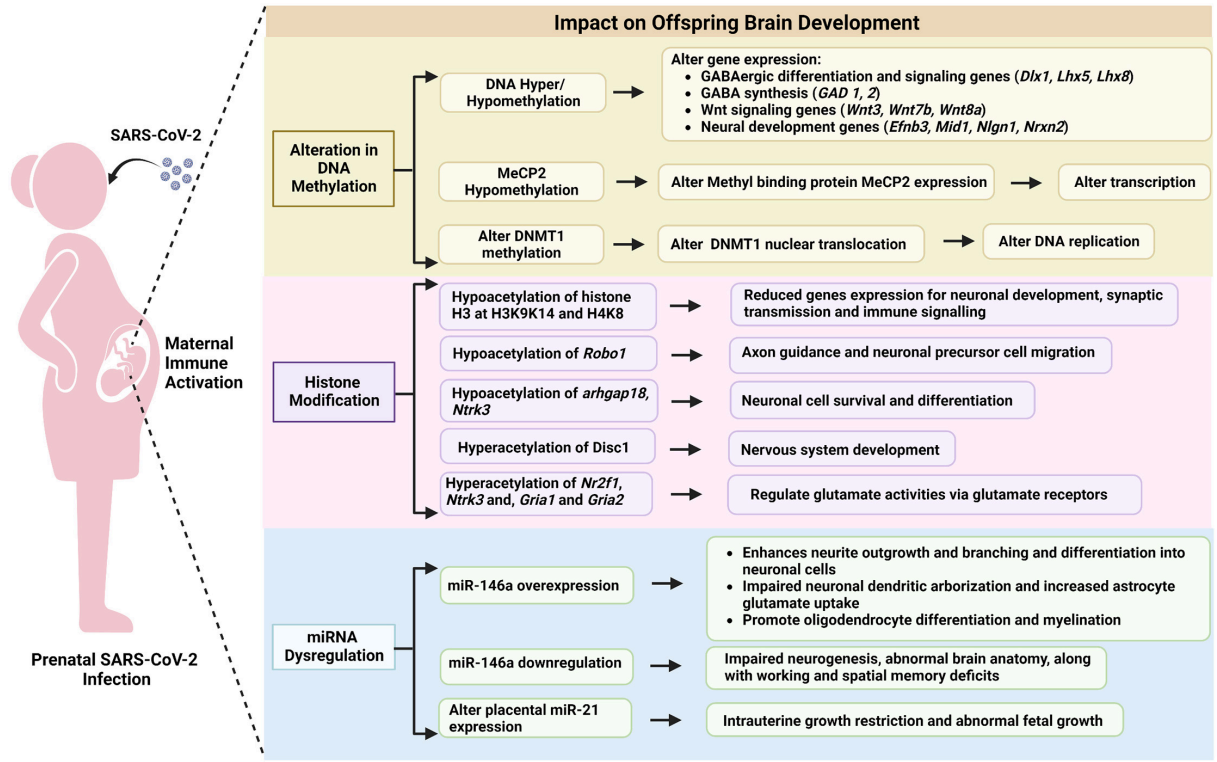

FIGURE 5
Potential epigenetic mechanisms linking prenatal SARS-CoV-2 infection to offspring brain development.

mothers infected with SARS-CoV-2 during gestation were identified as high risk for social and emotional problems at 3 months of age using the Ages and Stages Questionnaire: Social-Emotional, second edition (ASQ:SE-2) (63.6%) (Wang et al., 2020). In contrast, the proportion of children identified as high risk using the Ages and Stages Questionnaires, third edition (ASQ-3) was lower – 0% for communication and gross motor skills, 5.8% for fine motor skills and problem solving, and 9.6% for personal-social skills. No control group was included for comparison. Interestingly, gross motor, problem solving, personal–social, and social–emotional were negatively linked with the amount of time mothers and babies were separated after birth. More recent studies with larger sample sizes provide additional insights (Aldrete-Cortez et al., 2022; Ayed et al., 2022; Edlow et al., 2022). A prospective cohort study conducted in Kuwait (N = 298) reported developmental delays in around 10% of infants whose mothers had COVID-19 during pregnancy using the ASQ-3 (Ayed et al., 2022), which is similar to rates of developmental delay in healthy children in a similar geographical and cultural setting (Charafeddine et al., 2019). The ASQ:SE-2 was not included in this study. Risk of developmental delay was significantly higher in infants born to mothers infected during the first and second trimester than mothers infected in the third trimester, suggesting that adverse neurodevelopmental consequences of SARS-CoV-2 may be time-specific (Ayed et al., 2022). Key neurodevelopmental events occurring in the first and second trimesters include formation of the neural tube and neurogenesis (Stiles and Jernigan, 2010). A study of 254 infants born in New York City during the pandemic (114 exposed to COVID-19 *in utero* and 141 unexposed) and 62 infants born before the pandemic, revealed that birth during the pandemic was associated with significantly lower scores on gross motor, fine motor, and personal-social skills, with no significant differences between the exposed and unexposed groups (Shuffrey et al., 2022). This suggests that COVID-19-related stress may have a stronger impact on neurodevelopment than gestational exposure to SARS-CoV-2. Relatively few women had confirmed exposures in early pregnancy, but *post hoc* analyses suggested exposure in the first trimester might have adverse neurodevelopmental consequences, similar to the earlier Ayed study. The above studies all relied on the ASQ-3, a parent-response instrument used to screen for developmental delays, as an index of early neurodevelopment. Aldrete-Cortez et al. took a different approach using observations of early motor repertoires as their outcome. They showed that at 3-5 months of age, a motor optimality score was significantly lower in infants prenatally exposed to SARS-CoV-2 than unexposed controls, suggesting they are at higher risk for later neurological disorders (Aldrete-Cortez et al., 2022). Finally, Edlow et al.

TABLE 1 Effect of maternal SARS-CoV-2 infection on offspring neurodevelopment.

Transmission of SARS-CoV-2 mother to fetus	
SARS-CoV-2 virus infects fetus from direct transmission from infected mother to fetus in uterus	Dong et al., 2020; Kirtsman et al., 2020; Patanè et al., 2020; Sisman et al., 2020
No or very less chances of SARS-CoV-2 to vertical transmission from mother to fetus in uterus	Khan et al., 2020; Knight et al., 2020; Xiong et al., 2020; Zhu et al., 2020; Allotey et al., 2022
Neuroinvasiveness of SARS-CoV to human brain *via* binding to the endothelial ACE2 receptors of the blood brain barrier and subsequently entry into the central nervous system	Paniz-Mondolfi et al., 2020
Time dependent inflammatory response during pregnancy against SARS-CoV-2 infection	
Robust inflammatory responses in placenta tissue from third trimester COVID-19 infections including increased expression of pro-inflammatory genes and chemokines	Lu-Culligan et al., 2021
In decidual tissue from individuals with third trimester COVID-19 infections have increased macrophages, NK cells, and T cells, but reduced IL-8 levels and no variation in IFN-γ, IL-1β, IL-6, IL-10, and TNF levels	Juttukonda et al., 2022
In decidual tissue from individuals with second trimester infections showed a significant decrease in IL-6, IL-8, IL-10, and TNF-α and no change in abundance for IL-1β or IFN-γ	Juttukonda et al., 2022
Gender based differences, increased levels of IFN-α, IFN-γ, and IL-10 in placentas from individuals with third trimester infections, but only in males	Bordt et al., 2021
Effect of prenatal infection of SARS-CoV-2 on fetuses and infants	
Infants prenatally infected with SARS-CoV-2, diagnosed with neurodevelopmental disorders mainly related with motor function or speech and language disorders	Edlow et al., 2022
Children born to mothers infected with SARS-CoV-2 during gestation were identified as high risk for social and emotional problems	Wang et al., 2020
Infant born during the pandemic associated with significantly lower scores on gross motor, fine motor, and personal-social skills, with no significant differences between the exposed and unexposed groups.	Shuffrey et al., 2022
Infants born during the SARS-CoV-2 pandemic had similar rates of neurodevelopmental issues compared to those born before the pandemic, regardless of whether they were exposed *in utero*. Communication impairments were more common in children born during the pandemic and fine motor deficits were more common in those with *in utero* exposure	Hessami et al., 2022
Infants prenatally exposed to SARS-CoV-2 have low motor activity score and higher risk for later neurological disorders	Aldrete-Cortez et al., 2022
10% of infants whose mothers had COVID-19 during pregnancy exhibited developmental delays, which is similar to rates of developmental delay in healthy children in a similar geographical and cultural setting	Ayed et al., 2022

used electronic health records and a retrospective cohort design to test associations between SARS-CoV-2 exposure *in utero* and risk for neurodevelopmental disorders in the first year of offspring life (Edlow et al., 2022). This cohort included 7772 live births of which 222 were to SARS-Cov-2 positive mothers. Maternal SARS-CoV-2 positivity during pregnancy was associated with greater rate of neurodevelopmental diagnoses in both unadjusted models and models adjusted for race, ethnicity, insurance status, offspring sex, maternal age, and preterm status. In contrast to studies using the ASQ-3, third-trimester exposures appeared to confer greater risk. Finally, a recent meta-analysis suggested that infants born during the SARS-CoV-2 pandemic had similar rates of neurodevelopmental issues compared to those born before the pandemic, regardless of whether they were exposed *in utero*. However, communication impairments were more common in children born during the pandemic and fine motor deficits were more common in those with *in utero* exposure (Hessami et al., 2022). Overall, emerging evidence suggests maternal SARS-CoV-2 infection may impact neurodevelopment but is subject to several limitations. Sample sizes are relatively small, follow-up has been limited to the first year of life, and most studies have relied on a single parent-report instrument designed to screen for developmental delays. Thus, there is an urgent need for long term follow up in infants born during the pandemic.

Conclusion

Infection of pregnant women with SARS-CoV-2 could influence fetal brain development, potentially increasing risk for later cognitive and behavioral problems. SARS-CoV-2 infection can be transferred transplacentally from an infected mother to her fetus and the virus does have neuroinvasive potential. However, this appears to be a relatively rare event. Consequently, we anticipate that long-term neurodevelopmental effects, if they occur, are more likely to reflect indirect mechanisms. Like other viruses, SARS-CoV-2 infection may trigger maternal immune activation, which may disrupt fetal neurodevelopmental and lead to long term

cognitive and motor deficits, behavioral abnormalities, and, potentially, psychiatric illness in children. SARS-CoV-2 may also trigger preeclampsia, preterm birth, and/or intrauterine growth restriction, which are known risk factors for later neurodevelopmental issues. Key finding related to SARS-CoV-2 infection are summarized in **Table 1**. Given the high numbers of pregnant women that have been, and will be, exposed during this pandemic, the long-term impact of SARS-CoV-2 on fetal brain development needs to be investigated.

Author contributions

HD conceived the idea for the manuscript and wrote the first draft. RS, SK, and RK contributed to manuscript revision. All authors read and approved the submitted version.

Conflict of interest

The authors declare that this review was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Impulsive choice in two different rat models of ADHD—Spontaneously hypertensive and *Lphn3* knockout rats

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Introduction: Impulsivity is a symptom of attention-deficit/hyperactivity disorder (ADHD) and variants in the *Lphn3 (Adgrl3)* gene (OMIM 616417) have been linked to ADHD. This project utilized a delay-discounting (DD) task to examine the impact of *Lphn3* deletion in rats on impulsive choice. "Positive control" measures were also collected in spontaneously hypertensive rats (SHRs), another animal model of ADHD.

Methods: For Experiment I, rats were given the option to press one lever for a delayed reward of 3 food pellets or the other lever for an immediate reward of 1 pellet. Impulsive choice was measured as the tendency to discount the larger, delayed reward. We hypothesized that impulsive choice would be greater in the SHR and *Lphn3* knockout (KO) rats relative to their control strains - Wistar-Kyoto (WKY) and *Lphn3* wildtype (WT) rats, respectively.

Results: The results did not completely support the hypothesis, as only the SHRs (but not the *Lphn3* KO rats) demonstrated a decrease in the percent choice for the larger reward. Because subsequent trials did not begin until the end of the delay period regardless of which lever was selected, rats were required to wait for the next trial to start even if they picked the immediate lever. Experiment II examined whether the rate of reinforcement influenced impulsive choice by using a DD task that incorporated a 1 s inter-trial interval (ITI) immediately after delivery of either the immediate (1 pellet) or delayed (3 pellet) reinforcer. The results of Experiment II found no difference in the percent choice for the larger reward between *Lphn3* KO and WT rats, demonstrating reinforcement rate did not influence impulsive choice in *Lphn3* KO rats.

Discussion: Overall, there were impulsivity differences among the ADHD models, as SHRs exhibited deficits in impulsive choice, while the *Lphn3* KO rats did not.

KEYWORDS

externalizing behavior, response inhibition, delay-discounting, spontaneously hypertensive rat (SHR), *Adgrl3*, *Lphn3* KO rat, latrophilin 3, attention-deficit/hyperactivity disorder (ADHD)

Introduction

Attention-deficit/hyperactivity disorder (ADHD) is a highly prevalent neurodevelopmental disorder characterized by impulsivity, inattention, and hyperactivity (American Psychological Association [APA], 2013). ADHD is a commonly diagnosed in childhood but can continue into adulthood (Weibel et al., 2020) and is often comorbid with other externalizing disorders (Faraone et al., 2003; Palacio et al., 2004; Frick and Nigg, 2012; Hansen et al., 2018). Based on the 2016 National Parent Survey, about 9.8% of children are diagnosed with ADHD and 6 in 10 children with ADHD had at least one other mental, emotional, or behavioral disorder (Bitsko et al., 2022).

Although many factors in the environment can contribute to the development of ADHD, the literature has shown that genetics can help explain ADHD variability (Spencer et al., 2007). Recent data has shown a linkage of ADHD and other externalizing behaviors with markers on chromosome 4q13.2 (Arcos-Burgos et al., 2004; Acosta et al., 2008; Arcos-Burgos and Muenke, 2010). Mapping of this region has revealed that variants in the *Lphn3* (*Adgrl3*) gene (OMIM 616417) predispose individuals to ADHD (Acosta et al., 2008, 2011, 2016) and predict ADHD severity and response to treatment (Arcos-Burgos et al., 2010; Acosta et al., 2011; Bruxel et al., 2015). Similar studies in other populations have also found that *Lphn3* gene variants contribute to ADHD susceptibility (Ribases et al., 2011; Hwang et al., 2015; Gomez-Sanchez et al., 2016; Martinez et al., 2016; Huang et al., 2019; Kappel et al., 2019; Puentes-Rozo et al., 2019).

Research in animal models has also provided some corroborating evidence for the role of *Lphn3* in ADHD. For example, zebrafish that lack *lphn3.1* (one of two *Lphn3* orthologs) were found to be hyperactive (Lange et al., 2012, 2018)–an effect that was attenuated by the ADHD medications methylphenidate and atomoxetine (Lange et al., 2012). The down-regulation of *lphn3.1* in zebrafish caused a misplacement of dopamine (DA) (but not norepinephrine or serotonin) neurons (Lange et al., 2012) and decreased locomotor sensitivity to DA agonists and antagonists (Lange et al., 2018). Likewise, *Lphn3*$^{-/-}$ knockout (KO) mice and rats are also hyperactive (Wallis et al., 2012; Regan et al., 2019), and they have been shown to be impaired on a facet of impulsivity called impulsive action (Mortimer et al., 2019; Sable et al., 2021) which is the inability to inhibit a prepotent motor response (Bari and Robbins, 2013; MacKillop et al., 2016). The exact mechanism whereby alterations in *Lphn3* gene expression alters catecholamine neurotransmission is still being investigated. However, in both *Lphn3* mutant mice and rats, the expression levels of the DA transporter (DAT) gene (*Slc6a3*) and the protein itself differ from wildtype controls. Adult *Lphn3* KO rats have increased DAT expression (Regan et al., 2019) and increased reuptake of DA (i.e., functional implication for increase in DAT) (Regan et al., 2020) in the dorsal striatum. Likewise, adult *Lphn3*$^{-/-}$ mice demonstrate overexpression of *Slc6a3* in whole brain (Wallis et al., 2012) and in the dorsal striatum (Pramod et al., 2013), but downregulation of *Slc6a3* in the prefrontal cortex (PFC) (Orsini et al., 2016; Mortimer et al., 2019). DAT expression is associated with ADHD (see Pramod et al., 2013 for review), including the site of action for many ADHD medications (Fone and Nutt, 2005; Gerlach et al., 2013; Faraone, 2018), so these results are particularly noteworthy.

While previous research has demonstrated *Lphn3* contributes to hyperactivity and deficits in impulsive action, this project examined the impact of *Lphn3* deletion in rats on a different facet of impulsivity. Using a delay-discounting (DD) task, we assessed impulsive choice, which is the inability to delay gratification (Reynolds et al., 2002). We also report "positive control" measures for the same behavioral assay in spontaneously hypertensive rats (SHRs) which have also been proposed as an animal model of ADHD (Prediger et al., 2005; Sagvolden et al., 2005b, 2009; Kantak et al., 2008; Meneses et al., 2011; Sagvolden and Johansen, 2012; Garcia and Kirkpatrick, 2013; Natsheh and Shiflett, 2018) and have previously exhibited DD deficits (Fox et al., 2008; Aparicio et al., 2019; Sjoberg et al., 2021). Compared to their control strain, Wistar-Kyoto (WKY) rats, SHRs also exhibit hyperactivity and inattention (Russell, 2011; Sagvolden and Johansen, 2012) as well as deficits in impulsive action (Sable et al., 2021; González-Barriga and Orduña, 2022). Here, we expected to observe more impulsive choice in the SHR and *Lphn3* KO rats represented by their tendency to choose the small, immediate reward more often that the larger, delayed reward relative to their control strains, WKY and *Lphn3* WT rats, respectively.

Experiment I method

Subjects

Subjects consisted of 24 SHR (12 male, 12 female) and 24 WKY rats (12 male, 12 female) along with 32 *Lphn3* KO rats (16 male, 16 female) and 33 *Lphn3* WT rats (16 male, 17 female). The SHRs and WKYs were shipped in a single cohort from Charles River (Kingston, NY, USA) at 45 ± 2 days old. The *Lphn3*$^{-/-}$ rats were generated at the Cincinnati children's transgenic animal and genome editing core by using CRISPR/Cas9 technology (Regan et al., 2019). Once genotypes were confirmed, the KO rats were shipped in three cohorts to the University of Memphis, along with their WT controls, at 40 ± 10 days old.

All rats were housed in same-sex groups of 2–3 per cage in standard plastic cages with corn cob bedding and *ad libitum* tap water in a room with a 12 h reverse light/dark cycle (lights off 7:00 am). Rats were on free feed (Teklad, 2018) until 60 days old, after which they were put on a food restriction schedule to maintain 85–90% of their free-feeding weight so that they would respond for food rewards during behavioral testing. Body weights at the start of operant testing are presented in **Supplementary Table 1**.

Apparatus

Behavioral testing was performed in 10 automated, rat operant chambers (Med Associates Inc., St. Albans, VT, USA) housed in sound attenuating wooden boxes equipped with a fan for ventilation. The test chambers measured 17.5 cm tall with a 24 cm × 20 cm stainless steel grid floor resting above a tray filled with corn cob bedding. Dustless grain-based precision pellets (45 mg; Bio-Serv, Flemington, NJ, USA) were dispensed into a food magazine centered 2.5 cm above the floor. A retractable response lever with a cue light above was located on both sides of the food magazine and a house light was located on the opposite wall. White noise was presented during testing to minimize disruption from outside sounds. Med-PC V software (Med Associates) was used to conduct the testing programs and record data.

Procedure

Autoshaping and fixed ratio training

All rats were first trained to lever press for food using an autoshaping program, which was followed by a fixed ratio training program. The former was used to establish the lever press response, and the latter alternated the response requirement every five trials to ensure that no rat exhibited a side preference for either lever. Additional details about the autoshaping and fixed ratio training programs have been previously published (Sable et al., 2021).

Delay-discounting

During each DD session, the rat was given the choice to press one lever for one pellet delivered immediately or the other lever for three pellets delivered after 0, 4, 8, 12, or 16 s. As such, one lever was always the immediate lever (0 s) and the delay on the other lever progressively increased every 10 trials using the order of delays presented above for a total of 50 trials/session. Trial lengths were such that if the rat pressed the lever leading to the smaller, but immediate, reward, the next trial did not begin until the delay period on the other lever had elapsed. This ensured that the overall session length was the same for all rats. Rats completed 25 sessions.

Design and analyses

The data from the SHR/WKY rats were analyzed separately from KO/WT data.

Percent choice larger reward

The percent choice for the larger, delayed reward for the 25 sessions was averaged across blocks of 5 days to yield five, 5-day testing blocks. To simplify the omnibus analyses, only data from the first testing block (i.e., acquisition phase) and the last testing block (i.e., maintenance phase) were included. The independent variables included in the omnibus analysis were strain (SHR vs. WKY) or genotype (KO vs. WT), sex (male vs. female), delay (0, 4, 8, 12, and 16 s), and phase (acquisition vs. maintenance). Thus, each analysis was a mixed 2 (strain or genotype) × 2 (sex) × 5 (delay) × 2 (phase) mixed ANOVA where strain/genotype and sex were between-subjects factors and delay and phase were repeated-measures factors.

Slope/area under curve

The slope of the discounting curve and area under the curve (AOC) during the acquisition and maintenance phases were analyzed separately using a mixed 2 (strain or genotype) × 2 (sex) × 2 (phase) ANOVA where strain/genotype and sex were between-subjects factors and phase was a repeated-measures factor. The slope was determined by calculating rise/run based on the shortest (0 s) and longest delay (16 s) of the discounting curve for each rat. The AOC was the composite area of the parametric space beneath the percent choice for the larger reward at each delay. Unlike slope, AOC uses all delays and therefore accounts for fluctuations choice behavior more effectively than slope (Myerson et al., 2001).

Experiment I results

If a rat did not demonstrate 60% choice for the larger reward at the 0 s delay during the final maintenance phase, it was determined that the rat had not learned to differentiate between the levers associated with the small versus large reward (i.e., the rat had not learned the task). Thus, these data were not included in the final analyses. Specifically, data from 4 SHRs (1 male, 3 female), 3 WKY rats (3 male, 0 female), 3 *Lphn3* KO rats (2 male, 1 female), and 6 *Lphn3* WT rats (4 male, 2 female) were not included leaving final *n*'s of 20 SHRs (11 male, 9 female), 21 WKYs (9 male, 12 female), 29 *Lphn3* KOs (14 male, 15 female), and 27 *Lphn3* WTs (12 male, 15 female).

If a sphericity violation was found for any within-subjects effect, a Greenhouse–Geisser correction was used to reduce the risk of a Type I error because $\varepsilon < 0.75$ in all cases (Maxwell and Delaney, 1999). In the interest of brevity, only significant genotype- and strain-related main effects and interactions are reported. There were no significant strain × sex or genotype × sex interactions for any of the dependent variables, so the results are presented collapsed across sex.

SHR/WKY

The analysis of the percent choice for the larger reward revealed a significant main effect of strain [$F(1, 37) = 4.269$, $p = 0.046$] and significant interactions of strain × delay [$F(1.671, 61.840) = 3.813$, $p = 0.034$] and strain × delay × phase [$F(2.459, 90.975) = 2.984$, $p = 0.045$]. As can be seen in **Figure 1**, during acquisition (top panel) the SHRs discounted the larger reward significantly more than the WKY rats during the 12 ($p = 0.031$) and 16 ($p = 0.012$) s delays. During maintenance, this effect was present during the 4 ($p = 0.014$), 8 ($p = 0.018$), 12 ($p = 0.030$), and 16 ($p = 0.044$) s delays. The greater discounting by the SHRs was also evident in analyses of slope and AOC, where a main effect of strain was found in both cases [$F(1, 37) = 5.250$, $p = 0.028$ and $F(1, 37) = 5.016$, $p = 0.031$, respectively]. As seen in **Figure 2**, the slope of the discounting curve was significantly more negative, and the AOC was significantly smaller for the SHRs versus the WKY rats.

Lphn3 KO/WT

The analysis of the percent choice for the larger reward did not reveal a significant main effect of genotype [$F(1, 52) = 0.310$, $p = 0.580$], nor significant genotype × delay [$F(2.484, 129.150) = 2.334$, $p = 0.089$] or genotype × delay × phase [$F(2.614, 135.938) = 0.288$, $p = 0.807$] interactions. For comparison, **Figure 3** shows the results for each genotype across the various delays for both acquisition (top) and maintenance (bottom). As shown in **Figure 4**, the main effect of genotype was also not significant for the analysis of slope (top) or AOC (bottom) [$F(1, 52) = 1.693$, $p = 0.199$ and $F(1, 52) = 0.199$, $p = 0.657$, respectively].

Experiment I discussion

We hypothesized that impulsive choice would be greater in the SHR and *Lphn3* KO rats relative to their control strains–WKY and WT rats, respectively. However, the results only partially supported this hypothesis. While the SHRs demonstrated a decrease in the percent choice for the larger reward as well as a more negative slope and decreased area under the curve compared to WKY rats, the *Lphn3* KO and WT rats did not differ on these measures. These results

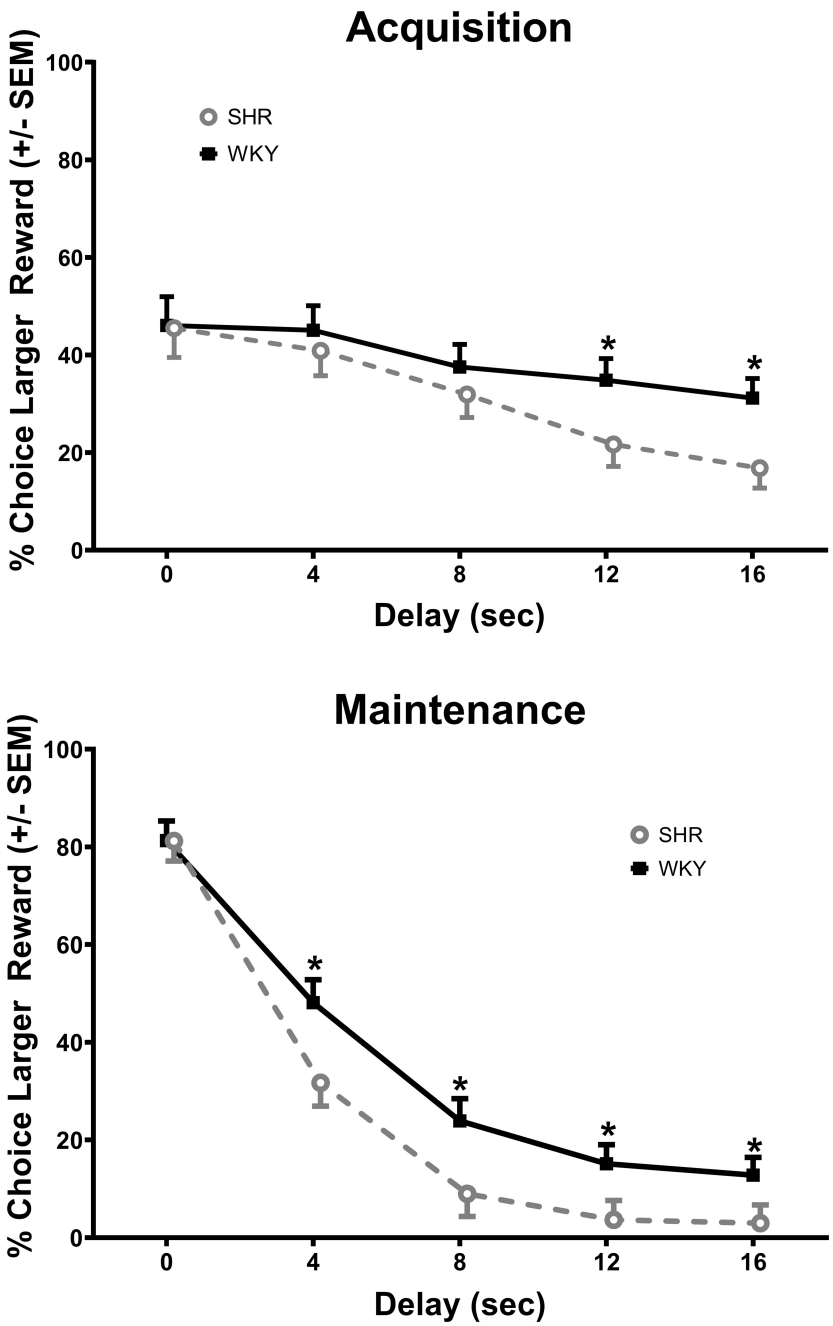

FIGURE 1
Percent choice for the larger reward during acquisition **(top)** and maintenance responding **(bottom)** for the spontaneously hypertensive rat (SHR) and WKY rats with equal trial lengths. The SHRs discounted the larger reward significantly more than the WKY rats at the longest two delays of acquisition and at all delays during maintenance, $*p < 0.05$.

were surprising as both SHRs and *Lphn3* KO rats have been shown to be impulsive when the task assessed impulsive action (Sable et al., 2021).

One possibility is that both the SHRs and *Lphn3* KO rats exhibit impulsive choice, but they present the impairment differently. As previously mentioned, DD deficits have been previously reported in SHRs (Fox et al., 2008; Sjoberg et al., 2021). These results and ours indicate that the SHRs have a substantial problem with delay of gratification. When the delay between response and reward was too long, the greater magnitude of the delayed reinforcer was not enough to entice them to choose that lever. Rather, the delivery of the reinforcer needed to occur soon after lever selection, so they choose the smaller, but immediate reward. As mentioned by Sjoberg et al. DD performance by SHRs strongly supports Dynamic Developmental Behavioral Theory, which argues the salience of a reinforcer decreases as it is separated in time from the response made to achieve it (Sagvolden et al., 2005a; Sjoberg et al., 2021).

Notably, in the version of the task conducted above, a subsequent trial did not begin until the end of the delay period, regardless of which lever was selected. In other words, because rats were required to "wait" for the next trial to start even if they picked the immediate lever, it is possible the *Lphn3* KO rats may have opted to pick the

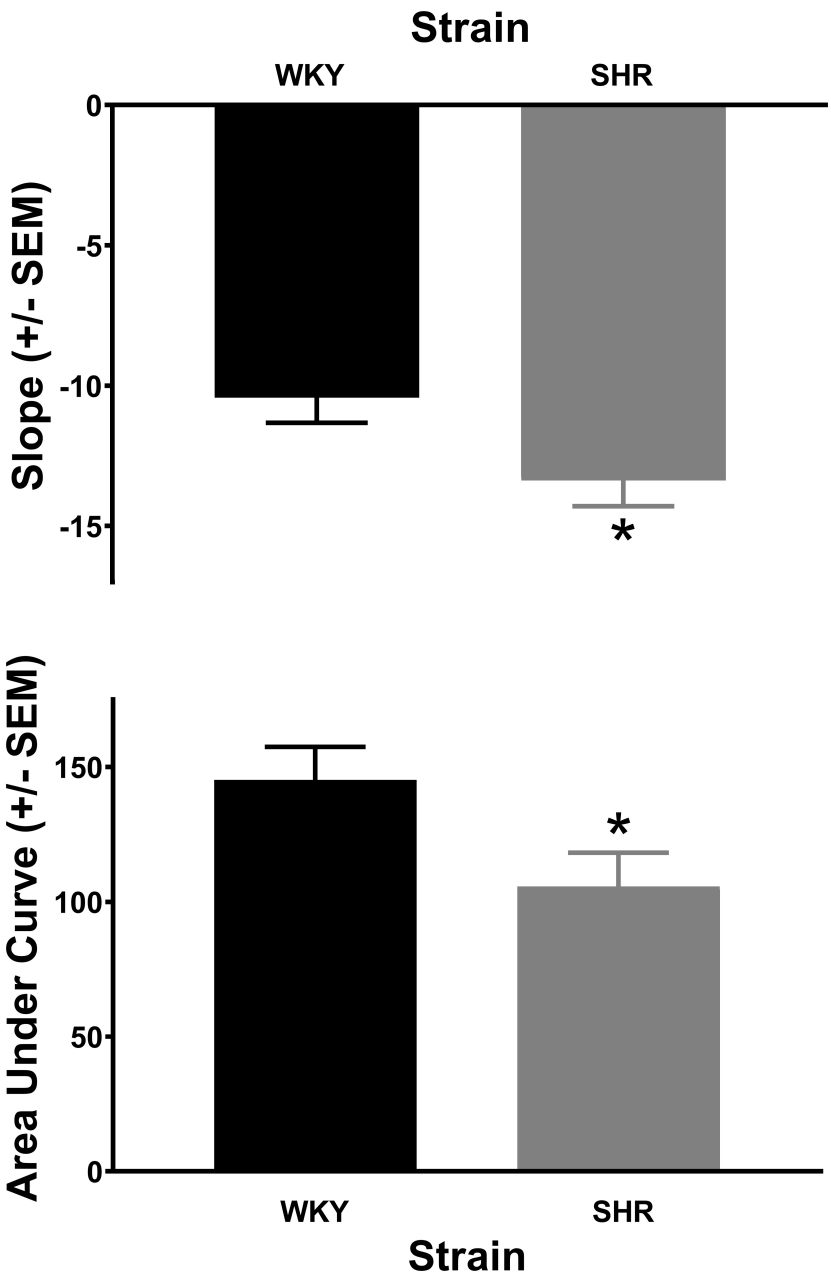

FIGURE 2
The slope of the discounting curve **(top)** was significantly more negative for the spontaneously hypertensive rat (SHR) versus WKY rats, while the area under the discounting curve **(bottom)** was significantly less for the SHR versus WKY rats, $*p < 0.05$. The trial lengths were the same regardless of which lever was selected.

larger magnitude reward. We decided to investigate this possibility in Experiment II.

Experiment II

Research that has examined whether consistency in trial length affects choice of the lever associated with the larger delayed reward has provided mixed results. For SHRs, the length of the inter-trial interval (ITI) does not appear to influence choice behavior within trials (Sjoberg et al., 2021). However, among ADHD children, when a subsequent trial begins as soon as the reinforcer from the previous trial is delivered/retrieved, this increase in relative response rate has been shown to shift an even greater percentage of responding to the immediate lever, thereby minimizing the impact of reward magnitude (Marco et al., 2009). This finding has also been shown to occur in research animals, especially when the post-reward delay was cued (Pearson et al., 2010). In Experiment II, we incorporated a 1-s ITI immediately after delivery of either the immediate (1 pellet) or delayed (3 pellet) reinforcer. Because previous research has already shown the length of the ITI does not appear to influence choice behavior in SHRs (Sjoberg et al., 2021), we only tested *Lphn3* KO and WT rats in Experiment II. We predicted that the *Lphn3* KO rats would choose the small, immediate reward more often that the larger, delayed reward relative to the WT rats, thereby demonstrating an increase

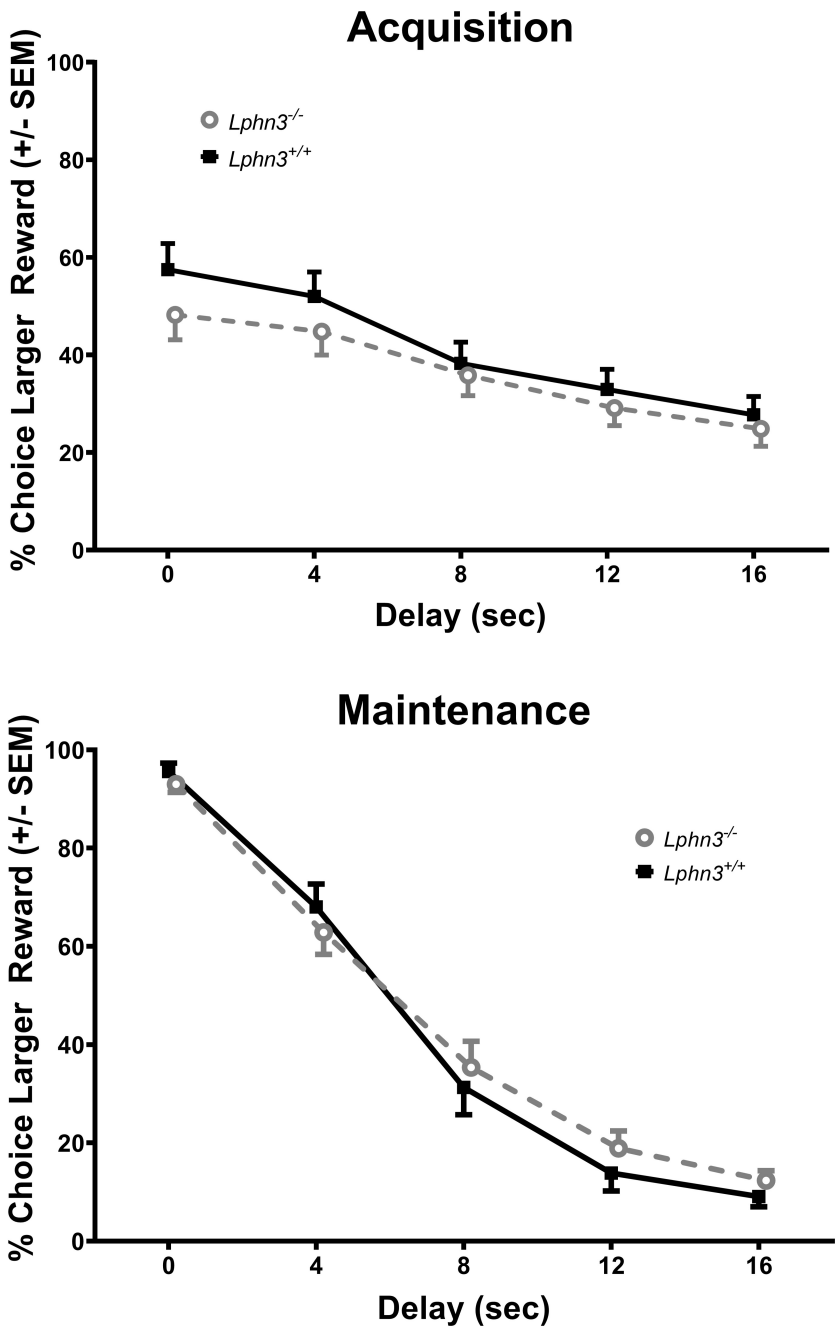

FIGURE 3
Percent choice for the larger reward during acquisition **(top)** and maintenance responding **(bottom)** for the *Lphn3*$^{-/-}$ (knockout) and *Lphn3*$^{+/+}$ (wildtype) rats with equal trial lengths. Genotype had no effect of on delay-discounting (DD) performance.

in impulsive choice associated with an increase in the rate of reinforcement.

Experiment II method

Subjects

The subjects consisted of an additional 22 *Lphn3* KO rats (11 male, 11 female) and 25 *Lphn3* WT rats (12 male, 13 female) from the Cincinnati children's transgenic animal and genome editing core that were generated using CRISPR/Cas9 technology as in Experiment I. They were shipped in three cohorts and housing feeding were identical to that employed in Experiment I.

Apparatus

Behavioral testing was performed in the same automated, rat operant chambers (Med Associates Inc., St. Albans, VT, USA) used in Experiment I. Likewise, white noise was again presented during testing to minimize disruption from outside sounds and Med-PC V software was used to conduct the testing programs and record data.

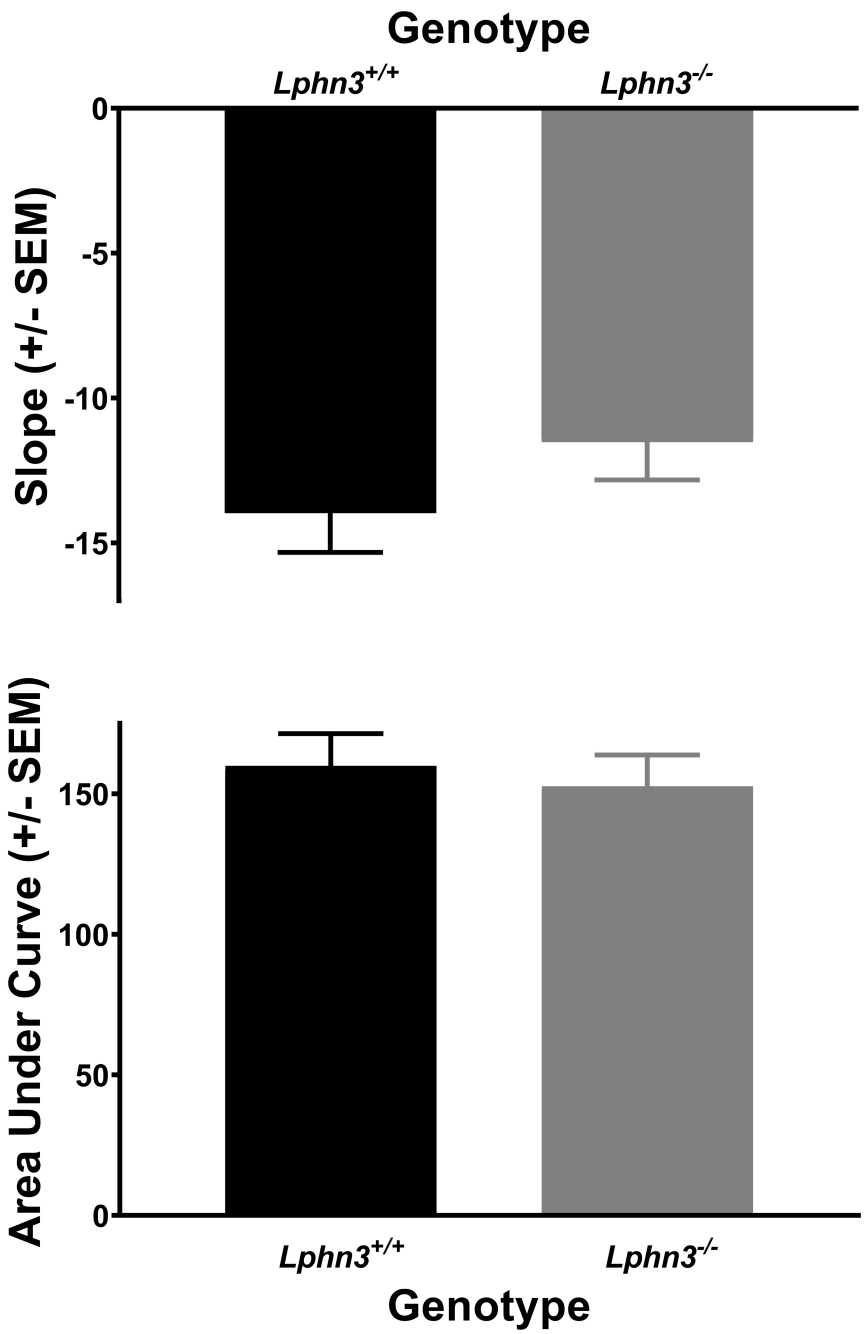

FIGURE 4
The slope of the discounting curve **(top)** and area under the discounting curve **(bottom)** did not differ between the *Lphn3*$^{-/-}$ (knockout) and *Lphn3*$^{+/+}$ (wildtype) rats with equal trial lengths.

Procedure

Autoshaping and fixed ratio training

The autoshaping and fixed ratio training programs were identical to those used in Experiment I.

Delay-discounting

The DD task used in Experiment II was the same as that used during Experiment I (delays = 0, 4, 8, 12, or 16 s; 10 trials/delay, 50 trials/session), with the exception that a 1 s ITI occurred after delivery of the food reinforcer but before the next trial began regardless of which lever was pressed. Thus, a tendency to respond on the immediate lever resulted in a shorter session duration. Rats completed 25 sessions.

Design and analyses

As in Experiment I, the percent choice for the larger, delayed reward for the 25 sessions was averaged across blocks of 5 days to yield five, 5-day testing blocks but only data from the first (i.e., acquisition phase) and last testing block (i.e., maintenance phase) were included. The analysis of the percent choice for the larger

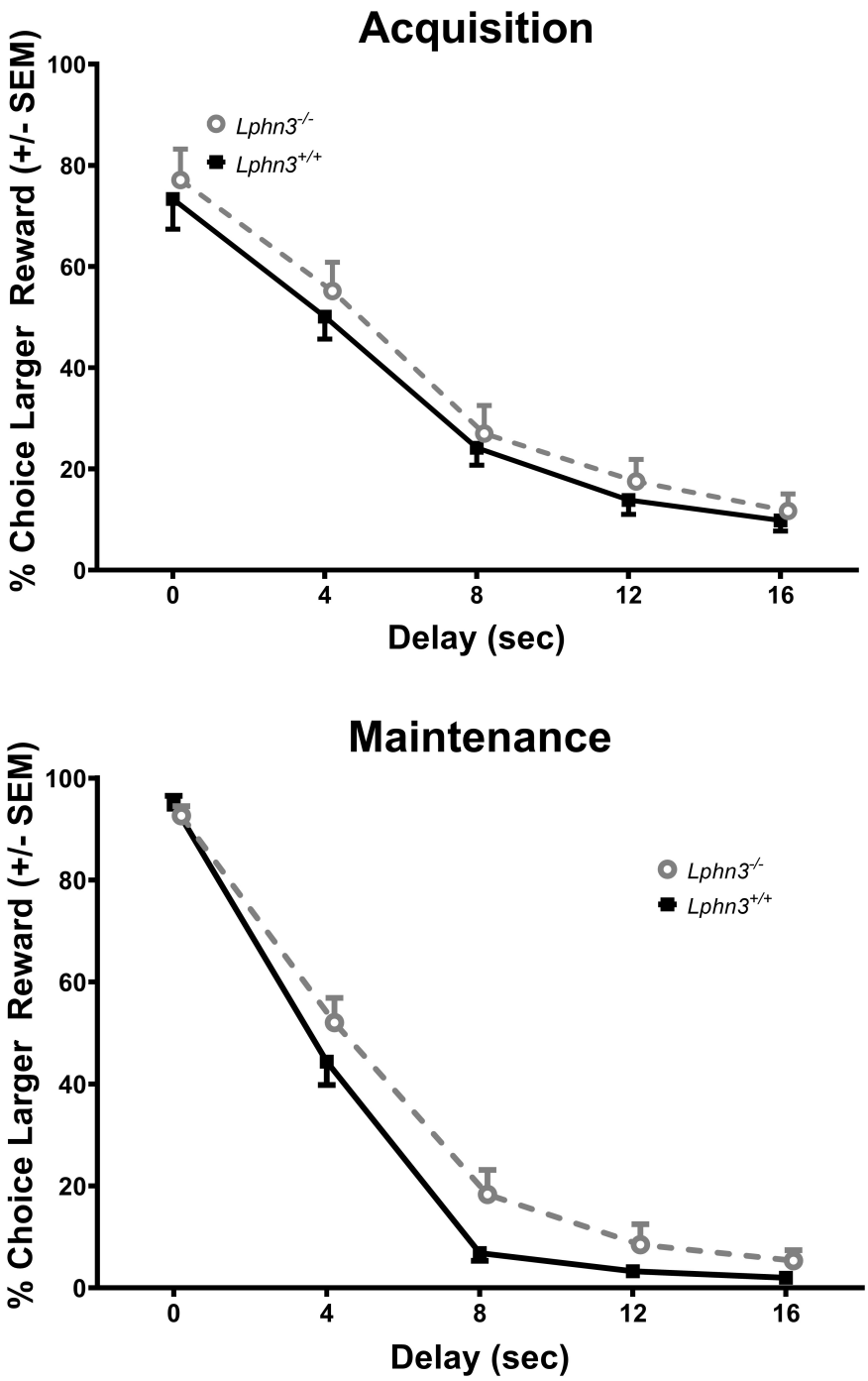

FIGURE 5
Percent choice for the larger reward during acquisition **(top)** and maintenance responding **(bottom)** for the *Lphn3*$^{-/-}$ (knockout) and *Lphn3*$^{+/+}$ (wildtype) rats with unequal trial lengths. Genotype had no effect of on delay-discounting (DD) performance.

reward was a 2 (genotype) × 2 (sex) × 2 (phase) × 5 (delay) mixed ANOVA, while slope and AOC were calculated as was done in Experiment I and analyzed separately *via* 2 (genotype) × 2 (sex) × 2 (phase) mixed ANOVAs.

Experiment II results

The inclusion criterion was the same as for Experiment 1. Data from 2 male *Lphn3* KO rats and 3 *Lphn3* WT rats (2 male, 1 female) were not included as they did not demonstrate 60% choice for the larger reward at the 0 s delay during the final maintenance phase. Thus, 20 *Lphn3* KOs (9 male, 11 female) and 22 *Lphn3* WTs (10 male, 12 female) were included in the final analyses. Greenhouse–Geisser corrections were again used for sphericity violations. Analysis of the percent choice revealed that discounting was evident, as the percent choice for the larger reward decreased overall with increasing delay [$F(2.034, 77.301) = 337.275$, $p < 0.001$]. However, analysis of the percent choice for the larger reward did not reveal a significant main effect of genotype [$F(1, 38) = 1.462$, $p = 0.234$] or sex [$F(1,$

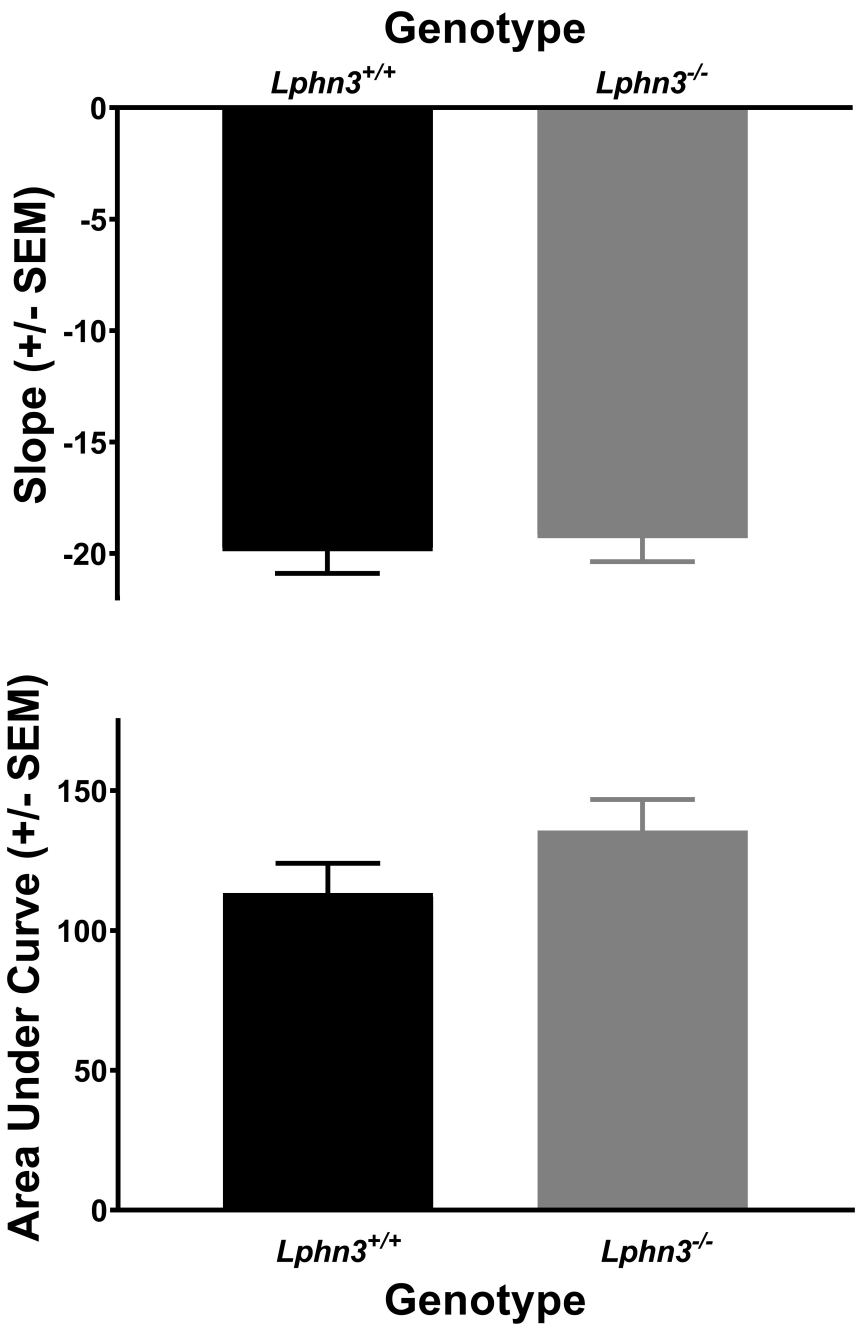

FIGURE 6
The slope of the discounting curve **(top)** and area under the discounting curve **(bottom)** did not differ between the *Lphn3*$^{-/-}$ (knockout) and *Lphn3*$^{+/+}$ (wildtype) rats when trial lengths were not equal.

38) = 0.007, $p = 0.934$], nor any significant genotype- or sex-related interactions (see Figure 5). The analyses of the slope and area under the discounting curve also did not reveal significant main effects of genotype [$F(1, 38) = 0.162$, $p = 0.690$ and $F(1, 38) = 2.070$, $p = 0.158$, respectively], nor any other significant genotype- or sex-related differences (see Figure 6).

Experiment II discussion

The results of Experiment II indicated that removing the post-reward buffer following selection of the lever associated with a smaller, but immediate reward did not differentially affect DD behavior for either genotype. Thus, the rate of reinforcement did not appear to affect impulsive choice in the KO rats. These results are in line with previous research demonstrating that the length of the ITI had little influence on impulsive choice in SHRs (Sjoberg et al., 2021).

Overall conclusion

Overall, SHRs had increased impulsive choice due to their inability to delay gratification after a response to obtain a reinforcer

had been elicited. *Lphn3* KO rats, on the other hand, did not appear to exhibit impulsive choice, even when the option to increase the rate of reinforcer delivery was available. Thus, while both ADHD models exhibit hyperactivity (Russell, 2011; Sagvolden and Johansen, 2012; Regan et al., 2019) and impulsive action deficits (Sable et al., 2021; González-Barriga and Orduña, 2022), impulsive choice appeared to be differentially affected between the models. Notably, our previous research found that while both models exhibited a deficit in impulsive action, the degree of impairment was much more profound in the SHRs than in the *Lphn3* KO rats (Sable et al., 2021). Thus, the overall degree of impulsivity appears to be much more substantial in the SHRs.

Dopamine regulation within the PFC is critically involved in impulsive behavior (Logue and Gould, 2014), and medications targeting the dopamine system are routinely prescribed to ADHD patients in an attempt to reduce impulsive behavior (Arnsten and Pliszka, 2011; Sharma and Couture, 2014). However, as previously mentioned impulsivity is a multi-faceted construct and behavioral deficits on tasks of impulsive choice do not always coincide with deficits on tasks of impulsive action (or vice-versa) in rats or in humans (Solanto et al., 2001; Broos et al., 2012; van den Bos et al., 2014).

Notably, there appears to be some degree of regional specificity that mediates impulsive action versus impulsive choice. While this is not yet entirely understood, in human subjects, gray matter volume in the right frontal pole (RFP) and left middle frontal gyrus (LMFG) were predictive of DD performance, while gray matter volume in the right inferior frontal gyrus (RIFG), supplementary motor area (SMA), and anterior cingulate cortex (ACC) predicted performance on an impulsive action task (Wang et al., 2016). Preclinical research also suggests the RIFG mediates impulsive action along with the ventrolateral prefrontal cortex (VLPFC), while the dorsal lateral prefrontal cortex (DLPFC) and is heavily involved in mediating impulsive choice (see Kim and Lee, 2011; Bari and Robbins, 2013 for reviews).

Our observed differences among the ADHD animal models has the potential to promote a better understanding of the underlying mechanism(s) responsible for the differential behavioral effects observed. The discrepant results between the SHRs and *Lphn3* KO rats presented above suggest more widespread disruption of frontal cortical regions involved in both impulsive action and impulsive choice in SHRs, with disruption limited only to those regions involved in impulsive action in the *Lphn3* KO rats. Ongoing research in our lab is currently investigating this possibility. These findings will be very important as they will have the potential to inform medication development, leading to a more targeted approaches to curb the facets of impulsivity that an ADHD individual may present (i.e., impulsive actions and/or impulsive choice), while sparing those that are not affected. This would undoubtedly reduce side effects and increase compliance.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

This animal study was reviewed and approved by the University of Memphis, Institutional Animal Care and Use Committee–protocol #0875.

Author contributions

MC: data collection, analysis, and manuscript preparation. AB, OH, and HN: experimental design, data collection, and manuscript preparation. SR: experimental design, data collection, and analysis. MW, CV, and HS: research idea, experimental design, data collection, analysis, and manuscript preparation. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnins.2023.1094218/full#supplementary-material

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Effects of neonatal fentanyl on late adolescent opioid-mediated behavior

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Introduction: Because of the steady increase in the use of synthetic opioids in women of childbearing age, a large number of children are at risk of exposure to these drugs prenatally or postnatally through breast milk. While there is older literature looking at the effects of morphine and heroin, there are relatively few studies looking at the long-term effects of high-potency synthetic opioid compounds like fentanyl. Thus, in the present study, we assessed whether brief exposure to fentanyl in male and female rat pups during a period roughly equivalent to the third trimester of CNS development altered adolescent oral fentanyl self-administration and opioid-mediated thermal antinociception.

Methods: We treated the rats with fentanyl (0, 10, or 100 μg/kg sc) from postnatal day (PD) 4 to PD 9. The fentanyl was administered daily in two injections given 6 h apart. After the last injection on PD 9, the rat pups were left alone until either PD 40 where they began fentanyl self-administration training or PD 60 where they were tested for morphine- (0, 1.25, 2.5, 5, or 10 mg/kg) or U50,488- (0, 2.5, 5, 10, or 20 mg/kg) induced thermal antinociception.

Results: In the self-administration study, we found that female rats had more active nose pokes than male rats when receiving a fentanyl reward but not sucrose alone solution. Early neonatal fentanyl exposure did not significantly alter fentanyl intake or nose-poke response. In contrast, early fentanyl exposure did alter thermal antinociception in both male and female rats. Specifically, fentanyl (10 μg/kg) pre-treatment increased baseline paw-lick latencies, and the higher dose of fentanyl (100 μg/kg) reduced morphine-induced paw-lick latencies. Fentanyl pre-treatment did not alter U50,488-mediated thermal antinociception.

Conclusions: Although our exposure model is not reflective of typical human fentanyl use during pregnancy, our study does illustrate that even brief exposure to fentanyl during early development can have long-lasting effects on mu-opioid-mediated behavior. Moreover, our data suggest that females may be more susceptible to fentanyl abuse than males.

KEYWORDS

fentanyl, self-administration, antinociception, opioid, ontogeny

Introduction

Opioid use has reached epidemic levels in the United States. Over a 20-year period (1999–2011) in the United States, the annual number of opioid painkiller prescriptions rose precipitously, and in 2009, drug overdose deaths exceeded those from car accidents (Gardner et al., 2022). There has been a 200% increase in opioid overdose (poisoning) deaths

since 2000, and 61% of all drug overdose deaths now involve opioids (Rudd et al., 2016). Drug overdose rates increased the most for persons aged 25–34 years, but there has been a sharp increase in the number of opioid overdoses in adolescents and young adults (15–24 years) in recent years. The synthetic opioid fentanyl has been particularly problematic with an 88% increase in fentanyl and fentanyl analog overdose deaths each year from 2013 to 2016 (Spencer et al., 2019; Han et al., 2022; Skolnick, 2022).

An unfortunate consequence of this crisis has been the increase in opioid use in women of childbearing age (Krans and Patrick, 2016; Hurley et al., 2020). Between 2008 and 2012, insurance records showed that roughly 30% of women in the childbearing age group filled a prescription for an opioid and the majority of people seeking treatment for opioid addiction are women (Yazdy et al., 2015; Krans and Patrick, 2016). Moreover, a review of over one million Medicaid enrollees showed that 21.6% of pregnant women had an opioid prescription filled, and 2.5% received prescriptions for greater than 30 days for chronic pain (Yazdy et al., 2015; Krans and Patrick, 2016).

Clinical studies have demonstrated that perinatal exposure to opioids can lead to long-lasting consequences such as cognitive deficits and sensorimotor impairments (Mactier and Hamilton, 2020). Recent imaging studies have also reported low-term changes in neural functioning after opioid exposure (Radhakrishnan et al., 2022; Vishnubhotla et al., 2022). These studies, however, were primarily focused on heroin and methadone and may not represent the effects of high-potency synthetic opioids like fentanyl. The available preclinical investigations of prenatal and postnatal exposure to fentanyl, however, suggest even brief can have seemingly permanent effects on mu-opioid functioning, affective behavior, and sensorimotor systems (Thornton and Smith, 1998; Medeiros et al., 2011; Alipio et al., 2021a,b; Rêgo et al., 2022).

The goal of the current investigation was to extend our understanding of fentanyl exposure during pregnancy and early development by assessing the effect of fentanyl administration from postnatal day (PD) 4 to PD 9 in late adolescent and young adult rats. Specifically, we examined morphine and U50, 488 thermal antinociception and oral fentanyl self-administration in both male and female rats. The fentanyl exposure time frame was chosen as it is roughly analogous to the last trimester of human pregnancy in terms of brain development (Schmitt and Barrow, 2022). While the third trimester only is not the most common exposure period for human women, it does represent a significant percentage of women who are prescribed opioids for pain during pregnancy (Cook, 2022).

Materials and methods

Animals

Male and female rats (N = 488) of Sprague–Dawley descent, born and raised at CSUSB, were used in both experiments. The day of parturition was considered PD 0, and litters were culled to a maximum of 10 rat pups at 3 days of age. Pups were kept with the dam until PD 23, at which time they were weaned and placed in group cages with same-sex litter mates. Only one rat from each litter was placed into a particular group. The colony room was maintained at 22–24°C and kept under a 12-h light/dark cycle. This study was approved by the Institutional Animal Care and Use Committee at California State University, San Bernardino. All studies were carried out in accordance with the "Guide for the Care and Use of Mammals in Neuroscience and Behavioral Research" (National Research Council, 2010).

Drugs

Fentanyl citrate salt, morphine sulfate salt, and (±)-trans-U50-488 methanesulfonate salt were purchased from Sigma-Aldrich. Fentanyl used for the neonatal pre-exposure was mixed in saline, given at a volume of 2.5 ml/kg, and injected subcutaneously (sc). Oral fentanyl was dissolved in distilled water or a sucrose solution. Morphine and U50-488 were mixed in saline and injected subcutaneously (sc) at a volume of 1 ml/kg. Drug doses were expressed in the forms listed above.

In vivo drug treatment and group assignment

On postnatal day (PD) 2, rats were sexed and assigned to fentanyl pre-treatment groups. In all cases, an equal number of male and female rat pups were allocated to each pre-treatment group. Drug assignments were coded, so experimenters were unaware of the drug dose administered. Starting at PD 4, male and female rats were weighed and injected with fentanyl (0, 10, or 100 μg/kg, sc). Fentanyl was given in two injections 6 h apart for 6 consecutive days.

Hot plate test

On PD 60, rats were habituated to the hot plate apparatus (Model 38D, Hot plate analgesia meter, IITC Inc., Woodland Hills, CA, USA). Habituation consisted of placing the rat on the unheated hot plate for 2 min. On the next day, rats were placed on the heated hot plate (54.0°C, ± 0.1°C), and latency to lick a hind paw or attempt to jump out of the chamber was measured. This procedure was repeated three times, with a 20-min interval between each trial. After these baseline trials, rats were injected with morphine (0, 2.5, 5, or 10 mg/kg, sc) or U50, 488 (0, 5, 10, and 20 mg/kg, sc) and returned to their home cage for 20 min. Rats were then tested three additional times with a 20-min interval between each trial. If no response was made, rats were removed from the hotplate for 30 s to avoid tissue damage.

Oral sucrose and fentanyl administration

Nose-poke training

Starting on PD 40, rats were pre-exposed to a 2% sucrose solution for 2 h in their home cage. Rats were then water deprived for 16 h. On the following day (i.e., PD 38), rats were placed in a self-administration chamber. Rats were allowed to nose poke for access to a 1% sucrose (w/v) solution on an FR1 schedule for 60 min each day until a criterion of >10 reinforces for 2 consecutive days was met. Nose-poke responses in the active hole resulted in the simultaneous presentation of a stimulus light and a sound cue (500 Hz, 10 dB above background) followed by a 30 s presentation of a liquid dropper that delivered 0.1 ml of the sucrose solution for 30 s. On nose-poke training days, water availability was restricted to 2 h day to accelerate

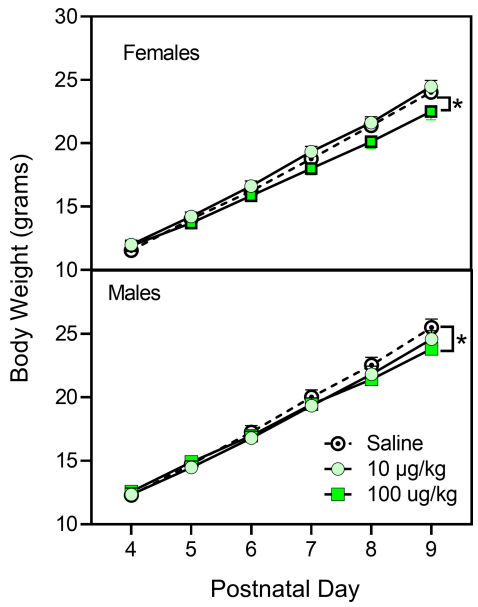

FIGURE 1
Mean (±SEM) body weight of male and female rats (n = 8–9/sex) treated with saline or fentanyl (10 or 100 µg/kg) from PD 4 to PD 9. *Significant difference between vehicle- and 100 µg/kg fentanyl-treated rats of the same sex.

the acquisition of operant responding. Rats returned to an ad-lib water schedule once the nose-poke criterion was met. Rats that fail to meet the training criteria were excluded from the study.

Self-administration

Once the criterion was met, fentanyl fade in and sucrose fade out began. Each nose-poke response in the active hole resulted in the simultaneous presentation of a stimulus light and a sound cue (500 Hz, 10 dB above background) and a 30 s presentation of a liquid dropper that delivered 0.1 ml of liquid solution. After each liquid dropper presentation, the active nose-poke hole became inactive for 20 s, which was indicated by the absence of the house light. Sessions 1–3 presented liquid solutions on a fixed ratio one (FR1) schedule where access to the liquid dropper occurred after every nose poke and sessions 4–5 presented liquid solutions on an FR2 schedule (i.e., two nose pokes were required before the presentation of the liquid dropper). Session 1 served as a baseline, where 1% sucrose solution was presented alone. In session 2, fentanyl (1 mg/L) was introduced into the sucrose solution. This dose of fentanyl is lower than that has been used in past studies (Shaham et al., 1993; Thornton et al., 2000) but was chosen to determine whether our early fentanyl exposure would increase the reinforcing value of fentanyl in adolescence. In session 3, sucrose fade out began with 0.5% sucrose presented in the liquid solution. In session 4, 0.25% sucrose was presented in the liquid solution. In session 5, no sucrose was present in the liquid solution. Sessions 1–4 were repeated until the criterion of >10 reinforces for 2 consecutive days is met. Session 5 was repeated for 7 days.

Statistical analysis

Body weight during the pre-treatment period was analyzed by 2 × 3 × 6 (sex × pre-treatment condition × pre-treatment day) repeated measures ANOVA. Adult weight was analyzed by 2 × 3 (sex × pre-treatment condition) ANOVA. The three baseline paw-lick trials were averaged and analyzed by 2 × 3 (sex × pre-treatment condition) ANOVAs. Data from the postdrug paw-lick assessment were also averaged over the three test trials but were analyzed by Kruskal–Wallis and Mann–Whitney tests because these data did not meet the normality of distribution or homogeneity of variance assumptions. The total nose pokes and days to criterion were analyzed for sucrose training using 2 × 3 (sex × pre-treatment condition) ANOVAs. Total nose pokes and days to criterion for acquisition training phases 1–4 were analyzed using 2 × 3 × 4 (sex × pre-treatment condition × training phase) repeated measures ANOVAs. Total nose pokes for the seven-phase 5 testing days (fentanyl-only sessions) were analyzed using 2 × 3 × 7 (sex × pre-treatment condition × day) repeated measures ANOVAs. Significant higher-order interactions were analyzed using lower-order ANOVAs. *Post hoc* analysis of data was made using Tukey's tests ($p < 0.05$). Effect sizes were reported as partial eta squared (η_p^2) and categorized based on the following scale: $\eta_p^2 \leq 0.03$ (small effect), $\eta_p^2 > 0.03$ and ≤ 0.10 (medium effect), and $\eta_p^2 > 0.10$ (large effect) (Labots et al., 2016).

Results

Body weight

During the injection period (i.e., PD 4–9), male rat pups were slightly larger than female rat pups (**Figure 1**) [sex main effect, $F_{(1,407)} = 8.828$, $p = 0.003$, $\eta_p^2 = 0.021$]. Weight increased progressively for all pups on the injection days, and pre-treatment with fentanyl (100 µg/kg) decreased weight in comparison with saline controls for both males and females on the last pre-treatment day (PD 9) [day main effect, $F_{(2,1713)} = 10{,}486.243$, $p < 0.001$, $\eta_p^2 = 0.963$; day × pre-treatment condition interaction, $F_{(4,1713)} = 6.99$, $p < 0.001$, $\eta_p^2 = 0.064$]. Weight for males and females did not differ from controls at the time of testing (data not shown).

Morphine- and U50-488-induced thermal antinociception

Early neonatal treatment with fentanyl (10 µg/kg) altered the baseline responses on the hotplate for both male and female rats on PD 60 (**Figure 2**) [fentanyl pre-treatment main effect, $F_{(2,256)} = 3.388$, $p < 0.035$; $\eta_p^2 = 0.026$; Tukey's test, $p < 0.05$]. As expected, treatment with morphine (2.5, 5.0, and 10 mg/kg) increased paw-lick latencies regardless of the pre-treatment group (**Figure 3**) [morphine post-treatment, $H(4) = 96.156$, $p < 0.001$; $\eta_p^2 = 0.368$; pairwise comparison with Bonferroni correction, $p < 0.05$]. The antinociceptive effects of morphine were altered by the early fentanyl treatment (**Figure 3**). Specifically, rats pre-treated with fentanyl (10 µg/kg) and tested with 5 mg/kg of morphine had greater paw-lick latencies than saline-treated rats, [5 mg/kg morphine × fentanyl groups, $H(2) = 8.583$, $p < 0.014$, $\eta_p^2 = 0.170$, Mann–Whitney U, $p = 0.033$]. In addition, rats treated with 100 µg fentanyl and tested with 10 mg/kg morphine had shorter paw-lick latencies than saline controls [10 mg/kg morphine × fentanyl groups, $H(2) = 6.372$, $p < 0.041$, $\eta_p^2 = 0.132$; Mann–Whitney U, $p = 0.0235$]. Sex did

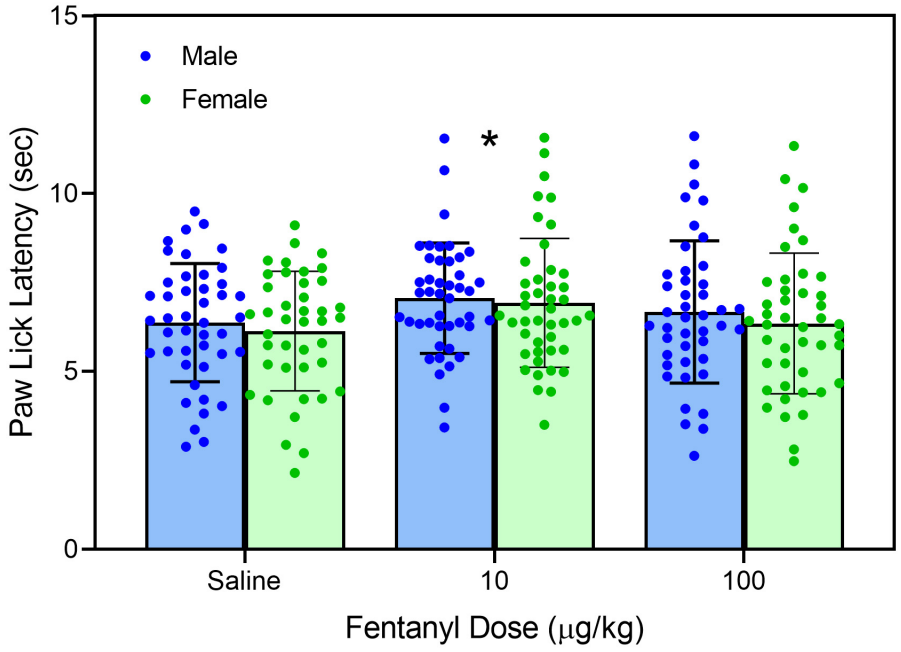

FIGURE 2
Mean (+SE) baseline paw-lick latencies of PD 60 male and female rats (n = 8–9/sex). Rats were pre-treated with fentanyl (10 or 100 μg/kg) or saline from PD 4 to PD 9. *Significantly different from rats in the saline-pre-treatment group.

not alter morphine-induced thermal antinociception. U50-488 (5, 10, and 20 mg/kg) also increased paw-lick latencies (Figure 4) [U50, 488 post-treatment, $H(3) = 48.371$, $p < 0.001$; $\eta_p^2 = 0.281$; pairwise comparison with Bonferroni correction, $p < 0.05$], but the effect of the kappa agonist was not altered by fentanyl pre-treatment (Figure 4).

Oral-fentanyl self-administration

Sucrose training

Active nose pokes, inactive nose pokes, sucrose solution consumed, or days to criterion were not altered by early fentanyl treatment. Only three rats failed to reach our criterion on the sucrose training procedure. Male rats, however, did have greater numbers of active and inactive lever presses [sex main effect $F_{(1,54)} = 5.779$, $p = 0.02$, $\eta_p^2 = 0.097$; $F_{(1,54)} = 4.343$, $p = 0.042$, $\eta_p^2 = 0.074$, respectively].

Acquisition of oral-fentanyl self-administration—Training phase

Pre-treatment with fentanyl did not alter active nose pokes, inactive nose pokes, or days to criterion (data not shown). However, female rats had a slightly greater number of active nose pokes during the stimulus presentation as compared to male rats (Figure 5) [sex main effects $F_{(1,39)} = 5.072$, $p = 0.030$, $\eta_p^2 = 0.079$]. Days to criterion were not affected by sex. In total, 11 rats (seven male rats and four female rats) failed to complete the training protocol.

Acquisition of oral-fentanyl self-administration—Test phase

Similar to the training phase, fentanyl pre-treatment did not alter active or inactive nose pokes during the 7-day test days (see Figure 6). Female rats, however, did have a greater number of active nose pokes, active nose pokes during the stimulus presentation, and inactive nose pokes [sex main effect $F_{(1,40)} = 4.295$, $p = 045$, $\eta_p^2 = 0.202$; $F_{(1,40)} = 6.253$, $p = 0.017$, $\eta_p^2 = 0.266$; $F_{(1,40)} = 5.746$, $p = 0.021$, $\eta_p^2 = 0.226$, respectively].

Discussion

In the present study, brief neonatal fentanyl exposure had long-term effects on opioid-mediated behavior in adolescent and young adult rats. Specifically, we found that fentanyl administered from PD 4 to PD 9 caused long-term changes in morphine-induced thermal antinociception of young adult rats. While we failed to find other studies that used the same injection period as in our study, the change in morphine thermal antinociception we reported is consistent with another study that did fentanyl infusions from PD 14 to PD 17 (Thornton and Smith, 1998). Moreover, these results are in agreement with prenatal opioid exposure investigations showing both an increase and decrease in response to morphine in adult offspring depending on injection interval (O'Callaghan and Holtzman, 1976, 1977; Kirby et al., 1982). Curiously, early fentanyl exposure did not alter nose-poke response to oral fentanyl in late adolescence.

The changes we found in morphine thermal antinociception were dose-dependent because exposure to a low dose (10 μg/kg) resulted in an augmented response to 5 mg/kg of morphine while pre-treatment with (100 μg/kg) caused an attenuated response to 10 mg/kg morphine. Decreased morphine sensitivity has been previously reported after early postnatal fentanyl exposure (Thornton and Smith, 1998) and is often found after prenatal or early postnatal exposure to other mu-opioids (O'Callaghan and Holtzman, 1976; Timár et al., 2010). In these studies, the decrease in morphine

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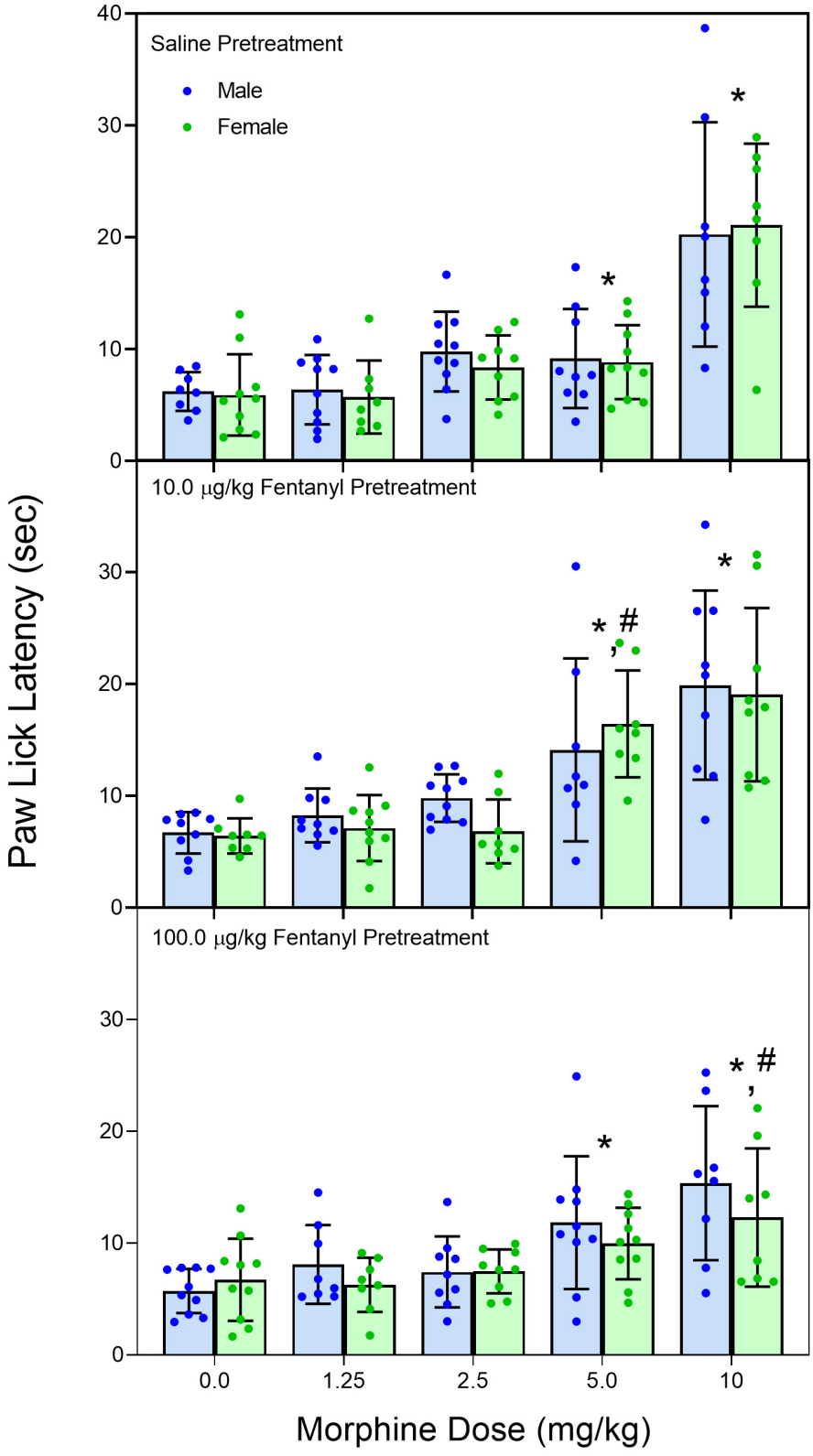

FIGURE 3
Mean (+SE) paw-lick latencies of PD 60 male and female rats (n = 8–9/sex). Rats were pre-treated with fentanyl (10 or 100 μg/kg) or saline from PD 4 to PD 9 and injected with morphine (0, 1.25, 2.5, 5.0, or 10 mg/kg, sc) on PD 60. *Significantly different from 0 mg/kg morphine. #Significantly different from saline-pre-treated rats in the same morphine drug condition.

sensitivity is seen after an early opioid exposure protocol that induces opioid dependence (Thornton and Smith, 1998); while the current study did not directly assess fentanyl dependence, our drug protocol (two 50 μg/kg injections; 6 h apart for 6 days) is similar to other protocols that have induced tolerance in young rats (Laferrière et al., 2005). The decrease in responsivity to morphine is likely due to

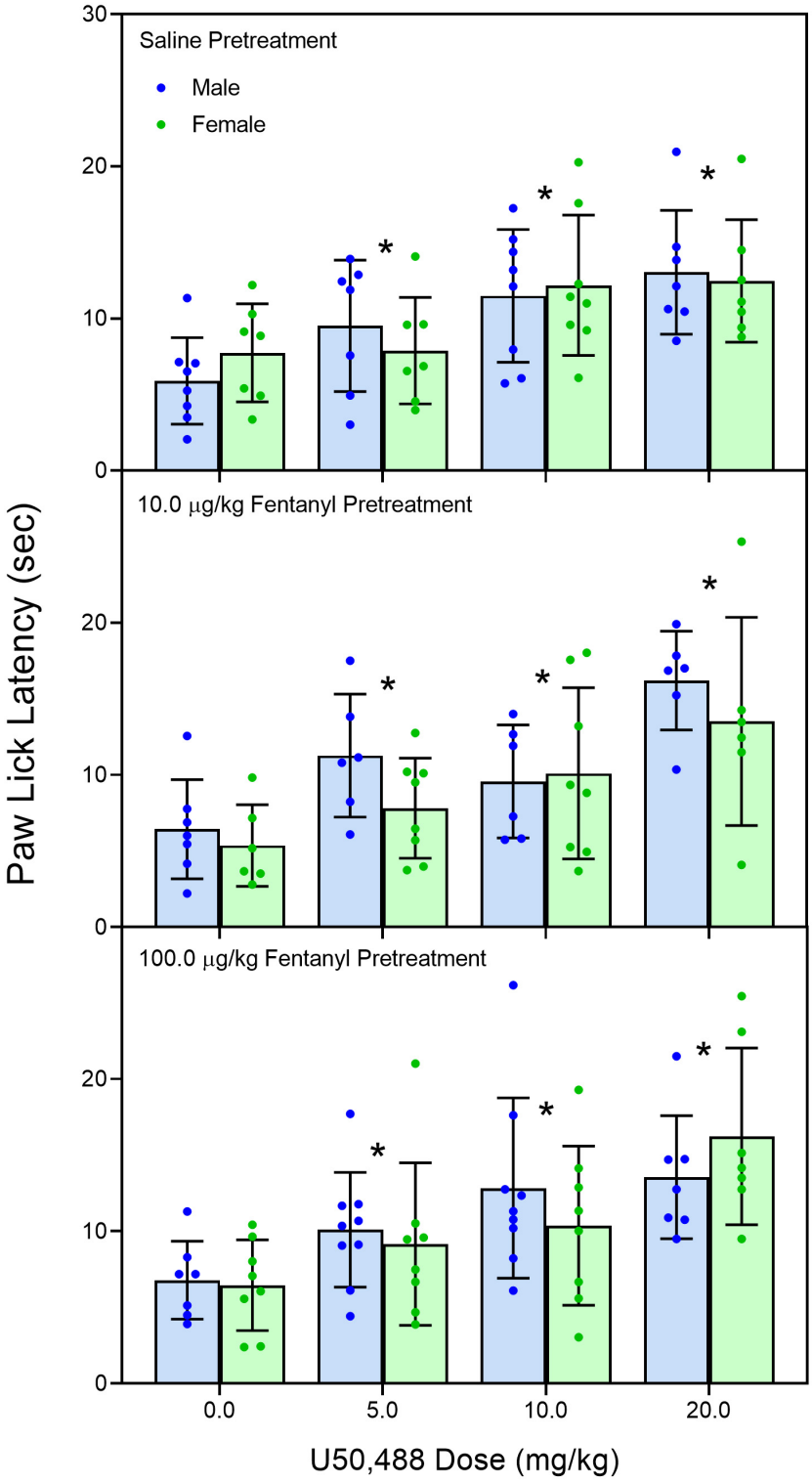

FIGURE 4
Mean (+SE) baseline paw-lick latencies of PD 60 male and female rats (n = 7–8/sex). Rats were pre-treated with fentanyl (10 or 100 μg/kg) or saline from PD 4 to PD 9 and injected with U50,488 (0, 5.0, 10, or 20 mg/kg, sc) on PD 60. *Significantly different from 0 mg/kg morphine.

long-lasting changes in mu-opioid receptors (i.e., receptor density, affinity, or G-protein coupling) as prior studies have demonstrated that morphine pharmacokinetics are not changed after early opioid exposure (O'Callaghan and Holtzman, 1976). Moreover, the current study showed that kappa receptors were unchanged as U50, 488 thermal antinociception was not affected by fentanyl pre-treatment.

The increased analgesic response to morphine after pre-exposure to the low dose of fentanyl was not anticipated based on most of the literature on early life exposure to mu-opioid agonists because these studies showed a decreased response to later opiate exposure or no change (O'Callaghan and Holtzman, 1976; Thornton and Smith, 1998; Timár et al., 2010). There was one published report

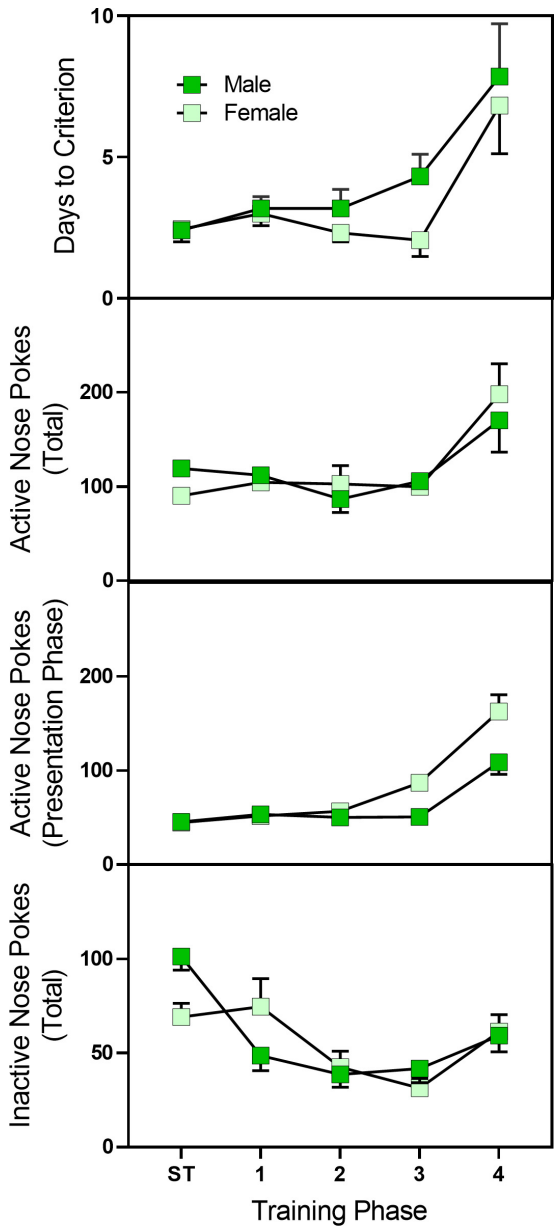

FIGURE 5
Mean (±SEM) active nose pokes, active nose pokes during the stimulus presentation, and inactive nose pokes in late adolescent male and female rats (n = 23–27/sex) during sucrose training and acquisition training for sucrose/fentanyl-rewarded nose pokes. Rats were pre-treated with fentanyl (10 or 100 μg/kg) or saline from PD 4 to PD 9 and started sucrose training on PD 40. Rats first were trained to nose poke for a sucrose solution and then underwent a four-phase sucrose fade out/fentanyl fade in procedure. ST, sucrose training.

where the morphine had a greater antinociceptive response after early fentanyl treatment, but that report used a protocol that should have induced tolerance and the nociceptive task (tail flick) measured spinal mediated thermal antinociception (Kirby et al., 1982). While again we did not assess the development of physical dependence on fentanyl, our protocol (two 5 μg/kg injections; 6 h apart for 6 days) was probably not sufficient based on other published reports. It is possible that instead of causing long-term desensitization of mu receptors, the low-dose fentanyl treatment upregulated or increased receptor sensitivity. This is of course speculative but not improbable and may be reflective of changes to mu receptor/G-protein coupling or intrinsic activity (Windh et al., 1995; Thornton et al., 1998).

Based on the thermal antinociception study, we expected that our fentanyl pre-treatment would alter oral fentanyl self-administration. However, we found almost no effect of early postnatal fentanyl pre-treatment on fentanyl-reinforced nose pokes. The one exception to this was a slight increase in inactive nose pokes in female rats treated with the low dose of fentanyl (10 μg/kg/day). Our failure to find enhanced fentanyl reward, however was consistent with another early fentanyl pre-treatment study that found that rat pups made dependent on fentanyl did not respond differently than saline-treated rats for oral fentanyl (Thornton et al., 2000) and a prenatal morphine paper where morphine- and saline-exposed rats did not differ on morphine-conditioned place preference task or morphine self-administration [Riley and Vathy, 2006; but see Timár et al. (2010)]. It is not known why morphine thermal antinociception was altered but fentanyl-rewarded responding was not, but it is possible that the difference is a result of morphine being a less efficacious agonist as compared to fentanyl (Grecksch et al., 2011; Cornelissen et al., 2018). It is possible that the desensitization caused by fentanyl was sufficient to blunt the morphine response but not the response to fentanyl. Alternatively, it is possible that the subset of receptors responsible for the antinociceptive effects was affected more by the early fentanyl treatment than the receptors necessary for reward. This explanation would agree with a study showing that early morphine treatment had opposite effects on morphine thermal antinociception and morphine-conditioned place preference (Timár et al., 2010). Finally, it is also possible that our procedure was not optimal for assessing differences in the rewarding properties of fentanyl. For example, we used a dose of fentanyl that was below the concentration typically used for self-administration, and it is conceivable that a higher concentration may have resulted in a more marked difference in response (Shaham et al., 1993; Thornton et al., 2000). In addition, while oral operant self-administration has proven to be effective at predicting the reinforcing value of a drug [see Wilson et al. (1997)], a two-bottle choice procedure may have given a better measure of drug preference.

Unlike the fentanyl pre-treatment, sex did alter nose-poke behavior on the oral fentanyl self-administration task because female rats consistently responded at higher rates for fentanyl. This finding is in agreement with other preclinical studies showing that female rats had a greater intake of intravenous fentanyl (Malone et al., 2021; Towers et al., 2022), heroin (George et al., 2021), and oral oxycodone (Fulenwider et al., 2020). Clinically, the role of sex in fentanyl and other opioid use is more complicated. Men have higher rates of opioid use disorder and opioid-related deaths as compared to women, but women show a stronger craving for drug cues, develop opioid use disorder faster than males, and are more likely to be prescribed opioid analgesics for pain management than men (Back et al., 2011; Wightman et al., 2021; Martin et al., 2022; Romanescu et al., 2022).

In summary, we found that exposure to fentanyl during the early neonatal period has long-lasting consequences on morphine thermal antinociception but did not enhance the rewarding effects of fentanyl in late adolescence. While our exposure period modeling third-trimester exposure only is not typical of human exposure, it does show even brief exposure to fentanyl can have a major impact on later functioning. Importantly, our findings also indicate that adolescent females are more vulnerable to fentanyl use than males.

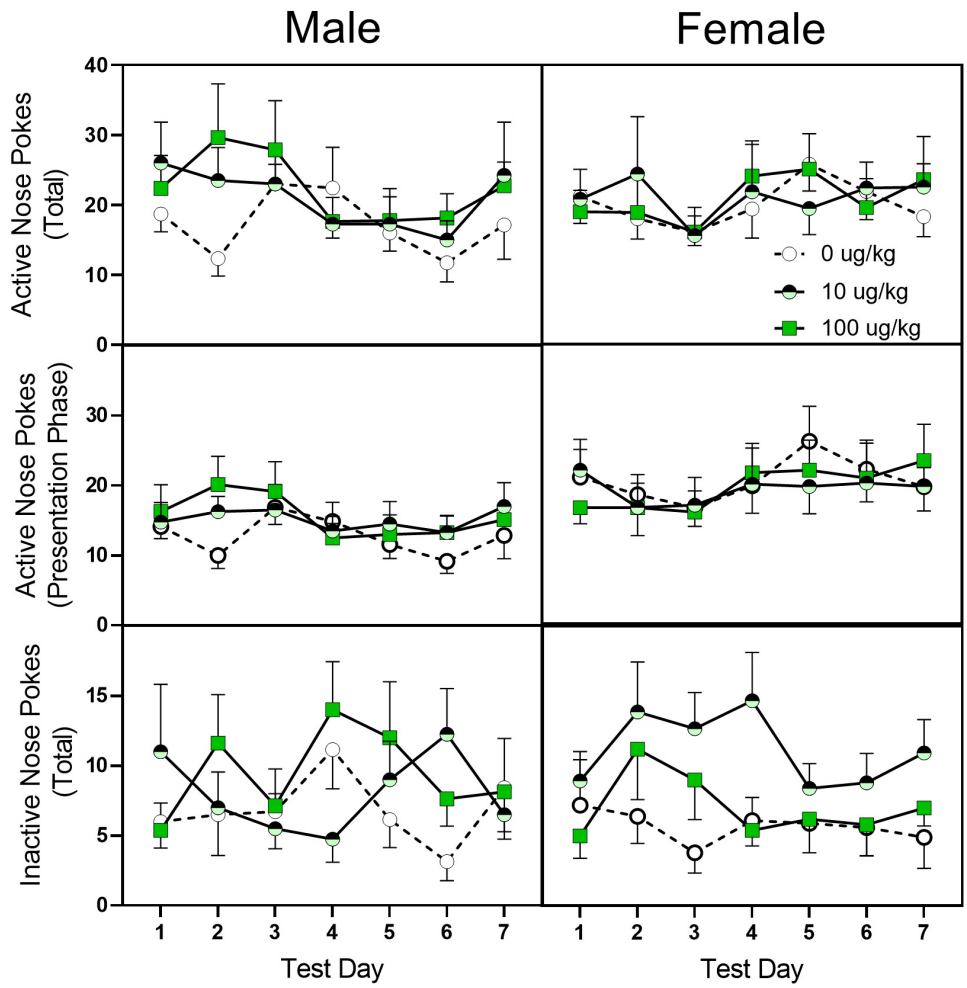

FIGURE 6
Mean (±SEM) active nose pokes, active nose pokes during the stimulus presentation, and inactive nose pokes in late adolescent male and female rats (n = 6–8/sex) during the seven fentanyl-only acquisition test days. Rats were pre-treated with fentanyl (10 or 100 μg/kg) or saline from PD 4 to PD 9 and started sucrose training on PD 40. After reaching the criterion for sucrose-rewarded nose pokes, rats underwent a four-phase sucrose fade out/fentanyl fade in procedure and then were assessed for 7 days for fentanyl-only reinforcement.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

This animal study was reviewed and approved by Institutional Animal Care and Use Committee, California State University, San Bernardino.

Author contributions

CC contributed to the conception and design of the study, performed the statistical analyses, and wrote the first draft. JT, GP, JR, JB, and CG conducted the morphine thermal antinociception and the self-administration experiments. JT, DL, and DG conducted the kappa thermal antinociception experiment. All authors contributed to the manuscript revision and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Insights into the role of intracellular calcium signaling in the neurobiology of neurodevelopmental disorders

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Calcium (Ca^{2+}) comprises a critical ionic second messenger in the central nervous system that is under the control of a wide array of regulatory mechanisms, including organellar Ca^{2+} stores, membrane channels and pumps, and intracellular Ca^{2+}-binding proteins. Not surprisingly, disturbances in Ca^{2+} homeostasis have been linked to neurodegenerative disorders, such as Alzheimer's and Parkinson's diseases. However, aberrations in Ca^{2+} homeostasis have also been implicated in neuropsychiatric disorders with a strong neurodevelopmental component including autism spectrum disorder (ASD) attention-deficit hyperactivity disorder (ADHD) and schizophrenia (SCZ). While plasma membrane Ca^{2+} channels and synaptic Ca^{2+}-binding proteins have been extensively studied, increasing evidence suggests a prominent role for intracellular Ca^{2+} stores, such as the endoplasmic reticulum (ER), in aberrant neurodevelopment. In the context of the current mini-review, we discuss recent findings implicating critical intracellular Ca^{2+}-handling regulators such as the sarco-ER Ca^{2+} ATPase 2 (SERCA2), ryanodine receptors (RyRs), inositol triphosphate receptors (IP_3Rs), and parvalbumin (PVALB), in the emergence of ASD, SCZ, and ADHD.

KEYWORDS

autism, SERCA2, ryanodine receptors, calcium, schizophrenia, attention-deficit hyperactivity disorder (ADHD), inositol triphosphate receptor (IP3)

1. Introduction

Neurodevelopmental disorders (e.g., autism spectrum disorder; ASD, and attention-deficit hyperactivity disorder; ADHD) and schizophrenia (SCZ), a neuropsychiatric disorder with a strong neurodevelopmental component (Birnbaum and Weinberger, 2017; Seidman and Mirsky, 2017; Rund, 2018), comprise debilitating diseases that are highly variable in their symptomatology and etiology (McGrath et al., 2008; Christensen et al., 2016; Hansen et al., 2018; Sayal et al., 2018). These disorders arise due to the complex interplay between genetic risk factors and early life environmental stressors, including prenatal complications, malnutrition, hormone imbalance, and exposure to environmental toxins (e.g., neurotoxic metals) (Wetmore and Garner, 2010; Lord et al., 2018; Li et al., 2019; Ijomone et al., 2020). Recent research efforts have sought to identify common disrupted molecular mechanisms that may lead to

abnormal neurodevelopment. One such candidate which has garnered interest is the disruption of intracellular calcium (Ca^{2+}) homeostasis.

Intracellular Ca^{2+} concentration is critical for orchestrating numerous cellular processes, including signal transduction and gene expression (Bootman et al., 2001; Naranjo and Mellström, 2012; Bononi et al., 2013; Brini et al., 2014; Britzolaki et al., 2018). Consequently, Ca^{2+} mishandling is implicated in the pathophysiology of neurodegenerative disorders (e.g., Alzheimer's and Parkinson's diseases) (Pchitskaya et al., 2018), while recent evidence suggests that aberrations in intracellular Ca^{2+} signaling may also underlie abnormal neurodevelopment (Pourtavakoli and Ghafouri-Fard, 2022). Of the major neuronal Ca^{2+}-handling players, plasma membrane voltage-gated Ca^{2+} channels (e.g., *Cacna1*) are well-reviewed with regards to their role in neurodevelopment (Breitenkamp et al., 2015; Cupertino et al., 2016; Pourtavakoli and Ghafouri-Fard, 2022). Readers are referred to recent excellent reviews discussing the implication of critical plasma membrane Ca^{2+} players (e.g., *CACNA1*) and Ca^{2+}-binding proteins involved in synaptic release (e.g., Synaptotagmin) in the pathophysiology of brain disorders (Breitenkamp et al., 2015; Cupertino et al., 2016; Pourtavakoli and Ghafouri-Fard, 2022). Interestingly, dysfunction of endoplasmic reticulum (ER) Ca^{2+} regulators such as the sarco-ER Ca^{2+} ATPase 2 (SERCA2), which sequesters cytosolic Ca^{2+} into the ER, and the Ca^{2+}-releasing channels inositol triphosphate receptors (IP_3Rs) and ryanodine receptors (RyRs) have recently garnered interest in the pathophysiology of brain disorders (Britzolaki et al., 2018, 2020). In the context of the current mini-review, we discuss recent findings implicating aberrant ER-dependent Ca^{2+} homeostasis as a convergent pathophysiological mechanism in brain disorders with a strong neurodevelopmental component.

2. Autism spectrum disorders (ASD)

2.1. Ryanodine receptors (RyRs) and the fragile X messenger ribonucleoprotein 1 (FMR1)

Autism spectrum disorders is a neurodevelopmental disorder which comprises a wide array of behavioral symptoms including impaired sociability and communication skills, repetitive behaviors, and intellectual disability (Christensen et al., 2016; Lord et al., 2018). Although no single genetic factor is responsible for ASD, RyRs have been identified as a potential contributor to ASD pathology. RyRs are homotetrameric Ca^{2+}-releasing channels expressed on the neuronal ER membrane; upon opening, the RyRs allow for the flux of Ca^{2+} ions from the ER stores into the cytosol (**Figure 1**; Abu-Omar et al., 2018). Notably, clinical studies suggest that mutations in genes coding for the different RyRs isoforms could possibly contribute to the pathophysiology of ASD. A copy number variation study has revealed a likely pathogenic duplication at 1q43, which encompasses the *RYR2* gene, thus identifying *RYR2* as a potential ASD risk gene (Soueid et al., 2016; Keil et al., 2019). Despite the fact that *Ryr3* has been shown to contribute to synaptic plasticity and cognitive flexibility in mice (Balschun et al., 1999), an earlier clinical study did not report an association between *RYR3* and ASD in a Japanese patient cohort (Tochigi et al., 2008). However, a more recent targeted sequencing and integrative analysis study of 3,195 Chinese patients with neurodevelopmental disorders exposed *RYR3* as one of the six novel candidate genes to preferentially contribute to ASD (Wang T. et al., 2021).

Preclinical studies have provided intriguing mechanistic insights into how RyR dysfunction could affect intracellular Ca^{2+} homeostasis and ASD-relevant phenotypes and endophenotypes in animal models. Interestingly, mutations in the *RYR1* and the fragile X messenger ribonucleoprotein 1 (*FMR1)* genes have both been associated with impaired Ca^{2+} signaling. Specifically, preclinical evidence suggests that the human T4826I-*RYR1* gain-of-function mutation and the human CGG-repeat expansion in the *FMR1* gene (i.e., *FMR1* premutation), are both associated with elevated intracellular Ca^{2+} signaling; indeed, the T4826I-*RYR1* gain-of-function mutation has been shown to result in increased intracellular Ca^{2+} concentrations in muscle cells (Barrientos et al., 2012), while murine cortical astrocytes with the *FMR1* premutation displayed enhanced asynchronous Ca^{2+} oscillations (Chen et al., 2010; Cao et al., 2013; Robin et al., 2017). Notably, Ca^{2+} signaling is critical for ensuring proper dendritic morphology and synaptic connectivity. Keil et al. (2019) assessed ASD-relevant behavioral and neurobiological correlates (i.e., dendritic morphology and social behavior) in adolescent mice with the humanized T4826I-*RYR1* gain-of-function mutation and with the *FMR1* premutation, as well as in double mutant (DM) mice (Keil et al., 2019). Interestingly, social deficits in T4826I male and DM female mice were both accompanied by abnormal dendritic morphology (Keil et al., 2019). Based on the authors, the observed changes in dendritic morphology in these mice could be attributed to altered intracellular Ca^{2+} dynamics, even though additional studies are needed to yield more conclusive results (Keil et al., 2019).

Sethi et al. (2021) conducted a follow-up study to understand the interaction of *Ryr1* and *Fmr1* and polychlorinated-biphenyls (PCBs) exposure in ASD-like behaviors (Sethi et al., 2021). PCBs comprise environmental contaminants with established neurodevelopmental consequences that exert their neurotoxic effects by binding to the RyRs (Pessah et al., 2010). In that study, dams were orally administered a PCB mixture from 2-weeks prior to mating until pup weaning (P21). Ultrasonic vocalizations at P7 were diminished in all three mutant pup genotypes while both male and female T4826I and DM pups exhibited high spontaneous grooming behavior (Sethi et al., 2021). Further, studies from the same group found that PCBs promote synaptogenesis in cultured hippocampal neurons, as evidenced by increased dendritic spines and miniature excitatory postsynaptic currents (Lesiak et al., 2014). Importantly, these effects were found to be RyR-dependent, as treatment with either the RyR inhibitor FLA365 or RyR siRNA both rescued these effects. Taken together, these preclinical studies suggest that mutations in *Ryr1* and *Fmr1*, two genes shown to be involved in neuronal Ca^{2+} handling, exert ASD-like behavioral and neuroarchitecture consequences in mice. Overall, these studies indicate that both genetic and environmental perturbation of neuronal Ca^{2+} homeostasis may contribute to aberrant synaptogenesis, dendritic arborization, and ultimately ASD-like behaviors.

2.2. Parvalbumin (PVALB)

Parvalbumin (PVALB) is a Ca^{2+}-buffering protein primarily expressed in the γ-aminobutyric acid (GABA) positive interneurons of the brain that exhibit rapid burst-firing activity and are heavily

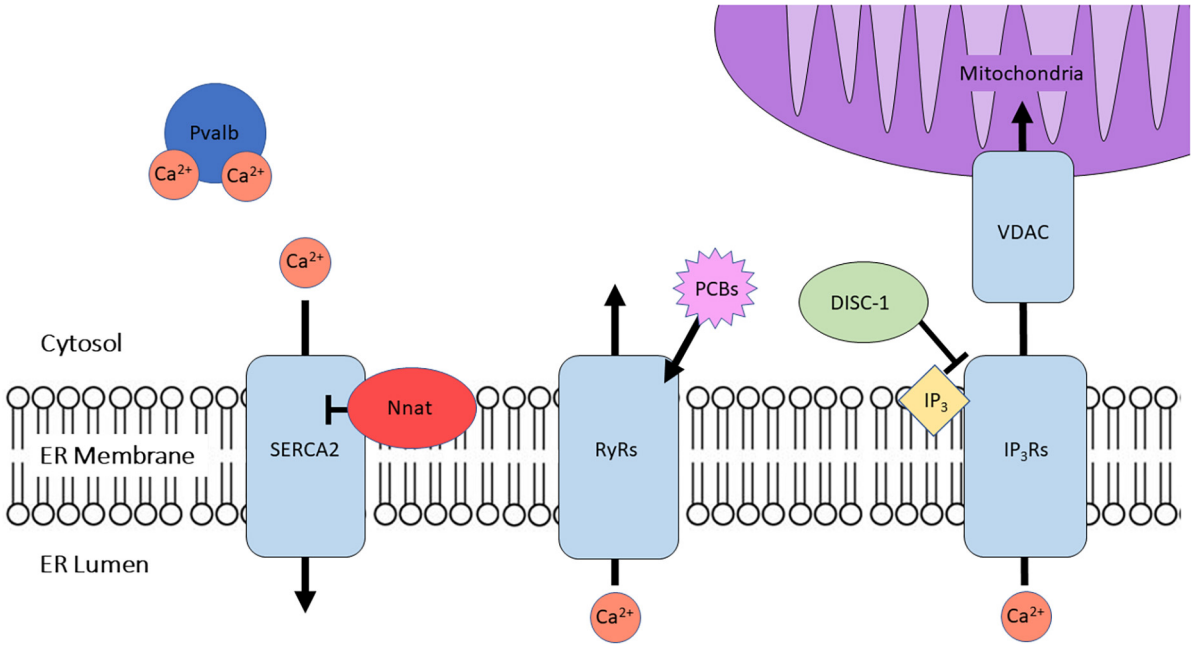

FIGURE 1
A summary of the proteins discussed herein and their primary role in regulating intracellular Ca^{2+} homeostasis. Proteins mediating ER Ca^{2+} efflux include ryanodine receptors (RyRs) and inositol triphosphate receptors (IP_3Rs), while cytosolic Ca^{2+} is handled by the sarco-endoplasmic reticulum Ca^{2+} ATPase 2 (SERCA2), which is negatively regulated by neuronatin (Nnat). Parvalbumin (Pvalb) regulates cytosolic Ca^{2+} buffering *via* directly binding Ca^{2+} ions. Polychlorinated biphenyls (PCBs) target RyRs to exert their effects on Ca^{2+} homeostasis. IP_3Rs associate with mitochondrial voltage-dependent anion channels (VDAC) in the mitochondrial-associated membranes (MAMs), and are regulated in part by disrupted in schizophrenia 1 protein (DISC-1).

dependent on intracellular Ca^{2+}-handling (Ruden et al., 2021). While dysfunction of $PVALB^+$ neurons is well-known to contribute to aberrant neurodevelopmental processes and ASD, the role of PVALB in maintaining the integrity of intracellular Ca^{2+} signaling pathways and its potential contribution to ASD has received less attention (Ruden et al., 2021). Interestingly, *Pvalb*$^{-/-}$ mice are known to exhibit an ASD-like behavioral phenotype (Wöhr et al., 2015). Recently, Janickova et al. (2020) explored the role of PVALB in regulating neuron morphology and dendritic arborization by utilizing a *Pvalb*$^{-/-}$ mouse strain in which EGFP expression was under the control of the *Pvalb* driver that allowed for visualization of $PVALB^+$ neurons even in the absence of functional *Pvalb* expression (Janickova and Schwaller, 2020; Janickova et al., 2020). Interestingly, loss of PVALB function resulted in increased cell soma and mitochondrial size primarily in regions rich in $PVALB^+$ neurons, such as the thalamic reticular nucleus (TRN), the molecular layer interneurons (MLI) of the cerebellum, the prefrontal cortex, and the striatum (Janickova and Schwaller, 2020; Janickova et al., 2020). Furthermore, loss of PVALB function was associated with dendritic hypertrophy in the dentate gyrus, the striatum, and the MLI, as well as by a shift of mitochondria from the central compartment of the cell to the subplasmalemmal region (Janickova and Schwaller, 2020). Taken together, these studies suggest that the impaired Ca^{2+} buffering brought about by the absence of PVALB may result in a compensatory proliferation and subplasmalemmal relocation of mitochondria to maintain the rapid Ca^{2+} dynamics these neurons rely on (Janickova and Schwaller, 2020; Ruden et al., 2021). Ultimately, this may result in enhanced dendritic arborization and oxidative stress. Although further studies are imperative, these data provide valuable insights into how PVALB-mediated Ca^{2+} dysfunction may induce ASD-relevant neurobiological correlates.

2.3. Inositol triphosphate receptors (IP_3R)

G protein-coupled receptor (GPCR)-mediated IP_3R Ca^{+2} signaling pathways comprise critical components of the intracellular Ca^{+2} handling machinery with potential implications in ASD (Berridge, 2009; Taylor and Tovey, 2010). For instance, the *IP3R2* has been shown to affected by *de novo* copy number variants in ASD patient cohorts, while recently *Ip3R2*$^{-/-}$ mutant mice and astrocyte-specific *Ip3R2* conditional knockout mice display ASD-like behaviors (Gilman et al., 2011; Wang Q. et al., 2021). Interestingly, *ex vivo* studies in human fibroblasts derived from patients with rare, monogenic forms of ASD (i.e., fragile X syndrome; FXS and tuberous sclerosis; TS) showed that ATP-evoked GPCR-mediated Ca^{2+} release from the IP_3Rs was diminished in ASD fibroblasts (Schmunk et al., 2015). In a follow-up study, Schmunk et al. (2017) extended their findings by using fibroblasts from patients with sporadic ASD, as well as two additional monogenic forms of ASD (i.e., Prader–Willi syndrome and Rett syndrome), and observed a similar impaired IP_3R-mediated Ca^{2+} signaling. Taken together, these studies suggest that depressed Ca^{2+} release through IP_3R signaling may disrupt neurodevelopment. To our knowledge these studies have not been replicated in neural cells or *in vivo* models, but provide mechanistic insights into the putative implication of IP_3Rs in the neurobiology of ASD.

2.4. Neuronatin (NNAT) and other genes

Neuronatin (NNAT) is a developmentally regulated ER resident protein and negative regulator of SERCA that is expressed in the brain's PVALB + GABAergic neurons; NNAT has also been

implicated in abnormal neurodevelopment, including ASD and Angelman Syndrome (AS) (Pitale et al., 2017; Vatsa et al., 2019). The miR-708, an NNAT downregulator, has been involved in the atypical Ca^{2+} signaling processes observed in the maternal-ubiquitin protein ligase E3A (*Ube3a*) deficient mouse model for AS (Vatsa et al., 2019). UBE3A plays a role in the proteasome-mediated degradation of proteins in neurons, and has thus been implicated in ASD and AS (Glessner et al., 2009; Williams et al., 2010; Yi et al., 2015; Xu et al., 2018; Lopez et al., 2019). Recently, Vatsa et al. (2019) identified miR-708 to be significantly downregulated in the cortex of maternal-*Ube3a*-deficient AS mice and showed that miR-708 regulates intracellular Ca^{2+} homeostasis by targeting NNAT (Vatsa et al., 2019). Taken together, these findings suggest that NNAT/miR-708-mediated aberrations in intracellular Ca^{2+} signaling may be involved in ASD/AS pathogenesis.

Interestingly, targeted sequencing and integrative analysis of 3,195 Chinese probands with several neurodevelopmental disorders exposed novel candidate genes involved in ASD, including three with relevance to Ca^{2+} homeostasis, namely: *RYR3* [*discussed in* the Section "2.1. Ryanodine receptors (RyRs) and the fragile X messenger ribonucleoprotein 1 (FMR1)"], ubiquitin protein ligase E3 (*UBR3*), and filamin A (*FLNA*) (Wang T. et al., 2021). UBR3 inhibits the function of alpha 1C subunit of L-type voltage-dependent Ca^{2+} channel ($Ca_v1.2$) *via* the ubiquitin-proteasome protein degradation pathway and has been identified as a modulator of Ca^{2+} -induced Ca^{2+} release (CIRC) (Ma et al., 2020). FLNA is an actin-binding protein which regulates cytoskeletal remodeling and is regulated by Ca^{2+} and calmodulin, and has been shown to interact with FMR1 in long term memory processes in *Drosophila* (Nakamura et al., 2005; Bolduc et al., 2010; Rosa et al., 2019). Overall, these findings further support a role for intracellular Ca^{2+} homeostasis in ASD pathogenesis, although further research is considered imperative to confirm the contribution of these genes in neurodevelopment.

2.5. $Ca2^{+}$ signaling in astrocytes

It is well established that Ca^{2+} signaling is also prevalent in astrocytes; while astrocytic dysfunction has been implicated in the pathophysiology of ASD, the precise mechanisms by which astrocytes contribute to disease progression and symptomatology remain elusive (Blanco-Suárez et al., 2017). The onset of ASD pathology is typically concurrent with neurodevelopmental astrocyte proliferation (Berger et al., 2013; Sigaard et al., 2016). Allen et al. (2022) sought to investigate the putative role of astrocytes in ASD pathologyClick or tap here to enter text. Upon harvesting astrocytes from organoids created by induced pluripotent stem cells (iPSCs) from ASD patients (Allen et al., 2022). Proteomic analysis revealed that "Ca^{2+} binding" processes were highly enriched in the altered protein networks observed in these ASD astrocytes. Follow-up two-photon live-cell imaging confirmed an exaggerated ATP-induced Ca^{2+} response in these ASD astrocytes. To investigate putative behavioral effects of Ca^{2+} disruption in ASD astrocytes, human-derived ASD astrocytes were implanted into mice at P1-3, thus generating ASD astrocyte chimeric mice. Engrafted human ASD astrocytes were found to exhibit aberrant Ca^{2+} fluctuations, as well as to result in ASD-relevant behaviors (i.e., enhanced repetitive behaviors in the marble burying test and impaired fear learning). Given the exaggerated Ca^{2+} response observed in ASD astrocytes, it was predicted that inhibition of IP_3Rs would possibly restore Ca^{2+} signaling and function. Intriguingly, IP_3R-knockdown in ASD astrocytes rescued the exaggerated Ca^{2+} response, hippocampal neuron network firing, and deficits in fear memory observed in chimeric mice (Allen et al., 2022). Overall, these data provide deep insights into the contribution of astrocytic Ca^{2+} dysregulation in the pathophysiology of ASD.

3. Schizophrenia (SCZ)

Schizophrenia is a brain disorder characterized by a constellation of symptoms including hallucinations, negative affect, and cognitive deficits (McCutcheon et al., 2020). The Disrupted in Schizophrenia–1 (DISC-1) protein is involved in numerous neuronal processes, including the regulation of dendrite morphology and neuronal migration during development (Balu and Coyle, 2011). Recent studies have suggested that DISC-1 is involved in Ca^{2+} regulation *via* the mitochondria-associated membranes (MAMs) which comprise physical connections formed between the ER IP_3Rs and mitochondrial voltage-dependent anion channels (VDAC) that are involved in the transfer of Ca^{2+} and molecular stress signals between these two organelles (Park et al., 2017, 2015; van Vliet and Agostinis, 2018; Barazzuol et al., 2021; Means and Katz, 2021). Recent findings suggest that DISC-1 localizes to the MAM in mouse neurons, and specifically binds IP_3R1 to reduce ligand-binding and subsequent Ca^{2+} transfer to the mitochondria in primary cortical neurons (Park et al., 2017). Upon DISC-1 dysfunction, IP_3R1-mediated Ca^{2+} release into the MAM is disinhibited, causing a buildup of mitochondrial Ca^{2+} that leads to oxidative stress that ultimately impairs mitochondrial function (Park et al., 2017). Interestingly, neuronal oxidative stress has been implicated in the pathogenesis of SCZ (Emiliani et al., 2014). Taken together, this experimental evidence suggests that DISC-1 is involved in the dysregulation of Ca^{2+} handling in the MAMs, causing downstream mitochondrial Ca^{2+} hyper-accumulation and oxidative stress, shining a light on a novel mechanism by which DISC-1 may contribute to SCZ pathogenesis.

Darier's disease is a skin condition characterized by persistent wart-like skin patches, which is due to a mutation in the *SERCA2* gene that subsequently leads to Ca^{2+} dysfunction (Cooper and Burge, 2003). Interestingly, Darier's disease patients have a significantly increased risk for SCZ, providing a causative link between *SERCA2* and neurodevelopmental processes (Tang et al., 2010). Recently, Nakajima et al. (2021) generated a brain-specific heterozygous *Serca2* loss-of-function mouse model (i.e., hetero cKO) to investigate how developmental hypofunction of *Serca2* may affect SCZ-relevant behavioral and neurobiological processes (Nakajima et al., 2021). As expected, both primary hippocampal neurons and ER membranes isolated from the brain of hetero cKO mice exhibited impaired Ca^{2+} uptake (Nakajima et al., 2021). Hetero cKO mice exhibited impaired fear memory and enhanced exploratory behavior; moreover, microdialysis studies suggested that *Serca2* hypofunction induces a hyperdopaminergic state in the nucleus accumbens (NAC) (Nakajima et al., 2021), echoing the neurochemical dopaminergic hallmarks of SCZ (McCutcheon et al., 2020). Taken together, these

TABLE 1 Studies focusing on notable genes implicated in the pathophysiology of neurodevelopmental disorders and summarized findings on intracellular Ca^{2+} signaling, neuronal function, and behavior.

Gene	Study	Gene manipulation	Disorder indication	Effect on Ca^{2+}	Effect on neuronal function	Effect on behavior
Ryr1	Keil et al., 2019	Humanized GoF *Ryr1* T4826I mutation	ASD	N/A	↑ Dendritic complexity	↓ Sociability
Fmr1	Keil et al., 2019	CGG-repeat GoF *Fmr1*	ASD	N/A	↑ Dendritic complexity	N/A
Pvalb	Janickova et al., 2020	*Pvalb* KO mouse	ASD	N/A	↑ Soma and dendrite size	N/A
Nnat	Vatsa et al., 2019	miR-207	ASD/AS	↓ Intracellular Ca^{2+} ↓ CaMKIIα phosphorylation	N/A	Maternal-*Ube3a* deficient mouse model for AS
DISC-1	Park et al., 2015, 2017	DISC-1 KD	SCZ	↓ MAM Ca^{2+} transfer	N/A	N/A
Serca2	Nakajima et al., 2021	Brain-specific heterozygous knockout		↓ER Ca^{2+} uptake	↑ NAc DA	↑ Exploratory behavior ↓ Fear memory
Gnb5	Xie et al., 2012	GNB5 KO mouse	ADHD	N/A	N/A	↑ Hyperactivity
GNB5	Kang et al., 2018	GNB5 overexpression in HEK293T cells	ADHD	↑ SOCE	N/A	N/A

GoF, gain of function; KD, knock-down; KO, knock-out.

findings support the notion that developmental hypofunction of the *Serca2* and subsequent aberrant intracellular Ca^{2+} handling induces SCZ-relevant behavioral and neurochemical effects.

Interestingly, recent evidence suggests an association between RyRs and SCZ. An exome sequencing study of childhood-onset SCZ patients, identified *de novo* variants of *RYR2*, which the authors highlight as a strong candidate gene given the role of RyRs in neurodevelopmental processes (Ambalavanan et al., 2016), further underscoring the putative role of RyRs in the neurobiology of neurodevelopmental disorders.

4. Attention-deficit hyperactivity disorder (ADHD)

Attention-deficit hyperactivity disorder is a neurodevelopmental disorder that is characterized by impaired attention, locomotor hyperactivity, and impulsive behaviors (Sharma and Couture, 2014). Preclinical evidence suggests that ADHD is associated with impaired intracellular Ca^{2+} handling; for instance, the spontaneously hypertensive rat (SHR) model of ADHD has been shown to exhibit impaired brain plasma membrane Ca^{2+} uptake (Horn et al., 1995; Lehohla et al., 2001). Further preclinical evidence has shown that knockout of the G-protein subunit Gβ5 (encoded by the gene *Gnb5*) elicits a pronounced ADHD-like hyperactive phenotype in mice (Xie et al., 2012). Moreover, a *GNB5* mutation (i.e., GNB5 S81L) associated with impaired termination of DA2 receptor signaling was reported in a Saudi family presenting speech impairments and a variable ADHD diagnosis, providing initial clinical evidence for the putative role of GNB5 in the neurobiology of ADHD (Shamseldin et al., 2016). Interestingly, a recent study highlighted the role of GNB5 in store-operated Ca^{2+} entry (SOCE) (Kang et al., 2018). Upon depletion of ER Ca^{2+} stores, stromal interaction molecule 1 (STIM1), an ER Ca^{2+} sensor, forms a complex with the plasma membrane calcium release-activated calcium channel protein 1 (ORAI1) to initiate extracellular Ca^{2+} entry (Srikanth and Gwack, 2012). Kang et al. (2018) found that GNB5 expression enhances SOCE *in vitro*. Notably, the ability of GNB5 to enhance SOCE was found to depend on STIM1 function suggesting that GNB5 may interact with the ER Ca^{2+}-sensing machinery to regulate Ca^{2+} homeostasis, although further studies are needed to determine the precise mechanisms that may underlie this process.

5. Conclusion

In the context of this mini-review, we have highlighted recent advances supporting the implication of prominent ER and cytosolic Ca^{2+} regulators (i.e., SERCA2, IP_3Rs, RyRs, PVALB, NNAT) in the neurobiology of brain disorders with a strong neurodevelopmental component (**Figure 1** and **Table 1**). Disease progression of monogenic brain disorders (e.g., AS, FXS) may be dependent on specific gene interactions with intracellular Ca^{2+} signaling mechanisms, whereas sporadic cases of SCZ, ASD, and ADHD may arise from polygenic variations that ultimately converge to the disruption of intracellular Ca^{2+} homeostasis and concomitant impairment of neuronal function. Further preclinical and clinical investigation is considered imperative to confirm and/or expand upon these intriguing discoveries in order to gain deep insights into the cellular and molecular Ca^{2+}-dependent neurodevelopmental processes that are compromised in these debilitating brain diseases.

Author contributions

BK conducted the primary literature search and wrote first draft of the manuscript. KK, JT, CM, and HO wrote sections of the manuscript. PP formulated the concept and supervised the writing

of the manuscript. All authors contributed to manuscript editing, revision, read, and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Alcohol and lactation: Developmental deficits in a mouse model

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It is well documented that prenatal ethanol exposure *via* maternal consumption of alcohol during pregnancy alters brain and behavioral development in offspring. Thus, the Centers for Disease Control (CDC) advises against maternal alcohol consumption during pregnancy. However, little emphasis has been placed on educating new parents about alcohol consumption while breastfeeding. This is partly due to a paucity of research on lactational ethanol exposure (LEE) effects in children; although, it has been shown that infants exposed to ethanol *via* breast milk frequently present with reduced body mass, low verbal IQ scores, and altered sleeping patterns. As approximately 36% of breastfeeding mothers in the US consume alcohol, continued research in this area is critical. Our study employed a novel murine LEE model, where offspring were exposed to ethanol *via* nursing from postnatal day (P) 6 through P20, a period correlated with infancy in humans. Compared to controls, LEE mice had reduced body weights and neocortical lengths at P20 and P30. Brain weights were also reduced in both ages in males, and at P20 for females, however, female brain weights recovered to control levels by P30. We investigated neocortical features and found that frontal cortex thickness was reduced in LEE males compared to controls. Analyses of dendritic spines in the prelimbic subdivision of medial prefrontal cortex revealed a trend of reduced densities in LEE mice. Results of behavioral tests suggest that LEE mice engage in higher risk-taking behavior, show abnormal stress regulation, and exhibit increased hyperactivity. In summary, our data describe potential adverse brain and behavioral developmental outcomes due to LEE. Thus, women should be advised to refrain from consuming alcohol during breastfeeding until additional research can better guide recommendations of safe maternal practices in early infancy.

KEYWORDS

alcohol, behavior, neocortex, lactation, anatomy, brain development, postnatal neocortical development, lactational ethanol exposure

Introduction

Alcohol is known as a developmental teratogen in mammalian systems. However, research in this area has primarily focused on exposures during the prenatal period. Maternal consumption of alcohol during pregnancy can result in Fetal Alcohol Spectrum Disorders (FASD) in offspring and children with FASD may exhibit physical, cognitive,

emotional, and behavioral phenotypes related to the exposure (May et al., 2009, 2014; Hoyme et al., 2016). Thus, Centers for Disease Control (CDC) have released a statement that no amount of alcohol is safe to consume during pregnancy (Centers for Disease Control and Prevention, 2022a). Generally, these recommendations are followed, as demonstrated by a reduction in alcohol consumption during pregnancy. However, consumption levels approach preconception levels shortly after birth in some populations (Little et al., 1990; Giglia and Binns, 2006). The prevalence of breastfeeding mothers consuming alcohol is high, ranging from 20% in Canada (Popova et al., 2013), 36% in the United States (May et al., 2016), and 60% in Australia (Tay et al., 2017). For a specific example, in Seattle, Washington, 80% of women consumed alcohol during the month before conception, 40% consumed alcohol during the last trimester of pregnancy, and 70% were drinking 3 months postpartum. Notably, this study also reported that 10% of breastfeeding mothers reported drinking more than once a day (>15 g alcohol) (Little et al., 1990).

Given the prevalence of maternal alcohol consumption during breastfeeding, it is important to understand how this can represent a teratogenic exposure for infants. Studies have shown that the levels of alcohol in the breast milk mirror the amount of alcohol in the blood (Lawton, 1985; Chien et al., 2005). These levels peak at 30–60 min after ethanol consumption and continue to be detected 2–3 h after consumption (Chien et al., 2005; Centers for Disease Control and Prevention, 2019). Although these levels are lower than the percentage in alcoholic beverages, they are non-zero values. In infants, exposure to breast milk containing alcohol may result in reduced body mass and verbal IQ scores (May et al., 2016). Congruently, exposure to alcohol *via* breast milk may result in a dose-dependent reduction of cognitive functions as seen when testing exposed children aged 6–7 years (Gibson and Porter, 2018) and dose-dependent reductions in children's academic abilities up to grade 5 (Gibson and Porter, 2020). Additionally, deficits in abstract reasoning skills are observed at age 7 in lactational-exposed children (Oei, 2019). Changes in sociability can also occur as exposed infants scored below, or within the monitoring zone, on the scale of the personal-social interactions at 12 months of age (Tay et al., 2017). Despite these potential negative effects of alcohol compromised breast milk on offspring development, there is a disconnect between conclusions drawn from scientific literature and behaviors in many new mothers.

In humans, there is variability in maternal behavior in terms of infant feeding preferences. In the US from 2012 to 2019, around 80% of mothers breastfed their infants, with just over half of them breastfeeding exclusively [from the National Immunization Survey (Centers for Disease Control and Prevention, 2019)]. Additionally, there is variability among women in their ability to metabolize alcohol and to respond to stressors, which can moderate infant exposure. Indeed, higher tolerance and stress may result in the increase of the consumption of alcohol, for certain populations (Guinle and Sinha, 2020). Women who consume alcohol during pregnancy are also more likely to drink while breastfeeding (May et al., 2016), suggesting certain populations may be considered high-risk for breast milk contamination. Additionally, unplanned, and drastic lifestyle changes may influence alcohol consumption levels. For example, the COVID-19 pandemic and subsequent "stay-at-home" orders, rapidly emerged as a public and/or personal health concern for many. In response to this novel stressor, women in the United States showed an increase in their Alcohol Use Disorders Identification Test scores during the COVID-19 "stay-at-home" order (Boschuetz et al., 2020). These results translate to an increase in frequency and quantity of alcohol ingested in those who already used alcohol; congruently, factors such as having children at home and a history of substance abuse were positively associated with an increase in alcohol use during the pandemic (Boschuetz et al., 2020). Similar results were observed in Australia (Bramness et al., 2021), Norway (Rossow et al., 2021), and Belgium (Vanderbruggen et al., 2020), and thus, the pandemic and "stay-at home" orders may have unintentionally increased infant alcohol exposure *via* increased maternal consumption. These studies show an increase in alcohol consumption in certain child rearing populations, elucidating the deleterious effects of postnatal ethanol exposure *via* breast milk, and bolster the importance of alcohol abstinence during breastfeeding. However, published postnatal alcohol exposure paradigms (*via* breast milk) tend to be uncontrolled, unstandardized, and often limited to humans. Much of the existing data leave questions of dosing, timing, and how the developing nervous system is affected by lactational ethanol exposure (LEE). Data from animal models are not always consistent, most likely due to the variability in postnatal ethanol exposure methods, ranging from direct ethanol exposure to combined prenatal and postnatal exposure. In one study, researchers exposed rat pups to ethanol *via* intragastric intubation from postnatal (P) day 4 to 8 and reported increased male body weights but no increases in cerebral cortex weight (Light et al., 1989). Another direct exposure study reported a reduction of stem cell progenitor cells in the hippocampus and reduced adult neurogenesis after a singular subcutaneous injection of alcohol at P7 (Ieraci and Herrera, 2007). A study from Vilaró et al. (1987) exposed rat pups to alcohol *via* an alcohol-treated mother and reported a reduction in weight of rat pups at age P15 compared to controls; however, this study exposed rats to ethanol during gestation as well as postnatally. These studies provide much-needed evidence toward the damaging effects of postnatal ethanol exposure; however, they do not target a particular time window in mammalian brain development. Hence, many of their results are contradictory. To combat this, an analogous age range for exposure must be established between mice and humans. To begin, the brain growth spurt (BGS) is a time window where the mammalian brain undergoes rapid growth (Dobbing and Sands, 1979). In humans this period ranges from the third trimester of pregnancy to about the first 2 years of life, peaking at the birth (Dobbing and Sands, 1979). In murine models, this period ranges from the first week postnatal to the third week, peaking around P7 (Dobbing and Sands, 1979). A study has shown that exposure to alcohol during the BGS induces deficits such as a reduction in long-term cerebellar growth and altered rotarod performance in a rat model (Goodlett et al., 1991). However, this study used artificial-rearing procedures to directly expose pups to ethanol during the P4–P9 time window and was a binge model (Goodlett et al., 1991). Furthermore, ethanol exposure has been shown to cause alterations in synaptic pruning (Kyzar et al., 2016). In mice, synaptic pruning reaches its peak 2–3 weeks postnatal (Lewis, 2011), this is within the BGS, providing further evidence of sensitivity toward perturbations early in postnatal development. Clearly, additional research is needed to illuminate the specific details of risk including dose-dependencies and the interaction

of developmental time and exposure. Here, we are specifically interested in how maternal drinking while breastfeeding impacts brain and behavioral development of offspring. The exposure period we targeted is within the BGS but begins on a postnatal day roughly equivalent to the day of human birth, to better mimic the time when breastfeeding would begin in humans.

In the current study, we targeted early LEE in our mouse model by estimating typical human birth in murine time. When making cross-species comparisons for developmental stage, the first postnatal week in mice relates to the third trimester in humans (Clancy et al., 2007). As our study did not aim to model human *prenatal* alcohol exposure, or FASD, we began our maternal dosing of ethanol at the end of the first week of murine life (evening of postnatal day 6). This way, offspring will have consumed alcohol *via* breast milk by P7. Estimates of human day of birth (full term) is between 245 and 265 days post conception with the mouse equivalent between 7 and 9 days postnatal (Clancy et al., 2007; Jukic et al., 2013). Specifically, we exposed CD-1 pups to breast milk contaminated with ethanol, *via* maternal consumption, at the end of the postnatal week until weaning. By mimicking human postpartum drinking behavior, our results revealed potential effects of LEE on offspring outcomes. We measured maternal blood ethanol content to assure exposure validity and blood osmolality to assess hydration. We analyzed several outcome measures in offspring to determine to what degree ethanol exposure *via* lactation altered key features of neuroanatomical development and whether these phenotypes were read out in behavior. As predicted, LEE resulted in abnormal brain and behavioral development.

Materials and methods

Animal care

All breeding and experimental studies were conducted in accordance with protocol guidelines approved by the Institutional Animal Care and Use Committee (IACUC) at the University of California, Riverside (UCR). CD-1 mice, initially purchased from Charles River Laboratories (Wilmington, MA, USA), were used for breeding. We chose to use the outbred CD-1 mouse strain in this lactational model because these mice show superior maternal care compared to inbred strains and because we had validated them as a model for prenatal ethanol exposure (PrEE) in our prior work (El Shawa et al., 2013). Mice were housed in animal facilities located at UCR that were kept at approximately 22°C on a 12-h light/dark cycle. Mouse chow and water (for controls), or mouse chow and a 25% ethanol solution in water, were provided *ad libitum* to the dams according to the dosing schedule.

Breeding and lactational ethanol exposure paradigm

Adult female and male mice, aged P90-150, were paired just before the start of the dark cycle. Once a vaginal plug was detected, the male was removed from the cage. Throughout pregnancy, mouse chow and water were provided *ad libitum* to all dams. Dams were undisturbed through pregnancy and birth until the pups were 6 days old, when litter sizes were recorded (**Figure 1**). During this time, we pseudo-randomly assigned each dam to the control or experimental group (Lactational Ethanol Exposed, LEE group). LEE dams had their water replaced with a 25% v/v ethanol in water solution throughout the exposure period from the evening of P6 to P20, while control dams remained on water. The liquid bottle tip was placed high in the cage so that developing pups could not reach it, thus, their only liquid intake was *via* dam breast. There were no alterations to the dam's food supply through the exposure period for any experimental condition. Measurements were taken daily for maternal liquid and food consumption during the exposure period for both conditions. At wean (P20), litter size was assessed, control and LEE pups were weighed and divided into two subsets. Subsets A and B had different sacrificial end dates of P20 and P30, respectively. Subset B control and LEE pups were weighed and subjected to no more than two behavioral assays. The division of the litters into subsets allowed us to evaluate the short and long-term effects of LEE with an array of techniques. To avoid litter effects, we distributed pups from multiple litters for each assay tested.

Dam and pup blood ethanol concentration and plasma osmolality measurements

To measure dam and pup blood ethanol concentration (BEC) and blood plasma osmolality (pOsm), a measure of hydration, animals from control and LEE groups were subjected to a whole blood collection protocol. Whole blood was collected at the time of weaning for dams and pups *via* cardiac puncture. After collection, blood was placed in an untreated 1.5 ml centrifuge tube and allowed to clot for 30 min at room temperature. The entire sample was then centrifuged at 4,000 × *g* for 15 min at 4°C to separate serum from whole blood. To determine BEC in control and LEE groups, an alcohol dehydrogenase (ADH) based enzymatic assay (Pointe Scientific, Canton, MI, USA) was employed. In brief, ethanol, and nicotinamide adenine dinucleotide (NAD+) become catalyzed by ADH and this interaction causes the oxidation of ethanol to acetaldehyde and reduces NAD+ to NADH. The modified sample was read on a Nanodrop 2000 Spectrophotometer (Thermo Fisher Scientific) at 340 nm. To determine pOsm, freshly extracted serum from control and LEE groups were subjected to testing using an osmometer.

Brain tissue preparation and collection

Pups from all conditions were randomly assigned for gross anatomical studies. Mice were weighed then sacrificed using a lethal dose of sodium pentobarbital (100 mg/kg) administered *via* intraperitoneal injection. Mice were transcardially perfused with 0.9% saline followed by 4% paraformaldehyde in PBS (PFA, pH: 7.4) for fixation. The skulls were post-fixed in a 4% PFA solution overnight, then the brains were extracted, weighed, and imaged. Dorsal views of whole brains were imaged using a Zeiss (Oberkochen, Germany) Axio high-resolution (HRm) camera attached to a dissecting microscope. Extracted brains were stored in 4% PFA for later use.

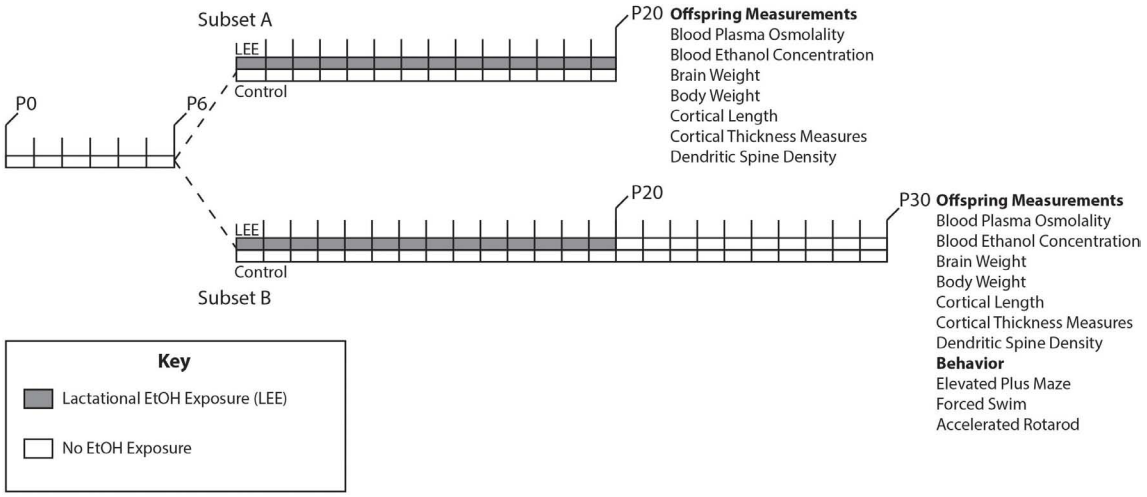

FIGURE 1
Experimental paradigm. Mice were designated as control or LEE at P6. LEE dams received 25% EtOH when pups were P6-P20. At P20 pups were weaned, divided into two subsets, and no longer exposed to EtOH. Subset A was subjected to a variety of measurements at P20. Subset B was subjected to measurements as well as behavioral tests at P30.

Anatomical measurements

Brain and body weights were assessed at P20 and P30 for both sexes and conditions. They were compared using statistical analyses and a brain/body weight ratio was computed to determine if any changes in brain or body weight were independent of one another. Typically, in normal development, brain and body size/weight are related. Larger animals within the same species tend to have larger brains. We calculated the ratio able to differentiate whether the exposure was causing a decrease in brain size alone, or whether decreases in brain size from our perturbation could be related to overall decrease in body size. Next, to measure cortical length of all brains, we used a digital micrometer in ImageJ (NIH, Bethesda, MD, USA), using the dorsal whole-brain images. To examine anatomical cortical areas, perfused brain tissues were hemisected and cryoprotected using a 30% sucrose (w:v) in PBS solution. Tissue was then sectioned using a Leica cryostat at 40 μm thick in the coronal plane, mounted on subbed slides, and stained for Nissl bodies using a 0.1% Cresyl Violet solution staining protocol then imaged using a Zeiss Axio Upright Imager microscope equipped with a Zeiss Axio HRm camera. To control for comparisons between groups, the Allen Mouse Brain Atlas (Allen Institute for Brain Science, 2004)[1] and the Paxinos Developing Mouse Brain Atlas (Paxinos et al., 2007) were used to determine matching planes of section between groups (anatomical landmarks used: corpus callosum, hippocampus, and subcortical structures). Once images were selected, regions of interest (ROIs) were measured using the ImageJ (NIH) electronic micrometer function by trained researchers blind to treatment conditions, as previously reported in Abbott et al. (2016). In brief, cortical thickness was measured with respect to the cortical sheet, by drawing perpendicular lines from the most superficial region of layer I to the deepest region of layer VI. Cortical regions measured include the frontal cortex (the boundary of layer $^{2/3}$ of the secondary motor area to boundary of layer $^{2/3}$ of the orbital area), prelimbic cortex, primary somatosensory cortex (S1), primary auditory cortex (A1), and primary visual cortex (V1).

Dendritic spine density measurements

P20 and P30 brains were hemisected and placed into a modified Golgi-Cox solution (Bayram-Weston et al., 2016; Zaqout and Kaindl, 2016) for 14 days in the dark at room temperature. Brains were then removed from the solution and placed in 30% sucrose in PBS for 2 days. Brains were then embedded in 5% agarose and sliced on a vibratome at 100 μm and mounted on subbed slides. Slides were allowed to dry for 2–3 days before developing. Slides were dipped in distilled water for 10 min, then 20% ammonia for 10 min, then distilled water for 10 min, then 70, 95, and 100% ethanol (EtOH) for 5 min each, and xylenes for 40 min. Slides were then immediately coverslipped with permount solution. Images of dendritic spines, of pyramidal cells in layer IV/V of the Prelimbic and Frontal cortices, were then imaged using a 630X oil immersion objective on a Leica Dmi8 bright field stereoscope using an attached Leica DFC 450C camera. Dendritic spine density was calculated for the entire length of the dendrites using ImageJ by an experimenter blind to condition. Counted spines were then divided by the length of the dendrite measured, then an average of dendritic spines was taken for each mouse as multiple neurons were sampled from each individual subject. In depth dendritic spine staining methodology has been previously described elsewhere (Bottom et al., 2022).

Behavioral assays

Due to higher than zero BEC levels in LEE pups at wean, behavioral assays were only performed at P30. Therefore the

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10-day post wean period was considered a "wash out" period in the paradigm. Mice were subjected to a maximum of two behavioral tests during the testing period with the forced swim test (FST) always being last due to the high-stress nature of the test. All behavioral analyses and scoring were performed and analyzed by trained researchers blind to experimental conditions. All apparatuses were cleaned using Virkon before and after each testing session.

Elevated plus maze

The elevated plus maze (EPM) has been historically employed to measure anxiety-like behaviors in rodents (Handley and Mithani, 1984; Rodgers and Dalvi, 1997). Notably, young CD-1 mice are known to contradict this measure of anxiety-like behaviors and they typically interact with the lower anxiety-associated metrics of this assay at higher portions; therefore, this behavior is thought to be considered risk-taking behavior (Macrì et al., 2002). This test has been used in our laboratory's PrEE mouse model (Bottom et al., 2022). In a dimly lit room, we employed the use of a plus "+" shaped apparatus that is designed to provide test mice with two different arm environments (arm specifications; 54 cm wide and 30 cm long). The first arm type (closed arms) shields the mouse from the testing room using 15 cm high non-transparent panels that laterally enclose the mouse, with an opening on top of the apparatus. This provides the mouse with a shaded semi-enclosed space. The second arm type (open arms) exposes the mouse to the testing room through omission of the non-transparent panels. These arm types are arranged adjacently to one another on the apparatus, such that each environment is flanked by the opposing environment. Additionally, the apparatus is lifted 50 cm above the ground using stilts. In sum, mice were subjected to a single 5-min trial on the EPM where the mouse was placed in the center of the apparatus and could move freely for the entire testing period. The amount of time spent in each arm, as well as entries and total time was recorded. Video recordings were made of each testing session. A longer time spent in the open arms may indicate increased risk-taking behavior or active exploratory behavior.

Forced swim test

Designed to assess the effects of antidepressant drugs in the late 1970s (Porsolt et al., 1978), the FST was originally used to measure depressive-like behaviors (Lucki et al., 2001). More recently, studies have re-evaluated the interpretation of the test. Mouse performance in the water (either actively swimming/attempting to climb or floating immobile) has been viewed as a response to the stressful environment; the mice could respond with a passive coping style (immobility) or an active stress-coping style (swimming/climbing). The active stress coping has also been hypothesized to be related to hyperactivity (Commons et al., 2017; Conner et al., 2020; Armario, 2021). This technique has been used in our laboratory previously in our PrEE mice (Abbott et al., 2018; Conner et al., 2020; Bottom et al., 2022). Mice were placed in an acrylic glass cylinder (30 cm in height and 12 cm in diameter) filled to two-thirds total volume with room temperature (27°C) water for 6 min. The initial 2 min were an acclimation period and the remaining 4 min (240 s) were video-recorded and the time in which the animal was immobile in the water was recorded. Mice had light placed directly above them throughout the testing period and no more than two experimenters were allowed to be present during the testing period. Percentage of time spent immobile was calculated for each mouse.

Accelerated rotarod

The accelerated rotarod (AR) test was used to examine motor ability, learning, grip strength, and coordination (Rustay et al., 2003; Buitrago et al., 2004). This test has been used in our laboratory's PrEE mouse model (Abbott et al., 2018; Bottom et al., 2022). Briefly, the mice were subjected to four, 5-min trials on the rotarod apparatus with each trial separated by a 10-min interval. The AR (Ugo Basile; Germonio, Italy) consists of a rod (diameter 28.5 mm) that rotates and gradually increases speed from 4 to 40 rpm. Mice are scored for the amount of time they are able to stay balanced on the AR. If they are able to maintain balance for the entire trial length, they are given a perfect score of 300 s.

Statistical analyses

All statistical analyses were completed using R (v4.1.2; R Core Team, 2021). Between-subjects tests were carried out using ANOVA with Type III sums of squares (*via* the car package, v3.0.12; Fox and Weisberg, 2019). Repeated measures tests were performed using multilevel models *via* the lme4 R package (v1.1.27.1; Bates et al., 2015). Planned comparisons and simple effect tests were carried out using the emmeans R package (v1.7.2; Lenth, 2022).

Results

Model verification: Blood ethanol concentration and blood plasma osmolality in dams and pups

To ensure adequate maternal intake of ethanol, we measured BEC at wean. As expected, at wean, LEE dams had significantly greater BEC when compared to control dams, $t(4) = 33.30$, $p < 0.001$ (**Figure 2**). Additionally, to assess maternal hydration during the ethanol self-administration period, we measured dam blood plasma osmolality (pOsm). No significant differences in pOsm were found between LEE and control dams at wean, $t(7.95) = 1.66$, $p = 0.1366$, suggesting similar levels of hydration in dams across conditions. Ethanol treated dams showed lower caloric consumption and body weights when compared to control dams. From P6 through P20, LEE dams consumed fewer calories from food and ethanol combined ($M = 75.1$, SD = 9.6, 95% CI [68.3, 82.0]) than control dams (from food alone; $M = 98.5$, SD = 11.6, 95% CI [87.8, 109.2]), $t(11.37) = 4.39$, $p = 0.001$. At wean, LEE dams ($M = 37.9$ g, SD = 4.4 g, 95% CI [35.5, 40.7]) weighed less than control dams ($M = 46.0$ g, SD = 4.7 g, 95% CI [42.7, 49.2]), $t(12.53) = 3.59$, $p = 0.003$. In the case of the ethanol treated dams in the current study, they engaged in higher rates of infanticide and cannibalism [from P6 through P20, more of the LEE dams' pups died ($M = 5.2$, SD = 3.3, 95% CI [3.0, 7.4]) than control dams' ($M = 1.3$, SD = 1.4, 95% CI [0.2, 2.4]), $t(14.08) = 3.49$, $p = 0.004$], which would reduce their requirements to produce milk, and, to some degree, compensate for lower food intake.

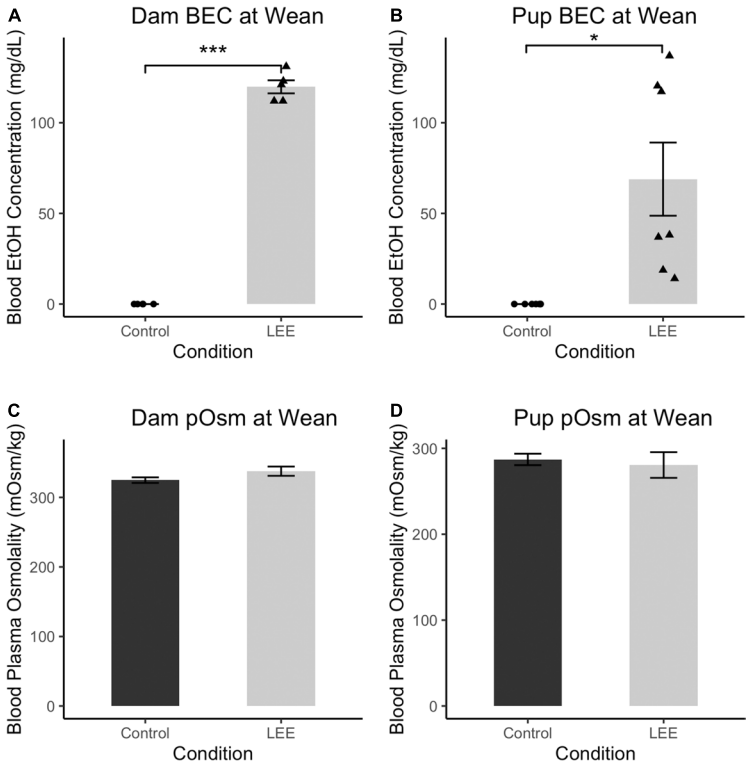

FIGURE 2
Blood ethanol concentration and pOsm measurements. **(A)** BEC measurements in Control and LEE dams at wean after a 14 day exposure to water (control) or 25% EtOH. LEE mice exposed to 25% EtOH had an average BEC of 119.8 mg/dL compared to controls which had a BEC of 0 mg/dL (N = 10). **(B)** BEC measurements in Control and LEE pups at wean after dams were exposed to water or 25% EtOH for 15 days. LEE pups had greater BECs (68.9 mg/dL on average) compared to controls at 0.0 mg/dL (N = 14). **(C)** No significant differences observed between control (M = 324.8 mOsm/kg, SD = 8.8 mOsm/kg) and LEE (M = 337.7 mOsm/kg, SD = 16.3 mOsm/kg) dam plasma osmolality (pOsm) at wean (N = 11). **(D)** No significant differences in pup pOsm at wean between control (M = 287.1 mOsm/kg, SD = 20.0 mOsm/kg) and LEE (M = 280.6 mOsm/kg) offspring (N = 16; *$p < 0.05$, ***$p < 0.001$). **(A,B)** Triangles represent individual data points taken for each experimental condition. Data expressed as mean ± SEM.

Lactational ethanol exposure pups at wean, as anticipated, had greater BEC than control pups, $t(6) = 3.41$, $p < 0.014$, although considerable variation was observed between individual measures. We endeavored to account for this variation by examining the relationship between both litter size and pups' sex on LEE pups' BEC at wean. Neither litter size [$t(6) = 0.34$, $p = 0.742$] nor sex [$t(6) = 0.49$, $p = 0.642$], however, was a significant predictor of BEC. Nevertheless, additional possible explanations for the increased variability are discussed in the section on study limitations and future directions.

There were no significant differences in blood plasma osmolality (pOsm) found between LEE and control pups at wean, $t(8.38) = 0.40$, $p = 0.700$, also suggesting similar levels of hydration in pups across conditions. These results confirm that non-zero levels of EtOH intoxication occur in LEE dams and pups at wean. Furthermore, these results indicate no disparity in dam or pup pOsm due to the exposure paradigm.

P20 and P30 pup gross measurements

To examine the ability of our exposure paradigm to produce gross alterations in pup central nervous system (CNS), and overall development, we evaluated body and brain weights, body-brain weight ratio (Figure 3), and cortical length measurements (Figure 5) at P20/P30 and by sex.

A three-way, condition (Control vs. LEE) × age (P20 vs. P30) × sex (male vs. female) ANOVA (Type III SS) identified a three-way condition × age × sex interaction on pups' weight, $F(1,302) = 4.22$, $p = 0.0409$ (Figure 3). In light of this three-way interaction, lower order interactions and main effects should be considered with caution. Nevertheless a two-way age × sex interaction was also present, $F(1,302) = 31.02$, $p < 0.001$, as was a main effect of condition, $F(1,302) = 58.45$, $p < 0.0001$, and age $F(1,302) = 434.23$, $p < 0.0001$. To examine the three-way interaction, the two-way condition × age interactions were examined separately for male and females. For males, the two-way interaction was significant, $F(1,302) = 12.36$, $p = 0.005$, indicating that the effect of condition was greater at P30 than at P20. For females, the two-way interaction failed to reach significance, $F(1,302) = 0.22$, $p = 0.637$. Sidak corrected planned comparisons were carried out to examine the difference between the weight of the control and LEE pups at each combination of age and sex. These indicated that control pups weighed more in all four combinations: P20 male, $t(302) = 7.343$, $p < 0.0001$, P30 male, $t(302) = 10.602$, $p < 0.0001$, P20 female, $t(302) = 7.646$, $p < 0.0001$, P30 female, $t(302) = 6.296$, $p < 0.0001$.

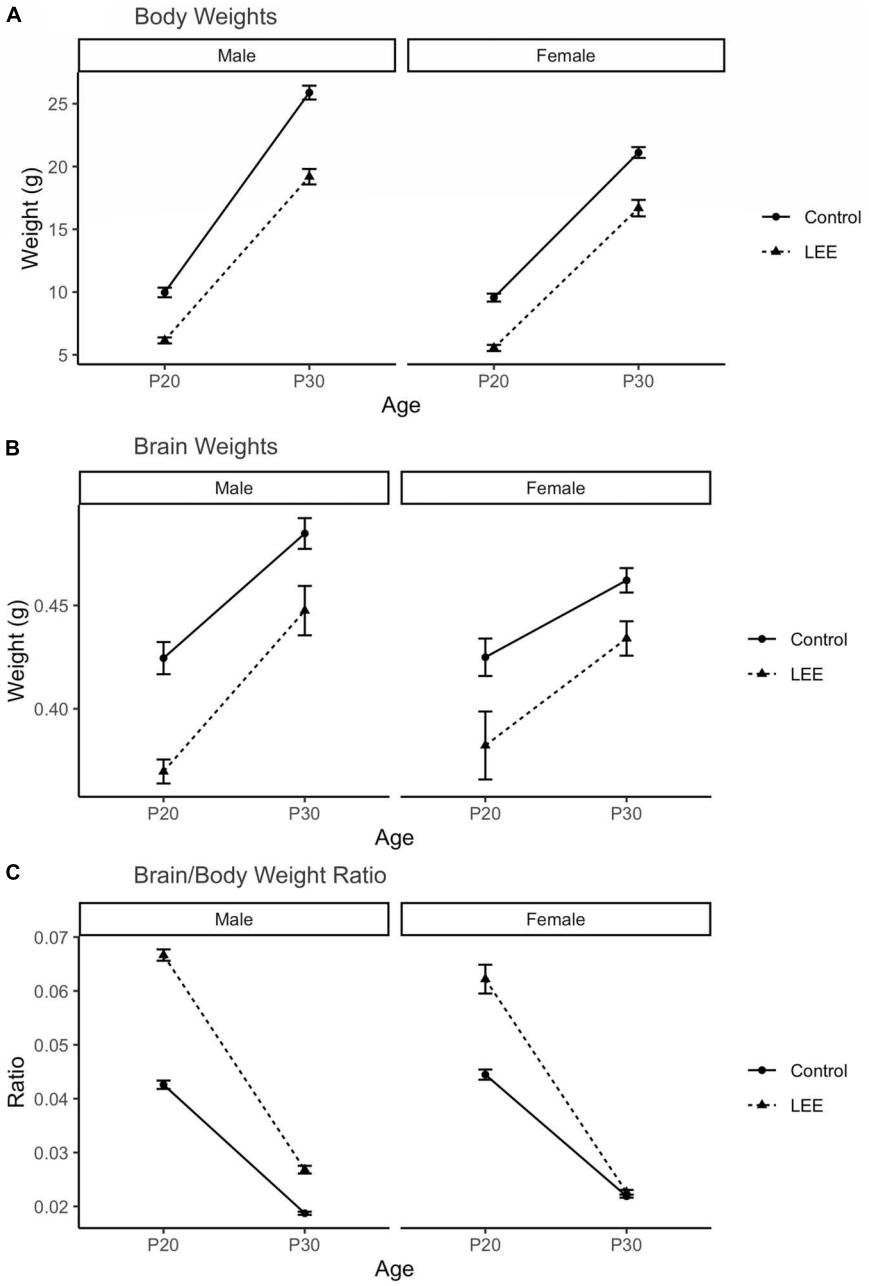

FIGURE 3
Offspring measures: Gross development. **(A)** A significant ($p < 0.001$) reduction in body weight for LEE pups was observed in every age and sex group, when compared to controls ($N = 310$). **(B)** Significant reductions in brain weights were observed for LEE males at P20 ($p = 0.003$) and P30 ($p = 0.0332$) developmental time points. However, significant reductions were only observed in P20 LEE females ($p = 0.0085$) and no significance is observed in P30 LEE females ($p = 0.1184$) compared to controls ($N = 70$). **(C)** Significant increases to the brain/body ratio are observed in LEE males at both developmental time points. Significant increases to the brain/body ratio were only observed in P20 LEE females and not P30 females as compared to controls ($N = 70$). Data expressed as mean ± SEM.

Next, a three-way, condition (Control vs. LEE) × age (P20 vs. P30) × sex (male vs. female) ANOVA (Type III SS) identified main effects of condition, $F(1,62) = 10.26$, $p = 0.002$, and age $F(1,62) = 9.12$, $p = 0.004$ on the weight of pups' brains. As described previously, Sidak corrected planned comparisons were carried out to examine differences between control and LEE pups weights at each combination of age and sex. Results indicated that control pups' brains weighed more in three of the four combinations: P20 male, $t(62) = 4.24$, $p = 0.0003$, P30 male, $t(62) = 2.72$, $p = 0.0332$, and P20 female, $t(62) = 3.20$, $p = 0.0085$, but not P30 female, $t(62) = 2.207$, $p = 0.1184$.

Lastly, to consider the relationship between body and brain weight, we examined the ratio of pups' brain to body weight *via* a three-way, condition (Control vs. LEE) × age (P20 vs. P30) × sex (male vs. female) ANOVA (Type III SS). This analysis identified main effects of condition, $F(1,62) = 128.75$, $p < 0.001$, and age $F(1,62) = 243.95$, $p < 0.0011$ on the on the ratio of pups' brain to body weight. These main effects, however, should be considered in

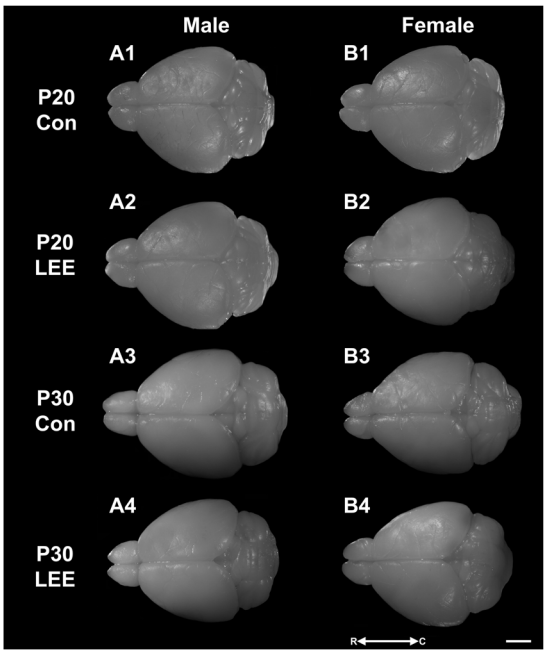

FIGURE 4
Dorsal views. Representative images of perfused and extracted brains of male **(A1–A4)** and female **(B1–B4)** pups at P20 **(A1,A2,B1,B2)** and P30 **(A3,A4,B3,B4)** after dams were exposed to water **(A1,B1,A3,B3)** or EtOH **(A2,B2,A4,B4)** for 14 days. Images oriented rostral (R) to the left and caudal (C) to the right. Scale bar, 1 cm.

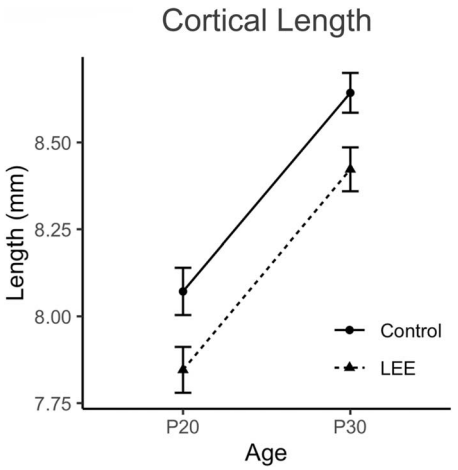

FIGURE 5
Cortical lengths. No significant differences in cortical length were observed between control and LEE pups at P20 or P30 (N = 70). Data expressed as mean ± SEM.

light of interactions between age and condition, $F(1,62) = 61.82$, $p < 0.001$, and sex and condition, $F(1,62) = 8.47$, $p = 0.005$. The age × condition interaction provided evidence that the effect of exposure to EtOH diminished between P20 ($M = 0.022$, 95% CI [0.018, 0.023]) and P30 ($M = 0.004$, 95% CI [0.002, 0.007]), and the sex × condition interaction provided evidence that the effect of exposure to EtOH was greater for male ($M = 0.016$, 95% CI [0.014, 0.019]) than female ($M = 0.009$, 95% CI [0.007, 0.012]) pups. As described previously, we carried out Sidak corrected planned comparisons to examine the difference between the ratio of pups' brain to body weight in the control and LEE pups at each combination of age and sex. These indicated that LEE pups' brain-body weight ratio was greater in three of the four combinations: P20 male, $t(62) = 15.88$, $p < 0.001$, P30 male, $t(62) = 5.03$, $p < 0.001$, and P20 female, $t(62) = 11.35$, $p < 0.001$, but not P30 female, $t(62) = 0.49$, $p = 0.981$.

To examine cortical length (Figures 4, 5), we performed a three-way, condition (Control vs. LEE) × age (P20 vs. P30) × sex (male vs. female) ANOVA (Type III SS) that identified main effects of condition, $F(1,68) = 5.52$, $p = 0.022$, and age $F(1,68) = 12.69$, $p = 0.001$ on the length of pups' brains. As described previously, Sidak corrected planned comparisons were carried out to examine the difference between the weight of the control and LEE pups at each combination of age and sex. None of these comparisons indicated a significant difference between the lengths of control and LEE pups' brains: P20 male, $t(68) = 1.00$, $p = 0.7860$, P30 male, $t(68) = 2.27$, $p = 0.1015$, P20 female, $t(68) = 2.35$, $p = 0.0843$, and P30 female, $t(68) = 1.37$, $p = 0.5370$.

Altogether, these results suggest that our exposure paradigm produces long-lasting gross alterations in CNS and general development in the LEE pups.

P20 and P30 pup cortical neuroanatomical measurements

To assess the effects of the exposure paradigm on cortical thickness development, we measured from five distinct regions (frontal, prelimbic, somatosensory, auditory, and visual cortices) in Nissl-stained coronal sections in both LEE and control pups at both milestone dates (Figures 6, 7). We carried out three-way condition (Control vs. LEE) × age (P20 vs. P30) × sex (male vs. female) ANOVAs (Type III SS) on the cortical thicknesses of pups' brains in each region. In these analyses, none of the main effects or interactions were significant although the main effect of age in the visual cortex trended toward greater thickness at age P30 ($M = 0.664 \pm 0.0173$) than at age P20 ($M = 0.590 \pm 0.0171$), $F(1,33) = 3.22$, $p = 0.0820$. The corresponding Sidak-corrected planned comparisons we carried out to examine the difference between the cortical thickness in the control and LEE pups at each combination of age and sex also failed to show significant differences with the exception of the frontal cortex in the P20 male pups, $t(34) = 2.94$, $p = 0.0235$ (Figure 6A5).

These results suggest that there were only modest alterations to frontal cortical thickness in the development of the LEE mice.

Dendritic spine measurements

An analysis on dendritic spine density (spines/um) was employed to explore the impact of our exposure paradigm on spine density at both milestone dates *via* Golgi-Cox-stained coronal sections (Figures 8, 9). Because we measured spinal density on multiple dendrites from individual mice, the data were analyzed using a multilevel model in which condition (Control vs. LEE) × age (P20 vs. P30) × sex (male vs. female)

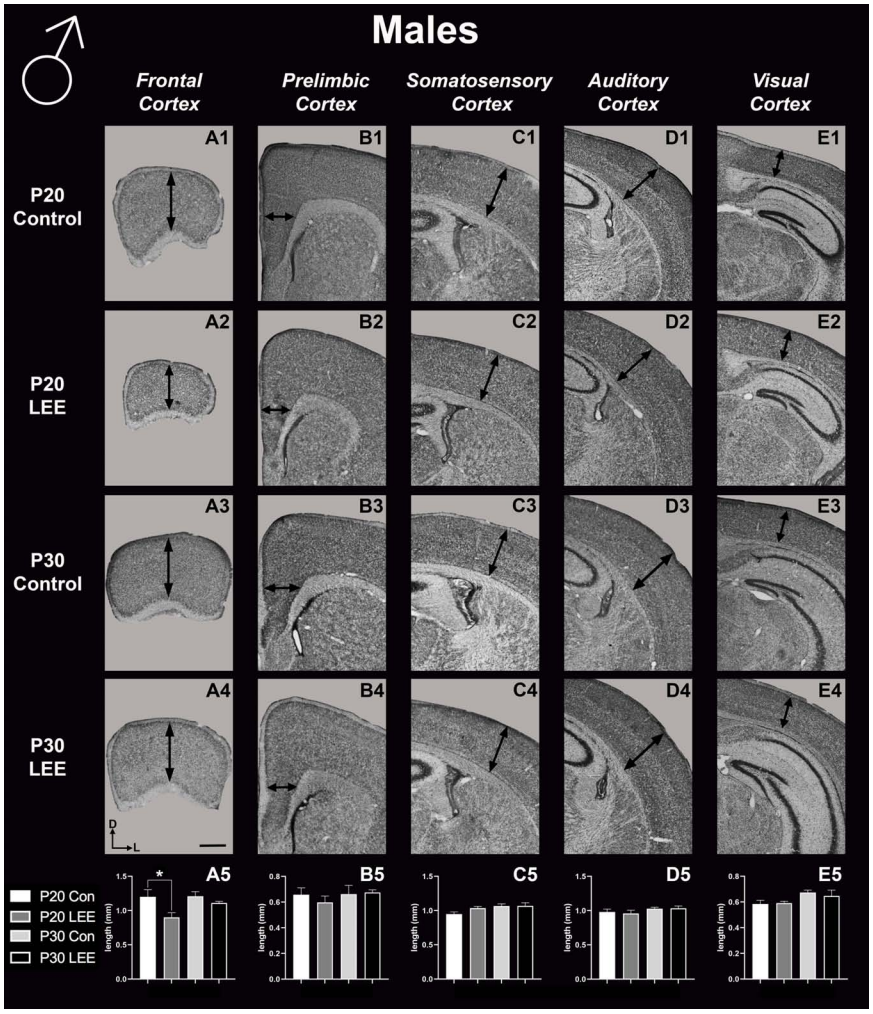

FIGURE 6
Cortical thickness measurements – males. High magnification coronal sections of Nissl-stained hemisections. Measurements include frontal cortex (**A1–A5**; *N* = 21), prelimbic cortex (**B1–B5**; *N* = 16), somatosensory cortex (**C1–C5**; *N* = 22), auditory cortex (**D1–D5**; *N* = 19), and visual cortex (**E1–E5**; *N* = 28). No significant differences between control and LEE males, except in the frontal cortex at P20 (A5, $p = 0.0235$). Data expressed as mean ± SEM. Images oriented dorsal (D) up and lateral (L) to the right. *Indicates $p < 0.05$. Scale bar, 1 mm.

were fixed factors and mouse was included as a random factor. In prelimbic cortex, this analysis indicated a main effect of sex on spinal density (male, $M = 0.662$ spines/μm ± 0.0305; female, $M = 0.719$ spines/μm ± 0.0304), $t(26.24) = 2.26$, $p = 0.0326$. There was also a trend toward an effect of condition (control, $M = 0.708$ spines/μm ± 0.0307; LEE, $M = 0.673$ spines/μm ± 0.0302), $t(23.73) = 2.05$, $p = 0.0517$, and an interaction between sex and condition (male LEE – control, $M = 0.0308$ spines/μm ± 0.0610; female LEE – control, $M = -0.1011$ spines/μm ± 0.0607), $t(24.97) = 1.73$, $p = 0.0954$. Sidak-corrected planned comparisons failed to show significant differences between the spinal densities of neurons in the prelimbic cortex of control and LEE pups for either male or female pups at either age. In frontal cortex, this analysis did not indicate any significant effects or interactions, nor did any of the planned comparisons show significant differences at any combination of sex and age. Overall, these results suggest a possible modest difference between the experimental group and controls moderated by sex in prelimbic cortex, but provided no evidence of differences in dendritic spine density in frontal cortex.

P30 behavioral analyses

To assess the impact of the exposure paradigm on behavioral development, we employed a number of behavioral tests to investigate potential differences. The included tests were: EPM, FST, and AR.

The EPM provides a measure of anxiety-like and risk-taking behaviors. We investigated the risk-taking behaviors by recording the percent of time mice spent in the open arms of the maze (Figure 10). A two-way, condition (Control vs. LEE) × sex (male vs. female) ANOVA (Type III SS) failed to identify a significant effect of condition or sex on the time pups spent in the open arms of the maze. There was, however, a trend toward LEE pups (23.0 ± 1.63%) spending more time in open arms than control pups (17.2 ± 1.59%), $F(1,37) = 3.40$, $p = 0.0733$ (Figure 10A). Sidak

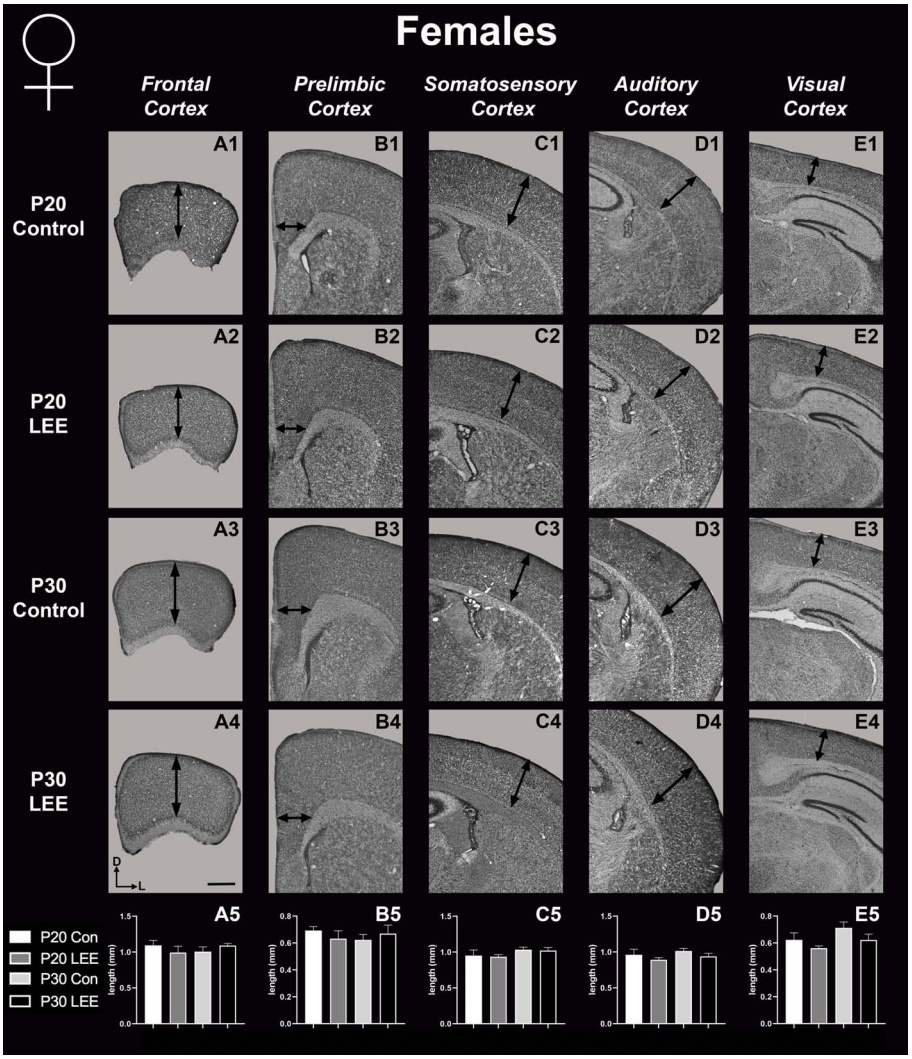

FIGURE 7
Cortical thickness measurements – females. High magnification coronal sections of Nissl-stained hemisections. Measurements include frontal cortex (**A1–A5**; *N* = 19), prelimbic cortex (**B1–B5**; *N* = 11), somatosensory cortex (**C1–C5**; *N* = 26), auditory cortex (**D1–D5**; *N* = 20), and visual cortex (**E1–E5**; *N* = 20). No significant differences between control and LEE pups. Data expressed as mean ± SEM. Images oriented dorsal (D) up and lateral (L) to the right. Scale bar, 1 mm.

corrected planned comparisons were carried out to examine the difference between the percent of time the control and LEE pups spent in open arms for male and female pups separately. These comparisons similarly failed to indicate significant differences (Figure 10B). The results suggest that LEE mice may spend more time on the uncovered arms of the apparatus compared to controls, regardless of sex (Figure 10A) suggesting the possibility of increased risk-taking behavior.

In the FST, immobility may be understood as a measure of passive coping behavior. A two-way, condition (Control vs. LEE) × sex (male vs. female) ANOVA (Type III SS) failed to identify a significant effect of condition or sex on the percent of time each mouse was immobile. Sidak corrected planned comparisons were carried out to examine the difference between the percent of time the control and LEE pups spent immobile for male and female pups separately. Here, it was found that male LEE pups spent less time immobile than male control pups, $t(30) = 3.31$, $p = 0.0049$ (Figure 11A). For female pups, however, the difference between the time spent immobile in the two groups was not significant, $t(30) = 1.31$, $p = 0.3588$.

The AR test measures motor ability, balance, coordination, and learning through repeated measures. Because the AR task extends across four trials for each mouse, the data were analyzed using a multilevel model in which condition (Control vs. LEE) × sex (male vs. female) × trial (1–4) were fixed factors, and mouse was included as a random factor. The analysis indicated a main effect of trial, $\chi^2(3) = 104.67$, $p < 0.001$. Planned polynomial contrasts showed significant linear [$t(114) = 3.54$, $p = 0.0006$] and quadratic [$t(114) = 2.15$, $p = 0.0338$] effects of trial as well as a three-way interaction between condition, sex, and the quadratic trial contrast [$t(114) = 2.07$, $p = 0.0405$]. This interaction can be understood by considering the pattern of the effect of condition across trials for males (trial 1 $M = 75.76$, 95% CI [−4.29, 155.81]; trial 2 $M = -74.39$, 95% CI [−154.44, 5.66]; trial 3

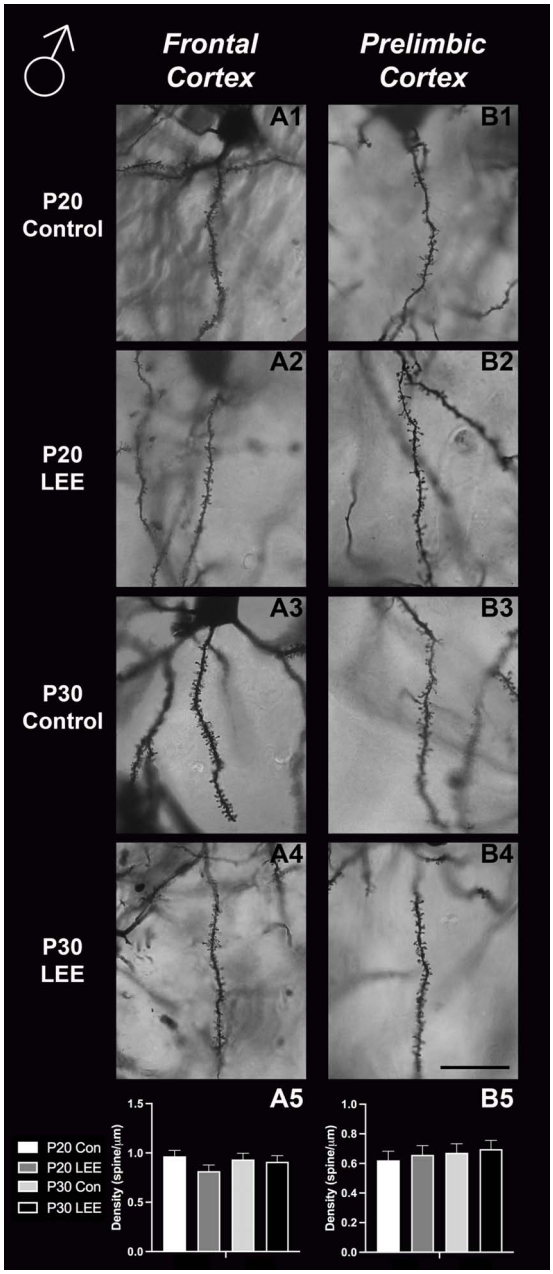

FIGURE 8
Dendritic spine density – males. Representative images of secondary dendrites of pyramidal cells in layers 4/5 of the frontal and prelimbic cortices of male control **(A1,B1,A3,B3)** and LEE **(A2,B2,A4,B4)** pups at P20 and P30. Comparison of dendritic spine density of males indicated no significant differences in frontal (**A5**; $N = 14$) and prelimbic (**B5**; $N = 16$) cortices. Data expressed as mean ± SEM. Scale bar, 250 µm.

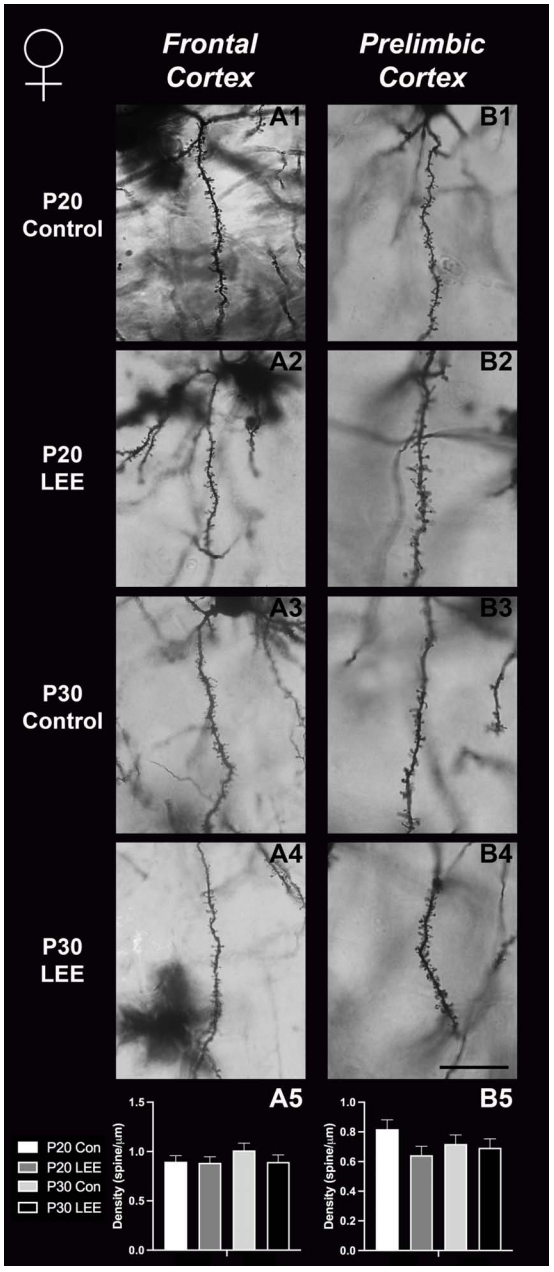

FIGURE 9
Dendritic spine density – females. Representative images of secondary dendrites of pyramidal cells in layers 4/5 of the frontal and prelimbic cortices of female control **(A1,B1,A3,B3)** and LEE **(A2,B2,A4,B4)** pups at P20 and P30. Comparison of dendritic spine density of males indicated no significant differences in frontal (**A5**; $N = 16$) and prelimbic (**B5**; $N = 16$) cortices. Data expressed as mean ± SEM. Scale bar, 250 µm.

$M = -31.77$, 95% CI [−111.82, 48.28]; trial 4 $M = -29.40$, 95% CI [−109.45, 50.65]), and for females (trial 1 $M = 12.576$, 95% CI [−71.04, 96.17]; trial 2 $M = 3.48$, 95% CI [−80.13, 87.09]; trial 3 $M = 35.05$, 95% CI [−48.56, 118.66]; trial 4 $M = -3.58$, 95% CI [−87.19, 80.03]). Other main effects and interactions did not reach significance (Figures 11B, C). Additional Sidak corrected planned comparisons between adjacent trials within each combination of sex and condition yielded significant differences between trials 1 and 2 for Control, $t(114) = 2.68$, $p = 0.0417$, and LEE, $t(114) = 2.97$, $p = 0.0191$, females (Figure 11C) and for LEE males, $t(114) = 6.61$, $p < 0.0001$ (Figure 11B).

Overall, these data suggest that our exposure paradigm generates behavioral aberrations at P30 including increased risk-taking behaviors in LEE mice regardless of sex as well as abnormal stress regulation, active stress-coping styles and/or hyperactivity in male LEE mice.

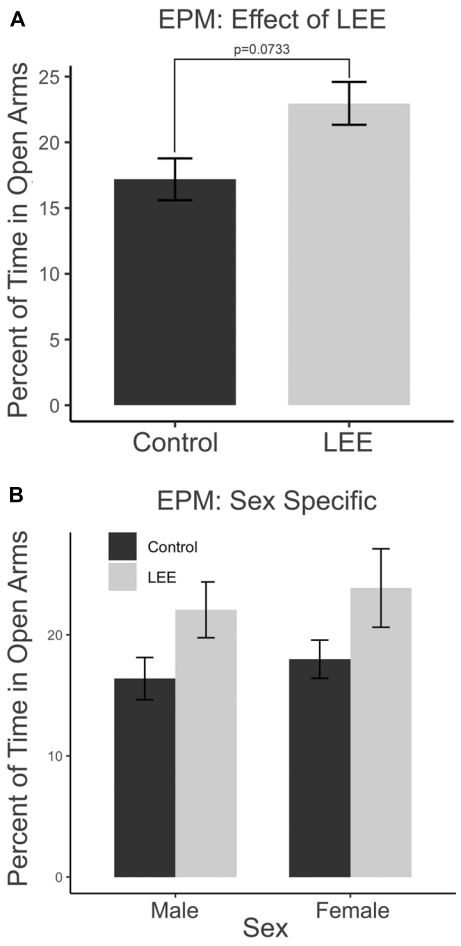

FIGURE 10
Behavioral assays at P30: EPM. **(A)** No significant differences in time spent in the open arms of the EPM when evaluated by sex and a marginal effect of condition (p = 0.07). **(B)** No effects observed in planned comparisons for male and female pups (N = 41). Data expressed as mean ± SEM.

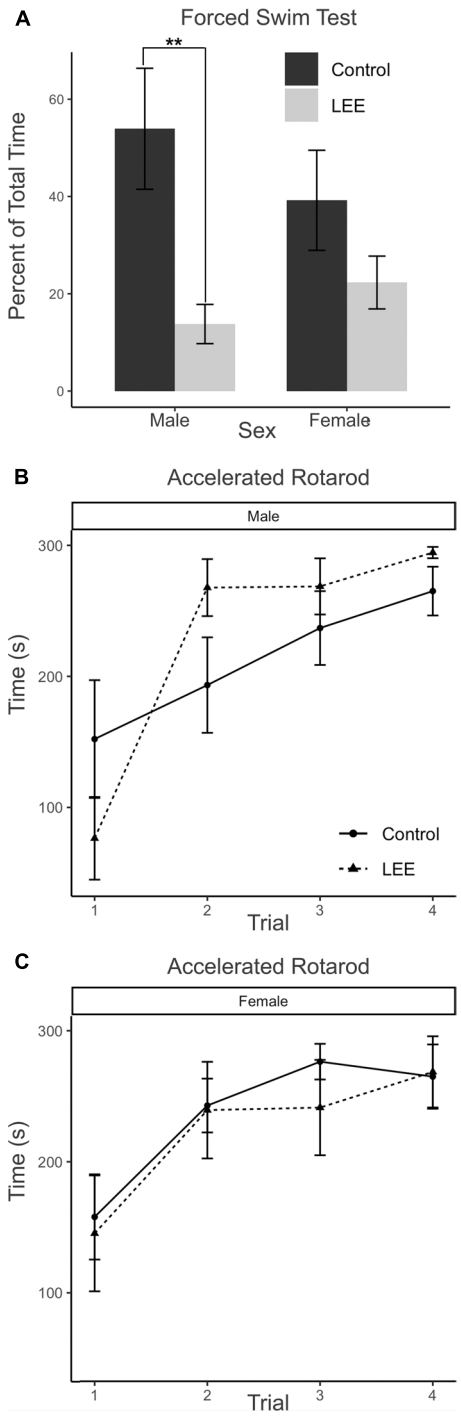

FIGURE 11
Behavioral assays at P30: FST and AR. **(A)** Male LEE pups (p = 0.0049) spent less time immobile than male controls in the FST. No significant differences in time spent immobile for females (p = 0.3588) on the FST (N = 34). No significant differences in performance on the AR for males **(B)** or females **(C)** (N = 42). Data expressed as mean ± SEM.

Discussion

Fifty years ago, several physicians at the University of Washington Medical School studied a small group of children who exhibited a particular set of developmental delays. The commonality among the children was that they were all born to alcoholic mothers. This was the first of many studies that aimed to identify and understand the condition that would be later known as Fetal Alcohol Syndrome (FAS) (Jones et al., 1973). Our laboratory has studied the effects of PrEE for over 10 years now and although we have gained insight on FAS, or its spectrum disorder, FASD, our work was limited to prenatal exposures. Unfortunately, maternal alcohol consumption may continue during pregnancy, or if the mother abstained from drinking while pregnant, it may begin in the early postnatal period. Many new mothers report that after 9 months of abstinence, they begin to drink again after the baby is born (Jagodzinski and Fleming, 2007). The advice by physicians for drinking alcohol while breastfeeding is quite variable, and this presents a possible health issue for infants of drinking mothers. In fact, the CDC warn against heavy drinking during breastfeeding but suggest that "moderate consumption of alcohol" is not harmful to offspring (Centers for Disease Control and Prevention, 2022b). Compared to research on prenatal alcohol exposure, studies examining the effects of maternal drinking during lactation are mostly limited to epidemiological reports

with a paucity of papers in animal models where changes in the developing nervous system are investigated. Thus, we developed a novel postnatal alcohol exposure model in breastfeeding mice, using the murine strain utilized in our PrEE studies. In this LEE model, we demonstrate that maternal consumption of alcohol while breastfeeding can induce gross developmental deficits in LEE pups including decreased body weights, brain weights, and cortical lengths. Additionally, we discovered some sex-specific, LEE-related phenotypes in the neuroanatomy of the frontal lobe and prelimbic cortex, as well as behavioral deficits in stress-coping styles and risk-taking behaviors in LEE offspring. Our findings that postnatal, indirect ethanol exposure (as modeled by our lactational experimental paradigm) can negatively impact various aspects of development represents an important advancement in solidifying the significance of conscientious, informed parental care.

A novel murine lactational ethanol exposure model: Impact of LEE on gross anatomical changes in offspring

Our results suggest that ethanol exposure *via* lactation is correlated with reduced body weights in both males and females at P20 and 30. These findings are consistent with human studies where children exposed to ethanol through contaminated breast milk can have consistently lower body weights and growth trajectories (May et al., 2016). Although there is a paucity of rodent data on offspring outcomes after ethanol exposure *via* lactation, a study from Vilaró et al. (1987) reported a reduction in body weight of ethanol-exposed rats after a period of maternal ethanol consumption while nursing her pups. In terms of brain size and morphology, we find some sex-specific effects of LEE in our model. Specifically, while LEE males show sustained low brain weights compared to controls at P20 and P30, LEE females only show deficits in brain weights at P20, with recovery to control weights by P30. Thus, LEE females show a faster rate of recovery when compared to males.

Few rodent models have examined brain weight changes in LEE mice; however, one study reported a decrease in weights of the forebrain, cerebellum, and brainstem in alcohol treated pups (Chen et al., 1998). When examining PrEE paradigms, sustained reductions in body weight and brain weights are observed from P0 to P50 in mice, consistent with findings in LEE offspring (Abbott et al., 2016; 2018). This suggests that LEE and PrEE may impact brain and body growth through similar mechanisms.

Considering the sustained growth retardation in PrEE and LEE mice, the reduction of body and brain weights might be due to the gut's inability to efficiently extract nutrients when alcohol is ingested. Acute and chronic ethanol administration results in a reduction of protein synthesis in the small intestine (Rajendram and Preedy, 2005) and can block absorption of micro- and macronutrients (Seitz and Homann, 2001; Seitz and Suter, 2002). Additionally, nutrient deficiency has the potential to manifest in epigenetic changes, as seen in the populations affected by the Dutch Hunger Winter (Dutch Famine) (Heijmans et al., 2008). We found that, in our PrEE model, epigenetic modifications occurred *via* changes in DNA methylation, which led to epigenetic and heritable phenotypes spanning three generations of mice (Abbott et al., 2018). It is possible that examination of epigenetic markers in LEE mice could provide further insight into mechanisms underlying LEE-induced phenotypes.

Impact of LEE on cortical length

In mammals, much of our sophisticated behavior, including language, sociability, decision making, and even fine motor skills and coordination, originates with complex functions of cells within the neocortex. In FASD or other alcohol-induced conditions, the abnormal phenotypes in humans are often related to presumed dysfunction within the neocortex (El Shawa et al., 2013). Thus, we chose to focus our study of the novel LEE model on development of the neocortex and the behaviors that are mediated, to some extent, by its function. To begin, we measured cortical length at both P20 and P30 ages in male and female LEE and control mice. We found that while the cortex expanded in length significantly from P20 to P30 in all mice, LEE cortices remained consistently smaller, regardless of sex. Few rodent models have examined the impact of LEE on cortical development, and, to our knowledge, there are no studies that specifically measure cortical length after LEE. Similarly, studies from our laboratory demonstrated a reduction in cortical length in PrEE mice (El Shawa et al., 2013; Abbott et al., 2018). As the cortex continues to grow and develop from birth to puberty in mice, we posit here that alcohol exposure *via* lactation may lead to apoptosis, increased oxidative stress, and interference with the activity of growth factors as is suggested for prenatal exposures (Goodlett and Horn, 2001).

Neocortical thickness

In mice, neocortical lamination is present by around P5, when barrels become apparent in later IV of somatosensory cortex. According to a comprehensive set of papers from our laboratory, the areal patterning period ends around this time, P5–6, when cortical areas have adult-like connections and lamination. Beyond P6, cortical thickness continues to increase, although the changes are minimal (Dye et al., 2011a,b). Here, we measured cortical thickness across several neocortical sensory and motor regions at P20 and P30 in LEE and control mice. Given that the frontal cortex develops later than other cortical regions, and that the time of exposure is after the areal patterning period closes, it is not surprising that the only LEE-related phenotype we found was a reduction in cortical thickness in the frontal cortex of P20 LEE males. This phenotype was recovered by P30 in the LEE male mice. Subsequent measurements in prelimbic, somatosensory, auditory, and visual cortices, at both milestone dates, produced no observable differences. Few rodent models have examined the effects of LEE on neocortex, and to our knowledge there are no studies that examine cortical thickness changes after LEE. There are, however, reports of alcohol-induced changes in cortical thickness measures after PrEE. Our laboratory demonstrated changes spanning from birth to P50 in cortical thickness measures in the brains of PrEE mice (Abbott et al., 2016). PrEE models impact cortical thickness at a higher extent due to exposure during gestation, as this is the primary time when the cortex develops layer-specific organization of cell types

and matures from a simply organized, single layer to a complex 6-layered structure. As the lactational exposure occurs after cortical areas subdivision and lamination, the exposure timing may be too late in development to induce significant changes in neocortical thickness.

LEE and dendritic spine densities in frontal cortex

Through Golgi-Cox staining we aimed to evaluate the impact of LEE on dendritic spine densities, as ethanol exposure has the potential to alter synaptogenesis (Adams et al., 2022) and synaptic pruning (Kyzar and Pandey, 2015; Kyzar et al., 2016). In typically developing mice, cortex wide synaptic pruning has been reported to reach its peak 14–21 days postnatal (Lewis, 2011). In early alcohol exposure models, acute exposures led to increased dendritic pruning in the prefrontal cortex, resulting in significant synapse loss (Socodato et al., 2020). Also, acute ethanol exposure during synaptogenesis (from P5 to P7) led to drastically decreased spine densities in the caudate/putamen, however, these densities recovered to normal levels by around P30 (Clabough et al., 2022).

Here, we exposed mice to ethanol *via* lactation within this postnatal sensitive period and conducted intensive spine counts in frontal lobe ROIs in male and female mice, aged P20 and P30. While we did not find any significant changes in our measured frontal cortex spine densities, we did find a trend toward significance for prelimbic cortex (a subregion of the medial prefrontal cortex) between LEE and control mice. There were no age- or sex-dependent effects observed, but the overall reduction in spine densities observed in the prelimbic cortex of LEE mice could impact later development, and this could be possibly caused by ethanol-induced impairment to synaptogenesis or to increased synaptic pruning as the insult take places during a sensitive period for both. Of note, whether spine densities in the prelimbic cortex decrease or increase is age dependent (Galaj et al., 2020); however, alterations due to alcohol exposure have been associated with altered behavior regardless of the direction of change (Fox et al., 2020). This is not surprising given that the prelimbic cortex is a region shown to play a role in alcohol-drinking reinforcement (Engleman et al., 2020). These data are consistent with other brain areas (basal ganglia) where reductions in spine densities observed immediately after exposure seemed to reverse by 1 month of age (Clabough et al., 2022). It is possible that alterations occurred in synaptogenesis and/or pruning earlier in the exposure period and recovered by weaning when the first measures were taken.

Impact of LEE on behavioral development

While it is important to uncover changes in the developing nervous system that are associated with ethanol exposure through lactation, understanding the potential behavioral effects of the postnatal exposure is critical. In our current study we implemented a battery of behavioral assays to examine LEE's effect on behavioral development. The EPM is a classic way to measure anxiety in rodents (Walf and Frye, 2007). However, researchers have also looked beyond the initial interpretation of the EPM and created alternative hypotheses about how time spent in open arms versus closed arms can be interpreted. Most importantly, if an animal spends more time in the open arm, it may indicate increased risk taking or increased exploratory behavior (Macrì et al., 2002, Kozanian et al., 2018). Also, as alcohol exposure impacts fear memory learning, affecting an animal's ability to learn a natural fear response, increased time in open arms could be from inhibited fear learning, as was observed in our PrEE model (Kozanian et al., 2018). Here, we found that, overall, LEE mice spent a significantly longer time in open arms when compared to control mice, without sex-specific effects. This suggests that exposure to ethanol *via* lactation may increase risk taking or exploratory behavior. This is consistent with exposure to ethanol *via* lactation in humans, as May et al. (2016) found that LEE children exhibited phenotypic variability consistent with FASD, with increased risk taking and cognitive deficits often present in children with FASD (Fast and Conry, 2009).

A hallmark of FASD and alcoholism is depression (Pei et al., 2011; Kuria et al., 2012) and the FST is a classic test used to detect depressive-like behaviors in animal models (Lucki et al., 2001). Like the EPM, behavioral results associated with the FST have been interpreted differently over time in the literature. Specifically, the FST test has been a successful method used to test for the effects of antidepressant drugs in that they increase the animal's activity in the swim well (Porsolt et al., 1978). Researchers who use the test for other model systems have identified that time immobile may represent a more complex measure than simple depressive behaviors. How the animal responds to being in the swim well, with floating (immobility) or active swimming/climbing can be viewed as different adaptive reactions to the stressful environment. For example, Armario (2021) determined that mice react according to their coping style, either passively or actively, and that the FST may be a more accurate measure of coping style rather than behavioral despair. This may also be correlated with hyperactivity or possibly response to fearful stimuli. Here, we found that LEE males demonstrated reduced time immobile when compared to control males in this task, with the effect not observed in female LEE mice. This indicates that LEE may cause abnormal stress regulation and hyperactivity in males, consistent with findings in humans with FASD (Hellemans et al., 2008). For example, alcohol compromised breast milk has been found to have an activating effect in humans, as behavioral states of infants showed increased variability, such as spending less time in quiet sleep and increased crying (Schuetze et al., 2002). It is also possible that increased time spent immobile during the FST for male LEE mice could indicate alteration in fear responsivity, as we showed abnormal fear learning in our FASD model mice (Kozanian et al., 2018). This behavioral phenotype may be related to reduced frontal lobe thickness in males (**Figure 6**), as the frontal cortex is likely to be involved in depression (Zhang et al., 2018) and fear responsivity (Gilmartin et al., 2014).

The AR test measures motor ability, balance, coordination and learning through repeated measures. Previously, we found that rotarod performance was altered in PrEE mice; specifically, first generation PrEE mice showed deficits in performance in the first two trials compared to controls at both P20 and P30 (Abbott et al.,

2018; Bottom et al., 2022). Additionally, postnatal alcohol exposure in rats can impact AR performance (Goodlett et al., 1991; Cebolla et al., 2009). In our LEE model, male LEE mice showed increased variability in performance in trials 1–2. Specifically, the change in performance was appreciably different from controls: the male LEE mice performed worse on trial 1 but showed a significantly greater degree of improvement between trials 1 and 2. After training, LEE mice performed similar to controls on the AR. In summary, male LEE mice show a greater deficit in trial 1 and showed an abrupt learning profile that differs significantly from both controls and female LEE mice.

Collectively, our results from our behavioral studies suggest LEE may impact offspring in ways similar to prenatal exposures, with increased risk-taking, hyperactivity, active stress-coping responses to environmental stressors, and transient deficits in motor coordination. Additionally, some of these LEE-induced deficits may be sex-specific.

Critical periods, pubescence, and plasticity

Developmental critical periods are described as times when systems are "plastic" or open to change from environmental experience, such as with learning, or insult, such as with early alcohol exposure. For brain development, these are precise time points where neuronal plasticity is heightened and cortical circuits are particularly susceptible to regulation by specific sensory modalities (Jeanmonod et al., 1981). Initial explorational work in somatosensory cortical reorganization found that the removal of mouse vibrissae at birth resulted in an absence of the associated barrels (Van der Loos and Woolsey, 1973). Since then, studies have refined these events and have assigned a critical period range (first week of life in mice) for proper barrel formation (Lo et al., 2017). Additionally, the critical period for the visual system has been extensively studied. A literature review from Hooks and Chen (2007), places the critical period prior to eye opening in mice, at P0–P10. Perturbations in this period may alter cortical retinotopic maps (Hooks and Chen, 2007) along with gene expression and intra neocortical connections (Dye et al., 2012). How perturbations, insults, or changes in input impact a developing animal depends on the critical period for development in the relevant system. If events occur after closure of a critical period, the animal may be protected from detrimental harm. Unfortunately, if these events occur outside the critical period, the ability of the brain to repair itself with plasticity mechanisms may also be reduced. Understanding critical periods when comparing the impact of prenatal versus postnatal alcohol exposure, on the developing nervous system, is critical.

Compared to the effects of prenatal alcohol exposure in our mouse model of FASD, LEE has more mild phenotypes associated with the exposure, although the changes we observed in our LEE mice could have debilitating consequences if mimicked in human systems. The difference in severity of outcomes between PrEE and LEE is possibly related to critical periods for development. As described previously, much of cortical development (lamination, arealization) in the mouse reaches an adult-like state by the first postnatal week, whereas during the prenatal period and the first few days of life, the developing brain is very susceptible to change. Thus, LEE animals may be somewhat protected, when compared to PrEE, from the more severe effects of the alcohol exposure because the key elements of cortical development, particularly those regulated by gene expression, such as the development of the intricate neuronal circuitry, are near complete.

Interestingly, there are sex differences revealed in our data. Specifically, we found that LEE females recovered brain and body weights more quickly when compared to LEE males, and that frontal cortex phenotypes and atypical behavior on the FST were observed only in LEE males. Also, LEE male rotarod performance demonstrated an abrupt learning pattern that was markedly different from controls and LEE females. One hypothesis as to why LEE females fare better, when compared to LEE males, related to differences in puberty onset compared to the timing of exposure and dependent measures. Typical onset of puberty for wild-type mice begins around P28 in males, and P25 for females (Ismail et al., 2011; Molenhuis et al., 2014). Alcohol exposure prior to this period may impact the milieu of hormones that regulate onset of puberty. For example, a gradual increase of Gonadotropin Releasing Hormone (GnRH) is responsible for the typical onset of puberty; its expression is diminished in the presence of alcohol, resulting in a pubertal onset delay (Srivastava et al., 2014; Dees et al., 2017). Therefore, our model can potentially delay puberty onset in LEE mice. Considering that female mice go through puberty earlier than males, it is not surprising that LEE has a greater impact on male behavior at P30.

Study limitation and future directions

With this study, we attempted to model offspring exposure to ethanol, naturally, *via* maternal consumption during lactation and active breastfeeding in an outbred mouse strain. With this comes limitations. For example, outbred mice have inherent variability, unlike inbred strains where genetics are controlled. However, inbred mice, such as C57BL/6 are less hardy than CD-1 mice and tend to provide inferior maternal care to their offspring. Additionally, the self-administration design of this experiment leads to variation in maternal ethanol consumption as well as milk production and composition. These factors could play influential roles in offspring outcome in addition to the impact that ethanol provides.

Another limitation is the variability in pup BEC we observed in our data. Although the variability in dam BEC was small, we believe there were several factors besides maternal ethanol levels that influenced pup BEC. The LEE pups were small at P20 and obtaining blood samples in a great enough volume for the assays was difficult. This resulted in a lower sample size. Also, by P20, some pups had begun eating chow in addition to nursing, possibly reducing ethanol intake and time from the last nursing event was variable from pups selected for analysis. Mice metabolize ethanol quickly, so increased variability in measured BEC is expected when time since the last dose is unknown. Additionally, competition for breast milk access can result in variability among pups. Also, timing of maternal alcohol consumption relative to the period of nursing that preceded the pup sampling could also introduce variability. Despite the observed variability in pup BEC, the BECs were

non-zero in all LEE pups and the level was significantly higher than controls in all LEE cases.

Future studies could include shorter time periods of exposure, as human mothers sometimes breastfeed for abbreviated periods of time post-partum. Also, additional studies of gene expression analyses in the frontal cortex as well as intraneocortical connectivity would be warranted and behavior tests of fear conditioning and learning as we observed phenotypes in these domains in our PrEE models. Finally, additional behavioral assays including tests to better assess hyperactivity, such as open field and assays that can detect cognitive deficits such as Morris water maze or radial arm maze.

Conclusion

A preponderance of evidence from researchers studying prenatal alcohol exposure and FASD led the CDC to correct its stance on drinking in pregnancy. They now clearly state "There is no known safe amount of alcohol use during pregnancy or while trying to get pregnant" (Centers for Disease Control and Prevention, 2022a). To date, the CDC has not made a similar statement regarding drinking while breastfeeding, despite research demonstrating high frequency of maternal alcohol consumption while nursing (Backstrand et al., 2004; Parackal et al., 2007; Giglia et al., 2008; Giglia, 2010; Lange et al., 2016). In their review, May et al. (2016) make a compelling argument that alcohol consumption during pregnancy can result in poor childhood outcomes.

Our data from our novel LEE model supports this notion, as our LEE model demonstrates similar phenotypes as our PrEE model; therefore, abstaining from alcohol consumption during BOTH the prenatal period and while breastfeeding is the safest option. Although the effects of LEE are mild compared to PrEE, most likely due to exposure outside critical periods for typical development, offspring exposure to ethanol *via* breast milk can have deleterious effects on developing brain and behavior and should be avoided.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

This animal study was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at the University of California, Riverside (UCR).

Author contributions

RP assisted with research design, conducted the experiments, collected, analyzed, and interpreted the data, and wrote the manuscript. KC conducted the experiments, collected the data, and wrote the manuscript. ME contributed to the statistical analysis and interpretation of data, and wrote the manuscript. MN conducted the experiments and collected the data. KH established the research design, interpreted the data, and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Maternal immune activation as an epidemiological risk factor for neurodevelopmental disorders: Considerations of timing, severity, individual differences, and sex in human and rodent studies

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Epidemiological evidence suggests that one's risk of being diagnosed with a neurodevelopmental disorder (NDD)—such as autism, ADHD, or schizophrenia—increases significantly if their mother had a viral or bacterial infection during the first or second trimester of pregnancy. Despite this well-known data, little is known about how developing neural systems are perturbed by events such as early-life immune activation. One theory is that the maternal immune response disrupts neural processes important for typical fetal and postnatal development, which can subsequently result in specific and overlapping behavioral phenotypes in offspring, characteristic of NDDs. As such, rodent models of maternal immune activation (MIA) have been useful in elucidating neural mechanisms that may become dysregulated by MIA. This review will start with an up-to-date and in-depth, critical summary of epidemiological data in humans, examining the association between different types of MIA and NDD outcomes in offspring. Thereafter, we will summarize common rodent models of MIA and discuss their relevance to the human epidemiological data. Finally, we will highlight other factors that may interact with or impact MIA and its associated risk for NDDs, and emphasize the importance for researchers to consider these when designing future human and rodent studies. These points to consider include: the sex of the offspring, the developmental timing of the immune challenge, and other factors that may contribute to individual variability in neural and behavioral responses to MIA, such as genetics, parental age, the gut microbiome, prenatal stress, and placental buffering.

KEYWORDS

neurodevelopmental disorders, maternal immune activation, perinatal period, development, individual differences, sex differences, autism spectrum disorder, schizophrenia

1. Introduction

According to the Centers for Disease Control and Prevention, the prevalence of neurodevelopmental disorders (NDDs) in the United States is 13.87% and yet the etiology of these disorders is not well understood. This rate has increased by about 9.5% in the last decade (Zablotsky et al., 2019), likely because our understanding of and ability to effectively diagnose various NDDs has improved over time. NDDs are similarly prevalent across most countries throughout the world, although the rates may vary due to socioeconomic factors, awareness, and diagnostic methods within each country (Chiarotti and Venerosi, 2020). Common epidemiological trends associated with NDDs include: the general age of onset within each disorder, symptom manifestation within each disorder, sex bias in the prevalence of certain NDDs, as well as the possible risk factors associated with many NDDs.

Epidemiological data suggest that genetic risk provides a foundation upon which other factors may precipitate or enhance the risk for many NDDs (Zawadzka et al., 2021). One of those other risk factors is prenatal infection associated with maternal immune activation, which slightly but significantly increases the risk of various NDDs. Maternal immune activation (MIA) is a term used in epidemiological studies that typically refers to maternal exposure to, or infection with, various immunogens (i.e., viral, bacterial, parasitic) during pregnancy. Some human studies have also considered increased levels of immune-related molecules (i.e., cytokines, chemokines) to serve as indicators of MIA. Animal studies are also commonly used to model MIA either *via* direct infection (i.e., of a virus, bacteria, or parasite) or *via* stimulation of the immune system (in the absence of infection) by utilizing a viral or bacterial mimetic, immune-related molecules, or other environmental stressors that are known to activate the immune system. Rodent models of MIA have been used extensively to model and better understand how activation of the immune system during gestation may affect the development of neurobiological systems underlying NDDs. Neuroscientists have only just begun to understand how the maturation of certain structures in the brain allows for the emergence of particular behaviors at specific ages (see Albani et al., 2014 for review). As such, it is still not well-understood how developing neural circuits or systems are disrupted by events such as immune activation that, in turn, increase the risk of NDDs or explain the underlying etiology of their symptoms.

In this review, we will (1) summarize epidemiological evidence that supports the role of MIA in the risk for NDDs, with a critical eye towards new emerging trends in the data, (2) introduce commonly used rodent models of MIA and their relevance for studying human NDDs, and (3) assess additional factors that should be considered when studying NDDs in rodents, including timing and severity of infection, sex differences in vulnerability for and symptomatology of NDDs, and individual differences associated with the maternal immune response. Thus, the overall goal of this review paper is to evaluate the epidemiological link between MIA and NDDs in order to identify factors that should be considered when designing future human and rodent studies. By considering additional dimensional criteria in their experimental design, researchers may begin to better address the immunological and neurobiological causes of NDDs and effectively identify possible treatments or therapies.

2. What are neurodevelopmental disorders?

The term "neurodevelopmental disorders" (NDDs) was first introduced as a diagnostic category in the *DSM-5,* to replace the more general term "developmental disorders" that was introduced in the *DSM-III* (Morris-Rosendahl and Crocq, 2020). These disorders affect one or several areas of development, including language, motor, social, and learning skills. More specifically, NDDs are a group of conditions that produce impairments of functioning during development and are associated with a *known* early-life medical, environmental, or genetic risk factor (Morris-Rosendahl and Crocq, 2020). Examples of NDDs defined in the *DSM-5* include, but are not limited to, autism spectrum disorder (ASD), attention-deficit/hyperactivity disorder (ADHD), intellectual disability (ID), and communication disorders. In 2019 and 2020 in the United States, the prevalence of ADHD was ~8.5%, ASD was ~2.9%, intellectual disability (ID) was ~1.4%, and various learning disabilities (LD) was ~6.4% (Yang et al., 2022). Notably, a higher prevalence of ADHD, ASD, ID, and LD have all been observed in boys relative to girls aged 3–17 (Yang et al., 2022). Schizophrenia is also considered by many researchers and clinicians to fall under the category of NDDs even though it is not defined as such in the *DSM-5*. This is because while the positive symptoms of schizophrenia (hallucinations, disorganized speech, etc.) typically manifest first during late adolescence, the etiology of these symptoms likely result from events that occur during perinatal or early postnatal development (Brašić and Holland, 2007; Fatemi and Folsom, 2009; Rapoport et al., 2012; Heyer and Meredith, 2017).

Interestingly, there are often overlapping symptoms experienced by people diagnosed with various NDDs. These include cognitive and learning disabilities (Pope and Kern, 2006; Gold et al., 2008; Cicero et al., 2014; Wang et al., 2017; Banker et al., 2021), decreased social behaviors (Nijmeijer et al., 2008; Hooley, 2010; Uekermann et al., 2010; Savla et al., 2013; Staikova et al., 2013; Supekar et al., 2013; Perepa, 2014; Cotter et al., 2018; Porcelli et al., 2019), altered sleep patterns or disrupted circadian rhythms (Williams et al., 2004; Cohrs, 2008; Konofal et al., 2010; Stein et al., 2012; Hvolby, 2014; Myles et al., 2016; Kaskie et al., 2017; Shelton and Malow, 2021), as well as metabolic or gastrointestinal disturbances (Richardson and Ross, 2000; Singh et al., 2020; Oyarzábal et al., 2021). It is important to note that symptoms shared across different NDDs can manifest differently based on the specific disorder and the individual person. For example, people diagnosed with ADHD, ASD, or schizophrenia may experience dysregulated sleep or circadian rhythm cycles, which can range from having delayed onset of sleep and melatonin peak to having decreased efficiency and total amount of sleep (Williams et al., 2004; Cohrs, 2008; Konofal et al., 2010; Stein et al., 2012; Shelton and Malow, 2021). People diagnosed with ADHD or schizophrenia may also experience sleep apnea or obstructed breathing during sleep (Konofal et al., 2010; Hvolby, 2014; Myles et al., 2016; Kaskie et al., 2017). Furthermore, particular modes of learning are differentially affected across NDDs. Schizophrenia is associated with difficulties in verbal learning, learning that requires self-correction, learning that happens on a rapid timescale, and reward or reinforcement learning (Pope and Kern, 2006; Gold et al., 2008; Cicero et al., 2014), whereas ASD is associated with impairments in spatial working memory, spatial navigation and reasoning, and memory retrieval tasks (Wang et al., 2017; Banker et al., 2021). Thus, the phenotype of a "shared"

NDD symptom may manifest differently depending on the distinct NDD of that individual.

It is currently unknown whether the neurobiology contributing to the overarching "shared" NDD symptoms is similarly impacted in humans across different disorders (see De Lacy and King, 2013 for review of neurobiological studies underlying ASD and schizophrenia). On one hand, it is possible that across different NDDs, a shared symptom may be caused by a disturbance in a *shared* neurobiological process. One hypothesis is that dysfunction across NDDs may be driven by alterations in the excitatory/inhibitory balance in the brain (Pearce, 2001; Foss-Feig et al., 2017). Additionally, Nair et al. (2020) theorize that reduced social behaviors in adolescents with ASD or psychosis are linked to disruptions in the default mode network. On the other hand, it is possible that for different NDDs, a shared symptom could be caused by a disturbance in *distinct* neurobiological processes. For instance, evidence suggests that different patterns of underconnectivity of long-range axons between multiple brain regions and of overconnectivity of short-range axons within a brain region are implicated in the pathogenesis of ASD, ADHD, and schizophrenia (see De Lacy and King, 2013 and Kern et al., 2015 for information about the underlying mechanisms and implications of these altered connectivity patterns). Specifically, underconnectivity between frontal cortex and posterior brain areas is associated with ASD whereas underconnectivity between parietal cortex and the cerebellum is associated with ADHD (Kern et al., 2015). In this case, even though a similar mechanism of dysfunction may be similar across disorders, the specific characteristics (and likely the etiology) of the disruption are distinct for each disorder. Finally, dysregulation of the immune system following MIA may contribute to the etiology of many NDDs, in that it elicits a core set of symptoms that are similar to sickness behavior and are consistent across different NDDs, including cognitive or learning deficits, decreased social behavior, metabolic or gastrointestinal disturbances, and dysregulated sleep.

In summary, age of onset, cause, severity, etiology, and manifestation of symptoms can be different amongst individuals, even within one type of NDD (e.g., ASD). This variability is not specific to any one NDD or to the class of NDDs in general, rather it applies to many mental health and psychiatric disorders where the diagnostic criteria reflect a collection of symptoms that often overlap. This variability and overlap in symptomatology across various disorders led the National Institutes of Mental Health (NIMH) to create the Research Domain Criteria (RDoC) framework, which focuses on a dimensional rather than categorical approach to preclinical research. The RDoC encourages researchers to study specific criteria (i.e., dimensions) related to a disorder (e.g., risk factors such as MIA or symptoms such as specific types of learning deficits), rather than attempting to model the entirety of a disorder. By focusing experimental designs -- of both epidemiological studies and rodent models -- on studying specific criteria related to an NDD, we may gain a better understanding of the underlying circuits and mechanisms pertaining to multiple NDDs that exhibit that criterion as a symptom or risk factor. This framework also highlights the need for researchers to consider *individual differences* in the expression of specific symptoms (or RDoC dimensions) when investigating risk factors for NDDs, which may ultimately provide us with a better understanding of how the symptoms, ontogeny, and severity of NDDs can be so distinct between one case to the next. There are many types of environmental factors and stressors that commonly and strongly predict the risk of NDDs, including genetic factors (Carter, 2009; McCarroll and Hyman, 2013), sex (Bargiela et al., 2016; Lai, et al., 2017), parental age, stress, diet, as well as prenatal and birth complications (summarized in Carlsson et al., 2021). That said, we will discuss the epidemiological evidence supporting that MIA is a well-known risk factor for many NDDs.

3. Maternal immune activation is an epidemiological risk factor for neurodevelopmental disorders

The developing brain is uniquely vulnerable to environmental insults and infections that can adversely impact the neurodevelopmental trajectory and ontogeny of behavior later in life (Bale, 2009; Deverman and Patterson, 2009; Schwarz and Bilbo, 2011b). Interestingly, the immune system has an important role in the various processes of typical neural development (Schwarz and Bilbo, 2011b; Tanabe and Yamashita, 2018; Zengeler and Lukens, 2021). Epidemiological data support that MIA increases the risk for NDDs in offspring. It is important to note that in both human and animal studies, MIA typically refers to any immune challenge that occurs during pregnancy. However, animal studies modeling gestational development can also encompass the perinatal period more broadly—occuring during gestation or around the time of birth—because the first 2 weeks of neonatal development in rodent pups is roughly equivalent to the third trimester of fetal development in humans (Guma et al., 2019). More specifically, third trimester neurodevelopmental processes such as immunogenesis, apoptosis, and synaptogenesis, occur during gestation in humans but continue post-birth in rodents (Estes and McAllister, 2016).

In humans, cohort and case–control studies are common experimental designs used to examine the relationship between MIA and NDD diagnosis (Song and Chung, 2010). Cohort studies first identify people that were exposed to an infectious agent during a specific time, and then either prospectively or retrospectively examine the likelihood that they are diagnosed with the disorder being studied. On the other hand, case–control studies first identify people diagnosed with the disorder of interest, and then retrospectively determine if they experienced an associated exposure or risk factor. Incidences of infection are typically confirmed *via* self-report, old medical records, or serological confirmation of infection. One limitation of these human studies is the necessity of an observational design, which prevents us from fully understanding the causal relationship between MIA and symptoms of NDDs. This highlights the importance of basic biomedical research and animal models to decipher the specific link between MIA and the ontogeny of NDDs, with the additional goal of identifying the underlying molecular, cellular, or neural circuit mechanisms or disruptions. Nevertheless, epidemiological studies of maternal exposure to various pathogens and environmental triggers—particularly viral and bacterial infections—during gestation provide some of the strongest data linking MIA and the risk of NDDs (see Han et al., 2021 for a more comprehensive review).

3.1. The epidemiological evidence with a focus on infection type, severity, febrile response, and medications

General infections during pregnancy have been associated with increased risk for ASD and schizophrenia in offspring (Nielsen et al.,

2013; Jiang et al., 2016; Zhou et al., 2021). More specifically, viral and bacterial infections during gestation are well-associated with later NDD diagnosis. Various viral infections during pregnancy are linked with ASD diagnosis (e.g., rubella, congenital cytomegalovirus, influenza) and schizophrenia diagnosis (e.g., influenza, rubella, Herpes simplex virus type 2 diagnosis) in offspring (see review articles: Boksa, 2008; Brown, 2012; Ornoy et al., 2015; Shuid et al., 2021; Cheslack-Postava and Brown, 2022; Massarali et al., 2022). Certain bacterial infections during gestation are also associated with ASD (e.g., urinary tract infection, genital infections) and schizophrenia (e.g., respiratory infections, pyelonephritis, and genital/reproductive infections) diagnoses in offspring (see review articles: Boksa, 2008; Brown, 2012; Cheslack-Postava and Brown, 2022; Massarali et al., 2022). Parasitic infections during pregnancy, particularly *Toxoplasmosis gondii* (*T. gondii*), have also been linked to schizophrenia in offspring (Khandaker et al., 2013; Cheslack-Postava and Brown, 2022). Some case–control studies have shown that individuals with schizophrenia were more likely to have IgG antibodies against *T. gondii* (Hamidinejat et al., 2010), be exposed to maternal Toxoplasma IgG antibodies during gestation (titer ≥1: 128) (Brown et al., 2005), or have increased levels of IgG antibodies against *T. gondii* as infants (Mortensen et al., 2007). The next subsections will discuss specific types of infections and factors most notably associated with the risk of NDDs, with an emphasis on ASD and schizophrenia.

3.1.1. Rubella

During the 1960s there was a rubella epidemic in the United States that resulted in various pregnancy and birth complications as well as physical and cognitive birth defects in the affected infants (Lindquist et al., 1965; Chess et al., 1979; Berger et al., 2011). While many cases of rubella infection have since been prevented by vaccination, rubella is estimated to still affect around 5% of pregnant persons worldwide (Berger et al., 2011; Hutton, 2016). Early links between rubella and ASD were identified from a New York cohort study of children in the United States that were part of the Rubella Birth Defect Evaluation Project (RBDEP). This study identified a significant correlation between congenital rubella syndrome (CRS) and autism diagnosis during childhood (Chess et al., 1979). Of particular note, CRS and ASD seem to overlap in their manifestation and symptomatology (Desmond et al., 1969; Swisher and Swisher, 1975; Hutton, 2016; Mawson and Croft, 2019). One mechanism thought to underlie the link between maternal rubella infection, particularly during the first trimester, and ASD risk in offspring is *via* liver dysfunction resulting in fetal exposure to high levels of vitamin A, which can be toxic to brain and other tissues of the developing fetus (Mawson and Croft, 2019).

Another cohort study of the RBDEP found that prenatal rubella exposure was associated with risk for nonaffective psychosis in young adulthood, regardless of hearing loss (Brown et al., 2000a). This association held true during a follow-up study with an updated assessment that allowed for a diagnosis of schizophrenia (Brown et al., 2001), which provided evidence that prenatal rubella is linked with an increased risk for schizophrenia in young adulthood. CRS and schizophrenia also overlap in brain dysmorphology, with both groups having reduced cortical gray matter volume and enlarged lateral ventricle volume, when adjusted for age and head size (Lim et al., 1995). In all, additional research still needs to be conducted to determine the underlying characteristics of prenatal rubella infection that may contribute to symptoms of ASD and schizophrenia, teased apart from other symptoms more specific to CRS.

3.1.2. Bacterial infections

In a Swedish cohort study, bacterial infection, not associated with a particular trimester of pregnancy, was linked with ASD without comorbid intellectual disability, ID (Lee et al., 2015). A significant association between ASD diagnosis and general bacterial infection during the third trimester was also reported in a Taiwanese case–control study (Fang et al., 2015). A meta-analysis similarly found that bacterial infection, particularly during the second or third trimester, was associated with ASD in offspring (Jiang et al., 2016). Moreover, bacterial infections requiring hospitalization during the second trimester (most commonly including urinary tract infection and genital infection) were linked to ASD diagnosis in a Danish cohort study (Atladóttir et al., 2010). Similarly, in a California case–control study in the United States, bacterial infections (such as urinary tract infection, amniotic infection at delivery, and major puerperal infection) diagnosed during a hospital stay, particularly during the third trimester of pregnancy, were significantly associated with risk for ASD in offspring (Zerbo et al., 2015). Additionally, in a Danish cohort study, genitourinary infections during weeks 33–36 of the third trimester were significantly linked with increased risk for ADHD in offspring (Werenberg Dreier et al., 2016).

Furthermore, a meta-analysis found increased risk of psychosis in offspring linked to general bacterial infections during pregnancy (Zhou et al., 2021). In a Danish cohort study, exposure to bacterial infection (including sinusitis, tonsillitis, pneumonia, cystitis, pyelonephritis, and bacterial venereal infection) during the first trimester of pregnancy was associated with an elevated risk for schizophrenia in offspring (Sørensen et al., 2009). Exposure to maternal genital/reproductive (G/R) infections during the periconceptual period (such as endometritis, cervicitis, pelvic inflammatory disease, vaginitis, syphilis, condylomata, "venereal disease," and gonorrhea) was also linked with increased risk for schizophrenia in offspring (Babulas et al., 2006). Further, pyelonephritis infection (kidney infection) that required hospitalization was associated with schizophrenia in offspring, but notably, only when there was a family history of psychosis (Clarke et al., 2009). Similarly, in a Swedish population-based cohort study, maternal infection during pregnancy was associated with later psychosis in offspring, only when there was also parental history of a psychiatric disorder (Blomström et al., 2016). While much of the above evidence supports that maternal bacterial infection increases the risk of NDDs, these last two studies, in particular, suggest that there may alternatively be an underlying susceptibility to perinatal infection in families with a history of NDDs, a concept that we will discuss in greater detail below.

3.1.3. Influenza

Influenza during pregnancy has been linked to an increased risk of schizophrenia in affected offspring (Khandaker et al., 2013). Two cohort studies reported an increased risk of schizophrenia associated with serologically confirmed maternal influenza infection, although this increased risk was ultimately not statistically significant (Brown et al., 2004; Ellman et al., 2009). In a prediction model, the number of influenza deaths in the general population of England was significantly associated with risk of schizophrenia in offspring that were in their 6th

or 7th month of gestation at the time (Sham et al., 1992). Similarly, the number of influenza infections in the general population of Denmark were linked with schizophrenia risk in offspring that were in their 6th month of gestation at that time (Barr et al., 1990; Takei et al., 1996). Moreover, using data from influenza epidemics in France between 1949 and 1981, Limosin et al. (2003) also reported that adults with schizophrenia were more likely to have been exposed to influenza during the 5th month of gestation as compared to controls.

Often, a major limitation of these studies is the lack of direct link between prenatal influenza exposure and schizophrenia outcomes within the same subjects, although a few studies *have* established this link. In a study examining outcomes of the 1957 influenza epidemic in Finland, admissions into psychiatric hospitals for schizophrenia in offspring was associated with a second-trimester gestational age at the time of the epidemic (Mednick et al., 1988). Prenatal infections were later confirmed *via* medical records, supporting that influenza exposure during the second trimester was associated with an increased risk for schizophrenia as compared to infection during the first or third trimesters (Mednick et al., 1994). Further, a Californian cohort study in the United States found that respiratory infections during the second trimester of pregnancy were significantly associated with schizophrenia spectrum disorder diagnosis in offspring (Brown et al., 2000b).

There is perhaps more limited evidence supporting an association between influenza infection during gestation, not specifically linked to a specific trimester, and increased risk for ASD (Atladóttir et al., 2012). In a United States study using a large patient dataset within Kaiser Permanente healthcare network in Northern California (Zerbo et al., 2017), and in a Norwegian cohort study (Mahic et al., 2017), there were no significant associations found between influenza infection during pregnancy and ASD diagnosis. On the other hand, influenza infection during the second trimester was significantly associated with risk for ASD in a Boston cohort study in the United States (Holingue et al., 2020). Interestingly, this association was only true when antibiotics were *not* taken at any point during pregnancy, and not necessarily that they were only avoided at the specific time of infection (Holingue et al., 2020). In all, there is limited and conflicting evidence for the link between prenatal influenza and NDDs, particularly schizophrenia and ASD, which suggests that the association may be more complex than initially reported.

3.1.4. Fever during pregnancy

Even though ASD and developmental delays were not associated with general influenza exposure in the Northern California cohort, the incidence of ASD and NDDs in offspring *were* significantly associated with fever during pregnancy in an earlier study (Zerbo et al., 2013). As expected, this fever-associated risk for ASD was attenuated in people that reported taking antipyretics to reduce their fever during pregnancy (Zerbo et al., 2013). Supporting these data, a meta-analysis examining the relationship between prenatal immune activation and ASD diagnosis found a significant association between maternal fever and ASD diagnosis in offspring; this association was not significant for prenatal infections without fever (Tioleco et al., 2021). A United States case–control study also found that there was a significant association between ASD risk and having a fever during the second trimester, even though there was no association between general prenatal infection and ASD risk (Croen et al., 2019). Thus, it is possible that links between maternal influenza during pregnancy and ASD risk may be obscured by unmeasured febrile response and unreported medication use (either antibiotics or antipyretics) in retrospective epidemiological studies, or through the lack of inclusion of such criteria in the original study design or analysis.

Furthermore, there is some evidence that maternal febrile response to influenza infection during pregnancy may be an important factor associated with increased risk for schizophrenia in offspring (Edwards, 2007). A Finnish cohort study found an increased odds ratio for schizophrenia in offspring that was associated with maternal fever during pregnancy, however the data were not statistically significant (Jones et al., 1998). Moreover, a Danish cohort study found that exposure to infections or maternal fever during gestation was associated with offspring having one psychosis-like experience by 11-years of age (Dreier et al., 2018). On another note, a Danish cohort study identified an association between maternal fever during weeks 9–12 of the first trimester and ADHD risk in offspring (Werenberg Dreier et al., 2016). Overall, it seems that the febrile response to infection during pregnancy or during parturition is linked with risk for NDDs in offspring and may serve as a potential mechanism to disrupt neurobiological development in the fetus and/or child.

3.1.5. Severity of infection

An additional nuance to the data presented thus far is the severity and duration of an infection or febrile response during pregnancy. A Danish cohort study reported that febrile episodes lasting more than 1 week, and that occurred prior to 32 weeks of gestation (roughly mid-third trimester), were associated with a threefold increase in risk for ASD in offspring (Atladóttir et al., 2012). Supporting this evidence, a study using data from the Norwegian Mother and Child Cohort Study (Magnus, et al., 2006) and Autism Birth Cohort Study (Stoltenberg, et al., 2010) found that maternal fever during the second trimester was associated with increased risk for ASD, and this risk was augmented with increased number of febrile episodes (Hornig et al., 2018).

Furthermore, a Swedish cohort study found an increased risk of ASD diagnosis associated with exposure to severe types of perinatal infections (such as sepsis, pneumonia, pyelonephritis, meningitis or encephalitis, influenza, and chorioamnionitis) versus a non-severe urinary tract infection (Al-Haddad et al., 2019). Having two or more infections during pregnancy, particularly during the third trimester, was also associated with increased risk for ASD (Zerbo et al., 2015). Along these lines, a Danish cohort study found that hospital admissions for viral infection during the first trimester (including influenza, gastroenteritis, rubella, etc.) and for bacterial infection (including urinary tract and genital infections) during the second trimester were significantly associated with an increased risk for ASD diagnosis (Atladóttir et al., 2010). Similarly, a meta-analysis showed that infection during pregnancy that required hospitalization was associated with increased risk for ASD, particularly during the first or second trimester of pregnancy (Jiang et al., 2016).

Psychosis risk in offspring has also been linked to hospital treatment for maternal infection during pregnancy (Zhou et al., 2021). Interestingly, schizophrenia in offspring has also been associated with both maternal and *paternal infections* that specifically required a visit to the hospital, regardless of whether the visit occurred before, during, or after pregnancy (Nielsen et al., 2013). This suggests that familial history of prior illness or general infection may also be an important factor to consider in the risk for NDDs, which we will discuss more

later in this review. Overall, these data indicate that, during gestation, the magnitude of the febrile response and the severity of infection may be specific factors important for consideration in the risk of ASD and schizophrenia diagnoses.

3.1.6. Cytokine and chemokine expression during pregnancy

As originally proposed by Dr. Paul Patterson and confirmed by many colleagues since, one mechanism by which influenza (or other infections) increases the risk of NDDs in offspring may be *via* the maternal immune response and its associated circulating cytokines, rather than *via* a direct infection of the fetus itself (Shi et al., 2005; Mahic et al., 2017). In the past few decades, human epidemiological studies have provided further evidence of this idea. A Californian case–control study in the United States found that elevated levels of interferon (IFN)-γ, interleukin (IL)-4, and IL-5 in maternal serum mid-pregnancy were associated with increased ASD risk in offspring relative to the general population (Goines et al., 2011). Importantly, cytokine levels were adjusted for covariates during analysis, including gestational age at the time of specimen collection and maternal weight, age, ethnicity and country of birth. Similarly, a Danish case–control study found increased levels of tumor necrosis factor (TNF)-α and TNF-β in amniotic fluid (collected during screening or diagnostic amniocentesis procedures) of individuals later diagnosed with ASD (Abdallah et al., 2013). The researchers also found an association between ASD and elevated levels of IL-4, IL-10, and monocyte chemoattractant protein (MCP)-1 in the amniotic fluid of individuals born after 1993, which is when an updated diagnostic code for autism was introduced (Abdallah et al., 2012, 2013).

Moreover, a Philadelphia case–control study in the United States analyzed maternal serum samples that were collected during various prenatal visits or at birth and found that elevated levels of pro-inflammatory cytokines TNF-α, IL-1β, and IL-6 were associated with the diagnosis of a psychiatric disorder (i.e., schizophrenia, schizoaffective disorder, and major depression or bipolar disorder with psychosis) decades later in adult offspring (Allswede et al., 2020). This association was particularly true for samples collected during the first half of pregnancy but was not significant for those collected during the second half of pregnancy. Similarly, a Rhode Island study in the United States found increased levels of TNF-α and IL-8 in serum collected at parturition that were linked with reports of maternal infection during the third trimester (Buka et al., 2001). The elevation in TNF-α was specifically associated with increased risk for psychosis in offspring.

Overall, these studies support that activation of the immune system (*via* infection) induces a concert of cytokines and chemokines that circulate throughout the body to fight the invading pathogen. In doing so, these pleiotropic immune molecules have powerful effects on the body; they can pass through the placental barrier and enter the fetal compartment, or they can trigger cytokine production in the placenta itself, which can produce similarly powerful effects on the developing fetus (Hsiao and Patterson, 2012). This inflammatory response can thus drive alterations in the fetal brain by disrupting processes important for typical neural development, which may underlie later vulnerability to NDDs and their symptoms.

As mentioned previously, the immune system is highly involved in regulating numerous processes important for neural development. Microglia, the innate immune cells of the brain, are involved in the pruning and maturation of appropriate synapses during development *via* phagocytosis of axonal terminals and dendritic spines (Schafer et al., 2012). Exposure to early-life immune activation (e.g., MIA) compromises these functions, and can subsequently affect the number and function of microglia, disrupt synaptic maturation and pruning, and result in neural circuit remodeling and deficits in neural function and behavior (Paolicelli et al., 2011; Zhan et al., 2014; Tay et al., 2017). The consequences of this dysregulation may also extend beyond fetal development and affect neurodevelopmental processes during the postnatal period (Matcovitch-Natan et al., 2016; de Cossío et al., 2017), such as disrupting synapse/neural circuit formation important for the ontogeny of specific behavioral phenotypes (e.g., language acquisition, social behaviors, learning, etc.). Thus, rodent models of MIA and NDDs are especially important to help elucidate the neural and molecular processes that can become disrupted by immune activation during sensitive periods of development. In particular, many rodent models have focused on measuring or manipulating the specific cytokines produced during a maternal infection or immune challenge, in order to examine their effects on offspring brain and behavior throughout the lifespan. (For a more detailed review of neurobiological processes associated with NDDs that can become disrupted by MIA, see: Knuesel et al., 2014; Estes and McAllister, 2016; Bergdolt and Dunaevsky, 2019).

3.2. Considerations for future epidemiological studies

There is significant overall evidence suggesting that maternal infection with viruses or bacteria significantly increases the risk of various NDDs. ASD seems to be associated with exposure to viral infections during the first/second trimesters and bacterial infections or fever during the second/third trimesters of pregnancy. On the other hand, schizophrenia seems to be associated with viral infections during the second/third trimesters and bacterial infections during the first trimester of pregnancy. However, many epidemiological studies are unable to account for the trimester of infection in their findings, either due to limitations of the data collected or not having enough statistical power. Therefore, one cannot exclude the possibility that infections or febrile episodes during other phases of gestation are not also risk factors for these NDDs and their behavioral symptoms.

The characteristics, timing, and severity of the maternal immune response to infection seem to matter greatly and can vary depending on the type of infectious agent and whether a robust febrile response occurs. In turn, medications that attenuate febrile response or cytokine production (such as antipyretics or antibiotics) may lessen the risk of NDDs associated with prenatal infection and may also be strong determinants in the outcomes of fetal neurodevelopment that determine NDD risk. Intriguingly, prenatal acetaminophen use has been linked to increased risk for ADHD (Avella-Garcia et al., 2016; Liew et al., 2016), and another study linked to ASD diagnosis in children that also had hyperkinetic symptoms (Liew et al., 2014). Additional data is needed to examine how these specific aspects of an infection may be a driving factor for how the fetal immune system or neural development is altered or compromised. For instance, is a febrile response the driving factor? What is the contribution of the maternal peripheral immune response, or the placental immune

response, or the fetal brain cytokine response to infection? In future human studies, serum or amniotic fluid samples should be collected and analyzed to better characterize the severity of the maternal or fetal immune and cytokine response, as originally proposed by Gilmore and Jarskog (1997). This may allow us to determine whether the immune response itself moderates the relationship between perinatal infection and the risk of NDDs in affected offspring. Moreover, the need for easily accessible serological and cell samples throughout gestation, in women with and without overt infection, will ultimately contribute to our better understanding of *how* the maternal immune reaction may be linked to NDDs.

Limited data from biological tissues and postmortem samples (Lintas et al., 2010) in humans has made it difficult to identify the biomarkers of disease that may be linked to MIA or perinatal infection. Human neuroimaging studies have begun to elucidate the association between elevated maternal cytokines during gestation and changes in brain structural and functional connectivity in offspring, which may or may not be associated with a particular NDD (see Guma et al., 2019 for data). The combined use of human neuroimaging and biological samples collected during gestation or from individuals with NDDs, may elucidate how long-term dysregulation of the immune system disrupts the development of important functional and/or structural brain regions and neural circuits in offspring. However, these methodologies cannot determine much about the cellular or molecular contributions toward NDDs, which are likely the first systems to be disrupted by an environmental or biological risk factor.

Furthermore, biological sex or possibly gender are likely critical factors that must be considered when examining the etiology of NDDs and their symptoms. Males are, on average, twice as likely than females to be diagnosed with developmental disorders, including ADHD, ASD, schizophrenia, and general learning disabilities (Polyak et al., 2015; Pinares-Garcia et al., 2018). Many of the epidemiological studies that are reviewed above did not include sex or gender as a factor in the study design nor statistical analysis. Although there are a few studies reviewed by Ardalan et al. (2019) that describe sex differences in cytokine expression of individuals diagnosed with ASD, their findings are not specifically related to MIA. For instance, males had a significantly higher risk for ASD in a Lebanese case–control study (Guisso et al., 2018) and for schizophrenia in a Finnish cohort study (Jones et al., 1998), independent of prenatal exposure to infection. There is one Boston case–control study in the United States that examined the association between cytokine levels in maternal serum collected throughout pregnancy and risk for schizophrenia in offspring (Goldstein et al., 2014). The researchers found a significant interaction between sex and subject group, such that females with schizophrenia were more likely to have decreased levels of maternal TNF-α as compared to males with schizophrenia and females in the control group. Overall, there is a need for future epidemiological studies to account for sex in their experimental design in order to better understand potential interactions between sex and specific risk factors associated with NDDs. It is also important to note here that a proper experimental design including sex as a variable requires sex to be statistically included in the analysis (i.e., testing for an *interaction* between sex and another factor of interest; if there is no significant sex effect then the analysis can be collapsed across sex). Researchers should also indicate in their publications whether sex was statistically analyzed, regardless of a significant effect.

Finally, many epidemiological studies identify links between exposure to infection with a general diagnosis of ASD or schizophrenia. Other studies have instead examined the link between MIA and specific symptoms of NDDs, like in the hyperkinetic symptom of ASD study mentioned above (Liew et al., 2014). In an Australian cohort study of children with ASD, symptoms of severe social impairment were associated with reports of the mother having a history of *chronic* immune activation such as asthma or allergies (Patel et al., 2018). Similarly, a Finnish cohort study found significant associations between maternal fever during the second trimester and several behavioral outcomes in children that are characteristic of NDDs, including distress to novel situations, difficulties with task persistence and orientation, and increased social inhibition (Dombrowski et al., 2003). These studies provide excellent examples of how we can tailor future human studies and animal models of MIA to the RDoC and examine specific behaviors that are implicated in many NDDs. The findings of these epidemiological studies can help to advance the field of neuroscience by providing the opportunity to bring rodent model research into better alignment with our current understanding of human NDDs and the associated presentation of their symptoms.

In all, the epidemiological studies are unable to show strong causal associations between any one particular infection type and a specific NDD outcome. The only possible exception being rubella, which produces its own congenital syndrome that mirrors or includes many of the symptoms of other NDDs. Furthermore, the epidemiological data do not always provide the most consistent results regarding the association between MIA and NDDs. It is possible that the relationship between prenatal infection and NDDs in studies with null findings may be clouded by other factors related to the infection or sickness response—such as fever, severity of infection, gestational timing, medication or treatment, genetic predisposition, individual immune response, fetal sex, lifestyle, etc.—that aren't always measured or accounted for in epidemiological studies. These factors may have a significant moderating effect in whether overt MIA results in the onset of NDDs later in life (Flinkkilä et al., 2016). That said, the majority of epidemiological evidence does support the hypothesis that MIA during gestation, in and of itself, is a risk factor for various NDDs, rather than being due to the unique biological characteristics of any one pathogen or immunogen.

4. The maternal immune system during pregnancy: Cytokines in the absence of infection

The immune system functions under very tight regulation, such that immune activation cannot be too robust but must also be sufficient to fight off infection, otherwise it can result in death. That said, there are genetic variations in the immune system and certain disorders that alter how the immune system functions across individuals. A recent example is individual differences in response to infection with the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), also known as the COVID-19 virus. For some individuals, COVID-19 produces a robust immune response with strong cytokine production that may increase the risk of acute respiratory distress (Cummings et al., 2020), whereas others may not experience any symptoms of infection. As another example, males and

females have different immune responses (Klein and Flanagan, 2016) and as a result, females are more likely to suffer from autoimmune disorders where the immune system is overactive and causes elevated cytokine levels and T-cell responses (Klein and Schwarz, 2018). The various factors that affect individual immune function can become the backdrop upon which pregnancy, and then subsequent infection, result in various outcomes in the developing fetus associated with risk for NDDs.

Many researchers in the field of developmental neuroimmunology, with a focus on MIA and NDDs, do not always account for the fact that the immune system is altered dramatically by pregnancy itself. The pregnant body goes through a process of immunosuppression in order to protect the non-self fetus from being attacked and rejected by the maternal immune system. As such, even a slight imbalance in this pregnancy-induced immunosuppression can result in early termination of the pregnancy. These changes in maternal immune function during pregnancy can allow for more severe infections to occur, particularly during late gestation, but can also temporarily alleviate autoimmune diseases for many women (Robinson and Klein, 2012; Sherer et al., 2017; Klein and Schwarz, 2018). Evidence for gestational immunosuppression has also been demonstrated in rat models. Within the 96 h before and 24 h after parturition, pregnant rats have a decreased febrile response following a *very low* dose of lipopolysaccharide (LPS; 25 μg/kg), as compared to unbred or lactating female rats. Moreover, within the 24-h period before expected time of parturition, *no* pregnant rat developed a fever and the majority became hypothermic; an effect that resulted in death in 80% of pregnant dams within 3–15 h (Martin et al., 1995). This shift in immune function is so robust that administering LPS (100 μg/kg) to a pregnant rat at embryonic day (E)11 (mid-gestation) attenuates IL-1β expression in the maternal spleen to 12%, IL-6 expression to 20%, and IFN-γ expression to 30% of the levels measured in a non-pregnant female administered the same dose of LPS (Sherer et al., 2017). By E22, the day prior to birth, the same dose of LPS produces virtually no significant cytokine production in the maternal spleen, highlighting just how dramatic the immunosuppression of pregnancy can be (Sherer et al., 2017). In the placenta and the fetal brain at E11, there is an upregulation of IL-1β and IL-6 that is modest (4-5-fold) following MIA with LPS, but then non-existent at E22, just prior to birth (Sherer et al., 2017). In conclusion, pregnancy significantly attenuates the function of the immune system, an effect that is necessary for a successful pregnancy.

In a typical healthy pregnancy, the developing fetus is exposed to very low levels of immune molecules, but there may be instances where cytokine production could become dysregulated even in the absence of infection. Fetal exposure to elevated levels of cytokines may increase the risk of NDDs and their symptoms. Supporting this theory, the human data summarized above shows that increased cytokine expression in maternal serum and/or the amniotic fluid is associated with NDDs in offspring, even in the absence of apparent, current infection. In these cases, it is possible that an underlying inflammatory condition, variations in immune function, stress-induced immune activation, or perhaps a slight shift in the typical immunosuppression associated with pregnancy may increase cytokine production and the associated risk of NDDs. This should be considered in future epidemiological and basic research.

5. Rodent models of immune activation to study neurodevelopmental disorders

As mentioned above, human studies are limited in their ability to establish a causal relationship between risk factors and NDD outcomes. Furthermore, it is difficult for researchers to determine the underlying neurobiology that may contribute to the disorder, as postmortem studies are limited by the number of donations and our current neuroimaging technology does not allow us to examine structural and functional neural changes at the cellular or molecular level. Therefore, animal models are necessary to understand the role of the immune system and immune activation in the perturbation of neurodevelopment.

In animal research, there are investigators that attempt to generate a model of a specific disorder (often *via* manipulation of a known genetic risk factor) and describe their research as using a rodent model of a specific NDD. However, these NDD-specific models often fail to capture the full range of symptoms and individual nuance of the disorder (Vigli et al., 2020). In recent years, researchers have begun to develop animal models that examine phenotype(s) shared across multiple disorders, rather than producing an animal model of a specific disorder. This practice is in line with the RDoC initiative from the NIMH, as described previously in Section 2. The reason for this is that symptoms of NDDs and other disorders are often overlapping, suggesting a potential for commonality in the neurobiological origins of the disorders (Conradt et al., 2021; Auerbach, 2022). MIA is often used as a solitary manipulation in animal models to examine how perturbations of the developing immune system (a risk factor for NDDs) may contribute to specific symptoms of NDDs, such as disturbances in learning, social, and sleep behaviors. Rodent models are especially important for our understanding of the neural processes that can become disrupted by MIA (particularly the inflammatory response in the maternal body and fetal compartments) and lead to NDD-associated outcomes in offspring.

However, we also know that the etiology of NDDs likely stems from a *combination* of genetic and environmental factors. Therefore, researchers have begun to utilize "two-hit" and "multi-hit" models of neurodevelopment whereby multiple inflammatory stressors (such as genetic mutations, immune challenges, diet manipulations, social stressors, etc.) are combined to examine specific phenotypes of NDDs. Utilizing genetic models of "specific disorders" that also examine other risk factors for NDDs may help us to better understand the interaction between biology and environment in the etiology of that specific disorder, rather than examining one factor by itself. Harvey and Boksa (2012) theorized that (1) the same risk factor can result in different NDDs and the characteristics of that risk factor (i.e., the dose, timing, immunogenic target, etc.) ultimately contribute to the distinct NDD phenotypes, OR (2) the same risk factor can cause different NDDs because it interacts with other vulnerability factors to contribute to the distinct NDD phenotypes. This theory should be kept in mind when considering basic biomedical research models for MIA and their associated outcomes. Next, we will discuss the various models of MIA that are commonly used by researchers in studying NDDs and evaluate their relevance to the human epidemiological data.

5.1. Rodent models of maternal immune activation

A range of different immunogens (any pathogens or molecules that can activate the immune system) are used in rat and mouse models to explore the underlying neurobiology implicated in the association between MIA and psychiatric phenotypes. For example, prenatal exposure to influenza in rodents produces altered expression of serotonergic and glutamatergic receptors, reduced exploration of the open arm of an elevated-plus maze (anxiety-like behavior), and deficits in prepulse inhibition (reduced sensorimotor gating; Shi et al., 2003; Moreno et al., 2011; Spini et al., 2021). Furthermore, exposure to *T. gondii* antigens during gestation in mice causes increased anxiety behaviors later in life (Webster et al., 2013; Spini et al., 2021). Exposure to diesel exhaust particles, which can also activate the immune system, during gestation and neonatal development produces learning and memory deficits in an elevated-plus and a Morris water maze, reduced social interactions, and alterations in ultrasonic vocalizations (communication deficits; Bolton et al., 2013; Chang et al., 2018; Ehsanifar et al., 2019). Further, prenatal infection with *E. coli* in rats impacts neonatal sensorimotor learning and adult spatial learning (Wallace et al., 2010). Perhaps some of the most commonly used rodent models of MIA include administration of polyinosinic:polycytidylic acid (Poly I:C) or lipopolysaccharides (LPS), which are mimetics for viral and bacterial infection, respectively.

5.1.1. Poly I:C

Poly I:C is a synthetic double-stranded RNA that is used to stimulate an innate immune response through the Toll-like receptor 3 (TLR3) pathway (Reisinger et al., 2015). As described by Bao et al. (2022), Poly I:C is recognized and internalized by TLR3, which causes downstream activation of nuclear factor-κB (NF-κB), activator protein 1 (AP-1), and interferon regulatory factor 3 (IRF3). This, in turn, induces the expression of inflammatory cytokines and type 1 interferon (IFN) genes which are produced and released by the immune system in order to, respectively, stimulate an innate immune response and inhibit replication of the viral Poly I:C RNA.

Mouse and rat models of MIA report using a range of doses of Poly I:C, from 250 μg/kg to over 20 mg/kg, with doses of 4 mg/kg and 20 mg/kg being the most commonly used (Boksa, 2010; Solek et al., 2018). The gestational timing of Poly I:C administration also varies widely from embryonic days (E)9 to E19, with administration most commonly reported on E12.5 and E15. Some labs also report using multiple exposures Poly I:C, such as across two consecutive days of gestation or on two different days such as E9 and E17 (Boksa, 2010; Solek et al., 2018).

5.1.2. Lipopolysaccharide

LPS is a cell-wall component of gram-negative bacteria (like *Escherichia coli*) that can also be used to stimulate an innate immune response *via* the TLR4 pathway (Reisinger et al., 2015). With the help of cluster of differentiation 14 (CD14), LPS binds to proteins on TLR4 which, similarly to Poly I:C, causes downstream gene expression and production of inflammatory cytokines and type 1 interferons (IFN) (Bae et al., 2010; Bao et al., 2022). Although interferons (particularly IFN-ɣ) are vital for the immune response to viruses, the activity of IFN-α and INF-β can also help the immune system fight off other pathogens such as bacteria (Bae et al., 2010).

Researchers report using dosages of LPS ranging from 25 μg/kg to over 1 mg/kg, with 100 μg/kg being more commonly used (Boksa, 2010; Solek et al., 2018). The timing of LPS administration varies across gestation, from E9 to E19, with administration most commonly reported around E15 and E18. Some models also employ multiple hits of LPS, such as subsequent injections on E15 and E16 or on E18 and E19. The utility of multiple hits of LPS is lessened by evidence that the peripheral cytokine response is attenuated after one previous LPS exposure (Wendeln et al., 2018). However, there is evidence to suggest the neural cytokine response is *augmented* to a second, but not a third or fourth, "hit" of LPS (Wendeln et al., 2018). Though, in the context of MIA, the maternal peripheral immune system is what influences the cytokine response in the fetal compartment; there is no evidence to support that the maternal *neural* immune response is implicated in the association between MIA and NDDs. Therefore, it is unclear how multiple hits of LPS during gestation would affect the developing fetal brain any differently than a single hit of LPS.

5.1.3. Relevance of rodent models to human studies of maternal immune activation

Several review articles have compiled data from rat and mouse MIA models that examine behavioral and neural outcomes following exposure to prenatal Poly I:C and LPS (see Meyer et al., 2009; Boksa, 2010; Solek et al., 2018; Kentner et al., 2019; Bauman and Van de Water, 2020). Some of the behavioral outcomes of rodent MIA studies that are summarized in these reviews include: deficits of latent inhibition and prepulse inhibition, reduced open-field exploration and novel object recognition, decreased social interactions, altered spatial memory in novel object location and Morris water maze tasks, and increased repetitive behaviors such as grooming or stereotyped behaviors in an open field. These rodent behavioral phenotypes are relevant to humans in that they align with the common overlapping symptoms of NDDs, such as deficits in sensorimotor gating, learning disabilities, and altered social behaviors, as we previously described. Such findings support the RDoC framework. Further, these data highlight how maternal inflammation during pregnancy, even in the absence of overt infection, can produce NDD-associated behavioral changes in offspring, likely through the disruption of neural processes necessary for brain development.

Similar to the human epidemiological data summarized above, the severity of the immune activation—related to immunogen dose in rodent models—may be implicated in neurodevelopmental outcomes related to NDDs. MIA with Poly I:C, and with lower doses of LPS (100–500 μg/kg), can cause subtle neural changes such as long-term alterations in cytokine expression, changes in neurotransmission, reduced proliferation of new neurons, and changes in microglia activation measured in adolescent and/or adult offspring (see Boksa, 2010; Solek et al., 2018; Kentner et al., 2019; Hameete et al., 2021 for more detailed information). On the other hand, larger doses of LPS (more than 1 mg/kg) administered during gestation can produce severe damage to white matter, axons, and dendrites (Fan et al., 2005). These MIA-driven neural changes may occur during fetal development but are often prolonged and measurable throughout offspring postnatal development and into adulthood. Again, many of these neural changes are thought to be driven by the maternal response to MIA, which can interact with the fetal compartment and disrupt

immune molecules and immune cells like microglia from performing their essential functions during neural development (i.e., synaptic pruning, neural circuit formation, etc.). Moreover, sex differences have been explored in rodent models of MIA and are detailed in other review articles (Ardalan et al., 2019; Bauman and Van de Water, 2020; Breach and Lenz, 2022), with the takeaway being that MIA differently affects the behavioral and neural phenotypes of male and female offspring. Below, we will further discuss the importance of offspring sex when studying the association between MIA and NDDs.

Taken together, rodent models of Poly I:C and LPS, which mimic viral and bacterial infections, respectively, seem to be effective in modeling the behavioral phenotypes of NDDs, particularly ASD, schizophrenia, and generalized learning deficits. Rather than employing a direct infection, Poly I:C and LPS are typically administered either intraperitoneally or subcutaneously, thereby stimulating the innate immune system in a similar manner to a peripheral infection in humans, but without the full infection (Ashdown et al., 2006). This is particularly relevant given the epidemiological data summarized above, which suggest that the maternal immune response (including fever, cytokine production, altered fetal microglia function, etc.) may precipitate NDDs in offspring rather than the direct infection itself. Interestingly, neonatal administration of LPS actually produces a broader and more robust neuroimmune response in rat hippocampus than *E. coli* (Schwarz and Bilbo, 2011a). Similarly, Poly I:C is more likely to result in activation of the rodent immune system, given that many common human viruses are not pathogenic in rodents. Though, many human viruses can be adapted for use in a rodent model if part of the immune system (e.g., IFN-ɣ) is knocked out of the rodent genome (Brehm et al., 2013; Sarkar and Heise, 2019). Thus, LPS and Poly I:C in rodent models may allow us to characterize the effects of MIA on neurobiological processes underlying symptoms of NDDs, independent of the infection itself.

One major limitation in our ability to interpret various rodent models of MIA is the variability in experimental design across labs, including: gestational timing and frequency of the immune challenge, serotype/strain/dose of the immune challenge, age at which behavioral measures are tested in offspring, the types of behaviors and neural outcomes measured within a lab, and mouse versus rat models (whose immune systems are quite distinct). More specifically, researchers should determine whether their model of MIA may be better studied in mice versus rats, depending on the intended experimental manipulation and measured outcomes. For instance, mouse models are currently better suited than rat models for manipulations whereby researchers can examine the role of particular genes in NDD risk. On the other hand, rats are often more adept at performing complex learning and behavioral tasks, deficits in which may be associated with NDDs (Parker et al., 2014). Furthermore, researchers should consider that different strains of mice and rats often display distinct biological and behavioral profiles, and that this can even be influenced by the vendor from which the animals are sourced (Kentner et al., 2019). Nonetheless, both mouse and rat models of MIA seem to yield similar patterns of behavioral and neural findings related to NDD risk and symptomatology, even with varying rodent strains, offspring age at the time of measured outcomes, and dosages/gestational timing of the immune challenge. This provides further support that rodent models can provide insight into the neurobiological mechanisms underlying MIA-driven disruptions in offspring neural and behavioral development.

There are also inconsistencies in the reports of maternal death and fetal resorption or pup death that are often an inevitable consequence of many of these models. For example, even administration of an "ultra-low" dose of LPS (0.5 μg/kg) early in gestation (E5) results in significantly smaller litters and resorbed fetuses (Xue et al., 2015). Thus, it is likely that experiments using higher doses of LPS or PolyI:C produce a much more severe immune activation in the dam and fetuses. The question then remains whether this associated fetal mortality is actually modeling simple maternal infection (MIA related to NDDs), or rather modeling more severe infections like chorioamnionitis or endometritis that are associated with serious birth complications like premature birth or stalled labor. On the other hand, one potential benefit of these experimental inconsistencies is that they mirror the in outcomes variability in outcomes that can occur within the human population, even in the severity of the maternal immune response during pregnancy. In all, there are similar neurobiological and behavioral consequences reported across multiple different epidemiological and rodent studies, even with variation in the factors contributing to the models; this provides strong evidence for an association between MIA and outcomes associated with NDDs. Beyond this, researchers have also begun to utilize "two-hit" and "multi-hit" models that incorporate MIA as just one of the multiple "hits" to examine the ontogeny or symptoms of NDDs.

5.2. "Two-hit" and "multi-hit" rodent models of neurodevelopment

Prenatal infection is not the only risk factor for NDDs. For example, environmental stressors such as negative social interactions or social exclusion during development, particularly adolescence, have also been linked to the onset of symptoms like psychosis or aberrant processing of social cues (Li et al., 2012; Davis et al., 2016). Indeed, many environmental and psychological stressors themselves—diet, pollutants, allergens, social stress, psychological stress, depression, etc. (Carlsson et al., 2021)—are able to trigger an inflammatory state in the brain and body. These inflammatory stressors can disrupt neural and behavioral development in offspring, both when experienced fetally during MIA and/or postnatally by the offspring. As such, the "two-hit" and "multi-hit" hypotheses of NDDs suggest that a *combination* of environmental, psychological social, or genetic "hits" throughout development significantly increases the overall risk for an individual to be diagnosed with NDDs such as ASD and schizophrenia (Davis et al., 2016), in addition to many comorbid disorders including general anxiety, depressive symptoms and learning disorders. Some risk factors associated with NDDs that have been examined in Poly I:C mouse models of MIA include genetic models of DISC1 (Disrupted in Schizophrenia 1) mutation, acute stress during juvenile development, and pubertal social isolation (Yee et al., 2011; Solek et al., 2018; Goh, 2020). Researchers should continue to develop more complex models of MIA that incorporate various other risk factors, in order to better understand how environmental and genetic factors mediate individual differences in the maternal and fetal immune responses and drive alterations in the behavioral and neurobiological development of offspring.

6. Factors to consider in rodent models of maternal immune activation

There are many factors that should be considered by researchers when studying NDDs, as they have an important role in the etiology and/or manifestation of symptoms of NDDs. These factors include (1) developmental timing of the immune challenge, (2) sex of the offspring, and (3) individual factors that may influence one's immune response, such as genetics, parental age, the gut microbiome, prenatal stress, and placental buffering.

6.1. Developmental timing of the immune challenge

The gestational timing of MIA may affect the fetal and maternal immune response differently throughout pregnancy. Individual differences in the immune response may be influenced by genetic predisposition to the infectious agent (Carter, 2009) and by the influence of pregnancy itself on the immune system (Sherer et al., 2017), as we described earlier. Furthermore, in both human and rodent studies, there is evidence that the timing of MIA can alter behavioral and neural outcomes in both the mother and offspring. For instance, maternal estradiol levels are lower during early stages of pregnancy compared to late stages, and ERβs begin to be expressed in fetal tissues around 16–18 weeks of gestation (Takeyama et al., 2001; Shepherd et al., 2021). Incubation with estradiol decreased levels of LPS-induced TNF and IL-6 cytokine production in infant cord blood mononuclear cells (Giannoni et al., 2011), suggesting that the circulating pregnancy hormones from the mother may impact the fetal immune response, in addition to the maternal immune response, as we already described. As another example, maternal infection with Zika virus during the first half of pregnancy is associated with greater rates of birth defects than during the latter half of pregnancy, likely due to the targeting of proliferative cells in the early developing brain (Honein, et al., 2017; Pomar et al., 2017).

The timing of MIA matters because it may affect different neurodevelopmental processes occurring during fetal development at that time. It is important to keep in mind that the gestational timing of animal models is shifted relative to that of humans, whereby the gestational period of mice is generally 21 days, of rats is 23 days, and of humans is 40 weeks. The first and second halves of gestation in rodents is approximately the equivalent of the first and second trimesters in humans, whereas the human equivalent to the third trimester in rodents occurs during the first 2 weeks of neonatal life, because rodent pups are born altricial. Therefore, many important neurodevelopmental processes—neurogenesis, immunogenesis, apoptosis, synaptogenesis—occur during gestation in humans but continue post-birth in rodents (Estes and McAllister, 2016; Guma et al., 2019). Moreover, some of these neurodevelopmental processes may be affected differently if the immune response occurs during early stages versus later stages of the developmental process (Bauman and Van de Water, 2020).

Overall, future research should take a more systematic approach to evaluate the effects of gestational timing within rodent models of MIA and try to better characterize which neurobiological processes are being studied and thus perturbed during fetal vs. postnatal neurodevelopment. In doing so, we should keep in mind that the later fetal developmental processes in humans are still being modulated by maternal biology, hormone production, and immune responses, whereas neurodevelopment in rodents continues postnatally, without these influences.

6.2. Sex of the offspring

As mentioned previously, NDDs—such as ASD, ADHD, and early-onset schizophrenia—are more commonly diagnosed in males than in females. There may be two reasons for such discrepancies: (1) the manifestation of symptoms in females is different than in males, and the current diagnostic criteria is more aligned with symptoms commonly presented in males, and/or (2) the disruption of neurobiological processes that cause NDDs are more likely to occur in males than in females. It is also possible that certain neural and behavioral processes mature at different rates between males and females, and therefore exposure to immunogens may differentially affect males and females depending on the developmental timing of the exposure. Despite the known sex-bias, there are limited epidemiological data investigating how sex may impact the role of MIA as a risk factor for NDDs, because research does not always seek out an equal female-matched comparison group (D'Mello, 2022). Moreover, rodent models themselves can contain sex biases in experimental design and analysis of results. For instance, many behavioral protocols were generated and validated when the use of only males in rodent studies was common (Beery and Zucker, 2011; Shansky and Murphy, 2021), which makes it difficult to assess the same behavioral endpoints in females. Studies that do now include the use of both male and female subjects often lack substantial power to statistically detect sex differences, or fail to examine the data for sex differences at all (Coiro and Pollak, 2019). Nevertheless, when properly designed to account for potential sex differences, rodent models can help us identify how MIA may impact the neurodevelopment of males and females differently and contribute to the variety of phenotypes relevant for NDDs. Sex differences have been successfully explored in rodent models of MIA and are detailed in other review articles (Ardalan et al., 2019; Bauman and Van de Water, 2020; Breach and Lenz, 2022).

One mechanism by which sex of the offspring may interact with MIA may be through estradiol receptors (ERα and ERβ). Estradiol regulates the activation of innate immune signaling pathways and can influence the synthesis of pro- and anti-inflammatory cytokines by the NF-κB pathway (Kovats, 2015; Liu et al., 2017). For example, estradiol (E2) can inhibit this pathway *via* increased production of IκBα mRNA (Xing et al., 2012). The expression and activation of ERs vary between males and females, which causes differences in the magnitude and duration of the innate inflammatory response between sexes (Kovats, 2015; Arnold and Saijo, 2021). For instance, females have a higher basal expression of ERα and ERβ than males in human blood monocytes-derived macrophages (MDMs) (Campesi et al., 2017). Similarly, female mice had a higher basal density of ERβ relative to male mice at postnatal day 21 (P21) in the anteroventral periventricular nucleus (AVPV), an area important for cardiovascular functions supporting female reproduction (Zuloaga et al., 2014; Saper and Stornetta, 2015). When human MDMs were incubated with 100 ng/ml of LPS for 24 h, both expression and phosphorylation ERα were upregulated to a larger degree in males than females (Campesi

et al., 2017). Thus, it is possible that the female immune response to pathogens may occur on a temporally faster timeline than males, due to a lesser need to express or phosphorylate ERs in response to the immunogenic insult.

Sex differences in the density, maturation, or activation of microglia, the innate immune cells of the brain, may also contribute to variability in immune response between males and females (Schwarz et al., 2012; Klein and Flanagan, 2016; Hanamsagar et al., 2017; Ardalan et al., 2019). As discussed above, microglia have an active role in the developmental pruning and maturation of synapses, and compromising these functions can lead to alterations in neural circuit development and deficits in learning (Paolicelli et al., 2011; Schafer et al., 2012; Zhan et al., 2014; Tay et al., 2017). One hypothesis may be that MIA alters the number or activational state of microglia differently in males and females, which may contribute to sex differences in the ontology and manifestation of various NDDs. Indeed, microglia with a stout and ameboid morphology—which commonly occur when microglia are activated during an immune response or insult—are more prevalent in females than males from P0–P4 and from P30–P60 (Schwarz et al., 2012; Hui et al., 2020). Differences in microglial activation state may potentiate differences in the neuroimmune response between males and females (Osborne et al., 2018). The role of microglia in the maternal neuroimmune response to MIA has been well-studied, however there are often conflicting findings due to differences in study design and analysis methods (see review: Smolders et al., 2018).

Overall, there is a need to examine sex as a factor in both human and rodent studies of MIA. It is important to mention there are sex biases in the experimental designs, the inclusion of male and female animal subjects, and the neurochemical analyses of MIA studies (Coiro and Pollak, 2019). It is again essential to note that a proper experimental design including sex as a variable requires sex to be statistically included in the analysis (i.e., testing for an interaction between sex and another factor of interest). Researchers should also report that sex was included in their analyses, even when there are no significant findings. In all, developing well-designed experiments that include sex as a variable can help us better identify how neurobiological processes are differently dysregulated by the maternal and fetal immune response and how sex may interact with MIA to contribute to differences in NDD diagnostic rates between males and females.

6.3. Individual differences in immune response

There is often high variability in the maternal and fetal immune response when examining cytokine expression in rodent models of MIA, which suggests that there may be individual differences in the immune response to MIA (Sherer et al., 2017). This individual susceptibility or resilience to MIA can also manifest in offspring behavioral outcomes. For example, one study found that MIA with Poly I:C resulted in two groups of adult offspring with distinct behavioral phenotypes: those with enhanced prepulse inhibition (PPI) and those with deficits in PPI, as compared to saline-exposed offspring (Chamera et al., 2021). Interestingly, only the MIA-exposed offspring with enhanced PPI had altered protein levels of CX3CL1-CX3CR1 (molecules involved in microglia–neuron signaling, important for synaptic organization) in the frontal cortex and hippocampus. It is also essential for researchers to consider how differences in the immune response at the litter level (maternal immune response) or at the offspring level (fetal or postnatal immune response) may impact their experimental and/or statistical design. A few articles (see Lazic and Essioux, 2013; Weber-Stadlbauer and Meyer, 2019) have been published to help guide researchers experimentally and statistically account for sources of variability in rodent models.

Individual susceptibility or resilience in the response to MIA, at both the maternal and fetal levels, indicate that other biological, environmental, and genetic factors may have an influence on offspring outcomes related to NDDs (Meyer, 2019; Herrero et al., 2023). It is also possible that these other factors—such as genetics, parental age, dietary deficiencies, stress, and placental buffering—may contribute to or account for some of the observed immune and behavioral variability in human and rodent studies of MIA and NDDs. It is therefore essential to take such factors into consideration when designing rodent and human studies of MIA and, rather than shy away from potential variability within the data, investigate the potential factors that may have individual or multiplicative effects on MIA and subsequent predisposition to NDDs.

6.3.1. Genetics

Twin studies have identified a high concordance among monozygotic (MZ) twins that is much lower in dizygotic (DZ) twins, demonstrating that many NDDs—namely ASD or schizophrenia—have a strong genetic link (Tick et al., 2016). That said, while thousands of genes, copy number variants, and *de novo* mutations have been associated with NDDs, to date there have been no risk loci identified that are common within each type of NDD or across all types of NDDs (Vorstman et al., 2017; López-Rivera et al., 2020). Rather, epidemiological data suggest that genetic risk provides a foundation upon which other factors may precipitate or enhance the risk for many NDDs (Zawadzka et al., 2021). It is possible that this genetic risk could be hereditary in nature, as both a familial history of psychiatric disorders and a parental history of severe infections seem to be involved in the association between MIA and offspring NDD risk, as discussed above.

Accordingly, genes that are implicated in schizophrenia may also impact how the body processes and fights off different pathogens, including influenza, rubella, and *T. gondii* (Carter, 2009), which suggests that individuals with these genes may be more prone to infections and, in turn, more at risk of NDDs as a consequence of the maternal infection. Similarly, people with ASD have an upregulation in genes that regulate neural cell development, but also in genes that regulate the immune response, the inflammatory response, antigen production and presentation, as well as immune cell signaling (Voineagu et al., 2011; Voineagu and Eapen, 2013). These genetic markers can also increase one's susceptibility to other inflammation-inducing factors, such as diet, physical stress, psychological stress, etc. There is also evidence that MIA produces transcriptional changes in expression of inflammatory markers, GABAergic signaling proteins, and myelin, and may drive epigenetic changes in the transcription of genes associated with NDDs (Woods et al., 2021). For example, MIA with Poly I:C in mice produced an integrated stress response (ISR) in male offspring, associated with increased phosphorylation of eIF2α which is important for cellular translation (Kalish et al., 2021). Therefore, genetic influences may impact both the maternal immune response as well as the neurodevelopmental and behavioral processes in offspring that are ultimately affected by the immune response.

6.3.2. Parental age

Maternal and paternal age have also been implicated in the risk for certain NDDs. For instance, older maternal and paternal ages have been linked with increased risk for ASD (Abdallah et al., 2012; Sandin et al., 2012; Carlsson et al., 2021). Advanced paternal and maternal ages have also been associated with increased risk for schizophrenia and psychosis (El-Saadi et al., 2004; Lopez-Castroman et al., 2010; Fountoulakis et al., 2018). On the other hand, younger maternal age has also been implicated in risk for psychosis, when controlling for paternal age (El-Saadi et al., 2004). Younger maternal and paternal ages have been associated with increased risk for ADHD diagnosis overall (Chang et al., 2014; Hvolgaard Mikkelsen et al., 2017). Interestingly, older maternal age and younger paternal age have been associated with hyperactivity/impulsivity symptoms of ADHD, whereas younger maternal and paternal ages have been linked to inattentive symptoms of ADHD (Ghanizadeh, 2014; Sciberras et al., 2017).

Not much is known about *how* parental age influences the risk of NDD in children. Maternal age may influence the immune response to MIA, given that immune function changes with age (though usually much older ages; see Haynes, 2020). Increased maternal age is also associated with an increased risk of pregnancy and obstetric or birth complications that are often associated with inflammation, such as preeclampsia, gestational diabetes, or general hypertension (Londero et al., 2019). The role of paternal age as a risk for NDDs implies that genetics may also have a role in this relationship. As the body ages, there is a greater risk for genetic mutations in the eggs or the sperm that would contribute to an increased risk of NDDs. Moreover, environmental exposure to toxins or infections throughout the lifespan may also result in *de novo* genetic mutations that can be passed to offspring and increase their risk for NDDs, an effect that appears to happen more frequently in sperm than in eggs (Kong et al., 2012; Jónsson et al., 2017).

Notably, the age of the dam or sire are not consistently reported or controlled for in animal models, nor how many litters that any one dam has had previously. Furthermore, there are few to no animal studies that have examined maternal or paternal age as a risk factor that may interact with MIA or developmental outcomes in the offspring. As with all controlled rodent studies, reporting the age of the mating pair is important, whether or not it has an effect on MIA or on the behavioral outcomes in the phenotype being examined. Human epidemiological studies often control for age as a covariate when examining the link between MIA and NDDs, however few studies actively include it as a variable in their overall analysis. As future studies characterize the role of aging in the risk of NDDs, they should consider use of a multivariate model that considers parental age, along with infection during or before pregnancy in either the mothers or the fathers, to get a better understanding of how these risk factors interact.

6.3.3. The gut microbiome and dietary factors

The gut microbiota can be impacted by diet and by metabolic conditions. Maternal metabolic conditions such as obesity, diabetes, and hypertension have been associated with increased risk for ASD (Van Lieshout and Voruganti, 2008; Krakowiak et al., 2012). In mice, maternal high-fat diet has been shown to produce microglia-associated changes in myelination and increase the number of perivascular microglia in the offspring brain (Bordeleau et al., 2021, 2022), as well as cause offspring to have less diverse gut communities, decreased oxytocin production in the paraventricular nucleus of the hypothalamus, and diminished synaptic plasticity in the ventral tegmental area (Buffington et al., 2016). Maternal high-fat diet itself has been used to model MIA in rodents, as it can trigger a chronic inflammatory profile in the dam and can produce behavioral phenotypes in offspring that are related to NDDs, including increased repetitive behaviors and disruptions in social and cognitive behaviors (Sullivan et al., 2015; Buffington et al., 2016; Penna et al., 2020; Bordeleau et al., 2021, 2022). Gestational diabetes may also interact with MIA to impact neurodevelopmental processes in offspring (Van Lieshout and Voruganti, 2008). Prenatal exposure to both gestational diabetes mellitus and Poly I:C in mice resulted in offspring with an altered transcriptional profile of genes that are associated with differentiation of dopamine neurons and the innate immune response (Money et al., 2018). Finally, antibiotic use can also alter the composition of the gut microbiota (Patrono et al., 2021). In humans, second trimester influenza infection associated with ASD risk was only apparent when antibiotics were *not* taken at any point during the pregnancy (Holingue et al., 2020), which suggests that the antibiotics may have altered the microbiome in a way that prevented the negative consequences of influenza from affecting the developing fetal brain.

The gut microbiota are essential in regulating the immune system, including the proliferation and differentiation of T- and B-cells that drive the maternal cytokine production implicated in MIA (Minakova and Warner, 2018). Certain forms of commensal gut bacteria, like segmented filamentous bacteria, are more likely to induce differentiation of T-cells that produce IL-17a, which is a cytokine that has consistently been associated with behavioral changes (particularly decreased social behaviors) and cortical abnormalities in various models of MIA (Kim et al., 2017). Colonization of Pregnant female mice that were colonized with segmented filamentous bacteria, then challenged with Poly I:C on E12.5, were more likely to produce TH-17 cells and have offspring with distinct behavioral phenotypes characteristics of NDDs (Kim et al., 2017), likely triggered by exposure to the enhanced IL-7 production from the maternal gut's adaptive immune cells (Kim et al., 2017). Similarly, increased levels of pro-inflammatory cytokines in the gut have also been associated with the positive symptoms of schizophrenia in humans (Patrono et al., 2021). Taken together, the gut microbiome may prove useful in providing additional biomarkers for immune dysregulation associated with NDDs or as targets for therapies against NDDs, particularly if the microbiome changes in concert with, or before the onset of, symptoms for many NDDs.

Maternal diet deficiencies of iron, omega-3 fatty acids, and folic acid may also impact neurodevelopmental outcomes in rodents and humans in the context of MIA. Long ago, researchers determined that folic acid was necessary as part of the maternal diet to ensure proper development of the fetal neural tube (Greenberg et al., 2011). In mice exposed to LPS on E17, omega-3 deficiency in the maternal diet caused increased IL-6 expression in maternal plasma, placenta, and fetal brain (Richardson and Ross, 2000; Labrousse et al., 2018). Adult offspring exposed to both the MIA and omega-3 deficiency during development had spatial memory deficits in a Y-maze task. Furthermore, in humans, anemia, with or without exposure to prenatal infection, is associated with an increased risk for

schizophrenia (Nielsen et al., 2016). In rats, dams fed an iron-deficient diet had increased serum levels of IL-6 and TNF-α following prenatal LPS on E15, as compared to typical chow-fed dams (Harvey and Boksa, 2014). Moreover, exposure to iron-deficiency and to MIA independently caused deficits in the offspring's development of various sensorimotor behaviors. In all, there is limited evidence of multiplicative effects between dietary iron deficiencies and MIA exposure, however both seem to be independently implicated in the risk for NDDs.

Perhaps with growing evidence such as that described here, future research should examine whether the gestational/developmental timing of dietary deficiencies or alterations in the gut microbiota may interact with MIA to increase the risk of NDDs. In turn, studies should examine whether diet-derived supplementations might mitigate the effects of MIA. For example, maternal dietary supplementation with choline, around the time of birth in rats, attenuated the splenic cytokine immune response of 3-week-old offspring to an *ex vivo* immune challenge (Richard et al., 2017). In addition, several dietary factors—including high maternal iron, zinc, and vitamin D—have been associated with resilience to effects of MIA *via* anti-inflammatory cytokine production and enhancement of antioxidant systems (Vuillermot et al., 2017; Meyer, 2019).

6.3.4. Prenatal stress and inflammation

Prenatal stress has long been associated with an increased risk of various NDDs, most notably schizophrenia, ADHD and autism (Ronald et al., 2011; Diz-Chaves et al., 2012, 2013; Chan et al., 2018; Minakova and Warner, 2018; Makris et al., 2022). More recently, this association has been further characterized by changes in inflammatory biomarkers in the maternal circulation that may increase the risk of various NDDs. For example, even socioeconomic disadvantage is a stressor that is associated with transcriptional indications of greater immune activation and slower tissue maturation in the placenta (Miller et al., 2017). This stress can lead to overproduction of pro-inflammatory cytokines by immune cells in response to additional immunostimulation (Miller et al., 2017). Stress-induced susceptibility to MIA may be linked to changes in baseline maternal cortisol levels (Van den Bergh et al., 2005), resulting in continuously elevated or stimulated pro-inflammatory cytokine levels that may impact fetal neurodevelopment associated with NDD risk.

Animal models of prenatal stress have demonstrated a pro-inflammatory cytokine response, particularly IL-6, with microglial activation similar to that elicited by MIA models. Specifically, prenatal stress in rodents enhanced cytokine levels in the hippocampus and increased the total number of immunoreactive microglial cells in the offspring compared to non-stressed animals, which exacerbated the inflammatory response to LPS (Diz-Chaves, et al., 2012). Behavioral phenotypes of anxiety, learning deficits, and depressive-like symptoms in prenatally stressed rat and non-human primate offspring are further associated with maternal and fetal HPA-axis alterations (Weinstock, 2005; Weinstock, 2008). Gestational stress and excess corticosterone in maternal and fetal plasma can impair feedback regulation of the HPA axis in both infancy and adulthood and can increase corticotropin-releasing hormone (CRH) activity in the amygdala (Van den Bergh et al., 2005; Weinstock, 2005; Weinstock, 2008). Excess amounts of CRH and cortisol that reach the fetal brain during periods of chronic maternal stress could thereby influence how the fetal brain responds in the presence of MIA, or how the brain is programmed to respond to subsequent stressors or immune challenges later in life.

Animal models often fail to report or account for unintended stressors in their models that may interact with MIA to exacerbate the neural and behavioral consequences in dams and offspring. For instance, stress associated with ambient noise levels, bedding levels, handling, injection procedures, behavioral tests, caging conditions, and nearby construction are all factors that may commonly occur throughout the course of an experiment. Researchers should take care to reduce exogenous stressors wherever possible, and when unable to control for such factors, should document and report them in the literature.

6.3.5. The placenta: Protector or instigator?

The placenta is an important organ that connects mother and fetus, providing oxygen and nutrition to the baby while protecting the delicate fetus from certain factors, most notably infections, that could harm it. That said, while it is well-known that many pathogens and larger immunogenic molecules *do not* cross the placental barrier, the placenta might also be implicated in the active transfer of immune molecules through the circulation to the fetus (Robbins and Bakardjiev, 2012). Unfortunately, research examining the site of the placental transfer of cytokines associated with MIA is sparse. Nevertheless, it is important to understand the role of the placenta as a site of cytokine transfer during MIA.

Decades ago, research indicated that monozygotic twins concordant for schizophrenia were more likely to have been monochorionic and to have shared a single placenta, whereas discordant monozygotic twins appear more likely to have been dichorionic with separate placentas (Davis et al., 1995). In human twin pregnancies with a conjoined placenta, the dividing membrane between the two placentae can be composed of four layers—the amnion and chorion of each twin—which allows some degree of shared circulation between the two fetuses (Benirschke, 1990). In this case, each twin may be exposed to similar circulating molecules, such as cytokines, from the mother. Maternal immune and endothelial cells come into contact with extravillous fetal cells at the uterine implantation site, allowing for maternal blood to surround the epithelial covering of placental cells, called syncytiotrophoblasts.

The syncytiotrophoblasts have been shown to be resistant to infections and thereby may contribute to the protective function of the placenta. At the same time, they are a type of immune cell that can initiate their own cytokine response in the presence of innate immune receptor activation. Due to their hemochorial nature, the placental buffer in rats and humans function in similar ways. Like in humans, the trophoblast epithelium of the rat placenta is directly bathed in maternal blood (Furukawa et al., 2019). In humans, this occurs at the decidua, the site of uterine implantation, which only has one dividing layer (the syncytiotrophoblasts). However, the rat has three layers at this site, which might imply differences in the fetal-maternal exchange processes between the two species. In both species, uterine natural killer (NK) cells are present in parts of the placenta, and help the uterus to adapt and accommodate for the fetus. In rats, MIA with Poly I:C can increase maternally-derived IL-6 protein directly in the placenta, which activates the JAK/STAT3 pathway and causes expression of acute phase immune genes in the

placenta that can enter into the fetal circulation (Hsiao and Patterson, 2011). In humans, IL-6 is transferred bidirectionally between maternal and fetal circulation (Zaretsky et al., 2004). While studies suggest that many immunogenic molecules, like LPS, do not cross the placental barrier (Ashdown et al., 2006; Ning et al., 2008), there remains some debate of whether fetal immune activation by way of MIA occurs *via* the reception of cytokines from the maternal circulation or *via* an immune response precipitated in the placenta itself. Thus, additional research should be performed to examine the rat placental barrier and its potential ability to transfer immune molecules from maternal circulation.

Moreover, early work has identified dichorionic monozygotic twins as having a *lower* rate of concordance for various NDDs (Davis et al., 1995), which should also be considered within the context of rodent pregnancies where each pup has its own placenta and might respond differently to MIA. Hormones can travel through the multiple placentae among fetuses due to the blood flow of the mother. More specifically, in pregnant rats, blood flows from the caudal to distal direction, or from cervix to ovaries. Thus, a rat fetus located at the cervical end of the uterus will receive maternal blood flow prior to fetuses in other uterine positions. In litter-bearing mammals that have multiple pregnancies, effects of intrauterine position on fetal development have been observed (Ryan and Vandenbergh, 2002). For instance, female fetuses that develop downstream from male fetuses have been shown to exhibit slightly masculinized anatomical, physiological, and behavioral characteristics as adults, including altered hormone levels and disrupted endocrine systems (Ryan and Vandenbergh, 2002). This is due to diffusion of testosterone from male fetuses to their uterine neighbors *via* amniotic fluid and the maternal circulation. Given this mechanism of hormonal transfer, it may be possible for the same type of transfer to occur with immune factors such as cytokines; this may result in differential exposure to MIA-associated molecules between fetuses based on their uterine position. It is also possible that some fetuses may be more exposed to the circulating maternal immune molecules from the pregnant dam in MIA models, particularly those located more caudally as they are the first to receive maternal blood flow.

In all, more research is needed to consider the role of the placenta in the fetal response and susceptibility to the inflammatory effects of MIA. Researchers should consider how differences in rodent and human pregnancies—particularly the number of fetuses, characteristics of the placental barrier, and maternal transference or fetal production of cytokines and other immunogens—may impact the generalizability of their findings to human NDDs and the translatability of rodent models of MIA to humans.

7. Discussion

We conclude this review with a figure that identifies the various factors that may influence the developing fetus in the context of MIA and the ontogeny of NDDs (Figure 1). Our conclusion is that every study need not examine every one of these factors in their experimental design. Rather, basic research that investigates the effect of MIA on NDDs should consider these factors when analyzing and interpreting their data. Kentner et al. (2019) have introduced a list of reporting guidelines for animal models of MIA in an effort to help standardize MIA models, to provide transparency in variability of these factors across labs, and to better enable reproducibility of findings across laboratories. Studies may still contain variability associated with these factors of consideration that we have introduced; however, this variability is similar to that observed in the risk factors and behavioral symptoms associated with human NDDs. Further investigation is still required for us to better understand the general effects of each of these factors, how they interact with perinatal immune activation (particularly with regard to the degree and severity of the MIA response), and how they contribute

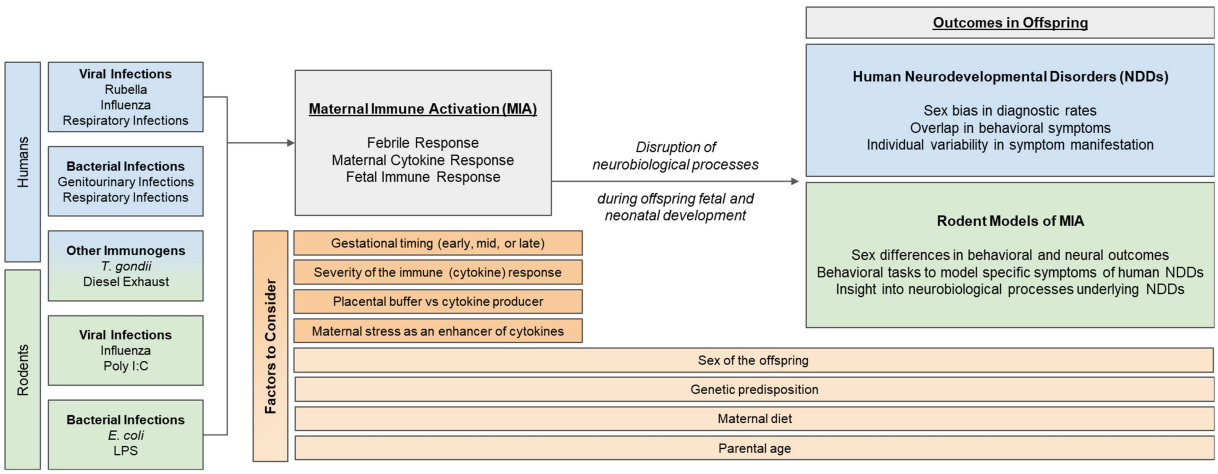

FIGURE 1
Proposed model of the association between maternal immune activation (MIA) and neurodevelopmental disorders (NDDs). Blue boxes represent human pathogens and outcomes related to MIA. Green boxes represent rodent immunogens and outcomes related to MIA. Orange boxes represent factors that should be considered in rodent models of MIA and their relevance for human NDDs. Dark orange boxes represent factors that are related to the gestational immune response, whereas light orange boxes represent factors that are related to the immune response both during gestation and in postnatal offspring.

to the ensuing manifestation or ontogeny of the behavioral and neural phenotypes associated with NDDs.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Brain effects of gestating germ-free persist in mouse neonates despite acquisition of a microbiota at birth

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At birth, mammals experience a massive colonization by microorganisms. We previously reported that newborn mice gestated and born germ-free (GF) have increased microglial labeling and alterations in developmental neuronal cell death in the hippocampus and hypothalamus, as well as greater forebrain volume and body weight when compared to conventionally colonized (CC) mice. To test whether these effects are solely due to differences in postnatal microbial exposure, or instead may be programmed *in utero*, we cross-fostered GF newborns immediately after birth to CC dams (GF→CC) and compared them to offspring fostered within the same microbiota status (CC→CC, GF→GF). Because key developmental events (including microglial colonization and neuronal cell death) shape the brain during the first postnatal week, we collected brains on postnatal day (P) 7. To track gut bacterial colonization, colonic content was also collected and subjected to 16S rRNA qPCR and Illumina sequencing. In the brains of GF→GF mice, we replicated most of the effects seen previously in GF mice. Interestingly, the GF brain phenotype persisted in GF→CC offspring for almost all measures. In contrast, total bacterial load did not differ between the CC→CC and GF→CC groups on P7, and bacterial community composition was also very similar, with a few exceptions. Thus, GF→CC offspring had altered brain development during at least the first 7 days after birth despite a largely normal microbiota. This suggests that prenatal influences of gestating in an altered microbial environment programs neonatal brain development.

KEYWORDS

cross-fostering, cell death, microglia, forebrain size, bacterial load, colonic content

1. Introduction

Microbiota from maternal and environmental sources rapidly colonize all epithelial surfaces of mammalian neonates at birth. Disruptions of the maternal microbiota during pregnancy, such as those resulting from a high fat diet or antibiotic treatment, alter the vertical transmission of microbes from mother to offspring and have long-term effects on

offspring physiology and behavior (Olszak et al., 2012; Bokulich et al., 2016; Leclercq et al., 2017; Schulfer et al., 2018; Chen et al., 2021a,b; O'Connor et al., 2021). In addition, several recent studies suggest *in utero* effects of the maternal microbiota on fetal development (Humann et al., 2016; Tochitani et al., 2016; Kim et al., 2017; Thion et al., 2018; Pronovost and Hsiao, 2019; Vuong et al., 2020), due to the presence of bacterial metabolites in maternal circulation that cross the placenta or other signaling mechanisms.

By far the largest population of microbes resides in the distal gastrointestinal tract (i.e., the colon), with bacteria comprising the vast majority of those microorganisms (Sender et al., 2016). The gut microbiota communicates reciprocally with the brain via the gut-microbiota-brain axis (Cryan and Dinan, 2012; Morais et al., 2021), and animals living in the absence of microbes [i.e., germ-free (GF)] have played a crucial role in establishing this link. GF mice have an altered neuroendocrine stress response, changes in hippocampal neurogenesis, reduced anxiety, and altered social behavior in adulthood compared to conventionally colonized (CC) controls (e.g., Sudo et al., 2004; Diaz Heijtz et al., 2011; Clarke et al., 2013; Ogbonnaya et al., 2015). Some of these changes are normalized by introducing a microbiota in adulthood or adolescence, but others persist, suggesting early life neural programming. However, the specific brain processes affected early in life by microbe exposure are largely unknown.

Microglia are the macrophages and primary innate immune cells of the brain, and they respond to the microbiota throughout life. GF adults have increased microglial numbers but decreased microglial responsiveness to immune challenges compared to controls (Erny et al., 2015; Matcovitch-Natan et al., 2016). The co-housing of GF dams and their litters with CC female mice soon after birth reduces microglial numbers in comparison to GF mice when examined in adulthood (Erny et al., 2021), suggesting a normalization of microglia in GF mice by long-term postnatal colonization. How quickly the normalization occurs, however, is unknown. This is an important question because current obstetric practices routinely alter the microbiota of pregnant mothers and their babies. For example, 40% of mothers in the United States are treated peripartum with antibiotics (Ledger and Blaser, 2013; Martinez de Tejada, 2014) that cause a marked depletion of their microbiota and that of their offspring. Even transient alterations in the microbiota during perinatal life could have lasting effects on offspring brain development, given the many important neurodevelopmental events that occur during the early postnatal period. In rodents, a depletion of the maternal/prenatal or postnatal microbiota by antibiotics alters social behaviors and anxiety-like behavior in the offspring in adolescence and adulthood (Tochitani et al., 2016; Leclercq et al., 2017; O'Connor et al., 2021; Lynch et al., 2023).

Microglial colonization and naturally occurring cell death are two of the most salient neurodevelopmental events occurring around the time of birth in mice. We recently showed that, compared to CC mice, those that are gestated and born into a GF environment have increased microglial labeling and altered neuronal cell death in the brain during the newborn period (Castillo-Ruiz et al., 2018a). It is unknown whether these changes are due solely due to the postnatal absence of microbes, or whether the maternal microbiota may program offspring brain development before birth. To test this, mice in the current study were gestated and born to a GF mother and then cross-fostered immediately after birth to CC dams; newborns fostered within microbial status served as controls. Colon contents and brains of offspring were collected 7 days later to compare bacterial colonization of the gut and several measures of brain development. Our results suggest that maternal microbial status *in utero* has a prolonged effect on neonatal brain development.

2. Materials and methods

2.1. Animals

Adult Swiss Webster GF and CC mice were purchased from Taconic Biosciences (Germantown, NY, USA). All mice were housed in our GF facility in an isolated, ventilated caging system (Isocage, Techniplast, Buguggiate VA, Italy). Mice were maintained on a 12:12 light-dark cycle with *ad libitum* access to autoclaved food and water. All animal procedures were approved by Georgia State University's Institutional Animal Care and Use Committee (protocol #A20013) and followed the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

2.2. Cross-fostering procedure

Females and males were housed together for 1–4 days. Beginning on the eve of the first possible embryonic day (E) 19, we performed hourly, around-the-clock checks for births, with checks during the dark period performed under red light illumination. Immediately upon observing the birth of a litter, cages were thoroughly sprayed with a sterilizing solution (1 part Exspor base: 1 part Expsor activator: 4 parts tap water; Ecolab Inc., Saint Paul, MN, USA) and placed within a biosafety cabinet that prior to the procedure had been UV treated and sprayed with the sterilizing solution. Offspring were gently transferred to a sterile container using a sterile set of tweezers before being assigned to a foster dam that had given birth within the previous 48 h. The foster dam's own pups were removed and experimental pups (whole litters) were then placed in the foster dam's cage under sterile conditions. We cross-fostered GF pups to CC dams (GF→CC group; $n = 34$), and, to control for the cross-fostering procedure, CC and GF pups to dams within the same microbiota status (CC→CC group, $n = 37$ and GF→GF group, $n = 15$) (**Figure 1**). In two additional cases, foster mothers were not available for control litters (one CC→CC $n = 17$ and one GF→GF litter $n = 10$) and these pups were sham cross-fostered; that is, they underwent all the procedural steps of cross-fostering (spraying of cages, placement of pups in sterile holding container) but pups were returned after a delay to the birth mother. Sham cross-fostered mice did not differ from pups fostered to an unrelated mother for any dependent variable tested (determined by ANOVA or t-tests within microbial status, as appropriate) and are therefore included in the analyses below and identified as sham cross-fostered on all figures. The total number of litters represented in each group was four for CC→CC, two for GF→GF and three for GF→CC. Note that due to low GF pregnancy rates, it was challenging to foster GF pups within microbial status; this explains the lower number of litters and subjects for the GF→GF group.

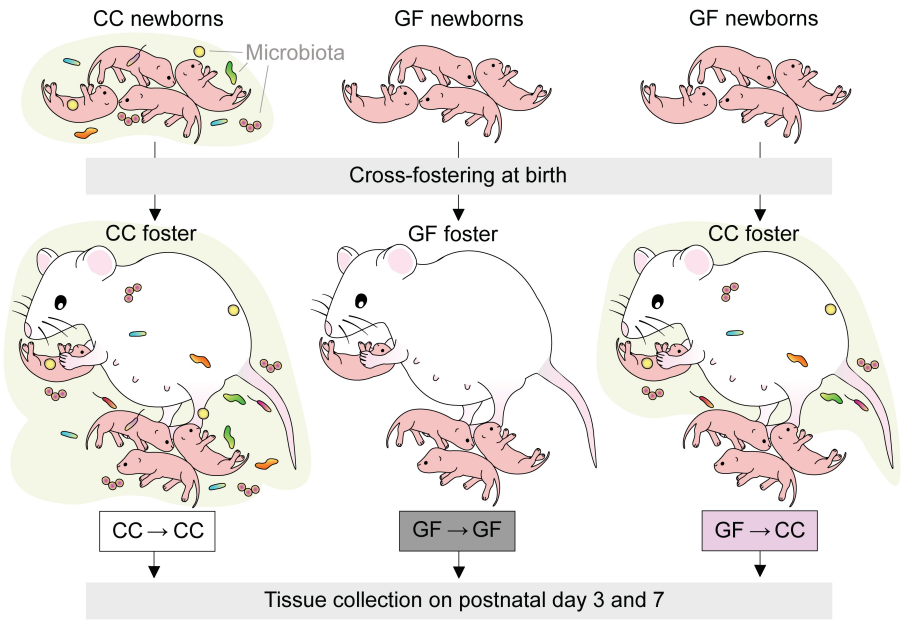

FIGURE 1
Experimental design. GF newborns were cross-fostered immediately after birth to CC dams (GF→CC group) and compared to offspring fostered within the same microbiota status (CC→CC, GF→GF groups).

2.3. Tissue collection

To assess how rapidly gut colonization takes place, we sacrificed half of each litter at P3 and collected colon contents from a subset of mice (CC→CC $n = 16$; GF→CC: $n = 12$; GF→GF $n = 14$). To assess brain effects related to bacterial colonization of the gut, we collected brains (CC→CC $n = 20$; GF→CC: $n = 12$; GF→GF $n = 10$) and colon contents (CC→CC $n = 17$; GF→CC: $n = 10$; GF→GF $n = 10$) of a subset of offspring at P7. On collection days, mice were weighed and immediately euthanized via rapid decapitation 8–10 h after lights on. Brains (P7) were fixed in 5% acrolein in 0.1 M phosphate buffer for 24 h at room temperature and then transferred to 30% sucrose at 4°C, followed by cryoprotection at −20°C until sectioning. Colon contents (P3 and P7) were collected by excising the colon and gently extruding contents with the flat surface of a curved, sterile tweezer. Contents were weighed, and stored at −80°C prior to processing.

2.4. Immunohistochemistry

Brains were coronally sectioned on a freezing microtome into four, 40 μm series. Sections were collected into cryoprotectant solution and stored at −20°C. One series was processed for the immunohistochemical detection of ionized calcium binding adaptor molecule 1 (Iba1) to label microglia, and two series for the detection of activated caspase-3 (AC3) to identify dying cells. Unless otherwise stated, tissue was washed between steps in 1X tris buffered saline (TBS) and all steps were carried out at room temperature. Epitope retrieval was performed with 0.05 M sodium citrate for 1 h for Iba1 or 30 min for AC3. Then, unreacted aldehyde was blocked via incubation with 0.1 M glycine for 30 min, followed by an incubation in a blocking solution (20% normal goat serum (NGS), 1% H_2O_2, 0.3% Triton X in TBS), and an overnight incubation with the primary antibody: rabbit anti-Iba1 (Wako, Chuo-Ku, Osaka, Japan; 1:3,000; 2% NGS, 0.3% Triton X in TBS) or rabbit anti-AC3 (Cell Signaling, Beverly, MA, USA; 1:5,000; 2% NGS, 0.3% Triton X in TBS). Sections were washed in a dilute blocking solution (1% NGS, 0.02% Triton X in TBS), incubated for 1 h in a goat anti-rabbit secondary antibody (Vector Laboratories, Burlingame, CA, USA; 1:1,000 for Iba1 or 1:500 for AC3; 0.32% Triton X in TBS), washed in 1X TBS-0.2% Triton X, and incubated for 1 h in an avidin–biotin solution (Vector Laboratories; 1:1,000 for Iba1 or 1:500 for AC3 in 1X TBS). Tissue was washed in acetate buffer and incubated in 0.02% diaminobenzidine tetrahydrochloride, 2% nickel sulfate, and 0.0025% H_2O_2 made in the same buffer. Sections were mounted onto gelatin-coated slides, counterstained with thionin in the case of AC3-immunoreacted tissue, dehydrated, and coverslipped.

2.5. Quantification of microglia, dying cells, and forebrain size

All analyses were performed on coded slides by an investigator blind to treatment group. We analyzed brain regions where we previously observed differences between neonatal GF and CC mice: the paraventricular nucleus of the hypothalamus (PVN), the CA1 oriens layer of the hippocampus, and the arcuate nucleus (ARC) (Castillo-Ruiz et al., 2018a). In addition, we included the primary somatosensory cortex (S1) in our analyses of microglia as microbiota-dependent effects have been previously reported for microglia in this region (Thion et al., 2018). For the PVN, we analyzed all available sections, starting when the nucleus has a tubular shape (Plates 127-131 in Paxinos et al., 2007). For the CA1 oriens, we included sections from the rostral-most appearance of

the dentate gyrus (Plate 128) to the point where the hippocampus starts to tip ventrally (Plate 131). For the ARC, sampling started at the point where the nucleus has a well-defined triangular shape (Plate 133) and ended when the nucleus was no longer visible (Plate 142). S1 was analyzed in three consecutive sections, starting where the dentate gyrus is clearly defined (Plate 128) and ending when the hippocampus tips ventrally (Plate 131), as described in Strahan et al. (2017).

Slides were scanned using a Hamamatsu Nanozoomer (Hamamatsu Photonics K. K. Hamamatsu City, Japan) and cell quantification was performed using Aperio Image Scope (Leica Biosystems Inc., Buffalo Grove, IL, USA). Contours were drawn around the regions of interest and the number of microglia and dying cells within those contours was recorded. The sum of AC3+ and Iba1+ counted cells across all sections in each animal was divided by total area sampled, and then multiplied by section thickness to obtain cell density per mm^3.

To assess forebrain size, we outlined the left side of the forebrain in one series of the AC3 labeled tissue, using six alternate sections, starting from the section where the medial border of the anterior commissure lies ventral to the tip of the lateral ventricle (Plate 117) and ending at the section with the rostral most appearance of the dorsomedial nucleus of the hypothalamus (Plate 133), as previously described (Castillo-Ruiz et al., 2018a). The sum of areas across all sections was multiplied by two and then by section thickness to obtain overall forebrain volume in mm^3 for each animal.

2.6. DNA extraction from colon contents

Deoxyribonucleic acid extraction from colon contents was performed using the QIAamp fast DNA stool mini kit (Qiagen LLC, Germantown, MD, USA) according to the manufacturer's instructions, with the addition of a bead beating step at the beginning of the procedure to aid with homogenization: samples were transferred to PowerBead Pro Tubes (Qiagen) and agitated for 2 min in the Mini-Beadbeater (Biospec Products, Inc., Bartlesville, OK, USA). The stock DNA was used for polymerase chain reaction (PCR) and sequencing analysis of the 16S rRNA gene.

2.7. 16S rRNA PCR for total bacterial load

Polymerase chain reaction was performed in the C1000 Touch Thermal Cycler (Bio-Rad, Hercules, CA, USA) (2 min at 95°C, followed by 40 cycles of 5 s at 95°C and 10 s at 60°C) using a QuantiNova SYBR green PCR kit (Qiagen) with universal 16S rRNA primers 8F: 5′-AGAGTTTGATCCTGGCTCAG-3′ and 338R: 5′-CTGCTGCCTCCCGTAGGAGT-3′. Negative controls were run concurrently and included clean paper towels used for sample collection and buffer from the DNA extraction kit. The quantitative cycle (Cq) values for negative control and GF samples were very close to the final cycle of the PCR run (mean = 38.47; SEM = 0.19; compare these values with the much earlier read outs from CC groups: mean = 22.51; SEM = 0.31). In order to calculate fold-increase in bacterial load in CC groups, we used the GF Cq values as reference. Bacterial load was calculated using the formula $2^{-(\Delta Cq)}$, where ΔCq was obtained by subtracting the Cq average of the GF→GF group from each individual animal's Cq value. Fold-change values were then obtained by dividing each experimental value by the average for the GF→GF group.

2.8. 16S rRNA gene sequencing and analysis

16S rRNA gene amplification and sequencing were performed using Illumina MiSeq technology (Illumina Inc., San Diego, CA, USA). The 16S rRNA genes, region V4, were PCR amplified from each sample using a composite forward primer and a reverse primer containing a unique 12-base barcode, designed using the Golay error-correcting scheme, which was used to tag PCR products from respective samples (Caporaso et al., 2012). We used the forward primer 515F 5′-*AATGATACGGCGACCACCGAGATCTACACGCT*XXXXXXXXXXXX**TATGGTAATT*GT***GTGYCAGCMGCCGCGGTAA-3′: the italicized sequence is the 5′ Illumina adaptor, the 12 X sequence is the Golay barcode, the bold sequence is the primer pad, the italicized and bold sequence is the primer linker, and the underlined sequence is the conserved bacterial primer 515F. The reverse primer 806R used was 5′-*CAAGCAGAAGACGGCATACGAGAT***AGTCAGCCAGCC***GGACTACNVGGGTWTCTAAT-3′: the italicized sequence is the 3′ reverse complement sequence of Illumina adaptor, the bold sequence is the primer pad, the italicized and bold sequence is the primer linker and the underlined sequence is the conserved bacterial primer 806R. PCR was performed using a Hot Master PCR mix (Quantabio, Beverly, MA, USA) in the C1000 Touch Thermal Cycler (3 min at 95°C, followed by 30 cycles of 45 s at 95°C, 60 s at 50°C and 90 s at 72°C). PCR products were purified with Ampure magnetic purification beads (Agencourt, Brea, CA, USA), and visualized by gel electrophoresis. Products were then quantified (BioTek Fluorescence Spectrophotometer; BioTek Instruments, SAS, France) using Quant-iT PicoGreen dsDNA assay (Invitrogen, Carlsbad, CA, USA). A master DNA pool was generated from the purified products in equimolar ratios. The pooled products were quantified using Quant-iT PicoGreen dsDNA assay and then sequenced using an Illumina MiSeq sequencer (paired-end reads, 2 × 250 bp) at Cornell University, Ithaca.

Sequences were demultiplexed and quality filtered using the Dada2 method (Callahan et al., 2016) with QIIME2 default parameters in order to detect and correct Illumina amplicon sequence data, and a table of QIIME2 artifact was generated. A tree was next generated, using the QIIME fragment-insertion sepp command, for phylogenetic diversity analyses, and alpha and beta diversity analyses were computed using the core-metrics-phylogenetic command. For taxonomy analysis, features were assigned to amplicon sequence variants (ASVs) with a 99% threshold of pairwise identity to the Greengenes reference database 13_8 (McDonald et al., 2012).

2.9. Statistics

We combined the data for males and females in all analyses below. Preliminary analyses did not identify significant

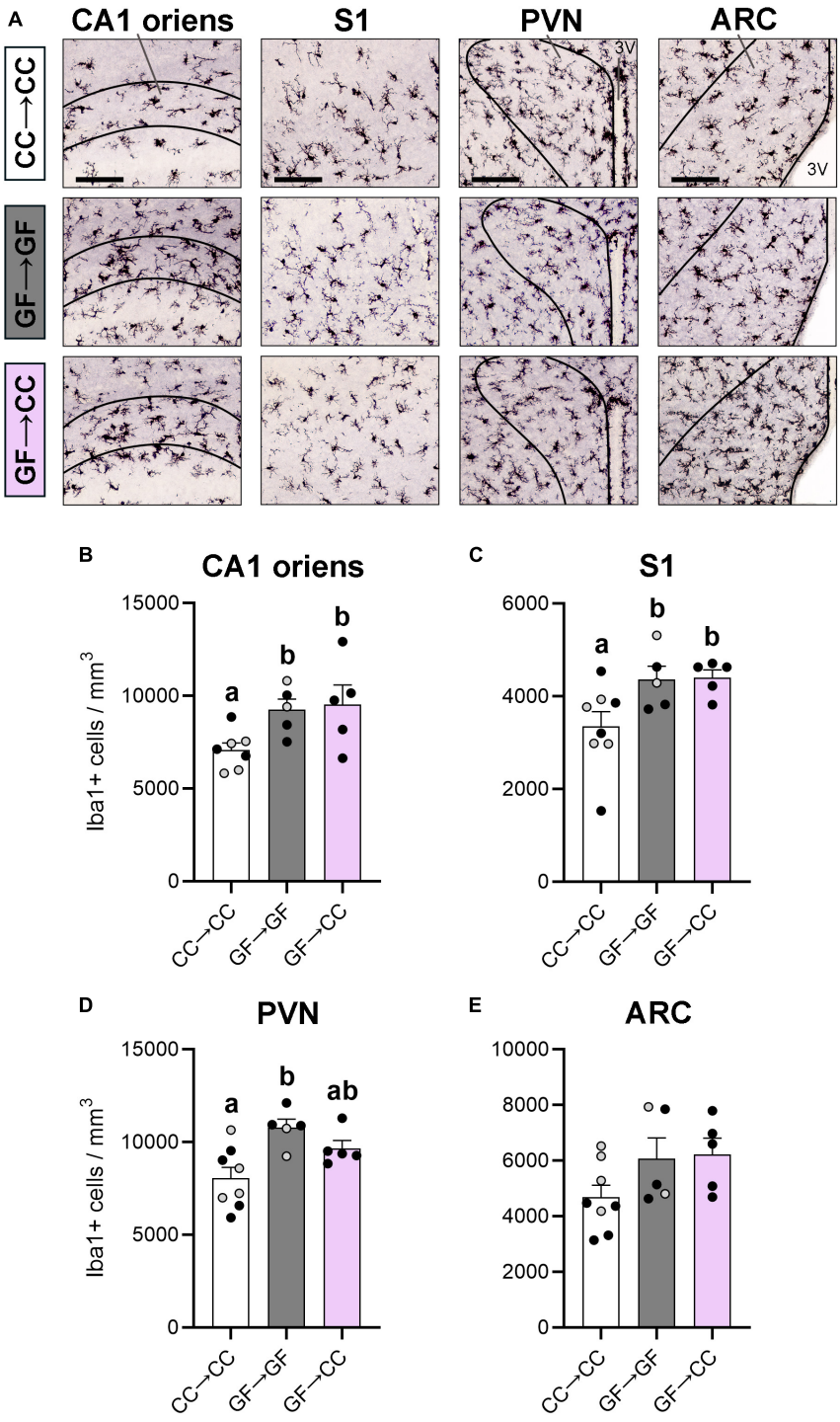

FIGURE 2
Microglial effects of gestating germ-free persist in mouse neonates despite introduction to a microbiota at birth. **(A)** Photomicrographs of Iba1 + stained tissue in representative CC→CC, GF→GF, and GF→CC mice, showing the brain regions analyzed: CA1 oriens, S1, PVN, and ARC (regions smaller than field of view indicated with black lines). 3V, third ventricle. Scale bar = 100 μm. **(B,C)** Microglial density was higher in groups gestated GF in the CA1 oriens **(B)** and S1 **(C)**, regardless of introduction to a microbiota at birth in the GF→CC group. **(D)** In contrast, microglial density in the PVN was no different between GF→CC and either control group, suggesting partial normalization of the microglial phenotype by microbiota introduction at birth. **(E)** No differences between groups were seen in the ARC. Group means with different letters are significantly different from each other. Mean + SEM and individual data points are depicted, with gray symbols representing sham cross-fostered mice in control groups.

effects of sex for any variable, although some comparisons may have been under-powered for identifying sex differences. One-way ANOVA was used to evaluate cross-fostering effects on microglial number, cell death, body weight, forebrain size, colon content weight, and bacterial diversity. When applicable, ANOVA was followed by Fisher's least significant difference.

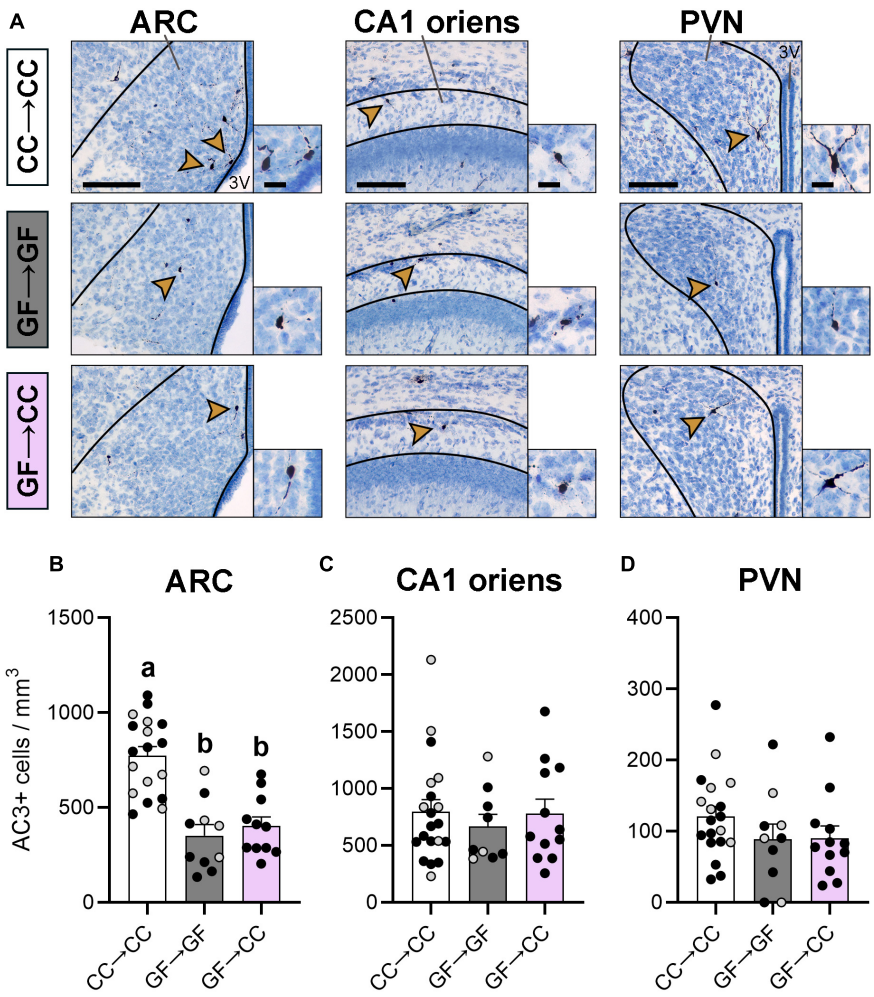

FIGURE 3
Cell death effects of gestating germ-free persist in the ARC of mouse neonates despite introduction to a microbiota at birth. **(A)** Photomicrographs of AC3 + stained tissue (counterstained with thionin) in representative CC→CC, GF→GF, and GF→CC mice, showing the brain regions analyzed: ARC, CA1 oriens, and PVN (all regions indicated with black lines). Arrowheads point to cells shown at higher magnification in the insets. 3V, third ventricle. Scale bar = 100 μm (main photomicrograph) and 20 μm (insets). **(B)** Cell death density was lower in groups gestated GF in the ARC, regardless of introduction to a microbiota at birth in the GF→CC group. **(C,D)** Cell death density did not differ between groups in the CA1 oriens **(C)** or PVN. **(D)** Group means with different letters are significantly different from each other. Mean + SEM and individual data points are depicted, with gray symbols representing sham cross-fostered mice in control groups.

Non-parametric tests (Kruskal–Wallis followed by Dunn's test) were performed for bacterial load as data did not conform to the homogeneity of variance assumption of ANOVA. Two-tailed independent samples *t*-tests were used to test the effects of cross-fostering on metrics of alpha diversity: ASVs and Shannon diversity index. Principal coordinate analysis (PCoA) plots of Bray Curtis distances were used to assess the variation between the experimental groups (beta diversity), which was further tested via Permutational analysis of variance (PERMANOVA). Analysis of composition of microbiomes (ANCOM) was used to identify differentially abundant species between groups, and one-tailed independent samples *t*-tests or Mann–Whitney tests were used to confirm differences. Statistical analyses were performed using GraphPad Prism (GraphPad software LLC, San Diego, CA, USA) and QIIME2 (Bolyen et al., 2019). Two immunohistochemical runs were performed per marker (Iba1, AC3), with half of the subjects per group included in each run. The second run for Iba1, however, was unsuccessful so animal numbers are lower for Iba1 than for AC3 analyses.

3. Results

3.1. Microglial effects of gestating germ-free persist in some brain regions despite introduction to a microbiota at birth

We first examined microglia in four brain regions in which we or others have reported effects of GF status. Specifically, microglial labeling is increased in the PVN, ARC, cortex, and CA1 oriens layer of the hippocampus in perinatal or adult GF mice

(Erny et al., 2015; Castillo-Ruiz et al., 2018a; Thion et al., 2018). Here, we found significant effects of group in the CA1 oriens [$F(2,14) = 4.40$, $p = 0.03$], S1 [$F(2,15) = 4.40$, $p = 0.03$], and PVN [$F(2,15) = 6.77$, $p = 0.008$] (Figures 2A–D). As seen previously when comparing GF and CC mice, GF→GF mice had more microglia than CC→CC mice in these brain regions ($p_s \leq 0.03$). Remarkably, the introduction of a microbiota at birth was not sufficient to change the GF phenotype in the CA1 oriens or S1, as microglial number in GF→CC mice remained significantly higher than in CC→CC mice ($p_s \leq 0.03$) and was no different from GF→GF mice at P7 (Figures 2B, C). In contrast, the PVN showed partial normalization of microglial phenotype as the GF→CC group did not differ from either the GF→GF or CC→CC groups (Figure 2D). For the ARC, there was no difference between groups in the overall ANOVA [$F(2,15) = 2.53$, $p = 0.11$] (Figure 2E).

3.2. Cell death effects of gestating germ-free persist despite introduction to a microbiota at birth

Compared to CC mice, we previously observed increased cell death in the CA1 oriens and PVN and reduced cell death in the ARC of GF mice on P0 and P3 (Castillo-Ruiz et al., 2018a). Here, we again found an effect in the ARC [$F(2,36) = 22.28$, $p < 0.0001$] and, as before, the GF→GF group had fewer dying cells than the CC→CC group (Figures 3A, B). Importantly, introduction to a microbiota at birth was not sufficient to change this phenotype as the GF→CC group did not differ from the GF→GF group and remained different from the CC→CC group at P7 ($p < 0.0001$). We did not find an effect of group in the CA1 [$F(2,38) = 0.30$, $p = 0.74$] or PVN [$F(2,38) = 1.33$, $p = 0.28$], perhaps because P7 is well after the peak of cell death in these regions (Figures 3C, D; Mosley et al., 2017).

3.3. Gross measurement effects of gestating germ-free persist in mouse neonates despite introduction to a microbiota at birth

Our previous study also showed greater body weight and forebrain size in GF neonates compared to CC controls (Castillo-Ruiz et al., 2018a). Here we again found significant effects of group for both measures [$F(2,39) = 16.41$, $p < 0.0001$ and $F(2,32) = 3.89$, $p = 0.03$, respectively], and similar to what we observed previously, the GF→GF group weighed more and had a larger overall forebrain size (Figures 4A, B) than the CC→CC group ($p_s \leq 0.04$). The GF→CC mice remained significantly different from CC→CC mice ($p_s \leq 0.02$) for both measures (Figures 4A, B) and were no different from GF→GF mice on either measure.

3.4. Cross-fostering largely normalizes gut bacterial load and composition

Persistence of the GF phenotype seen above in the GF→CC group could be related to differences in the amount (load) and/or identity (composition) of gut microbial species. To test these hypotheses, we first assessed bacterial load in colon contents 7 days after birth. Not surprisingly, a non-parametric one-way ANOVA revealed significant effects of group on bacterial load ($H_2 = 23.38$, $p < 0.0001$), with CC→CC and GF→CC groups having approximately 10^6 -fold greater bacterial load than the GF→GF group ($p_s \leq 0.0002$). Importantly, the CC→CC and GF→CC groups did not differ from each other on this measure (Figure 5A). This effect is unlikely driven by group differences in colon content size as there was no effect of group on this measure [$F(2,34) = 2.84$, $p = 0.07$] (Figure 5B).

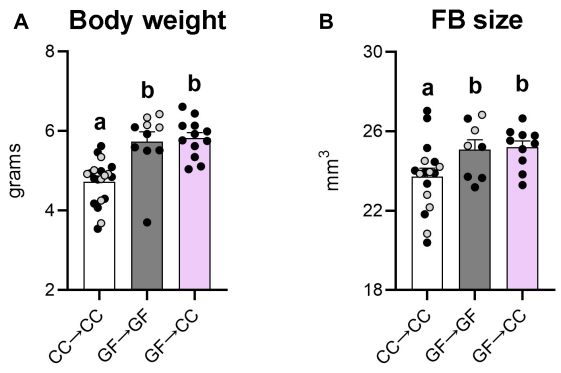

FIGURE 4
Effects of gestating germ-free on body weight and forebrain size persist in mouse neonates despite introduction to a microbiota at birth. Body weight **(A)** and forebrain size **(B)** were greater in GF→GF and GF→CC mice, in comparison to the CC→CC group. Group means with different letters are significantly different from each other. Mean + SEM and individual data points are depicted, with gray symbols representing sham cross-fostered mice in control groups.

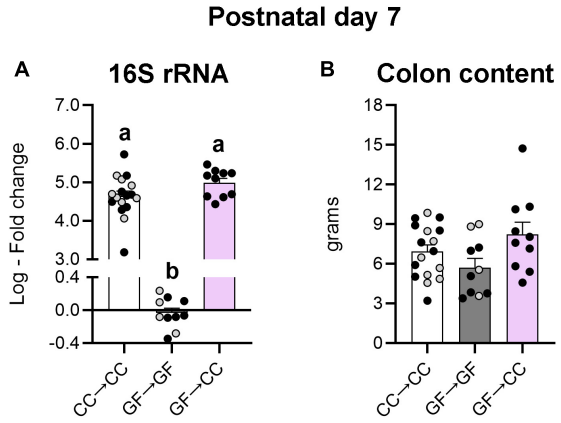

FIGURE 5
Introduction to a microbiota at birth normalizes the bacterial load of mice gestated germ-free at P7. **(A)** Relative quantification of the 16S rRNA gene from colon content showed similar levels of bacterial DNA in the groups harboring microbiota. The GF→GF group was used as reference group for fold change calculations. **(B)** Size of the colon content sample was unlikely to affect the assessment of bacterial load as there were no differences in this measure between groups. Group means with different letters are significantly different from each other. Mean + SEM and individual data points are depicted, with gray symbols representing sham cross-fostered mice in control groups.

We next assessed bacterial composition through 16S rRNA gene sequencing. Metrics of alpha diversity showed that there was no difference in ASVs (richness) between GF→CC and CC→CC groups (Figure 6A, top). However, there was a difference between these groups when richness and abundance (evenness) were considered using the Shannon diversity index: the GF→CC group had slightly lower diversity [$t(24) = 2.77$, $p = 0.01$] (Figure 6A, bottom). Taxon abundance assessment revealed that the colonic microbiota of GF→CC and CC→CC groups were remarkably similar but vastly distinct from negative control samples. The presence of a bacterial signal in 16S rRNA amplification of negative control samples is expected, as it captures any environmental contamination as well as the so-called "kit-ome," (i.e., bacterial presence in buffers and other reagents) (Grahn et al., 2003; van der Horst et al., 2013; Olomu et al., 2020). Interestingly, the profile observed in negative controls and GF→GF mice was very similar, further validating the absence of endogenous bacteria in the GF group (Figure 6B). *Lactobacillus*, *Proteus*, and *Staphylococcus* were predominant across GF→CC and CC→CC samples. In contrast, *Bacteroides* and *Enterobacteriaceae* were the contaminants that dominated in negative control and GF→GF samples.

Principal coordinate analysis of Bray Curtis distances was used to evaluate differences at the level of bacterial community composition (beta diversity). PCoA plots show that GF→CC and CC→CC samples cluster together but separately from negative control and GF→GF samples, suggesting that bacterial communities are similar in composition in the microbiota harboring groups (Figure 6C). Nonetheless, PERMANOVA found a significant difference between the GF→CC and CC→CC samples ($p = 0.03$). ANCOM was used to test for individual species that differed significantly in abundance between the GF→CC and CC→CC groups. Remarkably, just one species was identified: *Lactobacillus reuteri* ($W = 32$; $U = 45$, $p = 0.003$) was present in half of the GF→CC samples and absent in all CC→CC samples (Figure 6D). We also note that although CC→CC and sham CC→CC offspring overall had similar bacterial composition, ANCOM revealed that the sham CC→CC group had more *Proteus* ($W = 12$; also captured in Figure 6B). However, this comparison did not quite reach significance in a non-parametric *t*-test ($U = 19$, $p = 0.054$).

Thus, bacterial load and composition were largely identical between GF→CC and CC→CC mice at P7, but brain measures were not. Colon contents that were collected at P3 allowed us to test how quickly bacterial normalization occurs. Similar to what was seen at P7, bacterial load and colon content size did not differ between GF→CC and CC→CC groups at P3 (Figures 7A, B). However, colon contents of the CC→CC group had double the number of ASVs ($U = 18$, $p = 0.0004$) (Figure 8A, top), but similar values of the Shannon diversity index compared to the GF→CC group (Figure 8A, bottom). Taxon abundance assessment revealed that overall CC→CC and GF→CC were similar (and, again, vastly different from or negative controls of GF→GF samples), although *Streptococcus* appeared more predominant in CC→CC colons (Figure 8B). CC→CC and GF→CC groups at P3 clustered slightly further apart on PCoA plots than they did at P7 and PERMANOVA confirmed this difference ($p < 0.002$) (Figure 8C). However, ANCOM analysis again found only a single species that was significantly different in abundance between the groups: *Streptococcus acidominimus* [$W = 44$; $t(26) = 5.18$, $p < 0.0001$] was more predominant in the colons of CC→CC mice than in GF→CC mice at P3 (Figure 8D). In addition, we did not observe taxa abundance differences between CC→CC and sham CC→CC offspring at P3. Thus, when exposed to a normal microbiota on the day of birth, the neonatal gut microbiota was largely similar whether pups were gestated and born CC or GF, with some subtle differences, especially at the earlier timepoint (P3).

4. Discussion

We previously identified effects of the microbiota on microglia and neuronal cell death within hours after birth (Castillo-Ruiz et al., 2018a). In this study, a cross-fostering approach allowed us to test whether these effects are caused solely by the postnatal microbiota, or whether *in utero* exposure to the maternal microbiota plays a role. Overall, we find that the GF phenotype persists during the first postnatal week, despite successful acquisition of a microbiota at birth, suggesting a role for prenatal programming.

4.1. Microglia, cell death, and gross development effects

Microglial colonization of the brain and neuronal cell death are two of the most prominent neurodevelopmental events during the newborn period in mice. The number of microglia increases rapidly after birth and microglia undergo major morphological and gene expression changes during this period (Dalmau et al., 2003; Schwarz et al., 2012; Crain et al., 2013; Lai et al., 2013; Sharaf et al., 2013; Christensen et al., 2014; Matcovitch-Natan et al., 2016; Castillo-Ruiz et al., 2022). Similarly, developmental neuronal cell death is concentrated during the first postnatal week in mice (Ahern et al., 2013; Mosley et al., 2017). Microglia are quite sensitive to the microbiota. Erny et al. (2015) demonstrated increased microglial labeling in adult GF mice, and extended that to mice in which the microbiota was severely depleted in adulthood with antibiotics or which lacked a complex microbiome by virtue of being colonized by only three bacterial species. These findings suggest continuous regulation of microglia by the microbiome throughout life.

In the CA1 oriens and S1 we found that mice born GF, regardless of microbial status at P7, had more microglia than CC mice, suggesting persistence of the GF microglial phenotype in the GF→CC group. The ARC had a similar microglia pattern but we were underpowered to detect an effect. In contrast, in the PVN we observed partial normalization of the GF phenotype by the cross-fostering manipulation. The PVN is enriched in blood supply in comparison to neighboring regions (van den Pol, 1982), and this pattern develops during the first days postnatal in rats and mice (Menendez and Alvarez-Uria, 1987; Frahm et al., 2012). We speculate that microbial metabolites may be more accessible to the PVN via its nascent rich blood supply than to the other brain regions examined here. Consistent with this hypothesis, administration of bacterial metabolites to adult GF mice can rescue microglial numbers, morphology,

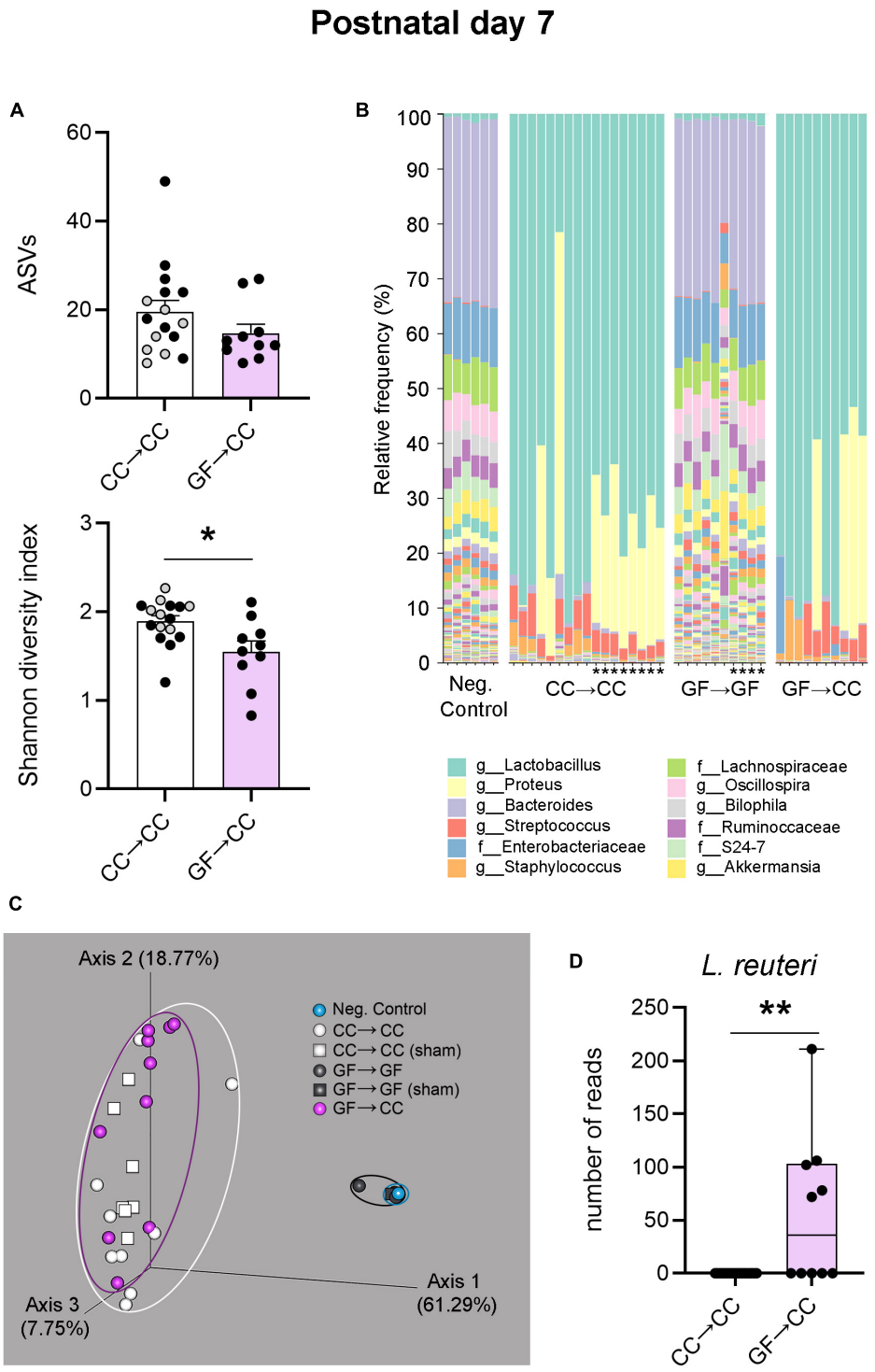

FIGURE 6
Introduction to a microbiota at birth largely normalizes bacterial composition of mice gestated germ-free by P7. **(A)** Measures of alpha-diversity revealed no difference between CC→CC and GF→CC groups in the number (richness) of ASVs (top). In contrast, when richness and abundance were considered by using the Shannon diversity index, the GF→CC group showed slightly lower diversity (bottom). Mean + SEM and individual data points are depicted, with gray symbols representing sham cross-fostered mice in control groups. *p = 0.01. **(B)** Relative abundance of bacterial groups per sample (columns), showing that overall bacterial composition was normalized in the GF→CC group as this group was similar to the CC→CC controls but markedly different from negative control samples and GF→GF controls. Asterisks identify the sham cross-fostered mice in control groups. The 12 most abundant taxa are shown in the color key. Sequences were classified to the lowest taxonomic level that could confidently be identified. f, family; g, genus. **(C)** PCoA plots based on Bray-Curtis dissimilarity, showing that GF→CC and CC→CC groups were similar in bacterial community composition as individual samples (symbols) clustered together but separate from controls (clustering indicated with ellipses). Note that most samples for negative control and GF→GF groups overlap due to tight clustering; n = 6 and 10 for those groups, respectively. Percent of variance explained by principal coordinates is indicated on the axes. **(D)** Boxplots of the number of reads per sample of the ASV identified as *Lactobacillus reuteri*. While the CC→CC group did not return positive *L. reuteri* reads, half of the samples in the GF→CC group did. **p = 0.003.

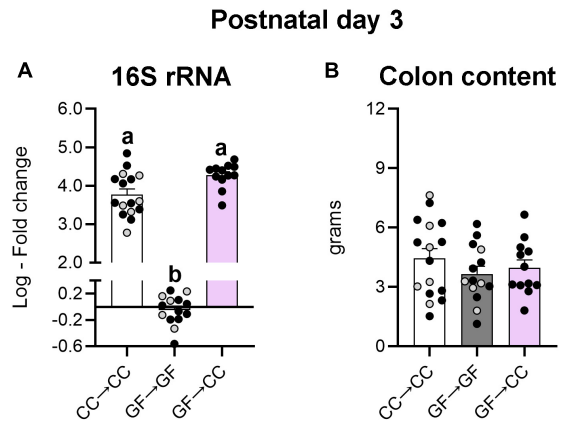

FIGURE 7
Introduction to a microbiota at birth normalizes the bacterial load of mice gestated germ-free at P3. **(A)** Relative quantification of the 16S rRNA gene from colon content showed similar levels of bacterial DNA in the groups harboring microbiota. GF→GF group was used as reference group for fold change calculations. **(B)** There were no differences in weight of the colonic content between groups. Group means with different letters are significantly different from each other. Mean + SEM and individual data points are depicted, with gray symbols representing sham cross-fostered mice in control groups.

and physiology (Erny et al., 2015, 2021). Moreover, gut-derived bacterial metabolites cross the blood-brain barrier *in vivo* (Frost et al., 2014) and influence microglia function *in vitro* (Erny et al., 2021).

For cell death, we found an effect of group in the ARC, with greater cell death in both GF→CC and GF→GF than in CC→CC mice. Interestingly, the ARC is involved in food intake, which is increased in GF mice (Bäckhed et al., 2004). In the CA1 oriens and PVN we did not find an effect of group on cell death, probably due to the fact that this process has tapered off in these regions by P7 (Ahern et al., 2013; Mosley et al., 2017). We did not assess the phenotype of the cells undergoing cell death in this study, however, they are likely to be mainly neurons based on previous reports in the neonatal brain (Zuloaga et al., 2011) and the neuron-like morphology shown by the cells we quantified.

Gross development was also affected by prenatal microbial absence, with GF→CC and GF→GF mice having greater forebrain size and body weight than CC→CC mice at P7. These measures may be dependent on mouse strain or diet, as they are found in some studies of GF mice but not others (Bäckhed et al., 2004; Fleissner et al., 2010; Khosravi et al., 2015; Selwyn et al., 2015; Kawase et al., 2017; Castillo-Ruiz et al., 2018a; Vuong et al., 2020). It is notable that most of the GF effects that we identified previously on microglia, cell death, and gross development in Swiss Webster mice at P0 and P3 (Castillo-Ruiz et al., 2018a) were replicated here at P7. Therefore, the GF phenotype persists throughout at least the first postnatal week. Because the brain undergoes extensive development during this time (Reemst et al., 2016), our past and current results could help explain why exposing GF rodents to microbes beyond the early postnatal window does not normalize some brain and behavior measures (Sudo et al., 2004; Clarke et al., 2013; Desbonnet et al., 2014). Similarly, introduction to a wild/more diverse mouse microbiota protects against diet-induced obesity if introduced to CC mice on P2, but not if the introduction is delayed to P15 (Hild et al., 2021).

As mentioned above, the co-housing of GF mice with CC mice at birth reduces microglial numbers in comparison to GF mice when examined in adulthood. Our results suggest that the normalization of brain measures is not immediate. Similarly, delayed effects of microbiota colonization have been reported in adult mouse colon (El Aidy et al., 2012; Johansson et al., 2015). Because microglia participate in diverse neurodevelopmental processes, including the phagocytosis of dying cells, neuro/gliogenesis, and synaptic pruning (Ferrer et al., 1990; Caldero et al., 2009; Paolicelli et al., 2011; Schafer et al., 2012; Cunningham et al., 2013; Shigemoto-Mogami et al., 2014; Lenz and Nelson, 2018), any deviations from their typical state could have significant effects on brain development.

4.2. Bacterial load and composition

Bacterial load and composition were largely identical between GF→CC and CC→CC mice at P7, suggesting rapid colonization of the gut in mice gestated GF and introduced to a microbiota at birth. In agreement, El Aidy et al. (2012) conventionalized adult GF mice and found that bacterial copy number reached its maximum after just 1 day. Overall, the species diversity observed in our study concurs with a previous report in neonatal mice showing low diversity at the end of the first week postnatal followed by a more stable and diverse community by weaning age (Pantoja-Feliciano et al., 2013). The predominant genera we observed are also in agreement with Pantoja-Feliciano et al. (2013), with dominance of *Lactobacillus* and *Streptococcus* during the first week postnatal. The prevalence of *Lactobacillus* may in part be due to its role inhibiting the growth of other bacterial communities via production of lactic acid from milk (Vandenbergh, 1993; Brownlie et al., 2022).

There were slight differences in alpha and beta diversity between CC→CC and GF→CC groups at P7 and ANCOM found a significant difference in one taxon: *L. reuteri* was greater in GF→CC than in CC→CC neonates. This finding is interesting given that administration of *L. reuteri* in its biofilm state normalizes microglia numbers in a mouse model of neonatal necrotizing enterocolitis (Wang et al., 2021). Therefore, it is tempting to speculate that *L. reuteri* may participate in the partial normalization of microglia seen in the PVN of GF→CC mice. However, *L. reuteri* was present in only half of the GF→CC mice at P7 and the presence of this species within the GF→CC group did not correlate significantly with microglial or cell death measures (not shown). We cannot rule out an association, however, as we may not have been sufficiently powered, especially for microglial measurements.

The GF→CC and CC→CC groups were already very similar in bacterial load and composition at P3. Nonetheless, we detected more pronounced differences between the groups at this age than at P7. The most notable was the predominance of *Streptococcus* in the CC→CC group, and as per the

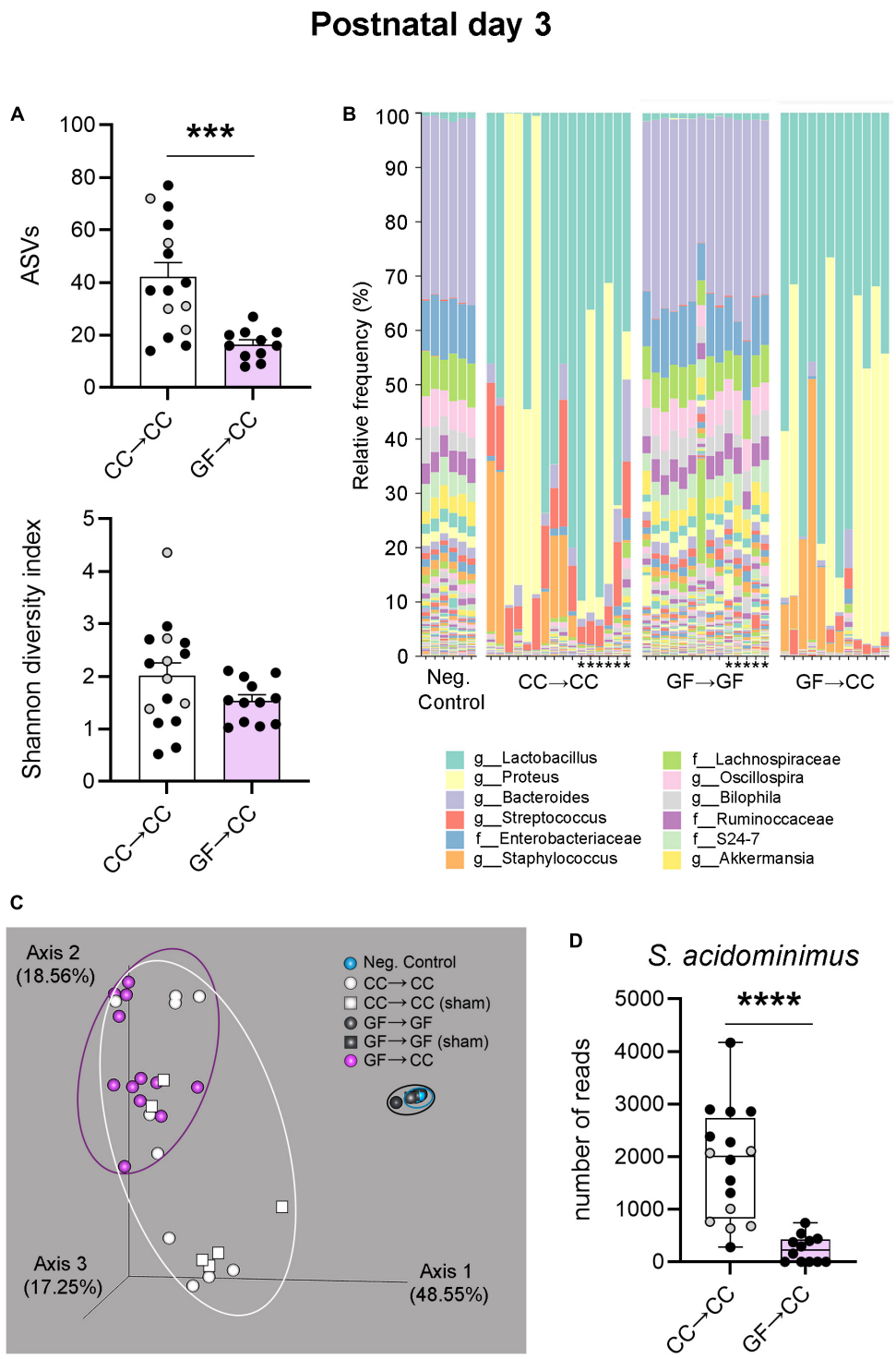

FIGURE 8
Introduction to a microbiota at birth largely normalizes bacterial composition of mice gestated germ-free by P3. **(A)** Measures of alpha-diversity revealed a difference between CC→CC and GF→CC groups in the number (richness) of ASVs: the CC→CC group showed doubled the number of ASVs (top). In contrast, when richness and abundance were considered by using the Shannon diversity index, there was no difference between groups (bottom). Mean + SEM and individual data points are depicted, with gray symbols representing sham cross-fostered mice in control groups. ***p = 0.0004. **(B)** Relative abundance of bacterial groups per sample (columns), showing that overall bacterial composition was similar between GF→CC and CC→CC groups, with the exception of higher abundance of *Streptococcus* in the CC→CC group. These two groups, however, were markedly different from negative and GF→GF groups. Asterisks indicate the sham cross-fostered mice in control groups. The 12 most abundant taxa are shown in the color key. Sequences were classified to the lowest taxonomic level they could confidently be identified. f, family; g, genus. **(C)** PCoA plots based on Bray-Curtis dissimilarity, showing that GF→CC and CC→CC individual samples (symbols) clustered somewhat further apart than at P7 but markedly separate from controls (clustering indicated with ellipses). Note that most samples for negative control and GF→GF groups overlap due to tight clustering; n = 6 and 14 in those groups, respectively. Percent of variance explained by principal coordinates is indicated on the axes. **(D)** Boxplots of the number of reads per sample of the ASV identified as *Streptococcus acidominimus*. Gray symbols represent sham, cross-fostering in control mice. ****p < 0.0001.

ANCOM results, this may in part relate to higher abundance of *S. acidominimus*. Interestingly, this species is sensitive to perinatal manipulations as shown by reduction in its numbers in the P2 mouse colon upon prenatal maternal stress (Jasarevic et al., 2018).

Overall, we did not find differences between true and sham cross-fostered mice for any of the variables assessed, with the exception of higher *Proteus* at P7 in the sham CC→CC group. Differences in gut microbiota composition due to cross-fostering were recently reported by Morais et al. (2020) in weanling and adult mice. However, in that study all non-cross-fostered pups remained undisturbed with the birth mother. Here, both sham and true cross-fostered pups experienced maternal separation and a disinfection regime, which may have more nearly equalized stress of the procedure across groups.

4.3. Does the maternal microbiota program brain effects?

The similarities between GF→CC and CC→CC bacterial communities are not surprising, given that the fetus develops in a sterile (or nearly sterile) womb and CC and GF offspring are expected to be on equal footing with respect to direct exposure to intestinal bacteria throughout gestation. Although we did not assess the maternal gut microbiota in our study, this microbiota was likely transferred promptly to GF→CC newborns via feces in the cage and foster dam behaviors: licking and grooming of pups after engaging in self-anogenital grooming and coprophagy. If so, our current results suggest that the maternal microbiota has programming effects on brain development *in utero*. Similarly *in utero* effects of the maternal microbiota have been reported for microglia and other neurodevelopmental events, including axonogenesis and sympathetic nervous system development (Thion et al., 2018; Kimura et al., 2020; Vuong et al., 2020). In fact, Thion et al. (2018) reported higher microglial numbers in GF mice as early as E14. We previously observed no differences in microglia and cell death between GF and CC mice in the hours just before birth (Castillo-Ruiz et al., 2018a) but this discrepancy may be due to the inflammation that occurs around time of parturition and that extends to the brain (Castillo-Ruiz et al., 2018b, 2022).

Alternatively, it is possible that our cross-fostering manipulation (GF→CC) did not fully mimic the vertical transmission of microbes that occurs at birth in CC animals, and that the subtle differences we found in the microbiota could explain the persistence of the GF phenotype for most brain measures. There are at least two ways that the initial colonization of pups gestated and born GF versus CC may differ. First, mice born to a GF dam are not exposed to a vaginal microbiota during parturition. However, the maternal gut microbiota most powerfully shapes the newborn's gut microbiota, and most maternal vaginal microbes are only very transiently found in the neonate's gut (Sakwinska et al., 2017; Ferretti et al., 2018; Jasarevic et al., 2021). Nonetheless, the transient presence of vaginally-derived species could alter subsequent stages of gut colonization and affect development (Jasarevic et al., 2021). The two species identified as significantly different between GF→CC and CC→CC mice in our study: *S. acidominimus* at P3 and *L. reuteri* at P7, inhabit the gut but also may be found in the vagina (Smith and Sherman, 1939; Rabe et al., 1988; Tannock, 1995; Oh et al., 2010; Leccese Terraf et al., 2016). Thus, it is plausible that initial inoculation by vaginal microbes could account for differences in the abundance of these species, although this explanation is difficult to reconcile with the *greater* presence of *L. reuteri* in GF→CC mice.

A second possible reason that colonization during the first 7 days postnatal might not be identical in GF→CC and CC→CC newborns is *in utero* effects of the maternal microbiota on development of the fetal intestine or immune system. If arriving bacteria encounter a different environment in the GF→CC versus CC→CC colon, this could affect the persistence of specific species. Indeed, the maternal microbiota *in utero* plays a role in the development of the immune system (Gomez de Agüero et al., 2016). Our results suggest that if there are differences, they have only subtle effects on colonization since bacterial load and composition were remarkably similar in the GF→CC and CC→CC groups.

Finally, we cannot ignore the possibility that the persistence of the GF phenotype in the GF→CC group may be related to differences in microbial populations that we did not assess (e.g., fungi, viruses, or protozoans). However, at least for microglia, bacterial normalization may be more important as bacterial metabolites rescue microglial effects in adult GF mice (Erny et al., 2015, 2021).

5. Conclusion

In sum, we find that brain effects of gestating GF persist during the first postnatal week, despite successful acquisition of a microbiota at birth. These findings argue for an important role of the maternal microbiota during fetal life on neonatal brain development. Because our results identify specific neurodevelopmental events that are sensitive to prenatal microbial exposure, this information could aid in interpretation of future studies that evaluate programming effects of microbiota on brain physiology and behavior. In addition, our work identifies two potential species: *L. reuteri* and *S. acidominimus*, to target in future experiments examining the role of specific bacteria in orchestrating neonatal brain development.

Data availability statement

The original contributions presented in this study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

Ethics statement

The animal study was reviewed and approved by the Georgia State University's Institutional Animal Care and Use Committee.

Author contributions

AC-R and NGF designed the experiments and wrote the manuscript. AC-R, AG, NMR, DNP, CJGD, and BC performed the experiments. AC-R, HS, CJGD, and BC analyzed data. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Developing brain under renewed attack: viral infection during pregnancy

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Living in a globalized world, viral infections such as CHIKV, SARS-COV-2, and ZIKV have become inevitable to also infect the most vulnerable groups in our society. That poses a danger to these populations including pregnant women since the developing brain is sensitive to maternal stressors including viral infections. Upon maternal infection, the viruses can gain access to the fetus via the maternofetal barrier and even to the fetal brain during which factors such as viral receptor expression, time of infection, and the balance between antiviral immune responses and pro-viral mechanisms contribute to mother-to-fetus transmission and fetal infection. Both the direct pro-viral mechanisms and the resulting dysregulated immune response can cause multi-level impairment in the maternofetal and brain barriers and the developing brain itself leading to dysfunction or even loss of several cell populations. Thus, maternal viral infections can disturb brain development and even predispose to neurodevelopmental disorders. In this review, we discuss the potential contribution of maternal viral infections of three relevant relative recent players in the field: Zika, Chikungunya, and Severe Acute Respiratory Syndrome Coronavirus-2, to the impairment of brain development throughout the entire route.

KEYWORDS

SARS-COV-2, CHIKV, ZIKV, vertical transmission, pregnancy, brain development, brain barrier, brain inflammation

1. Introduction

A sudden rise of a viral infection among populations can take a toll on societies by influencing daily life, economy, and public health as it continues to spread. In order to intervene and decelerate the spread as soon as possible, health organizations such as the World Health Organization (WHO) and the National Institutes of Health (NIH) promote research and development on viral pathogens that have the potential to cause widespread health issues (NIH, 2023; World Health Organization, 2023). Nonetheless, extensive spread of viral pathogens is inevitable, especially considering the fast-adapting nature of viruses, climate change, and expanded (inter)national travel (Abdul-Ghani et al., 2020; Pergolizzi et al., 2021). In this review, due to the (i) consistently reported high Zika virus (ZIKV) and Chikungunya virus (CHIKV) case numbers by numerous countries in the recent past, (ii) recent Severe Acute Respiratory Syndrome Coronavirus-2 (SARS-COV-2) pandemic, and (iii) known or potential mother-to-fetus transmission and subsequent neurodevelopmental impact, we have particularly focused on ZIKV, CHIKV and SARS-COV-2 within the context of exposure during brain development. Nevertheless, we realize that TORCH infections as well as HIV infections can still pose a threat to the developing brain in similar ways (NIH, 2023; World Health Organization, 2023); we focus

on the "renewed" attack of three of the most recent viral players in the field.

Historically, CHIKV and ZIKV viruses were identified in Africa between the 1940s and 1950s. With recurring outbreaks, CHIKV infections spread through the Indian ocean and Asian regions (2004–2007) reaching the Americas and subsequently leading to the 2013 Caribbean epidemic (Mugabe et al., 2018; Périssé et al., 2020). Travel-associated cases also contributed to its emergence in Europe, for example in Italy (Pierelli et al., 2018). Similarly, ZIKV drew the attention with outbreaks in Higuera and Ramírez (2019) and French Ginige et al. (2021) where 73% and 66% of the population were affected, respectively (Pergolizzi et al., 2021). Growing spread through the Pacific Islands and the Americas as well as reported striking negative pregnancy outcomes were followed by the declaration of ZIKV as an international health emergency by WHO in 2016. In the past 6 years, CHIKV and ZIKV infections have been consistently reported to health organizations reaching 115 and 87 countries, respectively, infecting thousands of people, especially in the Americas region. SARS-COV-2, on the other hand, emerged in Wuhan, China, causing a national outbreak in 2019. Not long after, it was declared as a global pandemic by WHO on 11 March 2020, and is the most recent example of the multifaceted devastating impacts of (global) viral spread. Within months of the emergence of SARS-COV-2, the virus has spread throughout the world, infected millions of people, and caused the death of thousands of people worldwide. These three viruses are still closely monitored by NIH (2023) and World Health Organization (2023).

Extensive spread of viral pathogens raises concern, particularly for pregnant women. Epidemiologic studies of previous pandemics and epidemics have shown that pregnant women and/or their offspring had higher rates of severe illness, morbidity, and mortality (Jamieson et al., 2009; Louie et al., 2010; Charlier et al., 2017; Van Campen et al., 2020; De St Maurice et al., 2021; Ginige et al., 2021). Though overall ZIKV, CHIKV, and SARS-COV-2 do not pose a major threat to pregnant women, upon SARS-COV-2 infection, pregnant women are more likely to require critical care and to have pregnancy complications (Musso et al., 2019; Pomar et al., 2019; Allotey et al., 2020; Schwartz and Morotti, 2020; Jacques et al., 2021; Jafari et al., 2021) and these may have indirect consequences for brain development (Vohr et al., 2017). In ZIKV and CHIKV infections, the main subject of concern had become the neonates due to the observed neurologic and neurodevelopmental abnormalities as a result of vertical transmission (CHIKV, ≥15.5%; ZIKV, 10.9%; Pomar et al., 2017; Contopoulos-Ioannidis et al., 2018; McEntire et al., 2021). Severe neonatal CHIKV cases involving central nervous system (CNS) manifestation was first time reported during Reunion Island outbreak in 2005 (Enserink, 2006; Josseran et al., 2006). Later, it was found that vertically transmitted neonates did not only develop encephalopathy, but also microcephaly and neurodevelopmental delay (Josseran et al., 2006; Borgherini et al., 2007; Couderc and Lecuit, 2009; Gérardin et al., 2014; Ramos et al., 2018; Waechter et al., 2020; Shukla et al., 2021). ZIKV causes congenital malformations which was first suspected during the French Polynesia outbreak but the association could be only made after its arrival to Brazil (Duffy et al., 2009; Hennessey et al., 2016; Mlakar et al., 2016; Moore et al., 2017). Prenatal ZIKV infection can result in a wide range of neurodevelopmental problems including microcephaly, cortical and cerebellar developmental impairment (Mlakar et al., 2016; Yoon et al., 2017; Yuan et al., 2017; Freitas et al., 2020). Despite ongoing debates, it has been generally accepted that SARS-COV-2 can be vertically transmitted during pregnancy (5.3%) and can result in neurological manifestation upon birth (Vivanti et al., 2020; Shook et al., 2022; Vivanti et al., 2022). Although longitudinal cohort studies are still in infancy due to the recent occurrence of the pandemic, emerging data have been pointing out that maternal SARS-COV-2 infection during pregnancy could affect neurodevelopment negatively and even increase the chance of neurodevelopmental and neurologic diagnosis later on (Chevalier and Poillon, 2022; Germano et al., 2022; Shook et al., 2022; Taquet et al., 2022; Wang et al., 2022).

Early brain development spanning from prenatal to postnatal is an intricate and sensitive period. The necessity of rapid but timely and precise changes in the developing brain as well as the relatively naive immune state of the neonates, make the developing brain susceptible to environmental insults including maternal viral infections (Vohr et al., 2017; Elgueta et al., 2022; Jash and Sharma, 2022). Throughout pregnancy, mother and the maternofetal barrier undergo a series of structural and immunological alterations to provide protection against pathogens and ensure healthy development of the fetus until term (Silasi et al., 2015; Cornish et al., 2020). Nonetheless, some viruses have the ability to directly circumvent protective mechanisms and/or induce inflammatory response which can create multi-level alterations. Maternal viral infections can directly and/or indirectly interfere with neurodevelopmental processes and increase the risk for brain injury and brain disorders including neurodevelopmental (NDDs) and neuropsychiatric disorders (NPDs) as well as neurodegenerative diseases (Tomonaga, 2004; Silasi et al., 2015; Zimmer et al., 2021; Elgueta et al., 2022; Ayesa-Arriola et al., 2023). In line with that, in 6% of the prenatally ZIKV-exposed neonates (Martins et al., 2021) and in 51% of the perinatally CHIKV-exposed neonates50 CNS-associated problems were reported, while the proportion of neurological manifestation among neonatal SARS-COV-2 infections was 18% (Raschetti et al., 2020).

Considering recurring outbreaks affecting multiple countries worldwide, and the danger to the developing brain with long-lasting effects, it is important to understand; (I) how viruses can bypass protective mechanisms and barriers, (II) how infections alter the developing brain, (III) how hosts (in this context, both mother and fetus) react to the infection, and (IV) how these reflect onto the developing brain such that it deviates from its developmental trajectory eventually disturbing normal functioning. In this review, by focusing on these points as well as the factors influencing susceptibility of the developing brain to viral infection, our aim is to provide stepwise insights into the effects of viral infection on protective barriers and the developing brain and highlight gaps in the current knowledge which could be helpful in future research of environmental insult-associated impairment of brain development.

1.1. Viruses, the maternofetal barrier and brain development

The maternofetal barrier with its two main components, placenta and the amniochorionic membrane, develop throughout gestation and create a multicellular complex structure to ensure healthy fetal development by allowing molecule transmission between mother and fetus (e.g., oxygen, nutrients, growth factors) and by providing fetal

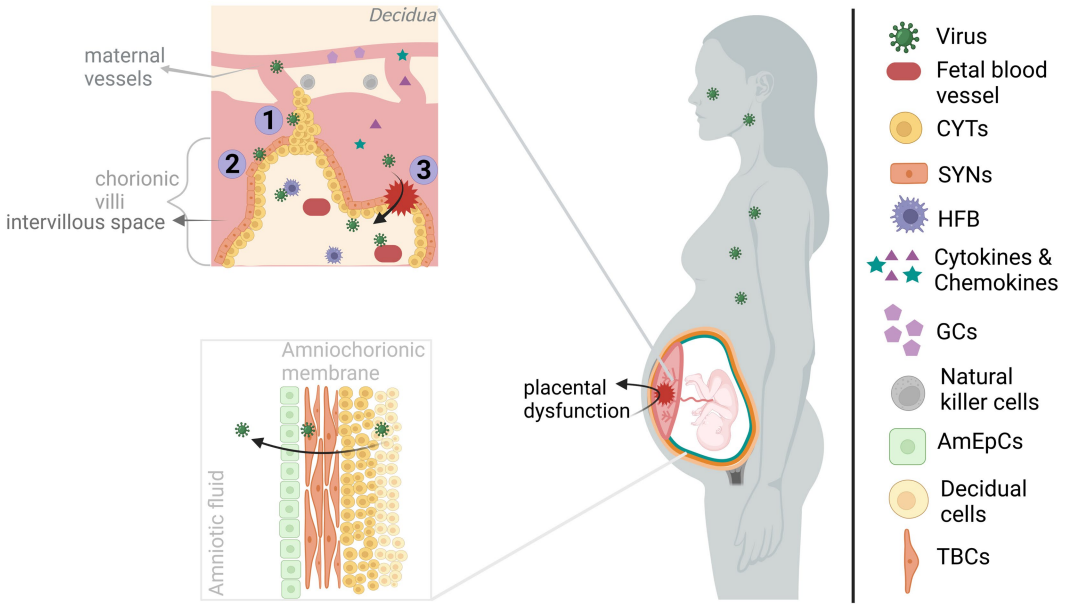

FIGURE 1
Viral infection of maternofetal barriers. The viruses infecting the mother during pregnancy could reach the fetus by acting on the maternofetal barriers. The maternal side of the placenta, consisting of basal decidua and maternal blood vessels, is in communication with the fetal side of the placenta, containing chorionic villi, mainly through maternal blood filling the intervillous space. However, maternal blood flow is blocked by EVTBs (trophoblastic plugs) during the 1st trimester, and it gradually disintegrates by the beginning of the 2nd trimester. Transplacental transmission of viral infection could occur through (I) infection of/diffusion through the plugs, (II) infection of/transportation across the trophoblasts of chorionic villous, and/or (III) through the structural alteration of the placenta (e.g., via inflammatory response). Upon entering the chorionic villi, viruses can gain access to fetal blood vessels and even directly the developing brain by infecting the HFBs. On the other hand, viruses reaching the amniochorionic membrane are able to elicit the release of viruses into the amniotic fluid, hence, paraplacental transmission. The viruses can directly alter the functioning of the placenta. Also, the maternal and placental inflammatory response might affect placental functioning in addition to allowing the transmission of inflammatory molecules (cytokines and chemokines) to the fetal side. Both cases will have harmful effects on the developing brain. Created with BioRender.com.

protection both structurally and immunologically (Figure 1; Pereira, 2018; Silini et al., 2020; Megli and Coyne, 2022). Vertical transmission, the passage of virus from mother to fetus, can occur transplacentally and/or paraplacentally through direct infection of maternofetal cell layers, cell-mediated transport and breach/diffusion.

From a closer perspective, chorionic villi are covered with trophoblast cells (e.g., cytotrophoblasts (CYT) and syncytiotrophoblasts (SYN)) located on the fetal side of the placenta and contain fetal blood vessels as well as fetal macrophages (Hofbauer cells; HFB). CYT and SYN which are in contact with the maternal blood filling the intervillous space, allowing nutrient and oxygen exchange (Megli and Coyne, 2022). Fundamentally, infection of these cells by CHIKV (Gérardin et al., 2014), ZIKV (Tabata et al., 2016; Pereira, 2018; Zanluca et al., 2018; Megli and Coyne, 2022), or SARS-COV-2 (Facchetti et al., 2020; Vivanti et al., 2020) indicates a transplacental viral passage. Especially, infection of the HFB cells by the viruses could be a direct threat to the developing brain due to their migrational ability. That way, they may mediate cell-associated transport into the brain (Tabata et al., 2016; Facchetti et al., 2020; Megli and Coyne, 2022; Vivanti et al., 2022) (see also section 1.3). ZIKV may also diffuse through trophoblastic plugs during the 1st trimester given the susceptibility of extravillous trophoblasts (EVTBs) (Adibi et al., 2016a,b). In addition, viral transportation across barriers can enable transmission. Even in the absence of infection of all cell types, virus-induced cytopathy and a strong immune response could disturb the placental architecture and/or functioning allowing viral transmission, resulting from ZIKV (Miner et al., 2016; Matusali et al., 2019; Cribiu et al., 2021; Ferreira et al., 2021), SARS-COV-2 (Vivanti et al., 2020; Cribiu et al., 2021; DeGrace et al., 2022) and possibly CHIKV (Ferreira et al., 2021) infections. Interestingly, in the earlier CHIKV studies, the absence of placental infection and the presence of perinatal maternal viremia led to the placental breach hypothesis. According to this hypothesis, CHIKV rather than infecting the maternofetal barrier, it infiltrates through the placental breaches during labor when maternal-fetal blood contact occurs (Gérardin et al., 2014; Matusali et al., 2019). The route and gestational time of vertical transmission have importance for the assessment of both preventative options and gestational time-dependent risk for brain development. Although SARS-COV-2 vertical transmission is a rare event, there is clear clustering of reported cases around the 3rd trimester-to-early postnatal period which may be reflective of entry receptor expression-dependent vulnerable period or reporting bias (Allotey et al., 2020; Facchetti et al., 2020; Fenizia et al., 2020; Hosier et al., 2020; Vivanti et al., 2020). Also, sparse CHIKV vertical transmission studies concluded contrasting findings (Gérardin et al., 2014; Contopoulos-Ioannidis et al., 2018; Honorio et al., 2019; Ferreira et al., 2021); hence, further research is required for both viruses.

Amniochorionic membrane attached to the uterine wall (decidua) encapsulates the fetus, thus, enhances fetal protection. The multi-layered membrane is formed with the alignment of the amniotic epithelial cells (AmEpCs), trophoblast cells (TBCs), CYT and decidual cells from most interior (fetal side) to outer surface (maternal side) (Silasi et al., 2015;

Silini et al., 2020). Susceptibility of AmEpCs and TBPCs to ZIKV suggests ZIKV diffusion into the amniotic fluid where it may infect fetal skin and/or placenta (Adibi et al., 2016a; Tabata et al., 2016). Similarly, detection of CHIKV in the endometrial epithelium, amniotic fluid, and AmEpCs (Tabata et al., 2016; Platt et al., 2018) and SARS-COV-2 in fetal membranes and amniotic fluid may suggest paraplacental transmission (Fenizia et al., 2020; Penfield et al., 2020; Cribiu et al., 2021).

It should be noted that maternal viral infections during pregnancy is a risk for brain development due to both vertical transmission and maternofetal barrier dysregulation (Yoon et al., 2017; Baines et al., 2020; Shukla et al., 2021; Ayesa-Arriola et al., 2023). As mentioned above, dysregulation can occur via virus-induced cytopathy and inflammatory response. For example, placental cell death and inflammation was reported among ZIKV-infected offspring with neurodevelopmental abnormality (Adibi et al., 2016a,b) and among SARS-COV-2-infected/exposed offspring some of which had a neurological manifestation (Fenizia et al., 2020; Vivanti et al., 2020; Favre et al., 2021). The contribution of placental dysfunction to neurodevelopmental outcome is partially due to its impaired secretory (e.g., neurotrophic factors, serotonin and glucocorticoids) function (Racicot and Mor, 2017; Narang et al., 2021; Megli and Coyne, 2022). Moreover, inflammatory response in the maternofetal barrier can not only cause placental dysregulation, but also affect neurodevelopmental processes in the fetal brain locally (see section 1.5). For instance, prematurity and chorioamnionitis both of which indicating placental dysfunction (Racicot and Mor, 2017; Narang et al., 2021) and were reported in maternal ZIKV (Garcia-Flores et al., 2022; Gomez-Lopez et al., 2022) and SARS-COV-2 infections (Jafari et al., 2021; Wong et al., 2021) could increase the risk for neurodevelopmental disorders (Allotey et al., 2020; Elgueta et al., 2022).

1.2. Susceptibility of the developing brain to viral infections

Gestational time of infection, viral tropism, exposed viral load, in combination with the balance between antiviral host immune response and pro-viral strategies are among the factors influencing susceptibility of developing brain to infection. Viral recognition of its entry mediators on host cells, with subsequent viral uptake via endocytosis initiates cellular infection (Agrelli et al., 2019; V'Kovski et al., 2021). The location of the mediators is important since they constitute the target of the viruses. In this way, their localization in maternofetal and brain barriers (e.g., ZIKV: TIM-1, AXL; CHIKV: DC-SIGN, MXRA8, TSPAN9; SARS-COV-2: ACE2) enables vertical transmission and access to the brain (Schnierle, 2019; Feng et al., 2020; Pellegrini et al., 2020; Teixeira et al., 2020; Varma et al., 2020; Xie et al., 2020). The expression pattern of the mediators creates cell-specific tropism of viruses and the distribution of the susceptible cells across the gestational period contributes to gestational time point-associated vulnerability. CHIKV, ZIKV and SARS-COV-2 show overlap in susceptible CNS cell populations, with different cell preference toward astrocytes, neural progenitor stem cell (NPSCs), and mature neurons, respectively (Retallack et al., 2016; Tang et al., 2016; Matusali et al., 2019; Pellegrini et al., 2020; Varma et al., 2020; Yi et al., 2020). It should be noted that none of the CHIKV entry mediators have been specifically associated with brain infectivity. But, the expression pattern of the mediators (e.g., PHB, AXL, FUZ, TIM-1, TSPAN9) some of which are common with ZIKV (Haddad-Tovolli et al., 2017; Garcez et al., 2018; Ramani et al., 2020; Kumari et al., 2021) and the brain injury pattern suggest that CHIKV can infect the brain (e.g., neurons, glial cells, neural stem cells; NSCs) (Das et al., 2015; Racicot and Mor, 2017; Schnierle, 2019; Baines et al., 2020). Interestingly, occurrence of viral infection in the absence of the mediators, especially for CHIKV (Schnierle, 2019; Kril et al., 2021) and ZIKV (Retallack et al., 2016; Hastings et al., 2017) implies employed other routes, presence of unidentified entry mediators and/or interchangeable use of mediators having multiple functions.

Viral infection initiates an antiviral host immune response through viral recognition by pattern recognition receptors (PRRs) such as RIG-I-like receptors (RLRs) and Toll-like receptors (TLRs) leading to release of interferons, inflammatory cytokines, and chemokines (Silasi et al., 2015; Racicot and Mor, 2017; Pereira, 2018). A sufficient level of antiviral response both on maternofetal barrier and in offspring is crucial for viral clearance and to prevent negative outcome. As an example, placental Type-3 interferon (IFN) response (IFN-λ) during the 3rd trimester can prevent ZIKV vertical transmission (Baines et al., 2020). During pregnancy, both maternofetal barrier and fetus have a tolerogenic immune state to prevent fetal rejection. The naïve immune state of the offspring extending to the neonatal period create immature immune responses (e.g., lower innate immune effector, lower IFN response), especially upon viral infection. Together, these create susceptibility to early life viral infections (Kollmann et al., 2017; Cornish et al., 2020). Indeed, insufficient antiviral type-1 IFN has been implicated in neonatal brain infection, developmental delay and severe vertical transmission cases of CHIKV as well as in vertical transmission and brain infection of ZIKV (Miner and Diamond, 2016; Van den Pol et al., 2017). Furthermore, the ability of ZIKV (Adibi et al., 2016a; Adams Waldorf et al., 2018; Lee et al., 2018), CHIKV (Priya et al., 2014; Kril et al., 2021), and SARS-COV-2 (Pellegrini et al., 2020; Song et al., 2021) to evade and/or inhibit Type-1,-2, and/or −3 IFN responses can contribute vertical transmission, BBB breakdown and/or CNS infection. For instance, SARS-COV-2, inducing metabolic changes in infected and neighboring neurons of cerebral organoids were accompanied with lack of IFN response implying potential contribution of immune response interference in SARS-COV-2 neuropathogenesis (Pellegrini et al., 2020; Song et al., 2021).

To establish successful infection, initial high viral load may not be as crucial for highly neurotrophic viruses like ZIKV (Halai et al., 2017; Adams Waldorf et al., 2018). On the other hand, high dose of exposure to the viruses with lower neurotropism (e.g., CHIKV, SARS-COV-2) together with impaired host immune response might increase the risk for brain infection (Vivanti et al., 2020; Wong et al., 2021). Nonetheless, dose-dependent CNS infectivity of SARS-COV-2 was not consistently reported (Ramani et al., 2020; Yi et al., 2020; Kumari et al., 2021), maybe suggesting a more prominent role of entry mediator expression level compared to viral load.

All three viruses could affect the fetus at any time during pregnancy, especially considering their ability to disturb placental (see section 1.1) and brain barrier (see section 1.3) homeostasis, and to interfere with the development through inflammatory factors (see section 1.5). However, within the context of viral tropism and (anti/pro-viral) immune responses, ZIKV has higher likelihood of affecting the brain during early pregnancy (Miner et al., 2016; Mittal et al., 2022), while CHIKV (Ramos et al., 2018; Waechter et al., 2020), and SARS-COV-2

are more likely to most harmful during late pregnancy (Pellegrini et al., 2020; Vivanti et al., 2020). In line with that, early neurodevelopmental processes (e.g., neurogenesis, migration) are more likely to be affected by ZIKV and late neurodevelopmental processes (e.g., neural circuitry formation and maturation) by CHIKV and SARS-COV-2.

1.3. Routes to developing brain and impact of viral infection

In order to affect the developing brain *in utero*, once the virus crosses maternofetal barrier, it needs to cross brain barriers to be able to infect neural tissue (Figure 2). Brain barriers start to form at very early stages, show early functionality, and continue to develop and mature after the postnatal period. Naturally, these structures are different than mature brain barriers: for example, the developing Blood–Brain Barrier (BBB) allows more restricted passage than the mature BBB (Obermeier et al., 2013; Haddad-Tovolli et al., 2017). The embryonic Blood-Cerebrospinal Fluid Brain barrier (e/BCSFB) differently structured than the adult BCSFB, transiently functions during embryonic and fetal stages. Together, the developing BBB and e/BCSFB provide protection from toxins and pathogens, and allow proper development of the brain by creating a controlled internal environment and by adjusting a molecule gradient specific to each developmental time point (Saunders et al., 2018, 2019). Nonetheless, they can be targeted by viruses from the mother during pre/perinatal period which access the developing brain. During that process, the viruses can adopt various strategies which can be generally categorized as with or without barrier disruption.

1.3.1. Blood–brain barrier

Endothelial cells, which are joined together via tight junctions (TJs) restricting paracellular permeability, as well as the basal membrane, which is in contact with pericytes, microglia, and astroglia end feet, constitute the two main components of cerebral blood vessels (Obermeier et al., 2013). By encircling the vessels, they create a highly selective adult BBB. During development, with the appearance of TJs and transporters at gestational week (GW) 12 and becoming more adult-like by GW18, it creates a barrier that pathogens need to cross (Obermeier et al., 2013; Goasdoue et al., 2017; Saunders et al., 2018, 2019). A growing body of evidence indicates that ZIKV can enter the brain mainly without overtly disturbing the BBB permeability via

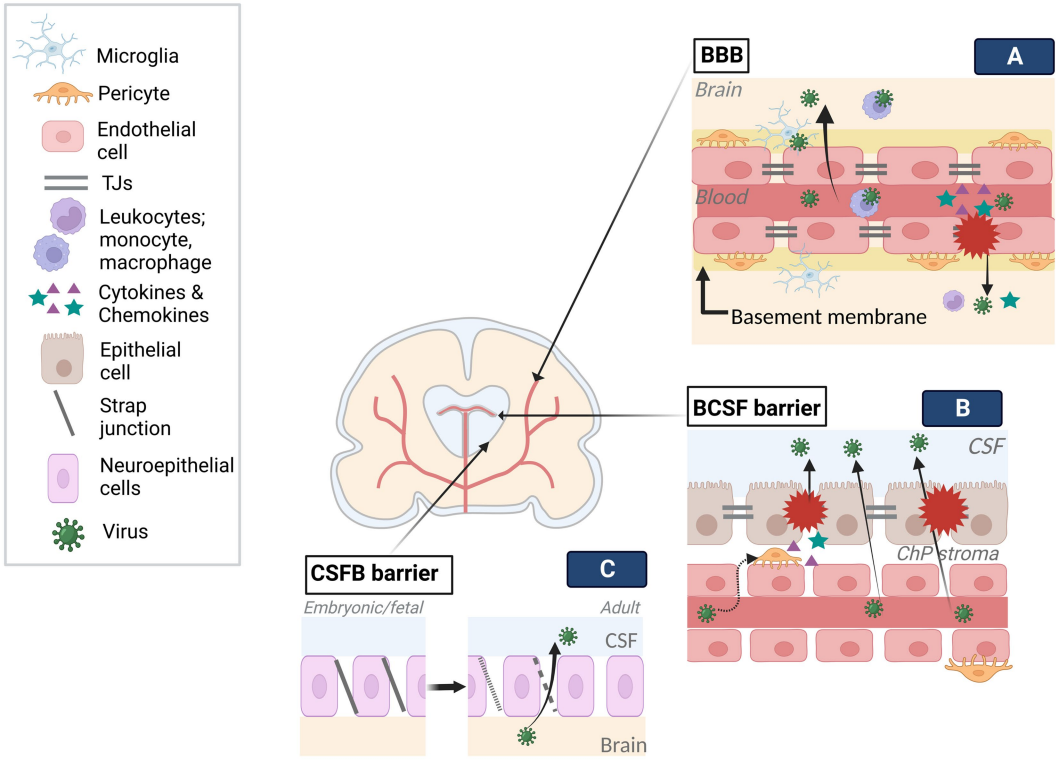

FIGURE 2
Viral infection of brain barriers. Brain barriers stand as obstacles in the way of viral access to the developing brain. To tackle the limited transmission across barriers, viruses employ various mechanisms. **(A)** Following viral entry into fetal blood circulation, viruses can travel into the blood lumen of the BBB. Infection of endothelial cells can result in viral release into the brain through distinct transcytosis and endocytosis-exocytosis mechanisms. Similarly, the use of immune cells (e.g., leukocytes, microglia) as Trojan horses, can enable viral transmission across the BBB without damaging the barrier. On the other hand, direct modulation of BBB components (e.g., TJs, endothelium) by the viruses and resulting inflammatory response on the barrier can give access to the brain as a result of interrupted barrier integrity. **(B)** Contrary to the BBB, viruses can traffic across fenestrated capillary of the BCSF and reach the ChP stroma. The infection of cells in the stroma, such as pericytes, and/or epithelium could disturb barrier integrity by direct modulation of BCSF components (e.g., TJs, epithelium) or inflammatory response. Trojan horse mechanisms may also be employed in BCSF transmission. **(C)** The strap junctions between neuroepithelial cells, specific to embryonic and fetal stages, form the first brain barrier, limiting the molecule transmission between CSF and the brain. Gradually disappearing strap junctions are replaced by gap junctions of ependyma throughout a period extending to the postnatal stage. The transition from strap to gap junctions may enable viral trafficking. Created with BioRender.com.

transcytosis (e.g., caveola-dependent transcytosis), endocytosis-exocytosis-dependent replication, and transinfection followed by basolateral release (Tomonaga, 2004; Papa et al., 2017; Leda et al., 2019; Chiu et al., 2020). Similarly, SARS-COV-2 crossing the endothelium via transcytosis (e.g., adsorptive) can breakdown the basement membrane by MMP9-mediated collagen degradation and taken up by several brain regions in an ACE2-dependent manner, which appears to be the main route in SARS-COV-2 encephalopathy cases (Leda et al., 2019; Song et al., 2021; V'Kovski et al., 2021). Limited studies are available showing that the effect of CHIKV on the BBB provides controversial findings: while in murine brain, the BBB was not affected, in zebra fish larvae, both brain vascular endothelium and parenchyma infection without BBB disruption were reported (Couderc et al., 2008; Passoni et al., 2017). However, the mechanism through which CHIKV crossed the BBB could not be assessed. The difference between these findings could be a result of different experimental paradigms (e.g., viral titer, model organism), limited brain endothelial infectivity of CHIKV, and/or low viral release into parenchyma.

The viruses can directly (e.g., cytopathy, interference with developmental processes) and indirectly (e.g., via the immune response) damage the BBB, enabling dissemination into the CNS. For example, (de)phosphorylation-dependent TJ modulation and endothelial cytotoxicity leading to cell death can affect endothelial permeability (Papa et al., 2017; Leda et al., 2019; Buzhdygan et al., 2020; Song et al., 2021). Neurovasculature development starting around GW8, sets the onset of BBB formation and it proceeds in parallel with neurogenesis and brain expansion during which it provides necessary oxygen and nutrients to the cells. Therefore, it is important to have a parallel development of the brain and a proper functioning of the BBB. As such, ZIKV-induced cerebral vasculature developmental delay associated with the reduced neurogenesis, indicated that it affected BBB function (Papa et al., 2017; Garcez et al., 2018; Leda et al., 2019). Likewise, altered protein levels connected to Rho family-associated pathways in CHIKV-infected neonate mice, could disturb blood vessel permeability (Couderc et al., 2008). Receptor ACE2 expression in cerebral vasculature and vascular injury in SARS-COV-2 infection, probably as a result of direct and immune-mediated effects, also pose as a risk factor for the developing brain (Leda et al., 2019; Buzhdygan et al., 2020; Wenzel et al., 2021).

Maternal and placental cytokines produced as an immune response to infection can have detrimental consequences for both the developing brain (see section 1.5) and the BBB. Initiated inflammatory responses against the viral attack, whether it is systemic or local, could disturb BBB permeability through its components (e.g., endothelium, astrocytes, transporters, TJs) resulting in viral entry into the CNS and exacerbation of brain injury. More specifically, activated endothelium upregulates adhesion molecules (e.g., ICAM-1) and inflammatory cytokines (e.g., CCL5, CXCL10, IL-1B, IL-6), allowing recruitment and docking of leukocytes to the BBB as well as increasing permeability (Adams Waldorf et al., 2018; Baines et al., 2020; Fenizia et al., 2020; DeGrace et al., 2022). Passage of leukocytes and viruses into the parenchyma further amplifies inflammatory mediators and BBB breach. Observation of such alterations along with increased matrix metalloproteinases and BBB permeability upon SARS-COV-2 infection indicates inflammation-associated BBB disturbance (Buzhdygan et al., 2020). Similar findings, albeit slight perturbations in TJs and BBB permeability upon ZIKV infection, imply that this may not be the main route of CNS entry, though induced local inflammation followed by subsequent events could amplify brain and barrier damage as well as viral entry (Cle et al., 2020). Indeed, within the brain, dysregulated immune responses associated with vascular damage resulted in leaky BBB and potentially brain calcification (Shao et al., 2016). A dysregulated neuroinflammatory response may also contribute to BBB breakdown in CHIKV infection (Dahm et al., 2016).

Leukocyte (e.g., macrophage, monocyte, microglia) recruitment during or after BBB breakdown confers as a risk factor since they can be hijacked by the viruses for CNS entry with a so-called Trojan horse mechanism (Mustafa et al., 2019). Peripheral monocyte and macrophage infectivity of the viruses further demonstrates their versatility in routes of dissemination and/or persistence (Silasi et al., 2015; Lang et al., 2018; Jafarzadeh et al., 2020; V'Kovski et al., 2021). As an example, increased number of alveolar macrophages with abundant ACE2 expression in severe elderly cases led to the hypothesis of, SARS-COV-2 infection of lungs may enable dissemination to other organs (e.g., brain) via infected macrophages (Abassi et al., 2020; Ferren et al., 2021). Though validity of this mechanism for the fetal stage is not known, in the vertical transmission case, lung infection and brain injury was however reported. Exceptionally, yolk sac-derived microglial cells appearing and migrating to the developing brain (GW4-24; Menassa and Gomez-Nicola, 2018) not only participate in brain development in critical stages but also are the resident macrophages of the brain acting as first-line defenders against pathogens (Tremblay et al., 2020). For example, ablation of microglias, which were localized at the embryonic murine cerebral vessels, decreased not only ZIKV load in brain but also fetal demise (Xu et al., 2020).

1.3.2. Blood-cerebrospinal fluid-brain barriers

Unlike the BBB, inner embryonic CSF (eCSF)-brain and blood-eCSF barriers are the first appearing transient barriers in the developing brain (Goasdoue et al., 2017; Saunders et al., 2019). Both the epithelial blood vessel TJs and the neuroepithelial strap junctions are impermeable to all except smallest lipid soluables as opposed to the adult CSF-brain barrier, hence, create a controlled internal environment and allow expansion of the developing brain until choroid plexus (ChP) becomes functional (Saunders et al., 2018). As these barriers progressively disappear, they become ependyma starting around late 2nd trimester and form the BCSF barrier on the ventricular system (Saunders et al., 2018, 2019). With the initiation of ChP differentiation between GW 6–8, the BCSFB barrier on ChPs forms the 4th, lateral and 3rd ventricles, respectively, until the end of pregnancy (Lun et al., 2015). BCSF barrier on the ChP consists of epithelial cells with TJs on the apical side (Saunders et al., 2018), while the ChP stroma contains endothelial fenestrae with attached pericytes around the blood vessels. ChPs show secretory, barrier, and transportation functions after differentiation, although, similar impermeability pattern as eCSF-brain barrier mentioned above, seem to apply to early differentiated ChPs as well.

Despite restricted molecule transmission between blood-CSF-brain early in development, structural alterations, transitional stages and long-lasting formation of protective ependymal layer may create vulnerability to viral infections (Coletti et al., 2018). As such, it was suggested that BCSF barrier could be vulnerable to ZIKV infection based on its developmental structure, susceptibility of NSCs to ZIKV infection which are closely located to CSF in ventricular zone (VZ) of

developing brain, and observed periventricular injury pattern (Nelson et al., 2020). Similarly, CHIKV-infected neonates were claimed to be affected by Trojan horse-associated CNS damage through ChP, leptomeninges, and ependyma due to the subcortical and periventricular damage (Ferreira et al., 2021). Infection on the level of BCSFB barrier can not only cause barrier dysfunction, but also enable viral access to the interior and outer surface of the brain upon viral release into CSF, in both cases there could be negative consequences for brain development. For example, ZIKV-infected pericytes in BCSF barrier disturb ChP epithelial barrier integrity and allow ZIKV CSF entry, likely by releasing factors (e.g., cytokines) (Kim et al., 2020). CHIKV can infect ChP ependymal and leptomeningeal cells and cause severe vacuolization of ChP epithelial cells which could affect its functionality. Productive SARS-COV-2 infection of ChP epithelium initiates cell death and inflammatory responses resulting in functional and structural deficits in the BCSF barrier. Also, decreased production of TTR protein, carrying thyroid hormone from the blood to CSF, may indicate developmental delay if it occurs during gestation (Richardson et al., 2015; Jacob et al., 2020; Pellegrini et al., 2020). Developmental stage-specific CSF volume and component adjustment provides necessary hydrostatic pressure and signaling factors (e.g., differentiation, guidance) to even distant regions. During this process, BCSFB barriers transport the factors from blood to CSF. ChPs play a crucial role by adjusting CSF volume and releasing ChP-derived factors which can affect the behavior of the neural stem cells on the ventricles. Moreover, even at postnatal stage, ChP continues to contribute to the development of the brain such as by modulating cerebral cortex plasticity. Therefore, virus-associated BCSFB damage and viral dissemination into the CSF could be detrimental for the developing brain.

1.4. Virus-induced direct damage to developing brain

The neurotrophic viruses entering the brain can interfere with the antiviral mechanisms (e.g., apoptosis, autophagy), cellular morphology (e.g., cell lysis, syncytia formation), and functionality (e.g., transcription and translation) through viral replication and/or interaction between viral components and the host. While benefiting from these interferences, such as by enhancing their replication and disseminating within the brain, the viruses create cytotoxicity during the process and temper cellular and molecular events which can damage cell populations and impair proper neurodevelopment (Figure 3). The extent of the impairment depends on several factors such as viral dissemination, targeted cell populations, and developmental time of interference.

1.4.1. Zika virus

ZIKV, having tropism to several cell populations (e.g., glial cells, early-late neurons) in addition to their well-known targets the neural progenitor/stem cells (NP/SCs), can affect the VZ where newborn neurons are generated of different brain regions such as hippocampus, cerebellum, thalamus, and hypothalamus (Cugola et al., 2016; Li et al., 2016; Van den Pol et al., 2017; Shelton et al., 2021). Apart from the presence of entry receptors, axonal transportation, infection through astrocytes and NPC pool, could enhance its dissemination ability (Retallack et al., 2016; Shelton et al., 2021). ZIKV replication and proteins modulating distinct mechanisms such as apoptosis, autophagy, and cell cycle create multi-level impairment in the developing brain spanning from structural defects to (sub−/extra-) cellular alterations (e.g., cytoplasmic vacuolization, mitochondria disruption, axonal rarefaction, and adherens junction impairment) (Miner and Diamond, 2016; Yoon et al., 2017; Lee et al., 2020; Yang et al., 2020). The high impact of ZIKV on neurodevelopment, more specifically on proliferation, differentiation, and migration processes, is due to higher tropism toward NP/SC populations. ZIKV-induced autophagy enhances viral replication by creating favorable conditions for its replication and inhibiting virus-targeted autophagy (virophagy). In the meantime, modulated pathways (e.g., Akt–mTOR, FA) in NSCs, having a dual role in autophagy and brain development, impair neurogenesis (Liang et al., 2016; Tiwari et al., 2020). Commonly observed apoptosis, probably as an antiviral host response, follow an incremental trend over the infection period attenuating brain growth (Cugola et al., 2016; Li et al., 2016). Apoptosis can be initiated in neuronal and glial lineages due to ZIKV-induced transcriptional dysregulation of related genes (Zhang et al., 2016), mitochondrial dysfunction (Yang et al., 2020), oxidative stress (Ledur et al., 2020), and DNA damage (Ledur et al., 2020). Regardless of intrinsic or extrinsic induction of apoptosis, it can be suppressed via stabilization of anti-apoptotic Bcl-2 family proteins by ZIKV (Turpin et al., 2019) demonstrating the extent of self-protective mechanisms especially during early infection while creating a catastrophic environment for the developing brain over time. Furthermore, ZIKV can dysregulate DNA damage repair- and cell cycle-associated (e.g., mitosis, cell cycle process) pathways (Tang et al., 2016; Zhang et al., 2016). As such, directly induced DNA damage combined with cell cycle arrest prevent host DNA replication thereby promoting ZIKV replication (Hammack et al., 2019). ZIKV protein-specific cell cycle arrest at different points with inhibition of differentiation can result in NPC pool depletion (Li et al., 2016; Hammack et al., 2019). During the process, mitotic and centrosomal alterations can interfere with the mode of NPC division (asymmetric/symmetric), chromosome segregation, and cell polarity which can result in chromosomal abnormalities, migration defects, and NPC pool depletion potentially due to chromosomal damage (Gabriel et al., 2017; Kesari et al., 2020).

1.4.2. Chikungunya virus

"The relative less tropism of CHIKV" is in comparison to ZIKV. While the ZIKV can extensively infect neuronal and glial cells, same level of infection was not reported for CHIKV (e.g., cerebral cortex, hippocampus, and cerebellum) during which axonal transportation and syncytia formation play a role (Das et al., 2015; Schnierle, 2019; Ferreira et al., 2021). CHIKV replication with subsequent induction of cell stress, apoptosis, and autophagy mediate its cytopathic effects resulting in cellular damage (e.g., cell lysis, death, cellular disintegration) likely contributing to brain injury (Ramos et al., 2018; Van Ewijk et al., 2021). Although cell type-specific CHIKV vulnerability is not known, higher susceptibility of immature neurons to cytopathy and potential involvement of NS/PCs infection in neurological manifestation was suggested. CHIKV replication and/or interfered cellular processes (e.g., antioxidant enzyme production) can induce endoplasmic reticulum (ER) stress and oxidative stress during which CHIKV can take different routes in modulating the antiviral responses for its benefit. For instance, during ER stress-associated unfolded protein response (UPR) activation, UPR can be suppressed via CHIKV NSP2-mediated host shut-off potentially to evade from UPR-associated antiviral mechanisms (Meshram et al., 2019; Law

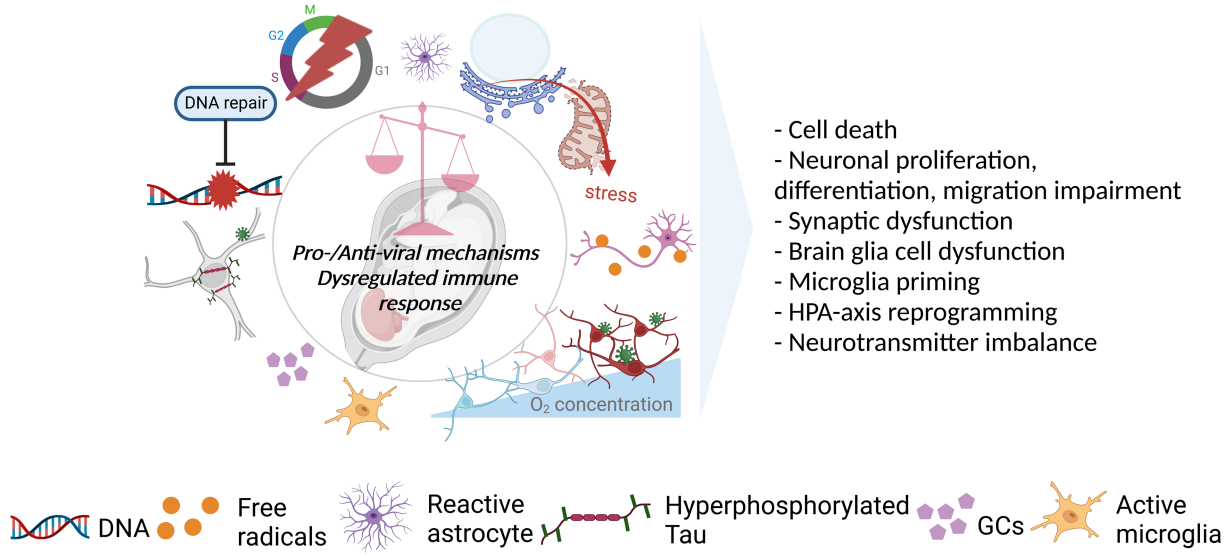

FIGURE 3
Viral acting mechanisms and consequences for the developing brain. Viral infection initiates a cascade of events in favor (pro-viral) of and against (antiviral) the establishment of persistent, productive infection in the host. The balance between pro-viral and antiviral mechanisms as well as the level and the length of the immune response could dictate the degree to which the developing brain is affected by the infection. As such, the shift in balance toward pro-viral mechanisms coupled with the cytopathic effects (CPEs) of the viruses and strong/prolonged immune response can affect several neurodevelopmental processes in a negative way. As illustrated, viruses can dysregulate several mechanisms including DNA repair, cell cycle, host proteins (e.g., Tau), and cellular metabolism managing the resources (e.g., oxygen), mostly, as a pro-viral strategy. CPEs, including the dysfunction/stress of cellular and sub-cellular structures (e.g., mitochondria, endoplasmic reticulum), in addition to immune response activation leading to, such as, the activation of glial cells (microglia, astrocytes) and release of GCs, can also be observed. These in turn, can have an impact on cell survival, neurodevelopmental processes, neuronal functioning, glial behavior, and neuroendocrine system activity. Created with BioRender.com.

et al., 2021). The interaction between CHIKV non-structural protein-2 (NSP2) and HSP90-associated PI3K/AKT/mTOR pathway, enables viral replication during early infection. While the occurrence and effects of these interferences in the developing brain is not known, the investigation of the PI3K/AKT/mTOR pathway within the context of CHIKV brain infection may provide information on neurodevelopmental aspects given its importance in neurodevelopment and cell death (Gérardin et al., 2014). Further, independent of stress-induced mTOR inhibition, CHIKV can activate autophagy and apoptosis. Specifically, the interaction of CHIKV NSP2 with human autophagy receptor NDP52, reduces cell death by limiting cell shut off and enhances viral replication by allowing anchorage of the viral replication complex to the Golgi complex (Verlhac et al., 2015). Early induction of autophagy in a glioblastoma cell line and prominent cell death especially in the late stages of the CHIKV brain infection may be the result of skewed autophagy and apoptosis toward a pro-viral role in the CNS (Abraham et al., 2013). Nonetheless, the possibility of host antiviral immune response-associated apoptosis induction cannot be overlooked given simultaneous activation of the immune response (Law et al., 2021). Also, hiding of CHIKV within apoptotic blebs could enable its cell-to-cell spread (e.g., neighboring cells, macrophages), hence, enhance its dissemination. In line with that, bystander apoptosis in murine brain with viral dissemination might be contributed by apoptotic bleb-associated infection (Abraham et al., 2013). Differential expression analyses have revealed modulation of several pathways including synaptic functioning, neurotransmission and neuronal cytoskeletal proteins in addition to cell death and stress response (Lim et al., 2017). However, specific functional connections of these modulations to the observed developmental delay in exposed neonates is not known. Further, despite an observed dysregulated immune response and its known negative influence on neurodevelopment (see section 1.5), a higher likelihood of direct CNS infection in neonates was implied. Therefore, investigation of CHIKV infection in the developing brain by focusing on functional consequences of the directly induced alterations could be informative for directly modulated neurodevelopmental processes.

1.4.3. Severe acute respiratory coronavirus-2

Despite the lower expression of SARS-COV-2 entry-associated proteins in the brain compared to lungs, several brain regions are vulnerable to infection with a preference toward mature neurons (e.g., excitatory, dopaminergic neurons; Jacob et al., 2020; Pellegrini et al., 2020; Lukiw et al., 2022). ZDHHC5, GOLGA7, and ATP1A1 are expressed abundantly during fetal brain development, especially in the 2nd and 3rd trimester, in (im)mature neurons and NPCs unlike widely investigated entry proteins (ACE2 and TMPRSS2; Varma et al., 2020; Chen et al., 2021). While showing the potential danger to the developing brain, the *in vivo* and *in vitro* assessment of newly identified interactors is still lacking. The dissemination within the brain might be contributed by syncytia formation allowing cell-to-cell spread (Jacob et al., 2020; Zhang et al., 2020) and axonal transport given its ability to mimic relevant transport proteins (Yapici-Eser et al., 2021). Cell death, particularly at the proximity of infected cells, has been commonly reported within the context of brain infection which could be contributed by inflammatory response (Jacob et al., 2020; Ferren et al., 2021) and cellular dysfunction (Song et al., 2021; Valeri et al., 2021). For example, neuronal metabolic alterations can manage cellular resources to both un/infected neurons probably for viral replication and lead to death of nearby neurons as a

result of hypoxic-state (Song et al., 2021). SARS-COV-2-induced oxidative stress, either as a result of mitochondrial manipulation (Clough et al., 2021) or facilitated infection, leads to DNA damage that cannot be repaired and enhances cortical neuronal death. Further, cell cycle impairment, which could be due to the DNA damage and/or oxidative stress, could induce neuronal senescence in which proliferation is permanently inhibited (Valeri et al., 2021). While SARS-COV-2 does not seem to have teratogenic effects similar to ZIKV, loss of neuronal populations during 2nd–3rd trimester when cortical growth continues may create subtle changes, contributing to cognitive and behavioral alterations (Andescavage et al., 2017). SARS-COV-2 interacting with host proteins could cause their dysfunction and affect relevant processes negatively (Idrees and Kumar, 2021; Yapici-Eser et al., 2021; DeGrace et al., 2022; Hok et al., 2022). For instance, the viral heparin binding site could assist binding of relevant proteins (e.g., Aβ, α-synuclein, tau, prion) and lead to their aggregation and neurodegeneration (Idrees and Kumar, 2021). Similarly, as the hallmark of adult-onset taupaties, mislocalized and aberrantly phosphorylated Tau was reported in cerebral organoids (Ramani et al., 2020, 2021). Given the role of Tau on axonal microtubule organization during neural differentiation as well as synaptogenesis and dendritic spine formation, dysfunctional Tau could have consequences for the developing brain (Rankovic and Zweckstetter, 2019). Additionally, viral interaction with MAO, growth factors, and the proteins having role in synaptic and neurotransmission could misbalance neurotransmitter levels, affect neuronal survival and neuronal differentiation (Yapici-Eser et al., 2021; Hok et al., 2022). Despite recent occurrence of the pandemic, emerging information suggests neurobiological interference of SARS-COV-2 creates a neurotoxic environment, though mechanisms and functional consequences especially for developing brain require further investigation.

1.5. Immune activation, inflammatory mediators and developing brain

Maternal viral infection inducing immune response which is mediated by inflammatory factors can change the homeostasis of the barriers and the developing brain, as mentioned in previous sections. During maternal infection, inflammatory cytokines in the fetus can increase due to the transplacental passage, placental production or fetal production posing as a developmental stressor for fetus (Fenizia et al., 2020; Ferreira et al., 2021; Han et al., 2021). Specifically, pathogen/damage-associated molecular patterns recognized by PRRs (e.g., TLRs) which are expressed in the CNS (e.g., neurons, microglia, astrocytes) and peripheral immune cells (e.g., macrophages) mediate cytokine release. Not only this molecular pathway was suggested to be the link between maternal inflammatory factors and immune-mediated disruption of brain development but also cytokines are recognized as the key modulators of developmental trajectories (Han et al., 2021).

Even a slight change in the balance between pro- and anti-inflammatory factors released upon pathogen encounter can be enough to deviate from normal neurodevelopment. Evidence suggests that all three viruses can induce immune activation and cause dysregulated immune response. Firstly, fever, associated with pyrogenic cytokines (IL6, IL1b, and TNFα) is among the symptoms in infected pregnant women and/or exposed neonates (Dahm et al., 2016; Raschetti et al., 2020; Ginige et al., 2021). Secondly, enhanced level of cytokines and chemokines (e.g.IL1B, IL6, CCL5-2, CXCL9-10, and TNFα) in the mother, placenta, and/or neonate was reported in SARS-COV-2 (Fenizia et al., 2020; Cribiu et al., 2021; DeGrace et al., 2022) and ZIKV (Lima et al., 2019; Rabelo et al., 2020) infections. Further, there is an association between SARS-COV-2 and cytokine storm (DeGrace et al., 2022) as well as between maternal cytokines and fetal brain abnormalities in ZIKV infection (Adams Waldorf et al., 2018). In case of CHIKV, maternofetal cytokine transmission is likely given the higher level of cytokine release during the acute phase of the infection compared to the convalescent phase (Kril et al., 2021). Prenatal exposure to some cytokines at high levels which are also seen in the viral infections (e.g., IL6, IL17) is sufficient to drive a behavioral outcome (Venugopalan et al., 2014; Chirathaworn et al., 2020). But maternal immune activation (MIA) is seen as a disease primer due to absence of neonatal neuropathology in most cases (Jiang et al., 2018). Thirdly, neuroinflammation and/or glial activation was demonstrated individually for all viruses mentioned (Dahm et al., 2016; Raschetti et al., 2020; Han et al., 2021; Elgueta et al., 2022). Finally, ZIKV (Cle et al., 2020; Rabelo et al., 2020), CHIKV, and potentially SARS-COV-2 (DeGrace et al., 2022) can cause prolonged immune activation. Several routes have been postulated through which immune activation and inflammatory responses may alter the developing brain, thus, exacerbate brain injury and/or predispose to NDDs, NPDs such as via glial cells, trained immunity, and HPA-axis (Figure 3).

Glial cells namely astrocytes and microglia play an important role during development enabling functional neural circuitry formation, maturation and maintenance (Lago-Baldaia et al., 2020; Eze et al., 2021). In response to infection and cytokine release, glial cells become activated showing a pro-inflammatory state to improve neuroprotection and homeostasis (Dahm et al., 2016; Cornish et al., 2020; Elgueta et al., 2022; Kim et al., 2022). However, such early life stresses can be damaging to the developing brain due to a dysregulated glial functioning (e.g., impaired phagocytic activity, over/prolonged activation) and blunted glial development. Phagocytosis, required for synaptic pruning by astrocytes and primarily by microglia, is one of the functions that is found to be impaired upon MIA and is associated with NDDs (Lago-Baldaia et al., 2020; Carloni et al., 2021). Astrocytes, having a role in synaptogenesis, synapse regulation and neurotransmitter turnover, upon overactivation can release neurotoxic molecules as well as causing excitotoxicity due to impaired neurotransmitter turnover function resulting in neuronal dysfunction and cell death (Inglis et al., 2016; Lago-Baldaia et al., 2020; Linnerbauer et al., 2020; Stasenko et al., 2023). Further, microglia can amplify not only excitotoxic activity of astrocytes but also fetal brain injury via, e.g., secreted cytokines and free radicals (Linnerbauer et al., 2020). For example, the myelinating cells, pre-oligodendrocytes, are vulnerable to cytokines partially due to their inability to scavenge free radicals efficiently. Thus, potential damage can result in hypomyelination and even white matter injury (Motavaf and Piao, 2021; Stasenko et al., 2023). That may contribute to brain injury and developmental abnormality in CHIKV-exposed infants considering oligodendrocyte susceptibility to the infection, and the presence of inflammatory response, demyelination, and white matter injury (Gérardin et al., 2014; Das et al., 2015; Mehta et al., 2018; Ramos et al., 2018). Moreover, microglial activity could contribute disruption of oligodendrocyte development in Zika infections (Li et al., 2016).

Microglias, as the primary immune cells of the CNS, as well as peripheral immune cells (e.g., macrophages, monocytes) are particularly relevant within the context of trained immunity and long-term impact of the prenatal immune activation. Developmental stressors (e.g.,

infection or cytokine exposure) (1st hit) can induce immune training by epigenetic and metabolic reprogramming immune cells (priming) thus enabling them to create strong inflammatory responses to the subsequent stimulus (2nd hit) (Netea et al., 2020; Carloni et al., 2021). Particularly microglia priming could be the key mediator of the negative consequences (e.g., neuronal and behavioral abnormalities) of the developmental stressor since MIA alters the function of microglia to the subsequent stimulus. Moreover, compared to adult microglias, neonatal microglias are more prone to priming (Carloni et al., 2021). For example, developmental stressor-induced inflammatory response, by priming microglias, created susceptibility to Alzheimer's disease. As such, late low-dose Aβ treatment exacerbated microglial activation contributing to synapse damage and cognitive impairment (Frost et al., 2019). A potential role of trained immunity in ASD onset and progression was suggested with the observations of altered immune response to the subsequent stimuli along with fluctuating neuropsychiatric symptoms in a subset of the ASD children in the cohort.

Infection and cytokines (e.g., IL-1/2/6, TNFα) by affecting hormone release from Hypothalamus, Pituitary and Adrenal glands activate maternal and/or fetal HPA axis leading to release of glucocorticoids (GCs) as an end product (Han et al., 2021). HPA-axis activity is controlled by a negative feedback loop during which produced GCs inhibit its continuous activation, preventing excess GC exposure. However, cytokines can not only downregulate placental GC inactivating enzyme (Cottrell and Seckl, 2009) but also create GC resistance upon prolonged exposure, thus, exposing the fetus to unrestrained GC. Also, GCs can suppress inflammation through production of anti-inflammatory cytokines (Han et al., 2021). Therefore, the ability of the developing brain to cope with inflammation partially depends on sufficient stress response generation through the HPA-axis. And that may differ before late gestation and during early postnatal period considering the functionality of the HPA axis throughout development (Sheng et al., 2020). Altogether, these can permanently alter HPA-axis response to stress (e.g., hyperactivation) which can contribute behavioral alteration (e.g., anxiety) and vulnerability to several diseases (e.g., psychiatric) in adulthood (Cottrell and Seckl, 2009; Han et al., 2021). Further, HPA-axis' hyperactivation could affect development of neurotransmitter systems and neurotransmitter levels in the developing brain due to the connection between neurotransmitter systems (e.g., serotonergic, dopaminergic) and the HPA-axis.

2. Conclusion and perspectives

Viruses whether it is due to viral receptor expression or placental breach can reach the fetus and move toward the fetal brain. But knowing the route, vulnerable developmental timing and the type of dysfunction can help to better assess the risk for brain development. The viruses inherently trying to establish productive and persistent infection can affect distinct developmental processes such as neuronal proliferation, differentiation as well as synaptic and brain barrier function especially considering the naïve immune state of offspring. Further, virus-induced dysregulated immune responses could have long-lasting effects on the developing brain. Better identification of the targeted cellular processes with respect to brain development for CHIKV and SARS-COV-2, additionally, the effects of dysregulated immune response upon CHIKV, ZIKV, and SARS-COV-2 infection on developing brain can help understanding the scope of neurodevelopmental impact. And that could enable development and/or application of the targeted therapies for the affected newborns.

Author contributions

HR wrote the manuscript with support from SK. All authors contributed to the article and approved the submitted version.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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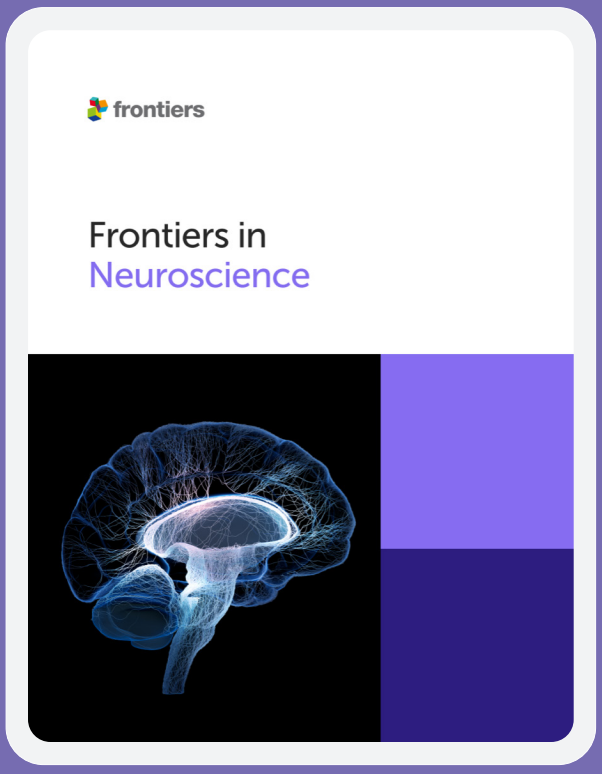

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