Brain, Behavior, and Immunity 120 (2024) 488–498

Contents lists available at ScienceDirect

Brain Behavior and Immunity

journal homepage: www.elsevier.com/locate/ybrbi

Full-length Article

Brain region-specific alterations in gene expression trajectories in the offspring born from influenza A virus infected mice

Stella Liong [a,1], K.H. Christopher Choy [b,1], Simone N. De Luca [a], Felicia Liong [a], Madison Coward-Smith [a], Osezua Oseghale [a], Mark A. Miles [a], Ross Vlahos [a], Celine Valant [b], Jess Nithianantharajah [c,d,*], Christos Pantelis [c,e,g,*], Arthur Christopoulos [b,f,g,*], Stavros Selemidis [a,*]

[a] Centre for Respiratory Science and Health, School of Health and Biomedical Sciences, RMIT University, Bundoora, VIC, Australia
[b] Drug Discovery Biology, Monash Institute of Pharmaceutical Sciences, Monash University, Melbourne, VIC, Australia
[c] The Florey Institute of Neuroscience and Mental Health, Melbourne, VIC, Australia
[d] Department of Florey Neuroscience, University of Melbourne, Melbourne, VIC, Australia
[e] Melbourne Neuropsychiatry Centre, Department of Psychiatry, University of Melbourne, Melbourne, VIC, Australia
[f] ARC Centre for Cryo-electron Microscopy of Membrane Proteins, Monash Institute of Pharmaceutical Sciences, Monash University, Melbourne, VIC, Australia
[g] Neuromedicines Discovery Centre, Monash Institute of Pharmaceutical Sciences, Monash University, Melbourne, VIC, Australia

ARTICLE INFO

Keywords:
Influenza A virus
Pregnancy
Maternal immune activation
Neuroinflammation
Viral infection
Offspring neurodevelopment
Neurodevelopmental Disorders
Microglia
Hippocampus

ABSTRACT

Influenza A virus (IAV) infection during pregnancy can increase the risk for neurodevelopmental disorders in the offspring, however, the underlying neurobiological mechanisms are largely unknown. To recapitulate viral infection, preclinical studies have traditionally focused on using synthetic viral mimetics, rather than live IAV, to examine consequences of maternal immune activation (MIA)-dependent processes on offspring. In contrast, few studies have used live IAV to assess effects on global gene expression, and none to date have addressed whether moderate IAV, mimicking seasonal influenza disease, alters normal gene expression trajectories in different brain regions across different stages of development. Herein, we show that moderate IAV infection during pregnancy, which causes mild maternal disease and no overt foetal complications *in utero*, induces lasting effects on the offspring into adulthood. We observed behavioural changes in adult offspring, including disrupted prepulse inhibition, dopaminergic hyper-responsiveness, and spatial recognition memory deficits. Gene profiling in the offspring brain from neonate to adolescence revealed persistent alterations to normal gene expression trajectories in the prefronal cortex, hippocampus, hypothalamus and cerebellum. Alterations were found in genes involved in inflammation and neurogenesis, which were predominately dysregulated in neonatal and early adolescent offspring. Notably, late adolescent offspring born from IAV infected mice displayed altered microglial morphology in the hippocampus. In conclusion, we show that moderate IAV during pregnancy perturbs neurodevelopmental trajectories in the offspring, including alterations in the neuroinflammatory gene expression profile and microglial number and morphology in the hippocampus, resulting in behavioural changes in adult offspring. Such early perturbations may underlie the vulnerability in human offspring for the later development of neurodevelopmental disorders, including schizophrenia. Our work highlights the importance of using live IAV in developing novel preclinical models that better recapitulate the real-world impact of inflammatory insults during pregnancy on offspring neurodevelopmental trajectories and disease susceptibility later in life.

1. Introduction

Severe influenza A virus (IAV) infection during pregnancy is associated with adverse maternal outcomes and, despite no evidence of vertical transmission by the virus from mother to foetus (Liong et al., 2020; Kanmaz et al., 2011), the infection can trigger both acute and

* Corresponding authors.
E-mail addresses: jess.n@florey.edu.au (J. Nithianantharajah), cpant@unimelb.edu.au (C. Pantelis), Arthur.christopoulos@monash.edu (A. Christopoulos), Stavros.selemidis@rmit.edu.au (S. Selemidis).
[1] These authors contributed equally.

https://doi.org/10.1016/j.bbi.2024.06.025
Received 13 March 2024; Received in revised form 4 June 2024; Accepted 23 June 2024
Available online 24 June 2024

chronic health consequences to the offspring (Mazumder et al., 2010; Li et al., 2014; Doyle et al., 2013; Khandaker et al., 2013). Short-term consequences to the offspring include being born small for gestational age (SGA), preterm birth and still birth (Doyle et al., 2013). Although severe IAV infection is well linked to adverse pregnancy complications, a recent epidemiological study has shown that seasonal influenza infection during pregnancy (without requiring hospitalisation) also increases the risk of late pregnancy loss and reduced mean birthweight among term infants. This highlights that even "mild" influenza infections that do not require hospitalisation can also carry adverse pregnancy risks (Dawood et al., 2021). In addition, epidemiological studies have reported long-term consequences, including reports of a 7-fold increase in the risk of developing schizophrenia in adulthood in children of mothers with serologically documented prenatal exposure to IAV during the first trimester of pregnancy (Brown et al., 2004). Preclinical rodent studies have also established a link between maternal IAV infection with altered behavioural, biochemical, and morphological changes in the adult offspring brain (Fatemi et al., 2002; Shi et al., 2005). It is postulated that these complications in offspring associated with maternal IAV infection are driven by maternal immune response hyperactivation rather than a direct cause of the virus, given that IAV does not directly infect the foetus *in utero* (Shi et al., 2005).

One of the first preclinical models to study the effect of maternal immune activation (MIA) on neonatal neurodevelopment utilised influenza viruses (Fatemi et al., 1999; Fatemi et al., 2002; Fatemi et al., 1998). However, more recently most of our knowledge on the effects of maternal immune activation (MIA) on offspring neuroinflammatory profiles and cognitive behaviour in adolescence has been derived from preclinical animal studies using the synthetic viral double-stranded RNA mimetic, polyinosinic-polycytidylic acid (poly(I:C)), rather than by use of actual live IAV (as reviewed by (Brown and Meyer, 2018; Meyer et al., 2009). One major difference between the two models is the route of administration, i.e., IAV is a respiratory infection occurring *via* the nasal passages, whereas poly(I:C) is given systemically via intravenous injection. Perhaps more pertinently, another significant difference between the models is that poly(I:C) is not a replicating stimulus, resulting in a transient immune response with no antibody generation and a relatively short-lived cytokine storm (Meyer et al., 2006; Mueller et al., 2018; Reisinger et al., 2015). In contrast, IAV infection drives an innate and adaptive immune system response characterised by a cytokine and vascular storm (Liong et al., 2020) and antibody generation, some of which can cross the placenta to the offspring. Furthermore, epidemiological studies have reported stronger links to schizophrenia risk associated with maternal exposure to single-stranded RNA viruses, such as IAV and rubella (Brown et al., 2006). More recently, there is also evidence to suggest that the choice of agent used to activate key pattern recognition receptors (PRR) in the immune system, specifically toll-like receptor-3 (TLR3) and toll-like receptor-7 (TLR7), results in different neurobiochemical changes and behavioural trajectories in the offspring (Kwon et al., 2021; Missig et al., 2020). For instance, although IAV is initially recognised via TLR7, double-stranded RNA produced by replicating IAV will also activate TLR3 (Le Goffic et al., 2007). In contrast, poly(I:C) specifically activates TLR3 only. For these reasons, preclinical rodent models should have greater translatability in understanding changes that affect the developing offspring brain, as well as the resulting impacts on behavioural trajectories, if they involve the use of live IAV, rather than synthetic analogues such as poly(I:C).

A recent study examining the effect of moderately pathogenic maternal IAV infection on foetal brain inflammation from embryonic day (E)17 offspring did not find evidence for whole brain neuroinflammation (Antonson et al., 2021). However, assessment of neuroinflammation in the whole brain at a single timepoint cannot address the potential of region-specific changes or gene transcriptional trajectories in the offspring brain in response to maternal IAV infection over time. Herein, we characterised the effect of maternal IAV infection on behaviour, measuring Y-maze spontaneous alternation, prepulse inhibition (PPI) of acoustic startle response, and amphetamine induced locomotor activity (LMA) in the adult offspring (P77 [week 11] – P98 [week 14]). We also characterised the temporal effects of moderate maternal IAV infection on genes involved in neuroinflammation in the offspring. The prefrontal cortex, hippocampus, hypothalamus and cerebellum were dissected from the offspring at 3 different age intervals (Laviola et al., 2003), representing neonatal (postnatal day [P]5), early adolescence (P21) and late adolescence (P56).

In the present study, we show that gestational IAV initiates long lasting perturbations in the neurodevelopmental trajectories in the offspring, characterised by neuroinflammatory gene expression alterations and microglial number and morphology changes in the hippocampus, which underpin behavioural modifications in adult offspring. These early perturbations are likely to influence the vulnerability of human offspring to neurodevelopmental disorders, including schizophrenia. Although poly(I:C)-based preclinical models have been instrumental in the MIA field, it is advantageous to compare different infection models to identify overlapping and/or immunogen-specific pathways when assessing MIA and its impact on offspring neurodevelopment. In this study, we utilised an IAV-based preclinical MIA model to assess the role of IAV-induced inflammation on offspring neurodevelopmental trajectories and disease susceptibility later in life. Our study also provides a unique pre-clinical, translational model to assess novel pharmacological interventions to restore the neurodevelopmental trajectories that have been perturbed by gestational influenza.

2. Materials and methods

2.1. Virus

Mouse-adapted influenza A virus strain HKx31 (H3N2), is a reassortment virus that contains the 6 internal proteins from PR/8/34 (PR8; H1N1) and the surface hemagglutinin and neuraminidase glycoproteins from A/Achi/2/68 (H3N2). HKx31 virus was grown in 10-day old embryonated chicken eggs and quantified by standard plaque assay using MDCK cells (expressed as plaque forming units (PFU/mL)).

2.2. Animals

The experiments were conducted at two sites, the Royal Melbourne Institute of Technology University (RMIT) and the Monash Institute of Pharmaceutical Sciences (MIPS). All animal experiments were conducted in compliance with the guidelines of the National Health and Medical Research Council (NHMRC) of Australia on animal experimentation and approved by the Animal Ethics Committee of each institution (RMIT AEC# 1801, 24336 and MIPS AEC # 22766). For the RMIT cohort, 8–12 weeks old time mated pregnant C57Bl/6J mice at E11 were sourced from Animal Research Centre (ARC; Western Australia, Australia), and followed the maternal immune activation (MIA) model reporting guidelines checklist based on the ARRIVE Guidelines and CONSORT (Kentner et al., 2019). For the MIPS cohort, 8–10 weeks old C57Bl/6J non-pregnant female mice were sourced from the Monash Animal Research Platform (MARP, VIC, Australia). They were time-mated within the MIPS animal facility near to the animal behavioural testing facilities. Dams were individually housed in microisolator cages and provided with standard mouse chow diet and water *ad libitum*. Mice were kept under standard 12-h light:12-h dark cycle (07:00–19:00) at an ambient temperature of 22 °C, with humidity between 40 and 60 %. Dams at E12 gestation were anesthetised with isoflurane and randomly assigned to be intranasally inoculated with either 35 μl of 10^3 PFU of HKx31 or mock infected with phosphate buffered saline (PBS) (Day 0) (RMIT: n = 10 PBS, n = 13 IAV; MIPS: n = 4 PBS, n = 7 IAV) as previously described (Liong et al., 2020). Dams were individually housed, and maternal body weights were recorded from Day 0 (E12 gestation) until Day 4 (E16 gestation) post-infection,

otherwise dams were left undisturbed until delivery (approx. E19-E20 gestation). Offspring were weaned at 3–3.5 weeks old. For the RMIT cohort, between 2 and 4 offspring per litter were randomly assigned for tissue collection at P5, P21 or P56 weeks of age. For the MIPS cohort, all animals underwent a battery of behavioural testing between 11 and 14 weeks of age. Each *n* refers to an individual mouse. It is noteworthy both cohorts of dams showed similar retarded growth rates post IAV inoculation (Supplementary Fig. 2C), thus showing consistency of the infection effect across the different institutions.

2.3. Bronchoalveolar lavage (BAL) fluid

To confirm airway inflammation, dams were euthanised at Day 6 post-infection via an intraperitoneal injection of ketamine (180 mg/kg) / xylazine (32 mg/kg) mixture. BAL fluid was collected as previously described (Liong et al., 2020). Briefly, a small incision made near the top of the trachea and a sheathed 21-Gauge needle was inserted into the lumen. The lungs were then lavaged with PBS. The number of total live cells from the collected BAL was counted on a hemocytometer.

2.4. Wire myography

Wire myography was performed as previously described (Liong et al., 2020). Briefly, perivascular adipose tissue was removed from the aorta. Vessels were cut into 2 mm ring segments and mounted onto pins on a wire myography bath. Endothelium-dependent relaxation to acetylcholine (ACh) was assessed in aorta that were sub maximally contracted with the thromboxane mimetic U46619.

2.5. RNA extraction and cDNA synthesis

Cerebellum, hippocampus, hypothalamus and the prefrontal cortex were dissected from offspring brains at different developmental stages: P5 (neonatal), P21 (early adolescent) and P56 (late adolescent). Brains from P56 offspring were sagittally hemisected through the midline. The left hemisphere was dissected over ice and key brain regions were immediately snap-frozen in liquid nitrogen and stored at −80 °C. The right hemisphere was fixed in 4 % paraformaldehyde in PBS overnight and then transferred into 20 % sucrose for cryoprotection. Brain samples were homogenized in 1 mL of TRIzol solution (ThermoFisher Scientific, MA USA) using the TissueLyserLT (Qiagen, Valencia, CA, USA). Total RNA was extracted using the RNeasy Mini kit (Qiagen) as per manufacturer's instructions. RNA samples were converted to cDNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, CA, USA) as previously described (Liong et al., 2020).

2.6. Quantitative real-time polymerase chain reaction (qPCR)

Gene expression was quantified using pre-designed TaqMan primers (ThermoFisher Scientific; Supplementary Table 1) and the TaqMan Universal PCR Master Mix (Applied Biosystems). The quantitative values were obtained from the threshold cycle (Ct) number. Gene expression analysis was performed using the comparative Ct method (Schmittgen and Livak, 2008). Each target gene expression was normalised against either GAPDH (hypothalamus and prefrontal cortex) or the ratio of GAPDH/PGK1 (cerebellum and hippocampus) mRNA expression as housekeeping controls. Supplementary Fig. 3 shows the stability of Ct values of the chosen housekeeping genes for each brain region. Determination of the expression ratio between two genes was performed using the method described in (Gordon et al., 2003). For genes that were differentially expressed with offspring sex, fold changes were calculated based on the PBS P5 male group set to 1. For genes that showed no interaction with offspring sex, fold changes were calculated either on the PBS P5 group (pooled male and female samples) set to 1.

2.7. Iba-1 immunofluorescence staining

Brains were coronally cut into 30 μm sections in a one-in-five series using a cryostat (Leica Biosystems, VIC, AUS). Iba-1 immunofluorescent staining was performed as previously described (De Luca et al., 2022). Briefly, sections were incubated with rabbit anti-Iba-1 primary antibody (1:1000, AB_2314666; Wako Chemicals, VA, USA) overnight at 4 °C and then incubated with a fluorescent anti-rabbit secondary antibody (1:400, AB_221544; Life Technologies, CA, USA). Two sections 60 μm apart between 1.70 and 2.18 mm relative to the bregma were analysed. Iba-1 images were assessed blindly for numbers and area per cell density using Image J software.

2.8. Behavioural testing and brain weight measurements

Behavioural tests (PBS: male = 11, female = 9; IAV: male = 14, female = 9) were conducted in the offspring raised within MIPS animal facility, where they were group-housed with same sex (2–5 mice/cage) in reverse light cycle (light on 7p.m.) post-weaning. All behavioural tests were conducted during the dark phase when the animals were active. Animals were tested in the following order: Y-maze spontaneous alternation, PPI of acoustic startle and LMA. All behavioural tests were conducted as previously described (Y-maze – (Suryavanshi et al., 2014); PPI and LMA – (Choy et al., 2021). In brief, for the Y-maze spontaneous alternation test, each mouse was placed at the end of one arm (pseudo-randomised between arm 1, 2 or 3), and allowed to explore the arm for 10 min. A successful alternation entry was counted when the arm entered was not the same as the previous two entries. Percent alternation in the Y-maze test was calculated as % alternation = number of successful alternations / (Total arm entries – 2) x 100 % (Suryavanshi et al., 2014). For assessment of PPI, the test sessions consisted of randomised trials of 'startle alone' pulses (120db), and pulses preceded by 6, 12 or 18db above the 65db background prepulses (pp6, 12 and 18). Inter-trial intervals (ITI) were, on average, 15 s (varying between 7 and 23 s), the prepulse–pulse (inter-stimulus) interval was 100 ms, length of the prepulse was 20 ms and the pulse was 40 ms, all as per our previous study (Choy et al., 2021). PPI was calculated using the formula [(pulse alone − prepulse-pulse)/ pulse alone] × 100 %. For LMA, animals were placed in a 40 cm x 40 cm square open field for 60 min to measure baseline activity, pseudo-randomly allocated a single i.p. injection of saline (Sal) or amphetamine (Amph, 2.5 mg/kg) (n value: PBS male: Sal = 4, Amph = 7; IAV male: Sal = 5, Amph = 9; PBS and IAV female: Sal = 3 and Amph = 6), and then tested for a further 90 min. Baseline activity was analysed for any potential effects of IAV treatment, and then the percentage (%) increase in amphetamine-induced hyperactivity was calculated relative to the baseline activity; the same calculation was applied to the saline treated group. After the last behavioural test, the animals were allowed a further 2–3 days of rest, and then humanely euthanised by cervical dislocation. Whole brain and hippocampus from each mouse were then immediately dissected and weighed.

2.9. Statistical analysis

Statistical tests were performed using GraphPad Prism Software Version 9 (San Diego, CA, USA) or SPSS Version 28 (IBM, Armonk, NY, USA). Data are expressed as the mean ± SEM. Unless specified otherwise, an unpaired Student's *t*-test was used for comparisons made between two groups, and a 3-way ANOVA was performed for the qPCR analyses, with maternal treatment, offspring age and sex as independent factors. For genes that showed no sex effect, male and female samples were pooled, and a 2-way ANOVA was performed with Tukey's post-hoc testing. Concentration response curve analysis for vascular reactivity studies was performed using 2-way ANOVA with repeated measures. Behavioural data were analysed by ANOVA, with sex and maternal treatment as independent factors. For PPI, the 3 prepulses (pp6, 12 and 18) were the 'within' factors (Choy et al., 2021) and average PPI (PPI%

averaging pp6, 12 and 18) is presented when interaction between prepulses and IAV and/or sex are absent. For LMA data, the distance travelled was presented with measurements made at 10-min intervals. The total distance travelled included a 60-min period to assess baseline activity, and a 90-min period following administration of amphetamine to address hyperreactivity. If there was no sex effect, the data were pooled to highlight the maternal IAV effect. Statistical significance was accepted at $P < 0.05$. All ANOVAs performed were corrected for multiple comparisons with the adjusted P-values reported.

3. Results

3.1. Moderate influenza during pregnancy causes localised and systemic inflammation while sparing offspring survival

To assess the effect of maternal influenza A virus infection on the neuroinflammatory profile across offspring postnatal development, pregnant C57BL6 mice at E12 gestation were intranasally inoculated with the moderately-pathogenic IAV strain HKx31 (H3N2) at two doses: high (10^4 PFU) and low (10^3 PFU). We have previously observed that dams infected with high IAV dose present with reduced maternal weight gain with advancing gestation, increased immune cell infiltration into the airways, severe aortic dysfunction, and reduced pup and placental weights at embryonic day E18 (Liong et al., 2020). In the present study, dams infected with the low dose of IAV presented with a more moderate systemic maternal disease (reduced body weight gain, increased airway immune cell infiltration, aortic dysfunction) without affecting E18 pup and placental weights *in utero* (Supplementary Fig. 1A-F). Litter size at time of delivery was not significantly affected by either high or low doses of maternal IAV infection (Supplementary Fig. 1G), however, offspring lethality was significantly increased with the high dose when compared to low dose of IAV (Supplementary Fig. 1H). Given that the offspring lethality was similar to PBS controls, we thus chose the low IAV dose for subsequent experiments to examine the effect of maternal IAV infection on offspring behaviour and early life neuroinflammation.

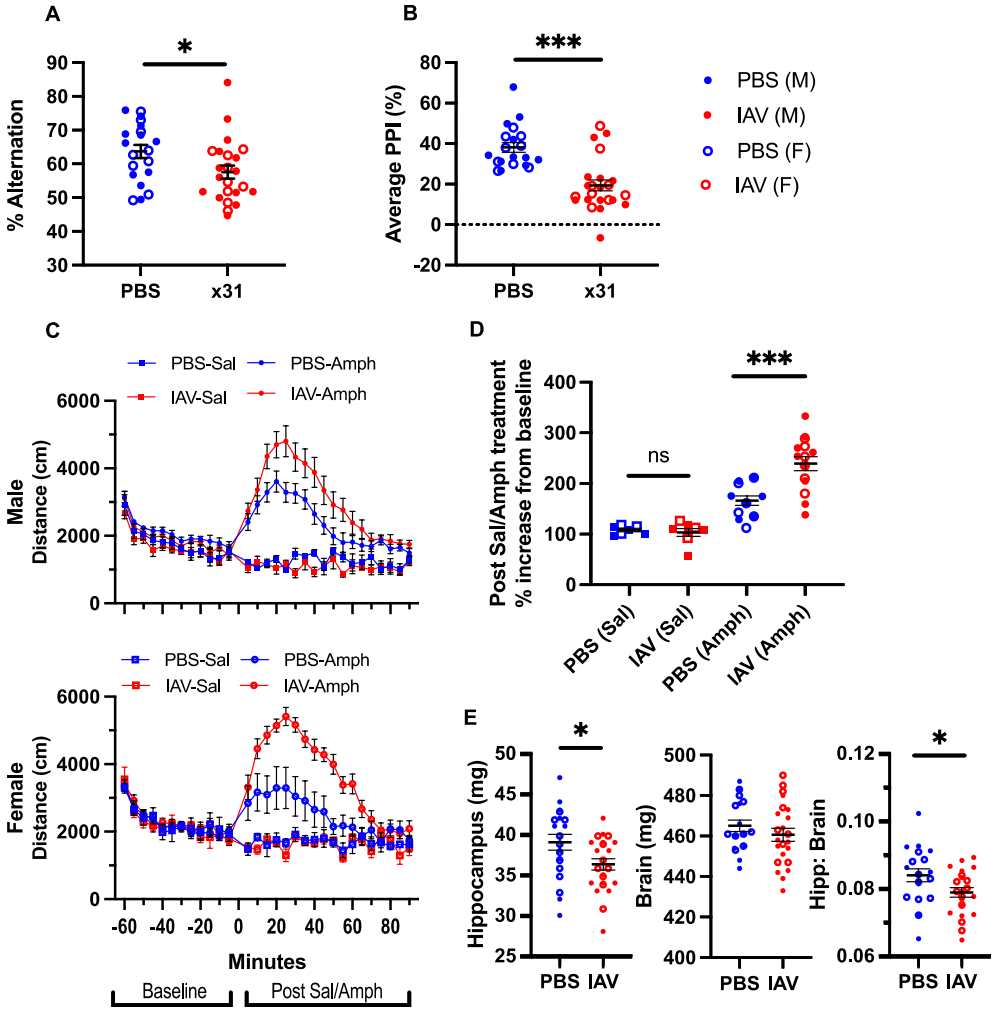

Fig. 1. Maternal IAV infection-induced behavioural changes in adult offspring. A battery of behavioural tests was conducted in adult offspring (n = 9 – 14/treatment/sex) at 11–14 weeks of age with 4–6 days' rest between tests. Tests were Y-maze spontaneous alternation, a measure of spatial recognition memory (A); prepulse inhibition (PPI) of acoustic startle, a measure of sensorimotor gating (B); and amphetamine (Amph 2.5 mg/kg, i.p.) induced locomotor activity in an open-field, a measure of responsiveness to a dopaminergic stimulant (C and D). Maternal IAV treated offspring displayed decreased Y-maze alternation (A) $F(1, 39) = 4.941$, $P = 0.032$) and robust deficits in sensorimotor gating of PPI (B) ($F(1, 39) = 23.311$, $P < 0.001$) 74 %). Maternal IAV treatment had no effect on offspring spontaneous locomotor activity (baseline), but a pronounced increase in response to amphetamine treatment (C). Further analysis of the % increase in locomotor activity following amphetamine relative to baseline ($F (1, 24) = 16.89$, $P < 0.001$) but not to saline treatment (D). Hippocampus weight was reduced in adult offspring from maternal IAV infected dams ($F(1,39) = 4.937$, $P = 0.032$) without any change to total brain weight (E). Statistical analysis was performed using ANOVA test; data were pooled from male and female mice if no sex effect was found; * $P < 0.05$, **$P < 0.01$ and *** $P < 0.001$ between PBS and IAV groups.

3.2. Maternal influenza infection reduces spontaneous alternation in the Y-maze test and disrupts PPI of acoustic startle in adult offspring

Adult offspring born from IAV-infected dams showed reduced Y-maze alternation, suggestive of impaired spatial recognition memory when compared to PBS offspring (Fig. 1A). Adult IAV offspring also exhibited sensorimotor gating deficits with reduced PPI compared to PBS offspring (Fig. 1B; PPI to individual prepulses (pp 6, 12 and 18) presented in Supplementary Fig. 2A).

3.3. Maternal influenza infection increases amphetamine induced locomotor activity without affecting baseline activity

Spontaneous locomotor activity under baseline conditions was analysed for the first 60 min habituation period (Fig. 1C) and showed no effect of maternal IAV infection on offspring baseline exploratory activity. Female mice travelled further distances in baseline exploratory activity compared to males, consistent with our previous findings (Supplementary Fig. 2B; Choy et al. 2021). To assess whether maternal IAV infection dysregulates dopaminergic transmission in adult offspring, the animals were administered either amphetamine or saline and monitored for a further 90 min (Fig. 1C). In the saline treated groups, there was no difference in locomotor activity between IAV and PBS offspring. However, in the amphetamine treated groups, IAV offspring showed a pronounced response to amphetamine induced LMA compared to PBS offspring (Fig. 1D). Of note, there were no sex differences in offspring responses to amphetamine between IAV and PBS cohorts.

3.4. Maternal influenza infection reduces hippocampal weight without affecting whole brain weight

There was no difference in total brain weight between IAV and PBS offspring, however, hippocampal weights from IAV offspring were significantly smaller than their age-matched PBS counterparts (Fig. 1E). Ratio calculations of hippocampal to total brain weights also demonstrated a similar reduction in the IAV offspring, suggesting that maternal IAV infection had a direct effect on hippocampal development, possibly via reduced neurogenesis rather than whole brain development.

3.5. Maternal influenza infection is associated with differential gene expression changes in late adolescent offspring

We next sought to investigate the effect of maternal IAV infection on genes with functional roles in mediating neuroinflammation in the prefrontal cortex, hippocampus, hypothalamus and cerebellum, at three ages during offspring development. Genes that were not affected by sex were pooled for subsequent analyses. PCA analyses of the four brain regions showed distinct clustering of neonatal offspring (P5) from juvenile (P21) and early adolescent (P56) offspring (Fig. 2). This suggests a more distinctive immunological profile during the early neonatal period, whereas the immunological profiles were more similar between the early (P21) and late adolescent (P56) offspring. Interestingly, PCA analyses revealed a clear separation between IAV and PBS offspring with regard to the hippocampus of neonates (P5) but not for any other brain regions. These findings indicate that neuroinflammation, which is part of healthy brain development, is distinct between the neonatal period and adolescence, and that the neonatal hippocampus (P5) is more vulnerable to perturbations by maternal IAV than the other brain regions studied.

3.6. Maternal influenza infection perturbs normal cytokine expression across development in the offspring brain

When compared to normal PBS offspring, the expression trajectories across development of pro-inflammatory cytokines and chemokines IL-1β, IL-6, TNF-α and CCL2 in the prefrontal cortex; IL-18, IFN-γ, CCL2, CCL3 and CCL5 in the hippocampus; IL-6, TNF-α, CCL3 and CCL5 in the hypothalamus; and IFN-γ, CCL2, CCL3 and CCL5 in the cerebellum were disrupted by maternal IAV infection (Fig. 3). Offspring born from IAV infected dams also demonstrated altered trajectories of anti-inflammatory cytokines, including IL-4, across development in the prefrontal cortex, hippocampus, and cerebellum (Fig. 4). IL-10 trajectory profiles across normal development were also altered in the hippocampus and hypothalamus of IAV offspring (Fig. 4).

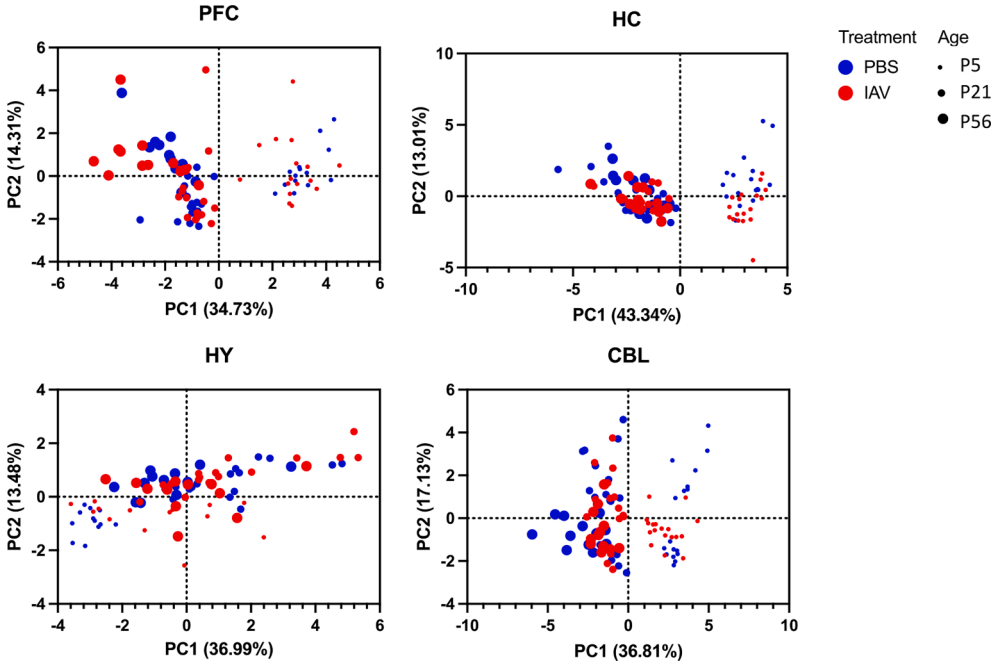

Fig. 2. Principal component analysis of gene expression data in IAV and PBS offspring brain across development. Prefrontal cortex (PFC), hippocampus (HC), hypothalamus (HY) and cerebellum (CBL) were dissected from offspring born from IAV-infected or PBS-mock-infected dams at postnatal days (P) 5, 21 or 56. PCA analysis was performed using the qPCR expressional data of 16 inflammatory genes of interest.

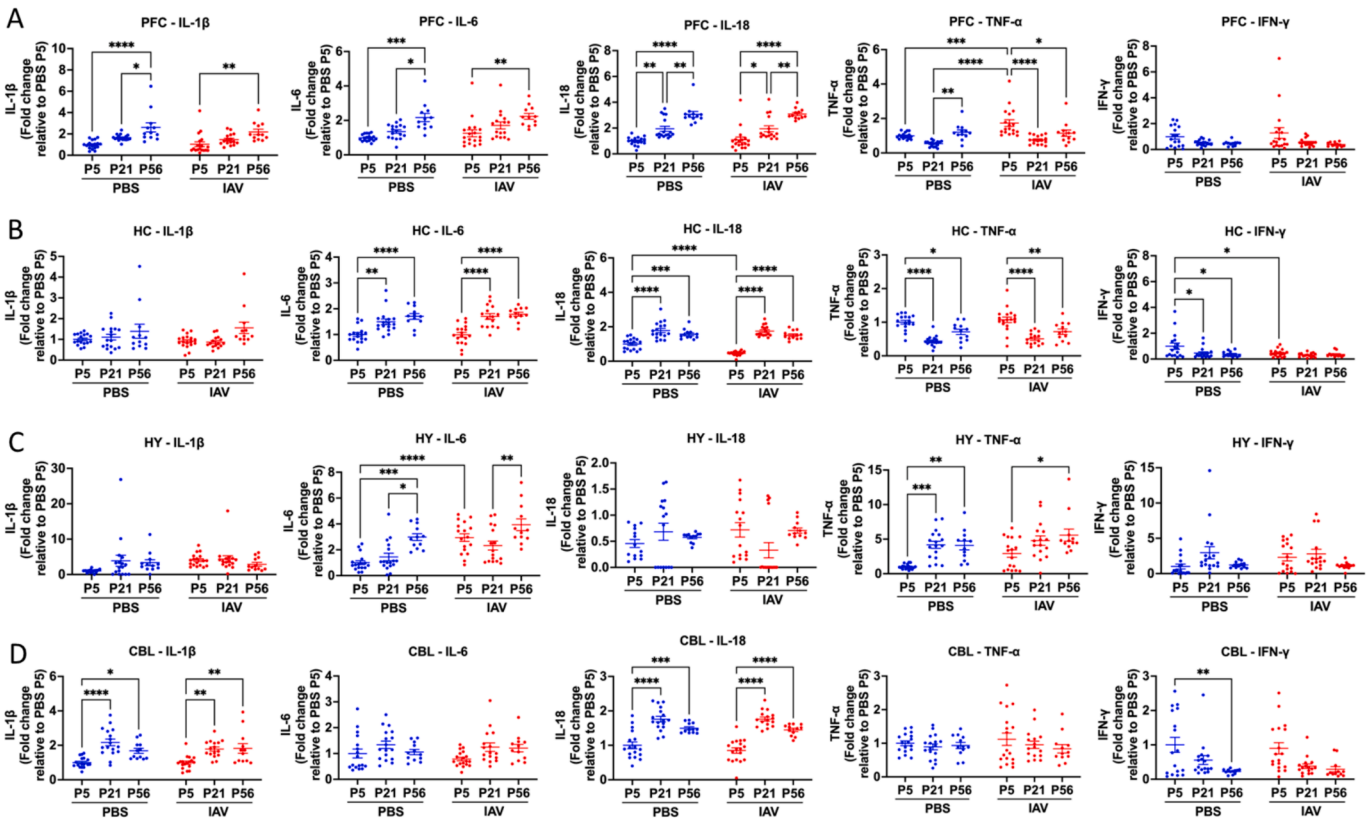

Fig. 3. Gene expressional changes of pro-inflammatory mediators in the brains of offspring born from IAV-infected dams. (A) Prefrontal cortex (PFC), (B) hippocampus (HC), (C) hypothalamus (HY), and (D) cerebellum (CBL) were dissected from offspring born from IAV-infected or PBS-mock-infected dams at postnatal days (P) 5, 21 or 56. Gene expression of the pro-inflammatory mediators IL-1β, IL-6, IL-18, TNF-α, IFN-γ, PTGS2 was assessed by qPCR. There was no interaction with sex, therefore male and female offspring were combined for 2-way ANOVA analysis with P5 PBS offspring as the reference group. Multiple comparisons between groups were performed using Tukey's post hoc tests. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

Given that inflammation is dependent on the balance between pro-inflammatory and anti-inflammatory mediators in the environment, we also assessed the ratio between pro- and anti-inflammatory cytokines, where a higher ratio indicates a more pro-inflammatory environment (Supplementary Fig. 4). The ratio profiles across development, in particular, IL-1β/IL-4, IL-6/IL-4 and TNF-α/IL-10 in the prefrontal cortex; TNF-α/IL-4, IL-1β/IL-10 and TNF-α/IL-10 in the hippocampus; IL-1β/IL-4, IL-6/IL-4, IL-6/IL-10 and TNF-α/IL-10 in the hypothalamus; and IL-1β/IL-4, IL-6/IL-4, IL-1β/IL-10, IL-6/IL-10 and TNF-α/IL-10 in the cerebellum were found to be different between PBS and IAV offspring. Overall, these changes in the ratio trajectories across development show an enhanced and sustained pro-inflammatory environment in IAV offspring brain, particularly across the adolescence period P21 and P56.

3.7. Maternal influenza infection enhances the expression of complement components in offspring brain during early postnatal and adolescent periods

A dysfunctional complement system has been shown to drive autoimmunity and inflammatory neurological disorders (Chen et al., 2010; Dalakas et al., 2020), as well as schizophrenia (Laskaris et al., 2019; Schizophrenia Working Group of the Psychiatric Genomics Consortium, 2014; Sekar et al., 2016). Thus, to assess whether maternal IAV infection alters offspring gene expression of components of the complement system, we measured the expression of C1qA, C3 and C4 of the complement system. Maternal IAV infection did not affect C1qA, C3 and C4 gene trajectories in the prefrontal cortex across development in the offspring (Fig. 5). In contrast, C3 and C4 expression in the hippocampus was significantly elevated in IAV neonatal (P5) and early adolescent (P21) offspring, respectively. In the hypothalamus, C1qA, C3 and C4 showed altered expression trajectories across development with upregulated C1qA and C4 expression in IAV neonates (P5). In the cerebellum, only C1qA showed altered gene trajectories between IAV and PBS offspring across development, demonstrating a significant peak in C1qA expression during early adolescence (P21).

3.8. Maternal influenza infection drives enhanced expression of microglial markers and altered microglial morphology in late adolescent offspring

Microglia, the brain's immune cells, play a central role in synaptic pruning and maturation which when perturbed in preclinical MIA models can lead to offspring neurogenic impairment and aberrant postnatal behaviours (Zhao et al., 2019). The complement system plays a central role in synaptic pruning by microglia (Gordon et al., 2003). Microglial markers TMEM119, AIF1 and CX3CR1 were measured to assess the effect of maternal IAV infection (Fig. 5). In comparison to PBS offspring, microglial markers TMEM119 and CX3CR1 in the prefrontal cortex, and TMEM119 and CX3CR1 in the cerebellum, were significantly elevated in late adolescent IAV offspring (P56). In the hypothalamus, TMEM119 was also significantly elevated during late adolescence (P56) when compared to neonates (P5). AIF1 expression in the hypothalamus was also significantly upregulated in IAV neonates (P5). Intriguingly, there were no expressional changes to microglial markers in the offspring hippocampus with maternal IAV.

To assess the long-term effects of maternal IAV infection on microglia numbers and morphology in the hippocampus of P56 offspring, immunofluorescent staining was performed against the ionized calcium-binding adaptor molecule-1 (Iba-1) (Supplementary Fig. 5A). IAV P56

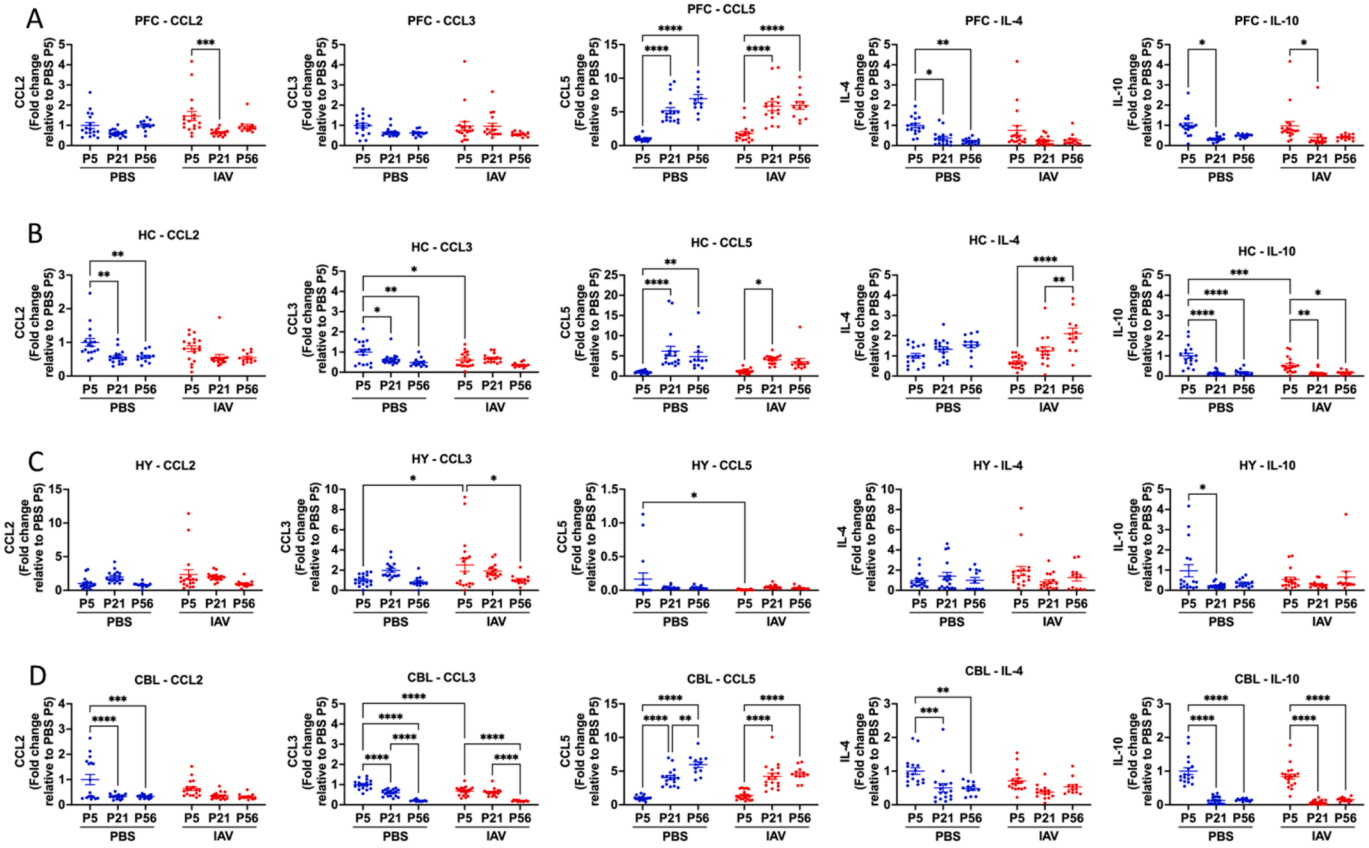

Fig. 4. Gene expression profiles of chemokines and anti-inflammatory cytokines in the offspring brain. (A) Prefrontal cortex (PFC), (B) hippocampus (HC), (C) hypothalamus (HY), and (D) cerebellum (CBL) were dissected from offspring born from IAV-infected or PBS-mock-infected dams at postnatal days (P) 5, 21 or 56. Gene expression of chemokines (CCL2, CCL3, CCL5) and the anti-inflammatory cytokines (IL-4, IL-10) was assessed by qPCR. There was no interaction with sex, therefore male and female offspring were combined for 2-way ANOVA analysis with P5 PBS offspring as the reference group. Multiple comparisons between groups were performed using Tukey's post hoc tests. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

offspring showed an increased number of Iba-1^{+} cells in the subgranular zone (SGZ) of the dentate gyrus with no changes in morphology (Supplementary Fig. 5B). In contrast, although the number of Iba-1^{+} cells in the CA1 region of the hippocampus of IAV P56 offspring was not increased, Iba-1^{+} cells in the IAV offspring displayed a more ramified morphology (i.e., reduced area per cell) compared to PBS offspring (Supplementary Fig. 5C).

3.9. The juvenile period in offspring brain development is most susceptible to neuroinflammation following maternal IAV infection

The juvenile period (P5) was found to be the most affected in terms of altered gene expression in the brain following maternal IAV infection. In particular, z-score analysis of the P5 hippocampus after prenatal IAV exposure identified 10 differentially expressed genes (IL-18, IFN-γ, CCL3, IL-4, IL-10, C1qA, C3, C4 AIF1, CX3CR1) compared to PBS offspring (Fig. 6). P5 hypothalamus had the next highest number of gene changes with IAV, with 6 differentially expressed genes (IL-6, IFN-γ, CCL3, CCL5, C1qA, C4). In contrast, the early adolescence period (P21) had the least overall change between IAV and PBS offspring, with P21 prefrontal cortex showing the most changes with 3 differentially expressed genes (TNF-a, C4, TMEM119). By late adolescence (P56), the cerebellum had the greatest number of changes following prenatal IAV exposure, with 4 differentially expressed genes (CCL5, C1qA, CX3CR1, TMEM119). P56 prefrontal cortex had the next highest number of genes (i.e., IFN-γ, C1qA and C3) being differentially expressed following IAV. Intriguingly, C4 expression in the hippocampus was the only gene that was consistently altered in the IAV offspring across all age groups.

4. Discussion

Gestational IAV infection can trigger not only maternal systemic inflammation but also a 'vascular storm' event in major blood vessels, consisting of vascular inflammation and dysfunction (Liong et al., 2020). This can lead to reduced blood flow to the placenta resulting in foetal hypoxia and growth restriction. These foetal complications are known risk factors for developing neurodevelopmental disorders (Wang et al., 2021), including schizophrenia. In this study, a moderate IAV infection during pregnancy produced adverse systemic outcomes (reduced maternal body weight gain, lung inflammation and vascular dysfunction), however, the foetal effects were more subtle compared to a high dose of IAV, characterised as no change in pup and placental weights at E18, and increased pup survival. Although we did not record birth weights (P0) in this study in order to minimise disturbance of litters soon after delivery, the similarities in E18 fetal weights between PBS and IAV groups would suggest that the neurodevelopmental effects are not likely to be attributed to low birth weight. The offspring from IAV infected dams also displayed no differences in body weights across development as well as no differences in whole brain weights by late adolescence (P56). Despite the lack of physical differences in the offspring exposed to moderate IAV infection across development, biochemical assessment of the developing offspring brain revealed altered trajectories in expression of genes involved in neuroinflammation and neurogenesis in specific regions of the brain. The offspring from IAV infected dams also had reduced hippocampus size and displayed disrupted behaviour in adulthood, including sensorimotor gating deficits, dopaminergic hypersensitivity, and deficits in spatial memory, all of which are commonly used as surrogates for modelling specific symptom domains associated with

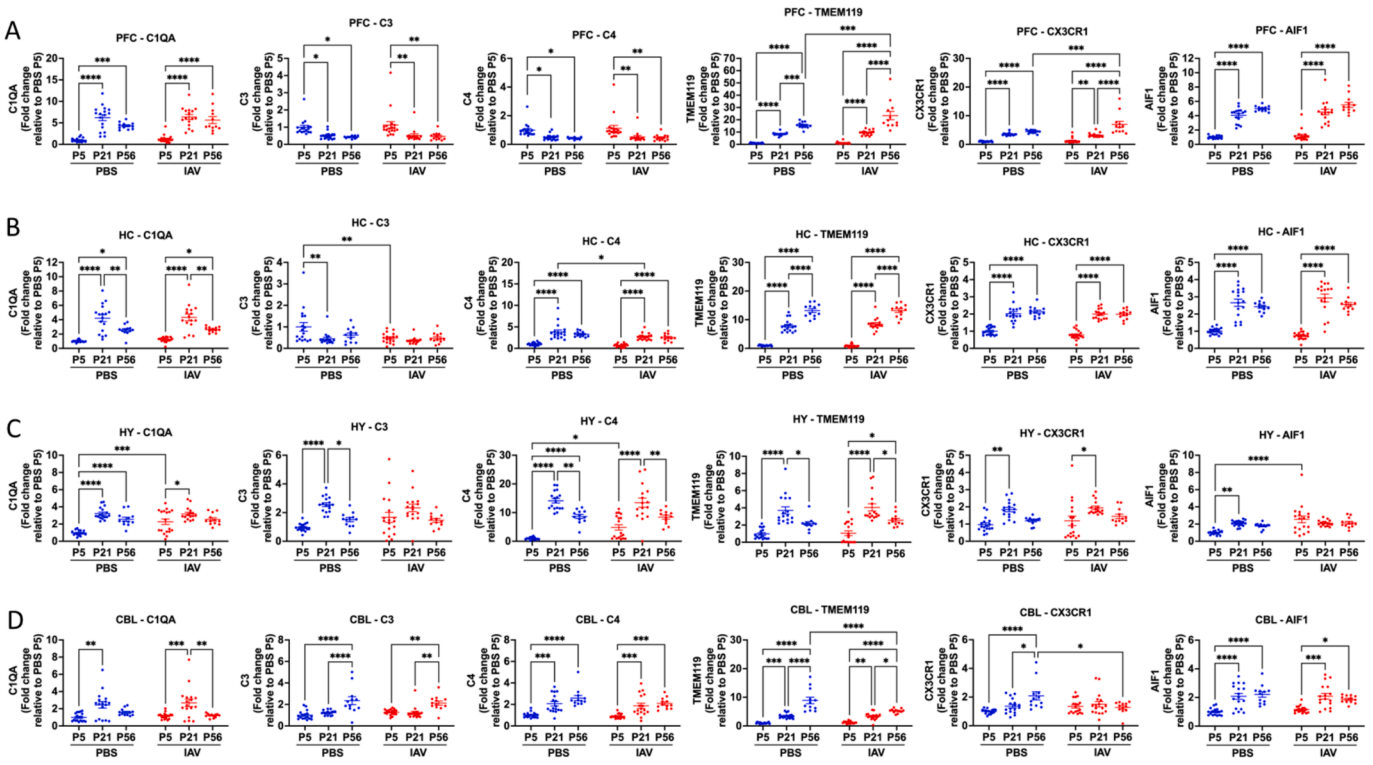

Fig. 5. Gene expression of complement system components and microglial markers in the offspring brain in response to prenatal IAV exposure. (A) Prefrontal cortex (PFC), (B) hippocampus (HC), (C) hypothalamus (HY), and (D) cerebellum (CBL) were dissected from offspring born from IAV-infected or PBS-mock-infected dams at postnatal days (P) 5, 21 or 56. Gene expression of the complement system components (C1qA, C3, C4) and microglial markers (TMEM119, CX3CR1, AIF1) was assessed by qPCR. There was no interaction with sex, therefore male and female offspring were combined for 2-way ANOVA analysis with P5 PBS offspring as the reference group. Multiple comparisons between groups were performed using Tukey's post hoc tests. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.

schizophrenia. These behavioural findings are to some degree consistent with a previous study that used live IAV (Shi et al., 2003). That study by Shi and colleagues reported PPI deficits in 6–8-week-old (P42-P56) offspring exposed to maternal IAV (Shi et al., 2003). However, we have recently shown that this age is not sufficiently mature developmentally to accurately represent a fully functional gating mechanism, as measured by PPI in healthy mice (Choy et al., 2021). Therefore, our current study is the first to accurately demonstrate PPI deficits in the mature adult brain (P84; 12 weeks old) in offspring born to IAV-infected dams.

The inflammatory processes in the developing brain are tightly regulated during critical developmental periods in early life and adolescence. In our model of moderate IAV infection, we did not observe overt increases in pro-inflammatory cytokines, but did note a significant reduction in anti-inflammatory cytokines IL-4 and IL-10, resulting in a skewed pro-neuroinflammatory phenotype in the IAV offspring. Assessment of cytokine gene expression across development also identified IAV offspring with altered expression of IFN-γ (cerebellum, hippocampus), TNF-α (prefrontal cortex) and IL-6 (hypothalamus) compared to uninfected offspring. Collectively, our findings indicate that a moderate IAV infection in pregnancy is associated with altered trajectories across development. Specifically, the trajectories across development for IL-1β, IL-6, TNF-α, CCL2, IL-4 in the prefrontal cortex; IL-18, IFN-γ, CCL2, CCL3, CCL5, IL-4, IL-10 in the hippocampus; IL-6, TNF-α, CCL3, CCL5, IL-10 in the hypothalamus; and IFN-γ, CCL2, CCL3, CCL5, IL-4 in the cerebellum were different between IAV and PBS offspring. Z-score analysis identified P5 offspring to be most susceptible to dysregulated cytokine and chemokine expression compared to P21 and P56 offspring following maternal IAV infection. As such, a cross-sectional or longitudinal analysis is better suited to detect subtle and long-term changes in the brain following moderate IAV infection that may otherwise be missed if looking at a single time point alone, particularly if we examined only the adolescent period when behavioural changes emerge.

Our study demonstrates that IAV infection during pregnancy alters the trajectories of inflammatory gene expression across development. Of note was the observation that IL-18 expression was reduced in the hippocampus of offspring from IAV infected dams. IL-18 is indispensable for sustained clearance of degenerative neural cells, neuronal maturation, and hippocampal function (Yamanishi et al., 2019). Mice deficient in IL-18 develop impaired learning, memory and motivation (Yamanishi et al., 2019). Given that gene transcription encodes the IL-18 precursor molecule and that cleavage by caspase-1 allows for the secretion of the biologically active mature form of IL-18, more studies are warranted to examine whether the reduction in IL-18 in the hippocampus of IAV offspring drives some of the neurobehavioral changes observed in these mice.

Although inflammation is frequently thought of as a deleterious process to healthy neurological function and is a hallmark of neurodegenerative diseases, activation of the inflammatory complement cascade is essential during postnatal development to eliminate inappropriate synapses in the CNS (synaptic pruning) occurring *via* microglia phagocytosis (Hua and Smith, 2004). Mice deficient in complement proteins C1q or the downstream complement C3 show impaired synaptic pruning activity (Schafer et al., 2012; Stevens et al., 2007). C3-deficient mice also display impaired neuronal migration resulting in disorganised cortical layers and abnormal positioning of neurons in the developing cortex (Gorelik et al., 2017). C4 complement protein sits upstream of C3 and is involved in the propagation of C3 activity. In the CNS, C1q is largely expressed by microglia, C3 is predominately synthesised by astrocytes and C4 is produced by oligodendrocytes in mice (Zhou et al., 2008). In the developing cortex of mice, C3 knockdown resulted in

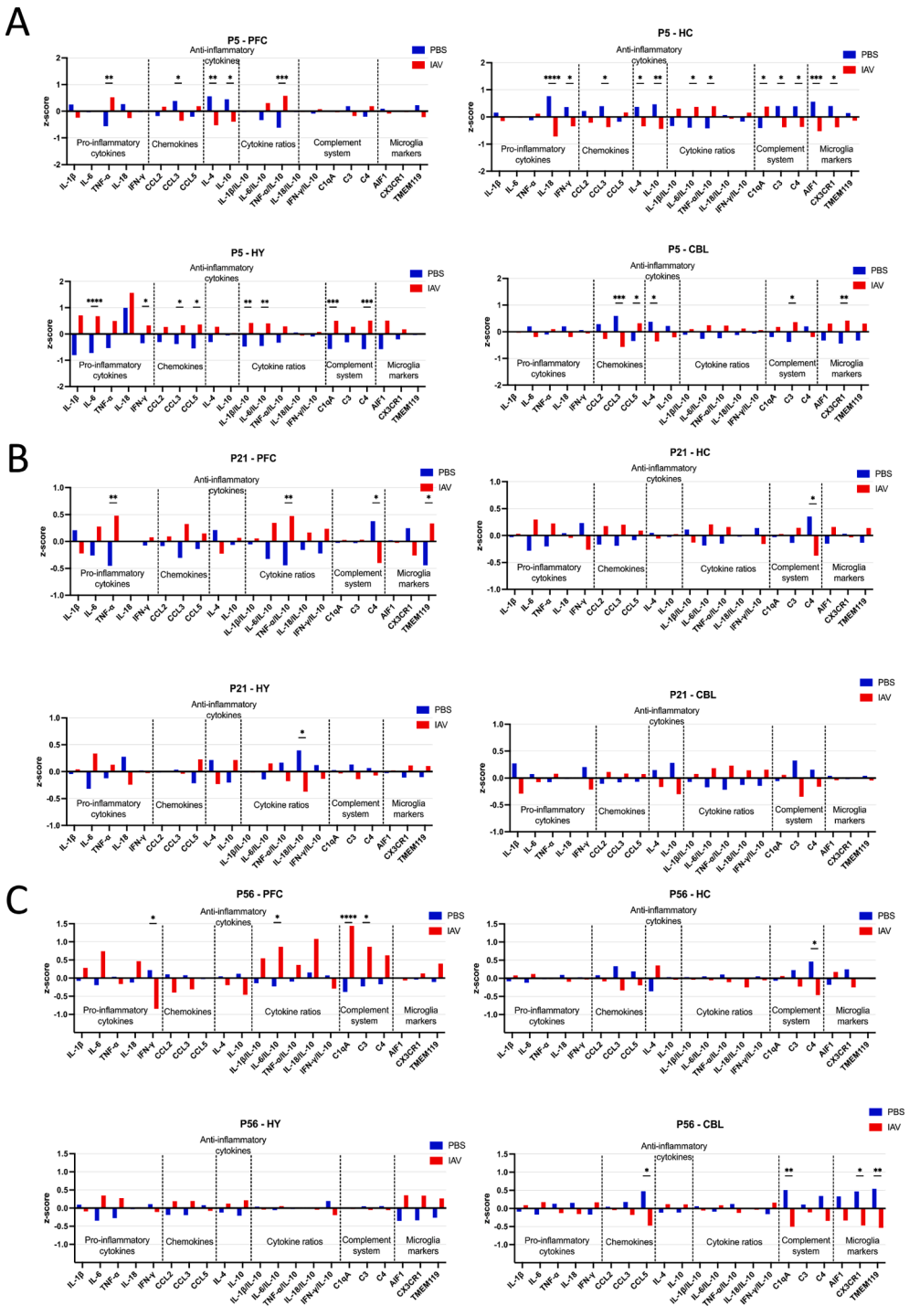

Fig. 6. Analysis of the association between brain regions and differential gene expression across development in the offspring. Mean z-scores of gene expression from the prefrontal cortex (PFC), hippocampus (HC), hypothalamus (HY), and cerebellum (CBL) in the offspring across different developmental ages. Student's *t* test was performed for comparisons between PBS vs IAV offspring. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

defective neuronal migration (Gorelik et al., 2017). C3 proteolysis was found to be central for neuronal migration with administration of molecular mimics of C3 cleavage products rescuing impaired migration (Gorelik et al., 2017). Others have hypothesised that neuronal migration deficits may be a common factor to the pathophysiology of autism spectrum disorder (ASD) (Reiner et al., 2016), while abnormalities in synaptic pruning are proposed as relevant to brain changes in schizophrenia (Feinberg, 1982). In this study, hippocampal expression of C3 is reduced in IAV offspring at P5 (brain development comparable to 33–36 weeks of gestation, a preterm infant) and P21 (comparable to 2–3-year-old child), which may be indicative of reduced synaptic pruning activity triggered by disrupted astrocyte and oligodendrocyte function during early life. C4 is encoded by 2 genes, C4A and C4B, which vary in expression levels and copy numbers. C4A expression is altered in brain samples of schizophrenic patients compared to healthy individuals (Sekar et al., 2016). In this study, the C4 primer used detects both C4A and C4B transcripts which reported an overall reduction in total C4 expression in IAV offspring at P5 and P21. It is unknown whether C4A or C4B are differentially expressed in the IAV offspring and thus warrants further investigation. Another limitation of this study was that all tissues

were processed for qPCR analysis, thus not allowing additional confirmation of protein expression changes using samples from the current cohorts. Additional protein expression effects will be investigated in future studies.

Microglia are specialised immune cells that reside in the CNS and are acutely sensitive to physiological stress with critical roles in neurological disease. In normal development, microglial morphology and properties change in a region-specific manner from an "amoeboid" to "ramified" state during the first 3 weeks of postnatal life (Perez-Pouchoulen et al., 2015). It is thought that microglial maturation beginning at 2–3 postnatal weeks (P14-21) coincides with changes to gene expression profiles in response to a large exposure to environmental stressors or stimuli that is absent during foetal development *in utero*. CX3CR1 is a microglia-specific chemokine receptor that sensors the chemokine fractalkine CX3CL1 expressed by neurons and is essential for microglial migration and regulating synaptic pruning from 2 weeks of postnatal life (P14) (Hoshiko et al., 2012). In this study, P5 IAV offspring show downregulation of CX3CR1 expression in the hippocampus compared to PBS offspring (z-scores). Loss of CX3CR1 in mice is associated with delayed functional maturation of postsynaptic glutamate receptors which occurs between 1–2 postnatal weeks of life (Hoshiko et al., 2012). Moreover, CX3CR1 deficient mice during postnatal development show a transient deficit in synaptic pruning in CA1 pyramidal neurons, showing more synapses than WT mice, which peaks at P15 and is recovered by P28 (Paolicelli et al., 2011), implicating the importance of CX3CR1 in hippocampal development during early postnatal life. In contrast, CX3CR1 deficiency in cerebral ischemia mouse models, was shown to be protective against ischemic damage, inflammation and smaller infarcts compared to WT mice (Denes et al., 2008). Given that our IAV infection model in pregnancy is linked to impaired maternal blood flow, the reduction in CX3CR1 in P5 IAV offspring could be a lingering response to protect against foetal cerebral ischemia *in utero*.

In mouse brain development, neurogenesis in the CA1 and CA3 regions occur rapidly during the embryonic period (E12.5–17.5) and is completed by birth. In contrast, neurogenesis in the dentate gyrus is a gradual and protracted process that begins at E12.5 and decreases by P14 and continues at low levels into adulthood (Bond et al., 2020). In adult hippocampus, the sub-granular zone (SGZ) is the site of neurogenesis. These newly generated cells then differentiate into mature neurons in the granular cell layer of the dentate gyrus where they are integrated into the hippocampal functional circuitry (van Praag et al., 2002). In rodents, approximately 50 % of these newly generated neurons survive to become functional neurons in the hippocampal circuitry (Cameron and McKay, 2001). Apoptotic newborn neurons are rapidly cleared by phagocytic unchallenged (ramified) microglia residing in the SGZ (Sierra et al., 2010). Furthermore, unlike the number of new neurons generated and apoptotic cells which decrease with age, the phagocytic activity of SGZ residing microglia is undeterred by increasing age or inflammatory stimuli (Sierra et al., 2010). Thus, increased number of microglial cells in the SGZ because of prenatal exposure to IAV infection may affect homeostatic clearance of newborn neurons and optimal integration of mature neurons into the hippocampal circuitry. Alternatively, an increase in microglial cells in the SGZ of late adolescent IAV offspring could be a remnant of increased apoptosis of newborn neurons during early life. In this study, we have identified increased number of microglia in the SGZ of the dentate gyrus, and an increased proportion of resting microglia (ramified morphology) in the CA1 region of the hippocampus in IAV late adolescent (P56) offspring. Coupled with reduced hippocampal expression of complement C3 and C4, this may result in an overall reduction in synaptic pruning leading to abnormal neuronal circuitry in the CA1 and deficits in hippocampal-dependent memory processes of IAV offspring. Collectively, these findings show that maternal IAV infection impacts offspring microglia numbers and function into late adolescence. Functional studies on the cortical microglia are required to confirm this observation and to delineate its impact on future disease processes and injury in the adult CNS.

In conclusion, we have established an IAV infection model in pregnant mice that did not cause significant offspring death during the early postnatal period with offspring survival being comparable to uninfected litters, nor did it affect overall brain weights or bodyweight trajectories of the surviving offspring. However, this model demonstrated a significant reduction in hippocampal size, which is important given the role of the hippocampus in schizophrenia (Velakoulis et al., 2016). In addition, this model has allowed us to assess acute (P5) and chronic (P21, P56) alterations in the normal trajectories of genes that regulate neuroinflammation that are regional-specific and reflect the behavioural deficits in IAV offspring. Therefore, our study highlights that even moderate gestational IAV infections, mimicking seasonal influenza disease severity, can result in brain-region specific alterations that can heighten the risk of offspring neuropsychiatric disorders. Our study also highlights an important translational pre-clinical model for future psychiatric drug discovery research, which will facilitate the development of novel pharmacotherapies to restore aberrant neurodevelopmental trajectories initiated by gestational viral insults that underpin disorders such as schizophrenia.

Funding

This work was supported by a National Health and Medical Research Council of Australia (NHMRC) Ideas grant to SS and SL (2002948) and Program Grant to AC and CP (1150083). CP was supported by a NHMRC L3 Investigator Grant (1196508). JN was supported by a One in Five McIver Research Fellowship and NHMRC Project Grant (1163504). CV was supported by an Australian Research Council Discovery Project (DP190102950) and a NHMRC Ideas Grant (2020289).

CRediT authorship contribution statement

Stella Liong: Writing – review & editing, Writing – original draft, Supervision, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **K.H. Christopher Choy:** . **Simone N. De Luca:** Writing – review & editing, Investigation, Formal analysis. **Felicia Liong:** Writing – original draft, Methodology, Investigation. **Madison Coward-Smith:** Writing – review & editing, Investigation, Formal analysis. **Osezua Oseghale:** Writing – review & editing, Investigation. **Mark A. Miles:** . **Ross Vlahos:** Writing – review & editing, Supervision. **Celine Valant:** Writing – review & editing, Supervision, Funding acquisition. **Jess Nithianantharajah:** . **Christos Pantelis:** . **Arthur Christopoulos:** Writing – review & editing, Writing – original draft, Supervision, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Stavros Selemidis:** Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbi.2024.06.025.

References

Antonson, A.M., Kenney, A.D., Chen, H.J., Corps, K.N., Yount, J.S., Gur, T.L., 2021. Moderately pathogenic maternal influenza A virus infection disrupts placental integrity but spares the fetal brain. Brain Behav. Immun. 96, 28–39.

Bond, A.M., Berg, D.A., Lee, S., Garcia-Epelboim, A.S., Adusumilli, V.S., Ming, G.L., et al., 2020. Differential Timing and Coordination of Neurogenesis and Astrogenesis in Developing Mouse Hippocampal Subregions. Brain Sci. 10 (12).

Brown, A.S., Meyer, U., 2018. Maternal Immune Activation and Neuropsychiatric Illness: A Translational Research Perspective. Am. J. Psychiatry 175 (11), 1073–1083.

Brown, A.S., Begg, M.D., Gravenstein, S., Schaefer, C.A., Wyatt, R.J., Bresnahan, M., et al., 2004. Serologic evidence of prenatal influenza in the etiology of schizophrenia. Arch. Gen. Psychiatry 61 (8), 774–780.

Brown, A.S., Schaefer, C.A., Quesenberry Jr., C.P., Shen, L., Susser, E.S., 2006. No evidence of relation between maternal exposure to herpes simplex virus type 2 and risk of schizophrenia? Am. J. Psychiatry 163 (12), 2178–2180.

Cameron, H.A., McKay, R.D., 2001. Adult neurogenesis produces a large pool of new granule cells in the dentate gyrus. J Comp Neurol 435 (4), 406–417.

Chen, M., Daha, M.R., Kallenberg, C.G., 2010. The complement system in systemic autoimmune disease. J. Autoimmun. 34 (3), J276–J286.

Choy, K.H.C., Luo, J.K., Wannan, C.M.J., Laskaris, L., Merritt, A., Syeda, W.T., et al., 2021. Cognitive behavioral markers of neurodevelopmental trajectories in rodents. Transl. Psychiatry 11 (1), 556.

Dalakas, M.C., Alexopoulos, H., Spaeth, P.J., 2020. Complement in neurological disorders and emerging complement-targeted therapeutics. Nat. Rev. Neurol. 16 (11), 601–617.

Dawood, F.S., Kittikraisak, W., Patel, A., Rentz Hunt, D., Suntarattiwong, P., Wesley, M. G., et al., 2021. Incidence of influenza during pregnancy and association with pregnancy and perinatal outcomes in three middle-income countries: a multisite prospective longitudinal cohort study. Lancet Infect. Dis. 21 (1), 97–106.

De Luca, S.N., Brassington, K., Chan, S.M.H., Dobric, A., Mou, K., Seow, H.J., et al., 2022. Ebselen prevents cigarette smoke-induced cognitive dysfunction in mice by preserving hippocampal synaptophysin expression. J. Neuroinflammation 19 (1), 72.

Denes, A., Ferenczi, S., Halasz, J., Kornyei, Z., Kovacs, K.J., 2008. Role of CX3CR1 (fractalkine receptor) in brain damage and inflammation induced by focal cerebral ischemia in mouse. J. Cereb. Blood Flow Metab. 28 (10), 1707–1721.

Doyle, T.J., Goodin, K., Hamilton, J.J., 2013. Maternal and neonatal outcomes among pregnant women with 2009 pandemic influenza A(H1N1) illness in Florida, 2009–2010: a population-based cohort study. PLoS One 8 (10), e79040.

Fatemi, S.H., Sidwell, R., Kist, D., Akhter, P., Meltzer, H.Y., Bailey, K., et al., 1998. Differential expression of synaptosome-associated protein 25 kDa [SNAP-25] in hippocampi of neonatal mice following exposure to human influenza virus in utero. Brain Res. 800 (1), 1–9.

Fatemi, S.H., Emamian, E.S., Kist, D., Sidwell, R.W., Nakajima, K., Akhter, P., et al., 1999. Defective corticogenesis and reduction in Reelin immunoreactivity in cortex and hippocampus of prenatally infected neonatal mice. Mol. Psychiatry 4 (2), 145–154.

Fatemi, S.H., Emamian, E.S., Sidwell, R.W., Kist, D.A., Stary, J.M., Earle, J.A., et al., 2002. Human influenza viral infection in utero alters glial fibrillary acidic protein immunoreactivity in the developing brains of neonatal mice. Mol. Psychiatry 7 (6), 633–640.

Fatemi, S.H., Earle, J., Kanodia, R., Kist, D., Emamian, E.S., Patterson, P.H., et al., 2002. Prenatal viral infection leads to pyramidal cell atrophy and macrocephaly in adulthood: implications for genesis of autism and schizophrenia. Cell Mol Neurobiol. 22 (1), 25–33.

Feinberg, I., 1982. Schizophrenia: caused by a fault in programmed synpatic eliminiation during adolescence? Psychiatr. Res. 17 (14), 319–334.

Gordon, G.J., Jensen, R.V., Hsiao, L.L., Gullans, S.R., Blumenstock, J.E., Richards, W.G., et al., 2003. Using gene expression ratios to predict outcome among patients with mesothelioma. J. Natl Cancer Inst. 95 (8), 598–605.

Gorelik, A., Sapir, T., Haffner-Krausz, R., Olender, T., Woodruff, T.M., Reiner, O., 2017. Developmental activities of the complement pathway in migrating neurons. Nat. Commun. 8, 15096.

Hoshiko, M., Arnoux, I., Avignone, E., Yamamoto, N., Audinat, E., 2012. Deficiency of the microglial receptor CX3CR1 impairs postnatal functional development of thalamocortical synapses in the barrel cortex. J. Neurosci. 32 (43), 15106–15111.

Hua, J.Y., Smith, S.J., 2004. Neural activity and the dynamics of central nervous system development. Nat. Neurosci. 7 (4), 327–332.

Kanmaz, H.G., Erdeve, O., Ogz, S.S., Uras, N., Celen, S., Korukluoglu, G., et al., 2011. Placental transmission of novel pandemic influenza a virus. Fetal Pediatr. Pathol. 30 (5), 280–285.

Kentner, A.C., Bilbo, S.D., Brown, A.S., Hsiao, E.Y., McAllister, A.K., Meyer, U., et al., 2019. Maternal immune activation: reporting guidelines to improve the rigor, reproducibility, and transparency of the model. Neuropsychopharmacology 44 (2), 245–258.

Khandaker, G.M., Zimbron, J., Lewis, G., Jones, P.B., 2013. Prenatal maternal infection, neurodevelopment and adult schizophrenia: a systematic review of population-based studies. Psychol. Med. 43 (2), 239–257.

Kwon, J., Suessmilch, M., McColl, A., Cavanagh, J., Morris, B.J., 2021. Distinct trans-placental effects of maternal immune activation by TLR3 and TLR7 agonists: implications for schizophrenia risk. Sci. Rep. 11 (1), 23841.

Laskaris, L., Zalesky, A., Weickert, C.S., Di Biase, M.A., Chana, G., Baune, B.T., et al., 2019. Investigation of peripheral complement factors across stages of psychosis. Schizophr. Res. 204, 30–37.

Laviola, G., Macri, S., Morley-Fletcher, S., Adriani, W., 2003. Risk-taking behavior in adolescent mice: psychobiological determinants and early epigenetic influence. Neurosci. Biobehav. Rev. 27 (1–2), 19–31.

Le Goffic, R., Pothlichet, J., Vitour, D., Fujita, T., Meurs, E., Chignard, M., et al., 2007. Cutting Edge: Influenza A virus activates TLR3-dependent inflammatory and RIG-I-dependent antiviral responses in human lung epithelial cells. J. Immunol. 178 (6), 3368–3372.

Li, M., Liu, Z., Lin, Y., Chen, X., Li, S., You, F., et al., 2014. Maternal influenza-like illness, medication use during pregnancy and risk of congenital heart defects in offspring. J. Matern. Fetal Neonatal Med. 27 (8), 807–811.

Liong, S., Oseghale, O., To, E.E., Brassington, K., Erlich, J.R., Luong, R., et al., 2020. Influenza A virus causes maternal and fetal pathology via innate and adaptive vascular inflammation in mice. PNAS 117 (40), 24964–24973.

Mazumder, B., Almond, D., Park, K., Crimmins, E.M., Finch, C.E., 2010. Lingering prenatal effects of the 1918 influenza pandemic on cardiovascular disease. J. Dev. Orig. Health Dis. 1 (1), 26–34.

Meyer, U., Feldon, J., Schedlowski, M., Yee, B.K., 2006. Immunological stress at the maternal-foetal interface: a link between neurodevelopment and adult psychopathology. Brain Behav. Immun. 20 (4), 378–388.

Meyer, U., Feldon, J., Fatemi, S.H., 2009. In-vivo rodent models for the experimental investigation of prenatal immune activation effects in neurodevelopmental brain disorders. Neurosci. Biobehav. Rev. 33 (7), 1061–1079.

Missig, G., Robbins, J.O., Mokler, E.L., McCullough, K.M., Bilbo, S.D., McDougle, C.J., et al., 2020. Sex-dependent neurobiological features of prenatal immune activation via TLR7. Mol. Psychiatry 25 (10), 2330–2341.

Mueller, F.S., Polesel, M., Richetto, J., Meyer, U., Weber-Stadlbauer, U., 2018. Mouse models of maternal immune activation: Mind your caging system! Brain Behav. Immun. 73, 643–660.

Paolicelli, R.C., Bolasco, G., Pagani, F., Maggi, L., Scianni, M., Panzanelli, P., et al., 2011. Synaptic pruning by microglia is necessary for normal brain development. Science 333 (6048), 1456–1458.

Perez-Pouchoulen, M., VanRyzin, J.W., McCarthy, M.M., 2015. Morphological and Phagocytic Profile of Microglia in the Developing Rat Cerebellum. eNeuro. 2 (4).

Reiner, O., Karzbrun, E., Kshirsagar, A., Kaibuchi, K., 2016. Regulation of neuronal migration, an emerging topic in autism spectrum disorders. J. Neurochem. 136 (3), 440–456.

Reisinger, S., Khan, D., Kong, E., Berger, A., Pollak, A., Pollak, D.D., 2015. The poly(I:C)-induced maternal immune activation model in preclinical neuropsychiatric drug discovery. Pharmacol. Ther. 149, 213–226.

Schafer, D.P., Lehrman, E.K., Kautzman, A.G., Koyama, R., Mardinly, A.R., Yamasaki, R., et al., 2012. Microglia sculpt postnatal neural circuits in an activity and complement-dependent manner. Neuron 74 (4), 691–705.

Schizophrenia Working Group of the Psychiatric Genomics Consortium, 2014. Biological insights from 108 schizophrenia-associated genetic. loci. Nature 511 (7510), 421–427.

Schmittgen, T.D., Livak, K.J., 2008. Analyzing real-time PCR data by the comparative C (T) method. Nat. Protoc. 3 (6), 1101–1108.

Sekar, A., Bialas, A.R., de Rivera, H., Davis, A., Hammond, T.R., Kamitaki, N., et al., 2016. Schizophrenia risk from complex variation of complement component 4. Nature 530 (7589), 177–183.

Shi, L., Fatemi, S.H., Sidwell, R.W., Patterson, P.H., 2003. Maternal influenza infection causes marked behavioral and pharmacological changes in the offspring. J. Neurosci. 23 (1), 297–302.

Shi, L., Tu, N., Patterson, P.H., 2005. Maternal influenza infection is likely to alter fetal brain development indirectly: the virus is not detected in the fetus. Int. J. Dev. Neurosci. 23 (2–3), 299–305.

Sierra, A., Encinas, J.M., Deudero, J.J., Chancey, J.H., Enikolopov, G., Overstreet-Wadiche, L.S., et al., 2010. Microglia shape adult hippocampal neurogenesis through apoptosis-coupled phagocytosis. Cell Stem Cell 7 (4), 483–495.

Stevens, B., Allen, N.J., Vazquez, L.E., Howell, G.R., Christopherson, K.S., Nouri, N., et al., 2007. The classical complement cascade mediates CNS synapse elimination. Cell 131 (6), 1164–1178.

Suryavanshi, P.S., Ugale, R.R., Yilmazer-Hanke, D., Stairs, D.J., Dravid, S.M., 2014. GluN2C/GluN2D subunit-selective NMDA receptor potentiator CIQ reverses MK-801-induced impairment in prepulse inhibition and working memory in Y-maze test in mice. Br. J. Pharmacol. 171 (3), 799–809.

van Praag, H., Schinder, A.F., Christie, B.R., Toni, N., Palmer, T.D., Gage, F.H., 2002. Functional neurogenesis in the adult hippocampus. Nature 415 (6875), 1030–1034.

Velakoulis, D., Wood, S.J., Wong, M.T., McGorry, P.D., Yung, A., Phillips, L., Smith, D., Brewer, W., Proffitt, T., Desmond, P., Pantelis, C., 2016. Hippocampal and amygdala volumes according to psychosis stage and diagnosis: a magenetic resonance imaging study of chronic schizophrenia, first-episode psychosis, and ultra-high-risk individuals. Arch. Gen. Psychiatry 63 (2), 139–149.

Wang, B., Zeng, H., Liu, J., Sun, M., 2021. Effects of Prenatal Hypoxia on Nervous System Development and Related Diseases. Front. Neurosci. 15, 755554.

Yamanishi, K., Doe, N., Mukai, K., Ikubo, K., Hashimoto, T., Uwa, N., et al., 2019. Interleukin-18-deficient mice develop hippocampal abnormalities related to possible depressive-like behaviors. Neuroscience 408, 147–160.

Zhao, Q., Wang, Q., Wang, J., Tang, M., Huang, S., Peng, K., et al., 2019. Maternal immune activation-induced PPARgamma-dependent dysfunction of microglia associated with neurogenic impairment and aberrant postnatal behaviors in offspring. Neurobiol Dis. 125, 1–13.

Zhou, J., Fonseca, M.I., Pisalyaput, K., Tenner, A.J., 2008. Complement C3 and C4 expression in C1q sufficient and deficient mouse models of Alzheimer's disease. J. Neurochem. 106 (5), 2080–2092.