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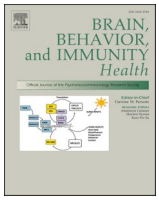

Maternal choline supplementation modulates cognition and induces anti-inflammatory signaling in the prefrontal cortices of adolescent rats exposed to maternal immune activation

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ABSTRACT

Maternal infection has long been described as a risk factor for neurodevelopmental disorders, especially autism spectrum disorders (ASD) and schizophrenia. Although many pathogens do not cross the placenta and infect the developing fetus directly, the maternal immune response to them is sufficient to alter fetal neurodevelopment, a phenomenon termed maternal immune activation (MIA). Low maternal choline is also a risk factor for neurodevelopmental disorders, and most pregnant people do not receive enough of it. In addition to its role in neurodevelopment, choline is capable of inducing anti-inflammatory signaling through a nicotinic pathway. Therefore, it was hypothesized that maternal choline supplementation would blunt the neurodevelopmental impact of MIA in offspring through long-term instigation of cholinergic anti-inflammatory signaling.

To model MIA in rats, the viral mimetic polyinosinic:polycytidylic acid (poly(I:C)) was used to elicit a maternal antiviral innate immune response in dams both with and without choline supplementation. Offspring were reared to both early and late adolescent stages (postnatal days 28 and 50, respectively), where anxiety-related behaviors and cognition were examined. After behavioral testing, animals were euthanized, and their prefrontal cortices (PFCs) were collected for analysis. MIA offspring demonstrated sex-specific patterns of altered cognition and repetitive behaviors, which were modulated by maternal choline supplementation. Choline supplementation also bolstered anti-inflammatory signaling in the PFCs of MIA animals at both early and late adolescent stages. These findings suggest that maternal choline supplementation may be sufficient to blunt some of the behavioral and neurobiological impacts of inflammatory exposures *in utero*, indicating that it may be a cheap, safe, and effective intervention for neurodevelopmental disorders.

1. Introduction

Maternal immune activation (MIA) by infectious diseases has been consistently linked to increased risk of autism spectrum disorders (ASD) (Atladóttir et al., 2010, 2012; Lee et al., 2015) and schizophrenia (Barr et al., 1990; Brown and Derkits, 2010) in offspring, and evidence suggests that it may also be linked to attention deficit hyperactivity disorder (ADHD) (Pineda et al., 2007; Silva et al., 2014), bipolar disorder (BD) (Canetta et al., 2014; Parboosing et al., 2013), and depression (Al-Haddad et al., 2019). In light of emerging infectious diseases like Zika and COVID-19, the identification of cost-effective, safe, and simple interventions to reduce the impact of MIA on the developing fetus is critical. Choline is an essential micronutrient that is actively transported across the placenta in large quantities to facilitate neural tube formation, hippocampal development, and epigenetic regulation (Zeisel, 2006a). Maternal choline levels are also predictive of levels of choline and its metabolites in offspring tissues (Howard et al., 2024; Yan et al., 2014). Despite its necessity and the fact that it can be synthesized endogenously, the overwhelming majority of pregnant and lactating females do not achieve the recommended levels of choline (Derbyshire and Obeid, 2020; Yan et al., 2012). Choline specifically plays a key role in neural tube formation, hippocampal development, and epigenetic regulation (Zeisel and Da Costa, 2009). Choline also exerts unique anti-inflammatory effects through the alpha-7 nicotinic acetylcholine receptor (α7nAChR), which activates PI3K-PKB/Akt cell-survival signaling, leading to the downstream expression of cell survival genes and antioxidant enzymes like heme oxygenase 1 (HO-1) (Alkondon et al., 1997; Egea et al., 2015; Youssef et al., 2020).

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1.1. The Poly(I:C) model

To model maternal infection in rodents, the synthetic double-stranded RNA (dsRNA) analog polyinosinic-polycytidylic acid (Poly(I:C)) is used to elicit an antiviral innate immune response in the dam. Poly (I:C) binds Toll-Like Receptor 3 (TLR3), a pattern recognition receptor that recognizes dsRNA, on the endosomal membrane (Reisinger et al., 2015). Once activated, TLR3 induces an intracellular cascade that eventually leads to the production of antiviral interferons and pro-inflammatory cytokines like IL-6 (Reisinger et al., 2015; Vercammen et al., 2008). IL-6 is capable of transfer across the placenta, and it has been identified as a key mediator of MIA (Ratnayake et al., 2013; Zaretsky et al., 2004). Indeed, MIA experiments using poly(I:C) with either an anti-IL-6 antibody or an IL-6 knockout mouse strain did not find the same behavioral abnormalities and transcriptional changes seen in offspring without IL-6 blocked (Smith et al., 2007). IL-6 acts directly on the developing brain in both protection and pathology and plays key roles in the differentiation of neurons and glial cells in both the central and peripheral nervous systems (Islam et al., 2009; Kummer et al., 2021; Reeh et al., 2019; Wong and Hoeffer, 2018). IL-6 also stimulates the differentiation of th17 cells and downstream release of IL-17A, which is also considered a core driver of MIA-induced pathologies (Choi et al., 2016).

1.2. Neurodevelopmental disorders and cognitive deficits

Neurodevelopmental disorders and intellectual disabilities are often characterized by dysfunction in the prefrontal cortex (PFC). The PFC is critical for higher-order cognition, information synthesis, and goal-directed behavior (Birrell and Brown, 2000; Ragozzino et al., 1999). It also modulates many cognitive processes through its wide array of connections throughout the brain (Ragozzino, 2007). Common PFC changes in neurodevelopmental disorders include disrupted functional connectivity to other brain regions, excitatory/inhibitory imbalances (Tendilla-Beltrán et al., 2021), loss of extracellular matrix (perineuronal nets) (Paylor et al., 2016), and dysregulated astrocytic and microglial activation (Mittli, 2023). Indeed, microglia are highly reactive to IL-6, the key MIA mediator, releasing pro-inflammatory cytokines and interferons when stimulated (Iitani et al., 2023; West et al., 2019). Therefore, infiltration of these cytokines into the fetal compartment during vulnerable periods of development may represent the beginning of a mechanism by which they induce long-term PFC changes (Cieślik et al., 2020; Loayza et al., 2023).

MIA models have identified vast and varied cognitive deficits consistent with several neurodevelopmental disorders. First, several MIA studies have identified alterations in object recognition and memory using the novel object recognition test (NORT), with some studies reporting deficiencies in novel object recognition (Gray et al., 2019; Guerrin et al., 2022; Osborne et al., 2017; Shi et al., 2003) and others reporting enhancements (Ito et al., 2010; Su et al., 2022). Additionally, MIA animals display impaired reversal learning, which tests animals' ability to flexibly adjust reward seeking behavior when reward contingencies are reversed in a discrimination test. Various paradigms of these tasks, which rely on the PFC, have revealed reversal learning deficits in adulthood (Amodeo et al., 2019; Han et al., 2011; Lins et al., 2019). These findings are consistent with those from other animal models and human studies, where difficulties in flexibly adapting to changing reward contingencies are observed in schizophrenia and ASD (Reddy et al., 2016; Yerys et al., 2009). Despite this, reversal learning has not been extensively tested in adolescent MIA animals while the PFC is still developing. Reversal learning deficits are also suggestive of perseverative and repetitive behaviors, which are particularly evident in ASD (D'Cruz et al., 2013). MIA studies have also identified increases in perseverative and repetitive behaviors, providing further face validity to MIA as a model of ASD in addition to schizophrenia (Estes et al., 2020; Vigli et al., 2020).

1.3. Hypotheses

It was hypothesized that MIA-exposed animals would exhibit cognitive dysfunction, increased anxiety behaviors, and poor motor coordination at juvenile and adolescent stages and that maternal choline supplementation would modulate these changes to an intermediate level between non-choline MIA offspring and saline controls. It was further hypothesized that MIA would decrease the activation of the α7nAChR-mediated cholinergic anti-inflammatory pathway in the PFC and that maternal choline supplementation would restore its activation to near-control levels.

2. Materials and methods

2.1. Animals and maternal immune activation

Timed pregnant Long-Evans rat dams were ordered from Charles River and arrived at the facility on gestational day (GD) 7. Immediately after arrival, dams were randomly assigned to one of three conditions: saline control, poly(I:C) with normal food, and poly(I:C) with choline-supplemented food. Those in the choline group were administered chow supplemented with choline chloride at a dose of 5 g/kg from arrival on GD 7 through weaning on postnatal day (P) 21 (Fig. 1). This dose has been repeatedly demonstrated to induce beneficial neural and behavioral changes in other neurodevelopmental animal models (Powers et al., 2017; Schulz et al., 2014; Stevens et al., 2014; Wu et al., 2015). On GD 15, all dams were injected intraperitoneally with either high molecular weight poly(I:C) (InvivoGen, catalog number tlrl-pic-5) at a dose of 10 mg/kg or saline. This dosage is consistent with previous MIA studies using the intraperitoneal route in rats (Gilmore et al., 2005; Monteiro et al., 2022; Su et al., 2022; Vorhees et al., 2015). Investigators were blind to condition and treatment. In total, 5 dams were assigned to the control condition, 7 to the poly(I:C) only condition, and 6 to the poly(I:C) plus maternal choline condition. To control for circadian fluctuations in immune status of the dams, all injections were carried out between the hours of 9:30–10:30 a.m. To verify maternal illness post-injection, maternal weight was measured 6, 24, and 48 h post-injection, as it has repeatedly been reported that dams injected with poly(I:C) display either slight weight loss or a lack of weight gain in this period, whereas control dams continue to gain weight (Kolmogorova et al., 2017). On P21, pups were weaned, sexed, randomly assigned an age cohort, and separated into double or triple housing with same-sex littermates, depending on numbers of each sex in the litter. Whenever possible, subjects were pair housed. Animals were housed on a 12-h light cycle (lights on at 8:30 a.m.) in open-top shoebox cages (46 × 24 × 20 cm) with pine bedding. All animal care, testing, and procedures were conducted in accordance with the requirements and guidelines of the Kansas State University Institutional Animal Care and Use Committee (IACUC). Additional reporting measures in compliance with the modified ARRIVE guidelines for MIA studies outlined by Kentner et al. (2019) are presented in Supplemental Table 1.

2.2. Reversal learning

2.2.1. Apparatus and stimuli

Reversal learning was assessed only in the adolescent cohort to examine cognitive flexibility. Animals were placed in a plexiglass arena (50 × 37 × 25 cm) with a sliding door between the holding chamber and testing arena. The door was lifted at the beginning of each trial to allow access to two flowerpots with opposing odor cues and digging media. Odor cues presented were vanilla/rum or cinnamon/anise, and media presented were aspen/shredded manilla folders or sequins/buttons. These odor and media combinations were selected based on the results of the lab's previous studies with similar tasks. Both the presentation order of the stimuli and which stimulus (odor or medium) constituted the relevant dimension were counterbalanced via Latin square. Digging

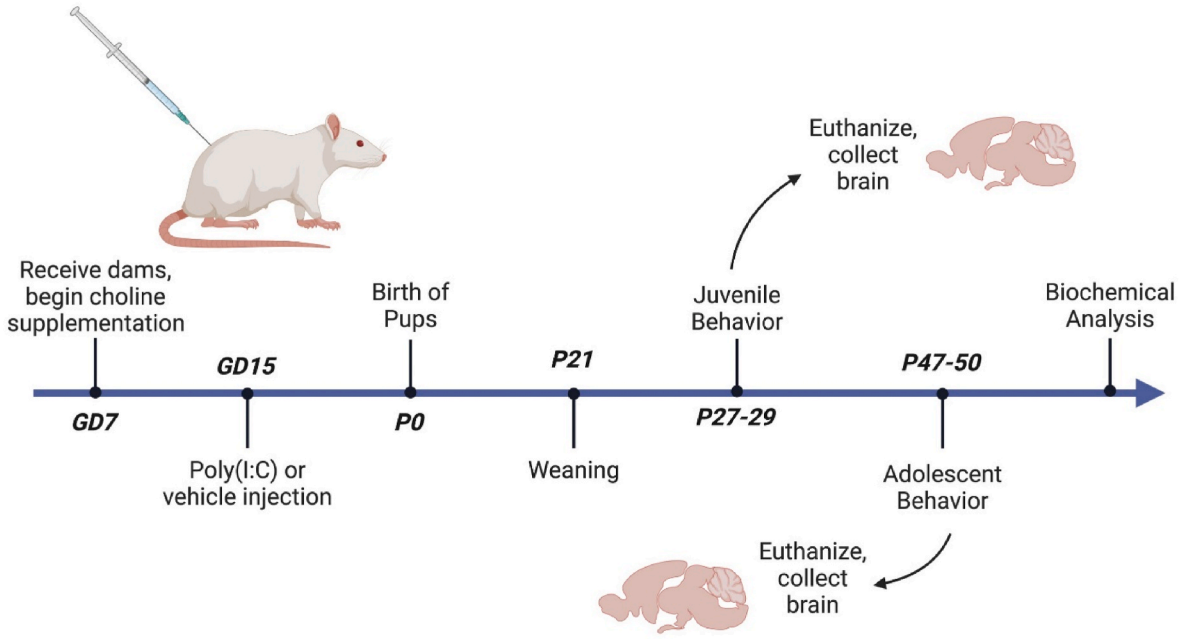

Fig. 1. Study Timeline: Timed pregnant dams arrived to the facility on GD 7 and were assigned a food condition. Dams were injected with 10 mg/kg poly(I:C) or saline on GD 15 and were then left undisturbed until weaning. Offspring were weaned on P21 and behavioral data were collected between P27-29 for the juvenile cohort (rotarod, NO, open field, EPM) and P47-50 for the adolescent cohort (rotarod, open field, NO, EPM and reversal learning). PFC samples were taken after behavioral testing (P30 for juveniles, P53 for adolescents) and frozen at -80°C for later use. Made in BioRender.com.

media were recycled between rats to disseminate scent cues, and pots were only ever scented with the same odor. Honey Nut Cheerios™ (General Mills) powder was dusted on all pots, and investigators touched each pot before the start of each trial to ensure that rats were discriminating on the basis of the odors and media presented to them, rather than based on outside cues. The locations of the pots were also counterbalanced to control for side preferences.

2.2.2. Training

About seven days before the full task, rats were food restricted to 12 g per day to ensure motivation for the food reward. Weights were taken daily for these animals to ensure that they did not fall below 80% of their free feeding-weight. If an animal was nearing 80% of its free-feeding weight, the animal was given more food, and its weight was recorded again the following day. After about five days of food restriction, rats were trained in basic digging and simple discrimination over two consecutive days before moving on to the full task. On day one of training, rats were trained to dig in flowerpots to receive food rewards, which were 1/4 to 1/3 pieces of Honey Nut Cheerios™ (General Mills). Training began with the cheerio pieces placed on top of the digging media, and they were buried progressively deeper with each consecutive trial. Once fully buried, the animal was required to retrieve fully buried food rewards with an average latency under 1 min to pass on to the second day of training. On the second day of training, rats were presented two simple discriminations (i.e., only odor or media cues presented): one odor (lavender vs nutmeg) in regular bedding, and one digging media (shredded paper vs pine shavings) in unscented pots. If the rat was able to complete these simple discriminations with six consecutive correct choices within 25 trials, it was allowed to continue to the full task the following day.

2.2.3. Reversal learning task

The reversal learning task was presented as a series of four phases, all of which were compound discriminations. That is, both different odors and different media were presented simultaneously. Rats were required to learn which dimension (odor or medium) to attend to, as well as which of the two exemplars was rewarded. For each phase, the first four trials were considered "discovery trials" where the animal was allowed to explore both pots, even if it made an incorrect choice. These trials were counted in the six consecutive correct trials, if applicable. After the first compound discrimination was completed with six trials to criterion, a reversal phase was presented where the rewarded exemplar was switched. After this, an intra-dimensional (ID) shift was presented, where the reward dimension stayed the same, but all exemplars were changed. After the ID shift, a second reversal was presented. Upon completion of each phase with six consecutive correct trials, the animal was returned to its home cage, and food restriction was immediately lifted.

2.3. Novel object recognition test (NORT)

To assess object memory, the novel object recognition test (NORT) was conducted. Testing occurred over two consecutive days. On the first day, animals were placed in a 65 × 50 cm arena for 5 min to habituate. Then, animals were removed and two identical objects (candle holder or wrench socket) were placed in opposite corners of the box. Animals were then placed back in the arena to explore both objects for 10 min. The next day, animals were again placed in the same arena, except with one identical object to those which it had encountered the day before and another, novel object. Objects were counterbalanced in their presentation and location to control for preference for one or the other location or object. Time spent investigating each object was recorded manually by blind-to-condition researchers in Actimetrics Limelight video coding software. Data were analyzed as percent exploration time spent with the novel object.

2.4. Rotarod test

Animals were placed on a rod rotating at 4 rpm, facing the same direction each time. Once the animal was securely on the rod, the trial began. Throughout the trial, the rod constantly accelerated to 40 rpm over the course of 5 min. Latency to fall off the rod was measured, except for those trials lasting the maximum 5 min. This was done a total of three times on the same day, with at least 10 min of rest between trials. To

control for fatigue effects, the maximum latency between trials two and three was considered and analyzed in reference to baseline performance, as it has been suggested that rapid short-term increases in latency may be reflective of repetitive motor behavior acquisition (Fuccillo et al., 2014).

2.5. Open field test (OFT)

For the OFT, animals were placed in a clear Plexiglass enclosure measuring 60 cm long on each side. Animals were placed in the center of the enclosure facing the same direction each time, after which they were free to explore the arena for 10 min. For analysis, the enclosure was divided into nine 20 × 20 cm zones. Time spent in the center zone, as well as crossings through the center zone were taken as measures of anxiety-like behaviors. Total locomotor activity, measured by distance traveled, was also assessed.

2.6. Elevated plus maze (EPM)

To further assess anxiety behaviors, animals were placed on an elevated four-armed maze with alternating open and closed arms, such that the open arms were opposite each other. Animals were placed on the maze facing the same direction at the start of each trial, after which they were free to explore the maze for 5 min. Videos of trials were collected and animal tracking was done with ANY-maze™ (Stoelting) software. Time spent in the center zone, open arms, and closed arms was assessed, as were the number of entries into the open and closed arms. If an animal fell off the maze during the trial, video acquisition was paused, the animal was placed back on the maze as at the beginning, and the trial was resumed. If the animal fell off a second time, it was excluded from analysis.

2.7. Tissue harvest and preparation

On P30 or P53, depending on age cohort, animals were deeply anesthetized under 5% isoflurane gas and euthanized by decapitation. Brains were immediately removed, medial prefrontal cortices were quickly dissected, placed into polypropylene microcentrifuge tubes, and flash frozen on dry ice or in liquid nitrogen. After freezing, tissue samples were stored at −80 °C until further use. All euthanasia was completed between the hours of 10:30 a.m. and 2:30 p.m. to control for circadian fluctuations in immune markers.

After all samples were collected, tissues were homogenized in different cell lysis buffers depending on downstream application. Left medial prefrontal cortices were taken for Milliplex cytokine and Akt/phospho-Akt assays and were homogenized in cell lysis buffer (Milliplex®, catalog number 43-040) containing phosphatase and protease inhibitors (Roche) via trituration through a 1000 μL pipette tip. Then, samples were incubated on an orbital plate shaker at 500 rpm for 2 h at 4 °C to maximize cell lysis. After 2 h, samples were centrifuged at 4500×*g* for 15 min, supernatants were collected, and aliquots were frozen at −80 °C for later use. The same process was followed for right medial prefrontal cortices, except that they were homogenized in another cell lysis buffer (Abcam Cell Extraction Buffer PTR, catalog number ab193170) for Heme Oxygenase 1 ELISA. To account for differences in total protein load between samples, a Qubit™ (Invitrogen) fluorometric assay was performed on an aliquot of each sample to determine total protein concentration. The results of each assay were normalized to their respective total protein concentrations for direct comparison.

2.8. Cytokine quantification

The cytokines IL-1β, IL-4, IL-6, IL-10, IL-17A, IFN-γ, and TNF-α were assayed with a magnetic bead panel (Milliplex®, catalog number RECYTMAG-65K). Samples were diluted to a concentration between 500 and 750 μg/mL (1000–1500 μg/mL in lysis buffer and then 1:2 in assay buffer) before assay per the manufacturer's recommendations. Samples and standards were then incubated with capture antibodies immobilized on magnetic beads in assay buffer at room temperature with agitation for 2 h. The remainder of the assay was conducted per the manufacturer's instructions. Median fluorescent intensity was then read for each analyte bead on a Luminex 200™ plate reader using Luminex xMAP™ technology. The median fluorescent intensity was then analyzed via a five-parameter standard curve fit by Intelliflex® software, and results were normalized to total protein concentration and analyzed directly.

2.9. Akt/phospho-Akt assay

Phosphorylation of Akt at Ser473 and total Akt were assayed with a Milliplex® Cell Signaling kit (Milliplex®, catalog number 48-618MAG). Samples were diluted to a concentration between 300 and 500 μg/mL (600-1000 μg/mL in lysis buffer and then 1:2 in assay buffer) per the manufacturer's recommendations and were incubated with capture antibodies immobilized on magnetic beads at 4 °C with agitation for 18 h overnight. The assay was conducted per manufacturer instructions, and median fluorescent intensity was read on a Luminex 200™ plate reader using Luminex xMAP™ technology.

2.10. Heme oxygenase 1 (HO-1) ELISA

Heme oxygenase 1 was assayed using a commercially available ELISA kit (Abcam SimpleStep®, catalog number ab279414). Samples were diluted to a concentration between 700-1000 μg/mL concentration (determined by a pilot experiment) in lysis buffer containing protease inhibitors and incubated with detection and capture antibodies for 1 h with agitation. Wells were then washed and incubated with TMB substrate for 10 min in the dark, followed by stop solution for 1 min. Optical densities were then read at 450 nm. Standard curves were fit using online GainData® ELISA analysis software, and the average of the two replicates was used to calculate analyte concentration in each well.

2.11. Animals included in experiments

Animals included in behavioral experiments completed the NORT, rotarod, OFT, and EPM at both age cohorts (juvenile and adolescent). In addition, some animals completed the reversal learning task in adolescence, which is presented as a separate column in Table 1. Numbers in biochemical experiments were balanced between groups to avoid excess contributions from any individual litter to the results.

2.12. Statistical analysis

Behavioral data were analyzed with generalized linear mixed models in R with appropriate distributional assumptions selected based upon data type and structure. Normal-, Gamma-, and Poisson-distributed data were analyzed using the lme4 package and beta-distributed data were analyzed using the glmmTMB package. Condition, sex, and the interaction between them were included as fixed-effect predictors, and litter was included as a random effect where appropriate to control for pseudoreplication due to multiple and unbalanced numbers of littermates within groups. *P*-values were adjusted to account for multiple comparisons using the Bonferroni method where appropriate. Molecular data were analyzed nonparametrically using a Kruskal-Wallis test followed by Dunn's test for pairwise comparisons. High variance between replicates was not observed for assays, so no samples were excluded for analysis. Only a small number samples fell below the limit of detection for IFN-γ and pAkt, and these samples were assigned a value of zero for analysis. No significant or near-significant sex effects were observed in the molecular data, so those data were analyzed with both sexes combined by condition.

Table 1
Number of Animals Included in Experiments. Animals were derived from 5 saline dams and 13 poly(I:C) dams, 6 of which received gestational choline supplementation. To account for group imbalances, measures were taken to account for litter as a covariate in all results.

	Behavioral			Biochemical					
	Juvenile	Adolescent		Juvenile			Adolescent		
	Total	Total	Reversal Learning	Cytokines	Akt	HO-1	Cytokines	Akt	HO-1
Saline Male	8	9	8	7	7	7	6	7	9
Saline Female	12	13	10	7	7	9	7	7	9
Poly(I:C) Male	14	15	13	7	7	9	7	7	9
Poly(I:C) Female	11	10	10	7	7	9	7	7	9
Poly(I:C) + Choline Male	16	16	13	7	7	9	7	7	9
Choline Female	14	13	12	7	7	9	7	7	9

3. Results

3.1. Maternal weight

Maternal weight was analyzed with a linear mixed model with a random effect of rat ID to account for repeated measures. As expected, choline intake had no differential impact on weight loss following injection, so both choline-supplemented and control chow dams were assessed as a single group. Following injection, both saline and poly(I:C) dams exhibited initial weight loss after 6 h ($t_{48} = 5.359$, $p < 0.001$). However, there was a significant difference between saline and poly(I:C) dam weight 24 h post-injection ($t_{44} = 3.187$, $p = 0.003$). At 48 h post-injection, though, poly(I:C) dams had generally recovered, and their weights were not significantly different from saline dams ($t_{44} = 1.511$, $p = 0.138$). There were no discernible differences in sickness behavior profiles between saline and poly(I:C) dams (Fig. 2).

3.2. Reversal learning

Reversal learning was only examined in adolescence as the lab has found that rats younger than P40 do not pass at sufficient rates for analysis. Sexes were analyzed separately based on the lab's previous findings with similar tasks (Mali et al., 2023). For males, there was a significant condition by phase interaction ($\chi^2_6 = 35.196$, $p < 0.001$). Planned pairwise comparisons revealed that poly(I:C) males took significantly fewer trials to learn on the initial compound discrimination of the task than saline controls, though this was only significant for the poly(I:C) + choline group (estimate = 0.566, $SE = 0.182$, z = 3.110, $p_{adj} = 0.013$; non-choline estimate = 0.415, $SE = 0.179$, $z = 2.317$, $p_{adj} = 0.123$). There were no significant differences in trials to criterion on any other phases. However, when considered in relation to baseline performance, poly(I:C) males took significantly more trials to reach criterion on the first reversal than on initial learning (non-choline estimate = 0.337, $SE = 0.101$, $z = 3.340$, $p_{adj} = 0.007$; choline estimate = 0.440, $SE = 0.107$, $z = 4.114$, $p_{adj} < 0.001$), while control animals did not (Fig. 3A). Additionally, despite no differences on the ID shift, the poly(I:C)-only males took more trials to complete the second reversal relative to their ID performance, while control and poly(I:C) + choline males did not, indicating a continued reversal impairment for the poly(I:C) group (estimate = 0.288, $SE = 0.106$, $z = 2.724$, $p_{adj} = 0.058$). In female poly(I:C) animals, there was also a significant interaction of poly(I:C) by phase ($\chi^2_6 = 14.457$, $p = 0.025$), but there were no significant pairwise comparisons in either absolute or relative performance on any phase after p-value correction for multiple comparisons (Fig. 3B).

3.3. Novel object recognition task (NORT)

The NORT was conducted at both ages. At the juvenile age, there were no significant effects of condition, sex, or the interaction between them. When examined as discrimination above chance levels, only control females displayed novel object recognition significantly above chance levels ($z = 2.696$, $p = 0.007$). However, poly(I:C) + choline

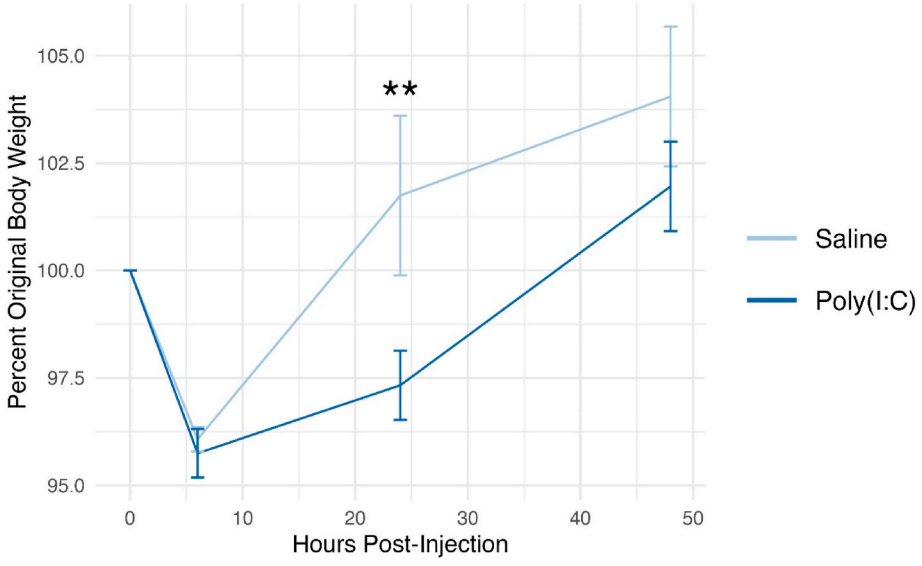

Fig. 2. Maternal Post-Injection Weight Loss
Weight trajectory of dams post-injection reported as percentage of weight at injection. Poly(I:C)-treated dams had significant weight loss at 24h post-injection compared to controls (p = 0.003). Though not significant, these weights were still reduced compared with controls at 48h post-injection (p = 0.14). Error bars represent SEM.

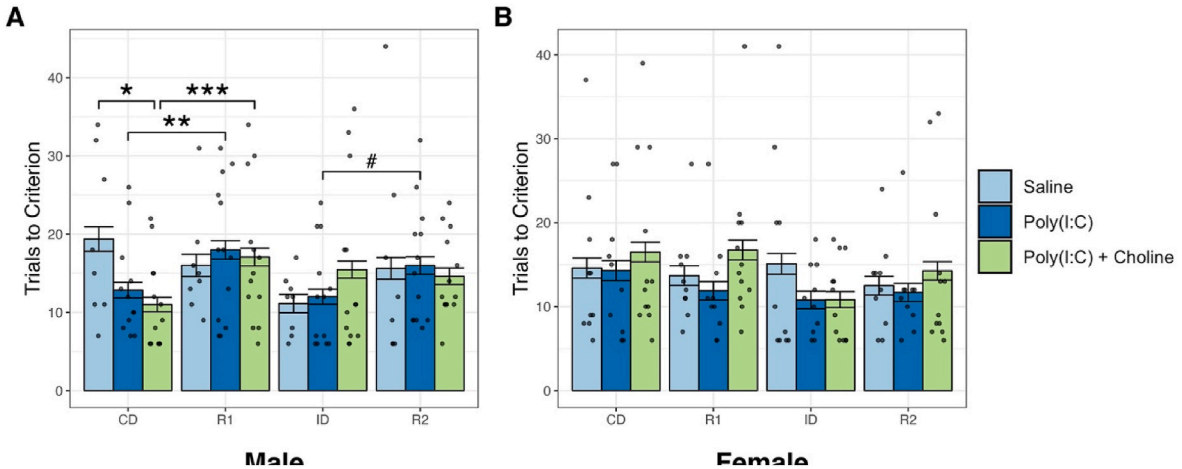

Fig. 3. Reversal Learning Trials to Criterion **(A)** On the reversal learning task, male poly(I:C) animals displayed improved basic learning on the first compound discrimination compared to controls (choline $p_{adj} = 0.013$, non-choline $p_{adj} = 0.123$). However, they performed worse on the first reversal phase relative to their baseline performance. This reversal learning deficit was present in the second compound discrimination-reversal combination only for the non-choline poly(I:C) males ($p = 0.058$). **(B)** There were no differences in performance in the female group. Error bars represent Poisson SEM. ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$, #$p < 0.10$. CD = compound discrimination, R1 = reversal 1, ID = intra-dimensional shift, R2 = reversal 2.

males trended toward above-chance performance ($z = 1.920$, $p = 0.055$) (Fig. 4A). In adolescent animals, there were likewise no significant effects of condition, sex, or the interaction between them. However, when analyzed as discrimination above chance, poly(I:C)-only ($z = 2.260$, $p = 0.024$) and poly(I:C) + choline ($z = 4.146$, $p < 0.001$) animals displayed greater than chance preference for the novel object, regardless of sex.

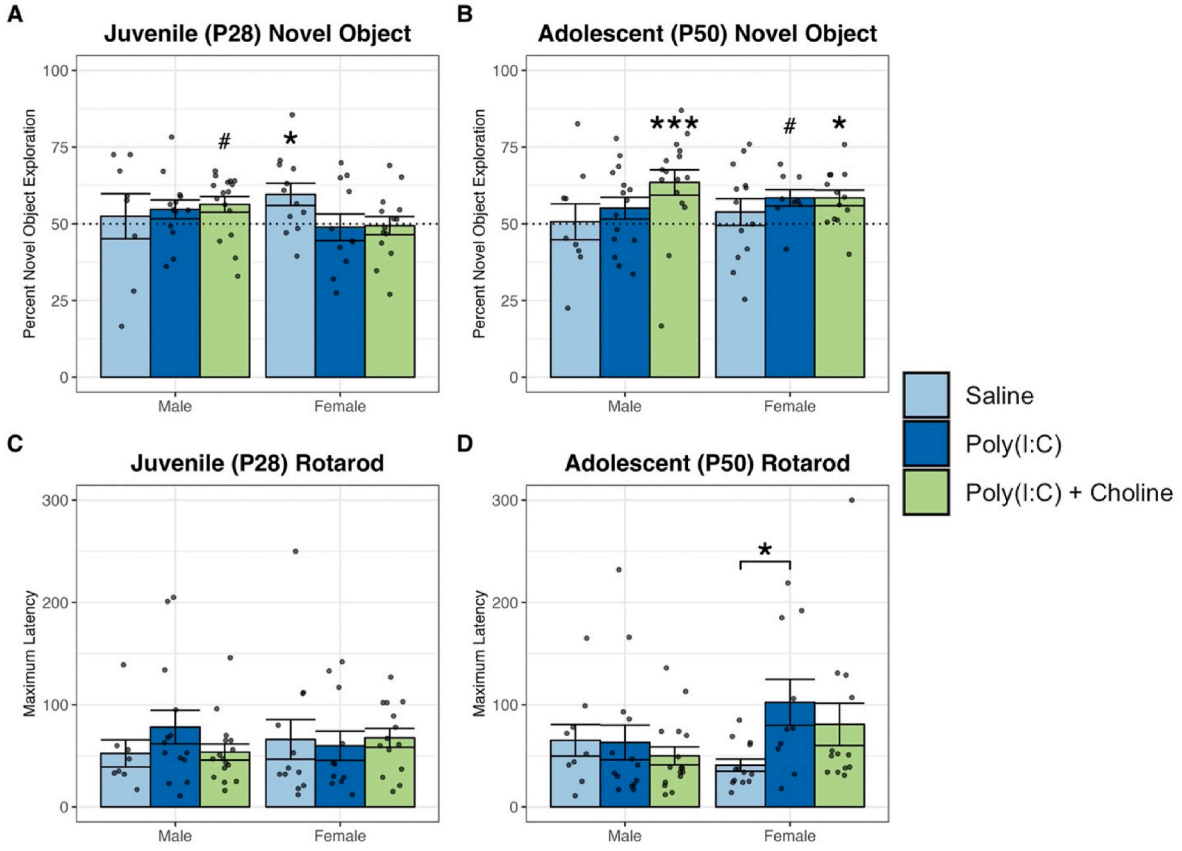

Fig. 4. Novel Object and Rotarod Results
(A) In juvenile animals, only control females discriminated the novel object above chance (50% time with each object) levels (p = 0.007), although poly(I:C) + choline males trended toward the same (p = 0.055). **(B)** In adolescence, however, poly(I:C) + choline males ($p < 0.001$) and females (p = 0.034) both demonstrated greater than chance discrimination of the novel object. Poly(I:C)-only females trended toward above chance discrimination of the novel object (p = 0.082). On the rotarod test, **(C)** juvenile animals displayed no condition or sex differences in maximum latency on the rotarod, but **(D)** in adolescence, poly(I:C)-only females had longer maximum latencies than controls (p = 0.027). Error bars represent SEM. Symbols without brackets indicate difference from chance performance. ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$, #$p < 0.10$.

When separated by sex, poly(I:C)-only females trended toward greater than chance discrimination (male $z = 1.442$, $p = 0.15$; female $z = 1.742$, $p = 0.082$), while males did not (Fig. 4B). In the poly(I:C) + choline animals, both males and females both discriminated above chance levels (male $z = 3.821$, $p < 0.001$; female $z = 2.124$, $p = 0.034$). There was no main effect of age on novel object discrimination, but there was a trend-level interaction between condition and age ($\chi^2_2 = 5.036$, $p = 0.081$). Pairwise analysis of this interaction revealed that poly(I:C) + choline animals, regardless of sex, had improved novel object discrimination in adolescence over juvenile animals, thus driving the interaction. However, this effect was only trend-level after adjustment for multiple comparisons ($z = 2.322$, $p = 0.061$).

3.4. Rotarod test

There were no differences in maximum latency to fall off the rod for juvenile animals (Fig. 4C). In adolescence, however, a condition*sex interaction was found for maximum latency to fall off the rod ($\chi^2_2 = 8.242$, $p = 0.016$) (Fig. 4D). Pairwise comparisons revealed that, after adjustment for multiple comparisons, only poly(I:C) females had significantly longer latencies to fall off the rod, though this only survived correction for multiple comparisons in the non-choline group (non-choline estimate = 0.874, $SE = 0.307$, $z = 2.844$, $p = 0.027$; choline estimate = 0.681, $SE = 0.292$, z = 2.330, $p = 0.099$). Further analysis showed that this difference was not due to significant differences in baseline performance at trial 1.

3.5. Open field test (OFT)

There were no group or sex differences in total distance traveled in the open field at either age. There were also no group or sex differences in time spent in the center zone at either age when analyzed as percentage of time spent in the center. Regarding crossings through the center zone, there were no group differences, but males crossed through the center zone more than females at in the younger cohort only ($\chi^2_1 = 7.623$, $p = 0.006$). These results are presented in in Supplemental Fig. 1.

3.6. Elevated plus maze (EPM)

There were no group or sex differences at either age for time spent in any zone. For zone entries, there were no group differences, but there were sex effects in the number of entries into the center zone in the juvenile animals only, where males entered the center zone more times than females did ($\chi^2_1 = 6.522$, $p = 0.011$). Entries were defined as crossing of the anterior half of the body into a zone, the default setting for the ANY-maze™ (Stoelting) software. These results are presented in in Supplemental Fig. 2.

3.7. Cytokine expression

No significant sex effects were observed for cytokine expression, so data are shown by condition only. Mean values and standard errors for cytokine measurements are given in Supplemental Table 2. In juvenile animals, a significant condition effect was found only for IL-4 ($\chi^2_2 =$

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Figure 5

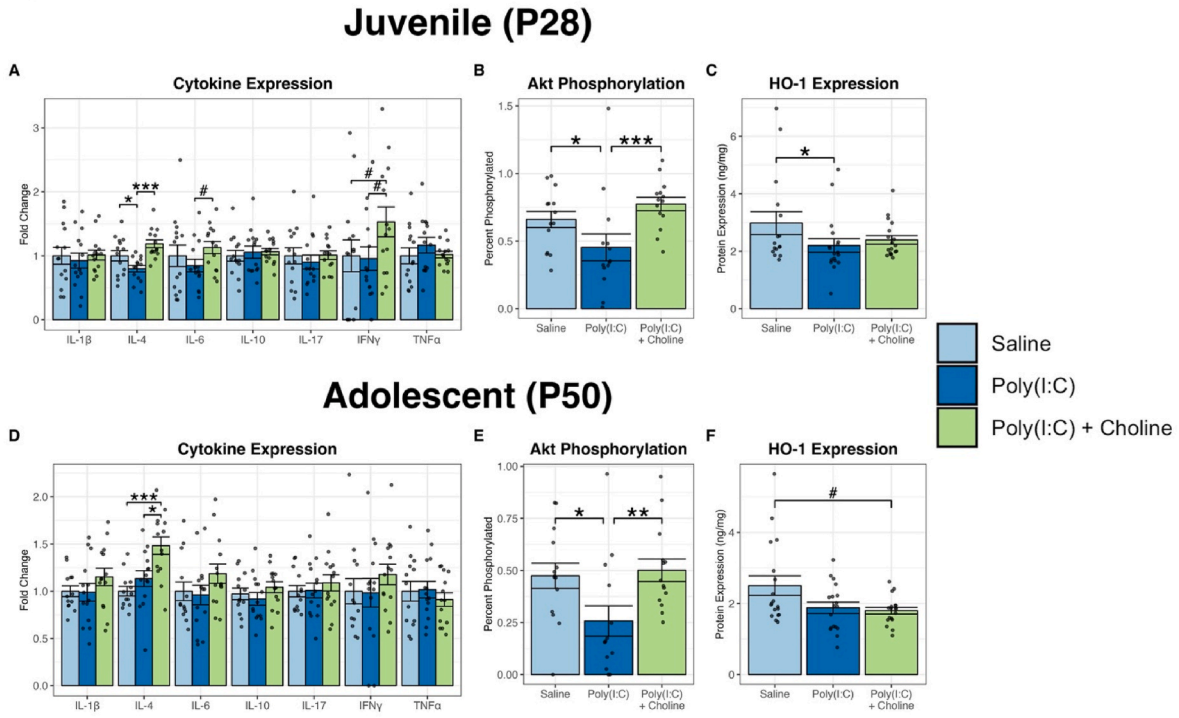

Fig. 5. Molecular Changes in the Prefrontal Cortex
Cytokine data were analyzed directly as normalized values, but data are presented as fold changes for ease of visualization. **(A)** In juvenile animals, poly(I:C)-only animals had significantly lowered expression of IL-4 compared to control animals (p = 0.036). Maternal choline reversed this (p < 0.001) and tended to increase IL-6 expression in the poly(I:C) group. Maternal choline also tended to increase IFN-γ expression over both controls (p = 0.093) and poly(I:C)-only (p = 0.081) animals. **(B)** Juvenile poly(I:C) animals had reduced relative pAkt expression compared to controls (p = 0.039), which was reversed by maternal choline (p < 0.001). **(C)** Juvenile poly(I:C) animals had reduced HO-1 expression relative to controls (p = 0.041), while the poly(I:C) + choline group did not. **(D)** In adolescence, only IL-4 was significantly increased in the poly(I:C) + choline animals compared to both controls (p < 0.001) and poly(I:C)-only (p = 0.032) animals. **(E)** In adolescence, poly (I:C) animals had reduced pAkt expression compared to controls (p = 0.018), which was reversed by maternal choline (p = 0.010). **(F)** In adolescence, despite no significant difference between groups, both poly(I:C) groups insignificantly tended toward reduced HO-1 expression (poly(I:C)-only p = 0.135, choline p = 0.053). Error bars represent SEM. ***p < 0.001, **p < 0.01, *p < 0.05, #p < 0.10.

13.969, $p < 0.001$) (Fig. 5A) Pairwise comparisons via Dunn's test revealed that poly(I:C)-only juvenile animals had reduced IL-4 expression compared to controls (estimate = 2.095, $p = 0.036$) and poly(I:C) + choline animals (estimate = 3.728, $p < 0.001$). Despite no significant differences, trend-level differences were observed in IL-6, where poly(I:C) + choline animals had higher PFC expression compared to poly(I:C)-only animals (estimate = 1.679, $p = 0.093$), and IFN-γ, where poly(I:C) + choline animals had higher levels than both control (estimate = 1.680, $p = 0.093$) and poly(I:C)-only (estimate = 1.742, $p = 0.081$) offspring. No significant or trend-level differences were observed for IL-1β, IL-10, IL-17A, and TNF-α. In adolescence, as with the juvenile animals, a significant condition effect was found for only IL-4 ($\chi^2_2 = 13.423$, $p < 0.001$). Similar to the juvenile animals, pairwise comparisons with Dunn's test showed that poly(I:C) + choline animals had higher IL-4 expression than poly(I:C)-only animals (estimate = 2.146, $p = 0.032$) and controls (estimate = 3.640, $p < 0.001$). Unlike juvenile animals, however, there were no trend-level differences in the levels of the other cytokines at this age (Fig. 5D). Point estimates and standard errors for each cytokine are given in Table 2.

Despite the general lack of group differences within ages, there were some striking differences in the expression of cytokines between juvenile and adolescent animals. First, there were several main effects and trends of age where, regardless of condition, the expression of IL-1β ($\chi^2_5 = 9.689$, $p = 0.085$), IL-17A ($\chi^2_5 = 13.546$, $p = 0.02$), and IFN-γ ($\chi^2_5 = 14.961$, $p = 0.011$) increased with age across all conditions. For IL-6, adolescence animals had elevated IL-6 across all groups ($\chi^2_5 = 14.245$, $p = 0.014$), but this was only significant in the poly(I:C)-only group ($p = 0.047$), with a trend-level increase in the poly(I:C) + choline group (estimate = 1.717, $p = 0.086$). Similarly, IL-4 expression significantly increased from with age ($\chi^2_5 = 33.47$, $p < 0.001$), but this elevation was only significant in the poly(I:C)-only (estimate = 3.473, $p = 0.001$) and poly(I:C) + choline (estimate = 2.046, $p = 0.041$) animals, but not in controls (estimate = 0.170, $p = 0.865$). Lastly, for IL-10, adolescent animals displayed increased expression compared to the younger animals ($\chi^2_5 = 13.267$, $p = 0.021$). However, this effect was only significant for the poly(I:C) + choline (estimate = 2.329, p = 0.020) and control (estimate = 2.209, $p = 0.027$) groups, but not the poly(I:C)-only group (estimate = 0.643, $p = 0.520$). The only cytokine that did not have increased expression with age was TNF-α ($\chi^2_5 = 5.446$, $p = 0.364$), which was not significantly different in any group between the two ages.

3.8. Protein Kinase B (Akt)

Protein Kinase B (Akt) phosphorylation results were analyzed both as total Akt expression and as the relative expression phosphorylated form of the protein (pAkt) at Ser473. As with the cytokine data, no sex effects were found, so data were analyzed by condition with both sexes combined. In the juvenile group, there was a significant condition effect ($\chi^2_2 = 11.098$, $p = 0.003$), and pairwise analysis via Dunn's test revealed that poly(I:C)-only animals had significantly reduced pAkt expression than poly(I:C) + choline ($p < 0.001$) and control ($p = 0.039$) animals (Fig. 5B). Likewise, there was also a significant condition effect in adolescence ($\chi^2_2 = 8.207$, $p = 0.017$). Pairwise comparisons showed that, as with the juvenile animals, poly(I:C)-only animals had reduced pAkt expression compared to poly(I:C) + choline ($p = 0.010$) and control ($p = 0.018$) animals (Fig. 5E). Beyond the increased pAkt expression relative to total Akt protein, poly(I:C)-only animals at both ages had decreased total Akt protein expression compared with poly(I:C) + choline animals (juveninle estimate = 2.819, $p = 0.005$; adolescent estimate = 3.204, $p = 0.001$).

3.9. Heme oxygenase 1 (HO-1) expression

Heme oxygenase 1 (HO-1) ELISA data were analyzed similarly to the cytokine data: by Kruskal-Wallis tests followed by Dunn's test post hoc. As with the other molecules, no significant sex effects were observed, so data were analyzed by condition with both sexes combined. At the juvenile age, poly(I:C)-only animals had reduced HO-1 expression compared to controls (estimate = 2.046, $p = 0.041$), despite no overall significant effect of condition ($\chi^2_2 = 4.436$, $p = 0.109$) (Fig. 5C). In adolescent animals, though, there was no effect of condition on HO-1 expression ($\chi^2_2 = 4.125$, $p = 0.127$) or any significant pairwise results, though both poly(I:C) groups trended toward decreased HO-1 expression compared with controls (poly(I:C) + choline estimate = 1.939, $p = 0.053$; poly(I:C)-only estimate = 1.494, $p = 0.135$) (Fig. 5F).

4. Discussion

4.1. Cognitive outcomes

Gestational choline modulated PFC-dependent reversal learning in males. Reversal learning requires flexible adaptation to changing reward contingencies and is often impaired in MIA animals (Amodeo et al., 2019; Han et al., 2011; Lins et al., 2018; Meyer et al., 2005; Wallace et al., 2014; Y. Zhang et al., 2012). PFC development is sensitive to early-life and prenatal stressors, which can induce alterations in excitatory/inhibitory balance in the PFC (Filarowska-Jurko et al., 2022; Powell et al., 2015). It is known that MIA alters excitatory/inhibitory activity in the PFC (Kaar et al., 2019; Zhang et al., 2023), but the developmental trajectory of these changes is unclear as very few MIA studies have examined reversal learning in adolescence. Therefore, detecting even subtle impairments in adolescence furthers the understanding of the trajectory of cognitive flexibility impairments as a result of MIA. The digging version of the task used in this study is a suitable way to examine reversal learning without introducing a large spatial working memory component, meaning that this version of the task can more easily isolate PFC-dependent reversal learning processes from hippocampus-mediated spatial working memory (Tait et al., 2014, 2021). This version of the reversal learning task is sufficient to recapitulate reversal learning impairments in adult MIA rats (Wallace et al.,

Table 2
Cytokine Measurements with Age Comparisons: Although there were relatively few group differences within each age cohort, there were several changes between ages that were different in the poly(I:C)-only and poly(I:C) + choline groups. Maternal choline supplementation in the poly(I:C) group blunted age-related increases in IL-6, IL-17A, and IFN-γ while facilitating increases in IL-1β and IL-10. Significant ($p < 0.05$) age-related increases are denoted by bolded numbers.

	Juvenile						Adolescent					
	Saline		Poly(I:C)		Poly(I:C) + Choline		Saline		Poly(I:C)		Poly(I:C) + Choline	
	pg/mg	*SE*	*pg/mg*	*SE*	*pg/mg*	*SE*	*pg/mg*	*SE*	*pg/mg*	*SE*	*pg/mg*	*SE*
IL-1β	308	41.1	287	36.8	**314**	**24.9**	364	21.2	361	34.6	**421**	**33.4**
IL-4	186	14.6	**149**	**9.33**	**220**	**11.2**	194	9.70	**219**	**16.2**	**286**	**17.7**
IL-6	3340	548	**2836**	**324**	3754	302	4181	403	**4008**	**422**	4936	417
IL-10	**352**	**29.2**	371	33.2	**374**	**17.4**	**437**	**25.5**	392	27.7	**459**	**22.7**
IL-17A	803	113	**728**	**94.3**	830	62.3	989	65.7	**1016**	**81.8**	1081	91.2
IFN-γ	5762	1126	**4779**	**896**	7577	1136	8045	1069	**9210**	**866**	9474	873
TNF-α	50.8	6.26	59.2	6.14	51.8	2.61	46.9	4.89	47.7	4.10	42.8	3.33

2014).

Male poly(I:C) animals, regardless of maternal choline, demonstrated improved basic learning in the task, but these animals took more trials to complete the reversal phase in reference to baseline (despite no overall difference in trials on the reversal phase), while saline controls took fewer. Analysis of reversal performance relative to baseline rule acquisition is a valid approach to detect reversal learning impairments when initial performance differs between groups (McBride and Morton, 2018; Rajalakshmi and Jeeves, 1965). The impaired reversal learning relative to baseline may be evidence of differential strategy in the task. Perhaps control male animals spent more trials sampling each pot to learn the rule, and their subsequent improvement on the reversal is more indicative of a flexible sampling strategy when reward conditions changed in the reversal phase, whereas the poly(I:C) animals had difficulty in adapting to the rule change. This same pattern was found on the second reversal only for the poly(I:C)-only animals. This suggests that prenatal choline supplementation may have modulated reversal learning such that the animals are better able to flexibly adapt over successive reversals. Indeed, maternal choline supplementation has been shown to facilitate reversal learning in non-MIA animal studies (Thomas et al., 2004; Waddell et al., 2020). There were no differences in females. This may be due to a strain-specific sex effect in reversal learning, which has been observed Long-Evans rats (the strain used in this study), regardless of poly(I:C) exposure (Gogos et al., 2020). Based on the historical underrepresentation of female subjects in preclinical research, these sex effects should be further examined in other strains and in clinical populations to determine if they are persistent across different paradigms.

MIA and gestational choline also facilitated object memory in the NORT over the control group in the adolescent cohort. The lack of strong preference for either object in the control animals, especially in the juvenile cohort, is typical. On the typical development trajectory, capacity for novel object recognition develops throughout adolescence into early adulthood (Cyrenne and Brown, 2011). This is in contrast to the trajectory of development observed the MIA group and in other MIA studies, where object memory in the NORT is better than controls in adolescence (Gray et al., 2019; Guerrin et al., 2022; Osborne et al., 2017; Shi et al., 2003) but is impaired in adulthood (Ito et al., 2010; Su et al., 2022). In line with the findings here, a study using both the NORT and an object location recognition task found that young adult MIA mice demonstrated heightened sensitivity to novel objects compared to control mice, but not objects placed in novel locations, suggesting that MIA may have altered object information processing, but not spatial processing (Ito et al., 2010). Supporting this, a different study identified enhanced novel object recognition in MIA rats at P45 (mid-adolescence), more closely aligning with the paradigm of this study (Su et al., 2022).

Prenatal choline supplementation, as in this study, has been found to improve hippocampus-dependent memory processes, such as those used in the NORT, in a variety of animal models (Meck et al., 1988; Waddell et al., 2020; Zeisel, 2006a). Since the maternal choline supplementation paradigm of this study spanned Embryonic days 11–18, the key gestational period for hippocampal development in rodents, choline-mediated neuroprotective mechanisms likely acted synergistically with MIA to produce strong object memory in the NORT used in this study (Bayer, 1980; Zeisel, 2006b).

4.2. Repetitive and anxiety-like behaviors

On the rotarod test, poly(I:C)-only females demonstrated significantly longer latencies to fall off the accelerating rod compared to controls in adolescence. This contrasts with findings that people with ASD and schizophrenia generally display impaired motor coordination, which has been replicated in MIA studies (Fatemi et al., 2012; Monteiro et al., 2022; Walther and Strik, 2012; Zhang and van Praag, 2015). However, many research groups using rotarod tests implement training over several days so that all groups of animals learn the repetitive motor routines necessary to stay on the rod before testing (Buitrago et al., 2004; Naviaux et al., 2013; Zhang et al., 2021). The present study conducted trials successively on the same day with only short breaks between trials, thus preventing long-term improvements in performance due to motor learning. This suggests that these results may be more indicative of repetitive behavior acquisition than motor learning, which may explain the blunting of MIA's effect by maternal choline. Supporting this, an elegant 2014 study by Fuccillo and colleagues (Fuccillo et al., 2014). demonstrated that improved performance on the rotarod over time is modulated by striatal circuits involved in repetitive and stereotyped behaviors instead of motor learning ones in neuroligin-3 knockout mice, a model of ASD. Many striatal circuits regulating repetitive behaviors are cholinergic, and the modulation of rotarod performance in the poly(I:C) + choline group may be due to modulation of circuits specifically (Bell et al., 2019; Williams and Christakou, 2022). Future studies should examine gait patterns on the rotarod and separate out the roles of cholinergic modulation of striatal circuitry related to motor learning that may facilitate repetitive behavior acquisition.

There were no differences in anxiety-like behaviors on the EPM or OFT at either age. This conflicts with previous findings of increased anxiety behaviors after P60, although the EPM and OFT have produced inconclusive results in the MIA model before this age (Bergdolt and Dunaevsky, 2019). A possible explanation may be that the EPM and OFT are locomotion-dependent, meaning that young animals may not locomote enough for apparent group differences to be reliably detected, although a sex effect was observed in juveniles only where males locomoted more than females (Börchers et al., 2022).

4.3. Cholinergic anti-inflammatory pathway

To assess the ability of maternal choline supplementation to blunt the neurodevelopmental insult of MIA, activation of the anti-inflammatory pathway downstream of the α7nAChR was assessed in the PFC. First, Protein Kinase B (Akt) was assayed as a measure of PI3K/Akt cell-survival pathway activation downstream of the α7nAChR (Youssef et al., 2020). It is known that maternal choline supplementation can increase the expression of the α7nAChR (Kelley et al., 2019; Stevens et al., 2008; Wu et al., 2015), and it was hypothesized that downstream PI3K/Akt signaling would be persistently upregulated through those receptors following maternal choline supplementation. This target was chosen because PI3K/Akt signaling is disrupted in neurodevelopmental disorders like ASD and schizophrenia (Enriquez-Barreto and Morales, 2016). As expected, the phosphorylated (i.e., active) form of Akt was significantly decreased in poly(I:C)-only animals at both ages, but was boosted to control levels by maternal choline supplementation. Akt-mediated pathways have been implicated in schizophrenia and have been found downregulated in the PFC of poly(I:C)-exposed mice (Bitanihirwe et al., 2010). Akt is also highly involved in regulating the development of dopaminergic circuitry in the PFC, and its phosphorylation is modulated by the antipsychotic drug haloperidol, further implicating it in schizophrenia (Emamian et al., 2004). This study provides evidence that dysfunctions in Akt-mediated processes may be evident as early as adolescence, which carries implications for it as a candidate biomarker for schizophrenia, especially since its levels are also altered in the periphery of people with schizophrenia (Emamian et al., 2004). The modulation of Akt in adolescence by maternal choline supplementation is a notable finding that further supports choline's critical role in proper neurodevelopment and anti-inflammatory signaling.

Downstream of Akt, HO-1 is an inducible antioxidant enzyme that converts heme into bilirubin, free iron, and carbon monoxide. Bilirubin is a strong antioxidant, and carbon monoxide exerts other protective effects (Salinas et al., 2004). Since cholinergic anti-inflammatory signaling through the α7nAChR inhibits NF-κB nuclear translocation through the JAK/STAT3 pathway and positively regulates NRF2 (a key upstream transcription factor) translocation via PI3K/Akt signaling, it

was hypothesized that the basal expression of HO-1 in the PFC would be decreased by MIA and modulated nearer to controls in the MIA + choline group (Fig. 6) (Saha et al., 2020; Youssef et al., 2020). However, poly(I:C) animals, regardless of maternal choline, had decreased PFC HO-1 protein expression with little evidence of modulation by maternal choline. However, this decreased expression of HO-1 in the PFC does not necessarily discount the role of cholinergic anti-inflammatory signaling. HO-1 transcription is regulated by a number of transcription factors beyond the control of the cholinergic anti-inflammatory pathway (Medina et al., 2020). Also, Akt can phosphorylate HO-1 directly, thereby mobilizing a rapid cellular response to oxidative stress using existing stores of the enzyme, meaning that upregulation of HO-1 gene expression per se may not be necessary for an antioxidant effect, and HO-1 can translocate to the nucleus and to other cellular locations to coordinate other antioxidant responses (Salinas et al., 2004; Wu and Hsieh, 2022). These mechanisms were unexplored in this study but would be consistent with the increase in Akt and pAkt expression in the poly(I:C) + maternal choline group and may explain the lack of difference between the poly(I:C)-only and poly(I:C) + maternal choline group. Future studies should examine this possibility.

4.4. PFC cytokines

To assess the impact of gestational choline on overall immune state in the PFC, a panel of cytokines were assessed in PFC homogenates. There were few changes at either age. Although human studies have observed increased levels of circulating pro-inflammatory cytokines in adolescents, this does not necessarily discount potential elevations locally in the PFC (Cieślik et al., 2020; Rodrigues-Amorim et al., 2018; Zhao et al., 2021). A prior study found that poly(I:C) animals at P30 had increased IL-1β and IL-6 in serum, but decreased levels of IL-1β, IL-4 IL-6, and IL-10 in the PFC. Though this finding was replicated significantly for IL-4 only, the directionality of the relationship for IL-1β and IL-6 was the same in the PFC. The slight differences in findings may be due to differences poly(I:C) administration paradigms or the use of different species. These findings support the idea that cytokine expression in the PFC is highly region- and time-specific throughout development.

Despite the lack of differences in many cytokines' expression, one cytokine whose expression was persistently elevated at both ages by maternal choline supplementation was IL-4. IL-4 is a canonical anti-inflammatory cytokine that drives the polarization of macrophages and microglia from the pro-inflammatory M1 state to the anti-inflammatory M2 state (He et al., 2020; Pu et al., 2023; Yu et al., 2020). M2 microglia scavenge debris, including amyloid protein aggregates, and further secrete IL-4 and IL-10 to maintain homeostasis (Muto et al., 2023; Onore et al., 2014). IL-4 is also upregulated following cholinergic stimulation of the α7nAChR, suggesting that maternal choline supplementation was sufficient to activate the cholinergic anti-inflammatory pathway (Q. Zhang et al., 2017). It is also known that choline metabolism is critical for shifts between the M1 and M2 phenotype in macrophages and microglia (Ghorbani et al., 2023; Okada et al., 2022).

Remarkably, PFC IL-4 expression increased with age in the poly(I:C) groups, regardless of maternal choline, but not in controls. Since poly(I:C)-only animals had decreased IL-4 expression relative to other groups in the juvenile animals, this increase only brought IL-4 to control levels. Poly(I:C) + choline animals had further increased their already elevated

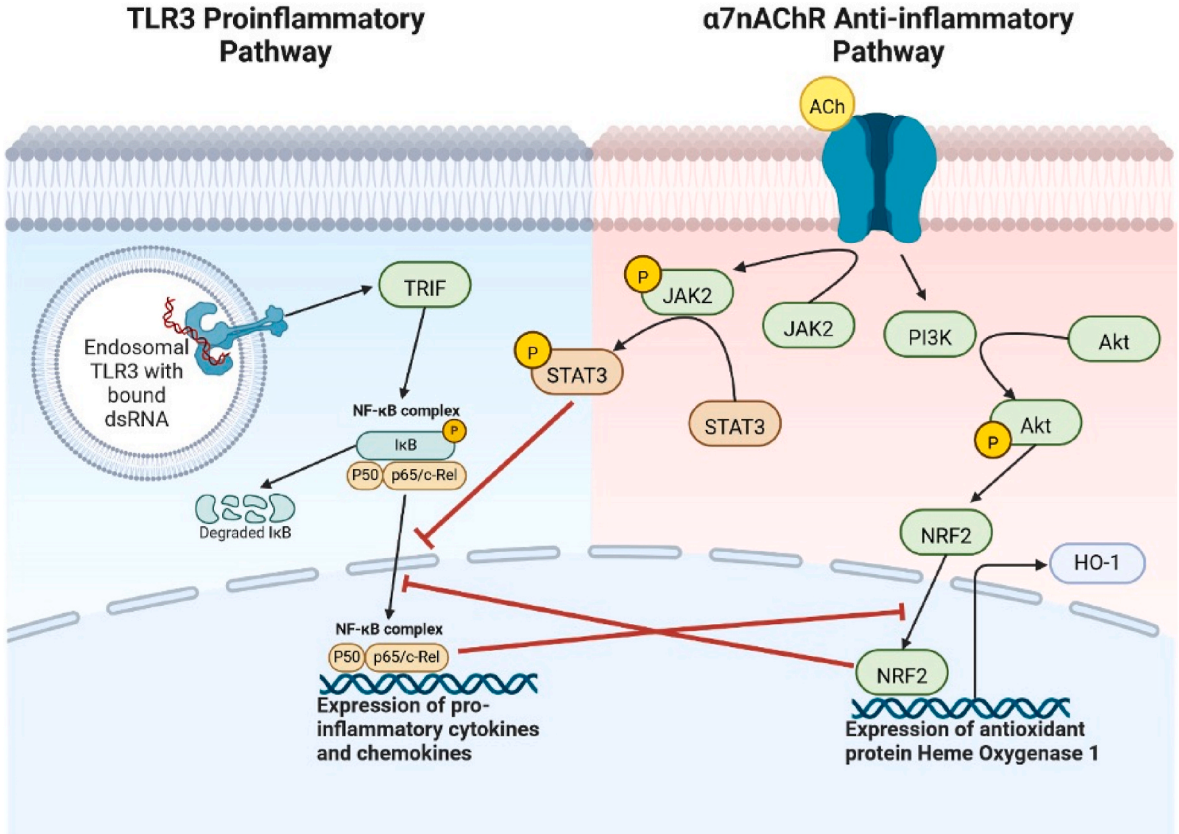

Fig. 6. Antagonism between a7nAChR and TLR3 Signaling.
Signaling downstream of the alpha-7 nicotinic acetylcholine receptor inhibits NF-κB-mediated proinflammatory signaling via two main mechanisms: JAK2/STAT3 signaling and PI3K/Akt signaling. JAK/STAT3 signaling inhibits the degradation of the IκB complex, thereby preventing NF-κB translocation to the nucleus. PI3K/Akt signaling induces antioxidant defense mechanisms in an Nrf2-dependent manner whereby Nrf2 competes with NF-κB for binding sites and inhibits pro-inflammatory responses. Made in BioRender.com.

IL-4 levels, suggesting a persistent, long-term anti-inflammatory impact of maternal choline supplementation. This was complemented by downstream increases in IL-10 levels in these animals, but not in poly(I:C)-only animals. Rather, this group displayed significant increases in IL-6 expression between with age, while the control and poly(I:C) + choline groups did not. Though not increased to levels above controls, this replicates previously observed increases in IL-6 from P30 through development (Garay et al., 2013). IL-6 is a front-line pro-inflammatory cytokine that underpins the neurodevelopmental impacts of MIA (Smith et al., 2007). It has also been found upregulated in humans with ASD and schizophrenia, and it is associated with several neuroinflammatory pathologies (Goines and Ashwood, 2013; Goldsmith et al., 2016; Wei et al., 2012).

4.5. Limitations and conclusion

Limitations include that maternal baseline immunoreactivity or the strength of the maternal immune response were not considered here. Future studies should take this into account, as it has been demonstrated that these factors greatly influence MIA outcomes (Estes et al., 2020). Additionally, the maternal choline supplementation regimen used in this study did not begin until GD7 since animals were not bred in-house. Beginning choline supplementation before mating may provide additional benefit as it would be elevated throughout pregnancy. Lastly, no saline + choline group was included in this study due to practical constraints. However, it is known that increasing maternal choline intake is safe and effective for bolstering proper development of higher-order brain regions and for modulating offspring behavior (Freedman et al., 2020; Jaiswal et al., 2023; Zeisel, 2006a). The results of this study are consistent with those findings.

This study contributes to the understanding of the biological underpinnings of neurodevelopmental disorders like ASD and schizophrenia in a number of ways. Behavioral findings observed in the poly(I:C) MIA model were replicated where poly(I:C) exposure led to reversal learning deficits and enhanced novel object performance. Importantly, this study identified inflammatory outcomes in the PFC that are consistent with those observed in ASD and schizophrenia, further highlighting the MIA model's translational validity for those conditions. This study provides evidence supporting maternal choline supplementation as an effective means to counteract the neurodevelopmental insult of MIA. In sum, these findings provide evidence that maternal choline supplementation is a cheap, simple, and effective intervention that bolsters neurodevelopment *in utero*, especially in the face of inflammatory insults.

CRediT authorship contribution statement

Cole King: Writing – review & editing, Writing – original draft, Visualization, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Bethany Plakke:** Writing – review & editing, Writing – original draft, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

None.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbih.2024.100836.

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