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14. ABSTRACT
Several important milestones were reached during the second year of this award. First, we were able to demonstrate that maternal inflammation during pregnancy, triggered by the viral-mimic poly(I:C), induces a significant increase of tryptophan metabolism in the placenta. This leads to a direct increased output of serotonin from the placenta to the fetal forebrain. Elevation of serotonin at these early stages of fetal brain development alters the development of the serotonergic system (blunting of axonal growth) and neuronal progenitor cell proliferation in specific forebrain regions. Thus our results demonstrate a direct molecular link between placental tryptophan metabolism and fetal brain development. A manuscript reporting these findings was submitted to the Journal of Neuroscience and is currently under review. In the second year of this award, we also started to investigate the possibility of pharmacologically interfering with this molecular pathway in order to potentially protect the fetal brain from the effects of maternal inflammation.

15. SUBJECT TERMS
Autism, placenta, tryptophan, serotonin, kynurenine, maternal immune activation, fetal brain

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INTRODUCTION

Maternal infections in humans increase the risk for autism spectrum disorders (ASD) in the offspring. In rodents, maternal infections cause behavioral, histological and transcriptional changes in adult offspring that are consistent with those seen in ASD. However, the anatomical and molecular pathways through which inflammation alters fetal brain development are not well understood. Serotonin (5-HT), which is synthesized from the essential amino acid tryptophan (TRP), is a trophic factor for the fetal brain before it acts as a neurotransmitter. In particular, 5-HT signaling modulates fetal brain wiring mechanisms and its disruption at early stages of pre- and postnatal development has long-term consequences on adult brain function and behavior. Thus, 5-HT is thought to be a critical mediator of the fetal programming of mental disorders such as ASD that appear later in life. In early pregnancy the placenta converts maternal TRP to 5-HT, through the tryptophan hydroxylase 1 (TPH1) pathway, thereby providing a source of the amine for the fetal brain. Therefore, altering maternal TRP metabolism in the placenta, and consequently placental 5-HT synthesis, may directly affect fetal brain development and constitute a new molecular pathway for the fetal programming of mental disorders. The goal of this project is to characterize the impact of inflammation during pregnancy on placental TRP metabolic pathways and the consequences on fetal brain development.

KEYWORDS

Autism, placenta, tryptophan, serotonin, kynurenine, maternal immune activation, fetal brain.

OVERALL PROJECT SUMMARY

Our objectives are to determine (1) whether maternal inflammation affects the balance of TRP metabolism through indoleamine deoxygenase (IDO1) and TPH1 placental pathways, resulting in compromised serotonergic modulation of fetal brain development as well as increased exposure to kynurenine, with long-term consequences on postnatal brain function; and (2) whether the timing of inflammation differentially impacts offspring brain development.

Placental TRP metabolism and transport of metabolites to the fetal blood stream and brain are tested using a technology unique to our laboratory that provides direct analytical capabilities of assessing mouse placenta metabolic pathways *ex vivo*. This new technology, combined with genetic and pharmacological *in vivo* approaches, is being applied to define the mechanisms by which inflammation in early and late gestation affect placental function and offspring brain development.

Aim I was fully completed and Aim II partially completed during the second year of this award. We focused on the effects of maternal inflammation triggered by the viral-mimic poly(I:C). Results from Aim I and Aim II are reported in a new manuscript that was submitted to The Journal of Neuroscience in July 2015. A copy of the manuscript is appended to this progress report.

Aim I: <u>To determine whether maternal inflammation alters placental synthesis and fetal exposure to 5-HT and kynurenine-pathway compounds.</u> This aim tested whether maternal inflammation affects placental TRP metabolism and 5-HT output to the fetus, and if these parameters are dependent on pregnancy stage. This was tested *ex vivo* and *in vivo*, by measuring changes in placental output of 5-HT and kynurenine-pathway compounds by HPLC at different gestational ages (E12-14; Aim 1A) following induction of maternal inflammation [using viral mimic RNA poly(I:C)] at E12. Short-term changes in placental expression of TRP metabolic genes (24 and 48h after immune activation) and of corresponding TRP metabolic enzyme activities *in situ* were assessed qualitatively by qRT-PCR and measure of enzymatic

activity. Results were correlated to the neurochemical measurements made in the fetal brain (Aim 1B).
All tasks proposed in Aim I regarding the effect of poly(I:C) injections in pregnant dams were completed (see 2014 Progress Report for detailed results).

Aim II: To characterize the inflammation-mediated alterations of TRP metabolism effects on fetal brain neurochemistry and postnatal brain structure. The effect of maternal inflammation induced in mice by poly(I:C) on fetal brain tissue concentrations of TRP and its metabolites generated through the IDO1/TDO2 (kynurenine) and TPH1 (5-HT) pathways were assessed by HPLC (Aim 2A). The consequences on thalamocortical and serotonergic neurons and axon pathway formation in the fetal brain were investigated by IHC (Aim 2B).

For clarity, we provide below a reminder of the major tasks that support the proposed specific aims (Aim II listed above, which was pursued during the second year of this award). For each task the methodology used, problems encountered and results obtained to date are described.

Task 1: *To determine whether maternal inflammation alters placental synthesis and fetal exposure to 5-HT and kynurenine-pathway compounds.*
Task 1a. *Changes in ex vivo placental output of 5-HT and kynurenine-pathway compounds following induction of maternal inflammation using the viral mimic RNA poly(I:C) at E12.*

These tasks were partially completed by focusing on poly(I:C) effects at 2 time points. We performed the proposed measures 24 and 48h after induction of maternal inflammation. We measured the impact on TPH1 enzymatic activity in the placenta and fetal brain, as well as measured the impact on placental 5-HT output in *ex vivo* perfused placentas harvested from control and poly(I:C)-injected dams.

Methodology: Poly(I:C) (2 mg/Kg) intraperitoneal injections were used to induce maternal inflammation in pregnant CD-1 mice at E12; saline (0.9% sterile solution) injections were used as control. For each time point, 3 to 5 dams were used. 24 or 48 hours after injection pregnant mice were anaesthetized through inhalation of isoflurane gas and sacrificed by cervical dislocation. Briefly, a single placenta per dam was harvested and quickly transferred to a thermostatically controlled incubation chamber at 37 °C. The uterine artery was cannulated with a 200 µm diameter catheter, and perfused at 20 µl/minute with Dulbecco's Modified Eagle Medium (DMEM; Life Technologies, cat. no. 11054-001), containing 200 µM L-tryptophan and 100 µM BH4. The umbilical artery was cannulated with a 105 µm diameter catheter and perfused at 5 µl/minute with DMEM. The eluate was collected from the umbilical vein for 90 minutes, and analyzed for 5-HT concentration with HPLC. See (*1*) for a detailed protocol.
The HPLC analysis of perfusion samples was performed on an Eicom 700 system (Eicom Corporation, Kyoto, Japan) consisting of an ECD-700 electrochemical detector, an Eicom 700 Insight autosampler, and Envision integration software. An Eicompak SC-3ODS 3 µm C18 reversed-phase column (3.0 x 100 mm I.D.) analytical column was used for separation. Samples were extracted in a 1:1 volume of 0.2 M perchloric acid with 100 µM EDTA, and a volume of 10 µL was injected into the column. The mobile phase used for separation consisted of 0.1M citric acid, 0.2M sodium phosphate dibasic, 7% methanol (JT Baker, cat. no. 9093-03; Center Valley, PA), and 5 mg EDTA in ultrapure water at a flow rate of 450 µL/minute. Unless otherwise noted, all reagents were purchased from Sigma-Aldrich.

Problems encountered in accomplishing tasks 1 & 1a: none.

Results:

1) As indicated in last year's progress report, 24h and 48h after poly(I:C)-induced inflammation, qRT-PCR analysis revealed significant effects on TRP metabolic gene expression in the placenta. Placental expression of monoamine oxidase A (*Maoa*), *Ido1* and *Tph1* genes were significantly up-regulated 24h after poly(I:C) injection. Interestingly, the levels and patterns of changes in gene expression were much different 48h after poly(I:C) injections when compared to 24h. *Maoa* gene expression was not significantly different from control level, whereas *Ido1* gene expression was still significantly elevated and *Tph1* gene expression was significantly reduced. There was no significant effect of treatment on any TRP metabolic gene expression (i.e. *Maoa, Ido1, Tph2*) in the fetal brain at either time point.

To examine whether altered gene expression ultimately impacts the placental conversion of maternal TRP to 5-HT, we directly measured TPH1 enzymatic activity in placentas harvested from a subset of the same litters. In these assays, placental extracts were incubated with TRP and TPH1 cofactors for 30 minutes. As an index of TPH1 enzymatic activity, the concentration of neo-synthesized 5-HTP - the first metabolic product, and rate-limiting step for the conversion of TRP to 5-HT - was quantified by HPLC. TPH1 enzymatic activity was significantly up-regulated in the placenta 48h after treatment with poly(I:C) when compared to saline injected dams ($p=0.0116$; Figure 1A). Similar enzymatic assays performed on the corresponding fetal brains revealed that in contrast to the placenta, maternal poly(I:C) treatment did not affect TPH2 enzymatic activity in the fetal hindbrain (**Figure 1A**).

To determine whether the increase in placental TPH1 enzymatic activity affects placental 5-HT synthesis and delivery to the fetus, we measured the real-time 5-HT output of *ex vivo* perfused placentas from saline and poly(I:C) injected dams. Forty-eight hours after exposure to poly(I:C), placentas were harvested from pregnant dams and re-perfused *ex vivo* with a cell culture medium supplemented with TRP and TPH1 cofactors. We observed a significantly increased ($p=0.020$) concentration of 5-HT released into the umbilical vein when comparing placentas from poly(I:C) exposed dams to placentas from saline exposed dams (**Figure 1B**). There was no significant difference between treatment groups for the tissue concentration of 5-HT remaining in the organ at the end of the perfusion. When taken together, these results demonstrate that a relatively mild maternal inflammation induces a cascade of genetic and enzymatic changes within the placenta that ultimately result in increased 5-HT output to the fetus.

Conclusions for Tasks 1 & 1a.

As previously reported (see 2014 Progress Report), the results show that maternal immune activation by poly(I:C) treatment induces a rapid increase of TRP metabolic gene expression in the placenta. The increase in placental *Ido1* gene expression in response to maternal immune activation is consistent with previously published studies (*2–5*) and was expected to lead to increased conversion of maternal TRP to kynurenine. Maternal inflammation should therefore elevate placental output of kynurenine to the fetus, which is in fact what we observed when measuring fetal brain kynurenine concentration (see previous report). Surprisingly, the results also show that placental *Tph1* gene expression is rapidly upregulated by maternal immune activation (in the first 24h of treatment). This initial rapid increase in placental *Tph1* gene expression leads to a delayed (48h post-injection) increase in TPH1 enzymatic activity. This is expected to increase placental output of 5-HT to the fetus. We directly tested this possibility in Task 1a using *ex vivo* dual perfusions of immune-activated placentas. Concomitantly to the

increased enzymatic activity, a significant increase of placental 5-HT output was measured following poly(I:C) exposure (**Figure 1B**).

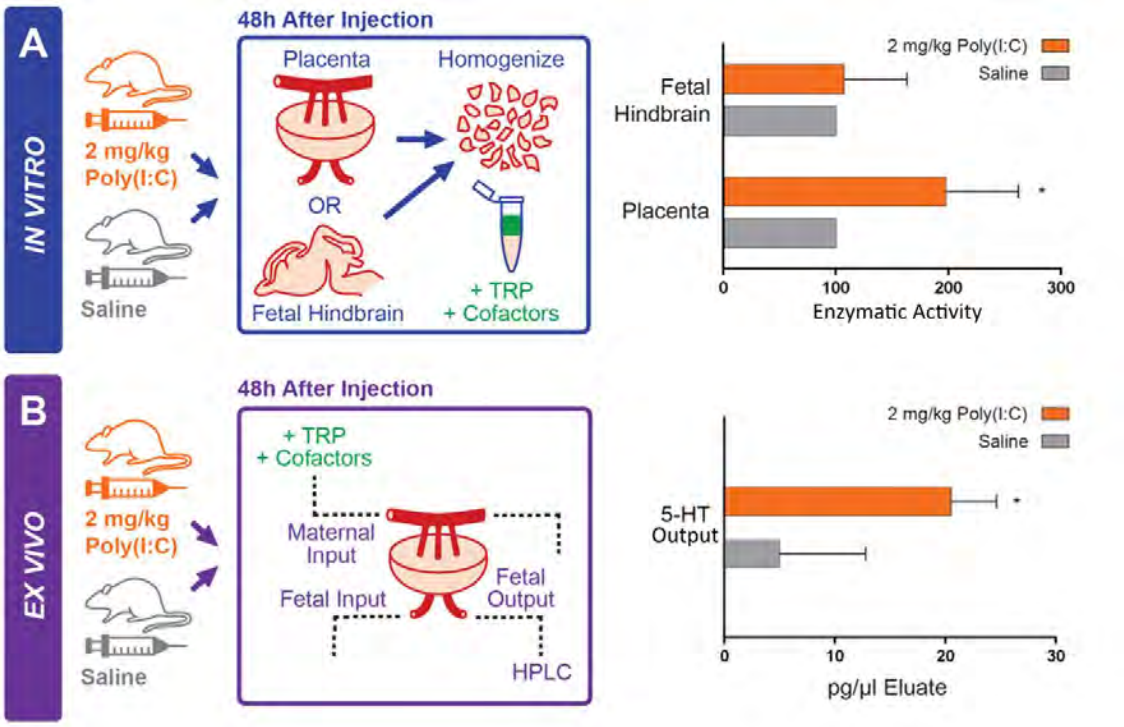

Figure 1: Effect of maternal immune activation on placental TPH1 enzymatic activity and *ex vivo* 5-HT output to the fetus. (A) 48h after poly(I:C) injections (2 mg/kg) at E12, placentas and fetal hindbrain were harvested, proteins extracted and TRP to 5-HTP conversion (enzymatic activity of TPH1: placenta; TPH2: hindbrain) was measured over a 30 min incubation time. Maternal immune activation induces a significant increase in TPH1 activity over control level (measured in placental extracts from saline-injected dams), but not of TPH2 in the fetal hindbrain. (B) 48h after poly(I:C) injections (2 mg/kg) at E12, a single placenta was quickly harvested, and its vasculature was reconnected inside of an ex vivo placental perfusion chamber. Cell culture medium supplemented with the 5-HT precursor tryptophan (TRP) and the TPH1 cofactor tetrahydrobiopterin, was perfused through the uterine artery into the placenta. The umbilical artery was perfused with basic culture medium, and the output from the umbilical vein was collected and analyzed by HPLC. Significantly more 5-HT is synthesized and released from the placenta following 2 mg/kg poly(I:C) exposure when compared to saline exposure. N = 4 dams (3 tissue samples pooled per dam) per treatment for the in vitro assay; n = 4 dams (1 placenta per dam) for the saline treatment and n = 3 dams for the poly(I:C) treatment for the ex vivo assay. *, p< 0.05. Error bars represent standard error of the mean percent change (A) or standard deviation (B). Statistical significance was determined using individual unpaired t-test comparisons, with the Holm-Sidak correction for multiple comparisons. α=0.05.

Task 2: *To characterize the inflammation-mediated alterations of TRP metabolism effects on fetal brain neurochemistry and postnatal brain structure.*

Task 2a. *The effect of maternal inflammation induced on fetal brain tissue concentrations of TRP and its metabolites generated through the IDO1 (kynurenine) and TPH1 (5-HT) pathways.* This task was completed (see 2014 Progress Report for detailed results).

Task 2b. *The consequences on 5-HT receptors expression, on thalamocortical and serotonergic neurons and axon pathway formation in the fetal and postnatal brain will be investigated by IHC and in situ hybridization.*

Task 2b was partially completed by focusing on maternal inflammation effects on serotonergic axon pathway formation, as well as neurogenesis throughout the fetal forebrain.

Methodology: Pregnant CD-1 dams were obtained from Charles-River laboratories (San Diego, CA), and allowed to acclimate to the vivarium. Saline (0.9% sterile solution; BD 297753) or 2 mg/kg poly(I:C) (Sigma-Aldrich P9582; St Louis, MO) intraperitoneal (i.p.) injections were performed at E12 (copulatory plug date is considered E1) using 5 µl per gram per pregnant dam. Dams were randomly assigned to each treatment group and time point. 24 or 48h after injection, pregnant dams were anaesthetized through inhalation of isoflurane gas (Western Medical Supply; Arcadia, CA) and sacrificed by cervical dislocation. The uterus was immediately dissected, and the resulting embryos were placed on ice in 1x phosphate buffered saline (PBS). The placenta, forebrain, and hindbrain (a precollicular coronal bisection was made to separate the forebrain + midbrain (termed forebrain) from the hindbrain) were removed, weighed, snap-frozen in liquid nitrogen, and stored at -80 °C until processing. For each embryo, the position in the uterus was recorded and a small tail biopsy was collected for sex determination by SRY genotyping. All procedures involving animals were approved by the Institutional Animal Care and Use Committee.

Fetal brains were dissected in ice cold PBS, immediately transferred to 4% paraformaldehyde (PFA) upon extraction, and incubated at 4 °C for 24 hours. The fetal brains were then incubated in 10%, 20%, and 30% sucrose (dissolved in PBS) for 24 hours each at 4 °C. Following incubation, the brains were embedded in cryomolds on dry ice using tissue tek (VWR, 25608-930; Radnor, PA) and stored at -80 °C until sectioning. Embedded brains were removed from the -80 °C freezer the night before sectioning, and allowed to warm to -20 °C. Fetal brains were sectioned coronally with a thickness of 20 µm, and sectioned tissue was stored at -80 °C until immunohistochemical analysis. Sections were permeabilized with 0.1% Triton X-100 in 2% fetal bovine serum in PBS, and incubated overnight at 4 °C with primary antibodies: rabbit anti-5HT (Final concentration 58 µg/ml; Sigma S5545, St. Louis, MO), rabbit anti-histone H3 phospho-s10 antibody (final concentration 5 µg/ml; abcam ab5176, Cambridge, MA). After extensive washing in PBS with 0.1% Triton X-100, slides were incubated at room temperature for 2 hours with secondary antibody: Rhodamine Red-X conjugated donkey anti-rabbit (1:800; Jackson ImmunoResearch Laboratories). Images were acquired with a Zeiss AxioCam MRm camera (Carl Zeiss, USA) using Zeiss AxioVision 4.8.2 software (Zeiss). 5-HT fluorescence intensity distribution was quantified using ImageJ (NIH, USA). In every section, the fluorescent intensity was normalized to the background.

Statistical Analyses

In all experiments, dams were treated as individual experimental groups (1 dam corresponds to n=1), and fetuses (n=3 to 5 per dam) as biological repeats. Hence, unless otherwise noted, for a single experimental data point multiple fetuses were pooled pre or post-analysis and counted as single data point. At each time point, the effects measured in treated vs untreated groups were compared using two-way ANOVA with correction for multiple comparisons. All analyses were performed using GraphPad Prism 6 (GraphPad Software, La Jolla, CA).

Problems encountered in accomplishing task 2b: none.

Results:

In order to test if the elevation of forebrain 5-HT tissue concentration measured in embryos from poly(I:C)-treated dams could result from increased endogenous serotonergic axon outgrowth into the region, we used immunohistochemistry to systematically measure serotonergic (5-HT immunoreactive, 5-HT+) axon density throughout the rostro-caudal extent of the fetal forebrain 48h after maternal poly(I:C) or saline injection at E12 (**Figure 2A**). Surprisingly, when comparing

sets of sections at matching rostro-caudal levels, densitometric analysis of fluorescence distribution showed a significant decrease of 5-HT+ axons density in the rostral 2/3rds of the forebrain in the poly(I:C)-treated group compared to saline (**Figure 2B**). The overall 5-HT+ axon density over the entire rostro-caudal axis was also significantly decreased in the poly(I:C)-treated group compared to saline (p=0.0009).

We next investigated whether other neurogenic processes modulated by 5-HT are impacted by maternal inflammation. Around the time-period chosen for maternal immune challenge (E12-14), active neuronal progenitor cell proliferation is taking place throughout the fetal brain (*6–8*). Since 5-HT signaling affects cell proliferation and maternal inflammation increases fetal forebrain 5-HT concentration during this time period (see 2014 Progress Report and **Figure 1**), we quantified the number of dividing basal progenitor cells in the ventricular/sub-ventricular zones (S/VZ) of several highly-proliferative regions (cortex, hippocampus, hypothalamus and thalamus). IHC for the M-phase marker phosphorylated histone H3 (PH3) was used to label and quantify the number of dividing progenitors throughout the rostro-caudal extent of the forebrain in E14 embryos (**Figure 2C**) harvested from dams injected at E12 with saline or poly(I:C). Statistical analysis of the distribution of PH3+ cell numbers at matching rostro-caudal levels showed a significant increase, restricted to the caudal part of the thalamus S/VZ, in the poly(I:C)-treated compared to saline-treated group (**Figure 2D**). There was also an overall difference in the number of PH3+ cells over the entire rostro-caudal extent of the thalamus S/VZ in the poly(I:C) treated group (p=0.0008). In contrast, no significant difference (overall or distribution) was observed in the other areas, including the cortex (**Figure 2E**), hypothalamus, or hippocampus (**Figure 3**).

Conclusions for Task 2b.

These data show that the elevated fetal forebrain 5-HT tissue concentration measured at E14 does not result from increased 5-HT axons growth in the region, but rather from increased placental 5-HT output. In addition, the results suggest that increased extracellular 5-HT during this period may inhibit serotonergic axon outgrowth, which is consistent with previous *in vitro* studies (*9*). Indeed, previous studies have suggested that subsets of 5-HT autoreceptors expressed either on dorsal raphe 5-HT neuron cell bodies or axons provide an intrinsic feedback mechanism, whereby extracellular 5-HT concentrations can regulate 5-HT axon outgrowth (*10*). Furthermore, it was demonstrated that 5-methoxytryptamine, a non-specific 5-HT receptor agonist, induced stunted axonal outgrowth when applied to dissociated raphe nuclei cells (*10*, *11*). This suggests that blunted rostral serotonergic axon outgrowth observed after maternal immune challenge may be a direct consequence of elevated concentrations of exogenous placental 5-HT reaching the forebrain.

Increased extracellular 5-HT has been previously shown to affect proliferation of various cell types by acting through a subset of 5-HT receptors (*12–15*). In particular, 5-HT signaling through the 5-HT1A (htr1a) receptor appears important for this process since 5-HT1A-specific receptor agonists and antagonists are sufficient to increase or decrease (respectively) neuronal progenitor cell proliferation (*12*, *16*). Given the observed increased concentration of 5-HT in the fetal forebrain following maternal inflammation, we hypothesized that cell proliferation would be affected in brain regions exhibiting high expression levels of 5-HT1A receptor. Previous work has shown that the 5-HT1A receptor is abundantly expressed from E12 to E14 in the thalamic ventricular zone in a caudal-high to rostral-low gradient (*17*). Paralleling these observations, the current results show that cell proliferation is increased following maternal inflammation specifically in and around the thalamic ventricular zone in a caudal-high to rostral-low fashion. This suggests that mild maternal inflammation impacts thalamic neuronal proliferation in the

fetus, potentially through increased 5-HT1A receptor signaling as a result of elevated placenta-derived 5-HT. Other important neurodevelopmental parameters, involving signaling through different receptor subtypes, may be specifically affected by the inflammation-mediated increase in fetal forebrain 5-HT. In particular, previous research demonstrated that extracellular 5-HT modulates thalamocortical axon guidance and circuit formation (*18*), through 5-HT1B/1D receptors which are also expressed in the fetal brain during this time period (*17*). The impact of maternal inflammation on the development of this axonal pathway is currently under investigation.

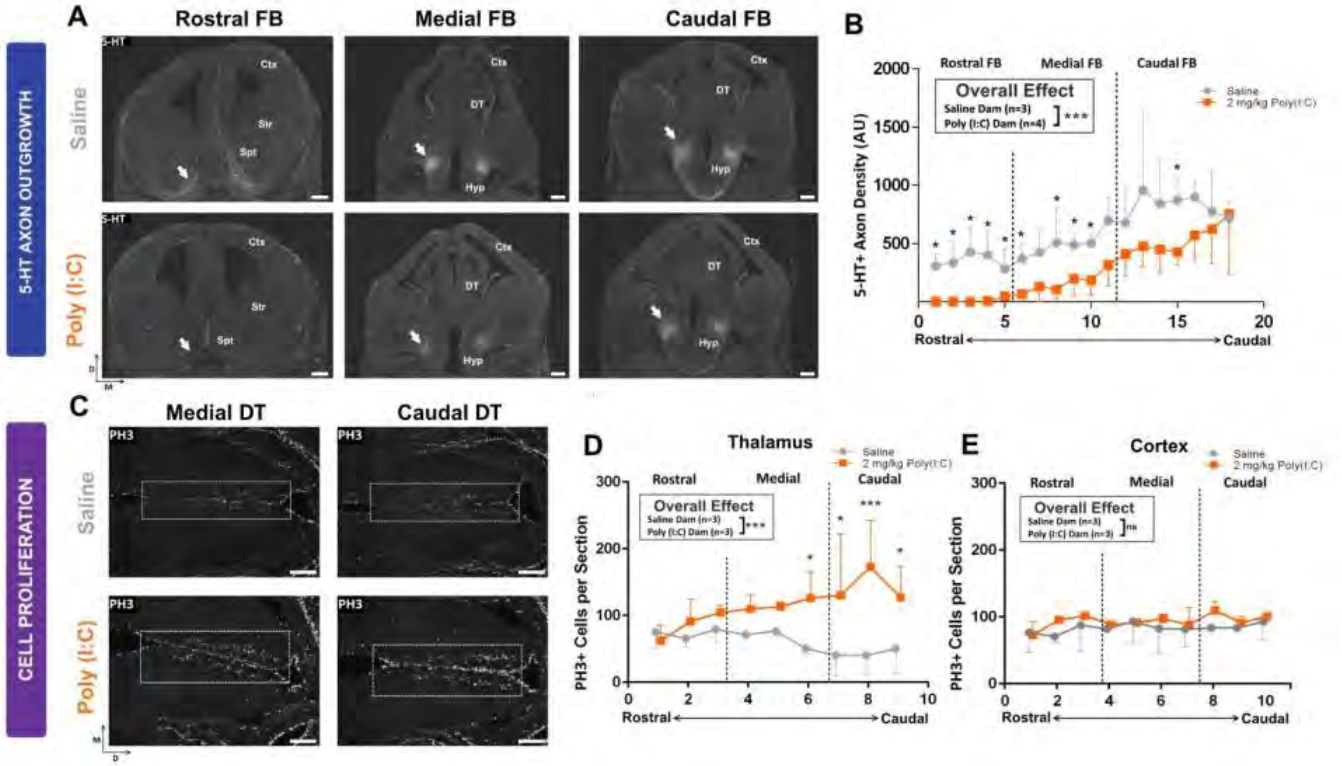

Figure 2: Maternal inflammation disrupts specific aspects of fetal neurodevelopment. (A) Immunohistochemical analysis of serotonergic axons in fetal brains taken from dams 48h after exposure to either saline or 2 mg/kg poly(I:C) reveals blunted outgrowth of serotonergic axons in a caudal to rostral gradient within the fetal forebrain when comparing saline to poly(I:C) treatments. (B) Quantification of 5-HT+ axons density (normalized fluorescence intensity) throughout the rostro-caudal extent of E14 fetal forebrains obtained from saline or poly (I:C) treated dams 48h after exposure. ***, p<.001; Mann-Whitney U test (overall effect: section versus experimental conditions). Individual section comparisons were performed between the two experimental conditions using the Fisher LSD post hoc test *, p< 0.05. (C) Analysis of PH3+ immunofluorescence in fetal brains taken from dams 48h after exposure to either saline or 2 mg/kg poly(I:C) reveals differential effects on cell proliferation in specific ventricular / subventricular (S/VZ) regions of the brain. The number of PH3+ progenitor cells in the thalamus S/VZ is increased following poly(I:C) treatment in a rostral to caudal gradient. In other brain regions, including the cortex, cell proliferation remains unaffected. (D and E) Quantification of PH3+ progenitor cell number in thalamic and cortical S/VZs throughout E14 fetal forebrains obtained from saline or poly (I:C) treated dams 48h after exposure. D, Dorsal; M, Medial; Ctx, Cortex; Str, Striatum; Spt, Septum; DT, dorsal thalamus; Hyp, hypothalamus. N = 3 dams per treatment (1 fetal brain analyzed per dam). ***, p<.001; ns, not significant, Mann-Whitney U test (overall effect: PH3+ cell number per section versus experimental conditions). Individual section comparisons were performed between the two experimental conditions using the Fisher LSD post hoc test (*, p < 0.05; ***, p<.001). Error bars represent standard deviation. Scale bars, 200 µm.

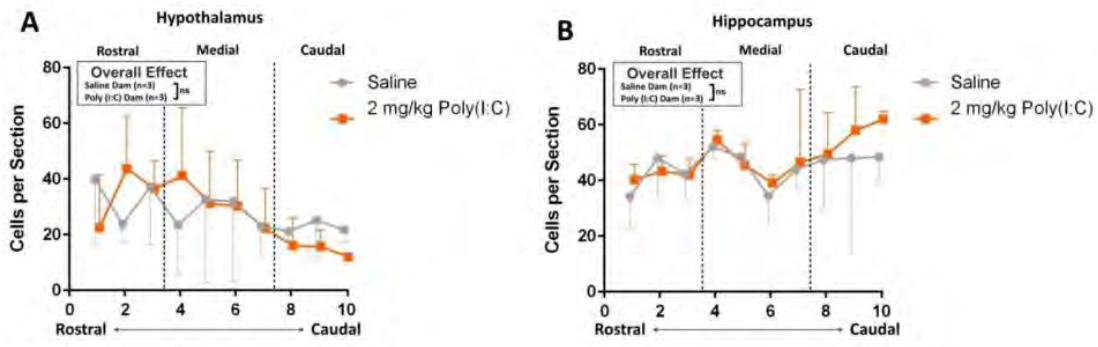

Figure 3: Cell proliferation in the hypothalamus and hippocampus. Analysis of PH3+ immunofluorescence distribution in fetal brains taken from dams 48h after exposure to either saline or 2 mg/kg poly(I:C) reveals no difference in cell proliferation in ventricular / subventricular (S/VZ) regions of the hypothalamus (A) and hippocampus (B). N = 3 dams per treatment (1 fetal brain analyzed per dam). ns, not significant; Mann-Whitney U test (overall effect: PH3+ cell number per section versus experimental conditions). Individual section comparisons were performed between the two experimental conditions using the Fisher LSD post hoc test. Error bars represent standard deviation.

Aim III: <u>To determine if genetic or pharmacological manipulations of placental TRP metabolic pathways *in vivo* can reduce inflammation effect on offspring brain development.</u> We propose that the severity of maternal inflammation effects on offspring brain development depends on the relative and absolute flux of TRP through the kynurenine and 5-HT pathway. We will investigate whether pharmacologically altering TRP metabolism during pregnancy (using *in vivo* injection of specific blockers for each pathway) can prevent or ameliorate inflammation-mediated effects on fetal and postnatal brain development (Aim 3A).
Task 3: *To determine if genetic or pharmacological manipulations of placental TRP metabolic pathways in vivo can reduce inflammation effect on offspring brain development.*

We started this task by co-injecting poly(I:C) and 4-Chloro-DL-phenylalanine methyl ester hydrochloride (pCPA; 10 μM) in pregnant dams at E12 and performing measures of 5-HT tissue concentration in the fetal forebrain. Preliminary results show that by blocking TPH1/2 enzymatic activity, pCPA prevents the inflammation-mediated increase in fetal forebrain 5-HT (**Figure 4**). Additional experiments are currently being performed in order to validate this result. In addition, we are in the process of analyzing the effects of this co-injection paradigm on serotonergic axon outgrowth and progenitor cell proliferation in the fetal forebrain, as described in Aim II, task 2b.

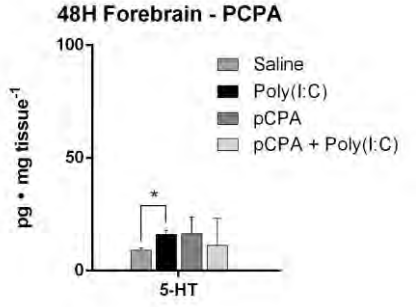

Figure 4: Effect of pCPA blocking of TPH activity on fetal forebrain 5-HT. HPLC analysis of 5-HT tissue concentration in fetal forebrains taken from dams 48h after exposure to either saline, 2 mg/kg poly(I:C), pCPA (10 μM) or pCPA + poly(I:C) reveals 1) the expected increase of fetal forebrain 5-HT following poly(I:C) exposure and 2) that blocking TPH1/2 activity prevent this effect. N = 2 dams per condition, 3 fetal forebrains pooled per dam.

KEY RESEARCH ACCOMPLISHMENTS

- Demonstration that poly(I:C) injections at E12 trigger a rapid increase of TRP metabolic gene expression and enzyme activity specifically in the placenta which directly leads to increased output of 5-HT from the placenta to the fetal forebrain.
- Demonstration that the increase in fetal forebrain 5-HT concentration triggered by maternal immune activation alters serotonergic axon outgrowth, as well as neurogenesis, in the fetal forebrain.

REPORTABLE OUTCOMES

- A manuscript reporting the results was submitted to The Journal of Neuroscience in July of 2015 and is currently under review.
- Dr Bonnin presented some of the results at the IFPA 2014 meeting in Paris, France (Sept. 9th-12th, 2014), at the annual meeting of the Federation of European Neuroscience (FENS; Milan, Italy, July 2014) as well as at the International Neuroplacentology Meeting (online venue hosted by Children's National Center for Neuroscience Research, Washington DC, USA; March 2015).
- Based on the work supported by this award Nick Goeden, a graduate student in Dr Bonnin's laboratory and key personnel in this award, received a predoctoral fellowship awarded in July 2014 by the Autism Science Foundation.
- This work will also be presented in poster form at the Society for Neuroscience annual meeting (Chicago, USA; Oct. 2015).

CONCLUSION

In animal models, maternal inflammation during pregnancy causes behavioral, histological, and transcriptional changes in adult offspring that are consistent with those seen in human disorders such as ASD. Our project tests the specific hypothesis that maternal inflammation in pregnancy alters the two main pathways of placental tryptophan (TRP) metabolism, namely the serotonin (5-HT) and kynurenine pathway. So far, our findings indicate that a mild and acute maternal immune challenge disrupts the steady-state placental conversion of TRP to 5-HT upstream of the fetal brain, which is a molecular pathway by which maternal inflammation ultimately impacts specific aspects of fetal neurodevelopment (*19*, *18*, *20*, *21*). Importantly, although the simultaneous increase of TRP metabolic flux through both IDO1 and TPH1 pathways in the placenta in response to maternal inflammation provides an important immune-suppressive protection for the fetus, it also leads to increased fetal brain exposure to elevated concentrations of 5-HT. Increased ligand concentration at these early stages of pregnancy impacts important developmental processes in the fetal brain that are normally modulated by 5-HT. Previous studies have already demonstrated that a mild maternal inflammation triggered by low to moderate dose poly(I:C) exposure, similar to the challenge used in this project, have long-term effects on offspring brain function and behavior, including disruption of latent inhibition, spatial working memory impairment and reduced spatial exploration (*22*). To what extent the specific disruptions of serotonergic circuit formation and progenitor cell proliferation reported here contribute to these long-term alterations of offspring brain function require further characterization. Further studies on the effects of other inflammatory and infectious agents on placentally-derived modulators of neurodevelopment are also warranted. The progress made in the first two years of this award provides strong support to the hypothesis that alteration of placental TRP metabolism by maternal inflammation during early gestation constitutes a new molecular pathway for the fetal programming of neurodevelopmental disorders such as ASD.

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APPENDICES

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SUPPORTING DATA

Figures and tables were included in the body of this report.

Maternal Inflammation Disrupts Fetal Neurodevelopment via Increased Placental Output of Serotonin to the Fetal Brain

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Abstract

Maternal inflammation during pregnancy affects placental function and is associated with increased risk of neurodevelopmental disorders in the offspring. The molecular mechanisms linking placental dysfunction to abnormal fetal neurodevelopment remain unclear. During typical development, serotonin (5-HT) synthesized in the placenta from maternal L-tryptophan (TRP) reaches the fetal brain. There, 5-HT modulates critical neurodevelopmental processes. We investigated the effects of maternal inflammation triggered in mid-pregnancy in mice by the immunostimulant polyriboinosinic-polyribocytidylic acid [poly(I:C)] on TRP metabolism in the placenta, and its impact on fetal neurodevelopment. We show that a moderate maternal immune challenge rapidly upregulates placental TRP conversion to 5-HT, leading to accumulation of 5-HT specifically within the fetal forebrain. Simultaneously, blunting of 5-HT axonal outgrowth in the rostral forebrain is observed. These results establish altered placental TRP conversion to 5-HT as a new mechanism by which maternal inflammation disrupts 5-HT-dependent neurogenic processes during fetal neurodevelopment.

Significance Statement:

The mechanisms linking maternal inflammation during pregnancy with increased risk of neurodevelopmental disorders in the offspring are poorly understood. Here we show that maternal inflammation in mid-pregnancy upregulates of tryptophan conversion to serotonin within the placenta. Remarkably, this leads to exposure of the fetal forebrain to increased concentrations of this biogenic amine and to specific alterations of serotonin-dependent neurogenic processes. More specifically we found altered serotonergic axon growth resulting from increased 5-HT in the fetal forebrain. The data provide a new understanding of placental function playing a key role in fetal brain development, and

how this process can be altered by adverse prenatal events. The results uncover important future directions for understanding the early developmental origins of mental disorders.

Introduction

Mechanistic links between maternal inflammation during pregnancy and the risk for developmental disorders in the offspring, including autism spectrum disorders (ASD), cognitive delay, and schizophrenia are being intensively investigated (Bonnin and Levitt, 2011; Stolp et al., 2011). Intra-uterine bacterial infection was shown to be an independent risk factor for early autistic features (Limperopoulos et al., 2008), and systemic maternal viral infection (e.g. influenza) has been reported as a risk factor for ASD and schizophrenia in the offspring (Patterson, 2002, 2009). These associations are observed for both vertically (from mother to fetus) and non-vertically transmitted pathogens, suggesting that maternal inflammation resulting from exposure to these pathogens in and of itself is sufficient to alter fetal neurodevelopment. In support of this possibility, elevated concentrations of soluble pro-inflammatory and chemo-attractive cytokines in the maternal serum have been linked to neurodevelopmental disorders in the offspring (Bell and Hallenbeck, 2002). Studies in rodents have demonstrated that systemic exposure to high doses of the immunostimulant poly(I:C) induces a strong and sustained elevation in several pro-inflammatory cytokines, notably interleukin-6 (IL-6), in the maternal serum. This dramatic induction of the maternal inflammatory pathway alters several neurodevelopmental processes and results in abnormal adult behavior in the offspring (Patterson, 2002, 2009; Wang et al., 2009). Intrauterine inflammation is observed in approximately 20% of all pregnancies, and a staggering 85% of very premature births, the latter of which has also been associated with a number of neurodevelopmental disorders (Elovitz et al., 2011). This highlights the need to investigate the causal mechanisms underlying maternal inflammation and fetal neurodevelopment.

During pregnancy, a fraction of the maternal TRP is converted to 5-HT through the placental tryptophan hydroxylase (TPH1) enzymatic pathway, providing an exogenous source of 5-HT to the fetus. Prenatally, 5-HT modulates key neurodevelopmental processes, including axonal circuit formation (Bonnin et al., 2011; Goeden and Bonnin, 2012). Another important placental TRP metabolic pathway is the conversion of TRP to kynurenine (KYN) by indoleamine 2,3-dioxygenase (IDO1). This pathway protects the developing fetus from the maternal immune system at the early stages of pregnancy (Munn et al., 1998; Mellor and Munn, 2001). While effects of IDO1 induction on the KYN pathway have been studied, effects of maternal inflammation on placental 5-HT synthesis and delivery to the fetus have not been investigated. Although moderate induction of the IDO1 and TPH1 pathway during pregnancy is important from an immunosuppressive (countering maternal rejection) standpoint, altered 5-HT output to the fetus may significantly impact neurodevelopment.

Here we used a well-established prenatal immune challenge in mice to study the effects of maternal inflammation in mid-pregnancy on TRP metabolism in the placenta, and the downstream consequences on fetal brain development. Moderate doses of poly(I:C) were used to induce a mild maternal immune challenge which was previously shown to induce significant long-term brain and behavioral deficits in the adult offspring (Meyer et al., 2005; Meyer and Feldon, 2012). We found that mild maternal inflammation during pregnancy leads to a transient upregulation of *Tph1* gene expression, which results in increased TPH1 enzymatic activity in the placenta and increased placental 5-HT output to the fetus, and ultimately increased 5-HT tissue concentration specifically within the fetal forebrain. Simultaneously, serotonergic axon outgrowth was disrupted specifically in this region, suggesting that fetal brain development is highly sensitive to maternal inflammation through disruptions of placental TRP metabolism to 5-HT.

Methods and Materials

Animals and Maternal Immune Activation

Pregnant CD-1 dams (Charles-River laboratories) were injected intraperitoneally (i.p.) at E12 (copulatory plug date = E1) with saline (0.9% sterile solution; BD 297753) or 2 mg/kg poly(I:C) (Sigma-Aldrich P9582) using 5 µl per gram per pregnant dam. Dams were anesthetized and fetal forebrain, hindbrain, placenta, and serum harvested as previously described (Bonnin et al., 2011). All procedures involving animals were approved by the Institutional Animal Care and Use Committee.

qRT-PCR Analysis

Brain and placenta tissue samples fresh-frozen in liquid nitrogen were ground into a fine powder using a mortar and pestle cooled with liquid nitrogen. Three fetal tissue samples from each dam were pooled, and RNA was extracted and processed in triplicate as described previously (Nolan et al., 2006) to analyze T*ph1* (Mm01202614_m1)*, Tph2* (Mm00557715_m1)*, Ido1* (Mm00492586_m1)*, Maoa* (Mm00558004_m1)*,* and reference gene TATA box binding protein (*Tbp*) (Mm00446973_m1). Completed reactions were analyzed using the 2(-Delta Delta C(t)) method.

In Vitro 5-hydroxytryptophan (5-HTP) Synthesis Assays

Harvested tissues (placenta, fetal forebrain and hindbrain) were analyzed according to previously published techniques (Bonnin et al., 2011).

HPLC Analysis

The HPLC analysis of perfusion samples was performed on an Eicom 700 system (Eicom Corporation) consisting of an ECD-700 electrochemical detector, an Eicom 700 Insight autosampler, and Envision

integration software. An Eicompak SC-3ODS 3 µm C18 reversed-phase column (3.0 x 100 mm I.D.) analytical column was used for separation. Samples were extracted in a 1:1 volume of 0.2 M perchloric acid with 100 µM EDTA, and a volume of 10 µL was injected into the column. The mobile phase used for separation consisted of 0.1M citric acid, 0.2M sodium phosphate dibasic, 7% methanol (JT Baker, cat. no. 9093-03), and 5 mg EDTA in ultrapure water at a flow rate of 450 µL/minute. For measures of biogenic amine concentrations in fetal tissues, see (Hervé et al., 1996; Brand and Anderson, 2011) for detailed methodology.

Cytokine Analysis

The maternal and placental inflammatory response to poly(I:C) or saline was quantified in every mouse according to previously published techniques (Lund et al., 2004). Tissue concentrations were measured in the maternal serum, placenta and fetal brains for the following cyto/chemokines: CCL2-5, CXCL1, CXCL9, IL-1a/b, IL-2, IL-4, IL-6, IL-10, IL-17, IL-23, IFNɣ, TNFα.

Ex-vivo Placental Perfusion 5-HT Synthesis Assay

See (Goeden and Bonnin, 2012) for detailed methodology

Neurodevelopmental Assays

Fetal brains were sectioned throughout the entire rostro-causal axis. Sections were processed for anti-5HT immunostaining (anti-5-HT antibody, final concentration 58 µg/ml; Sigma S5545) and analyses of fluorescent staining intensity were performed according to previously published techniques (Bonnin et al., 2007, 2011).

Statistical Analyses

In all experiments, dams were treated as individual experimental groups (1 dam, n=1), and fetuses (n=3 to 5 per dam) as biological repeats. For cytokine analysis, a large panel of cyto- and chemokines were measured, and at each time point the effects in treated vs untreated groups were compared using two-way ANOVA with correction for multiple comparisons. In all other experiments, individual outcomes in treated and untreated groups were compared using repeated-measures t test or Mann-Whitney *U* test, and all comparisons were planned prior to analysis of the data. Where appropriate, post-hoc correction for multiple comparisons was utilized. All analyses were performed using GraphPad Prism 6 (GraphPad Software).

Results

Mild inflammation increases placental TRP metabolism and 5-HT delivery to the fetus

Pregnant dams were injected at E12 with 2 mg/kg poly(I:C) or saline solution (control), and the maternal, placental, and fetal inflammatory response was quantified in every mouse at time of harvesting (i.e. E12 + 24 or 48h). Of all the cytokines tested, only IL-6 was significantly different in the maternal serum of poly(I:C) treated dams compared to controls (Two-way ANOVA, $F_{(2,15)}$=13.54, p=0.0004; Bonferroni post-tests: control vs poly(I:C), p<0.0001), and only at the 24h time point. Importantly, despite the increased concentration of IL-6 in the maternal serum following poly(I:C) exposure, there was no change in fetal brain or placenta levels at 24 or 48 hours (Figure 1A, B). QRT-PCR analysis revealed significant effects of maternal inflammation on TRP metabolic gene expression in the placenta, i.e. monoamine oxidase A (*Maoa*), *Ido1* and *Tph1* gene expression was significantly up-regulated 24h after poly(I:C) injection (Figure 1C). The most significant increase over basal expression measured in controls obtained from saline injected dams was observed for *Ido1* (*t* test with repeated-measures, $t_{(6)}$=43.4, p=8.9E-10), followed by *Tph1* gene expression ($t_{(6)}$=11.6, p=7.7E-6) and *Maoa* ($t_{(6)}$=5.8, p=6.3E-4). The changes in

gene expression were different 48h after poly(I:C) injections, i.e. *Maoa* gene expression was not significantly different from control level, whereas *Ido1* gene expression was still significantly elevated ($t_{(6)}$=7.0, p=2.1E-4) and *Tph1* gene expression was significantly reduced ($t_{(6)}$=6.9, p=2.2E-4) (Figure 1D). There was no significant effect of treatment on any TRP metabolic gene expression (i.e. *Maoa, Ido1, Tph2*) in the fetal brain at either time point (not shown).

To examine whether altered gene expression ultimately impacts the placental conversion of maternal TRP to 5-HT, we directly measured TPH1 enzymatic activity in placentas harvested from a subset of the same litters. TPH1 enzymatic activity was significantly up-regulated in the placenta, but not fetal brain, 48h after treatment with poly(I:C) when compared to saline (Figure 2A; $t_{(5)}$=3.87, p=0.0116).

To determine whether the increase in placental TPH1 enzymatic activity affects placental 5-HT synthesis and delivery to the fetus, we measured the real-time 5-HT output of *ex vivo* perfused placentas from saline and poly(I:C) injected dams. We observed a significantly increased concentration of 5-HT released into the umbilical vein when comparing placentas from poly(I:C) exposed dams to placentas from control dams (Figure 2B; $t_{(5)}$=2.7, p=0.020). These results demonstrate that a mild maternal inflammation induces a cascade of genetic and enzymatic changes within the placenta that result in increased 5-HT output to the fetus.

Fetal forebrain 5-HT rapidly increases after mild maternal immune activation

Consistent with previous findings that placental 5-HT synthesis impacts fetal forebrain 5-HT concentration (Bonnin et al., 2011; Goeden and Bonnin, 2012), there was a significant increase in fetal forebrain, but not hindbrain, 5-HT tissue concentration (Figure 3A, C; 24h forebrain: $t_{(6)}$=3.4 p=0.0353, 48h forebrain: $t_{(4)}$=4.8, p=0.0307) in embryos from poly(I:C) exposed dams. In contrast, fetal brain tissue concentrations of 5-HIAA, the primary metabolite of 5-HT, remained unchanged at both time points.

Poly(I:C) treatment also induced an elevation of KYN in the fetal brain at both time points compared to saline injected controls, although a statistically significant increase was observed only in the hindbrain at 24h ($t_{(6)}$=2.5, p=0.0464; Figure 3B) and forebrain at 48h ($t_{(4)}$=15.7, p=5.5E-4; Figure 3C). No significant differences were observed when comparing male to female embryos for any of the treatments at either time point.

Decreased 5-HT axon outgrowth in the fetal forebrain

In addition to increased placental output, the elevation of forebrain 5-HT tissue concentration measured in embryos from poly(I:C)-treated dams could result from increased endogenous serotonergic axon outgrowth into the region. In order to test this possibility, we used immunohistochemistry to systematically measure serotonergic (5-HT immunoreactive, 5-HT+) axon density throughout the rostro-caudal extent of the fetal forebrain 48h after maternal poly(I:C) or saline injection at E12 (Figure 4A). Surprisingly, when comparing sets of sections at matching rostro-caudal levels, densitometric analysis of fluorescence distribution showed a significant decrease of 5-HT+ axons density in the rostral 2/3^{rds} of the forebrain in the poly(I:C)-treated group compared to saline (Figure 4B). The overall 5-HT+ axon density over the entire rostro-caudal axis was also significantly decreased in the poly(I:C)-treated group compared to saline (Figure 4B; Mann-Whitney test, U=60, p=0.0009). This result shows that the elevation of 5-HT in the forebrain after mild inflammation is not due to increased serotonergic axon outgrowth into the region. Rather, the results all together suggest that changes in placental output of 5-HT after mild maternal immune activation lead to increased amount of 5-HT in the fetal forebrain that negatively impact serotonergic axon rostral outgrowth.

Discussion

Our results identify a novel molecular pathway by which maternal inflammation during pregnancy alters specific aspects of fetal brain development. We used a mild maternal immune challenge (2 mg/kg poly(I:C) administered i.p. at E12) which presents the following characteristics: 1) it mimics clinically common mild inflammation with transient cytokine elevations in the maternal serum (Giovanoli et al., 2013; Meyer, 2014), and 2) it induces significant long-term brain and behavioral deficits in the adult offspring (Meyer et al., 2005; Meyer and Feldon, 2012). Importantly, while the mild immune challenge induces the expected transient IL-6 increase in the maternal serum, there is no evidence of cytokine accumulation in the fetal brain tissue (Figure 1). This suggests that mild sub-clinical maternal inflammation primarily involves indirect effects on the fetal brain (Bergström, 2003; Tincani et al., 2005; Seong et al., 2008; Romero et al., 2014) which may be mediated in part by placental cytokine and toll-like receptors (Girard et al., 2010).

Consistent with an indirect action of maternal inflammation on the fetal brain through alteration of placental function, we found that low dose poly(I:C) exposure affects placental TRP metabolism directly upstream of the fetus. The increase in placental *Ido1* gene expression in response to inflammation demonstrated here is consistent with previously published studies (Shayda et al., 2009; Wang et al., 2010) and is critical for the localized suppression of the maternal immune response in order to protect the semi-allogeneic fetus from rejection (Munn et al., 1998). It was hypothesized recently that such upregulation would lead to decreased TRP substrate availability for the competing TPH1 pathway, ultimately decreasing placental output of 5-HT to the developing fetal brain (Sato, 2013). Contrary to this hypothesis, our results demonstrate an increased flux of TRP metabolism through the TPH1 pathway following poly(I:C) exposure which coincides with increased placental 5-HT output to the fetus, elevated

5-HT concentrations in the fetal forebrain, and disrupted neurogenic processes. Importantly, the increase of forebrain 5-HT is not associated with a decrease in 5-HIAA concentration, indicating that it does not result from changes in MAOA activity. It can be concluded that placental TRP metabolism through both TPH1 and IDO1 enzymatic pathways is rapidly upregulated by a maternal immune challenge (24-48h post-treatment). Interestingly, placental *Tph1* gene expression is transiently elevated 24h and significantly decreased 48h post-treatment (Figure 1C, D), whereas TPH1 enzymatic activity remains significantly elevated (Figure 2A). This suggests that feedback control mechanisms between TPH1 protein activity and *Tph1* gene transcription allow the fine-tuning of placental 5-HT synthesis, similar to what was observed for TPH2 in the brain (Boadle-Biber et al., 1983; Gutknecht et al., 2009).

The data indicate that the elevation of fetal forebrain 5-HT concentration measured after the maternal immune challenge directly results from increased placental 5-HT output. First, maternal inflammation upregulates TPH1 enzymatic activity in the placenta, but not TPH2 activity in the fetal hindbrain (Figure 2A). Second, the increase of 5-HT tissue concentration is significant in the fetal forebrain, but not hindbrain, where endogenous 5-HT neuronal cell bodies and proximal axons are located. Third, immunohistochemical analysis shows that maternal inflammation induces a significant reduction, not increase, of 5-HT axons outgrowth specifically into the rostral forebrain (Figure 4B). This effect took place between E12 and E14, a period of particularly active serotonergic axon outgrowth into the rostral forebrain (Lidov and Molliver, 1982; Wallace and Lauder, 1983). It was shown that extracellular 5-HT concentrations can regulate 5-HT axon outgrowth through subsets of 5-HT autoreceptors expressed either on dorsal raphe 5-HT neuron cell bodies or axons (Daubert and Condron, 2010). This suggests that blunted rostral serotonergic axon outgrowth observed after maternal immune challenge may be a direct consequence of elevated concentrations of exogenous placental 5-HT reaching the forebrain.

Together, these findings indicate that a mild and acute maternal immune challenge disrupts placental conversion of TRP to 5-HT upstream of the fetal brain, which ultimately impacts specific aspects of fetal neurodevelopment (Figure 4A, B). Importantly, although the simultaneous increase of TRP metabolic flux through both IDO1 and TPH1 pathways in the placenta in response to maternal inflammation provides an important immune-suppressive protection for the fetus, it also leads to increased fetal brain exposure to elevated concentrations of 5-HT. Such increase in ligand concentration impacts important developmental processes in the fetal brain that are normally modulated by 5-HT at early stages of pregnancy. A mild maternal inflammation triggered by low dose poly(I:C) exposure, similar to the challenge used in the current study, was shown to have long-term effects on offspring brain function and behavior, including disruption of latent inhibition, spatial working memory impairment and reduced spatial exploration (Meyer and Feldon, 2012). To what extent the specific disruptions of serotonergic circuit formation reported here contribute to these long-term alterations of offspring brain function require additional characterization. Further studies on the effects of other inflammatory and infectious agents on placentally-derived modulators of neurodevelopment are also warranted.

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Figure Legends

Figure 1 | Maternal inflammation affects placental TRP metabolic gene expression. A, 24h after 2 mg/kg poly(I:C) injection at E12, there is a significant increase of the proinflammatory cytokine IL-6 in the maternal serum, but not placenta or fetal forebrain/hindbrain. **B,** 48 hours after poly(I:C) exposure, maternal serum IL-6 levels have returned to baseline. **C,** *Maoa*, *Ido1* and *Tph1* placental gene expression is significantly up-regulated 24h after poly(I:C) injection compared to saline. **D,** 48h after poly(I:C) injections, *Maoa* gene expression is not significantly different from control level, whereas *Ido1* gene expression is still significantly elevated and *Tph1* gene expression is significantly reduced. **A, B**: n=5 to 7 dams (3 fetal tissues per dam), two-way ANOVA, Sidak correction; α=0.05. **C, D**: n= 4 to 5 dams (3 placentas per dam), unpaired t-test, Holm-Sidak correction. *, $p< 0.05$ and **$p<0.005$. Error bars represent standard deviation.

Figure 2 | Maternal inflammation increases placental TPH1 enzymatic activity and *ex vivo* 5-HT output to the fetus. A, 48h after saline or poly(I:C) (2 mg/kg) injections at E12, TRP to 5-HTP conversion, which reflects enzymatic activity of TPH1 (placenta) or TPH2 (hindbrain), was measured in placentas and fetal hindbrains. Maternal inflammation induces a significant increase in TPH1 activity

over control level (measured in placental extracts from saline-injected dams), but not of TPH2 in the fetal hindbrain. **B**, *Ex vivo* placental perfusions show that significantly more 5-HT is synthesized and released from the placenta 48h after maternal poly(I:C) exposure when compared to saline exposure. N= 3 to 4 dams [(**A)** 3 or (**B**) 1 placenta per dam], unpaired t-test, Holm-Sidak correction. *, $p< 0.05$. Error bars represent standard error of the mean percent change (**A**) or standard deviation (**B**).

Figure 3 | Maternal inflammation leads to increased tryptophan metabolites tissue concentration in the fetal brain. Inflammation was induced by poly(I:C) injection (2 mg/kg) at E12. Fetal brain tissue concentration of 5-HIAA, 5-HT, and KYN were measured by HPLC. **A, B,** Maternal inflammation induces a significant increase of 5-HT in the fetal forebrain (**A**), but not hindbrain (**B**) 24h after injection. KYN concentration is significantly increased in the fetal hindbrain only. **C, D,** 48h after maternal poly(I:C) injection, 5-HT and KYN are significantly increased in the fetal forebrain (**C**), but not hindbrain (**D**). Brain 5-HIAA concentration is not significantly altered at any time point in both regions. N=3 dams per treatment (3-5 embryos per dam), unpaired t-test, Holm-Sidak correction. *, $p< 0.05$; **, $p<0.005$. Error bars represent standard deviation.

Figure 4 | Maternal inflammation disrupts fetal serotonergic axon outgrowth. A, IHC analysis of serotonergic axons in fetal brains 48h after maternal exposure to either saline or 2 mg/kg poly(I:C) reveals blunted outgrowth of serotonergic axons in a caudal to rostral gradient within the fetal forebrain when comparing saline to poly(I:C) treatments. **B,** Quantification of 5-HT+ axons density (normalized fluorescence intensity) throughout the rostro-caudal extent of E14 fetal forebrains obtained from saline or poly (I:C) treated dams 48h after exposure. D, Dorsal; M, Medial; Ctx, Cortex; Str, Striatum; Spt, Septum; DT, dorsal thalamus; Hyp, hypothalamus. N= 3 to 4 dams (3 fetal brains per dam), Mann-Whitney *U* test, Fisher LSD post hoc text. *, $p < 0.05$; **, $p<.005$; ns, not significant. Error

bars represent standard deviation. Scale bars, 200 μm. **C, D,** Schematic representation of the indirect effects of maternal inflammation on fetal brain development through tryptophan placental metabolism. **C,** The placenta metabolizes maternal tryptophan (TRP) to kynurenine (KYN) and serotonin (5-HT) through IDO1 and TPH1 enzymatic pathways, respectively. **D,** A mild maternal inflammation results in the elevation of IL-6 in the maternal blood, but not placenta and fetal brain. This immune challenge induces a rapid increase in TRP metabolism through placental TPH1 enzymatic pathway, resulting in a significant increase in 5-HT in the fetal forebrain. The data suggests that this elevation in fetal forebrain 5-HT during critical developmental periods directly impact 5-HT-dependent neurogenic processes such as serotonergic axonal circuit formation.

Figure 1

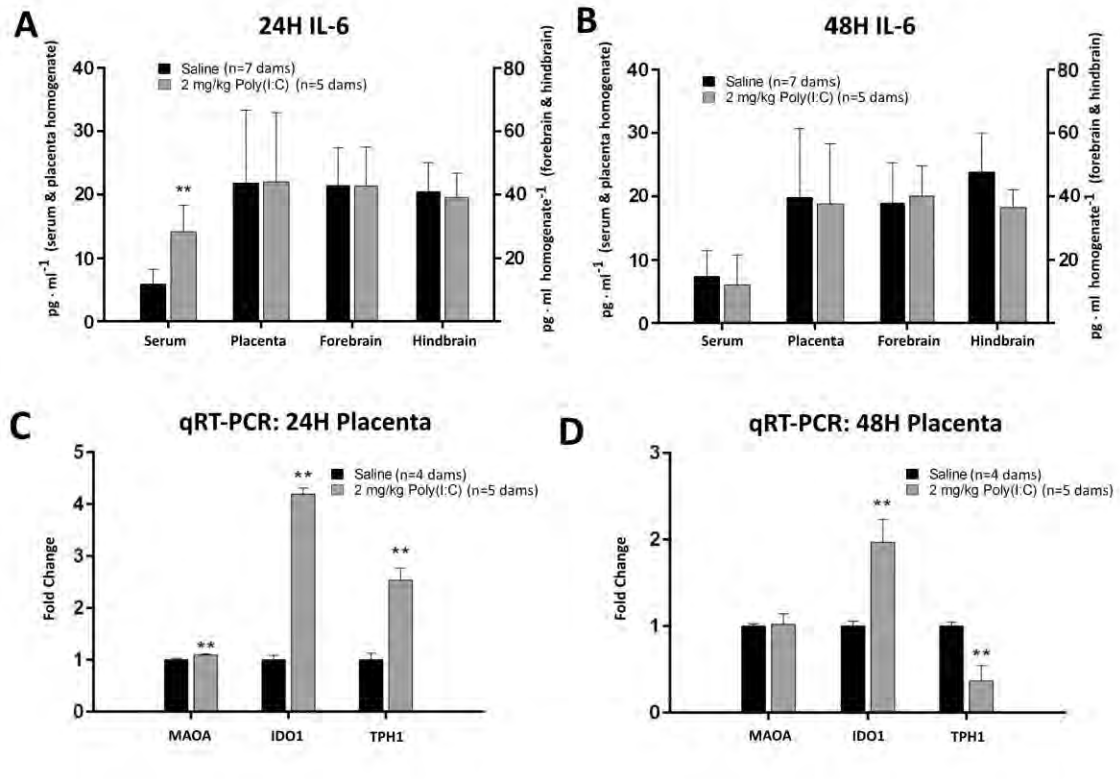

A
24H IL-6
Saline (n=7 dams)
2 mg/kg Poly(I:C) (n=5 dams)
pg · ml-1 (serum & placenta homogenate)
pg · ml homogenate-1 (forebrain & hindbrain)
Serum
Placenta
Forebrain
Hindbrain
B
48H IL-6
C
qRT-PCR: 24H Placenta
Saline (n=4 dams)
2 mg/kg Poly(I:C) (n=5 dams)
Fold Change
MAOA
IDO1
TPH1
D
qRT-PCR: 48H Placenta

Figure 2

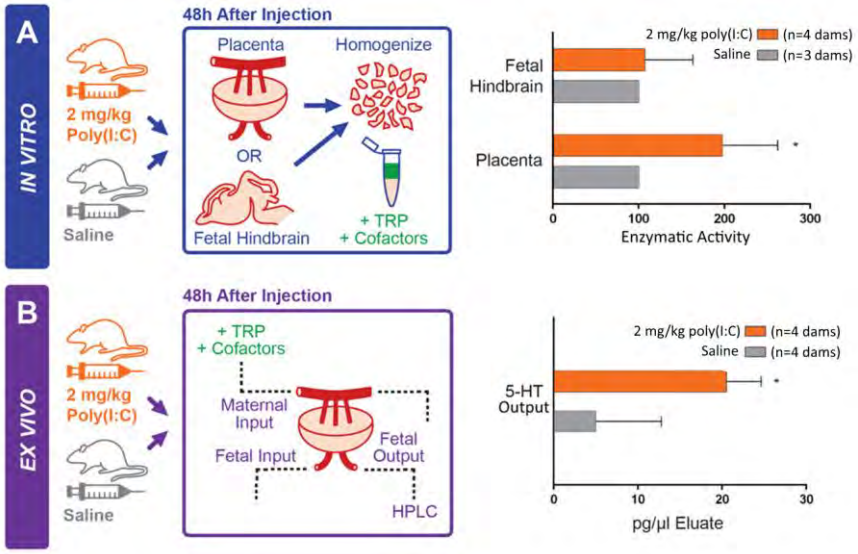

A
IN VITRO
2 mg/kg
Poly(I:C)
Saline
48h After Injection
Placenta
Homogenize
OR
Fetal Hindbrain
+ TRP
+ Cofactors
2 mg/kg poly(I:C) (n=4 dams)
Saline (n=3 dams)
Fetal
Hindbrain
Placenta
0
100
200
300
Enzymatic Activity
B
EX VIVO
2 mg/kg
Poly(I:C)
Saline
48h After Injection
+ TRP
+ Cofactors
Maternal
Input
Fetal Input
Fetal
Output
HPLC
2 mg/kg poly(I:C) (n=4 dams)
Saline (n=4 dams)
5-HT
Output
0
10
20
30
pg/µl Eluate

Figure 3

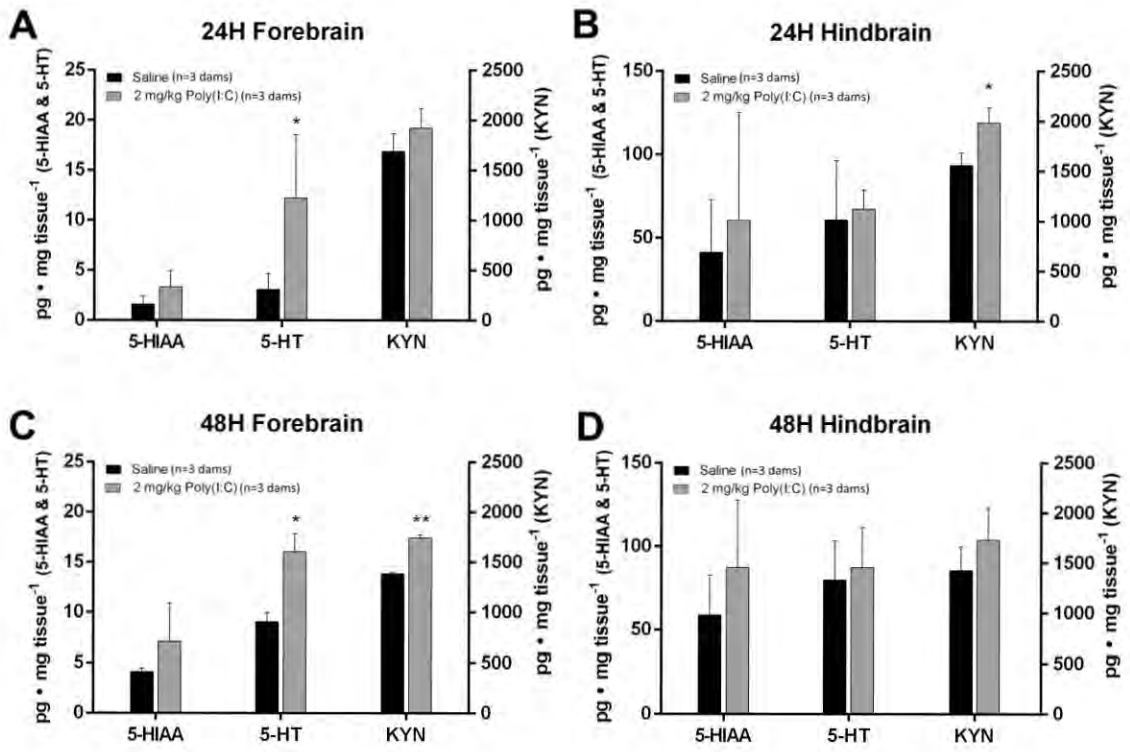
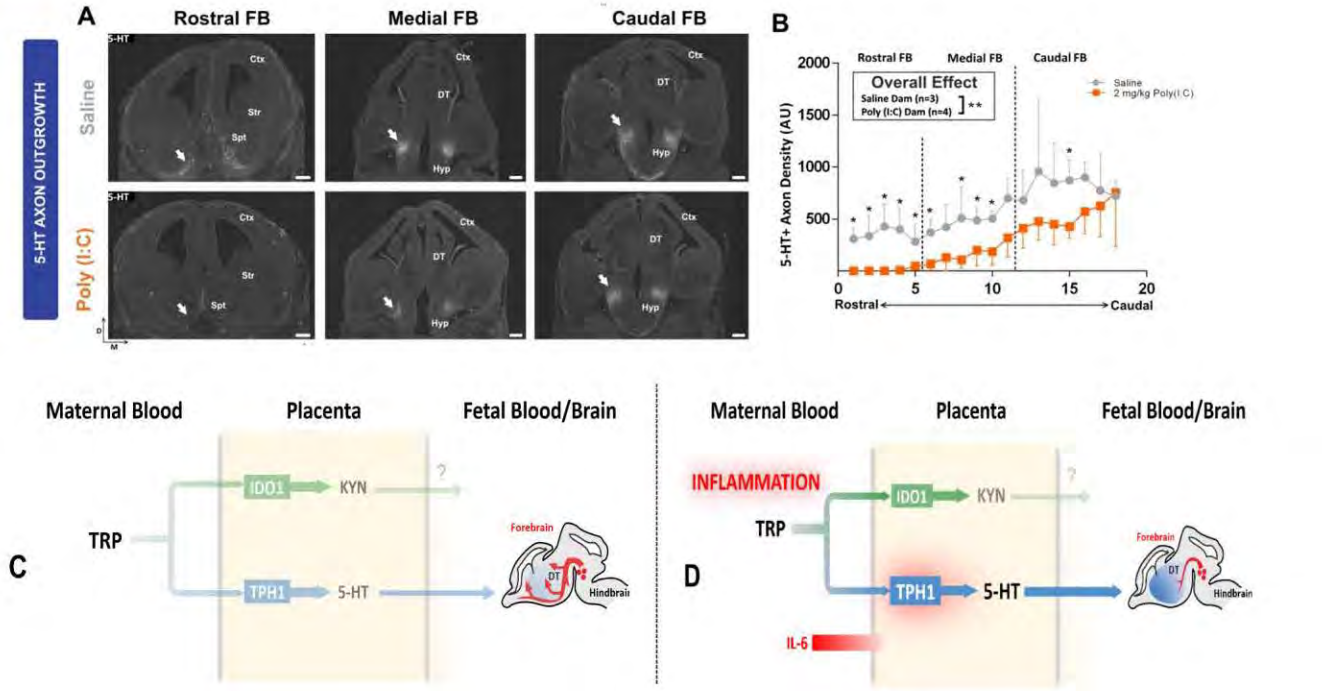

A
24H Forebrain
Saline (n=3 dams)
2 mg/kg Poly(I:C) (n=3 dams)
pg • mg tissue-1 (5-HIAA & 5-HT)
pg • mg tissue-1 (KYN)
5-HIAA
5-HT
KYN
B
24H Hindbrain
C
48H Forebrain
D
48H Hindbrain

Figure 4


A
Rostral FB
Medial FB
Caudal FB
5-HT AXON OUTGROWTH
Saline
Poly (I:C)
5-HT
Ctx
Str
Spt
DT
Hyp
B
Rostral FB
Medial FB
Caudal FB
Overall Effect
Saline Dam (n=3)
Poly (I:C) Dam (n=4)
**
Saline
2 mg/kg Poly(I:C)
5-HT+ Axon Density (AU)
2000
1500
1000
500
0
5
10
15
20
Rostral
Caudal
C
Maternal Blood
Placenta
Fetal Blood/Brain
TRP
IDO1
KYN
TPH1
5-HT
?
Forebrain
DT
Hindbrain
D
Maternal Blood
Placenta
Fetal Blood/Brain
INFLAMMATION
TRP
IDO1
KYN
TPH1
5-HT
IL-6
?
Forebrain
DT
Hindbrain