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Award Number: W81XWH-13-1-0135

TITLE: Altered Placental Tryptophan Metabolism: A Crucial Molecular Pathway for the Fetal Programming of Neurodevelopmental Disorders

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REPORT DATE: July 2014

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
July 2014	Annual	1 July 2013 - 30 Jun 2014

4. TITLE AND SUBTITLE

Altered Placental Tryptophan Metabolism: A Crucial Molecular Pathway for the Fetal Programming of Neurodevelopmental Disorders

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-13-1-0135

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

University of Southern California

Los Angeles, CA 90089-2821

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

In the first year of work supported by this award, we were able to demonstrate that injections of bacterial (LPS) and viral-like (poly(I:C)) immunostimulants in pregnant dams at E12 trigger different maternal and placental inflammatory responses. In addition, maternal immune activation triggered by either treatment induces a rapid increase of TRP metabolic gene expression and enzyme activity specifically in the placenta. Most importantly, maternal immune activation triggered by poly(I:C) and LPS treatment induces rapid, differential alterations of the TRP metabolites 5-HT and kynurenine tissue concentrations in the fetal forebrain. Therefore results so far demonstrate that maternal inflammation in early pregnancy affects the balance of TRP metabolism through IDO1 and TPH1 placental pathways, resulting in increased fetal brain exposure to 5-HT and kynurenine. These effects are likely to result in compromised serotonergic modulation of fetal brain development and have direct, long-term consequences on fetal brain circuit formation and postnatal brain function. The progress made in the first year 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.

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

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	15	19b. TELEPHONE NUMBER *(include area code)*

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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.

Two aims were initiated during the first year of this award:

Aim I: <u>To determine whether maternal inflammation alters placental synthesis and fetal exposure to 5-HT and kynurenine-pathway compounds.</u> This aim tests whether maternal inflammation affects placental TRP metabolism and 5-HT output to the fetus, and if these parameters are dependent on pregnancy stage. This is tested *ex vivo* and *in vivo*, by measuring changes in placental output of 5-HT and kynurenine-pathway compounds by HPLC following induction of maternal inflammation [bacterial endotoxin lipopolysaccharide (LPS) or viral mimic RNA polyI:C (poly(I:C))] at different gestational ages (E12-14 and E16-18; Aim 1A). Short-term changes in TRP metabolic enzyme activities *in situ* and long-term changes in placental expression patterns of the corresponding genes (E12 to birth) are also being assessed qualitatively by immunohistochemistry (IHC) and quantitatively by western blot and qRT-PCR and related to the neurochemical measurements (Aim 1B).

Aim II: <u>To characterize the inflammation-mediated alterations of TRP metabolism effects on fetal brain neurochemistry and postnatal brain structure.</u> The effect of maternal inflammation induced in mice by LPS or poly(I:C) on fetal brain tissue concentrations of TRP and its metabolites generated through the IDO1/TDO2 (kynurenine) and TPH1 (5-HT) pathways are being assessed by HPLC (Aim 2A). 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 (Aim 2B).

For clarity, we provide below a reminder of the major tasks that support the proposed specific aims (Aims I & II listed above, which were initiated during the first 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 will be measured by HPLC following induction of maternal inflammation [bacterial endotoxin lipopolysaccharide (LPS) or viral mimic RNA polyI:C (poly(I:C))] at different gestational ages (E12-14 and E16-18).*

This task will be initiated in August 2014; targeted completion date: October 2014.

Task 1b. *Short-term changes in TRP metabolic enzyme activities in situ and long-term changes in placental expression patterns of the corresponding genes (E12 to birth) will be assessed qualitatively by immunohistochemistry (IHC) and quantitatively by western blot and qRT-PCR and related to the neurochemical measurements.*

Methodology: Saline (0.9% sterile solution), LPS (10 μg/Kg) or poly(I:C) (2 mg/Kg) intraperitoneal injections were used to induce maternal inflammation in pregnant CD-1 mice at E12. For each immunostimulant and each time point, 1 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. The uterus was immediately dissected and the resulting embryos were placed on ice in 1x PBS. The placenta, forebrain, and hindbrain were removed, snap-frozen in liquid nitrogen, and stored at -80 °C until processing.
<u>1) qRT-PCR Analysis</u>
The samples were transferred from the -80 °C freezer to liquid nitrogen, pooled by dam, and ground into a fine powder using a mortar and pestle cooled with liquid nitrogen. 40 mg of powder was transferred to a 1.5 mL Eppendorf tube, and placed in liquid nitrogen until all samples were pulverized. RNA was extracted from the powdered tissue using the Bio-Rad Aurum Total RNA Mini Kit, according to the manufacturer specifications.
Extracted RNA was quantified and tested for purity through the use of a spectrophotometer, and RNA quality was assessed using the Agilent Technologies 2100 bioanalyzer. Only samples with an RNA integrity number of 8.5 or higher, and a 260/280 ratio of 1.8-2.2 were used for downstream qRT-PCR analysis. Following quality assessment and quantification, RNA was reverse-transcribed to cDNA using the Invitrogen SuperScript III First-Strand Synthesis SuperMix for qRT-PCR kit according to manufacturer specifications.
qRT-PCR reactions were performed in triplicate using the Invitrogen Single Tube TaqMan Gene Expression Assays and TaqMan Gene Expression Master Mix, and cycled and analyzed using the Invitrogen StepOnePlus Real-Time PCR System. Briefly, for each gene and template

combination, 20 µl reactions were set up in triplicate containing 10 µl TaqMan Gene Expression Master Mix, 1 µl TaqMan Gene Expression Assay, and 9 µl cDNA diluted in ultrapure water to a concentration of 10ng template per well. Once all reactions were setup in 96-well plates, they were transferred to the StepOnePlus thermocycler, and run using standard cycling conditions of a 2 minute hold at 50 °C, a 10 minute hold at 95 °C, and 50 cycles of 15 seconds at 95 °C followed by 60 seconds at 60 °C.

Completed reactions were analyzed using the 2(-Delta Delta C(t)) method, previously described in (*1*). The $2^{-\Delta\Delta CT}$ method for quantifying relative changes in gene expression involves comparing expression of a target gene to that of a reference gene that maintains stable expression throughout treatment groups and time points. Several reference genes were tested, and TPB was found to be the most stable between treatment groups and time points in our samples. In order for the $2^{-\Delta\Delta CT}$ method to be valid, the amplification efficiencies of both the target and reference genes must be similar. To test this, efficiency curves for each TaqMan Assay were performed across a 100-fold template dilution range, and compared for each primer-template combination to ensure similarity. After amplification, the relative expression of the untreated samples was compared to that of the treated samples and expressed in terms of fold change, which can be represented numerically as: $2^{-(CT, Target– CT, Reference)}$untreated $^{-(CT, Reference – CT, Target)}$treated or, $2^{(-\Delta\Delta CT)}$.

<u>*2) In vitro* 5-HTP synthesis assays</u>

The collected tissues (placenta, forebrain, and hindbrain) were thawed on ice, and ultrasonically homogenized in, respectively, 400, 100, and 80 µl of extraction buffer composed of 0.05 M Tris buffer, pH 7.5, containing 1 mM dithiothreitol and 1 mM EGTA. Homogenates were centrifuged at 29,000xg for 15 min at 4 °C and then assayed for tryptophan hydroxylase activity. Briefly, 20 µl of supernatant was added to tubes containing 80 µl reaction buffer to give a final concentration of: 0.05 M Tris buffer, pH 7.5, 1 mM EGTA, 50 µg/mL catalase, 200 µM L-tryptophan, 100 µM ammonium iron (II) sulfate, and 100 µM tetrahydrobiopterin (BH4; a cofactor required for TPH1 and TPH2 activity). Reactions were performed for 30 min at 37 °C and terminated through protein denaturation by adding 100 µl of 0.2M percholoric acid with 100 µM EDTA. Samples were stored on ice for 15 minutes to allow for complete protein denaturation, and then centrifuged for 15 min at 20,000xg. L-tryptophan metabolism was determined by measuring 5-hydroxytryptophan (5-HTP) concentration using HPLC with electrochemical detection. Protein concentration of the supernatants was determined using a DC protein assay (Bio-Rad), and enzymatic activity was quantified as pg 5-HTP · ug protein^{-1} · 30 min^{-1}. Results are expressed as percent of activity measured in saline-injected controls.

Problems encountered in accomplishing task 1b: none.

Results:

1) <u>Effects of maternal inflammation triggered by poly(I:C) on placental gene expression.</u>

Maternal immune activation leads to differential up-regulation of TRP metabolic genes expression in the placenta 24 and 48h after poly(I:C) injection at E12. As shown Figure 1A, placental expression of monoamine oxydase A (*Maoa*), *Ido1* and *Tph1* genes is significantly up-regulated 24h after poly(I:C) injection. The most significant increase is observed for *Ido1* gene expression (increase of ~320% over basal expression level measured in controls obtained from saline-injected dams), followed by *Tph1* gene expression (increase of ~153%) and *Maoa* (increase of ~9%). Interestingly, the levels and patterns of changes in gene expression become different 48h after poly(I:C) injections. As shown Figure 1B, *Maoa* gene expression is not significantly different from control level, whereas *Ido1* gene expression is still

significantly elevated (increase of ~96% over saline-injected controls) and *Tph1* gene expression becomes significantly reduced (decrease of ~64%).

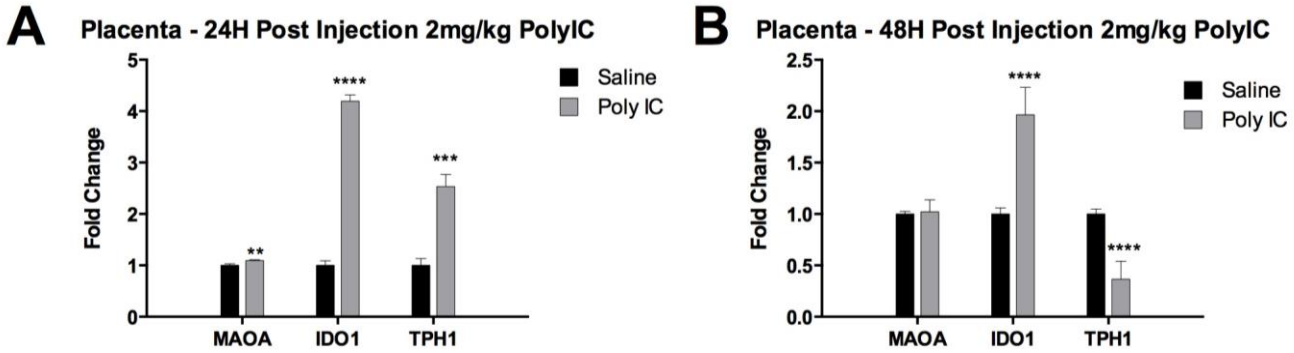

Figure 1: Effects of maternal inflammation triggered by poly(I:C) on placental gene expression. Changes in placental expression 24 and 48h after poly(I:C) (2 mg/Kg) intraperitoneal injection in the dams at E12 were measured by qRT-PCR. **(A)** Placental expression of monoamine oxydase A (*Maoa*), *Ido1* and *Tph1* genes is significantly up-regulated 24h after poly(I:C) injection compared to saline. **(B)** 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. N= 4 dams (3 placentas pooled per dam) for saline, N = 5 dams (3 placentas pooled per dam) for poly(I:C). **, p< 0.005; ****, p<0.001, Statistical significance determined using unpaired t-test comparison and Holm-Sidak correction, with alpha=5.0%.

2) Effects of maternal inflammation triggered by LPS on placental gene expression. Maternal immune activation triggered by LPS injection at E12 leads to differential up-regulation of TRP metabolic genes expression in the placenta 24 and 48h later. As shown Figure 2A, placental expression of *Ido1* and *Tph1* genes is significantly up-regulated 24h after LPS injection.

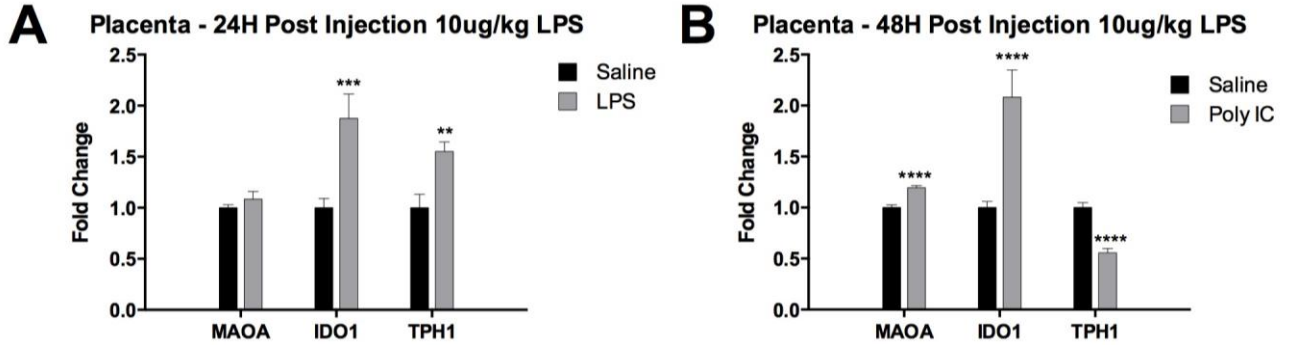

Figure 2: Effects of maternal inflammation triggered by LPS (10 μg/Kg) on placental gene expression. **(A)** Placental expression of *Ido1* and *Tph1* but not *Maoa* genes is significantly up-regulated 24h after LPS injection in comparison to saline. **(B)** 48h after LPS injections, *Maoa* gene expression is significantly increased over control level, *Ido1* gene expression is still significantly elevated and *Tph1* gene expression is significantly reduced. N= 4 dams (3 placentas pooled per dam) for saline, N = 3 dams (3 placentas pooled per dam) for LPS. **, p< 0.005; ***, p<0.001, Statistical significance determined using unpaired t-test comparison and Holm-Sidak correction, with alpha=5.0%.

The most significant increase is observed for *Ido1* gene expression (increase of ~87% over basal expression level measured in controls obtained from saline-injected dams), followed by *Tph1* gene expression (increase of ~55%). *Maoa* gene expression is not significantly altered by treatment. Similar to the effects of poly(I:C), the levels and patterns of changes in gene expression become different 48h after LPS injections. As shown Figure 2B, *Maoa* gene expression is significantly increased (~19%) over control level, *Ido1* gene expression is still significantly elevated (increase of ~108% over saline-injected controls) and *Tph1* gene expression becomes significantly reduced (decrease of ~45%).

3) Effects of maternal inflammation on placental TRP metabolic enzymes activity. The changes in placental gene expression reported above may impact maternal TRP conversion to 5-HT.

This possibility was directly assessed by measuring TPH1 enzyme activity in subsets of placentas harvested from the same litters. In these assays, placental extracts are incubated with TRP and a co-factor necessary for TPH1 enzyme activity (tetrahydrobiopterin, BH4) for 30 min. As an index of TPH1 enzyme activity, concentration of neo-synthesized 5-hydroxytryptophan (5-HTP), the first metabolic product of TRP and immediate precursor of 5-HT, was quantified by HPLC. Results indicate that placental TPH1 enzyme activity is not significantly different from control 24h after maternal immune activation with either LPS or poly(I:C) (not shown). However, as shown Figure 3A, placental TPH1 enzyme activity is significantly up-regulated 48h after treatment with poly(I:C); LPS treatment also induces a non-significant trend toward increased placental TPH1 activity. Importantly, neither treatment induces a significant change in the rate of TRP conversion to 5-HTP in the fetal forebrain and hindbrain (not shown). In addition, 48h after poly(I:C) injection a significant increase in placental, but not fetal forebrain or hindbrain, MAOA activity over control level (measured in placental extracts from saline-injected dams) is observed (Figure 3B).

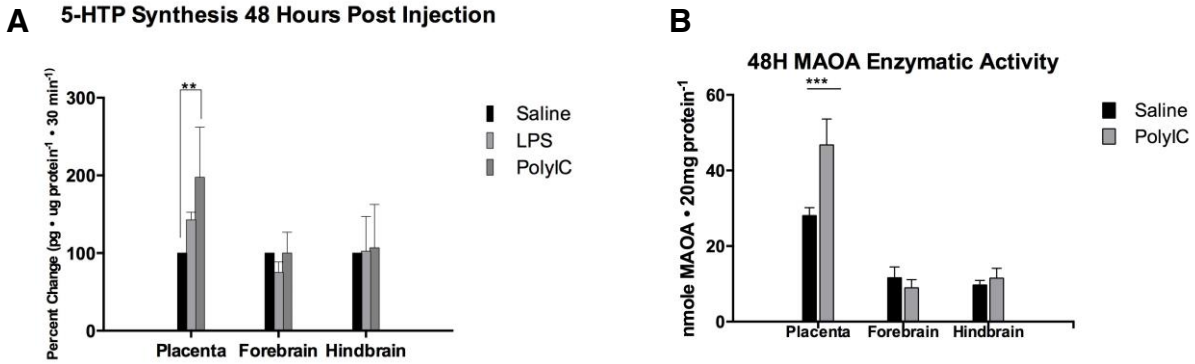

Figure 3: Effects of maternal inflammation on placental TPH1 and MAOA enzymatic activity. 48h after LPS or poly(I:C) injections, placentas were harvested, proteins extracted and TRP to 5-HTP conversion (TPH1 activity) or 5-HT to 5-HIAA conversion (MAOA activity) measured over a 30 min incubation time. **(A)** Maternal immune activation triggered by poly(I:C) injection induces a significant increase in TPH1 activity over control level (measured in placental extracts from saline-injected dams). LPS injection induces a trend toward increased TPH1 activity. Neither poly(I:C) nor LPS treatment induces a change in fetal forebrain or hindbrain TPH2 activity. **(B)** Maternal immune activation triggered by poly(I:C) injection induces a significant increase in placental, but not fetal forebrain or hindbrain, MAOA activity over control level (measured in placental extracts from saline-injected dams). N= 5, 3 and 4 dams (2 placentas, 3 brains pooled per dam) for saline, LPS and poly(I:C) treatments respectively. **, p=0.027; Statistical significance determined using 2-way ANOVA, with alpha=5.0%.

Conclusion for Task 1b.

The results show that maternal immune activation by either poly(I:C) or LPS 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 together with cytokine measures (see Task 2 below) confirms that injection of these immunostimulants at E12 induces a maternal inflammatory response. Up-regulation of *Ido1* metabolic pathway, either at the gene or enzymatic activity level, is expected to lead to increased conversion of maternal TRP to kynurenine. These treatments should therefore elevate placental output of kynurenine to the fetus. As proposed in the main project narrative, subsequent studies will determine the impact of elevated placental kynurenine exposure on fetal brain expression of NMDA receptors (NR1, NR2a, NR2b) for which kynurenine is a specific ligand. 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, a possibility that will be directly tested in Task 1a using *ex vivo* dual perfusions of immune-activated placentas. Concomitantly to this increased enzymatic activity, a significant decrease of *Tph1* gene expression is observed 48h post-injection, suggesting a tight activity-dependent regulation of *Tph1* gene expression in the placenta that is modulated by maternal inflammatory status. Another interesting observation is the small but significant increase in placental *Maoa* gene expression, either 24h after poly(I:C) injection or 48h after LPS injection. Following poly(I:C) exposure specifically, this increase in gene expression is followed, 24h later, by an increase in MAOA enzymatic activity. The simultaneous increases in TPH1 and MAOA enzyme activity suggest that both elevated 5-HT neosynthesis and elevated 5-HT degradation occur simultaneously in the placenta. This may seem paradoxical, however it is thought that placental MAOA activity primarily degrades maternal blood 5-HT (*6*, *7*), whereas placentally derived 5-HT (generated by placental TPH1) may not be a direct substrate for placental MAOA. This would suggest a precise compartmentalization and regulation of neosynthesis and degradation of maternal *vs* placental and fetal stores of 5-HT. Taken together, the results suggest that specific triggers (poly(I:C) and LPS) of maternal immune activation have differential effects on placental gene expression and enzymatic activity related to TRP metabolism. In particular our results suggest that, at the doses used, poly(I:C) injections have more potent effects on placental TRP metabolic gene expression and enzymatic activity than LPS. The difference in potency could be simply proportional to the doses of immunostimulant used, which will be tested by using higher LPS concentrations in follow-up studies. Alternatively, the difference could be related to the distinct signaling pathways triggered in the placenta by poly(I:C) (Toll Like Receptor 3, TLR3) vs LPS (TLR2). These possibilities will be tested in future studies.

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 in mice by LPS or poly(I:C) on fetal brain tissue concentrations of TRP and its metabolites generated through the IDO1 (kynurenine) and TPH1 (5-HT) pathways will be assessed by HPLC.*

Methodology: Saline (0.9% sterile solution), LPS (10 μg/Kg) or poly(I:C) (2 mg/Kg) intraperitoneal injections were used to induce maternal inflammation in pregnant CD-1 mice at E12. For each immunostimulant and each time point, 1 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. The uterus was immediately dissected and the resulting embryos were placed on ice in 1x PBS. The placenta, forebrain, and hindbrain (a precollicular coronal bisection is made to separate the forebrain+midbrain (termed forebrain) from the hindbrain) were removed, snap-frozen in liquid nitrogen, and stored at -80 °C until processing.
1) HPLC Analysis
Measurements of TRP and its metabolites in samples harvested *in vivo* (placentas, fetal forebrains and hindbrains) are performed by HPLC, a technique routinely used in Dr Anderson's laboratory at Yale University.
2) Cyto/chemokine measures
The maternal and placental inflammatory response to LPS or poly(I:C) are quantified in every mouse, at time of harvesting, by measuring the concentration of the cytokines IL-1β, IL-6, IFNγ and TNF and of the chemokines MCP-1, MIG, RANTES in serum or tissue homogenates. These data provide a comprehensive overview of the inflammatory response that occurs both in the pregnant dam and in the placenta and fetal brain and allow for us to control for variations

in the inflammatory response in individual animals. We use commercially available cytometric bead arrays (CBA) from BD Biosciences that allow us to simultaneously detect each of these solutes in the low pg/ml range. Each assay includes standards and positive controls, as per manufacturer instructions. Solute concentrations are determined in a 50 µl volume and all tests are run in triplicate, in a blinded fashion. The CBA is processed using a BD FACS Canto II cytometer and data analyzed using the commercially available FCAP Array Software (BD Biosciences) in the laboratory of Dr Lund at USC. The mean of sample concentration from serum is given as pg/ml and from tissue is given both pg per mg of tissue and pg per µg of total protein.

Problems encountered in accomplishing task 2a: We purchase timed-pregnant mice from Charles River Laboratories in batches throughout the year. Upon delivery, pregnant mice are randomly assigned to control (saline-injected) or immunostimulant (LPS or poly(I:C)-injected) treatment groups at E12. Our tissue harvest method includes weighting each placenta, forebrain and hindbrain collected. This is done in order to 1) normalize HPLC measures of biogenic amines to tissue weight (expressed as pg biogenic amine per mg tissue), and 2) ensure homogeneity in terms of the pregnancy stages at which tissues are collected. Although all timed-pregnant mice should be delivered at similar pregnancy stages, we noticed significant variations in fetal brain tissue weights in several cohorts (Figure 4). In particular some outlier cohorts showed abnormally high fetal brain weights (spread indiscriminately among embryos harvested at 24h or 48h from saline-, LPS- or poly(I:C)-injected dams). This suggested that some timed-pregnant mice might have been delivered at slightly more advanced pregnancy stages than expected. Since there is no other independent method to precisely determine pregnancy stage, we decided to exclude these particular cohorts from subsequent analyses. This problem led us to repeat several of the harvests and measures, which were not originally planned.

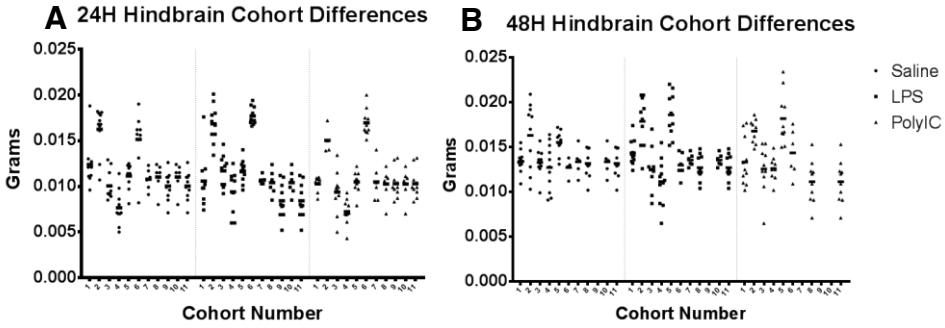

Figure 4: Unexpected variations observed in fetal hindbrain weights of embryos harvested at 24h (**A**) or 48h (**B**) from saline-, LPS- or poly(I:C)-injected dams.

Results:

1) Effects of maternal inflammation induced by poly(I:C) on fetal brain neurochemistry. Our investigation focused on fetal brain tissue concentrations of TRP metabolites generated through the IDO1 (kynurenine (Kyn)) and TPH1/MAOA (5-HT, 5-HIAA) pathways. Results show that poly(I:C)-mediated maternal immune activation induces a significant increase of fetal forebrain, but not hindbrain, Kyn tissue concentration 24h post-injection (Figure 5A, B). In both brain areas, 5-HT and 5-HIAA tissue concentrations show non-significant trends of increase in comparison to saline controls. Forty-eight hours after maternal poly(I:C) injection, fetal forebrain, but not hindbrain, 5-HT and Kyn tissue concentrations are significantly increased compared to saline-injected controls (Figure 5C, D).

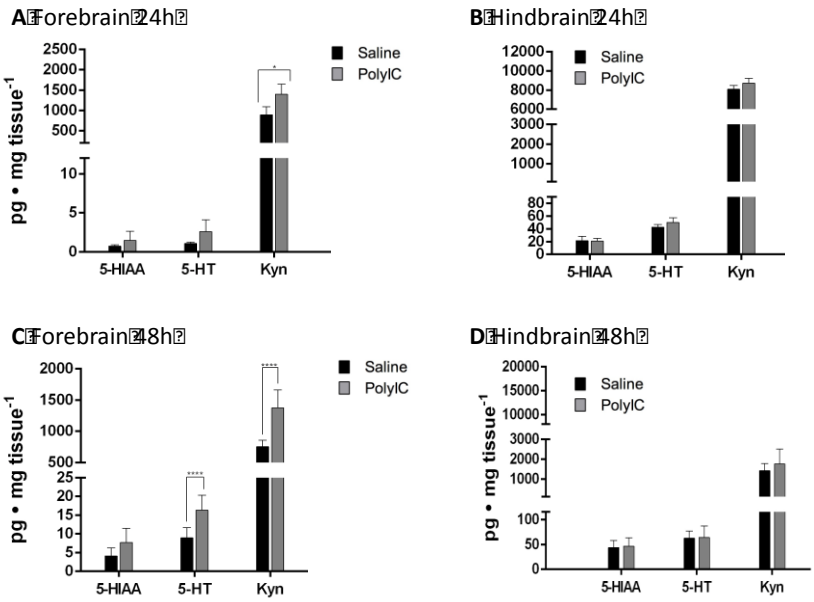

Figure 5: Effects of maternal inflammation induced by poly(I:C) on fetal brain 5-HIAA, 5-HT and Kyn concentrations. **(A, B)** Maternal immune activation triggered by poly(I:C) induces 24h later a significant increase of Kyn concentration in the fetal forebrain (**A**), but not hindbrain (**B**). **(C, D)** 48h after maternal poly(I:C) injection, fetal forebrain (**C**), but not hindbrain (**D**) 5-HT and Kyn concentrations are increased compared to saline-injected controls. N= 3 dams per time point and per treatment (4 or 5 fetal brains analyzed per dam). *, p< 0.05; ****, p<0.001, Statistical significance determined using unpaired t-test comparison, with alpha=5.0%.

2) Effects of maternal inflammation induced by LPS on fetal brain neurochemistry. In comparison to saline controls, LPS-mediated maternal immune activation induces a significant increase of fetal forebrain, but not hindbrain, 5-HT and 5-HIAA tissue concentrations 24h post-injection (Figure 6A, B). Kyn tissue concentration is not affected by treatment in either brain area. Forty-eight hours after maternal LPS injection, fetal forebrain but not hindbrain Kyn tissue concentration is significantly increased compared to saline-injected controls (Figure 6C, D). At this time point, fetal forebrain and hindbrain 5-HT and 5-HIAA tissue concentrations are not significantly different from concentrations measured in saline controls (Figure 6C, D).

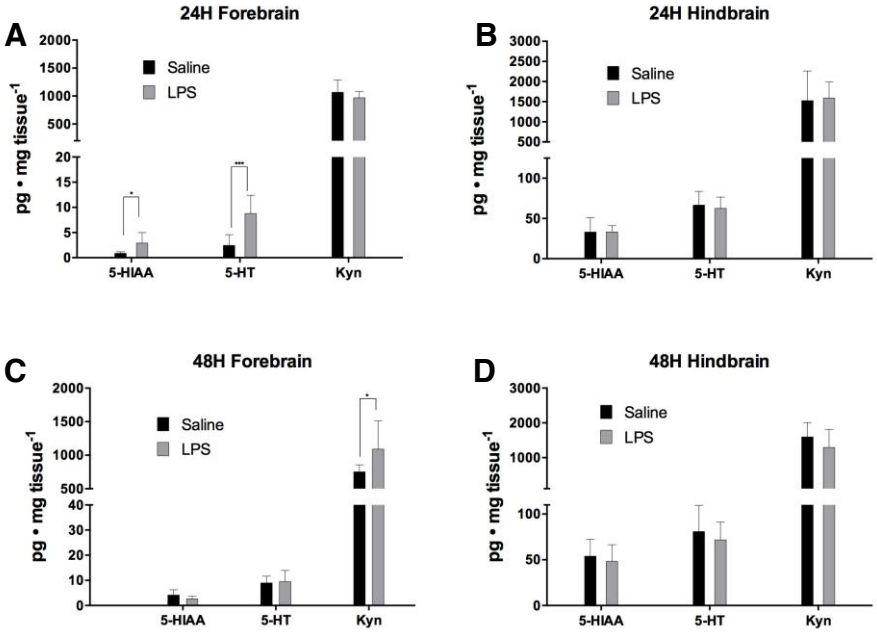

Figure 6: Effects of maternal inflammation induced by LPS on fetal brain 5-HIAA, 5-HT and Kyn concentrations. **(A, B)** Maternal immune activation triggered by LPS induces 24h later a significant increase of 5-HIAA and 5-HT concentrations in the fetal forebrain (**A**), but not hindbrain (**B**). **(C, D)** 48h after maternal LPS injection, fetal forebrain (**C**), but not hindbrain (**D**) Kyn concentration is increased, whereas 5-HIAA and 5-HT concentrations are similar to saline-injected controls. N= 3 dams per time point and per treatment (4 to 5 fetal brains analyzed per dam). *, p< 0.05; ****, p<0.001, Statistical significance determined using unpaired t-test comparison, with alpha=5.0%.

3) Effects of LPS and poly(I:C) injections on maternal blood and placenta cytokine/chemokine concentrations. The maternal and placental inflammatory response to LPS or poly(I:C) were quantified in every mouse, at time of harvesting (i.e. E12 + 24 or 48h), by measuring the concentration of various cytokines and chemokines; at the doses of immunostimulant used, the most significant changes in maternal blood serum and placental homogenates concentrations were observed for interleukin 6 (IL-6), monocyte chemotactic protein 1 (MCP-1), monokine induced by gamma interferon (MIG) and regulated on activation normal T cell expressed and secreted (RANTES) (Figure 7). Results show that 24h post-injection, maternal blood serum levels of these cyto/chemokines are either significantly elevated or show trends toward increase compared to the effect of saline injections. Interestingly, LPS and poly(I:C) injections induce differential responses in terms of maternal blood serum cyto/chemokine concentrations; for instance, LPS exposure induces a significant increase of IL-6 cytokine in the blood serum (Figure 7A) and placenta (Figure 7B) 24h post-injection whereas poly(I:C) exposure induces a significant increase of RANTES chemokine in these tissues. LPS exposure also induces trends toward increased maternal serum level of MCP-1, MIG and RANTES, whereas poly(I:C) exposure induces significant increases of these chemokines. Forty-eight hours after maternal immune activation, serum concentration of cyto/chemokines returned to basal control level (no significant difference in concentration for any of these molecule was observed – Figure 7C). In contrast, the effects appear to persist in the placenta, albeit with different patterns (Figure 7D). MIG and RANTES placental tissue concentrations are still significantly elevated compared to saline controls 48h after exposure to LPS or poly(I:C); however, tissue concentration of MCP-1 is significantly decreased after both LPS and poly(I:C) exposure, and IL-6 concentration is either unchanged 48h post LPS injection or significantly decreased for poly(I:C).

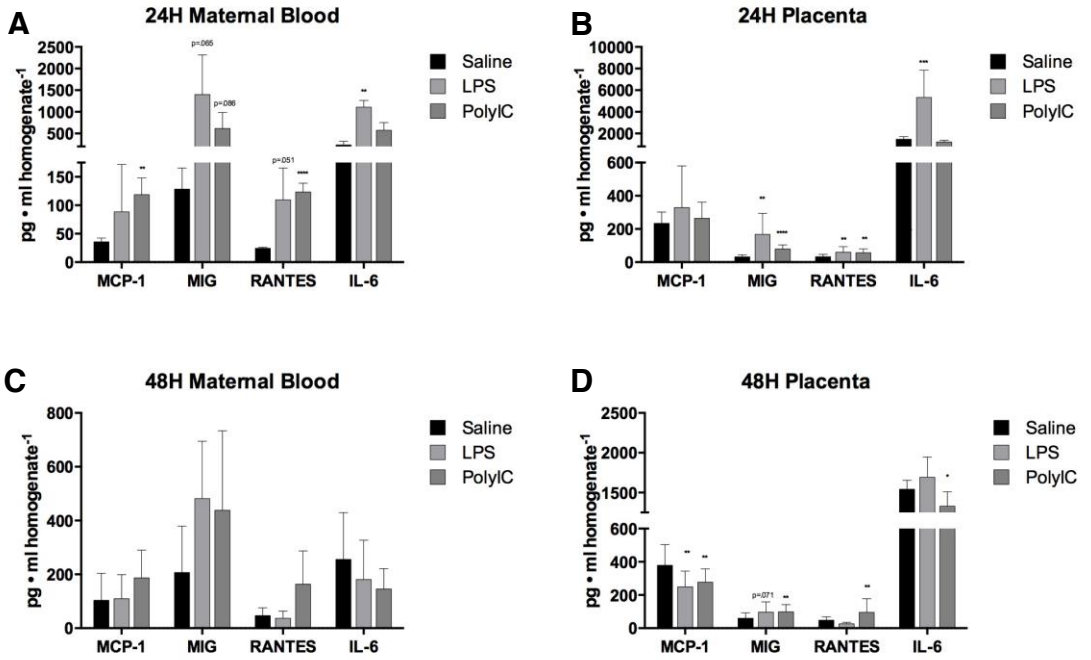

Figure 7: Effects of LPS and poly(I:C) injections at E12 on maternal blood serum and placenta tissue concentrations of cyto- and chemokines. **(A, B)** Maternal immune activation triggered by LPS and poly(I:C) induces 24h later a significant and differential increase of IL-6 cytokine and MCP-1, MIG and RANTES chemokines. **(C, D)** 48h after maternal LPS or poly(I:C) injection, maternal blood serum concentrations of these cyto- and chemokines are not significantly different from saline-injected controls, whereas placental tissue concentration still show differential patterns of activation. N= 2 (IL-6) to 3 (MCP-1, MIG, RANTES) dams per time point and per treatment (3 placentas analyzed per dam). *, p< 0.05; **, p<0.01, Statistical significance determined using unpaired multiple t-test comparison, with alpha=5.0%.

Conclusion for Task 2a.
The results show that LPS and poly(I:C) injections at E12 trigger maternal and placental inflammatory responses within 24h, a response that persists 48h later specifically in the placenta. HPLC measures of biogenic amines reveal that maternal immune activation affects fetal forebrain tissue concentrations of TRP metabolites such as kyn and 5-HT. In addition, the kinetics of these effects are dependent on the specific trigger of maternal immune activation; poly(I:C) injected to pregnant dams at E12 induces the most significant changes in fetal forebrain concentrations of kyn, 5-HT and 5-HIAA 48 h post-injection, whereas for LPS, the most significant changes are observed 24 h post injection. This suggests that poly(I:C) and LPS have different kinetics of activation of inflammatory pathways, which is consistent with previously published studies [REFs]. Furthermore, our measures of inflammatory responses in the maternal blood serum and placenta reveal a differential activation of specific chemo- and cytokines by LPS and poly(I:C); this suggest that specific subclasses of chemo- and cytokines may differentially affect placental TRP metabolic pathways. Most importantly, the increases in fetal forebrain kyn, 5-HT and 5-HIAA in response to maternal inflammation appear directly correlated to increased TRP metabolic gene expression and enzymatic activity in the placenta measured in Task 1b. This is illustrated in Table 1 for the effect of maternal poly(I:C) injection on placental gene expression, enzymatic activity, fetal forebrain biogenic amine concentrations and fetal brain TRP and 5-HT metabolic enzymes activity. Although at this point the relationships between increased placental gene expressions 24h post injection, and increased enzymatic activities and fetal brain metabolites tissue concentrations 48h post injection are still correlative, Task 1a experiments will determine causation.

Time post-injection	*Placental gene expression*			*Placental enzymatic activity*	
	Tph1	*Maoa*	*Ido1*	TPH1	MAOA
24h	↑ ***	↑ **	↑ ****	nc	nc
48h	↓ ****	nc	↑ ****	↑ **	↑ ***
	Fetal forebrain concentration			*Fetal brain enzymatic activity*	
	5-HT	5-HIAA	Kyn	TPH2	MAOA
24h	nc	nc	↑ *	nc	nc
48h	↑ ****	↑ ns	↑ ****	nc	nc

Table 1: effects of maternal poly(I:C) injection at E12 on placental and fetal brain TRP metabolic pathways activity. Arrows denote increased or decreased expression, activity or tissue concentration. Asterisks refer to the significance level of the effect (detailed in Figure 1 to 6). nc = no change; ns = not significant.

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.*
We have collected, embedded and frozen 3 fetal brains per dam from every cohort used in Task 2a. This tissue bank is awaiting sectioning and staining which will be initiated in August 2014. Tissue collection and processing for the postnatal time period will be initiated in the fall of 2014.

Task 3: *To determine if genetic or pharmacological manipulations of placental TRP metabolic pathways in vivo can reduce inflammation effect on offspring brain development.*
This task was not yet initiated.

KEY RESEARCH ACCOMPLISHMENTS

- Demonstration that LPS and poly(I:C) injections at E12 trigger different maternal and placental inflammatory responses within 24h, a response that persists 48h later specifically in the placenta.
- Demonstration that maternal immune activation by either poly(I:C) or LPS treatment induces a rapid increase of TRP metabolic gene expression and enzyme activity specifically in the placenta.
- Demonstration that maternal immune activation triggered by poly(I:C) and LPS treatment induces rapid, differential alterations of fetal forebrain TRP metabolite tissue concentrations, such as 5-HT and kynurenine which play important roles in neurogenesis and circuit formation.

REPORTABLE OUTCOMES

- A manuscript reporting the results above is in preparation and will be submitted upon completion of Task 1a (October 2014).
- Dr Bonnin will present some of the above results at the IFPA 2014 meeting in Paris, France (Sept. 9th-12th, 2014).
- Based on the work supported by this award Nick Goeden, a graduate student in Dr Bonnin's laboratory and key personnel in this award, successfully applied for a predoctoral fellowship awarded in July 2014 by the Autism Science Foundation.

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. Due to the neurotrophic effects of 5-HT and the neuroactive effects of kynurenine-pathway compounds, this alteration is postulated to have substantial consequences for neurodevelopment. In the first year of work supported by this award, we were able to demonstrate that injections of bacterial (LPS) and viral-like (poly(I:C)) immunostimulants in pregnant dams at E12 trigger different maternal and placental inflammatory responses. In addition, maternal immune activation triggered by either treatment induces a rapid increase of TRP metabolic gene expression and enzyme activity specifically in the placenta. Most importantly, maternal immune activation triggered by poly(I:C) and LPS treatment induces rapid, differential alterations of the TRP metabolites 5-HT and kynurenine tissue concentrations in the fetal forebrain. Therefore results so far demonstrate that maternal inflammation in early pregnancy affects the balance of TRP metabolism through IDO1 and TPH1 placental pathways, resulting in increased fetal brain exposure to 5-HT and kynurenine. These effects are likely to result in compromised serotonergic modulation of fetal brain development and have direct, long-term consequences on fetal brain circuit formation and postnatal brain function; these possibilities will be investigated in Year 2 and 3 of this award. In addition, the possibility that timing of inflammation (e.g. early pregnancy (E12, when the placenta provides the main source of 5-HT to the fetal brain) vs late pregnancy (E16, when 5-HT is endogenous to the fetal brain)) may differentially impact offspring brain development will be tested.

The progress made in the first year 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.

REFERENCES

1. K. J. Livak, K. J. Livak, T. D. Schmittgen, T. D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods*. **25**, 402–8 (2001).

2. Y. Kudo, C. a Boyd, Human placental indoleamine 2,3-dioxygenase: cellular localization and characterization of an enzyme preventing fetal rejection. *Biochim. Biophys. Acta*. **1500**, 119–24 (2000).

3. Y. Kudo, C. a Boyd, I. L. Sargent, C. W. Redman, Tryptophan degradation by human placental indoleamine 2,3-dioxygenase regulates lymphocyte proliferation. *J. Physiol.* **535**, 207–15 (2001).

4. P. Dharane Neé Ligam, U. Manuelpillai, E. Wallace, D. W. Walker, P. Dharane, NFκB-dependent increase of kynurenine pathway activity in human placenta: inhibition by sulfasalazine. *Placenta*. **31**, 997–1002 (2010).

5. U. Manuelpillai *et al.*, Identification of kynurenine pathway enzyme mRNAs and metabolites in human placenta: up-regulation by inflammatory stimuli and with clinical infection. *Am. J. Obstet. Gynecol.* **192**, 280–8 (2005).

6. D. Balkovetz, C. Tiruppathi, Evidence for an Imipramine-sensitive Serotonin Transporter in Human Placental Brush-border Membrane. *J. Biol. ….* **264**, 2195–2198 (1989).

7. A. Bonnin *et al.*, A transient placental source of serotonin for the fetal forebrain. *Nature*. **472**, 347–50 (2011).

APPENDICES

N/A

SUPPORTING DATA

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