Got Milk? Maternal immune activation during the mid-lactational period affects nutritional milk quality and adolescent offspring sensory processing in male and female rats

Running title: Maternal immune activation during mid-lactation

Holly DeRosa[1], Salvatore G. Caradonna[2], Hieu Tran[1], Jordan Marrocco[2,3], Amanda C. Kentner[1]*

[1]School of Arts & Sciences, Health Psychology Program, Massachusetts College of Pharmacy
and Health Sciences, Boston Massachusetts, United States 02115.
[2]Laboratory of Neuroendocrinology, The Rockefeller University, 1230 York Ave, New York, NY 10065.
[3]Department of Biology, Touro University, 227 W 60th St, New York, NY 10023.

*Corresponding author:
Amanda Kentner
amanda.kentner@mcphs.edu
Office #617-274-3360
Fax # 617-732-2959

Word Counts:
Abstract: 247
Main Text: 5000
Figures: 6
Tables: 1
Supplementary Methods: 1
Supplementary Figures: 1
Supplementary Tables: 3

Abstract

Previous studies have underscored the importance of breastfeeding and parental care on offspring development and behavior. However, their contribution as dynamic variables in animal models of early life stress are often overlooked. In the present study, we investigated how lipopolysaccharide (LPS)-induced maternal immune activation (MIA) on postnatal day (P)10 affects maternal care, milk, and offspring development. MIA was associated with elevated milk corticosterone concentrations on P10, which recovered by P11. In contrast, both milk triglyceride and percent creamatocrit values demonstrated a prolonged decrease following inflammatory challenge. Adolescent MIA offspring were heavier, which is often suggestive of poor early life nutrition. While MIA did not decrease maternal care quality, there was a significant compensatory increase in maternal licking and grooming the day following inflammatory challenge. However, this did not protect against disrupted neonatal huddling or later-life alterations in sensorimotor gating, conditioned fear, mechanical allodynia, or reductions in hippocampal parvalbumin expression in MIA offspring. MIA-associated changes in brain and behavior were likely driven by differences in milk nutritional values and not by direct exposure to LPS or inflammatory molecules as neither LPS binding protein nor interleukin-6 milk levels differed between groups. These findings reflected comparable microbiome and transcriptomic patterns at the genome-wide level. Animal models of early life stress can impact both parents and their offspring. One mechanism that can mediate the effects of such stressors is changes to maternal lactation quality which our data show can confer multifaceted and compounding effects on offspring physiology and behavior.

Introduction

Epidemiological studies have shown that prenatal exposure to maternal immune activation (MIA) is a leading risk factor for developing psychiatric disorders such as autism and schizophrenia (1-3). Administering immunogens such as lipopolysaccharide (LPS) or polyinosinic:polycytidylic acid (poly I:C) to pregnant animals induces MIA by promoting a cascade of inflammatory cytokines and activating the hypothalamic-pituitary adrenal (HPA) axis of the mother (4-7). Together, these physiological responses are recognized to affect the long-term development of offspring (4, 7). However, the impact on mothers and their offspring when MIA occurs during lactation is relatively unexplored. This is surprising because infections during the postnatal period are common, particularly in low resource settings, and can result in severe morbidity and mortality (8-11).

LPS administered to rodent dams during the lactational period can impact maternal care by reducing the amount of time spent in efficient arched back nursing postures and the number of pups retrieved and returned back to the nest when displaced (12). In line with the behavioral alterations, MIA in lactating dams may influence maternal milk quality. For example, maternal LPS treatment on postnatal days 4, 11, and 18 reduced the mRNA expression of milk nutrient precursors such as glucose and fatty acid transporters in the rat mammary gland (13). Exposure to MIA during the lactational period can also impact offspring behavior. For example, Nascimento and colleagues (14) observed a reduction in ultrasonic vocalizations in male neonates shortly after their dams were treated with LPS. Although this presumably involved changes in various forms of maternal input signals (e.g., parental care and/or milk quality), the mechanisms of this MIA-derived effect on offspring behavior remain unclear. While there is a small amount of evidence suggesting how breastfeeding can transmit certain pathogenic bacteria and viral infections that lead to infant disease (15-17), to our knowledge no epidemiological study has assessed how maternal infection may impact the trajectory of infant development.

Other exogenous factors have been shown to alter contents in breastmilk and subsequent offspring development in animals and humans (18-22). For example, psychological stress in humans reduces milk microbial diversity (23). Further, cortisol in maternal milk was found to predict temperament and promote greater body weights in infant primates, although milk energy content was also associated with body weight (24). Together, this evidence suggests that 1) disturbances to the maternal-neonatal environment alters the developmental trajectory of offspring, and 2) these alterations are mediated by changes in maternal care and/or nutritional milk quality. To explore these ideas further, we assessed the effects of MIA challenge during the mid-lactational period on maternal care, maternal milk composition, and offspring brain development and behavior in Sprague-Dawley rats. While a majority of studies have focused on adult behavioral outcomes following gestational MIA exposure, longitudinal evidence suggests that differences in MIA-associated alterations in brain development emerge in adolescence (25-26). Therefore, we curated a battery of behavioral measures with proven translational potential (27-28) to uncover how the developing brain may be disrupted by maternal infection. We also examined hippocampal expression of parvalbumin given the role of this critical neuronal population in the pathophysiology of neuropsychiatric disorders in humans and in animal models of MIA (29).

Methods and Experimental Overview

All animal procedures were performed in accordance with the Association for Assessment and Accreditation of Laboratory Animal Care with protocols approved by the Massachusetts College of Pharmacy and Health Sciences Institutional Animal Care and Use Committee. **Figure 1A** and Table 1 outline the experimental procedures and groups evaluated in this study. Please see the **Supplementary Methods** and **Supplementary Table 1** for a more detailed description of the methodological protocols and statistical analyses used in this study. Briefly, on postnatal day 10 (P10; equivalent to the mature milk phase), Sprague-Dawley rat mothers were removed from their litters and placed into a clean cage located in a separate procedure room. Dams were challenged intraperitoneally (i.p.) with either 100 μg/kg of the inflammatory endotoxin, lipopolysaccharide (LPS; Escherichia coli, serotype 026:B6; L-3755), or pyrogen-free saline between 08:00hrs and 10:00hrs. During this period the offspring stayed in their regular holding room and were placed into a smaller clean cage positioned on top of a heating pad to facilitate body temperature maintenance. Offspring body weights were collected immediately before returning to their dams and again 2 and 24 hours later, alongside the inspection of milk bands, to monitor health. Exactly 2 hours after receiving either a saline or LPS injection, dams were anesthetized with isoflurane in O2 and administered 0.2 mL of oxytocin (20 USP/mL i.p.) to facilitate milk production. The pharmacokinetic profile of isoflurane suggests it is not absorbed by offspring and that breastfeeding can resume immediately after anesthesia (30-31). Similarly, oxytocin is not expected to affect offspring given its short plasma half-life of 1-6 minutes which is reduced further during lactation (32). Teats were prepared by moistening the collection areas with distilled water. Milk was collected by gently squeezing the base of each teat, and 20uL of expelled milk was immediately processed to measure levels of creamatocrit while an additional ~500uL was stored at -80°C for additional processing. On P11, dams were separated from their litters again for 2 hours prior to milk collection which occurred 24 hours after LPS or saline treatment (see 33).

Milk quality was determined through the use of several assays (see 20, 33) to measure levels of milk creamatocrit, lactose (Sigma-Aldrich Cat. #MAK017), triglycerides (Abcam, Cat. #ab65336), protein (Thermo Fisher Scientific, Cat. #23227), corticosterone (#ADI-900–097, Enzo Life Sciences, Farmingdale, NY), immunoglobulin (Ig) A (Bethyl Laboratories, Cat. #E111-102), interleukin (IL)-6 (Thermo Fisher Scientific, Cat. #BMS625) and lipopolysaccharide binding protein (LBP; Abcam, Cat. #ab269542). Commercially available immunoassays were performed according to the manufacturer's instructions.

The milk microbiome was examined by sequencing the V3-V4 regions of the 16S gene (Zymo Research, Irvine, CA). Following preparation, the final library was quantified using TapeStation® (Agilent Technologies, Santa Clara, CA) and Qubit® (Thermo Fisher Scientific, Waltham, WA), and sequenced using an Illumina® MiSeq™ with a v3 reagent kit (600 cycles) and 10% PhiX spike-in. Taxonomic profiling of was performed with Uclust (Qiime v.1.9.1) and the Zymo Research Database (Zymo Research, Irvine, CA).

Following RNA-isolation (see 20, 33; QIAGEN, Cat. #217004), milk samples were more broadly analyzed using RNA Sequencing (RNA-seq). After determining fragment size and concentration using TapeStation® (Agilent Technologies, Santa Clara, CA), an Illumina NovaSeq 6000 was used to obtain 100-bp reads and samples were read at a sequencing depth of approximately 50 million reads.

Maternal behavior was assessed in the afternoon (15:00-17:00 hrs) and evening (20:00-22:00 hrs) of P10 and in the morning (07:30-9:30 hrs) on P11. Dams were evaluated for 1-minute intervals over 6 observation periods on behaviors including the frequency of pup-directed behaviors, self-directed behaviors, nest building/digging behavior, and the total time spent on the nest. The offspring of saline and MIA treated dams completed an assessment of neonatal huddling, a form of social thermoregulation, 2 hours after being reunited with their mother on P10 and P11. Starting on P43, one male and one female offspring from each litter were assessed in several behavioral measures including the open field and social preference tasks, as well as assessments of mechanical allodynia (the von Frey test), sensorimotor gating (prepulse inhibition; PPI), and conditioned fear (see 34-37). On P46, whole hippocampus was collected and stored at -80°C for future processing.

To measure hippocampal parvalbumin (PV) expression using western blotting, 20µg of protein was loaded into each well of MiniProtean® gels (Bio Rad Laboratories, Cat. #4568101). Gels were transferred onto nitrocellulose membranes (Bio Rad Laboratories, Cat. #1620147) and blocked in 5% nonfat milk with TBS + 0.05% Tween 20 (TBST) for 1 hour at room temperature. Membranes were then washed with TBST and incubated in a 1:1000 dilution of parvalbumin (PV) antibody (RnD Systems, Cat. # AF5058) plus TBS overnight at 4°C. The next morning, membranes were washed and incubated in an HRP-conjugated secondary antibody (1:1000, RnD Systems, Cat., #HAF016) made in 1% nonfat milk with TBS for 1 hour at room temperature. Membranes were then washed and exposed to a chemiluminescent substrate (Thermo Fisher Scientific, Cat. #34580) for 5 minutes prior to being scanned. After imaging, membranes were stripped (Thermo Fisher, Cat. # 21062) for 15 minutes at 37°C, blocked, washed, and incubated in beta actin primary antibody (1:1000, Thermo Fisher Scientific, Cat. #MA515739) for 1 hour at room temperature. Membranes were exposed again to the chemiluminescence substrate and imaged. Densitometry measures were used to obtain a ratio of PV/β-actin in order to quantify differences between groups.

Two-way repeated measure ANOVAs (MIA x Time) were used to evaluate milk and behavioral measures across P10 and P11. Violations to the assumption of sphericity were addressed using the Huyndt Feldt correction. One-way ANOVAs were used as appropriate for all other measures unless there were violations to the assumption of normality (Shapiro-Wilk test) in which case Kruskal-Wallis tests were employed (expressed as X^2). The mechanical allodynia threshold data was assessed using body weight as a covariate. The partial eta-squared (n_p^2) is reported as an index of effect size for the ANOVAs (38). Because the dataset was not powered to evaluate sex-differences directly, male and female animals were evaluated separately (39). All data are expressed as mean ± SEM. Offspring data are depicted as each sex separately and collapsed across males and females for display purposes.

For microbiome sequencing, samples underwent composition visualization, in addition to alpha-diversity and beta-diversity analyses (Qiime v.1.9.1) and statistical comparisons were performed using Kruskal-Wallis (40). Linear discriminant analysis effect size (LEfSe; http://huttenhower.sph.harvard.edu/lefse/) was utilized to determine significant differences in taxonomy abundance between each group as previously described (41-42).

For RNA-Seq, DESeq2 was used to determine differentially expressed genes based on a $p < 0.05$, Benjamini–Hochberg false discovery rate corrected (FDR) and fold change (FC) > 1.3 (43). Heatmaps were generated using the MultiExperiment Viewer (National Library of Medicine, USA), while gene ontology was determined using the Database for Annotation, Visualization and Integrated Discovery functional annotation cluster tool (https://david.ncifcrf.gov/).

Results

MIA challenge affected maternal behavior and offspring huddling

Immune challenge during lactation significantly impacted the number of maternal care bouts directed towards pups. For example, a MIA by time interaction was identified for the number of licks pups received following maternal LPS exposure (**Figure 1B**). While the number of licks received was not significantly affected on P10, there was a general pattern of reduced care provided by MIA treated dams on the afternoon of P10. This was followed by a rebound of maternal pup licking by MIA dams on the morning of P11, possibly to compensate for overall reduced maternal care due to illness. The display of active and low crouch nursing behaviors and time on nest were affected by the circadian cycle (**Figure 1C, D, E**), but only time on nest was impacted by MIA experience. Maternal self-directed care was impacted as a function of sickness. There was a significant MIA by time interaction for total number of self-grooms (**Figure 1F**); MIA dams had fewer bouts of grooming on the evening of P10, which reversed substantially by the morning of P11. Similarly, the number of maternal eating bouts was elevated on P11, although there were no significant effects of time (**Figure 1G**) there was a main effect of MIA (**Figure 1G**). Additional maternal care data are presented in **Supplementary Figure 1A-D**.

Huddling behavior was significantly reduced in the offspring of MIA dams compared to saline (main effect of MIA; time spent huddling, **Figure 1H**; average number of clusters: **Figure 1I)**. See **Figure 1J** for representative displays of huddling behavior in saline and MIA offspring.

MIA challenge impacted nutritional composition of milk and microbiome communities

For milk collection (see photographs in **Figure 2A** depicting the milk collection procedure), there was a significant MIA by time interaction for maternal milk levels of corticosterone. This stress-associated hormone was elevated in milk samples 2-hours following MIA challenge and was recovered by P11 (**Figure 2B)**. A MIA by time interaction was also present for % creamatocrit (**Figure 2C**), which is linearly related to the fat concentration and energy content of milk (44, 45; see **Supplementary Figure 2A, B**). These measures were significantly lower in the milk of MIA exposed mothers compared to saline on P11. Triglycerides were also reduced in the milk of MIA treated dams on P11 (**Figure 2D**).

In order to examine the possibility that MIA challenge increased expression inflammatory cytokines that could be transferred to nursing offspring, we analyzed the concentration of milk IL-6. While there were no significant differences in IL-6 concentration between saline and MIA treated dams, there was a main effect of time on this measure (**Figure 2E).** We also tested the idea that bound LPS could cross into maternal milk and be absorbed by offspring.

LPS binds directly to LBP which triggers a downstream pro-inflammatory response and the release of acute phase proteins like IL-6 (46-47). LBP levels were measured in blood plasma and P10 milk samples. LPB levels were significantly greater in the blood plasma of MIA dams (**Figure 2F)**. In milk, LPB was absent in both MIA and saline dams. Additional data on maternal milk composition (i.e., protein, lactose, and IgA concentrations) can be found in **Supplementary Figure 2C-E.**

Microbiome sequencing revealed no main effect of treatment group on alpha (**Figure 2G**) or beta diversity in milk samples (**Figure 2H, I**). Milk samples collected on P11 demonstrated significantly greater alpha diversity along the Shannon index compared to samples collected on P10 (**Figure 2G**). Between treatment groups, LEfSE analysis identified nine differently abundant taxa on P10 and six significantly abundant taxa on P11 **(Figure 2J, K)**. These analyses revealed a greater abundance of *Pseudomonadaceae* and *Christensenellaceae* on P10, and a greater expression of *Stenotrophomonas maltophilia*, *Ruminococcaceae* and *Lachnospiraceae* on P11 in the milk from MIA dams. For additional data on the composition of the milk microbiome, see **Supplementary Figures 3-6.**

RNA-seq of the milk was performed to characterize the effects of mid-lactational MIA at the genome-wide level. A total of 829 genes were differently expressed on P10 between saline and MIA milk samples (**Figure 3A**). We analyzed clusters of gene networks that are involved in maternal milk nutrition, immune activation, and offspring brain development (48-55, **Figure 3B).** Heatmap clustering revealed a general upregulation of genes related to nutrient transport and inflammation, while genes related to epigenetic modifications, parvalbumin development, and glucocorticoid signaling were largely downregulated by MIA on P10 (**Figure 3B**). In P11 samples (**Figure 4A, B),** a total of 893 genes were differentially affected between the milk of saline and MIA dams. Heat clustering analysis of these samples demonstrated a general downregulation in genes responsible for inflammation, glucocorticoid signaling, epigenetic modifiers, and prolactin in MIA samples, while genes related to oxytocin, nutrient transport, triglycerides, and parvalbumin development were largely upregulated. For more information on the genes indicated in the heatmap clustering, see **Supplementary Tables 2 and 3.**

The combination of MIA challenge and nutritional deficit affected adolescent offspring physiology and behavior.

Male, but not female MIA offspring were significantly heavier than saline controls on P43 (**Figure 5A**). See **Supplementary Figure 7** for additional data demonstrating the trajectory of weight gain in both offspring and dams across early development. Using body weight as a covariate, female offspring from MIA dams were found to have higher mechanical allodynia thresholds on the von Frey test compared to their saline counterparts (**Figure 5B**). This is suggestive of reduced sensitivity to tactile stimulation.

Although there were no differences in distance traveled (cm), percent time spent in the center, duration of time (seconds) spent in the center, or perimeter of the open field arena (see **Supplementary Figure 8A-D**), male offspring of MIA exposed mothers displayed an increased number of crosses into both the center (**Figure 5C**) and perimeter (**Figure 5D**) of the arena. Social preference was not affected by the early life experience for either sex (**Figure 5E**).

Sensorimotor gating was evaluated using PPI of the acoustic startle reflex. A significant MIA by time interaction (**Figure 5F**) and a significant main effect of time (**Figure 5G, H**, collapsed across males and females for display purposes) confirmed that all groups demonstrated an increased % PPI as the intensity was raised from 69 to 85 dB. While females were unaffected, male offspring originating from MIA exposed mothers had attenuated PPI values compared to saline controls at the 69dB, 73dB, and 81dB intensities (**Figure 5F**). The mean percent prepulse inhibition across all five prepulse intensities was not affected as a function of maternal MIA exposure (**Figure 5I**).

With respect to conditioned fear, there was a main effect of MIA for % PPI during the trials without fear (**Figure 5J, K, L)**. % PPI for trials containing the conditioned stimulus was not significantly different between saline and MIA animals for either sex (**Supplementary Figure 8E**, collapsed across males and females for display purposes). One-way ANOVA revealed a main effect of trial type (**Supplementary Figure 8E**), which was expected given that the added element of fear increases % PPI (56-57). Conditioned fear did not lead to significant changes in total mean % PPI when collapsed across all dB, regardless of MIA or sex (**Supplementary Figure 8F**, data collapsed across males and females for display purposes), suggesting that fear modulated % PPI similarly regardless of sex or MIA exposure.

In addition to these behavioral effects, PV/β-actin ratios were significantly reduced in the hippocampus of male MIA offspring compared to saline dams (**Figure 5M**).

Discussion

In the United States, postpartum bacterial infections develop in approximately 5-7% of mothers (58), and yet, little research is dedicated toward understanding how postnatal infection affects parental input signals like lactation and subsequent infant outcomes. We demonstrate that variations in milk quality may be part of a mechanism that programs offspring physiology and behavior following a transient stress exposure during the lactational period (see **Figure 6** for summary of proposed mechanisms). In the present study, acute MIA exposure on P10 was associated with sustained changes in both the nutritional content and microbial profile of maternal milk. Moreover, the MIA-induced changes in milk fat and corticosterone levels were accompanied by long-term physiological and behavioral alterations in offspring. For example, neonatal offspring from MIA-challenged dams spent less time huddling compared to controls. In adolescence, MIA offspring were heavier, demonstrated deficits in sensorimotor gating, threat detection, hippocampal PV expression, and had increased mechanical allodynia thresholds. These MIA-associated effects in offspring were unlikely due to an overexposure to inflammatory cytokines. Instead, offspring behavior and bodyweight differences likely reflected the unique expression of the nutritional elements and metabolically relevant bacterial taxa found in P10 and P11 milk samples. While most of the MIA-related literature has been dedicated to uncovering the physiological and behavioral effects on offspring gestationally exposed to MIA, fewer studies have focused on the impact of MIA exposure after birth. Importantly, the timing of MIA administration is critical to the translatability of these animal models to human studies, which have identified the second trimester as a sensitive window in cases of prenatal MIA exposure (2, 4). In rats, P10 corresponds to the perinatal period of development in humans (59), making our MIA model most relevant to maternal infections that occur soon after birth. Future studies are needed to extrapolate how MIA affects development when administered at later points during the lactational window.

MIA was associated with multiple changes in the composition of maternal milk content. We observed a significant increase in milk corticosterone 2-hours post LPS treatment that subsided by P11. Milk corticosterone is expressed linearly to plasma corticosterone (60), validating our MIA model. Importantly, milk corticosterone can pass from a mother to her offspring where these glucocorticoids can remain active in the periphery and in the brain (61-62). Increased corticosterone exposure from breastmilk has previously contributed to HPA axis dysfunction as well as altered learning and memory abilities in rat pups of both sexes, independent of maternal care (63-64). In addition to its modulatory actions within the HPA axis, recent evidence has demonstrated the regulatory role of corticosterone in breastmilk where the presence of this hormone (the human equivalent of cortisol) can modulate nutritional elements including sodium, potassium (65-66) and fat (24). This evidence suggests that the temporary rise in corticosterone on P10 may have preceded downstream changes in milk nutritional quality. In line with this, MIA reduced % creamatocrit and milk triglycerides on P11. However, higher levels of glucocorticoids in milk alone are not a sufficient predictor of offspring bodyweight (24, 62), suggesting that the actions of corticosterone in neonates are likely interwoven with milk nutrition.

Microbiome sequencing did not reveal significant differences in alpha or beta diversity between saline and MIA treated dams. While general diversity was not affected, LEfSe analysis uncovered significant differences in the expression of certain taxa on P10 and P11. Specifically, MIA dams exhibited a higher abundance of bacterial families including *Pseudomonadaceae*, which was previously shown to be over expressed in the ceca of pregnant mice with low serum triglycerides (67). Further, milk from MIA dams also exhibited elevated levels of *Stenotrophomonas maltofilia*, which has the ability to degrade hydrocarbon chains and therefore could prevent triglyceride synthesis (68). Given that the microbes in breast milk directly colonize the infant gut (69-70), it is likely that these differently expressed taxa are being passed to the offspring to influence future metabolic processes. However, microbiome sequencing of the offspring duodenum was not performed, and this is an important limitation to the present study. Future experiments assessing the relationship between milk and the offspring microbiome will be critical to determine the contribution of these MIA-induced microbial differences to offspring development. Clinical research exploring potential therapeutic interventions, such as targeting the microbiome (71) which may rescue changes in milk composition and subsequent offspring developmental outcomes in contexts where postnatal infections are common, would highly benefit from these future investigations.

Differences in microbial and nutritional markers paralleled MIA-mediated changes found in the whole-transcriptome of the milk using RNA-seq. Notably, we observed a significant upregulation in aldosterone-promoting *Ace* and a downregulation of the mineralocorticoid receptor-coding *Nr3c2* in MIA milk on P10 (**Figure 3B, Supplementary Table 2**). These changes, coupled with the increase in P10 milk corticosterone, corroborated differences observed in milk nutrition given that aldosterone is another crucial mediator of breastmilk nutrition and ion exchange (65). These results were further supported by the differential genomic profiles between milk from MIA and saline dams for gene clusters related to triglycerides and nutrient transport. RNA-seq also revealed a general upregulation of genes involved in the inflammatory response in maternal milk on P10 in MIA-treated dams. Although LBP and IL-6 were equally regulated between saline and MIA dams, the possibility that other inflammatory cytokines may be active in milk and subsequently affect offspring development cannot be entirely ruled out. There was an unexpected significant upregulation of several genes within the inflammation pathway on P11 (**Figure 4B, Supplementary Table 3**), however this may be reflective of the simultaneous downregulation of epigenetic modifiers since cytokine release is often associated with aberrant epigenetic activity (72). While the function of breastmilk RNA once it is absorbed by offspring is still being unpacked, fluorescently tagged RNA-containing milk exosomes were shown to reach the offspring brain *in vivo*, and these exomes have the ability to influence cellular processes *in vitro* (73, 74). Our data identified several key genes related to brain development within maternal milk including Foxp2, Homer1, Tnr, and Gabbr2, necessitating a more focused investigation into how these genes may have been differently expressed in the offspring brain following mid-lactational MIA. Overall, these data demonstrate the extensive effects of MIA on maternal milk at the genome-wide level.

We used several behavioral measures to explore the role of lactational MIA exposure on offspring development. Neonatal 'huddling' is described as a form of social thermoregulation (75). One previous study found a reduction in nest seeking behavior in rat pups prenatally exposed to LPS (76). Undernourishment has also been shown to reduce neonatal huddling in rat pups (77), and huddling efficiency may be related to serum triglycerides in newborn rabbits (78). This evidence suggests that MIA-modulated huddling may emerge due to differences in the fat content of maternal milk. Disruptions in huddling can also arise following changes to maternal care (79-81). Although maternal care in the present study did not drastically differ between MIA and saline dams, we did observe differences in maternal self-directed care. Therefore, reduced pup huddling may not be exclusively promoted by MIA-associated changes in milk quality.

Prenatal MIA has been shown to modulate offspring performance on certain tasks, such as the open field, social preference, PPI, measures of mechanical allodynia, and fear conditioning paradigms (4, 36, 82-84). Here, we observed significant MIA induced reductions in % PPI that were exacerbated in male adult offspring while females demonstrated deficits in mechanical allodynia. MIA was also associated with a significant reduction in % PPI in trials without fear, following the foot shock and conditioned stimulus (CS) pairing. These findings suggest MIA contributes to an impairment in the offspring's ability to discriminate between "safe" trials (without CS presentation) and "stressful" trials (with CS presentation). Early life stress has been shown to impair this form of contextual threat detection in adolescence, and this is especially true for females (85-86). These results, in addition to reduced neonatal huddling, point to generally impaired sensory processing and threat responses in the offspring of dams exposed to MIA during lactation. While we might expect these behavioral changes in our offspring to be maintained into adulthood, neonatal LPS exposure has been associated with altered behavior during specific developmental windows (e.g., adolescence) which can remit by later ages (e.g., adulthood; 87). Therefore, future studies should consider assessing both adolescents and adults in order to better understand the trajectory of these effects. The lactational period is an important time for neonates and here we underscore the value of maternal inputs (e.g., maternal milk quality and behavior) in offspring behavioral development.

We measured the expression of GABAergic PV in the offspring hippocampus since altered PV expression in this region is a common theme in prenatal MIA models and is associated with many MIA-associated behavioral and sensory processing changes (88-90). Further, poor nutrition in early life can prevent the development of PV in rat hippocampus (91) although this has only been explored in males. The emergence of neonatal huddling behavior is time-locked to the maturation of GABAergic signaling (92). Therefore, mid-lactational MIA may disrupt this early form of social behavior by attenuating the development of PV cells. Importantly, MIA dams showed significant increases in pup-directed licking and grooming behavior the day following MIA challenge. This heightened maternal care demonstrated by MIA dams on P11 may have been reflected by the upregulated expression of milk genes related to oxytocin on P11, but also may have acted as a compensatory mechanism to buffer the future development of more severe social deficits in adolescent offspring. This is plausible, given that tactile stimulation through the licking and grooming of pups is one form of maternal behavior that promotes brain development and modulates future offspring behavior (93-96). In adolescence, male MIA offspring demonstrated a reduced expression in hippocampal PV. In vivo inhibition of PV in the hippocampus has previously been shown to reduce PPI in male mice (97). Mechanistically, gestational exposure to MIA results in a more reactive HPA axis and altered hippocampal circuitry in male animals (36). In this study, we show that mid-lactational MIA may target similar mechanisms, as our male offspring demonstrated reduced PV expression and greater bodyweights, which may suggest greater stress levels compared to females. Together, these results demonstrate one neuronal target by which MIA during mid-lactation contributes to lasting changes in offspring brain development in males. More work is needed to discern the neuronal targets of mid-lactational MIA in females.

Although baseline maternal care was not assessed prior to milking procedures in the present study, future studies should consider how milking procedures may confound maternal care and affect offspring. Aside from a general pattern of decreased (but not significant) licking and grooming of pups on the afternoon of P10, we did not observe a drastic reduction in pup directed behaviors in MIA dams. The effects of LPS are highly dose dependent (98). While Vilela and colleagues (12) observed more dramatic deficits in maternal care following MIA, they implemented a dose of LPS 5-times higher than the dose used in the present study. Moreover, lactational exposure to LPS using the same dose as that used in the current study also resulted in improved maternal care (14). Importantly, LPS is a bacterial memetic that acts via the Toll-like receptor 4 pathway (99). Therefore, caution should be taken when interpreting the clinical relevance of our finding since work is needed in this field to establish if viral and other types of infections may affect lactation in a similar manner. Together, the results of our study suggest that stressors experienced during the neonatal period may interact with maternal care and lactation quality, affecting offspring physiology and behavior. Indeed, early life stress models affect both the parent(s) and the offspring; the dynamic impacts on each need to be considered as part of the mechanistic programming of the developmental trajectory.

Conclusions

Maternal care and milk quality are important components for offspring development, brain health, and behavior. Here, we demonstrate that an acute maternal immune response during mid-lactation is sufficient to trigger changes in breastmilk quality and its microbiome profile, in addition to the physiology and behavior of adolescent offspring. These results contribute to the broader literature by suggesting that the effects of MIA on offspring development are not restricted to the prenatal window. Moreover, this study ties in the characteristics of lactation and nutrition as part of a mechanism contributing to the trajectory of offspring development following early life stress. Given that animal models of early life stress can impact both parents and their offspring, the quality of maternal milk should be considered among the variables investigated in future studies. Finally, this work underscores the importance of research focused on potential therapeutic interventions (e.g., nutritional lactation supplements) and necessitates a better representation of pregnant and nursing people to aim for increased equity and inclusivity in both basic and clinical research.

Funding and Disclosures

This project was funded by NIMH under Award Number R15MH114035 (to ACK) and the Massachusetts College of Pharmacy and Health Sciences (MCPHS) Center for Undergraduate Research (H.T). The authors wish to thank Mary Erickson, Sahith Kaki, Ada Cheng, Dr. Mattia Migliore, and Dr. Theresa Casey for their technical support in the early phases of this project. The authors would also like to thank the MCPHS Schools of Pharmacy and Arts & Sciences for their continual support, the Bioinformatic Resource Center at the Rockefeller University where the RNA-seq was performed, and the University of Massachusetts Boston where HD is a graduate student. Figure 6 made with BioRender.com and an earlier version of this manuscript was posted on the preprint server bioRvix. The content is solely the responsibility of the authors and does not necessarily represent the official views of any of the financial supporters.

Author Contributions

H.D., S.G.C., H.T., and A.C.K., ran the experiments; H.D., S.G.C., J.M., & A.C.K. analyzed and interpreted the data; H.D. and A.C.K. wrote the manuscript; A.C.K., designed and supervised the study.

Conflict of Interest

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

References

1. Mahic, M., Che, X., Susser, E., Levin, B., Reichborn-Kjennerud, T., Magnus, P., ... & Lipkin, W. I. (2017). Epidemiological and serological investigation into the role of gestational maternal influenza virus infection and autism spectrum disorders. *Msphere*, *2*(3), e00159-17. https://doi.org/10.1128/mSphere.00159-17
2. Brown, A. S., Sourander, A., Hinkka-Yli-Salomäki, S., McKeague, I. W., Sundvall, J., & Surcel, H. M. (2014). Elevated maternal C-reactive protein and autism in a national birth cohort. Molecular psychiatry, 19(2), 259-264.
3. Meyer, U. (2019). Neurodevelopmental Resilience and Susceptibility to Maternal Immune Activation. *Trends in Neurosciences*, *42*(11), 793–806. https://doi.org/10.1016/j.tins.2019.08.001
4. Kentner, A.C., Bilbo, S.D., Brown, A.S., Hsiao, E.Y., McAllister, A.K., Meyer, U., Pearce, B.D., Pletnikov, M.V., Yolken, R.H., Bauman, M.D. (2019). Maternal immune activation: reporting guidelines to improve the rigor, reproducibility, and transparency of the model. *Neuropsychopharmacology 44*, 245-258.
5. Brown, R., Imran, S. A., & Wilkinson, M. (2009). Lipopolysaccharide (LPS) stimulates adipokine and socs3 gene expression in mouse brain and pituitary gland in vivo, and in N-1 hypothalamic neurons in vitro. Journal of *Neuroimmunology*, *209*(1-2), 96-103. https://doi.org/10.1016/j.jneuroim.2009.02.001
6. Arsenault, D., St-Amour, I., Cisbani, G., Rousseau, L.-S., & Cicchetti, F. (2014). The different effects of LPS and poly I:C prenatal immune challenges on the behavior,development and inflammatory responses in pregnant mice and their offspring. *Brain, Behavior, and Immunity*, *38*, 77–90. https://doi.org/10.1016/j.bbi.2013.12.016
7. Estes, M.L. & McAllister, A.K. (2016). Maternal immune activation: Implications for neuropsychiatric disorders. *Science, 353*(6301):772-7. doi: 10.1126/science.aag3194.
8. Karolinski A, Mazzoni A, Belizan JM, Althabe F, Bergel E, Buekens P (2010). Lost opportunities for effective management of obstetric conditions to reduce maternal mortality and severe maternal morbidity in Argentina and Uruguay. Int J Gynaecol Obstet, 110, 175–180.
9. Kutlesic, V., Breinski Isaacs, M., Freund, L.S., Hazra, R., Raiten, D.J. (2017). Executive summary: research gaps at the intersection of pediatric neurodevelopment, nutrition, and inflammation in low resource settings. Pediatrics, 139, S1-S11.
10. John, C.C., Black, M.M., Nelson, C.A III. (2017). Neurodevelopment: the impact of nutrition and inflammation during early to middle childhood in low-resource settings. *Pediatrics*, 139, e 20162828.
11. Hussein J, Mavalankar DV, Sharma S, D'Ambruoso L. (2011). A review of health system infection control measures in developing countries: what can be learned to reduce maternal mortality. *Global Health*, 7, 14.

12. Vilela, F.C., Antunes-Rodrigues, J., Elias, L.L., & Giusti-Paiva, A. (2013). Corticosterone synthesis inhibitor metyrapone preserves changes in maternal behavior and neuroendocrine responses during immunological challenge in lactating rats. *Neuroendocrinology. 97*(4):322-30. doi: 10.1159/000346354
13. Ling B, Alcorn J. LPS-induced inflammation downregulates mammary gland glucose, fatty acid, and L-carnitine transporter expression at different lactation stages. (2010). *Research in Veterinary Science, 89*(2):200-2. doi: 10.1016/j.rvsc.2010.03.004
14. Nascimento, A. F., Alves, G. J., Massoco, C. O., Teodorov, E., Felicio, L. F., & Bernardi, M. M. (2015). Lipopolysaccharide-induced sickness behavior in lactating rats decreases ultrasonic vocalizations and exacerbates immune system activity in male offspring. *Neuroimmunomodulation, 22*(4), 213-221. https://doi.org/10.1159/000363350
15. Hamprecht K, Maschmann J, Vochem M, Dietz K, Speer CP, Jahn G. (2001). Epidemiology of transmission of cytomegalovirus from mother to preterm infant by breastfeeding. *Lancet, 357*(9255):513-8. doi: 10.1016/S0140-6736(00)04043-5.
16. Jones, C. (2001). Maternal transmission of infectious pathogens in breast milk. *Journal of Paediatrics and Child Health*, 37: 576-582. https://doi.org/10.1046/j.1440-1754.2001.00743.x
17. Desgraupes S, Hubert M, Gessain A, Ceccaldi PE, Vidy A. (2021). Mother-to-child transmission of arboviruses during breastfeeding: From epidemiology to cellular mechanisms. *Viruses, 13*(7):1312. doi: 10.3390/v13071312.
18. Lesorogol, C., Bond, C., Dulience, S. J. L., & Iannotti, L. (2018). Economic determinants of breastfeeding in Haiti: the effects of poverty, food insecurity, and employment on exclusive breastfeeding in an urban population. *Maternal & Child Nutrition, 14*(2), e12524. https://doi.org/10.1111/mcn.12524
19. Mäkelä, J., Linderborg, K., Niinikoski, H., Yang, B., & Lagström, H. (2013). Breast milk fatty acid composition differs between overweight and normal weight women: the STEPS Study. *European Journal of Nutrition, 52*(2), 727-735. https://doi.org/10.1007/s00394-012-0378-5
20. Chen, Y., Wang, J., Yang, S., Utturkar, S., Crodian, J., Cummings, S., Thimmapuram, J., San Miguel, P., Kuang, S., Gribskov, M., Plaut, K., Casey, T. (2017). Effect of high fat diet on secreted milk transcriptome in midlactation mice. *Physiology Genomics, 49*, 747-762, doi:10.1152/physiolgenomics.00080.2017.
21. Grazia Di Benedetto, M., Bottanelli, C., Cattaneo, A., Pariante, C.M., Borsini, A. (2020). Nutritional and immunological factors in breast milk: a role in the intergenerational transmission from maternal psychopathology to child development. *Brain, Behavior, and Immunity*, 85, 57-68.
22. Edwards, P.D., Lavergne, S.G., McCaw, L.K., Wijenayake, S., Boonstra, R., McGowan,
P.O., Holmes, M.M. (2021). Maternal effects in animals: broadening our understanding of offspring programming. *Frontiers in Neuroendocrinology*, 62, 100924, doi: 10.1016/j.yfrne.2021.100924
23. Browne, P. D., Aparicio, M., Alba, C., Hechler, C., Beijers, R., Rodríguez, J. M., ... & de Weerth, C. (2019). Human milk microbiome and maternal postnatal psychosocial distress. *Frontiers in Microbiology, 10,* 2333. https://doi.org/10.3389/fmicb.2019.02333
24. Hinde, K., Skibiel, A. L., Foster, A. B., Del Rosso, L., Mendoza, S. P., & Capitanio, J. P. (2015). Cortisol in mother’s milk across lactation reflects maternal life history and predicts infant temperament. *Behavioral Ecology, 26*(1), 269-281. https://doi.org/10.1093/beheco/aru186

25. Crum, W. R., Sawiak, S. J., Chege, W., Cooper, J. D., Williams, S., & Vernon, A. C. (2017). Evolution of structural abnormalities in the rat brain following in utero exposure to maternal immune activation: A longitudinal in vivo MRI study. *Brain, Behavior, and Immunity*, *63*, 50–59. https://doi.org/10.1016/j.bbi.2016.12.008
26. Piontkewitz, Y., Arad, M., & Weiner, I. (2011). Abnormal trajectories of neurodevelopment and behavior following in utero insult in the rat. *Biological Psychiatry*, *70*(9), 842-851. https://doi.org/10.1016/j.biopsych.2011.06.007
27. Kirlic, N., Young, J., & Aupperle, R. L. (2017). Animal to human translational paradigms relevant for approach avoidance conflict decision making. *Behaviour Research and Therapy*, *96*, 14–29. https://doi.org/10.1016/j.brat.2017.04.010
28. Powell, S. B., Zhou, X., & Geyer, M. A. (2009). Prepulse inhibition and genetic mouse models of schizophrenia. *Behavioural Brain Research*, *204*(2), 282–294. https://doi.org/10.1016/j.bbr.2009.04.021
29. Canetta S, Bolkan S, Padilla-Coreano N, Song LJ, Sahn R, Harrison NL, Gordon JA, Brown A, Kellendonk C. (2016). Maternal immune activation leads to selective functional deficits in offspring parvalbumin interneurons. *Molecular Psychiatry*. *21*(7):956-68. doi: 10.1038/mp.2015.222.
30. Drugs and Lactation Database (LactMed) [Internet]. Bethesda (MD): National Library of Medicine (US); 2006-. Isoflurane. [Updated 2020 Nov 16]. Bookshelf URL: https://www.ncbi.nlm.nih.gov/books/
31. Lee, J.J. & Rubin, A.P. (2993). Breast feeding and anesthesia. Anaesthesia, 48, 616-625, doi: 10.1111/j.1365-2044.1993.tb07130.x.
32. Par Pharmaceutical, Inc, Pitocin [Label], Par Pharmaceutical, Chestnut Ridge, NY, (2020), https://dailymed.nlm.nih.gov/dailymed/fda/fdaDrugXsl.cfm?setid=6d4b2c25-2e5d- 49b5-93bc-2ae8a20916d1&type=display
33. DeRosa, H., Caradonna, S.G., Tran, H., Marrocco, J., & Kentner, A.C. (2022). Milking it for all it's worth: The effects of environmental enrichment on maternal nurturance, lactational quality, and offspring social behavior. *bioRxiv.* doi: https://doi.org/10.1101/2021.12.13.472509
34. Yan S. & Kentner A.C. (2017). Mechanical allodynia corresponds to Oprm1 downregulation within the descending pain network of male and female rats exposed to neonatal immune challenge. *Brain, Behavior, and Immunity*, *63*, 148-159. doi: 10.1016/j.bbi.2016.10.007.
35. Connors, E. J., Shaik, A. N., Migliore, M. M., & Kentner, A. C. (2014). Environmental enrichment mitigates the sex-specific effects of gestational inflammation on social engagement and the hypothalamic pituitary adrenal axis-feedback system. *Brain, Behavior, and Immunity*, *42*, 178-190. https://doi.org/10.1016/j.bbi.2014.06.020
36. Zhao, X., Mohammed, R., Tran, H., Erickson, M., & Kentner, A. C. (2021a). Poly (I: C)-induced maternal immune activation modifies ventral hippocampal regulation of stress reactivity: prevention by environmental enrichment. *Brain, Behavior, and Immunity*. https://doi.org/10.1016/j.bbi.2021.03.018
37. Núñez Estevez, K.J., Rondón-Ortiz, A.N., Nguyen, J.Q.T., & Kentner, A.C. (2020). Environmental influences on placental programming and offspring outcomes following maternal immune activation. *Brain, Behavior, and Immunity*, *83*, 44-55. doi: 10.1016/j.bbi.2019.08.192.
38. Miles, J., Shevlin, M., 2001. Applying regression and correlation: A guide for students and researchers. Sage.

39. Ordoñes Sanchez, E., Bavley, C.C., Deutschmann, A.U., Carpenter, R., Peterson, D.R.,
Karbalaei, R….Bangasser, D.A. (2021). Early life adversity promotes resilience to opioid addiction-related phenotypes in male rats and sex-specific transcriptional changes. *PNAS USA, 118*, e2020173118. doi: 10.1073/pnas.2020173118.
40. Caporaso, J.G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F.D., Costello, E.K. et al. (2010) QIIME allows analysis of high-throughput community sequencing data. *Nature Methods, 7*: 335-336. https://doi.org/10.1038/nmeth.f.303
41. Segata, N., Izard, J., Waldron, L., Gevers, D., Miropolsky, L., Garrett, W.S., and Huttenhower, C. (2011) Metagenomic biomarker discovery and explanation. *Genome Biology, 12*: R60. doi: 10.1186/gb-2011-12-6-r60.
42. Schellekens, H., Torres-Fuentes, C., van de Wouw, M., Long-Smith, C. M., Mitchell, A., Strain, C., ... & Cryan, J. F. (2021). Bifidobacterium longum counters the effects of obesity: Partial successful translation from rodent to human. *EBioMedicine*, *63,* 103176.
43. Love, MI, Hogenesch, JB, & Irizarry, RA (2016). Modeling of RNA-seq fragment sequence bias reduces systematic errors in transcript abundance estimation. *Nature Biotechnology*. 34(12):1287-91. 10.1038/nbt.3682.
44. Lucas, A., Gibbs, J.A., Lyster, R.L., Baum, J.D. (1978). Creamatocrit: simple clinical technique for estimating fat concentration and energy value of human milk. Br Med J., 1 (6119):1018-1020, doi: 10.1136/bmj.1.6119.1018.
45. Paul, H.A., Hallam, M.C., Reimer, R.A. (2015). Milk collection in the rat using capillary tubes and estimation of milk fat content by creamatocrit. *Journal of Visualized Experiments, 106,* 1-6, doi: 10.3791/53476.
46. Wurfel, M.M. & Wright, S.D. (1997). Lipopolysaccharide-binding protein and soluble CD14 transfer lipopolysaccharide to phospholipid bilayers: preferential interaction with particular classes of lipid. *Journal of Immunology. 15;158*(8):3925-34. PMID: 9103463.
47. Park, B. & Lee, J.O. (2013). Recognition of lipopolysaccharide pattern by TLR4 complexes. Experimental Molecular Medicine 45, https://doi.org/10.1038/emm.2013.97
48. Quintana, DS, Rokicki, J, van der Meer, D, Alnæs, D, Kaufmann, T, Córdova-Palomera, A, Dieset, I, Andreassen, OA, & Westlye, LT. (2019). Oxytocin pathway gene networks in the human brain. Nat Commun 10, 668. https://doi.org/10.1038/s41467-019-08503- 8
49. Oakley, RH & Cidlowski, JA (2013). The biology of the glucocorticoid receptor: new signaling mechanisms in health and disease. J Allergy Clin Immunol., 132(5), 1033-1044. https://doi.org/10.1016/j.jaci.2013.09.007
50. Polman JA, Welten JE, Bosch DS, de Jonge RT, Balog J, van der Maarel SM, de Kloet ER, Datson NA. (2012). A genome-wide signature of glucocorticoid receptor binding in neuronal PC12 cells. BMC Neurosci.13:118. doi: 10.1186/1471-2202-13-118.
51. Zhu X, Girardo D, Govek EE, John K, Mellén M, Tamayo P, Mesirov JP, Hatten ME. (2016). Role of Tet1/3 Genes and Chromatin Remodeling Genes in Cerebellar Circuit Formation. *Neuron*. 89(1):100-12. doi: 10.1016/j.neuron.2015.11.030
52. Ozkan, H, Tuzun, F, Taheri, S, Korhan, P, Akokay, P, Yılmaz, O, Duman, N, Özer, E, Tufan, E, Kumral, A, & Özkul, Y (2020). Epigenetic programming through breast milk and its impact on milk-siblings mating. Front. Genet., 11, 569232. https://doi.org/10.3389/fgene.2020.569232
53. Zhang, TY, Labonté, B, Wen, XL, Turecki, G, & Meaney, MJ (2013). Epigenetic

mechanisms for the early environmental regulation of hippocampal glucocorticoid receptor gene expression in rodents and humans. NPP, 38(1), 111–123. https://doi.org/10.1038/npp.2012.149

54. Bowen, W.S., Gandhapudi, S.K., Kolb, J.P., Mitchell, T.C. (2013). Chapter Three- Immunopharmacology of Lipid A Mimetics, *Advances in Pharmacology, 66,* 81-128. https://doi.org/10.1016/B978-0-12-404717-4.00003-2
55. Borish, L.C. & Steinke, J.W. (2003). Cytokines and chemokines. *Journal of Allergy Clinical Immunology. 111*, 460-745. doi: 10.1067/mai.2003.108.
56. Ishii, D., Matsuzawa, D., Fujita, Y., Sutoh, C., Ohtsuka, H., Matsuda, S., ... & Shimizu, E. (2010). Enhancement of acoustic prepulse inhibition by contextual fear conditioning in mice is maintained even after contextual fear extinction. *Progress in Neuro-Psychopharmacology and Biological Psychiatry*, *34*(1), 183-188.
57. Balogh, S. A., Radcliffe, R. A., Logue, S. F., & Wehner, J. M. (2002). Contextual and cued fear conditioning in C57BL/6J and DBA/2J mice: context discrimination and the effects of retention interval. *Behavioral Neuroscience*, *116*(6), 947. doi: 10.1037//0735-7044.116.6.947.
58. Boushra M & Rahman O. (2021). Postpartum Infection. In: StatPearls. Treasure Island (FL): *StatPearls Publishing*; PMID: 32809639.
59. Clancy, B., Finlay, B. L., Darlington, R. B., & Anand, K. J. S. (2007). Extrapolating brain development from experimental species to humans. NeuroToxicology, 28(5), 931–937. doi:10.1016/j.neuro.2007.01.014
60. Patacchioli, F. R., Cigliana, G., Cilumbriello, A., Perrone, G., Capri, O., Alemà, S., ... & Angelucci, L. (1992). Maternal plasma and milk free cortisol during the first 3 days of breast-feeding following spontaneous delivery or elective cesarean section. *Gynecologic and Obstetric Investigation*, *34*(3), 159-163. https://doi.org/10.1159/000292751
61. Angelucci, L. (1985). A model for later-life effects of perinatal drug exposure: maternal
hormone mediation. *Neurobehavioral Toxicology & Teratology*.
62. Brummelte, S., Schmidt, K.L., Taves, M.D., Soma, K.K. & Galea, L. A. (2010). Chronic corticosterone during pregnancy and postpartum affects maternal care, cell proliferation and depressive-like behavior in the dam. *Hormones and Behavior, 58*(5), 769-779. https://doi.org/10.1016/j.yhbeh.2010.07.012
63. Catalani, A., Marinelli, M., Scaccianoce, S., Nicolai, R., Muscolo, L. A., Porcu, A., ... & Angelucci, L. (1993). Progeny of mothers drinking corticosterone during lactation has lower stress-induced corticosterone secretion and better cognitive performance. *Brain Research, 624*(1-2), 209-215. https://doi.org/10.1016/0006-8993(93)90079-3
64. Catalani, A., Casolini, P., Cigliana, G., Scaccianoce, S., Consoli, C., Cinque, C., ... & Angelucci, L. (2002). Maternal corticosterone influences behavior, stress response and corticosteroid receptors in the female rat. *Pharmacology Biochemistry and Behavior*, *73*(1), 105-114. https://doi.org/10.1016/S0091-3057(02)00755-4
65. Zietek, M., Sochaczewska, D., Swiatkowska-Freund, M., Celewicz, Z., & Szczuko, M. (2021). The possible role of corticosterone in regulating sodium and potassium concentrations in human milk. *Ginekologia Polska*. doi: 10.5603/GP.a2021.0072.
66. Sullivan, E. C., Hinde, K., Mendoza, S. P., & Capitanio, J. P. (2011). Cortisol concentrations in the milk of rhesus monkey mothers are associated with confident temperament in sons, but not daughters. *Developmental Psychobiology, 53*(1), 96–104. https://doi.org/10.1002/dev.20483
67. Connor, K. L., Chehoud, C., Altrichter, A., Chan, L., DeSantis, T. Z., & Lye, S. J. (2018). Maternal metabolic, immune, and microbial systems in late pregnancy vary

with malnutrition in mice. *Biology of Reproduction*, *98*(4), 579-592. https://doi.org/10.1093/biolre/ioy002

68. Singh, D., & Fulekar, M. H. (2010). Biodegradation of petroleum hydrocarbons by Pseudomonas putida strain MHF 7109. *CLEAN–Soil, Air, Water, 38*(8), 781-786. https://doi.org/10.1002/clen.200900239
69. Sheard, N. F., & Walker, W. A. (1988). The role of breast milk in the development of the gastrointestinal tract. *Nutrition Reviews (USA).* doi: 10.1111/j.1753-4887.1988.tb05343.x.
70. Pannaraj, P. S., Li, F., Cerini, C., Bender, J. M., Yang, S., Rollie, A., ... & Aldrovandi, G. M. (2017). Association between breast milk bacterial communities and establishment and development of the infant gut microbiome. JAMA Pediatrics, 171(7), 647-654.
71. Holingue, C., Brucato, M., Ladd□Acosta, C., Hong, X., Volk, H., Mueller, N. T., ... & Fallin, M. D. (2020). Interaction between Maternal Immune Activation and Antibiotic Use during Pregnancy and Child Risk of Autism Spectrum Disorder. *Autism Research*, *13*(12), 2230-2241. https://doi.org/10.1002/aur.2411
72. Obata, Y., Furusawa, Y. and Hase, K. (2015). Epigenetic modifications of the immune system in health and disease. *Immunology & Cell Biology*, *93*: 226-232. https://doi.org/10.1038/icb.2014.114
73. Golan-Gerstl, R., Elbaum Shiff, Y., Moshayoff, V., Schecter, D., Leshkowitz, D., Reif, S., (2017). Characterization and biological function of milk-derived miRNAs. *Molecular. Nutrition & Food Research*, *61*, 1700009. https://doi.org/10.1002/mnfr.201700009
74. Manca, S., Upadhyaya, B., Mutai, E., Desaulniers, A. T., Cederberg, R. A., White, B. R., & Zempleni, J. (2018). Milk exosomes are bioavailable and distinct microRNA cargos have unique tissue distribution patterns. *Scientific Reports*, *8*(1), 1-11. DOI:10.1038/s41598-018-29780-1
75. Gilbert, C., McCafferty, D., Le Maho, Y., Martrette, J. M., Giroud, S., Blanc, S., & Ancel, A. (2010). One for all and all for one: the energetic benefits of huddling in endotherms. *Biological Reviews*, *85*(3), 545-569. https://doi.org/10.1111/j.1469-185X.2009.00115.x
76. Baharnoori, M., Bhardwaj, S. K., & Srivastava, L. K. (2012). Neonatal behavioral changes in rats with gestational exposure to lipopolysaccharide: a prenatal infection model for developmental neuropsychiatric disorders. *Schizophrenia Bulletin*, *38*(3), 444–456. https://doi.org/10.1093/schbul/sbq098
77. Soriano, O., Regalado, M., Torrero, C., & Salas, M. (2006). Contributions of undernutrition and handling to huddling development of rats. *Physiology & Behavior, 89*(4), 543-551. https://doi.org/10.1016/j.physbeh.2006.07.021
78. García-Torres, E., Hudson, R., Castelán, F., Martínez-Gómez, M., & Bautista, A. (2015). Differential metabolism of brown adipose tissue in newborn rabbits in relation to position in the litter huddle. *Journal of Thermal Biology*, *51*, 33-41. https://doi.org/10.1016/j.jtherbio.2015.03.003
79. Alberts, J.R. & May, B. (1984), Nonnutritive, thermotactile induction of filial huddling in rat pups. *Developmental Psychobiology*, *17:* 161-181. https://doi.org/10.1002/dev.420170207
80. Kojima, S., & Alberts, J. R. (2009). Maternal care can rapidly induce an odor-guided huddling preference in rat pups. *Developmental Psychobiology, 51*(1), 95–105. https://doi.org/10.1002/dev.20349

81. Miele, J., Makin, J.W., Russo, S., Cameron, K., Costantini, F., & Deni, R. (1983). Huddling behavior of spiny mouse pups toward foster siblings from another species. *Bulletin of the Psychonomic Society, 21(6), 479-482.* doi: 10.3758/BF03330014
82. Fortier, M. E., Luheshi, G. N., & Boksa, P. (2007). Effects of prenatal infection on prepulse inhibition in the rat depend on the nature of the infectious agent and the stage of pregnancy. *Behavioural Brain Research, 181*(2), 270-277. https://doi.org/10.1016/j.bbr.2007.04.016
83. Inceoglu, B., Jinks, S. L., Schmelzer, K. R., Waite, T., Kim, I. H., & Hammock, B. D. (2006). Inhibition of soluble epoxide hydrolase reduces LPS-induced thermal hyperalgesia and mechanical allodynia in a rat model of inflammatory pain. *Life Sciences*, *79*(24), 2311–2319. https://doi.org/10.1016/j.lfs.2006.07.031
84. Howland, J. G., Cazakoff, B. N., & Zhang, Y. (2012). Altered object-in-place recognition memory, prepulse inhibition, and locomotor activity in the offspring of rats exposed to a viral mimetic during pregnancy. *Neuroscience*, *201*, 184-198. https://doi.org/10.1016/j.neuroscience.2011.11.011
85. Manzano-Nieves, G., Gaillard, M., Gallo, M., & Bath, K. G. (2018). Early life stress impairs contextual threat expression in female, but not male, mice. *Behavioral Neuroscience*, *132*(4), 247. https://doi.org/10.1037/bne0000248
86. Gerhard, D. M., Meyer, H. C., & Lee, F. S. (2021). An Adolescent Sensitive Period for Threat Responding: Impacts of Stress and Sex. *Biological psychiatry*, *89*(7), 651–658. https://doi.org/10.1016/j.biopsych.2020.10.003
87. MacRae, M., Macrina, T., Khoury, A., Migliore, M. M., & Kentner, A. C. (2015). Tracing the trajectory of behavioral impairments and oxidative stress in an animal model of neonatal inflammation. *Neuroscience*, *298*,455-466. https://doi.org/10.1016/j.neuroscience.2015.04.048
88. Zhao, X., Tran, H., DeRosa, H., Roderick, R.C., & Kentner, A.C. (2021b) Hidden talents: Poly (I:C)-induced maternal immune activation improves mouse visual discrimination performance and reversal learning in a sex-dependent manner. *Genes, Brain, and Behavior*, *20*(7). doi: 10.1111/gbb.12755.
89. Nakamura JP, Gillespie B, Gibbons A, Jaehne EJ, Du X, Chan A, Schroeder A, van den Buuse M, Sundram S, Hill RA. (2021). Maternal immune activation targeted to a window of parvalbumin interneuron development improves spatial working memory: Implications for autism. *Brain, Behavior, & Immunity*, 91:339-349. doi: 10.1016/j.bbi.2020.10.012.
90. Vojtechova, I., Maleninska, K., Kutna, V., Klovrza, O., Tuckova, K., Petrasek, T., & Stuchlik, A. (2021). Behavioral Alterations and Decreased Number of Parvalbumin-Positive Interneurons in Wistar Rats after Maternal Immune Activation by Lipopolysaccharide: Sex Matters. *International Journal of Molecular Sciences*, *22*(6), 3274. https://doi.org/10.3390/ijms22063274
91. Callahan, L. S., Thibert, K. A., Wobken, J. D., & Georgieff, M. K. (2013). Early-life iron deficiency anemia alters the development and long-term expression of parvalbumin and perineuronal nets in the rat hippocampus. Developmental neuroscience, 35(5), 427-436. https://doi.org/10.1159/000354178
92. Naskar, S., Narducci, R., Balzani, E. et al. (2019). The development of synaptic transmission is time-locked to early social behaviors in rats. *Nature Communications, 10*, 1195 https://doi.org/10.1038/s41467-019-09156-3
93. Champagne, F. A., Francis, D. D., Mar, A., & Meaney, M. J. (2003). Variations in maternal care in the rat as a mediating influence for the effects of environment on development. *Physiology & Behavior*, *79*(3), 359-371. https://doi.org/10.1016/S0031-9384(03)00149-5

94. Francis, D. D., Young, L. J., Meaney, M. J., & Insel, T. R. (2002). Naturally occurring differences in maternal care are associated with the expression of oxytocin and vasopressin (V1a) receptors: gender differences. *Journal of Neuroendocrinology, 14*(5), 349-353.
95. van Hasselt, F. N., Cornelisse, S., Yuan Zhang, T., Meaney, M. J., Velzing, E. H., Krugers, H. J., & Joëls, M. (2012). Adult hippocampal glucocorticoid receptor expression and dentate synaptic plasticity correlate with maternal care received by individuals early in life. *Hippocampus*, *22*(2), 255-266. https://doi.org/10.1002/hipo.20892
96. Francis, D. D. & Kuhar, M. (2008). Frequency of maternal licking and grooming correlates negatively with vulnerability to cocaine and alcohol use in rats. *Pharmacology Biochemistry and Behavior, 90*(3), 497-500. https://doi.org/10.1016/j.pbb.2008.04.012
97. Nguyen, R., Morrissey, M.D., Mahadevan, V., Cajanding, J.D., Woodin, M.A., Yeomans, J.S. Takehara-Nishiuchi K, Kim JC. (2014). Parvalbumin and GAD65 interneuron inhibition in the ventral hippocampus induces distinct behavioral deficits relevant to schizophrenia. *Journal of Neuroscience*. *34*(45):14948-60. doi: 10.1523/JNEUROSCI.2204-14.2014.
98. Bison, S., Carboni, L., Arban, R., Bate, S., Gerrard, P. A., & Razzoli, M. (2009). Differential behavioral, physiological, and hormonal sensitivity to LPS challenge in rats. *International Journal of Interferon, Cytokine and Mediator Research*, *1*, 1-13. https://doi.org/10.2147/IJICMR.S4273
99. Pålsson-McDermott, E.M. and O'Neill, L.A.J. (2004), Signal transduction by the lipopolysaccharide receptor, Toll-like receptor-4. Immunology, 113: 153-162. https://doi.org/10.1111/j.1365-2567.2004.01976.x

Figure Captions

Figure 1. Maternal care and offspring huddling behaviors following maternal immune activation (MIA) during the mid-lactational period. (A) Timeline of experimental procedures. Maternal care was evaluated in the afternoon and night of postnatal day (P) 10 and the morning of P11. (B) MIA x Time interaction for the total number of pup licks (F(2, 26) = 31.435, p = 0.001, n_p^2 = 0.701). Pup licking and grooming were elevated in MIA dams on P11 (p = 0.001). The circadian cycle influenced the display of (C) active nursing bouts (F(2, 26) = 7.752, p = 0.0022, n_p^2 = 0.374), which were elevated in both groups on P10. (D) Low crouch nursing bouts (F(2, 26) = 3.960, p = 0.032, n_p^2 = 0.233), and (E) total time (seconds) that the dam spent on nest were also affected by the circadian cycle, as dams spent less time in the low crouch nursing posture and overall less time on the nest during the evening on P10. Total number of (F) self-groom bouts demonstrated a MIA x Time interaction (F(1.322, 26) = 12.33, p = 0.001, n_p^2 = 0.485); Huyndt Feldt correction. MIA was associated with a reduction in self-grooming bouts on the evening of P10 (p = 0.025), which were increased on the morning of P11 (p = 0.0001). A main effect of MIA was observed for (G) the number of eating bouts (F(1, 13) = 10.64, p = 0.006, n_p^2 = 0.450). (H) Total time pups spent huddling (seconds) was reduced by MIA (F(1, 12) = 30.079, p = 0.001, n_p^2 = 0.715) as was the (I) average number of pup clusters across P10 and P11 (F(1, 12) = 8.33, p = 0.014, n_p^2 = 0.410). (J) Shows representative photographs of the huddling behavior of saline and MIA litters. Data are expressed as mean ± SEM; Saline: n = 8; MIA: n = 7. *p < 0.05, ***p

<0.001, MIA versus Saline; $^{a}p < 0.05$, $^{aa}p < 0.01$, main effect of time; $^{b}p < 0.05$, $^{bb}p < 0.01$, $^{bbb}p < 0.001$, main effect of MIA.

Figure 2. Nutritional profile and microbiome community distribution of milk following maternal immune activation (MIA) during the mid-lactational period. (A) Photographs of maternal milk collection. A significant MIA x Time interaction was observed for maternal milk levels (Saline: n = 8; MIA: n = 7) of (B) corticosterone (ng/mL; F(1, 13) = 8.496, p = 0.013, $n_p^2 = 0.415$), which was elevated in the milk of MIA dams on P10 (p = 0.009). A MIA x Time interaction was observed for (C) percent (%) creamatocrit (F(1, 13) = 5.86, p = 0.032, $n_p^2 = 0.858$), which was significantly lower in MIA samples on P11 (p = 0.002). A significant main effect of MIA was observed for (D) milk triglycerides (mg/dL; F(1, 13) = 6.496, p = 0.026, $n_p^2 = 0.351$). There was a main effect of time on (E) milk interleukin (IL)-6 (pg/mL) concentrations (F(1,13) = 33.01, p < 0.001, $n_p^2 = 0.717$) in that IL-6 was increased in both groups on P11. Levels of (F) LPS binding protein (LPB; ug/mL) were increased in the blood plasma of MIA dams ($X^2(1)=5.00$, p=0.025), but there were negligible levels in maternal milk (p>0.05). (G) Alpha diversity of P10 and P11 milk samples along the Shannon Index was increased in both groups on P11 compared to P10 ($X^2(1) = 5.33$, p = 0.021). (H) Beta diversity using principal coordinate analysis (PCoA) of P10 and (I) P11 milk samples were created using the matrix of pair-wise distance between samples determined by Bray-Curtis dissimilarity using unique amplicon variants. (J) Microbial composition of taxonomy at the family level for saline and MIA dams at P10 (Saline: n = 6; MIA: n = 6) and P11 (Saline: n = 6; MIA: n = 7). (K) LEfSe biomarkers plots. These figures display taxa that are significantly more abundant in the milk of saline-treated dams (blue bars) versus MIA-treated dams (red bars) on P10 and P11. These taxa were identified based on their significant distributions (p < 0.05) and effect sizes (LDA score) larger than 2 for P10 (Saline: n = 6; MIA: n = 6) and for P11 (Saline: n = 7; MIA: n = 7). Among these taxa, MIA was associated with a higher level of *Pseudomonadaceae* (LDA score = 2.94, p=0.02) and *Christensenellaceae* (LDA score = 2.82, p = 0.02) on P10, and a greater expression of *Stenotrophomonas maltophilia* (LDA score= 3.45, p=0.025), *Ruminococcaceae* (LDA score = 3.35, p = 0.02) and *Lachnospiraceae* (LDA score= 3.85, p = 0.01) on P11. Data are expressed as mean ± SEM. *p < 0.05, ***p <0.001, MIA versus Saline; $^{a}p < 0.05$, $^{aa}p < 0.01$, main effect of time; $^{b}p < 0.05$, $^{bb}p < 0.01$, $^{bbb}p < 0.001$, main effect of MIA. LPS – lipopolysaccharide.

Figure 3. Transcriptomic analyses of milk samples obtained on P10 from rat mothers exposed to either saline or maternal immune activation (MIA). (A) Volcano plot depicting the distribution of 829 genes based on log2 fold change and -log10 p values. Grey dots are genes, and the dots highlighted in blue represent genes that displayed the highest magnitude of significance (padj<0.05, FC>1.3). (B) Heatmaps of differentially expressed genes related specifically to milk nutrient transport, triglyceride concentration, parvalbumin development, aldosterone/MR signaling, inflammation, LPS binding, oxytocin, prolactin, glucocorticoid signaling (events following glucocorticoids binding to the GR receptor), glutamate/GABA, and epigenetic modifiers. Expression is represented with the log2 transformation of counts recorded with a z-score based on the average across experimental groups. Data are expressed as *p <0.05 or **adjusted p <0.05, or ***FC>1.3, MIA versus Saline. GR- glucocorticoid receptor. MR- mineralocorticoid receptor.

Figure 4. Transcriptomic analyses of milk samples obtained on P11 from rat mothers exposed to either saline or maternal immune activation (MIA). (A) Volcano plot depicting the distribution of 893 genes based on log2 fold change and -log10 p values. Grey dots are genes, and the dots highlighted in blue represent genes that displayed the highest

magnitude of significance (padj<0.05, FC>1.3). (B) Heatmaps of differentially expressed genes related specifically to milk nutrient transport, triglyceride concentration, parvalbumin development, aldosterone/MR signaling, inflammation, LPS binding, oxytocin, prolactin, glucocorticoid signaling (events following glucocorticoids binding to the GR receptor), glutamate/GABA, and epigenetic modifiers. Expression is represented with the log2 transformation of counts recorded with a z-score based on the average across experimental groups. Data are expressed as *p <0.05 or **adjusted p <0.05, or ***FC>1.3, MIA versus Saline. GR- glucocorticoid receptor. MR- mineralocorticoid receptor.

Figure 5. Adolescent offspring physiology and behavior following maternal immune activation (MIA) during the mid-lactational period. A main effect of MIA was observed in males for (A) postnatal day 43 body weights (grams; F(1,13) = 19.037, p = 0.001, n_p^2 = 0.594. (B) Mechanical paw withdrawal thresholds (grams) on the von Frey test evaluating mechanical allodynia were significantly greater in female offspring (F(1, 12) = 8.925, p =0.011, n_p^2 = 0.427). Male offspring made a greater number of crosses into the (C) center (F(1, 13) = 5.744, p = 0.032, n_p^2 = 0.306); and (D) perimeter (F(1, 13) = 6.513, p = 0.024, n_p^2 = 0.334) of an open field arena. There were no effects of MIA on (E) social preference index. Line plots displaying percent prepulse inhibition (PPI) as a function of increasing prepulse intensities for (F) males (significant MIA by time interaction F(4, 52) = 4.578, p = 0.003, n_p^2 = 0.260). Male offspring from MIA dams demonstrated significant reductions in PPI at the 69dB (F(1, 13) = 17.009, p = 0.001, n_p^2 = 0.743), 73dB (F(1, 13) = 5.927, p =0.030, n_p^2 = 0.581), and 81dB (F(1, 13) = 8.315, p = 0.013, n_p^2 = 0.633) intensities. (G) PPI performance for females (main effect of dB F(4, 52) = 80.357, p = 0.001, n_p^2 = 0.861), and (H) male and female PPI combined for display purposes. (I) The bar plot shows the mean percent prepulse inhibition across all five prepulse intensities. Conditioned fear % PPI for (J) males (main effects of MIA for 73dB (F(1, 13) = 6.787, p = 0.022, n_p^2 = 0.343), 81dB (F(1, 13) = 6.787, p = 0.022, n_p^2=0.343) and 85 dB (F(1, 13) = 13.124, p = 0.003, n_p^2=0.502)). Conditioned fear % PPI for (K), females (main effects of MIA for 69dB (F(1, 13) =12.969, p = 0.003, n_p^2=0.495) and 81dB (F(1, 13) = 25.117, p = 0.001, n_p^2=0.659)). (L) Males and females combined for display purposes. (M) Hippocampal parvalbumin (PV) and densitometric ratios for male and female offspring from saline (black) and MIA (grey)-treated dams. PV/β-actin ratios were significantly reduced in male MIA offspring (F(1, 13) =6.96, p = 0.022, n_p^2 = 0.367). Data are expressed as mean ± SEM; Saline: n = 8; MIA: n = 7 for the behavioral measures and Saline: n = 7; MIA = 7 for hippocampal PV. *p <0.05, **p<0.01, ***p <0.001, MIA versus Saline. ^{a}p <0.05, ^{aa}p<0.01, ^{aaa}p <0.001, main effect of trial type.

Figure 6. Summary of mechanisms affecting milk quality and offspring brain and behavior following maternal immune activation (MIA) during the mid-lactational period. Mid-lactational MIA experienced on postnatal day (P)10 is associated with changes in the milk transcriptome on both P10 and P11, in addition to increased milk corticosterone concentrations and microbial expression of *Christensenellaceae* on P10. In concert, these effects may lead to reduced LPS binding protein in milk on P10 and fat content on P11. We propose that this altered milk profile is one mechanism by which mid-lactational MIA contributes to the emergence of sex-specific behavioral and physiological changes across the early life of offspring. LPS – lipopolysaccharide; MR- mineralocorticoid receptor.

Table 1. Litter treatments and endpoints.

Supplementary Methods.

Supplementary Table 1. Maternal immune activation model reporting guidelines checklist.

Supplementary Table 2. Differentially expressed genes in P10 milk samples from the selected pathways. Genes with "NA" padj values indicate that FDR was not calculable due to fewer normalized counts than the optimal threshold.

Supplementary Table 3. Differentially expressed genes in P11 milk samples within the targeted pathways.

Supplementary Figure 1. Maternal care behaviors following maternal immune activation (MIA) during the mid-lactational period. Total number of (A) pup retrievals, (B) passive nursing bouts, (C) nest building behaviors, and (D) maternal drinking bouts. Maternal care was evaluated in the afternoon and night of postnatal day (P) 10 and the morning of P11. Saline: n = 8, MIA: n = 7. Data are expressed as mean ± SEM. $^{a}p < 0.05$, main effect of time.

Supplementary Figure 2. Nutritional profile of milk following maternal immune activation (MIA) during the mid-lactational period. Maternal milk (A) fat concentration (g/L), (B) energy value (kcal/L), (C) protein concentration (mg/mL), (D) lactose concentration (ng/μL), and IgA concentration (mg/mL). Saline: n = 8; MIA: n = 7. Data are expressed as mean ± SEM. $^{*}p < 0.05$, $^{***}p < 0.001$, MIA versus Saline; $^{a}p < 0.05$, main effect of time.

Supplementary Figure 3. Cladogram of milk biomarkers associated with treatment group on P10 (determined by LEfSe). Diameter of the nodes indicates relative abundance of taxa for saline (green) and MIA (red) samples. Placement indicates the classification of taxa, where nodes decrease in rank the closer to the center of the diagram. (Saline: n = 6; MIA: n = 6).

Supplementary Figure 4. Taxonomy heatmap demonstrating the top fifty most abundant species identified in samples on P10. Treatment group is indicated by the colored bar at the top of the figure (red = saline, blue = MIA). Each row represents the abundance for each taxon, with the taxonomy ID shown on the right. Each column represents the abundance for each sample. (Saline: n = 6; MIA: n = 6).

Supplementary Figure 5. Cladogram of milk biomarkers associated with treatment group on P11 (determined by LEfSe). Diameter of the nodes indicates relative abundance of taxa for saline (green) and MIA (red) samples. Placement indicates the classification of taxa, where nodes decrease in rank the closer to the center of the diagram. (Saline: n = 7; MIA: n = 7).

Supplementary Figure 6. Taxonomy heatmap demonstrating the top fifty most abundant species identified in samples on P11. Treatment group is indicated by the colored bar at the top of the figure (red = saline, blue = MIA). Each row represents the abundance for each taxon, with the taxonomy ID shown on the right. Each column represents the abundance for each sample. (Saline: n = 7; MIA: n = 7).

Supplementary Figure 7. Offspring and maternal body weights across early development following maternal immune activation (MIA) during the mid-lactational period. A) Male body weights demonstrated a significant effect of time (F(2, 26)= 1078.59, $p<0.001$) and MIA (F(1,13)= 7618.25, $p<0.001$) while B) female offspring and C) dam body weights only differed significantly across time (females: F(2, 26)=1491.4, $p<0.001$; maternal body weight: F(1, 13)=21.64, $p<0.001$). P10 offspring body weights were obtained by taking the average weight from the weight of all pups of the same sex for each litter (Saline: n = 8; MIA n = 7). P21 and P43 body weights were obtained from one male and one female per litter (Saline: n = 8; MIA n = 7). Data are expressed as mean ± SEM. ***$p < 0.001$, main effect of MIA; $^{aaa}p < 0.001$, main effect of time.

Supplementary Figure 8. Adolescent offspring behavior following maternal immune activation (MIA) during the mid-lactational period. (A) Distance traveled (cm), (B) percent time spent in the center, and duration of time (seconds) spent in the (C) center and (D) perimeter of an open field arena. (E) Conditioned fear significantly increased %PPI regardless of MIA or sex (F(4, 52)= 8.19, p=0.008, n_p^2=0.226). (F) %PPI for trials primed with conditioned fear collapsed across males and females for display purposes. Saline: n = 8; MIA n = 7. Data are expressed as mean ± SEM. Data are expressed as mean ± SEM, *$p<0.05$.

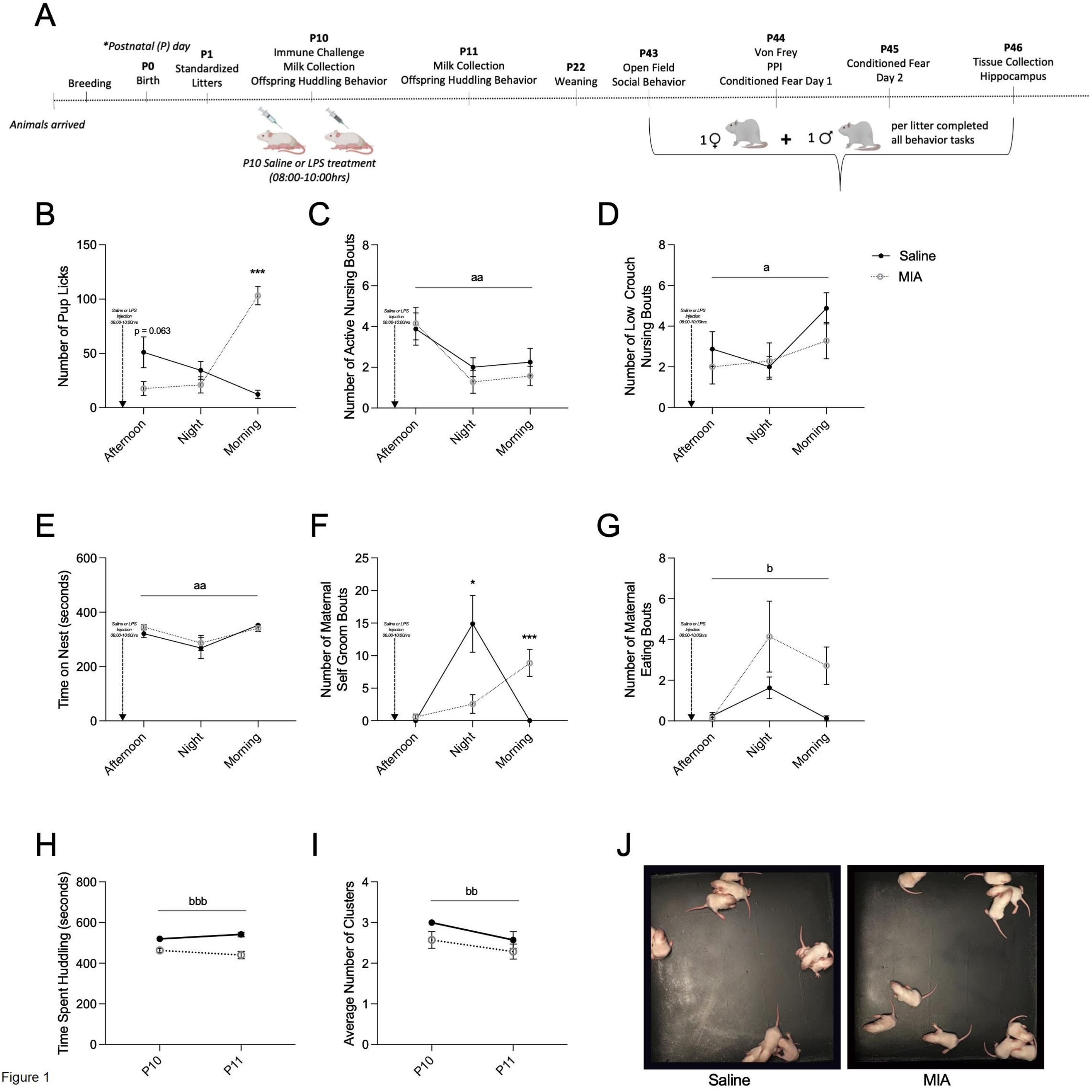
A
*Postnatal (P) day
Animals arrived
Breeding
P0
Birth
P1
Standardized
Litters
P10
Immune Challenge
Milk Collection
Offspring Huddling Behavior
P11
Milk Collection
Offspring Huddling Behavior
P22
Weaning
P43
Open Field
Social Behavior
P44
Von Frey
PPI
Conditioned Fear Day 1
P45
Conditioned Fear
Day 2
P46
Tissue Collection
Hippocampus
P10 Saline or LPS treatment
(08:00-10:00hrs)
1♀ + 1♂ per litter completed all behavior tasks
B
Number of Pup Licks
Saline or LPS Injection 08:00-10:00hrs
p = 0.063
***
Afternoon
Night
Morning
C
Number of Active Nursing Bouts
aa
D
Number of Low Crouch Nursing Bouts
a
Saline
MIA
E
Time on Nest (seconds)
aa
F
Number of Maternal Self Groom Bouts
*
***
G
Number of Maternal Eating Bouts
b
H
Time Spent Huddling (seconds)
bbb
P10
P11
I
Average Number of Clusters
bb
J
Saline
MIA

Figure 1

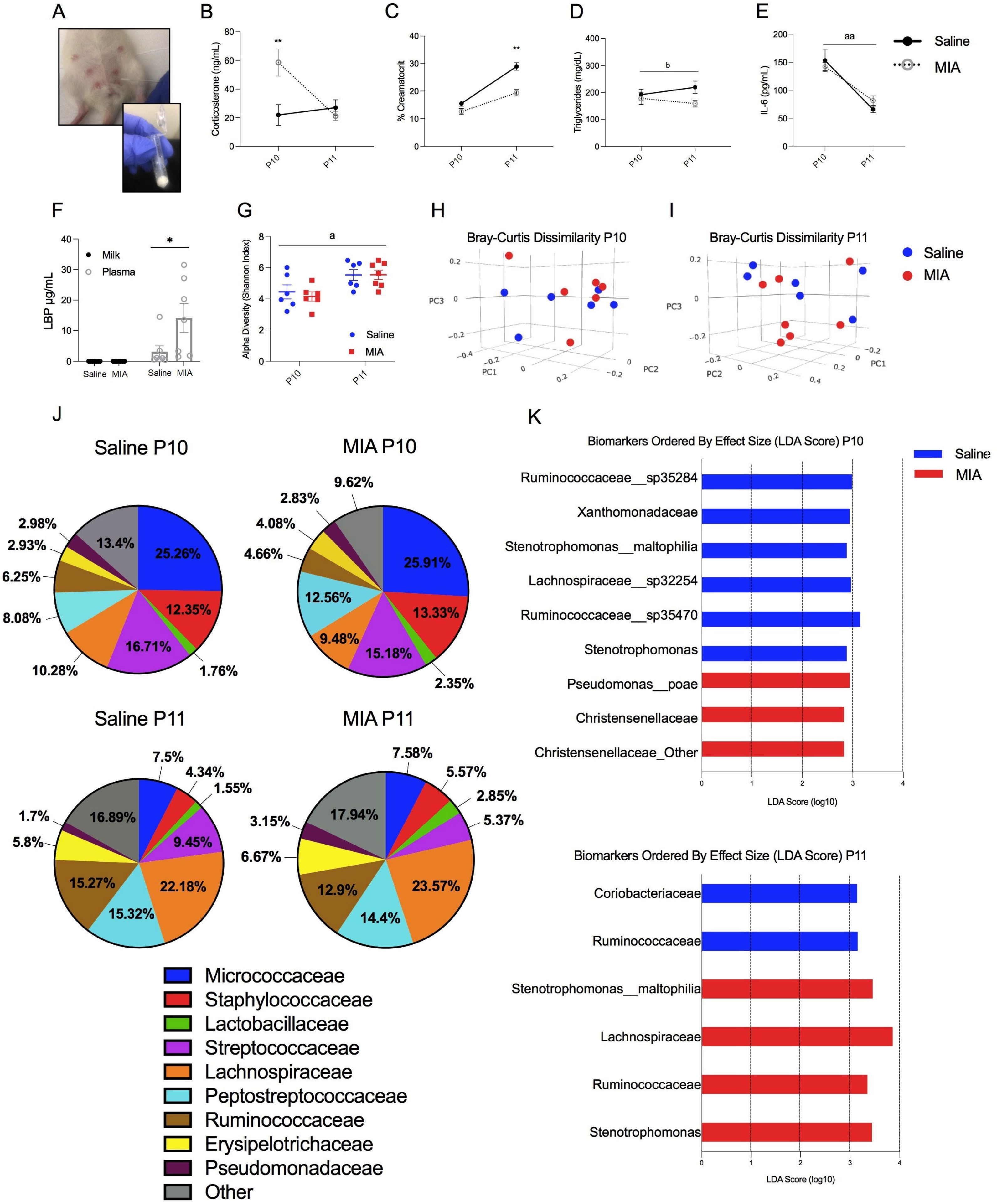
A
B
Corticosterone (ng/mL)
80
60
40
20
0
**
P10
P11
C
% Creamatocrit
40
30
20
10
0
**
P10
P11
D
Triglycerides (mg/dL)
400
300
200
100
0
b
P10
P11
E
IL-6 (pg/mL)
200
150
100
50
0
aa
P10
P11
Saline
MIA
F
LBP μg/mL
40
30
20
10
0
Milk
Plasma
*
Saline
MIA
Saline
MIA
G
Alpha Diversity (Shannon Index)
8
6
4
2
0
a
Saline
MIA
P10
P11
H
Bray-Curtis Dissimilarity P10
PC3
PC1
PC2
0.2
0
−0.2
−0.4
−0.2
0
0.2
0
−0.2
I
Bray-Curtis Dissimilarity P11
PC3
PC2
PC1
0.2
0
−0.2
−0.2
0
0.2
0.4
−0.2
0
0.2
Saline
MIA
J
Saline P10
25.26%
12.35%
1.76%
16.71%
10.28%
8.08%
6.25%
2.93%
2.98%
13.4%
MIA P10
25.91%
13.33%
2.35%
15.18%
9.48%
12.56%
4.66%
4.08%
2.83%
9.62%
Saline P11
7.5%
4.34%
1.55%
9.45%
22.18%
15.32%
15.27%
5.8%
1.7%
16.89%
MIA P11
7.58%
5.57%
2.85%
5.37%
23.57%
14.4%
12.9%
6.67%
3.15%
17.94%
Micrococcaceae
Staphylococcaceae
Lactobacillaceae
Streptococcaceae
Lachnospiraceae
Peptostreptococcaceae
Ruminococcaceae
Erysipelotrichaceae
Pseudomonadaceae
Other
K
Biomarkers Ordered By Effect Size (LDA Score) P10
Saline
MIA
Ruminococcaceae__sp35284
Xanthomonadaceae
Stenotrophomonas__maltophilia
Lachnospiraceae__sp32254
Ruminococcaceae__sp35470
Stenotrophomonas
Pseudomonas__poae
Christensenellaceae
Christensenellaceae_Other
0
1
2
3
4
LDA Score (log10)
Biomarkers Ordered By Effect Size (LDA Score) P11
Coriobacteriaceae
Ruminococcaceae
Stenotrophomonas__maltophilia
Lachnospiraceae
Ruminococcaceae
Stenotrophomonas
0
1
2
3
4
LDA Score (log10)

Figure 2

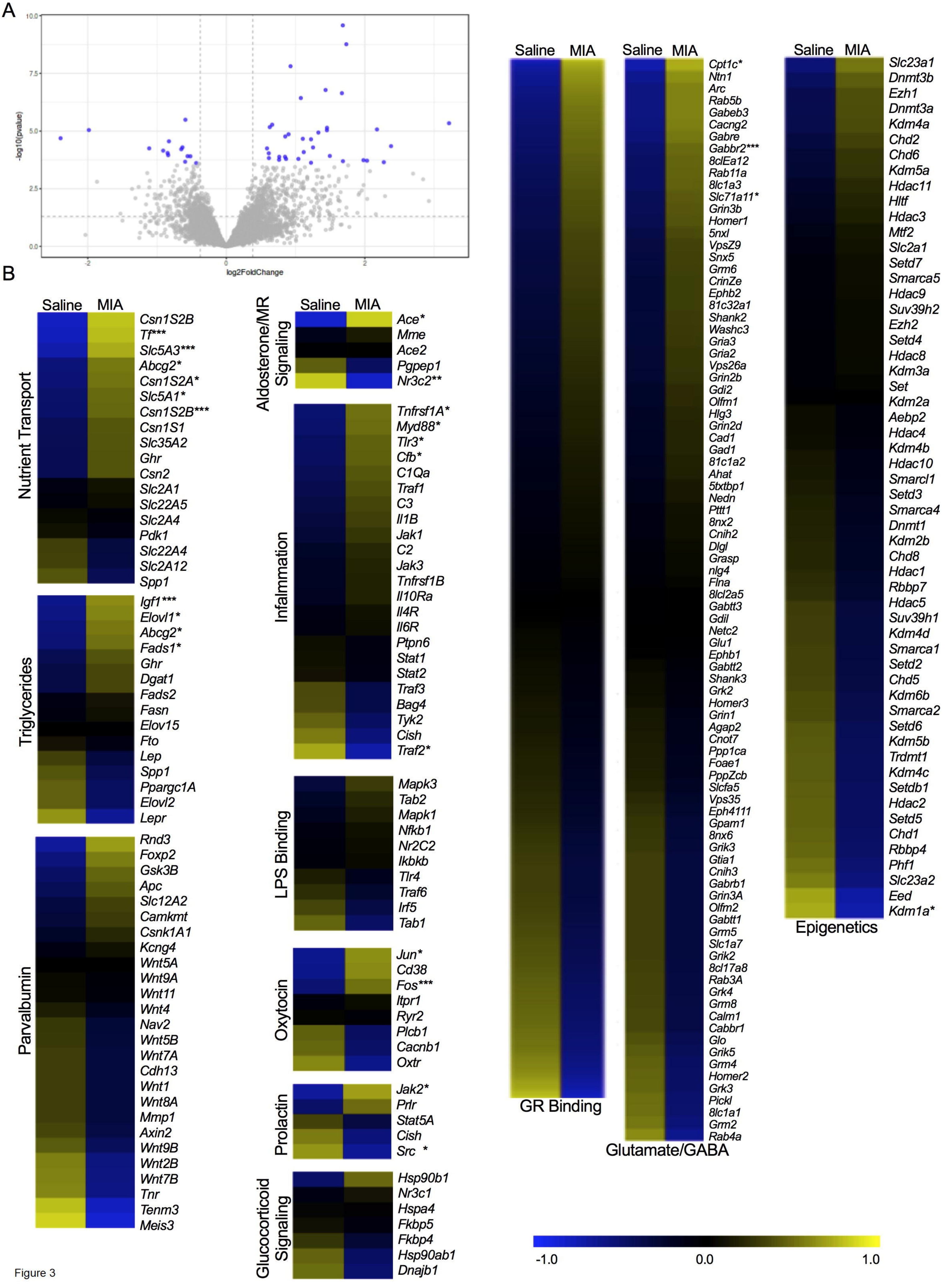
A
10.0
7.5
5.0
2.5
0.0
-log10(pvalue)
-2
0
2
log2FoldChange
B
Saline
MIA
Nutrient Transport
Csn1S2B
Tf***
Slc5A3***
Abcg2*
Csn1S2A*
Slc5A1*
Csn1S2B***
Csn1S1
Slc35A2
Ghr
Csn2
Slc2A1
Slc22A5
Slc2A4
Pdk1
Slc22A4
Slc2A12
Spp1
Triglycerides
Igf1***
Elovl1*
Abcg2*
Fads1*
Ghr
Dgat1
Fads2
Fasn
Elov15
Fto
Lep
Spp1
Ppargc1A
Elovl2
Lepr
Parvalbumin
Rnd3
Foxp2
Gsk3B
Apc
Slc12A2
Camkmt
Csnk1A1
Kcng4
Wnt5A
Wnt9A
Wnt11
Wnt4
Nav2
Wnt5B
Wnt7A
Cdh13
Wnt1
Wnt8A
Mmp1
Axin2
Wnt9B
Wnt2B
Wnt7B
Tnr
Tenm3
Meis3
Aldosterone/MR Signaling
Saline
MIA
Ace*
Mme
Ace2
Pgpep1
Nr3c2**
Infalmmation
Tnfrsf1A*
Myd88*
Tlr3*
Cfb*
C1Qa
Traf1
C3
Il1B
Jak1
C2
Jak3
Tnfrsf1B
Il10Ra
Il4R
Il6R
Ptpn6
Stat1
Stat2
Traf3
Bag4
Tyk2
Cish
Traf2*
LPS Binding
Mapk3
Tab2
Mapk1
Nfkb1
Nr2C2
Ikbkb
Tlr4
Traf6
Irf5
Tab1
Oxytocin
Jun*
Cd38
Fos***
Itpr1
Ryr2
Plcb1
Cacnb1
Oxtr
Prolactin
Jak2*
Prlr
Stat5A
Cish
Src *
Glucocorticoid Signaling
Hsp90b1
Nr3c1
Hspa4
Fkbp5
Fkbp4
Hsp90ab1
Dnajb1
Saline
MIA
GR Binding
Saline
MIA
Cpt1c*
Ntn1
Arc
Rab5b
Gabeb3
Cacng2
Gabre
Gabbr2***
8clEa12
Rab11a
8lc1a3
Slc71a11*
Grin3b
Homer1
5nxl
VpsZ9
Snx5
Grm6
CrinZe
Ephb2
81c32a1
Shank2
Washc3
Gria3
Gria2
Vps26a
Grin2b
Gdi2
Olfm1
Hlg3
Grin2d
Cad1
Gad1
81c1a2
Ahat
5txtbp1
Nedn
Pttt1
8nx2
Cnih2
Dlgl
Grasp
nlg4
Flna
8lcl2a5
Gabtt3
Gdil
Netc2
Glu1
Ephb1
Gabtt2
Shank3
Grk2
Homer3
Grin1
Agap2
Cnot7
Ppp1ca
Foae1
PppZcb
Slcfa5
Vps35
Eph4111
Gpam1
8nx6
Grik3
Gtia1
Cnih3
Gabrb1
Grin3A
Olfm2
Gabtt1
Grm5
Slc1a7
Grik2
8cl17a8
Rab3A
Grk4
Grm8
Calm1
Cabbr1
Glo
Grik5
Grm4
Homer2
Grk3
Pickl
8lc1a1
Grm2
Rab4a
Glutamate/GABA
Saline
MIA
Slc23a1
Dnmt3b
Ezh1
Dnmt3a
Kdm4a
Chd2
Chd6
Kdm5a
Hdac11
Hltf
Hdac3
Mtf2
Slc2a1
Setd7
Smarca5
Hdac9
Suv39h2
Ezh2
Setd4
Hdac8
Kdm3a
Set
Kdm2a
Aebp2
Hdac4
Kdm4b
Hdac10
Smarcl1
Setd3
Smarca4
Dnmt1
Kdm2b
Chd8
Hdac1
Rbbp7
Hdac5
Suv39h1
Kdm4d
Smarca1
Setd2
Chd5
Kdm6b
Smarca2
Setd6
Kdm5b
Trdmt1
Kdm4c
Setdb1
Hdac2
Setd5
Chd1
Rbbp4
Phf1
Slc23a2
Eed
Kdm1a*
Epigenetics
-1.0
0.0
1.0

Figure 3

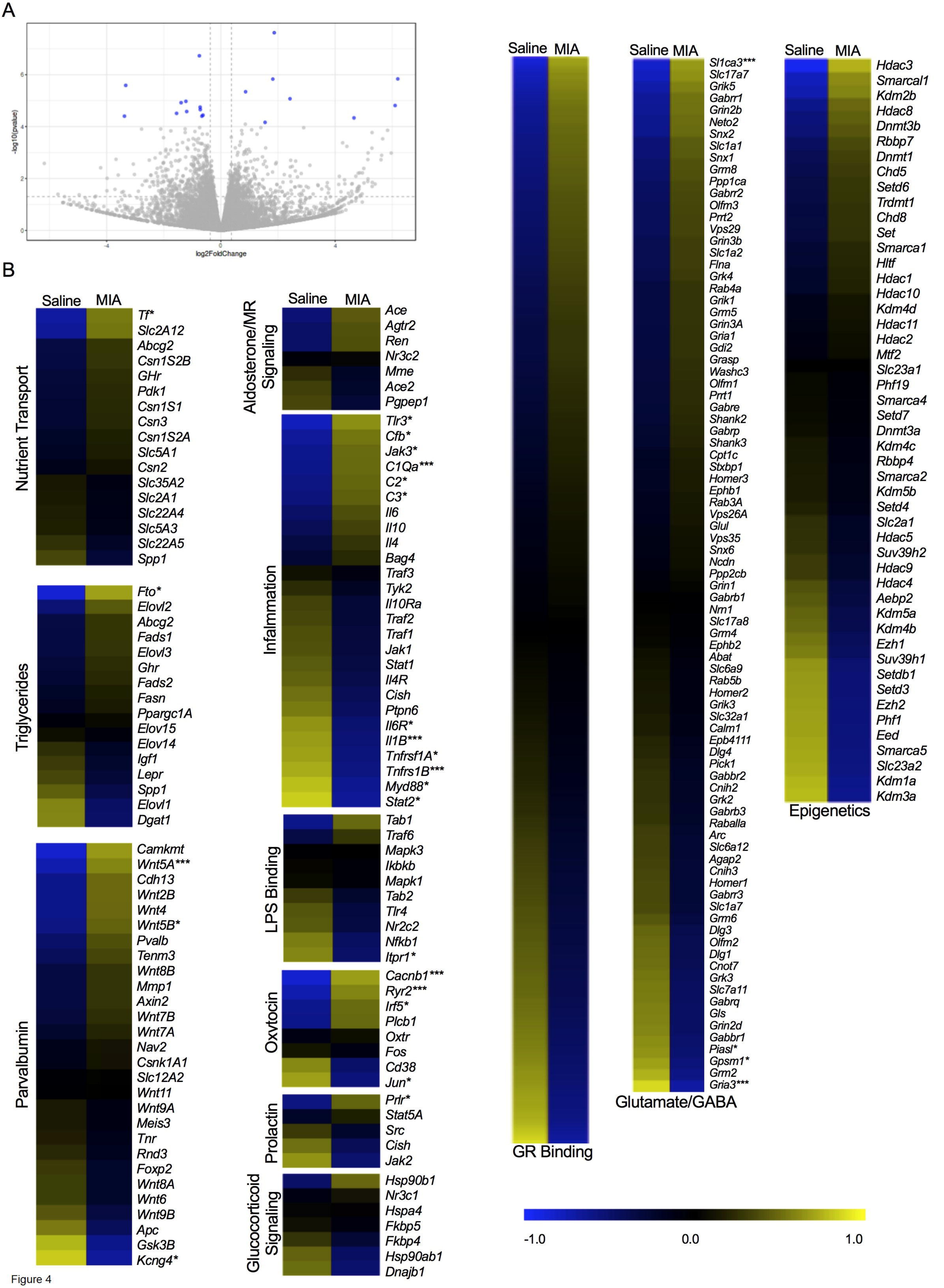
A
-log10(pvalue)
0
2
4
6
log2FoldChange
-4
0
4
B
Saline
MIA
Nutrient Transport
Tf*
Slc2A12
Abcg2
Csn1S2B
GHr
Pdk1
Csn1S1
Csn3
Csn1S2A
Slc5A1
Csn2
Slc35A2
Slc2A1
Slc22A4
Slc5A3
Slc22A5
Spp1
Triglycerides
Fto*
Elovl2
Abcg2
Fads1
Elovl3
Ghr
Fads2
Fasn
Ppargc1A
Elov15
Elov14
Igf1
Lepr
Spp1
Elovl1
Dgat1
Parvalbumin
Camkmt
Wnt5A***
Cdh13
Wnt2B
Wnt4
Wnt5B*
Pvalb
Tenm3
Wnt8B
Mmp1
Axin2
Wnt7B
Wnt7A
Nav2
Csnk1A1
Slc12A2
Wnt11
Wnt9A
Meis3
Tnr
Rnd3
Foxp2
Wnt8A
Wnt6
Wnt9B
Apc
Gsk3B
Kcng4*
Saline
MIA
Aldosterone/MR Signaling
Ace
Agtr2
Ren
Nr3c2
Mme
Ace2
Pgpep1
Infalmmation
Tlr3*
Cfb*
Jak3*
C1Qa***
C2*
C3*
Il6
Il10
Il4
Bag4
Traf3
Tyk2
Il10Ra
Traf2
Traf1
Jak1
Stat1
Il4R
Cish
Ptpn6
Il6R*
Il1B***
Tnfrsf1A*
Tnfrs1B***
Myd88*
Stat2*
LPS Binding
Tab1
Traf6
Mapk3
Ikbkb
Mapk1
Tab2
Tlr4
Nr2c2
Nfkb1
Itpr1*
Oxytocin
Cacnb1***
Ryr2***
Irf5*
Plcb1
Oxtr
Fos
Cd38
Jun*
Prolactin
Prlr*
Stat5A
Src
Cish
Jak2
Glucocorticoid Signaling
Hsp90b1
Nr3c1
Hspa4
Fkbp5
Fkbp4
Hsp90ab1
Dnajb1
Saline
MIA
GR Binding
Saline
MIA
Sl1ca3***
Slc17a7
Grik5
Gabrr1
Grin2b
Neto2
Snx2
Slc1a1
Snx1
Grm8
Ppp1ca
Gabrr2
Olfm3
Prrt2
Vps29
Grin3b
Slc1a2
Flna
Grk4
Rab4a
Grik1
Grm5
Grin3A
Gria1
Gdi2
Grasp
Washc3
Olfm1
Prrt1
Gabre
Shank2
Gabrp
Shank3
Cpt1c
Stxbp1
Homer3
Ephb1
Rab3A
Vps26A
Glul
Vps35
Snx6
Ncdn
Ppp2cb
Grin1
Gabrb1
Nrn1
Slc17a8
Grm4
Ephb2
Abat
Slc6a9
Rab5b
Homer2
Grik3
Slc32a1
Calm1
Epb4111
Dlg4
Pick1
Gabbr2
Cnih2
Grk2
Gabrb3
Raballa
Arc
Slc6a12
Agap2
Cnih3
Homer1
Gabrr3
Slc1a7
Grm6
Dlg3
Olfm2
Dlg1
Cnot7
Grk3
Slc7a11
Gabrq
Gls
Grin2d
Gabbr1
Piasl*
Gpsm1*
Grm2
Gria3***
Glutamate/GABA
Saline
MIA
Hdac3
Smarcal1
Kdm2b
Hdac8
Dnmt3b
Rbbp7
Dnmt1
Chd5
Setd6
Trdmt1
Chd8
Set
Smarca1
Hltf
Hdac1
Hdac10
Kdm4d
Hdac11
Hdac2
Mtf2
Slc23a1
Phf19
Smarca4
Setd7
Dnmt3a
Kdm4c
Rbbp4
Smarca2
Kdm5b
Setd4
Slc2a1
Hdac5
Suv39h2
Hdac9
Hdac4
Aebp2
Kdm5a
Kdm4b
Ezh1
Suv39h1
Setdb1
Setd3
Ezh2
Phf1
Eed
Smarca5
Slc23a2
Kdm1a
Kdm3a
Epigenetics
-1.0
0.0
1.0

Figure 4

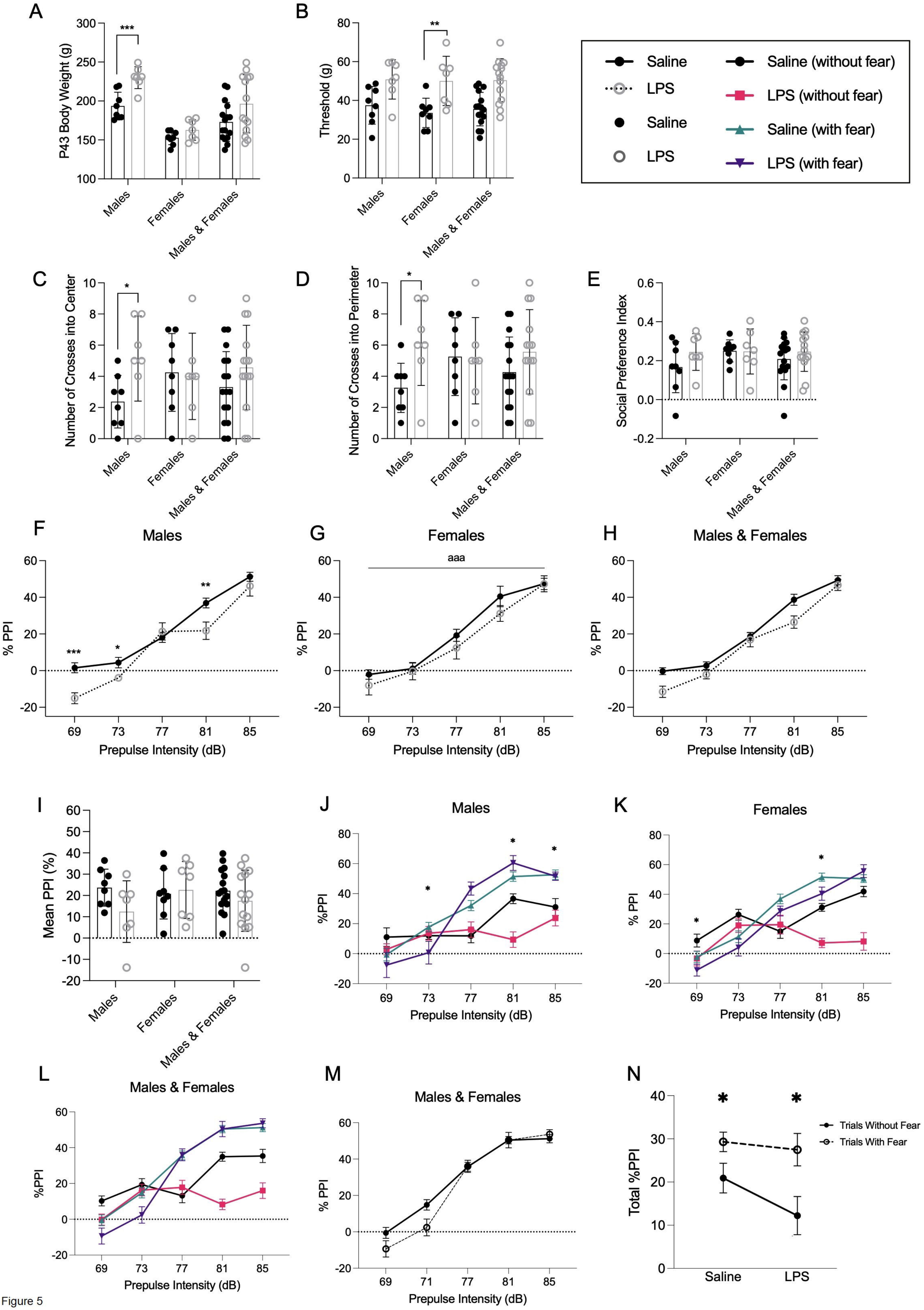
A
P43 Body Weight (g)
***
Males
Females
Males & Females
B
Threshold (g)
**
C
Number of Crosses into Center
*
D
Number of Crosses into Perimeter
*
E
Social Preference Index
Saline
LPS
Saline
LPS
Saline (without fear)
LPS (without fear)
Saline (with fear)
LPS (with fear)
F
Males
% PPI
***
*
**
Prepulse Intensity (dB)
69
73
77
81
85
G
Females
aaa
H
Males & Females
I
Mean PPI (%)
J
Males
%PPI
K
Females
L
Males & Females
M
Males & Females
71
N
Total %PPI
Trials Without Fear
Trials With Fear
Saline
LPS

Figure 5

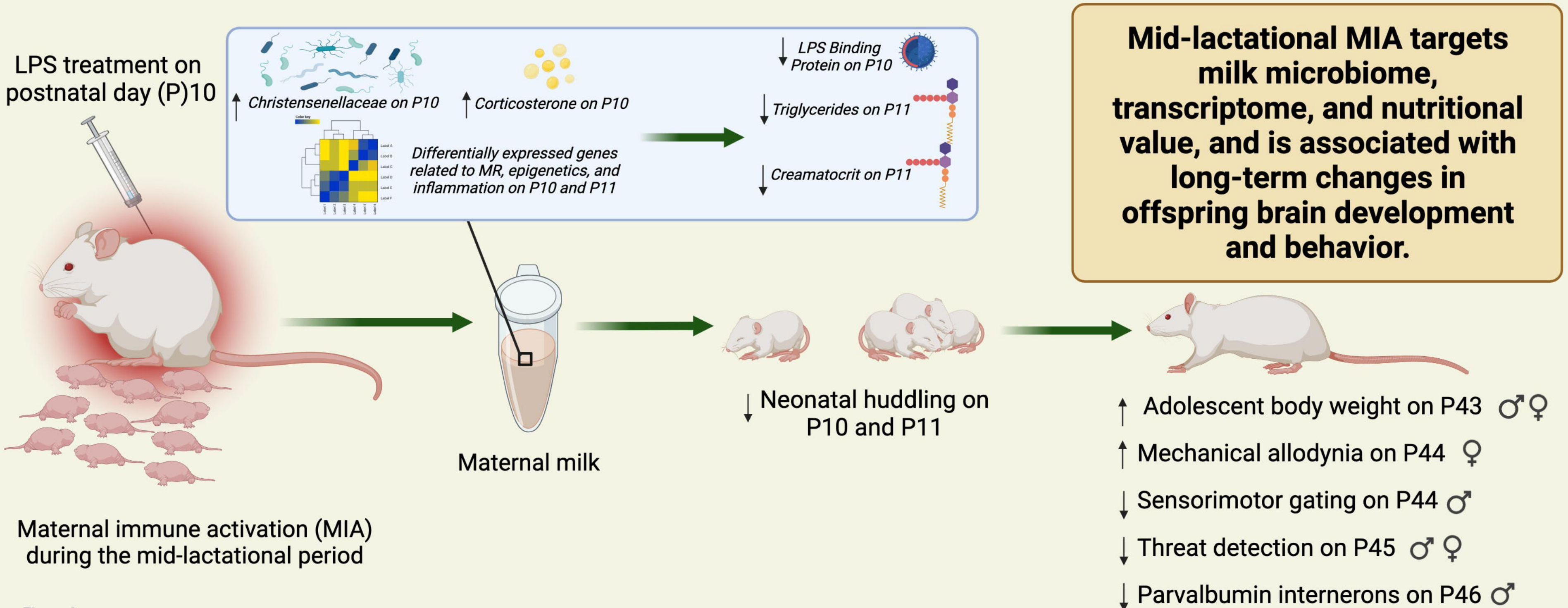
LPS treatment on postnatal day (P)10
↑ Christensenellaceae on P10
↑ Corticosterone on P10
Differentially expressed genes related to MR, epigenetics, and inflammation on P10 and P11
↓ LPS Binding Protein on P10
↓ Triglycerides on P11
↓ Creamatocrit on P11
Mid-lactational MIA targets milk microbiome, transcriptome, and nutritional value, and is associated with long-term changes in offspring brain development and behavior.
Maternal milk
↓ Neonatal huddling on P10 and P11
Maternal immune activation (MIA) during the mid-lactational period
↑ Adolescent body weight on P43 ♂♀
↑ Mechanical allodynia on P44 ♀
↓ Sensorimotor gating on P44 ♂
↓ Threat detection on P45 ♂ ♀
↓ Parvalbumin internerons on P46 ♂

Figure 6

Table 1. Litter Treatments and Endpoints.

Treatment	Number of Litters Included in Maternal Behavior Measures	Number of Whole Litters* included in P10 and P11 Huddling Test	Number of Offspring** Included in P43 Behavior Analyses	Number of Offspring** Included in P44 Behavior Analyses	Number of Offspring** Included in P45 Behavior Analyses	Number of Offspring** Included in Hippocampal PV Analyses
Saline	8 Litters	7 Litters[#]	8 Males; 8 Females	8 Males; 8 Females	8 Males; 8 Females	7 Males; 7 Females[#]
LPS	7 Litters	7 Litters	7 Males; 7 Females	7 Males; 7 Females	7 Males; 7 Females	7 Males; 7 Females

[*] Whole litter refers to all 10 pups (5 males and 5 females).

[**] Only one male and one female pup were evaluated from each litter.

[#] One Saline litter was not evaluated on this measure

P = Postnatal day

PV- Parvalbumin interneurons.