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

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Abstract

Background: 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. **Methods:** 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. **Results:** 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 interneuron 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. **Conclusions:** 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 (Mahic et al., 2017; Meyer, 2019). Administrating 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 (Kentner et al., 2019; Brown et al., 2009; Arsenault et al., 2014; Estes & McAllister, 2016). Together, these physiological responses are recognized to affect the long-term development of offspring (Kentner et al., 2019; Estes & McAllister, 2016). 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 (Karolinski et al., 2010; Kutlesic et al., 2017; John et al., 2017; Hussein et al., 2011).

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 (Vilela et al., 2013). 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 (Ling & Alcorn, 2010). Exposure to MIA during the lactational period can also impact offspring behavior. For example, Nascimento and colleagues (2015) 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.

Other exogenous factors have been shown to alter contents in breastmilk and subsequent offspring development in animals and humans (Lesorogol et al., 2018; Mäkelä et al., 2013; Chen et al., 2017; Grazia Di Benedetto et al., 2020; Edwards et al., 2021). For example, psychological stress in humans reduces milk microbial diversity (Browne et al., 2019). 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 (Hinde et al., 2014). 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, 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.

Methods and Experimental Overview

All animal procedures were conducted 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 (MCPHS) Institutional Animal Care and Use Committee. **Figure 1A** outlines the experimental procedures and groups evaluated in this study. Please see the **Supplementary Methods, Supplementary Table 1,** and **Key Resources Table** for a more detailed description of the methodological protocols and statistical analyses used in this study. Briefly, on postnatal day 10 (P10), female 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) or pyrogen-free saline. Milk samples were collected 2 hours later at the height of the inflammatory response and 24 hours later on P11 during the recovery period (see DeRosa et al., 2022).

Milk quality was determined through the use of several assays to measure levels of milk creamatocrit, lactose, triglycerides, protein, corticosterone, immunoglobulin (Ig) A, interleukin (IL)-6 and lipopolysaccharide binding protein (LBP). Milk quality was also more broadly examined using microbiome and RNA Sequencing (RNA-seq). Maternal behavior was assessed in the afternoon and evening of P10 and in the morning on P11. 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 completed several behavioral measures including the open field and social preference tasks, as well as assessments of mechanical allodynia, sensorimotor gating (prepulse inhibition; PPI), and conditioned fear (see Yan & Kentner, 2017; Connors et al., 2014; Zhao et al., 2021a; Núñez Estevez et al., 2020). On P46, whole hippocampus was collected and later processed to assess the expression of parvalbumin (PV) interneurons using western blotting.

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 von Frey 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 (Miles and Shevlin, 2001). Because the dataset was not powered to evaluate sex-differences directly, male and female animals were

evaluated separately (Ordoñes Sanchez et al., 2021). 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 using Qiime (v.1.9.1) and statistical comparisons were performed using Kruskal-Wallis (Caporaso et al., 2010). 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 (Segata et al., 2011; Schellekens et al., 2021).

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 (**Figure 2B**). This stress-associated hormone was elevated in milk samples 3-hours following MIA challenge (**Figure 2B)** and was recovered by P11. 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 (Lucas et al., 1978; Paul et al., 2015; 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 (Wurfel & Wright, 1997; Park & Lee, 2017). 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 (Quintana et al., 2019; Oakley & Cidlowski, 2013; Polman et al., 2012; Zhu et al., 2016; Ozkan et al., 2020; Zhang et al., 2013; Bowen et al., 2013; Borish & Steinke, 2003; **Figure 3B).** Heatmap clustering revealed a general upregulation of genes related to nutrient transport and inflammation, while genes related to epigenetic modifications, parvalbumin interneuron 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 interneuron 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**). Using body weight as a covariate, female offspring from MIA dams were found to have higher thresholds in 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 7A-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 3G, 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. 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 7E**, collapsed across males and females for display purposes). One-way ANOVA revealed a main effect of trial type (**Supplementary Figure 7E**), which was expected given that the added element of fear increases % PPI (Ishii et al., 2010; Balogh et al., 2002). Conditioned fear did not lead to significant changes in total mean % PPI when collapsed across all dB, regardless of MIA or sex (**Supplementary Figure 7F**, 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

We demonstrate that variations in milk quality may be part of a mechanism that programs offspring physiology and behavior following stress exposure during the lactational period (see **Figure 6** for summary of proposed mechanisms). In the present study, MIA 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 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. Importantly, 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. The lactational period is an important time for neonates and here we underscore the value of maternal inputs (e.g., maternal milk quality) to offspring development.

MIA was associated with multiple changes in the composition of maternal milk content. We observed a significant increase in milk corticosterone 3-hours post LPS treatment that remitted by P11. Milk corticosterone is expressed linearly to plasma corticosterone (Patacchioli et al., 1992), 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 (Angelucci et al., 1985; Brummelte et al., 2010). 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 (Catalani et al., 1993; Catalani et al., 2002). 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 (and the human equivalent, cortisol) can modulate nutritional elements including sodium, potassium (Zietek et al., 2021; Sullivan et al., 2011) and fat (Hinde et al., 2015). 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 (Brummelte et al., 2010; Hinde et al., 2015), 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 (Connor et al., 2018). 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 (Singh & Fulekar et al., 2010). Given that the microbes in breast milk directly colonize the infant gut (Sheard & Walker, 1988; Pannaraj et al., 2017), it is likely that these differently expressed taxa are being passed to the offspring to influence future metabolic processes. Additional studies are needed to explore potential therapeutic interventions, such as targeting the microbiome (Holingue et al., 2020) which may rescue changes in milk composition and subsequent offspring developmental outcomes.

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 (Zietek et al., 2021). 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. While there was an unexpected significant upregulation of several genes within the inflammation pathway on P11 (**Figure 4B, Supplementary Table 3**), this may be reflective of the simultaneous downregulation of epigenetic modifiers since cytokine release is often associated with aberrant epigenetic activity (Obata et al., 2015). Overall, these data demonstrate the extensive effects of MIA on maternal milk at the genome-wide level. Future investigations are needed to understand the action of these transcriptomic profiles within offspring after they are absorbed.

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 (Gilbert et al., 2009). One previous study found a reduction in nest seeking behavior in rat pups prenatally exposed to LPS (Baharnoori et al., 2012). Undernourishment has also been shown to reduce neonatal huddling in rat pups (Soriano et al., 2006), and huddling efficiency may be related to serum triglycerides in newborn rabbits (García-Torres et al., 2015). This evidence suggests that MIA-modulated huddling may emerge due to differences in the fat content of maternal milk. Indeed, pups from MIA-treated mothers displayed disorganized clumping behavior.

Prenatal MIA has been shown to modulate offspring performance on certain tasks, such as the open field, social preference, von Frey, PPI, and fear conditioning paradigms (Kentner et al., 2019; Fortier et al., 2007; Zhao et al., 2021a; Inceoglu et al., 2006; Howland et al., 2012). 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 (Manzano-Nieves et al., 2018; Gerhard et al., 2020). 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.

We measured the expression of GABAergic PV interneurons 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 (Zhao et al., 2021b; Nakamura et al., 2021; Vojtechova et al., 2021). Further, poor nutrition in early life can prevent the development of PV interneurons in rat hippocampus (Callahan et al., 2013) although this has only been explored in males. The emergence of neonatal huddling behavior is time-locked to the maturation of GABAergic signaling (Naskar et al., 2019). Therefore, mid-lactational MIA may disrupt this early form of social behavior by attenuating the development of PV cells. In adolescence, male MIA offspring demonstrated a reduced expression in hippocampal PV interneurons. In vivo inhibition of PV in the hippocampus has previously been shown to reduce PPI in male mice (Nguyen et al., 2014). 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.

In our study, MIA dams showed significant increases in pup-directed licking and grooming behavior the day following MIA challenge. The heightened maternal care demonstrated by MIA dams on P11 may have been a compensatory mechanism to buffer the effects of lactational MIA exposure. Tactile stimulation through the licking and grooming of pups is one form of maternal behavior that promotes brain development and modulates future offspring behavior (Champagne et al., 2003; Francis et al., 2002; Van Hasselt et al., 2012; Francis & Kuhar, 2008). 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 (Bison et al., 2009). While Vilela and colleagues (2013) 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 (Nascimento et al., 2015). 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 to 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.

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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.

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

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 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. n = 7-8 for all measures. *$p < 0.05$, ***$p < 0.001$, 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 of milk and microbiome community distribution following maternal immune activation (MIA) during the mid-lactational period. (A) Photographs of maternal milk collection. A significant time interaction was observed for levels of maternal milk levels 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 by 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 ($p = 0.002$) along with (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 principle 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 and P11 (n = 6-7). (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 (n = 6-7). 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 represented 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 interneuron 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 represented 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 interneuron 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 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 effect 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 effect 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 effect 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) interneuron 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=.367). Data are expressed as mean ± SEM, n = 7-8. *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 is associated with changes in the milk transcriptome and increased milk corticosterone concentrations, in addition to elevated microbial expression of *Christensenellaceae*. In concert, these effects may lead to reduced levels of milk LPS binding protein and fat content. 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 in offspring. LPS – lipopolysaccharide; MR- mineralocorticoid receptor.

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.

Key Resources Table.

Supplementary Methods.

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. Data are expressed as mean ± SEM, n = 7-8. $^{a}p < 0.05$, 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). Data are expressed as mean ± SEM, n = 7-8. $^{*}p < 0.05$, $^{***}p < 0.001$, 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.

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.

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.

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.

Supplementary Figure 7. 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 drug or sex ($F(4, 52)= 8.19$, $p=0.008$, $n_p^2=.226$). (F) % PPI for trials primed with conditioned fear collapsed across males and females for display purposes. Data are expressed as mean ± SEM, n = 7-8.

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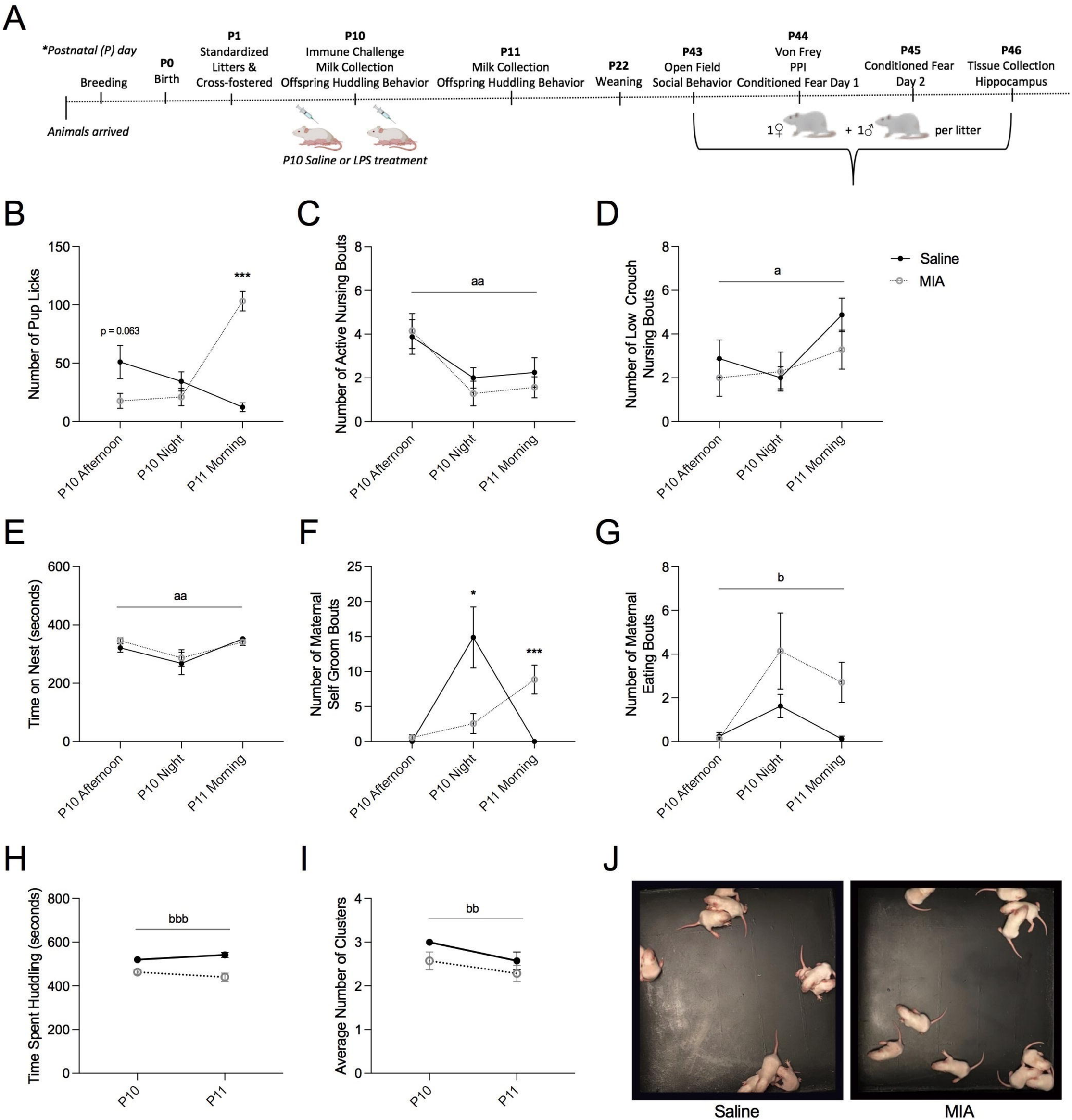

A
*Postnatal (P) day
Breeding
P0
Birth
P1
Standardized
Litters &
Cross-fostered
P10
Immune Challenge
Milk Collection
Offspring Huddling Behavior
P11
Milk Collection
Offspring Huddling Behavior
P22
Weaning
P43
Open Field
Social Behavior
P44
Von Frey
PPI
Conditioned Fear Day 1
P45
Conditioned Fear
Day 2
P46
Tissue Collection
Hippocampus
Animals arrived
P10 Saline or LPS treatment
1♀ + 1♂ per litter
B
Number of Pup Licks
p = 0.063
***
P10 Afternoon
P10 Night
P11 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.

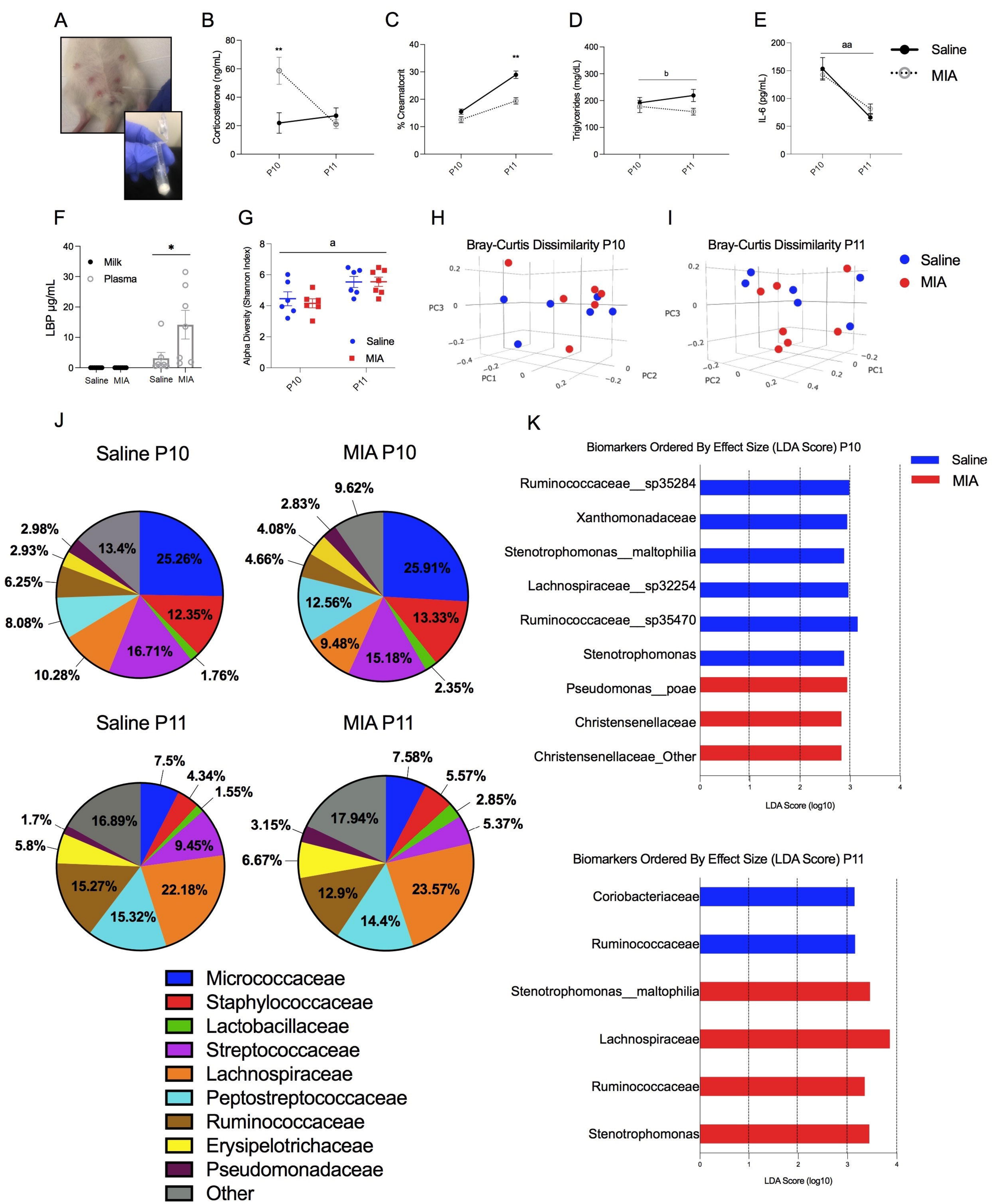
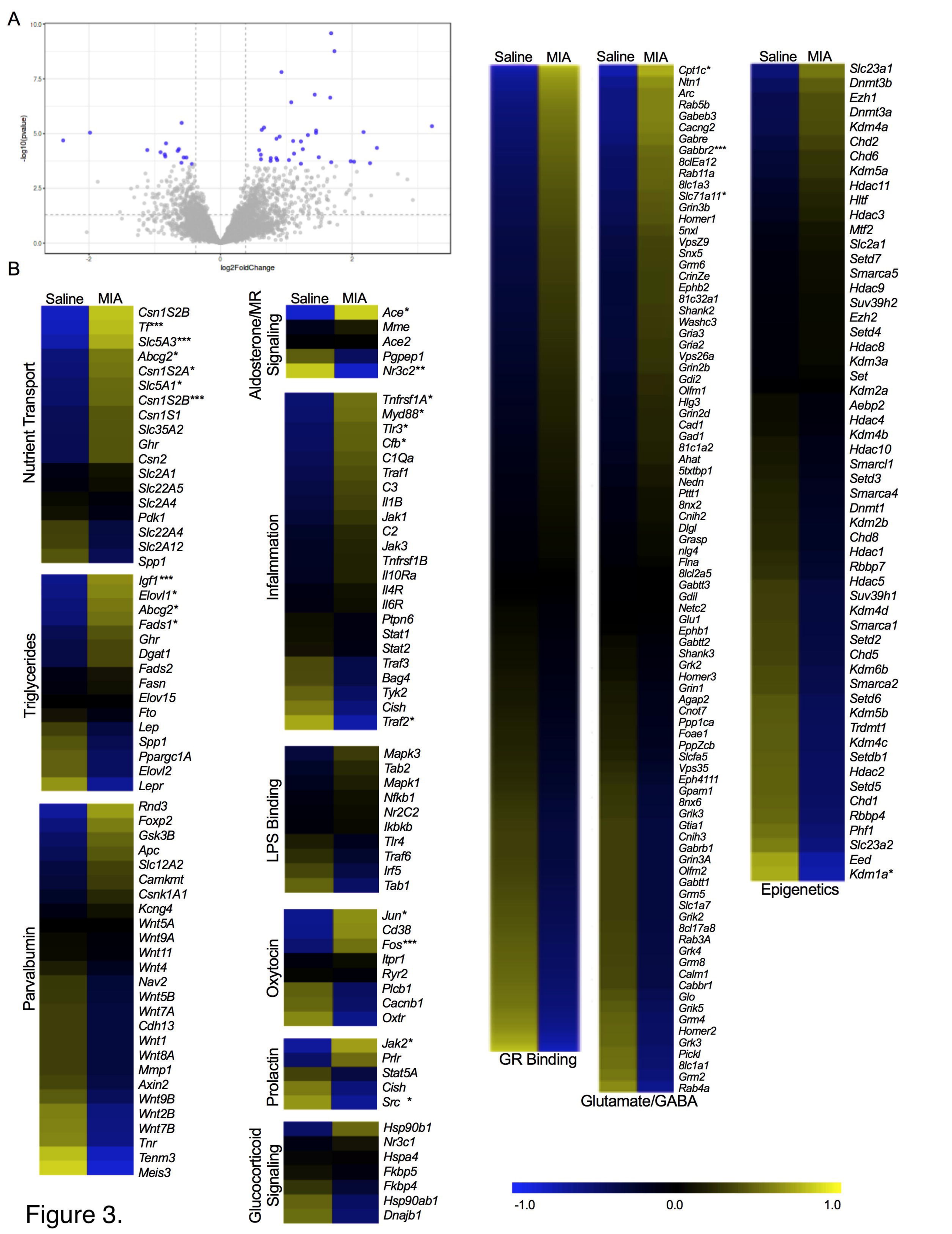

A
B
Corticosterone (ng/mL)
P10
P11
C
% Creamatocrit
D
Triglycerides (mg/dL)
b
E
IL-6 (pg/mL)
aa
Saline
MIA
F
LBP µg/mL
Milk
Plasma
Saline
MIA
G
Alpha Diversity (Shannon Index)
a
H
Bray-Curtis Dissimilarity P10
PC1
PC2
PC3
I
Bray-Curtis Dissimilarity P11
J
Saline P10
MIA P10
Saline P11
MIA P11
Micrococcaceae
Staphylococcaceae
Lactobacillaceae
Streptococcaceae
Lachnospiraceae
Peptostreptococcaceae
Ruminococcaceae
Erysipelotrichaceae
Pseudomonadaceae
Other
K
Biomarkers Ordered By Effect Size (LDA Score) P10
Ruminococcaceae__sp35284
Xanthomonadaceae
Stenotrophomonas__maltophilia
Lachnospiraceae__sp32254
Ruminococcaceae__sp35470
Stenotrophomonas
Pseudomonas__poae
Christensenellaceae
Christensenellaceae_Other
LDA Score (log10)
Biomarkers Ordered By Effect Size (LDA Score) P11
Coriobacteriaceae
Ruminococcaceae
Stenotrophomonas__maltophilia
Lachnospiraceae
Ruminococcaceae
Stenotrophomonas
LDA Score (log10)

Figure 2.


A
-log10(pvalue)
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
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
GR Binding
Glutamate/GABA
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
Epigenetics
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
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Phf1
Slc23a2
Eed
Kdm1a*
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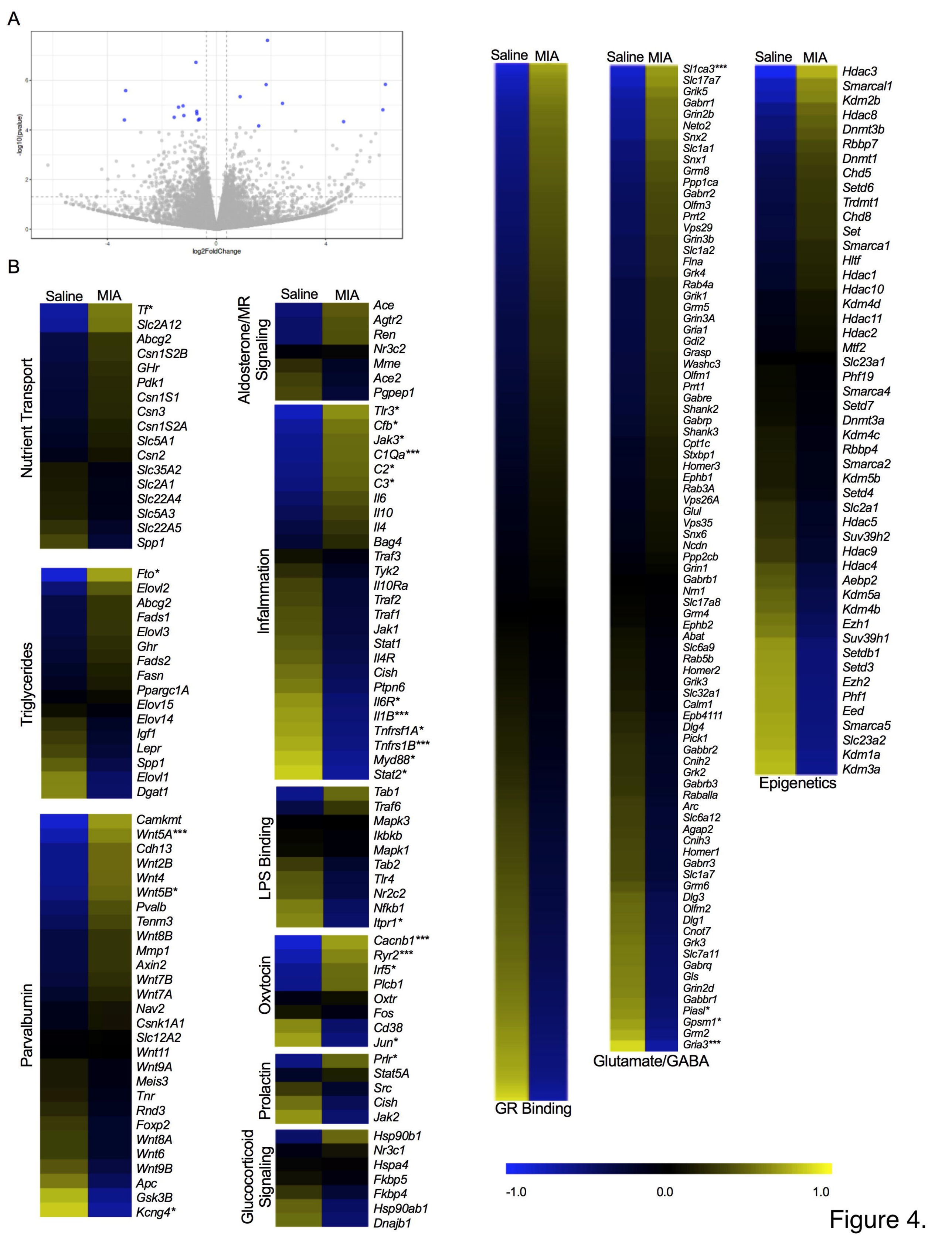
0.0
1.0

Figure 3.


A
-log10(pvalue)
log2FoldChange
B
Saline
MIA
Nutrient Transport
Tf*
Slc2A12
Abcg2
Csn1S2B
GHr
Pdk1
Csn1S1
Csn3
Csn1S2A
Slc5A1
Csn2
Slc35A2
Slc2A1
Slc22A4
Slc5A3
Slc22A5
Spp1
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Fto*
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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*
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
GR Binding
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
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
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Epigenetics
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1.0

Figure 4.

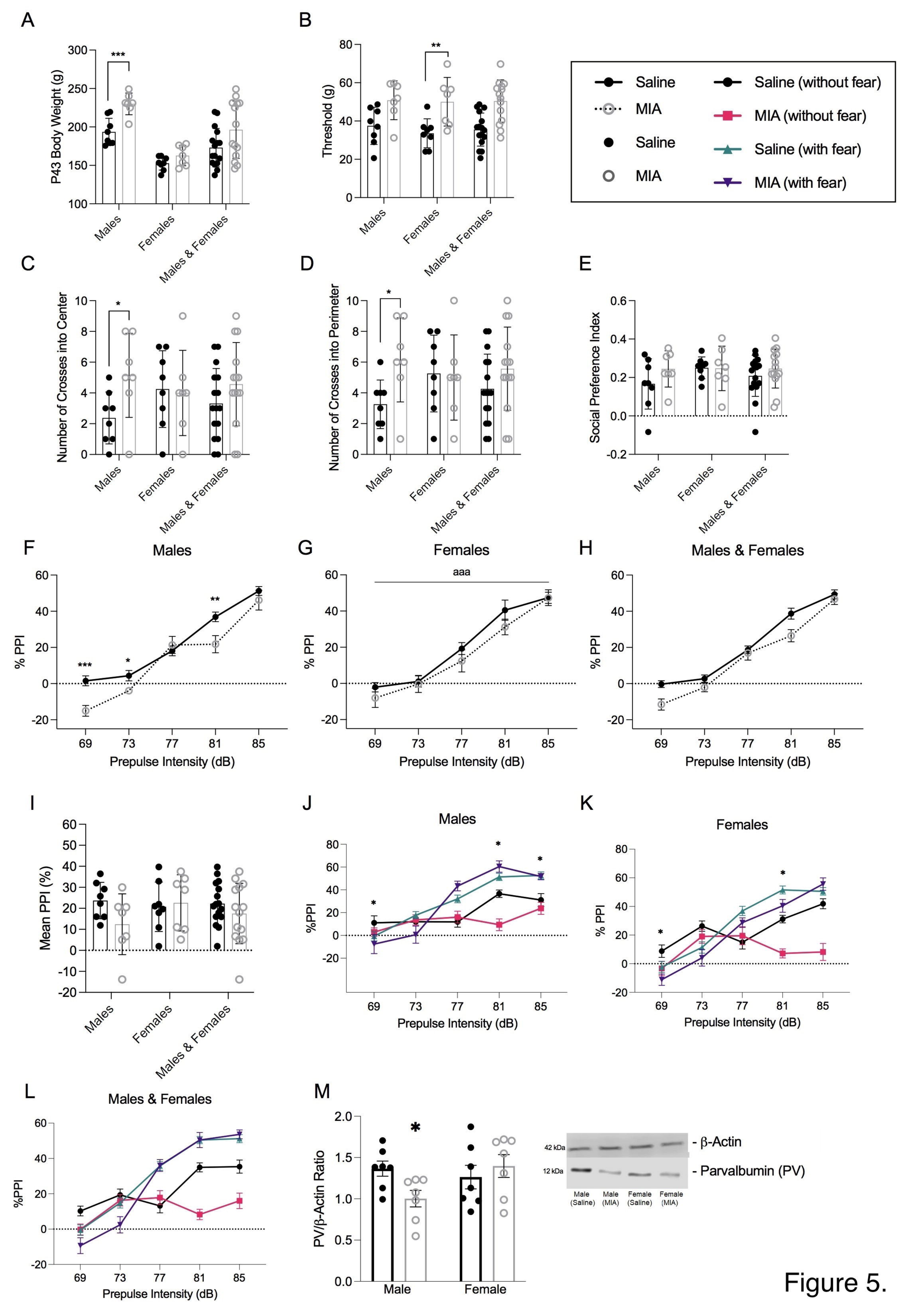
A
P43 Body Weight (g)
Males
Females
Males & Females
B
Threshold (g)
C
Number of Crosses into Center
D
Number of Crosses into Perimeter
E
Social Preference Index
Saline
MIA
Saline
MIA
Saline (without fear)
MIA (without fear)
Saline (with fear)
MIA (with fear)
F
Males
G
Females
H
Males & Females
% PPI
Prepulse Intensity (dB)
I
Mean PPI (%)
J
Males
K
Females
L
Males & Females
%PPI
M
PV/β-Actin Ratio
Male
Female
42 kDa
12 kDa
- β-Actin
- Parvalbumin (PV)
Male (Saline)
Male (MIA)
Female (Saline)
Female (MIA)

Figure 5.

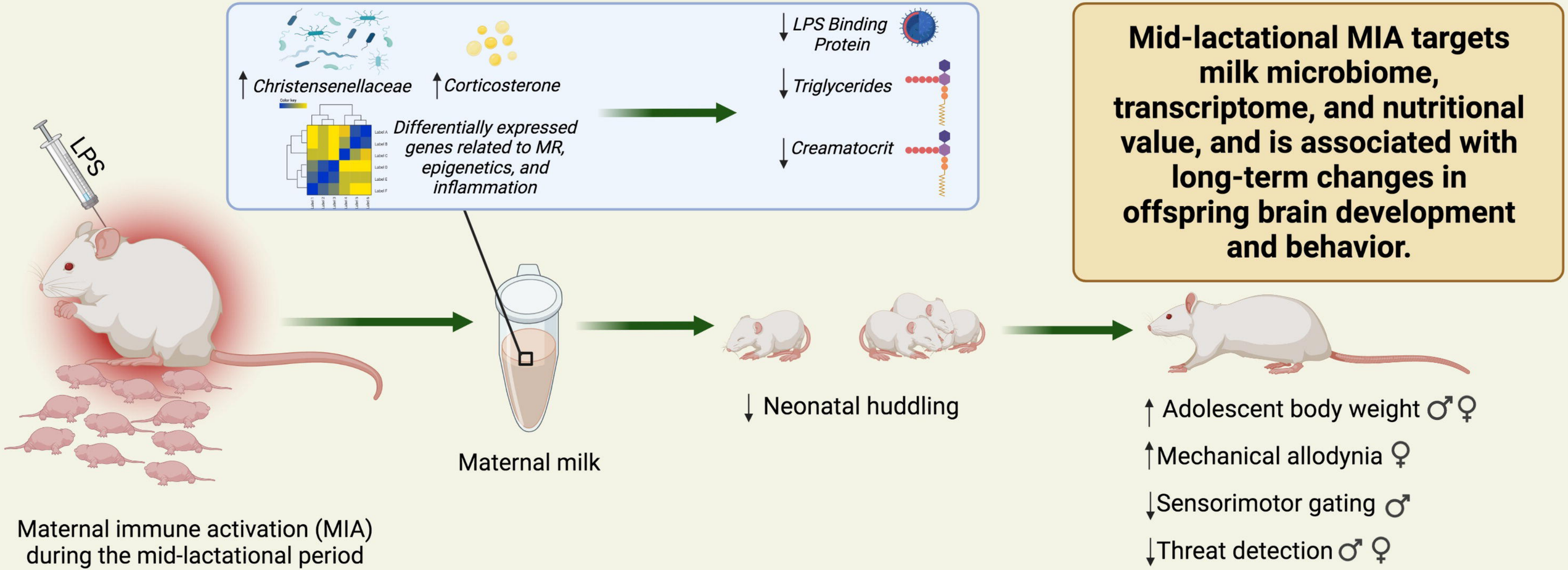
Mid-lactational MIA targets milk microbiome, transcriptome, and nutritional value, and is associated with long-term changes in offspring brain development and behavior.
LPS
Maternal immune activation (MIA) during the mid-lactational period
↑ Christensenellaceae
↑ Corticosterone
Differentially expressed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

Figure 6.