EFFECTS OF LPS-INDUCED MATERNAL IMMUNE ACTIVATION ON EXPRESSION OF AUTISM-LINKED GENES AND AUTISM-LIKE BEHAVIOURS IN NEONATES AND ADULTHOOD

TOWARDS A MATERNAL INFECTION MODEL OF IDIOPATHIC AUTISM

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ABSTRACT

Epidemiological studies have revealed a link between maternal immune activation (MIA) and autism-spectrum disorder (ASD) risk. In support of this, the offspring of dams exposed to LPS during pregnancy develop ASD-like behaviours that persisted into adulthood. To date, the literature has primarily focused on the effects of LPS-MIA in adult male mice; there are few reports describing the behavioural effects of LPS-MIA in female mice, especially early in development. Further, the involvement of the mammalian target of rapamycin (mTOR) signaling pathway, which integrates both the humoral and cellular immune response, and a risk factor for ASD, has not been studied in this model. In the current study, we injected pregnant C57BL/6J mice with 75µg/kg of LPS on embryonic day 11.5 and 12. Male and female offspring were tested during development and at adulthood for the three core symptoms of ASD: social communication, social interaction, and repetitive behaviours. Additionally, the expression, protein abundance and activity-induced phosphorylation of mTOR and its downstream effect on post-synaptic density 95 (PSD95) was analysed in hippocampal and cortical areas of offspring at postnatal day zero (P0) and at P130. Lastly, the hippocampal expression of *Mtor* in the LPS-MIA model was compared to the valproic acid (VPA) model of ASD. Female LPS-MIA mice displayed an impairment in social communication as displayed by a reduced number of USVs emitted at P8, and male LPS-MIA mice displayed deficits in social exploration and restrictive and repetitive behaviours in adulthood displayed by reduced response to social odours in the olfactory habituation/dishabituation task and increased grooming. A reduction in *Mtor* expression was observed in the P0 hippocampus which was associated with an increase in phosphorylated mTOR(ser2481). At P130 there was a reduction in mTOR activity resulting in reduced PSD95 in the ventral and dorsal hippocampus. In the VPA model, an increase in *Mtor* gene expression was observed, which was previously associated with reduced mTOR activity similar to the P130 LPS-MIA hippocampus. Overall, novel sex- and age- specific insights were observed behaviourally and in the mTOR pathway in the LPS-MIA model of ASD, comparable to the VPA model and idiopathic ASD, furthering our understanding of ASD and developing new avenues of future research.

LIST OF ABBREVIATIONS USED

Units:

% – percentage

°C – degrees centigrade

h – hour

min – minute

s – second

ms – millisecond

ml – millilitre

µl – microlitre

w – watt

cm – centimetre

nm – nanometre

nM – nanomolar

kg – kilogram

g – gram

µg – microgram

kHz – kilohertz

Hz – hertz

dB– decibels

RPM– rotations per minute

Cq– quantification cycle value

Reagents and Acronyms:

4EBP1 – eukaryotic translation initiation factor 4E-binding protein 1

ABN1-4 – Arched-back nursing 1-4

ADOS-G – Autism Diagnostic Observation Schedule-Generic

ANOVA – analysis of variance

ASD – Autism Spectrum Disorder

BSA – Bovine serum albumin

DH – Dorsal hippocampus

E – Embryonic day

eIF4E – eukaryotic translation initiation factor 4E

F – Feeding

HPRT – Hypoxanthine-guanine phosphoribosyltransferase

HRP – Horseradish peroxidase

IKKα – Inhibitor of nuclear factor kappa-B kinase subunit alpha

IL – interleukin

LG – Licking and grooming

MIA – Maternal immune activation

mPFC – medial prefrontal cortex

mTOR – Mechanistic target of rapamycin

mTORc1/2 – mTOR complex 1/2

MZ – Monozygotic

n – number of (e.g., subjects)

NCP – No contact with pups

P – Postnatal day

PDK1 – pyruvate dehydrogenase lipoamide kinase isozyme 1

PI3K – Phosphoinositide 3-kinase

PIP_3 – Phosphatidylinositol (3,4,5)-trisphosphate

pmTOR – Phosphorylated mTOR

PolyI:C – Polyinosinic:polycytidylic acid

PSD95 – Post-synaptic density 95

PTEN – Phosphatase and tensin homolog

PVDF – polyvinylidene difluoride

Rheb – Ras homolog enriched in brain

RT-qPCR– Real-time quantitative polymerase chain reaction

S6K – S6 kinase beta-1

SG – Self-grooming

TBST – Tris-Buffered Solution with Tween

TSC1/2 – Tuberous sclerosis 1 and 2

USV – Ultrasonic vocalization

UV – Ultraviolet

VH – Ventral hippocampus

VPA – Valproic Acid

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Chapter 1

Introduction

1.1. *Autism Spectrum Disorder*

Autism Spectrum Disorder (ASD) is a multifactorial developmental disorder, which results in life-long deficits in social communication and social interaction, and the development of restrictive and repetitive behaviours of an obsessive nature (DSM-5, 2013). The prevalence of ASD has increased in the past several years, rising from 1 in 68 children in 2012 (Centers for Disease Control, 2014) to 1:59 in 2016 (Baio et al., 2018). This has been attributed largely to broader diagnostic criteria and increased awareness and detection (Hansen, Schendel, & Parner, 2015). In Nova Scotia, this results in an estimated 13,900 individuals on the autism spectrum, representing 1.5% of the province's population (Autism Nova Scotia, 2017). The combination of early onset, poor treatment response and persistent disability makes ASD one of the most burdensome illnesses to individuals and society, with Nova Scotia's annual governmental expenditure being $14.6 million on support services not including additional health care costs (Autism Nova Scotia, 2017).

1.2. *Human Diagnositcs of ASD*

The symptoms of ASD are manifest in early childhood, as early as 6 months of age, and persist into adolescence and adulthood (Chawarska, Macari, Volkmar, Kim, & Shic, 2014). Although infants can be reliably diagnosed behaviourally at 24 months (Johnson, Myers, & American Academy of Pediatrics Council on Children With Disabilities, 2007), the average age of diagnosis for ASD is around 38-120 months depending on access to proper diagnostic testing (Daniels & Mandell, 2014). ASD symptoms range from mild to severe in terms of functional impact. The symptoms of high-functioning ASD are less impeding on early social development, and are therefore often diagnosed later in child development as well as in adulthood (Happé et al., 2016). The lifelong nature of the condition suggests the need to adopt a life-course perspective and a multi-disciplinary approach for the study of ASD.

The current diagnostic criteria for ASD is derived from the The Diagnostic and

Statistical Manual of Mental Disorders (DSM-5), and focuses on two major behavioural criteria: social deficits and restrictive and repetitive behaviours (DSM-5, 2013). The social deficits in communication and interaction are characterized by deficits in social-emotional reciprocity, non-verbal communication and the development and maintenance of social relationships (DSM-5, 2013; Lord et al., 2000; Sigman, Ungerer, & Sherman, 1986; Travis & Sigman, 1998). The restrictive and repetitive behaviours are characterized by stereotyped or repetitive motor patterns, an insistence on adherence to ritualized routines, fixated interests and hypo- or hyper-active sensory input or output (DSM-5, 2013; Szatmari et al., 2006; Wigham, Rodgers, South, McConachie, & Freeston, 2015).

The final criterion for an ASD diagnosis is that it must not be explained by another disorder. This is a result of the many ASD-related genetic disorders such as the more frequently studied fragile X syndrome, tuberous sclerosis and Rett syndrome, and other rarer syndromes such as Joubert syndrome, Timothy syndrome and Smith-Lemli-Opitz syndrome (for a review of these disorders see: de la Torre-Ubieta, Won, Stein, & Geschwind, 2016). This is supported by behavioural evidence showing a high rate of an ASD-diagnosis or ASD-like behaviours amongst these disorders. What differentiates these disorders is they have a known genetic origin and display a genetic homogeneity amongst affected individuals, which is not observed in all forms of ASD (Abrahams & Geschwind, 2010a). Although behavioural evidence shows a high rate of an ASD-diagnosis or ASD-like behaviours amongst these disorders, what differentiates them is that they have a known genetic origin and display a genetic homogeneity amongst affected individuals, which is not observed in all forms of ASD. This differentiation has led to the development of two subtypes of ASD: syndromic ASD referring to a related disorder where there is a comorbid presence of ASD explained by a specific genetic alteration, and idiopathic ASD which refers to any ASD diagnosis where the etiology is unknown (Rogers, Wehner, & Hagerman, 2001).

1.3. *Etiology of ASD*

With the increasing efficiency and accessibility of genome-wide sequencing, many risk genes have been implicated in both syndromic and idiopathic ASD. Collectively, variations in these high-effect genes can be observed in about 15% of all ASD

cases, however, individually, each gene only accounts for 1-2% of all cases. (Abrahams & Geschwind, 2010b). This genetic heterogeneity, combined with evidence that the concordance (0.58-0.88) amongst monozygotic (MZ) twins raised together is not 100% (Hallmayer et al., 2011; Rosenberg et al., 2009), suggests other environmental factors, such as the uterine and early postnatal period, may also effect the development of ASD. Taken together this suggests both a polygenic and environmental etiology to ASD.

1.3.1. Environmental Etiology of ASD

Epidemiological studies have been providing increasing evidence of a prenatal environmental role in ASD. The *in utero* developing brain is particularly vulnerable to environmental effects (Grandjean & Landrigan, 2014). Many environmental toxicants can pass freely through the placenta (Needham et al., 2011), and the blood-brain barrier is also more susceptible during embryological development than in later life (Zheng, Aschner, & Ghersi-Egea, 2003). Moreover, many noxious environmental substances are neurotoxic at lower doses in the developing brain compared to adult tissue (Grandjean & Landrigan, 2014). Apart from direct environmental effects on developing neural tissue, environmental dysregulation during prenatal development can lead to variations in gene expression (Jirtle & Skinner, 2007; Sng & Meaney, 2009).

There is increasing evidence for a critical period of ASD environmental susceptibility during the perinatal period of development. For example, the earliest teratogenic cause of ASD revolved around thalidomide, where a proportion of offspring born to mothers taking thalidomide during pregnancy developed ASD particularly when taken during the fourth week post-conception (Strömland, Nordin, Miller, Akerström, & Gillberg, 2008). Other early teratogens which have been epidemiologically associated with ASD during the first and second trimester are the anticonvulsant valproic acid (VPA; Moore et al., 2000), ethanol (Nanson, 1992) and misoprostol (Bandim, Ventura, Miller, Almeida, & Costa, 2003).

The development of ASD has also been heavily associated with increased immune activity during pregnancy, as indicated by prenatal exposure to maternal infection, prolonged fever, and inflammation (Chess, 1976.; Atladóttir et al., 2010; Fox et al., 2012;

Zerbo et al., 2013). Specifically, ASD has been associated with hospitalization for bacterial infection and viral infection in the first and second trimester respectively (Atladóttir et al., 2010). In particular, mothers of children with ASD have been reported to be four times more likely to have been hospitalized for a bacterial infection during the second trimester of their pregnancy and were more likely to have multiple recurring infections (Zerbo et al., 2015). Cases of ASD associated with hospitalization for bacterial infection during pregnancy were 1.5 times more likely to be of increased severity with comorbidity of intellectual disability (Lee et al., 2015). Furthermore, maternal viral influenza and the administration of antibiotics such as penicillins and sulfonamides result in a 1.6-2.2-fold increase for the likelihood of autism particularly in the second trimester of development (Atladottir, Henriksen, Schendel, & Parner, 2012). Prolonged fever (greater than a week) accounted for a 3-fold increase in ASD in the first trimester and a fourfold increase during the second trimester (Atladottir et al., 2012), particularly if there was an incidence of three or more fever episodes (Hornig et al., 2018). Interestingly the increased risk of ASD associated with these factors was dramatically reduced if they occurred during the third trimester (Atladottir et al., 2012; Zerbo et al., 2015) suggesting a critical period of ASD susceptibility from immune factors focused on the earlier months of pregnancy. Throughout the second trimester is a period of critical cortical migration and proliferation where signalling by transcription factors regulates cortical layering required for normal brain development (Murayama, Matsuzaki, Kawaguchi, Shimazaki, & Okano, 2002). As such this process is highly susceptible to the uterine and exterior environment and is thought to be the earliest origin of ASD (for a review see: Pardo & Eberhart, 2007). Of particular relevance is that the number of neural precursor cells available during early cortical development has been shown to be regulated by microglia (Paolicelli et al., 2011), which are activated in the prenatal brain following an immune response to lipopolysaccharide, which acts by stimulating an immune response via toll-like receptor 4 (Tronnes et al., 2016).

The association between ASD and maternal immune activation is hypothesized to be the result of pro-inflammatory cytokines, which are able to cross the placental barrier into the developing fetus (Patterson, 2011; Zaretsky, Alexander, Byrd, & Bawdon, 2004). In particular, interleukin (IL)-1β and IL6 have been shown to be upregulated in

lymphoblast cell lines of MZ twins with ASD (Hu, Frank, Heine, Lee, & Quackenbush, 2006), and in the circulating plasma of 2-5 year old regressive ASD children compared to typically developing controls (Ashwood et al., 2011). Additionally, following viral immune activation during pregnancy, an upregulation of IL-6 mRNA is observed in the placenta (Hsiao & Patterson, 2011). IL-6 is also a direct pyrogenic, acting on brain endothelium to induce the production of prostaglandins which stimulate a febrile response, providing further evidence for a role of fever in ASD (Eskilsson et al., 2014). The causal role of interleukins has been suggested by experiments demonstrating that IL-1 receptor antagonists, given simultaneously during a bacterial immune activation, inhibit the development of an ASD-phenotype in the offspring (Girard, Tremblay, Lepage, & Sebire, 2010). Although the mechanisms are poorly understood, there is increasing evidence for the involvement of inflammatory pathways in the pathogenesis of ASD.

In summary, these data suggest that certain environmental factors affecting immune responses in early prenatal development increase the risk of ASD. With the recent identification of several ASD risk alleles and the high concordance amongst MZ twins, these environmental factors likely interact with genetic predispositions and further raise ASD risk.

1.3.2. Genetic Etiology of ASD

Despite there being strong evidence for an environmental etiology to ASD, the relatively high concordance amongst MZ and dizygotic (DZ) twins with ASD (0.58-0.88) suggest that there is a likely a genetic component (Hallmayer et al., 2011). Additionally, infants born to a sibling with ASD, with the same biological parents, have a reported 18.7% recurrence rate further supporting this genetic role. However, recent re-sequencing whole-genome studies have implicated immense heterogeneity amongst individuals with idiopathic ASD (Abrahams & Geschwind, 2010a). To amass all of the novel whole-genome sequencing data and to sort through the heterogeneity of the population, the Simons Foundation Autism Research Initiative (SFARI) has developed a collaborative process which amalgamates peer-reviewed evidence on genetic influences of ASD to evaluate a score for each gene of interest in terms of its strength and likelihood of playing a role in ASD (Abrahams et al., 2013). In total SFARI has indicated 25 genes of high confidence,

including *Pten*, *Shank3*, and *Reln* and 59 strong candidate genes including *Shank2*, *Mecp2* and *Nlgn3*. This has helped associate idiopathic and syndromic ASD by showing the role that a specific gene has within both ASD and its related disorders. For example, in Rett syndrome, missense mutations in the methyl binding domain of *MECP2* results in motor disabilities such as ataxia and loss of hand movement, microcephaly, seizure and the development of ASD around 6-18 months of age (Amir et al., 1999). *Mecp2* is considered a strong candidate gene as 3`-untranslated regions and missense mutations in this gene result in Rett syndrome and are also strongly associated with idiopathic ASD (Shibayama et al., 2004).

In comparison, *Pten* is considered a gene of high confidence as alteration to *Pten* expression has been extensively observed in both syndromic and idiopathic ASD at high rates. *PTEN* is translated into phosphatase and tensin homolog (PTEN), a phosphatase in the phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) signalling cascade. PTEN was initially associated with ASD, as many individuals with ASD presenting with extreme macrocephaly, 2.5-8 standard deviations from the mean, have germline heterozygous mutations to *PTEN* at three loci (Butler et al., 2005; Buxbaum et al., 2007). In idiopathic ASD, *Pten* was identified as a risk gene through transmission and *de novo* association analysis of whole-exome sequencing data, which identifies risk genes by statistically weighing novel and inherited genetic mutations (De Rubeis et al., 2014). This association with loss of function mutations in *Pten,* a result of haploinsufficiency, was further confirmed in whole-genome studies of idiopathic ASD, where *Pten* mutations were associated with downstream mutations in *Akt, Tsc1/2* and *mTOR*, identifying a key pathway in ASD pathophysiology (C Yuen et al., 2017; Yeung et al., 2017).

1.4. *PTEN- Akt-TSC1/2-mTOR pathway in ASD*

Downstream of PTEN, mutations to *Tsc1* and *Tsc2*, the genes that encode tuberous sclerosis 1 (TSC1) and tumour suppressor complex 2 (TSC2), respectively, contribute to tuberous sclerosis, a developmental disorder which results in multi-organ benign tumors causing seizures, facial abnormalities, intellectual impairment and in many cases ASD (Smalley, 1998). *Tsc1* and *Tsc2* are not considered candidate genes within the SFARI database; however, *Tsc1* is considered syndromic in the SFARI gene database, as

it results in ASD in about 50% of the population with this syndrome. This mutation has yet to be observed with high homogeneity in sample populations of idiopathic ASD (Spurling Jeste et al., 2014). Figure 1 displays the converging pathway between PTEN and TSC1/TSC2 on mTOR in the brain. The loss of function mutations to *Pten* com-

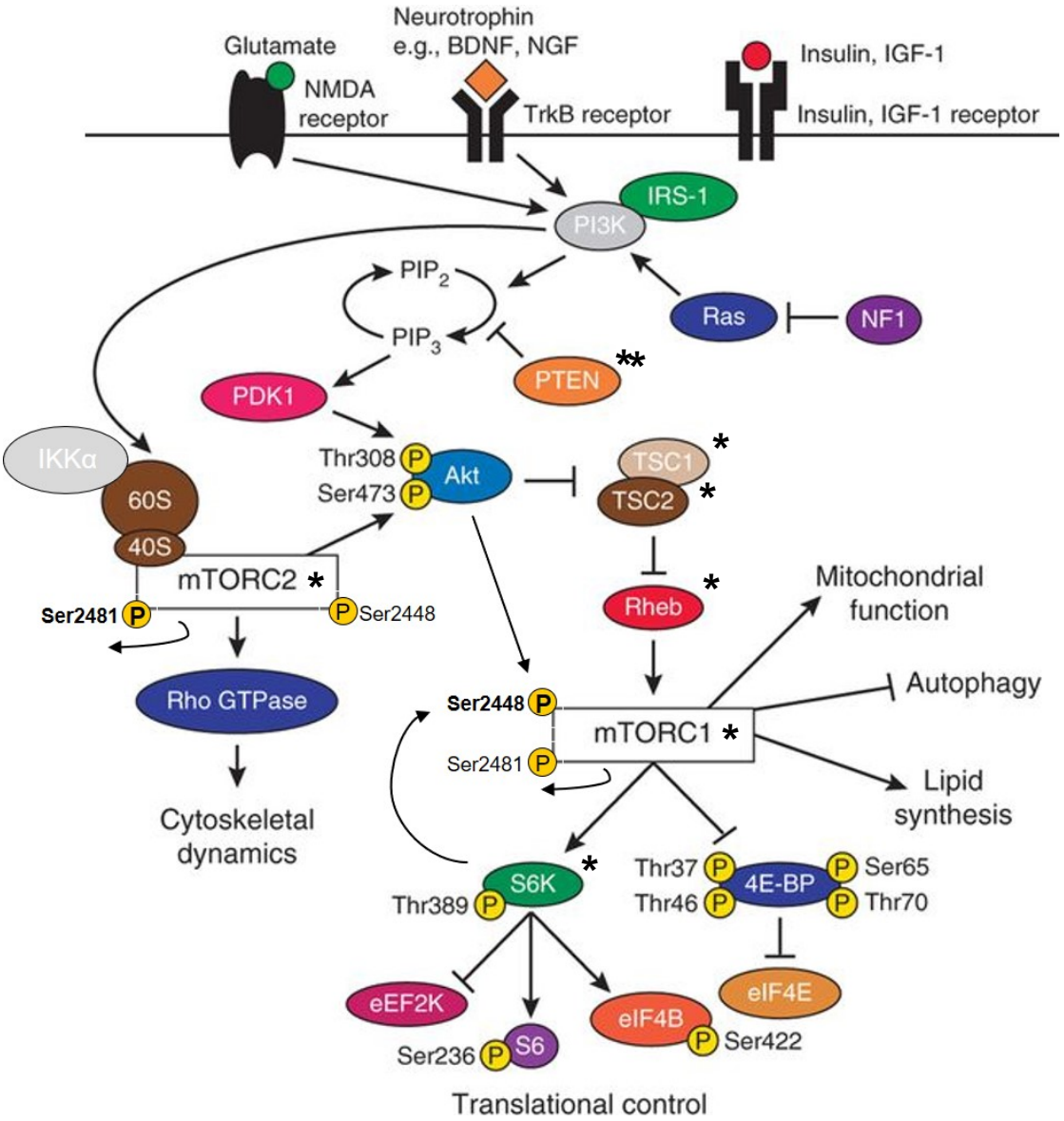

Figure 1. Neuronal mTOR pathway. Adapted from Costa-Mattioli and Monteggia (2013). *gene included in SFARI database.

monly observed in ASD, result in reduced PTEN inhibition of phosphatidylinositol (3,4,5)-trisphosphate (PIP_3) regulated pyruvate dehydrogenase lipoamide kinase isozyme 1 (PDK1) activation. This leads to increased protein kinase B (Akt) activity and

subsequent constitutive inhibition of TSC1/TSC2 (Costa-Mattioli & Monteggia, 2013). Although *Tsc1* is considered syndromic, reduced TSC1 and TSC2 activity and expression have been observed in idiopathic ASD, and are common as loss of function mutations in tuberous sclerosis (Kalsner et al., 2018; Smalley, 1998) The relationship between mutations in PTEN and TSC1, and their associated syndromes further supports both the polygenic nature of ASD and a role for this pathway.

TSC1 and TSC2, are upstream negative regulators of the multi-action protein mechanistic target of rapamycin (mTOR). As such, loss of function to either TSC1/TSC2 or PTEN would result in increased mTOR catalytic kinase activity (Figure 1). In the SFARI database, *Mtor* is one of 176 genes scored as genes of suggestive evidence. *Mtor* receives this designation due to the reduced hippocampal mTOR observed in idiopathic ASD (Nicolini, Ahn, Michalski, Rho, & Fahnestock, 2015) and due to its associations with syndromic forms of ASD including tuberous sclerosis (Meikle et al., 2008), Smith Kingsmore syndrome (Gordo et al., 2018) and a specific gain-of-function germline induced missense mutation of *Mtor* resulting in ASD (Mroske et al., 2015).

mTOR is known to form two separate complexes, mTORC1 and mTORC2, which are distinguished functionally through upstream and downstream regulators, and molecularly through their composition. The role of mTORC2 is poorly understood as it was only recently identified; however, it has been associated with both the indirect regulation of mTORC1 via Akt phosphorylation, and actin cytoskeletal dynamics. Specifically, mTORC2 has been linked to actin polymerization during long-term potentiation (LTP) in the hippocampus during learning and memory (Huang et al., 2013). Indirectly, mTORC2 regulates mTORC1 via a feedforward mechanism leading to Akt phosphorylation via PI3K, insulin dependent signalling. This is achieved through inhibitor of nuclear factor kappa-B kinase subunit alpha (IKKα), which has been shown to activate mTORC2 kinase activity on Akt thereby inhibiting the inhibitor of mTORC1, TSC1 and TSC2 (Figure 1; Dan et al., 2016). In contrast

Contrarily to mTORC2, mTORC1 has been extensively studied as it is the primary complex responsible for the effects of mTOR on protein translation and subsequent

cell growth and autophagic processes (Costa-Mattioli & Monteggia, 2013) and is directly activated via association with Ras homolog enriched in brain (Rheb) at mTOR's catalytic domain (Long, Ortiz-Vega, Lin, & Avruch, 2005; Soliman et al., 2010). The role of the mTORC1 pathway is quite broad as it regulates cell growth by affecting global ribosomal translation through ribosomal protein S6 kinase beta-1 (S6K) and the eukaryotic translation initiation factor 4E-binding protein 1/eukaryotic translation initiation factor 4E (4EPB1/eIF4E) complex. mTORC1 also regulates cell growth indirectly via the initiation of autophagic processes and is involved in many lipid and mitochondrial metabolic pathways (Costa-Mattioli & Monteggia, 2013). Herein, aberrant activation of mTORC1 is often associated with cancer cell proliferation (Saxton & Sabatini, 2017). Considering the role of mTOR in cell growth and proliferation, it is not surprising that mTOR plays a pivotal role during neurodevelopment particularly in regulating the pruning of synapses, a critical element of typical brain development (Tang et al., 2014).

The activation of mTOR in either complex is regulated by phosphorylation at predominately two sites, ser2448 and ser2481. Ser2448 is phosphorylated upstream via Akt (Atherton et al., 2005) and downstream via S6K to promote mTOR kinase activity, and as such occurs predominantly in mTORC1 (Chiang & Abraham, 2005; Copp, Manning, & Hunter, 2009). Ser2481 is a site of mTOR autophosphorylation which was initially thought to only occur in mTORC2 (Copp et al., 2009). More recent evidence has shown that this autophosphorylation is a marker of mTOR intrinsic catalytic activity in either complex, as it was lost following the removal of upstream activators of both mTORC1 and mTORC 2 (Soliman et al., 2010).

Interestingly, increased mTORC1 activity is observed in idiopathic ASD resulting in reduced autophagy and subsequent impairment of dendritic spine pruning (Tang et al., 2014). This increase in spine density has been previously reported in ASD and is common in many animal models of ASD in which increased mTOR activity is reported (Bhattacharya et al., 2012; Santini et al., 2012). The increased spine-density is associated with increased ribosomal translational activity through the downstream activation of S6K and 4EBP1/eIF4B via mTORC1 kinase activity in tuberous sclerosis and mutative PTEN syndromic ASD (Santini et al., 2012; Spinelli, Black, Berg, Eickholt, & Leslie, 2015). In

turn, aberrant cell growth via mTORC1-mediated mechanisms may underlie the macrocephaly commonly observed in ASD, particularly those associated with mutations to *Pten* (Butler et al., 2005; Yeung et al., 2017). Although mTORC2 has been less associated directly with ASD development, inhibiting all mTOR activity with prolonged rapamycin treatment reduces the increased mTORC2 activity seen in animal models of syndromic mutative PTEN and tuberous sclerosis (Meikle et al., 2008; Zhou et al., 2009). Considering that increases in mTOR activity are present in idiopathic ASD and many genes associated with syndromic ASD converge on this pathway, alterations in mTOR expression and activity serve as a key target of analysis when studying new populations or animal models of ASD and may serve as a potential bio-marker in ASD diagnosis.

1.5. *Rodent Models of ASD*

Rodent models of ASD are often developed from genetic knowledge of ASD or the environmental factors revealed in epidemiological studies. Despite there being many different existing rodent models, they often use a similar battery of social tests to determine the ASD-like phenotype. Although many of the clinical behaviours of ASD may seem complex and specific to humans, similar disruptions to sociability and increases in repetitive and restrictive behaviours can be analogous species-specific behaviours in rodents. Across all models, the battery of behavioural assays conducted on ASD-representative models focus on the three primary symptoms of ASD: impairments in social communication and social interaction and the development of repetitive and restrictive behaviours. Mice are social animals, typically living in demes with a hierarchical social structure that consists of dominant and subordinate males and multiple females (Berry, 1981). The interactions between members of the same-sex, between males and females as part of reproduction, and mother-infant social interactions that are necessary to develop and maintain this social organization, have led to the evolution of behaviours involving tactile (J. B. Panksepp & Lahvis, 2007) and olfactory (Brennan & Kendrick, 2006) cues, as well as ultrasonic vocalizations (Portfors, 2007).

Social interaction behaviours in mice are tested with a variety of behavioural assays which primarily rely on their investigative and affiliative behaviour towards novel social stimuli. Perhaps the most naturalistic assay is one of reciprocal social interaction

where the ASD-model animal and an unfamiliar conspecific are placed together in an open field and the frequency or duration of various affiliative or pro-social behaviours (e.g., time together or following) and of non-social behaviours (e.g., time spent exploring the arena) can be measured in the ASD-model animal (Bolivar, Walters, & Phoenix, 2007). In this assay, a reduction in the frequency or duration of social behaviours is observed in various strains associated with ASD such as the inbred BTBR T+ tf/J mouse model (Bolivar et al., 2007). Simpler, but less ethologically valid, is a measure of social approach using the 3-chamber assay. In this assay, there are three compartments separated by small entrances in which the testing mouse can choose to explore either an unfamiliar conspecific in an inverted wire cup in one chamber, the novel area in the centre chamber or a novel object similarly in an inverted wire cup in the third chamber (McFarlane et al., 2008). This assay relies on the animal's natural preference for a social stimulus over a novel stimulus and a reduction in this can be associated with the ASD phenotype of reduced sociability (McFarlane et al., 2008). Due to separation of the three chambers this task can be easily automated which allows for a higher throughput. The earlier two assays described contain visual, tactile, auditory and olfactory stimuli between the mouse being tested and the stimulus animal. Another common assay, the olfactory habituation/dishabituation task relies solely on olfactory cues, which are essential for typical rodent social behaviour and the formation of social memory (Restrepo, Arellano, Oliva, Schaefer, & Lin, 2004). This assay serves two purposes when testing for sociability, first by assessing the general olfactory ability of the animal through the use of non-social odours; habituation to repeated presentation of the same odour and dishabituation to a novel odour naturally occurs if the animal is able to discriminate between odours (Crawley et al., 2007). The second aspect of this assay is the use of social odours acquired via swabs of unfamiliar conspecific cage bedding. Here, an increased interest demonstrated by a larger dishabituation to a social odour following a non-social odour occurs under normal conditions (Crawley et al., 2007). In ASD mice, assuming there are no impairments in olfaction, as observed with the non-social odours, reduced interest in social odours could be indicative of an ASD-like behavioural phenotype in mice (Silverman, Yang, Lord, & Crawley, 2010). Common to all these assays is determining the ASD-like phenotype via the use of assays which rely on species-specific measures of

sociability as a comparison to complex human social behaviour.

Measures of social communication via ultrasonic vocalizations (USVs) are less commonly assayed in ASD mouse models likely due to the complexity of analysis and the specific equipment required. Additionally, there is limited evidence on the intentional communicative nature of the USVs being produced in mice, and in the variations in the type of calls being made (Silverman et al., 2010). As such, due to the complex nature of human communication and the vast evidence on the specific language deficits observed in ASD, this measure in rodents is less analogous to ASD than the assays for social behaviour. However, deficits in USVs have been commonly reported during development and in adult interactions in rodent models of ASD when compared to controls, thus providing evidence for USVs as a measure of social communication that may be a species-specific comparison to the communication deficits observed in ASD (Jules B. Panksepp et al., 2007; M. L. Scattoni, Ricceri, & Crawley, 2011; Wöhr, Roullet, & Crawley, 2011). The most common assay of communication is maternal separation induced USVs in pups (Branchi, Santucci, & Alleva, 2001). Reductions and variations in various features such as the number, frequency, duration, amplitude and call type are often used to determine an ASD-like phenotype (Maria Luisa Scattoni, Gandhy, Ricceri, & Crawley, 2008). Although this assay is quite informative, these maternal separation induced USVs only occur during the early post-natal period and are reduced throughout development (Branchi et al., 2001). Although USVs are also emitted during adult same-sex social interactions they are more infrequent than during early development and their purpose is poorly understood(Silverman et al., 2010).

Additionally, the development of stereotyped behaviours in mice are often used as an assay for the restrictive and repetitive behaviours observed in ASD. One commonly used assay is marble burying, which represent both restrictive and repetitive activity, as the animal repeatedly buries marbles presented in a grid (Thomas et al., 2009). This assay has been validated as a measure of repetitive activity when conducted in darkness (Thomas et al., 2009) and increases in marbles buried is often reported in ASD mouse models (Malkova, Yu, Hsiao, Moore, & Patterson, 2012; Xuan & Hampson, 2014). Another more ethologically valid measure of restrictive and repetitive behaviours in rodents

is repetitive self-grooming (Lewis, 2011) where increases in the duration and frequency of grooming is representative of an ASD-like phenotype in representative mouse models. Similar to the social interaction and communication deficits, rodent assays for restrictive and repetitive behaviours take a species-specific approach for behaviours that are analogous to those observed in ASD. Overall, if many ASD behavioural assays are conducted in rodent models a strengthened assessment of the ASD-like phenotype can be evaluated when determining the role that a particular genetic or environmental factor can have on ASD etiology and the development of the behavioural phenotype.

1.5.1. mTOR related Genetic Models of ASD

PTEN, an upstream negative regulator of mTOR, and a gene of interest in ASD (De Rubeis et al., 2014) has been used to model an ASD-like phenotype in rodent models (Figure 1). Heterozygous mutations to *Pten* commonly observed in ASD were modeled by inducing a deletion at the catalytic phosphatase domain of exon 5, thereby inactivating the phosphatase action of PTEN (Page, Kuti, Prestia, & Sur, 2009). Although homozygous offspring of this deletion are not viable, the heterozygous offspring ($Pten^{+/-}$) survive into adulthood and display macrocephaly. They also display reduced social interaction as measured by the three-chamber sociability apparatus, where the observed mouse has the option to enter a chamber with another sex- and age-matched conspecific or an alternative chamber with a novel object (Page et al., 2009). Interestingly, only the male $Pten^{+/-}$ mice displayed a significant reduction in time spent in the social chamber compared to females. A similar reduction in sociability was observed in the female $Pten^{+/-}$ mice only after a second heterozygous mutation to *Slc6a4*, a gene encoding for a serotonin transmitter, was introduced (Page et al., 2009). *Slc6a4* has been previously implicated as a risk gene in ASD (Bartlett, Gharani, Millonig, & Brzustowicz, 2005), and has been associated with brain overgrowth (Wassink et al., 2007) which is commonly observed in PTEN mutative syndromic and idiopathic ASD (Butler et al., 2005; Buxbaum et al., 2007). Along with macrocephaly, $Pten^{+/-}$ mice display hypertrophic dendrites and axonal tracts with increased synaptic density in the hippocampus, conferring further similarities to ASD (C.-H. Kwon et al., 2006). Furthermore, these mice display significant increased phosphorylation to Akt and S6 indicative of increased mTORC1 and mTORC2 activity (Guertin et

al., 2009; C.-H. Kwon et al., 2006).

Downstream of PTEN, another negative regulator of mTOR, TSC1/2, has also been implicated in syndromic ASD (Spurling Jeste et al., 2014). *Tsc1*$^{+/-}$ and *Tsc2*$^{+/-}$ mice used as models of the developmental disorder tuberous sclerosis, also display social deficits as measured by free-social interaction (Sato et al., 2012). This deficit was not dependent on sex, which is not surprising as no sex ratio exists within TSC1/2-related ASD (J. C. Lewis, Thomas, Murphy, & Sampson, 2004). Importantly, these mice displayed equal social dominance in the tube test when compared to their wild-type litter mates indicating that the deficits in social interaction was not related to social dominance (Sato et al., 2012). These mice also displayed an increase in rearing, which may be indicative of an increase in repetitive behaviours. At a molecular level, reduction in TSC1 and TSC2 in these mice resulted in increased phosphorylated-S6K, the primary downstream effector of mTOR, which was reversed when rapamycin, a potent inhibitor of mTOR activity, was administered (Sato et al., 2012; Zeng, Xu, Gutmann, & Wong, 2008). The administration of rapamycin in adulthood also rescued the deficits in social interaction in these mice (Sato et al., 2012). Overall, it is apparent that mouse models of syndromic ASD can accurately model an ASD-like behavioural phenotype and display the representative increase in mTOR activity commonly observed. However, despite the intrinsic experimental control available with genetic models, they fail to assess the environmental influence on ASD, which may elucidate the role of mTOR signalling in idiopathic ASD.

1.5.2. mTOR in Environmental Models of ASD

Although genetic models can directly assess the role that risk genes play in the development of ASD, environmental models represent a more ethological depiction of ASD development. An animal model of ASD recently associated with mTOR activity is the VPA model (Murakami-Kotajima, Ikeda, & Uchino, 2016; Nicolini et al., 2015; Qin, Dai, & Yin, 2016). VPA exposure *in utero* has been associated with ASD based on evidence that children exposed to this teratogen develop a fetal valproate syndrome resulting in ASD and additional delays in global behavioural development (Moore et al., 2000). Prenatal exposure to VPA in rodent models have linked this treatment with the three core symptoms of ASD. Male mice born to dams treated prenatally with VPA at E12.5,

display a significant increase in repetitive behaviours, as evidenced by time spent grooming and marbles buried, was observed when compared to sham-injected controls (Mehta, Gandal, & Siegel, 2011). Prenatally VPA-exposed mice also show a significant reduction in social preference in the three-chamber task when compared to sham-injected controls (Kim et al., 2013). Moldrich et al. (2013) observed a reduction in the number and complexity of ultrasonic vocalizations (USV) in mouse pups separated from their dam. Postnatal day 24 (P24) VPA-pups displayed reduced sociability in the 3-chamber apparatus and in free-social interaction, and increased grooming and digging when compared to the vehicle sham-injected controls (Moldrich et al., 2013). At a molecular level there is conflicting data as VPA-treated rats have been shown to have both reduced mTOR activity (Nicolini et al., 2015) and increased mTOR activity in the hippocampus (Qin et al., 2016). Administration of rapamycin, a negative regulator of mTOR, to adolescent and adult prenatally VPA-treated mice rescued the deficits in social interaction observed, suggesting that increased mTOR activity is a contributing factor to this behavioural deficit (Murakami-Kotajima et al., 2016). Interestingly, VPA has been shown to directly activate the PI3K/Akt/mTOR pathway (Gurpur, Liu, Burkin, & Kaufman, 2009) which may result in increased synaptic density as indicated by increased post-synaptic density 95 (PSD95) in the hippocampus observed in this model (Kim et al., 2013). In further support of the connection between mTOR and PSD95, rapamycin treatment in the hippocampus has been shown to reduce expression and protein levels of PSD95 (Li et al., 2010; Ma et al., 2010). Apart from the VPA model of environmentally induced ASD, the maternal immune activation (MIA) models of ASD are also commonly studied and have been recently associated with alterations in mTOR activity.

1.5.3. Maternal Immune Activation, mTOR and ASD

Pregnant dams prenatally exposed to lipopolysaccharide (LPS), the molecule on the exterior surface of gram-negative bacteria which stimulates a bacterial immune response via toll-like receptor 4 (Takeuchi et al., 1999), or the double stranded RNA polyinosinic:polycytidylic acid (Poly I:C), to stimulate a viral immune response via toll-like receptor 3 (Alexopoulou, Holt, Medzhitov, & Flavell, 2001), have offspring which display an ASD-like phenotype (Fernández de Cossío, Guzmán, van der Veldt, & Luheshi,

2017; Malkova et al., 2012; Xuan & Hampson, 2014). Male PolyI:C treated mice display reduced complexity and frequency of USVs following maternal separation and reduced sociability in the 3-chamber in adulthood (Malkova et al., 2012). These mice also show increased grooming and marble burying further supporting the ASD phenotype (Malkova et al., 2012). Interestingly, when prenatal PolyI:C was combined with *Tsc2*$^{+/-}$ mice, slightly greater deficits in sociability were observed (Ehninger et al., 2012; Ehninger, 2014), suggesting mTOR pathway involvement in the PolyI:C model. Recently adult cannabidiol treatment, a known positive regulator of mTOR (Giacoppo, Pollastro, Grassi, Bramanti, & Mazzon, 2017), has been shown to improve deficits in social interaction and recognition in males prenatally treated with PolyI:C (Osborne, Solowij, Babic, Huang, & Weston-Green, 2017) further supporting the possible role of mTOR deficits in this model. Surprisingly, this differs from results from *Tsc2*$^{+/-}$ mice not treated with PolyI:C, where adult rapamycin treatment, a negative regulator of mTOR, improved the phenotype (Sato et al., 2012), suggesting possible antagonistic mechanisms on mTOR activity between PolyI:C and *Tsc2* heterozygotes.

Offspring from dams treated with LPS also show ASD-like deficits in social behaviours and increased repetitive behaviours however there is some inconsistency in the results. This has been observed quite consistently in females, but not in males. Reduced time with the social stimulus in a three-chamber sociability task in both lipopolysaccharide-treated maternal immune activation (LPS-MIA) males and females has been reported (Fernández de Cossío et al., 2017) but a female specific effect has also been observed (Xuan & Hampson, 2014). Furthermore, a female-specific effect in an increase in marble burying was reported in Fernández de Cossío et al. (2017) whereas a similar increase in both males and females was observed in Xuan and Hampson (2014). A reduction in the average duration of maternal separation-induced USVs was observed in both male and female LPS-MIA offspring at P8 (Fernández de Cossío et al., 2017). Interestingly, a significant reduction in mTOR expression was observed in whole prenatal mice brains at 1 hr and 3 hrs post LPS inoculation (Lombardo et al., 2018; Oskvig, Elkahloun, Johnson, Phillips, & Herkenham, 2012). *Tsc1* and *Tsc2*, upstream regulators of mTOR, were also reduced in expression 3 hrs post LPS inoculation, and a subsequent increase in eIF4B was observed; however, this study failed to look at possible sex differences (Lombardo et

al., 2018). This increase in downstream mTOR activity, known to result in deficits in synaptic pruning (Tang et al., 2014), resulted in increased hippocampal granule cell spine number in male P15 LPS-MIA mice. This was associated with a reduction in microglial fractalkine receptor (CX3CR1) mRNA, a known molecular pruning signal (Fernández de Cossío et al., 2017). This ultimately supports a possible role of the TSC1/2-Akt-mTOR pathway in the LPS-MIA model of ASD.

Although MIA seems to result in an ASD-like phenotype in exposed offspring, existing research has primarily focused on adult male mice. Considering that ASD presents as early as 6 months in human development, there is a surprising lack of research into the early development of ASD-like symptoms in pups and juvenile mice, particularly in the LPS-MIA model. There is also a lack of evidence regarding the expression of ASD-like symptoms in females. Although males are 4.5 times more likely to be diagnosed with ASD than females (Centers for Disease Control, 2014), in moderate to severe ASD, this ratio is only about 1-2:1, depending on the classification of severe (Werling & Geschwind, 2013). In ASD, the lack of literature on females with ASD is likely due to a sex-bias in the sample population and the subsequent lack of statistical power in females to determine an effect. In LPS-MIA models, females are either excluded to attempt to represent the sex bias in ASD or there is conflicting evidence regarding potential sex-effects. The potential sex effects, and the loss of a sex-bias in severe ASD, suggests the need for increased research on severe ASD, and its representative animal models, particularly in females. This is increasingly relevant as MIA-related ASD is often associated with more severe cases of ASD, as is the case with females with ASD (Baker & Milivojevich, 2013; Lee et al., 2015; Tsai, Stewart, & August, 1981). The differing sex biases contingent on ASD severity may be indicative of pathophysiologies underlying both female and male ASD possibly contributing to many of the more severe behavioural symptoms.

1.6. *Current Research*

The majority of the current knowledge surrounding LPS-MIA, and the subsequent development of an ASD-like phenotype, has focused on adult male offspring or has shown conflicting results. Thus, the current study compared the behavioural

characteristics of ASD between male and female LPS-MIA offspring from birth into adulthood to better characterize the sex effects that occur in this model. Additionally, the current study focused on determining an activity profile of mTOR in this model at P0 and in adulthood which has yet to be described post-birth and may serve as a key diagnostic and therapeutic target for this form of ASD.

After LPS-MIA offspring and sham-injected controls were generated via intraperitoneal injection of LPS at E11.5 and 12, alterations in maternal care were initially assessed because maternal care has been shown to program certain behavioural and molecular components of the stress response in adult rodents (Weaver et al., 2004). Following this, the three primary domains of ASD (deficits in social communication and social interaction and the development of restrictive and repetitive behaviours) were assessed in the LPS-MIA mice compared to sham-injected controls. In early development, deficits in social communication were evaluated using maternal separation-induced USVs and spontaneous grooming was observed as a measure of repetitive and restrictive behaviours. In adulthood, olfactory ability as well as the response to social stimuli was assessed using an olfactory habituation and dishabituation test, and social interaction and spontaneous grooming was measured using the three-chamber sociability task. Lastly, repetitive and restrictive behaviours was assessed using the marble burying task. Overall, we hypothesized that LPS-MIA mice would present an ASD-like phenotype across all tasks, possibly dependent on sex, as observed by deficits in social interaction and communication and the presence of repetitive and restrictive behaviours in previous MIA literature (Malkova et al., 2012; Xuan & Hampson, 2014).

At a molecular level, we assessed the offspring at P0 and in adulthood (P130) targeting cortical and hippocampal, ASD-implicated, brain regions, and measured alterations in the expression of *mTOR* and subsequent protein levels. We hypothesized that there would be reduced *mTOR* at P0 as it was previously observed 3-hours post LPS induction (Lombardo et al., 2018). Functionally, the activity of mTOR was assessed through the auto phosphorylation marker of intrinsic activity at serine 2481. Finally, we assessed possible downstream structural alterations via analysis of PSD95 as increased mTOR activity is associated with an increase in PSD95 (Kim et al., 2013; Ma et al.,

2010). Considering the increased downstream activity of mTOR observed in whole brain 3hrs-post LPS inoculation (Lombardo et al., 2018) we projected an increase in mTOR phosphorylation and subsequent synaptic density as measured by PSD-95.

Overall, we aimed to better characterize the LPS-MIA model in terms of sex-dependent ASD-related behaviours and mTOR pathway functionality.

1.7. *Main Objectives:*

I. Evaluate the ASD-like phenotype in LPS-MIA mice using a battery of behavioural tasks from early development to adulthood.

II. Evaluate *Mtor* mRNA at P0 and P130 in frontal and hippocampal brain areas between LPS-MIA and vehicle-injected controls to determine reduced expression as observed in earlier time periods (Lombardo et al., 2018)

III. Compare adult hippocampal mTOR expression in the VPA model of ASD to the LPS-MIA model of ASD.

IV. Evaluate phosphorylation of mTOR to determine the functional effect of LPS-MIA on downstream the catalytic kinase activity of mTOR, where a decrease was observed in idiopathic ASD (Nicolini et al., 2015).

V. Observe possible downstream functional results of altered mTOR via analysis of PSD-95 in adult hippocampal regions to determine alterations in synaptic density.

VI. Evaluate any sex-effects in behaviour and physiology in the LPS-MIA model to support and develop previous evidence on males and female MIA-associated ASD.

Chapter 2

Behavioural effects of LPS-MIA

2.1. *Animals*

2.1.1. Animal Husbandry

8-week-old male and female C57Bl/6J mice (Jackson Laboratories, Bar Harbor, ME) were used for breeding in this study. Mice were housed in standard housing which consisted of a translucent plastic cage (18.75cm x 28cm x 12.5cm) with wood chip bedding and a wire top in a climate controlled (20 ± 2ºC) vivarium. A small black PVC pipe was present in all cages as a source of environmental enrichment. Animals were provided *ad libitum* access to food (Purina Laboratory Chow, Strathy, Ont., Canada) and tap water. Breeding females were housed in groups of 2-3 until they were separated for breeding. Breeding males were individually housed. Offspring were housed in same-sex groups of 2-4. Animals were kept on a reverse light dark cycle (12h:12h) with lights off at 08:30. All treatments and behavioural tests were approved by the Dalhousie University Committee of Laboratory Animals (protocol #16-049) using guidelines of the Canadian Council of Animal Care.

2.1.2. Pregnancy and LPS Injections

Males and females were paired in dyads and the presence of a copulatory plug in the females designated possible pregnancy and marked E0.5. Following the presence of a copulatory plug the male breeder was removed and re-paired with another female breeder in order to reduce the total number of male breeder mice used. Pregnant females were then left undisturbed until E11.5 when weight was recorded, and a morphological assessment of pregnancy was conducted. Pregnancy was determined quantitatively by increased weight gain and qualitatively through a visual assessment of weight gain in the lower abdomen. If no pregnancy was detected the animal was re-paired with a novel male to conduct another breeding. If pregnant, the animal received a 150µg/kg intraperitoneal (IP) injection of LPS (L2630; Sigma-Aldrich, Oakville, Ont., Canada) in 0.9% sodium chloride saline solution (Braun, Irvine, CA, USA) at a 5µl/g injection volume. 12 hours later, at E12, a second LPS IP injection was given. Sham-injected controls received a vehicle intraperitoneal injection with an identical injection schedule and injection volume as the

LPS group. Following the second injection, a cage change was conducted after which the mice were left undisturbed until pups were present. At birth, the health and size of the litter was visually assessed. All statistical analyses were conducted using R (r-project.org) and p-values<0.05 were considered statistically significant. Any values greater than two standard deviations from the mean were considered outliers and removed from analysis.

2.1.3. Offspring Demographics

Overall, we conducted 59 pairings and had 30 pregnant dams which received injections (Table 1). Table 1 shows the number of injections given, successful pregnancies, infanticides and spontaneous abortions. It also displays the 1.36:1 female to male sex bias observed in the LPS treated mice compared to the 0.9:1 female to male ratio observed in the vehicle injected controls (Table 1). There was also more infanticide in the LPS-treated mice compared to the controls.

Table 1. Breeding and Offspring demographics following treatment with LPS at E11.5 and 12 in C57Bl/6J mice.

	LPS	Vehicle
Received Injections	18	12
Gave Birth	12 (67%)	9 (75%)
Spontaneous Abortions/Not Pregnant	3 (17%)	2 (17%)
Infanticide	3 (17%)	1 (0.083%)
Number of Offspring	5.5±1.2	5.4±2.3
Number of Male Offspring	2.5±1.5	3±1.5
Number of Female Offspring	3.4±1.3	2.8±1.1

Animals were weighed at P0, 8, 12 and P130 (Figure 2). P0 weight was recorded from the animals used for the P0 molecular analysis, and the P8, 12 and 130 weights were recorded from the animals used in the behavioural testing. A three-way ANOVA was conducted comparing weight with sex, treatment and litter size as between-subject factors at each age point separately. There was a significant effect of treatment at P0 ($F_{(1,37)}$ = 6.550, p = 0.0147) with reduced weight observed in the LPS animals (1.26±0.025g) compared to the vehicle animals (1.34±0.020g; Figure 2a), and an expected effect of litter size ($F_{(4,37)}$ = 4.086, p = 0.00766), where weight decreased as litter size increased (R^2=0.137, p = 0.00896; Figure 2b). The treatment effect on weight observed at P0, was not mediated by sex ($F_{(1,37)}$ = 1.082, p = 0.305) nor was there an interaction between sex

and litter size ($F_{(4,37)} = 0.128$, $p = 0.971$). There was no effect of sex at P0 ($F_{(1,37)} = 2.449$, $p = 0.126$), P8 ($F_{(1,20)} = 0.292$, $p = 0.595$) or P12 ($F_{(1,20)} = 0.073$, $p = 0.790$). There was no significant difference in weight between treatments at P8 ($F_{(1,20)} = 0.549$, $p = 0.467$), P12 ($F_{(1,20)} = 3.363$, $p = 0.0816$) or P130 ($F_{(1,20)} = 1.415$, $p = 0.248$).

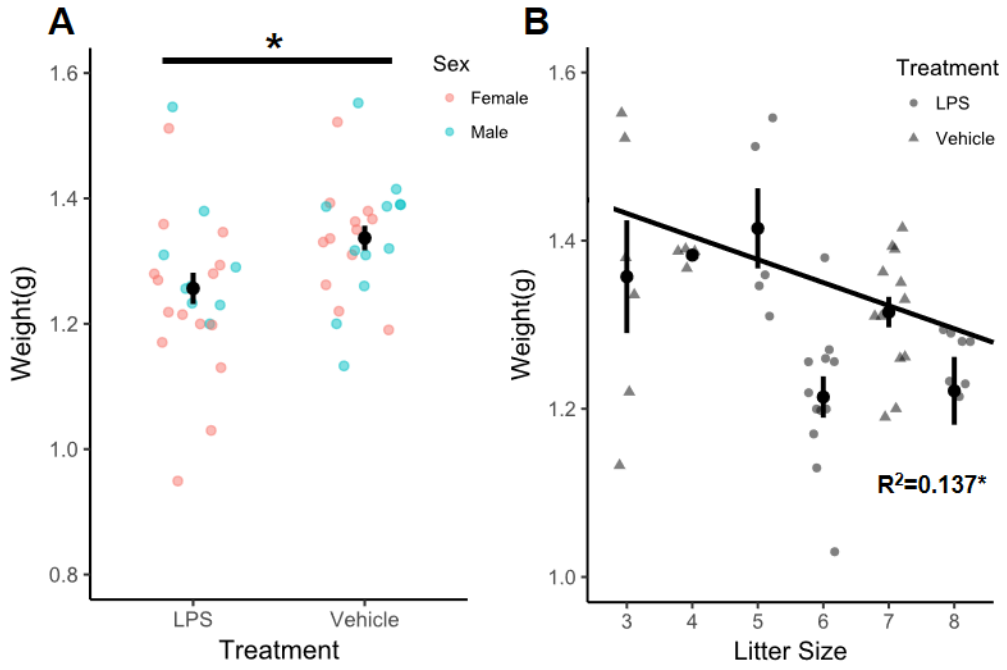

Figure 2. Weight of C57Bl/6J offspring prenatally treated with lipopolysaccharide (LPS) or saline-vehicle controls at (a/b) P0. Error bars are mean ± SEM. ***p<0.05.**

Similar to P0, weight decreased as litter size increased in P8 (main effect of litter size: $F_{(1,20)} = 16.362$, $p = 1.31 \times 10^{-5}$) and P12 mice (main effect of litter size: $F_{(1,20)} = 14.016$, $p = 3.77 \times 10^{-5}$). At P130 there was only the typical significant increase in weight in males (28.0g ± 0.394g) compared to females (22.0g ± 0.259; $F_{(1,20)} = 142.69$, $p = 1.47 \times 10^{-9}$).

2.2. *Methods*

Approximately half of the successfully bred animals were used for behavioural analysis (n = 38; $n_{(F\text{-}LPS)}$=8, $n_{(M\text{-}LPS)}$=10, $n_{(F\text{-}Vehicle)}$=9, $n_{(F\text{-}Vehicle)}$ = 11 from 5 LPS-MIA and 4 vehicle litters). These animals were undisturbed until P7 to avoid disrupting maternal care. Any animals that were singly housed at weaning due to a lack of sex-, treatment- and age-matched animals available, were not used in the adult behavioural testing (P90-P130).

Figure 3 displays the timeline of behavioural assays conducted on the mice from P0-P130. Briefly, maternal care was first observed, followed by measurements of social communication via USVs and spontaneous grooming. The animals were then weaned into age- sex- and treatment-matched mixed litter cages and were reared until P90. At P90 the animals were tested on olfactory ability and social interest in the olfactory habituation/dishabituation task. They were then tested on spontaneous grooming and sociability in the 3-chamber task. Lastly, the animals were tested for restrictive and repetitive behaviours in the marble burying task. All behavioural testing was conducted during the dark phase.

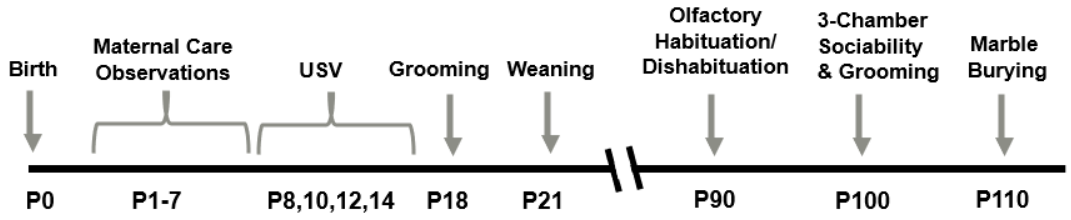

Figure 3. Timeline of behavioural tests conducted on prenatally treated lipopolysaccharide C57Bl6 mice and vehicle controls.

2.2.1. Maternal Care

Analysis of maternal care was carried out from P1-P7 (Figure 3). Maternal care during this period has been shown to result in behavioural and genetic programming in adulthood (Champagne, Weaver, Diorio, Sharma, & Meaney, 2003; Weaver et al., 2004) and can be easily quantified in C57Bl/6J mice (Champagne, Curley, Keverne, & Bateson, 2007). Three 1hr observations during the dark phase and two 1hr observations during the light phase took place. Every three minutes the behaviour occurring was scored by trained observers with a high degree of interrater reliability (>0.90). The scored behaviours can be broken down into two categories: active and passive maternal behaviours. Active behaviours consisted of licking and grooming (LG) and the four degrees of arched back nursing (ABN1-4) which have been previously described (Champagne, Francis, Mar, & Meaney, 2003). Passive behaviours consisted of no contact with pups (NCP), self-feeding (F) and self-grooming (SG). Because of the reported difficulty in determining the difference between higher ABN postures in mice (Champagne et al., 2007), instances of ABN3 and ABN4 were combined. Behaviours were scored on the

MouseWatch application (I.C.G. Weaver; Novum Scientific, Halifax, NS., Canada). The frequency of each behaviour over the 5 daily observation periods and across all days observed was calculated. Instances where LG and ABN2 or ABN3/4 occurred simultaneously were scored as additional active behaviours. An ANOVA was used to determine any significant differences in the individual maternal care behaviours between treatments.

2.2.2. Ultrasonic Vocalizations

In preparation for USV testing, following the final maternal care observation on P7, the pups were removed from their dam and tattooed on the tail using a 28-gauge needle and green animal tattoo paste. This allowed experimenters to identify the pup throughout the behavioural testing. USVs were quantified every other day from P8-P14 following maternal separation as previously described (Hofer, Shair, & Brunelli, 2002). Initially, the dam was removed from the home cage for 15 minutes and placed in a fresh cage. Following removal of the dam the pups were transported to a separate room lit by a 60-watt red light bulb and the cage was placed on a heat pad on low heat. Following the 15-minute separation, a single pup was randomly selected from the litter, weighed, transported in a 200mL plastic beaker and placed into a background sound attenuating Styrofoam box testing chamber. The lid of the testing chamber had a small hole to pass through the audio cable attached to an UltraSoundGate condenser microphone (CM16, Avisoft Bioacoustics, Berlin) which was suspended 10cm above the pup. The microphone detected frequencies of 0-250kHz and was connected to a UltraSoundGate 416Hb, 16bit, analog to digital converter (Avisoft Bioacoustics, Berlin, Germany) which was further connected to a computer. Each pup was recorded for 3 minutes using the AvisoftSASLab Pro recorder software (Version 3.2; Avisoft Bioacoustics, Berlin, Germany) at a sampling rate of 250kHz. Once each pup in the litter was tested, they were returned to the colony room where the dam was returned until the next day of USV testing.

The USVs were analysed using AvisoftSASLab Pro software (Version 3.2; Avisoft Bioaccoustics, Berlin, Germany) where a spectrogram was developed following a fast Fourier transformation with a 1024 FFT-length, 100% frame, Hamming window and a 75%-time window overlap. The spectrograms had a frequency resolution of 488Hz with a time resolution of 1ms. A frequency threshold of 60kHz was used to reduce background

noise. Calls were detected with an automatic threshold-specific algorithm with a hold time between calls of 10ms. Because the highest number of calls was observed on P8, following the removal of animals who made no calls, the number of calls was classified by an experienced observer into the 10 differing call-types with parameters similar to those previously described (Figure 4, Table 2; Scattoni et al., 2008).

Three-way ANOVAs comparing number, average frequency and average duration of calls was compared between sex, age and post-natal day. The proportion of each call type compared to the total number of calls was determined and compared between sex and treatment.

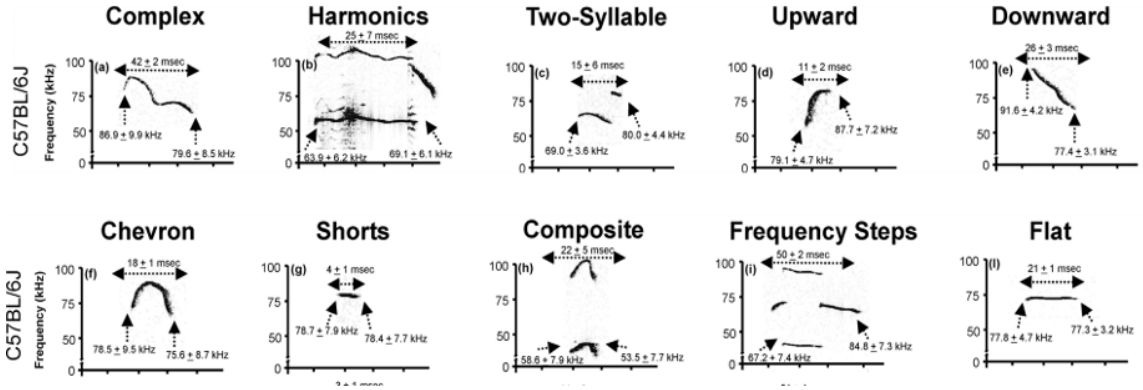

Figure 4 Examples of various call types analysed following maternal separation induced USVs in C57Bl/6J mouse pups. Adapted from Scattoni et al., 2008.

Table 2 List of USV call types and their associated parameters for classification.

Call type	Parameter
Complex	Single syllable, containing two or more directional changes in pitch of at least 3kHz
Harmonics	Single syllable with multiple frequencies
Two-Syllable	A primary single syllable, with a second shorter syllable to follow
Upward	Single syllable, continuous increase in pitch of at least 6kHz
Downward	Single syllable, continuous decrease in pith of at least 6kHz
Flat	Single syllable, constant frequency
U-shaped calls	Single syllable, resembles a 'U' with an initial decrease in frequency, followed by an increase
Chevron	Single syllable, opposite of U-shaped calls
Composite	Two simultaneous differing syllables with varying frequencies
Frequency Step	Increasing changes in frequency appearing as a discontinuous 'step' without a time delay.

2.2.3. Juvenile Grooming

On P18, the dam was separated from her pups and placed into a new standard cage without a wire top, food or water bottle. Immediately after, the pups were individually placed into a new standard cage which contained only a thin layer of wood chip bedding, enough to cover the floor. A camera was set 30cm away from the cage so that the whole side of the cage was within view. The pup was recorded for 20 minutes under a 60-watt red light. The video recordings were analyzed using Media Player Classic (Version 1.7.13) and the frequency and duration of each grooming bout was recorded. Only grooming bouts longer than 1s were included and any break in grooming shorter than 2s was considered a single bout. Two-way ANOVAs comparing overall grooming duration, number of bouts and average grooming bout length was compared between sex and treatment to determine any significant effects.

2.2.4. Olfactory Habituation and Dishabituation

On P90, the animals were placed into a clean standard cage with wood chip bedding lining the floor for the olfactory habituation and dishabituation test. The olfactory habituation and dishabituation protocol was similar to those previously described (Silverman et al., 2010; Yang & Crawley, 2009).The cage had a wire top but no food or water bottle. Through the water bottle hole a fixed cotton swab (Puritan, Guilford, ME., USA) was suspended approximately 5cm from the ground so that the animal could investigate it without rearing (Figure 5a). A camera was placed in front of the cage to record the mouse and all testing was carried out under a 60w red light. Initially the animals were placed in the cage with a fresh cotton swab for 10 minutes to habituate to the testing apparatus. Following habituation, the mouse was presented with three sequential two-minute presentations of the following odours in this order: blank (distilled water), almond (1:100; ClubHouse, London, Ont., Canada), banana (1:100; ClubHouse, London, Ont., Canada), social odour 1, social odour 2. The social odours were acquired by wiping the cotton swabs across the bottom of two different unchanged cages (each day for 6 days) from sex-and age-matched, group-housed unfamiliar conspecifics. The same set of social odours was used for all mice tested and was stored at -20ºC between cohorts. The time spent investigating the cotton swab for each presentation was recorded, as determined by the orientation of the mouse towards the tip of the swab within a 1cm radius using

Viewer (Biobserve, Bonn, Germany). Any instances where the swab was accidentally dislodged were removed from analysis. To determine the amount of habituation taking place, the difference between the time spent investigating the

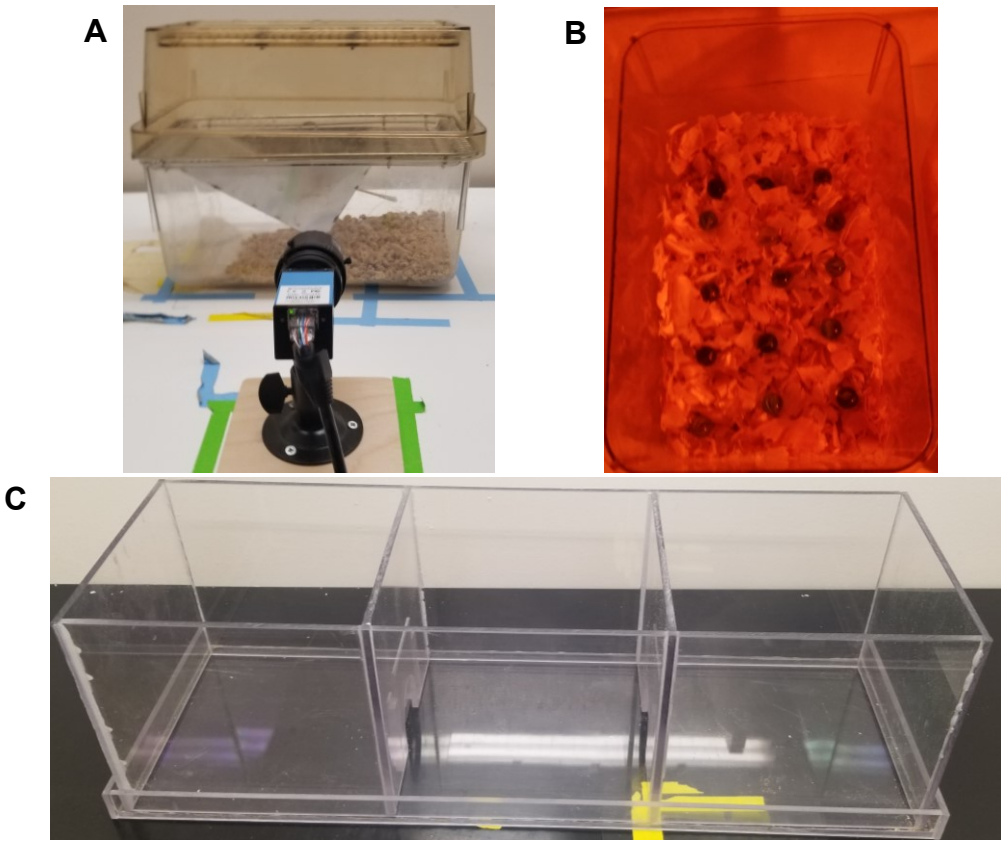

Figure 5. Photos of the behavioural apparatus used for the adult behavioural testing: (a) Olfactory habituation/dishabituation, (b) Marble Burying and (c) 3-Chamber Sociability.

first and the third presentation was calculated. To score dishabituation the difference between the third presentation of an odour and the first presentation of the subsequent odour was calculated.

2.2.5. Three-Chamber Sociability

At P100 the animals were removed from their housing groups and tested in the 3-chamber apparatus. The protocol was similar to that previously described (Silverman et al., 2010). The animal was initially placed in the center of an empty apparatus (each chamber is 22cm x 20.5cm x 20.5cm) with the floor covered in wood chip bedding (Figure 5c). The left and right chamber were separated by the centre chamber via a removable

plastic door. In the centre of the left and right chamber was an inverted wire pencil cup with a plastic 1L bottle of water on top to discourage climbing. The doors to the left and right chamber were removed and the animal freely explored the apparatus for 10 minutes to acclimatize to the novel environment. Following the habituation, the animal was confined into the middle chamber with the plastic barriers and a sex-and age-matched untreated unfamiliar conspecific was randomly placed into one of the wire cups located in the left and right chambers. A novel object was placed into the opposite to the stimulus mouse. The plastic barriers were reopened, and the testing mouse freely explored the apparatus once again for 10 minutes. Following testing, the mouse was removed from the apparatus and placed into a novel home-cage solely with cage-mates that had already been tested. The habituation and testing phase was recorded with a camera above the apparatus so that all areas of the floor were visible and a camera to the side for more accurate scoring of behaviours. Using the Event Recorder plugin of Viewer (Biobserve, Bonn, Germany), the frequency and duration of grooming during the habituation phase was recorded. During the testing phase using the automatic mouse tracking feature of Viewer, the distance travelled, frequency of entries to each chamber and the time spent in each chamber was recorded. Using Event Recorder, the time spent directly investigating each stimulus (object or mouse) was manually recorded as indicated by an orientation with 2cm towards the stimulus. A Two-way ANOVA comparing the measures grooming, distance travelled, and stimulus preference between sex and treatment was conducted to determine any significant effects.

2.2.6. Marble Burying

At P110, each animal was removed from its housing groups and placed individually in a novel standard cage which was covered with a 4cm layer of wood shaving bedding and allowed to freely explore for 5 minutes to habituate to the novel bedding. Following the habituation phase, the animal was removed and a three by five grid of 15, 0.5cm radius glass marbles was placed on top of the bedding (Figure 5b). To ensure accuracy of comparison, a photo was taken of the grid prior to the testing phase. The animal was then returned to the cage for a subsequent 7 minutes, where marble burying occurred. After this, the animal was carefully and quickly removed so not to disturb any of the marbles and a second photo was taken of the resulting cage. Marbles were cleaned with water

and Sparkleen (Fisherbrand, Ottawa, Ont., Canada) between animals. Buried marbles were scored as either 100% buried, where the marble was completely covered and obstructed from view, or as greater than 50%, where at least half of the marble was covered or more. A two-way ANOVA comparing marbles buried at 100% and 50% between sex and treatment was conducted to determine any significant effects.

2.3. *Results*

2.3.1. Maternal Care

There was no significant difference in the overall frequency of any of the active maternal behaviours between treatments ($F_{(1,30)} = 0.008$, $p = 0.928$) or the varying litter sizes ($F_{(1,34)} = 0.491$, $p = 0.742$; Figure 5a). This was also the case in the frequency of the non-maternal behaviours (treatment: $F_{(1,20)} = 0.023$, $p = 0.880$, litter size: $F_{(1,20)} = 0.004$, $p = 0.951$; Figure 6b).

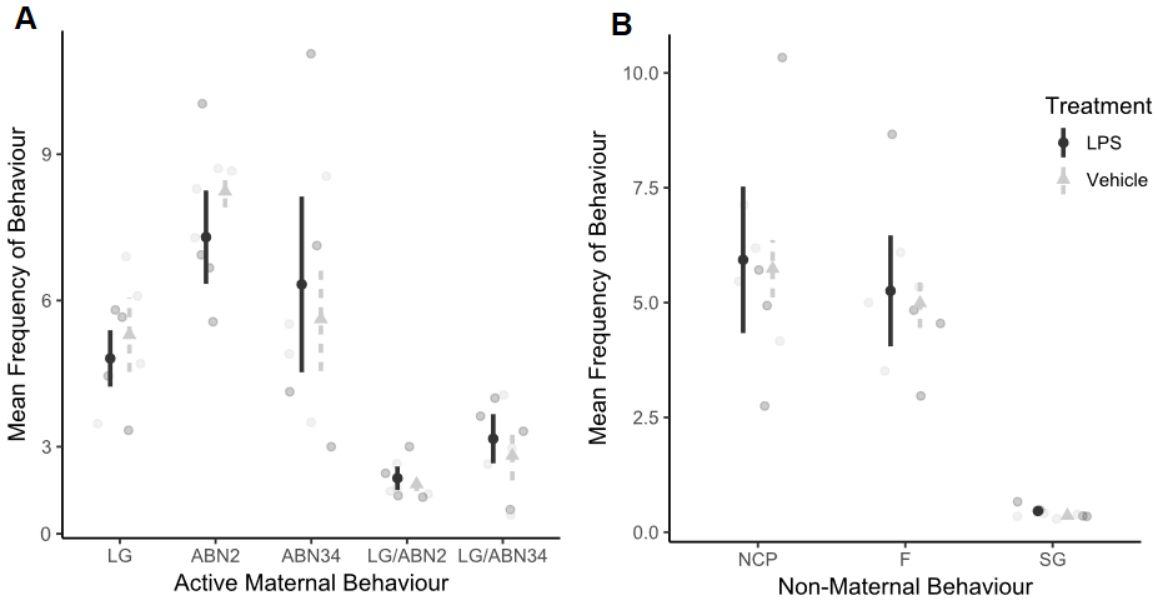

Figure 6. Mean frequency of (a) active maternal behaviours: licking and grooming (LG), arched back nursing posture (ABN2, ABN34) and instances where they cooccur (LG/ABN2, LG/ABN34) and (b) non-maternal behaviours: no contact with pups (NCP), feeding (F) and self-grooming (SG) in C57Bl/6J dams following lipopolysaccharide (LPS) injection at E11.5 and 12 or vehicle control. Error bars are mean±SEM.

2.3.2. Ultrasonic Vocalizations

LPS treated animals made significantly fewer calls at P8 (15.8±4.15) and P10

(7.53±2.30) compared to vehicle controls (P8:33.5±7.21, P10:17.2±4.74; $F_{(1,168)} = 6.693$, $p = 0.0105$; Figure 7a). The number of calls decreased significantly across all four days independent of treatment ($F_{(3,168)} = 14.246$, $p = 2.58x10^{-8}$; Figure 7a) and there was no sex by treatment interaction in the full model containing both sexes ($F_{(1,168)} = 2.67$, $p =$ 104129). Despite this, the difference in the number of calls between treatments was

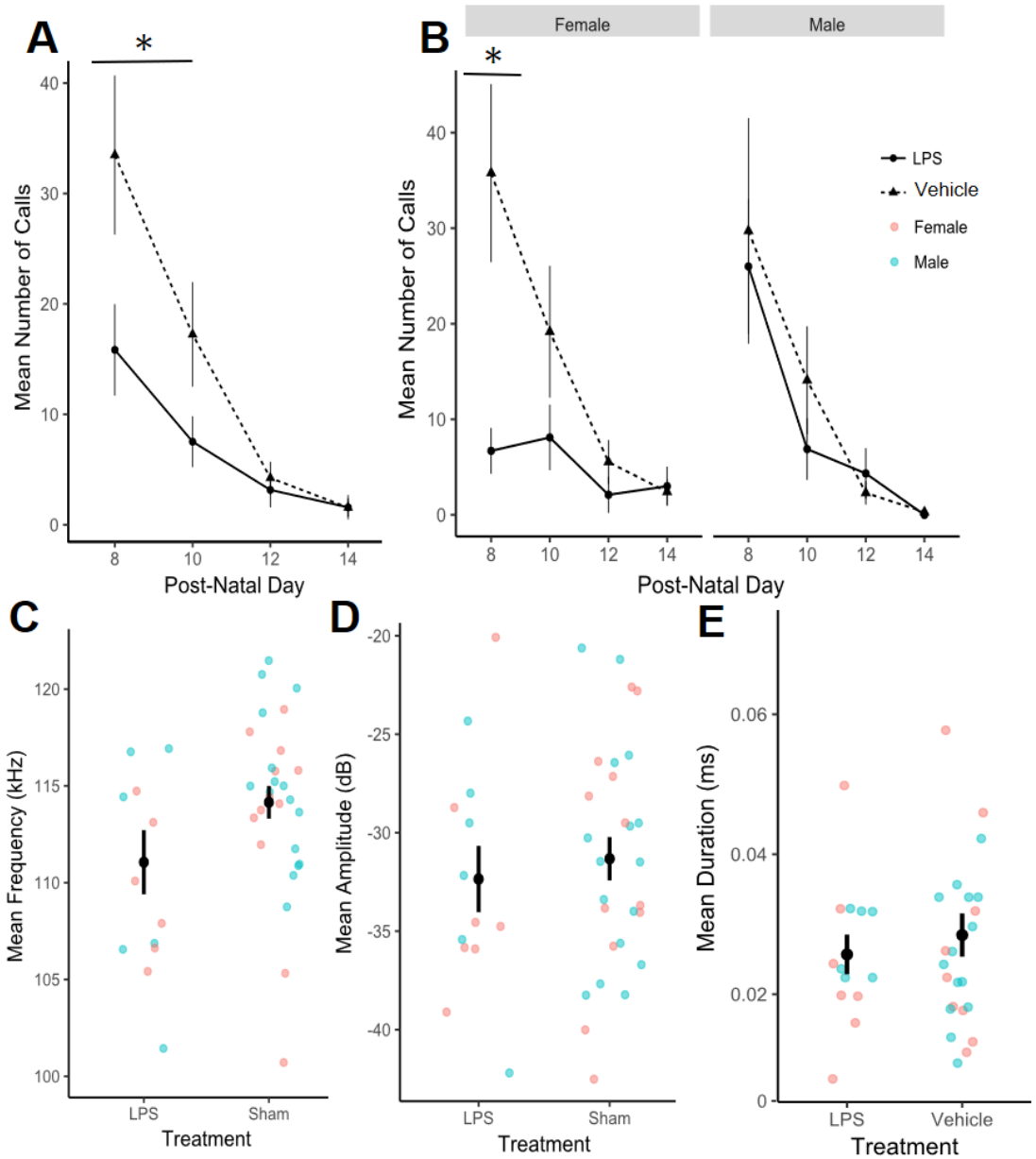

Figure 7: Ultrasonic vocalizations in C57BL/6J mice prenatally treated with lipopolysaccharide or vehicle control. (a) Mean number of calls across all tested days (b) separated by sex. (c) mean frequency (kHz), (d) amplitude (dB) and (e) mean duration (ms) of calls on P8. Error bars are mean ± SEM. ***p<0.05**.

driven by females as there was a significant difference in the number of USVs between LPS (6.7±2.40) and Sham (35.8±9.32; $F_{(1,98)} = 6.644$, $p = 0.011438$) at P8 (Figure 7b), which was not observed in the males (LPS: 26±7.06, Vehicle: 29.7±11.8; $F_{(1,70)} = 0.319$, $p = 0.574$; Figure 7b). Because the significant effect in call number was only apparent at P8, the more in-depth analysis of frequency (kHz), amplitude (dB) duration (ms) and call classification was only done using these data. In these P8 data, the LPS-treated animals had a slightly lower pitch (111.056±1.657kHz) than the vehicle treated controls (114.153±0.847 kHz; Figure 7c); however, this was not significant ($F_{(1,37)}=3.316$, $p=0.0767$), nor was there a difference in frequency between males and females ($F_{(1,24)}=0.231$, $p=0.6336$). There was also no significant effect of treatment ($F_{(1,33)}=0.339$, $p=0.564$) or sex ($F_{(1,33)}=0.350$, $p=0.558$; Figure 7d) for the amplitude of calls or for the mean duration of calls (treatment: $F_{(1,37)}=0.257$, $p=0.615$, sex: $F_{(1,33)}=0.0.26$, $p=0.874$; Figure 7e) .

The mean percentage of each type of call out of the total number of calls per treatment and sex is displayed in Figure 8. Harmonic, Two-Syllable, Flat, U-Shaped and

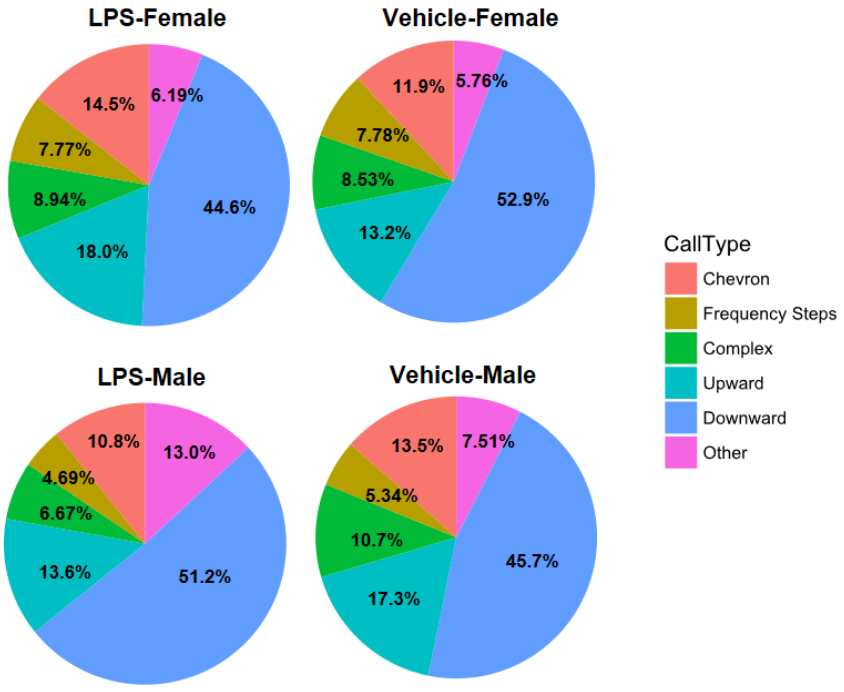

Figure 8. Mean proportion of types of calls made at P8 following maternal seperation in male and female C57Bl/6J mice prenatally treated with lipopolysaccharide (LPS) or vehicle control.

Composite call types occurred in less than 50% of the animals and were thereby grouped as 'Other'. Despite fewer call, those made by the female LPS pups had a similar vocal repertoire to controls.

2.3.3. Juvenile Grooming

Following a 2-way ANOVA, it was determined that there was no main effect of treatment ($F_{(1,36)}$=1.054, p=0.312) or sex ($F_{(1,36)}$=0.091, p=0.765) on the percent of time spent grooming with LPS mice grooming for slightly longer periods (22.4±2.33s) than the vehicle treated controls (19.0±2.24s; Figure 9a). In the average bout duration there

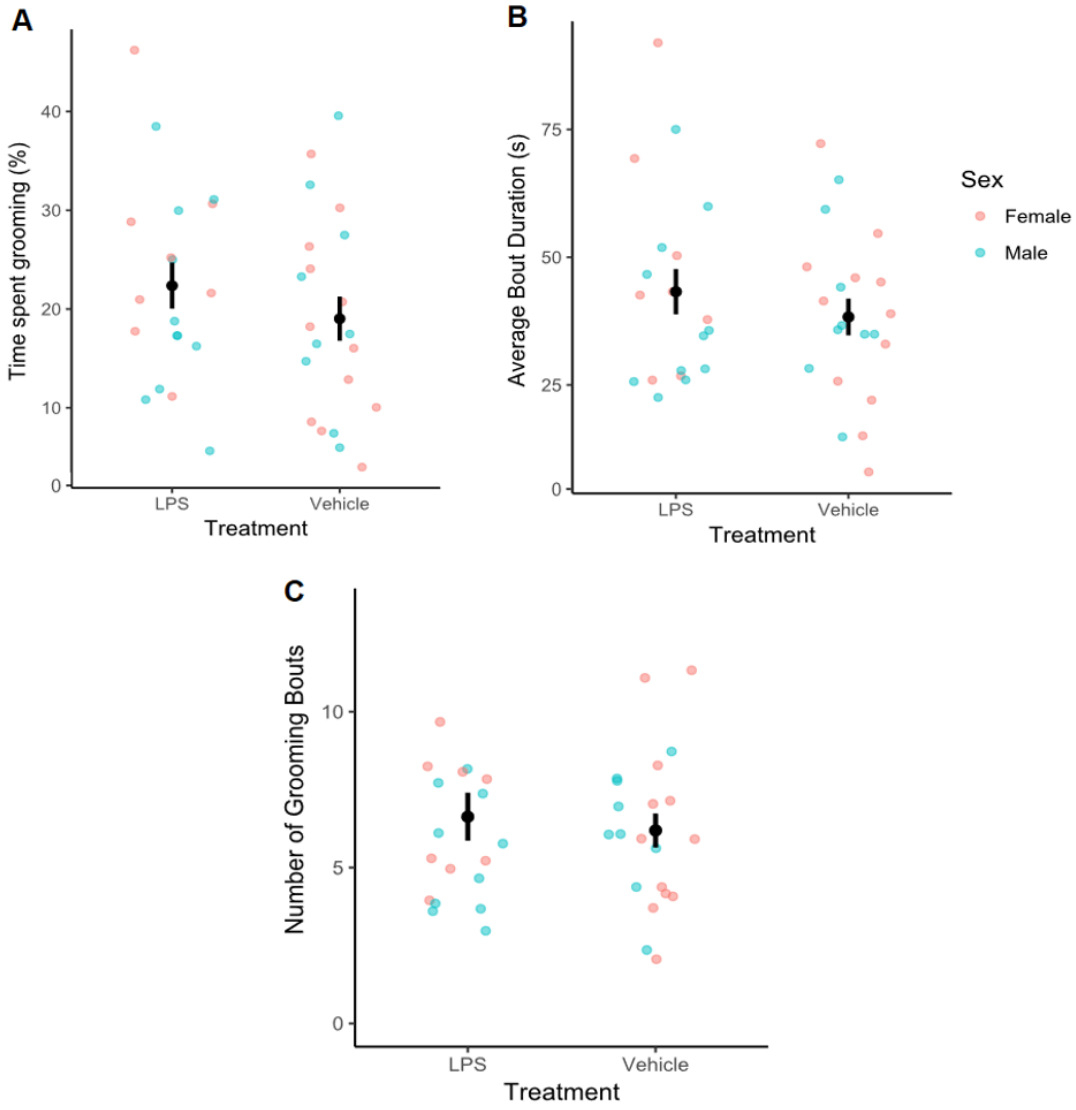

Figure 9. (a) Time spent grooming (%), (b) average bout duration and (c) number of grooming bouts in male and female C57Bl/6J P18 mice prenatally treated with lipopolysaccharide (LPS) or vehicle control. Error bars are mean ± SEM

was similarly no effect of treatment ($F_{(1,36)}$=0.752, p=0.391) or sex ($F_{(1,36)}$=0.333, p=0.568) and this was also the case for the number of grooming bouts (treatment: $F_{(1,36)}$=0.213, p=0.647; sex: $F_{(1,36)}$=0.001, p=0.568). The slight increase in average bout duration in the LPS animals (43.3±4.43s) compared to the vehicle controls (38.3±3.59s; Figure 9b) was contributing to the small variability observed in the percent time spent grooming as there was relatively no difference between LPS (6.63± 0.769) and the controls (6.19± 0.546) in the number of grooming bouts (Figure 9c). There was no effect of sex in time spent grooming ($F_{(1,36)}$ = 1.380, p = 0.248), average bout duration ($F_{(1,36)}$ = 0.880, p = 0.355) or number of grooming bouts ($F_{(1,36)}$ = 0.001, p = 0.982).

2.3.4. Olfactory Habituation and Dishabituation

In the olfactory habituation, males and females of both groups showed habituation over the three odour presentations and dishabituation following the presentation of a novel odour (Figures 10a, 11a). When measuring the 95% confidence intervals of the effect of habituation and dishabituation there was an overlap between treatments across all habituations and dishabituations to every odour in the females (Figure 10b). In the males there was also an overlap of all confidence intervals apart from the dishabituation from banana to social odour 1 (cage 1-1), where LPS males (34.976s, 95%CI:14.757-54.510s) displayed less dishabituation than the vehicle controls (61.915s, 95%CI:54.74724-68.86175s; Figure 11b).

2.3.5. Three-Chamber Sociability and Grooming

In the habituation phase of the 3-chamber sociability test there was a main effect of treatment on time spent grooming ($F_{(1,24)}$=7.718, p=0.0104), where the LPS animals spent more time grooming (12.5±1.64s) compared to the vehicle treated animals (6.99±1.26s; Figure 12a), which was being driven by the males (LPS:14.8±2.34s, Vehicle:5.88±1.68s, p=0.01), but not the females (LPS:9.80±1.9s, Vehicle:5.88±1.68s, p=0.5; Figure 12b) however there was no sex by treatment interaction ($F_{(1,24)}$=3.09, p=0.0915).

In the 3-Chamber test of sociability there was no significant effect of treatment ($F_{(1,27)}$=1.430, p=0.242) or sex ($F_{(1,27)}$=0.908, p=0.349; Figure 13a) on the time spent investigating the social stimulus or on the time spent investigating the object (treatment:

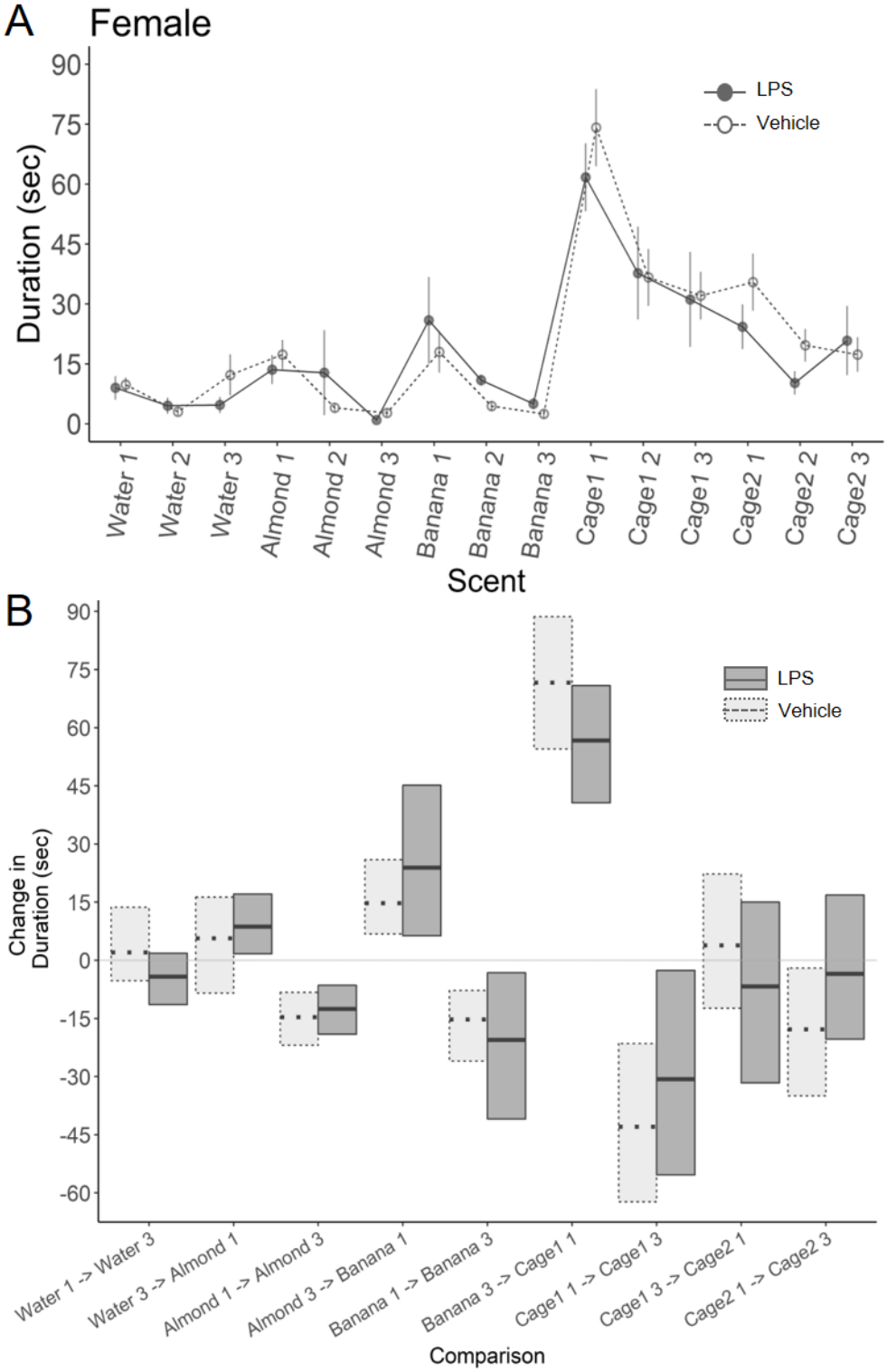

Figure 10. Olfactory habituation and dishabituation to three successive presentations of water, almond, banana and 2 different social odours in P90 female C57Bl/6J mice prenatally exposed to lipopolysaccharide (LPS) or vehicle injections. (a) change in duration across each presentation. Error bars are mean ± SEM. (b) habituation and dishabituation effect size. Error bars are mean ± 95%CI.

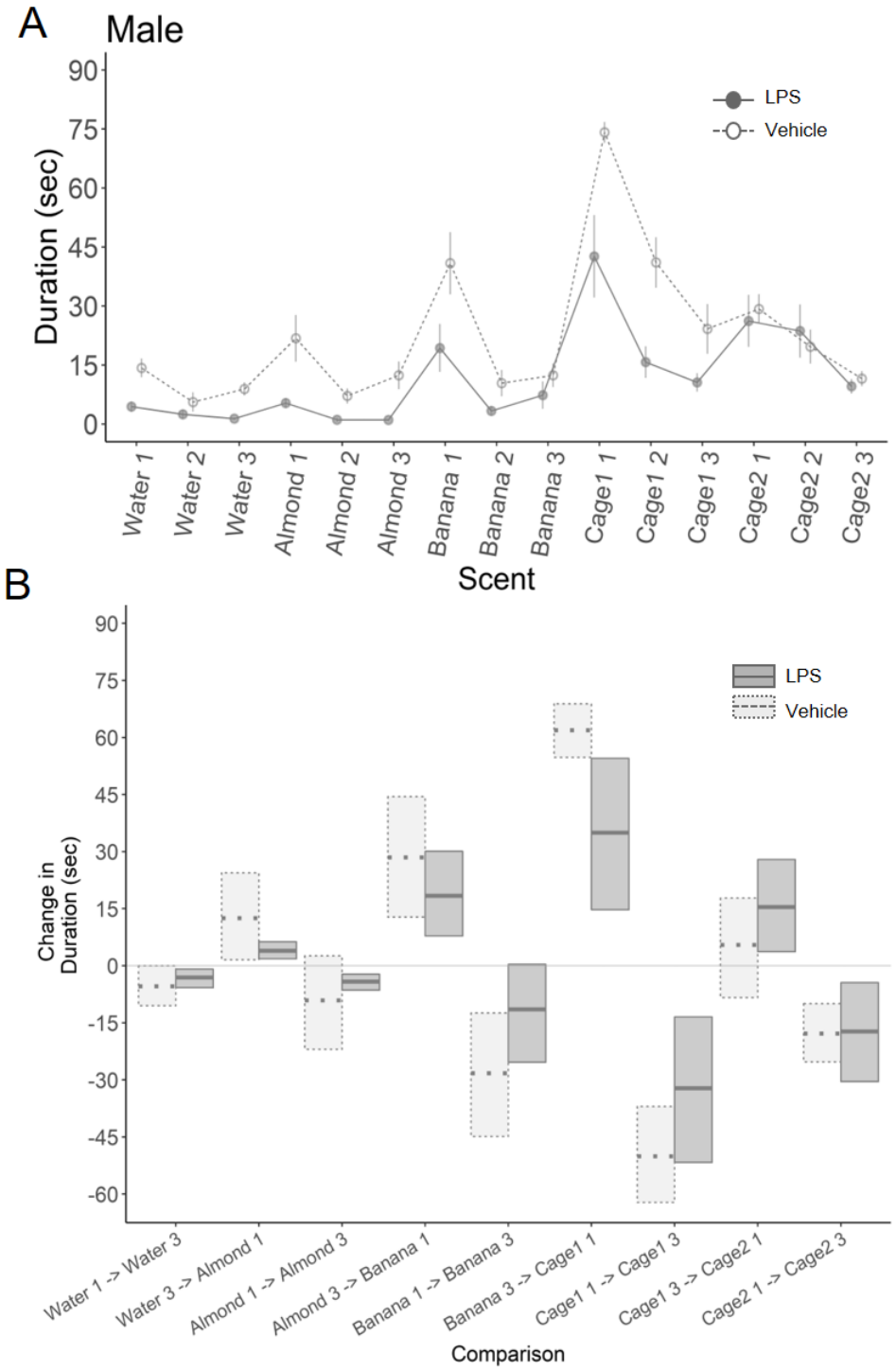

Figure 11. Olfactory habituation and dishabituation to three successive presentations of water, almond, banana and 2 different social odours in P90 male C57Bl/6J mice prenatally exposed to lipopolysaccharide (LPS) or vehicle injections. (a) change in duration across each presentation. Error bars are mean ± SEM. (b) habituation and dishabituation effect size. Error bars are mean ± 95%CI.

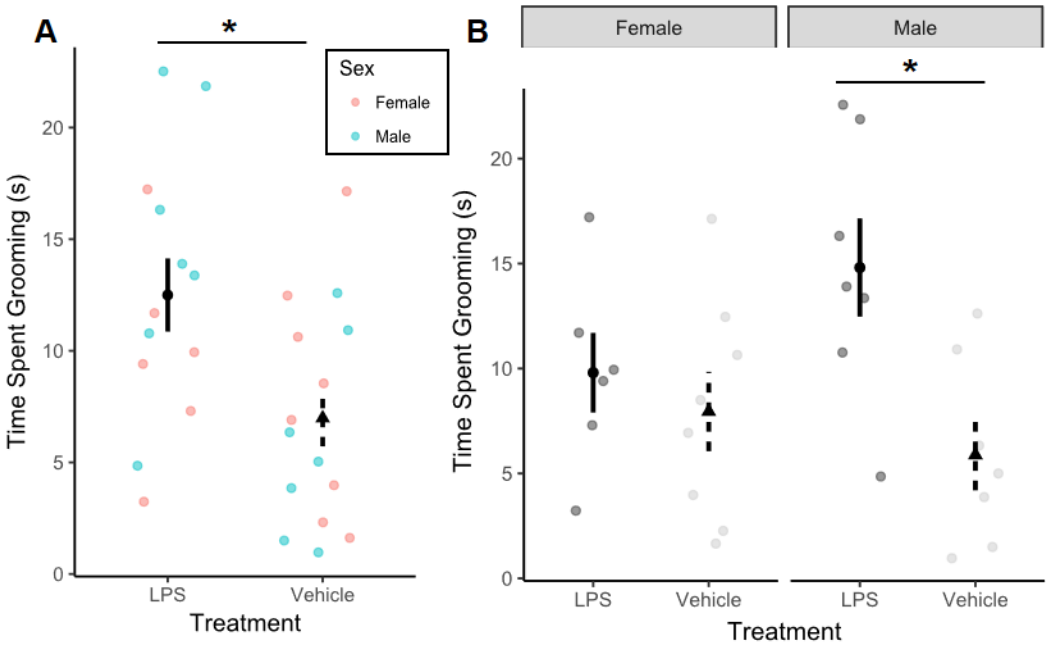

Figure 12. Time spent grooming during the habituation phase of the 3-chamber sociability test in (a) Male and Female, and (b) Male vs. Female C57Bl/6J mice prenatally treated with lipopolysaccharide (LPS) or vehicle control. Error bars are mean ± SEM. ***p<0.05.**

$F_{(1,27)}$=1.372, p=0.253, sex: $F_{(1,27)}$=2.00, p=0.1682; Figure 13b). When calculated as a preference ratio (time spent investigating social stimulus/total time investigating social and object) there was similarly no significant effects of treatment ($F_{(1,27)}$=1.80, p=0.191) or sex ($F_{(1,27)}$=0.264, p=0.612; Figure 13c). There was less investigation of the social stimulus in the LPS animals (92.9±8.47s) compared to the vehicle controls (105±5.22s; Figure 13a) which resulted in a similar difference observed in the preference ratio of investigation (LPS: 0.672±0.0304, Vehicle: 0.718±0.0207; Figure 13c). Since both LPS and vehicle had a preference ratio of investigation greater than chance (0.5) it is indicative of a preference for the social stimulus. This was not observed further in the preference ratio of visits (LPS: 0.546±0.0240, Vehicle:0.540±0.0137; Figure 12d) where there was consequently no effect of treatment ($F_{(1,27)}$=0.041, p=0.841) or sex ($F_{(1,27)}$=0.540, p=0.469). Although, LPS-treated animals (4409.98±209.21cm) travelled less distance than vehicle controls (4932.94±204.30cm; Figure 12e), this was not significant ($F_{(1,27)}$=3.249, p=0.0827) nor was there any effect of sex ($F_{(1,27)}$=1.699, p=0.203)

indicating that distance travelled did not influence the results observed in time spent investigating the social stimulus or the object.

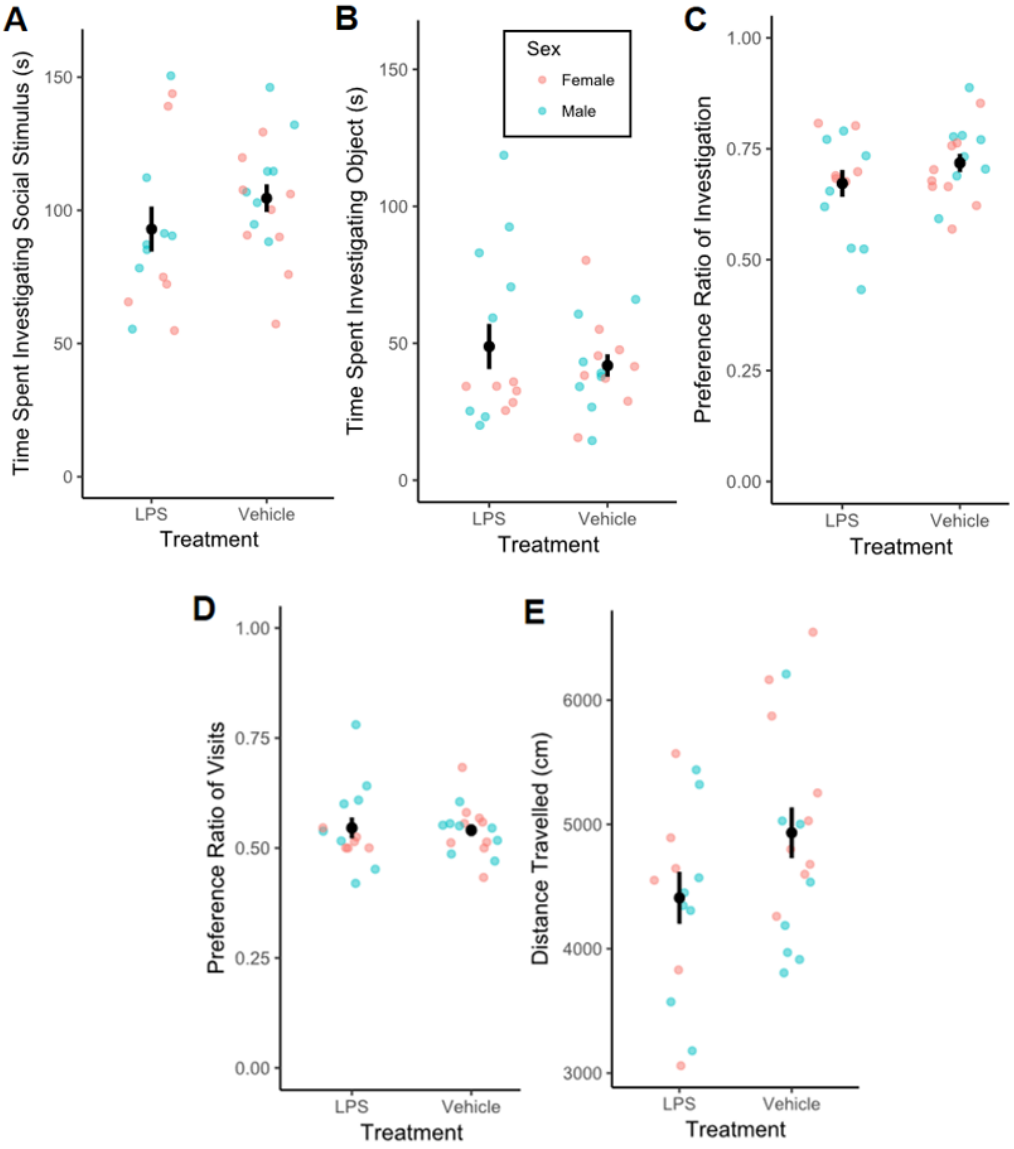

Figure 13. Measures of (a) time spent investigating social stimulus (s), (b) time investigating object (s), (c) preference ratio of investigation, (d) preference ratio of visits and (e) distance travelled in the 3-chamber sociability apparatus between C57Bl/6J mice prenatally treated with lipopolysaccharide (LPS) or vehicle. Error bars are mean ± SEM.

2.3.6. Marble Burying

When scoring for marbles that were completely (100%) buried, there was no significant effect of treatment ($F_{(1,28)} = 1.570$, $p = 0.221$) or sex ($F_{(1,28)} = 0.001$, $p = 0.978$).

However, there was a slight increase in marbles buried completely in the LPS animals (8.29±0.934) compared to the vehicle controls (6.67±0.828; Figure 14a). This difference was also observed when scoring for marbles buried greater than fifty percent (LPS: 11.1±0.691, Vehicle: 9.67±0.848; Figure 14b) but was not significant ($F_{(1,28)} = 1.427$, $p = 0.242$) nor was there a main effect of sex ($F_{(1,28)} = 0.001$, $p = 0.978$). In both cases, any difference observed in treatment was not mediated by a significant interaction with sex (100%: $F_{(1,28)} = 0.053$, $p = 0.820$, 50%: $F_{(1,28)} = 0.052$, $p = 0.821$).

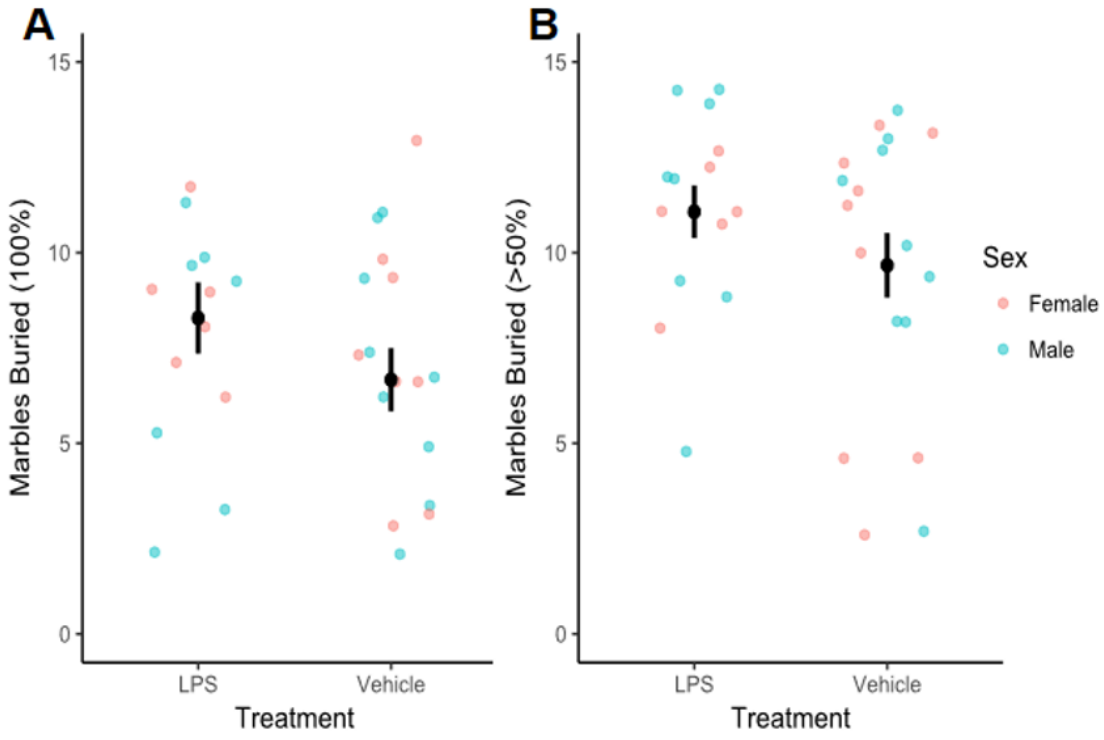

Figure 14. Marbles buried either (a) 100% from view or (b) greater than 50% in 7-minutes by P130 male and female C57Bl/6J mice prenatally treated with lipopolysaccharide (LPS) or vehicle control. Error bars are mean ± SEM.

2.4. *Summary of Behavioural Effects of LPS-MIA*

There was no observable difference in the maternal care provided to LPS and vehicle pups (Figure 6). Overall, there were sex-dependent ASD-like phenotypic behaviours observed. In terms of social interaction, reduced habituation to the first presentation of social odour was observed in the LPS-treated males (Figure 11) but this was not observed

further in the 3-chamber sociability task (Figure 13). Similarly, an increase in repetitive grooming was also observed in the LPS-males at P100 indicative of the increase in restrictive and repetitive behaviour as observed in ASD (Figure 12), but not during the juvenile period (Figure 9). This increase in restricted and repetitive behaviour in the LPS mice was not displayed further in the marble burying task, where although more marbles on average were buried, it was not significant (Figure 14). Lastly, deficits in social communication were observed in the maternal separation-induced USVs at P8 specifically in females where a fewer USVs were made (Figure 7). However, this did not alter the overall repertoire of calls made (Figure 8).

Chapter 3

Molecular Effects of LPS-MIA

3.1. *Methods*

At both P0 and P130, expression and phosphorylation of mTOR and downstream PSD95 was assessed in the hippocampal and frontal cortex brain regions of LPS-MIA offspring via real-time quantitative polymerase chain reaction (RT-qPCR) and Western blot. All statistical testing was carried out in R (r-project.org) p-values<0.05 were considered significant.

3.1.1. Animals and Tissue Collection

Approximately half of the animals bred were used for P0 tissue analysis ((n = 39), $n_{(F\text{-}LPS)}$=10, $n_{(M\text{-}LPS)}$=9, $n_{(F\text{-}Vehicle)}$=10, $n_{(F\text{-}Vehicle)}$=10 from 4 LPS-MIA and 5 vehicle control litters). P0 animals were sacrificed via cervical dislocation and the heads were immediately flash frozen on dry ice. Animals tested in the behavioural assays were sacrificed at P130 via cervical dislocation and brains were extracted and flash frozen on dry ice. In both the P0 and P130 animals the brain tissue was flash frozen before dissection so that the entire litter or cage was sacrificed consecutively with no interruption between animals. This was especially important in the P0 animals to reduce the effects of separation from the dam.

The P0 and P130 brains were free-hand dissected for relevant brain regions. Specifically, in the P0 brain, bilateral sections of the frontal cortex and hippocampus were removed. The P0 hippocampus was identified and dissected as previously described (Nunez, 2008). In the P130 mice, bilateral samples of the medial prefrontal cortex (mPFC) and the dorsal and ventral hippocampus (DH, VH) were dissected. Because of their small size, the bilateral samples of the mPFC were pooled. All brain regions were immediately flash frozen in liquid nitrogen and stored at -80ºC until further processing.

3.1.2. RT-qPCR

RNA, in 30µl elutions, was extracted from the P0 frontal cortex and hippocampus using an RNA/DNA/Protein Purification Plus Micro Kit (Cat. 51600, Norgen Biotek Corp. Thorold, Ont., Canada). In the P130 ventral and dorsal hippocampus, RNA, in 40µl

elutions was extracted using an RNA/DNA Purification Kit (Cat. 48700, Norgen Biotek Corp. Thorold, Ont., Canada). All extractions underwent a DNAse treatment to reduce any possible contamination with an RNase-Free DNase I Kit (Cat. 25710, Norgen Biotek Corp. Thorold, Ont., Canada). RNA purity (via 280/260nm ratio) and concentration was evaluated using 2µl nanodrop with the Take3 micro-volume plate and Epoch reader (Biotek, Winooski, VT., USA). Any samples with abnormally low ratios and subsequent concentrations were excluded from further analysis. 4µl of RNA was then converted into cDNA via reverse transcriptase (RT) with the iScript™ cDNA Synthesis Kit (Bio-Rad Laboratories). A pooled RNA sample from each set of extractions was used as a no-RT control, containing the nRT reagent in place of the RT, and underwent the same thermocycling as the RT samples.

Following synthesis of cDNA, real time qPCR (RT-qPCR) was conducted on a 96-well CFX Connect™ Real-Time PCR detection system (Bio-Rad, Hercules, CA, USA). Primers for HPRT, forward primer 5'- AAGCTTGCTGGTGAAAAGGA -3' and reverse primer 5'-TTGCGCTCATCTTAGGCTTT-3', yielded a 186bp fragment. An annealing temperature gradient was conducted on a pooled 1:10 diluted cDNA sample and the optimal range was discovered to be 60-62ºC. A pre-validated and optimized PrimePCR™ mTOR primer was obtained from Bio-Rad (qMmuCED0047795) and yielded a 111bp fragment with an optimized annealing temperature of 60 ºC. For the P0 and P130 derived tissue a separate concentration gradient in a 1:4 serial dilution from a pooled 1:10 cDNA concentration was used, and an optimal cDNA dilution of 1:20 was determined for all tissues examined and primer efficiencies between 95-105% were observed. The total reaction volume for the HPRT analysis per well was 10µl with 2µl of cDNA template, 0.3ul of 20nM forward and reverse primers, 2.4µl of PCR-grade H_20 and 5µl of SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad, Hercules, CA, USA). For the mTOR PrimePCR™ assay, a similar 10µl reaction took place with 2µl of template, 2.5µl of PrimePCR™ primer, 2.5µl of PCR-grade H_2O water and 5µl of the SYBR Green Supermix. For each assay, a no-template and no-RT control was used to ensure the lack of contamination in the samples or reagents. The thermocycling began with a 2min activation at 95ºC, followed by 40 cycles of 5s of denaturation at 95 ºC and annealing/extension at 60 ºC for 30s. This was finished with a melt curve from 65-95 ºC

which increased by 0.5 ºC every 5sec. Each sample was run in triplicate and any replicate with deviation of greater than 0.2Cq from the mean was excluded from the analysis. A no-template control was run on each blot and no contamination in the master mix was observed.

Analysis of RT-qPCR cycling was conducted using the CFX™ Maestro software (version 1.1, Bio-Rad, Hercules, CA, USA). When required, where gene-, treatment- and region-matched samples crossed over multiple plates, the average Cq for a pooled sample common to each plate served as an interplate calibrator and was adjusted to match across plates. Following this, using HPRT as the reference gene, the ΔCq was determined. The ΔCq, or expression, was then compared with a two-way ANOVA with sex and treatment as between-subject factors separately for each region analyzed. Any values greater than two standard deviations from the mean were considered outliers and were removed from analysis.

3.1.3. Western Blot

To extract protein, P0 and P130 brain samples were homogenized in RIPA buffer (R0278, Sigma-Aldrich, Oakville, Ont., Canada) containing phophatase (PhosSTOP, Roche, Indianapolis, IN, USA) and protease (Complete, Roche, Indianapolis, IN, USA) inhibitor cocktail tablets. 30µl of the buffer was added to the P0 hippocampal, frontal cortex and P130 mPFC samples. 50µl of the buffer was added to the P130 ventral and dorsal hippocampal samples. Samples were sonicated at 4ºC, 35% amplitude at a pulse rate of 20s ON, 40s OFF, until clear. The samples were then centrifuged at 10,000 RPM for 10min at 4ºC to pellet any insoluble debris. The supernatants were collected and protein concentration was determined using a Bradford analysis as previously decribed (Bradford, 1976), using a bovine serum albumin (BSA) standard curve and Bradford Reagent (B6916, Sigma-Aldrich, Oakville, Ont., Canda) read at 595nm using an Epoch plate reader (BioTek, Winooski, VT., USA).

The wells were loaded with 30µg of protein with 4x Laemmli sample buffer in β-mercaptoethanol and separated on 4-15% mini-PROTEAN® TGX Stain-Free™ gels (Bio-Rad, Hercules, CA, USA) via electrophoresis. These gels activate all protein loaded per sample and are used for more accurate normalization of the protein of interest to the

amount of protein loaded compared to the traditional housekeeping protein method which can be differentially regulated (Gürtler et al., 2013). The stain-free gel was then activated under 1min of UV light before transferring the gels onto polyvinylidene difluoride (PVDF) membranes (Bio-Rad, Hercules, CA, USA) using the Trans-Blot® Turbo™ Transfer system (Bio-Rad, Hercules, CA, USA) for 7min at 25mV (mixed molecular weight). The PVDF blots were then imaged for total protein using a ChemiDoc™ Imaging system (Bio-Rad, Hercules, CA, USA). The blots were blocked for 1hr at room temperature with 3% BSA in 1xTris-buffered saline containing 0.1% Tween 20 (TBST). Following blocking the blots were probed overnight at 4ºC with the following primary antibodies in 1%BSA in 1xTBST: mTOR (1:1000; Cell Signalling Technology, Danvers, MA., USA), pmTORser2481 (1:500; Santa Cruz Biotechnology, Dallas, TX., USA) and PSD95 (1:2000; Thermo-Fisher, Ottawa, ON., Canada). Following 3, 10min washes in 1xTBST, blots were probed with the following corresponding secondary antibodies in 1%BSA in 1xTBS: goat anti-rabbit horseradish peroxidase (HRP) conjugate, goat anti-mouse HRP conjugate (1:1000; Bio-Rad, Hercules, CA., USA) and Cy™5-conjugated AffiniPure donkey anti-rabbit IgG (H+L; 1:1000; Jackson ImmunoResearch, West Grove, PA., USA). The membranes were then washed in TBST and those conjugated to HRP were developed with Clarity Western ECL substrate (Bio-Rad, Hercules, CA, USA). Bands were detected using a ChemiDoc™ Imaging system (Bio-Rad, Hercules, CA, USA) and quantified, normalizing to a section of total protein between 20-50kDA on each sample, using Image Lab (version 6.0, Bio-Rad, Hercules, CA, USA). The density of the bands was measured following a background rolling disk subtraction of 12mm for mTOR and PSD95 and of 20mm for mTOR(ser2481) which had a weakened antibody signal and stronger background signal. The normalized absorption for each sample was normalized further to the mean absorption of the sham-injected controls for each sex per blot. A non-parametric Mann-Whitney-Wilcoxon rank test was then conducted for percent control between treatments.

3.2. *Results*

3.2.1. P0 analysis of mTOR

Following the RT-qPCR, a significant decrease in mTOR relative normalized expression was observed in the P0 hippocampus of LPS (0.263±0.0268) compared to

vehicle injected controls (0.535±0.0655; $F_{(1,30)} = 14.036$, $p = 7.62x10^{-4}$; Figure 15a). This effect was not observed in the frontal cortex ($F_{(1,32)} = 1.563$, $p = 0.220$; Figure 16a). There was no effect of sex on mTOR expression in the hippocampus ($F_{(1,30)} = 0.411$, $p = 0.526$) or frontal cortex ($F_{(1,32)} = 0.220$, $p = 0.656$). When comparing to the Western blot (Figure 15b), the hippocampus showed no effect of treatment on mTOR in the LPS (97.32±6.54)

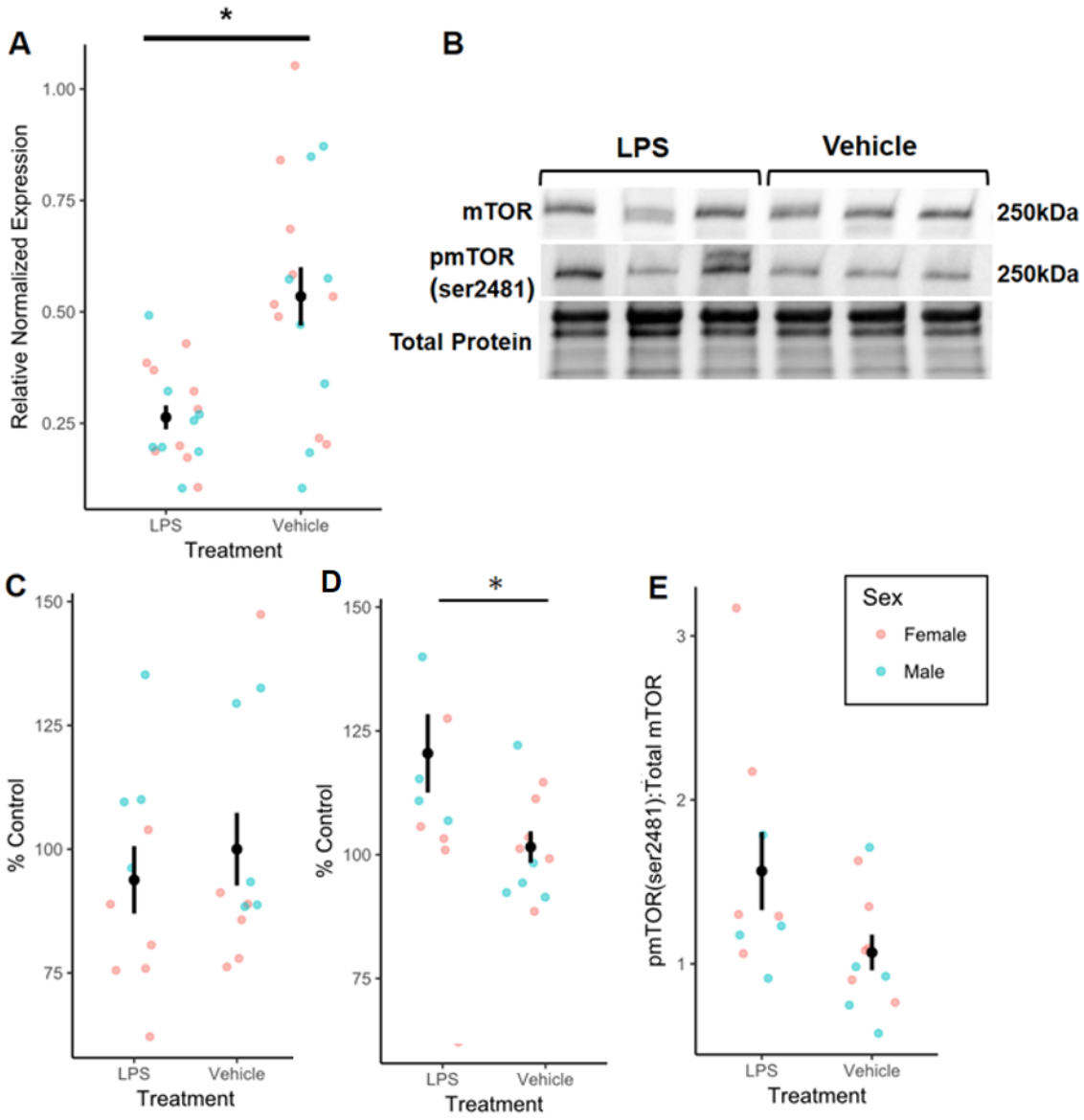

Figure 15. (a) Relative Normalized Expression of *Mtor* from RT-qPCR and (b) western blot of (c) mTOR. (d) phosphorylated mTOR (ser2481; pmTOR) and (e) ratio in P0 Hippocampus from C57Bl/6J mice prenatally treated with lipopolysaccharide (LPS) or vehicle saline. Lanes in western blot (b) represent different individual samples. Error bars are mean±SEM. ***p<0.05.**

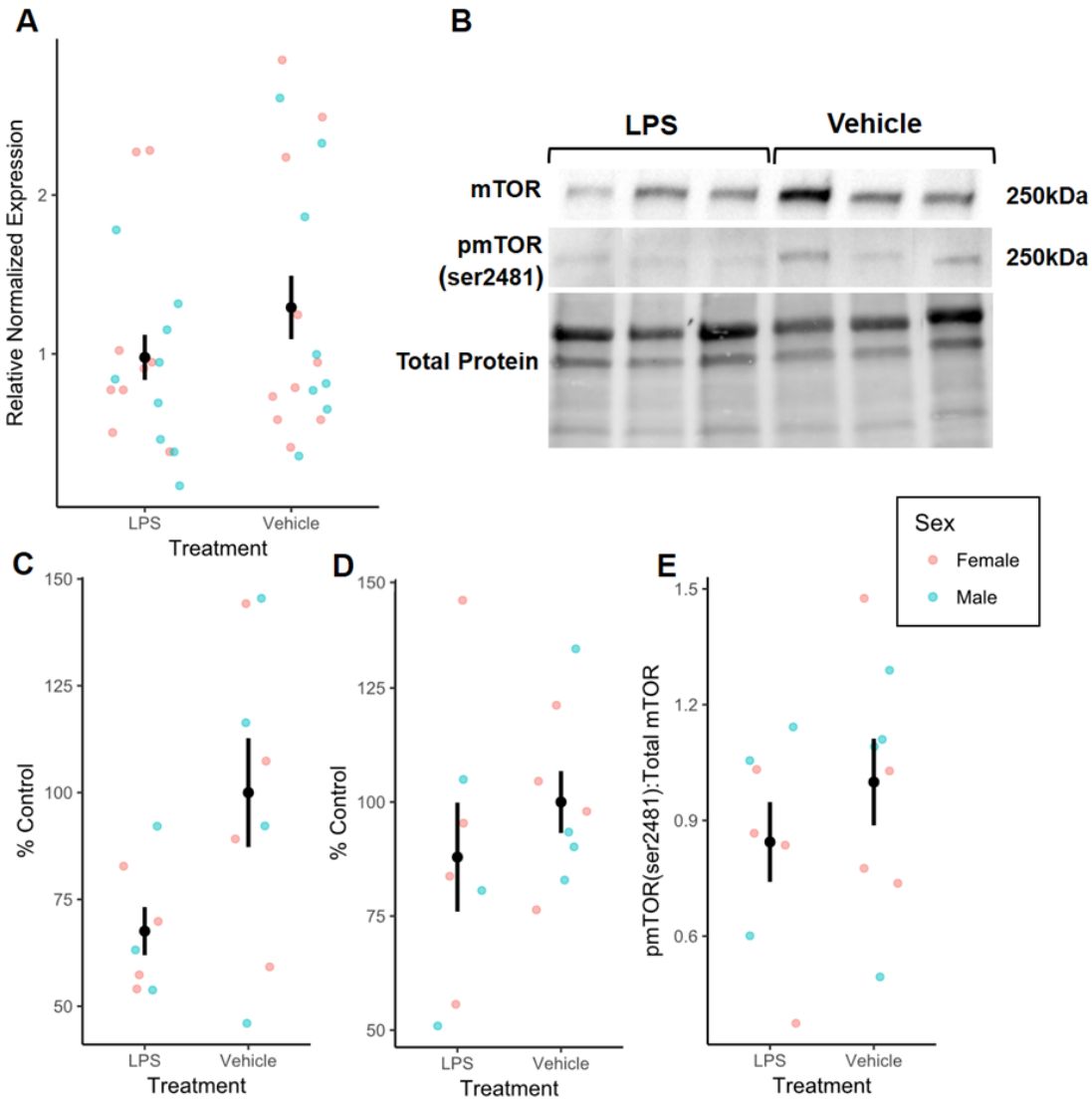

Figure 16. (a) Relative Normalized Expression of *Mtor* from RT-qPCR and (b) western blot of (c) mTOR. (d) phosphorylated mTOR (ser2481; pmTOR) and (e) ratio in P0 Frontal Cortex from C57Bl/6J mice prenatally treated with lipopolysaccharide (LPS) or vehicle saline. Lanes in western blot (b) represent different individual samples. Error bars are mean±SEM.

compared to the vehicle controls (100±7.35; W=49, p=1.00; Figure 15c). The levels of pmTOR(ser2481) were significantly higher in the LPS hippocampus (147.38±19.82) compared to the vehicle animals (100±8.00; W=80, p=0.0200; Figure 15d). The ratio of pmTOR(ser2481) to mTOR was subsequently increased in the hippocampus of the LPS animals (1.57±0.24) compared to the controls (1.07±0.109) but this did not reach statistical significance (W=75, p=0.0562; Figure 15e). In the frontal cortex, (Figure 16b) a decrease in mTOR and pmTOR(ser2481) was observed in the LPS animals

(mTOR:67.58±5.64, pmTOR:87.92±11.957) compared to the vehicle controls (mTOR:100±12.75, pmTOR:100±6.79; Figure 16c,d) but it was not statistically significant (mTOR: W=12, p=0.0721; pmTOR: W=21, p=0.463). A similar decrease in the ratio between mTOR and pmTOR was observed in the P0 frontal cortex in the LPS (0.844±0.103) compared to the controls (0.999±0.112; Figure 16e) but was not statistically significant (W=21, p=0.463).

3.2.2. P130 analysis of mTOR

In the DH and VH, no effect of treatment in mTOR expression was observed

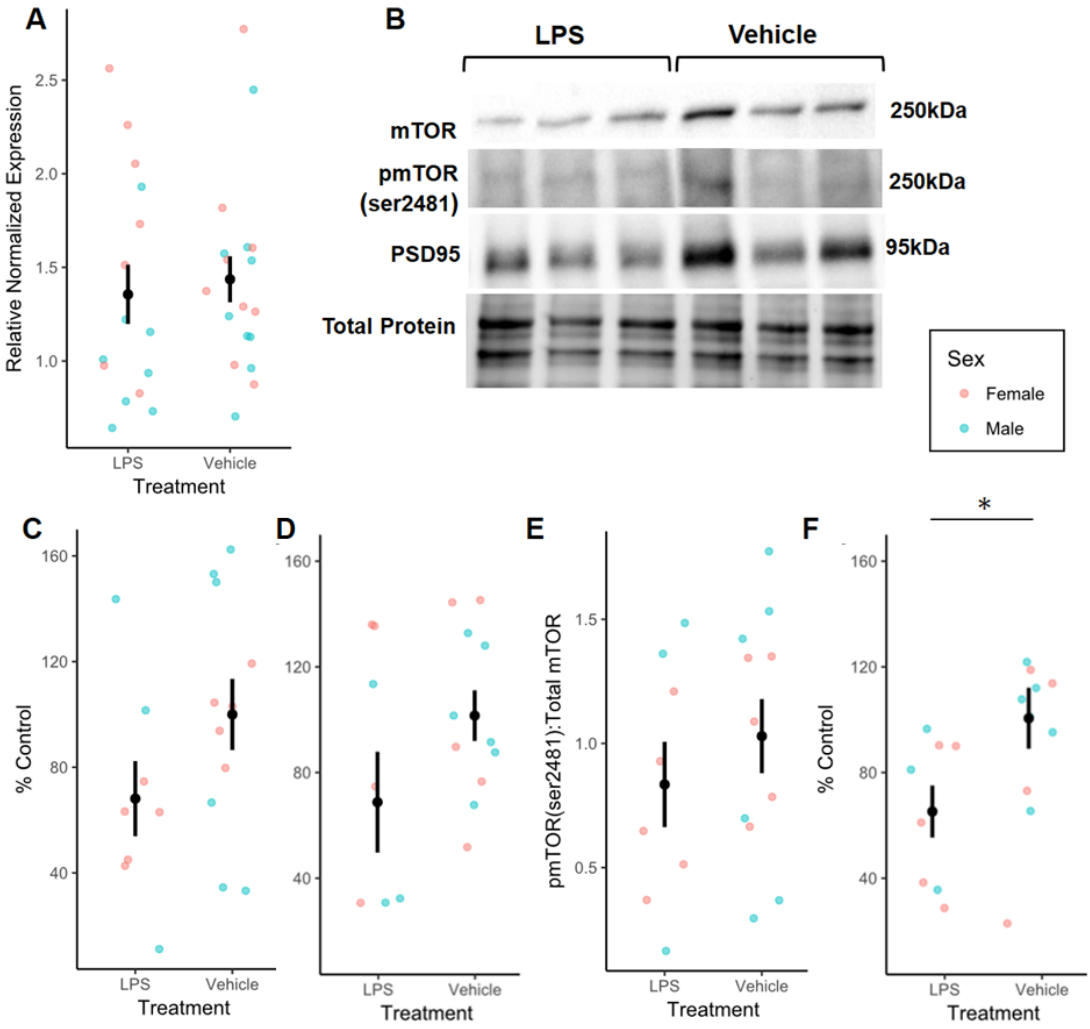

Figure 17. (a) Relative Normalized Expression of *Mtor* from RT-qPCR and (b) western blot of (c) mTOR. (d) phosphorylated mTOR (ser2481; pmTOR), (e) ratio and (f) PSD95 in P130 Ventral Hippocampus from C57Bl/6J mice prenatally treated with lipopolysaccharide (LPS) or vehicle saline. Lanes in western blot (b) represent different individual samples. Error bars are mean±SEM. ***p<0.05.**

(DH: $F_{(1,27)}$=0.469, p=0.499; VH: $F_{(1,29)}$=0.188, p=0.6678) nor was there an effect of sex (DH: $F_{(1,27)}$=3.301, p=0.0804; VH: $F_{(1,29)}$=3.926, p=0.0571; Figures 17a, 18a). In the DH there was a significant interaction between sex and treatment ($F_{(1,27)}$=9.388, p=0.00491) where female animals had higher *Mtor* expression when treated with LPS (0.959±0.0856) than when treated with saline vehicle (0.641±0.103) and the opposite was observed in males (LPS: 0.766±0.139, Vehicle:1.15±0.0823; Figure 18a).

When comparing to the Western blot analysis (Figures 17b, 18b), in the VH we

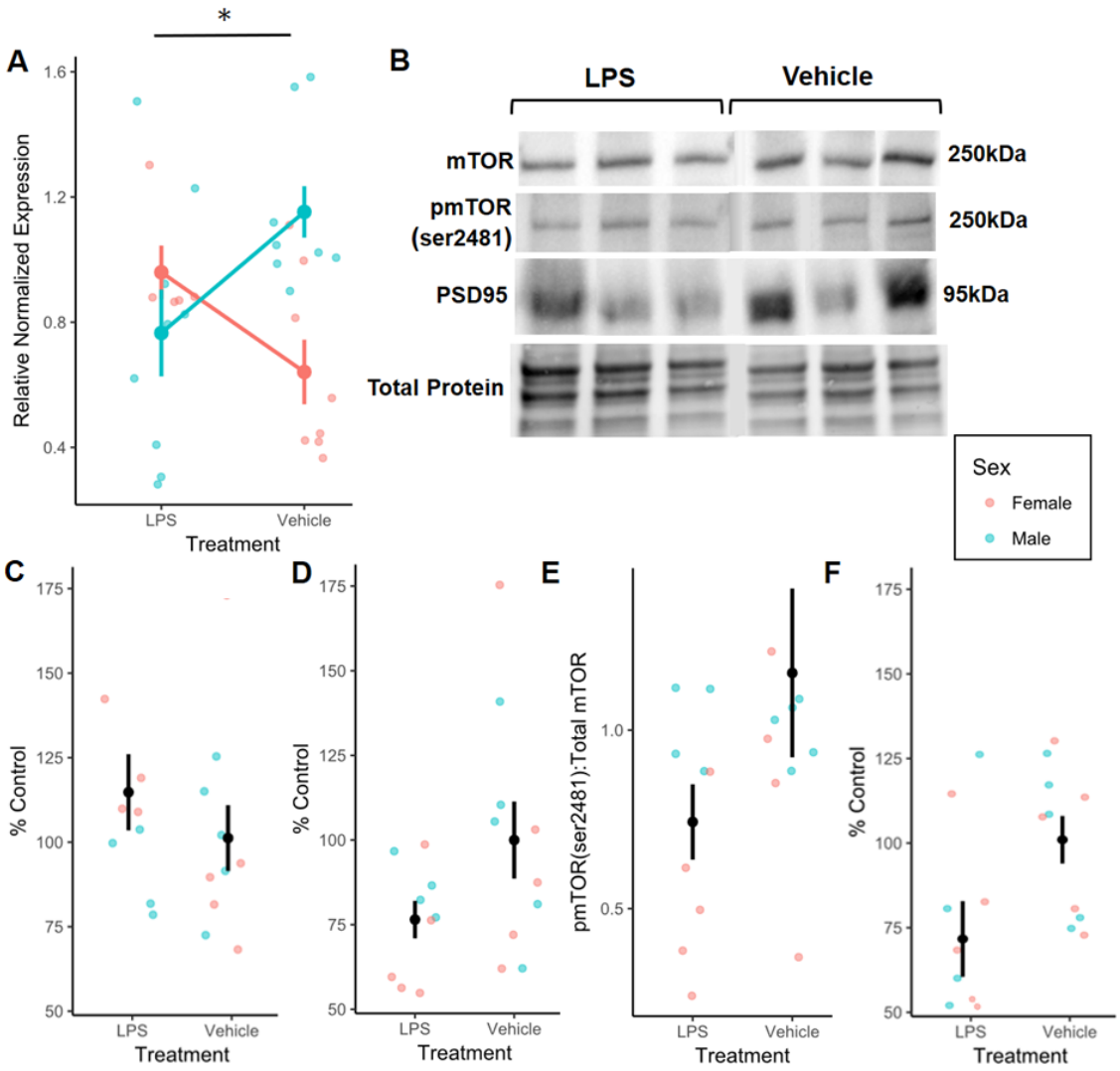

Figure 18. (a) Relative Normalized Expression of *Mtor* from RT-qPCR and (b) western blot of (c) mTOR. (d) phosphorylated mTOR (ser2481; pmTOR), (e) ratio and (f) PSD95 in P130 Dorsal Hippocampus from C57Bl/6J mice prenatally treated with lipopolysaccharide (LPS) or vehicle saline. Lanes in western blot (b) represent different individual samples. Error bars are mean±SEM. ***p<0.05.**

observed no significant difference in mTOR, pmTOR and the ratio between pmTOR mTOR: W=24, p=0.109; pmTOR: W=27, p=0.177; ratio: W=34, p=0.442; Figure 17c,d,e) in the LPS animals (mTOR:68.12±14.23, pmTOR:85.06±8.70, ratio:0.84±0.17) compared to the vehicle injected controls (mTOR:100±13.47, pmTOR:100±4.37, ratio:1.03±0.15). There was however a significant decrease in a postsynaptic marker, PSD95 (W=17, p=0.0259; Figure 17f), in the LPS (70.89±8.13) compared to the controls (100±9.50).

In the DH, there was no statistically significant difference in mTOR between LPS and vehicle animals (W=59, p=0.278). Interestingly, a similar pattern to the one observed in the expression of *Mtor* was observed with the LPS females having more mTOR (144.46±20.69) compared to the vehicle females (100±25.22) and the opposite observed in the males (LPS: 86.06±8.59, Vehicle:100±12.61; Figure 18c). This was not observed in pmTOR where there was no significant difference between treatments (W=24, p=0.095; Figure 18d). Furthermore, there was no statistically significant difference between treatments in the ratio between mTOR and pmTOR (W=26, p=0.22: Figure 18e). Similar to the VH, there was lower PSD95 in the LPS (70.33±11.30) compared to the vehicle controls (100±7.12) however this was not significant (W=23, p=0.0789; Figure 18f).

In the mPFC (Figure 19a) there was an increase in mTOR, pmTOR and the ratio between mTOR and pmTOR in the LPS (mTOR:125.33±19.61, pmTOR:152.67±29.64, ratio:1.23±0.16) compared to the vehicle controls (mTOR:100±20.75, pmTOR:100±19.55, ratio:1.20±0.23; Figure 19b,c,d), however it was not significant (mTOR: W=39, p=0.470; pmTOR: W=41, p=0.351, ratio:W=32, p=1.000). Similarly, an increase in PSD95 was observed in the LPS animals (129.46±19.97) compared to the controls (100±15.18) which was not significant (W=44, p=0.211; Figure 19e)

3.3. *Summary of Molecular effects in LPS-MIA*

Overall, we observed some interesting alterations to mTOR in the LPS model. At P0, there was decreased *Mtor* expression in the hippocampus (Figure 15a), which was associated with an increase in phosphorylated mTOR at serine 2481 (Figure 15d). We did

not observe any significant changes to mTOR in the FC at P0 (Figure 16). In adulthood, we observed a similar situation where significant alteration to mTOR was only observed in the hippocampus but not the mPFC (Figure 19). Interestingly, we observed an increase in *Mtor* expression in the DH of female LPS mice compared to vehicle controls (which is opposite from the P0 findings), and the opposite effect in males. The effect observed in

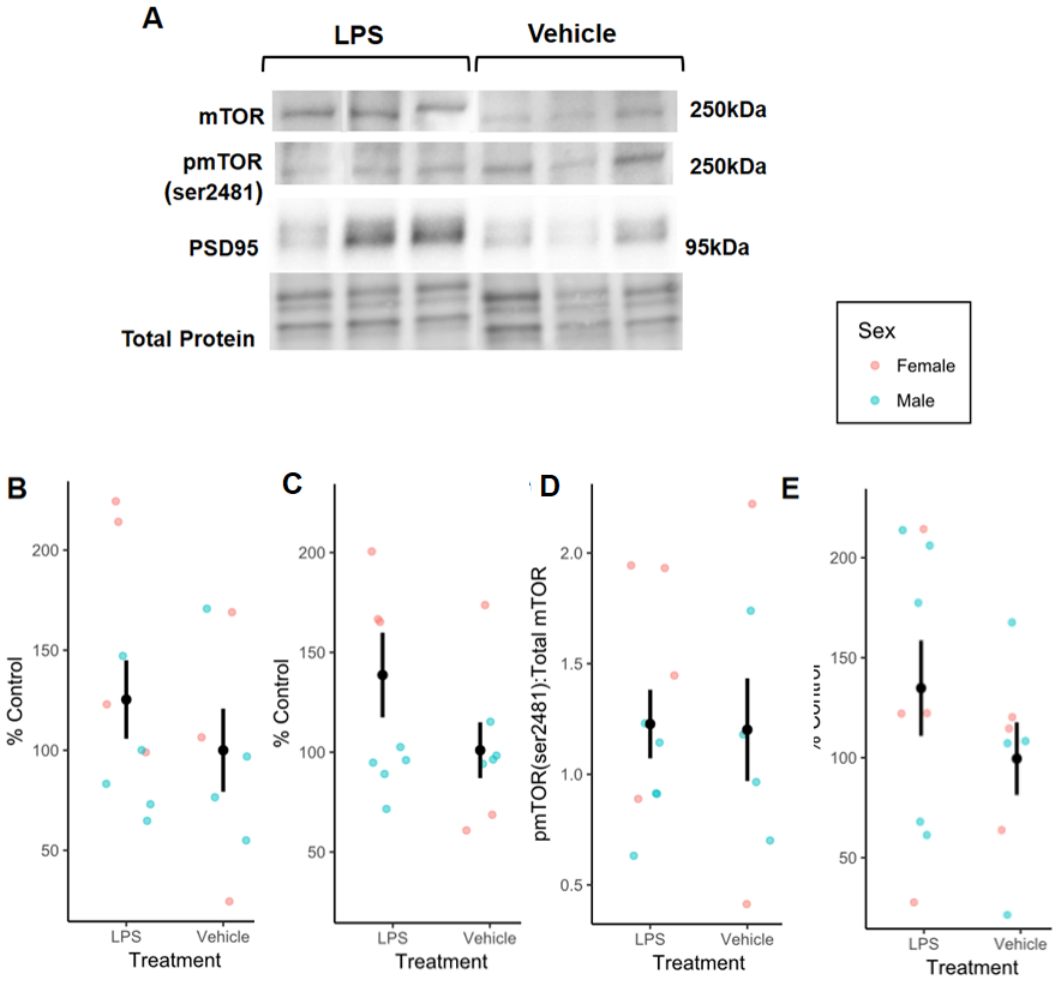

Figure 19. (a) western blot of (b) mTOR. (c) phosphorylated mTOR (ser2481; pmTOR), (d) ratio and (e) PSD95 in P130 Medial Prefrontal Cortex from C57Bl/6J mice prenatally treated with lipopolysaccharide (LPS) or vehicle saline. Lanes in western blot (b) represent different individual samples. Error bars are mean±SEM.

male DH at P130 was, however, comparable to that observed in P0 male hippocampus (Figure 18a). Changes present at the level of *Mtor* expression were not observed at the protein level (Figure 18c). In the VH, we observed a significant reduction in PSD95 in the LPS mice (Figure 17f) which was not associated with significant decreases to pmTOR and the associated ratio in the LPS mice (Figure 17d, e)

Chapter 4

mTOR expression in the VPA model of ASD

4.1. *Methods*

Whole hippocampal sections from male (n=6) and female (n=7) prenatally (E12.5) VPA-treated C57Bl/6J mice and male (n=4) and female (n=5) vehicle (saline) controls were obtained on dry ice from Dr. M. Fahnestock (McMaster) and stored at -80ºC until further processing.

4.1.1. Droplet Digital PCR

A 50µl elution of RNA was extracted from the whole hippocampal samples using an RNA/DNA Purification Kit (Cat. 48700, Norgen Biotek Corp. Thorold, Ont., Canada). All extractions underwent a DNAse treatment to reduce any possible contamination using a RNase-Free DNase I Kit (Cat. 25710, Norgen Biotek Corp. Thorold, Ont., Canada). After checking the purity and concentration on the Take 3 nanodrop plate (Biotek), an optimal 16µl of RNA template was determined and converted into cDNA with the iScript™ cDNA Synthesis Kit (Bio-Rad, Hercules, CA., USA). A no-RT control was derived from a pooled RNA sample.

The same HPRT primer set used in the RT-qPCR analysis was used in the droplet digital PCR analysis (ddPCR™; Bio-Rad, Hercules, CA., USA), however the optimal annealing temperature range was determined to be 51-58 ºC following an annealing temperature gradient of a pooled 1:10 dilution cDNA sample. A pre-validated and optimized PrimePCR ddPCR mTOR primer set (dMmuEG5080770; Bio-Rad, Hercules, CA., USA) was also used. An initial concentration gradient of a pooled sample determined that a 1:1 cDNA concentration was optimal for these primers. In the 20.56µl reaction for HPRT there was 5µl of cDNA template, 10µl of QX200™ ddPCR™ EvaGreen Supermix (Bio-Rad, Hercules, CA., USA), 1µl of 2nM forward and reverse primers and 3.56µl of PCR grade H_20. The reaction for the mTOR was identical except for having only 1µl of the PrimePCR ddPCR mTOR primer and 4.56µl of PCR grade H_20. The reactions were pipetted into the DG8™ Cartridges (Bio-Rad, Hercules, CA., USA) and 65µl of Droplet Generation Oil for EvaGreen (Bio-Rad, Hercules, CA., USA) was added to the appropriate wells. Cartridges were covered with the DG8™ gaskets and droplet generation took

place using the QX200™ droplet generator (Bio-Rad, Hercules, CA., USA). Following droplet generation, 42µl of droplets were pipetted into a 96-well plate, sealed, and placed into a thermocycler under the following protocol: enzyme activation for 5min at 95ºC, denaturation for 30s at 95ºC followed by annealing and extension for 1min at 58ºC for 40cycles and signal stabilization for 5min at 4ºC and 5min at 95ºC. The ramp rate for all cycling steps was 2ºC/s. After thermocycling, the plate was placed into a QX200™ droplet reader (Bio-Rad, Hercules, CA., USA) and the ratio of positive droplets to total droplets was measured (concentration). Expression of mTOR was normalized to HPRT concentration. A two-way ANOVA comparing mTOR expression between sex and treatment was conducted to determine any significant effects.

4.2. *Results*

Following normalization to HPRT, a main effect of treatment as observed by a significant increase in mTOR expression was observed in the VPA animals (0.116±0.0229) compared to saline vehicle controls (0.0577±0.0775; $F_{(1,16)}$=5.826, p = 0.0281; Figure 20). There was no significant effect of sex ($F_{(1,16)}$=1.796, 0.1989).

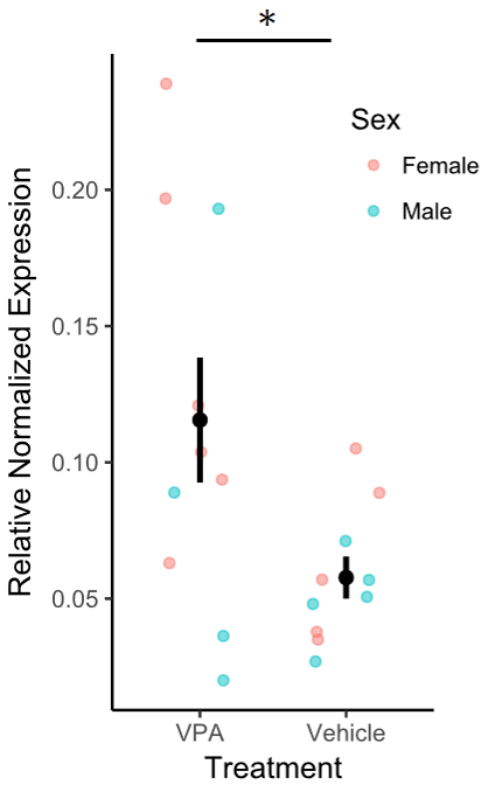

Figure 20. Expression of *Mtor* using droplet digital PCR in the hippocampus of mice prenatally treated with valproic acid (VPA) or saline vehicle control. Error bars are mean±SEM. ***p<0.05**

Chapter 5

Discussion

Overall, we observed that MIA via LPS results in a number of behavioural and physiological changes in C57Bl/6J mice. The prenatal LPS injection resulted in changes to the offspring throughout postnatal development, the juvenile period and in adulthood. Specifically, we observed alterations in the offspring health as demonstrated through increases in infanticide and spontaneous terminations of pregnancy (Table 1) as well as with reduced birth weight (Figure 2a). These alterations in offspring health did not translate to differences in maternal care in the animals that had healthy offspring (Figure 6). Behaviourally we observed a female-specific reduction in social communication as demonstrated by a reduction the number of USVs at P8 in the LPS females, but no change in the males or between treatments at P10,12 or 14 (Figure 7). In the juvenile grooming there was no alteration in the frequency of bouts, average bout duration or total time spent grooming between treatments or sex indicative of no increases in restrictive or repetitive behaviours in the model at this time point. Following weaning, at adulthood the male LPS animals displayed reduced social interest in the olfactory habituation task indicative of reduce sociability. However, this did not translate to the 3-chamber task where there was no sex or treatment effects on all measures of sociability. In the adult measurement of grooming a significant increase in grooming was observed specific to the LPS males, which was not observed at the juvenile phase. This increase in restrictive and repetitive behaviour did not translate to the marble burying where there was no sex or treatment specific effects.

Molecularly, there was a significant decrease in *Mtor* expression in the P0 hippocampus, which did not translate to an associated decrease in protein levels of mTOR but an unexpected significant increase in mTOR activity. These effects at P0 were restricted to the hippocampus as no effects on mTOR physiology were observed in the frontal cortex. At adulthood there was a sex by treatment interaction in *Mtor* expression in the DH and a significant decrease in PSD95 in the VH. Similarly to P0, these effects on the mTOR pathway were restricted to the hippocampus as there was no significant effects observed in the medial prefrontal cortex. Lastly, in the VPA model, at adolescence a

significant increase in hippocampal *Mtor* expression was observed.

5.1. *Breeding and Offspring Health*

There were increase in incidences of infanticide and in the LPS offspring compared to the vehicle-injected offspring as displayed in Table 1. This has not been previously reported in the E11.5 and E12 LPS model of ASD; however, following a subcutaneous LPS inoculation at E17, there was a reported 20% increase in pup mortality (Pujol Lopez et al., 2015), which is associated with infanticide (Weber, Algers, Hultgren, & Olsson, 2013). This was also observed following 3 consecutive intraperitoneal LPS injections on E15-17 which resulted in a 70-80% decrease in the number of surviving pups per dam (Arsenault, St-Amour, Cisbani, Rousseau, & Cicchetti, 2014). Considering that more invasive intraperitoneal injections were used in this study, and during an earlier gestational period, it is likely that the LPS response induced the increase in pup mortality and resultant infanticide observed. Conversely, Pujol Lopez et al., (2015) reported a decrease in overall litter size at birth, which was not observed in the current study.

Along with increases in infanticide, a significant decrease in weight was observed at P0 (Figure 2a). This finding is relevant as low birthweight in mice has been associated with the development of many characteristics of metabolic disease in adulthood (Beauchamp et al., 2015). Lower weight in LPS pups was similarly reported at P4 in this model (Xuan & Hampson, 2014), and was normalized in adulthood apart from the typical sex-difference (Fernández de Cossío et al., 2017), consistent with our findings. Considering that at P8, P12 and in adulthood there was no treatment-associated effect on weight, the difference observed at P0 likely did not result in the development of a metabolic disorder in the LPS mice but may have been possibly due to delayed development. Future studies would benefit from a more thorough assessment of pup development such as through eye-opening to ensure delayed development was not influencing the behavioural phenotypes observed.

5.2. *Behevioural Consequences of LPS-MIA*

Following generation of the LPS-MIA offspring, we initially investigated if there were any changes in maternal care due to the treatment, as changes in maternal care have

been shown to alter adult behaviour in rats (Weaver et al., 2004) and in mice (Champagne et al., 2007) particularly in mediating stress response. Overall, no changes in maternal care were observed (Figure 6), suggesting that behavioural alterations associated with a fear response were likely not mediated by variations in maternal care. However, it is important to note that variability in maternal care is typically low amongst inbred strains, such as the C57Bl/6J mice used in this study, and alterations to maternal care have little effect on shifting the adult anxiety phenotype. This has been observed via cross-fostering experiments where variations of maternal care exert less effect in shifting the adult anxiogenic phenotype in inbred mouse strains when compared to outbred strains (Champagne et al., 2007; Francis, Szegda, Campbell, Martin, & Insel, 2003). Our findings in the LPS model differ from those observed in the PolyI:C model where a reduction in licking and grooming and nest building behaviours were observed compared to vehicle controls in C57Bl/6J and C3H/HeNCrl mice (Berger, Ronovsky, Horvath, Berger, & Pollak, 2018; Schwendener, Meyer, & Feldon, 2009). However, although the maternal care does not differ between treatments in the present study, pup preference could alter the amount of care received as LPS mice have been shown to have a reduced preference for maternally scented nest bedding (Thiago Berti Kirsten et al., 2011). In the current study this does not have major implications as the maternal care analysis showed equal levels of maternal care between treatments.

Following maternal care observations, behavioural assays to determine deficits in social communication, social interaction and repetitive and restrictive behaviours were administered throughout the juvenile period and in adulthood (Figure 3). First, deficits in social communication were observed in the female LPS-MIA mice as demonstrated by a significant reduction in the number of USVs emitted on P8; this was not observed in the males (Figure 7b). This is in contrast to past work describing findings that in male rats prenatally treated with LPS on E9.5, there was a reduction in the number of USVs emitted, although they did not test females (Thiago B. Kirsten et al., 2012). Fernández de Cossío et al., (2017) however, observed no change in the overall number of USVs between LPS and vehicle treatment mice in either sex, but did observe a decrease in the average duration of USVs that was not observed in the present study (Figure 7e). The previously reported lack of a significant difference in the number of calls may be due to

limited detection as Fernández de Cossío et al., (2017) were only detecting calls at 50kHz, and Kirsten et al., (2012) were detecting at 30-50kHz, both of which are far below the average 110-115 kHz call frequency that we observed (Figure 7c), and that which is typically observed in other mouse studies (Malkova et al., 2012; Maria Luisa Scattoni et al., 2008). In comparison to our study, a previous study using the PolyI:C model found a significant reduction in the number of calls and average duration, and this lasted from P8-12; however, only male pups were assayed (Malkova et al., 2012). Malkova et al., (2012) further found a decrease in harmonic calls and an increase in complex and short calls in the male animals at P8. Conversely, in the current study we observed a reduction in the mean proportion of complex calls in the LPS-males but had too few harmonic or short calls to analyse alone (Figure 8). However, similar to our study, complex calls are reduced in the BTBR T+ tf/J mouse model of ASD (Maria Luisa Scattoni et al., 2008). These variations are likely due to the difference between treatments; PolyI:C evokes a viral immune response which produces the ASD-like phenotype specifically in males via a single prenatal injection at E12.5, a finding that is more similar to the sex-bias observed in idiopathic ASD (Malkova et al., 2012; Werling & Geschwind, 2013). Our finding of greater deficits in social communication in females is consistent with other evidence, where females with ASD have been shown to have greater global deficits in communication on the Autism Diagnostic Observation Schedule-Generic (ADOS-G) and deficits in expressive and receptive language skills on the Vineland Adaptive Behavior Scales (Carter et al., 2007; Hartley & Sikora, 2009).

In the domain of repetitive and restrictive behaviours, we only observed an effect in adult males: there were no effects of treatment or sex in juvenile grooming (Figure 9). This age has not been previously described in the LPS or MIA literature; however, in the BTBR T+ tf/J model of ASD, an increase in grooming was continuously observed from P18-P60 when compared to C57Bl/6J mice (McFarlane et al., 2008). The BTBR T+ tf/J model, however, differs greatly from our current model. In this model, different genetics, rather than a prenatal intervention, cause the ASD-like phenotype. Interestingly, we observed an increase in grooming specific to the males at P100 in the habituation phase of the three-chamber task (Figure 12). This effect only occurred post weaning and post sexual maturity and may be due to increases in testosterone which have been shown to

induce grooming in rodents (Aloisi, Ceccarelli, Fiorenzani, De Padova, & Massafra, 2004). Nevertheless, this effect replicates that previously observed in males of this model (Fernández de Cossío et al., 2017; Thiago B. Kirsten & Bernardi, 2017).

Interestingly, Xuan and Hampson (2014) did not observe a significant increase in grooming in the LPS model during the habituation phase of their three-chamber task but did see a male-specific increase in marble burying. In the current study, neither the males nor females displayed an increase in marbles buried (Figure 14). This may be due to differences in time spent investigating as Xuan and Hampson (2014) observed their effect after 30 minutes of exploration with the marbles, where our procedure lasted only 7 minutes. Additionally, Xuan and Hampson only had marbles in 2/3 of the cage and had an area without marbles to one side, this may have acted as an escape zone, thus requiring the longer period of exploration. Lastly, Xuan and Hampson (2014) used corn-cob bedding, whereas we used wood-shavings which may have resulted in more marbled being accidentally covered up and a loss of any effect.

In the PolyI:C model of ASD, an increase in adult grooming and marble burying was observed in males (Malkova et al., 2012). Similar to our study, the marble burying took place in a 10-minute period of exploration; however, in their study, the testing took place under bright light during the light-period of the animal's circadian rhythm which may have resulted in an additive anxiety effect which was not accounted for in their animals. Although Malkova et al., (2012) referenced the Thomas et al., (2009) paper which determined that marble burying was due to repetitive activity and not a fear response, this was determined under dark light with white noise, further adding to the possible discrepancy between data. Overall, marble burying has not been standardized, resulting in discrepancies in the outcomes of our study and others, and thus, any outcome must be carefully scrutinized.

Our sex-specific finding is consistent with the human literature suggesting that boys with ASD have higher ADOS-G scores in restricted/repetitive/stereotyped interests compared to girls (Hartley & Sikora, 2009). However, unlike ASD, this behaviour only occurred in adult LPS mice, which may enforce that LPS, or the particular injection

schedule used in this study is not sufficient to fully replicate an ASD-like phenotype. This also serves as a potential caveat for other studies which only looked at restrictive and repetitive behaviours in adulthood to establish the ASD phenotype (Fernández de Cossío et al., 2017; Xuan & Hampson, 2014).

Finally, the domain of social interaction was tested in adulthood. First, in the olfactory habituation and dishabituation task, males and females showed normal habituation to each odour and dishabituation to the new odour, except for the LPS males, which displayed a reduced dishabituation to the first presentation of a social odour (Figures 10 and 11). The habituation/dishabituation task has not been previously conducted in the LPS model or any maternal immune activation models and as such this is a novel finding. Similarly, male VPA mice also showed a reduction in social investigation during the social odour portion of the olfactory habituation/dishabituation task which was only observed when the mice were weaned and housed with other VPA animals, similar to the LPS animals in our study (Campolongo et al., 2018). In our 3-chamber task we observed an overall increase in preference for the social stimulus over the novel object and no alterations in the time spent investigating either (Figure 13). In contrast, a reduction in sociability in LPS male and female mice was observed previously, but the dams received a stronger, 100ug/kg LPS intraperitoneal injection, at a later gestational period, E15, a time at which more embryonic neural differentiation and migration has taken place (Murayama et al., 2002). Similarly, Xuan and Hampson (2014) observed an effect specific to the LPS males, however it was in an adapted three-chamber task where the chambers were not separated by walls and only the percent of time in zones was measured. Under this paradigm the experimental animal can receive visual, olfactory and auditory cues from the stimulus mouse distracting the animal from the novel object.

Anxiety may have contributed to both significant findings in males. The reduced dishabituation to social odours in the male LPS animals is likely due to reduced motivation to explore and increased fear of the stimulus. Anxiety may also play a role in the observed increase in grooming during the habituation phase of the 3-chamber, particularly because this is in a novel environment. LPS mice have been shown to spend less time in the open arms of the elevated plus maze, the centre of the open field, and on the light side

of the light/dark box when compared to vehicle controls (Depino, 2015). This indicates the need for baseline anxiety testing in these animals in order to evaluate the contribution of anxiety to the development of the behavioural phenotype.

Our results indicate that, in males, the increased grooming occurs only post-sexual maturity, as there was no effect at P18. This is also when reduced social investigation is observed in the olfactory habituation/dishabituation task, possibly indicating that there is a role for hormones in the development of this phenotype. However, currently we can only definitively say that an increase in repetitive grooming occurs post-puberty in the males. To better compare the juvenile period to the adult period, a measure of social interaction and investigation should be assessed during the juvenile phase to determine when this behavioural phenotype develops. This could be done via a juvenile play investigation, where free social interaction occurs between age- sex- and treatment- matched conspecifics and the frequency of investigative, affiliative, play soliciting, and non-social behaviours are recorded (McFarlane et al., 2008). Additionally, an adult measure of social communication should be assessed to determine the continuity in the presence and lack of this phenotype in female and males, respectively. This could be done via analysis of the USVs emitted as a response to female pheromones in male mice (Holy & Guo, 2005) and in female dyads (Moles, Costantini, Garbugino, Zanettini, & D'Amato, 2007). Additionally, these behaviour assays should be conducted throughout the lifespan beyond adulthood to observe any effects of aging on the ASD-like phenotype observed.

Across all behavioural tests, there was typically higher variability observed in the LPS animals. Variations in gestational day at the time of injection, considering that the two injections are only 12 hours apart, may play a role in some of the observed variability particularly as this is a period of embryonic neurogenesis and differentiation of neural stem cells (Murayama et al., 2002). However, a phenotype can still be observed with a single injection on E15 suggesting that developmental stage and not the frequency of injections introduces the majority of the variability (Fernández de Cossío et al., 2017). Although variations in uterine position are known to result in sex differences in behaviour and physiology (Drickamer, 1996), there is evidence that individual differences in the up-regulation of α-crystallin in the brains of a litter following prenatal influenza treatment

(Garbett, Hsiao, Kálmán, Patterson, & Mirnics, 2012). α-Crystallin is a key component of the vertebrate lens which is also a chaperone protein that has been shown to be upregulated following an immune response to LPS (Horwitz, 1992; Masilamoni et al., 2006). This suggests that although uterine position may have introduced some variability in this study, it is more likely that the variability was introduced by individual differences in the response to the LPS.

5.3. *mTOR pathway in the LPS-MIA and VPA models of ASD*

To better characterize the effects of LPS-MIA on a known ASD-associated genetic pathway, we examined mTOR regulation and activity in the hippocampal and forebrain regions of LPS and control mice at P0 and P130. At P0 we observed a significant decrease in *Mtor* expression in the hippocampus of LPS pups (Figure 15). This is consistent with a prior finding 3-hours post-LPS inoculation at E12.5 at a dose of 60µg/kg in whole brain samples (Lombardo et al., 2018), and suggests that altered *Mtor* expression occurs rapidly after LPS injection and persists postnatally. The decrease in *Mtor* expression did not result in an associated decrease in protein levels of mTOR. This is surprising, but not entirely unlikely, as cellular concentrations of proteins have been shown to correlate with their respective mRNAs at only about 40% (de Sousa Abreu, Penalva, Marcotte, & Vogel, 2009). This could be explained by protein degradation or post-transcriptional and translational mechanisms. In mammalian cells, fewer copies of a given mRNA are produced per hour, compared to their respective proteins leading to this low correlation (Schwanhäusser et al., 2011). Alongside this, after transcription, mRNA may go through alternative and differential splicing and is subject to degradation with a half-life of about 2.6-7 hrs (Sharova et al., 2009). Following translation, alterations in protein stability, which can alter the average 46 hr half-life observed in proteins (Sharova et al., 2009). Recently, it has been suggested that taking a ratio of RNA-to-protein for a given gene so that an average ratio of expression to translation can be observed for that gene within that animal (Edfors et al., 2016). These factors need to be especially considered within mTOR physiology as inhibition of mTOR activity leads to the recruitment of a ubiquitin proteasome system to promote overall protein degradation (Zhao, Zhai, Gygi, & Goldberg, 2015). Interestingly, there was an observed significant upregulation in mTOR activity at P0 via an increase in pmTOR(2481), which may have inhibited this global degradation of

protein, leading to this counterintuitive effect we observed in the unchanged mTOR protein levels between treatments. Looking at multiple time points both before birth and shortly post birth at the mRNA and protein levels of mTOR would provide more evidence to better understand this counterintuitive result. Despite this lack of correlation, this effect was restricted at P0 to the hippocampus as no effect in mTOR activity was observed in the frontal cortex of the LPS-MIA animals (Figure 16). Further studies at P0 should look at phosphorylated S6K, the downstream target of mTOR to confirm that this increased phosphorylation is enhancing pathway activation (Costa-Mattioli & Monteggia, 2013).

Comparable to the P0 animals, all the significant effects regarding the mTOR pathway in the P130 animals were restricted to the hippocampal areas studied, as no significant effects were observed in the mPFC (Figure 19). Specifically, a significant reduction in PSD95 was observed in the VH in the LPS animals (Figure 17) and a sex by treatment interaction in the expression of *Mtor* was observed in the DH (Figure 18). This sex by treatment interaction is novel and more animals would need to be added to see if this results in a significant effect in the mTOR protein analysis. The current findings, though not statistically significant, support a similar direction. The significant decrease in PSD95 observed in the VH may point to an earlier decrease in mTOR activity. Inhibition of mTOR activity via rapamycin treatment has previously been shown in hippocampal slice cultures to reduce the expression and protein levels of PSD95 (Li et al., 2010; Ma et al., 2010). In the current study, an increase in mTOR activity was only observed at P0 with no change at P130, however this may have been observed at an earlier age as adolescent animals of the similar VPA model display reduced mTOR activity (Nicolini et al., 2015). As such, an analysis of mTOR should be additionally carried out at adolescence in the LPS-MIA model to observe for a decrease in mTOR activity driving this reduction in PSD95 at P130. This finding is opposite to the reduced pruning observed in tuberous sclerosis and PTEN mutative syndromes (C.-H. Kwon et al., 2006; Tang et al., 2014). However, in those syndromic cases of ASD a constitutive increase in mTOR activity is observed throughout the lifespan resulting in this downstream loss of pruning. Of course, if there was increased PSD95 in the P0 hippocampus following the observed increase in mTOR activity it would provide further support to this mechanism in this model.

Furthermore, it would provide evidence for a switch in mTOR physiology occurring between P0 and P130 possibly driving changes in structure and age-related alterations in behaviour. Future studies should look at PSD95 in the P0 hippocampus as well as a histological assay of spine density as previously observed to be increased in the hippocampal granule cell in males of this model at E15 (Fernández de Cossío et al., 2017).

Traditionally, the DH has been associated primarily with analysis of spatial location as it has more place fields (Jung, Wiener, & McNaughton, 1994), and lesions to the DH result in deficits on the Morris water maze (Moser, Moser, Forrest, Andersen, & Morris, 1995). The VH was traditionally associated with emotional and fear responses due to its connections with the amygdala (Henke, 1990), which have been more recently associated with regulating social behaviour (Felix-Ortiz & Tye, 2014) which is consequently where we observed a significant reduction in PSD95 in adult LPS mice. In further support of our finding of reduced PSD95 in the adult VH, a similar reduction in PSD95 was observed in the whole hippocampus in the PolyI:C model of ASD at P40 and P90 (Giovanoli, Weber-Stadlbauer, Schedlowski, Meyer, & Engler, 2016). Conversely, an increase in hippocampal granule cell spine density was observed in the LPS model specific to the males however this was at P15 and PSD95 was not assayed directly (Fernández de Cossío et al., 2017). Ultimately, a histochemical analysis of spine density and PSD95 localization would serve as a strong piece of supporting evidence for this finding at P130 and is necessary for further interpretation of the P0 finding of increased mTOR activity.

Of interest is the differences in mTOR and downstream PSD95 between P0 and P130. Histone modifications may be underlying this alteration as recent evidence has shown that *Mtor* expression is regulated by NrF2 binding, which occurs following oxidative stress and inflammation, resulting in reduced H3-K27me3 (marker of gene repression) and increased H3-K4me3 binding (marker of gene activation) and a resultant increase in *Mtor* expression (Bendavit, Aboulkassim, Hilmi, Shah, & Batist, 2016). However, when mTOR activity is increased, as shown by an increase in phosphorylated S6K and 4EBP-1, the opposite occurs and *Mtor* expression is reduced via an associated increase in H3-K27me3 (Bendavit et al., 2016). This may be underlying the effect observed

prenatally and at birth when an increase in mTOR activity, evidenced by an increase in *Eif4e* expression in whole brain 3-hrs post-LPS injection (Lombardo et al., 2018) and an increase in pmTOR(ser2481) in P0 LPS hippocampus (Figure 15), is associated with a downregulation of *Mtor* at these time points. Conversely, if a decrease in activity occurred before P130, it may negate this process. This is further supported by evidence which shows that *NrF2* expression is upregulated specifically following birth in the P0 cortex, possibly driving this mechanism of *Mtor* suppression specific to P0, which would not occur later due to the reduced *NrF2* expression (Bell et al., 2015). Future studies should measure both NrF2 levels at P0 and P130 and perform a chromatin-immunoprecipitation assay to look for changes of H3K9-me3 and H3K27-me3 associated with *Mtor* expression.

These age-specific findings in mTOR activity and PSD95 are partially consistent with evidence from idiopathic ASD, where reduced hippocampal volume is observed at birth and into adolescence (Schumann et al., 2004), with a reduction in hippocampal functional connectivity observed via fMRI in adulthood following a memory retrieval task (Cooper et al., 2017). Interestingly, reduced PSD95 has been observed in the hippocampus of adult males with idiopathic ASD possibly underlying this reduction in functional connectivity (Nicolini et al., 2015). As previously mentioned, the analysis of P0 hippocampal PSD95 in the LPS-MIA model, and measures of hippocampal size and density throughout the lifespan would be required to associate this link further in the LPS-MIA model.

In light of the extensive hippocampal-specific alterations, future studies should explore possible cognitive effects of LPS-MIA. Previous research has observed deficits in spatial memory on the Morris water maze in rats prenatally treated with LPS on E8, 10 and 12 (Labrousse et al., 2018). Assaying for spatial memory deficits is increasingly important for the current study considering the sex by treatment interaction in *Mtor* expression and the reduction in PSD95 in the DH. Similarly, the significant reduction in PSD95 in the VH provides further evidence that general anxiety should be assayed in this model.

Contrary to our findings in the LPS-MIA model, a significant increase in *Mtor*

expression was observed in the hippocampus of the VPA model of ASD however this tissue was extracted at adolescence (P36) and may represent a specific time period of alteration to mTOR signalling (Figure 20). This model has previously been associated with a reduction in pmTOR(2448):mTOR (Nicolini et al., 2015), which suggests an inverse relationship of mTOR activity with expression. This reduction in activity was not observed in the adult VH and DH of the LPS-MIA model likely due to a difference between adulthood and adolescence. As such, the adolescent period should be further explored in the LPS-MIA model to observe if an increase in *Mtor* expression and decrease in activity occurs similar to the VPA model. Further studies in the VPA model would also benefit by an evaluation of both the ventral and dorsal hippocampus at P0 and P130 as the current study shows differences between these time periods and regions in the LPS-MIA model. The many similarities between these two models, and other models of ASD indicate a role for age-related increases and decreases in mTOR physiology in the pathophysiology of ASD. In this particular model it seems as though a switch in mTOR activity at adolescence leading to changes in downstream PSD95, possibly mediated by Nrf2 expression mediated changes in mTOR expression as described earlier, or via hormonal changes at puberty, results in reduced PSD95 at adulthood.

5.4. *Future Directions*

With the novel insights gained from this study, there are many future pursuits that could be undertaken to understand the sex-specific phenotypic behavioural differences and to associate the alterations in the mTOR pathway with the behaviour observed.

To further explain the sex effects observed, particularly considering they occurred pre-sexual maturity in the females and post-sexual maturity in the males, the four-core genotypes mouse model should be used in the LPS-MIA paradigm employed in the current study. This transgenic model results in XX and XY mice with testis and XX and XY mice with ovaries by deletion of the testis-determining gene from one Y chromosome and the addition to an X chromosome (Itoh et al., 2015). This would allow for the separation of a chromosomal role in developing the phenotype and associated mTOR pathophysiology, from a hormonal role. This has not been previously attempted in a model of maternal immune activation, however the four core genotype mouse model has shown that a

chromosomal and hormonal interaction alters the development of juvenile social behaviour which may be inducing the behavioural changes observed in the adult males of this study (Cox & Rissman, 2011).

To associate the reduced *Mtor* expression at P0 in the hippocampus and at early gestational points in the whole brain (Lombardo et al., 2018), with the altered behaviour observed, *Mtor* expression would have to be upregulated in the prenatal period or at P0, either by transduction of viral vectors via *in utero* electroporation (Navarro-Quiroga, Chittajallu, Gallo, & Haydar, 2007), or via the novel dgRNA trans-epigenetic CRISPR/Cas9 system of gene activation at P0 (Liao et al., 2017). If this upregulation of *Mtor* results in improvements in social communication, as displayed by an increase in USVs in the females at P8, and in improvements in social interest, displayed by an increase in dishabituation to the first social odour in the odour dishabituation/habituation task and reduced self-grooming in adult males, this would implicate mTOR in the development of the ASD behavioural phenotype. Furthermore, an associated reduction in pmTOR(ser2481) in the P0 hippocampus and the normalization of PSD95 in the adult hippocampus would add additional evidence supporting the role of mTOR in the development of this behavioural phenotype observed. Apart from a genetic manipulation, a pharmacological manipulation using prenatal rapamycin treatment in the dam could also be used to validate the role of the mTOR pathway in developing the ASD-like behaviours observed in the LPS-MIA model however this would result in reduced specificity compared to a genetic manipulation. This treatment has been previously validated with a single subcutaneous dose of rapamycin rescuing mortality at birth due to hyperactivation of the mTOR pathway in a *Tsc1* knockout model (Anderl, Freeland, Kwiatkowski, & Goto, 2011).

Similarly, adult cannabidiol treatment, which is a known up regulator of mTOR activity (Giacoppo et al., 2017), could be used to normalize the possible reduction in mTOR activity observed in adolescents exposed to LPS and VPA. This is because the upregulation in *Mtor* observed in the hippocampus of the VPA model has been previously associated with a reduction in pmTOR(2448):mTOR (Nicolini et al., 2015) and an ASD-behavioural phenotype (Moldrich et al., 2013). Similarly, lower PSD95 was observed in

the hippocampus of the LPS-MIA model, which may be due to a reduction in pmTOR(2481) earlier than P130 around the time period in which the males developed a phenotype in restrictive and repetitive behaviours. This would strengthen the argument that in the VPA and LPS models of ASD there is convergence on a similar pathway at adulthood. Of course, further analysis of the mTOR physiology at weaning, during the adolescent phase of development, would be required to determine when this switch in mTOR activity is taking place.

Lastly, a multiple insult model may be warranted, suggesting that future studies should look at combining mutations in the mTOR pathway that have been previously linked to ASD (e.g., in *Tsc1*, *Tsc2* and *Pten*) with MIA, to induce a strengthened ASD phenotype. This may better reflect human idiopathic ASD, which has several risk alleles, none of which directly cause ASD, but which interact with various immune factors.

5.5. *Conclusions*

Overall, the LPS-MIA model displays some of the behavioral characteristics associated with ASD. Most interestingly, the model induced these characteristics in a sex-bias similar to those observed in human ASD with females generally showing greater communicative deficits and males displaying greater deficits in restrictive and repetitive behaviours (Carter et al., 2007; Hartley & Sikora, 2009; Lord, Schopler, & Revicki, 1982). These behavioural effects may also be regulated by age, as the effects in males was predominantly observed post-weaning suggesting this model of LPS does not fully recapitulate ASD in the domain of restricted and repetitive behaviours. Molecularly, the LPS model showed a reduction in *Mtor* expression specific to the hippocampus at P0, which was associated with an increase in activity as assayed by phosphorylation. This is commonly observed in both *Pten* mutative syndromes and tuberous sclerosis associated ASD (C. H. Kwon et al., 2006; Sato et al., 2012). Interestingly, this effect was reversed in the adult hippocampus, where a decrease in PSD95 was observed. This is comparable to adult idiopathic ASD (Nicolini et al., 2015), suggesting a shared pathway in this model and ASD. The observed increase in *Mtor* expression and previously reported decrease in activity in the adolescent hippocampus of VPA mice (Nicolini et al., 2015) points to a period where mTOR activity may be reduced despite increased expression. This may

possibly result in the lowered adult hippocampal PSD95 observed in the LPS-MIA model, although parallel studies must be conducted between the two models before definitive comparisons can be made. Despite more evidence being required to connect the alterations in mTOR physiology to the observed behavioural phenotype, this study indicates a novel route of investigation in ASD while highlighting some of the sex effects commonly observed in ASD patients.

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