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Beyond infection - Maternal immune activation by environmental factors, microglial development, and relevance for autism spectrum disorders

Staci D. Bilbo[1,*], **Carina L. Block**[2], **Jessica L. Bolton**[3], **Richa Hanamsagar**[1], and **Phuong K. Tran**[1]

[1]Pediatrics and Neuroscience, Harvard Medical School, Lurie Center for Autism, Massachusetts General Hospital *for* Children, Boston, MA 02126

[2]Psychology and Neuroscience, Duke University, Durham NC, 27708

[3]Pediatrics and Anatomy/Neurobiology, University of California-Irvine, Irvine, CA 92697

Abstract

Immune molecules such as cytokines and chemokines and the cells that produce them within the brain, notably microglia, are critical for normal brain development. This recognition has in recent years led to the working hypothesis that inflammatory events during pregnancy, e.g. in response to infection, may disrupt the normal expression of immune molecules during critical stages of neural development and thereby contribute to the risk for neurodevelopmental disorders such as autism spectrum disorder (ASD). This hypothesis has in large part been shepherded by the work of Dr. Paul Patterson and colleagues, which has elegantly demonstrated that a single viral infection or injection of a viral mimetic to pregnant mice significantly and persistently impacts offspring immune and nervous system function, changes that underlie ASD-like behavioral dysfunction including social and communication deficits. Subsequent studies by many labs – in humans and in non-human animal models - have supported the hypothesis that ongoing disrupted immune molecule expression and/or neuroinflammation contributes to at least a significant subset of ASD. The heterogeneous clinical and biological phenotypes observed in ASD strongly suggest that in genetically susceptible individuals, environmental risk factors combine or synergize to create a tipping or threshold point for dysfunction. Importantly, animal studies showing a link between maternal immune activation (MIA) and ASD-like outcomes in offspring involve different species and diverse environmental factors associated with ASD in humans, *beyond infection*, including toxin exposures, maternal stress, and maternal obesity, all of which impact inflammatory or immune pathways. The goal of this review is to highlight the broader implications of Dr. Patterson's work for the field of autism, with a focus on the impact that MIA by diverse environmental factors has on fetal brain development, immune system development, and the pathophysiology of ASD.

[*]To whom correspondence should be addressed: Staci D. Bilbo, PhD, Director of Research, Lurie Center for Autism, MassGeneral Hospital *for* Children, Associate Professor of Pediatrics and Program in Neuroscience, Harvard Medical School, Lab: Charlestown Navy Yard Building 114, 114 16th Street (Mail Stop 114-3400), Charlestown, MA 02129, P: 617-643-9887, F: 617-724-1731, sbilbo@mgh.harvard.edu.

Introduction

Autism spectrum disorder (ASD) is a complex continuum of neurodevelopmental disorders with early childhood-onset. These disorders, for which there is presently no cure and only limited treatments, are typically associated with significant lifelong cognitive, social, communication and behavioral impairments for the individual (Birtwell et al., 2016; Magiati et al., 2016). The prevalence of ASD has been progressively increasing, and is more common than previously thought (Centers for Disease Control and Prevention, 2014). Though a component is certainly genetic, hundreds of diverse genes are now linked to ASD, each of which contributes to only a very small percentage of the affected population. The heterogeneous clinical and biological phenotypes observed in ASD strongly suggest that in genetically susceptible individuals, environmental risk factors also combine or synergize to create a tipping or threshold point for dysfunction. Multiple prenatal/maternal exposures, most notably infection, have been linked to an increased risk of ASD in offspring (Ashwood and Van de Water, 2004; Becker, 2007; Blaylock and Strunecka, 2009; Lauritsen et al., 2005; Volk et al., 2014). A large body of work by the highly influential Dr. Paul Patterson, and colleagues, has illustrated some of the mechanisms by which maternal immune activation (MIA) with viral infection or viral mimetics can persistently alter offspring immune function, disrupt fetal brain development, and induce the onset of ASD-like behaviors in animal models. The immune system is our interface with the environment, and a role for immunological involvement in at least a subtype of ASD has been hypothesized for some time (Money et al., 1971). Family studies document a significant relationship between familial autoimmune disorders and ASD (Comi et al., 1999; Molloy et al., 2006a; Molloy et al., 2006b; Sweeten et al., 2003a; Sweeten et al., 2003b). Results from post-mortem and neuroimaging studies utilizing PET/MRI have identified stable and persistent inflammation in the brains of some subjects with ASD compared to control subjects (Pardo et al., 2005; Suzuki et al., 2013; Vargas et al., 2005). Findings from multiple animal models have demonstrated marked immune abnormalities that correlate with abnormalities in behavior (Bauman et al., 2013; Bauman et al., 2014; Borrell et al., 2002; Braunschweig et al., 2012; Brimberg et al., 2016; Dalton et al., 2003; Malkova et al., 2012; Martin et al., 2008; Shi et al., 2003; Singer et al., 2009; Smith et al., 2007a). These studies involve different species and diverse environmental factors associated with ASD in humans, *beyond infection*, including toxin exposures, maternal stress, and maternal obesity, all of which impact inflammatory or immune pathways (Krakowiak et al., 2012; Volk et al., 2014; Zerbo et al., 2013). The goal of this review is to highlight the broader implications of Dr. Patterson's work for the field of autism, with a focus on the impact that MIA by diverse environmental factors may have on fetal brain development, immune system development, and the risk of ASD.

The Immune System is a Regulator of Normal Brain Development

Immune molecules are critical for normal brain development (Bilbo and Schwarz, 2012; Deverman and Patterson, 2009; Schwarz and Bilbo, 2012). A novel role for major histocompatibility (MHC) class I proteins in activity-dependent synapse formation within the visual cortex was identified over two decades ago (Corriveau et al., 1998), and a role for complement proteins in synapse elimination was described several years later (Stevens et al.,

2007), two pivotal findings that fundamentally changed our concept of “immune privilege” within the healthy brain. Several chemokines and cytokines have now been identified for their critical roles in neuronal and glial cell migration, differentiation, synaptic maturation, and many other processes (Deverman and Patterson, 2009; Nawa and Takei, 2006). Time-dependence and regional specificity has been demonstrated for cytokines during brain development (Pousset, 1994), suggesting distinct roles for individual cytokines in the development of specific brain circuits. For instance, interleukin (IL)-1β is expressed at high levels throughout the late prenatal/early postnatal hippocampus and cortex, but at very low (constitutive) levels in the adult (Giulian et al., 1988; Schmitz and Chew, 2008). Microglia, the primary immune and cytokine-producing cells of the central nervous system (CNS), are also important for normal brain development via the phagocytosis of extraneous synapses (Paolicelli et al., 2011; Schafer et al., 2012) and apoptotic cells (Sierra et al., 2010), and for aspects of axonal growth and angiogenesis (Rakic and Zecevic, 2000; Streit, 2001). Microglia colonize the fetal brain as primitive myeloid precursor cells from the yolk sac, starting around embryonic (E) day 9 in the rodent and late 1st trimester in humans (Chan et al., 2007; Ginhoux et al., 2010). They enter the parenchyma via the blood stream and ventricles, and are initially clustered around subcortical regions such as the hippocampus and corpus callosum (Cuadros and Navascues, 1998; Wang et al., 2002; Xu et al., 1993). From there, microglia migrate throughout the brain where they continue to proliferate throughout the first postnatal weeks in humans and rodents. They are long-lived cells that are now accepted to be self-renewing, e.g. without contribution from the hematopoietic cells in the periphery under normal conditions (Elmore et al., 2014; Schulz et al., 2012). Microglia largely appear thick, reactive or amoeboid during the early perinatal period, in contrast to the ramified morphology found in adults (Fujita et al., 1981; Ling and Wong, 1993; Rezaie and Male, 2002), though little is known about what this means for function. Interestingly, the developmental peak in cytokine concentrations within distinct brain regions depends on the presence of amoeboid microglia in the rat (Giulian et al., 1988). Due to the critical role that immune molecules play in normal brain development, many, notably Dr. Patterson (Deverman and Patterson, 2009), have hypothesized that aberrant expression of these molecules in response to immune activation is harmful for neural development. That is, it is *because* immune moleculesand glia are critical for normal development that they are also implicated in abnormal development (Bilbo and Schwarz, 2009, 2012).

Immune Activation and Neural Dysfunction

We now recognize that immune activation or abnormalities within the brain may play a pivotal role in the etiology and/or progression of neuropsychiatric conditions as diverse as Alzheimer's disease, schizophrenia, ASD, and depression. The link between influenza virus in pregnancy and increased risk for ASD and other neurodevelopmental disorders such as schizophrenia has been documented for many years (Brown, 2012; Canetta and Brown, 2012). Severe bacterial infections in pregnancy, in particular those associated with fever, are similarly associated with increased risk of ASD in children (Atladottir et al., 2010). Very recent data show the association extends to parasites, as low circulating maternal immunoglobulin (Ig) levels against *Toxoplasma gondii*, a common parasite, is linked to increased odds of ASD in offspring, whereas high levels of Ig predict the opposite,

suggesting a protective effect of adequate maternal Ig (Spann et al., 2017). Conversely, fetal brain-specific antibodies have been identified in a subset of mothers of autistic children in several different studies (Brimberg et al., 2016; Careaga et al., 2013; Fox-Edmiston and Van de Water, 2015; Piras et al., 2014; Rossi et al., 2014). These same isolated antibodies produce ASD-like symptoms in naïve rhesus monkey offspring following injection of the mothers during pregnancy (Bauman et al., 2013). These data strengthen the intriguing evidence that autoimmune disorders are more common in individuals and first degree relatives with ASD, broadly suggesting a critical role of immune dysregulation.

The developing CNS may be especially vulnerable to inflammatory disruption (Rodier, 1980), in large part due to its remarkable plasticity within discrete critical windows, perturbations of which may produce effects on brain and behavior that manifest much later in life, or persist throughout an organism's life span (Cai et al., 2000; Pang et al., 2003; Richardson-Burns and Tyler, 2004; Urakubo et al., 2001; Yu et al., 2004). For instance, the neural circuitry underlying sensory systems such as vision and audition require environmental input during discrete developmental windows for adult function to emerge intact – disruption of this input (e.g. via sensory deprivation) results in severe functional deficiencies throughout the remainder of the lifespan. Though less well characterized, there is emerging evidence that immune molecules such as complement proteins and cytokines guide the normal development of neural circuits (Stevens et al., 2007), and thus their abnormal expression in response to immune challenge – during a critical period – is likely to have devastating consequences.

Indeed, altered cytokine expression early in life is increasingly associated with the risk of neurodevelopmental disorders including ASD and severe developmental delay. Concentrations of IL-1β, IL-6, and tumor necrosis factor (TNF) are elevated in infants with severe perinatal complications (Miller et al., 1990), and children with bacterial meningitis have elevated levels of IL-1β that strongly correlate with the occurrence of neurological disorders (Mustafa et al., 1989). Increased levels of IL-6 in amniotic fluid have also been a clinically useful marker of increased risk for neurological disorders and morbidity (Yoon et al., 1995). Lipopolysaccharide (LPS), the cell wall component of gram-negative bacteria, has been used to mimic bacterial infection in many pre-clinical studies because it initiates a rapid and well-characterized immune response, notably robust cytokine expression (see (Wang et al., 2006) for review). Prenatal LPS injections in mice or rats induce long-term changes in immune function, synaptic plasticity, white matter development, and in social, cognitive, and motor behaviors of offspring (Batinic et al., 2016; Fernandez de Cossio et al., 2017; Fidel et al., 1994; Foley et al., 2015; Gao et al., 2015; Golan et al., 2005; Urakubo et al., 2001). Similarly, influenza virus administered to pregnant mice increases cytokine levels in placental tissue and amniotic fluid, in particular IL-6, and results in abnormal development of the offspring (Shi et al., 2003), as does the viral mimic polyinosinic:polycytidylic acid (poly IC) (Shi et al., 2009). Importantly, the infectious agents themselves most often do not enter the fetal circulation, and experiments demonstrating that specific cytokine antagonists in the presence of infection protect the fetal brain from adverse outcomes have implicated the critical role of the maternal immune response itself (Smith et al., 2007b). Moreover, the strong association between infection and developmental disorders

spans a diverse number of bacterial, viral, and mimetic agents, suggesting a common mechanism such as cytokine release.

Evidence of immune system abnormalities in autism

Immune system abnormalities have been reported throughout the body and brain of many autistic individuals. As described briefly above, these include evidence of brain specific auto-antibodies, altered lymphocyte responses to antigen, altered cytokine production, and an increased incidence of allergies and other autoimmune disorders (Ashwood and Wakefield, 2006; Ashwood et al., 2006; Blaylock, 2009; Blaylock and Strunecka, 2009; Derecki et al., 2010; Enstrom et al., 2010; Maezawa and Jin, 2010; Pardo et al., 2005). Recent epidemiological studies have shown statistical correlations between risk for ASD and either maternal or infantile atopic diseases, such as asthma, eczema, food allergies and food intolerance (Theoharides et al., 2016), and mouse models of allergic asthma priming in the mother results in ASD-like behavior in the offspring (Schwartzer et al., 2015). Microglia appear to be persistently activated or altered in a significant subset of individuals with ASD (Morgan et al., 2010; Pardo et al., 2005; Vargas et al., 2005). These cells are critical for host defense in response to any perturbation of body or brain homeostasis, including infection, trauma, or hypoxia, in part via their production of cytokines, chemokines, and reactive oxygen species (e.g., nitric oxide) (Graeber and Streit, 2010). In response to injury or immune stimulation, microglia up-regulate multiple surface receptors, including those for complement proteins, MHC II (important for antigen presentation), and cytokines, which in turn initiate both repair and cytotoxic processes via interactions with numerous other CNS cell types (e.g., astrocytes, neurons, pericytes) (Rostene et al., 2011). Microglia are a good candidate for long-term or persistent changes and/or pathology within the brain, because these cells are long-lived and can become and remain activated chronically (Town et al., 2005). The molecular mechanisms of “activation” are not well defined, though they have been characterized *in situ* by changes in morphology consistent with injury, e.g. enlarged cell bodies and short, thick processes, a phenotypic switch that has been observed in post-mortem ASD brains in multiple studies (Morgan et al., 2010; Pardo et al., 2005; Vargas et al., 2005). Note that there is also evidence that microglia become *dystrophic* with disease progression, in contrast to sensitized or reactive, which exhibit stripped or disembodied processes and impairments in their normal homeostatic functions such as phagocytosis (Streit et al., 2009; Streit and Xue, 2009). In either case, because microglia are believed to be long-living cells, glial pathology has the capacity to significantly alter neural function and behavior, perhaps over the entire lifespan.

Because the prenatal period is a time of such intense microglial proliferation and activity, we have hypothesized that it is particularly vulnerable to the induction of long-term changes in microglial cell number or function (Bilbo and Schwarz, 2009, 2012; Bilbo et al., 2011). Indeed, our work has demonstrated significant and persistent impacts of perinatal infection or inflammation on microglial development and function in rodents, which produce sensitized inflammatory responses to subsequent insults later in life that are directly implicated in behavioral abnormalities (Bilbo et al., 2005a; Bilbo and Tsang, 2010; Williamson et al., 2011). For the remainder of this review, we focus on the working hypothesis that microglial activation by *non-infectious* environmental factors in pregnancy,

resulting in MIA, is a critical mechanism in the pathophysiology of a significant subtype of ASD, which if true has implications for a wide segment of the population, but also for treatment and prevention strategies. Air pollution, one of the most relevant and pervasive environmental toxins in the modern world, may be a particularly important threat to child health, and increasing evidence links exposure early in life to increased risk of ASD (Carter and Blizard, 2016; Roberts et al., 2013; Volk et al., 2013), a topic we expand upon in the sections that follow.

Environmental Factors and ASD

Diverse exposures to environmental factors, beyond infection, are increasingly recognized as a risk factor for ASD (reviewed in (Carter and Blizard, 2016)). These factors include pesticides, various components of air pollution (diesel exhaust, NO2, heavy metals), phthalates, polychlorinated biphenyls (e.g., BPA), and even dietary components such as common food allergens (e.g., gluten, casein) (Lin et al., 2016). All of these chemicals can impact immune or inflammatory pathways. Though the evidence is primarily derived from large scale epidemiological studies with their inherent caveats regarding causation, the animal literature in support of these associations at a mechanistic level is steadily increasing. Roadway exposures account for the majority of air pollution in the environment, of which diesel exhaust is a primary toxic component (Block and Levesque, 2009; Hartz et al., 2008; Inoue et al., 2006a; Inoue et al., 2006b; Inoue et al., 2006c). Air levels of diesel exhaust at the time and place of birth is one of the strongest and most consistent environmental links to ASD (Roberts et al., 2013; Rossignol et al., 2014). In animal studies, maternal air pollutant chemical exposures during pregnancy have been linked with asthma in offspring, even independent of postnatal exposures (Gilliland et al., 2000; Jedrychowski et al., 2004), suggesting similar impacts are present for the brain. Mechanistic studies have revealed that diesel exhaust particles markedly activate microglia in vitro and in vivo, in adult rodents and canines (Block and Levesque, 2009; Block and Calderon-Garciduenas, 2009; Block et al., 2012; Block et al., 2007; Costa et al., 2017) though less is known about its impact during the prenatal period.

We have recently determined the impact of maternal exposure to diesel exhaust on microglial development in the offspring mouse brain. Pregnant mice received aspirations of vehicle (VEH) or diesel exhaust particles (DEP) intermittently (every 2-3 days) throughout gestation, a regimen which induces significant lung inflammation in the mother similar to that observed in humans (Ghio et al., 2012). We tested the hypothesis that DEP-induced MIA would impact fetal microglial development, with significant consequences for brain development and long-term function. Microglial morphology is dynamic throughout development, and exhibits a stereotypical pattern of maturation in most brain regions, with a transition from a predominantly round or “amoeboid” morphology early in development (E10-14) to one with small cell bodies and long, thin processes by around postnatal day (P) 30 (Figure 1A). In the E18 cortex, maternal DEP exposure increased the number of round microglia compared to offspring of VEH-exposed dams, without any changes in the total number of cells, but notably only in males (Figure 1B; (Bolton et al., 2017)). By P30, when the majority of microglia in normal cortex exhibit small cell bodies with long, thin processes, the number of microglia with thick processes was increased by DEP, once again

independent of changes in total number, and again only in males. These data suggest the maturation of microglia within the cortex was delayed by DEP in males. We saw the same effect in other brain regions including the hippocampus and hypothalamus (not shown), suggesting a global effect on the CNS. Notably, all of the DEP-induced changes in microglial morphology in males were dependent on the presence of the innate immune receptor toll-like-receptor (TLR) 4, as TLR4$^{-/-}$ mice did not exhibit DEP-induced effects in any brain region (Figure 1C). The impact of diesel particles on the airway inflammatory response is dependent on TLR4 (Inoue et al., 2006b), which is classically defined for its recognition of pathogen-associated molecular patterns (PAMPs, e.g. LPS). Increasing evidence shows the function of some TLRs extend beyond that of pathogen recognition to the discrimination of "danger" associated molecules (DAMPs) (Bianchi and Manfredi, 2009; Matzinger, 2002; Seong and Matzinger, 2004), including a class of endogenous molecules known as "alarmins" (e.g., hyaluronic acid, high mobility box group 1 protein (HMGB1), heat shock proteins, fibrinogen) which are released systemically in response to sterile inflammation or in response to multiple chemicals or toxins, e.g. as part of an inflammatory cascade or in response to cell death or tissue distress (Bianchi, 2007) (Figure 2). Interestingly, sensitized monocyte responses to TLR ligands are widely reported in individuals with ASD (Enstrom et al., 2010; Nadeem et al., 2017; Nazeen et al., 2016). *These combined data suggest a model in which prenatal inflammation by air pollution delays the maturation of microglia particularly within the male brain, which we hypothesize leads to long-term alterations in their function, ultimately leading to abnormal neural circuit development and ASD-like behaviors*.

Based on the hypothesis that perturbations of normal microglial development have significant consequences for neural development and thus behavioral function throughout the lifespan, we assessed learning and memory using a fear conditioning test, and anxiety using an elevated zero maze test, in young adult mice exposed prenatally to DEP or VEH; however, there was no impact of DEP on either measure (Bolton et al., 2013). These data suggest that maternal exposure to air pollution, even at a relatively high dose that induces inflammation in the mother and alters microglial development in the fetal (male) brain, is insufficient to cause changes in behavior. Notably, the effect sizes of even the largest single environmental factor associations with ASD are quite small. This strongly suggests that it is the *combination* of multiple perinatal exposures that increases vulnerability in the offspring. Most exposure models from both the pre-clinical and epidemiological literatures suffer from oversimplification, with single factors in isolation, which simplifies models but lends little insight into mechanisms by which diverse environmental exposures combine or synergize to cause pathology. A growing body of research suggests that maternal well-being during pregnancy is a crucial determinant of lifelong physical and mental health of the offspring (Case et al., 2005; Hackman et al., 2010; Susser et al., 1999). Chemical toxins such as lead exposure are well known to adversely affect brain development (Needleman et al. 1990; Weiss and Landrigan 2000). However, "social toxins", such as violence, poverty, and other factors that generate psychological stress in mothers and children, have in recent years gained recognition as risk factors that can also alter the trajectory of brain development (Tamayo et al., 2017; Wright et al., 2010). For example, one study noted an association between air pollution and asthma only in children that were also living with a chronic

stressor (e.g. domestic violence) (Clougherty et al. 2007). Similarly, parental stress can increase the effect of *in utero* toxin exposures (i.e., tobacco smoke) on childhood asthma risk (Shankardass et al. 2009). In such cases, stress may increase vulnerability, permitting a toxin to initiate significant injury to physiological systems when it would have been insufficient to do so in isolation. We hypothesize these synergistic effects of stress and pollutants are possible because they likely act on common biological systems, such as innate immune pathways (Frank et al. 2007; Levesque et al. 2011) within the developing nervous system. Notably, glucocorticoids may upregulate TLRs on microglia, augmenting subsequent neuroinflammatory responses (Frank et al. 2010; Gárate et al. 2012). These findings could explain why a single exposure or risk factor in isolation is a modest predictor of ASD risk (Kinney et al., 2008a; Kinney et al., 2008b; Magnusson et al., 2012; Roberts et al., 2014).

Based on this intriguing literature, we developed a mouse model that combines prenatal exposure to DEP, as described previously, with a novel model of maternal stress (MS), in which the nesting material within the cage is restricted during the final week of pregnancy, a psychological stressor designed to model poor housing conditions (Rice et al., 2008). We predicted the combination of prenatal exposures would activate the maternal immune system, and thereby impact the developing fetal brain (Figure 3). We found significant impacts of combined maternal DEP+MS on offspring communication and social behavior early in life, two of the hallmark behavioral deficits observed in ASD (Towbin et al., 2005; Truitt et al., 2007; White et al., 2009). We assessed ultrasonic vocalizations (USVs) at P5, during a 1 min task in which pups were separated from dam and littermates, and found a significant impact of DEP+MS only in males (Figure 3B). We next assessed social exploratory behavior at P15 (in a behaviorally naïve cohort), a time period when pups become more independent, in a task that assesses time spent in home cage bedding vs. unfamiliar bedding (collected from a conspecific dam and pups). Control pups explored both areas of the arena equally, whereas DEP+MS males and females spent time almost exclusively in home cage bedding. Moreover, male DEP+MS pups took significantly longer to enter the unfamiliar bedding, and overall activity was higher in DEP+MS pups compared to controls (Figure 3C). Thus, DEP+MS males immediately entered the home nest bedding, and were hyperactive in that area once they arrived, with virtually no exploration of the unfamiliar bedding, likely indicative of profound anxiety. Taken together, there are clear social and communication alterations in DEP+MS pups, especially males, at ages similar to the emergence of ASD in humans.

We also assessed learning and memory in young adult (P60) mice from each prenatal treatment group using a fear-conditioning paradigm, as before. Cognitive deficits are a core component of many neurodevelopmental disorders, including ASD (DeLong, 1992; Pellicano et al., 2006; Piven and Palmer, 1997; Rutter, 1983; Sheinkopf, 2005; Solomon et al., 2009). In this task, mice receive a series of foot shocks in a novel context, and increased freezing in that context on a later test day is indicative of intact memory (more freezing = better memory). This type of contextual memory (i.e., memory for the conditioned context) depends on the hippocampus, which is densely populated with glucocorticoid and innate immune receptors (e.g., TLRs), and is often vulnerable to environmental perturbation (stress, trauma) (Williamson and Bilbo, 2013). Connectivity between the hippocampus and cortex is impaired in an animal model of ASD (Zhan et al., 2014). Males but not females exposed

prenatally to combined exposures (DEP+MS) showed contextual memory deficits on the test day. We tested the same animals for anxiety using an elevated zero maze, in which increased time in the dark arms is indicative of greater anxiety. Again, anxiety-like behavior is significantly increased only in DEP+MS males (Bolton et al., 2013) (Figure 3D).

Mechanisms Underlying Long-term Changes in Brain and Behavior

What are the potential mechanisms underlying these persistent behavioral deficits in combined exposure mice? DEP exposure alone impacts the maturation of microglia within the male brain, as described previously, but this is insufficient to impact behavior. We have demonstrated in a different animal model that bacterial infection in newborn rats similarly induces changes in microglial activation and development, and results in profound cognitive deficits in adulthood. However, the deficit is *only* observed if the rats infected as newborns receive a systemic injection of LPS around the time of learning (Bilbo et al., 2005a; Bilbo et al., 2005b). This low dose of LPS is insufficient to impact memory in control rats, as is the neonatal infection in isolation, without the “second hit”. Subsequent experiments demonstrated the systemic LPS causes the microglia of previously infected rats to respond more vigorously, even months later, with an overproduction of the inflammatory cytokine IL-1β, which impairs memory (Williamson et al., 2011), potentially via the downregulation of brain-derived-neurotrophic-factor (Bilbo et al., 2008), which is important for memory consolidation. These data bear a striking similarity to the phenomenon of immunological or glial “priming” that is well described in the neurodegeneration and aging literatures (Cunningham et al., 2009; Godbout and Johnson, 2006; Hennessy et al., 2017; Perry, 2004; Perry et al., 2007; Perry et al., 2010), and increasingly within the delirium literature as well (Maclullich et al., 2013; Sanders et al., 2014). A central characteristic of glial priming is the transition to and maintenance of an “activated” morphology (e.g. amoeboid or reactive) in response to an initial stimulus, e.g. infection, trauma, or cumulative aging alone. However, primed glia do not chronically produce cytokines and other pro-inflammatory mediators typical of cells in an acutely activated state. Upon challenge, however, such as infection or injury in the periphery, these primed cells will over-produce cytokines within the brain compared to cells that were not previously primed or sensitized (Perry et al., 2002). Notably, we have already demonstrated that adult mice born to DEP-exposed dams gain more weight when placed on a high fat “inflammatory” diet as adults, and exhibit greater central and peripheral measures of inflammation; and these effects are once again greater in males than females (Bolton et al., 2014; Bolton et al., 2012).

These collective data suggested to us that microglia may be sensitized or “primed” by DEP, and subsequently over respond to the secondary challenge of maternal stress, which would have persistent consequences for neural development and function. To broadly assess neuroinflammatory changes within the brains of mice in each condition, we measured inflammatory gene expression in whole forebrain of offspring from each group at P30 using a pathway-focused qPCR Array. We analyzed candidate genes implicated within the peripheral immune system in autoimmune disorders such as asthma, which are known to be impacted by DEP and which we suspected may also be altered in the brain. Only 2 genes were significantly altered, and only in the male offspring of DEP+MS dams: **toll-like-receptor (TLR) 4** and **caspase 1**, the enzyme which converts the pro-inflammatory cytokine

IL-1β into its active form downstream of TLR4 ligation (Figure 3E). As introduced previously, TLRs are a family of highly conserved pattern recognition receptor proteins that transduce signals through cytosolic adapter proteins (e.g. myeloid differentiation factor 88 (MyD88)) following ligation by PAMPS (e.g. LPS) or DAMPS (e.g. HMGB1), that link to downstream signaling cascades, notably the activation of the transcription factor NF-kB, which is key to subsequent cytokine release. TLRs 2, 3, and 4 are the best characterized within the mammalian CNS, and growing evidence demonstrates their recognition of putative DAMPS, including a wide array of environmental factors that are hypothesized to directly or indirectly activate TLRs (Seong and Matzinger, 2004); Figure 2; notably TLR2 and 4 are primarily expressed by microglia within the CNS (Zhang et al., 2014).

The “double hit” of DEP plus maternal stress exposure required to lend significant, persistent gene changes in male mice is intriguing given the critical function of TLRs within the inflammasome pathway. IL-1β is transcribed by immune cells including microglia as a larger pro-hormone, pro-IL-1β, which must be cleaved by caspase-1 to form the biologically active, mature form of the protein. The process of cleaving pro-IL-1β often requires the formation of an inflammasome, an intracellular multiprotein complex that mediates processing and maturation of IL-1β via activation of caspase-1 (Martinon et al., 2009). Importantly, signaling at TLR4 initiates the intracellular signaling cascade to activate NF-kB and the subsequent production of Nod-Like-Receptor family Pyrin domain containing 3 (NLRP3) (Kawai and Akira, 2010), which is a critical step in the formation of the inflammasome (Figure 4). The inflammasome is of particular interest for our model because it requires a priming step and an activation step (Hornung and Latz, 2010), and there is evidence that glucocorticoids can act as a priming stimulus (Busillo and Cidlowski, 2013; Frank et al., 2016). Our data suggest the opposite may also occur; i.e. that DEP exposure (or, presumably, a broad array of environmental exposures) is the priming stimulus, and maternal stress-induced DAMP production is the activation step. Both DEP and stress are likely activating the inflammasome within microglia indirectly via the release of an endogenous DAMP(s), which remain to be characterized. Notably, a recent report shows multiple inflammasome complexes are activated in peripheral blood cells of individuals with ASD, along with increased IL-1β (Saresella et al., 2016b), suggesting that regardless of initial priming/activation stimuli, the inflammasome pathway may represent a novel target for treatment.

Consistent with the hypothesis of inflammasome activation, male offspring exposed to DEP +MS exhibit consistently increased levels of IL-1β protein along with increased caspase-1 mRNA, and *decreased* levels of anti-inflammatory IL-10 protein within the hypothalamus, hippocampus, and cortex, whereas the same brain regions in females showed the opposite pattern. Overall, DEP+MS males exhibit a greater pro-inflammatory bias (IL-1β/IL-10 ratio) than all other groups (Figure 3F), while females exhibit an intriguing anti-inflammatory bias driven by high IL-10 (Bolton et al., 2013). This constitutive change in cytokine protein was nonetheless quite surprising, as cytokine expression is exquisitely and tightly regulated within the CNS (Yirmiya and Goshen, 2011); however, a persistent neuroinflammatory process would agree with the stable pathophysiology thought to underlie ASD and associated behavioral dysfunction. Notably, elevated circulating levels of IL-1β have often been reported in ASD, though it is unclear if this is true for the brain (Masi et al., 2015;

Saresella et al., 2016a). Subsequent analyses in our experiments revealed striking correlations between the behavioral deficits in male mice, and their brain levels of IL-1β. Specifically, high brain levels of IL-1β in DEP+MS males were significantly correlated with impaired memory and increased anxiety (Bolton et al., 2013). Moreover, these same mice produce exaggerated levels of peripheral IL-1β in response to a systemic LPS injection (Bolton et al., 2014), similar to that reported in ASD patients (Masi et al., 2015; Saresella et al., 2016a).

In sum, these data suggest a persistent priming or sensitization of the TLR/IL1/inflammasome pathway in this multiple exposure MIA model, especially in males, which lends enduring ASD-relevant behavioral deficits. These data are compelling given a recent transcriptomic meta-analysis of control vs. ASD genetic data sets showing a significant convergence onto innate immune genes - and TLR genes in particular - between ASD and co-morbid conditions such as asthma, inflammatory bowel disease, and others (Nazeen et al., 2016), and lend further strength to the hypothesis that there is a meaningful, neuroinflammation-linked subtype of ASD.

Conclusions

Taken together, both the associations between immune system dysfunction and ASD, and the list of environmental contributors to ASD, appear to be increasing. Existing epidemiology and association studies in humans are intriguing but difficult to ascribe causality; thus there is an urgent need for animal models that incorporate multiple factors in a rigorous and mechanistic way. Dr. Paul Patterson laid the critical groundwork for the hypothesis that MIA by infection leads to altered neural development and thus behavioral dysfunction throughout life, with relevance to ASD and other neurodevelopmental disorders. The important work of many labs has just begun to build upon this foundation, both to unravel the mysteries of how and why ASD rates continue to rise, and to determine if and how immune system (dys) function in response to diverse environmental factors and gene × environment interactions during neurodevelopment may hold an important key. We believe this knowledge will be critical for prevention and treatment going forward.

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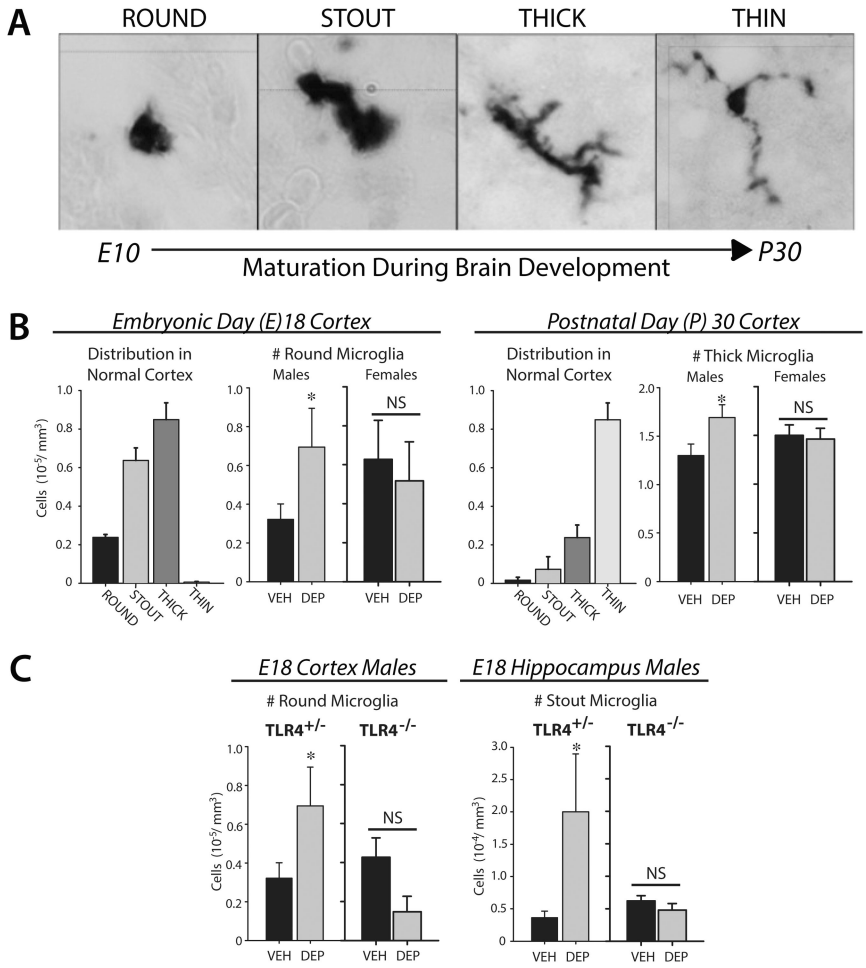

Figure 1.

(A) Microglia transition from a round/amoeboid morphology early in development (~E10, when they first begin to colonize the CNS) to a ramified morphology with small cell bodies and thin processes by P30. **(B)** DEP increases the number of round microglia in cortex at E18, and the number of thick microglia at P30, in males only, suggesting DEP delays their maturation and/or leads to their activation. There are similar effects in other brain regions including hippocampus (not shown) **(C)** All of the effects of DEP in males are dependent on TLR4. *p<0.05 for all; NS = not significantly different.

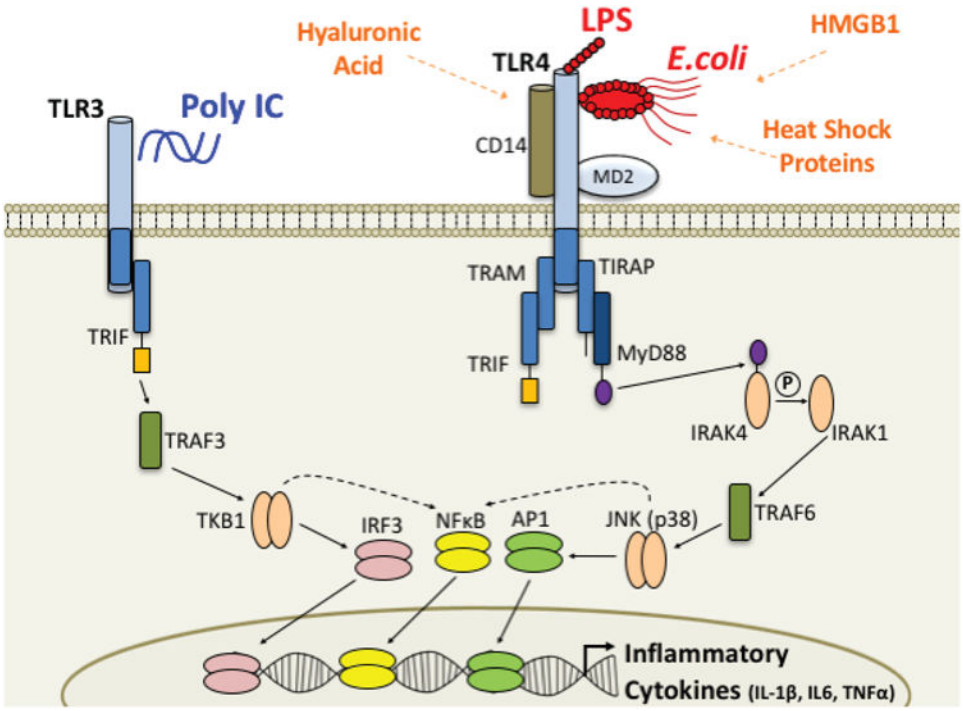

Figure 2.

Toll-like receptors (TLR)s are the primary innate immune receptors which recognize pathogens in the body and brain. TLR4 recognizes bacteria, such as E. coli, and lipopolysaccharide (LPS), which is the cell wall component. TLR3 recognizes double-stranded RNA, the genetic code for viruses, as well as viral mimetics, such as Poly IC. Though TLR4 and TLR3 activation induces very different intracellular signaling pathways, activation of either receptor results in the transcription and translation of many similar inflammatory cytokines. The role of these receptors has recently expanded to include the recognition of more general “danger” associated molecular patterns (DAMPs). TLRs, including both TLR4 and TLR2, can identify and respond to DAMPs that are produced and released by nearby cells undergoing cell death or distress. To date, proteins that have been identified as DAMPs include hyaluronic acid, Heat Shock Proteins, and high mobility group box (HMGB) 1. CD14 = cluster of differentiation 14, TRIF = Toll-like receptor adaptor molecule, TIRAP = Toll-interleukin 1 receptor (TIR) domain containing adaptor protein, MyD88 = Myeloid differentiation primary response gene (88), IRAK = interleukin-1 receptor-associated kinase, TRAF = TNF receptor-associated factor, JNK = c-Jun N-terminal kinase, TKB1 = a tyrosine kinase, AP1 = activator protein 1 transcription factor, NFκB = nuclear factor kappa-light-chain-enhancer of activated B cells transcription factor, IRF3 = interferon regulatory factor 3 transcription factor. Adapted from (Schwarz and Bilbo, 2011).

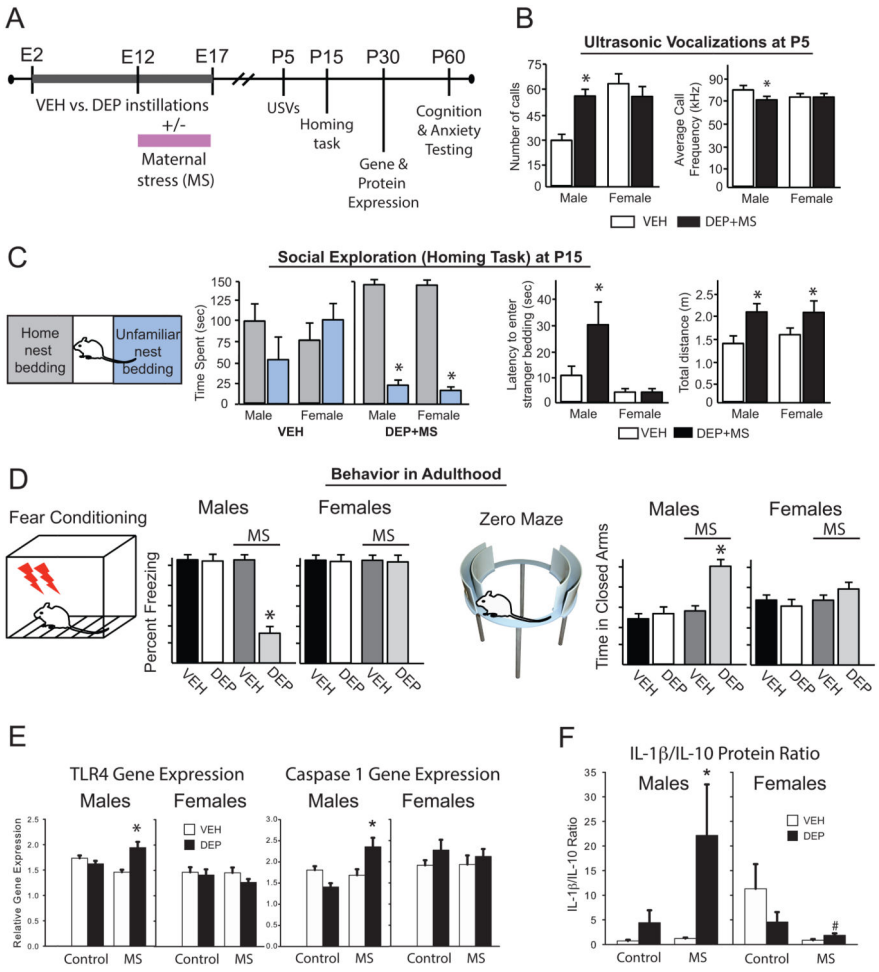

Figure 3.

(A) Experimental timeline, treatments, and outcomes assessed. **(B)** USVs were collected during a 1 min separation from dam and littermates. **(C)** P15 pups were placed into the center of a novel arena with equal portions of home cage vs. unfamiliar nest material, and total activity, latency to enter and time spent in each portion were assessed. **(D)** Cognitive and anxiety testing in adulthood, see text for details. **(E)** Inflammatory gene expression and **(F)** cytokine protein in whole brain at P30; *p<0.05. VEH = vehicle control, DEP = diesel exhaust particles, MS = maternal stress.

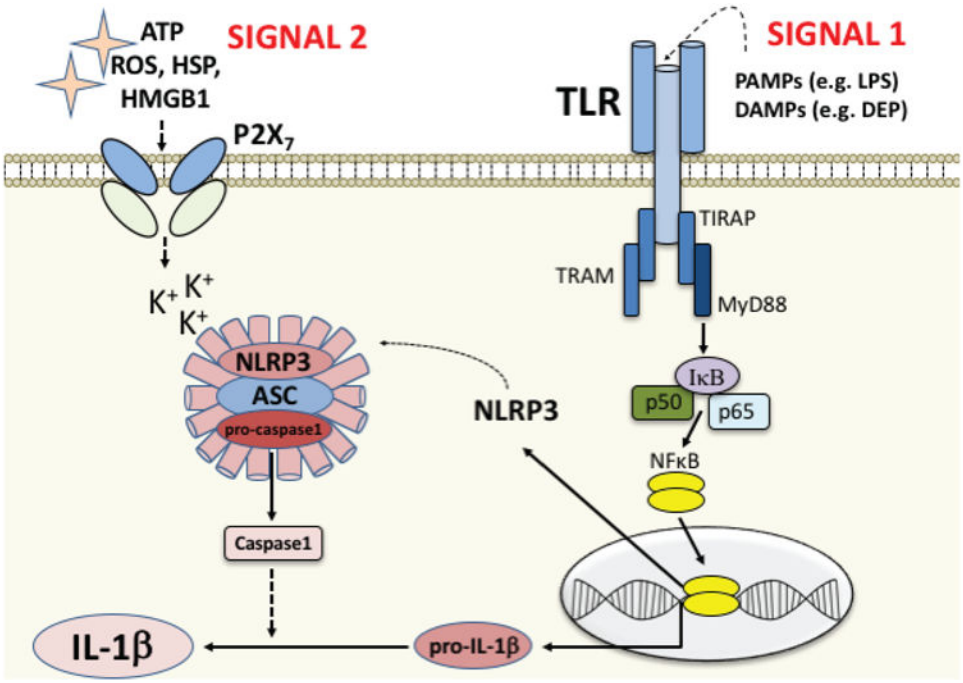

Figure 4.

The process of cleaving pro-IL-1β into the mature, active protein often requires the formation of an inflammasome, an intracellular multiprotein complex that mediates processing and maturation of IL-1β via activation of caspase-1. This signaling cascade requires both a *priming* step (**SIGNAL 1**, acting on the TLR), and an *activation* step (**SIGNAL 2**, acting on, e.g. a purinergic receptor such as $P2X_7$ or a variety of other scavenger receptors, see Hornung and Latz, 2010). SIGNAL 1 candidates include both exogenous ligands/PAMPs such as LPS, and endogenous "danger" signals/DAMPs that are released in response to toxins such as DEP (or cellular damage induced by DEP). Ligation of the TLR by SIGNAL 1 initiates the intracellular signaling cascade to activate the transcription factor NFkB and the subsequent production of pro-IL-1β, as well as Nod-Like-Receptor family Pyrin domain containing 3 (NLRP3). NLRP3 then translocates to the cytosol to form a complex with the proteins ASC and pro-caspase1, a critical step in the formation of the inflammasome. SIGNAL 2 candidates include ATP, reactive oxygen species (ROS), heat shock proteins (HSP), HMGB1, and many others, released in response to cellular distress or tissue damage. This second signal activates the inflammasome complex for cleavage of pro-IL-1β into IL-1β and release.