MATERNAL EXPOSURE TO AIR POLLUTANT $PM_{2.5}$ INDUCES AUTISTIC-LIKE BEHAVIOR IN ADULT OFFSPRING MICE

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A thesis submitted in partial fulfilment of the requirements for the degree of Master of Philosophy

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CERTIFICATE OF ORIGINALITY

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Abstract of thesis of entitled

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Abstract

Autism spectrum disorders (ASD) is a neurodevelopmental condition with heterogeneous etiology. Core behavioral deficits commonly exhibited by individuals with ASD include impaired social communication and interaction skills, repetitive behavior, and intellectual disability with wide degrees of severity among individuals. ASD not only hinders an individual's physical and psychological development, it also adversely affects their families' daily life. Numerous studies undertaken in Asia, Europe, and North America have estimated the incidence rate of ASD to be between 1% and 2%, with a higher frequency in males than in females (4:1).

Emerging studies have indicated the significant influence of environmental factors on increasing ASD susceptibility. According to a recent study, approximately 20% of 215 ASD candidate genes are epigenetic regulators, highlighting the importance of the interaction between genetic and environmental variables on the etiology of ASD. It has been speculated that gene and environment interactions are contributors to the increased prevalence of ASD.

Amongst the various environmental factors, the surge in air pollutants in the environment is an emerging concern. Epidemiological studies have divulged into the relationship between long-term prenatal or postnatal exposure to hazardous air pollutants

being potentially linked to ASD. Ambient air pollutant particulate matter (PM) has been associated with adversely affecting neurodevelopment. Prenatal and postnatal exposure to PM with sizes less than 2.5 μm ($PM_{2.5}$) is significantly correlated with an increased risk of ASD, suggesting the potential role of $PM_{2.5}$ exposure in increasing ASD susceptibility.

Maternal exercise has been suggested that a long-lasting improvement and transgenerational neuroplasticity could be induced by maternal exercise in human brains. In this project, I aim to investigate the potential effects of maternal exposure to $PM_{2.5}$ on causing autism-like behaviors in offspring, and to examine whether maternal running could protect the offspring from developing autistic phenotypes. The results have revealed that maternal exposure to $PM_{2.5}$ induced core ASD behavior, including impaired social recognition memory and increased repetitive behavior, as well as, impairment in hippocampal dependent learning and memory performance. Maternal running was able to reverse core ASD-like behavior including social recognition memory and repetitive behavior, but not learning and memory impairment. The results also showed that maternal exposure to $PM_{2.5}$ impaired the dendritic development of immature neurons in the hippocampal dentate gyrus but did not affect hippocampal neurogenesis and neuroinflammation. I hypothesize it may be due to difference in maturation and integration into neural circuitry as indicated by the reduction in total dendritic length of immature neurons in offspring with maternal PM2.5 exposure. Other mechanisms including changes in oxidative stress, gene expression and gut microbiota profile could also play a role in underlying behavioral deficits as we observed in our model. The underlying mechanisms warrant further investigation.

Publications

1. **Ahadullah***, Yau, S. Y*., Lu, H. X., Lee, T. M., Guo, H., & Chan, C. C. (2021). PM2. 5 as a potential risk factor for autism spectrum disorder: its possible link to neuroinflammation, oxidative stress and changes in gene expression. *Neuroscience & Biobehavioral Reviews, 128*, 534-548.
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2. Lee, T. H, **Ahadullah**, Christie, B. R., Lin, K., Siu, P. M. F., Zhang, L., Yuan, T. F., Komal, P., Xu, A., So, K. F., & Yau, S. Y. (2021). Chronic AdipoRon Treatment Mimics the Effects of Physical Exercise on Restoring Hippocampal Neuroplasticity in Diabetic Mice. *Molecular Neurobiology*, 1-16.

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Table of Contents

Chapter 1

Literature Review[1]

Autism spectrum disorder

Autism spectrum disorder (ASD) is a neurodevelopmental condition with heterogeneous etiology. Individuals with ASD display core behavioral deficits, including impaired social communication and interaction skills, repetitive behavior, and intellectual disability with wide degrees of severity among individuals (Happé & Ronald, 2008; Lintas & Persico, 2009). Apart from hindering an individual's physical and psychological development, ASD also adversely affects the daily life of individuals and their families (Rao & Beidel, 2009). The prevalence rate of ASD has been reported to be in the range of 1–2% according to numerous studies conducted in Asia, Europe and North America, with a higher prevalence in males than in females (4:1) (Elsabbagh et al., 2012). The increasing prevalence rate could be partly due to the increase in awareness, reclassification, or improved diagnosis of ASD (Faras et al., 2010). Previous twin studies by Folstein and Rutter (1977) have offered initial evidence showing the contribution of genetic factors to ASD. Emerging studies have indicated the significant influence of environmental factors on increasing ASD susceptibility. It was recently reported that approximately 20% of 215 ASD candidate genes are epigenetic regulators (Roberts et al., 2013), suggesting the importance of the interaction between genetic and environmental factors on the etiology of ASD. Gene and environment interactions are thought to contribute to the increased prevalence in ASD (Persico & Merelli, 2014). In particular, prenatal exposure to air pollution, dietary nutrients, heavy teratogenic drugs or congenital viral infections have been linked to an increased incidence of ASD. In addition, prenatal exposure to folic acid (Bakulski, 2019) or certain heavy metals that can pass through the placenta to the fetus (Sakamoto et al., 2004) increases DNA methylation, providing evidence of possible epigenetic changes involved in ASD.

Among the environmental factors, the increase in air pollutants in our living environment is an emerging concern. Epidemiological studies have revealed that long-term prenatal or postnatal exposure to hazardous air pollutants is potentially linked to ASD. Ambient air pollutants, such as nitrogen dioxide (NO_2) and particulate matter (PM), could

[1] Portion of this literature review was partly adopted from a previously published review paper:
Ahadullah, Yau, S. Y., Lu, H. X., Lee, T. M., Guo, H., & Chan, C. C. (2021). $PM_{2.5}$ as a potential risk factor for autism spectrum disorder: its possible link to neuroinflammation, oxidative stress and changes in gene expression. *Neuroscience & Biobehavioral Reviews*. *128,* 534-548

adversely affect neurodevelopment. Several studies have shown that prenatal and postnatal PM_{10} exposure has no correlation with the increased risk of developing ASD (Ritz et al., 2018; Heather E Volk et al., 2013). Some studies have shown a positive correlation between prenatal exposure to NO_2 and the incidence of ASD (Becerra et al., 2013; Raz et al., 2015), while other studies have reported no correlation between prenatal and postnatal NO_2 exposure and ASD (Fortoul et al., 2015; Heather E Volk et al., 2013). Therefore, the effects of NO_2 exposure on ASD remain uncertain. In contrast, prenatal and postnatal exposure to PM with sizes less than 2.5 μm are significantly correlated with an increased risk of ASD (Heather E Volk et al., 2013), suggesting the potential role of $PM_{2.5}$ exposure in increasing ASD susceptibility. This could be because $PM_{2.5}$ contains polycyclic aromatic hydrocarbons, metals, organic matter, and elemental carbon, which are potentially neurotoxic. Exposure to these neurotoxic contents induces inflammation, generates reactive oxygen species (ROS), and alters gene expression, which could possibly contribute to the development of ASD (Association, 2013; J. L. Silverman et al., 2010).

Behavioral abnormalities in individuals with ASD

According to the *Diagnostic and Statistical Manual of Mental Disorders* (5th ed. DSM-5), the diagnostic manual of the American Psychiatric Association, and the 10th revision of the International Statistical Classification of Diseases and Related Health Problems (ICD-10) by the World Health Organization, individuals with ASD often demonstrate three main core deficits: social impairment, communication difficulties, and rigid and repetitive interests and activities (Redcay & Courchesne, 2005). The *DSM-5* includes the behavioral symptoms along a severity continuum and several diagnostic types falling under the umbrella of ASD (*i.e.* autistic disorder, Asperger's disorder, childhood disintegrative disorder, and the catchall diagnosis of pervasive developmental disorder not otherwise specified). An individual with atypical social communication and interactions can be classified as having a lack of interest in others and exhibiting reduced eye contact and facial expression during communication. Additionally, affected individuals could also have poor language comprehension and responses. Moreover, repetitive behaviors are common, with diagnosed individuals often repeating their movement and usage of objects. The symptoms may present before the age of 2 and affect daily functioning (Courchesne et al., 2011). Rodent models of ASD display certain behavioral abnormalities that are equivalent to behavioral phenotypes of ASD in individuals, including impairments in social interaction

and communication, cognitive impairment and repetitive behaviors.

Hippocampal abnormalities in individuals with ASD

Behavioral abnormalities could be associated with changes in brain volume in individuals with ASD. At birth, infants with ASD were found to have a lower total brain volume than healthy infants, followed by overgrowth within the first year of life (Aylward et al., 2002). Up until the age of 4, children diagnosed with ASD showed a larger brain volume than healthy individuals. Progressing to late-childhood, puberty, and adolescence, individuals with ASD experience delayed brain growth (Bailey et al., 1993; Brambilla et al., 2003; Tsatsanis et al., 2003). The region of the hippocampus in children with ASD have provided inconsistent result; some studies reported an increase in volumes(Schumann et al., 2004; Sparks et al., 2002), and some reported decrease in volume (Aylward et al., 1999). Some studies have even reported no change in the hippocampus (Nickl-Jockschat et al., 2012; Saitoh et al., 2001; Sparks et al., 2002). In the study by Nicolson et al. (2006), they also reported no difference in traditional measures of hippocampal volume. However, using computational mapping methods, the three-dimensional parametric surface meshes, and shape analysis revealed subtle reduction. Further investigation of the morphology of hippocampal neurons in autistic children revealed that cornu ammonis (CA) 1 and CA4 neurons were small and densely packed, as well as, reduced dendritic branching and complexity (Bailey et al., 1998; Bauman & Kemper, 1985; Raymond et al., 1995). Single concentration ligand binding studies indicate that the GABAergic receptor system is significantly reduced, marking an abnormality in the inhibitory network in autism. In contrast, the density and distribution of the serotoninergic [5-HT], cholinergic and glutamatergic receptors studied in the hippocampus did not demonstrate any statistically significant differences in binding (Blatt et al., 2001). Some evidence suggests that human variants of the serotonin transporter gene SLC6A4 may be associated with ASD and in particular with the presence of rigid-compulsive behaviors and tactile hypersensitivity (Sutcliffe et al., 2005).

The post-mortem studies of two fragile-X syndrome brains revealed abnormalities in the hippocampus, particularly in the CA1 region (Greco et al., 2011). The CA1 pyramidal cell layer displayed an increase in the number of cells in an undulating pattern, whilst the surrounding region had a reduction in pyramidal cells (Greco et al., 2011). In fragile X syndrome, fragile X mental retardation protein (FMRP) is credited to be the cause of the

condition (Guo et al., 2011). Extensive studies in the knock-out models of FMRP revealed similar phenotypes as ASD. Thus, knock-out model of FMRP is commonly used to study the characteristics and treatment of ASD. The hippocampal region has shown abnormalities in the knock-out models, exhibiting abnormal neurogenesis and impaired synaptic plasticity (Guo et al., 2011; Guo et al., 2012). Guo et al. (2012) reported a decrease in neuronal differentiation but an increase in astrocyte differentiation. Apart from this, they also reported dendritic development is also impaired in fragile X syndrome. The dendrites observed showed a reduction in length and complexity and an increase in the density of spines. Guo et al. (2011) also reported an increase in immature spines in the DG.

In mice lacking tryptophan hydroxylase 2, the rate-limiting enzyme in serotonin synthesis in the brain, deficits in social behavior and cognitive flexibility have been observed (Guo & Commons, 2017), supporting the possibility that serotonin is a common factor in ASD. Another autism mice model, BTBR, exhibits reduced social approach behavior and restricted-repetitive behaviors, common diagnosed ASD behavior. It has been speculated that the behavior ASD phenotype in the BTBR model is also caused by an imbalance in the serotonergic system. This is supported by the reported reduction in serotonin tissue content, density of serotonin axons and levels of 5-HT in the hippocampus (Guo & Commons, 2017; Onaivi et al., 2011).

Clinical evidence showing a linkage between $PM_{2.5}$ exposure and risk of ASD

Several clinical studies have found that both prenatal and postnatal exposure to $PM_{2.5}$ could increase the risk of developing ASD in offspring (Becerra et al., 2013; Raz et al., 2015; Talbott et al., 2015). Prenatal exposure to $PM_{2.5}$ is associated with an increased risk of ASD (Becerra et al., 2013). Becerra et al. included 7,603 children with ASD, and 75,782 children without ASD in their study in Los Angeles, California. This study reported an increase in the adjusted odds ratio (AOR) with $PM_{2.5}$ exposure during the entire pregnancy period, suggesting that maternal inhalation of $PM_{2.5}$ increases the risk of ASD in humans. AOR is a measure of association between an exposure and an outcome with controlled variables. Likewise, Talbott et al. (2015) affirmed that prenatal exposure to $PM_{2.5}$ could increase the risk of ASD. Their study involved 245 children with ASD and 1,522 children without ASD from 14 states in the United States. The AOR for ASD during pregnancy per 4.4 $\mu g/m^3$ increase in $PM_{2.5}$ is 1.57. Among the three trimesters of pregnancy, the AOR was largest in the third trimester (1.42), followed by the first and second trimester

(1.06 and 1.00), concluding that exposure to $PM_{2.5}$ in the third trimester could lead to the highest risk of ASD. In addition, H. E. Volk et al. (2013) found that both prenatal and postnatal exposure to $PM_{2.5}$ is associated with an increased risk of ASD. By adjusting for the sex and ethnicity of the participants, as well as their parents' educational levels, maternal age, and prenatal smoking, the AORs of ASD for the 279 children with ASD and the 245 control children without ASD in California were 2.08 (during entire pregnancy) to 2.12 (during the first year of life) per 8.7 $\mu g/m^3$ increase in $PM_{2.5}$ concentration during the period. These results suggest that early-life exposure could increase the risk of ASD as much as prenatal exposure to $PM_{2.5}$.

Similarly, Chen et al. (2018) reported that both prenatal and postnatal exposures to $PM_{2.5}$ are associated with an increased risk of ASD. The AOR of ASD per 2.84 $\mu g/m^3$ increase in $PM_{2.5}$ during the second year of life is the highest (1.45), followed by the first year of life (1.37). The AOR during pregnancy (1.20) was relatively lower, and the differences in odds ratios between the first (1.07), second (1.04) and third trimesters (1.04) were smaller. Taken together, the increased risk of ASD associated with $PM_{2.5}$ is more significant during the postnatal period than during the prenatal period, consistent with the findings of Talbott et al. (2015). Similarly, a recent case control study in China studying the effect of $PM_{2.5}$ exposure during the first 3 years of infancy revealed consistent findings of an increased risk of developing ASD (Chen et al., 2018). The aforementioned case control studies have also revealed that both prenatal and postnatal exposure to $PM_{2.5}$ could increase the risk of ASD. The AOR of postnatal studies are larger than those of prenatal studies, indicating that postnatal exposure to $PM_{2.5}$ may impose a higher risk of developing ASD than prenatal exposure (Raz et al., 2015; Talbott et al., 2015). Based on prenatal studies, exposure to $PM_{2.5}$ in the first trimester and the third trimester (Chen et al., 2017; Power et al., 2018) may have a more prominent impact. According to the postnatal studies, exposure to $PM_{2.5}$ in the second year of life has a larger impact than that in the first year (Chen et al., 2018). However, the convergent findings on the $PM_{2.5}$ exposure and risks of ASD from the above studies should be interpreted with caution, given that the composition of the $PM_{2.5}$ studied could be different across countries or regions.

$PM_{2.5}$ exposure induced hippocampal abnormalities

The current understanding of ASD is associated with brain abnormalities observed in individuals with ASD. Power et al. (2019) found that long-term exposure to $PM_{2.5}$ is

associated with reduced deep-grey volume, indicating that $PM_{2.5}$ might induce cumulative brain damage and atrophy. MRI imaging was employed on subjects on a regular basis to examine their brain structures. Monthly $PM_{2.5}$ exposure was predicted by validated spatiotemporal statistical models using addresses of the participants. The results showed an association between higher mean $PM_{2.5}$ exposures in the past 5 to 20 years and a smaller brain volume. Furthermore, elderly women who reside in areas with high $PM_{2.5}$ levels showed a decrease in whole brain volume (Atladóttir et al., 2010). There were significant volume reductions in the white matter and grey matter of the frontal, parietal, temporal lobes, and corpus callosum. The association between $PM_{2.5}$ and changes in brain volume did not demonstrate a correlation with the sociodemographic factors, socioeconomic status, lifestyle factors, or other clinical characteristics, suggesting that postnatal exposure to $PM_{2.5}$ may act on the brain directly during brain development to induce neurological changes in ASD.

Emerging animal studies have confirmed that exposure to $PM_{2.5}$ can induce neuronal atrophy in various brain regions. Mice exposed to $PM_{2.5}$ caused an increase in phosphorylation of tau protein and malondialdehyde (MDA) in the hippocampus, but not amyloid-beta protein in Alzheimer disease mice models (Lee et al., 2021). $PM_{2.5}$ exposure increased hippocampal expression of miR-3560 and let-7b-5p, which are the translating proteins that regulate genes Oxct1 and Lin28. Oxct1 and Lin28 regulate ketogenesis and glycosylation, and neural cell differentiation (Chao et al., 2017). The disruption in a metabolic pathway supports the notion that $PM_{2.5}$ induces neuronal degeneration, alters neurogenesis, hence leading to a disruption in hippocampal function. The changes observed could be caused by an increase in $PM_{2.5}$ induced apoptosis as reported by Q. Zhang et al. (2018), showing an increase in apoptosis related protein increase myelin sheaths damage. The transgenerational effect of $PM_{2.5}$ has also been shown, although the mechanism(s) are still largely unknown. T. Zhang et al. (2018) showed that chronic intratracheal instillation of $PM_{2.5}$ at medium (1.56695 μg/μL) and high dosages (3.456 μg/μL) during maternal pregnancy significantly reduced the number and diameter of neurons in the cerebral cortex of offspring mice. High dose $PM_{2.5}$ exposure also reduces the number of presynaptic vesicles in offspring mice, suggesting detrimental effects of $PM_{2.5}$ exposure on synaptic plasticity in offspring. Raymond et al. (1995) reported that mice with short-term $PM_{2.5}$ exposure displayed a significant reduction in total apical dendritic length in the CA1 region of the hippocampus. K. Li et al. (2018) exposed mice to $PM_{2.5}$ at a dosage of 16.85 $\mu g/m^3$ using a mobile trailer exposure system for 5 days per week continuously for 10 months. The results revealed that long-term exposure to $PM_{2.5}$ significantly reduced apical spine density in the

CA1 region, decreased apical dendritic length and reduced dendritic complexity of pyramidal neurons in the CA3 region of the hippocampus. The hippocampus plays an important role in learning and memory formation and emotional control. Since hippocampal impairment is observed in individuals with ASD (Davis III, 2014), these findings may support the notion that chronic exposure to $PM_{2.5}$ could impair learning and memory, and induce emotional dysregulation associated with ASD.

$PM_{2.5}$ induced autism-like phenotypes in rodents

Emerging clinical studies have suggested a possible linkage between $PM_{2.5}$ exposure and the risk of developing ASD. Recent animal studies have supported the potential harmful effect of $PM_{2.5}$ on brain health and behavioral deficits that resembles some symptoms in individuals with ASD. T. Zhang et al. (2018) found that $PM_{2.5}$ exposure in young pups resulted in autistic-like behaviors. Experimental rats were subjected to intranasal instillation once daily during postnatal days 8 to 22 with two different dosages of $PM_{2.5}$ (2 µg or 20 µg/g of body weight). Their results showed a significantly lower intensity of sound generated by $PM_{2.5}$-exposed pups through ultrasonic vocalization analysis. Pups exposed to 20 µg/g $PM_{2.5}$ also showed significantly less interaction time to stimulus rats and spent less time sniffing social odors than unexposed groups. The behavioral data suggested that exposure to $PM_{2.5}$ could induce communication and social interaction deficits in young pups. In addition, $PM_{2.5}$-exposed rats spent significantly less time exploring new objects than control animals in the novel object recognition test, indicating an increase in anxiety-like behavior and repetitive behavior. Rats exposed to an increased concentration of $PM_{2.5}$ buried fewer marbles. Although contradictory to ASD behavior, the authors hypothesized that $PM_{2.5}$-exposed rats could have exhibited novelty avoidance due to increased anxiety levels.

Zheng et al. (2019) reported that maternal $PM_{2.5}$ exposure during pregnancy-induced autistic-like behaviors in offspring mice. Pregnant mice were subjected to $PM_{2.5}$ at three different concentrations [0.2592 µg/µL (low dose), 1.56695 µg/µL (intermediate dose) and 3.456 µg/µL (high dose)] via intratracheal instillation throughout pregnancy. The results showed a significant decrease in the number and diameter of neurons in a dose dependent manner. However, only maternal treatment with $PM_{2.5}$ at intermediate or high doses impaired the ultrastructure of mitochondria, including broken and partly blurred mitochondrial cristae, fuzzy and broken nuclear membranes, and autophagic bodies. Moreover, the high-dose treatment group displayed synaptic impairment with decreased

presynaptic vesicles in the synapses; increased cell apoptosis; and increases expression of apoptotic proteins, including Caspase-8 and Caspase-9; and decreased expression of Bcl2/Bax. Apart from apoptotic proteins, cell proliferation was decreased in the cerebral cortex. Likewise, $PM_{2.5}$ exposure induces neuronal damage and apoptosis in the CA3 region of the hippocampus (Church et al., 2018). This study also showed that $PM_{2.5}$ exposure increased depression- and anxiety-like behavior, but decreased locomotor activity. Further studies have shown that maternal exposure to $PM_{2.5}$ impairs spatial learning and memory in offspring mice (Vargas et al., 2005). These changes could be due to neuroinflammation in the hippocampus as indicated by the increased levels of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB), tumor necrosis factor αlpha (TNF- α), and interleukin 1 beta (IL-1β).

The effects of prenatal and postnatal exposure to $PM_{2.5}$ have also been reported by (Church et al., 2018), who exposed pregnant mice to 135.8 ug/m^3 $PM_{2.5}$ for 6 hours/day from gestational day 1 to day 17, followed by postnatal exposure for 2 hours/day for 10 days continuously. Compared to the control group exposed to filtered air with a $PM_{2.5}$ concentration of 3.1 ± 1.04 μg/m^3, the $PM_{2.5}$-exposed group displayed a reduction in sociability score in the social approach task. Male offspring mice showed significantly reduced time in social interaction, total social time, anogenital sniff and body sniff time. Moreover, male mice spent significantly more time self-grooming than the control group. These data suggested that $PM_{2.5}$ could induce autistic-like behavior in mice similar to the behavioral deficits observed in individuals with ASD.

$PM_{2.5}$ induced neuroinflammation in ASD

Clinical studies have shown that individuals with ASD differ in levels of inflammatory biomarkers compared to healthy controls. Vargas et al. (2012) reported remarkable reactivity of astrocytes in the cerebral cortex and cerebellum, as well as an increase in the volumes of perikarya and glial processes in the cerebral cortex, white matter, and cerebellum of individuals with ASD. The findings suggest an increase in astroglia reactions in association with ASD. Chronic neuroinflammation has also been found in postmortem examinations of individuals with ASD (Morgan et al., 2010). ASD patients display an increase in neuroinflammation as indicated by the increased number of microglia in the fronto-insular and visual cortices (Vargas et al., 2005), the dorsolateral prefrontal cortex (Al-Ayadhi, 2005), and the cerebellum (Ashwood et al., 2011).

Aside from cellular changes, proinflammatory cytokines; IL-1β, IL-6, IL-8, T helper type 1 (th1), T helper type 2 (th2), interferon gamma (IFN-γ), TNF- α, and transforming growth factor beta 1 (TGF-β1) are elevated in various brain regions and in the serum of individuals with ASD (Al-Ayadhi, 2005; Basheer et al., 2018; Chez et al., 2007; Emanuele et al., 2010; Hu et al., 2018; Li et al., 2009; Molloy et al., 2006; Ricci et al., 2013; Suzuki et al., 2011; Tonhajzerova et al., 2015; Vargas et al., 2005; Wei et al., 2011; Xie et al., 2017). IL-1β levels are elevated in the frontal cortex (Xie et al., 2017) and the serum (Ricci et al., 2013; Vargas et al., 2005; Wei et al., 2011) of individuals with ASD. In addition, IL-6 levels are elevated in the cerebellum (Vargas et al., 2005), mid-frontal (Li et al., 2009), cingulate gyrus (Al-Ayadhi, 2005), frontal cerebral cortex (Basheer et al., 2018), and serum (Li et al., 2009; Ricci et al., 2013; Vargas et al., 2005), and IL-8 levels are increased in the frontal cerebral cortex (Suzuki et al., 2011), cerebrospinal fluid (Ashwood et al., 2011) and plasma (Ashwood et al., 2011; Tonhajzerova et al., 2015; Vargas et al., 2005). Notably, IL-1β, IL-6 and IL-8 cytokines have been shown to be associated with behavioral impairment (Vargas et al., 2005), suggesting that dysfunction in the immune system could be a contributor to behavioral abnormalities in individuals with ASD. IL-2 and IFN-γ levels are significantly increased in cerebrospinal fluid (Molloy et al., 2006). IL-4 and IL-10 levels are increased in the anterior cingulate gyrus (Gupta et al., 1998), and IL-3/IL-10 and IFN-γ/IL-10 are increased in peripheral blood mononuclear cells (Giulian & Baker, 1986). An increase in th1 and th2 cytokines suggest increased activation of the chronic adaptive T immune response in ASD. This is further supported by the skewed ratio of CD4+ and CD8+ cells (Hanisch, 2002). Immune cells and their respective cytokines can alter neurophysiology and induce brain changes associated with ASD. Activated microglial cells promote opsonization and phagocytosis as well as the release of proinflammatory cytokines (Block & Hong, 2007; Marín-Teva et al., 2011). Microglia play an important role in brain development through cell death regulation, axonal guidance and synaptogenesis (Lull & Block, 2010); however, excessive activation of microglia can induce cytotoxicity and neuronal cell death (Glynn et al., 2011; Shatz, 2009). Cytokines can also directly interact with major histocompatibility complex class I (MHC I), which negatively regulates activity-dependent synaptic pruning and formation (Babadjouni et al., 2018; Fenoglio et al., 2006). Furthermore, certain cytokines, such as IL-1, IL-6 and TNF-α can regulate neuroplasticity, because these cytokines can induce activation of the hypothalamic-pituitary-adrenal (HPA) axis, which in turn regulates the secretion of adrenocorticotropic hormone (ACTH), corticotropin-releasing hormone (CRH), arginine vasopressin, and corticosterone (Babadjouni et al., 2018). $PM_{2.5}$

exposure is known to induce neuroinflammation in the corpus callosum (Wang et al., 2019). $PM_{2.5}$ exposure can also increase microglial cell activation, and the levels of inflammatory cytokines including TNF-α, NF-κB, IL-1β, IL-6 and macrophage chemoattractant protein-1 (MCP-1**)**. Increases in the number of microglia also occur in the fronto-insular and visual cortices, dorsolateral prefrontal cortex, and cerebellum (Hertz-Picciotto et al., 2005; Liu et al., 2016; Lovett et al., 2018; Zheng et al., 2019). Increases in activated microglia are known to adversely affect the normal development of neuronal connectivity. Exposing neonatal cord blood to $PM_{2.5}$ can reduce the number of CD3+, CD4+ and CD8+ cells, but increase the number of CD19+ cells, which are markers for T cells and B cells in the adaptive immune system (K. Li et al., 2018), suggesting changes in the immune response with exposure to $PM_{2.5}$ in the neonatal stage. Similarly, Chen (2017) found that maternal $PM_{2.5}$ exposure during pregnancy at a dosage of 15 mg/kg significantly increases peripheral blood mononuclear cells, platelets and levels of IL-6 in Sprague–Dawley rats. Likewise, intranasal administration of two dosages of 2 μg and 20 μg of $PM_{2.5}$ per body weight (in grams) once daily from postnatal days 8 to 22 significantly increases the levels of proinflammatory cytokines IL-1b and TNF-α levels, and the neuroinflammatory biomarkers GFAP and Iba-1 in the hippocampus and prefrontal cortex (Chao et al., 2017). Interestingly, prenatal but not postnatal exposure to $PM_{2.5}$ significantly decreased the expression of several proinflammatory cytokines, including TNF-α, IL-1β and IL-6, in the hypothalamus (Sandin et al., 2014). Similarly, Chao et al. (2017) found that the white blood cell count increased drastically after $PM_{2.5}$ intratracheal instillation in 6- to 8-week-old pregnant rats. Taken together, these findings show that the neuroinflammation response induced by $PM_{2.5}$ exposure could be one of the possible mechanisms underlying behavioral deficits as mentioned above. However, it is still unclear whether severe behaviors or other factors associated with them could result in the cytokine changes, given that the causal relation between inflammation and behavioral deficits in ASD is still unclear**.**

Maternal exercise on offspring's brain health

The beneficial effects of maternal exercise have been reported in numerous studies. It has been reported that maternal physical exercise lowers the risk of cancer, cardiovascular diseases and metabolic disorders, as well as elicit long-term positive effects on the offspring brain (Blaize et al., 2015; Rahimi et al., 2018; Robinson & Bucci, 2012; Yau et al., 2019). Certain cytokines associated with improvement in brain development have also been reported to be increased in pups after maternal exercise (Dayi et al., 2012; Kim et al., 2007; Rahimi et al., 2018).

Maternal physical exercise during pregnancy lowers the risk of cancer, cardiovascular diseases, and metabolic disorders of the offspring (Blaize et al., 2015). Additionally, maternal exercise can elicit long-lasting and positive effects on the offspring brain during the critical period of fetal brain development (Robinson and Bucci, 2012). In humans, maternal exercise not only improves the growth of fetus and placenta, but also promotes brain development, connectivity, and enhances cognitive functions in offspring in their later life. For example, maternal exercise during pregnancy has been shown to improve intelligence and the language skills of children when they are 5 years old (ClappIII, 1996). Also, maternal physical exercise training including jogging, yoga, weight-lifting, and aerobics during pregnancy promotes language skills in the offspring as assessed when they are 15 months old (Jukic et al., 2013). The results have suggested a long-lasting improvement and transgenerational neuroplasticity induced by maternal exercise in human brains.

In animal maternal exercise models, maternal exercise has been shown to enhance learning and memory, whilst reducing anxiety-like behavior in offspring. Aksu et al. (2012) reported an increase in locomotor activity in the open field test and reduce in anxiety in the elevated plus maze. These behavioral changes were positively correlated with the increase in vascular endothelial growth factor (VEGF) and brain-derived neurotrophic factor (BDNF) levels in the prefrontal cortex. BDNF is an important protein in regulating neurogenesis and neuroplasticity, regulating neuronal survival, differentiation and integration in the nervous system (Chao et al., 2006). BDNF has been associated with increased likelihood of neuropsychiatric disorders such as depression, anxiety and bipolar disorders (Lang et al., 2005). On the other hand, dysregulation of VEGF has only been reported in individuals with depressive and anxiety disorders (Gormanns et al., 2011). A similar result in a reduction in anxiety-like behavior by maternal exercise has been reported by Uysal et al. (2011), which is also positively correlating with BDNF and VEGF levels.

The pro-cognitive function of maternal exercise that has been reported limits to improvement in spatial learning and memory (Dayi et al., 2012; Parnpiansil et al., 2003; Yau et al., 2019) and cognitive (Lee et al., 2006; Robinson & Bucci, 2012, 2014) learning and memory. The improvement in cognitive function was associated with increase in neurogenesis, neurotrophic factors and neuronal activity. The improvement reported in adult neurogenesis include increases in cell survival, cell proliferation and differentiation in the hippocampus (Kim et al., 2007; Lee et al., 2006; Robinson & Bucci, 2012, 2014; Yau et al., 2019). Increase in neurogenesis leads to increase in neuronal number and, hence, neuronal activation, which has been reported as well (Chun et al., 2020; Robinson & Bucci, 2014).

Maternal running has also been shown to rescue neurocognitive impairments against certain pathological conditions. Prenatal exposure to 6-propyl-2-thiouracil (PTU) induces hypothyroidism-associated impairment of spatial learning and memory, as well as, reduced hippocampal BDNF levels in both male and female rats (Shafiee et al., 2016). Maternal running was able to reverse the behavioral and neurochemical deficits induced by developmental thyroid hormone insufficiency in both male and female rat offspring (Shafiee et al., 2016)

The neuronal system involved in maternal exercise-induced pro-neurocognitive effects could be attributed to the noradrenergic and serotonergic neurotransmitters. Systemic lesion of pathways of the noradrenergic and serotonergic pathways through para-chloroamphetamine (PCA) and Dizocilpine (MK-801) resulted in a reverse in the beneficial effects on learning and memory in the pups of maternal exercise but not in the offspring of the maternal sedentary group. BDNF has been said to be a key neurotrophic factor for regulating noradrenergic neurons plasticity through the downstream upregulation of cAMP-signaling (Akbarian et al., 2002; Matsunaga et al., 2004). Blocking the NMDA receptor abolishes learning and memory in offspring of both maternal exercise and sedentary groups. The results suggest that offspring's brain serotonergic and noradrenergic signaling may play an important role in maintaining the effects of maternal exercise on pups' cognitive function whereas the action of NMDA receptors apparently has a non-selective role in regards to the pups' learning and memory.

References

Chapter 2

Maternal exposure to $PM_{2.5}$ induced autistic-like behavior

Introduction

With mounting clinical studies correlating prenatal exposure of $PM_{2.5}$ to increase incidence of developing ASD, there is a growing concern that prenatal exposure of $PM_{2.5}$ may lead to adverse outcomes of fetal neurodevelopment. As ASD is an idiopathic disease, behavior phenotypes are commonly used for diagnosis. Although ASD is along a severity continuum, it displays 3 main core deficits, namely impaired social communication, social interaction and increase in repetitive behavior. Certain phenotypes of ASD also display impaired cognitive function, although not always presented. In epidemiological studies, prenatal and/or postnatal exposure to $PM_{2.5}$ increase the odds of an offspring developing ASD (Becerra et al., 2013; Raz et al., 2015; Talbott et al., 2015), possible doubling the risk of developing in ASD (Heather E Volk et al., 2013). In animal studies, maternal exposure to $PM_{2.5}$ has been linked to the development of neurodevelopmental impairment, including spatial learning and memory, cognitive learning and memory, reduced social interactions, communication and increase in anxiety-like behavior (Wang et al., 2019; M. Zhang et al., 2018; Q. Zhang et al., 2018; Zheng et al., 2019). Furthermore, maternal $PM_{2.5}$ exposure reduced the number, diameter of neurons and synaptic damage, causing negative effects on the synaptic plasticity (T. Zhang et al., 2018; Zheng et al., 2019). These results also indicate cross-generation detrimental effects of maternal exposure to $PM_{2.5}$.

In contrast, numerous pro-cognitive function of maternal exercise has been reported, including improvement in depression (Yau et al., 2019), anxiety (Aksu et al., 2012), spatial learning and memory (Dayi et al., 2012; Parnpiansil et al., 2003; Yau et al., 2019) and cognitive learning and memory (Lee et al., 2006; Robinson & Bucci, 2012, 2014). Maternal running has also been shown to be able to rescue neurocognitive impairments against certain pathological conditions in offspring. For example, maternal running was able to reverse the prenatal induced hypothyroidism associated impairments, i.e. spatial learning and memory, as well as, the reduced hippocampal BDNF levels in both male and female rats.

In this section, we aim to (1) investigate whether maternal exposure to $PM_{2.5}$ leads to the development of ASD-like behavior in adult male and female offspring in a sex-specific manner; and (2) examine whether maternal running is effective in preventing ASD-like behavior in offspring. Our results reported that chronic maternal exposure to $PM_{2.5}$ is able to induce ASD-like behavior in offspring, whereas, running was able to ameliorate core ASD-like behavior but not cognitive impairment.

Methodology

Animal handling

All experimental procedures were approved and followed the guidelines of the Animal Subjects Ethics Sub-Committee from The Hong Kong Polytechnic University. C57BL/6J mice had standard chow and water ad libitum in the animal holding room in a 12-h light-dark cycle (lights on at 8 a.m.). The mice were housed 2 mice per cage from 4 weeks old. Female mice were randomly assigned to control, treatment ($PM_{2.5}$ instillation) and treatment and intervention ($PM_{2.5}$ + running) group. During the mating period, the mice were placed in a 2:1 female to male ratio. Prior to the behavioral testing, the offspring mice were handled for 7 days. Animal bedding was changed regularly once every week except during the behavior experiment.

Treatment

$PM_{2.5}$ was dissolved into artificial lung fluid (ALF) to a desired concentration of 2.5 ug/ul to produce an ALF/$PM_{2.5}$ solution. The concentration was derived from corresponding a literature showing detrimental effects of $PM_{2.5}$ on the brain (T. Zhang et al., 2018). From the age of 5 weeks old, the female mice were subjected to intratracheal instillation of ALF alone for control group and ALF/$PM_{2.5}$ solution for $PM_{2.5}$ and running group once every 3 days until parturition. At 7 weeks old, the male mice were introduced for mating. For the $PM_{2.5}$ + running group, running wheels were also introduced at 7 weeks old. The running activity of the mice were measured to ensure that the mice were running. Male mice were removed once impregnation was confirmed. The running wheels were removed after parturition. The treatment lasted for a total of 42 days. The offspring mice were weaned at 3 weeks old, sex separated at 4 weeks old. Behavioral tests were conducted at 5-weeks old. The offspring from the dams subjected to the treatments were labelled as follows; ALF group as Control, the $PM_{2.5}$ group as $PM_{2.5}$, and $PM_{2.5}$ with running group as Running. From each dam, 1-3 male(s) and female(s) were taken for behavior tests respectively.

Behavioral tests

At 5 weeks old, the offspring were subjected to a series of behavioral tests in dim light during the light cycle to assess the sociability, social novelty preference, spatial learning and

memory, working learning and memory, locomotor activity, anxiety-like behavior and depression-like behavior. Each behavior test was conducted on a separate day for a total of 7 days in order of open field test, novel object recognition test, Y-maze, three chamber social interaction test and forced swim test. All behaviors were recorded and analyzed in sample blinded manner.

3 chamber social interaction

The social interaction protocol was adopted from the protocol of Kaidanovich-Beilin et al. (2011). The 3-chamber social interaction test was carried out in a box with acrylic 3 chambers separated by a partition. One wired enclosure was placed in the left and right respectively. A cup was placed on top of the wire enclosure to prevent the mice from climbing. The test is divided into 3 phases. In phase 1, the mice were allowed to roam freely to get used to the 3 chambers for 10 mins. In phase 2, a mouse of the same sex was introduced into the left chamber for 10 mins. In phase 3, another mouse of the same sex was placed into the wire enclosure in the right chamber. In phase 2, the enclosure with the mouse was recorded as mice chamber, while the empty mesh was recorded as empty chamber. In phase 3, the mesh with the mice from phase 2 was recorded as familiar, while the new mice were recorded as novel. The total time interacted with the enclosures was recorded. In phase 2, the empty chamber exploration ratio was calculated with the formula: (Time spent with empty enclosure/Total interaction time with the two enclosures). The mice chamber exploration ratio was calculated with the formula (Time spent with enclosure with mice)/(Total interaction time with the two wire enclosures). The exploration index was calculated with the formula: (Time spent with enclosure with mice-Time spent with empty enclosure)/(Total interaction time with the two enclosures). In phase 3, the familiar exploration ratio was calculated with the formula: (Time spent with familiar mice/Total interaction time with the two mice). The novel exploration ratio was calculated with the formula (Time spent with novel mice)/(Total interaction time with the two mice). The exploration index was calculated with the formula: (Time spent with mesh with novel mice-Time spent with enclosure with familiar mice)/(Total interaction time with the two enclosure)

Marble burying test

In a 40 cm x 28 cm x 18 cm cage, 20 marbles were evenly arranged on the surface of clean bedding. The mice was introduced into the cage for 1 hour. The number of marbles buried after the hour were recorded. Marbles were considered buried if >70% of the marble is buried.

Y Maze

The Y-maze protocol was adopted as previously done (Yau et al., 2019). The Y maze test was carried out in a Y maze with each arm having the dimensions of 30 cm x 15 cm X 6 cm. The test was divided into 2 phases. In phase 1, the mice were allowed to explore for 10 mins with one arm was block (Novel arm). Phase 2 was conducted after an interval of 2 hours. In phase 2, the block arm was open and the mice were allowed to explore freely for 10 mins. The familiar exploration ratio was calculated with the formula: (Time spent in familiar arm/time spent in novel and familiar arm). The novel exploration ratio was calculated with the formula (Time spent in novel arm/time spent in novel and familiar arm). The exploration index was calculated with the formula: (Time spent in novel arm-Time spent in familiar arm)/(time spent in novel and familiar arm).

Novel object recognition test

The novel object recognition test was conducted in a 40 cm x 40 cm x 40 cm acrylic box. The test was divided into 3 phases. In phase 1, a set of objects (object A) were placed in the box 6cm away from the edge of box. In phase 2, object A were replaced with another set of objects (object B) of similar dimensions. In phase 3, the objects were replaced with a different object A and object C. The time spent with object A (familiar object) and object C (novel object) were recorded. The familiar exploration ratio was calculated with the formula: (Time spent with familiar object/time spent with novel and familiar objects). The novel exploration ratio was calculated with the formula (Time spent with novel object/time spent in novel and familiar objects). The exploration index was calculated with the formula: (Time spent with novel object-Time spent with familiar object)/(time spent with novel and familiar objects).

Open field test

The open field test was developed from a protocol in our lab (Yau et al., 2019). The open field test was carried out in an acrylic 40 cm x 40 cm x 40cm box. The mice were allowed to explore freely for 10 mins and recorded. The recording was analyzed by AnyMaze software (USA) for the time spent in the center region, entries in the center region and distance travelled. A center region was defined as a 20 cm x 20 cm box and drawn using the software.

Forced Swim Test

The forced swim test was developed from an existing protocol in our lab (Yau et al., 2019). The forced swim test was carried out in an acrylic cylindrical chamber of radius 15cm and height 60cm. The cylinder was filled with water up to 3/4 of the tank the night before. The time spent immobile was recorded and analyzed.

Statistical analysis

The analysis was carried out using Graphpad Prism software version 7.0 (Graphpad, USA). For the exploration analysis, t-test was carried out between the novel exploration and familiar exploration. For the other intergroup analysis, two way-ANOVA was carried out, followed by Fisher-LSD Post-hoc test.

Results

According to the DSM-5 and ICD-10, individuals diagnosed with ASD lie on a continuum severity and often demonstrate three main behavioral core deficits: social impairment, communication difficulties, and rigid and repetitive behaviors and may exhibit cognitive impairment. With no valid proteomic or genetic biomarkers to test for all types of ASD, behavioral test is the consistently used for diagnosis (Redcay & Courchesne, 2005). From our results, we observed social novelty avoidance, increase in repetitive behavior, object recognition memory impairment and spatial memory impairment in the offspring mice. As the result of cognitive memory impairment, social impairment and increase in repetitive behavior are consistent with commonly observed in individuals with ASD, the results show that prenatal exposure to $PM_{2.5}$ induces autism-like behavior in mice offspring.

Table 1. Mean and standard deviation of behavior data results

	Mean (±SD)					
	Male			Female		
3CST (phase 2)	Familiar exploration	Novel exploration	Exploration index	Familiar exploration	Novel exploration	Exploration index
Control	0.24(±0.05)	0.76(±0.05)	0.53(±0.11)	0.27(±0.04)	0.73(±0.04)	0.46(±0.09)
PM2.5	0.14(±0.01)	0.86(±0.01)	0.72(±0.03)	0.11(±0.05)	0.89(±0.05)	0.77(±0.10)
Running	0.33(±0.12)	0.67(±0.12)	0.35(±0.25)	0.29(±0.05)	0.71(±0.05)	0.41(±0.10)
3CST (phase 3)						
Control	0.64(±0.09)	0.36(±0.09)	0.27(±0.17)	0.59(±0.06)	0.41(±0.06)	0.18(±0.12)
PM2.5	0.57(±0.03)	0.43(±0.03)	0.14(±0.06)	0.52(±0.08)	0.48(±0.08)	0.04(±0.16)
Running	0.30(±0.11)	0.70(±0.11)	0.43(±0.27)	0.42(±0.17)	0.58(±0.17)	0.13(±0.44)
Y-Maze						
Control	0.61(±0.04)	0.39(±0.04)	0.23(±0.07)	0.59(±0.06)	0.41(±0.06)	0.18(±0.13)
PM2.5	0.58(±0.02)	0.42(±0.02)	0.17(±0.05	0.46(±0.18)	0.41(±0.16)	0.07(±0.11)
Running	0.45(±0.18)	0.55(±0.18)	0.10(±0.36)	0.39(±0.11)	0.61(±0.11)	0.21(±0.22)
NORT						
Control	0.59(±0.08)	0.41(±0.08)	0.31(±0.27)	0.54(±0.08)	0.46(±0.08)	0.08(±0.17)
PM2.5	0.54(±0.07)	0.46(±0.07)	0.08(±0.14)	0.48(±0.08)	0.52(±0.08)	-0.04(±0.16)
Running	0.53(±011)	0.47(±0.11)	-0.05(±0.23)	0.49(±0.12)	0.51(±0.12)	0.03(±0.23)
Marble burying						
Control		3.68(±1.93)			4.40(±1.96)	
PM2.5		8.18(±3.46)			8.92(±4.59)	
Running		5.0(±3.0)			4.88(±3.80)	
OFT	Distance Travelled	Time spent in center	Distance travelled in center	Distance Travelled	Time spent in center	Distance travelled in center
Control	17.21(±5.33)	0.09(±0.03)	0.17(±0.05)	18.92(±6.75)	0.12(±0.06)	0.18(±0.06)
PM2.5	18.39(±4.42)	0.12(±0.03)	0.15(±0.03)	18.05(±4.57)	0.09(±0.04)	0.14(±0.03)
FST						
Control		84.25(±23.18)			101.93(±20.84)	
PM2.5		104.91(±18.27)			117.72(±26.05)	

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3 Chambers social interaction

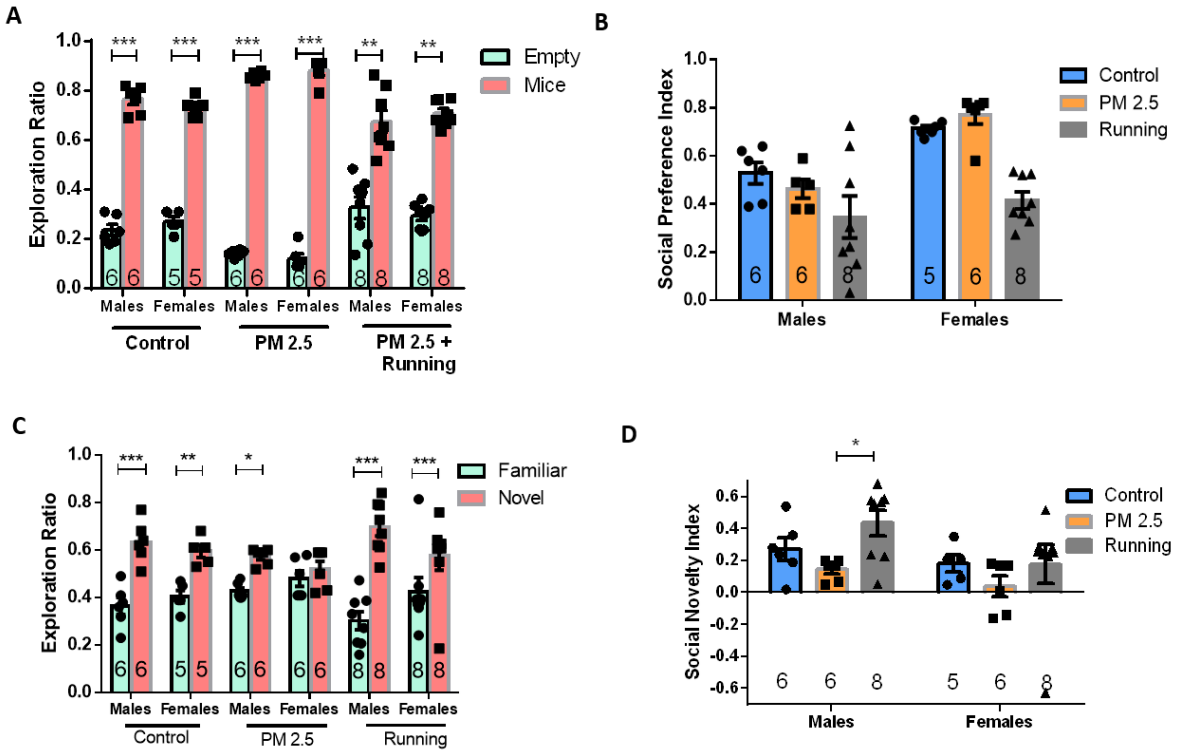

Figure 1. Sociability and social novelty in 3 Chambers Social Interaction Test. (A&B) Behavioral analysis for sociability, there was no significant difference among groups. (C&D) Behavior analysis for social novelty. (C) Male offspring from $PM_{2.5}$ exposed dam showed significant impairment in social novelty recognition test, but female offspring showed no significant preference towards novel mice *p <0.05, **p <0.01, ***p <0.001.

The exploration ratio in the sociability phase of 3 chambers social interaction showed greater preference towards chamber with mice over empty chamber [Figure 1A; *p <0.05, **p <0.01, ***p <0.001]. The two-way ANOVA analysis of the social preference index showed similar results, with significant sex effect [Figure 1B: interaction F (1, 33) =17, p =0.0002], but no significant treatment effect [Figure 1B: interaction F (1, 19) = 2.9, p =0.1067]. The exploration ratio in the social novelty phase showed significant preference to chamber with novel mice over familiar mice in all (Figure 1C; *p <0.05, **p <0.01, ***p <0.001) but female offspring mice of the maternal $PM_{2.5}$ exposed group (Figure 1C; p =0.415). Two-way ANOVA revealed no significant sex [F (1, 27) = 4.1, p =0.0540] or treatment [F (2, 27) = 2.0, p =0.1530] effect. No significant changes were observed in the social novelty index [Figure 1D; Interaction F (1, 19) = 0.018, p =0.8948]. Post-hoc analysis revealed no significant changes in the maternal Running group and control and $PM_{2.5}$ group.

Marble burying

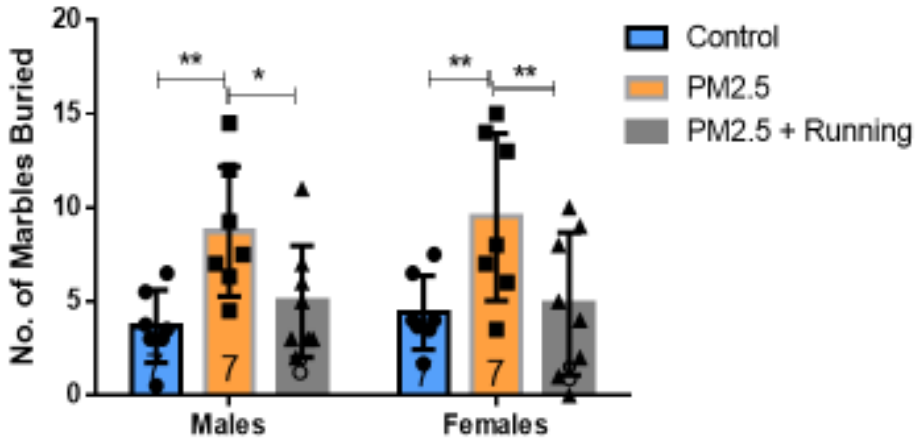

Figure 2. Marble Burying Test. Male and female offspring mice showed a significant increase in number of marbles buried in the maternal $PM_{2.5}$ exposed group compared to the control group. Maternal Running was able to significantly reduce number of marbles buried in both female and male offspring, indicating increased repetitive behavior. *p <0.05, **p <0.01, ***p <0.001.

Two-way ANOVA revealed that the offspring of the $PM_{2.5}$ group had an treatment effect in number of marbles buried [Figure 2; interaction F (2, 38) = 9.8, p =0.004] but have no effect for sex [interaction F(1, 38) = 0.22, p =0.643]. In the male and female offspring mice, $PM_{2.5}$ group buried significantly more marbles compared to the offspring of the control group. (Figure 2; *p <0.05). Post-hoc analysis revealed maternal running was able to significantly reduce the number of marbles buried, suggesting the effects of maternal exercise on rescuing repetitive behavior induced by maternal exposure to $PM_{2.5}$ (Males; p =0.033; Females; p =0.009).

Novel object Recognition

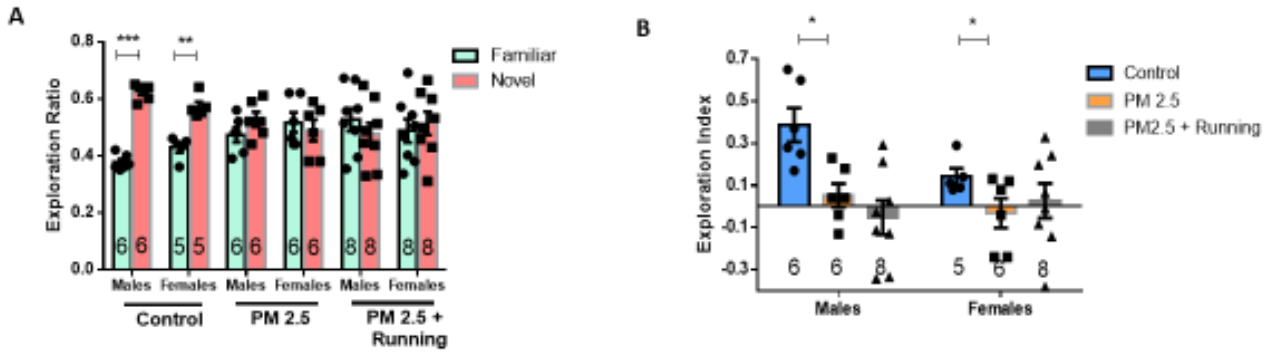

Figure 3 Novel Object Recognition Test. (A) No significant intra-group changes observed in the offspring of the maternal $PM_{2.5}$ exposed or $PM_{2.5}$+Running exposed group. (B) Male and female offspring mice showed a significant decrease in exploration index in the maternal $PM_{2.5}$ exposed group compared to control group. *p <0.05, **p <0.01, ***p <0.001.

The novel object recognition tests for the object recognition memory in the mice. The exploration ratio revealed that only control offspring mice had significant exploration towards the novel object over the familiar object (Figure 3A; *p <0.05, **p <0.01, ***p <0.001). Male and female offspring from the $PM_{2.5}$ and Running group showed no significant difference. Two-way ANOVA analysis revealed a main effect of $PM_{2.5}$ treatment on the exploration index (Interaction $F(1,19) = 15.17$, $p = 0.001$). In the male and female offspring mice, $PM_{2.5}$ group had a significant reduction in exploration index compared to the offspring of the control group. (Figure 3B; *p <0.05). Post-hoc analysis revealed maternal running did not restore the behavior impairment (Males; p =0.315; Females; p =0.573). The results indicate that maternal exposure to PM2.5 impaired the learning and working memory of the offspring mice, while maternal running was unable to reverse the impairment.

Y maze

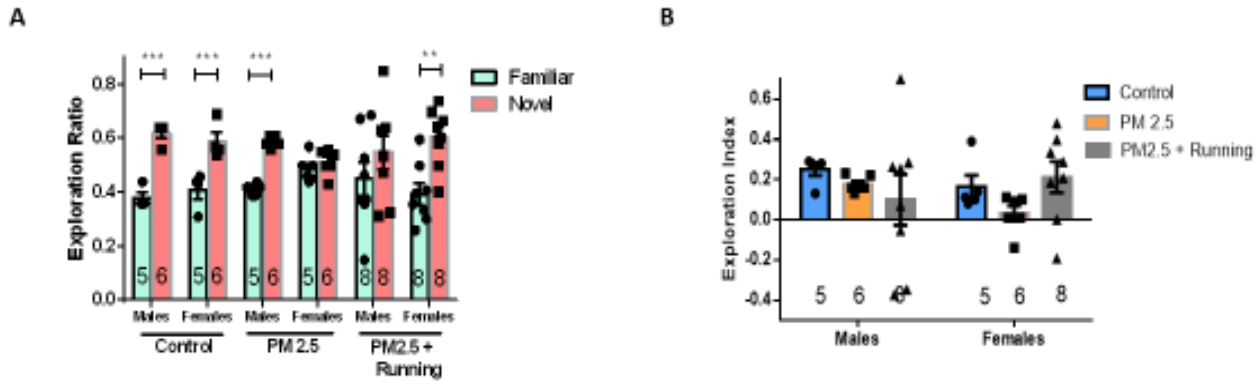

Figure 4. Y Maze Test. (A) Female but not male offspring from dams exposed to $PM_{2.5}$ showed no preference for novel arm, indicating impaired hippocampal dependent learning and memory. However, maternal running restored memory impairment in female but not male offspring. (B) No significant changes observed in exploration index in the 3 groups. *p <0.05, **p <0.01, ***p <0.001.

The Y Maze test examines the spatial learning and memory of the mice. The exploration ratio revealed that control offspring, $PM_{2.5}$ Male offspring and Running female offspring mice had significant exploration in the novel arm (Figure 4A; *p <0.05, **p <0.01, ***p <0.001). Female, but not male offspring from the maternal $PM_{2.5}$ group did not show any significant difference in exploration between novel and familiar arms, indicating impairment in spatial memory. In contrast, male offspring mice from the Running dams did not show significant difference between novel and familiar arms, whereas female offspring with maternal running showed restored spatial learning. Two-way ANOVA analysis of the exploration index revealed no significant difference between sex [$F(2, 32) = 1.5$, p =0.234] and treatment [$F(1,32) = 0.34$, p =0.5635] effect (Figure 4B). $PM_{2.5}$ group had no significant reduction in exploration index compared to the offspring of the control group in the male (Figure 4B; p = 0.52) and female (Figure 4B; p = 0.29) offspring mice. Post-hoc analysis revealed maternal running showed no significant difference in exploration index (Males; p =0.491; Females; p =0.113).

Open Field Test

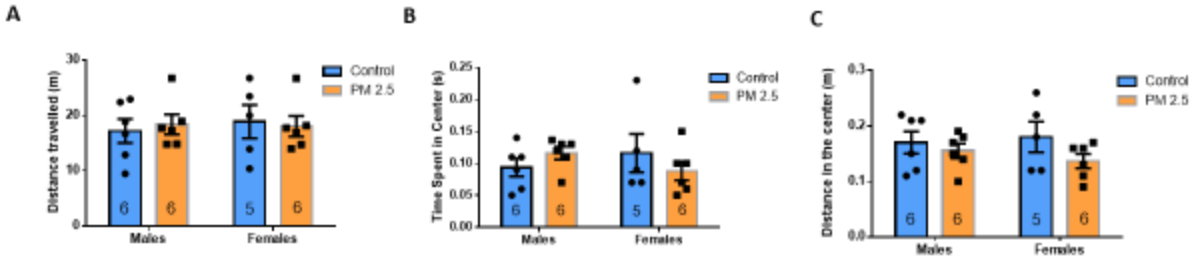

Figure 5. Open Field Test. (A) Male and female offspring mice showed no significant changes observed in the locomotor activity between the control and maternal $PM_{2.5}$ exposed group. (B&C) Male and female offspring mice showed no significant changes observed in the anxiety-like behavior between the control and maternal $PM_{2.5}$ exposed group.

The open field test was assessed for the distance travelled (Figure 5A), Time spent in center (Figure 5B) and Distance travelled in center (Figure 5C). The distance travelled revealed that maternal exposure to $PM_{2.5}$ did not significantly affect the locomotor activity of the offspring mice (Males $p = 0.99$, Females $p = 0.97$). The time spent in center (Males $p = 0.78$, Females $p = 0.69$) and distance travelled in the center (Males $p = 0.93$, Females $p = 0.40$) revealed no significant changes in anxiety-like behavior in the mice between the groups.

Forced Swim Test

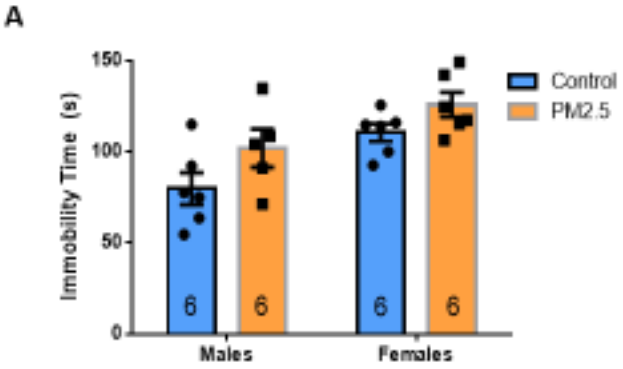

Figure 6. Forced Swim Test for depressive-like behavior. Male and female offspring mice showed no significant changes observed in the immobility time between the control and maternal $PM_{2.5}$ exposed groups. No significant changes in immobility time was recorded in the male ($p = 0.23$) and female ($p = 0.50$) offspring mice between groups.

Discussion

With mounting clinical evidence supporting the increase in the risk of offspring developing ASDs after early postnatal or prenatal exposure to $PM_{2.5}$, the urgent need for proving evidence for this link is required. The results of the present study consolidate the hypothesis that maternal exposure to $PM_{2.5}$ increases the risk of the offspring developing ASD-like behavior.

In order to test for ASD-like behavior, three chambers social interaction and marble burying test were used. The three-chamber social interaction experiment was conducted to assess sociability and social novelty. Maternal exposure to $PM_{2.5}$ did not affect sociability in the offspring in the social preference phase. However, during the social novelty phase, female offspring mice of the $PM_{2.5}$ group showed no preference when presented to the novel and familiar mice, suggesting an impairment in social novelty, a behavior that has been previously reported to be a characteristic of autism-like behavior (Schopler & Mesibov, 1986). In order to assess repetitive behavior, a marble burying test was used. Our results showed that maternal exposure to $PM_{2.5}$ increased the number of marbles buried in both male and female offspring. The increase in the number of marbles buried indicates an increase in repetitive behavior (Angoa-Pérez et al., 2013; Chang et al., 2017).

Apart from social communication impairment and repetitive behaviors, certain types of ASD also have impairment in learning and memory (Minshew & Goldstein, 2001), as well as, an increase in anxiety and depression (Kerns & Kendall, 2012; Stewart et al., 2006). To assess difference in object recognition memory, the novel object recognition test was used for working memory, whilst Y Maze was used to test for spatial memory. Our results showed that the offspring showed no preference between the familiar and novel object in the novel object recognition test, indicating the inability to distinguish between the novel and familiar object. The inability to distinguish the objects may reflect the inability of the mice to retain the familiar objects encountered in the earlier phase (Leger et al., 2013; Lueptow, 2017), therefore demonstrating the impairment in working memory of the mice. There was no observed difference in locomotor activity, anxiety-like or depression-like behavior in the offspring after maternal exposure to $PM_{2.5}$.

Mouse models of ASD have shown the 3 main core behavior for diagnosis of ASD, namely abnormal reciprocal social interactions, communication deficits, and repetitive behaviors with restricted interests, as well as other associated symptoms of ASD, such as anxiety, mental retardation and depression (Jill L Silverman, Mu Yang, et al., 2010). For

example, the *Fmr1* knock out mouse model of Fragile X syndrome, the best characterized single gene mutation caused mouse model of ASD thus far, also shows altered social interaction, increased repetitive behavior and learning deficits (Bakker et al., 1994; McNaughton et al., 2008; Spencer et al., 2005). Another model of ASD, the BTBR T+tf/J mouse model, also shows abnormal social interactions, communication deficits, increased repetitive behavior and increased in anxiety (Jill L Silverman, Seda S Tolu, et al., 2010). Our results have shown similar behavioral abnormalities as commonly observed in ASD model, indicating maternal exposure to $PM_{2.5}$ induces ASD-like behavior in male and female offspring.

Our behavioral findings echoed to findings from other groups (Church et al., 2018; Emam et al., 2020; K. Li et al., 2018; Wang et al., 2019). Church et al. (2018) exposed female dams to a concentration of 135.8 $\mu g/m^3$ of PM $\leq$2.5μm (CAPs) daily from the duration of gestation to postpartum day 10. They also reported an impairment in social interaction and an increase in repetitive behavior in both male and female offspring, which coincides with the results produced in our experiment. Similar results were observed by Wang and colleagues (2019) showing that pregnant mice subjected to 30 μl of 3.456 μg/μL $PM_{2.5}$ displayed impaired social communication and an increase in repetitive behavior. Other repetitive behaviors, such as self-grooming, examined by M. Zhang et al. (2018) has shown an increase in self-grooming. Taken together, it can be concluded that prenatal exposure to $PM_{2.5}$ causes an increase in impairment in social interaction and an increase repetitive behavior in offspring. Apart from the main core behavior impairments in ASD, the offspring mice also exhibited learning and memory impairment.

Whether prenatal exposure to $PM_{2.5}$ induces anxiety and depression remains to be debated. Numerous studies have shown that maternal exposure to $PM_{2.5}$ increased anxiety and depression (Wang et al., 2020; M. Zhang et al., 2018; T. Zhang et al., 2018), although our results indicated no difference in anxiety and depression between the control group and $PM_{2.5}$ group of offspring. In the study by Cui et al. (2019), it was noted that there was a significant increase in time spent in center, indicating a decrease in anxiety-like behavior. In order to probe the relationship between prenatal exposure to $PM_{2.5}$ and anxiety, further investigation will be needed to include more sensitive behavioral tests.

The mechanisms underlying the detrimental effects of $PM_{2.5}$ on the offspring is still largely unknown. A hypothesis of activated maternal immune system is proposed earlier. As previously described, $PM_{2.5}$ is inflammatory inducer and lead to systemic inflammation. Previous work has shown that maternal immune activation (MIA) can lead to a neurodevelopmental disorder in mouse model (Boulanger-Bertolus et al., 2018). Additionally,

studies by Dr. Patterson and his colleagues have shown that maternal immune activation in mice induce the 3 main core ASD-like behaviors (Hsiao et al., 2012; Malkova et al., 2012).

In our study, it has been noted that male and female offspring are affected differently, with female mice being affected in more behavioral tests. Female offspring mice displayed social novelty impairment, increase in repetitive behavior, impairment learning and memory, whilst male offspring mice only displayed repetitive behavior and impairment in learning memory. The difference in male and female effect is replicated in other studies as well. Zhou et al. (2020) reported neurodevelopmental impairment only in female offspring mice. Church et al. (2018) reported a male specific effect in the reciprocal social interaction test, whilst (Wang et al., 2020) a male specific effect in Morris water maze for spatial learning memory. The gender difference may be due the $PM_{2.5}$ concentration, $PM_{2.5}$ constituents, exposure duration, exposure method and mice strain. Since there are a number of variations in the studies conducted, it should be noted to take the sex-specific results with a grain of salt. However, all of the results point out that there is an effect on ASD behavior after prenatal and postnatal exposure to $PM_{2.5}$.

A possible hypothesis for the difference in the sex difference effect is the two hit model. The two-hit model proposes the first hit incites the development of a vulnerable CNS system, where as a second hit induces the condition. In the study by Bilbo et al. (2018), only prenatal diesel exposure in combination with maternal stressor, but not diesel exposure alone, induced ASD-like behaviors in offspring. The authors hypothesized a two-hit model in which prenatal exposure to air pollutant (the first hit) renders more reactive immune response to the second hit (e.g. psychological stressor, maternal inflammation). Apart from environmental x environmental two hit model, other two hit models may also exist. Another possible two hit model would be the first hit being genetic predisposition, with the second hit being environment influences, e.g. exposure to $PM_{2.5}$, diet, maternal inflammation. The influence on individual may vary depending on the effect of the two-hit model. Moreover, whether $PM_{2.5}$ exposure is the sole cause of ASD or if it follows the two-hit model warrants further investigation.

As a treatment intervention for the maternal exposure to $PM_{2.5}$ induced behavioral changes in the offspring, running during the gestational period was carried out. Gestational running was able to ameliorate the core ASD-like behavior but was unable to recover the detrimental effect of $PM_{2.5}$ on the learning and memory deficits in offspring except for spatial learning and memory. Unlike our study, other studies reported maternal exercise can improve behaviors related to learning and memory or even a protective effect against certain pathogenesis. Parnpiansil et al. (2003) and Yau et al. (2019) reported an improvement in spatial

memory in rat pups. Whereas, Kim et al. (2007) and Robinson and Bucci (2014) reported maternal running improving short-term memory. The positive effect of maternal running is not limited to only those, there has also been reported improvement in anxiety-like and depression-like behavior. Prenatal stress has been shown to induce neurocognitive impairments, including short-term and long-term spatial memory and learning disturbances. Pregnant dams subjected to running as a prevention to detrimental effects of prenatal stress showed that maternal voluntary running was able to prevent spatial memory and learning impairments in prenatally stressed offspring mice. Sevoflurane, a general anesthetic agent, exposure during gestational period have been reported to cause learning and memory impairment, which could be restored by maternal running. In our study, maternal running was able to restore the spatial learning and memory deficit in the Y maze test in offspring from exercised dams. However, maternal running showed no significant difference in the working memory in novel object recognition task. A possible reason for the indifference is due to involvement serotonergic system. The serotonergic system is dysregulated in ASD individuals, with the brain serotonin synthesis and synaptogenesis being highly disrupted (Zafeiriou et al., 2009). Until the age of 5, individuals with ASD demonstrate a reduced capacity for synthesis of serotonin, which later increases and overcome the values of a healthy adult by the age of 15. According to the report by Akhavan et al. (2008), serotonergic lesions can suppress the enhancing effects of maternal exercise during pregnancy in pups with regards to learning and memory. Thus, a possibility for inability for maternal running to prevent cognitive dysfunction may be due to the dysfunction of the serotonergic system. Future experiments will be needed to prove this hypothesis.

Chapter 3

Maternal Exposure to $PM_{2.5}$ impaired adult Neurogenesis in the hippocampus

Introduction

$PM_{2.5}$ is a potentially neurotoxic particle due to its composition containing polycyclic aromatic hydrocarbons, metals, organic matter, and elemental carbon. These compounds have the ability to induce an inflammatory response, introducing damage to surrounding tissues, including the brain.

In the previous section, we were able to establish a link between maternal exposure to $PM_{2.5}$ and ASD-like behavior in offspring. These behavioral changes may be associated with adult neurogenesis. Adult neurogenesis is the process in which new neurons are born and integrated into the existing hippocampal neural circuitry. An increase in adult neurogenesis has been associated with cognitive improvements, including spatial learning and memory, and working memory, and anti-depression. The process of adult neurogenesis occurs in the dentate gyrus (DG) of the hippocampus (Zhao et al., 2008).

The hippocampus region has shown abnormalities in ASD models, exhibiting abnormal neurogenesis and impaired synaptic plasticity, including a decrease in neuronal differentiation but an increase in astrocyte differentiation (Bailey et al., 1998; Bauman & Kemper, 1985; Guo & Commons, 2017; Raymond et al., 1995). The neuronal maturation has also been shown to be disrupted with reduction in dendritic length and complexity, and increase in the density of immature spines in the DG.

Studies have shown that prenatal exposure to $PM_{2.5}$ reduces the number, diameter of neurons and causes synaptic damage (T. Zhang et al., 2018). Apart from this, gestational $PM_{2.5}$ exposure can lead to dysfunction in the synaptic synthesis, e.g. increase in synaptic cleft, thinned postsynaptic density thickness, and shorter length of synaptic active area (Wang et al., 2019; Zheng et al., 2019). Taken together, the data suggest a dysfunction in neuronal maturity by $PM_{2.5}$.

Limited evidence has shown that maternal exercise improves neurocognitive function via increasing neurogenesis, neuronal maturation, and neuronal activation. The beneficial effects on neurogenesis include increase in cell proliferation, cell survival, neuronal maturation, immature neurons and neuronal activation (Akhavan et al., 2008; Aksu et al., 2012; Lee et al., 2006; Robinson & Bucci, 2012, 2014; Yau et al., 2019).

In this section, we aim to investigate (1) whether maternal $PM_{2.5}$ exposure impairs adult hippocampal neurogenesis and (2) whether maternal running elicits its effect in association with restored adult neurogenesis in the hippocampus of offspring. We observed a gender difference in response to maternal PM2.5 exposure in which there was an increase in hippocampal cell proliferation in male offspring only, an increase in neuronal differentiation in both male and female offspring, but decrease in total dendritic length of immature neurons in female offspring only. In contrast, maternal running significantly increased hippocampal cell proliferation, and neuronal differentiation in both male and female offspring. There was no change in the number of immature neurons between control and $PM_{2.5}$ offspring, whereas maternal running increased the number of immature neurons in male, but not female offspring. The data suggest that there is a gender difference in response to maternal $PM_{2.5}$ exposure. Both male and female offspring showed an increase in hippocampal cell proliferation and neuronal differentiation, however, there was delayed neuronal maturation of newborn neurons in female offspring, suggesting abnormalities in neuronal development in the hippocampus. These structural changes would be linked to hippocampal dysfunction associated with ASD induced by air pollutant $PM_{2.5}$.

Methodology

Animal handling

All experimental procedures were approved and followed the guidelines of the Animal Subjects Ethics Sub-Committee from The Hong Kong Polytechnic University. C57BL/6J mice had standard chow and water ad libitum in the animal holding room in a 12-h light-dark cycle (lights on at 8 a.m.). Female mice were randomly assigned to control, treatment ($PM_{2.5}$ instillation) and treatment and intervention ($PM_{2.5}$ + Running) group, with 2 female mice grouped per cage. During the mating period, the mice were placed in a 2:1 female to male ratio.

Treatment

$PM_{2.5}$ was dissolved into artificial lung fluid (ALF) to a desired concentration of 2.5 µg/µl. The concentration was derived corresponding literature showing detrimental effects of $PM_{2.5}$ (T. Zhang et al., 2018). From the age of 5 weeks old, the female mice were subjected to intratracheal instillation of ALF/ $PM_{2.5}$ solution once every 3 days until parturition. At 7 weeks old, the male mice were introduced for mating. For the $PM_{2.5}$+Running group, running wheels

were introduced at 7 weeks old. Male mice were removed once impregnation was confirmed. The running wheels were removed after parturition. The offspring mice were weaned at 3 weeks old, sex separated at 4 weeks old. Behavior test conducted at 5 weeks old and sacrificed at 6 weeks old. The offspring from the dams subjected to the treatments were labelled as follows; ALF group as control, the $PM_{2.5}$ group as $PM_{2.5}$ and $PM_{2.5}$ and running group as Running.

Tissue preparation

Offspring mice were deeply anesthetized with 10 mg/kg Ketamine and 4 mg/kg Xylazine cocktail (USA). Upon anesthesia, mice were perfused with 60 ml of 1x PBS. Afterwards, the mice were perfused with 60 µl of 4 % PFA. The brains of the mice were collected and stored in 4 % PFA for 48 hours. The brains were then transferred to 30% sucrose solution until the brain sinks. The brains containing hippocampal region were sliced and collected in a 1 in 6 series, with each section thickness of 30 µm. The brain sections were stored in a cyroprotectant solution, composed of 30 % glycerol and 30 % ethylene glycol in 1x PBS, until immunostaining.

Immunostaining

Immunohistochemistry analysis was conducted using free floating DAB (3, 3'-diaminobenzidine) method. Antigens were exposed using citric acid heat retrieval method (pH 6.0; 95^{o}C; 10 mins). After 3 times with10 mins washes in 1x PBS, the brain slices were incubated overnight with the respective primary antibodies; rabbit anti-KI67 (1:1000, Abcam, United Kingdom), mouse anti-doublecortin (DCX) (1:200, Vector Laboratories, CA, United States) or mouse anti-NeuroD (1:200, Vector Laboratories, CA, United States). Then, the slices were incubated in the respective secondary antibody, goat anti-mouse (1:200, Vector Laboratories, CA, United States). for the slices of mouse anti-DCX or mouse anti-NeuroD and goat anti-rabbit (1:200, Vector Laboratories, CA, United States) for the rabbit anti-KI67. The positive cells were visualized using the VECTASTAIN ABC kit (HRP) (1:200, Vector Laboratories, CA, United States) and the DAB peroxidase substrate kit (1:200, Vector Laboratories, CA, United States).

Image analysis

Quantification of proliferation cells, immature neurons and neuronal differentiation was carried out by counting Ki67, DCX and NeuroD immunopositive cells from 8 sections across -1.34 to -3.80 mm bregma position. Only cells residing in the sub-granular zone and the granule cell layer of the DG were counted using microscope (H600L, Nikon, Japan).

Sholl analysis

Immature neurons were selected based on the following criteria (1) The immature neuron must at least be a tertiary immature neuron (with 3 or more dendrite branches). (2) The immature neuron must extend towards the molecular layer of the dentate gyrus with intact dendritic branching. (3) The cell body of the immature neuron should be in subgranular zone of the dentate gyrus. (4) The immature neurons can be selected in superior or inferior blade of the dentate gyrus. A total of 5 immature neurons are traced per brain region, a total of 3 dorsal and 3 ventral regions, resulting in a total of 30 immature neurons traced. The immature neurons were selected under 400x magnification to measure the total dendritic length and perform sholl analysis by Neurolucida (MicroBrightField Bioscience, VT, United States).

Statistical analysis

The analysis was carried out using Graphpad Prism software version 7.0 (Graphpad, USA). For the analysis of intergroup, 2 way-ANOVA was carried out, followed by intragroup analysis using Fisher-LSD Post-hoc test. For analysis of sholl radius, Wilcoxon test was carried out.

Results

From the anatomical studies of the brain, it has been reported that the brain growth in individuals with ASD is abnormal. Infants with ASD have been found to have a lower total brain volume than healthy infants, followed by overgrowth within the first year of life (Aylward et al., 2002). Up until the age of 4, children diagnosed with ASD showed a larger brain volume than healthy individuals. Progressing to late-childhood, puberty, and adolescence, individuals with ASD experience delayed brain growth (Bailey et al., 1993; Brambilla et al., 2003; Tsatsanis et al., 2003). Neurogenesis was examined as reflected by changes of cell proliferation (Ki67), number of immature neurons (DCX) and neuronal differentiation (NeruoD). The results indicate that cell proliferation and neuronal maturation were increased in male and female offspring. Contrary to the hypothesis, an increase in neurogenesis was observed in young adult offspring mice. To further investigate it, we examined the immature neurons dendritic branching in the hippocampus. The results show that the total dendritic length is reduced in female offspring. To summarize, although neurogenesis may be increased, it appears that the development of the neurons is affect by $PM_{2.5}$.

Table 2. Mean and standard deviation of cell quantification of adult neurogenesis.

	Mean (±SD)			
	Male		Female	
Ki67	Dorsal	Ventral	Dorsal	Ventral
Control	544(±85)	639(±97)	716(±153)	787(±254)
PM2.5	960(±286)	1265(±329)	893(±148)	898(±398)
Running	1486(±253)	1509(±306)	2075(±508)	2178(±170)
DCX				
Control	6876(±978)	7372(±1395)	7486(±1553)	7767(±1376)
PM2.5	7723(±1990)	7728(±1311)	7771(±1140)	7815(±1997)
Running	10589(±2401)	10313(±2577)	7983(±1426)	7047(±644)
NeuroD				
Control	3085(±227)	4449(±522)	2919(±383)	4479(±170)
PM2.5	4744(±541)	4892(±839)	4936(±529)	5387(±1291)
Total Dendritic Length				
Control	157.1(±40.3)		146.3(±33.1)	
PM2.5	138.8(±39.9)		114.3(±44.2)	
Running	196.9(±44.3)		190.3(±33.2)	

Ki67-Proliferation

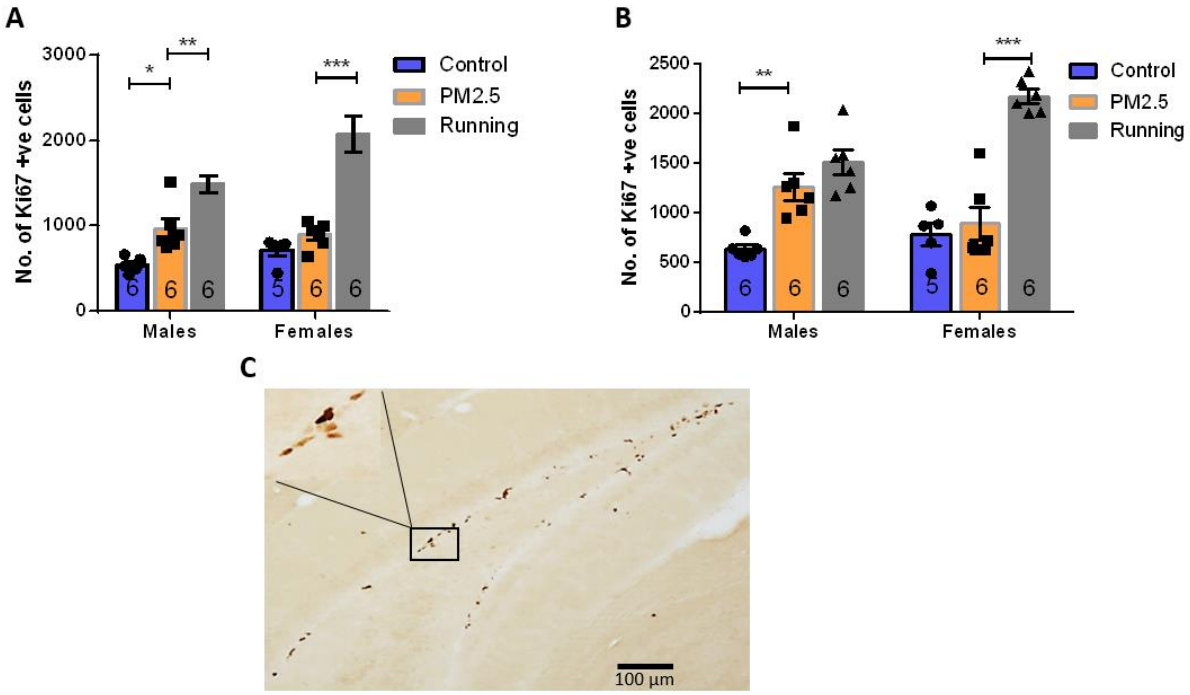

Figure 7. Number of Ki67 positive cells in the (A) dorsal and (B) ventral hippocampal dentate gyrus region. (C) Representative image. * p <0.05, **p <0.01, ***p <0.001, ****p <0.0001.

The density of $Ki67^+$ cells in male offspring was significantly increased after maternal exposure to $PM_{2.5}$ compared to the control group in the dorsal region (Figure 7A & 7B, *p <0.05, **p <0.01), indicating increase in proliferating cells. There was an observed significant treatment interaction [Figure 7A; F (2, 29) = 53, p <0.05; Figure 7B; interaction F (2, 29) = 50, p <0.05] but no significant sex effect [Figure 7A; F (1, 29) = 6.0, p =0.20; Figure 7B; interaction F (1, 29) = 2.5, p =0.12]. No significant difference was observed between the control and $PM_{2.5}$ group in the females in the dorsal region or ventral region (Figure 7A; p =0.30; Figure 7B; p =0.52). Post-hoc analysis of the $PM_{2.5}$+Running revealed that maternal running was able to increase the density of $KI67^+$ cells compared to the $PM_{2.5}$ group in the dorsal region (Males; p <0.05, Females; p <0.001) and ventral region (Females; p <0.001), except for the males in ventral region (Males; p =0.14).

DCX-immature neurons

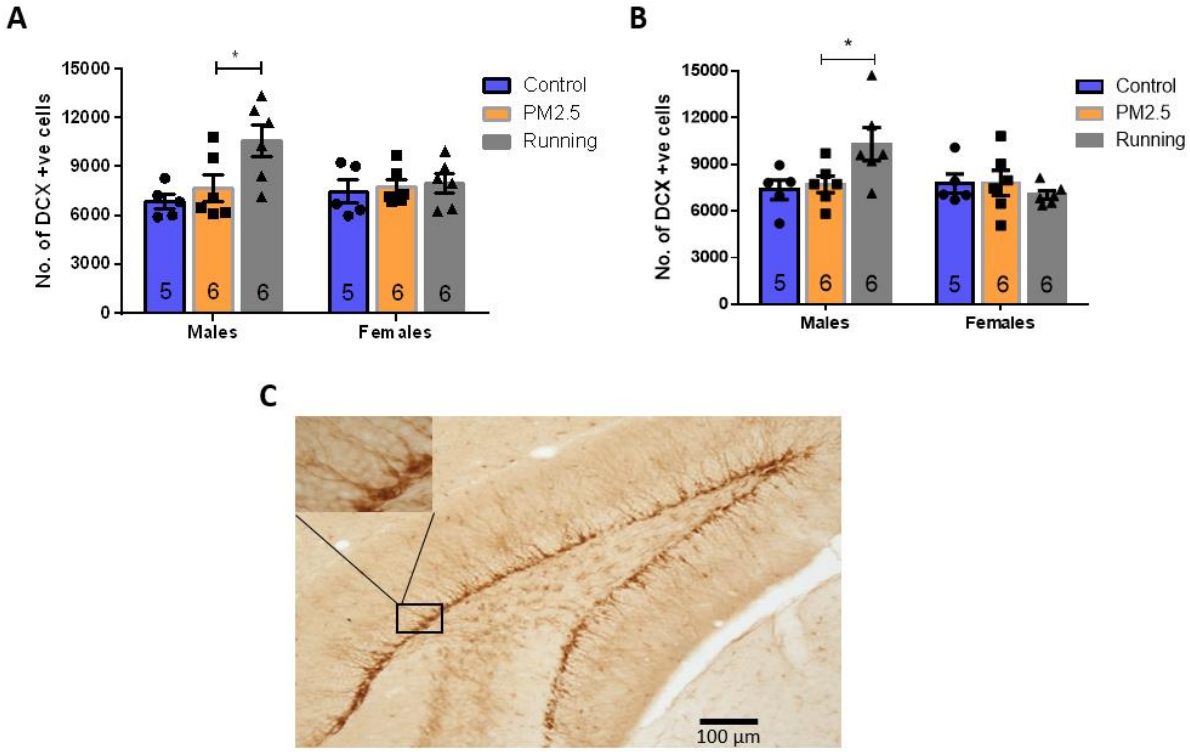

Figure 8. No. of DCX positive cells in the in the (A) dorsal and (B) ventral hippocampal dentate gyrus region. (C) Representative image. *p <0.05, **p <0.01, ***p <0.001.

The density of DCX^+ cells revealed significant treatment interaction in the dorsal but not ventral region [Figure 8A; interaction F (2, 28) = 4.8, p <0.05; Figure 8B; interaction F (2, 28) = 1.4, p =0.26], as well as, no significant sex effect [Figure 8A; interaction F (1, 28) = 1.3, p =0.27; Figure 8B; interaction F (1, 28) = 2.6, p =0.12] in the dorsal or ventral region. The density of DCX^+ cells revealed no significant difference between the control and $PM_{2.5}$ group in the dorsal region (Figure 8A; Males; p =0.41, Females; p =0.78 and ventral region (Figure 8B; Males; p =0.73, Females; p =0.96). Post-hoc analysis of $PM_{2.5}$+Running revealed that maternal running was able to increase the density of DCX^+ compared to the $PM_{2.5}$ group in the dorsal region in male but not female offspring (Males; p <0.05, Females; p =0.83) and ventral region (Males; p <0.05, Females; p =0.44).

NeuroD-Neuronal maturation

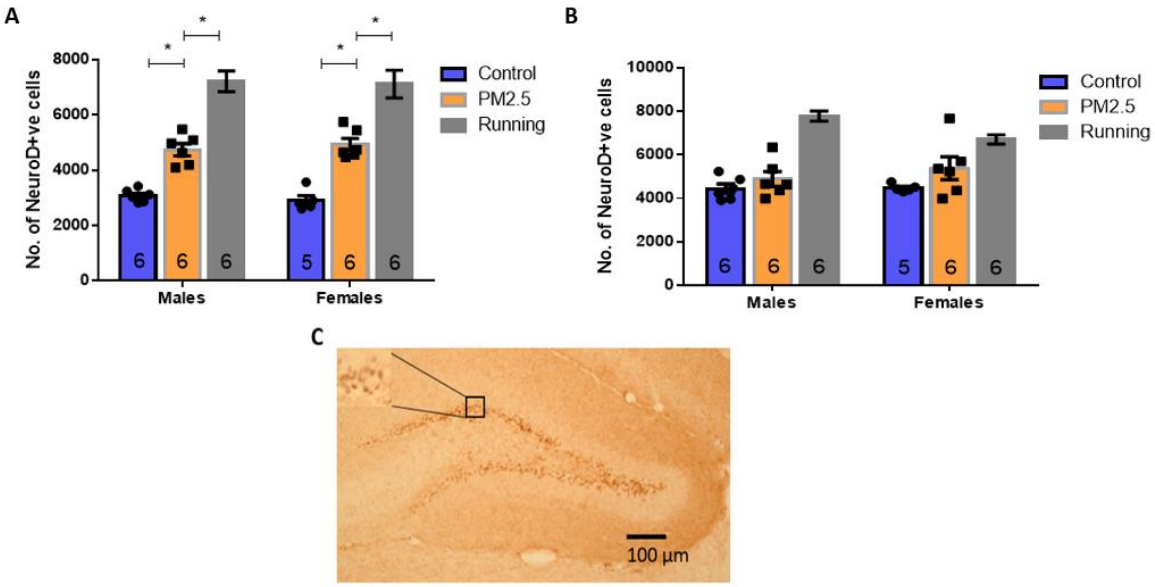

Figure 9. Number of NeuroD positive cells in the (A) dorsal and (B) ventral hippocampal dentate gyrus region. (C) Representative image. *p <0.05, **p <0.01, ***p <0.001.

The density of NeuroD$^+$ cells revealed significant treatment interaction [Figure 9A; interaction F (1, 19) = 99, p <0.05; Figure 9B; interaction F (1, 19) = 3.7, p <0.05] but no significant sex effect [Figure 9A; interaction F (1, 19) = 0.0049, p =0.94; Figure 9B; interaction F (1, 19) = 0.56, p =0.46]. The density was significantly increased in the dorsal region after maternal exposure to $PM_{2.5}$ compared to the control group (Figure 9A, *p <0.05). No significant difference in the ventral region was observed between the control and $PM_{2.5}$ group in the males and females (Figure 7B; Males; p =0.37; Females; p =0.09). Post-hoc analysis of $PM_{2.5}$+Running revealed that maternal running was able to increase the density of NeuroD$^+$ cells compared to the $PM_{2.5}$ group in the dorsal region (Males; p<0.05, Females; p<0.05) but ventral offspring.

Dendritic branching of immature neurons: DCX morphology

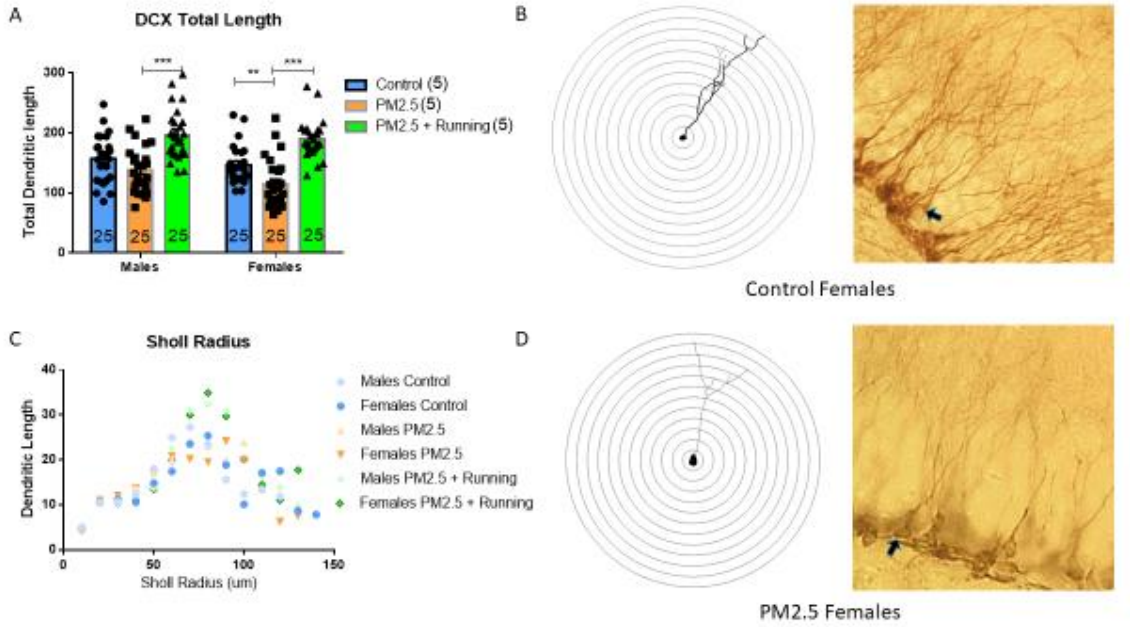

Figure 10. Morphologic analysis of immature neurons. (A) $PM_{2.5}$ female offspring had reduced total dendritic length of doublecortin labelled immature neurons when compared to the control group. (B&D) Representative images of sholl analysis and doublecortin labeling of control and $PM_{2.5}$ female offspring mice. (C) There was no significant difference in dendritic length at different sholl radius.

Two-away ANOVA revealed that there was a main treatment specific effect [$F(2, 143) = 38$, $p < 0.001$] and a sex-specific effect [$F(1, 143) = 4.9$, $p < 0.05$] in the total dendritic length. Males offspring revealed no significant difference (Figure 10A; $p = 0.10$), whilst the female offspring revealed a significant reduction in $PM_{2.5}$ group (Figure 10A; $p < 0.001$). Post-hoc analysis of maternal running showed a significant increase in total dendritic length compared to the $PM_{2.5}$ group. (Males; $p < 0.001$; Females; $p < 0.001$). No significant difference was observed in the male and female offspring group in the dendritic length in sholl analysis with radius interval of 10 um.

Discussion

Neuronal maturation defects have also been observed in multiple regions in multiple regions in ASD, indicating dysregulated adult neurogenesis, neuronal migration and/or maturation (Wegiel et al., 2010). Our results showed an increase in proliferation and neuronal differentiation, whilst no difference in number of immature neurons observed after the exposure.

Similar animal studies conducted have also shown disruption in neurodevelopment, including neurogenesis and/or synaptic plasticity (Wang et al., 2019). Wang et al. (2019) reported a decrease in the number of EDU+ve cells and NeuN+ve cells in offspring after maternal $PM_{2.5}$ exposure. EDU is used as a marker for cell survival, whereas NeuN is a marker for mature neurons. The data from Wang and his colleagues suggest that maternal exposure to $PM_{2.5}$ decreases neurogenesis, which is opposite to our result. A possible explanation is the age difference of the mice used between our study at 42 days old (mainly measuring adult neurogenesis) and Wang's study at 14 days (undergo active developmental stage).

Numerous studies have shown that hippocampal cell proliferation in ASD is affected (Courchesne et al., 2011; Marchetto et al., 2017; Mariani et al., 2015). For example, in cell culture analysis of reprogrammed fibroblasts displayed an increase in cell proliferation of the induced pluripotent stems cells, neural progenitor cells and neurons from ASD individuals (Marchetto et al., 2017). The increase in proliferation coincides with our result. It was also reported that the increase in cell proliferation was linked to the dysregulation of β-catenin/BRN2 transcriptional cascade. Similarly, three dimensional neural cultures derived from induced pluripotent stem cells from ASD individuals revealed increase in upregulation of genes involved in cell proliferation and neuronal differentiation. The increase in proliferation is supported by further analysis of shorten cell-cycle length observed (Mariani et al., 2015). Mariani et al. (2015) also reported an imbalance in glutamate/GABA neuro ratio, with an increase in GABAergic neurons and no change in glutamatergic neurons. It was also noted that there was an increase GABAergic inhibitory neurons associated with the overexpression of the transcription FOXG1 (Mariani et al., 2015). This was consistent with the ASD post-mortem studies which showed an increase in three types of GABA interneurons subtypes in the hippocampal region (Lawrence et al., 2010). We have noted that there is an increase in neuronal differentiation, however, the type of neuron requires further investigation.

Another study with $PM_{2.5}$ prenatal exposure showed that genetic expression of the subunit Lin28 is downregulated (Chao et al., 2017). Lin28, consisting of 2 subunits Lin28A

and Lin 28B, is a RNA-binding protein that is involved in the miRNA biogenesis and translation associated with neuronal differentiation (Nowak et al., 2014). During the process of neuronal differentiation, Lin28A inhibits the biogenesis of miR-9 by binding to pre-miR-9, ultimately decreasing differentiation. Whereas Lin28B expression decreases along with the downstream regulator STAU1 when neuronal differentiation increases. Taken together with the result in our study, the changes of epigenetic expression Lin28 may be a cause for the increase in neuronal differentiation.

Although no significant changes can be observed in the number of immature neurons, the profile of the immature neurons revealed that there is a decrease in the total dendritic length in female offspring. Additionally, the ultrastructural analysis of neurons revealed synaptic membrane proteins, synaptophysin and PSD-95, were increased, as well as, increased mitochondrial degeneration (Wang et al., 2019; Zhang et al., 2021; T. Zhang et al., 2018; Zheng et al., 2019). The ultrastructural analysis of the neurons in other studies revealed that gestational $PM_{2.5}$ exposure leads to an increase in the synaptic cleft, postsynaptic density thickness thinning, and length of synaptic active area shortening, as well as matrix swelling in mitochondria, partial vagueness in mitochondrial cristae, vacuolar degeneration in mitochondria in the hippocampal neurons of mice offspring (Wang et al., 2019; Zheng et al., 2019). Furthermore, the serotonergic system has also been reported to be affected by maternal exposure to PM2.5. Zhang et al. (2021) reported increased the receptors of 5-hydroxytryptamine (5-HT), namely 5-$HT_{2A,}$ in the offspring. 5-HT_{2A} is a G-coupled protein receptor for the neurotransmitter involved in the serotonergic system. The serotonergic system plays a vital role in the development of learning and memory (Harvey, 2003; Jiang et al., 2020; Meneses, 2017). With intervention, alleviated behavioral deficits observed were associated of decreased levels of 5-HT_{2A}, as well as, improved neuronal synaptic membrane proteins and mitochondrial health (Wang et al., 2019; Zhang et al., 2021). Taken together, this data set suggests that neuronal connectivity is affected by maternal exposure to PM2.5.

Our data showed that there is a significant increase in number of proliferating cells and immature neuron after maternal running. This is consistent with the data conducted by other studies (Kim et al., 2007; Yau et al., 2019). Despite running being able to increase in the number of proliferating cells and immature neurons significantly, we observed that running was not able to improve learning and memory impairment (as discussed in chapter 3). This suggests the underlying cause may not the number of cells but the integration of the cells into the network and differentiation of the cells into neuronal type, i.e. serotonergic and/or GABAergic neurons.

These studies along with our data have provided strong evidence to support the theory of dysregulated proliferation and neuronal maturation from neural progenitors as an underlying pathology for some core behavioral deficits in ASD and neurological learning and memory impairment.

Chapter 4

Maternal Exposure to $PM_{2.5}$ did not induce neuroinflammation in the hippocampus of offspring

Introduction

Various disease conditions, including ASD, are caused or exacerbated by the presence of inflammation. Neuroinflammation in the hippocampus regulates adult hippocampal neurogenesis. In chronic models of neuroinflammation, inhibitory neurogenesis has been reported. Furthermore, suppressing interleukine-6 (IL-6) in the chronic models of IL-6 reversed the inhibitory neurogenesis effect caused. On the other hand, tumor necrosis factor-alpha (TNF-α) has an inhibitory effect on neuronal survival and differentiation. Apart from cytokines, glial cells maintain a hemostatic environment, and regulate neurogenesis. Prior studies have indicated that microglial activation negatively affects neuronal function, especially in chronic activated conditions. Recent studies have demonstrated that microglia plays a key role in neuro-regeneration and neuroprotection (Czeh et al., 2011). Activating microglial cells into pro-inflammatory (M1) phenotype results in a release of pro-inflammatory cytokines, including IL-6 and TNF-α. Anti-inflammatory (M2) microglial cells have been reported to release neurotrophic factors, such as, basic fibroblast growth factor (bFGF) and brain-derived neurotrophic factor (BDNF), thus promoting neurogenesis. Elevated levels of IL-1β have been shown to block hippocampal long-term potentiation (LTP) via activation of c-Jun N-terminal kinases (JNK) and p38 MAPK pathways (Kelly et al., 2003; Minogue et al., 2003; Vereker et al., 2000). Taken together, the studies conclude that active neuroinflammation can interfere with neuronal development.

Exposing neonatal cord blood to $PM_{2.5}$ can reduce the number of CD3+, CD4+ and CD8+ cells, but increase the number of CD19+ cells, which are markers for T cells and B cells in the adaptive immune system (Li et al., 2018), suggesting changes in the immune system in the neonatal stage. Studies have also shown that gestational $PM_{2.5}$ exposure significantly increases inflammatory cells, i.e. peripheral blood mononuclear cells (Chen, 2017), Glial fibrillary acidic protein (GFAP) and Iba-1 (Chao et al., 2017), and levels of proinflammatory cytokines IL-1b, IL-6 and TNF-α (Chao et al., 2017; Sandin et al., 2014)

In this section, we aim to investigate if maternal exposure to $PM_{2.5}$ induces autism-like behavior in association with neuroinflammatory response in offspring. Our results show that there was no observable inflammatory response in terms of no significant changes of high mobility group box-1 protein (HMGB1), TNF-α and Matrix metallopeptidase 9 (MMP-9)

protein levels, as well as number of ionized calcium-binding adapter molecule 1 (Iba-1) positive microglial cells in the hippocampus.

Methodology

Animal handling

All experimental procedures were approved and followed the guidelines of the Animal Subjects Ethics Sub-Committee from The Hong Kong Polytechnic University. C57BL/6J mice had standard chow and water ad libitum in the animal holding room in a 12-h light-dark cycle (lights on at 8 a.m.). Female mice were randomly assigned to control and treatment group with two female mice grouped per cage. During the mating period, the mice were placed in a 2:1 female to male ratio.

Treatment

$PM_{2.5}$ was dissolved in artificial lung fluid (ALF) to a desired concentration of 2.5ug/ul. The concentration was derived corresponding literature showing detrimental effects of $PM_{2.5}$ (T. Zhang et al., 2018). From the age of 5 weeks old, the female mice were subjected to intratracheal instillation of ALF/ $PM_{2.5}$ solution once every 3 days until parturition. At 7 weeks old, the male mice were introduced for mating. Male mice were removed once impregnation was confirmed. The offspring mice were weaned at 3 weeks old, sex separated at 4 weeks old. Behavior test conducted at 5 weeks old and sacrificed at 6 weeks old. The offspring from the dams subjected to the treatments were labelled as follows; ALF group as control and the ALF/$PM_{2.5}$ group as $PM_{2.5}$.

Tissue preparation

Offspring mice were deeply anesthetized with 10 mg/kg Ketamine and 4 mg/kg Xylazine cocktail (USA). Upon anesthesia, mice were perfused with 60ml of 1x PBS. Afterwards, the mice were perfused with 60 µl of 4% PFA. The brains of the mice were collected and stored in 4% PFA for 48 hours. The brains were then transferred to 30% sucrose solution until the brain floats. Brains containing hippocampal region were sliced and collected in a 1 in 6 series, with each section thickness of 30 µm. The brain sections were stored in a cyroprotectant solution, composed of 30% glycerol and 30% ethylene glycol in 1x PBS, until immunostaining.

Mice were decapitated and the hippocampus was retrieved and immediately snap frozen in liquid nitrogen. The samples were stored in -80 ^{o}C until used for ELISA.

Immunostaining

Immunohistochemistry analysis was conducted using free floating DAB (3, 3'-diaminobenzidine) method. Antigens were exposed using citric acid heat retrieval method (pH 6.0; 95^{o}C; 10 mins). After 3 10mins 1x PBS washes, the brain slices were incubated overnight with the primary antibody; rabbit anti-Iba-1 (1:1000, Abcam, United Kingdom. Then, the slices were incubated in the respective secondary antibody, goat anti-rabbit (1:200, Vector Laboratories, CA, United States). The positive cells were visualized using the VECTASTAIN ABC kit (HRP) (1:200, Vector Laboratories, CA, United States) and the DAB peroxidase substrate kit (1:200, Vector Laboratories, CA, United States).

Image analysis

Quantification of inflammatory cells was carried out by counting Iba-1 immunopositive cells were counted from 8 sections across -1.34 to -3.80 mm bregma position. The sections were scanned at 200x magnification using microscope (United States). The hippocampus was divided into the DG, CA1 and CA3 region respectively using ImageJ (NIH, University of Wisconsin, United States) and Iba-1 cells in the regions were counted.

Enzyme-linked immunosorbent assay (ELISA)

After collection of fresh tissue sample, the hippocampal region was homogenized ad centrifuged at 13,000 rpm. The supernatant was collected and ELISA was performed. The ELISA protocols were followed as described in the commercial kits. A total of 3 commercial kits including High Mobility Group Box 1 protein (HMGB1) (CUSABIO, Houston, USA), TNF-α (CUSABIO, Houston, USA) and Matrix metallopeptidase 9 (MMP9) (CUSABIO, Houston, USA). The supernatant was added to the ELISA plate and incubated for 2 hours, followed by incubation with Biotin antibody for 1 hour and Avidin antibody for 1 hour respectively. The 3,3′,5,5′-Tetramethylbenzidine (TMB) substrate was added for 15 mins. Finally, the stop solution was added and the plate was immediately read at 450nm and 570nm.

Statistical analysis

The analysis was carried out using Graphpad Prism software version 7.0 (Graphpad, USA). For the exploration analysis, t-test was carried out between the novel exploration and familiar exploration. For the other analysis, two-way ANOVA was carried out.

Results

Studies analyzing post-mortem brain of individuals with ASD revealed an increase in inflammatory biomarkers compared to healthy brains, as indicated by the increase in proinflammatory microglial cells increase and cytokines (Ashwood et al., 2011; Morgan et al., 2010). The changes in inflammatory profile in ASD have been associated with behavioral impairments (Yau et al., 2021). To investigate whether $PM_{2.5}$ induced neuroinflammatory response in the offspring may the cause of neurological associated behavioral changes (as discussed in Chapter 3. Our results indicate an absence of neuroinflammation in the hippocampus region of offspring.

Table 3. Mean and standard deviation data of number of Iba-1 positive cells in the DG, CA1 and CA3.

No. of cells	Mean (±SD)	
DG	Male	Female
Control	7298(±905)	6640(±493)
PM2.5	7942(±1071)	7875(±970)
CA1		
Control	3953(±512)	3619(±409)
PM2.5	4055(±1132)	4220(±887)
CA3		
Control	3691(±731)	3548(±250)
PM2.5	3708(±668)	4068(±835)

Table 4. Mean and standard deviation data of number of primary projections of Iba-1 positive cells in the DG, CA1 and CA3.

No. of primary projections	Mean (±SD)	
DG	Male	Female
Control	4.67(±0.44)	4.61(±0.41)
PM2.5	4.11(±0.25)	4.16(±0.27)
CA1		
Control	4.59(±0.37)	4.75(±0.42)
PM2.5	4.12(±0.22)	4.22(±0.28)
CA3		
Control	4.57(±0.36)	4.64(±0.40)
PM2.5	4.01(±0.22)	4.15(±0.27)

Number and morphology of Microglial cells in the hippocampus

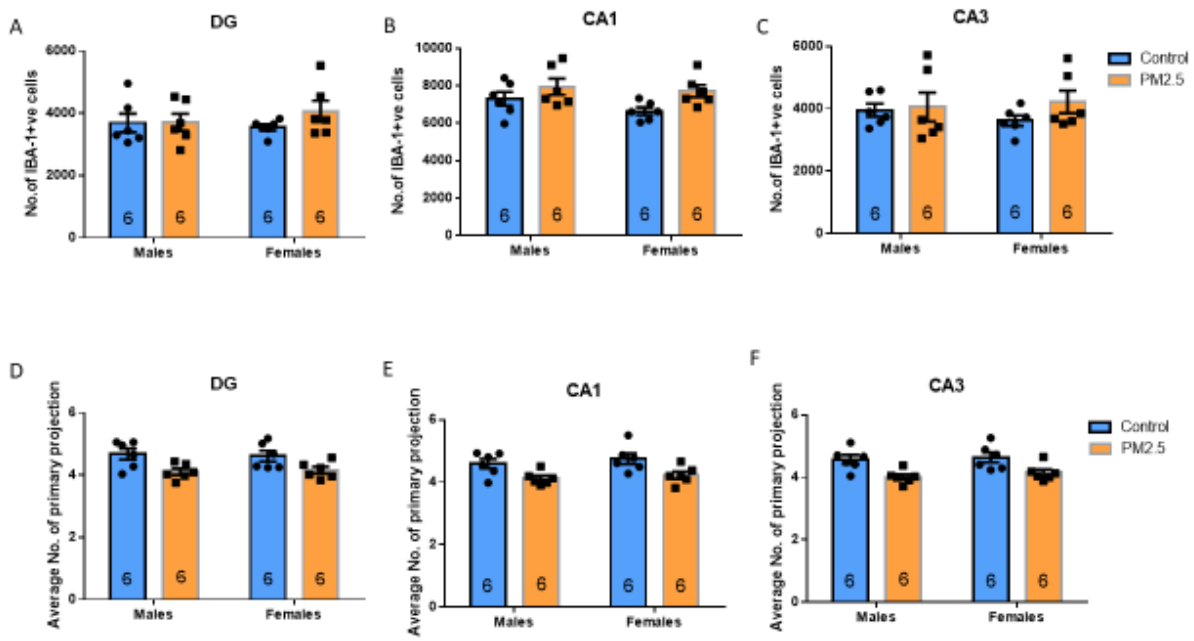

Figure 11. Number of Iba-1 positive cells in different regions of the hippocampus. No significant change in Iba-1 positive cells was observed after maternal $PM_{2.5}$ instillation in the DG (A), CA1 (B) and CA3 (C). No significant changes in the number of primary projections in the DG (D), CA1 (E) and CA3 (F).

The analysis of Iba-1 cells revealed no significant changes in neuroinflammation in the hippocampal subregions, namely the DG (Males; p =0.87, Females; p =0.67), CA1 (Males; p =0.71, Females; p =0.62), and CA3 (Males; p =0.81, Females; p =0.52). The was no observed main treatment in the DG [F(1,20)=0.99, p =0.33], CA1 [F(1,20)=6.2, p =0.57] and CA3[F(1,20)=1.2, p =0.29] region or gender in the DG [F(1,20)=0.16, p =0.69], CA1 [F(1,20)=1.7, p =0.21] and CA3 [F(1,20)=0.068, p =0.79] effect.

Inflammatory cytokines in the hippocampus: HMGB1, TNF-α, NF-kb & MMP-9 levels

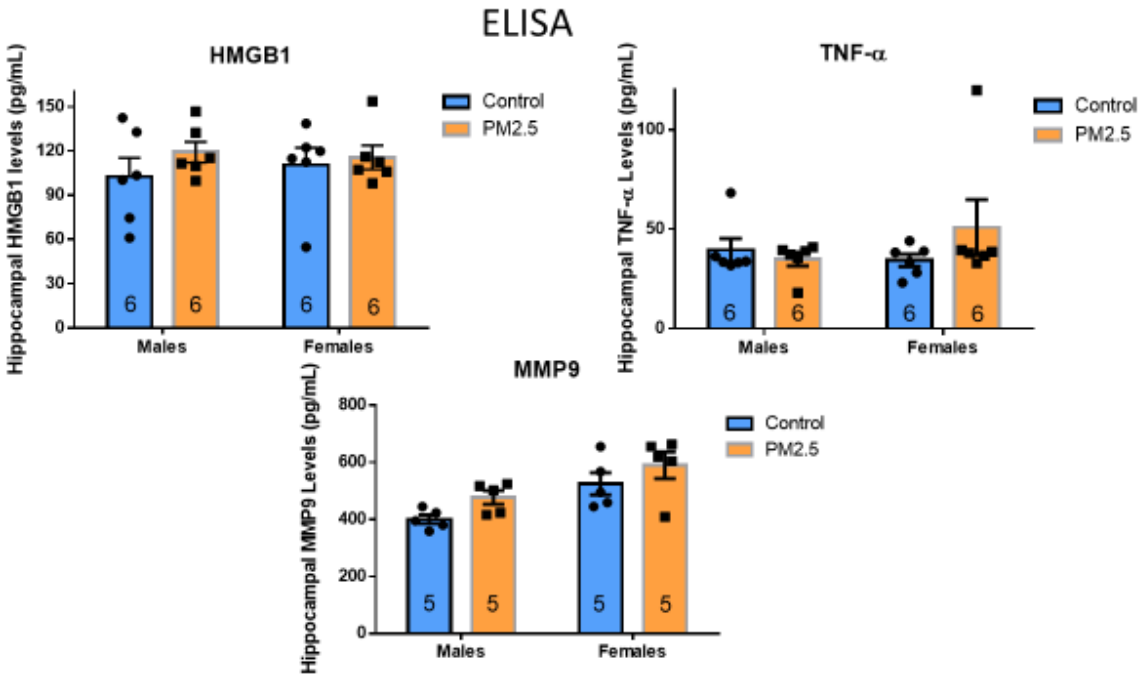

Figure 12. There was no difference in the hippocampal levels of HMGB1, TNF-α and MMP-9 among groups.

There was no significant change in hippocampal levels of HMGB1 (Males; p =0.42, Females; p =0.62), TNF-α (Males; p =0.78, Females; p =0.51) and MMP-9 (Males; p =0.31, Females; p =0.22) among all groups.

Discussion

Numerous studies have suggested inflammation, oxidative stress and genetic mutation or changes as possible causes of autism as discussed above. We examined neuroinflammation in the hippocampal region by using ELISA. The results revealed no significant changes in neuroinflammation as indicated by no significant increases in activated microglial cells or pro-inflammatory cytokines, namely HMGB1, TNF-α and MMP-9.

Contradictory to our results, other gestation $PM_{2.5}$ studies have revealed an increase in inflammation in the hippocampal region. Elevated pro-inflammatory cytokine profile have been reported, including NF-kB and TNF-α, HMGB1, IL-6 and IL-1b (K. Li et al., 2018; Wang et al., 2019; T. Zhang et al., 2018; Zheng et al., 2019). In the study by (T. Zhang et al., 2018), they examined the effect of three dosages of $PM_{2.5}$, including low dosage at 0.2592 mg/kg, medium dosage at 1.728 mg/kg and high dosage groups at 3.456 mg/kg. They only observed an increase in inflammation in the high dosage group. The dosage used in our study is comparatively lower than the largest dosage than they have examined. This may be a possible reason for the difference observed.

Even though we did not observe any inflammation in the hippocampal region, our data have indicated increased in some core ASD behaviors induced by maternal exposure to $PM_{2.5}$. A comprehensive study (Kemper & Bauman, 1998) also revealed no observable inflammation in the hippocampus, but abnormally small and densely distributed neurons with reduced complexity and length of dendritic arbors in the hippocampal region. Similarly, another post-mortem brain study also reported no signs of inflammation in the hippocampus region (Vargas et al., 2005). This puts forward the conjecture that hippocampal inflammation may not be the underlying mechanism of induced ASD by gestational exposure to PM2.5, but rather changes in neuronal maturation and differentiation may contribute to the behavioral deficits (as discussed in chapter 4).

Although Vargas et al. (2005) reported an increase in neuroinflammation in the cerebellum, cortical regions and white matter of autistic patients, there was no difference in the hippocampal region. To add, post-mortem studies of ASD individuals reveal inflammation to be a systemic issue rather a localized one (Onore et al., 2012). With the limitation of our study to investigate inflammation in one brain region, it is inconclusive that neuroinflammation plays a crucial role in autistic-like behavior induced by $PM_{2.5}$.

Although we have shown that the offspring do not have increased levels of HMGB1, it has been reported that maternal intervention with glycyrrhizin (GL), an inhibitor of HMGB1, was

able to ameliorate the behavior deficits observed by PM2.5 exposure (Zhang et al., 2021). The data presented suggests that the HMGB1-NLRP3 pathway in the maternal system plays a vital role in the induced cognitive impairment in the offspring. Moreover, this data set also supports the possible mechanistic pathway of development of ASD being the maternal inflammatory profile. However, this data should be taken with caution as the offspring profile also indicated presence of neuron inflammation.

As previously mentioned, $PM_{2.5}$ could induce neuroinflammatory and it could induce ASD through two possible pathways through direct or indirect pathway. As $PM_{2.5}$ are relatively small in size, it can enter the systemic circulation through inhalation and reach various parts of the body. Thus, for its direct action, $PM_{2.5}$ or its constituents can reach the placenta and certain compounds may pass through. This is supported by the presence of $PM_{2.5}$ carbon particles in the placenta (T. Zhang et al., 2018). As $PM_{2.5}$ or its constituent may pass through the placenta and induce systemic and/or central inflammatory response. This response may be responsible for the pathological condition in ASD. It has been reported that $PM_{2.5}$ reduces cognitive learning abilities with associated increase in lead, manganese and aluminum content of the hippocampus (Q. Li et al., 2018). Increased levels of the metals has been associated with neurotoxicity. For example, increased exposure to Pb during prenatal studies have reported to lead to intellectual deficits in animal studies (Goyer, 1996; Needleman et al., 1984). As the constituents of $PM_{2.5}$ can pass through the placenta, it may be able to act in the neurodevelopment.

On the other hand, indirect action involves the exposure to $PM_{2.5}$ inducing an inflammatory response in the mother. The study by Hogan et al. (2015) reported that maternal inflammation during the first trimester has been associated with an increased risk of ASD. The inflammatory response causes an increase in the production of proinflammatory cytokines, which can cross the placenta to the embryo or induce a *de novo* inflammatory response in offspring. This is supported by the presence of maternally derived IL-6 protein in the placenta. Maternal inflammation activation (MIA) by lipopolysaccharide (LPS) or synthetic double-stranded RNA poly(I:C) injection has also been associated with behavioral abnormalities and neuropathology. The behavioral abnormalities include social communication, a common feature observed in ASD. Current hypothesis of MIA induced ASD releases pro-inflammatory cytokines, especially IL-6, which activates the Janus kinase (JAK) and activator of transcription 3 (STAT3) pathway (Patterson, 2011). JAK/STAT3 pathway is involved in neuronal proliferation, survival and differentiation (Yadav et al., 2005). This hypothesis also explains the increase in the data of neurogenesis (Chapter 3) we observed.

Conclusion

Although we observed no significant changes in neuroinflammation in the hippocampal region in regards to Iba-1 cells and inflammatory cytokines, maternal exposure to $PM_{2.5}$ induced autistic-like behavior (including impairment in learning and memory, social novelty avoidance, as well as, increase in repetitive behavior) in association with abnormality in adult neurogenesis and neuronal maturation of newborn neurons with gender-specific effects in offspring. Despite no significant changes observed in the number of immature neurons in both female and male offspring, dendritic analysis of the immature neurons revealed a significant reduction in total dendritic length in female offspring, suggesting a linkage between change in neuronal maturation and differentiation and behavioral deficits observed in offspring. Our results are consistent with other $PM_{2.5}$ exposure models, showing that maternal $PM_{2.5}$ exposure induces core ASD-like behavior in offspring associated with alteration in adult hippocampal neurogenesis.

Maternal running was able to ameliorate the core ASD-behavior changes observed but not the impairment in learning and memory. Maternal running also significantly increased cell proliferation and neuronal differentiation in both male and female offspring but the number of immature neurons in the male mice only. In spite of the fact that both maternal running and $PM_{2.5}$ exposure increased cell proliferation and neuronal differentiation, the effects of two vary. According to studies conducted, as well as our study, maternal running has been shown to improve learning and memory associated with increased neurogenesis. On the other hand, maternal exposure to PM2.5 increased neurogenesis but impaired learning and memory. We hypothesize it may due to difference in maturation and integration into neural circuitry as indicated by the reduction in total dendritic length of immature neurons in offspring with maternal $PM_{2.5}$ exposure. Other mechanisms including changes in oxidative stress, gene expression and gut microbiota profile could also play a role in underlying behavioral deficits as we observed in our model. Further investigation is required for validating the hypothesis.

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Abbreviations

5-HT – Serotonin

ACTH – Adrenocorticotropic hormone

ALF – Artificial lung fluid

ANOVA – Analysis of variance

AOR – Adjusted odds ratio

ASD – Autism spectrum disorder

BDNF – Brain-derived neurotrophic factor

bFGF – Basic fibroblast growth factor

BTBR – Black and Tan BRachyury mouse model

CA – Cornu Ammonis

cAMP – Cyclic adenosine monophosphate

CRH – Corticotropin-releasing hormone

DAB – 3, 3'-diaminobenzidine

DCX – Doublecortin, a marker for immature neurons

FMRP – Fragile X mental retardation protein

GABA – γ-Aminobutyric acid

GFAP – Glial fibrillary acidic protein

GL – glycyrrhizin

HMGB1 – High mobility group box-1 protein

HPA – Hypothalamic-pituitary-adrenal

IBA-1 – Ionized calcium-binding adapter molecule 1

IFN-γ – interferon gamma

IL-10 – Interleukin 10

IL-1β – Interleukin 1 beta

IL-2 – Interleukin 2

IL-3 – Interleukin 3

IL-4 – Interleukin 4

IL-6 – Interleukin 6

IL-8 – Interleukin 8

JAK – Janus kinase

JNK – c-Jun N-terminal kinases

Ki67 – a maker for proliferation

LPS – lipopolysaccharide

LTP – Long-term potentiation

MAPK – mitogen-activated protein kinase

MCP-1 – Macrophage chemoattractant protein-1

MDA – Malondialdehyde

MHC I – Major histocompatibility complex class I

MIA – Maternal inflammation activation

MK-801 – Dizocilpine

MMP9 – Matrix metallopeptidase 9

NeuroD – a marker for neuronal maturation

NF-kB – Nuclear factor kappa-light-chain-enhancer of activated B cells tumor

NMDA – N-methyl-D-aspartate receptor

NO2 – Nitrogen dioxide

PCA – Para-chloroamphetamine

PM2.5 – Particulate matter of less than 2.5 µm

PTU – 6-propyl-2-thiouracil

ROS – Reactive oxygen species

STAT3 – activator of transcription 3

TGF-β1 – transforming growth factor beta 1

Th1 – T helper type 1

Th2 – T helper type 2

TNF- α – Necrosis factor αlpha

VEGF – Vascular endothelial growth factor