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Chronic maternal exposure to low-dose $PM_{2.5}$ impacts cognitive outcomes in a sex-dependent manner

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ABSTRACT

There is no safe level of air pollution for human health. Traffic-related particulate matter ($PM_{2.5}$) is a major *in-utero* toxin, mechanisms of action of which are not fully understood. BALB/c dams were exposed to an Australian level of traffic $PM_{2.5}$ (5 μg/mouse/day, intranasal, 6 weeks before mating, during gestation and lactation). Male offspring had reduced memory in adulthood, whereas memory was normal in female littermates, similar to human responses. Maternal $PM_{2.5}$ exposure resulted in oxidative stress and abnormal mitochondria in male, but not female, brains. RNA-sequencing analysis showed unique sex-related changes in newborn brains. Two X-chromosome-linked histone lysine demethylases, *Kdm6a* and *Kdm5c*, demonstrated higher expression in female compared to male littermates, in addition to upregulated genes with known functions to support mitochondrial function, synapse growth and maturation, cognitive function, and neuroprotection. No significant changes in *Kdm6a* and *Kdm5c* were found in male littermates, nor other genes, albeit significantly impaired memory function after birth. In primary foetal cortical neurons, $PM_{2.5}$ exposure suppressed neuron and synaptic numbers and induced oxidative stress, which was prevented by upregulation of *Kdm6a* or *Kdm5c*. Therefore, timely epigenetic adaptation by histone demethylation to open DNA for translation before birth may be the key to protecting females against prenatal $PM_{2.5}$ exposure-induced neurological disorders, which fail to occur in males associated with their poor cognitive outcomes.

1. Introduction

Air pollution particulate matter with diameters of 2.5 μm ($PM_{2.5}$) was identified as a major threat to human health, with robust data being derived from both epidemiological and experimental human and animal exposure studies, in addition to *in-vitro* studies (Chen et al., 2021; Chen et al., 2022; Fuller et al., 2022; Khomenko et al., 2021; Pryor et al., 2022). Vehicle-emitted $PM_{2.5}$ consist of carbon, metals, and other toxins that exert greater and longer-term harm to body systems in comparison to the gaseous component of the emission (Pryor et al., 2022; HEI Panel, 2022). This is because gas dissipates rapidly, whereas $PM_{2.5}$ remain in the atmosphere and, upon inhalation, can enter the circulation by

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crossing the endothelial barrier in distal lung segments (Chen et al., 2022; Pryor et al., 2022; Thangavel et al., 2022).

The exposure hazards are unevenly distributed among the general population, with the unborn child being particularly vulnerable. Fine PMs have been shown to cross the blood-placental barrier and enter foetal blood, which may have a direct effect on developing organs (Bové et al., 2019; Liu et al., 2021; Valentino et al., 2016). However, the precise interaction of these PM with foetal organs, and questions around the dose effect of $PM_{2.5}$ being sufficient to directly affect foetal development, need further validation (Bongaerts et al., 2021; Holder et al., 2021). The latter can be difficult in humans due to confounding factors, such as maternal smoking (HEI Panel, 2022). Besides, cytokines and chemokines produced in the lung in response to $PM_{2.5}$ inhalation (Wang et al., 2021), can also be detected systemically and can amplify the damage. As a result, $PM_{2.5}$ are considered a significant intrauterine environmental toxin (Chen et al., 2021; Wang et al., 2020; Rich et al., 2015). However, the general public is alarmingly ignorant of the dangers of traffic PM exposure and, when asked, refuse to actively avoid or protect themselves from exposure to polluted air (Walker et al., 2023); such as walking along a busy road even during pregnancy.

The health risks of living in a heavily polluted environment are readily appreciated, with numerous epidemiological studies demonstrating adverse effects in countries like Mexico, India, and China (Chen et al., 2022; Yi et al., 2022). In particular, exposure to high ambient $PM_{2.5}$ levels increases the risk of neurocognitive disorders, from poor memory and learning difficulties in school-aged children to depression and dementia in the ageing population (Altug et al., 2020; Cowell et al., 2015; Harris et al., 2015; Clifford et al., 2016; Lertxundi et al., 2019). Emerging evidence supports the long-term health risks of chronic exposure to even a 'safe level' of $PM_{2.5}$ (Yazdi et al., 2021; Khomenko et al., 2021). While individual vehicle emissions have been reduced due to the advancement of technology, those who live and work close to major traffic corridors are still affected due to high vehicle numbers and traffic congestion, especially during peak hours (HEI Panel, 2022). For example, those living within 500 m of main roads are still subjected to chronic exposure to low-level $PM_{2.5}$ produced by traffic, rendering them at a higher risk of $PM_{2.5}$-related health conditions (WHO Regional Office for Europe, 2013). However, the health impacts of prenatal exposure to low levels of $PM_{2.5}$ are often overlooked, especially in countries seen as having good air quality, such as Australia. We previously modelled exposure to what is widely considered a 'safe level' of $PM_{2.5}$, in mouse dams and showed that their offspring had significantly lower birth weight and lung pathology, akin to humans with prenatal exposure to heavily polluted air (Wang et al., 2021; Johnson et al., 2021). Low birth weight is a well-accepted risk factor for cognitive dysfunction in both childhood and during ageing (Mosing et al., 2018; Upadhyay et al., 2019), suggesting that any level of $PM_{2.5}$ exposure may affect growing foetuses.

$PM_{2.5}$ is a potent oxidant and can directly impair mitochondrial function *in vitro* (Chen et al., 2021; Wang et al., 2021). Mitochondria not only produce ATP, but have an important role in regulating oxidative stress, neural development, neural protection, as well as cognitive function (Yi et al., 2022; Keller et al., 1998; Huang et al., 2012). Mitochondrial impairment is a common mechanism in the pathogenesis of diseases of multiple organs and systems (including the brain) in response to various *in-utero* toxins (such as maternal smoking and maternal e-vaping) as our previous studies revealed (Chen et al., 2021; Li et al., 2018; Chan et al., 2020; Li et al., 2019; Li et al., 2019; Li et al., 2020). There is a distinct sex difference in the susceptibility to prenatal $PM_{2.5}$ exposure, with males more vulnerable than females (Cowell et al., 2015; Lertxundi et al., 2019; Chiu et al., 2013). However, these conclusions are drawn from studies on individuals living in heavily polluted regions and may not predict the neurological outcome of a low 'safer' level of $PM_{2.5}$ exposure. Furthermore, it is difficult to isolate *in-utero* exposure from direct $PM_{2.5}$ exposure after birth in humans, as the parents normally live in the same environment after babies are born (Cowell et al., 2015). Developing brains are also vulnerable to directly inhaled PMs (Alvarez-Pedrerol et al., 2017; Forns et al., 2017; Sunyer et al., 2015), which may obscure the effects of *in-utero* exposure (Chen et al., 2009). Therefore, we exposed mice to a 'safe' low $PM_{2.5}$ level (similar to the typical levels – year average $PM_{2.5}$ 8 μg/m^3 (IQAir, 2024) with higher exposure (e.g. 13 μg/m^3) along roads (Wadlow et al., 2019) – in Australia where air quality is considered good) to examine the impact on cognitive outcomes in young (pre-puberty age) and adult offspring without the confounding effects of direct $PM_{2.5}$ inhalation after birth. We also used RNA sequencing to analyse newborn mice brains and investigate the potential 'key-regulator(s)' affected by maternal $PM_{2.5}$ exposure, and examined their impacts on neural integrity in primary neurons using gene manipulation.

2. Materials and methods

2.1. Animal experiments

$PM_{2.5}$ was collected along a busy roadside in Sydney, NSW Australia, using Smart Particulate Flow Samplers (Total Suspended Particles (TSP) $PM_{2.5}$, 8 L/min) and a 47 mm Teflon filter (Pall Life Sciences, Ann Arbor, MI), and then extracted from the filters using 90 % ethanol and sonication as previously described (Wang et al., 2021).

The animal experiments were approved by the Animal Care and Ethics Committee at the University of Technology Sydney (ETH18-3175), following the Australian National Health and Medical Research Council Guide for the Care and Use of Laboratory Animals. Virgin female BALB/c mice (6 weeks, Animal Resources Centre, WA, Australia) were subjected to the intranasal instillation of $PM_{2.5}$ (5 μg/day, PM group) or saline (SHAM group) for 6 weeks prior to mating, during gestation and lactation (Wang et al., 2021; Chan et al., 2019). This yielded four offspring groups: SHAM-male (male offspring from control dams), PM-male (male offspring from $PM_{2.5}$ exposed dams), SHAM-female (female offspring from control dams), and PM-female (female offspring from $PM_{2.5}$ exposed dams).

The dose was determined assuming $PM_{2.5}$ levels of 10 μg/m^3 in Sydney, Australia; humans have a total daily exposure of around 145 μg of $PM_{2.5}$. The total volume of air breathed by a mouse over 24 h is 278 times lower than in a human. Therefore, the equivalent mouse exposure dose is 0.5 μg/day. We used 5 μg/day to account for losses due to the route of administration (up to 50 % (Southam et al., 2002) and the convention of using approximately 10 times higher doses of agonists in mouse models (Nair and Jacob, 2016).

2.2. Behavioural studies

Behavioural tests were performed on male and female offspring at 4–5 weeks (before puberty) and 10–12 weeks (adulthood). These tests are based on the natural ability of mice to explore novel objects. The results of all tests were expressed as the proportion of time spent on the new object or new position during the test phase, calculated as: *new objective/position (seconds)/(new objective/position (seconds) + familiar objective/position (seconds))*. If a mouse was curious about the new object/position, it would spend more time on it than the old objective, resulting in a value > 0.05.

Novel objective recognition test – assessing short term memory. During the acquisition session, mice were placed in the empty arena for 5 min for two consecutive days to familiarise themselves with the environment. In the Sample Phase, mice were placed in the arena containing two objects and allowed to explore the arena for 2 x 5 min sessions (60 min apart between the Sample Phase and Test Phase). During the Test Phase, one of the objects is replaced with a new one. Increased exploration of the novel object (proportion of time > 0.5) indicates a functioning short-term memory (Chan et al., 2017; Chen et al., 2018; Zakarya et al., 2020).

Episodic memory test – assessing learning memory. In the

acquisition session, mice were placed in the empty arenas for 5 min for two consecutive days to familiarise themselves with the environment. In Sample Phase 1, mice were placed in the arena containing two objects and allowed 3 x 3 min sessions (Sample Phase 1, Sample Phase 2, and Test Phase, with 2 min between phases) to explore the arena using a published protocol (McLean et al., 2018). During Sample Phase 2, the position of the objects was changed in a new arena. During the Test phase, two identical objects (seen in Sample Phase 1) were placed in the old arena (used in Sample Phase 1); however, one, whilst familiar in its position and context, is novel in its position-context configuration. Increased exploration of the novel object (proportion of time > 0.5) indicates a functioning episodic memory.

Spatial memory test – assessing working memory. In the Sample Phase, mice were placed in the arena containing two different objects. Each animal was allowed to explore the box for 2 x 3 min sessions (2 min apart). During the Test Phase, one of the objects is replaced with a new object that is identical to one of the previous objects. Increased exploration of the object in a novel position (proportion of time > 0.5) indicates a functioning spatial memory (McLean et al., 2018).

2.3. Western blot

The protein levels were measured in the whole brains of P1 offspring, including oxidative phosphorylation (OXPHOS) complexes I-V, mitophagy fission marker dynamin-related protein (Drp)-1 and fusion marker optic atrophy (Opa)-1, and endogenous antioxidant manganese superoxide dismutase (MnSOD) following our published methods (Chan et al., 2017; Chan et al., 2016). Whole brains were homogenised using lysis buffer for whole protein and mitochondrial protein fractions, as previously described (Nguyen et al., 2015). Protein concentration was measured by DC protein assay, and samples were separated in the Criterion™TGX Stain-Free Precast Gel (Bio-Rad, USA) and then transferred onto PVDF membranes (Bio-Rad) followed by blocking with 2 % BSA. Membranes were incubated with primary antibodies (OXPHOS complexes [1:2500, Abcam, Cambridge, UK], Drp-1 [1:2000, Novus Biotechnology, Colorado, USA], Opa-1 [1:2000, Novus Biotechnology, Littleton, USA], MnSOD [1:2000, Millipore, MA, USA], cytochrome *c* oxidase subunit [Cox] IV [1:10000, Novus Biological], β-actin [1:3000, Santa Cruz Biotechnology, Texas, USA]) overnight at 4 °C and incubated with secondary antibodies (goat anti-rabbit or rabbit anti-mouse IgG horseradish peroxidase-conjugated secondary antibodies, 1:5000 for OPA-1, MnSOD, OXPHOS complexes; 1:2000 for Drp-1; 1:1000 for β-actin, Santa Cruz Biotechnology; 1:10000 for CoxIV, Bio-Rad) for 1 h at room temperature. Protein expression was detected by SuperSignal West Pico Chemiluminescent substrate (Thermo, MA, USA) and Fujifilm LAS-3000 (Fujifilm, Tokyo, Japan). ChemiDoc (Bio-Rad, USA) was used to capture the images, and Image J was used to measure band density. CoxIV was used as a housekeeping protein for OXPHOS complexes in mitochondria, and β-actin was used as a housekeeping protein for Drp-1, Opa-1, and MnSOD in the whole brain.

2.4. Immunofluorescent staining

Paraffin-embedded brain tissues were sectioned (5 μm) in the coronal plane. After deparaffinisation and rehydration through graded ethanol solutions, antigen retrieval was performed by immersing all slides in citrate antigen retrieval solution buffer (Sangon Biotech, China) (pH=6.0) at 100 °C for 15 min. Immunohistochemistry was carried out using the following primary antibodies: mouse anti-Tuj1 (1:500, MMS-435P, Biolegend, USA), rabbit anti-MAP2 (1:500, 4542s, CST, USA), rabbit anti-Synapsin1 (1:1000, 5297s, CST, USA), rabbit anti-PSD95 (1:500, GTX133091, GeneTex, USA), rabbit anti-Cleaved caspase3 (1:1000, 9661s, CST, USA), rat anti-MBP (1:500, GTX133091, Millipore, USA), and goat anti-Iba-1 (1:500, ab5076, Abcam, USA). Prior to primary antibody incubation, slides were incubated in 5 % normal goat serum in phosphate-buffered saline with Triton X-100 at pH 7.4 (PBST) for 30 min. Primary antibodies were diluted with 5 % normal goat serum in phosphate buffer (PBG) and incubated overnight at 4 °C. Slides were washed in PBST and incubated in goat anti-rabbit Alexa Fluor 488 (1/200, Invitrogen, USA), goat anti-rat Alexa Fluor 488 (1:200, Invitrogen, USA), goat anti-mouse Alexa Fluor 555 (1:200, Invitrogen, USA), or donkey anti-goat Alexa Fluor 555 (1/200, Invitrogen, USA) in 5 % PBG for 2 h at room temperature. Slides were washed with PBST and counterstained by DAPI for 10 min. All slides were cover-slipped in a fluorescent mounting medium (Dako, Denmark). Imaging was carried out using a confocal microscope LSM900 (Zeiss) or slice scanner VS200 (Olympus). Image analysis was carried out on samples taken from the cortex and hippocampus using ImageJ software (National Institutes of Health, USA).

Mitochondrial density was evaluated by labelling formalin-fixed, paraffin-embedded tissue sections with MitoTracker Orange dye (Thermo Fisher Scientific, CA, USA). After deparaffinisation and rehydration through graded ethanol solutions, the sections were incubated with MitoTracker Orange (1:5000 in PBS) for 30 min at room temperature. The sections were then rinsed with PBS for 3 times, cover-slipped with Vectashield mounting medium (Vector Laboratories, CA, USA), and examined by fluorescence microscopy using a fluorescein filter. ImageJ software was used to quantify the density of the MitoTracker staining.

2.5. RNA sequencing and data analysis

At postnatal day 1 (P1), one male and one female pup were selected from every litter to obtain n = 6 male and n = 6 female offspring for analysis. Total RNA extraction was performed with NucleoSpin TriPrep (Macherey-Nagel, Cat. No. 740966.50) following the manufacturer's protocol. Briefly, whole brains (n = 6 per group) were homogenised with the substitution of β-mercaptoethanol with DTT and filtered through the NucleoSpin Filter followed by multiple washing steps. DNA was removed by rDNase mixture. RNA was eluted with RNase-free H_2O. RNA sequencing was performed at the Ramaciotti Centre at UNSW Sydney, NSW, Australia. Alignment to the *mus musculus* genome GRCm38 was performed using the Ensembl gene annotation (release 99, https://ensembl.org/). Quality control was assessed using R (v3.6.3) and *limma* (v3.42.2) by conducting a principal component analysis. Differential gene expression analysis was performed with DESeq2 (v1.26.0) to assess the $PM_{2.5}$-induced gene expression within and/or between stimulations and/or sex. Differentially expressed genes (DEGs) between female and male SHAM were assessed by adding *Sex* in the equation. DEGs between $PM_{2.5}$ and SHAM disregarding gender were determined by correcting for the first three principal components (PCs) and *Sex,* adding the *Treatment* as a variable of interest. Investigating the DEGs of the delta (PM vs SHAM) between the genders, correcting for the first three PCs, *Sex*, *Treatment* and adding the interaction term *Sex*:*Treatment* as a variable of interest. Lastly, examine the effects of $PM_{2.5}$ on gene expression within sex, correcting for the first three PCs and adding *Treatment* as a variable of interest. P values were adjusted for multiple tests using the Benjamini-Hochberg procedure (Benjamini and Hochberg, 1995).

2.6. In vitro gene manipulation and mitochondrial function assay

Gene knock-in and knockdown were performed in primary BALB/c mouse foetal neurons. Primary neurons were isolated from the cortex of a 17.5-day-old female embryo (E17.5), and treated with $PM_{2.5}$ for 16 h. A CCK8 assay was used to measure the cell viability of Day 3 and Day 7 cultured neurons to identify the most suitable $PM_{2.5}$ concentration, and 50 μg/mL was selected as the concentration for subsequent experiments (Supplementary Fig. S1a). Gene knock-in and knockdown were performed using lentivirus infection. Primary neurons were transfected with empty carrier lentivirus (Lenti-Ctrl, OBiO Technology (Shanghai) Corp., Ltd) or pSLenti-U6-shRNA (Kdm5c)-CMV-EGFP-F2A-Puro-WPRE, pSLenti-U6-shRNA (Kdm6a)-CMV-EGFP-F2A-Puro-WPRE, pcSLenti-EF1-Puro-CMV-Kdm6a-3xFLAG-WPRE and pcSLenti-CMV-Kdm5c-

3xFLAG-P2A-Puro-WPRE for 48 h and ready for subsequent experiments. Real-time PCR was used to identify the most efficient lentiviruses for overexpression and knockdown of *Kdm6a* and *Kdm5c* (Supplementary Fig. S1B, C).

Mitochondrial respiration was measured by the Seahorse XF24 Extracellular Flux Analyzer and the XF Cell Mito Stress Test kit (Seahorse Bioscience, North Billerica, MA, USA) according to the manufacturer's instructions. Mitochondrial respiration was reflected by oxygen consumption rates (OCRs) (Wang et al., 2021). Oligomycin (1.5 μM) was used to inhibit ATP synthase. Carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP, 2 μM) was used to shuttle protons across the mitochondrial inner membrane independent of AT synthesis and induce maximal activity of the electron transport chain to re-establish the proton gradient across the mitochondrial inner membrane. OXPHOS complexes I (Rotenone, 0.5 μM) and III (Antimycin A, 0.5 μM) inhibitors were added at the end of the experiment to correct the oxygen consumption from non-mitochondrial oxidases.

2.7. Statistical methods

Results are expressed as mean ± SEM. All data were first analysed for parametric distribution. If the data were not normally distributed, they were log-transformed. The data were analysed using one-way or two-way ANOVA where applied, followed by *post hoc* Tukey's test (GraphPad Prism 10, CV, USA). $P<0.05$ was considered significant.

3. Results

3.1. Maternal $PM_{2.5}$ exposure resulted in lower at-birth body and brain weights in males only

We found a sex difference in birth weights of the SHAM offspring (Fig. 1A), where SHAM-females were born with smaller brains than male littermates ($P<0.001$ for net weight, Fig. 1B), albeit without statistical significance after normalisation to body weight (Fig. 1C). However, in adulthood, SHAM-female brain size (as % of body weight) was larger than male counterparts (Fig. 1F). Maternal $PM_{2.5}$ exposure reduced both birth body weight ($P<0.01$) and brain weights ($P<0.01$ for net weight and % of body weight) at P1 in males only (Fig. 1A-C). This difference disappeared in adulthood, suggesting postnatal catch-up growth (Fig. 1D-F). In females, maternal $PM_{2.5}$ exposure did not affect birth body weight between PM-female and SHAM-female mice, although the adult body weight of females born by $PM_{2.5}$-exposed mothers appeared smaller (Fig. 1D). The brain weight as % of body weight was significantly larger in adult females born by $PM_{2.5}$–exposed mothers ($P<0.01$ vs SHAM-female, Fig. 1F) without significant change in net brain weight (Fig. 1E), suggesting preservation of the brain size.

3.2. Effects of $PM_{2.5}$ exposure on neuronal numbers and memory

Before puberty at 4–5 weeks of age, SHAM-females had better short-term memory than their male littermates (SHAM-male), as shown in the Novel Objective Recognition Test (Fig. 2A). Maternal $PM_{2.5}$ exposure significantly impaired the spatial memory (working memory) of female progeny ($P<0.05$, PM-female vs SHAM-female, Fig. 2C), with a marginal adverse effect on their episodic memory (learning memory, Fig. 2B). In adulthood, malfunctions in all memory types were observed in male offspring from dams exposed to $PM_{2.5}$ ($P<0.05$ PM-male vs SHAM-male, Fig. 2D-F). There was no difference in memory functions between the females born by $PM_{2.5}$–exposed mothers and SHAM-female groups in adulthood (Fig. 2D-F).

MAP2 stains mature neurons; its fluorescence intensity was not different between SHAM-males and SHAM-females (Fig. 2G). The PM-male group displayed significantly lower neuronal density (MAP2 staining) observed in the hippocampus and cortex of those born by $PM_{2.5}$-exposed mothers compared to those born by control mothers

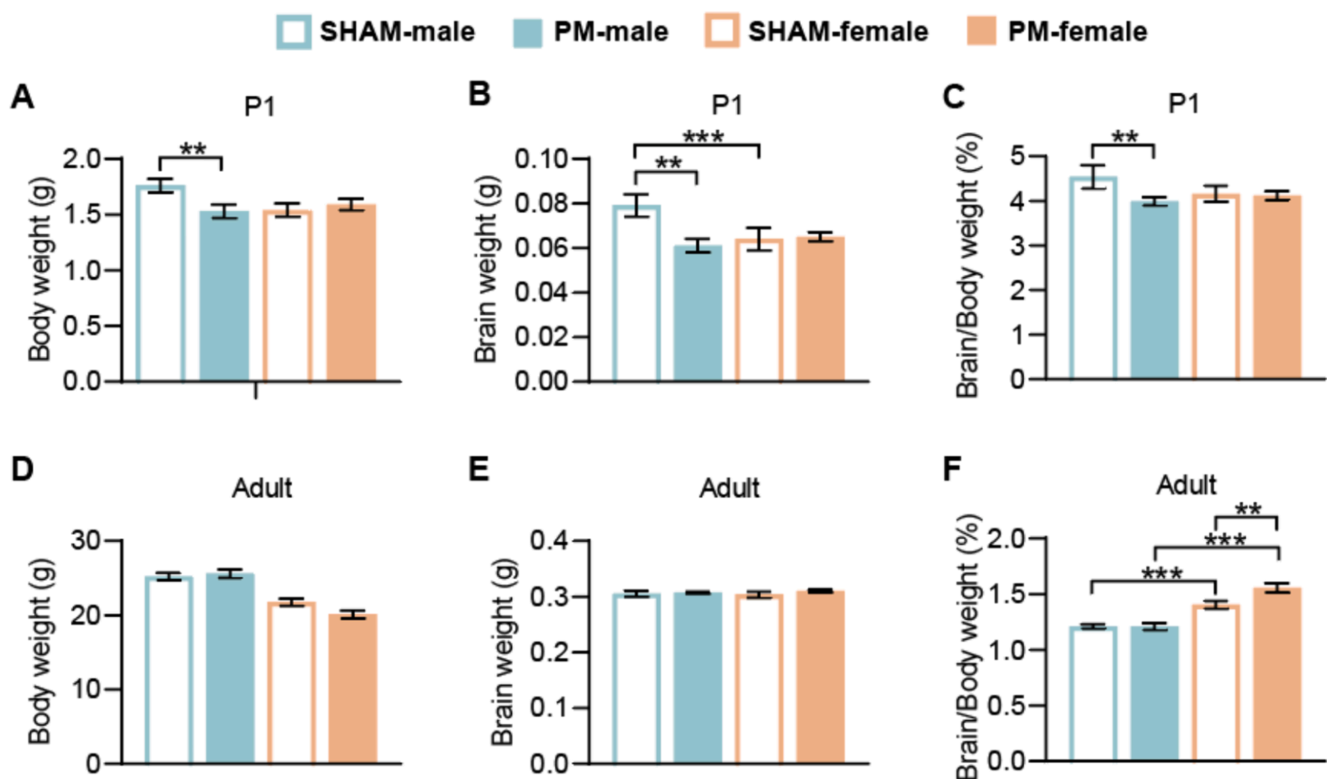

Fig. 1. Body weight and brain weight at postnatal day 1 and adulthood. Body weight (A, D), brain weight (B, E) and brain weight standardised to body weight (C, F) at P1 and adulthood. Results are expressed as mean ± SEM. The differences were two-way ANOVA followed by Tukey post hoc tests. SHAM-male, n = 15; PM-male, n = 14; SHAM-male, n = 11; SHAM-male, n = 16. * $P<0.05$, ** $P<0.01$, *** $P<0.001$.

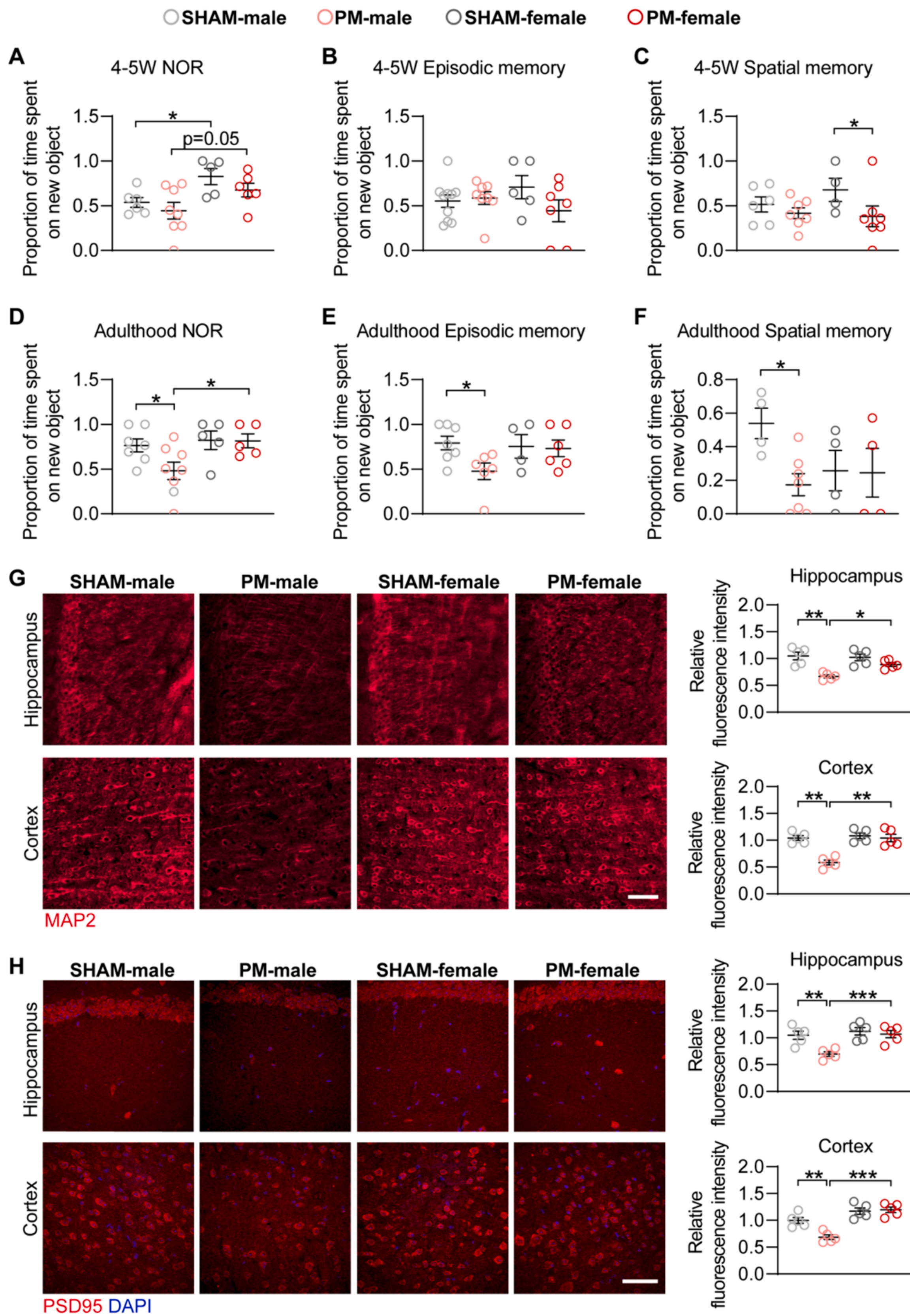

Fig. 2. Cognitive behaviours and neuronal markers. Novel Objective Recognition (NOR) test for short memory, spatial memory, and episodic memory tests for working memory function in male and female offspring at 4–5 weeks (4–5 W, A-C) and adulthood (D-F, n = 4–8). Immunostaining of MAP2 (G), and PSD95 (H) in the hippocampus and cortex in in male and female offspring at adulthood (scale bar = 50 μm, n = 5). Results are expressed as mean ± SEM. Data were analysed by two-way ANOVA followed by Tukey post hoc tests, *P<0.05, **P<0.01, *** P<0.001.

(both $P<0.01$ PM-male vs SHAM-male, Fig. 2G). The synaptic protein marker PSD95 was also reduced in males born by $PM_{2.5}$-exposed mothers compared to SHAM-males in both brain regions (both $P<0.01$, Fig. 2H). Neither MAP2 nor PSD95 density was significantly changed in female mice by maternal $PM_{2.5}$ exposure. The numbers of the Iba1-positive microglia within the hippocampus and cortex were not different between all groups and sexes (Supplementary Figure S2).

3.3. Maternal $PM_{2.5}$ exposure affects brain mitochondrial functional and mitophagy markers in male offspring

At P1, SHAM-females have higher levels of complexes I and IV than their male littermates, suggesting a higher capacity to receive and use metabolic substrates (Fig. 3A, B, E). Maternal $PM_{2.5}$ exposure decreased protein levels of complexes II, III, and V in the PM-male offspring (Fig. 3A, C, D, F), while increasing protein levels of complexes II and V in female offspring (Fig. 3C, F). Overall, females born by $PM_{2.5}$-exposed mothers have higher protein levels of complexes I, II, IV, and V than their male littermates, which may give females some advantages in generating sufficient ATP to meet energy demand. Females also have an increased capacity to renew injured mitochondria and defend against oxidative stress, reflected by higher protein levels of mitophagy fission (Drp-1, Fig. 3G, H) and fusion (Opa-1, Fig. 3G, I) markers, and endogenous manganese superoxide dismutase (MnSOD) (Fig. 3G, J). Maternal $PM_{2.5}$ exposure suppressed MnSOD levels in the male offspring ($P<0.05$, PM-male vs SHAM-male, Fig. 3G, J), suggesting increased oxidative stress in their brains.

3.4. Maternal $PM_{2.5}$ exposure differentially affects gene expression in newborn male and female brains

The RNA sequencing data were generated from 6 biologically independent brain samples in each group, which provided robust statistical power without the need to verify the changes by real-time PCR. We found no significant differences in gene expression between PM-male and Sham-male offspring (data not shown), suggesting a lack of response to maternal $PM_{2.5}$ exposure. We first analysed gene expression between PM-females and SHAM-females. Compared with SHAM-females, 4 genes were significantly downregulated, and 23 genes were significantly upregulated in PM-female's brains (Fig. 4A). The upregulated genes include *Kdm5c, Kdm6a, Slc16a7, Slc17a6, Slc38a4*, and *Ntng1*. The downregulated genes include *Ntn1* and *Dgat1*. The differentially expressed genes are involved in histone lysine demethylation (Kdm5c, Kdm6a), nervous system development, neurogenesis, and neuron differentiation (e.g., *Ntn1, Ntng1, Tcf7l2, Nefl, Cdkl5*). These data show a clear sex difference, PM-female and PM-male mice had different expression of genes in response to maternal $PM_{2.5}$ exposure (Fig. 4B). There were 53 genes that were significantly downregulated and 75 genes that were significantly upregulated. Among the upregulated genes were *Eif2s3x, Kdm5c, Kdm6a, Neurod6*, and *Erdr1*. Among the downregulated genes were *Nrsn2, Nap115*, and *Ntn1*. When adjusting for maternal PM exposure and sex, there were two significantly differentially expressed genes, *Slc38a4* and *Cldn11*, (adjusted FDR value of 0.05 and 0.05, Log2 Foldchange of 1.147 and 2.141, respectively). *Slc38a4* is a Na^{+}-dependent neutral amino acid transporter, which, in particular, transports astrocytic glutamine, an obligatory precursor for glutamate and GABA (Morotti et al., 2021). *Cldn11* plays a critical role in the formation and maintenance of tight junctions between cells in the myelin sheaths, important for nerve axons and fibre protection (Devaux et al., 2010).

Here, *Eif2s3x* may drive X-inactivation escaping in newborn female brains, where two X-chromosome-linked histone lysine demethylase genes – *Kdm6a* and *Kdm5c*, were both highly expressed, resulting in a cascade expression of key genes involved in early brain development and neural protection, insulating the female foetal brain from the adverse effect of *in-utero* $PM_{2.5}$ exposure.

3.5. Overexpressing Kdm6a and Kdm5c protects female neurons from the adverse effects of $PM_{2.5}$ exposure

Kdm6a and Kdm5c are histone demethylases located on the X-chromosome and were among the top upregulated genes identified by RNA sequencing (Fig. 4). *Kdm5c* mutation in humans is linked to intellectual disability due to its role in normal foetal brain development. Therefore, Kdm6a and Kdm5c may play a key role in protecting female offspring's cognition during maternal $PM_{2.5}$ exposure (Karwacki-Neisius et al., 2024). Re-analysis of a previously published single-cell RNA sequence database of the human foetal brain showed that *Kdm5c* and *Kdm6a* are significantly involved in brain development, neuronal differentiation, and neuronal mitochondrial activities (Supplementary Figure S3). However, the role and sex-dependent effects of these two histone demethylases in neurons are unknown. Therefore, we hypothesised that Kdm6a and Kdm5c are important for regulating gene responses in female neurons. The *in vitro* experiments focused on investigating their roles in neuronal response to $PM_{2.5}$ exposure.

We used primary cortical neurons to examine the upregulation and downregulation of these two genes on cellular response to $PM_{2.5}$ exposure, which better represents *in vivo* conditions where complete gene deletion due to $PM_{2.5}$ exposure is unlikely. In the presence of $PM_{2.5}$ (50 μg/mL), we used a lentivirus transfection system to upregulate or knock down Kdm6a and Kdm5c expression (Supplementary Fig. S1C-F).

Inhaled PMs can cross the blood–brain barrier to deposition in various brain regions (Vanbrabant et al., 2024). The same phenomenon is expected for foetal brains in pregnant mothers inhaling PM during pregnancy. Therefore, we investigated the direct neurotoxicity of $PM_{2.5}$ in primary foetal neurons. Cortical neurons overexpressing *Kdm6a* or *Kdm5c* were resistant to $PM_{2.5}$-induced neurotoxicity *in vitro*. The suppressed pre-synaptic marker Synapsin1 and neurite length by $PM_{2.5}$ exposure were restored in cells with *Kdm6a* or *Kdm5c* overexpression (Fig. 5A-C). In addition, $PM_{2.5}$ exposure induced a marked increase in oxidative stress and associated apoptotic marker caspase-3 in neurons, which was also significantly lower in neurons overexpressing *Kdm6a* or *Kdm5c* (Fig. 5D, E). On the other hand, the knockdown of either *Kdm6a* or *Kdm5c* in cortical neurons did not exacerbate $PM_{2.5}$-induced oxidative stress, apoptosis, synapse loss and neurite damage in cortical neurons *in vitro* (Supplementary Figure S4), suggesting Kdm6a and Kdm5c may compensate for each other when one is downregulated. However, knockdown of both *Kdm6a* and *Kdm5c* in neurons exaggerated the neural toxicity of $PM_{2.5}$ exposure in primary neurons, reflected by decreases in the levels of Synapsin-1 and neurite length (Fig. 6A-C), as well as further increased oxidative stress and apoptosis markers in $PM_{2.5}$ exposed cells with both *Kdm5c* and *Kdm6a* knockdown (Fig. 6D-G).

3.6. Primary neurons overexpressing Kdm6a or Kdm5c were resistant to $PM_{2.5}$ exposure-induced impairment of mitochondrial respiratory function

Mitochondria are vulnerable to oxidative stress, including increased ROS accumulation, as shown in Fig. 5D and 6D. As expected, when $PM_{2.5}$ significantly reduced cell viability in primary neurons, mitochondrial function was also suppressed, maybe due to oxidative stress injury caused by $PM_{2.5}$, reflected by the Seahorse assay measuring mitochondrial function (Fig. 7A), including respiration at baseline and under stress, as well as the ability to produce ATP (Fig. 7B-F). Overexpression of *Kdm6a* or *Kdm5c* partially restored protein leak and maximum respiratory function (Fig. 7D, E); however, there were no effects on spare respiratory function (Fig. 7F). Knockdown of *Kdm6a* did not exacerbate $PM_{2.5}$-induced mitochondrial malfunction (Fig. 7G-L); however, when *Kdm5c* was knocked down, maximal respiration was further reduced, and spare respiratory function was decreased (Fig. 7K, L). Double knockdown of *Kdm6a* and *Kdm5c* nearly diminished mitochondrial respiration and ATP production in response to the cytotoxicity of $PM_{2.5}$ (Fig. 7M-R).

These findings suggest that both Kdm5c and Kdm6a support

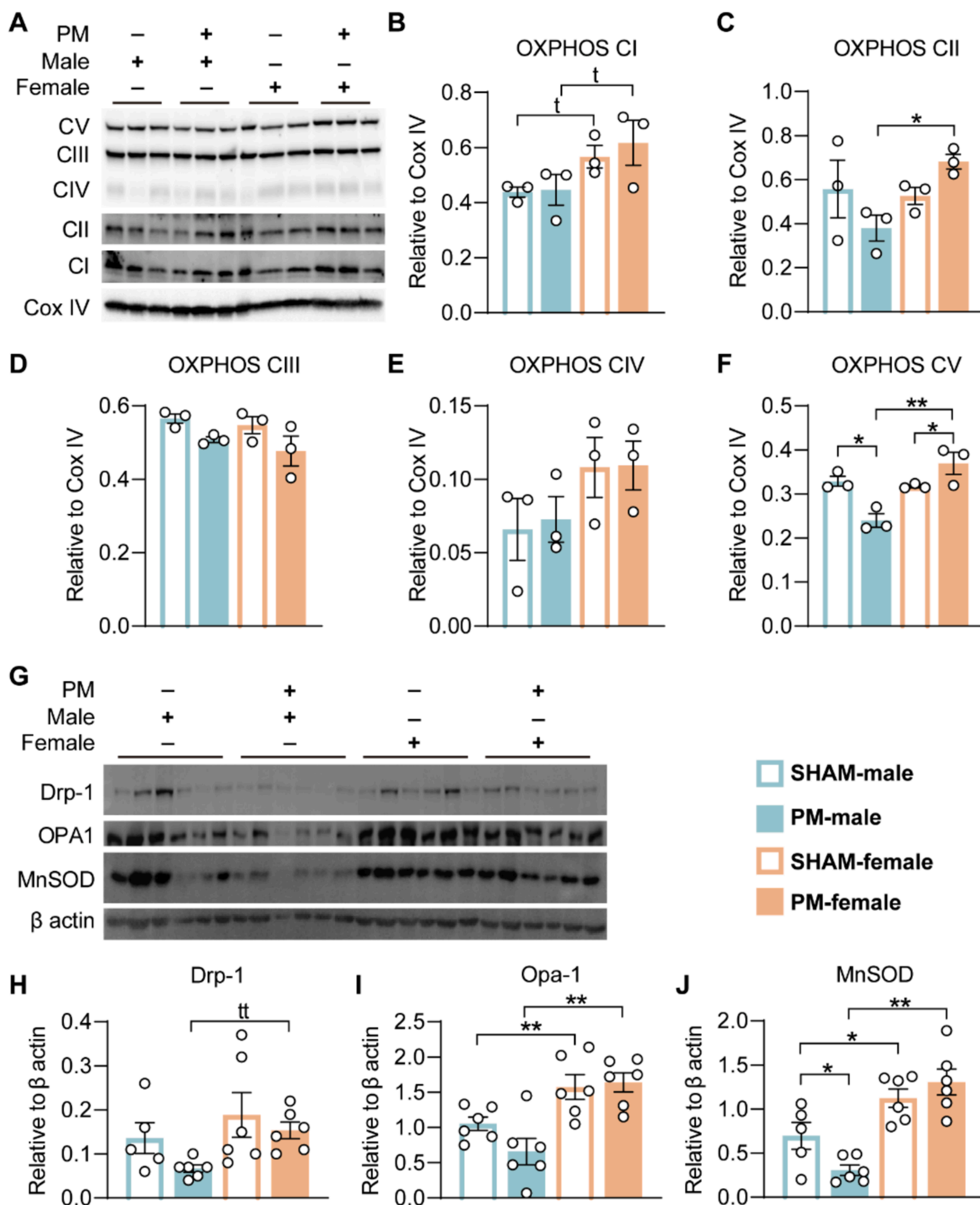

Fig. 3. Mitochondrial markers. Protein levels of mitochondrial OXPHOS complex (C) I-V (n = 3, A-F), mitophagy markers Drp-1 (n = 6, G, H) and Opa-1 (n = 6, G, I), and endogenous antioxidant MnSOD (n = 6, G, J) in the brains of male and female offspring at birth. Results are expressed as mean ± SEM. Data were analysed by two-way ANOVA followed by Tukey post hoc tests, *P<0.05, **P<0.01. Conditional *t*-test, *t* P<0.05, *tt* P<0.01.

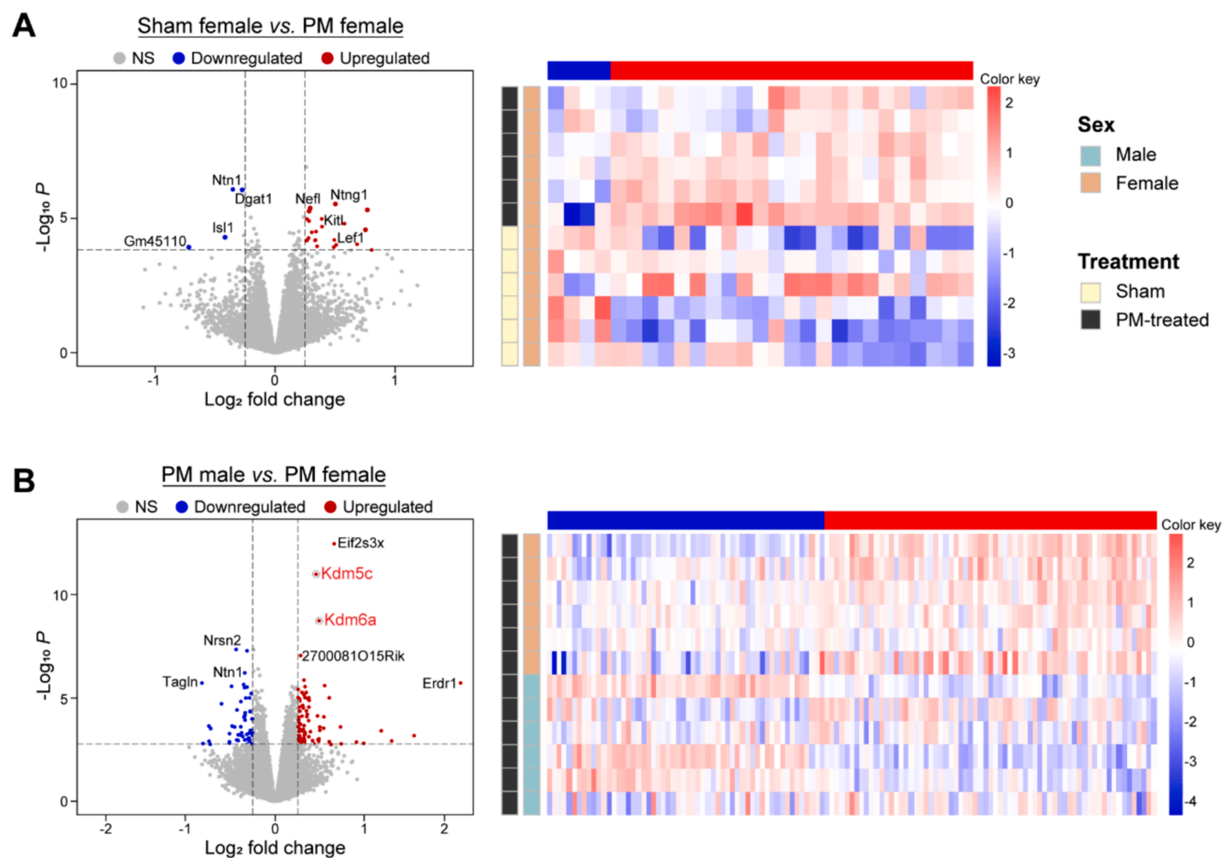

Fig. 4. RNA-sequencing analysis. Volcano plot of −log10 (p-value) against log2 fold change in gene expression and heatmap of significantly differentially expressed genes between the PM-female and SHAM-female (A), PM-females and PM-male (B) groups (n = 6). Heatmap data were filtered to have an FDR-adjusted p < 0.05 & |LFC| > 1.2. Red and blue dots are the differentially expressed genes exceeding the threshold (FDR adjusted p-value < 0.05 and |LFC| > 1.2).

mitochondrial function under the stress of $PM_{2.5}$ exposure, yet Kdm5c is less dispensable than Kdm6a. Increasing Kdm5c or Kdm6a can be protective to the neurons, which may be the key to female resilience towards maternal $PM_{2.5}$ exposure compared with their male littermates.

4. Discussion

Here, we have revealed several paradigm shifting discoveries. Firstly, continuous low -'safe' - levels of $PM_{2.5}$ exposure in mouse mothers can have long lasting adverse impacts on offspring's memory function, which is more prominent in male mice. Secondly, the *in-utero* period is a critical window for sex-hormone-independent genetic adaptations in mice brains. Here, we hypothesise that epigenetic programming acts as a 'main switch' that diverges from conventional single-gene-centric approaches. For the first time, we report a brain histone-related adaptation to preserve brain cognitive function in female mouse offspring mediated predominantly by an upregulation of neuronal *Kdm5c* and *Kdm6a* in response to *in-utero* $PM_{2.5}$; however, the impaired cognitive function in male mice was not due to the loss of Kdm5c or Kdm6a functions, suggesting a different mechanism by foetal programming from that concluded from loss of function mice in a recent study (Karwacki-Neisius et al., 2024). This is possibly linked to unique X-inactivation escape patterns, functioning as the genetic basis of cognitive behavioural outcomes influenced by environmental factors, whereas, in the literature (Hüls et al., 2022; Guo et al., 2022; Caramaschi et al., 2022), this has been frequently attributed to DNA epigenetic modifications (Fig. 8).

All stages of foetal development and birth outcomes are vulnerable to any interruption to the intrauterine environment, which can make the foetus and newborns susceptible to postnatal non-communicable illnesses, such as respiratory disorders, metabolic disorders, and cardiovascular diseases (Chen et al., 2013; Zhang et al., 2006). In terms of neurological malfunction, different *in-utero* toxins (e.g. maternal smoking and maternal e-cigarette vapour exposure) appear to share a similar mechanism involving oxidative stress, inflammation, and mitochondrial disorders in newborn brains, which can persist into adulthood (Chen et al., 2021). In this study, we administered low-level $PM_{2.5}$, while potential health/neurological issues due to 'low-level' $PM_{2.5}$ exposure are often overlooked in scientific studies. We also identify the critical genes (namely, two X-chromosome-linked histone lysine demethylases – Kdm5c and Kdm6a) that contribute to the sex difference in response to such a stimulus, protecting the female brain from deleterious effects of continuous maternal $PM_{2.5}$ exposure. PM is a strong oxidant, which drives adverse health effects (Daellenbach et al., 2020). Its effects on the newborn brain can be as profound as those on the foetal brain due to *in-utero* $PM_{2.5}$ exposure (Clifford et al., 2016; Pardo et al., 2020; Wang et al., 2009). In this study, we separated maternal exposure from direct postnatal inhalation, which is difficult in human studies. We used three widely recognised cognitive tests to measure short-term memory (Novel Objective Recognition Test), learning memory (episodic memory test: What-Where-Which learning tasks), and working memory (spatial memory: What-Where) using the nature of rodents to explore a new object (McLean et al., 2018). In our model of maternal exposure to low-level traffic $PM_{2.5}$, PM-female mice had a transient impairment of memory function in childhood, which recovered in adulthood. However, their male littermates had a delayed cognitive impairment persisting in adulthood. Indeed, this finding in our animal model is akin to the findings in humans exposed to heavily polluted air, suggesting no safe limit of $PM_{2.5}$ exposure for human health, especially that of unborn children (Yi et al., 2022; Clifford et al., 2016).

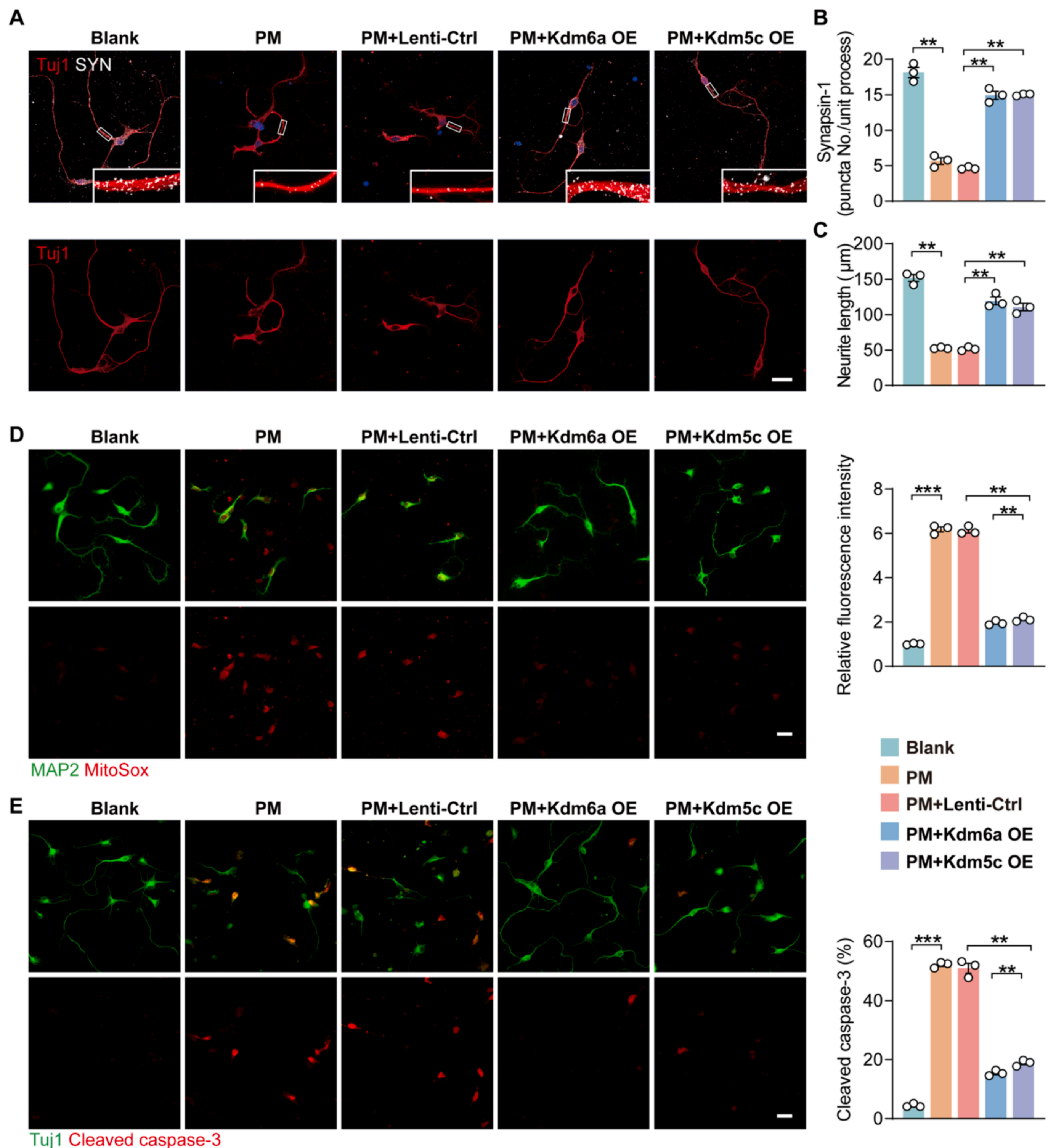

Fig. 5. Primary cortical neurons overexpressing Kdm6a or Kdm5c were resistant to $PM_{2.5}$ induced neurotoxicity, oxidative stress, and apoptosis *in vitro*. Co-immunostaining of Tuj1 (neuronal marker) and Synapsin1 (SYN, a pre-synaptic protein marker), representative images (A), quantification of Synapsin1 (B) and neurite length (C); co-immunostaining of MAP2 and MitoSox (Mitochondrial Superoxide Indicator), and quantification of MitoSox staining (D), as well as co-immunostaining of Tuj1 and cleaved caspase-3 (apoptosis-related marker) co-staining and quantification of caspase-3 staining (E) in cells with Kdm6a or Kdm5c overexpression. Results are presented as mean ± SEM, n = 3, scale bare = 25 μm. *P<0.05, **P<0.01, ***P<0.001, analysed by one-way ANOVA followed by post hoc Tukey tests.

Sex is an important factor in influencing the foetal development and formation of cognitive function (Li and Singh, 2014). Females were born smaller than males, yet the net weight of their brains was comparable to males in adulthood, suggesting a minimal brain volume is required to support normal neurological functions in adulthood. On the other hand, both birth weight and brain weight are critical for the maturation of cognitive functions (Clifford et al., 2016). PM-male mice have reduced birth weight and brain weight, accompanied by impaired cognitive

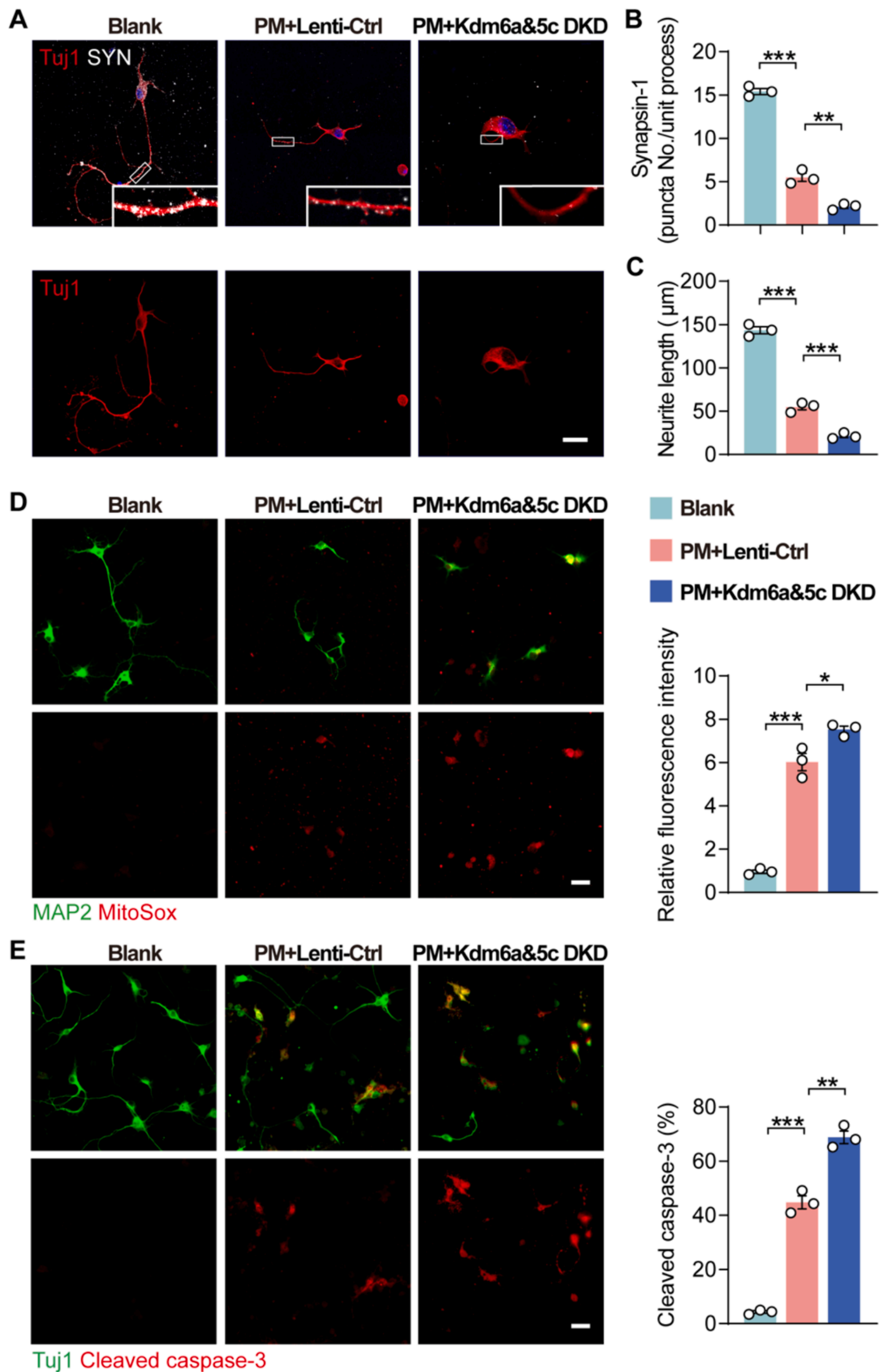

Fig. 6. Knockdown of both Kdm6a and Kdm5c in primary cortical neurons exacerbated $PM_{2.5}$-induced neurotoxicity, oxidative stress, and apoptosis *in vitro*. Co-immunostaining of Tuj1 and Synapsin1 (A, B) and neurite length (A, C), MAP2 and MitoSox co-staining (D), as well as Tuj1 and cleaved caspase-3 co-staining (E) in cells with Kdm6a and Kdm5c downregulation. Results are presented as mean ± SEM, n = 3 independent experiments, scale bare = 25 μm. *P<0.05, **P<0.01, ***P<0.001, analysed by one-way ANOVA followed by post hoc Tukey tests.

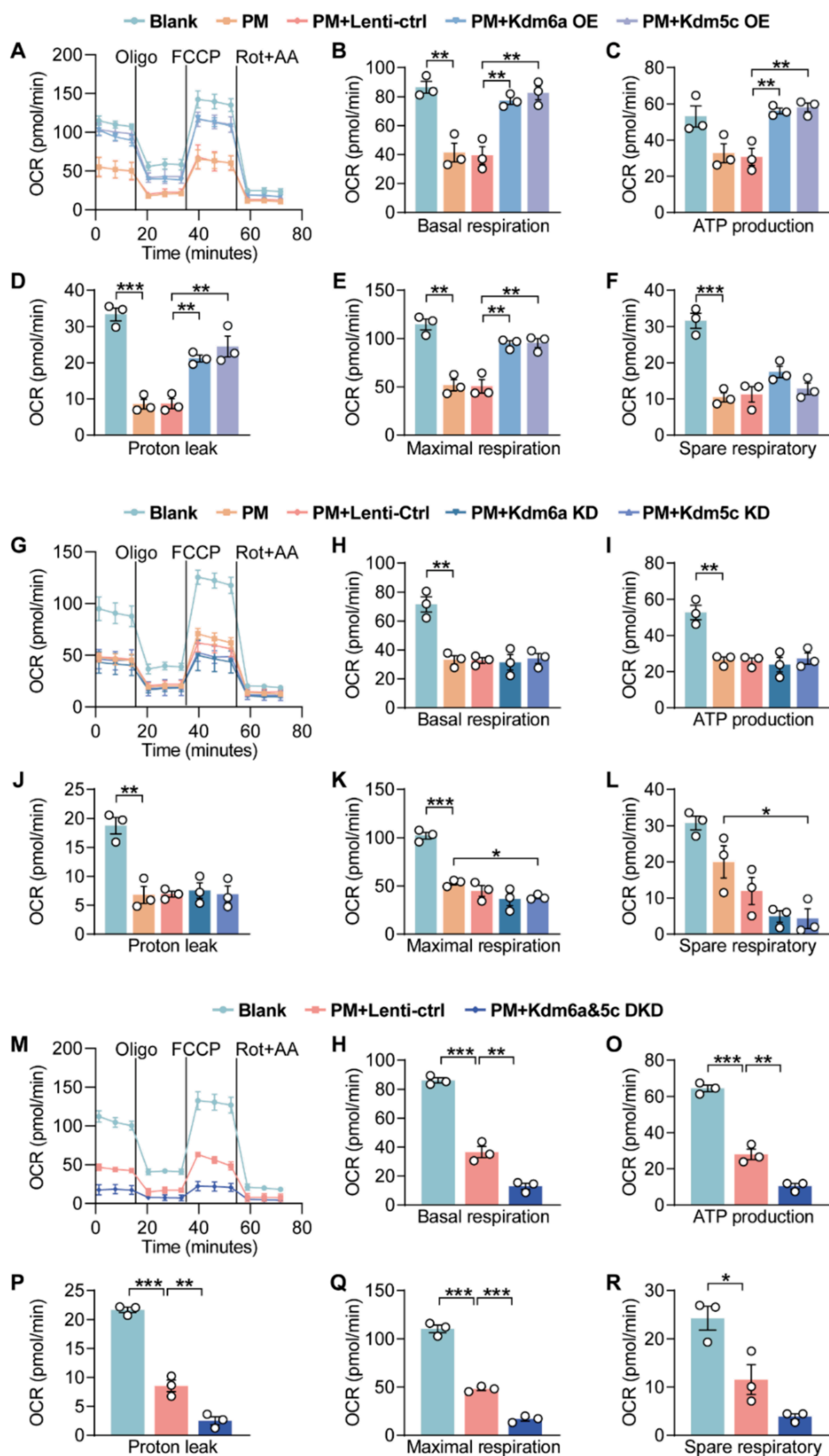

Fig. 7. Mitochondrial function in primary neurons with Kdm6a or Kdm5c overexpression and knockdown. Mitochondrial respiratory function in primary neurons with Kdm6a or Kdm5c overexpression (A-F) and knockdown (G-L), as well as with both Kdm6a and Kdm5c knockdown (M−R). Results are presented as mean ± SEM, n = 3, *P<0.05, **P<0.01, ***P<0.001, analysed by one-way ANOVA followed by post hoc Tukey tests. FCCP: *carbonyl cyanide-p-trifluoromethoxyphenylhydrazone*; RO: Rotenone; AA: Antimycin A; OCR: Oxygen consumption rate.

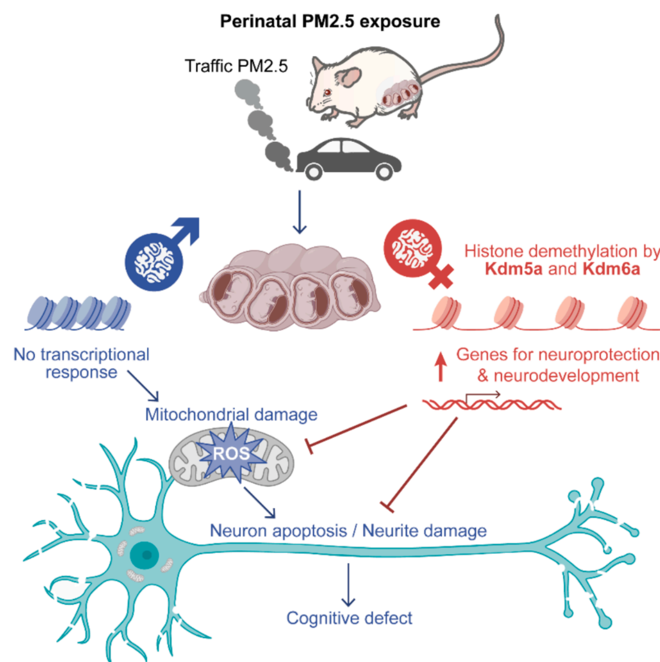

Fig. 8. The diagram describes the working mechanism of how epigenetic response protects female brains from maternal $PM_{2.5}$ exposure (elements from BioRender.com).

performance. As the powerhouse, mitochondria play a key role in neural development and cognitive function, and mitochondrial malfunction is a crucial factor in some pathological situations where oxidative stress is increased, e.g., dementia and neurodegenerative diseases (Chen et al., 2022; Yi et al., 2022; Fernandez et al., 2019; Zhao et al., 2020). Mitochondrial complexes I and II in the mitochondrial inner membrane are the substrate entry points in the last stage of ATP synthesis in OXPHOS complex V (Dudkina et al., 2010). ROS are produced as a by-product in complex V during ATP synthesis. Endogenous MnSOD scavenges excess ROS, to prevent ROS-induced cellular injury. Here, we observed that females have more protein levels of mitochondrial OXPHOS complexes, better mitochondrial repairing machinery, and a greater level of endogenous antioxidant MnSOD than their male littermates, meaning that under the same *in-utero* environmental stress, female cells are innately protected from oxidative stress. This is further exemplified by the observation of normal *in-utero* development and expression of mitochondrial markers in PM-female mice, whereas significant defects occurred in their male littermates. Therefore, brain mitochondrial health may also determine the cognitive outcome of *in-utero* PM exposure.

Sex hormones and their effects are often used to explain sex-related disease susceptibility; however, this is not applicable to newborns, as sex hormones are only present post-puberty. In PM-male's brains, there was an absence of gene response to *in-utero* $PM_{2.5}$ exposure, as shown by the RNA-sequencing results, whereas genes involved in neurodevelopment were upregulated in their female littermates to support normal brain development. Among the genes markedly upregulated in PM-female's brains, there are two histone demethylases, Kdm6a and Kdm5c. Kdm5c and Kdm6a can act as molecular keys, which open the chromatin for transcription and thus regulate genes on the other chromosomes to enable brain adaptability to $PM_{2.5}$ exposure. Kdm6a increases gene transcription by removing the repressive methylation mark on histone 3. Mutation of *Kdm5c* is associated with X-chromosome-linked neurodevelopmental disorders, as Kdm5c is involved in fine-tuning enhancer activity during neuronal maturation (Poeta et al., 2019; Scandaglia et al., 2017). Kdm5c knockout mice show adaptive and cognitive abnormalities, impaired social behaviour, memory deficits, aggressive behaviour, and seizure susceptibility (Karwacki-Neisius et al., 2024; Scandaglia et al., 2017). Such evidence in the literature strongly suggests epigenetic modification on histone may be the key to foetal response to maternal $PM_{2.5}$ exposure. Indeed, in this study, there were changes in multiple genes in the same female brains that were critical for normal brain development and function, contrasting with no significance in male littermates. Particularly, Kdm5c in the pseudo-autosomal region (meaning its Y-chromosome allele has the same coding) seems only responsive in females to *in-utero* $PM_{2.5}$ exposure. The functional significance was verified by upregulating *Kdm5c* or *Kdm6a* in foetal primary neurons *in vitro*, effectively mitigating $PM_{2.5}$-induced oxidative stress and neural death. Therefore, the high activities of Kdm5c and Kdm6a in neurons in early life may be critical in maintaining normal numbers of neurons and synapses, as well as preserving normal memory functions in PM-female mice. On the other hand, either Kdm5c or Kdm6c was significantly changed in male mice with *in-utero* $PM_{2.5}$ exposure, albeit with impaired cognitive function, suggesting different 'main switch' mechanisms are involved which can be followed up in future studies. Furthermore, we propose for the first time that as a safeguard, the functions of Kdm5c and Kdm6a seem to be redundant, meaning there exists an unknown mechanism to maintain the baseline function for survival when both genes are knocked down. This warrants investigation in future studies.

Disruption in placental blood perfusion and physiology has been proposed to contribute to foetal underdevelopment caused by prenatal $PM_{2.5}$ exposure (Li et al., 2022). Evidence also suggests differential changes in placental development and gene expression due to foetal sex in response to adverse environmental factors (Kalisch-Smith et al., 2017). These genes changed by maternal $PM_{2.5}$ exposure are involved in nutrient metabolism, DNA damage, mRNA splicing, mitochondrial function, oxidative stress, inflammation, transmembrane transport, and neurotransmitter signalling (Enquobahrie et al., 2022; Kaur et al., 2022; Broséus et al., 2024), which may in turn influence foetal programming in a sex-dependent manner. However, the placenta is a complex organ vital in supporting foetal development. How placental gene changes affect epigenetic response in male and female foetal brains requires further studies, which is beyond the scope of this study.

5. Conclusion

Traffic-derived $PM_{2.5}$ is a significant *in-utero* toxin regardless of the dose of exposure. Epigenetic mechanisms, namely X-chromosome-linked histone demethylases, may function as drivers behind the sexual dimorphism of cognitive outcomes in response to *in-utero* $PM_{2.5}$ exposure.

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CRediT authorship contribution statement

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Declaration of competing interest

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

Data availability

Data will be made available on request.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2024.108971.

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