NEUROLOGICAL EFFECTS IN THE OFFSPRING AFTER SWITCHING FROM TOBACCO CIGARETTES TO E-CIGARETTES DURING PREGNANCY IN A MOUSE MODEL

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KEY WORDS:
Electronic nicotine delivery devices
Addiction
Cotinine
Nicotine
Non-cigarette tobacco products

ABSTRACT

Background: Maternal smoking is currently a public health concern and has been associated with a number of complications in the offspring. E-cigarettes are gaining popularity as a 'safer' alternative to tobacco cigarettes during pregnancy, however, there are a limited number of studies to suggest that they are actually 'safe'.
Study Design: Balb/C female mice were exposed to ambient air (n=8; Sham), or tobacco cigarette smoke (n=8; SE) before gestation, during gestation and lactation. A third group was exposed to cigarette smoke before gestation followed by e-cigarette aerosols during gestation and lactation (n=8; Switch). Male offspring (12-week old, n=10-14/group) underwent behavioural assessments to investigate short-term memory, anxiety and activity using the novel object recognition (NOR) and elevated plus maze (EPM) tests. Brains were collected at postnatal day (P)1, P20 and Week13 for global DNA methylation, epigenetic gene expression, and neuronal cell counts.
Results: The offspring from mothers switching to e-cigarettes exhibited no change in exploration/activity, but showed a decrease in global DNA methylation, Aurora Kinase (Aurk) A and AurkB gene expression and a reduction in neuronal cell numbers in the cornu ammonis 1 region of the dorsal hippocampus compared to the SE group.
Conclusions: Continuous tobacco cigarette smoke exposure during pregnancy resulted in marked neurological deficits in the offspring. Switching to e-cigarettes during pregnancy reduced these neurological deficits compared to cigarette smoke exposure. However, neurological changes were still observed, so we therefore conclude that e-cigarette use during pregnancy is not advised.

INTRODUCTION

Electronic cigarettes (e-cigarettes) are becoming increasingly popular worldwide and are attracting more users. E-cigarettes are battery-powered devices that convert an oily, flavoured liquid into an aerosol. Due to the variety of different flavours available, such as chocolate and cinnamon, e-cigarettes are particularly appealing to young people and they are gaining popularity among pregnant women, primarily as a smoking cessation aid or as a perceived healthier alternative to tobacco smoking (1, 2).

Maternal exposure to tobacco cigarettes during pregnancy is associated with premature birth, low birth weight, cardiovascular problems, respiratory problems and sudden infant death syndrome (3-8). Moreover, children from mothers who smoked during their pregnancy exhibit behavioural changes such as hyperactivity, aggression and anti-social behaviour (9-11). According to the Centers for Disease Control and Prevention, in 2016, the prevalence of maternal smoking was highest among women aged between 20-24 (12). To assist these women to quit smoking, nicotine replacement therapies such as nicotine patches, gum and inhalers are available, however, many studies did not find a significant increase in abstinence (13-16). Therefore, there is a need to find new, innovative ways to assist pregnant women with their nicotine addiction. Although there is currently no evidence to prove that maternal 'vaping' is safe, pregnant women are starting to use e-cigarettes in higher numbers. A study of 445 pregnant women in the United States in 2017 found that 7% of these women used e-cigarettes, and a further 8% were dual users of tobacco and e-cigarettes. Of the e-cigarette users, 74.6% reported that they switched to e-cigarettes once they had learnt that they were pregnant (2). Another review also suggested that the percentage of pregnant women who used an e-cigarette may be as high as 15% (17). Although they are promoted as a 'safer' alternative to smoking

tobacco cigarettes, studies in animals have shown that exposure to e-cigarette aerosols in adult male mice resulted in physiological and behavioural changes (18-20).

We have previously reported the effects of maternal exposure to e-cigarette aerosols (with and without nicotine) on anxiety, exploration/activity and epigenetic gene expression in male offspring (21) and that replacing tobacco cigarette smoke with e-cigarette aerosols during pregnancy showed some benefits for energy homeostasis and inflammation in the offspring brain at weaning (22). The current study investigated whether switching to e-cigarette exposure from tobacco cigarette smoke during gestation, changes offspring behaviour and epigenetics.

MATERIALS AND METHODS

For the full materials and methods, please refer to the Supplementary document 1.

Animal experiment procedure and treatment exposure

All animal experimental procedures were conducted in accordance with the guidelines of the Australian National Health and Medical Research Council with approval from the University of Technology Sydney Animal Care and Ethics Committee (ETH15-0025). Twenty-four female Balb/c mice (7 weeks old, Animal Resource Centre, Perth, WA, Australia) had *ad libitum* food and water with a 12:12 hour light/dark light cycle.

Animals were divided into three treatment groups (n=10-14 per group): (i) Ambient air (Sham); (ii) Tobacco cigarette smoke exposure (SE); and (iii) SE prior to gestation followed by e-cigarette aerosol exposure during gestation and lactation (Switch). Animals from each treatment group were exposed to their condition six weeks prior to pregnancy, during pregnancy and lactation (Figure 1). Animals from the SE and Switch groups were exposed to two cigarettes twice daily (Winfield Red^{TM}, ≤16mg tar, ≤1.2mg nicotine, and ≤15mg of CO; VIC, Australia) in an automated InExpose system (Scireq®, Montreal, QC, Canada). In the Switch group, animals were exposed to e-cigarette aerosols (Tobacco flavour; 18mg nicotine) using the KangerTech NEBOX e-cigarette device (KangerTech, Shenzhen, China). In the Switch group, animals were exposed to e-cigarette aerosols (Tobacco flavour; 18mg nicotine) using the KangerTech NEBOX e-cigarette device (KangerTech, Shenzhen, China) in accordance to the protocol outlined in our previous e-cigarette exposure study (21).

Behavioural assessments

Behavioural assessments were performed on offspring at 12 weeks old and all tests were performed between 0900 and 1400h. Each behavioural test was described in our previous study (21). The novel object recognition (NOR) test was used to assess short-term memory in the animals. The NOR test included a familiarisation and a test phase. The familiarisation phase involved an animal exploring two identical objects for 5 minutes before being returned to its home cage. After a one-hour interval, each animal underwent the test phase where they explored one 'familiar' object and one 'novel' object. The total time spent investigating each object was recorded and calculated as a recognition index using the following equation:

$$Recognition\ Index = \frac{Tn}{(Tn + Tf)}$$

where *Tn* is the time spent exploring the novel object and *Tf* is the time spent exploring the familiar object. Unimpaired animals spend more time exploring the novel object compared to an object that it has seen before (Recognition index > 0.5).

The elevated plus maze (EPM) test was used to measure anxiety and exploration. The EPM consists of an open arm and an enclosed arm. Each animal was placed on the EPM apparatus in the same direction to ensure consistency within each test. Animals were allowed to move freely on the EPM for two minutes before being returned into its home cage. The total time spent in the open arm and the number of centre crosses were recorded. In addition, the total number of head dips and whole body stretches in the close arm (protected) and the open arm (unprotected) were also recorded.

Tissue collection and extraction

Brain tissue and plasma were collected from offspring at P1 (birth), P20 (weaning) and Week13 (adulthood) as previously described (21). Offspring were weighed before tissue collection. The right hemisphere of each P1 and P20 brain was snap frozen. The hippocampus was micro-dissected from the right hemisphere from the Week13 offspring. Total DNA and RNA were extracted using the Isolate II DNA/RNA/protein extraction kit (Bioline, MA, USA) and quantified. RNA integrity was determined using the Experion RNA StdSens Analysis Kit (BioRad, CA, USA).

Plasma cotinine

Whole blood was collected in a heparin-rinsed syringe from mothers and offspring at P20 via cardiac puncture. Each plasma sample was collected by centrifuging the blood at 13k rpm for 5 minutes. Plasma cotinine, a major metabolite of nicotine, was measured using the cotinine ELISA kit (Abnova, Taipei, Taiwan) following the manufacturer's protocol. Samples and control standards (provided by the kit) were processed and the absorbance was read at 450nm using the Tecan's Infinite® M1000 PRO (Thermo Fisher Scientific, MA, USA).

Global 5-mC DNA methylation assay

Global DNA methylation was analysed using the DNA 5-methylcytosine (5-mC) methylation ELISA kit (Zymo Research, CA, USA). DNA samples (100ng) from each offspring at P1, P20 and Week 13 were processed according to the manufacturer's protocol. Anti-5-methylcytosine monoclonal antibody was provided by the kit to detect 5-mC. DNA methylation of each DNA sample was quantified and expressed as a percentage using a standard curve generated by the control standards provided by the kit. Absorbance was read at 450nm using the Tecan's Infinite® M1000 PRO (Thermo Fisher Scientific, MA, USA).

Analysis of epigenetic mRNA gene expression levels

Chromatin modifying mRNA gene expression levels were investigated using RT-qPCR. Total RNA (1µg) was extracted from P1 and P20 brains and Week13 hippocampus. RNA was reverse-transcribed using the Tetro cDNA synthesis kit (Bioline, MA, USA). qPCR amplification of cDNA was performed using the SensiFAST SYBR No-ROX kit (Bioline, MA, USA). All epigenetic primer sequences were listed in our previous study (21). mRNA gene expression was determined using the ΔΔCt method (23) and normalization of mRNA gene expression was adjusted using glyceraldehyde 6-phosphate dehydrogenase (GAPDH) as the reference gene.

Histological tissue preparation

The left hemisphere of P20 and Week13 brains were fixed in 4% paraformaldehyde and paraffin embedded. Coronal sections (5µm) of the brains were made at Bregma; -1.9mm to -2.06mm (24). The sections were stained with cresyl violet (Sigma Aldrich, MO, USA). High power images of the cornu ammonis (CA)1, CA2, CA3 at the dorsal hippocampus were

captured (NDP.view2, Hamamatsu, Shizuoka, Japan) and cell counts per region of interest (ROI) were completed using ImageJ (NIH, NY, USA).

Statistical analysis

All data analysis was conducted blindly and all data were presented as mean ± standard deviation. For the NOR test, a *paired t-test* was used to analyse the recognition index of the familiarisation and test phase. For the EPM test, the cotinine ELISA, the DNA methylation assay, and the mRNA gene expression, a *one-way analysis of variance (ANOVA) with Bonferroni's post-hoc test* was used to compare each treatment group. A *p*-value of less than 0.05 was considered statistically significant and the *p* value for Bonferroni post-hoc tests was reported in the text and figure legends to indicate differences between each group.

RESULTS

Maternal exposure to e-cigarette aerosols after smoke exposure did not have any effect on litter size

There was no significant difference in litter size among the three groups ($p=0.229$) [data not shown]. Only male pups were used as per our previous study (21). There was no evidence of pups being born dead or cannibalised by the mothers. Sample sizes of offspring at each time point ranged between 10-14 depending on the size of the litter.

Plasma cotinine levels from previous and current exposure systems delivered comparable levels of cotinine in the offspring and mothers

The average cotinine level of the mothers from the Sham group was 2.95±0.93ng/ml. Mothers from the SE and Switch groups had a significant increase in plasma cotinine levels of 19.36±10.17ng/ml ($p<0.001$) and 21.97±12.76ng/ml ($p<0.01$), respectively, compared to the Sham group (figure 2a). In the P20 offspring, the average cotinine level in the Sham group was 3.56±1.60ng/ml. Offspring from the SE and Switch groups had a significant increase in cotinine levels of 9.62±3.66ng/ml ($p<0.001$) and 10.93±2.38ng/ml ($p<0.001$), respectively (figure 2b).

Offspring from mothers that switched to e-cigarette aerosol exposure or exposed to continuous cigarette smoke during pregnancy had a low birth weight

In the P1 offspring, the average body weight in the Sham group was 1.69±0.29g. Body weight in the SE and Switch groups were significantly reduced to 1.39±0.10g ($p<0.05$) and 1.41±0.12g ($p<0.01$), respectively, compared to the Sham group (figure 2c). In the P20 offspring, the average body weight in the Sham group was 10.63±1.41g. Body weight in the SE and Switch groups were significantly reduced to 9.45±0.58g ($p<0.01$) and 9.55±0.58g ($p<0.001$), respectively, compared to the Sham group (figure 2d). In the Week13 offspring, there was no significant difference in the body weight between the Sham (26.26±1.82g), SE (25.91±1.60g) and Switch groups (25.81±1.01g) (figure 2e).

Switching to e-cigarette aerosol exposure showed changes in offspring memory and hyperactivity

Behavioural assessments were conducted on Week12 offspring from each treatment group to determine if switching to e-cigarette alters offspring memory (NOR), exploration/activity (EPM) and anxiety (EPM) compared to SE. For the NOR test, in an unimpaired animal, there should be a significant increase in the recognition index from the familiarisation and test phase as shown in the Sham group ($p<0.001$;figure 3a). This result was also observed in the SE group ($p<0.001$;figure 3b). Interestingly, in the Switch group, there was no significant change

between the familiarisation and test phase, indicating short-term memory deficits within these offspring (figure 3c).

In the EPM test, offspring showed a significant increase in the time spent in the open arm in the SE ($p<0.001$) and Switch groups ($p<0.001$) compared to the Sham group, indicating less anxiety (figure 3d). The number of centre crosses is an indication of exploration/activity. There was a significant increase in the number of centre crosses from SE ($p<0.05$) and Switch groups ($p<0.05$; figure 3e) compared to the Sham group. This indicates that offspring are more active and more likely to explore different environments. Other sensitive measures of anxiety such as head dipping and whole body stretches were not different. However, there was a significant increase in the number of unprotected stretches in offspring from the SE group compared to the Sham ($p<0.001$) and Switch groups ($p<0.001$; Supplementary figure 1).

Switching to e-cigarette aerosol exposure reduced offspring global DNA methylation

DNA methylation activates/inactivates genes by controlling the transfer of a methyl group on CpG sites of the genome (25). In the P1 offspring brain, global DNA methylation was 5.6±0.6% in the Sham group. DNA methylation in the SE and Switch groups were significantly increased to 44.3±5.7% ($p<0.001$) and 12.6±1.2% ($p<0.01$), respectively (figure 4a). In the P20 offspring brain, DNA methylation was 5.1±0.6% in the Sham group. DNA methylation in the SE and Switch groups were significantly increased to 39.0±3.6% ($p<0.001$) and 12.3±2.2% ($p<0.001$), respectively (figure 4b). In the Week13 hippocampus, DNA methylation was 9.5±1.1% in the Sham group. DNA methylation in the SE and Switch groups were significantly increased to 40.8±3.2% ($p<0.001$) and 15.9±2.5% ($p<0.001$), respectively (figure 4c). Interestingly, despite the marked increase in global DNA methylation in both the SE and Switch groups, the Switch group showed a lower global DNA methylation when compared to the SE group, regardless of offspring age. Our findings suggest that switching to e-cigarettes during pregnancy reduced DNA methylation in the offspring brain.

Changes in mRNA gene expression of key chromatin modifying genes in offspring from mothers that switched to e-cigarette aerosol exposure

Epigenetic gene expression was analysed by RT-qPCR and outlined in Table 1. Here, we investigated epigenetic gene expression of representative chromatin modification enzymes that were identified and altered in our previous study (21).

Table 1. mRNA gene expression of key chromatin modifiers in the offspring brains.

Time points	Encoded protein	Sham	Smoke Exposure	Switch
Postnatal Day 1 (Whole brain)	Dnmt3a	1.0 ± 0.4	0.1 ± 0.1***†	0.6 ± 0.2
	Dnmt3b	1.0 ± 0.5	0.5 ± 0.1*	1.0 ± 0.3
	Kdm5c	1.0 ± 0.2	8.4 ± 3.2***†††	1.0 ± 0.4
	Kdm6b	1.0 ± 0.1	46.1 ± 30.1**††	0.9 ± 0.3
	Atf2	1.0 ± 0.2	3.7 ± 0.9***†††	0.6 ± 0.2
	Hdac1	1.0 ± 0.1	2.6 ± 0.8**†††	0.8 ± 0.1
	Aurka	1.0 ± 0.2	1.4 ± 0.6	0.3 ± 0.0*###
	Aurkb	1.0 ± 0.1	2.9 ± 0.4***†††	0.4 ± 0.1***
	Aurkc	1.1 ± 0.4	2.0 ± 0.7	5.0 ± 3.5
Postnatal Day 20 (Whole brain)	Dnmt3a	1.0 ± 0.1	0.6 ± 0.1	0.8 ± 0.3
	Dnmt3b	1.0 ± 0.3	0.7 ± 0.2	0.8 ± 0.4
	Kdm5c	1.0 ± 0.2	0.4 ± 0.1***†††	0.9 ± 0.3
	Kdm6b	1.0 ± 0.2	0.3 ± 0.1***†††	0.9 ± 0.3
	Atf2	1.0 ± 0.1	0.4 ± 0.1***†††	1.2 ± 0.3
	Hdac1	1.0 ± 0.1	1.3 ± 0.2*	1.1 ± 0.2
	Aurka	1.0 ± 0.2	0.4 ± 0.1**††	1.0 ± 0.3
	Aurkb	1.0 ± 0.2	0.5 ± 0.1**†††	1.1 ± 0.3
	Aurkc	1.0 ± 0.2	0.5 ± 0.1†	1.0 ± 0.5
Week13 (Hippocampus)	Dnmt3a	1.0 ± 0.1	0.6 ± 0.1**†	0.9 ± 0.3
	Dnmt3b	1.0 ± 0.2	0.2 ± 0.0***††	0.7 ± 0.3
	Kdm5c	1.0 ± 0.2	1.5 ± 0.3	1.6 ± 0.6
	Kdm6b	1.0 ± 0.2	1.6 ± 0.3*	1.5 ± 0.5
	Atf2	1.0 ± 0.1	1.5 ± 0.2***†††	0.9 ± 0.1
	Hdac1	1.0 ± 0.1	1.3 ± 0.1**†††	0.9 ± 0.1
	Aurka	1.0 ± 0.1	1.2 ± 0.3††	0.7 ± 0.2
	Aurkb	1.0 ± 0.2	1.4 ± 0.1*†††	0.6 ± 0.2**
	Aurkc	1.0 ± 0.4	0.3 ± 0.1*	0.9 ± 0.5

*The Sham, Smoke Exposure and Switch group data expressed as the percentage of gene expression normalised to the Sham group. Data is represented as mean ± standard deviation, *p<0.05, **p>0.01, ***p<0.001 vs. Sham, †p<0.05, ††p<0.01, †††p<0.001 vs. Switch, ###p<0.001 vs. Smoke Exposure.*

DNA methyltransferases, Dnmt3a and Dnm3b, are *de novo* enzymes that are important in controlling the expression of certain genes in the genome. At P1, Dnmt3a and Dnmt3b gene expression was significantly decreased to 15.0±8.8% and 53.4±15.6%, respectively, in the SE group compared to the Sham ($p<0.001$) and Switch groups ($p<0.05$). In the P20 offspring whole brain, no significant difference was observed between treatment groups. In the Week13 hippocampus, Dnmt3a and Dnmt3b gene expression was significantly decreased to 57.0±8.9% and 23.7±5.0%, respectively, in the SE group compared to the Sham ($p<0.01$) and the Switch groups ($p<0.01$).

Histone demethylases, Kdm5c and Kdm6b, are important in the removal of methyl groups on histones. In the P1 offspring brain, Kdm5c gene expression was significantly increased to 841.8±327.0% in the SE group compared to the Sham ($p<0.001$) and Switch groups ($p<0.001$). Similarly, Kdm6b gene expression was also significantly increased to 4609.4±3014.6%

compared to the Sham ($p<0.001$) and Switch groups ($p<0.001$). In the P20 offspring brain, Kdm5c gene expression was significantly decreased to 36.4±5.4% in the SE group compared to the Sham ($p<0.001$) and Switch groups ($p<0.001$). Similarly, Kdm6b gene expression was significantly decreased to 31.2±6.0% in the SE group compared to the Sham ($p<0.001$) and Switch groups ($p<0.001$). In the Week13 hippocampus, Kdm6b gene expression was significantly increased to 161.4±31.8% in the SE group compared to the Sham group ($p<0.05$).

Histone acetylase, Atf2, and deacetylase, Hdac1, are important in adding and removing acetyl groups from histones. I the P1 offspring brain, Atf2 gene expression was significantly increased to 370.4±93.7% in the SE group compared to the Sham ($p<0.001$) and Switch groups ($p<0.001$). Similarly, Hdac1 gene expression was significantly increased to 256.8±80.2% in the SE group compared to the Sham ($p<0.01$) and Switch groups ($p<0.001$). In the P20 offspring brain, Atf2 gene expression was significantly decreased to 36.2±5.9% in the SE group compared to the Sham ($p<0.001$) and Switch groups ($p<0.001$). In addition, Hdac1 gene expression was significantly increased to 131.2±18.1% in the SE group compared to the Sham group ($p<0.05$). In the Week13 offspring hippocampus, Atf2 gene expression was significantly increased to 152.9±17.2% in the SE group compared to the Sham ($p<0.001$) and Switch groups ($p<0.001$). Moreover, Hdac1 gene expression was significantly increased to 126.7±9.6% in the SE group compared to the Sham ($p<0.01$) and Switch groups ($p<0.001$).

Aurora Kinases, AurkA, AurkB and AurkC, are important in chromosomal alignment and segregation during mitosis. In the P1 offspring brain, AurkA gene expression was significantly decreased to 31.7±4.4% in the Switch group compared to the Sham ($p<0.05$) and SE groups ($p<0.001$). AurkB gene expression was significantly increased to 295.7±36.2% in the SE group compared to the Sham ($p<0.001$) and Switch groups ($p<0.001$). However, AurkB gene expression was shown to be significantly decreased to 40.5±10.7% in the Switch group compared to the Sham group ($p<0.001$). No significant difference was observed in AurkC gene expression in any treatment groups. In the P20 offspring brain, AurkA gene expression was significantly decreased to 47.1±9.1% in the SE group compared to the Sham ($p<0.01$) and Switch groups ($p<0.01$). Similarly, AurkB gene expression was significantly decreased to 49.8±14.4% in the SE group compared to the Sham ($p<0.01$) and Switch groups ($p<0.001$). AurkC gene expression was also significantly decreased to 51.1±10.4% in the SE group compared to the Switch group ($p<0.05$). In the Week13 offspring hippocampus, AurkA gene expression was significantly increased to 124.6±27.8% in the SE group compared to the Switch group ($p<0.01$). AurkB gene expression was significantly increased to 138.5±10.9% in the SE group compared to the Sham ($p<0.05$) and Switch groups ($p<0.001$). However, AurkB gene expression in the Switch group was significantly decreased to 55.5±22.9% compared to the Sham group ($p<0.01$). AurkC gene expression was significantly decreased to 34.4±10.9% in the SE group compared to the Sham group ($p<0.05$).

Adult offspring from mothers exposed to tobacco smoke, but not e-cigarettes, have reduced neuronal counts in the hippocampus

The dorsal hippocampus is a region of the brain that is important for cognitive functions such as learning and working memory. In the P20 offspring, the neuronal count at CA1 in the Sham group was at 170.43±8.83 cells/ROI (figure 5a). There was a significant reduction in neuronal counts in the SE and Switch groups at 154.42±4.16 cells/ROI ($p<0.01$) and 158±8.67 neurons/ROI ($p<0.05$), respectively. At the CA2 and CA3 regions, no significant change in neuronal cell counts was observed in the offspring at P20 (figure 5b&c). In the Week13 offspring, the neuronal cell count in the CA1, CA2, and CA3 in the Sham group was 161.86±17.80, 100.00±8.74 and 123.00±14.73 cells/ROI, respectively. In the SE group, there

were marked reductions in the neuronal count at 143.00±10.56 cells/ROI ($p<0.05$) in CA1 (figure 5d), 70.00±12.83 cells/ROI ($p<0.001$) in CA2 (figure 5e), and 103.25±8.65 cells/ROI ($p<0.01$) in CA3 (figure 5f). No significant difference in neuronal counts was observed in the hippocampus between Sham and Switch groups in the offspring at this time point.

DISCUSSION

We recently showed that the offspring from mice exposed to e-cigarette aerosols (with and without nicotine) during pregnancy had significant behavioural and epigenetic changes (21). In this study, we investigated whether maternal exposure to cigarette smoke (with nicotine) followed by a switch to e-cigarette aerosols (also with nicotine) changed the responses seen by continuous maternal smoking.

The effects of maternal smoking on offspring body weight have been well-documented (5, 26). We found a reduction in body weight from the smoke-exposed group, as expected. However, we showed that switching to e-cigarettes did not ameliorate this change in body weight in a murine model. By adulthood, however, all the offspring had returned to normal body weight and the drop in birth weight is likely due to a nicotine effect, as both the tobacco and the e-fluids contained nicotine in this study. A review of smokeless tobacco and nicotine replacement therapies also concluded that nicotine alone can reduce birth weight (27), however, another study examining nicotine replacement therapy has reported otherwise (28). In addition, elevated serum and urine cotinine levels have been shown to be closely correlated to the reduction in body weight following maternal exposure to cigarette smoke and e-cigarette aerosols (5, 18, 29-31).

The experiments examining exploration/activity in offspring showed a similar pattern with both the maternal smoking and switching to e-cigarettes displaying increased activity and reduced anxiety. This is characteristic of hyperactivity which has been reported in human children from smoking mothers (32-36). The changes that we observed could be due to the nicotine exposure which has been shown to cause hyperactivity and impulse-decision making in offspring (30). A number of studies have investigated working memory in animals exposed to cigarette smoke and e-cigarette aerosols, with contradictory findings (37-39). These differences may be due to methodological factors such as differences in the dose and delivery of the cigarette compounds. We found that maternal smoking did not affect memory in the offspring compared to the Sham, whereas switching to e-cigarettes did. As both products contained nicotine, it could be concluded that the aerosol itself may be having an effect on memory in the offspring.

Epigenetic changes often result in an alteration of the expression and regulation of genes that can play key roles during stages of normal development. Maternal smoking has been known to cause inter-generational alterations to DNA methylation in cord blood and the placenta (40, 41). Our results showed that there were more marked changes in DNA methylation in offspring from mothers exposed to cigarette smoke only, which was reduced when switching to e-cigarettes during pregnancy. However, this reduction in DNA methylation did not return to Sham levels. This suggests that chemical compounds that are found in cigarette smoke are likely to be the cause of these changes. Cigarette smoke consists of over 4000 known toxins such as arsenic, polychromatic hydrocarbons and acrolein which have shown to alter DNA methylation (42-44). E-cigarettes have also been known to release volatile organic compounds such as formaldehyde and tobacco-specific nitrosamines (45-47), however, the concentration of these compounds that are released from the e-cigarettes are minimal (48, 49).

DNA methylation can lead to changes in the expression of key genes that play a crucial role in development and cellular signalling and subsequently that may result in devastating consequences. In our previous study, we showed that mothers exposed to e-cigarette aerosol alone (with and without nicotine) resulted in changes to chromatin modifiers (21). These chromatin modifiers include DNA methyltransferases, histone demethylases, histone acetylase, histone deacetylase and Aurora Kinases. We showed that mothers exposed to e-cigarettes alone (with and without nicotine) had significant changes in Dnmt3a, Dnmt3b, Kdm5b, Kdm6b, Atf2, and Hdac1 gene expression in the offspring brain at birth, weaning and adulthood (21). In the current study, significant changes in gene expression were mainly observed in offspring from mothers exposed solely to cigarette smoke. However, gene expression of AurkA and AurkB, which are involved in mitotic division (50), were significantly decreased in in offspring from mothers switching to e-cigarette exposure, and significantly increased in offspring from mothers exposed to cigarette smoke only. This suggests that switching to e-cigarettes may alter epigenetic gene expression levels compared to continual smoke exposure, however, it can subsequently induce changes that are independent of smoking. It is likely that alterations to genes involved in generating epigenetic changes can result in physiological consequences, however, this remains to be investigated.

The neuronal cell numbers in the dorsal hippocampus of the adult offspring were reduced in CA1 following maternal smoking, but there were no changes in this group in the NOR test. Conversely, offspring from mothers that switched to e-cigarette exposure after smoke exposure had an effect on memory, but not cell numbers. This highlights the difficulty of teasing out the effects of tobacco chemicals, nicotine and the chemicals in the e-fluids and how they may alter neurological function at different post-natal time-points. It is known that nicotine, when injected into pregnant rats, compromises offspring neuronal maturation, leading to long-lasting alterations in the structure of the hippocampus (51). A study that investigated the effects of prenatal nicotine exposure in a rat model found a decrease in cell size and a higher cell packing density in juvenile and adolescent offspring (52). Although many studies have shown that nicotine causes significant changes to regions of the hippocampus, there were no significant changes observed in the adult offspring for the Switch group in our study. Again, this may be due to the difference in nicotine delivery methods or possibly due to dose effects since previous studies have either administered nicotine orally through the water bottles or via a pump (52-54). One study that investigated prenatal e-cigarette exposure in the offspring hippocampus found no changes to neuron numbers (55). However, they did find an increase in IBA-1 protein expression, a marker for microglia (55). This suggests that brain cells other than neurons can be affected by prenatal e-cigarette exposure.

It is important to note that there are some limitations to the current study. The 5-mC DNA ELISA provides an overview of DNA methylation within the brain and does not show the specific genes that are methylated. Future experiments will be needed to focus on locating the genes that are methylated which will allow us to identify the physiological changes that are associated with those genes. Furthermore, a detailed analysis of the specific neuronal cell populations and different glial cells in the hippocampus and other brain regions would be beneficial to determine the cellular responses in the offspring.

Overall, we conclude that in this murine model, lower levels of DNA methylation, lower levels of induced epigenetic modification of selected genes and no decrease in neuronal cell numbers were observed in the offspring of mothers that switched from tobacco cigarette smoke to e-cigarette aerosol exposure, compared to those from mothers continuously exposed to tobacco smoke during pregnancy. However, despite this, short-term memory deficits and hyperactivity

in the offspring were not affected by switching to e-cigarette during pregnancy. The results from this study, therefore, confirm that e-cigarette use during pregnancy is not a safe option. it may not be ‘safer’ to use than tobacco cigarette in terms of neurocognitive outcome, while the other physiological aspect remains to be determined. We conclude that abstinence from all nicotine delivery products during pregnancy should still be advised.

ACKNOWLEDGEMENTS
The authors do not have any potential conflicts of interest. The authors would like to thank Fiona Ryan and Lalit Overlunde for helping with the care of the animals as well as the members of the Molecular Biosciences Team in the School of Life Sciences for their support in the project.

COMPETING INTEREST
We declare that there is no competing interest in this study.

FUNDING
This study was funded by the Centre for Health Technologies and Faculty of Science, University of Technology Sydney. Mr Gerard Li is supported by a Strategic Scholarship by Faculty of Science, University of Technology Sydney. Associate Professor Hui Chen is supported by a Research Fellowship for International Young Scientists (81750110554) awarded by National Natural Science Foundation of China.

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