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Maternal E-cigarette exposure results in cognitive and epigenetic alterations in offspring in a mouse model

Key words

Anxiety, DNA methylation, E-cig, memory, vaping

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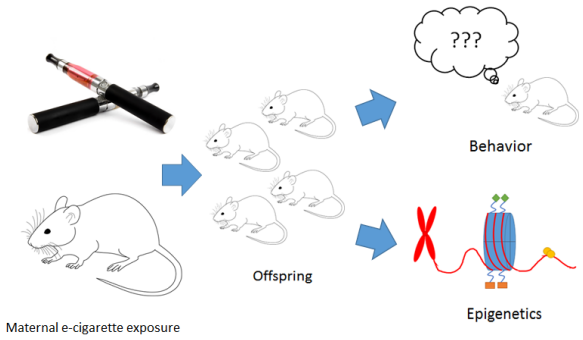

Abstract

Electronic cigarette (e-cigarette) use is on the rise worldwide and is particularly attractive to young people and as a smoking substitute by pregnant woman. There is a perception in pregnant woman and women of child-bearing age that the use of e-cigarettes (vaping) is safer than smoking tobacco cigarettes during pregnancy. However, there is little evidence to support this perception. Here, we examined the offspring from mouse dams that had been exposed during and after pregnancy to ambient air (sham) (n=8), e-cigarette aerosols with nicotine (n=8) or e-cigarette aerosols without nicotine (n=8). Offspring underwent cognitive testing at 12 weeks of age and epigenetic testing of brain tissues at 1 day, 20 days and 13 weeks after birth. The findings showed deficits in short-term memory, reduced anxiety and hyperactivity in offspring using the novel object recognition and elevated plus maze tests. In addition, global DNA methylation was increased in the brains of offspring soon after birth. Using a quantitative-PCR array specific to chromatin modification enzymes on genomic DNA and histones, 13 key genes were identified to be significantly altered in the offspring brains from the e-cigarette groups compared to the non-exposed groups. The changes to genes Aurka, Aurkb, Aurkc, Kdm5c, Kdm6b, Dnmt3a, Dnmt3b and Atf2, all associated with modulating neurological activity were validated using RT-qPCR. In conclusion, in a mouse model, maternal exposure to e-cigarette aerosols resulted in both cognitive and epigenetic changes in offspring. This suggests that the use of e-cigarettes during pregnancy may have hitherto undetected neurological consequences on newborns.

Introduction

Smoking tobacco cigarettes is the leading cause of preventative disease worldwide. Prenatal exposure to tobacco cigarette smoke has been linked to a number of pathological changes in the offspring, such as lower birth weight [1, 2], respiratory complications [3, 4] and sudden infant death syndrome [5, 6]. Concerningly, maternal smoking has also been linked to psychological changes such as attention deficit hyperactive disorder, aggressiveness, decrease social behaviour and susceptibility to mental health illnesses such as depression, anxiety and anti-social behavior in children [7-9]. Moreover, DNA methylation studies, one of the most studied epigenetic modification factors, have shown changes to DNA methylation in fetal cord blood [10] and placenta [11, 12] following maternal cigarette smoking [13].

To aid the delivery of nicotine whilst eliminating exposure to harmful chemicals in tobacco smoke, electronic cigarettes (e-cigarettes) were introduced in 2003 as an electronic nicotine delivery system (ENDS). E-cigarettes are battery-powered devices that convert an oily-flavoured liquid (e-liquid) into an aerosol using a super-heated coil. Due to its non-combustible property, wide range of flavours (over 7000) [14] and aggressive marketing, e-cigarette use is on the rise, particularly amongst young people. In the United States, the use of e-cigarettes in young people has been reported to be as high

as 5.3% in middle school and 16% in high schools [15]. Pregnant woman are another vulnerable group where e-cigarette use is increasing with a recent report showing 13% of pregnant woman reported using e-cigarettes during their pregnancy [16].

There is a perception in woman of child-bearing age, as well as pregnant woman, that e-cigarettes are less harmful than smoking tobacco cigarettes [17-19]. However, studies investigating the effects of e-cigarette exposure in mice models of pregnancy are limited [20-23]. Although there are numerous studies that focus on offspring from maternal smoking in humans and animals [1, 7, 10, 24, 25], the effects of maternal exposure to e-cigarette aerosols on offspring behavior and epigenetics are lacking and epidemiological data is not yet available. In this study, we aimed at investigating the effects of maternal e-cigarette aerosol exposure on offspring in a mouse model. Murine offspring underwent behavioural assessments and brain tissues were collected to assess if e-cigarette affects global DNA methylation.

Experimental procedures (materials and methods)

Animals and treatment

All animal experiments were conducted in accordance to the guidelines described by the Australian National Health and Medical Research council code of conduct for animals with approval from the institutional Animal Care and Ethics Committee (ACEC#ETH15-0025). Balb/C female mice (n=24) were obtained from the Animal Resource Center (Perth, WA, Australia). Mice were housed in groups of four with ad libitum food and water and a 12:12 hour light dark cycle. All animals were provided with environmental enrichment as part of the housing conditions.

Delivery of e-cigarette aerosols matched similar protocols used to deliver cigarette smoke reported previously[20]. Animals were randomly divided into 3 groups (n=8) and exposed to the following conditions for 6 weeks before pregnancy, during pregnancy and lactation; ambient air (sham), e-cigarette aerosols with nicotine [Ecig(+nic)] and e-cigarette aerosols without nicotine [Ecig(-nic)]. Mice in each group were placed in a 9L chamber filled with e-cigarette aerosols twice daily for thirty minutes (2 x 15 minutes' exposure with a 5-minute aerosol-free washout interval. The amount of nicotine exposure used in this study is equivalent to smoking two tobacco cigarettes twice daily based on our previous maternal smoking studies[25, 26])[20]. The KangerTech NEBOX^{TM} e-cigarette device (KangerTech, Shenzhen, China) was used for all experiments. A commercially sourced E-cigarette liquid was used that consisted of 50% propylene glycol, 50% vegetable glycerin tobacco flavour additives with or without nicotine at a concentration of 18mg/mL. The nicotine dosage that was used for this study was adopted by previous maternal smoking experiments [25, 27]. Offspring were randomly divided into 3 groups for culling at postnatal day (D) 1, D20 (weaning), and Week 13.

Behavioral assessments

At 12 weeks of age the offspring underwent behavioral assessments. Mice were acclimatized to each of the testing apparatus for 5 minutes a day for 5 days prior to the assessment. All testing sessions were conducted between 0900 and 1400 hours and were recorded.

The *novel object recognition* (NOR) test has been used in many studies to measure short-term memory [28]. It consists of a *familiarization phase* where the animal is placed in a container with two identical objects for 5 minutes. The animals are then removed for an interval of 1 hour before a *test phase* where the animal was placed back into the container with one 'familiar' object and one 'novel' object. The total time spent investigating each object was recorded. The outcome measure is shown as a recognition Index calculated from the time spent on the novel object divided by the time spent on both objects.

$$Recognition\ Index = \frac{Novel\ (sec)}{Novel\ (sec) + Familiar\ (sec)}$$

Typically, an unimpaired animal spends equal time exploring two identical objects (RI = 0.5) and, after an interval, spends more time exploring a novel object compared to a familiar object (RI >0.5). Statistical significance between the familiarisation and test phase were calculated using an unpaired two-tailed *t-test*.

The *elevated plus maze* (EPM) is used as a test for anxiety [29-32]. Each animal was placed in the same position at the center of the EPM and allowed to freely move for two minutes. The time spent investigating the open arms was recorded. In addition, the amount of head dips and whole body stretches in the open arm (unprotected) and close arm (protected arm) was also recorded. Finally, the number of times each animal crosses the centre of the EPM was recorded.

Tissue collection, DNA and RNA extraction

Pups were euthanized at three time points: at D1 (postnatal day 1), D20 (weaning), and Week 13. Behavioural assessments were performed on Week 13 pups a week before euthanasia. For this study, we were specifically interested in the changes occurring in the hippocampus but it was technically challenging to accurately dissect the hippocampus in the earlier time points. Therefore, at D1 and D20, whole brains were collected and snap frozen for epigenetic analysis. The hippocampus were micro-dissected from Week 13 pups before being snap frozen and stored at -80^{o}C. The following time points were used to investigate neurological changes during gestation (D1), lactation (D20) and at maturity (Week 13). Genomic DNA and total RNA were extracted from frozen brain tissue using Isolate II RNA/DNA/protein kit (Bioline, MA, USA) following the manufacturer's protocol. The concentration of the extracted DNA and total RNA was then quantified with the Nanodrop^{TM} 2000 Spectrophotometer (Thermo fisher scientific, MA, USA).

Global DNA methylation assay

DNA was assessed for global DNA methylation levels using a DNA 5-methylcytosine (5-mC) methylation ELISA kit (Zymo Research, Irvine, CA, USA). DNA samples (100 ng) were denatured at 98°C for 5 minutes before incubation in the 5-mC coated well plates provided for an hour at 37°C. Wells were washed with 5-mC ELISA buffer before a mixture of anti-5-methylcytosione and secondary antibody was added to the wells. Horseradish peroxidase reagent was then added and the absorbance was measured between 405-450 nm using the Infinite M1000 PRO Microplate Reader (Tecan Group Ltd, Männedorf, Switzerland). Global DNA methylation was determined using a standard curve generated using a positive control that had been previously methylated.

Epigenetic PCR array and gene expression

RNA Integrity of the total RNA (1 µg) isolated from brain tissue was assessed using the Experion RNA StdSens Analysis Kit (Biorad, CA, USA) before they were reverse transcribed. The cDNA generated was then added as templates to the Mouse Epigenetic Chromatin Modification Enzymes RT^{2} Profiler^{TM} PCR Array (Table 1) according to manufacturer's protocol (Qiagen, CA, USA). PCR arrays were performed in the QuantStudio^{TM} 12K Flex Real-Time 384 well-plate (Applied Biosystems, CA, USA) and relative changes in mRNA levels were determined by the $\Delta\Delta$Ct method using the Qiagen Excel-based PCR array data analysis center (http://www.qiagen.com/shop/genes-and-pathways/data-analysis-center-overview-page/#Excel). Fold-change calculations were performed by methods established on the Qiagen website. Fold change between each test group is considered significantly different by a fold change of more than two. Ct cut-off was set to 35.

Total RNA (1 µg) was reverse transcribed to cDNA using Tetro cDNA synthesis kit (Bioline, MA, USA) following the manufacturer's protocol. cDNA was amplified by real-time PCR in reaction mixtures containing primers (20 pmol each) and SensiFast™ SYBR® No-ROX kit (Bioline, CA, USA). Amplification was performed in a CFX96™ Real-Time System (BioRad, CA, USA) using the following protocol: 95°C for 5 secs, Tm of specific primer set for 15 secs. Relative changes in mRNA gene expression were determined by the $\Delta\Delta$Ct method [33] using glyceraldehyde 6-phosphate dehydrogenase (GAPDH) as the reference gene. All primer pair's sequences are listed in Table 2.

Statistical analysis

Data are expressed as mean ± SD. For the analysis of the NOR test, the familiarisation and test phase was compared by using an unpaired two-tailed t-test. For the EPM, DNA methylation and gene expression, treatment groups were compared using one-way ANOVA with *Bonferroni's post-test analysis* to determine statistical significance.

Results

E-cigarette aerosol exposure

The experiments were undertaken without incident and the dams tolerated the aerosol exposure without discomfort. Litter sizes of 3-6 offspring were obtained from each dam and all male offspring were included in this study (n=126). There was no difference in birth weights or morbidity between the exposure status of the dams.

Maternal exposure to e-cigarette aerosol with nicotine causes short-term memory deficits

To test for short-term memory deficits, each offspring was assessed by the NOR test. The expected NOR result for a unimpaired animal is that there will be a significant increase in the recognition index in the *test phase* ($p<0.05$) as shown for the sham mice (Figure 1A) and the Ecig(-nic) group (Figure 1C). However, offspring from Ecig(+nic) group did not show an increase in the recognition index during the test phase ($p=0.535$, Figure 1B) indicating that offspring from maternal exposure to e-cigarette aerosols with nicotine may have short-term memory deficits.

Maternal exposure to e-cigarette aerosols causes anxiety and hyperactivity

To observe anxiety and exploratory activity, each offspring was assessed in the EPM test. The expected EPM result for an unimpaired animal is shown for the sham mice where only a small percentage of time is spent in the open arms of the maze (Figure 2). This provides a baseline level of anxiety and exploratory behaviour in our study. Both the Ecig(+nic) group ($p<0.05$) and Ecig(-nic) group ($p<0.05$) group spent a higher percentage of time in the open arms compared to the sham group indicating reduced anxiety levels.

Head dipping and whole body stretches are measures of exploration and a sensitive measure of anxiety in rodents [34, 35]. These characteristics can be considered as risk assessment responses as they indicate the tendency of a mouse to remove itself from its safe surroundings and enter a space that is 'risky'. The sham offspring showed little exploratory behaviour (Figure 3A-D) whereas the Ecig(+nic) group had an increased number of both protected ($p<0.05$) and unprotected head dips ($p<0.05$) compared to the sham group (Figure 3A-B). There were no significant differences in protected stretches (Figure 3C) between test groups. However, there was a significant increase in the number of unprotected stretches (Figure 3D) in the Ecig(-nic) group compared to the sham group ($p<0.05$).

The number of times an animal completely crosses the center platform of the EPM is a measure of exploration and locomotor activity. The sham mice crossed the center relatively infrequently (Figure

4) providing a baseline level of motor activity and exploration in this study. Both the Ecig(+nic) group ($p<0.05$) and Ecig(-nic) group ($p<0.05$), however, showed an increase in the number of center crosses compared to the sham group (Figure 4).

E-cigarette aerosols without nicotine increases global DNA methylation at D1 and D20

DNA methylation plays an important role in controlling gene expression that can subsequently affect the developmental changes observed. As cigarette smoking has been associated with modification of DNA methylation [36], we next assessed whether e-cigarettes also affects global DNA methylation levels in the brain. D1 Ecig(-nic) and D20 Ecig(-nic) samples showed significantly higher global DNA methylation compared to the sham group ($p<0.05$ and $p<0.05$, respectively; Figure 5A&B). At week 13, global DNA methylation was investigated only in the hippocampus and we observed no significant global DNA methylation change between the control and treatment groups in these older-aged mice (Figure 5C).

E-cigarettes aerosols induce changes to mRNA expression profile of chromatin modification enzyme-related genes

The changes in global DNA methylation in the whole brain indicated that e-cigarette aerosols induce epigenetic changes. Therefore, we investigated whether exposure to e-cigarette aerosols affects the gene expression of key chromatin modification enzymes that have been known, or are predicted to modify, the epigenome in the offspring brain. From our PCR array, we have identified 13 genes that showed significant change by a fold change of more than two (Figure 6&7). In the Ecig(+nic) group at D1, gene expression of Aurkc, Setd3, Smyd1 showed significant fold changes of 3.9, 29.7 and -2.2 respectively compared to the sham group ($p<0.05$) (Figure 6). In the Ecig(-nic) group at D1, gene expression of Ash1l, Atf2, Aurka, Aurkc, Carm1, Asco1, Setd3 had a significant fold changes of 2.2, 3.0, 2.1, 3.5, 2.3, 2.2, 30.4 respectively compared to the sham group ($p<0.05$) (Figure 6). However, at Week 13 in the hippocampus, gene expression of Aurkb, Aurkc, Csrp2bp, Dnmt3a, Dnmt3b, Kdm5c, Smyd1 had significant fold changes of -2.1, 3.7, 31.1, 2.2, 2.4, 2.7, 2.5 respectively compared to the sham group ($p<0.05$) (Figure 7). In addition, in the Week 13 Ecig(-nic) group, genes expression of Aurkb, Aurkc, Csrp2bp had significant fold changes of -9.2, -2.1 and 16.4, respectively, compared to the sham group ($p<0.05$) (Figure 7). The gene expression levels of enzymes involved in epigenetic changes included those responsible for DNA methylation in the D1 whole brains and Week 13 hippocampus samples. These PCR array results were confirmed by RT-qPCR (Table 3).

Although we did not see significant changes in DNA methylation in the hippocampus, this does not necessarily mean that we are not seeing any epigenetic changes elsewhere. Therefore, we analysed epigenetic changes in the hippocampus of the brain in Week 13 offspring and the whole brains at D1 and D20 since hippocampus collection at these time points would not provide sufficient RNA yield to investigate epigenetic changes in this study.

Maternal exposure to e-cigarette aerosol affects offspring DNA methyltransferases

DNA methyltransferases (Dnmt3a, Dnmt3b) are involved in *de novo* DNA methylation which determines whether a gene is expressed or not [37]. Validation of PCR array results by RT-qPCR shows that for Dnmt3a gene expression, a significant decrease of 41.0 ($p = 0.036$) was shown in the Ecig(+nic) group compared to the sham group at D20 (Table 3). In addition, Dnmt3a gene expression at D20 showed a significant increase of 73.9% ($p < 0.005$) in the Ecig(-nic) compared to the Ecig(+nic) group. No changes were observed in offspring at D1 and Week 13 (Table 3). For Dnmt3b gene expression, a significant decrease of 46.2 ($p < 0.005$) and 34.0% ($p = 0.006$) were observed in the Ecig(+nic) and Ecig(-nic) groups respectively, compared to the sham group. No change in Dnmt3b gene expression was observed in offspring at D1.

Maternal exposure to e-cigarette aerosol effects offspring histone-lysine demethylases

Histone-lysine demethylases (KDM) control the access of transcriptional enzymes to parts of the DNA and to remove a methyl group from histones [38]. In addition, KDMs are known to be important in embryogenesis in zebra fish [39]. Validation of PCR array results by RT-qPCR shows that for Kdm5c gene expression, a significant increase of 46.4% ($p = 0.021$) in Ecig(-nic) compared to the Ecig(+nic) group at D20 (Table 3). Kdm5c gene expression was significantly decrease of 38.8% ($p = 0.018$) and 40.4% ($p = 0.018$) in Ecig(+nic) and Ecig(-nic) respectively compared to the sham group at Week 13. No changes were observed in offspring at D1. For Kdm6b gene expression, a significant increase of 47.1% ($p = 0.007$) was shown in the Ecig(+nic) group compared to the Ecig(-nic) group at D20. No change in Kdm5c gene expression was observed at D1 and Week 13 (Table 3).

Maternal exposure to e-cigarette aerosol effects offspring histone acetyltransferases but not histone deacetylase

Activating transcription factor-2 (Atf2) is a histone acetyltransferase that activates transcription in a sequence-specific manner and is important in neuronal development in the brain [40-42]. Validation of PCR array results by RT-qPCR shows that for Atf2 gene expression, a significant decrease of 19.7% ($p < 0.005$) and 24.1% ($p < 0.005$) was shown in Ecig(+nic) and Ecig(-nic) groups, respectively, compared to the sham group at D1 (Table 3). At D20, Atf2 gene expression was significantly increased by 26.6% ($p = 0.343$) in the Ecig(-nic) group compared to the Ecig(+nic) group. At Week 13, Atf2 gene expression was significantly decreased by 7.6% ($p < 0.005$) and 12.8% ($p < 0.005$) in the Ecig(+nic) and Ecig(-nic) group, respectively, compared to the sham group. No change in histone deacetylase (Hdac1) gene expression was observed in offspring at any time point.

Maternal exposure to e-cigarette aerosol effects offspring histone phosphorylation

Aurora kinases (Aurka, Aurkb, Aurkc) are involved in chromatin condensation, alignment and segregation during mitosis by phosphorylation of histones [43]. Validation of PCR array results by RT-qPCR shows that Aurka gene expression was significantly decreased by 50.8% ($p < 0.005$) and 31.8% ($p < 0.005$) in the Ecig(-nic) group compared to the sham and Ecig(+nic) group respectively. Similarly, at D20, Aurka expression was significantly decreased by 38.1% ($p < 0.005$) in the Ecig(-nic) group compared to the sham group. No change in Aurka gene expression was observed in offspring at Week 13. At D1, gene expression of Aurkb was significantly decreased by 38.3% ($p < 0.005$) and 37.3% ($p < 0.005$) in the Ecig(+nic) and Ecig(-nic) group, respectively, compared to the sham group. At D20, Aurkb gene expression was significantly decreased by 19.7% ($p < 0.005$) in the Ecig(+nic) group compared to the sham group. No change in Aurkb gene expression was observed in offspring at Week 13. For Aurkc gene expression, we found a significant decrease by 22.7% ($p < 0.005$) in the Ecig(+nic) group compared to the sham group at D20. In addition, Aurkc gene expression was significantly increased by 30.1% ($p = 0.023$) in the Ecig(-nic) group compared to the Ecig(+nic) group. No change in Aurkc gene expression was observed in offspring at D1 and Week 13.

Discussion

E-cigarettes are generally considered safer than conventional tobacco cigarettes, however little is known about the effects of maternal exposure to e-cigarette aerosols on offspring. In this study, we used a mouse model to investigate behavioral (memory, anxiety and hyperactivity) and epigenetic changes occurring in the brains of offspring after maternal exposure to e-cigarette aerosols with and without nicotine. The offspring from mothers exposed to e-cigarette aerosols showed short-term memory deficits, reduced anxiety and hyperactivity in adulthood. In addition to the neurological changes observed, global DNA methylation and alterations in chromatin modification enzymes were found in all stages of the developing offspring.

The present study is the first to show that offspring from mothers exposed to e-cigarette aerosols with nicotine showed short-term memory deficits, while those without nicotine did not. Short-term memory deficits have been previously identified in the offspring from smoke-exposed dams [25] and in mice exposed to nicotine via drinking water [44] suggesting that the memory deficits seen here in the offspring are solely due to the intrauterine nicotine exposure.

The EPM test revealed that offspring from dams exposed to e-cigarette aerosols with and without nicotine had reduced anxiolytic-like behavior. These offspring tended to take more risks exploring new environments and were more hyperactive. This change in behavior might be due to other constituents within the e-cigarette aerosols apart from nicotine. E-cigarette aerosols are produced by superheating e-liquids containing propylene glycol, vegetable glycerin, flavourings and different concentrations of nicotine. The effects of propylene glycol have been investigated by Smith and colleagues and they have found that offspring showed a significant increase in head dipping from animals exposed to propylene glycol and nicotine compared to pre- and postnatal exposure to ambient air [23]. In addition, intraperitoneal injections of propylene glycol in the developing mouse brain showed neuronal apoptosis and degeneration at D7 [45]. Although propylene glycol was approved by the Food and Drug Administration to be used as a solvent for pharmaceuticals, cosmetics and food, studies have reported airway and ocular irritability from acute propylene glycol 'mist' exposure in the entertainment and aviation industry [46, 47]. Given that the e-liquids tested in this study has propylene glycol as one of the main ingredients (50%), this suggests that propylene glycol could be potentially toxic to the central nervous system in offspring from dams exposed to e-cigarette aerosols. This is reflected in the Nor and EPM tests. The other component, vegetable glycerine, makes up the other 50% in the e-liquid tested but not much is known about the effects of vegetable glycerin inhalation.

There are over 7000 different flavoured e-liquids available on the market. In addition, e-cigarette users can make their own concoction of e-liquids. There is very little regulation surrounding the content and manufacture of e-cigarette flavorings. Moreover, the exact chemical makeup of these fluids vary from product to product. There are examples of respiratory issues arising in cohorts of people working in certain food industries that have been linked to flavouring products e.g. *bronchiolitis obliterans* has been linked to 'popcorn' lung disease which is effected by the flavourant *diacetyl* which imparts a buttery flavour [48]. Moreover, there are 34 substances that are listed as 'high priority' substances that may cause respiratory hazards in flavour-manufacturing workplaces [49]. Potentially hazardous chemicals that are on the list include acetaldehyde, acetoin, and diacetyl; all of which have been present in numerous e-liquids [50-52]. While it is possible, and even likely, that some of the chemicals in e-liquids may be detrimental when heated and inhaled, we cannot attribute any of the neurological deficits seen in the offspring in the current study directly to the tobacco flavorings, although this is certainly an area that needs more investigation.

It has been reported that maternal tobacco smoking causes changes to DNA methylation in the offspring in cord blood [53], and placenta [11, 12, 24]. We report here for the first time that e-cigarette exposure in a mice model of pregnancy resulted in changes in global DNA methylation in offspring brain. This finding is in line with our recent study that showed a global DNA methylation in the lungs of mice offspring after maternal exposure to e-cigarette aerosols [20]. Although, global DNA methylation does not specifically show which genes are methylated, it provides a snapshot of epigenetic changes that occur during a specific time. Our data showed that DNA hypermethylation were observed in the brain of offspring from maternal exposure to e-cigarette aerosols without nicotine right after birth and weaning but was no longer detected as the offspring reaches adulthood. In the early stages of life, offspring were housed with their mothers during lactation. This

suggests that substances from the e-cigarette aerosol are being transferred in the breastmilk which may be causing DNA hypermethylation only at these young ages. This also correlates with why we are not seeing similar changes in adulthood once the offspring were weaned from their dam. There are many drugs that can be transferred in the breastmilk which includes, but not limited to, nicotine, caffeine, alcohol and cocaine.[54-57].

We have shown that DNA hypermethylation in offspring from maternal exposure to e-cigarette aerosol. This can potentially induce changes to the epigenome that may lead to imprinting of genes that can play a role in the development of pathophysiological conditions later in life. For example, other studies have shown changes to the epigenome is associated with risk of late-onset Alzheimer's disease[58-60]. Moreover, this effect was independent of nicotine. Therefore, future directions for this study are to determine which regions of the brain have alterations in DNA methylation and pinpoint which genes, if any, are being methylated.

Further exploration of our DNA methylation result observed significant changes in chromatin modification enzymes in the offspring brains. Moreover, gene expression levels of the epigenetic enzyme family, namely DNA methyltransferase, histone demethylase, histone acetyltransferase and histone phosphorylation, were significantly altered in offspring brain after birth, weaning and into adulthood.

DNA methyltransferases, Dnmt3a and Dnmt3b, have been shown to play a key role in neurogenesis and are dynamically present in different regions of the brain such as the cortex, cerebellum and hippocampus [61]. From our results, maternal exposure to e-cigarette aerosols, with or without nicotine, resulted in a trend towards an overall reduced Dnmt3a/Dnmt3b gene expression level, particularly in adulthood. Okano and colleagues generated Dnmt3-deficient mice and found the mice with heterozygous expression of Dnmt3a and Dnmt3a gene were 'grossly normal'[37]. However, a homozygous Dnmt3a deletion resulted in death by 4 weeks and a homozygous Dnmt3b gene deletion was lethal at birth[37]. In addition, mouse embryonic stem cell studies by Li and colleagues suggested that ablation of Dnmt3a and Dnmt3b results in a significant reduction in genes Oct4 and Nanog, both important in the regulating development[62]. This suggests that maternal exposure to e-cigarettes could result in DNA methylation changes in the epigenome that can lead to developmental changes in the offspring.

Histone demethylase plays an important role in gene activation and supression[63], although the functional role of histone demethylases is not well understood. However, mutations of the histone demethylase Kdm5c gene may cause mental retardation in humans [64, 65]. Here we demonstrated that mice dams exposed to e-cigarette aerosols before and during pregnancy causes a reduction in Kdm5c gene expression in adult offspring. Iwase and colleagues reported Kdm5c knockdown in mice showed learning deficits as well as a reduction in dendritic spine density[66]. In addition, Kdm5c knockdown in zebrafish resulted in neuronal loss, abnormal neural tube restructuring and decreases in lengths of granule neuronal dendrites in the cerebellum [67]. From the ample links between Kdm5c and the brain, our data suggest maternal exposure to e-cigarette aerosols may result in a delay in mental development and, therefore, working memory. This is consistent with the NOR results from offspring from mothers exposed to aerosols containing nicotine (Figure 1).

Histone acetyltransferases and histone deacetylases are important in the turnover of acetyl-groups that control gene transcription within the genome [68]. Histone acetyltransferase Atf2 have been found to be expressed in granule cells in hippocampal neurons, cerebral cortex, substantia nigra and the cerebellum [41, 69]. Our results show an overall decrease in Atf2 gene expression in mice offspring directly after birth, at weaning and at adulthood. Down-regulation of Atf2 has been shown in

neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease [41]. Our results may indicate that mothers exposed to e-cigarettes may alter the development of granule cells in these particular regions of the brain which ultimately can lead to neurodegenerative conditions.

Histone phosphorylation enzymes such as those in the family of aurora kinases, are essential in the regulation of cell division and embryonic development. Here we show an overall decrease in gene expression of the aurora kinases in offspring brain directly after birth, at weaning and at adulthood. *In vitro* studies have shown that the knockdown of AurkB reduces telomere activity which is important in cell mitosis[70, 71]. siRNA knockdown of Aurka has been shown to cause multiple mitotic defects such as instability in microtubules[72], improper completion of cytokinesis[73] and chromosome condensation delays[74]. Moreover, a depletion in Aurka expression in zebrafish resulted in developmental retardation[75]. This may suggest that a reduction in aurora kinases in offspring from dams exposed to e-cigarette aerosols may cause instability in cell division that could lead to neurological changes that can carry on into adulthood. This is consistent with our behavioural data.

We conclude that the exposure of mouse dams to e-cigarette aerosol leads to behavioral changes in the adult offspring. The effect may be due to inhaling aerosols containing nicotine or inhaling aerosols alone. We also demonstrated significant changes in global DNA methylation that was associated, in part, with significant changes in chromatin modification enzymes in the brains of the offspring. Whether these epigenetic changes can potentially cause developmental delays and cognitive deficits in offspring requires further study. Future research should therefore include investigating downstream genes that are affected by DNA methyltransferases, histone demethylase, acetyltransferase and phosphorylation, and to precisely determine the contributing chemical factors found in e-cigarette aerosols and their mechanism of interaction with the brain. Overall, this study highlights our limited understanding of the potential neurological risks that e-cigarettes can have if mothers use e-cigarettes during their pregnancy.

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Abbreviations

ANOVA, analysis of variance; D, postnatal day; E-cigarettes, electronic cigarettes; EPM, elevated plus maze; nic, nicotine; NOR, novel object recognition

Reference

(1) Chiolero, A., Bovet, P., and Paccaud, F. (2005) Association between maternal smoking and low birth weight in Switzerland: the EDEN study. *Swiss medical weekly 135*, 525-530.

(2) Ricketts, S. A., Murray, E. K., and Schwalberg, R. (2005) Reducing low birthweight by resolving risks: results from Colorado's prenatal plus program. *American journal of public health 95*, 1952-1957.

(3) McEvoy, C. T., and Spindel, E. R. (2017) Pulmonary Effects of Maternal Smoking on the Fetus and Child: Effects on Lung Development, Respiratory Morbidities, and Life Long Lung Health. *Paediatric respiratory reviews 21*, 27-33.

(4) Zacharasiewicz, A. (2016) Maternal smoking in pregnancy and its influence on childhood asthma. *ERJ open research 2*.
(5) Shah, T., Sullivan, K., and Carter, J. (2006) Sudden Infant Death Syndrome and Reported Maternal Smoking During Pregnancy. *American journal of public health 96*, 1757-1759.
(6) Zhang, K., and Wang, X. (2013) Maternal smoking and increased risk of sudden infant death syndrome: a meta-analysis. *Legal medicine (Tokyo, Japan) 15*, 115-121.
(7) He, Y., Chen, J., Zhu, L. H., Hua, L. L., and Ke, F. F. (2017) Maternal Smoking During Pregnancy and ADHD: Results From a Systematic Review and Meta-Analysis of Prospective Cohort Studies. *Journal of attention disorders*, 1087054717696766.
(8) Paradis, A. D., Shenassa, E. D., Papandonatos, G. D., Rogers, M. L., and Buka, S. L. (2017) Maternal smoking during pregnancy and offspring antisocial behaviour: findings from a longitudinal investigation of discordant siblings. *Journal of epidemiology and community health 71*, 889-896.
(9) Wakschlag, L. S., Pickett, K. E., Cook, E., Benowitz, N. L., and Leventhal, B. L. (2002) Maternal Smoking During Pregnancy and Severe Antisocial Behavior in Offspring: A Review. *American journal of public health 92*, 966-974.
(10) Joubert, B. R., Felix, J. F., Yousefi, P., Bakulski, K. M., Just, A. C., Breton, C., Reese, S. E., Markunas, C. A., Richmond, R. C., Xu, C. J., Kupers, L. K., Oh, S. S., Hoyo, C., Gruzieva, O., Soderhall, C., Salas, L. A., Baiz, N., Zhang, H., Lepeule, J., Ruiz, C., Ligthart, S., Wang, T., Taylor, J. A., Duijts, L., Sharp, G. C., Jankipersadsing, S. A., Nilsen, R. M., Vaez, A., Fallin, M. D., Hu, D., Litonjua, A. A., Fuemmeler, B. F., Huen, K., Kere, J., Kull, I., Munthe-Kaas, M. C., Gehring, U., Bustamante, M., Saurel-Coubizolles, M. J., Quraishi, B. M., Ren, J., Tost, J., Gonzalez, J. R., Peters, M. J., Haberg, S. E., Xu, Z., van Meurs, J. B., Gaunt, T. R., Kerkhof, M., Corpeleijn, E., Feinberg, A. P., Eng, C., Baccarelli, A. A., Benjamin Neelon, S. E., Bradman, A., Merid, S. K., Bergstrom, A., Herceg, Z., Hernandez-Vargas, H., Brunekreef, B., Pinart, M., Heude, B., Ewart, S., Yao, J., Lemonnier, N., Franco, O. H., Wu, M. C., Hofman, A., McArdle, W., Van der Vlies, P., Falahi, F., Gillman, M. W., Barcellos, L. F., Kumar, A., Wickman, M., Guerra, S., Charles, M. A., Holloway, J., Auffray, C., Tiemeier, H. W., Smith, G. D., Postma, D., Hivert, M. F., Eskenazi, B., Vrijheid, M., Arshad, H., Anto, J. M., Dehghan, A., Karmaus, W., Annesi-Maesano, I., Sunyer, J., Ghantous, A., Pershagen, G., Holland, N., Murphy, S. K., DeMeo, D. L., Burchard, E. G., Ladd-Acosta, C., Snieder, H., Nystad, W., Koppelman, G. H., Relton, C. L., Jaddoe, V. W., Wilcox, A., Melen, E., and London, S. J. (2016) DNA Methylation in Newborns and Maternal Smoking in Pregnancy: Genome-wide Consortium Meta-analysis. *American journal of human genetics 98*, 680-696.
(11) Suter, M., Abramovici, A., Showalter, L., Hu, M., Shope, C. D., Varner, M., and Aagaard-Tillery, K. (2010) In utero tobacco exposure epigenetically modifies placental CYP1A1 expression. *Metabolism: clinical and experimental 59*, 1481-1490.
(12) Suter, M., Ma, J., Harris, A., Patterson, L., Brown, K. A., Shope, C., Showalter, L., Abramovici, A., and Aagaard-Tillery, K. M. (2011) Maternal tobacco use modestly alters correlated epigenome-wide placental DNA methylation and gene expression. *Epigenetics 6*, 1284-1294.
(13) Lee, K. W., and Pausova, Z. (2013) Cigarette smoking and DNA methylation. *Frontiers in genetics 4*, 132.
(14) Zhu, S. H., Sun, J. Y., Bonnevie, E., Cummins, S. E., Gamst, A., Yin, L., and Lee, M. (2014) Four hundred and sixty brands of e-cigarettes and counting: implications for product regulation. *Tobacco control 23 Suppl 3*, iii3-9.
(15) Singh, T., Arrazola, R. A., Corey, C. G., Husten, C. G., Neff, L. J., Homa, D. M., and King, B. A. (2016) Tobacco Use Among Middle and High School Students--United States, 2011-2015. *MMWR. Morbidity and mortality weekly report 65*, 361-367.
(16) Mark, K. S., Farquhar, B., Chisolm, M. S., Coleman-Cowger, V. H., and Terplan, M. (2015) Knowledge, Attitudes, and Practice of Electronic Cigarette Use Among Pregnant Women. *Journal of addiction medicine 9*, 266-272.

(17) Baeza-Loya, S., Viswanath, H., Carter, A., Molfese, D. L., Velasquez, K. M., Baldwin, P. R., Thompson-Lake, D. G., Sharp, C., Fowler, J. C., De La Garza, R., 2nd, and Salas, R. (2014) Perceptions about e-cigarette safety may lead to e-smoking during pregnancy. *Bulletin of the Menninger Clinic 78*, 243-252.

(18) England, L. J., Tong, V. T., Koblitz, A., Kish-Doto, J., Lynch, M. M., and Southwell, B. G. (2016) Perceptions of emerging tobacco products and nicotine replacement therapy among pregnant women and women planning a pregnancy. *Preventive medicine reports 4*, 481-485.

(19) Wagner, N. J., Camerota, M., and Propper, C. (2017) Prevalence and Perceptions of Electronic Cigarette Use during Pregnancy. *Maternal and child health journal 21*, 1655-1661.

(20) Chen, H., Li, G., Chan, Y. L., Chapman, D. G., Sukjamnong, S., Nguyen, T., Annissa, T., McGrath, K. C., Sharma, P., and Oliver, B. G. (2017) Maternal E-cigarette Exposure in Mice Alters DNA Methylation and Lung Cytokine Expression in Offspring. *American journal of respiratory cell and molecular biology*.

(21) Lauterstein, D. E., Tijerina, P. B., Corbett, K., Akgol Oksuz, B., Shen, S. S., Gordon, T., Klein, C. B., and Zelikoff, J. T. (2016) Frontal Cortex Transcriptome Analysis of Mice Exposed to Electronic Cigarettes During Early Life Stages. *International journal of environmental research and public health 13*, 417.

(22) McGrath-Morrow, S. A., Hayashi, M., Aherrera, A., Lopez, A., Malinina, A., Collaco, J. M., Neptune, E., Klein, J. D., Winickoff, J. P., Breysse, P., Lazarus, P., and Chen, G. (2015) The Effects of Electronic Cigarette Emissions on Systemic Cotinine Levels, Weight and Postnatal Lung Growth in Neonatal Mice. *PLOS ONE 10*, e0118344.

(23) Smith, D., Aherrera, A., Lopez, A., Neptune, E., Winickoff, J. P., Klein, J. D., Chen, G., Lazarus, P., Collaco, J. M., and McGrath-Morrow, S. A. (2015) Adult Behavior in Male Mice Exposed to E-Cigarette Nicotine Vapors during Late Prenatal and Early Postnatal Life. *PLOS ONE 10*, e0137953.

(24) Maccani, J. Z., Koestler, D. C., Houseman, E. A., Marsit, C. J., and Kelsey, K. T. (2013) Placental DNA methylation alterations associated with maternal tobacco smoking at the RUNX3 gene are also associated with gestational age. *Epigenomics 5*, 619-630.

(25) Chan, Y. L., Saad, S., Machaalani, R., Oliver, B. G., Vissel, B., Pollock, C., Jones, N. M., and Chen, H. (2017) Maternal Cigarette Smoke Exposure Worsens Neurological Outcomes in Adolescent Offspring with Hypoxic-Ischemic Injury. *Frontiers in molecular neuroscience 10*, 306.

(26) Chan, Y. L., Saad, S., Al-Odat, I., Zaky, A. A., Oliver, B., Pollock, C., Li, W., Jones, N. M., and Chen, H. (2016) Impact of maternal cigarette smoke exposure on brain and kidney health outcomes in female offspring. *Clinical and experimental pharmacology & physiology 43*, 1168-1176.

(27) Nguyen, L. T., Stangenberg, S., Chen, H., Al-Odat, I., Chan, Y. L., Gosnell, M. E., Anwer, A. G., Goldys, E. M., Pollock, C. A., and Saad, S. (2015) L-Carnitine reverses maternal cigarette smoke exposure-induced renal oxidative stress and mitochondrial dysfunction in mouse offspring. *American journal of physiology. Renal physiology 308*, F689-696.

(28) Antunes, M., and Biala, G. (2012) The novel object recognition memory: neurobiology, test procedure, and its modifications. *Cognitive processing 13*, 93-110.

(29) Carola, V., D'Olimpio, F., Brunamonti, E., Mangia, F., and Renzi, P. (2002) Evaluation of the elevated plus-maze and open-field tests for the assessment of anxiety-related behaviour in inbred mice. *Behav Brain Res 134*, 49-57.

(30) Hata, T., Nishikawa, H., Itoh, E., and Funakami, Y. (2001) Anxiety-like behavior in elevated plus-maze tests in repeatedly cold-stressed mice. *Japanese journal of pharmacology 85*, 189-196.

(31) Komada, M., Takao, K., and Miyakawa, T. (2008) Elevated Plus Maze for Mice. *Journal of Visualized Experiments : JoVE*, 1088.

(32) Walf, A. A., and Frye, C. A. (2007) The use of the elevated plus maze as an assay of anxiety-related behavior in rodents. *Nature protocols 2*, 322-328.
(33) Livak, K. J., and Schmittgen, T. D. (2001) Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2−ΔΔCT Method. *Methods 25*, 402-408.
(34) Blanchard, D. C., Griebel, G., and Blanchard, R. J. (2003) The Mouse Defense Test Battery: pharmacological and behavioral assays for anxiety and panic. *European journal of pharmacology 463*, 97-116.
(35) Wall, P. M., and Messier, C. (2001) Methodological and conceptual issues in the use of the elevated plus-maze as a psychological measurement instrument of animal anxiety-like behavior. *Neuroscience & Biobehavioral Reviews 25*, 275-286.
(36) Zeilinger, S., Kühnel, B., Klopp, N., Baurecht, H., Kleinschmidt, A., Gieger, C., Weidinger, S., Lattka, E., Adamski, J., Peters, A., Strauch, K., Waldenberger, M., and Illig, T. (2013) Tobacco Smoking Leads to Extensive Genome-Wide Changes in DNA Methylation. *PLOS ONE 8*, e63812.
(37) Okano, M., Bell, D. W., Haber, D. A., and Li, E. (1999) DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development. *Cell 99*, 247-257.
(38) Spannhoff, A., Hauser, A. T., Heinke, R., Sippl, W., and Jung, M. (2009) The emerging therapeutic potential of histone methyltransferase and demethylase inhibitors. *ChemMedChem 4*, 1568-1582.
(39) Xu, J., Deng, X., and Disteche, C. M. (2008) Sex-Specific Expression of the X-Linked Histone Demethylase Gene Jarid1c in Brain. *PLOS ONE 3*, e2553.
(40) Kawasaki, H., Schiltz, L., Chiu, R., Itakura, K., Taira, K., Nakatani, Y., and Yokoyama, K. K. (2000) ATF-2 has intrinsic histone acetyltransferase activity which is modulated by phosphorylation. *Nature 405*, 195-200.
(41) Pearson, A. G., Curtis, M. A., Waldvogel, H. J., Faull, R. L., and Dragunow, M. (2005) Activating transcription factor 2 expression in the adult human brain: association with both neurodegeneration and neurogenesis. *Neuroscience 133*, 437-451.
(42) Reimold, A. M., Grusby, M. J., Kosaras, B., Fries, J. W. U., Mori, R., Maniwa, S., Clauss, I. M., Collins, T., Sidman, R. L., Glimcher, M. J., and Glimcher, L. H. (1996) Chondrodysplasia and neurological abnormalities in ATF-2-deficient mice. *Nature 379*, 262.
(43) Fu, J., Bian, M., Jiang, Q., and Zhang, C. (2007) Roles of Aurora kinases in mitosis and tumorigenesis. *Molecular cancer research : MCR 5*, 1-10.
(44) Balsevich, G., Poon, A., Goldowitz, D., and Wilking, J. A. (2014) The effects of pre- and post-natal nicotine exposure and genetic background on the striatum and behavioral phenotypes in the mouse. *Behav Brain Res 266*, 7-18.
(45) Lau, K., Swiney, B. S., Reeves, N., Noguchi, K. K., and Farber, N. B. (2012) Propylene glycol produces excessive apoptosis in the developing mouse brain, alone and in combination with phenobarbital. *Pediatric research 71*, 54-62.
(46) Grana, R., Benowitz, N., and Glantz, S. A. (2014) E-cigarettes: a scientific review. *Circulation 129*, 1972-1986.
(47) Wieslander, G., Norback, D., and Lindgren, T. (2001) Experimental exposure to propylene glycol mist in aviation emergency training: acute ocular and respiratory effects. *Occupational and Environmental Medicine 58*, 649-655.
(48) Kovacic, P., and Cooksy, A. L. (2010) Electron transfer as a potential cause of diacetyl toxicity in popcorn lung disease. *Reviews of environmental contamination and toxicology 204*, 133-148.
(49) FEMA. (2004) Respiratory Health and Safety in the Flavor Manufacturing Workplace, (States, T. F. a. E. M. A. o. t. U., Ed.), Washington, D.DC. 20006.

(50) Ogunwale, M. A., Li, M., Ramakrishnam Raju, M. V., Chen, Y., Nantz, M. H., Conklin, D. J., and Fu, X.-A. (2017) Aldehyde Detection in Electronic Cigarette Aerosols. *ACS Omega 2*, 1207-1214.
(51) Allen, J. G., Flanigan, S. S., LeBlanc, M., Vallarino, J., MacNaughton, P., Stewart, J. H., and Christiani, D. C. (2016) Flavoring Chemicals in E-Cigarettes: Diacetyl, 2,3-Pentanedione, and Acetoin in a Sample of 51 Products, Including Fruit-, Candy-, and Cocktail-Flavored E-Cigarettes. *Environmental health perspectives 124*, 733-739.
(52) Klager, S., Vallarino, J., MacNaughton, P., Christiani, D. C., Lu, Q., and Allen, J. G. (2017) Flavoring Chemicals and Aldehydes in E-Cigarette Emissions. *Environmental science & technology 51*, 10806-10813.
(53) Joubert, B. R., Haberg, S. E., Nilsen, R. M., Wang, X., Vollset, S. E., Murphy, S. K., Huang, Z., Hoyo, C., Midttun, O., Cupul-Uicab, L. A., Ueland, P. M., Wu, M. C., Nystad, W., Bell, D. A., Peddada, S. D., and London, S. J. (2012) 450K epigenome-wide scan identifies differential DNA methylation in newborns related to maternal smoking during pregnancy. *Environmental health perspectives 120*, 1425-1431.
(54) Cressman, A. M., Koren, G., Pupco, A., Kim, E., Ito, S., and Bozzo, P. (2012) Maternal cocaine use during breastfeeding. *Canadian Family Physician 58*, 1218-1219.
(55) Calvaresi, V., Escuder, D., Minutillo, A., Bastons-Compta, A., Garcia-Algar, O., Pallas Alonso, C. R., Pacifici, R., and Pichini, S. (2016) Transfer of Nicotine, Cotinine and Caffeine Into Breast Milk in a Smoker Mother Consuming Caffeinated Drinks. *Journal of analytical toxicology 40*, 473-477.
(56) Haastrup, M. B., Pottegard, A., and Damkier, P. (2014) Alcohol and breastfeeding. *Basic & clinical pharmacology & toxicology 114*, 168-173.
(57) Dahlstrom, A., Ebersjo, C., and Lundell, B. (2008) Nicotine in breast milk influences heart rate variability in the infant. *Acta paediatrica (Oslo, Norway : 1992) 97*, 1075-1079.
(58) Mosconi, L., Berti, V., Swerdlow, R. H., Pupi, A., Duara, R., and de Leon, M. (2010) Maternal transmission of Alzheimer's disease: Prodromal metabolic phenotype and the search for genes. *Human Genomics 4*, 170-193.
(59) Chaudhry, M., Wang, X., Bamne, M. N., Hasnain, S., Demirci, F. Y., Lopez, O. L., and Kamboh, M. I. (2015) Genetic variation in imprinted genes is associated with risk of late-onset Alzheimer's disease. *Journal of Alzheimer's disease : JAD 44*, 989-994.
(60) Bassett, S. S., Avramopoulos, D., Perry, R. T., Wiener, H., Watson, B., Jr., Go, R. C., and Fallin, M. D. (2006) Further evidence of a maternal parent-of-origin effect on chromosome 10 in late-onset Alzheimer's disease. *American journal of medical genetics. Part B, Neuropsychiatric genetics : the official publication of the International Society of Psychiatric Genetics 141b*, 537-540.
(61) Feng, J., Chang, H., Li, E., and Fan, G. (2005) Dynamic expression of de novo DNA methyltransferases Dnmt3a and Dnmt3b in the central nervous system. *Journal of neuroscience research 79*, 734-746.
(62) Li, J. Y., Pu, M. T., Hirasawa, R., Li, B. Z., Huang, Y. N., Zeng, R., Jing, N. H., Chen, T., Li, E., Sasaki, H., and Xu, G. L. (2007) Synergistic function of DNA methyltransferases Dnmt3a and Dnmt3b in the methylation of Oct4 and Nanog. *Molecular and cellular biology 27*, 8748-8759.
(63) Outchkourov, N. S., Muino, J. M., Kaufmann, K., van Ijcken, W. F., Groot Koerkamp, M. J., van Leenen, D., de Graaf, P., Holstege, F. C., Grosveld, F. G., and Timmers, H. T. (2013) Balancing of histone H3K4 methylation states by the Kdm5c/SMCX histone demethylase modulates promoter and enhancer function. *Cell reports 3*, 1071-1079.
(64) Tahiliani, M., Mei, P., Fang, R., Leonor, T., Rutenberg, M., Shimizu, F., Li, J., Rao, A., and Shi, Y. (2007) The histone H3K4 demethylase SMCX links REST target genes to X-linked mental retardation. *Nature 447*, 601-605.

(65) Jensen, L. R., Amende, M., Gurok, U., Moser, B., Gimmel, V., Tzschach, A., Janecke, A. R., Tariverdian, G., Chelly, J., Fryns, J. P., Van Esch, H., Kleefstra, T., Hamel, B., Moraine, C., Gecz, J., Turner, G., Reinhardt, R., Kalscheuer, V. M., Ropers, H. H., and Lenzner, S. (2005) Mutations in the JARID1C gene, which is involved in transcriptional regulation and chromatin remodeling, cause X-linked mental retardation. *American journal of human genetics 76*, 227-236.

(66) Iwase, S., Brookes, E., Agarwal, S., Badeaux, A. I., Ito, H., Vallianatos, C. N., Tomassy, G. S., Kasza, T., Lin, G., Thompson, A., Gu, L., Kwan, K. Y., Chen, C., Sartor, M. A., Egan, B., Xu, J., and Shi, Y. (2016) A Mouse Model of X-linked Intellectual Disability Associated with Impaired Removal of Histone Methylation. *Cell reports 14*, 1000-1009.

(67) Iwase, S., Lan, F., Bayliss, P., de la Torre-Ubieta, L., Huarte, M., Qi, H. H., Whetstine, J. R., Bonni, A., Roberts, T. M., and Shi, Y. (2007) The X-linked mental retardation gene SMCX/JARID1C defines a family of histone H3 lysine 4 demethylases. *Cell 128*, 1077-1088.

(68) Legube, G., and Trouche, D. (2003) Regulating histone acetyltransferases and deacetylases. *EMBO reports 4*, 944-947.

(69) Yamada, T., Yoshiyama, Y., and Kawaguchi, N. (1997) Expression of activating transcription factor-2 (ATF-2), one of the cyclic AMP response element (CRE) binding proteins, in Alzheimer disease and non-neurological brain tissues. *Brain research 749*, 329-334.

(70) Mallm, J.-P., and Rippe, K. (2015) Aurora Kinase B Regulates Telomerase Activity via a Centromeric RNA in Stem Cells. *Cell reports 11*, 1667-1678.

(71) Cerone, M. A., Burgess, D. J., Naceur-Lombardelli, C., Lord, C. J., and Ashworth, A. (2011) High-throughput RNAi screening reveals novel regulators of telomerase. *Cancer research 71*, 3328-3340.

(72) Wysong, D. R., Chakravarty, A., Hoar, K., and Ecsedy, J. A. (2009) The inhibition of Aurora A abrogates the mitotic delay induced by microtubule perturbing agents. *Cell cycle (Georgetown, Tex.) 8*, 876-888.

(73) Marumoto, T., Honda, S., Hara, T., Nitta, M., Hirota, T., Kohmura, E., and Saya, H. (2003) Aurora-A kinase maintains the fidelity of early and late mitotic events in HeLa cells. *The Journal of biological chemistry 278*, 51786-51795.

(74) Portier, N., Audhya, A., Maddox, P. S., Green, R. A., Dammermann, A., Desai, A., and Oegema, K. (2007) A microtubule-independent role for centrosomes and aurora a in nuclear envelope breakdown. *Developmental cell 12*, 515-529.

(75) Jeon, H. Y., and Lee, H. (2013) Depletion of Aurora-A in zebrafish causes growth retardation due to mitotic delay and p53-dependent cell death. *The FEBS journal 280*, 1518-1530.

Figures and Tables

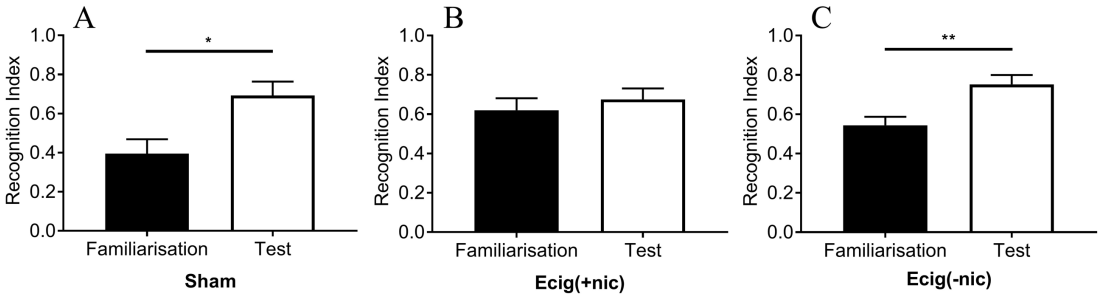


Figure 1. Novel object recognition (NOR) test. Recognition index for Week 13 offspring from dams exposed to A) ambient air (sham) (n=14), B) Ecig(+nic) (n=14) and C) Ecig(-nic) groups (n=14) in the familiarization phase and test phase. Data represents average ± standard deviation, *p<0.05, **p<0.01. Ecig = Electronic cigarette, ±nic = ±nicotine.

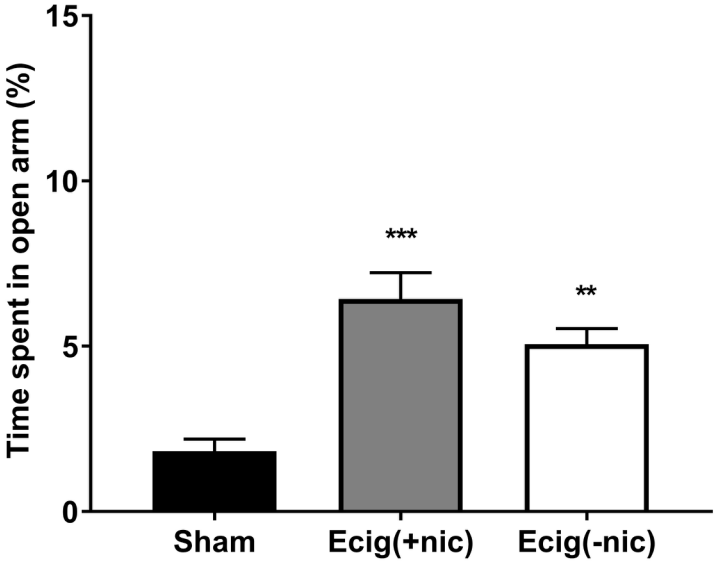
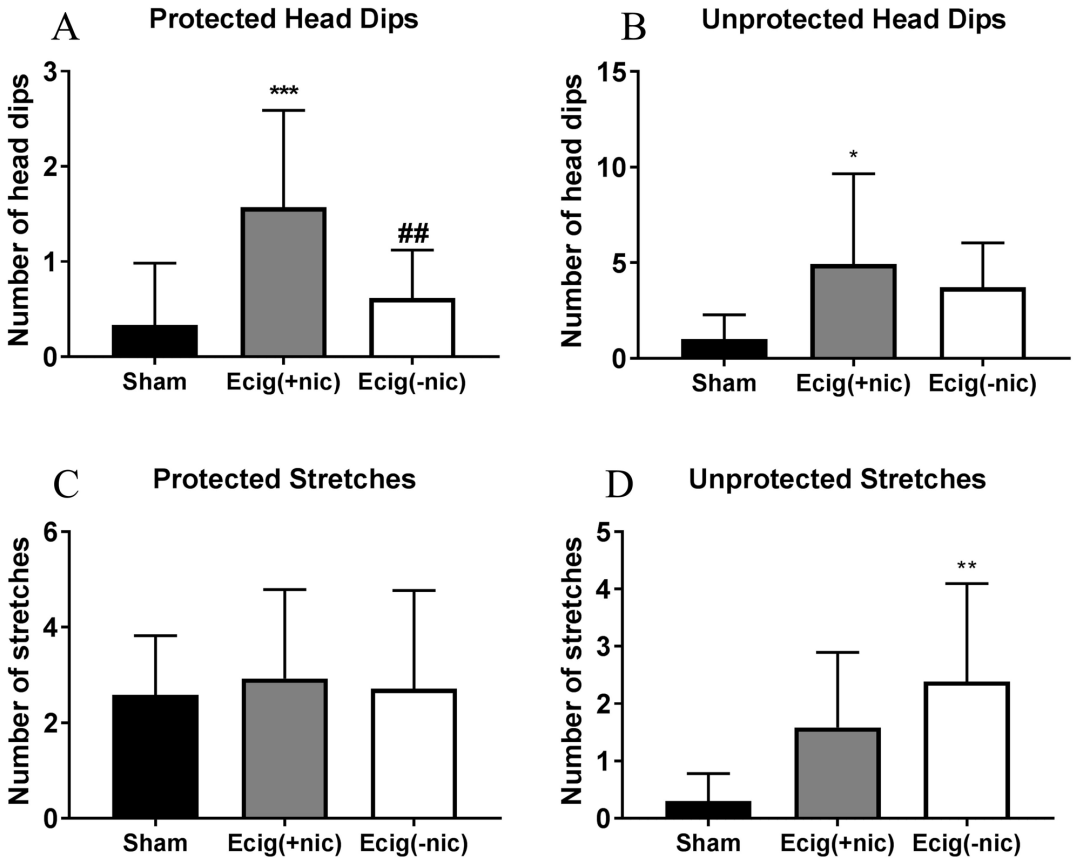
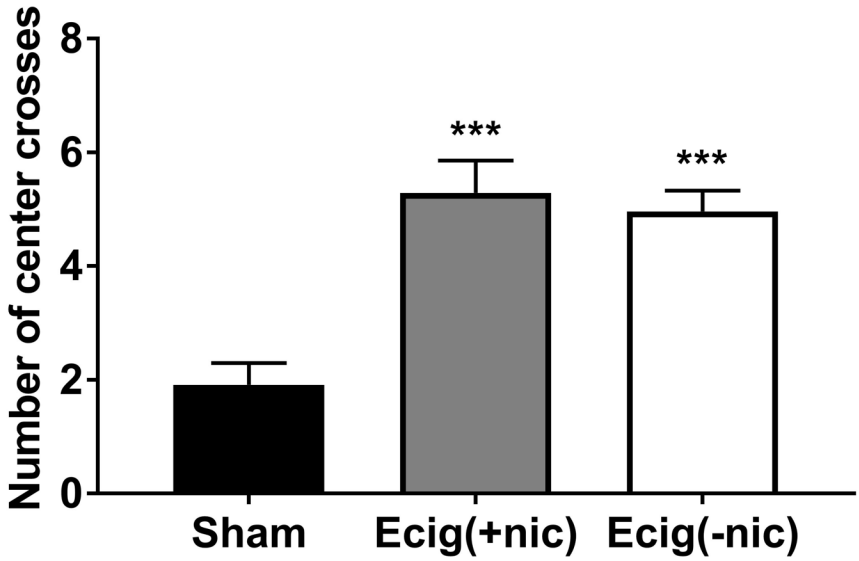


Figure 2. Elevated plus maze (EPM) test –closed versus open arms. The percentage of time spent in the open arm for Week 13 offspring from dams exposed to ambient air (sham) (n=14), Ecig(+nic) (n=14) and Ecig(-nic) group (n=14). Data represents average ± standard deviation, **p<0.01, ***p<0.001. Ecig = Electronic cigarette, ±nic = ±nicotine.



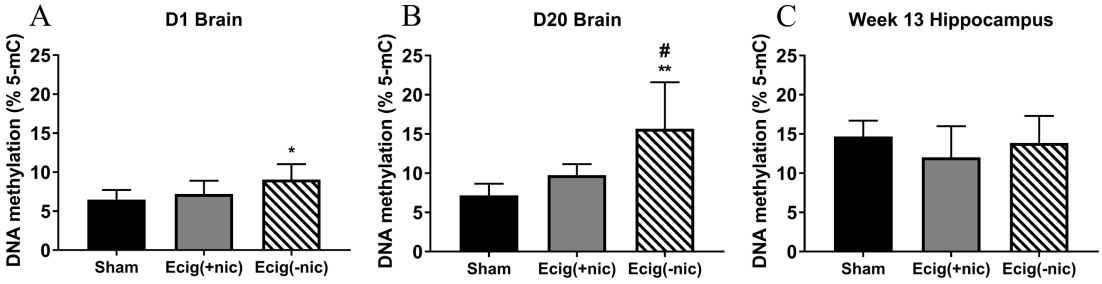
677

678 **Figure 3.** Elevated plus maze (EPM) test – head dips and body stretches. Total number of A)
679 protected head dips, B) unprotected head dips, C) protected stretches and D) unprotected stretches
680 observed in the elevated plus maze for Week 13 offspring from dams exposed to ambient air (sham)
681 (n=14), Ecig(+nic) (n=14) and Ecig(-nic) (n=14). Data represents average ± standard deviation,
682 *p<0.05, **p<0.01, ***p< 0.001 vs. sham, ##p<0.01 vs. Ecig(+nic). Ecig = Electronic cigarette, ±nic =
683 ±nicotine.



684

685 **Figure 4.** Elevated plus maze (EPM) test – center crosses. Number of center crosses on the elevated
686 plus maze for Week 13 offspring from dams exposed to ambient air (sham) (n=14), Ecig(+nic) (n=14)
687 and Ecig(-nic) group (n=14). Data represents average ± standard deviation, ***p<0.001. Ecig =
688 Electronic cigarette, ±nic = ±nicotine.



689

690 **Figure 5.** Percentage of global DNA methylation in the brains of offspring from dams exposed to
691 ambient air (sham) (n=8), Ecig(+nic) (n=6) and Ecig(-nic) (n=8) at A) postnatal day 1 (D1), B) postnatal
692 day 20 (D20) and C) in the hippocampus at Week 13. Data represents average ± standard deviation,
693 *p<0.05, **p<0.01 vs. sham, #p<0.05 vs. Ecig(+nic). Ecig = Electronic cigarette, ±nic = ±nicotine.

694

695 **Figure 6.** Heat map of epigenetic genes fold changes in Ecig(+nic) (n=3) and Ecig(-nic) group (n=3)
696 normalized to the sham group (n=3) at Postnatal Day 1. Fold changes of greater than two were
697 considered significantly changed.

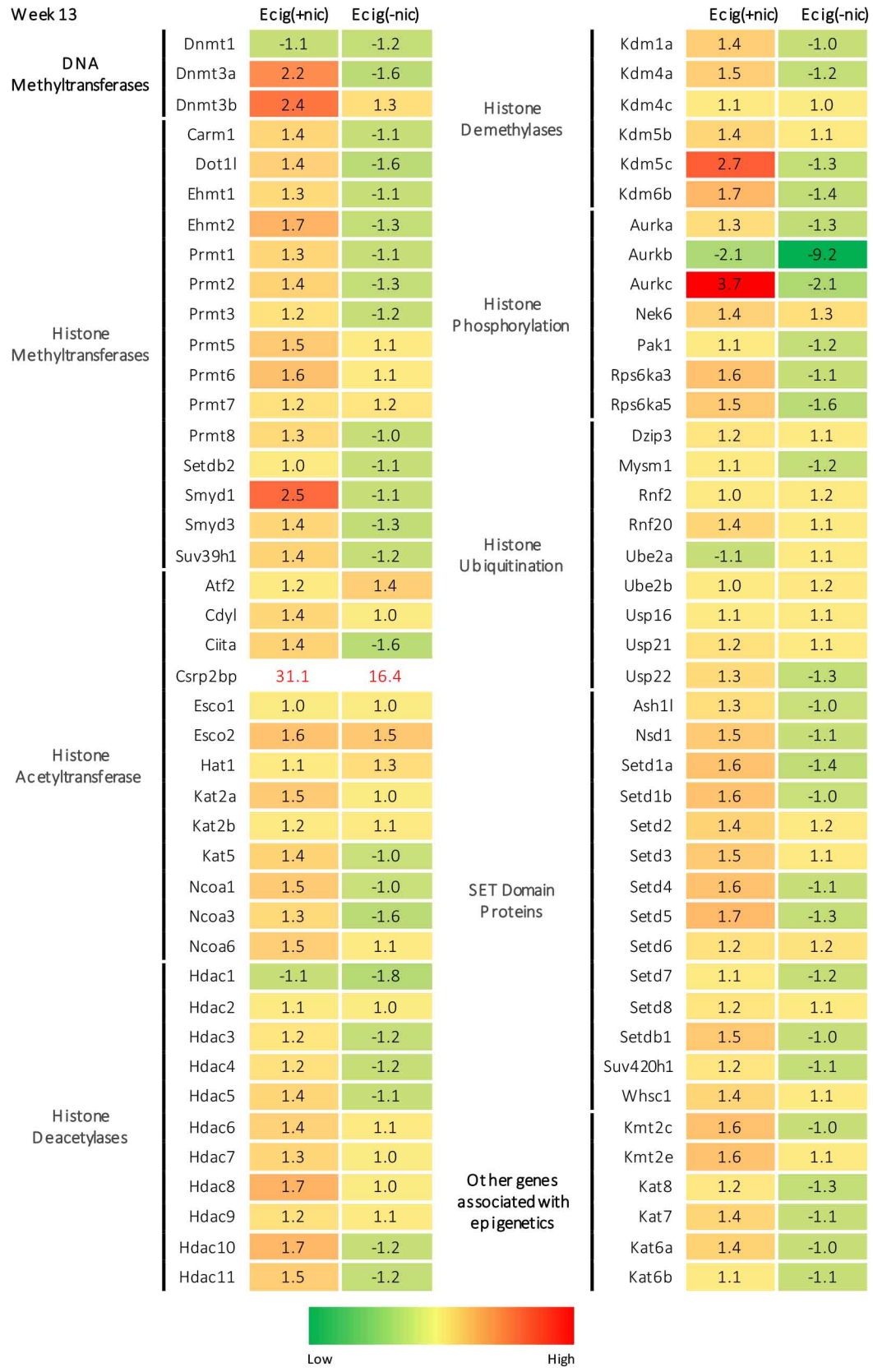


Figure 7. Heat map of epigenetic genes fold changes in Ecig(+nic) (n=3) and Ecig(-nic) group (n=3) normalized to the sham group (n=3) at Week 13. Fold changes of greater than two were considered significantly changed.

Class	Gene
DNA Methyltransferases	Dnmt1, Dnmt3a, Dnmt3b
Histone Methyltransferases	Carm1, Dot1l, Ehmt1, Ehmt2, Mll3, Prmt1, Prmt2, Prmt3, Prmt5, Prmt6, Prmt7, Prmt8, Setdb2, Smyd1, Smyd3, Suv39h1
Histone Demethylases	Kdm1a, Kdm5b, Kdm5c, Kdm4a, Kdm4c, Kdm6b
Histone Acetyltransferases	Atf2, Cdyl, Ciita, Csrp2bp, Esco1, Esco2, Hat1, Kat2a, Kat2b, Kat5, Myst1, Myst2, Myst3, Myst4, Ncoa1, Ncoa3, Ncoa6
Histone Deacetylases	Hdac1, Hdac2, Hdac3, Hdac4, Hdac5, Hdac6, Hdac7, Hdac8, Hdac9, Hdac10, Hdac1
Histone Phosphorylation	Aurka, Aurkb, Aurkc, Nek6, Pak1, Rps6ka3, Rps6ka5
Histone Ubiquitination	Dzip3, Mysm1, Rnf2, Rnf20, Ube2a, Ube2b, Usp16, Usp21, Usp22
SET Domain Proteins	Ash1l, Mll5, Nsd1, Setd1a, Setd1b, Setd2, Setd3, Setd4, Setd5, Setd6, Setd7, Setd8, Setdb1, Suv420h1, Whsc1

Table 1. Genes associated or predicted to influence epigenetic modification enzymes and gene expression included in the PCR array kit, listed by class.

Gene ID	Description	F/R	Primer sequence (5'→3')
Atf2	Activating transcription factor 2	Forward	CTTCACTGATAAAACACGAC
		Reverse	TGTTTATGGACAGCCAAATG
AurkA	Aurora kinase A	Forward	CAGAGAACAGCTACTTACATC
		Reverse	GTCTGCAATTCTTCAACTCTC
AurkB	Aurora kinase B	Forward	AAGTCTCAGATTGAGAAGGAG
		Reverse	GAAGGATGTTGGGATGTTTC
AurkC	Aurora kinase C	Forward	TCTCTCAGGAGAAAGACAATG
		Reverse	AAACTTAAAATCCACCTGGC
Dnmt3a	DNA methyltransferase 3a	Forward	ACCAGAAGAAGAGAAGAATCC
		Reverse	CAATGATCTCCTTGACCTTAG
Dnmt3b	DNA methyltransferase 3b	Forward	GACTTCATGGAAGAAGTGAC
		Reverse	TATCATCCTGATACTCTGTGC
Kdm5c	Lysine (K)-specific demethylase 5c	Forward	AATGCCCAGTTTATTGAGTC
		Reverse	TAATTGTCATCACAGCCATC
Kdm6b	Lysine (K)-specific demethylase 6b	Forward	CTGGCATGTTGAAGTAATCAG
		Reverse	TCAAGATGATCAAGTCTGC
Hdac1	Histone deacetylase 1	Forward	CAATCTGACCATCAAAGGAC
		Reverse	CCGGTCCAAAGTATTCAAAG

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706 **Table 2.** PCR primer sequences.

Time point	Encoded protein	Sham	Ecig(+nic)	Ecig(-nic)
Postnatal Day 1	Dnmt3a	1.1 ± 0.5	0.8 ± 0.1	0.7 ± 0.1
	Dnmt3b	1.0 ± 0.4	1.2 ± 0.2	1.1 ± 0.2
	Kdm5c	1.0 ± 0.2	1.0 ± 0.3	0.7 ± 0.2
	Kdm6b	1.0 ± 0.2	1.0 ± 0.1	0.9 ± 0.2
	Atf2	1.0 ± 0.1	0.8 ± 0.1**	0.8 ± 0.1***
	Hdac1	1.0 ± 0.2	0.9 ± 0.1	0.8 ± 0.2
	Aurka	1.0 ± 0.2	0.8 ± 0.2	0.5 ± 0.1**†
	Aurkb	1.0 ± 0.1	0.6 ± 0.1***	0.6 ± 0.0***
	Aurkc	1.1 ± 0.5	2.2 ± 1.3	3.5 ± 1.7
Postnatal Day 20	Dnmt3a	1.0 ± 0.2	0.6 ± 0.2*	1.3 ± 0.3†††
	Dnmt3b	1.0 ± 0.2	0.6 ± 0.1***	0.8 ± 0.1
	Kdm5c	1.0 ± 0.3	1.0 ± 0.2	1.4 ± 0.3†
	Kdm6b	1.0 ± 0.2	0.8 ± 0.2	1.2 ± 0.2††
	Atf2	1.0 ± 0.2	1.0 ± 0.2	1.3 ± 0.1†
	Hdac1	1.0 ± 0.2	0.8 ± 0.1	1.1 ± 0.4
	Aurka	1.0 ± 0.2	0.9 ± 0.1	0.6 ± 0.2**
	Aurkb	1.0 ± 0.1	0.8 ± 0.1**	0.6 ± 0.3
	Aurkc	1.0 ± 0.2	0.8 ± 0.1**	1.1 ± 0.4†
Week 13	Dnmt3a	1.0 ± 0.1	0.9 ± 0.1	0.9 ± 0.1
	Dnmt3b	1.0 ± 0.1	0.7 ± 0.1**	0.8 ± 0.2
	Kdm5c	1.0 ± 0.3	0.6 ± 0.1*	0.6 ± 0.1**
	Kdm6b	1.0 ± 0.2	0.8 ± 0.1	0.9 ± 0.1
	Atf2	1.0 ± 0.1	0.9 ± 0.1*	0.9 ± 0.0**
	Hdac1	1.0 ± 0.1	0.8 ± 0.1	0.9 ± 0.1
	Aurka	1.0 ± 0.1	0.8 ± 0.1	1.0 ± 0.1
	Aurkb	1.0 ± 0.2	0.7 ± 0.2	0.8 ± 0.1
	Aurkc	1.0 ± 0.3	0.8 ± 0.3	1.2 ± 0.3

Table 3. Confirmation of genes associated or predicted to influence epigenetic modification enzymes in postnatal day 1 brains, postnatal day 20 brains, and Week 13 micro-dissected hippocampus tissues by RT-qPCR. Ecig(+nic) (n=6) and Ecig(-nic) (n=6) data is expressed as the percentage gene expression normalised to the sham group (n=6). Data represents average ± standard deviation, *p<0.05, **p<0.01, ***p<0.001 vs. sham, †p<0.05, ††p<0.01, †††p<0.001 vs. Ecig(+nic).