Electronic Cigarettes: Neurological Effects on Murine Offspring and the Response of Neuronal Cells

Tara Nguyen

Doctor of Philosophy

University of Technology Sydney

Faculty of Science

June 2020

Certificate of Original Authorship

I, Tara Nguyen declare that this thesis, is submitted in fulfilment of the requirements for the award of Doctor of Philosophy, in the School of Life Science, Faculty of Science at the University of Technology Sydney.

This thesis is wholly my own work unless otherwise reference or acknowledged. In addition, I certify that all information sources and literature used are indicated in the thesis.

This document has not been submitted for qualifications at any other academic institution. This research is supported by the Australian Government Research Training Program.

Production Note:
Signature removed
prior to publication.

Signature .....

1/5/20

Date.....

Acknowledgements

First and foremost, I would like to acknowledge my principal and co-supervisor, Associate Professor Catherine Gorrie, Dr. Kristine McGrath and Dr. Charles Cranfield for their continuous support and encouragement throughout my PhD. Without their advice and expertise, I would not have produced the quality of research for this dissertation.

This study would not have been completed without the vast support from many research students and support staffs. I would like to thank the members from the Neural Injury Research Unit, Theresa Sutherland, Neus Gomila Pelegri and Rebecca Keppel for their constant support and high-quality banter during our meetings. In addition, I would like to thank the members from the Molecular Biosciences Team in the School of Life Sciences for their support at the beginning of the project.

I want to express my sincere appreciation to Associate Professor Hui Chen and Gerard Li for their invaluable support during the animal experiments. In addition, I would also like to extend my appreciation to Fiona Ryan and Lal Overlunde for taking care of my animals and their continuous support in the Ernst Facility. I would like to thank Dr. Valery Combes and Benjamin Sealy for their guidance and providing me with the primary human brain microvascular endothelial cells for my *in vitro* blood brain barrier models.

I would like to extend my gratitude to the support staffs from the UTS Level 7 laboratories, Mercedes Ballesteros, Sarah Osvath and Luke Beebe for their continuous technical support in the laboratories. Thank you to the undergraduate research student, Thomas Hueneburg, and the Singapore interns, Si Xing Quah and Fadhullah Fauzi for their assistance in cell culture, tissue collection, tissue processing and behavioural analysis.

Finally, I would like to thank my family and friends. Thanks Mum and Dad for your unconditional love and support throughout my PhD. Words cannot describe how much I appreciate all the hard work and dedication you have given to my brother and I. I love you both very much. I would like to thank my brother, David, for your love and hard-core advice at the gym during the stressful

times. To my research friends, Charlotte, Claire, Daniel, Alison, Gihani, Claudia, Amani and Que, thank you so much for just being there for me. Thank you for your constant banter during our lunch breaks and advice during practise runs of my presentation. Last but not least, to David Ramjan, thank you so much for supporting me throughout my PhD. You have always been optimistic when I am feeling stress and you always know what to do to make me feel better. THANK YOU EVERYONE!

This dissertation is a thesis by compilation.

Publications arising from PhD research

Nguyen, T.; Li, G.E.; Chen, H.; Cranfield, C.G.; McGrath, K.C.; Gorrie, C.A. Neurological effects in the offspring after switching from tobacco cigarettes to e-cigarettes during pregnancy in a mouse model. *Toxicological Sciences.* 2019, 172(1):191-200.

Nguyen, T.; Li, G.E.; Chen, H.; Cranfield, C.G.; McGrath, K.C.; Gorrie, C.A. Maternal E-cigarette exposure results in cognitive and epigenetic alterations in offspring in a mouse model. *Chem. Res. Toxicol*. 2018, 31(7):601-611.

Other publications during PhD candidature

Li, G., Chan, Y.L., Nguyen, L.T., Mak, C., Zaky, A., Anwer, A.G., Shi, Y., **Nguyen, T.**, Pollock, C.A, Oliver, B.G.[2], Saad, S., Chen, H. Impact of maternal e-cigarette vapor exposure on renal health in the offspring. *Annals of New York Academy and Sciences.* 2019. doi: 10.1111/nyas.14174.

Chen, H., Li, G., Chan, Y. L., **Nguyen, T.**, van Reyk, D., Saad, S., Oliver, B. G. Modulation of neural regulators of energy homeostasis, and of inflammation, in the pups of mice exposed to e-cigarettes. *Neuroscience Letter*. 2018, Volume: 684, Pages 61-66, https://doi.org/10.1016/j.neulet.2018.07.001.

Chen, H., Li, G., Chan, Y. L., Chapman, D. G., Sukjamnong, S., **Nguyen, T.**, Annissa, T., McGrath, K. C., Sharma, P., Oliver, B. G. Maternal E-Cigarette Exposure in Mice Alters DNA Methylation and Lung Cytokine Expression in Offspring. *Am J Respir Cell Mol Biol*. 2018, 58(3):366-377.

Conference proceedings arising from PhD research

Society for Neuroscience 2019 Conference

Nguyen, T.; Li, G.E.; Chen, H.; Cranfield, C.G.; McGrath, K.C.; Gorrie, C.A. The effects of maternal electronic cigarette exposure on offspring behavior and epigenetics. In: Neuroscience 2019; Chicago; US; 2019.

Australian Society for Medical Research NSW meeting 2019

Nguyen, T.; Li, G.E.; Chen, H.; Cranfield, C.G.; McGrath, K.C.; Gorrie, C.A. Switching to electronic cigarette from tobacco cigarette during pregnancy mildly improves offspring neurological outcomes. In; AMSR NSW meeting 2019; Sydney; Australia, 2019.

Australasian Neuroscience Society 2018 Conference

Nguyen, T.; Li, G.E.; Chen, H.; Cranfield, C.G.; McGrath, K.C.; Gorrie, C.A. Exposure to e-cigarette aerosols results in neurological changes in both *in vivo* and *in vitro* models. In; Australasian Neuroscience Society 2018 Conference; Brisbane; Australia, 2018.

New Horizons 2017

Nguyen, T.; Li, G.E.; Chen, H.; Cranfield, C.G.; McGrath, K.C.; Gorrie, C.A. Behavioural and epigenetic changes in offspring in a mouse model of maternal vaping. In; New Horizons 2017; Centenery Institute; Sydney; Australia, 2017.

Funding and Scholarships

This research was funded by the:

Centre for Health and Technologies to the Molecular Biosciences Team, Faculty of Science, University of Technology Sydney.

PhD Strategic Scholarship to the Molecular Biosciences Team, Faculty of Science, University of Technology Sydney.

Table of Contents

Certificate of Original Authorship……i

Acknowledgements ii

Publications arising from PhD research iv

Other publications during PhD candidature iv

Conference proceedings arising from PhD research v

Funding and Scholarships vi

List of Figures xv

List of Tables xx

Abbreviations xxi

Abstract xxiv

Chapter 1 - Literature review

1.1 Introduction to electronic cigarettes 1

1.2 Tobacco cigarettes vs. e-cigarettes 3

1.3 E-cigarette as a cessation aid for tobacco smokers 4

1.4 Policy around e-cigarettes 6

1.5 Epidemiology of e-cigarette use in society 10

1.5.1 Adults 11

1.5.2 Young people 13

1.5.3 Women of child-bearing age and pregnant woman 14

1.6 Electronic liquids used in e-cigarettes 15

1.6.1 Propylene glycol and glycerine 16

1.6.1.1 Safety of inhaling propylene glycol and glycerin 17

1.6.2 Nicotine....20
1.6.3 Flavouring in e-cigarettes....22
1.7 In vitro and in vivo studies of e-cigarettes....23
1.7.1 *In vivo* animal models of e-cigarette exposure....23
1.7.1.1 E-cigarette exposure in normal animals....23
1.7.1.2 E-cigarette exposure in pregnancy models....25
1.7.2 *In vitro* cell culture models....27
1.8 Effects of vaping in humans....29
1.9 Brain development and cognitive effects....30
1.9.1 Brain regions affected by nicotine and chemical flavourings....30
1.10 Project aims and thesis....33
Chapter 2 - The effects of e-cigarette aerosol exposure on offspring during pregnancy
2.1 Introduction....34
2.1.1 Maternal e-cigarette exposure and epigenetic changes....34
2.2 Hypothesis and aims....38
2.2.1 Hypothesis....38
2.2.2 Aims....38
2.3 Materials and Methodology....39
2.3.1 E-cigarette device....39
2.3.2 Animal experimental procedure....40
2.3.3 Aerosol exposure procedure....43
2.3.4 Behavioural assessments....44
2.3.4.1 The Novel Object Recognition (NOR)....45
2.3.4.2 The Elevated Plus Maze (EPM)....47
2.3.5 Euthanasia and tissue collection....48
2.3.6 Plasma cotinine....48

2.3.7 DNA and RNA extraction 49
2.3.8 RNA quality check 52
2.3.9 Global 5-mC DNA methylation 54
2.3.10 Epigenetics 54
2.3.10.1 RT^2 First Strand kit 56
2.3.10.2 RT^2 SYBR Green Mastermix 56
2.3.11 PCR array validation 58
2.3.11.1 Reverse transcription 60
2.3.11.2 Real time PCR assay 60
2.3.12 Tissue fixation, processing, embedding and cutting 61
2.3.12.1 Tissue staining 61
2.3.12.2 Image analysis and neuronal cell counting 62
2.3.13 Statistical analysis 63
2.4 Results 64
2.4.1 Plasma cotinine 64
2.4.2 Anthropometry data 64
2.4.3 Maternal exposure to e-cigarette aerosol with nicotine causes short-term memory deficits in adult offspring 66
2.4.4 Maternal exposure to e-cigarette aerosol with nicotine showed reduced anxiety and hyperactivity in adult offspring 68
2.4.5 Maternal exposure to e-cigarette aerosols without nicotine resulted in changes to global DNA methylation at P1 and P20 71
2.4.6 E-cigarette aerosols induce changes in the mRNA expression profile of chromatin modification enzyme-related genes 73
2.4.7 Validation of epigenetic genes from the chromatin modification enzyme PCR array 76
2.4.7.1 Maternal exposure to e-cigarette aerosols showed changes in Dnmt3a and Dnmt3b gene expression in offspring at P20 and Week 13 76
2.4.7.2 Maternal exposure to e-cigarette aerosols showed changes in Kdm5c and Kdm6b gene expression in offspring at P20 and Week 13 79

2.4.7.3 Maternal exposure to e-cigarette aerosols showed changes in Atf2 gene expression in offspring but not Hdac1 81

2.4.7.4 Maternal exposure to e-cigarette aerosols showed an overall decrease in AurkA, AurkB and AurkC gene expression in offspring 83

2.4.8 Offspring from mothers exposed to e-cigarette aerosols showed no changes in neuronal cell counts at P20 and Week 13 85

2.5 Discussion 87

2.5.1 Offspring from mothers exposed to e-cigarette aerosols with nicotine have a decrease in whole body weight, and without nicotine, had an increase in body weight 87

2.5.2 Offspring from mothers exposed to e-cigarette aerosols shows short-term memory deficits, increase risk-taking, increase exploration and less anxiety-like behaviour 88

2.5.3 Offspring from mothers exposed to e-cigarette aerosols had increased levels of DNA Methylation 90

2.5.4 PCR array and PCR validation 92

2.5.5 Maternal e-cigarette aerosol exposure did not affect neuronal cell counts in regions of the dorsal hippocampus and the lateral amygdala nucleus 94

2.6 Conclusion 96

Chapter 3 - Behavioural and epigenetic changes in offspring from maternal exposure to e-cigarette aerosols and tobacco smoke

3.1 Introduction 97

3.1.1 Tobacco cigarette use during pregnancy 97

3.2 Hypothesis and aims 100

3.2.1 Hypothesis 100

3.2.2 Aims 100

3.3 Materials and Methodology 101

3.3.1 E-cigarette device 101

3.3.2 Animal experimental procedure 101

3.3.3 Aerosol and smoking procedure 104

3.3.3.1 Smoke exposure group procedure 105

3.3.4 Behavioural assessments 106

3.3.4.1 The Novel Object Recognition 107

3.3.4.2 The Elevated Plus Maze 107

3.3.5 Euthanasia and tissue collection 107

3.3.6 Plasma cotinine 107

3.3.7 DNA and RNA extraction 107

3.3.8 RNA quality check 107

3.3.9 Global 5-mC DNA methylation 107

3.3.10 mRNA expression of chromatin modification genes 108

3.3.11 Tissue fixation, processing and embedding and cutting 108

3.3.12 Statistical analysis 108

3.4 Results 109

3.4.1 Cotinine analysis of breeders and offspring 109

3.4.2 Anthropometry data 110

3.4.3 Switching to e-cigarette aerosol exposure during pregnancy with nicotine causes short-term memory deficits 112

3.4.4 Switching to e-cigarette aerosol exposure during pregnancy showed reduced anxiety and hyperactivity in adult offspring 114

3.4.5 Maternal smoking and switching to e-cigarette aerosols during pregnancy showed an increase in global DNA methylation in offspring at all time points 116

3.4.6 Maternal smoking showed decreases to Dnmt3a and Dnmt3b gene expression in offspring at P1 and Week 13 118

3.4.7 Maternal smoking showed changes to Kdm5c and Kdm6b gene expression in offspring at all time points 120

3.4.8 Maternal smoking showed changes to Atf2 and Hdac1 gene expression in offspring at all time points 122

3.4.9 Maternal smoking and switching to e-cigarette aerosols showed changes to AurkA, AurkB and AurkC gene expression in offspring at all time points 124

3.4.10 Maternal smoking showed a significant reduction in neuronal cell counts in the adult offspring in the hippocampus and amygdala 126

3.5 Discussion 128

3.5.1 Plasma cotinine levels were comparable in mothers and offspring exposed to cigarette smoke and e-cigarette aerosol from different exposure systems 128

3.5.2 Offspring from mothers exposed to cigarette smoke and e-cigarette aerosols showed a reduced body weight at birth and at weaning 129

3.5.3 Offspring from mothers exposed to e-cigarette aerosols containing nicotine showed short-term memory deficits and increased exploration and increased motor activity 129

3.5.4 Offspring from mothers exposed to e-cigarette aerosols increased levels of DNA Methylation compared to offspring exposed to ambient air 131

3.5.5 DNA methyltransferases and histone demethylase, acetyltransferase and deacetylase but not aurora kinases showed minimal changes to gene expression in offspring from mothers that switched to e-cigarette aerosols compared to offspring exposed to cigarette smoke 133

3.5.6 Maternal smoking affects neuronal counts in regions of the dorsal hippocampus and the lateral amygdala nucleus.....136

3.6 Conclusion 138

Chapter 4 - *In vitro* effects of e-cigarette aerosol condensate using neuronal cell culture

4.1 Introduction..... 139

4.1.1 *In vitro* blood brain barrier model.....139

4.2 Hypothesis and aims 142

4.2.1 Hypothesis.....142

4.2.2 Aims 142

4.3 Materials and Methodology 143

4.3.1 Experimental cell lines and conditions 143

4.3.2 SHSY5Y cell differentiation – Optimisation 146

4.3.2.1 Differentiation with phorbol 12-myristate 13-acetate (PMA) 146

4.3.2.2 Validation of PMA treated SHSY5Y cells via RT-qPCR 146

4.3.3 E-cigarette aerosol condensation.....149

4.3.4 Monoculture assays.....151

4.3.4.1 Methylthiazolydiphenyl-tetrazolium bromide assay 151

4.3.4.2 2', 7' Dichlorofluorescein assay 152

4.3.4.3 JC-10 Mitochondrial membrane potential assay.....152

4.3.5 Differentiation of SHSY5Y cells with PMA in the presence of ECAC.....153

4.3.6 *In vitro* blood brain barrier model.....154

4.3.6.1 Creating the *in vitro* blood brain barrier – Fluorescein isothiocyanate (FITC)-dextran and TEER measurements 154

4.3.6.2 Blood Brain Barrier (BBB) co-culture model with HBECs and diff-SHSY5Y cells 155

4.3.6.3 BBB co-culture model with HBEC and BV2 cells 158

4.3.7 Conditioned media experiment 161

4.3.8 *In vivo* experiment of e-cigarette aerosol exposure 162

4.3.8.1 E-cigarette device, animal experimental procedure, aerosol exposure, euthanasia and tissue collection 162

4.3.8.2 RNA extraction, quality check, reverse transcription and real time PCR assay 162

4.3.9 Statistical analysis 163

4.4 Results 164

4.4.1 SHSY5Y are differentiated with PMA 164

4.4.1.1 SHSY5Y differentiation optimisation 164

4.4.1.2 Differentiation of SHSY5Y cells is reduced following treatment with e-cigarette aerosol condensate 166

4.4.2 Cell viability is lower in diff-SHSY5Y and BV2 cells treated with flavoured ECAC without nicotine 168

4.4.3 Reactive oxygen species production was highest in diff-SHSY5Y, BV2 cells and HBEC treated with flavoured e-cigarette aerosol condensate without nicotine 171

4.4.4 Mitochondrial membrane potential depolarisation was increased in diff-SHSY5Y cells and BV2 cells treated with flavoured e-cigarette aerosol condensate without nicotine 174

4.4.5 Co-culture 177

4.4.5.1 HBEC only co-culture experiments 177

4.4.5.2 HBEC co-cultured with diff-SHSY5Y cells 181

4.4.5.3 HBEC co-cultured with BV2 cells 185

4.4.5.4 Conditioned media experiment 188

4.4.6 *In vivo* gene expression of inflammatory markers in the offspring brain 190

4.5 Discussion 193

4.5.1 Differentiation of SHSY5Y cells with e-cigarette aerosol condensate stunts SHSY5Y maturation 193

4.5.2 E-cigarette aerosol condensate effects cell viability, ROS release and mitochondrial potential in SHSY5Y cells, BV2 cell and HBEC monoculture 194

4.5.3 Treatment with e-cigarette aerosol condensate causes increased permeability and decreased integrity in endothelial membrane. 196

4.5.4 Inflammatory gene expression after e-cigarette aerosol condensate exposure of diff-SHSY5Y and BV2 cells co-cultured with HBEC. 200

4.5.5 Treatment with e-cigarette aerosol condensate with conditioned media but not HBEC alone showed changes to epigenetic gene expression in diff-SHSY5Y cells 201

4.5.6 *In vivo* treatment of e-cigarette exposure showed increase inflammatory gene expression of IL-6, IL-10 and TNF-α in offspring at postnatal day 20 203

4.6 Conclusion 204

Chapter 5 - Summary and conclusions

5.1 Concluding remarks 205

List of Figures

Figure 1. Several generations of e-cigarette devices, e-cigars, e-pipes and JUULs.. 1

Figure 2. Image of the different parts in a typical e-cigarette...... 2

Figure 3. A map of the global status of e-cigarette regulation around the world...... 7

Figure 4. Total sales of electronic nicotine delivery systems for each quarter of the year between 2011-2017.. 10

Figure 5. E-cigarette device used for animal experiments.. 40

Figure 6. A timeline summary of the animal experiments for Chapter 2......42

Figure 7. Schematic of the experimental treatment groups for this study......44

Figure 8. A schematic of the novel object recognition test......45

Figure 9. Images of animals exploring the identical objects in the novel object recognition test......46

Figure 10. A schematic of the elevated plus maze with the apparatus dimensions.. 47

Figure 11. Step by step schematic of the DNA, RNA and protein extraction protocol.. 51

Figure 12. A representative virtual gel and an electropherogram generated by the Experion RNA StdSens Analysis Kit.. 53

Figure 13. RT^2 Profiler^{TM} PCR Array 384 PCR plate layout for Format 'E'......57

Figure 14. Coronal section of a Week 13 brain stained with cresyl violet showing the dorsal hippocampus and the lateral amygdala nucleus......63

Figure 15. Weights of offspring at (A) postnatal day (P) 1, (B) P20 and (C) Week 13 from the Sham (n = 14), Ecig(+nic) (n = 14) and Ecig(-nic) groups (n = 14).. 65

Figure 16. Recognition index from the novel object recognition test in the Sham (n = 14), Ecig(+nic) (n = 14) and Ecig(-nic) groups (n = 14).. 67

Figure 17. The time spent in the open arm and the number of centre crosses performed by week 12 offspring in the elevated plus maze in the Sham (n = 14), Ecig(+nic) (n = 14) and Ecig(-nic) groups (n = 14)......68

Figure 18. The total number of head dips and whole body stretches in the closed arm (protected) and open arm (unprotected) in offspring from the Sham (n = 14), Ecig(+nic) (n = 14) and Ecig(-nic) groups (n = 14).. 70

Figure 19. Global DNA methylation in offspring brains at postnatal day 1 (P1), postnatal day 20 (P20) and postnatal Week 13 (n = 10/group).. 72

Figure 20. Heat map of epigenetic gene fold changes (ΔΔCt) in whole brains of P1 offspring in the Ecig(+nic) (n = 3, pooled) and Ecig(-nic) groups (n = 3, pooled) normalised to the Sham group (n = 3, pooled)......74

Figure 21. Heat map of epigenetic gene fold changes (ΔΔCt) in the hippocampus of Week 13 offspring in the Ecig(+nic) (n = 3, pooled) and Ecig(-nic) groups (n = 3, pooled) normalised to the Sham group (n = 3, pooled)......75

Figure 22. Real-time PCR verification of DNA methyltransferases (Dnmt3a and Dnmt3b) mRNA expression levels of offspring at postnatal day 1 (P1) whole brains, postnatal day 20 (P20) whole brains and Week 13 hippocampus......78

Figure 23. Real-time PCR verification of histone-lysine demethylases (Kdm5c and Kdm6b) mRNA expression levels of offspring at postnatal day 1 (P1) whole brains, postnatal day 20 (P20) whole brains and Week 13 hippocampus......80

Figure 24. Real-time PCR verification of histone acetyltransferase (Atf2) and histone deacetylase (Hdac1) mRNA expression levels in offspring at postnatal day 1 (P1) whole brains, postnatal day 20 (P20) whole brains and Week 13 hippocampus......82

Figure 25. Real-time PCR verification of histone phosphorylation (AurkA, AurkB, AurkC) mRNA expression levels of offspring at postnatal day 1 (P1) whole brains, postnatal day 20 (P20) whole brains and Week 13 hippocampus......84

Figure 26. Neuronal cell counts of pyramidal neuronal cells in the postnatal day 20 (P20) [n = 8] and Week 13 offspring [n = 8] dorsal hippocampus and the lateral amygdala nucleus.. 86

Figure 27. A Timeline summary of the animal experiments for Chapter 3......102

Figure 28. Schematic of the treatment groups for the animal experiment in Chapter 3...103

Figure 29. Image of the Scireq® InExpose system that was used to expose animals to tobacco cigarette smoke......106

Figure 30. Plasma cotinine levels of breeders and offspring at postnatal day 20 (P20)......110

Figure 31. Offspring whole body weight at (A) P1, (B) P20 and (C) Week 13 in the Sham, Smoke Exposure (SE) and Switch groups (n = 14)......111

Figure 32. Recognition index from the novel object recognition test in the Sham (n = 14), Smoke Exposure (SE) (n = 14) and the Switch group (n = 14)......113

Figure 33. The time spent in the open arm and the number of centre crosses performed by week 12 offspring in the elevated plus maze in the Sham, Smoke Exposure (SE) and Switch group groups (n = 8-14)......114

Figure 34. The total number of head dips and whole body stretches in the closed arm (protected) and open arm (unprotected) in offspring from the Sham (n = 12), Smoke Exposure (SE) (n = 10) and Switch groups (n = 14).. 115

Figure 35. Global DNA methylation in offspring brains at postnatal day 1 (P1), postnatal day 20 (P20) and postnatal Week 13. From the Sham, Smoke Exposure (SE) and Switch groups (n = 4-8).. 117

Figure 36. mRNA gene expression levels for DNA methyltransferases (Dnmt3a and Dnmt3b) in offspring at postnatal day 1 (P1) whole brain, postnatal day 20 (P20) whole brain and Week 13 hippocampus.. 119

Figure 37. mRNA gene expression levels for histone methyltransferases (Kdm5c and Kdm6b) in offspring at postnatal day 1 (P1) whole brains, postnatal day 20 (P20) whole brains and Week 13 hippocampus......121

Figure 38. mRNA gene expression levels for histone acetyltransferase (Atf2) and histone deacetylase (Hdac1) in offspring at postnatal day 1 (P1) whole brains, postnatal day 20 (P20) whole brains and Week 13 hippocampus.. 123

Figure 39. mRNA gene expression levels for histone phosphorylation (AurkA, AurkB and AurkC) in offspring at postnatal day 1 (P1) whole brains, postnatal day 20 (P20) whole brains and Week 13 hippocampus......125

Figure 40. Neuronal cell counts of pyramidal neuronal cells in the postnatal day 20 (P20) (n = 8/group) and Week 13 offspring (n = 8/group) dorsal hippocampus and the lateral amygdala nucleus.. 127

Figure 41. Schematic of in vivo and in vitro blood brain barrier illustrations. The in vivo blood brain barrier model shows the endothelial cells forming tight junctions.. 140

Figure 42. Timeline summary of the in vitro experiments for Chapter 4......145

Figure 43. A schematic of the set up to create the e-cigarette aerosol condensate.. 150

Figure 44. Fluorescein isothiocyanate (FITC) dextran and trans-endothelial electrical resistance (TEER) measurements after ECAC treatment on HBECs seeded in transwells. 154

Figure 45. Experimental set-up for the co-culture of HBEC cells with diff-SHSY5Y cells.. 156

Figure 46. Experimental set-up for the co-culture of HBEC cells with differentiated BV2 cells.. 158

Figure 47. E-cigarette aerosol condensate treatment of a co-culture of HBEC cells with BV2 cells.. 161

Figure 48. Validation of SHSY5Y cells treated with PMA (n = 3)......165

Figure 49. E-cigarette aerosol condensate treatment during SHSY5Y cell differentiation with PMA. (n = 3).....167

Figure 50. The MTT assay to measure cell viability of (A) diff-SHSY5Y cells, (B) BV2 cells and (C) HBEC, following exposure to e-cigarette aerosol condensate at dilutions of 1 in 12.5, 1 in 25, 1 in 50 and 1 in 100 (n = 4).. 170

Figure 51. The DCF assay to measure reactive oxygen species released from (A) diff-SHSY5Y cells, (B) BV2 cells and (C) HBEC, following exposure to e-cigarette aerosol condensate at dilutions of 1 in 200, 1 in 100 and 1 in 50 (n = 4).. 173

Figure 52. The JC-10 assay measures mitochondrial membrane depolarisation of (A) diff-SHSY5Y cells, (B) BV2 cells and (C) HBEC, following treatment with e-cigarette aerosol condensate at dilutions 1 in 200, 1 in 100 and 1 in 50 (n = 4).. 176

Figure 53. Permeability of tracer molecule FITC-Dextran through the human brain endothelial cell membrane treated with e-cigarette aerosol condensate with the appropriate controls (n = 3)...... 178

Figure 54. TEER measurements across the human brain endothelial cell (HBEC) monolayer over 11 days (n = 3).. 180

Figure 55. TEER measurements across the human brain endothelial cell (HBEC) monolayer over 11 days co-cultured with diff-SHSY5Y cells (n = 3).. 182

Figure 56. TEER measurements across the human brain endothelial cell (HBEC) monolayer over 11 days co-cultured with BV2 cells (n = 3).. 186

Figure 57. Schematic of the tri-culture using human brain microvascular endothelial cells (HBEC), glioblastoma (U87MG) cells, and differentiated neuroblastoma (diff-SHSY5Y) cells. 199

List of Tables

Table 1. List of epigenetic chromatin modification genes provided by the Mouse Epigenetic Chromatin Modification RT^{2}Profiler^{TM} PCR array. 55

Table 2. The cycling condition for cDNA synthesis according to the manufacturer's protocol......57

Table 3. List of epigenetic genes selected for RT-qPCR validation from the RT^{2}Profiler^{TM} PCR Array results......59

Table 4. Details of GAP43 gene to validate PMA-treated SHSY5Y cells using GAPDH as the reference gene......148

Table 5. E-cigarette aerosol condensate treatments for the study. 150

Table 6. PCR primer sequences for the in vitro experiments. 157

Table 7. PCR primer sequences for the in vitro experiments. 160

Table 8. PCR primer sequences for the in vivo experiments. 163

Table 9. mRNA expression levels of epigenetic and inflammatory genes in diff-SHSY5Y co-cultured with HBEC after e-cigarette aerosol condensate exposure (n=3)......184

Table 10. mRNA expression levels of inflammatory genes of BV2 cells co-cultured with HBEC after e-cigarette aerosol condensate exposure (n = 3)......188

Table 11. mRNA expression levels of epigenetic and inflammatory genes in diff-SHSY5Y cells treated with media from BV2 cells co-cultured with HBEC after e-cigarette aerosol condensate exposure (n = 3). 189

Table 12. mRNA expression levels of inflammatory genes in offspring brain at Postnatal day (P) 1, P20 and Week 13 in the Sham, PGVG+Flavour and PGVG+Flavour+nic groups (n = 3). 191

Table 13. A summary table of the results from Chapter 4......192

Abbreviations

ADHD:	Attention deficit hyperactive disorder
ANOVA:	Analysis of variance
BBB:	Blood brain barrier
BDNF:	Brain derived neurotrophic factor
BV2:	Microglial cells
CA:	Cornu ammonis
CNS:	Central nervous system
COPD:	Chronic obstructive pulmonary disease
CpG:	Cytosine-phosphate-guanine
C_T:	Threshold cycle
DCF:	2' 7'-Dichlorofluorescein
Diff-SHSY5Y:	Differentiated SHSY5Y
DMSO:	Dimethyl sulfoxide
E-cigarettes:	Electronic cigarettes
E-liquids:	Electronic liquids
ECAC:	E-cigarette aerosol condensate
ENDS:	Electronic nicotine delivery systems
EPM:	Elevated plus maze
EU:	European Union

FBS:	Foetal bovine serum
FDA:	Food and Drug Administration
FITC:	Fluorescein isothiocyanate
GABA:	Gamma-aminobutyric acid
GAP43:	Growth associated protein 43
GAPDH:	Glyceraldehyde-3-phosphate dehydrogenase
H_2O_2:	Hydrogen peroxide treatment
HBEC:	Human brain microvascular endothelial cells
IBA-1:	Ionised calcium-binding adaptor molecule -1
IL-:	Interleukin
iNOS:	Inducible nitric oxide synthase
nAChR:	Nicotinic acetyl-choline receptor
NEAA:	Non-essential amino acids
NGFR:	Nerve growth factor receptor
NNK:	4-Methylnitrosamino-1-(3-pyridyl)-1-butanone
NOR:	Novel object recognition
NOS2:	Nitric oxide synthase 2
NRT:	Nicotine replacement therapies
MCP-1:	Monocyte chemoattractant protein-1
MTT:	Methylthiazolydiphenyl-tetrazolium bromide

P1:	Postnatal day 1
P20:	Postnatal day 20
PBS:	Phosphate buffer with saline
PECAM-1:	Platelet endothelial cell adhesion molecule-1
PFA:	Paraformaldehyde
PMA:	Phorbol 12-myristate 13-acetate
ROI:	Region of interest
ROS:	Reactive oxygen species
SE:	Smoke exposure
SHSY5Y:	Neuroblastoma cells
TEER:	Trans-endothelial electrical resistance
TGA:	Therapeutics Goods Administration
THP-1:	Human monocyte leukaemia
TNF-α:	Tumour necrosis factor alpha
U87MG:	Glioblastoma
UK:	United Kingdom
US:	United States
VEGF-A:	Vascular endothelial growth factor-A
ZO-1:	Zonula occludens-1

Abstract

Electronic cigarettes (e-cigarettes) are battery-powered devices that convert an oily-flavoured liquid into an aerosol. E-cigarette liquids contain propylene glycol, glycerin, flavouring and varying concentrations of nicotine. Due to aggressive marketing, e-cigarettes are attractive to a number of vulnerable groups such as young people and pregnant women. It is perceived within these populations that e-cigarettes are a safer alternative to smoking tobacco cigarettes although there is limited evidence proving this.

In this thesis, Chapter 1 provides an extensive review on what is currently known about e-cigarettes within the literature. Chapter 2 describes a mouse pregnancy model of e-cigarette exposure and examines the offspring at three time-points; postnatal day 1 (right after birth), postnatal day 20 (right after weaning) and at week 13 (adulthood). Chapter 3 describes a pregnancy model of switching from tobacco cigarette to e-cigarette exposure during pregnancy. Behavioural assessments using the novel object recognition and the elevated plus maze tests were conducted in both Chapter 2 and 3 to determine changes to short-term memory, anxiety and exploration. In addition, epigenetic changes investigating DNA methylation and epigenetic gene expression on offspring brain were investigated. Finally, Chapter 4 investigated the effects of e-cigarette condensate on differentiated neuroblastoma cells (diff-SHSY5Y), microglial (BV2) cells and human brain endothelial cells (HBEC) in monoculture and in co-culture using a blood brain barrier (BBB) model.

The results showed that offspring from mothers exposed to e-cigarette aerosols with and without nicotine had significant changes to memory, anxiety, hyperactivity, DNA methylation and epigenetic gene expression compared to normal offspring. Continuous tobacco cigarette exposure showed significant effects on offspring behaviour and epigenetics, however, switching to e-cigarettes during pregnancy reduced some of these changes but not all to normal levels. In the cell culture experiments, e-cigarette exposure on diff-SHSY5Y, BV2 and HBEC showed reduced cell-

viability and an increase in oxidative stress in monoculture. In a co-culture model of the BBB, significant epigenetic gene changes were observed in diff-SHSY5Y cells after treatment with conditioned media from BV2 cells. All of these results are summarised in Chapter 5.

In summary, the *in vivo* experiments showed that neurological changes including behavioural and epigenetics occurred in the offspring after maternal e-cigarette exposure. The *in vitro* experiments showed that this may be due to a direct effect of e-cigarette constituents on neuronal cells, or through an indirect inflammatory response involving microglia. Overall, this study concluded that e-cigarettes are not safe to be used during pregnancy.

Chapter 1- Literature Review

1.1 Introduction to electronic cigarettes

Electronic cigarettes (e-cigarettes) are battery-powered devices that heat a flavoured liquid into an aerosol. This aerosol is then inhaled by the user in a process known as '*vaping*'. Electronic liquids (e-liquids) are comprised of two main base liquids, namely propylene glycol and glycerine (vegetable glycerin), a small concentration of flavouring agents and varying concentrations of nicotine. E-cigarettes delivering nicotine are also often referred to as an electronic nicotine delivery systems (ENDS). E-liquids can come in a wide range of sweet, fruity, tobacco and cannabis flavours that can be refillable so users can try more flavours easily, and therefore, adding to their marketability.

Ever since their introduction into the global market in 2004, the evolution of e-cigarettes have dramatically advanced over the years to give users a wider range of customisable options (Figure 1).

Figure 1. Several generations of e-cigarette devices, e-cigars, e-pipes and JUULs. Image taken and modified from the Food and Drug Administration website [1].

First generation e-cigarettes were thin and cylindrical in shape just like conventional tobacco cigarettes and were known as '*cigalikes*'. E-cigarettes consist of four main parts; a main device that holds the battery, a tank that holds the e-liquid, a coil that is super-heated to convert the e-liquid into an aerosol, and finally the mouthpiece (Figure 2). E-cigarettes have evolved overtime and has modified in device design, pricing and customisation [2-4]. Manufacturers have designed e-cigarettes that allow users to modify the heating coil, increased e-liquid holding volume and the power of the battery source [4]. This allow users to personalise their e-cigarettes for prolonged usage. The expansion of ENDS is increasing at an alarming rate, which raises concerns as to how the manufacturing process of e-cigarettes is being regulated and how they are distributed in society.

Figure 2. Image of the different parts in a typical e-cigarette. Image was modified from the King County government website [5].

1.2 Tobacco cigarettes vs. e-cigarettes

The reason why e-cigarettes were introduced into the market was to aid smokers to quit. Tobacco cigarette smoking is currently the leading cause of preventable deaths all over the world where over 7 million people die every year from tobacco-related diseases, and this is expected to rise [6, 7]. Tobacco smoking is estimated to result in 15,500 deaths every year in Australia and 480,000 deaths in the United States (US) [7, 8]. Smoking is known to be associated with a number of complications such as cardiovascular diseases, respiratory diseases, kidney complications, cancer and perinatal complications [7, 9-17]. In Australia, at least 1 in 10 people will develop a lung disease such as chronic obstructive pulmonary disease (COPD) and/or lung cancer [18]. Smoking tobacco cigarettes has been linked to diseases like lung cancer, where 90% of lung cancer in males and 65% of lung cancer in females has a direct link to tobacco smoking [19]. Although the number of smokers is high, there is a decline in the rate of smoking in Australia over the past two decades. According to the Australian Bureau of Statistics, the percentage of current smokers between the ages of 18-34 has gradually declined from 33.5% to 18.4% for males and 28.1% to 10.5% for females from 1995 to 2017-18 [20]. The reduction in the number of smokers over the past 20 years is evidence that Australia's quit smoking campaigns are helping smokers to quit and preventing new smokers from starting.

Cigarette smoke releases numerous toxic chemicals which include carcinogens like tar, formaldehyde, reactive oxygen species (ROS) and acrolein [21, 22]. Originally, e-cigarettes were invented to assist smokers to quit smoking tobacco cigarettes. Therefore, the device was designed to mimic the shape of a cigarette, and thus, mimic the behavioural habit that accompanies smoking. E-cigarettes have been promoted as a safer alternative to smoking tobacco cigarettes since they require no combustion of tobacco and are made of as few as four ingredients. In addition, e-cigarettes are also made attractive by being cost effective and currently there are over 7500 different flavours of e-liquids on the market [23]. Instead of burning tobacco, e-cigarettes use a metal coil that superheats to convert the oily-flavoured liquid into an aerosol. Although e-cigarettes were branded as a non-combustible product, harmful substances such as carbon

monoxide, acrolein, aldehydes, volatile organic carbons, polycyclic aromatic hydrocarbons, heavy metals and tobacco-specific nitrosamines were reported to be found in e-cigarette aerosols [24-28].

E-cigarettes are more versatile than tobacco cigarettes. The evolution of e-cigarettes allow users to custom-design their own devices. This could include creating personal coils to accommodate each individual's vaping preference. In addition, users could create their own e-liquid with varying concentrations of propylene glycol, glycerine or nicotine to introduce more 'clouds' of aerosols or to get a stronger hit of nicotine depending on the user's preference [29-31]. Puff topography of e-cigarette users are specific to each user. Some factors that can affect the e-cigarette puff topography include, but not limited to e-cigarette device design (coil design and power setting) [32], user's cigarette smoking behaviour (whether they are heavy or light smokers) [33], and flavouring of e-liquids [34]. Overall, although e-cigarettes were originally designed to mimic tobacco cigarettes to aid cessation in current cigarette smokers, e-cigarettes have also been designed for recreational use to produce larger clouds of aerosols, this sort of e-cigarette behaviour has been coined the term '*cloud chasers'*.

1.3 E-cigarette as a cessation aid for tobacco smokers

Difficulties in quitting smoking are due to a number of factors. These include established behavioural habits such as bringing a cigarette towards the mouth and drawing from the cigarette, smoking-associated social activities and nicotine addiction. Although nicotine has a highly addictive effect, excessive nicotine intake can cause a number of symptoms such as dizziness, vomiting and palpitations [35, 36]. Smokers who are in the early stages of quitting find it highly difficult to remain smoke-free since they become more anxious, moody and stressed [37, 38]. A combination of behavioural support and nicotine replacement therapies (NRT) such as nicotine patches, nicotine gum, nicotine lozenges and nicotine mouth sprays have been proven to help smokers quit [39-41]. However, some users experience mild to severe side effects when using

NRT such as nausea, vomiting, heart complications, skin irritation and insomnia [42]. Therefore, the introduction of e-cigarettes can be seen as a more favourable alternative to NRT as they can assist with the delivery of nicotine in addition to the behavioural addiction of drawing a cigarette to the mouth.

A number of studies have investigated the effectiveness of using e-cigarettes and smoking cessation. Brady and colleagues did a 7-month follow up on 2204 callers using a Quitline and found no correlation between e-cigarette use and smoking cessation [43]. In contrast, Weaver and colleagues did a one-year follow up study on 858 adult smokers (between 2015-2016) and found no association between ENDs use and the rate of quitting in these participants [44]. Recently, Farsalinos and Niaura investigated quitting duration in current smokers (n = 9935) and former smokers (n =14,754) by analysing the 2016 and 2017 National Health Interview surveys. They found that the daily use of e-cigarettes showed a high correlation with smoking cessation [45]. In terms of quitting in pregnant women, Chiang and colleagues found no correlation between e-cigarette use during pregnancy and quitting outcomes [46]. There are inconsistencies within the literature as to whether e-cigarette can be used as a quitting aid for current smokers (including pregnant women), and urgent studies are needed to determine the risks and benefits involved when they are used in vulnerable populations.

The evolution of e-cigarettes has drastically changed over the years, providing users with a more powerful device that can increase temperature and wattage use with a longer battery life [47]. E-cigarette use as a quitting aid for smoking is still a controversial topic since there is new evidence arising on the harmful effects of e-cigarette use on systematic organs such as the heart, liver, lung and the brain in murine models [48-55]. Moreover, the impacts of using e-cigarettes in vulnerable groups such as young people and pregnant mothers have not been widely investigated and this needs to be further addressed.

1.4 Policy around e-cigarettes

E-cigarettes are closely associated with tobacco cigarettes giving that it can deliver nicotine. With the growing use of e-cigarettes in young people [56-58] and adults [59-62], regulation around the manufacture and distribution of e-cigarettes has become more important to control. The regulation of e-cigarettes are different throughout many countries [63] (Figure 3). A summary of the global status of e-cigarettes are summarised in the No Fire, No Smoke, Global State of Tobacco Harm Reduction Report [64]. Countries such as Thailand, Singapore, Brazil and Uruguay have completely banned the use, distribution and manufacturing of e-cigarettes in their countries. Countries such as the US and Canada have placed some regulation around the use of e-cigarettes such as age limit restrictions and packaging requirements and this is dependent on each state. Countries such as the United Kingdom (UK) and New Zealand, however, have a more positive outlook on e-cigarettes compared to countries like the US and Canada. Currently, the policy makers from the UK and New Zealand are making future plans for a tobacco-free population which involves utilising ENDS products such as e-cigarettes. This section will discuss e-cigarette regulations in Australia, the US, the UK and New Zealand.

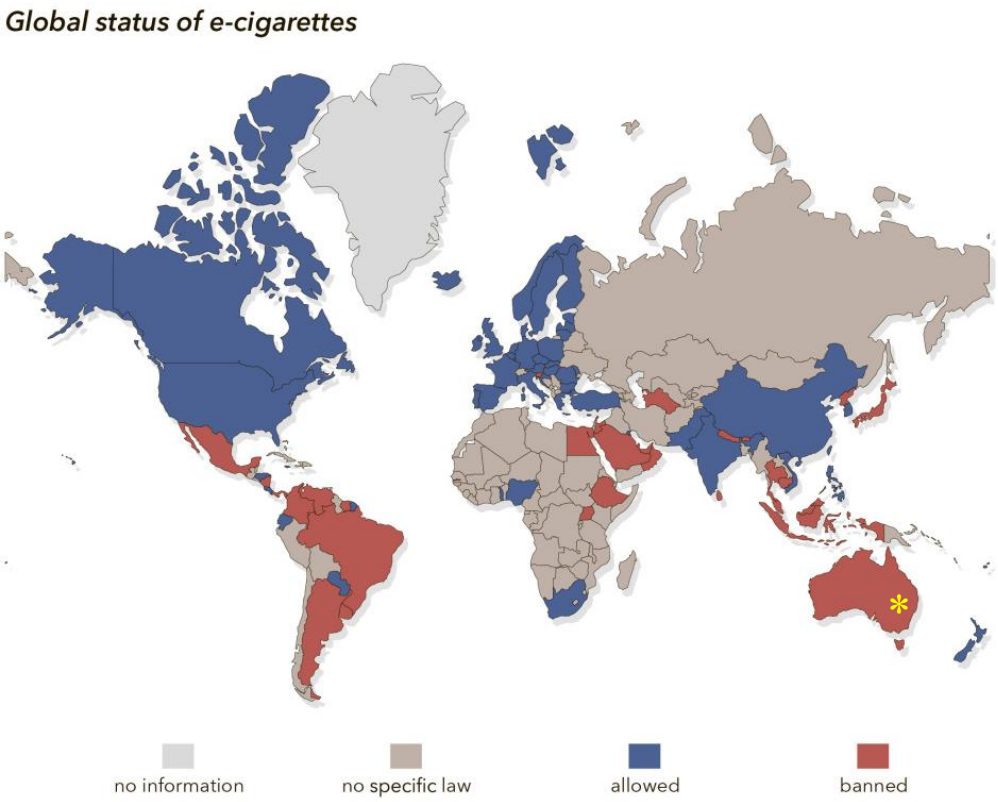

Figure 3. A map of the global status of e-cigarette regulation around the world. * = Refers to the banning of e-cigarettes with nicotine only i.e. e-cigarettes without nicotine could still be used. Images was modified from the No fire, No smoke, global State of tobacco Harm reduction report [64].

The introduction of e-cigarettes was somewhat delayed in Australia compared to other countries such as the US and the UK. During Australia's early exposure to e-cigarettes, laws regulating the advertisement, distribution or manufacturing of e-cigarettes were unclear. As the use of e-cigarettes exponentially increased in Australia, stricter laws were put in place to better regulate e-cigarettes. In all states of Australia, it is now illegal to display, advertise and promote e-cigarette devices and accessories. It is illegal to sell e-cigarette devices and accessories to children under the age of 18, have e-cigarettes displayed at the point of sale, and make health claims about e-cigarette use such as promoting e-cigarettes as a tool to quit smoking [65-72]. All of these restrictions are in place under the Public Health (Tobacco) Act 2008 (New South Wales), the Tobacco and E-cigarette Products Regulation 2019 (South Australia), and the Tobacco and Other

Smoking Products Act 1998 (Tobacco Act) (Queensland). Nicotine products associated with e-cigarettes such as liquid nicotine is classified as a 'Dangerous Poison' under the federal Poisons Standards [73, 74], and therefore, are regulated differently to e-cigarette devices and their accessories. Under federal law, it is illegal to obtain, purchase, distribute or manufacture liquid nicotine in Australia. The only NRT products approved by the Therapeutics Goods Administration (TGA) such as nicotine patches, gum, lozenges or mouth sprays are considered to be a Schedule 4 Prescription Only Medicine classification and can be marketed as a cessation aid to quit smoking [73, 75]. Since e-cigarettes contains nicotine, this means that the regulation of e-cigarettes falls under tobacco regulation, and therefore, the Food and Drug Administration (FDA).

Due to the dramatic increase in e-cigarette sales and use in the US, the FDA have issued a final rule to categorise "smokeless tobacco" devices which includes all ENDS and e-cigarette devices as tobacco products under the Food, Drug, and Cosmetic Act [76]. This gives FDA authorities the power to regulate the maintenance, distribution and selling of e-cigarettes in the US [76]. As of March 2019, all US states, except for Pennsylvania and Michigan, have laws that restrict the distribution of e-cigarette products to youths (Under 18 or 21 depending on each state). Moreover, over 26 US states (e.g. California, New York, Texas) require a level of e-cigarette packaging requirement and 20 US states (e.g. California, Louisiana, Washington) have State laws that require a license to sell e-cigarette devices and accessories along with strict product packaging. In addition, as of January 2020, the FDA has started a priority enforcement on flavour bans on ENDS products (except for menthol and tobacco flavours) without pre-market authorisation [77, 78]. This enforcement aims to reduce the number of youths in the US trying e-cigarettes since the majority of young people (93.2%) who tried an ENDS product for the first time was flavoured [77, 79].

The UK government have pushed the benefits of using e-cigarettes as a cessation tool for smokers wanting to quit. With this positive outlook on e-cigarettes, laws have been created to control and standardise the products associated with e-cigarettes. The Tobacco Products Directive 2014/14/EU outline guidelines for new distributors on the legal requirements for selling e-

cigarette products. This law enforces restrictions on the volume of nicotine-containing e-liquid i.e. e-cigarette tanks can hold up to a maximum of 2 mL of e-liquid and e-liquid cartridges can hold up to 10 mL of e-liquid only [80]. It is important to note that The Tobacco Products Directive 2014/14/EU only applies to the UK as long as the UK is part of the EU. In addition, the UK is the first country to enforce laws that control certain ingredients in e-liquids, e.g. e-liquids are not allowed to contain caffeine or colouring [80]. The UK government has developed a report known as the 'Towards a Smoke Free Generation – A Tobacco Control Plan for England 2017-2022' [81]. This report extensively addresses the key plans to reduce the rate of smoking in individuals including pregnant women in England as well as a smoking prevention plan to achieve a 'smoke free generation' [81]. Evidence on whether e-cigarettes are effective long-term solutions for quitting smoking was compiled by West and colleagues, and Beard and colleagues [82, 83]. These two studies, taken together, have estimated that out of the number of current smokers, approximately 22,000 individuals have successfully stopped smoking with the help of e-cigarettes [84]. Although this data was collected in 2014, there are positive opinions that this quit rate could get higher over the coming years.

New Zealand are also moving towards legislating e-cigarettes under their tobacco laws [85]. The New Zealand government aims to move the population of New Zealand to a "smoke-free" country by 2025 [86]. The Medicines Act 1981 regulates the selling of e-cigarettes and accessories (including liquid nicotine). In addition, the Smoke free Environments Act 1990 bans smoking and vaping indoors, bans sales to minors and bans the advertisement of e-cigarettes. However, there are no specific laws that regulate the quality and safety of e-cigarettes. Legislation on the selling of e-cigarettes and accessories (including liquid nicotine) has been implemented. In addition, the Ministry of Health in New Zealand are currently discussing plans of banning all flavours of ENDS product except mint, menthol and tobacco [87]. This announcement of flavour bans came after the death of six people in the US who used THC-flavoured ENDs products [88].

Overall, the effectiveness of e-cigarettes for smoking cessation remains controversial, however, there are now stricter laws that are specifically made for e-cigarettes in countries that deem them

as useful for smoking cessation. Countries like New Zealand and the UK have been 'pro' e-cigarettes since there are evidence that showed e-cigarettes being more effective for smoking cessation compared to NRT [89].

1.5 Epidemiology of e-cigarette use in society

Since their introduction in the US market, the sales of ENDS have skyrocketed from $775 million in 2015 to $1.3 billion in 2017 [90] (Figure 4). With the entry onto the market of new e-cigarette companies such as JUUL, NJOY Inc., Imperial Tobacco and Japanese Tobacco Inc., there has been an exponential increase in e-cigarette sales [90]. E-cigarette use in society remains a controversial issue [44, 86, 91-93]. The perception of e-cigarettes, especially in vulnerable populations like young people and pregnant women, is that e-cigarettes cause less harm than tobacco cigarettes, even though not much scientific evidence is available to back up the claim [57, 91-93]. It is important to investigate the percentage of e-cigarette users in different age populations and identify the groups in society who are more susceptible to using e-cigarettes since, it is too soon to understand the long-term health effects of e-cigarette use. This section reviews the vaping status among adults, young people and pregnant women.

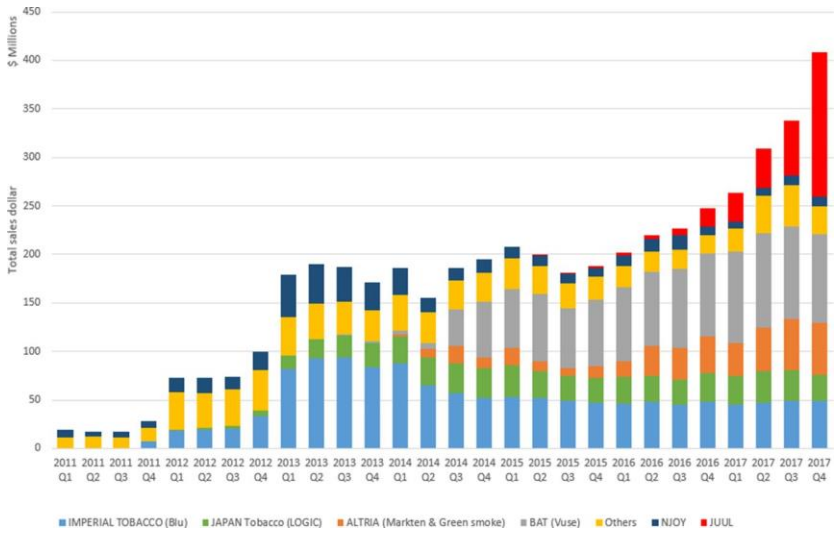

Figure 4. Total sales of electronic nicotine delivery systems for each quarter of the year between 2011-2017. Graph was taken from Huang et al 2019 [90].

1.5.1 Adults

E-cigarettes have not been as popular in Australia compared to the US or in European countries such as the UK, however, the awareness of the existence of e-cigarettes and their use has been gradually increasing, especially in adults. A report in 2013 has investigated the awareness of e-cigarettes in four different nations including Australia [59]. The awareness of e-cigarettes in Australia was 20% which was the lowest percentage compared to the UK, US and Canada, which were at 73%, 54% and 40%, respectively [59]. However, Adkison and colleagues have found that e-cigarette use was the highest among daily smokers [59]. In Australia, a cross-sectional study in 2016 by Twyman and colleagues discovered that there was an association between smokers from low socio-economic background who tried e-cigarettes and the belief that vaping was safer than smoking tobacco cigarettes [61]. In addition, another cross sectional study in 2018 by Twyman and colleagues found that over half the number of adult participants who took the survey uses e-cigarettes to quit smoking [62] This suggests that smokers are choosing e-cigarettes to quit smoking or to maintain their nicotine dosage. Although switching to e-cigarettes may help people to quit smoking, they may not stop people from 'quitting' nicotine. There have been studies that investigated the success rates of smokers switching to e-cigarettes and more importantly, the rates of people quitting e-cigarettes completely after they have weaned off tobacco cigarettes. Hajek and colleagues showed that using e-cigarette were more effective than using NRT [89]. Russel and colleagues collated advice about quitting smoking from 4912 people who have stopped smoking by switching to e-cigarettes [94]. The overall general advice from these former smokers was choose the appropriate e-cigarette device, flavours and nicotine strength that suits them and the continue use of tobacco cigarettes while using e-cigarettes was considered part of the process for quitting completely [94]. In contrast, studies in New Zealand showed no significant quitting attempts in smokers who used e-cigarettes [95, 96]. Although the quit rates were taken at 6 months for both studies, which may not be enough time for former smokers to completely quit.

While the rate of e-cigarette use is low in Australia, the use of e-cigarettes is steadily rising. A study that analysed the 2016 National Drug Strategy Household Survey in Australia found that

there are 227,000 Australian adults (over 18 years of age) who are current users of e-cigarettes [60]. In addition, daily e-cigarette users were comprised of mainly former smokers (38%), current smokers (32%) and never smokers (18%) [60]. Indigenous people in Australia are more likely to use e-cigarettes compared to non-indigenous people [60]. Moreover, online threads about e-cigarette use and people with a mental illness showed both positive (such as quit smoking and online community connection) and negative attributes (such as nicotine addiction and unsatisfactory substitute) of using e-cigarettes [97]. The rate of smoking in Australia has been steadily declining in both men and women [98]. This could suggest that smokers who are transitioning to using e-cigarettes are attempting to quit smoking, even though distribution of e-liquids containing nicotine is illegal in Australia.

In the US, there is an increase in the number of adults that are ever-users of e-cigarettes. A study that was conducted between 2014-2016 revealed that there was a significant increase in former smokers and never-smokers that are using e-cigarettes by 12.6% in 2014, 13.9% in 2015 and 15.3% in 2016 [99]. Moreover, e-cigarettes are the third highest tobacco product that are used by adults in the US after tobacco cigarettes and cigars [100]. In 2017, there were 6.9 million adults using e-cigarettes in the US [100]. However, the use of e-cigarettes among former smokers suggest that adult smokers are changing over from smoking tobacco cigarettes to using e-cigarettes. Moreover, there is a concern that there are adults who have never-smoked that are currently using e-cigarettes, even though e-cigarette aerosols can release harmful chemicals such as volatile organic compounds, polycyclic aromatic hydrocarbons and nicotine [101]. Interestingly, 39% of adults who are attempting to quit smoking are choosing e-cigarettes over FDA approved NRT such as the nicotine patch and nicotine gums (25%) [102]. A follow-up investigation from this research is needed to investigate the effectiveness of using e-cigarettes as a cessation tool for cigarette smoking.

In the UK, 5.5% of the population are current e-cigarette users, this equates to 2.8 million people. The number of adults that have ever-used an e-cigarette have increased between 2014-2017 [103]. The Office for National Statistics in the UK have reported the highest use of e-cigarette used in

men and women aged between 25-34 [103]. In addition, the percentage of adults in this age bracket who have ever-used an e-cigarette has risen from 22.2% in 2014 to 29.5% in 2017. Moreover, current users of e-cigarettes have also increased from 5.3% in 2014 to 7.3% in 2017 [103].

1.5.2 Young people

Another demographic of e-cigarette users are young people. E-cigarette awareness, perceptions and use are different in young people aged between 14-25 compared to adults. Adults who are over 25 are more likely to use e-cigarettes as a way to quit smoking, but there may be other motivations as to why younger people may choose to vape. In the UK, a study that investigated the motivation and reasons why young people vape have found that 28% of young people use e-cigarettes as a cessation aid for tobacco smoking [57]. Other motivations include the variety of flavours that are available on the market and the ability to manipulate the aerosols and perform 'tricks'. Such tricks are commonly known as *cloud chasing* [57]. The percentage of young people smoking in Australia has gradually declined to 16% [98]. Similar trends have been found in the US and the UK [56, 104]. In contrast, popularity and awareness of e-cigarettes have increased in major countries such as Australia, the UK and the US [105-107]. In Australia, young people are in support of placing more restrictions on the availability of e-cigarettes, however, they oppose the complete ban of e-cigarettes in society and its use in smoke-free zones [108].

There is a high prevalence of young people who perceive there is a lower risk of nicotine addiction arising from vaping compared to tobacco smoking. This raises concern that e-cigarette use may lead to a new generation of nicotine addicts or could be a gate-way to smoking tobacco cigarettes [109]. In the US, the most common tobacco products used by middle and high school students are e-cigarettes [56]. Currently, there are a total of 4.9 million middle and high school student using e-cigarettes with the highest use found among high school students [56, 110]. The use of e-cigarettes in high school students has significantly increased from 1.5% in 2011 to 11.3% in 2016

[56]. In contrast, the use of combustible-base tobacco products such as cigarettes, cigars, hookahs, and pipe tobacco has significantly declined from 21.8% in 2011 to 13.8% in 2016 [56].

Multiple studies in the US have shown that there is a correlation between the use of e-cigarettes in the younger generation and smoking initiation [58, 111, 112]. In the UK, one study conducted a 6-month follow-up study in young people aged between 11-18 about smoking and e-cigarette use and found that the percentage of young people who have ever-used an e-cigarette almost doubled after the follow up [113]. No such studies on e-cigarette use and smoking initiation have been conducted in Australia and this may be due to the low percentage of young users. However, seeing that young people in the UK and the US are more likely to smoke after using e-cigarettes, we can speculate that young people in Australia may take the same path. Overall, e-cigarette use in young people is becoming a gateway for smoking tobacco cigarettes. With no long-term data on e-cigarette use and the potential health effects in the second generation from the young e-cigarette users, this can be a major concern. Further research is required to determine the causality of this.

1.5.3 Women of child-bearing age and pregnant woman

Another vulnerable population in society who are using e-cigarettes are women of child-bearing age (20-35 years old) and pregnant women. The perception of e-cigarettes in pregnant women has changed drastically over the past 6 years. In 2013, women perceived ENDS products as a better option than using NRT, describing ENDS as ‘cost-effective’ with ‘fewer health effects’ and NRT as ‘ineffective’ with ‘undesired side effects’ [114]. Currently, many pregnant women still perceive e-cigarettes to be a safer alternative to smoking and a potential cessation aid during pregnancy [115-118]. This perception in conjunction with the aggressive marketing approach by the e-cigarette companies has led to an increase in the number of pregnant women using e-cigarettes [119]. A study that investigated e-cigarette and tobacco cigarette use in 194 women (pregnant and non-pregnant) who were current or former smokers, found that women who use e-

cigarettes are also heavy smokers [118]. In addition, half of the women within this study who are users of e-cigarettes were also pregnant [118]. A study conducted on 3277 women in Oklahoma who had a live-birth in 2015 found that 10.3% of mothers used an e-cigarette product with 7.0% using an e-cigarette product during pregnancy [120]. Moreover, 45.2% of these women chose to use e-cigarettes during their pregnancy because they perceived them to be less harmful than smoking cigarettes [120]. Bowker and colleagues found that women from the UK who smoked chose to switch to e-cigarettes once they have discovered that they were pregnant [115].

There is a great deal of uncertainty by medical practitioners on the use of e-cigarettes during pregnancy. A study surveying Obstetricians and Gynaecologists from the US have reported that only 5% felt that they were well-informed about the health risks of using e-cigarettes during pregnancy [121]. In addition, 14% reported that e-cigarettes poses no health risk, 39% believed that e-cigarette use is safer than smoking tobacco cigarettes and 14% believes the health risks are the same as smoking cigarettes [121].

Overall, maternal vaping has become a growing public health concern. With little consensus on the health risk e-cigarette poses in pregnant women from medical professionals, research is urgently needed to assess the potential risk and health implications of e-cigarette use during pregnancy.

1.6 Electronic liquids used in e-cigarettes

One major concern about e-cigarettes is that there is a lack of research that investigates the effects of aerosolised e-liquid constituents. E-liquids are the substrate for the thick white clouds of aerosols formed when someone uses an e-cigarette. The ingredients that makes up and e-liquid includes varying concentrations of propylene glycol, glycerin (vegetable glycerin), chemical flavouring and different concentrations of nicotine ranging from 0 mg/mL (no nicotine) up to 36 mg/mL. Other ENDS on the market such as JUUL, nicotine concentrations can be as high as 50 mg/mL [122]. Most of the ingredients that make up an e-liquid such as propylene glycol and

glycerine were approved by the FDA to be ingested. However, this does not necessarily mean they are safe to be inhaled.

1.6.1 Propylene glycol and glycerine

Propylene glycol is widely used in the food and cosmetic industry. It is found in cosmetics, alcoholic beverages, dairy products and in seasonings which are consumed regularly [123]. Propylene glycol has become increasingly popular over the years and has become one of the main ingredients in e-liquids.

On the other hand, glycerine is a natural, oily-liquid that is widely used in the food and beauty industry. It is a natural sweetener and has a higher viscosity than propylene glycol, hence, it is the other main ingredient used in e-liquids. Due to its higher viscosity, a high ratio of glycerine, compared to propylene glycol, allow users to produce larger clouds of aerosols while propylene glycol is used to mimic the so-called 'throat hit', which conventional tobacco cigarette users prefer when smoking. Propylene glycol and glycerine serves as a *humectant* i.e. a substance that provides moisture and carries the flavouring from the e-cigarette. In addition, propylene glycol is found in the filter of tobacco cigarettes where it also carries out the same purpose [124]. In addition, propylene glycol carries the flavour within the e-liquid more efficiently compared to glycerin. Overall, propylene glycol and glycerine can be adjusted at different ratios to provide users with different levels of throat sensations and aerosol volume. However, the safety and efficacy of inhaling propylene glycol and glycerine still needs to be elucidated.

1.6.1.1 Safety of inhaling propylene glycol and glycerin

Although propylene glycol and glycerine are considered safe to be consumed by the FDA [125], the safety of propylene glycol and glycerine when heated and vaporised is not extensively known. A number of researchers have suggested that contents that can be consumed may not be suitable for constant inhalation [25, 126, 127]. There is a rise in the number of studies that have investigated the safety of propylene glycol and glycerine (with or without nicotine) *in vitro* [128, 129] and *in vivo* studies [130-136]. An extensive search by Hua and colleagues on a number of e-cigarette forums have found that the main symptoms that were present after inhaling the aerosols included throat irritation, dizziness and a dry mouth [137]. Moreover, human subjects that were exposed to propylene glycol mists in a chamber for a minute caused throat and ocular symptoms [138], although the aerosol delivery method is more of a passive exposure rather than a deep inhalation that is typically delivered from an e-cigarette. Studies are limited on the effects of glycerine inhalation *in vivo* or *in vitro*. However, there has been a case report of a 42-year-old woman developing lipoid pneumonia from using e-cigarettes [139].

In addition, animal studies have been conducted to investigate the effects of propylene glycol in rats, monkeys and nematodes [140, 141]. Although no major physiological effects of propylene glycol aerosols were found in rats and monkeys, an increase in oxidative stress was found in nematodes [140]. In addition, Phillips and colleagues exposed Sprague-Dawley rats to propylene glycol and glycerine aerosols with and without nicotine have shown no adverse systematic effects [142]. In contrast, Lau and colleagues injected propylene glycol in mice and shown an increase in apoptosis in developing brain [143]. E-cigarette aerosol exposure containing only propylene glycol and glycerine showed alterations in circadian molecular clock genes in mouse lung tissues [132]. However, it is important to note that the inconsistencies in each study are due to different administration and percentage of propylene glycol and glycerine. Recent studies have used other forms of propylene glycol and glycerine mixtures such as liquid distillates [140], vapour inhalation [141] or oral administration [141]. The effects of propylene glycol aerosols have been

extensively reported dating back to the 70's when the effects of propylene glycol aerosols were compared to the ingestion of ethylene glycol which has been known to cause renal failure and excessive build-up of toxic metabolites in the liver [144]. Propylene glycol, compared to ethylene glycol, is the less harmful option as a food additive, however, no longitudinal studies of excessive propylene glycol vaping from e-cigarettes have been reported.

The effects on different cultured cell lines have been reported [125, 127, 145-147]. Cervellati and colleagues [145] have found no morphological cytotoxicity when skin cells and lung cells were exposed to the humectants but there were significant increases in pro-inflammatory cytokines like interleukin (IL-) 8 and IL-12 suggesting that there may be an underlying inflammatory response. Although the result shows that there is an effect on the skin and lung cells, the study does not clearly define what ratio of propylene glycol to glycerine was applied to experimental cells. Human lung fibroblasts treated with either propylene glycol, glycerine, 0mg/mL nicotine or 24mg/mL nicotine have shown to cause morphological changes such as cell enlargement and hyper-vacuolisation [147].

In contrast, some studies have shown no cytotoxicity in skin, lung and heart cells when exposed to humectants at different concentrations [126, 145]. The parameters for each study, such as the ratio of propylene glycol to glycerine, e-cigarette device and voltage level that was used were not mentioned. This makes comparing studies difficult since there is no standardisation in delivery methodology of ENDS products. Aerosols with a higher ratio of propylene glycol have been shown to create more carbonyl compounds when vaped at a higher voltage level of up to 4.8 volts [148]. In addition, Kosmider and colleagues found 8 out of the 13 e-liquids tested contained acetaldehyde and formaldehyde [148]. Therefore, vaping different ratios of propylene glycol and glycerine at different voltages have the potential to release toxic chemicals. One study that investigated the treatment of varying ratios of propylene glycol and glycerine in mice has shown significant changes to genes associated with the circadian molecular clock in the lung, kidney, skeletal muscle and brain [132]. In addition, one study investigated no-flavoured e-cigarette aerosols, with and without nicotine, on offspring from postnatal day 1 to postnatal day 10 and

found a decrease in the proliferation marker, *Ki67*, indicating an impaired lung growth in infant mice [134]. Therefore, more studies are needed to investigate the safety of inhaling propylene glycol and glycerine since there is not much in the literature to prove that it is safe to inhale, especially if they are used in combination with flavourings and nicotine which are found in most e-liquids.

1.6.2 Nicotine

Nicotine is another main ingredient that is found in e-cigarettes. Unlike tobacco cigarettes, e-liquid packaging contains the nicotine concentration on the e-liquid cartridge. However, a number of studies have shown that the amount of nicotine can be significantly higher than what is specified on the label [27, 149-153]. Moreover, 25 out of 41 different flavoured e-liquids labelled with 0 mg/mL nicotine were revealed to have trace amounts of nicotine in the liquid ranging from 5-330 μg/g [151]. Recently, Jackson and colleagues assayed a number of e-liquids with low concentrations of nicotine and discovered that the majority of the e-liquids did not have the proper concentration of nicotine labelled [154]. In addition, analysis of nicotine in e-liquids by Chun and colleagues discovered that there were inconsistencies with nicotine concentrations in each bottle of e-liquid as well as nicotine-free e-liquids having low levels on nicotine in them [155]. Recently, Chivers and colleagues analysed e-liquids that claimed to be nicotine free, and found that some of the e-liquids tested to have nicotine levels of 1.3-2.9 mg/mL [156]. This is a great concern since many e-cigarette users use e-liquids without nicotine with the expectation that they won't get any nicotine in their aerosols. This may create an unintentional addiction to e-cigarettes by the accidental inhalation of nicotine. Stricter regulation on the production and manufacturing of e-liquids may avoid this problem.

Nicotine is absorbed in the lungs when a person uses an e-cigarette, similar to how nicotine is absorbed from smoking conventional tobacco cigarettes. Nicotine can also be absorbed readily through skin contact [157]. This means that people who are in contact with liquid nicotine have a higher likelihood of getting nicotine poisoning if the e-liquid is not handled or stored properly. An overdose in nicotine can lead to vomiting, dizziness, nausea and in some cases death [158, 159]. Lethal doses of nicotine vary between children and adults. The lethal dose of nicotine for children and adults are approximately 10 mg/mL and between 30-60 mg/mL, respectively [160]. Therefore, it takes only one e-liquid containing 10 mg/mL of nicotine to induce nicotine poisoning in children. In Australia, between 2009-2016, there were almost 80 cases of nicotine poisoning in children reported to the Australia Poison Information Centre [161]. In 2019, a toddler died from

nicotine poisoning in Victoria, Australia [162]. In addition, nicotine poisoning has also been reported in a number of children, as well as four cases of attempted suicides in adolescents and adults [133, 159, 163, 164]. It is hazardous for children to come into close contact with e-liquid especially if the concentration of liquid nicotine in e-liquid can be high as 36 mg/mL.

Although selling and obtaining liquid nicotine is considered illegal in Australia under federal law, it is legal to import e-liquids containing nicotine under the TGA Personal Importation Scheme for an individual's own personal use [165]. However, there is no guarantee about the quality and safety of the products being imported. What is more concerning is that a recent study that had tested 94 e-liquids from different brands and stores have found that 65% of e-liquid packaging were not child-resistant [166]. The Australian government may need to consider regulating importation laws for e-liquids that include a limit to the amount of e-liquid that can be imported, better protective packaging to avoid accidental breakage and leakage, and prevent unauthorised selling of e-liquids to minors.

Some studies showed the effects of nicotine are not always detrimental. Nicotine itself, while considered addictive, and usually associated with damage caused by smoking, may not have such a direct adverse effect on individual cells as generally perceived. Nicotine has been shown to have anti-inflammatory effects [167, 168]. This suggests that any inflammatory responses from exposure to e-cigarette aerosol elements may be masked if they contain nicotine. A study that compared cigarette smoke extracts and nicotine metabolites showed an increase in activation of neutrophils in the cigarette smoke extract that is independent of nicotine metabolites [169]. Similar results have been found in a number of studies showing non-nicotine-containing e-liquids having a greater effect than nicotine-containing e-liquids, suggesting that nicotine is not the only ingredient that is causing negative effects *in vivo* and *in vitro* [127, 170, 171].

1.6.3 Flavouring in e-cigarettes

Under the Family Smoking Prevention and Tobacco Control Act 2009, the US has prohibited the addition of flavourings in tobacco cigarettes except menthol flavour to avoid younger people being attracted to sweet flavoured cigarettes [172]. No laws in any nation, except for the UK, have any regulation around chemical constituents that makes up the flavouring compounds in e-liquids. This may result in harmful chemicals being added into the flavouring which can be inhaled by the user. As of 2015, there are over 7500 different e-liquid flavours (with and without nicotine) available on the market [173]. E-liquid flavours can range from different blends of tobacco flavours to sweet and fruity flavours such as butterscotch, chocolate and apple. With so many e-liquids available on the market, only a small percentage of the flavours are tobacco [174]. Thus, the large selection of different flavoured e-liquids other than tobacco, such as dessert and fruity flavours, are likely to attract the younger populations such as school children and teenagers [175]. Muthumalage and colleagues investigated the toxicity of flavourings used in e-cigarettes such as diacetyl, cinnamaldehyde, acetoin and o-vanillin on human monocytic cell lines and elevated secretion of IL-8 and ROS [176]. What was interesting was different combinations of e-liquid flavours such as strawberry zing, pineapple coconut, cinnamon roll and American tobacco, taken together, had a more marked effect on cell toxicity [176]. Some flavourings have been known to be harmful when inhaled and have been reported to be an ingredient in e-liquids. An example of this is the chemical called diacetyl which has been used as a flavouring in popcorn factories that resulted in factory workers developing *bronchiolitis obliterans* [177]. Clapp and colleagues have shown that e-cigarette liquids containing cinnamaldehyde showed macrophage phagocytosis impairment in the lungs of female mice [178]. Hua and colleagues have showed also showed e-liquids containing ethyl maltol exhibited high cytotoxicity in murine neural stem cells and human bronchial epithelial cells [179]. These studies suggest that e-cigarette users who are experimenting with a variety of flavours available on the market could potentially have a higher risk of adverse effects, and thus, requires further toxicological evaluation.

There have also been reports that certain flavoured e-liquids have an effect on cell toxicity *in vitro* [126, 145, 147, 179-182]. This is further discussed in **section 1.7.2**.

1.7 In vitro and in vivo studies of e-cigarettes

1.7.1 *In vivo* animal models of e-cigarette exposure

1.7.1.1 E-cigarette exposure in normal animals

Over the last three years, there has been an increase in the number of studies investigating the effects of e-cigarette exposure in animal models. Animal experiments currently in the literature focuses on the effects of e-cigarettes in the lung [48, 131-133, 147, 183-185], the heart [130, 135, 186, 187], the brain [49, 52, 188], the kidney [130, 189, 190], liver [130, 191] and the male reproductive system [49, 192].

The majority of murine studies that investigated e-cigarette exposure were conducted on the lungs. The lungs are the first surface that reacts to the e-cigarette aerosols. Although there are many studies investigating the effects of e-cigarettes in the lungs, the delivery and type of e-cigarette exposure differs in each study. Garcios-Arcos and colleagues exposed mice to nicotine-containing e-cigarette aerosol without flavouring and showed changes to mouse lung reactivity with enlargement of alveoli space and an increase mucin production [131]. However, no changes were seen in e-cigarette aerosols without nicotine [131]. Garcios-Arcos method of exposure was nebulisation of the e-cigarette liquids, therefore, this method of exposure is unrealistic compared to the chemical changes released from heating an e-liquid in an e-cigarette. Lim and colleagues diluted e-liquids from e-cigarette cartridges with saline solution before exposing asthma-induced mice via intratracheal administration [133]. They found that after 10 weeks of exposure, mice exhibited airway hyper responsiveness, increase infiltration of eosinophils and an increase in cytokines like IL-4, IL-5 and IL-13 -[133]. In addition, Lerner and colleagues exposed mice to

tobacco-flavoured e-cigarette aerosols with nicotine (16 mg/mL) for 5 hours/day for 3 days and found an increase in pro-inflammatory cytokines such as MCP-1, IL-6, IL-1α and IL-13 [147]. In contrast, Larcombe and colleagues showed no inflammatory response after 8 weeks of exposure to e-cigarette aerosols in the lungs, however, they did see a reduction in lung function [184].

A subsection of the literature that focused on e-cigarette exposure in the lungs was viral and bacterial clearance in mice studies. Sussan and colleagues investigated anti-bacterial and anti-viral defences on alveolar macrophages in the mouse lungs after e-cigarette aerosol exposure with flavouring and found an impaired bacterial clearance after infection with *Streptococcus pneumonia*, and increase viral titers after infection with Influenza A [183]. Hwang and colleagues investigated the effects of e-cigarette aerosol exposure (1 hour/day for 4 weeks) on *Staphylococcus aureus* colonisation in the mice lungs and showed biofilm formation and an increase in pro-inflammatory cytokine production of TREM-1 [185]. Moreover, Miyashita and colleagues investigated the effects of e-cigarette aerosols with nicotine on pneumococcal adhesion in the mouse nasopharynx and found an increase in pneumococcal colonization [193].

E-cigarette exposure has also been investigated in the cardiovascular system and showed adverse changes. Olfert and colleagues exposed e-cigarette aerosols with flavouring and nicotine in mice and showed an increase in aortic arterial stiffness [186]. Although not in a mouse model, Palpant and colleagues treated zebrafish eggs with e-cigarette extracts with flavouring and nicotine and found developmental changes to the heart, pericardial oedema and overall reduced heart function [187]. In contrast, Shi and colleagues treated mice with e-cigarette aerosols with nicotine and no flavouring and showed an increase in angiogenesis, which can be beneficial during a heart attack or could induce tumour growth [135].

The effects of e-cigarette on the brain are slowly emerging in the literature. Cardinia and colleagues investigated e-cigarette aerosol exposure with flavour and nicotine and showed an increase in saturated fatty acids in the brain of rats [52]. Ponzoni and colleagues investigated e-

cigarette aerosol with nicotine in mice and found changes to neurochemicals in the brain as well as physiological and behavioural changes [188].

The effects of e-cigarettes were also investigated in the kidney. Drummond and colleagues investigated e-cigarette aerosols with nicotine and no flavouring and showed an increase in renal fibrosis and reduced glomerular filtration rate [189]. El Golli and colleagues performed intraperitoneal injections of e-liquid with flavouring and nicotine, and showed changes to collecting duct morphology, decreased urea, uric acid and anti-oxidant function in the kidney [190]. In addition, another study from El Golli and colleagues administered intraperitoneal injections of e-liquid with flavouring and nicotine and showed a decrease in sperm density, sperm viability and an increase in oxidative stress [192].

One study that investigated the effects of e-cigarettes on multiple organs found an increase in pro-inflammatory and pro-fibrotic proteins in the blood, decrease renal filtration rates and increased fibrosis in the kidney, heart and the liver in CD-1 mice [130]. In addition, to the murine models, one study that treated *Caenorhabditis elegans* nematode with e-cigarette constituents observed developmental impairment after propylene glycol treatment and an increase in oxidative stress after tobacco-flavoured e-cigarette aerosol treatment [140]. Moreover, another study investigated the treatment of e-cigarette extracts on zebrafish development and showed heart malformation, pericardial oedema and overall reduced heart function [187].

1.7.1.2 E-cigarette exposure in pregnancy models

The effects of maternal smoking in mouse models have been well documented in the literature [194-198]. Animal models of maternal e-cigarette exposure have been slowly emerging in the literature [48-51, 134, 136, 191, 199-202].

It has been widely known that smoking during pregnancy effects the development of the foetus [203]. Smoking during pregnancy have been associated with a number of detrimental

complications of the newborn such as lower birth weight, preterm birth, respiratory complications, cardiac complications, kidney complications and sudden infant death syndrome [204-210]. The effects of smoking during pregnancy is widely known to be detrimental to the foetus, thus, there is a growing concern that e-cigarettes, a product that is inhaled into the lungs, may show similar effects during pregnancy. The effects of maternal e-cigarette exposure on offspring lung have been investigated. We have shown that maternal exposure to e-cigarette aerosols change offspring DNA methylation and cytokine expression of IL-1β, IL-6 and tumour necrosis factor alpha (TNF-α) in the lungs [48]. In addition, McGrath and colleagues also showed significant reduction in lung growth in mice offspring after maternal e-cigarette exposure [134].

The effects of maternal e-cigarette exposure on offspring brain was also studied. We have showed changes to energy homeostatic gene expression of neuropeptide Y and inducible nitric oxide synthase (iNOS) in offspring brains after maternal e-cigarette exposure [49]. Lauterstein and colleagues investigated the effects of maternal e-cigarette exposure on the frontal cortex and found significant transcriptomes alterations that are associated with neurological conditions [50]. Following this study, Zelikoff and colleagues showed a significant increase in ionised calcium-binding adaptor molecule -1 (IBA-1) protein expression (a marker for microglia) and a decrease in nerve growth factor receptor (NGFR) and brain derived neurotrophic factor (BDNF) gene expression in the hippocampus of offspring from mothers exposed to e-cigarette exposure [51]. Smith and colleagues have investigated behavioural changes in offspring after maternal e-cigarette exposure and showed significant behavioural alterations when assessed on the open field test and elevated zero maze test [136]. Wetendorf and colleagues investigated the effects of e-cigarette aerosol exposure on female mice during pregnancy and found a significant delay in the first litter [202]. In addition, they also showed a delay in implantation of embryos in female mice that was exposed to e-cigarette aerosols [202].

1.7.2 *In vitro* cell culture models

Over the past few years there has been a surge of research investigating the effects of e-cigarettes using *in vitro* models. E-cigarette constituents have also been tested on a variety of cells from the respiratory system [127, 128, 131, 145, 147, 170, 180, 181, 211], cardiovascular system [126, 171, 187, 212], central nervous system (CNS) [129], gastrointestinal tract [213], integumentary system [145, 185], immune system [214] and urinary system [215]. In addition, varying types of stem cells were also used to investigate e-cigarette cytotoxicity [170, 187]. Currently, there is no standardisation on the treatment of e-cigarette constituents; therefore, different researchers have adapted their own methodologies to treat cells with e-cigarettes. The three main ways that researchers are treating e-cigarettes in cell culture includes applying the e-liquids directly [127, 180-182], using e-cigarette aerosols as an extract (either as a condensate or bubbled through cell culture media or phosphate buffer) [126, 129, 170, 171, 187, 212, 214, 216], or exposing e-cigarette aerosols using a chamber/air liquid interface model [131, 145, 147, 185, 211, 217].

Testing cell cultures by directly applying the e-liquid content does not provide an accurate physiological response from the cells being treated. Studies have found that aerosolised e-liquid and non-aerosolised e-liquids have different chemical properties. For example, propylene glycol, when super-heated, could form carcinogens such as formaldehyde [218]. Moreover, the flavouring constituent cinnamaldehyde has been shown to release benzaldehyde when aerosolised [219]. Thus, treating cell cultures using e-liquids directly is not necessarily the main substrate that is being inhaled by an e-cigarette user.

Reports investigating e-cigarette extracts on cell culture models have shown a reduced viability in airway-related cell lines [216], myocardial cells [126], human embryonic stem cells [170] and lung microvascular endothelial cells [212], as well as a reduction in vascular and differentiation markers in mammalian neural crest cells [129], a delayed differentiation of human embryonic stem cells to cardiac cells [187] and a reduced endothelial barrier and barrier function in lung microvascular endothelial cells [212]. Erythropel and colleagues studied the stability of adding flavouring to base liquids. They found that heating flavoured e-liquids mixed with propylene

glycol formed propylene glycol acetals, which are molecules that bind to transient receptor potential ion channels that are irritant receptors in HEK-293T cells [215]. Collecting the e-cigarette extract via a condensate or bubbling through a collecting liquid has been extensively used in the literature and has been extensively performed with tobacco cigarette extracts [220-225]. The use of extracts is a widely accepted method for these types of studies and hence used in the current study to interpret the effects of e-cigarette aerosols *in vitro*.

Finally, the most accurate model of testing e-cigarette vapours is using an air-liquid interface model of aerosol exposure which has been investigated recently in the literature. The air-liquid interface cell cultures involves the use of primary airway epithelial cells differentiated into specialised epithelial cell types such as goblet cells, ciliated cells and basal cells [226]. Studies that investigated the cell cultures using air-liquid interface systems or exposure chambers showed a reduced cell viability and vacuolisation in keratinocytes [145]. Epithelial cells and alveolar macrophages after exposure to e-cigarette aerosols showed a decrease anti-microbial activity against *Staphylococcus aureus* [185]. The majority of air-liquid interface experiments that were conducted, investigated the effects primarily on human bronchial epithelial cells. Studies have shown that e-cigarette aerosol exposure on these cells exhibited an increase in cytotoxicity, an increase in ROS production [147, 211], increases in inflammatory cytokines such as IL-6 ad IL-8 [147], reduced ciliary beat frequency [131] and a reduction in cystic fibrosis transmembrane regulator ion channels [131]. Interestingly, e-cigarette aerosol exposure have also shown to alter transcriptomes in human bronchial epithelial cells which were independent from tobacco cigarette smoke exposure [217]. Overall, the air-liquid interface experiment is a better representation of the respiratory system when reacting to external compounds released from e-cigarette aerosols and, therefore, provides a better result more akin to that when a person uses an e-cigarette.

Another experimental design similar the air-liquid interface experiment is a blood brain barrier (BBB) model compromising of brain-derived endothelial cells. Using transwell inserts, endothelial cells are able to grow and form tight junctions in the inner chamber, thus, creating an

endothelial barrier that is representative of the BBB [227]. This section will be further discussed in **Chapter 4 section 4.1.1**.

1.8 Effects of vaping in humans

There have been a number of reports that have recruited human participants on e-cigarette use [228-233]. New users using e-cigarettes exhibited moderate physiological effects such as dry mouth, throat irritation, nausea and headaches, particularly after trying e-cigarettes for the first time [228-231]. In addition, Liu and colleagues investigated studies that recorded adverse effects during e-cigarette use (16,000 total participants) and found the highest common adverse effects included mouth and throat irritation followed by anxiety and depression [234]. Two studies showed an increase in e-cigarette user's heart rate, blood pressure and arterial stiffness, similar to users who smoke tobacco cigarettes [232, 235]. Other physiological effects in the lungs, such as respiratory flow, impedance, and exhaled nitric oxide, are altered after acute exposure [233]. Although all of these acute negative effects have also been found in acute inhalation of tobacco cigarette smoke [236], one report did not find any adverse effects after participant's use of e-cigarettes [95].

Human studies of e-cigarette exposure *ad libitum* have also shown effects in gene activity [237]. Gene alterations in immune-regulating genes, which are involved in defending the body against microbial infections, were reported in donated human nasal epithelial cells by non-smokers, smokers, and e-cigarette users [237]. E-cigarette users had the highest level of gene alterations with a reduced expression in altered genes compared to smokers [237]. Moreover, Martin and colleagues found that e-cigarette use suppressed genes that are involved in the immune and inflammatory response, which were comparable to tobacco cigarette users [237].

Clinical trials are the most accurate model for identifying physiological effects of e-cigarettes in human participants. However, testing human participants presents many other challenges. This

can include creating the appropriate protocol, recruitment, ethics and follow-up of patients for longitudinal studies [228]. Another limitation is *puff topography*. Puff topography refers to the measurement of the duration and volume of a single inhalation of aerosol or smoke [238]. Puff topography in non-experienced e-cigarette users is significantly different to experienced e-cigarette users and cigarette smokers [239]. Being able to make a standardisation in terms of investigating the effects of e-liquids is difficult since all e-cigarette users have their own way of vaping, where some may enjoy the smaller puffs to get a 'throat hit', or some may enjoy the longer puffs to produce larger clouds of aerosols.

Although studies have focused on the effects of nicotine in e-liquids, more studies are needed to explore other avenues that are being affected by the flavouring constituents in the lung, such as epigenetics and the effects in a disease-state lung, such as COPD and asthma. Moreover, studies on the effects of e-cigarette aerosols on developing brains are also lacking. It is widely accepted in the literature that nicotine has been known to affect the developing brain [92, 197]. In addition, flavourings in the e-liquid have been shown to induce stronger effects in the absence of nicotine compared to the presence of nicotine [140]. Therefore, it is necessary to determine what flavouring constituents that can cause harm to the brain, whether it is affecting the user behaviourally, or whether it has an adverse effect in vulnerable populations such as young people and pregnant mothers.

1.9 Brain development and cognitive effects

1.9.1 Brain regions affected by nicotine and chemical flavourings

The effects of e-cigarette on the brain, particularly in a maternal model of e-cigarette exposure, are largely unknown. This section reports what is known about the effects of the individual e-cigarette constituents on different regions of the brain.

There are a number of reports in the literature that have investigated the effects of nicotine in the developmental brain [240, 241]. Nicotine is linked to changes in behaviour in the offspring such as hyperactivity and neurobehavioural changes in infants and early childhood [241]. Adverse effects of nicotine on the brain have been extensively investigated in the literature [240, 242, 243]. A study that compared human smokers who have used nicotine-containing e-cigarettes to those who used non-nicotine-containing e-cigarettes showed an improved prospective memory and a reduced urge to smoke [244], suggesting that nicotine-containing e-cigarettes may be able to help smokers quit tobacco cigarettes and improve neurological performance. This study is consistent with another human study that showed improved memory performance after smokers use nicotine gum compared to a placebo [245].

Investigations on the effects of nicotine in adult human brain regions have been reported [246-249]. Kobiella and colleagues investigated brain region activations from acute and chronic nicotine exposure in human smokers and non-smokers, with non-smokers chewing nicotine gum or a placebo gum by detecting changes in blood flow using magnetic resonance imaging [246]. They have found that smokers who were chronically exposed to nicotine had less brain activation in the parietal/occipital regions, the hippocampus and the cerebellum. Decreased activation from acute exposure of nicotine was also found in the same regions from smokers who were chronically exposed to nicotine but was more prominent in the left hippocampus, amygdala and the anterior insula [246]. This is in line with results from the hippocampus and amygdala since those regions of the brain have a high density of nicotinic acetyl-choline receptor (nAChR) [250], suggesting that they are more sensitive to an acute exposure to nicotine. The parietal/occipital regions of the cortex, the hippocampus and the amygdala are important in sensory/visual processing, memory and fear respectively [251, 252]. These regions may be more sensitive to e-cigarette aerosols.

The expression of inflammatory and oxidative stress markers from various brain regions in rats were found to be altered after exposure to flavouring 2, 3-pentanedione [253]. A study have reported 23 of the 51 flavoured e-liquids tested contain 2, 3-pentanedione such as peach schnapps, vanilla, cherry crush, tutti frutti and classic tobacco [173]. 2, 3-pentanedione exposure have shown

to upregulate the gene expression of IL-6, nitric oxide synthase-2 (NOS2) and vascular endothelial growth factor-A (VEGF-A) at the olfactory bulb, striatum, hippocampus and cerebellum [253]. These markers in the brain are important in regulating inflammatory mediation, neurotransmitters, angiogenesis, neurogenesis and neuroprotection although more studies are required to determine any other possible changes that are occurring in the brain [254].

IL-6 has widely been used as a marker for a number of inflammatory diseases [255]. NOS2 regulates neurotransmitters in neurons located at the entorhinal cortex which is the gateway region between the hippocampus and neocortex [256]. VEGF-A is important in angiogenesis, neurogenesis and neuroprotection in the CNS and messenger ribonucleic acid of VEGF-A is highly expressed at the cerebellar granule layer of the cerebellum [257-259]. Therefore, IL-6, NOS2 and VEGF-A might be possible markers to investigate the effects of e-cigarette aerosols in the brain. It is important to understand which regions of the brain have high expression of these markers and whether it is altered in second generation offspring after maternal vaping.

The effects of propylene glycol exposure in the brain have also been investigated in the literature [143]. Lau and colleagues administered intraperitoneal injection of non-heated propylene glycol at varying concentrations in a mouse model and have shown cell apoptosis in the brain [143]. Regions of the brain that was affected the most included the caudate putamen and different segments of the cortical regions. Other areas of the brain that were also affected included the nucleus accumbens, hippocampus, and specific regions of the thalamus [143].

Overall, there are limited numbers of studies that investigated the effects of e-cigarette constituents (other than nicotine) in the brain. Therefore, there is a need to investigate the effects of e-cigarette aerosols on the developing brain. More research is required to understand the regions of the brains that are being affected and whether there are any altered gene expressions and cognitive affects within those targeted regions.

1.10 Project aims and thesis

To conclude, e-cigarettes are an emerging product that are not highly regulated within Australia. E-cigarettes are attracting vulnerable populations in society such as young people and pregnant women, even though there is little evidence to prove that they are in fact safe to use during these time periods of life [260]. In addition, studies on the effects of e-cigarette exposure on the brain are lacking. Hence, this thesis will use *in vivo* and *in vitro* models of e-cigarette exposure to investigate neurological changes. **Chapter 2** of this thesis will examine the effects of e-cigarette aerosol exposure during pregnancy on offspring behaviour and epigenetics. **Chapter 3** of this thesis will examine the effects of switching from tobacco cigarette exposure pre-pregnancy to e-cigarette aerosol exposure during pregnancy, and will investigate changes offspring behaviour and epigenetics compared to continuous tobacco smoke exposure. Finally, **Chapter 4** of this thesis will examine the effects of e-cigarette aerosol condensate exposure using cells from the CNS in monoculture and co-culture to create an *in vitro* BBB model.

Chapter 2 – The effects of e-cigarette aerosol exposure on offspring during pregnancy

2.1 Introduction

2.1.1 Maternal e-cigarette exposure and epigenetic changes

E-cigarettes were originally introduced into the market in 2003 as a cessation aid to quit smoking. E-cigarettes have been attracting younger people of reproductive age due to low costs, excessive marketing and having access to over 7500 different flavours available on the market. Moreover, e-cigarettes are attracting younger people of reproductive age that have never smoked tobacco cigarettes before [23, 261, 262]. Currently, there are growing concerns that the number of young people using e-cigarettes outranked the number of adult users. In the US in 2017, 6.9 million adults were users of e-cigarettes [100] whereas in 2018, the percentage of middle school and high school students using e-cigarettes was at 570,000 and 3.05 million students, respectively [263]. In addition, a study by Mirbolouk and colleagues investigated e-cigarette use in the US population are commonly used in younger adults [264]. Furthermore, they also found that the prevalence of e-cigarette users was the highest in young people between the ages of 18 and 24 [264].

There is a perception in young people that e-cigarettes are safe to use and adolescents often consider e-cigarettes to be ‘cleaner’ and ‘safer’ compared to smoking tobacco cigarettes [262]. Compared to adults, many young people engage in ‘vaping’ differently. Generally, adult users of e-cigarettes are former or current tobacco cigarette smokers [264], whereas young people uses e-cigarettes because they perceived them as being ‘cool’, ‘trendy’ and ‘fun’ with ‘great flavourings’ [91, 262]. In addition, many young people are attracted to the ‘tricks’ that can be created when

using an e-cigarette, and this has been influenced by social media giants like Twitter, Facebook and YouTube [91]. From all the popularity of e-cigarette use in young people, there is a growing fear that youths who uses e-cigarette may be at risk at smoking tobacco cigarettes [265].

There is a growing popularity in e-cigarette use among vulnerable groups in our society. E-cigarette use in young people is a continuing public health concern. Among young people using e-cigarettes, pregnant women are also using e-cigarettes [46, 115, 117]. Currently, more women are currently using e-cigarettes compared to the male population. A 2018 online survey that had over 2,200 respondents from the US found that 60% women had ever-used or are current users of e-cigarettes compared to men which was at 45% [266]. Although, in 2017, a study that reviewed articles on e-cigarette use among adolescents between 2012-2017 showed that over half the number of studies reviewed showed e-cigarette use was higher in males than females [267] This could be due to the excessive marketing of e-cigarettes such as advertising e-cigarettes as a safer alternative to tobacco cigarette, as they release a non-combustible product, and they come in a variety of sweet and fruity flavours. In 2015, a study that interviewed 316 participants found that 13% of pregnant women reported to have used an e-cigarette [117]. In addition, a study in 2017 that conducted an online survey with 445 participants found that 6.5% of pregnant women reported to have ever-used an e-cigarette with 8.5% reported to have used both e-cigarette and tobacco cigarette [268]. From the limited number of studies currently available, there is a perception in pregnant women and women of childbearing age that e-cigarettes are safer to use compared to tobacco cigarette smoke and some had a positive attitude about ‘vaping’ [115, 268, 269].

Ultimately, there are a limited number of studies to prove that e-cigarettes are safe to use during pregnancy. Currently, there are studies that have investigated e-cigarette exposure on the respiratory [48, 130, 132, 134, 147, 183, 270], cardiovascular [130, 135, 186, 271] and urinary [130, 132, 189] system. There is a slow emergence of studies investigating e-cigarette exposure in the brain of rodents [50-52, 132, 136, 188]. In addition, there are limited studies that investigated the effects of e-cigarette exposure on gene expression in the brain. Lauterstein and

colleagues investigated transcriptome changes in one-month old murine offspring at the prefrontal cortex and found significant changes to prefrontal cortex transcriptomes that were associated with neurological diseases and psychiatric disorders [50]. In addition, Zelikoff and colleagues investigated gene expression of neurotrophins and transcriptome changes in the murine hippocampus and found a decrease in NGFR and BDNF gene expression and transcriptome changes that were associated with inflammation [51].

One specific marker of epigenetic change is DNA methylation. DNA methylation involves the addition of a methyl group on a cytosine-phosphate-guanine (CpG) island on the genome. The addition or removal of a methyl group at the CpG island determines whether the downstream gene is expressed. A number of environmental factors such as air pollution and tobacco cigarette smoke exposure have been known to cause changes to DNA methylation [272-274]. Maternal exposure to tobacco cigarette smoke has been known to cause changes to DNA methylation in the foetal cord blood [275], the placenta [276] and leukocytes [277]. Studies are sparse when investigating DNA methylation and maternal e-cigarette exposure. Only one study showed significant changes to DNA methylation and an increase in inflammatory cytokine gene expression in the offspring lung after maternal e-cigarette exposure [48]. Neurobehavioural changes within the offspring after maternal smoking have also been documented. Maternal smoking has been closely linked to a number of behavioural and psychological changes in the offspring. This includes attention deficit hyperactive disorder (ADHD), aggressiveness, decreased social behaviour, anxiety and anti-social behaviour [278-280]. In animal studies, only one study investigated the effects of e-cigarette exposure in male mice behaviour and found changes to episodic memory and emotional responses [188]. In addition, only one study investigated behavioural changes in adult offspring after prenatal exposure to e-cigarettes and found that offspring had an increase in exploration activity evident by the increase in rearing [136].

Although there are a limited number of studies that investigate maternal e-cigarette exposure on offspring, the effects of maternal e-cigarette exposure on offspring behaviour and epigenetic changes in the brain are still to be elucidated. In addition, epidemiological data on long-term

effects of maternal e-cigarette exposure in humans will not be available for another two decades, when the children of e-cigarette users grow into adulthood.

2.2 Hypothesis and aims

2.2.1 Hypothesis

That e-cigarette aerosol exposure during pregnancy in a mouse model will affect offspring behaviour and epigenetics in the brain compared to offspring from mothers exposed to ambient air.

2.2.2 Aims

To investigate the effects of e-cigarette aerosol exposure during pregnancy on offspring by:

1. Assessing behavioural changes in the adult offspring using the novel object recognition test and the elevated plus maze test.
2. Determining the global DNA methylation in offspring brain right after birth, weaning and adulthood using the 5-mC DNA ELISA kit.
3. Determining any epigenetic gene changes in the offspring brain using the Mouse Epigenetic Chromatin Modification RT^{2}Profiler^{TM} PCR Array.
4. Validating the epigenetic genes that were changed using RT-qPCR.
5. Determining the counts of neuronal cells in the offspring dorsal hippocampus and the lateral amygdala nucleus.

2.3 Materials and Methodology

2.3.1 E-cigarette device

The e-cigarette device used for the experiment was the Kanger Tech MINIBOX starter kit. It is one of the third-generation devices that are available on the market. It consists of a mouthpiece (drip tip), a stainless steel organic cotton coil (0.5 Ω), a Subtank mini which encases the organic cotton coil and where the e-liquid is poured into, and the device itself that holds an 18650 battery with an organic light-emitting diode display (Figure 5). The Kanger Tech minibox starter kit was chosen as the device of choice for this study since it has a top removable tank that allows for changes to different e-liquid flavours with ease and avoids contamination between e-liquid flavours when using the same tank. Furthermore, the Kanger Tech minibox starter kit has a controllable temperature and wattage setting. The e-liquids that were used in this experiment was tobacco-flavoured with 0 mg/mL of nicotine and tobacco-flavoured with 18 mg/mL of nicotine. The ratio of propylene glycol and glycerine that was used for the experiment was 20 % and 80 %, respectively. Enough e-liquids were purchased online (www.vaperempire.com.au) to avoid different batches of e-liquids being given to the animal cohorts during the experiment.

Figure 5. E-cigarette device used for animal experiments. The e-cigarette device consists of the main device that has a fire button, LED display and wattage variations. The subtank mini sits on top of the main device that holds the e-liquid containing the organic cotton coil and the mouth piece.

2.3.2 Animal experimental procedure

All animal handling experiments were conducted with the assistance of Mr. Gerard Li (University of Technology Sydney). All experimental procedures were conducted strictly under the guidelines described by the National Health and Medical Research Council code of conduct for animals with approval from the institutional Animal Care and Ethics Committee (ethics approval number ETH15-0025). All animal experiments were completed in the Ernst Facility at the University of Technology Sydney. The timeline of the animal experiments is outlined in Figure 6. Twenty-four 7-week old female BALB/c mice were obtained from the Animal Resources Centre in Perth. Mice were housed in groups of four in cages with *ad libatum* food and water with a 12:12 light/dark light cycle. All animals were provided with environmental enrichment as part of the standard housing conditions. To ensure female mice are adapted to the new facilities, acclimatisation occurred that involved no experimental disturbances for one week.

After acclimatisation to the Ernst facility, animals were randomly assigned to one of three groups and were exposed to the following; (i) **Sham:** Ambient air (n = 8), (ii) **Ecig(+nic):** E-cigarette aerosols with of nicotine (18 mg/ml) (n = 8) and (iii) **Ecig(-nic):** E-cigarette aerosols without nicotine (0 mg/ml) (n = 8), twice daily, seven days a week, for six weeks. The number of animals for each group provided the least number of animals for a statistical significance based on previous maternal smoking experiments in our laboratory [196, 197, 281]. Animals were exposed once in the morning between 0900-1000 and once in the afternoon between 1500-1600. Animals were continued to be exposed to their corresponding treatment during pregnancy and lactation until pups were weaned from their mothers. No animals were exposed to their corresponding treatment during the birth of pups to reduce animal stress during labour. It is important to note that offspring were not placed in the chamber with their mothers during aerosol exposure. However, offspring can be exposed to treatment once mothers return to the home cage by licking their mother's fur or suckling for breastmilk.

After six weeks of exposure to treatment, female mice were caged with 8-week old male BALB/c mice for mating. All female mice became pregnant between 1-2 weeks being housed with the male mice. Females were considered pregnant when they have significant weight increase. Once we have established that the females were pregnant, each mouse were separated into individual cages for nesting purposes to reduce maternal stress. Generally, pregnant mice gave birth to pups at around 2 weeks after getting pregnant for all test groups. No pups were lost or cannibalised after birth. Depending on the size of the litter, male pups were randomly selected from each litter to be culled at three time points; Postnatal day 1 (P1) [n = 14], Postnatal day 20 (P20) [n = 14] and Week 13 (n = 14). Time points were chosen to determine changes to offspring right after birth (P1), after weaning (P20) and at adulthood (Week 13). Offspring were weighed regularly each week and recorded for analysis. Male offspring were included in the experimental work. Female pups were subsequently transferred to other researchers to investigate the effects of e-cigarette aerosols in an asthma model of e-cigarette exposure under ethics approval number ETH15-0025. It is important to study the differences between sexes and this will be considered in future studies.

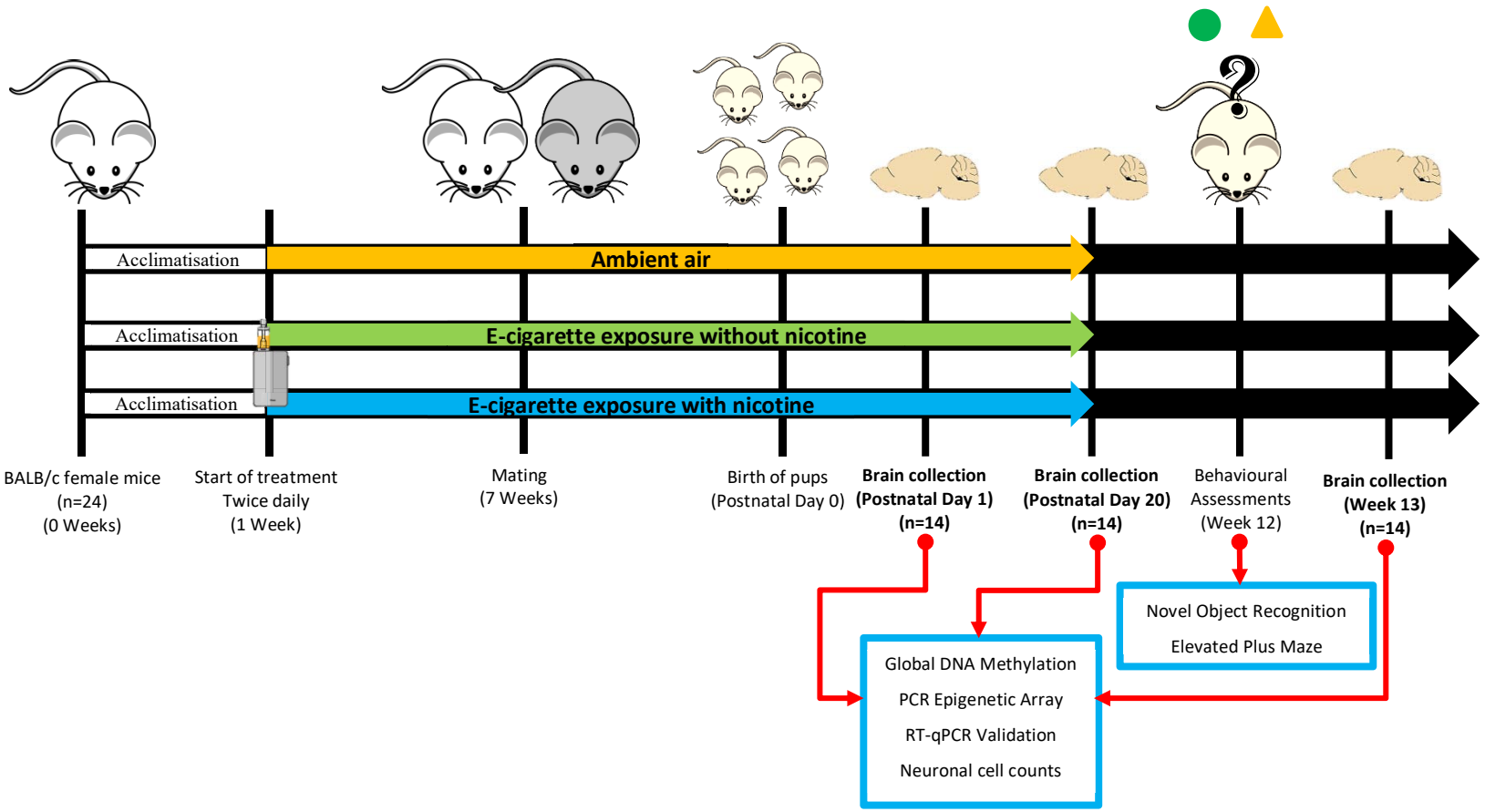

Figure 6. A timeline summary of the animal experiments for Chapter 2. Twenty-four female BALB/c mice were divided into three treatment groups; Sham, Ecig(+nic) and Ecig(-nic). Animals were exposed to the ambient air or e-cigarette aerosols with or without nicotine for 6 weeks before pregnancy, during mating, pregnancy and lactation. Tissue collection occurred at postnatal day 1, postnatal day 20 and Week 13 to investigate global DNA methylation, PCR epigenetic array, RT-qPCR and neuronal cell counts. Behavioural assessments were performed at 12 weeks prior to Week 13 collection.

2.3.3 Aerosol exposure procedure

To produce the e-cigarette aerosol, a manufactured third generation e-cigarette device was used for all of our animal experiments (KangerTech NEBOX). To avoid inconsistencies in aerosol exposure, only one flavour of e-liquid with different nicotine concentrations was used for the experiment. The flavoured that was used for this experiment was tobacco flavoured e-liquid with nicotine concentrations of either 18 mg/mL or 0 mg/mL (Vaper Empire, Gordon, New South Wales, Australia). The e-cigarette aerosol exposure procedure has been adapted from a cigarette smoke exposure model that was established previously [197]. To avoid cross contamination of e-cigarette aerosols, each test group had their own experimental chamber, e-cigarette device and expelling system.

Before the commencement of aerosol exposure, animals from each group were placed into a 9 L chamber. E-cigarette aerosols were created by pressing the 'fire button' on the e-cigarette device to activate the heating coil. E-cigarette aerosols were collected using a 30 mL syringe before being released into the chamber (Figure **7**). E-cigarette aerosols were created for 5 x 5 seconds with a 20 second interval for each 'vape' produced to ensure that the coil doesn't overheat. The e-cigarette aerosols remain in the chamber for 20 minutes before the remaining aerosols were released from the chamber. This process was repeated after a 5-minute break. The amount of e-cigarette aerosols being delivered to the chamber equates to a nicotine delivery of two cigarettes per treatment, twice daily, i.e. each animal mice had a total of 5 x 5 seconds x 2 sessions x 2 times a day = 100 seconds of active puffing each day. Heating coils were changed every 2-3 weeks due to coil degradation after prolonged use.

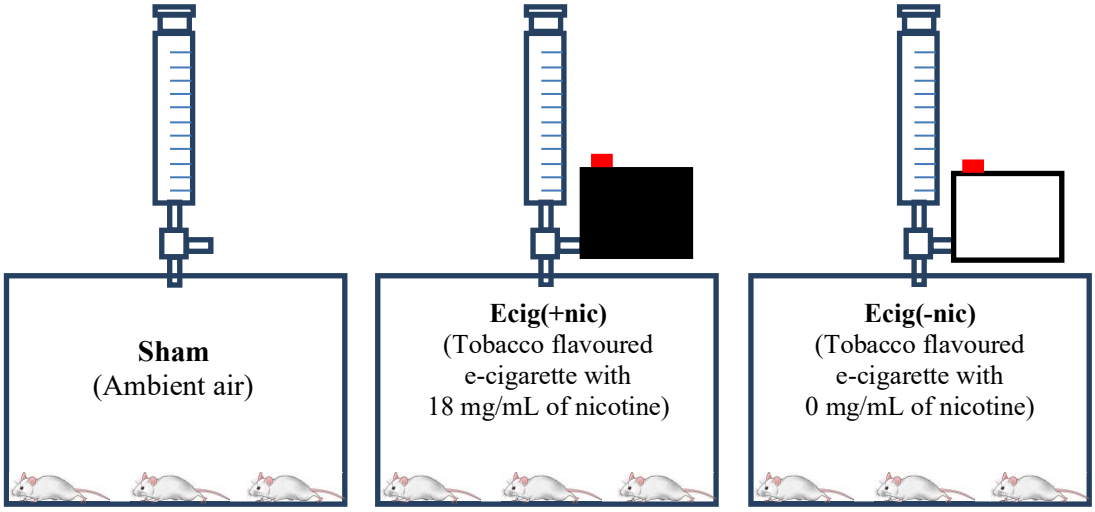

Figure 7. Schematic of the experimental treatment groups for this study. Animals were divided into three treatment groups; Sham (exposed to ambient air), Ecig(+nic) (exposed to tobacco flavoured e-cigarette with 18 mg/mL of nicotine), Ecig(-nic) (exposed to tobacco flavoured e-cigarette with 0 mg/mL of nicotine).

2.3.4 Behavioural assessments

Behavioural assessments were conducted on male pups at 12-weeks old. Behavioural assessments were performed to investigate whether there were any cognitive deficits occurring after maternal exposure to vaping in terms of anxiety and memory. Each behavioural test was conducted between the hours of 0900-1200 hours and behavioural tests were conducted from less-stressful to most stressful to avoid each mice being stagnant or overwhelmed in each test [282]. Each behavioural assessment was video recorded and analysed once all pups had completed all assessments. This is to avoid investigator interference which may cause alterations in anxiety and stress levels in the animal being tested [283].

2.3.4.1 The Novel Object Recognition (NOR)

The novel object recognition (NOR) was the first assessment to be performed. The NOR test measured deficits in short-term memory. There are three phases to the NOR test. The *acclimatisation phase* involved placing the mice in the apparatus without any objects present for five minutes (Figure 8A). This is to ensure that the test is not affected by the animal's anxiety from being exposed to a new environment. The second phase is the *familiarisation phase*. On the second day, each animal is placed into the same apparatus for five minutes containing two identical objects (Figure 8B). The final phase is known as the *test phase*. After an hour, each animal was exposed to a previous object from the *familiarisation phase* and a novel object for five minutes (Figure 8C). Between each test, the apparatus and object were cleaned with 80% ethanol. A camera was set up on top of the container to record the animal's exploration. The assessment ended once the animal was returned to their cage.

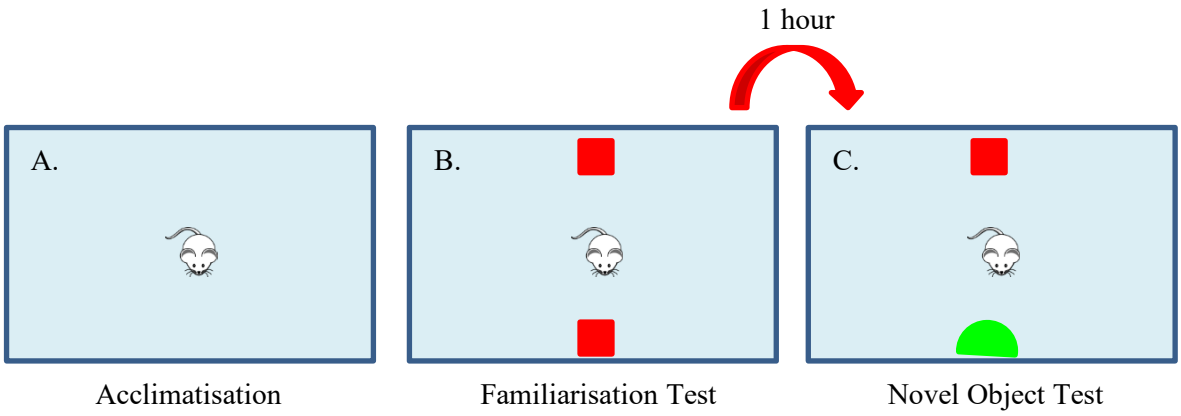

Figure 8. A schematic of the novel object recognition test. (A) Animals were acclimatised to the apparatus before starting the experiment. (B) On the day of the experiment, animals were placed in the apparatus with two identical objects. (C) After an hour, animals were placed in the same apparatus with a novel object.

It was expected that a normal animal would spend equal amounts of time on the identical objects in the familiarisation phase but more time on the novel object in the test phase. This would indicate

that an animal can recognise an object that it had seen previously, and therefore, does not explore the object as often compared to an object that it has never seen before.

Analysis of the NOR videos was guided by published criteria [284]. The length of time it took the animal to spend on an object was determined when the direction of the nose, facing the object, was within 2 cm and/or physically touching the object with its nose and/or fore limbs (Figure 9). The animal was not considered to be exploring the object when the animal climbed onto the object, ran past the object or sat facing away from the object unless the animal was facing and/or sniffing the object. An animal was excluded if they did not explore any of the objects or if they remained stationary for the majority of the test.

Figure 9. Images of animals exploring the identical objects in the novel object recognition test. Animals were considered to be exploring the objects when the animal's nose was touching the object (arrow) and/or forelimbs were touching the objects (asterisk).

All data from the NOR test was calculated using the *recognition index* as a score of short-term memory retention [285]. The recognition index is represented as a percentage of the amount of time exploring a novel object (Tn) over the overall time spent exploring both the familiar and novel object (Tf). The recognition index was determined by the equation shown below:

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

2.3.4.2 The Elevated Plus Maze (EPM)

The elevated plus maze (EPM) test is a measure of anxiety in animals. The EPM test is a plus (+) shaped apparatus that consists of four arms. The east and west arms consisted of only the platform (open arms), whilst the north and south arms consisted of the platform and tall black walls (closed arms) (Figure 10). No acclimatisation was required since this may alter the anxiety levels of each animal.

To keep the assessment consistent, each animal was placed directly in the middle of the EPM before the timer was set. Each animal had two minutes to explore the closed and open arms. After two minutes, the animal was placed back into their cage. Video recording was analysed after all animals went through the EPM test. From the EPM video recordings, the time spent exploring the open arm was analysed. In addition, the number of head dips and stretches in the closed arm (protected) and open arm (unprotected) were also analysed. Finally, the number of times the animal crosses the centre of the EPM was also analysed. To ensure consistency when analysing each video recording, arm entries were defined as when the animal has more than three limbs in the newly chosen arm on the apparatus.

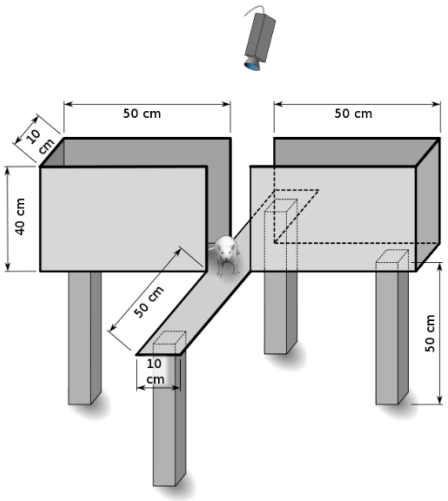

Figure 10. A schematic of the elevated plus maze with the apparatus dimensions. Each animal was placed in the same direction at the centre of the apparatus. Exploration was recorded using a camera that was attached on top of the apparatus. Image was taken from Wikipedia [286].

2.3.5 Euthanasia and tissue collection

All bench surfaces and surgical tools were decontaminated with 80% ethanol before collection commenced. Mothers were sacrificed after all offspring were weaned at P20. The brain, heart, liver, kidneys, lungs and brain of each mother was collected and stored appropriately for later analysis. Offspring were sacrificed at P1, P20 and Week 13. At P1, pups were euthanized by decapitation and blood was collected from the decapitation site. Animals that were culled at P20 and Week 13 were placed in an induction chamber with 4% isofluorane and 1 L of oxygen for five minutes. Hind limbs, eye cornea and mucosa were checked to ensure that animals were deeply anaesthetised before cervical dislocation. After euthanasia, an incision was made at the midline of the animal's body and the skin was pulled back to reveal the abdominal cavity. Whole blood was collected via cardiac puncture using a heparin-rinsed syringe before being centrifuged at 13,000 rpm for five minutes to collect plasma. For each time point, the left cerebral hemisphere was fixed in 4% paraformaldehyde (PFA) in 0.1 M of phosphate buffer (pH 7.4) for 24 hours before being placed in 70% ethanol for long-term storage. The right cerebral hemisphere from P1 and P20 pups were snap frozen using dry ice. For the Week 13 brains, the hippocampus was a particular region of interest to determine the effects of maternal e-cigarette exposure since this part of the brain is an important region involved in short-term memory retention. Therefore, Week 13 offspring brains were micro-dissected to collect the hippocampus. All offspring tissue samples and plasma were stored at -80ºC for later processing. Offspring lungs and kidneys were used in a separate study [48, 200].

2.3.6 Plasma cotinine

Plasma from the breeders and P20 offspring were collected right after the last e-cigarette exposure and used to analyse cotinine levels. Cotinine is a widely known biomarker to detect the amount of nicotine being delivered from tobacco cigarette smoking [287]. Cotinine levels were measured

using the *cotinine ELISA* kit (Abnova, Taipei, Taiwan, KA0930) according to the manufacturer's protocol. Briefly, standards and plasma samples were pipetted in duplicates to the bottom of a 96 well-plate coated with rabbit anti-cotinine antibody. Cotinine-alkaline phosphatase conjugate was added to each well and incubated at room temperature for 40 minutes. After incubation, the conjugate solution was removed and each well was washed with buffer three times. Excess solution from the plate was removed by inverting and shaking the plate. p-Nitrophenyl phosphate substrate was then added to each well and incubated for 20 minutes at room temperature. Stop solution was subsequently added to stop the reaction, and the absorbance from each well was read at 405nm using the infinite M1000 PRO Microplate reader (Tecan group Ltd, Männedorf, Switzerland).

2.3.7 DNA and RNA extraction

DNA, RNA and protein were extracted from the brain samples at P1 (whole brain), P20 (whole brain) and Week 13 (hippocampus) using the *ISOLATE II RNA/DNA/Protein* Kit (Phenol free) (Bioline, MA, USA) following the manufacturer's protocol. The step by step process of each extraction is outlined in Figure 11. Briefly, lysis buffer from the kit was added to brain tissues and homogenised at 5000 rpm for 15 seconds using the Precelley's 24 homogenizer (ThermoFisher Scientific, MA, USA). The homogenised samples were then centrifuged at 14,000 g before each lysate was collected and placed into a DNA column. DNA columns were spun down for two minutes at 14,000 g and the flow-through that contained RNA and protein was left on ice until required for further extraction. DNA columns were washed with buffers provided by the kit before DNA was eluted into a fresh Eppendorf tube by centrifugation. For RNA extraction, the RNA/protein flow-through was transferred into the RNA column and spun down at 14,000 g at room temperature. The flow-through that contained proteins were stored at -80^{o}C. To ensure that there was no genomic DNA contamination, the RNA columns were treated with DNase (provided by the kit) at room temperature for 15 minutes. RNA columns were washed with buffer twice

before RNA was then eluted from the column with RNase-free water into a fresh tube. RNA concentration was quantified using a Nanodrop® (Thermo Fisher Scientific, MA, USA). RNA quality was checked using the *Experion RNA StdSens Analysis* Kit (Bio-Rad, CA, USA) following the manufacturer's protocol. Purity of DNA and RNA was based of the nucleic acid absorbance at 260 nm and 280 nm. Therefore, the 260/280 ratio cut off for DNA and RNA was 1.8 and 2.0, respectively. DNA and RNA were stored in at -80^{o}C until ready for reverse transcription.

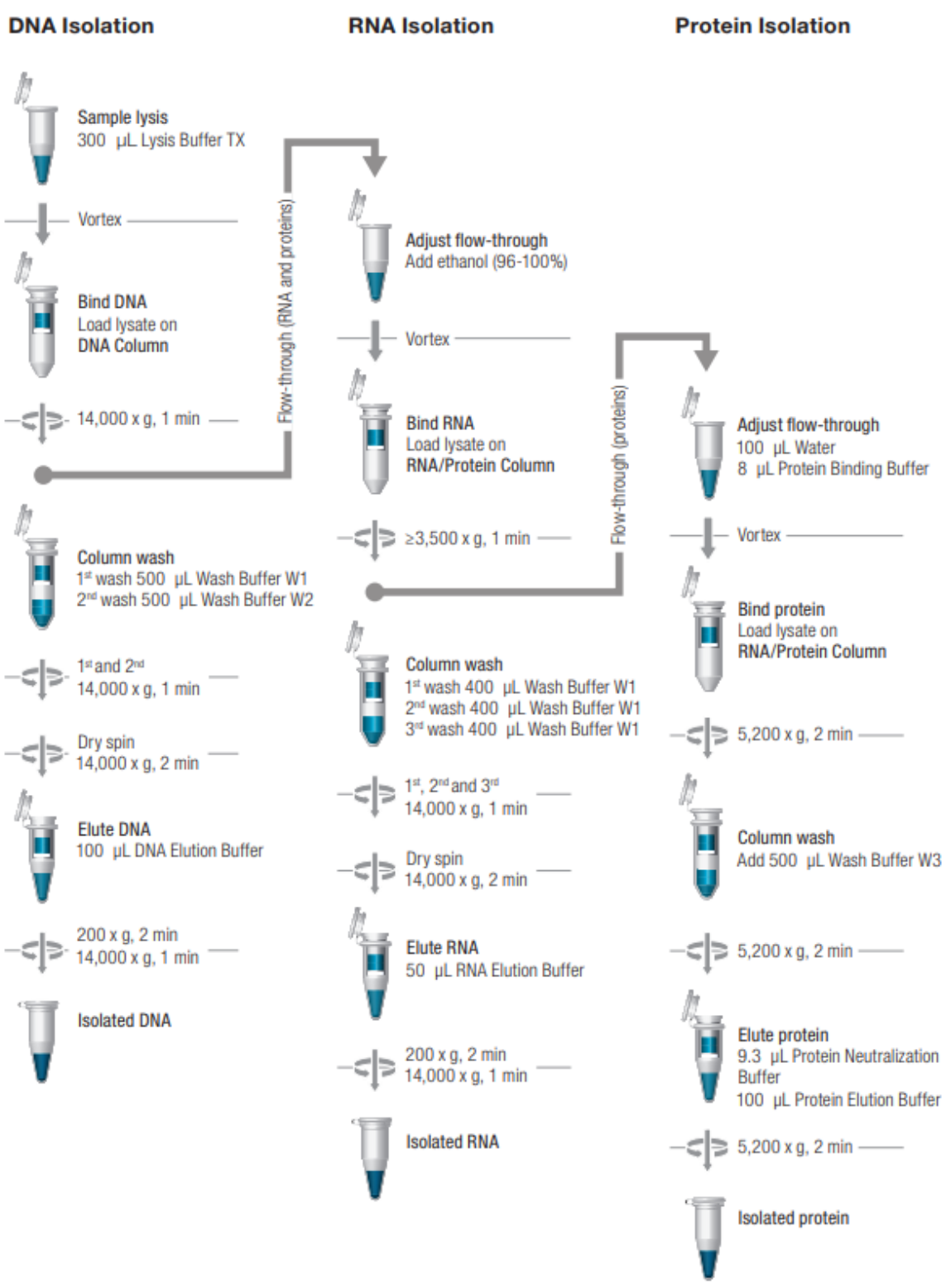

Figure 11. Step by step schematic of the DNA, RNA and protein extraction protocol. Image was taken from the ISOLATE II RNA/DNA/Protein Kit product manual [288].

2.3.8 RNA quality check

The quality and integrity of the RNA from each sample was checked using the *Experion™ RNA StdSens Analysis* Kit (Bio-Rad, CA, USA) following manufacturer's protocol. The RNA quality was checked using an *Automated Electrophoresis System* (Bio-Rad, CA, USA). Total RNA (3 µl) and the RNA ladder (provided by the kit) was denatured at 70°C for two minutes. After denaturation, RNA samples and the RNA ladder were immediately transferred onto an ice block for five minutes. To prime the *Experion™ RNA StdSens Chip* (Bio-Rad, CA, USA), gel stain (9 µl) was loaded in the appropriate well of the chip and placed into a *Priming Station* (Bio-Rad, CA, USA) at setting '1B' for 30 seconds. After the priming step, RNA loading buffer (5 µl) was added to each well followed by 1 µl of RNA sample and RNA ladder in the marked wells. The chip was then placed in an *Automatic Vortex Station* (Bio-Rad, CA, USA) for one minute before being transferred onto the *Experion™ Electrophoresis Station* (Bio-Rad, CA, USA) for analysis. The RNA ladder and samples were visualised as bands (virtual gel) and peaks (electropherogram).

Data analysis was presented as a virtual gel and an electropherogram. A representation of the virtual gel and the electrophoresis are shown in Figure 12. The virtual gel showed the ladder in the first lane followed by two bands representing 18S and 28S ribosomal RNA in the next 12 lanes (Figure 12A). To identify the quality and integrity of the RNA, the electropherogram showed nine peaks representing the different sizes of the RNA ladder and the two highest peaks representing the 18S ribosomal RNA and the 28S ribosomal RNA, respectively (Figure 12B).

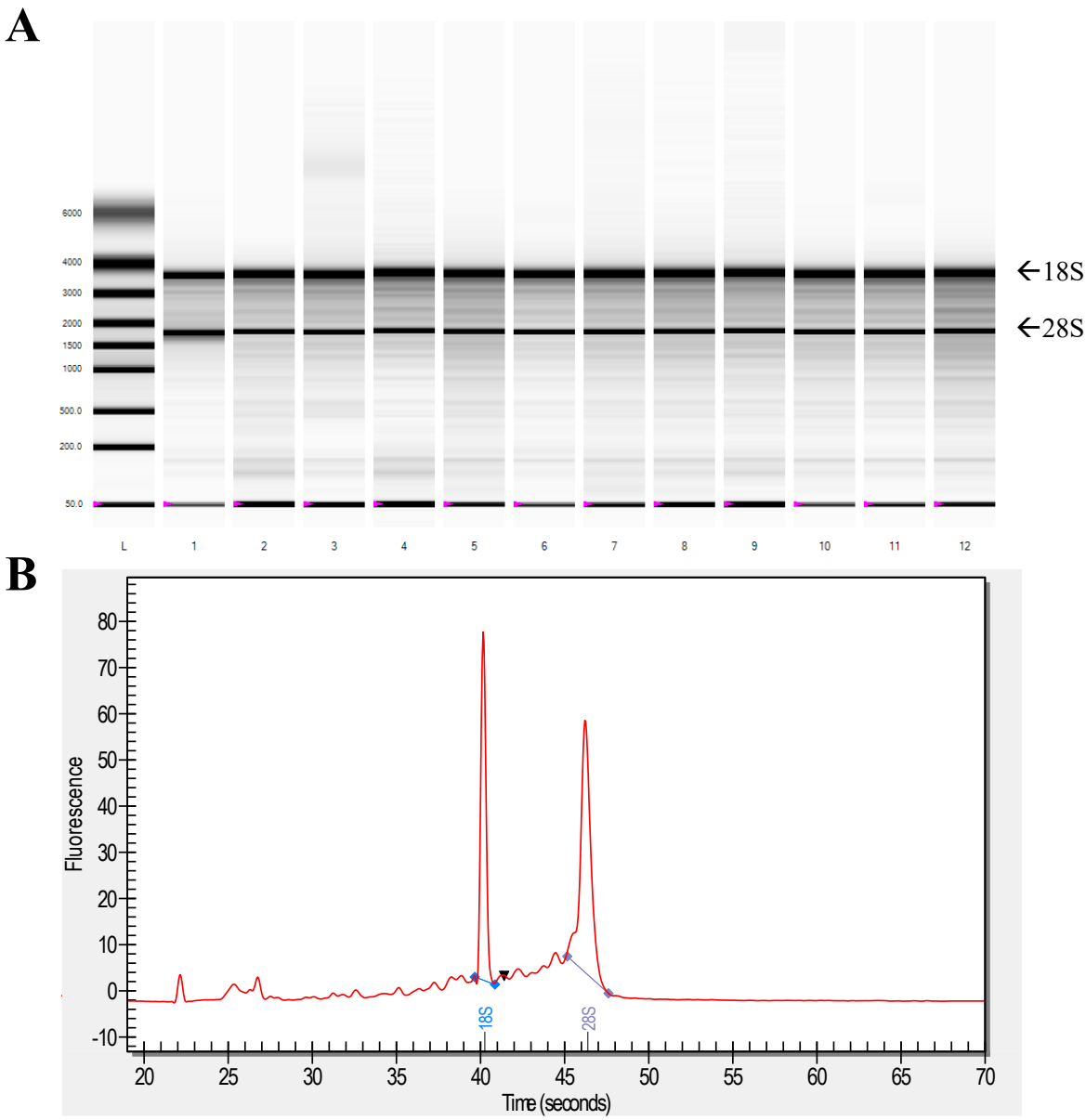

Figure 12. A representative virtual gel and an electropherogram generated by the Experion RNA StdSens Analysis Kit. (A) A virtual gel showing two bands representing 18S and 28S ribosomal RNA. (B) An electropherogram showing two peaks representing 18S and 28S ribosomal RNA.

2.3.9 Global 5-mC DNA methylation

To investigate global DNA methylation in the brain, DNA samples from P1, P20, and Week 13 offspring were investigated using the *5-mC DNA ELISA* kit (Zymo Research, CA, USA) following the manufacturer's protocol. Briefly, DNA (100 ng) from each sample and DNA standards were denatured at 95°C for five minutes before being placed on ice for 10 minutes. The denatured DNA was then transferred to a coated 96 well-plate provided with the kit and incubated at 37°C for one hour. After the incubation, the plate was washed and incubated with 5-mC ELISA buffer for a further 30 minutes. Each well was then treated with a mixture containing Anti-5-methylcytosine (1:2000) and secondary antibody (1:1000) diluted in 5-mC ELISA buffer for another hour. After three more washes with 5-mC ELISA buffer, horseradish peroxide developer was added to each well and incubated for another hour or until a green colour developed. Absorbance was measured at 405-450nm using the Tecan's Infinite® M1000 PRO (Thermo Fisher Scientific, MA, USA).

2.3.10 Epigenetics

To assess if there were any effects on the epigenome, an array was performed using the *Mouse Epigenetic Chromatin Modification RT²Profiler™ PCR Array* (Qiagen, CA, USA). The PCR array consisted of two kits; *RT² First Strand* Kit for reverse transcription and the *RT² SYBR Green Mastermix* kit for gene expression amplification. The PCR array consisted of 84 genes associated or predicted to influence epigenetic modification enzymes, and thus, gene expression (Table 1). These included DNA methyltransferases, histone methyltransferases, histone demethylases, histone acetyltransferase, histone deacetylases, histone phosphorylation, histone ubiquitination and SET domain proteins.

Table 1. List of epigenetic chromatin modification genes provided by the Mouse Epigenetic Chromatin Modification RT^{2}Profiler^{TM} PCR array.

Epigenetic chromatin modification enzymes	Epigenetic chromatin modification genes
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 Acetyltransferase	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

Genes for normalization included β-actin, β-2-microglobulin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), β-glucuronidase, and heat shock protein 90 α (cytosolic) class B member 1. Moreover, mouse genomic DNA contamination, reverse transcription control and positive PCR control were also profiled in the kit to ensure the efficacy of the PCR array.

2.3.10.1 RT^2 First Strand kit

Reverse transcription was performed using the *RT^2 First Strand* Kit (Qiagen, CA, USA) following the manufacturer's protocol. For each treatment group, three RNA samples (within each time point) with the highest $A_{260/280}$ ratio (highest integrity) were chosen to be pooled together for the PCR arrays. There was a total of six pooled samples for each treatment group [Sham, Ecig(+nic) and Ecig(-nic)] at two time points (P1 and Week 13). For reverse transcription of RNA, genomic DNA elimination mix was prepared using an RNA concentration of 333.33 ng/µl, 2 µl of Buffer GE and a variable amount of RNase-free water to give a total volume of 10 µl. The genomic DNA elimination mix was incubated at 42°C for five minutes and immediately transferred on ice for one minute. A mixture containing 10 µl of treated DNA elimination mix, 16 µl of 5X Buffer BC3, 4 µl Control P2, 8 µl RE3 reverse transcriptase mix and 12 µl RNase-free water was then used to prepare the reverse-transcription mix. Once all reagents and DNA elimination mix were combined, the reverse-transcription mix was incubated at 42°C for 15 minutes followed by 95°C for five minutes to stop the reaction. Each reaction was left on ice after the addition of 91 µl RNase-free water.

2.3.10.2 RT^2 SYBR Green Mastermix

Amplification of gene expression was performed using the *RT^2 SYBR Green Mastermix* (Qiagen, CA, USA) following the manufacturer's protocol. At room temperature (15-25°C), the PCR components mix consisted of 650 µl 2x RT2 SYBR Green mastermix, 102 µl cDNA synthesis reaction (reverse-transcribed samples) and 548 µl RNase-free water. The components mix was loaded in a 384 (4 x 96) PCR plate provided by the PCR array kit with the Format 'E' plate layout containing the housekeeping gene, genomic DNA control, reverse transcription controls and positive PCR controls (Figure 13). All genes and controls were already included in each well in the form of dried assays. After loading, the PCR plate was sealed with an optical adhesive film

and centrifuged at 1000 g for one minute. Amplification was performed using the *Quant Studio*^{TM} *12K Flex Real-Time* 384 well-plate (Applied Biosystems, CA, USA) using the following cycling conditions found in Table 2. All samples that were tested for PCR array reproducibility, RT efficiency and genomic DNA contamination passed all quality checks. A dissociation melt curve was also performed to ensure PCR specificity. The melt curve cycling condition was performed by increasing the temperature from 65ºC to 95ºC at 2ºC per minute.

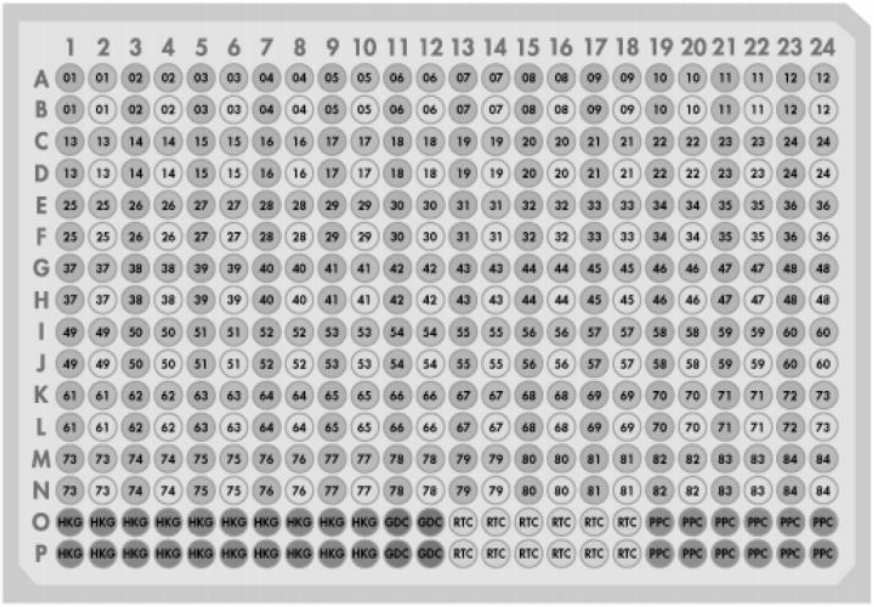

Figure 13. RT^2 Profiler^{TM} PCR Array 384 PCR plate layout for Format 'E'. The PCR plate consists of 4 x 96 replicates of key chromatin epigenetic genes, housekeeping genes (HKG), Genomic DNA control (GDC), Reverse transcription controls (RTC) and positive PCR controls (PPC). Image taken from the RT^2 Profiler PCR array Handbook [289].

Table 2. The cycling condition for cDNA synthesis according to the manufacturer's protocol.

Cycles	Duration	Temperature	Program
1	10 minutes	95ºC	DNA Taq Polymerase activation
40	15 seconds 1 minute	95ºC 60ºC	Fluorescence data collection

The threshold value (C_T) from the PCR run was collected and analysed using the Qiagen PCR Data Analysis Center [290]. C_T values were entered into the Excel® spreadsheet provided by the Qiagen website before being uploaded to the Data Analysis Center for analysis [290]. Fold changes in gene expression levels were calculated based off the methods provided by the Qiagen website. The C_T threshold was set to 35 before gene expression analysis. Gene expression was considered significant if a fold change of greater than ±2 was recorded.

2.3.11 PCR array validation

To verify the PCR array results, real-time PCR was performed for genes that showed significant fold changes in the treatment groups. The full list of primer pairs is listed inTable 3. Changes in gene expression levels were also assessed in all three time points (P1, P20 and Week 13).

Table 3. List of epigenetic genes selected for RT-qPCR validation from the RT^{2}Profiler^{TM} PCR Array results.

Epigenetic chromatin modification enzymes/reference gene	Gene ID	Description	Forward/ Reverse	Primer sequence (5'→3')
Histone Acetyltransferase	Atf2	Activating transcription factor 2	Forward	CTTCACTGATAAAACACGAC
			Reverse	TGTTTATGGACAGCCAAATG
Histone Phosphorylation	AurkA	Aurora kinase A	Forward	CAGAGAACAGCTACTTACATC
			Reverse	GTCTGCAATTCTTCAACTCTC
	AurkB	Aurora kinase B	Forward	AAGTCTCAGATTGAGAAGGAG
			Reverse	GAAGGATGTTGGGATGTTTC
	AurkC	Aurora kinase C	Forward	TCTCTCAGGAGAAAGACAATG
			Reverse	AAACTTAAAATCCACCTGGC
DNA Methyltransferases	Dnmt3a	DNA methyltransferase 3a	Forward	ACCAGAAGAAGAGAAGAATCC
			Reverse	CAATGATCTCCTTGACCTTAG
	Dnmt3b	DNA methyltransferase 3b	Forward	GACTTCATGGAAGAAGTGAC
			Reverse	TATCATCCTGATACTCTGTGC
Histone Demethylases	Kdm5c	Lysine (K)-specific demethylase 5c	Forward	AATGCCCAGTTTATTGAGTC
			Reverse	TAATTGTCATCACAGCCATC
	Kdm6b	Lysine (K)-specific demethylase 6b	Forward	CTGGCATGTTGAAGTAATCAG
			Reverse	TCAAGATGATCAAGTCTGC
Histone Deacetylases	Hdac1	Histone deacetylase 1	Forward	CAATCTGACCATCAAAGGAC
			Reverse	CCGGTCCAAAGTATTCAAAG
Reference gene	GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	Forward	GCTCACTGGCATGGCCTTCCG
			Reverse	GTAGGCCATGAGGTCCACCAC

2.3.11.1 Reverse transcription

First strand cDNA synthesis from RNA extraction was performed using the *Tetro cDNA synthesis* kit (Bioline, MA, USA) according to the manufacturer's protocol. Briefly, a reaction mixture containing random hexamer primer, 10 mM dNTP mix, RT buffer, Ribosafe RNase Inhibitor (10 units/µl), Tetro Reverse Transcriptase (200 units/µl) and RNA (1 µg) in a total volume of 20 µl were incubated using a Thermo Cycler (Bio-Rad, CA, USA) at 25ºC for 10 minutes. This was followed by 45ºC for 30 minutes before the reaction was terminated at 85ºC for 5 minutes. Samples were chilled on ice and stored at -20ºC until real-time PCR amplification was performed.

2.3.11.2 Real time PCR assay

PCR amplification was performed on cDNA using the *SensiFAST™ SYBR® No-ROX* kit (Bioline, CA, USA) according to the manufacturer's protocol. A reaction mixture containing SensiFAST™ SYBR® No-ROX mix, forward and reverse primers (20 pmol each) was added with the cDNA template in a total volume of 20 µl. Amplification was performed on the CFX96 Real-Time System (Bio-Rad, CA, USA) using the following two step cycling; 95ºC for 5 seconds, T_m of a specific primer set for 15 seconds. A melt curve was added at the end of the amplification cycles by increasing the melt temperature from 60ºC at a rate of 1ºC per 20 seconds. Relative changes in mRNA expression levels were determined by the $\Delta\Delta C_T$ method [291] using GAPDH as the housekeeping gene. The average C_T for each treatment group was subtracted to the C_T of the housekeeping gene, this value provides the baseline ΔC_T. Relative mRNA gene expression levels of the gene of interest were then assessed using the formula $2^{-\Delta\Delta CT}$ as a percentage.

2.3.12 Tissue fixation, processing, embedding and cutting

The left hemisphere of the offspring brains were collected at P20 and Week 13 and were fixed in 4% PFA (Sigma, MO, USA) for 24 hours at room temperature. After fixation, brains were rinsed in PBS before being stored in 70% ethanol for one week. Brain tissues were then placed in cassettes and processed using the *Excelsior™ AS Tissue Processor* (Thermo Fisher Scientific, MA, USA) for an overnight process involving two changes of formalin, increasing grades of ethanol and two changes of paraffin wax. After processing, brain tissues were embedded in paraffin wax and cut in coronal sections using a microtome (Thermo Fisher Scientific, MA, USA). Brain sections were cut at a thickness of five microns in coronal sections through the region of interest (ROI). Sections were then left in an oven to dry overnight at 37°C.

2.3.12.1 Tissue staining

For quantification of neurons in the hippocampus and the amygdala, brain tissues from P20 and Week 13 offspring were stained with cresyl violet stain. Slides were placed in two changes of xylene followed by decreasing grades of ethanol (100%, 95%, 70%) for three minutes each. They were then hydrated completely in water for ten minutes. Slides were then immersed in cresyl violet stain [0.1% cresyl violet powder (w/v) (Sigma-Aldrich, MO, USA)] and 0.25% glacial acetic acid (Sigma-Aldrich, MO, USA) (pH 3.4-3.5). for 3½ minutes. Slides were rinsed in water for another five minutes followed by dehydration in two changes of 100% ethanol and two changes of xylene. Finally, slides were cover-slipped using dibutyl phthalate polystyrene xylene and left to dry overnight in the fume hood.

2.3.12.2 Image analysis and neuronal cell counting

The region of the brain at the level of the hippocampus at Bregma -1.9mm to -2.06mm was identified using a mouse brain atlas [292]. High power images of the left cerebral hemisphere sections were taken using the *NanoZoomer-AQ Digital Slide Scanner* (Hamamatsu, Shizuoka, Japan). Digital slides of each brain section were viewed using the *NDP.view 2* software program. Three different regions of the dorsal hippocampus and one region of the lateral amygdala nucleus were identified on each brain and imaged at 40X magnification (Figure 14). The ROI for this study included the pyramidal layer of *cornu ammonis* (CA) 1, CA2, and CA3, and the lateral amygdala nucleus. The area of neurons that were counted had dimensions of 400 µm x 50 µm. This area covered 80% (CA1), 100% (CA2), and 80% (CA3) of the CA regions. Cell counts were performed on the ROI using ImageJ (NIH, NY, USA). Neurons were identified as a large pale cell with dense nucleoli located in the nucleus (Figure 14). All neuronal cell counts were done blind with one representative image per mouse was scored.

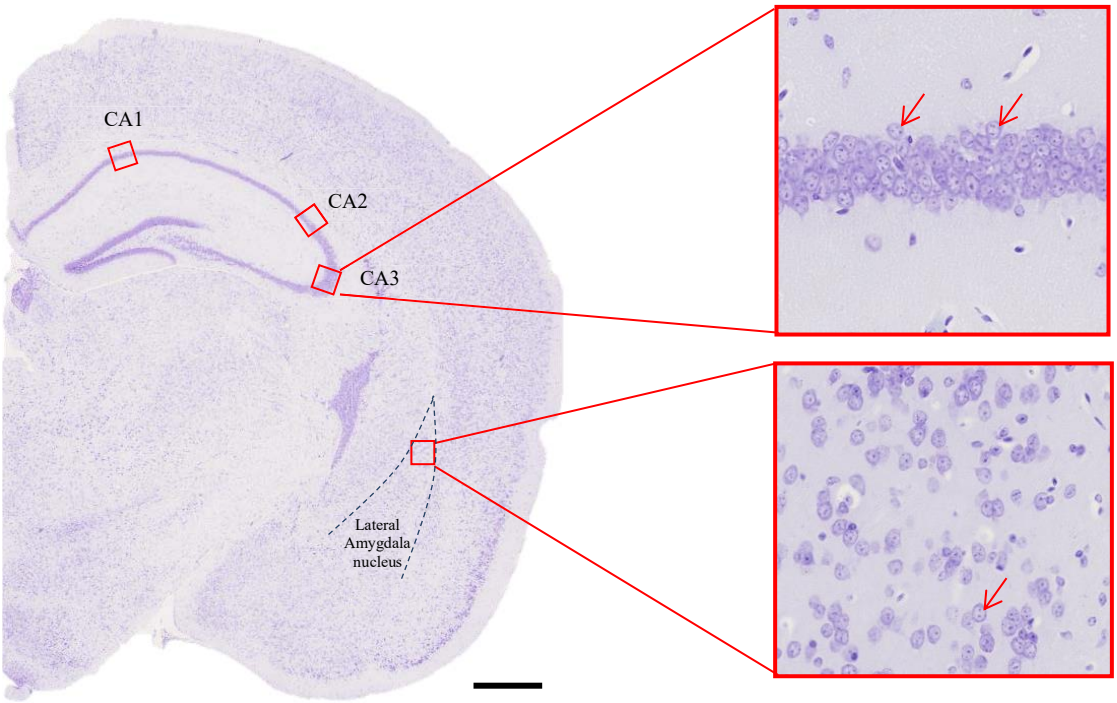

Figure 14. Coronal section of a Week 13 brain stained with cresyl violet showing the dorsal hippocampus and the lateral amygdala nucleus. Red squares show the representative images taken at CA1, CA2, CA3 and the lateral amygdala nucleus. Neurons were recognised by the pale nucleus with multiple nucleoli (arrows). Dotted blue line on the brain section represents the region of the lateral amygdala nucleus Scale bar = 500 µm.

2.3.13 Statistical analysis

All data was normally distributed. One-way analysis of variance (ANOVA) with *Bonferroni's* post-hoc test was used as statistical analysis for the whole-body weight, EPM, global DNA methylation, RT-qPCR and neuronal cell counts. A paired *t*-test was used to analyse the recognition index for the NOR test. All the following results were expressed as mean ± standard deviation and analysed using Prism 7 (GraphPad, CA, USA). Treatment groups were considered significantly different if the *p value* was less than 0.05. Population sizes for each group were between 6-14 samples. Some subjects were excluded from the statistical analysis due to being clear outliers (Unpaired *t*-test; $p < 0.05$) to the rest of the data points. PCR array statistical analysis was performed using the Data Analysis Center on the Qiagen website [290].

2.4 Results

During e-cigarette aerosol exposure, all breeders tolerated the aerosols without any incident. During the birth of the offspring, litter sizes were between 3-6 offspring from each breeder. There was no significant difference between the number in each litter between each treatment group ($p = 0.61$). There we no significant difference in sex ratio between treatment groups ($p = 0.54$).

2.4.1 Plasma cotinine

In order to measure the amount of nicotine being delivered into the offspring, plasma cotinine levels were analysed in P20 offspring. P20 offspring from the Sham and Ecig(-nic) group had low plasma cotinine levels of 2.83±0.63 ng/mL and 3.31±0.57 ng/mL, respectively. In contrast, P20 offspring from the Ecig(+nic) group had a significant increase in plasma cotinine levels of 9.12±1.17 ng/mL compared to the Sham after weaning.

2.4.2 Anthropometry data

Offspring whole body weights were taken at P1, P20 and Week 13. P1 Offspring showed no significant changes in body weight between the Sham (1.6±0.3 g; n = 14), Ecig(+nic) [1.5±0.2 g; n = 15] and Ecig(-nic) [1.6±0.2 g; n = 23] groups (Figure 15A). In the offspring at P20, the average body weight in the Sham group was 10.1±1.0 g (n = 15). Body weight in the Ecig(+nic) group was significantly reduced at 9.4±0.4 g (n = 23) compared to the Sham group ($p<0.001$, n = 15; Figure 15B). In contrast, body weight in the Ecig(-nic) group was significantly increased to 11.2±0.7 g compared to the Sham group ($p<0.01$) and the Ecig(+nic) group ($p<0.05$). Offspring at Week 13 showed no significant difference in body weight in the Sham (26.5±2.0g; n = 14), Ecig(+nic) [26.0±1.2 g; n = 14] and Ecig(-nic) [26.0±1.3 g; n = 14] groups (Figure 15C).

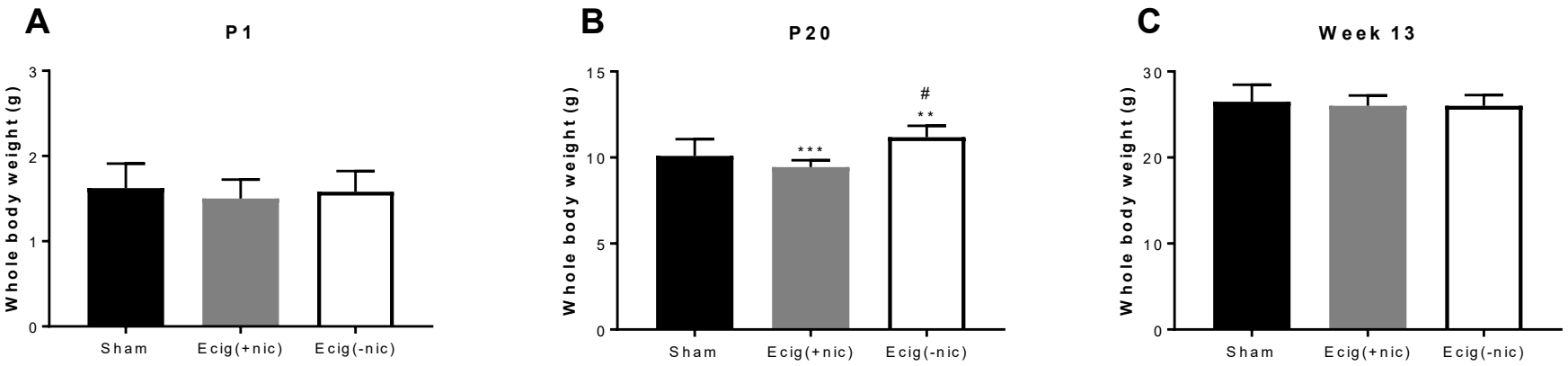

Figure 15. Weights of offspring at (A) postnatal day (P) 1, (B) P20 and (C) Week 13 from the Sham (n = 14), Ecig(+nic) (n = 14) and Ecig(-nic) groups (n = 14). Data presented as mean ± standard deviation, **p<0.01, ***p<0.001 vs. Sham, #p<0.05 vs. Ecig(+nic), Ecig = electronic cigarette, nic = nicotine.

2.4.3 Maternal exposure to e-cigarette aerosol with nicotine causes short-term memory deficits in adult offspring

To analyse short-term memory, the NOR test was conducted on week 12 offspring in each treatment group. The expected NOR result for an unimpaired animal is a significant increase in the *recognition index* between the *familiarisation phase* and the *test phase*. This result was represented in the Sham group (paired *t*-test, $p<0.001$; Figure 16A). Offspring from the Ecig(-nic) group showed a similar result with a significant difference between the familiarisation phase and test phase (paired *t*-test, $p<0.01$; Figure 16C). However, offspring from the Ecig(+nic) group showed no significant difference (Figure 16B) indicating that offspring from mothers exposed to e-cigarette without nicotine lead to short-term memory deficits.

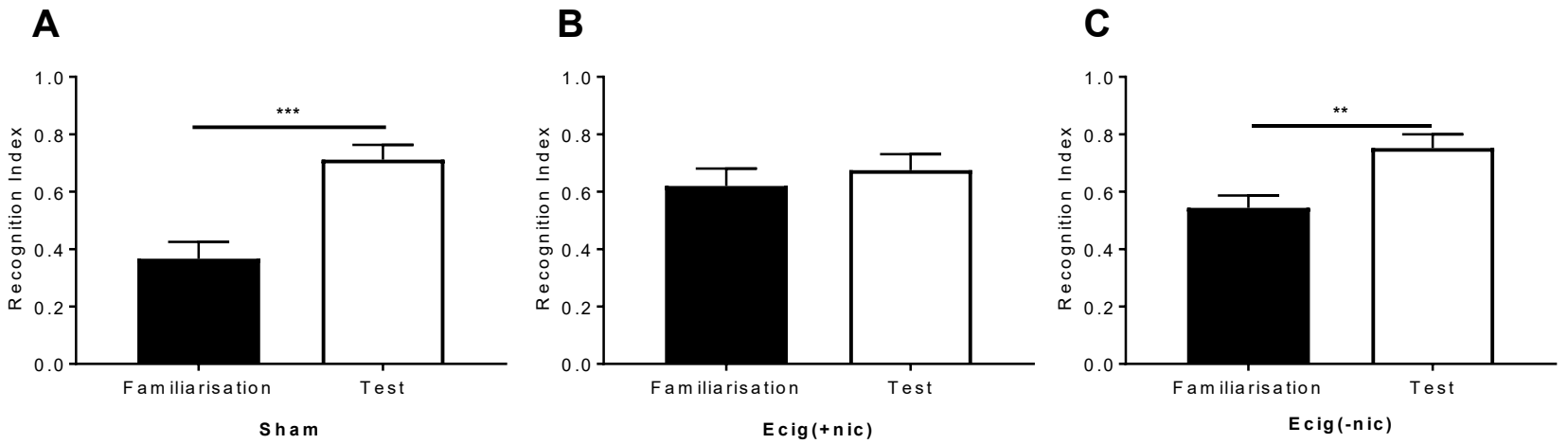

Figure 16. Recognition index from the novel object recognition test in the Sham (n = 14), Ecig(+nic) (n = 14) and Ecig(-nic) groups (n = 14). (A) Offspring exposed to ambient air (Sham, n = 14) showed a significant difference between the familiarisation and test phase. (B) Offspring from the Ecig(+nic) group showed no significant difference between both phases. (C) Offspring from the Ecig(-nic) group showed a significant difference between both phases. Data presented as mean ± standard deviation, paired t-test, **p<0.01, ***p<0.001 vs. Sham. Ecig = electronic cigarette, nic = nicotine.

2.4.4 Maternal exposure to e-cigarette aerosol with nicotine showed reduced anxiety and hyperactivity in adult offspring

To analyse anxiety and exploratory behaviour, the EPM test was conducted on week 12 offspring in each treatment group. The baseline for the percentage of time an unimpaired animal spends in the open arm of the EPM is represented by the Sham group (Figure 17). There was an increase in the time spent in the open arm in both the Ecig(+nic) ($p=0.001$; Figure 17A) and Ecig(-nic) group ($p<0.001$) compared to the Sham group. To investigate exploration, the total number of centre crosses from each animal were analysed (Figure 17B). The results showed a significant increase in the number of centre crosses in the Ecig(+nic) ($p<0.001$) and Ecig(-nic) ($p<0.001$) group compared to the Sham group. This indicates that maternal exposure to e-cigarette aerosols with and without nicotine may cause increases in offspring exploration, indicative of a hyperactivity state.

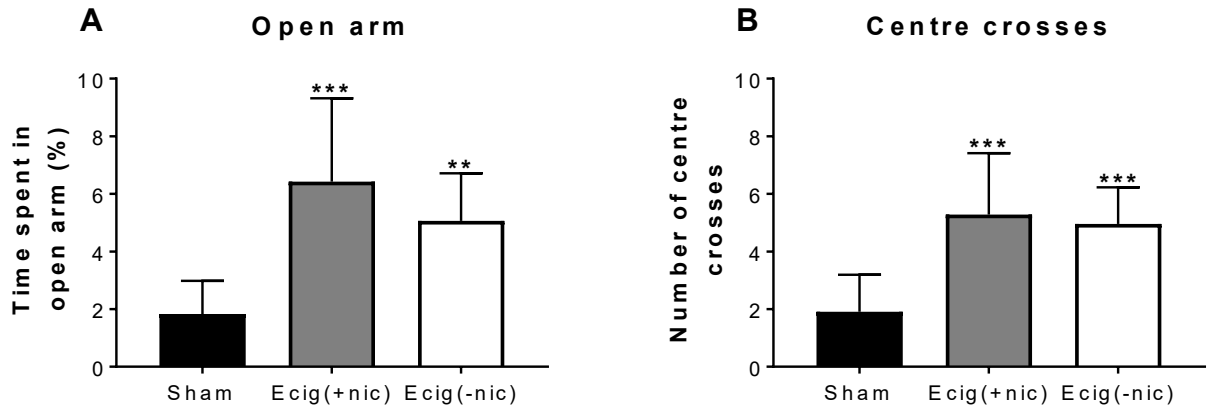

Figure 17. The time spent in the open arm and the number of centre crosses performed by week 12 offspring in the elevated plus maze in the Sham (n = 14), Ecig(+nic) (n = 14) and Ecig(-nic) groups (n = 14). Offspring from the Ecig(+nic) and Ecig(-nic) group showed a significant increase in the amount of time spent in the open arm compared to the Sham group. This pattern was also seen in the number of centre crosses in the Ecig(+nic) and Ecig(-nic) group. Data presented as mean ± standard deviation, **p<0.01, ***p<0.0001vs. Sham. Ecig= electronic cigarette, nic=nicotine.

Other sensitive measures of anxiety such as head dipping and whole body stretches in the closed arm (protected) and open arm (unprotected) were also analysed. The number of head dips and whole-body stretches are characteristics of a mouse exploring and assessing risk in the closed arm (protected) and open arm (unprotected). Therefore, assessing unexplored environments such as the open arm was considered a 'risky' behaviour. From the results, offspring from the Sham group showed minimal exploration in the EPM. However, offspring from the Ecig(+nic) group showed a significant increase in the number of protected head dips compared to offspring from the Sham group ($p<0.001$; Figure 18A) and the Ecig(-nic) group ($p<0.001$). In the unprotected head dips, there was also a significant increase in unprotected head dips from the Ecig(+nic) group ($p<0.01$;Figure 18B) compared to the Sham group. These results indicate that maternal exposure to nicotine from e-cigarette may increase 'risky' behaviour in offspring.

In the protected stretches, there was no significant difference between each treatment group (Figure 18C). In the unprotected stretches, offspring from the Ecig(-nic) group showed a significant increase in the number of unprotected stretches compared to the Sham group ($p<0.001$; Figure 18D). Overall, the EPM results suggest that maternal e-cigarette exposure with and without nicotine results in less anxiety but an increase in exploratory behaviour in the offspring in adulthood, and nicotine may also be encouraging more 'risky behaviour'.

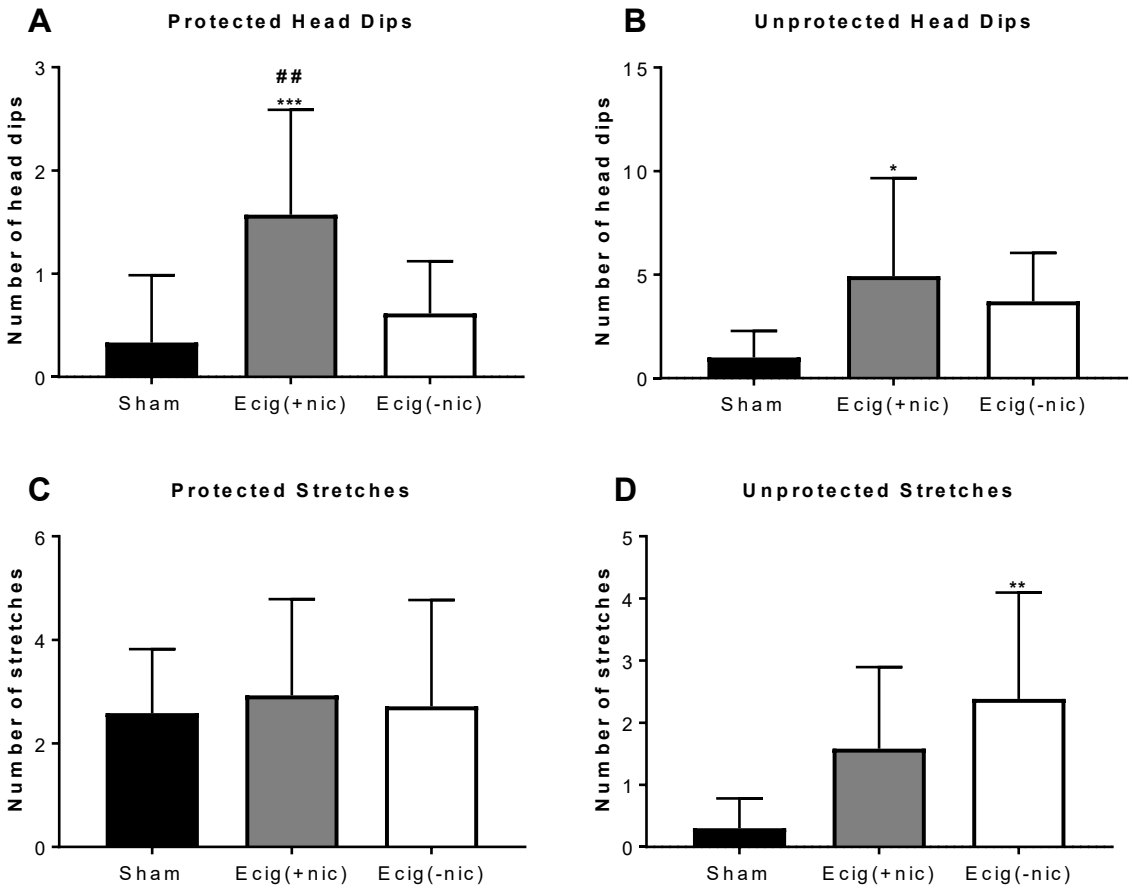

Figure 18. The total number of head dips and whole body stretches in the closed arm (protected) and open arm (unprotected) in offspring from the Sham (n = 14), Ecig(+nic) (n = 14) and Ecig(-nic) groups (n = 14). (A) There was a significant increase in protected head dips from the Ecig(+nic) group compared to the Sham group, and a significant decrease in Ecig(-nic) group compared to the Ecig(+nic) group. (B) There was a significant increase in unprotected head dips in the Ecig(+nic) group compared to the Sham group. (C) There was no significant difference in protected stretches between each treatment group. (D) There was an increase in unprotected stretches in the Ecig(-nic) group compared to the Sham group. Data presented as mean ± standard deviation, *p<0.05, **p<0.01, ***p<0.0001 vs. Sham, ##p<0.01 vs. Ecig(-nic). Ecig= electronic cigarette, nic=nicotine.

2.4.5 Maternal exposure to e-cigarette aerosols without nicotine resulted in changes to global DNA methylation at P1 and P20

Global DNA methylation plays an important role in controlling epigenetic gene expression that can affect development. Global DNA methylation was analysed in offspring brain tissues at three different time points; P1 (right after birth), P20 (right after weaning) and Week 13 (adulthood). At P1, the Sham group had a DNA methylation of 6.5±1.2 %. There was a significant increase in DNA methylation in the Ecig(-nic) group to 9.1±2.0 % compared to the Sham group ($p<0.01$; Figure 19A). No significant difference in DNA methylation was observed in the Ecig(+nic) group (7.2±1.7 %). At P20, DNA methylation was significantly increased in the Ecig(-nic) group (15.7±5.9 %) compared to the Ecig(+nic) group (9.8±1.4 %) ($p<0.05$) and the Sham group (7.2±1.5%) ($p<0.01$) (Figure 19B). The hippocampus is an important region of the brain that is involved in short-term memory retention, and therefore, was a particular region of interest to investigate in the adult offspring. Interestingly, at Week 13 in the hippocampus, there were no significant difference in DNA methylation in the Sham (14.7±2.0 %), Ecig(+nic) (12.0±4.0 %) and Ecig(-nic) groups (13.8±3.5 %) (Figure 19C). No difference in DNA methylation in the hippocampus could be suggestive of age-related effects since the hippocampus were not investigated in the P1 or P20 offspring, and therefore, there were brains from different age-groups to compare it with.

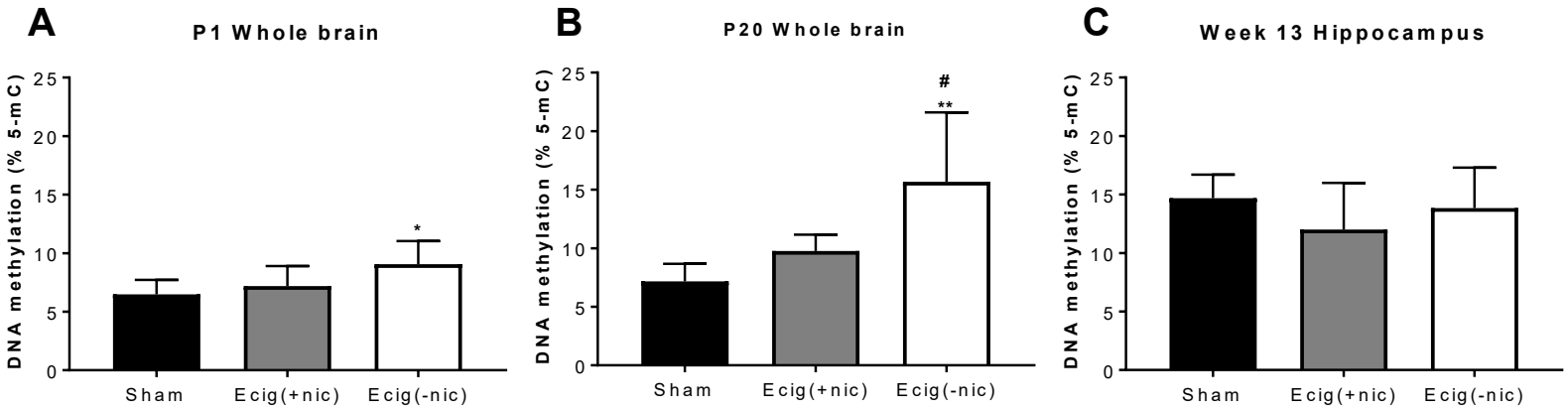

Figure 19. Global DNA methylation in offspring brains at postnatal day 1 (P1), postnatal day 20 (P20) and postnatal Week 13 (n = 10/group). (A) At P1, there was a significant increase in DNA methylation at Ecig(-nic) group compared to the Sham group. (B) At P20, there was a significant increase in DNA methylation in the Ecig(-nic) group compared to the Sham group as well as the Ecig(+nic) group. (C) At Week 13, there was no significant difference between any treatment groups. Data presented as mean ± standard deviation, *p<0.05, **p<0.01 vs. Sham, #p<0.05 vs. Ecig(+nic). Ecig= electronic cigarette, nic=nicotine.

2.4.6 E-cigarette aerosols induce changes in the mRNA expression profile of chromatin modification enzyme-related genes

Given the changes observed in the overall global DNA methylation, key chromatin enzymes involved in epigenetic modification were investigated using the *Mouse Epigenetic Chromatin Modification RT²Profiler™ PCR Array* (Qiagen, CA, USA). Changes in chromatin modification enzymes were investigated at P1 and Week 13. Due to limited resources and constraints, pooled (n = 3 from each group were combined as n = 1) samples from these two time-points were first chosen to obtain a broad overview of gene changes that were occurring in the epigenome of the whole brain and the hippocampus. At P1 in the whole brain, there were a total of eight chromatin modification enzymes that had significant fold changes of more than ± 2 fold (Figure 20). In the Ecig(+nic) group at P1, significant epigenetic gene changes were observed for Smyd1, AurkC and Setd3 by a fold change of -2.1, 4.0 and 30.3, respectively. In the Ecig(-nic) group at P1, significant epigenetic gene changes were observed for Carm1, Atf2, Esco1, AurkA, AurkC, Ash1l and Setd3 by a fold change of 2.3, 3.1, 2.2, 2.1, 3.6, 2.3 and 31.1, respectively. At Week 13 in the hippocampus, there were a total of seven chromatin modification enzymes that had significant fold changes of ± 2 folds (Figure 21). In the Ecig(+nic) group at Week 13, significant epigenetic changes were observed for Dnmt3a, Dnmt3b, Smyd1, Csrp2bp, Kdm5c, AurkB and AurkC by a fold change of 2.2, 2.4, 2.5, 31.1, 2.7, -2.1, and 3.7, respectively. In the Ecig(-nic) group at Week 13, significant epigenetic changes were observed for Csrp2bp, AurkB and AurkC by a fold change of 16.4, -9.2, and -2.1, respectively.

Postnatal Day 1		Ecig (+nic)	Ecig (-nic)			Ecig (+nic)	Ecig (-nic)
DNA Methyltransferases	Dnmt1	1.1	-1.0	Histone Demethylases	Kdm1a	1.2	1.1
	Dnmt3a	-1.3	1.8		Kdm4a	1.2	1.2
	Dnmt3b	1.0	2.0		Kdm4c	1.0	1.1
Histone Methyltransferases	Carm1	1.1	2.3		Kdm5b	1.2	1.2
	Dot1l	-1.0	-1.7		Kdm5c	-1.1	1.1
	Ehmt1	1.0	1.5		Kdm6b	1.1	1.1
	Ehmt2	1.0	1.5	Histone Phosphorylation	AurkA	1.1	2.1
	Prmt1	1.1	1.0		AurkB	-1.2	1.7
	Prmt2	-1.2	-1.1		AurkC	4.0	3.6
	Prmt3	1.1	1.3		Nek6	1.2	1.1
	Prmt5	1.1	1.3		Pak1	1.3	1.1
	Prmt6	1.1	-1.0		Rps6ka3	1.1	1.2
	Prmt7	1.2	1.2		Rps6ka5	1.1	1.2
	Prmt8	1.1	1.1	Histone Ubiquitination	Dzip3	1.2	1.3
	Setdb2	-1.2	-1.3		Mysm1	1.1	1.2
	Smyd1	-2.1	1.0		Rnf2	1.6	1.3
	Smyd3	1.1	1.2		Rnf20	1.2	1.3
	Suv39h1	1.2	1.1		Ube2a	1.3	1.2
Histone Acetyltransferase	Atf2	1.4	3.1		Ube2b	1.5	1.3
	Cdyl	-1.1	2.0		Usp16	1.3	1.4
	Ciita	-1.5	1.9		Usp21	1.0	1.3
	Csrp2bp	-1.1	1.7		Usp22	1.2	1.2
	Esco1	1.0	2.2	SET Domain Proteins	Ash1l	-1.0	2.3
	Esco2	1.1	2.0		Nsd1	1.2	1.1
	Hat1	1.1	1.2		Setd1a	-1.1	1.0
	Kat2a	-1.0	-1.0		Setd1b	1.1	1.1
	Kat2b	1.0	1.1		Setd2	1.4	1.4
	Kat5	1.0	-1.1		Setd3	30.3	31.1
	Ncoa1	1.5	1.3		Setd4	1.1	1.0
	Ncoa3	-1.2	-1.1		Setd5	1.1	1.0
	Ncoa6	1.3	1.4		Setd6	1.5	1.4
Histone Deacetylases	Hdac1	1.1	1.0		Setd7	1.2	1.4
	Hdac2	1.3	1.0		Setd8	1.3	1.8
	Hdac3	-1.2	-1.2		Setdb1	1.2	1.2
	Hdac4	-1.3	1.2		Suv420h1	1.2	1.4
	Hdac5	1.2	1.2		Whsc1	1.2	1.7
	Hdac6	-1.1	1.0	Other genes associated with epigenetics	Kmt2c	1.3	1.3
	Hdac7	1.1	1.3		Kmt2e	1.2	1.3
	Hdac8	1.0	1.1		Kat8	1.1	1.1
	Hdac9	1.1	-1.2		Kat7	1.3	1.3
	Hdac10	1.1	1.0		Kat6a	1.2	1.2
	Hdac11	-1.4	-1.3		Kat6b	1.3	1.3

Figure 20. Heat map of epigenetic gene fold changes (ΔΔCt) in whole brains of P1 offspring in the Ecig(+nic) (n = 3, pooled) and Ecig(-nic) groups (n = 3, pooled) normalised to the Sham group (n = 3, pooled). Fold changes of more than ± 2 fold were considered significantly different. Colour scale was based on the lowest and highest fold in gene changes.

Week 13		Ecig (+nic)	Ecig (-nic)			Ecig (+nic)	Ecig (-nic)
DNA Methyltransferases	Dnmt1	-1.1	-1.2	**Histone Demethylases**	Kdm1a	1.4	-1.0
	Dnmt3a	2.2	-1.6		Kdm4a	1.5	-1.2
	Dnmt3b	2.4	1.3		Kdm4c	1.1	1.0
Histone Methyltransferases	Carm1	1.4	-1.1		Kdm5b	1.4	1.1
	Dot1l	1.4	-1.6		Kdm5c	2.7	-1.3
	Ehmt1	1.3	-1.1		Kdm6b	1.7	-1.4
	Ehmt2	1.7	-1.3	**Histone Phosphorylation**	AurkA	1.3	-1.3
	Prmt1	1.3	-1.1		AurkB	-2.1	-9.2
	Prmt2	1.4	-1.3		AurkC	3.7	-2.1
	Prmt3	1.2	-1.2		Nek6	1.4	1.3
	Prmt5	1.5	1.1		Pak1	1.1	-1.2
	Prmt6	1.6	1.1		Rps6ka3	1.6	-1.1
	Prmt7	1.2	1.2		Rps6ka5	1.5	-1.6
	Prmt8	1.3	-1.0	**Histone Ubiquitination**	Dzip3	1.2	1.1
	Setdb2	1.0	-1.1		Mysm1	1.1	-1.2
	Smyd1	2.5	-1.1		Rnf2	1.0	1.2
	Smyd3	1.4	-1.3		Rnf20	1.4	1.1
	Suv39h1	1.4	-1.2		Ube2a	-1.1	1.1
Histone Acetyltransferase	Atf2	1.2	1.4		Ube2b	1.0	1.2
	Cdyl	1.4	1.0		Usp16	1.1	1.1
	Ciita	1.4	-1.6		Usp21	1.2	1.1
	Csrp2bp	31.1	16.4		Usp22	1.3	-1.3
	Esco1	1.0	1.0	**SET Domain Proteins**	Ash1l	1.3	-1.0
	Esco2	1.6	1.5		Nsd1	1.5	-1.1
	Hat1	1.1	1.3		Setd1a	1.6	-1.4
	Kat2a	1.5	1.0		Setd1b	1.6	-1.0
	Kat2b	1.2	1.1		Setd2	1.4	1.2
	Kat5	1.4	-1.0		Setd3	1.5	1.1
	Ncoa1	1.5	-1.0		Setd4	1.6	-1.1
	Ncoa3	1.3	-1.6		Setd5	1.7	-1.3
	Ncoa6	1.5	1.1		Setd6	1.2	1.2
Histone Deacetylases	Hdac1	-1.1	-1.8		Setd7	1.1	-1.2
	Hdac2	1.1	1.0		Setd8	1.2	1.1
	Hdac3	1.2	-1.2		Setdb1	1.5	-1.0
	Hdac4	1.2	-1.2		Suv420h1	1.2	-1.1
	Hdac5	1.4	-1.1		Whsc1	1.4	1.1
	Hdac6	1.4	1.1	**Other genes associated with epigenetics**	Kmt2c	1.6	-1.0
	Hdac7	1.3	1.0		Kmt2e	1.6	1.1
	Hdac8	1.7	1.0		Kat8	1.2	-1.3
	Hdac9	1.2	1.1		Kat7	1.4	-1.1
	Hdac10	1.7	-1.2		Kat6a	1.4	-1.0
	Hdac11	1.5	-1.2		Kat6b	1.1	-1.1

Figure 21. Heat map of epigenetic gene fold changes (ΔΔCt) in the hippocampus of Week 13 offspring in the Ecig(+nic) (n = 3, pooled) and Ecig(-nic) groups (n = 3, pooled) normalised to the Sham group (n = 3, pooled). Fold changes of more than ± 2 fold were considered significantly different. Colour scale was based on the lowest and highest fold in gene changes.

2.4.7 Validation of epigenetic genes from the chromatin modification enzyme PCR array

The epigenetic genes that showed significant fold changes and are associated with neurological development, behaviour, neurodegeneration and cell division were identified from the PCR array. Although there were not many significant gene changes in the hippocampus, this does not necessarily rule out other forms of epigenetic gene changes. To verify the findings from the PCR array data, mRNA levels of the epigenetic genes that showed significant fold changes were measured by real-time PCR. These changes include chromatin modification enzymes such as DNA methyltransferases (Dnmt3a, Dnmt3b), histone-lysine demethylases (Kdm5c, Kdm6b), histone acetyltransferases (Atf2, Hdac1) and histone phosphorylation (AurkA, AurkB, AurkC) were validated in all treatment groups at each time point i.e. the right hemisphere from P1 and P20 offspring, and the hippocampus from Week 13 offspring. In this section, the right hemisphere is referred to as the 'whole brain' for the P1 and P20 offspring and the 'hippocampus' for the Week 13 offspring.

2.4.7.1 Maternal exposure to e-cigarette aerosols showed changes in Dnmt3a and Dnmt3b gene expression in offspring at P20 and Week 13

DNA methyltransferases, Dnmt3a and Dnmt3b, are involved in *de novo* DNA methylation which is important in determining the expression of genes in the genome [293]. RT-qPCR results for the P1 whole brain showed no significant difference in Dnmt3a and Dnmt3b gene expression in the Ecig(+nic) and Ecig(-nic) groups compared to the Sham group (Figure 22A&D). However, for the P20 whole brain, there was a decrease in Dnmt3a gene expression in the Ecig(+nic) group to 60.2±21.5 % compared to the Sham group ($p<0.05$; Figure 22B) and the Ecig(-nic) group ($p<0.001$). Similarly, the Ecig(+nic) group showed a significant decrease to 55.6±12.9 % in Dnmt3b gene expression compared to the Sham group ($p<0.001$; Figure 22E).

In the Week 13 hippocampus, there was no significant difference in Dnmt3a gene expression in any of the treatment groups (Figure 22C). However, Dnmt3b gene expression was found to be significantly decreased in the Ecig(+nic) group to 66.6±8.7 % compared to the Sham group ($p<0.001$; Figure 22F).

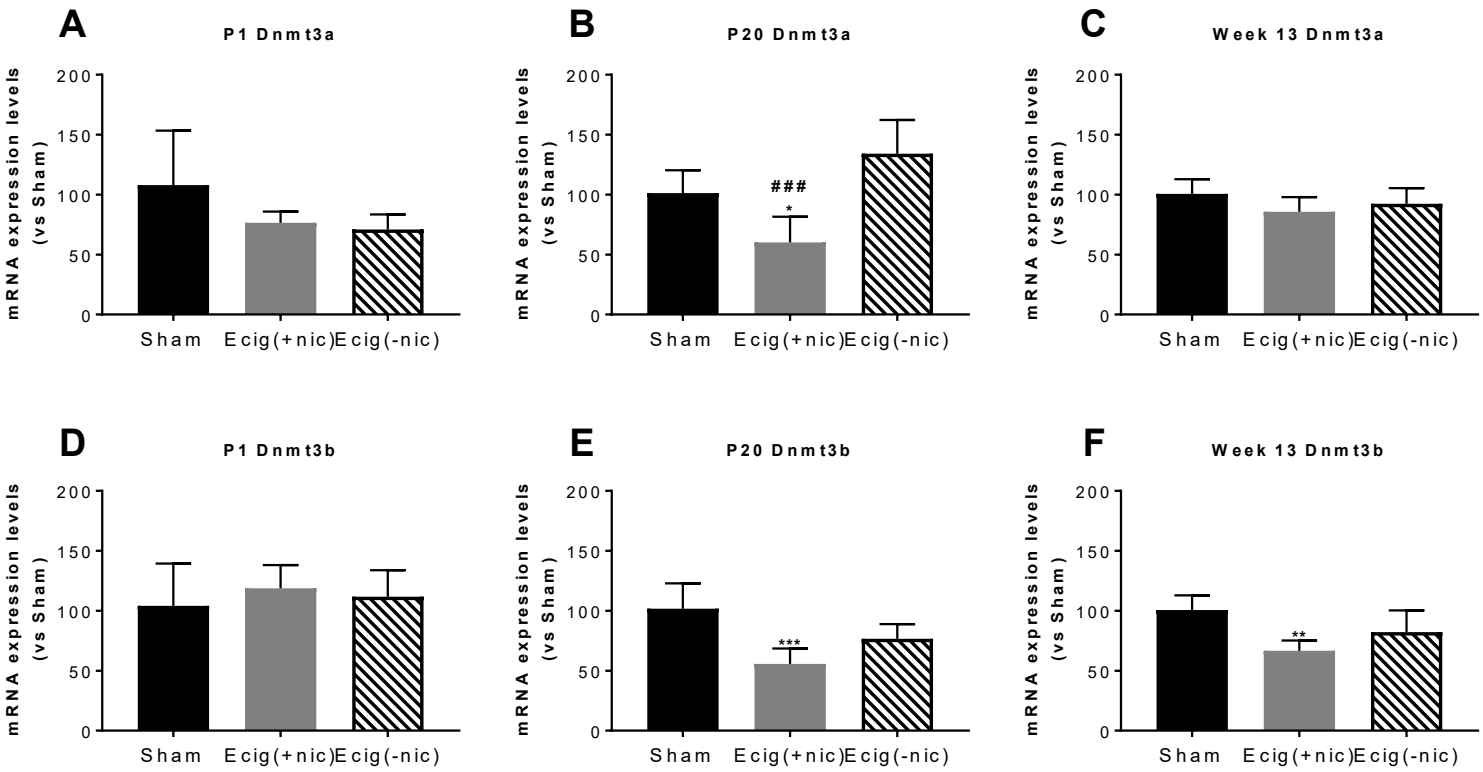

Figure 22. Real-time PCR verification of DNA methyltransferases (Dnmt3a and Dnmt3b) mRNA expression levels of offspring at postnatal day 1 (P1) whole brains, postnatal day 20 (P20) whole brains and Week 13 hippocampus. mRNA expression levels of the Ecig(+nic) (n = 6) and Ecig(-nic) groups (n = 6) were expressed as a percentage compared to the Sham group (n = 6). Data presented as mean ± standard deviation, *p<0.05, **p<0.01, ***p<0.0001 vs. Sham, ###p<0.001 vs. Ecig(-nic). Ecig= electronic cigarette, nic=nicotine.

2.4.7.2 Maternal exposure to e-cigarette aerosols showed changes in Kdm5c and Kdm6b gene expression in offspring at P20 and Week 13

Histone-lysine demethylases, Kdm5c and Kdm6b, are chromatin modification enzymes that are important in the maintenance of transcriptional enzymes to different regions of the DNA [294]. This process happens when histone-lysine demethylases remove a methyl group from histones in the DNA. In the P1 whole brain, there was no significant difference in Kdm5c and Kdm6b gene expression between each treatment group (Figure 23A&D). In the P20 whole brain, there was a significant increase in Kdm5c gene expression in the Ecig(-nic) group to 141.8±31.1 % compared to the Ecig(+nic) group ($p<0.05$; Figure 23B). This result was also consistent in the Kdm6b gene expression in the Ecig(-nic) group where Kdm6b gene expression was increased to 123.3±20.9 % compared to the Ecig(+nic) group ($p<0.001$; Figure 23E). In the Week 13 hippocampus, there was a significant decrease in Kdm5c gene expression in the Ecig(+nic) group to 64.7±10.4 % ($p<0.01$; Figure 23C) and Ecig(-nic) group to 63.1±10.3 % ($p<0.01$) compared to the Sham group. No significant difference in Kdm6b gene expression was found at Week 13 (Figure 23F).

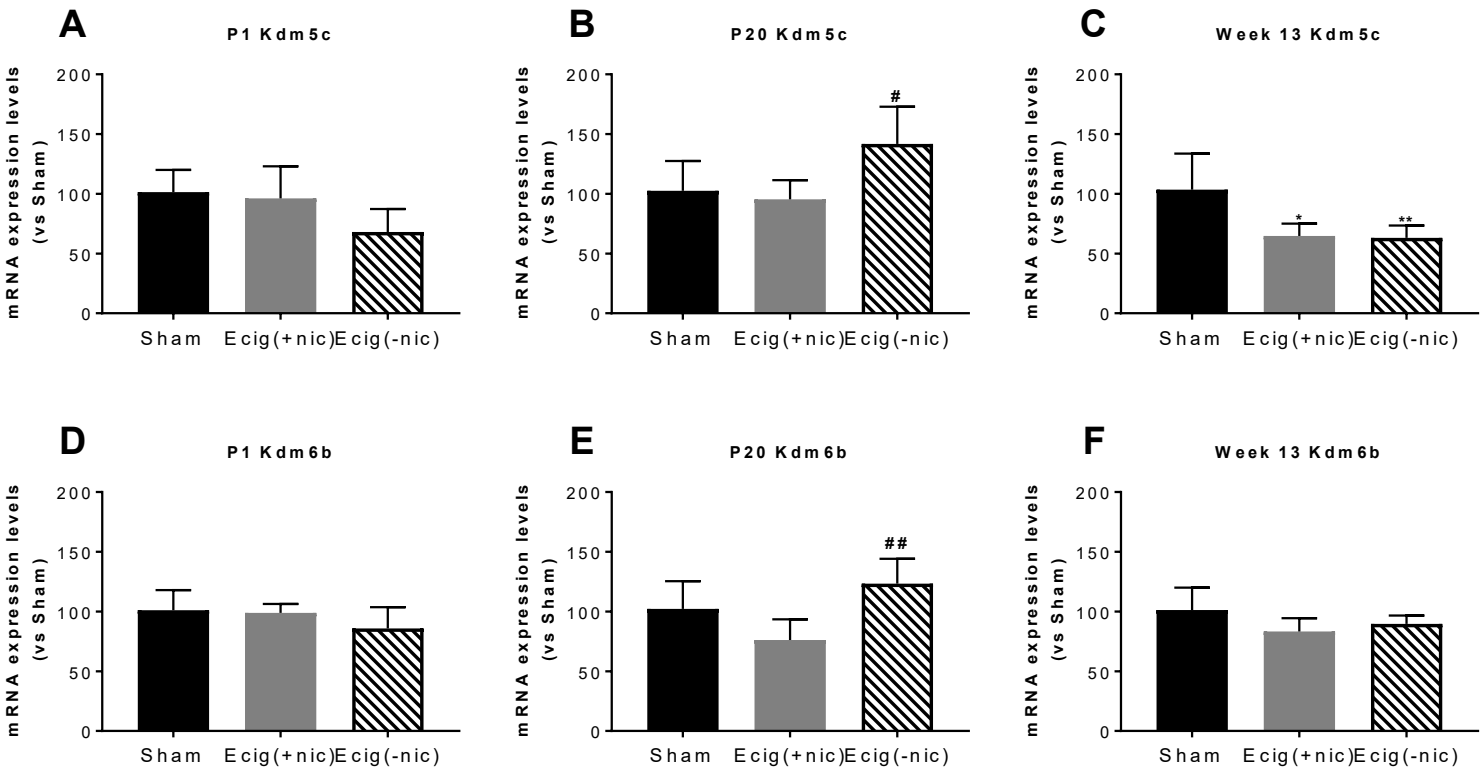

Figure 23. Real-time PCR verification of histone-lysine demethylases (Kdm5c and Kdm6b) mRNA expression levels of offspring at postnatal day 1 (P1) whole brains, postnatal day 20 (P20) whole brains and Week 13 hippocampus. mRNA expression levels of the Ecig(+nic) (n = 6) and Ecig(-nic) groups (n = 6) were expressed as a percentage compared to the Sham group (n = 6). Data presented as mean ± standard deviation, *p<0.05, **p<0.01 vs. Sham, #p<0.05, ##p<0.01 vs. Ecig(+nic). Ecig= electronic cigarette, nic=nicotine.

2.4.7.3 Maternal exposure to e-cigarette aerosols showed changes in Atf2 gene expression in offspring but not Hdac1

Histone acetyltransferases, like Atf2, is a type of chromatin modification enzyme that is important in transcription activation in a sequence specific manner and is important in neuronal development [295, 296]. Atf2 was also found to have a role in neuronal development. In the P1 whole brain, there was a significant decrease in Atf2 gene expression in the Ecig(+nic) group to 80.6±7.5 % ($p=0.001$; Figure 24A) and Ecig(-nic) group to 76.2±7.9 % ($p<0.001$) compared to the Sham group. In the P20 brain tissue, there was a significant increase in Atf2 gene expression in the Ecig(-nic) group to 125.7±10.1 % compared to the Ecig(+nic) group ($p<0.001$; Figure 24B), but no significant difference compared to the Sham group. In the Week 13 hippocampus, there was a significant decrease in Atf2 gene expression in the Ecig(+nic) group to 90.7±3.9 % ($p<0.05$; Figure 24C) and Ecig(-nic) group to 87.4±4.3 % ($p<0.001$) compared to the Sham group.

In contrast to Atf2, histone deacetylases, like Hdac1, remove acetyl groups from the histone on the DNA. From the results, there was no significant difference between treatment groups at all time points (Figure 24D-F).

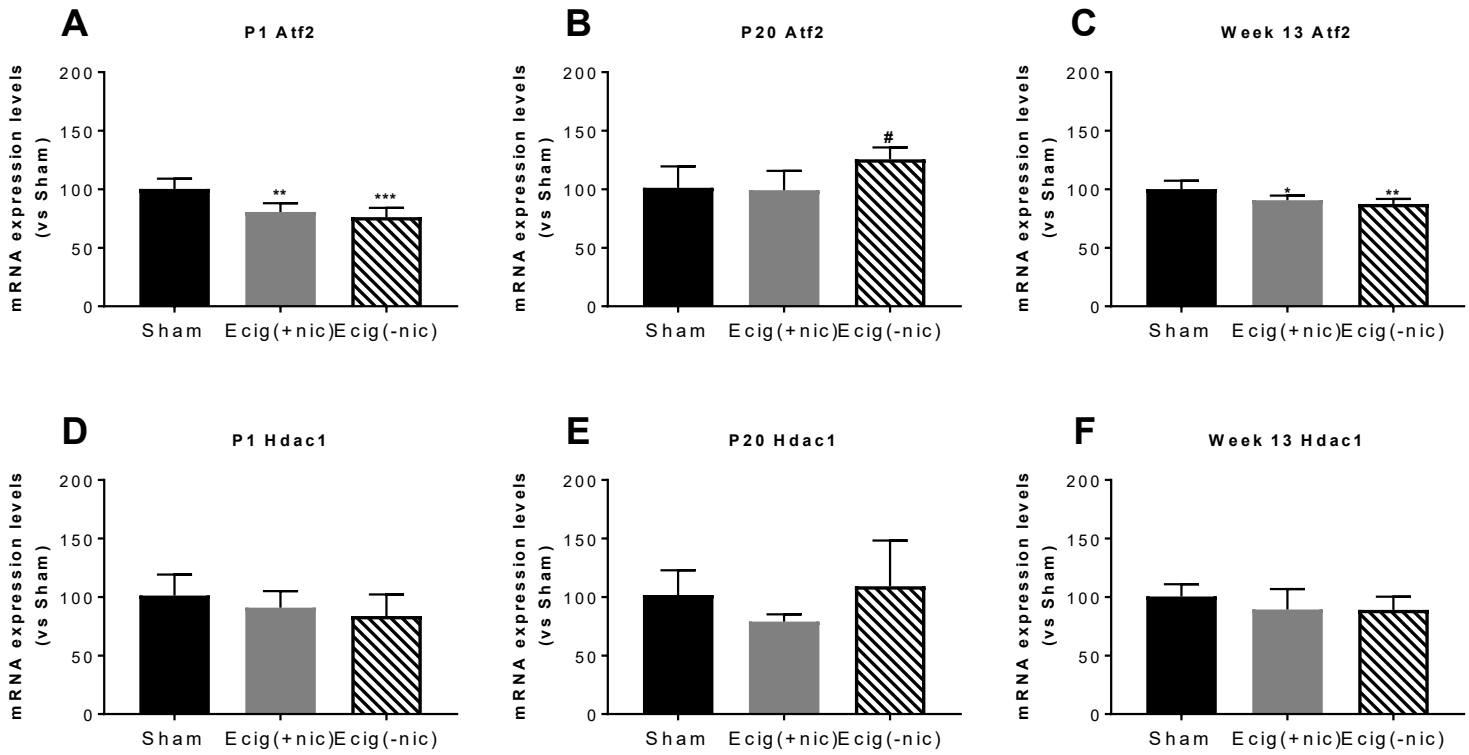

Figure 24. Real-time PCR verification of histone acetyltransferase (Atf2) and histone deacetylase (Hdac1) mRNA expression levels in offspring at postnatal day 1 (P1) whole brains, postnatal day 20 (P20) whole brains and Week 13 hippocampus. mRNA expression levels of the Ecig(+nic) (n = 6) and Ecig(-nic) groups (n = 6) were expressed as a percentage compared to the Sham group (n = 6). Data presented as mean ± standard deviation, *p<0.05, **p<0.01, ***p<0.001 vs. Sham, #p<0.05 vs. Ecig(+nic). Ecig= electronic cigarette, nic=nicotine.

2.4.7.4 Maternal exposure to e-cigarette aerosols showed an overall decrease in AurkA, AurkB and AurkC gene expression in offspring

Histone phosphorylation, like AurkA, AurkB and AurkC, are important in chromatin condensation, chromatin alignment and chromatin segregation through phosphorylation of the histone on the DNA [297]. In the P1 whole brain, there was a significant decrease in AurkA gene expression in the Ecig(-nic) group to 51.3±11.9 % compared to the Sham group ($p=0.001$; Figure 25A) and the Ecig(+nic) group ($p<0.05$). In addition, there was a significant decrease in AurkB gene expression at P1 in Ecig(+nic) group to 62.1±11.1 % ($p<0.001$; Figure 25D) and Ecig(-nic) group to 63.2±4.8 % ($p<0.001$; Figure 25D) compared to the Sham group. No significant difference was found in AurkC gene expression at P1 (Figure 25G).

In the P20 whole brain, there was a significant decrease in AurkA gene expression in the Ecig(-nic) group to 64.6±8.7 % compared to the Sham group ($p<0.001$; Figure 25B). In addition, there was a significant decrease in AurkB expression in the Ecig(+nic) group to 81.0±14.0 % compared to the Sham group ($p<0.001$; Figure 25E). Similarly, AurkC gene expression in the Ecig(+nic) group was significantly decreased to 57.2±15.0 % compared to the Sham group ($p<0.001$; Figure 25H) and Ecig(-nic) group ($p<0.05$). Finally, in the Week 13 hippocampus, there were no changes AurkC gene expression between treatment groups (Figure 25I).

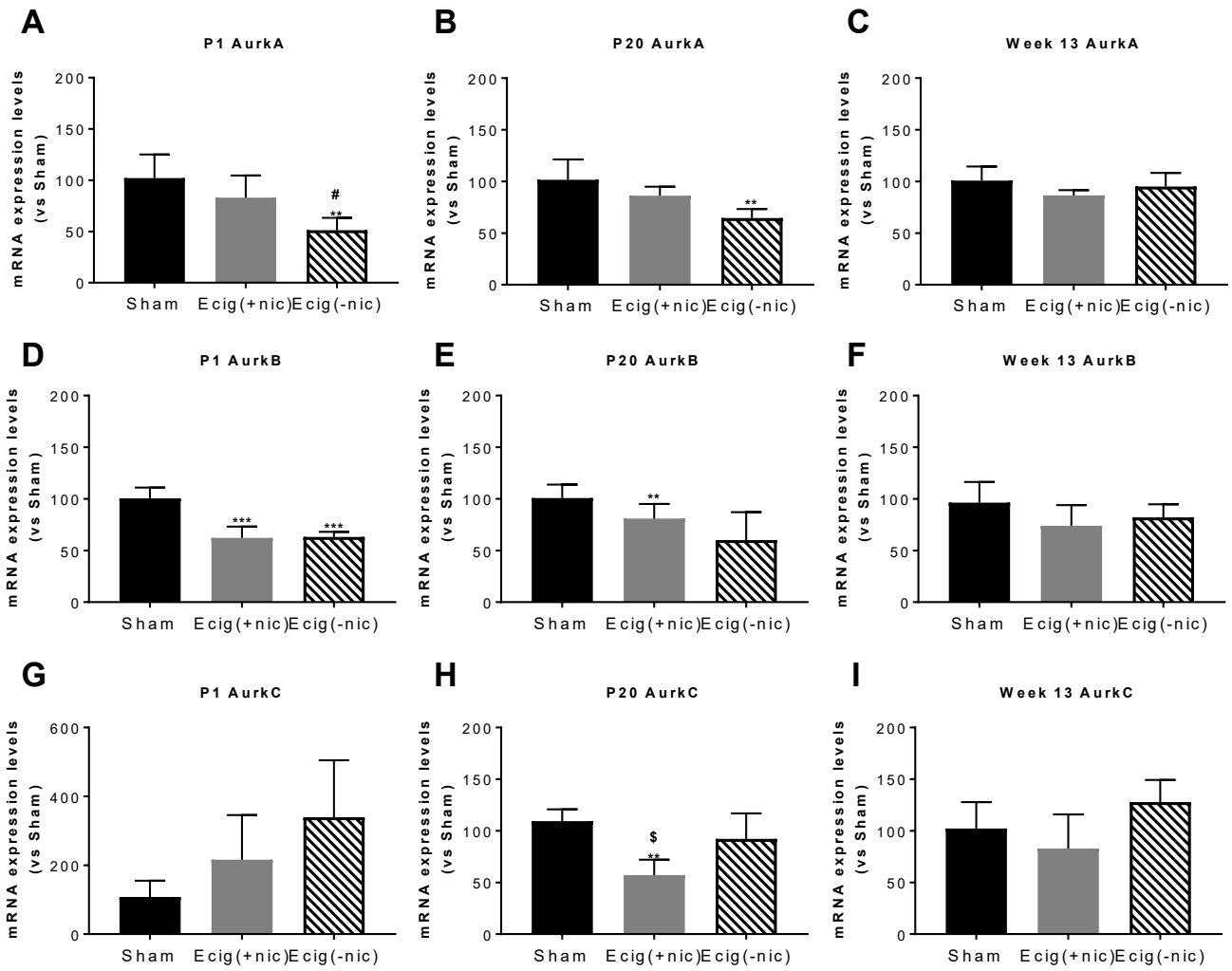

Figure 25. Real-time PCR verification of histone phosphorylation (AurkA, AurkB, AurkC) mRNA expression levels of offspring at postnatal day 1 (P1) whole brains, postnatal day 20 (P20) whole brains and Week 13 hippocampus. mRNA expression levels of the Ecig(+nic) (n = 6) and Ecig(-nic) groups (n = 6) were expressed as a percentage compared to the Sham group (n = 6). Data presented as mean ± standard deviation, **p<0.01, ***p<0.001 vs. Sham, #p<0.05 vs. Ecig(+nic), $p<0.05 vs. Ecig(-nic). Ecig= electronic cigarette, nic=nicotine.

2.4.8 Offspring from mothers exposed to e-cigarette aerosols showed no changes in neuronal cell counts at P20 and Week 13

Neurons from representative areas of the dorsal hippocampus and the dorsal-lateral end of the amygdala were counted in offspring from each treatment group at P20 and Week 13 (Figure 26). Neuronal counts in the P1 brains were not completed since cutting the size of the P1 brains were deemed technically difficult. At the dorsal hippocampus and the dorsal-lateral amygdala of the P20 and Week 13 offspring brain, there were no significant differences in regions CA1, CA2, CA3 and the amygdala in any of the treatment groups (Figure 26A-H).

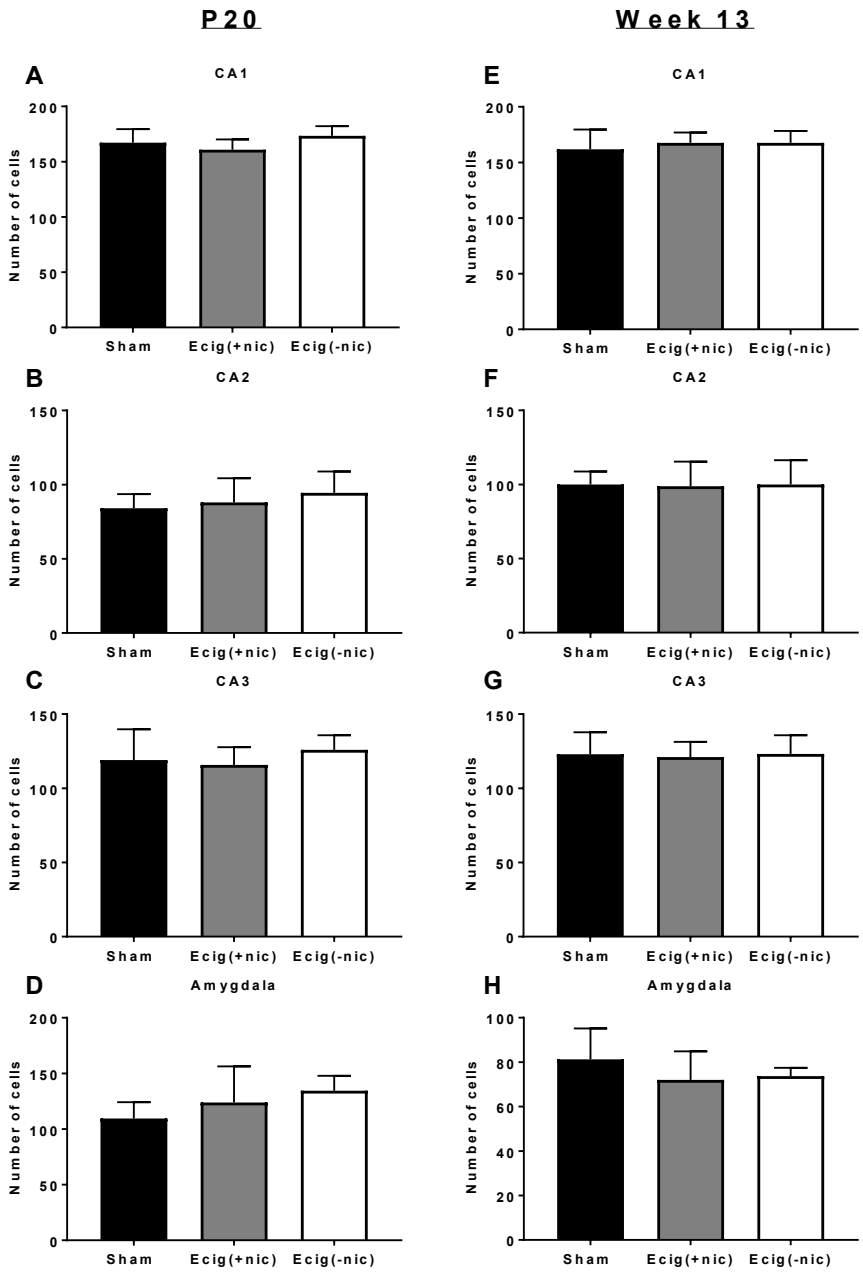

Figure 26. Neuronal cell counts of pyramidal neuronal cells in the postnatal day 20 (P20) [n = 8] and Week 13 offspring [n = 8] dorsal hippocampus and the lateral amygdala nucleus. Neuronal cells were counted at (A) CA1, (B) CA2, (C) CA3 and (D) the amygdala at postnatal day 20, and (E) CA1, (F) CA2, (G) CA3 and (H) the amygdala at Week 13 in offspring from the Sham group, Ecig(+nic) group and Ecig(-cig) group. Data represented as cells/region of interest; mean ± standard deviation, one-way ANOVA with Bonferroni post-hoc test.

2.5 Discussion

Smoking tobacco cigarettes has been known to be associated with a number of health conditions during prenatal development on offspring in animal models [196, 197, 281, 298-300] and human studies [203, 301-304]. Prenatal exposure to nicotine has been widely known to effect neurological outcomes in children such as ADHD, aggressiveness, decreased social behaviour, anxiety and anti-social behaviour [278-280]. There is not much known about the neurological effects of e-cigarettes, particularly, the effects in the offspring after maternal exposure. Therefore, this study investigated the effects of intrauterine e-cigarette exposure on behaviour and epigenetics in offspring.

2.5.1 Offspring from mothers exposed to e-cigarette aerosols with nicotine have a decrease in whole body weight, and without nicotine, had an increase in body weight

A number of prenatal nicotine exposure studies have been reported in the literature [305-309]. Zhu and colleagues showed a decrease in offspring body weight after mothers were exposed to nicotine via drinking water [309]. Lee and colleagues also showed a reduction in offspring weight compared to the non-nicotine exposed offspring after prenatal nicotine exposure [305]. From this study, the whole-body weight data showed that offspring from intrauterine exposure to e-cigarette aerosol with nicotine had a reduced body weight at weaning only. This is suggestive of the intake of nicotine from offspring *in utero*, nicotine from the mother's fur and nicotine through the mother's breastmilk. Interestingly, offspring from mothers exposed to e-cigarette aerosols without nicotine showed an increase in body weight at weaning. While only whole-body weight was measured in the current study, we have reported an increase in retroperitoneal fat and epididymal fat in offspring from mothers exposed to e-cigarette aerosols without nicotine (data from a previous study [48]). This could suggest that there is an additional fat being deposited in the

offspring and, therefore, an increased intake of lipids from the oil-based aerosols. This data was also consistent with a number of studies that investigated lipids after e-cigarette aerosol exposure. A study conducted by Cardinia and colleagues investigated the lipid profile of rats exposed to e-cigarette aerosols and found that there was an increase in lipid infiltration in the brain after eight weeks of e-cigarette exposure [52]. Another study that measured plasma triglycerides from rats after intraperitoneal injections of e-liquid showed elevated triglycerides in rat plasma [190]. Although the mechanisms for an increase in body weight and fat are unknown, this may suggest that there is a disturbance in the offspring metabolism from intrauterine exposure to e-cigarette aerosols that could possibly lead to metabolic disorders.

2.5.2 Offspring from mothers exposed to e-cigarette aerosols shows short-term memory deficits, increase risk-taking, increase exploration and less anxiety-like behaviour

From the behavioural results, only offspring from mothers exposed to e-cigarette with nicotine showed signs of short-term memory deficits. Two reasons can be reasonably suggested for this: 1) The nicotine from the e-cigarette aerosol is having an effect on offspring short-term memory or 2) The addition of nicotine to the e-liquid is having a culmination effect. Nicotine has been known to increase alertness and memory retention in normal rats after behavioural assessment in the NOR [310, 311]. However, the effects of nicotine on the developing brain varies depending on which period during gestation the mothers were exposed [312]. Zhang and colleagues investigated the effects of prenatal nicotine exposure in offspring and found ADHD-like behaviour in the offspring in a sex-dependent manner, however, no changes to short-term memory were observed [312]. Zhu and colleagues investigated the effects of prenatal exposure on offspring behaviour and found hyperactivity in the offspring after observation during the active night phase of the day, however, nicotine exposure was through the drinking water and not inhalation [309]. Lee and colleagues also found anxiety-like behaviour and impulsive decision

making in offspring after prenatal exposure in the dams [305]. In contrast, prenatal exposure to nicotine via subcutaneous perfusion in rats showed deficits in spatial memory of the hidden platform in the Morris water maze test [313]. This may suggest that prenatal exposure to nicotine alone can have an effect on spatial memory but not short-term memory. However, the current study showed deficits in short-term memory from adult offspring and this could be due a cumulative effect of the nicotine with other e-cigarette constituents being released in the aerosol such as propylene glycol, glycerine, flavouring and metals. Prenatal exposure to aerosolised propylene glycol and glycerine is not extensively known. In addition, non-specific labelling of the flavouring on the e-liquids make it difficult to identify the flavouring composition, and thus, the exact chemical that could be having an effect on behaviour. Metals such as nickel and lead have also been detected in e-cigarette aerosols from eroding coils [314, 315] and a study have shown chronic administration of nickel exhibited impaired learning and working memory in rats [316].Therefore, understanding the effects of different combinations of aerosolised constituents within e-cigarettes would further elucidate the effects of e-cigarette aerosols on behaviour. Our laboratory is currently analysing gas chromatography–mass spectrometry data of the chemicals that were released from a number of flavoured e-liquids (including the e-liquids used for this study), and will be published soon.

Offspring from mothers exposed to e-cigarette aerosols with and without nicotine showed an increase in exploration/activity and signs of reduction in anxiety-like behaviour. These results are consistent in studies conducted in children from smoking mothers showing behavioural changes such as hyperactivity, anxiety, anti-social and aggressive-like behaviour [278-280]. This could suggest that other constituents within the aerosols other than nicotine may also be having an effect on the offspring activity levels and anxiety. It is well documented that nicotine is associated with hyperactivity in children [197]. Propylene glycol, which is one of the main constituents in e-liquids, has not been extensively studied once aerosolised. In addition, the effects of aerosolised glycerine have yet to be extensively studied. A report on oral administration of glycerine have been reported and showed moderate side effects such as tremors and diarrhoea in rabbits and rats

[317]. Heating up propylene glycol at high temperatures release harmful chemicals such as formaldehyde, which has been shown to cause anxiety-like and depression-like behaviour in mice [318]. Reports of heavy metals such as cadmium were present in e-cigarette aerosols [319]. A study that investigated environmental cadmium exposure in urine from prenatal and Bangladeshi children found an association with poor IQ and altered behaviour [320]. Although the mechanisms are unclear on how these changes are occurring in the offspring after maternal e-cigarette exposure, from the results, there seems to be an effect on behaviour following intrauterine exposure to e-cigarette that is independent of nicotine that can potentially result in behavioural changes in the offspring.

2.5.3 Offspring from mothers exposed to e-cigarette aerosols had increased levels of DNA Methylation

DNA methylation is the most studied epigenetic marker in the literature. DNA methylation can be used as a screening tool to detect any DNA methylation on the genome potentially leading to imprinting of genes, which can lead to pathophysiological conditions later in life. During embryogenesis, DNA methylation plays a crucial role in assisting transcription during neural development and maturation [321]. Therefore, any environmental exposure during the early stages of gestation has the potential to affect epigenetic modulation [322, 323]. For instance, maternal environmental exposure *in utero* such as diet, infection or cigarette smoke exposure has been known to alter DNA methylation in cord blood [275, 324-329]. In this study, global DNA methylation was investigated in offspring brains right after birth, at weaning and adulthood. Surprisingly, the results from this study showed no significant global DNA methylation changes in the offspring from mothers exposed to e-cigarette with nicotine compared to the control, which contradict the studies reported [275, 321-329]. However, global DNA methylation was significantly increased in offspring from mothers exposed to e-cigarette without nicotine at birth and weaning, and this suggests somehow nicotine might be playing a neuroprotective role during

intrauterine exposure of e-cigarette aerosols. Nicotine has been known to be neuroprotective and neurotoxic in the brain [330]. However, there are numerous studies that investigated the effects of prenatal nicotine exposure in the offspring [136, 241, 308, 331, 332]. Nicotine exposure via the drinking water showed changes to DNA methylation in the male mice spermatozoa leading to transgenerational behavioural impairment [331]. Ke and colleagues exposed pregnant mice to nicotine using an osmotic pump and found an increase in Dnmt3a in the offspring left ventricle [333]. In addition, smoking tobacco cigarettes has been known to alter DNA methylation in aborted foetal prefrontal cortex in humans [332]. Even though these studies contradict the current findings, this could mean that the effects of nicotine on DNA methylation is specific between tissue types.

In contrast to DNA methylation in the whole brain, there were no changes in DNA methylation in the hippocampus in adulthood. Although these results cannot be comparable to other age groups in this study since we did not investigate the hippocampus right after birth or after weaning, this may suggest that these results could be age-related effects. Moreover, this could be due to the fact that there was no exposure to e-cigarette aerosols after weaning, or may be an issue with the current study's design. The hippocampus was selected for investigation in the adult offspring only to determine changes in memory seen in the behavioural test. However, it may be that global DNA methylation changes are occurring in other regions of the brain. Unfortunately, other regions of the brain that are important in memory such as the prefrontal cortex and the amygdala could be affected, but during the initial planning of the animal experiment, these brain regions were not dissected. It is important to note that only global DNA methylation was investigated in the offspring brain, therefore, it does not report on the specific genes that are methylated on the DNA.

Overall, these results provide a wide overview that intrauterine exposure to e-cigarettes without nicotine results in global epigenetic changes in the offspring at various stages in life. There are studies that investigated DNA methylation from prenatal exposure to smoking [324, 334-336], however, this was not specific to Dnmt3a or Dnmt3b. For future experiments, it is important to locate the genes that are methylated in the DNA such as using chromatin immunoprecipitation.

Identifying the genes that are affected by methylation can potentially detect conditions that the gene is linked to.

2.5.4 PCR array and PCR validation

Epigenetic investigations were done to identify up or down-regulation of chromatin modification enzymes that are important in controlling the expression genes on the DNA. To determine if e-cigarette aerosols during pregnancy had an effect on the offspring brain, a PCR array was conducted and a subset of samples. Of the epigenetic genes that were tested, a selected number of these genes were then validated at all time points with more samples.

Dnmt3a and Dnmt3b are key genes that are important in transferring a methyl group onto the DNA and so are important in gene expression. Changes to Dnmt3a and Dnmt3b gene expression can ultimately alter the activation and suppression of genes that the DNA methyltransferases controls. Moreover, Dnmt3a and Dnmt3b have been known to be important in neurogenesis at different regions of the brain such as the hippocampus. The current study showed no significant changes to Dnmt3a and Dnmt3b gene expression right after birth. However, there is a considerable decrease in Dnmt3a and Dnmt3b gene expression in offspring from mothers exposed to e-cigarette aerosols with nicotine right after weaning. Moreover, in the adult offspring, Dnmt3b but not Dnmt3a gene expression was shown to be significantly decreased in the e-cigarette with nicotine group. Mutations in Dnmt3a has been known to be associated with intellectual retardation [337]. In addition, knockout of Dnmt3b have been shown to be lethal embryonically in mice [293] and mutations within the Dnmt3b gene showed immunodeficiency in patients [338]. One study conducted a genome-wide study to identify genes that contribute to nicotine dependence in 38,602 smokers and found Dnmt3b to be associated with a higher rise of nicotine dependency [339]. The reduction in Dnmt3b gene expression in the adult offspring is consistent with the behavioural data showing short-term memory deficits and hyperactivity since these offspring were from mothers exposed to e-cigarette aerosols with nicotine. However, hyperactivity was also observed in

offspring from mothers exposed to e-cigarette aerosols without nicotine. Therefore, this suggests that the reduction in Dnmt3a and Dnmt3b gene expression could be a nicotine effect, or a cumulative effect alongside the other e-cigarette constituents and the effects during prenatal exposure, but this is yet to be determined.

Kdm5c and Kdm6b plays a role in activating and suppressing gene expression by removing a methyl group from the histone on the DNA. Mutations in the Kdm5c gene have been associated with mental retardation in humans [340, 341]. Moreover, knockdown of Kdm5c showed neuronal loss and decrease lengths in neuronal dendrites in zebrafish [342]. The results show that in the adult offspring, there is a reduction in Kdm5c gene expression in both e-cigarette exposure with and without nicotine that could suggest intrauterine exposure to e-cigarettes may result in changes to mental development. These could manifest in the type of behavioural changes such as hyperactivity that was seen in the offspring from maternal exposure to e-cigarette aerosols.

Atf2 is important in controlling the transcription of genes on the DNA by adding an acetyl group to the histone on the DNA. Downregulation of Atf2 has been known to be associated with neurodegenerative diseases such as Huntington's, Alzheimer's and Parkinson's disease [295, 343]. Intrauterine exposure to e-cigarette aerosols causes a reduction in Atf2 in the offspring at P1 and in adulthood. If this has the potential to be a neurodegenerative condition in later life, then it won't show up for 60-70 years and this highlights the uncertainty surrounding the use of new tobacco products.

AurkA, AurkB, and AurkC are a family of aurora kinases that play a key role in chromosomal alignment and segregation by phosphorylating a histone on the DNA [297]. Changes to aurora kinase gene expression can result in defects in mitotic division. The results show that there were significant alterations in AurkA, AurkB and AurkC in offspring from mothers exposed to e-cigarette aerosols with and without nicotine at birth and at weaning. Although there were no changes to AurkA, AurkB, and AurkC gene expression in adulthood in the offspring, the changes that were observed were most likely as a result of e-cigarette aerosol exposure *in utero* and from

breastfeeding. Therefore, for future studies, it would be very interesting to investigate the concentration of leptin in the breastmilk as well as cytokines and proteins that is important in the growth of the offspring. For all of these epigenetic changes, there are some variations in the time and effect. The results were unable to state what the overall effect will be, but this is the first time these changes have been described at all.

It is also important to note that we are seeing changes to mRNA gene expression in offspring from mothers exposed to e-cigarette aerosols that are independent of nicotine. Therefore, changes to chromatin modification enzymes from e-cigarette exposure without nicotine are likely due to other ingredients that were created in the e-liquid such as propylene glycol, glycerine and flavouring compounds may be resulting neurological changes in the offspring from maternal exposure.

2.5.5 Maternal e-cigarette aerosol exposure did not affect neuronal cell counts in regions of the dorsal hippocampus and the lateral amygdala nucleus

Maternal exposure to e-cigarette aerosols, with and without nicotine, did not have an effect on the number of neurons in the dorsal hippocampus and the lateral amygdala nucleus indicating whatever epigenetic changes are occurring could lead to a change in cell number. One study has previously investigated the effects of prenatal e-cigarette aerosol exposure on the hippocampus. Zelikoff and colleagues investigated neurotoxicity in the developing mouse brain using an *in vivo* model. They did not see any changes in cell number but reported a significant increase in the microglial population at the hippocampus at CA1 in offspring compared to their controls and in gene expression of NGFR and BDNF that was significantly reduced [51]. A recent study investigating the effects of e-cigarette aerosol exposure on offspring brain transcriptomes that are associated with neurological outcomes have shown more dramatic changes from offspring

exposed to e-cigarettes without nicotine compared to offspring exposed to e-cigarette aerosol with nicotine [50]. From these studies, and compared to the DNA methylation data in the current study, it seems that nicotine is neuroprotective to epigenetic changes in the brain which has been evident in a number of studies [344, 345].

Nicotine exposure by itself has been reported to affect the developing hippocampus in a number of studies, although in these studies, the mode of delivery was generally quite direct, being administered orally [306, 346, 347]. The hippocampal changes reported following pre-natal nicotine exposure included a decrease in cell size and a higher cell packing density in juvenile and adolescent offspring hippocampus and somatosensory cortex [306], a decrease proportion in pyramidal neurons [348], a decrease in pyramidal neuron spine density at hippocampal region CA1 and CA3 in the offspring [348], a decrease number of gamma-aminobutyric acid (GABA) receptor which are important hippocampal synaptic activity [347], a decrease in neurogenic activity in the dendate gyrus [346], and an increase in cell apoptosis [346]. In contrast, other studies have reported similar findings to our study, with no significant changes to the hippocampal neurons [349, 350]. In addition, prenatal exposure to low doses of nicotine showed an increase in neuronal population in the central nucleus of the amygdala, an area involved in nicotine 'craving' [349]. Subacute and chronic exposures of nicotine effect the amygdala by altering a number of gene changes that are involved in cell (β-tubulin, β-actin) and protein degradation (ubiquitins) [351], but there were no changes in the lateral amygdala, an area more involved in fear conditioning.

While there was no effect of nicotine in the reduction of selected hippocampal neuronal cell numbers, full detailed examination of sub-populations of cells or changes in cell structures were not conducted, and this may be warranted in the future. It is also likely that the mode of delivery (and the concentration) of nicotine is critical for *in utero* neurotoxicity. This further reinforces the importance of understanding the chemical compositions that are being released from e-cigarette aerosols without the presence of nicotine, and the mechanisms of how it is affecting the brain. There are limited studies that investigated the effects e-cigarette base liquids propylene glycol

and glycerine when used in combination with other chemicals, and therefore, the effects of e-cigarette aerosols without nicotine should not be underestimated.

2.6 Conclusion

Overall, maternal exposure to e-cigarette aerosols with and without nicotine showed changes to behaviour and brain epigenetics within the offspring at birth, at weaning and adulthood. This study provides an insight into the effects of e-cigarette aerosols on the offspring, highlighting that e-cigarette use during pregnancy is not as safe as many people think. While there is considerable variation in age-related changes, differences between e-cigarette aerosol exposure with and without nicotine, provides evidence that *in utero* e-cigarette aerosol exposure results in neurological changes to the offspring. Future experiments are required to compare the effects of prenatal e-cigarette exposure and smoking and whether these changes are comparable to each other, but the results in themselves are reason for concern.

The results shown in this chapter have been published:

Nguyen, T., Li, GE., Chen, H., Cranfield, CG., McGrath, KC., Gorrie, CA. *Maternal E-Cigarette Exposure Results in Cognitive and Epigenetic Alterations in Offspring in a Mouse Model.* Chem Res Toxicol, 2018. **31**(7): p. 601-611.

Chen, H., Li, G., Chan, YL., Chapman, DG., Sukamnong, S., **Nguyen, T.**, Annissa, T., McGrath KC., Sharma, P., Oliver, BG. *Maternal E-Cigarette Exposure in Mice Alters DNA Methylation and Lung Cytokine Expression in Offspring.* Am J Respir Cell Mol Biol, 2018.**58**(3): p. 366-377.

Chapter 3 – Behavioural and epigenetic changes in offspring from maternal exposure to e-cigarette aerosols and tobacco smoke

3.1 Introduction

3.1.1 Tobacco cigarette use during pregnancy

Tobacco cigarettes are the leading cause of preventable deaths in the world. Smoking has been associated with a number of detrimental conditions such as cancer (lung, throat, mouth), respiratory (emphysema, COPD, bronchitis), cardiovascular, and kidney diseases (chronic kidney disease) [9-14]. Among the number of young people and adults who smoke tobacco cigarettes, pregnant women are also known to smoke during their pregnancy and this has become a major public health concern. In 2016, there is an average of 7.2 % of women who smoked during their pregnancy in the US [352]. The highest rate of maternal smoking occurred in West Virginia (25 %). Kentucky (18 %) and Missouri (15 %) [352]. Smoking during pregnancy have been associated with a number of newborn complications such as lower birth weight, preterm birth, respiratory complications, cardiac complications, kidney complications and sudden infant death syndrome [204-210]. In addition, neurobehavioral changes have also been associated with maternal smoking such as ADHD, aggressive behaviour, anxiety and anti-social behaviour [279, 353, 354]. Moreover, studies have showed that children from mothers who have smoked during pregnancy were more likely to take up smoking [355-357].

Maternal smoking is known to be associated with epigenetic changes, with DNA methylation changes seen in cord blood, placenta and leukocytes [334, 358, 359]. In addition, alterations in

DNA methylation in children from mothers who smoked during their pregnancy can persist into adulthood [334]. Currently in the US, 10% of pregnant women continue to smoke during their pregnancy. Australia is similar with the overall percentage of pregnant women smoking during pregnancy, at 11.7%, and maternal smoking is highest in the indigenous population at 20% [360, 361].

Although health officials encourage pregnant women to abstain from smoking, there are NRTs approved by the FDA that are available for the general population who want to quit smoking. NRTs that are available on the market include nicotine patches, nicotine gum, nicotine lozenges and nicotine inhalers. Although NRTs are promoted as a positive method of smoking cessation in the general population, there is still some inconsistencies within the literature as to whether it is safe for women to use NRTs to quit smoking during pregnancy. Prescribing NRTs to pregnant women by clinicians is not popular [362-365]. Medical organisations such as the Royal Australian and New Zealand College of Obstetricians and Gynaecologists, United States Preventative Services Task Force and the National institute for Health and Care Excellence have given their own detailed guidelines on how to appropriately prescribe NRTs to pregnant women [365-368]. However, the World Health Organisation does not provide any guidelines on using NRTs during pregnancy. There are limited studies that investigated the efficacy and effectiveness of NRTs use during pregnancy. Studies have shown that NRTs improve the smoking cessation rate when using nicotine patches during the first trimester of gestation compared to the placebo [369, 370]. However, limitations within each study such as the type and dosage of the NRT, insufficient recording of individual's withdrawal symptoms and no tailoring of treatments to each individual could be some reasons why clinicians do not often prescribe NRTs [365]. Therefore, there is a need to provide innovative ways of assisting pregnant women to quit smoking.

Although e-cigarettes were originally designed and marketed for consumers who are current smokers, there are women that have been reported to have used e-cigarettes during pregnancy [46, 114, 116, 117]. Wagner and colleagues have reported that 6.5% of women used e-cigarettes during their pregnancy and 8.4% of women are dual users of e-cigarettes and tobacco cigarettes

[268]. Of the e-cigarette users, 74.6% reported that they switched to e-cigarettes once they had learnt that they were pregnant [268]. Oncken and colleagues reported 13.5% of women using e-cigarettes during their pregnancy with the majority of them using e-cigarettes as an attempt to quit smoking [371].

The perceptions of e-cigarettes in pregnant women are somewhat contradictory. While some pregnant women believe that e-cigarettes do pose a risk, they also think that e-cigarettes could potentially be used as a substitute to cigarette smoking [115, 118, 269]. These studies suggest that pregnant women believe that e-cigarettes are 'unsafe', however, it is considered 'safer' than smoking tobacco cigarettes. This is a major cause of concern. It suggests that women, who might otherwise 'give up' smoking, will consider 'vaping' during pregnancy even though they do not know how that will impact their child.

There are currently no studies that investigate the effects of replacing tobacco cigarettes with e-cigarettes during pregnancy. From the study discussed in **Chapter 2**, there were neurological effects that were observed in mice offspring from mothers exposed to e-cigarette with and without nicotine, before gestation, during gestation and lactation [199]. There are numerous studies that investigate the effects of tobacco cigarette smoking, however, changes that were found in individual's who smoked could not be compared to intrauterine exposure of tobacco cigarettes.

3.2 Hypothesis and aims

3.2.1 Hypothesis

That switching to e-cigarette aerosol exposure will not reduce behavioural and epigenetic changes found in offspring from mothers exposed to continuous cigarette smoke.

3.2.2 Aims

To investigate the effects of switching to e-cigarette aerosol exposure during pregnancy on offspring by:

1. Determining the serum cotinine levels in the mothers and offspring.
2. Assessing behavioural changes in the offspring in adulthood using the novel object recognition test and the elevated plus maze test.
3. Determining the global DNA methylation in offspring brain right after birth, weaning and adulthood using the 5-mC DNA ELISA kit.
4. Determining epigenetic gene changes in the offspring brain using RT-qPCR.
5. Determining the neuronal counts in the offspring dorsal hippocampus and the lateral amygdala nucleus.

3.3 Materials and Methodology

3.3.1 E-cigarette device

Refer to **Chapter 2 section 2.3.1**.

3.3.2 Animal experimental procedure

Similar to the animal experiment outlined in **Chapter 2**, all experimental procedures were conducted strictly under the guidelines described by the National Health and Medical Research Council code of conduct for animals with approval from the institutional Animal Care and Ethics Committee. Ethics approval was obtained by the Animal Ethics Committee with the ethics approval number ETH15-0025. The timeline of the animal experiments is outlined in Figure 27.

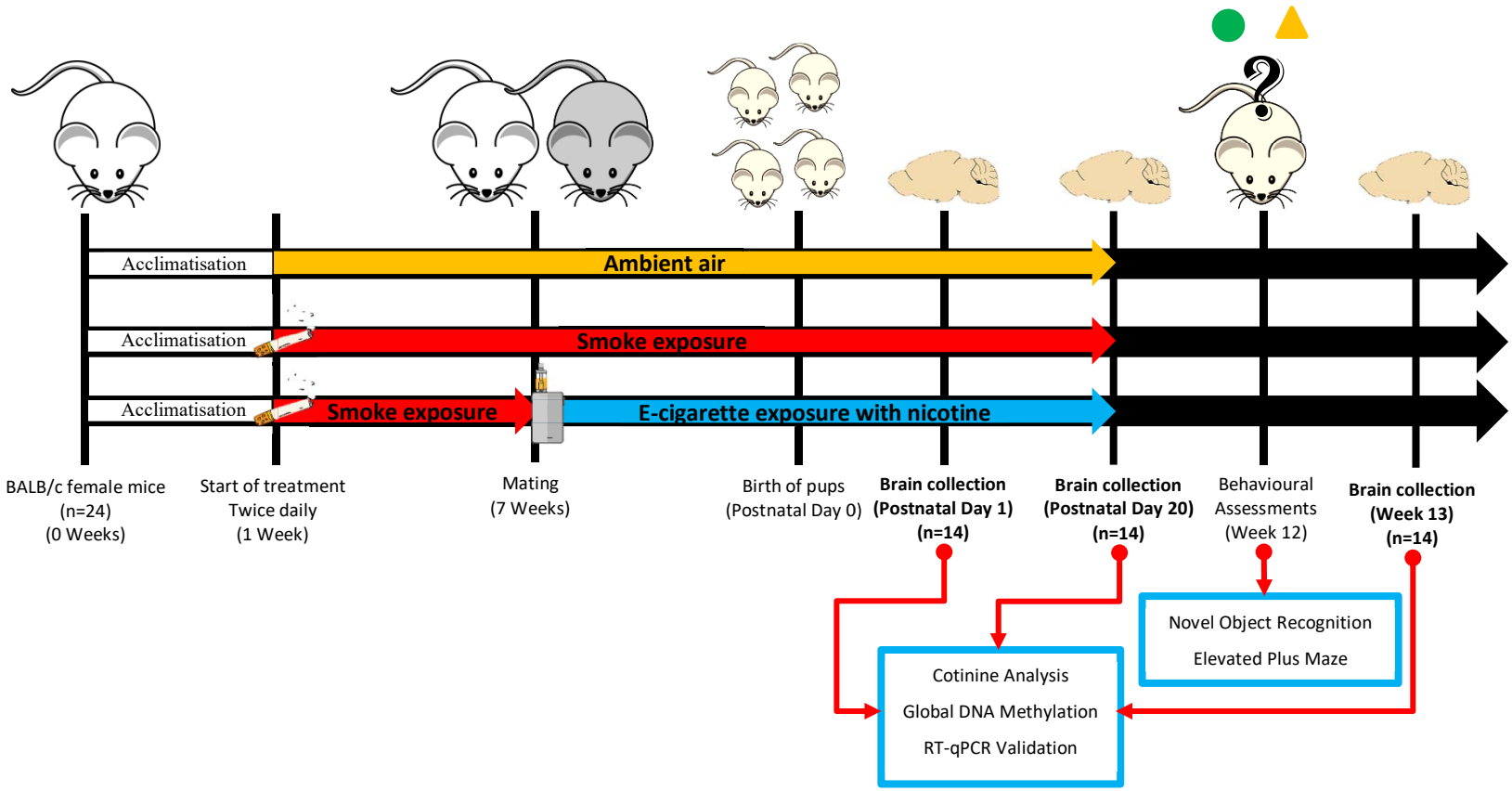

Figure 27. A Timeline summary of the animal experiments for Chapter 3. Twenty-four female BALB/c mice were divided into three treatment groups; Sham, smoke exposure only and smoke exposure followed by switch to e-cigarette exposure with nicotine. Animals were exposed for 6 weeks before pregnancy, during mating, pregnancy and lactation. Tissue collection occurred at postnatal day 1, postnatal day 20 and Week 13 to investigate global DNA methylation, PCR epigenetic array, RT-qPCR and neuronal counts. Behavioural assessments were performed at 12 weeks old prior to Week 13 collection.

Twenty-four 7-week old female BALB/c mice were obtained from the Animal Resources Centre in Perth. Mice were housed in groups of four in cages with *ad libatum* food and water with a 12:12 light dark light cycle. All animals were provided with environmental enrichment as part of the standard housing conditions. To ensure female mice are adapted to the new facilities, acclimatisation occurred that involved no experimental disturbances for one week.

Animals were randomly assigned to one of three groups and were exposed to the following; **Sham**: animals were exposed to ambient air (n = 8), **Smoke exposure (SE)**: animals were exposed to tobacco cigarette smoke (n = 8), and **Switch**: animals exposed to cigarette smoke pre-pregnancy then switched to e-cigarette exposure with nicotine during gestation and lactation (Figure 28).The Sham and SE group were exposed to their treatment using a modified protocol. This is discussed further in **section 3.3.3.1**. E-cigarette aerosol exposure with nicotine were selected for two reasons; 1) This mimics a common scenario seen in pregnant women and 2) This controls for the effects attributed to nicotine and allows for the effects of aerosol products to be compared to cigarette products alone.

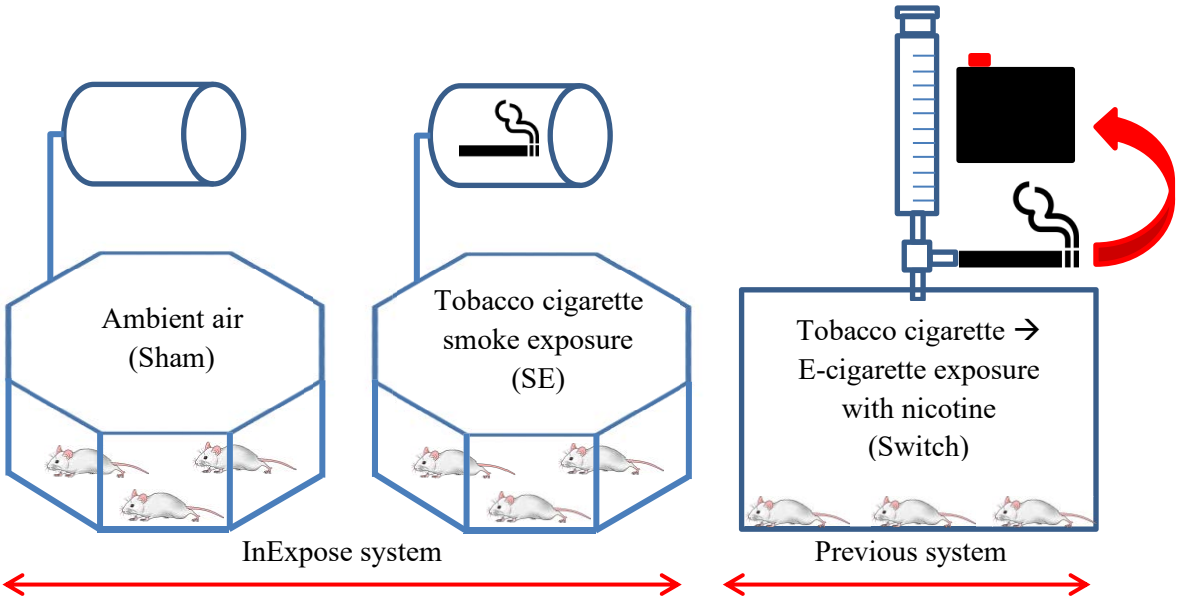

Figure 28. Schematic of the treatment groups for the animal experiment in Chapter 3. There are three treatment groups, the Sham and Smoke Exposure group which was treated in the InExpose system (Scireq®, Montreal, QC, Canada), and the Switch group which was treated according to the system from the previous protocol outlined in **Chapter 2 section 2.3.3**. SE = smoke exposure.

Once animals arrived at the Ernst Facility, animals were acclimatised to the facility for one week before being exposed to the designated treatment twice daily for five weeks. Breeders were exposed once in the morning between 09:00-10:00 and once in the afternoon between 15:00-16:00. Animals were further exposed to their corresponding treatment during pregnancy and lactation until pups were weaned from their mothers. Breeders were not exposed to their corresponding treatment during the birth of pups to reduce the stress on the mothers during labour. It is important to note that offspring were not placed in the chamber with their mothers for any treatment exposure. However, offspring can be exposed to treatment once mothers return to the home cage by licking their mother's fur or suckling for breastmilk.

After six weeks of exposure to treatment, female breeders were caged with 8-week old male BALB/c mice for mating. Pregnant mice were separated into individual cages for nesting purposes to reduce maternal stress. Depending on the size of the litter, male pups were randomly selected from each litter to be culled at three time points; P1 [n = 14], P20 (P20) [n = 14] and Week 13 (n = 14). Time-points were chosen to determine changes to offspring right after birth (P1), after weaning (P20) and at adulthood (Week 13). Offspring were weighed regularly each week and recorded for analysis. Female pups were donated to the Ernst Facility for training purposes.

3.3.3 Aerosol and smoking procedure

Please refer to **Chapter 2 section 2.3.3** for the e-cigarette aerosol exposure protocol. Tobacco cigarette exposure was conducted for six weeks until the animal became pregnant. After that, the mothers were continued to be exposed to the same treatment until the pups were weaned. The smoking procedure was developed according to the laboratory method described previously [197]. The smoke exposure procedure was further outlined in **section 3.3.3.1**.

3.3.3.1 Smoke exposure group procedure

The smoke exposure procedure for the Switch group was performed using a similar protocol outlined in section **Chapter 2 section 2.3.3**. Breeders from the Switch group were placed into a 9 L chamber. After acclimatisation to the chamber, animals were exposed to tobacco cigarette smoke (Winfield Red, ≤ 16 mg tar, ≤ 1.2 mg/mL nicotine, and ≤ 15 mg of CO; VIC, Australia). Animals were exposed twice daily for six weeks prior to mating, during gestation and lactation. For each session, animals were exposed to two cigarettes for 15 minutes/cigarette with a smoke-free washout period for five minutes in between cigarettes to avoid asphyxiation. The SE group exposure was performed after the completion of the Sham and Switch group animal experiments. The protocol for the smoke exposure was modified from using the smoke exposure procedure outlined in **Chapter 2**, to using the *InExpose system* (Scireq®, Montreal, QC, Canada). Due to changes to the exposure protocols, the procedure for the smoke exposure in the InExpose system was adapted to closely resemble the previous protocol where possible. The InExpose system was calibrated to 35 psi to ensure all tube connections were tightly sealed. Breeders from the SE group were placed into the InExpose system chamber (Figure 29). Using the Scireq® flexiWare 9 software, the exposure protocol was set up so that cigarette smoke was expelled in the chamber at a rate of two puffs/minute. Breeders were left in the chamber for 15 minutes followed by a smoke-free washout period for 5 minutes. After the washout period, a second cigarette was placed in the InExpose system for an additional 15-minute exposure. To ensure that the Sham, SE and Switch groups are comparable to each other, plasma cotinine levels from breeders and P20 offspring were analysed to ensure that the same amount of nicotine was being delivered by both exposure systems.

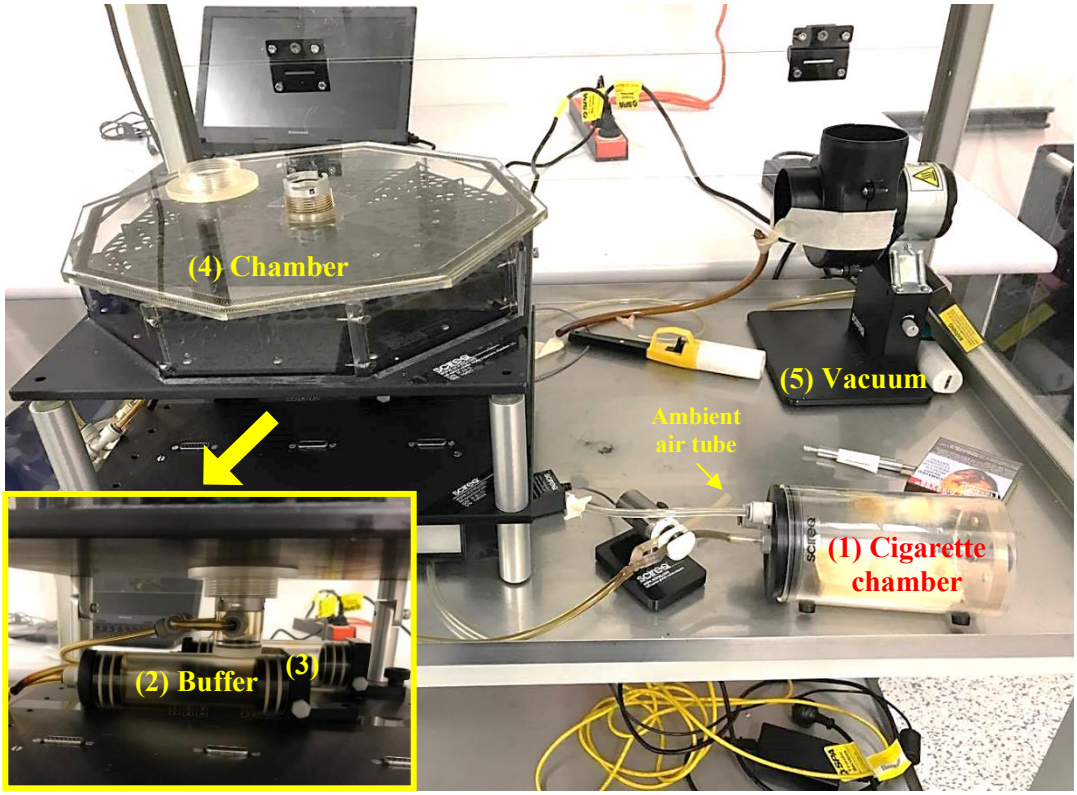

Figure 29. Image of the Scireq® InExpose system that was used to expose animals to tobacco cigarette smoke. The system was controlled using the Scireq® flexiWare 9 program software. To create the cigarette smoke, a cigarette is placed into the cigarette chamber (1) and the puffs of cigarette smoke travelled to the buffer chamber to mix with ambient air (2). The smoke/air mixture then enters the filter chamber (3) before being expelled into the chamber (4). Gradually, cigarette smoke from the chamber was released by the vacuum (5).

3.3.4 Behavioural assessments

All behavioural assessments were conducted on male offspring at 12-weeks old. Behavioural assessments were conducted to determine if there were any changes to offspring from mothers that switched to e-cigarette aerosols (with nicotine) compared to offspring from mothers exposed to tobacco cigarette smoke or ambient air. Each behavioural test was conducted between the hours of 09:00-12:00 and behavioural tests were conducted from least stressful to most stressful to avoid each mouse being stagnant or overwhelmed in each test [282]. Each behavioural assessment was video recorded and analysed once all of the offspring had completed all assessments.

3.3.4.1 The Novel Object Recognition

Refer to **Chapter 2 section 2.3.4.1** for the full protocol.

3.3.4.2 The Elevated Plus Maze

Refer to **Chapter 2 section 2.3.4.2** for the full protocol.

3.3.5 Euthanasia and tissue collection

Refer to **Chapter 2 section 2.3.5** for the full protocol.

3.3.6 Plasma cotinine

Refer to **Chapter 2 section 2.3.6** for the full protocol.

3.3.7 DNA and RNA extraction

Refer to **Chapter 2 section 2.3.7** for the full protocol.

3.3.8 RNA quality check

Refer to **Chapter 2 section 2.3.8** for the full protocol.

3.3.9 Global 5-mC DNA methylation

Refer to **Chapter 2 section 2.3.9** for the full protocol.

3.3.10 mRNA expression of chromatin modification genes

The current animal experiment aimed to determine any epigenetic genes that were altered when exposed to e-cigarette aerosols. Therefore, those epigenetic genes from **Chapter 2** were investigated to determine if any changes in those epigenetic genes were observed in offspring from mothers exposed to cigarette smoke or e-cigarette aerosols. Refer to **Chapter 2 section 2.3.11** for the full protocol. The full list of primer pairs is listed in **Table 3** within **Chapter 2 section 2.3.11**.

3.3.11 Tissue fixation, processing and embedding and cutting

Refer to **Chapter 2 section 2.3.12** for the full protocol.

3.3.12 Statistical analysis

One-way ANOVA with *Bonferroni's post-hoc* test was used to determine statistical significance in the whole body weight, EPM, global DNA methylation and RT-qPCR, and neuronal cell counts per ROI. A paired *t-test* was used to determine statistical significance in the NOR data. All statistical analyses were performed using Prism 7 (GraphPad, CA, USA) and the results were expressed as mean ± standard deviation. Treatment groups were considered significantly different if the *p value* was less than 0.05. The sample size for each group was between 6-14 samples. Some subjects were excluded from the statistical analysis due to being clear outliers to the rest of the data points or the data points were of insufficient quality.

3.4 Results

During experimental treatment, all breeders tolerated the smoke and aerosol exposure without any incident. During the birth of the offspring, litter sizes were between 3-7 offspring for each breeder. There was no significant difference between the number in each litter between each treatment group ($p=0.99$). There was no significant difference in sex ratio between treatment groups ($p=0.76$).

3.4.1 Cotinine analysis of breeders and offspring

To ensure the Sham, SE and Switch groups are comparable to each other, breeders and P20 offspring plasma cotinine levels were measured to ensure that the previous and current cigarette smoke and aerosol exposure protocols delivered the same amount of nicotine to each animal. Breeders and P20 offspring plasma were taken right after the last exposure session on weaning day. The results showed that breeder's plasma cotinine levels in the Sham group were 2.95±0.93 ng/mL (Figure 30). Plasma cotinine levels from breeders in the SE and Switch group was significantly increased at 19.36±10.17 ng/mL ($p<0.001$) and 21.97±12.76 ng/mL ($p<0.001$), respectively, compared to the Sham group. In the P20 offspring, plasma cotinine levels in the Sham group were 3.56±1.60 ng/mL. Similarly, like the breeders, P20 offspring plasma cotinine levels from the SE and Switch group was significantly increased at 9.62±3.66 ng/mL ($p<0.001$) and 10.93±2.38 ng/mL ($p<0.001$), respectively, compared to the Sham group. These results indicate that both the InExpose system and our previous protocol delivered similar amounts of nicotine to each group, and therefore, each treatment group were comparable to each other for this study.

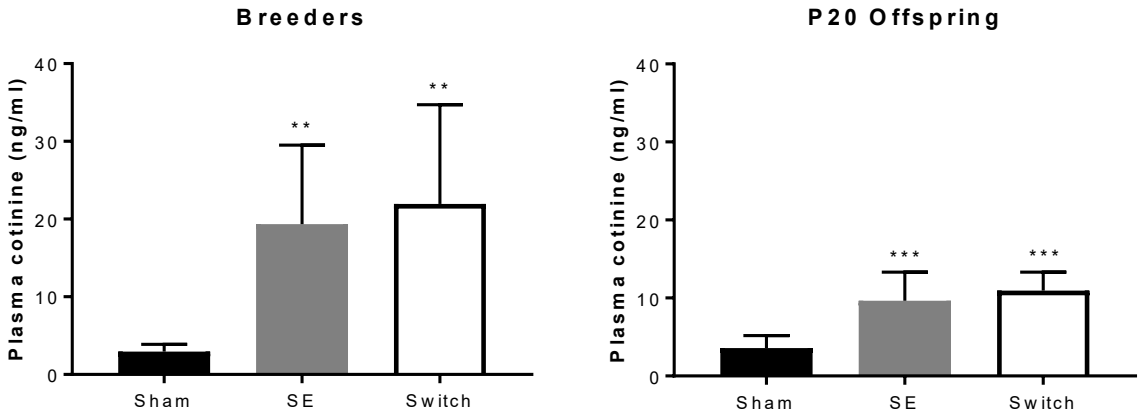

Figure 30. Plasma cotinine levels of breeders and offspring at postnatal day 20 (P20). There was a significant increase in cotinine levels in both the Smoke Exposure (SE) group and Switch group compared to the Sham group in both breeders and P20 offspring plasma (n = 8). Data represented as mean ± standard deviation, one-way ANOVA with Bonferroni post-hoc test, **p<0.01, ***p<0.001 vs. Sham.

3.4.2 Anthropometry data

Offspring body weights were measured at P1, P20 and Week 13. At P1, the Sham group had a body weight of 1.8±0.2 g. The SE group and the Switch group had a significantly decrease body weight of 1.3±0. 1g ($p<0.05$); Figure 31A) and 1.4±0.1 g ($p<0.001$), respectively, compared to the Sham group. In the P20 offspring, the Sham group had a body weight of 10.6±1.4 g. The SE group and the Switch group had a significantly decreased body weight of 9.5±0.6 g ($p<0.01$; Figure 31B) and 9.5±0.6 g ($p<0.01$), respectively, compared to the Sham group. At Week 13, there were no significant differences in body weights between the Sham (26.5±2.0 g), SE (25.9±1.6 g) and Switch groups (25.9±1.0 g) (Figure 31C). This indicates that the reduction in body weight at P1 and P20 was likely caused by nicotine exposure *in utero* and during breast-feeding.

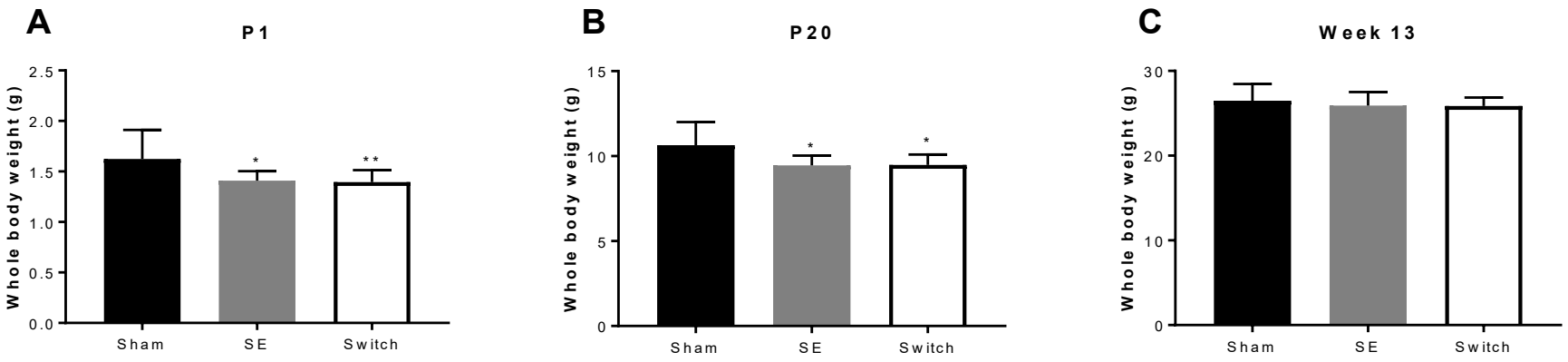

Figure 31. Offspring whole body weight at (A) P1, (B) P20 and (C) Week 13 in the Sham, Smoke Exposure (SE) and Switch groups (n = 14). Data represented as mean ± standard deviation, one-way ANOVA with Bonferroni post-hoc test, *p<0.05, **p<0.01 vs. Sham.

3.4.3 Switching to e-cigarette aerosol exposure during pregnancy with nicotine causes short-term memory deficits

The NOR test is a measure for short-term memory. The expected result in an unimpaired animal is a significant difference between the recognition index in the familiarisation and test phase which is shown in the Sham group (paired t-test, $p<0.001$; Figure 32A). There was a significant difference in the recognition index in the SE group (paired t-test, $p<0.001$; Figure 32B). In contrast, the Switch group had no significant difference in the recognition index between the familiarisation and test phase (Figure 32C). This result was also observed in the Ecig(+nic) group from **Chapter 2 section 2.4.3**, suggesting that maternal exposure to tobacco-flavoured e-cigarette aerosols with nicotine is causing short-term memory deficits in offspring, and that it may be an interaction between nicotine and the e-cigarette constituents.

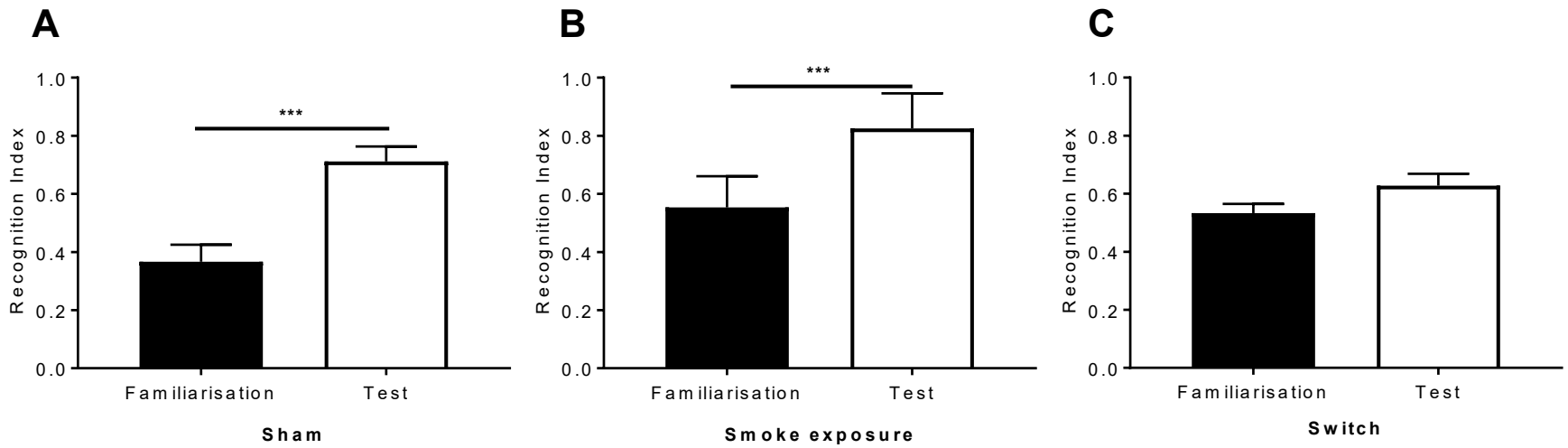

Figure 32. Recognition index from the novel object recognition test in the Sham (n = 14), Smoke Exposure (SE) (n = 14) and the Switch group (n = 14). (A) Offspring exposed to ambient air (Sham) showed a significant difference between the familiarisation and test phase. (B) Offspring from the SE group showed a significant difference between both phases. (C) Offspring from the Switch group showed no significant difference between both phases. Data represented as mean ± standard deviation, paired t-test, ***p<0.001 vs. Familiarisation.

3.4.4 Switching to e-cigarette aerosol exposure during pregnancy showed reduced anxiety and hyperactivity in adult offspring

The EPM test investigates an animal's level of anxiety, exploration and activity [372]. The baseline for the percentage of time an unimpaired animal spent in the open arm of the EPM was represented by the Sham group (Figure 33A). Compared to the Sham group, there was an increase in the time spent in the open arm in both the SE group ($p<0.001$) and the Switch group ($p<0.001$). To investigate exploration, the total number of centre crosses from each animal were analysed (Figure 33B). The results showed that there was a significant increase in the number of centre crosses in the SE group ($p<0.05$) and Switch group ($p<0.01$) compared to the Sham group. These results suggest that exposure to nicotine is likely the cause of an increase in offspring activities, which is an indication of hyperactivity, and a reduction in anxiety. Furthermore, these results are consistent with the short-term memory deficits observed in **Chapter 2**.

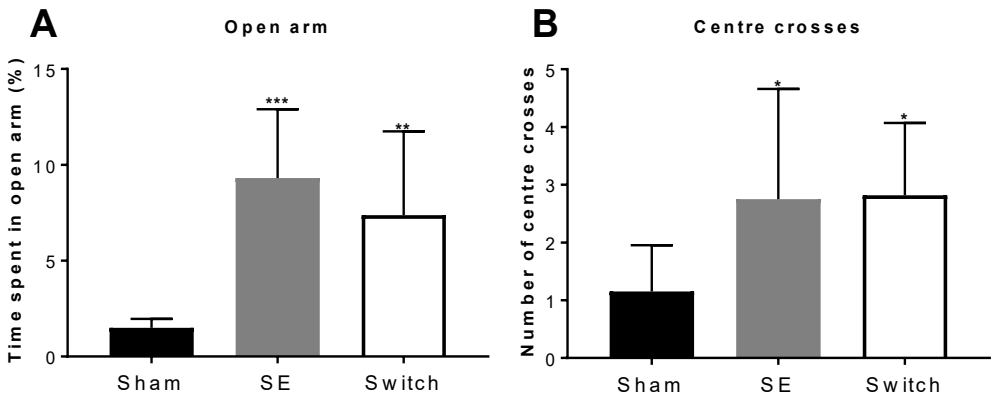

Figure 33. The time spent in the open arm and the number of centre crosses performed by week 12 offspring in the elevated plus maze in the Sham, Smoke Exposure (SE) and Switch group groups (n = 8-14). (A) The SE and Switch groups showed significant increases in the time spent in the open arm compared to the Sham group. (B) The SE and Switch groups showed significant increases in the number of centre crosses compared to the Sham group. Data represented as mean ± standard deviation, one-way ANOVA with Bonferroni post-hoc test, *$p<0.05$, **$p<0.01$, ***$p<0.001$ vs. Sham.

Other sensitive measures of anxiety, such as head dipping and whole body stretches in the closed arm (protected) and open arm (unprotected), were also analysed. For all treatment groups, there was no significant difference between the amount of protected and unprotected head dips (Figure 34A&B). For the number of protected and unprotected stretches, there was no significant difference in the number of protected stretches between each treatment group (Figure 34C). However, for the number of unprotected stretches, there was a significant increase in the SE group compared to the Sham group ($p<0.001$; Figure 34D and the Switch group ($p<0.001$).

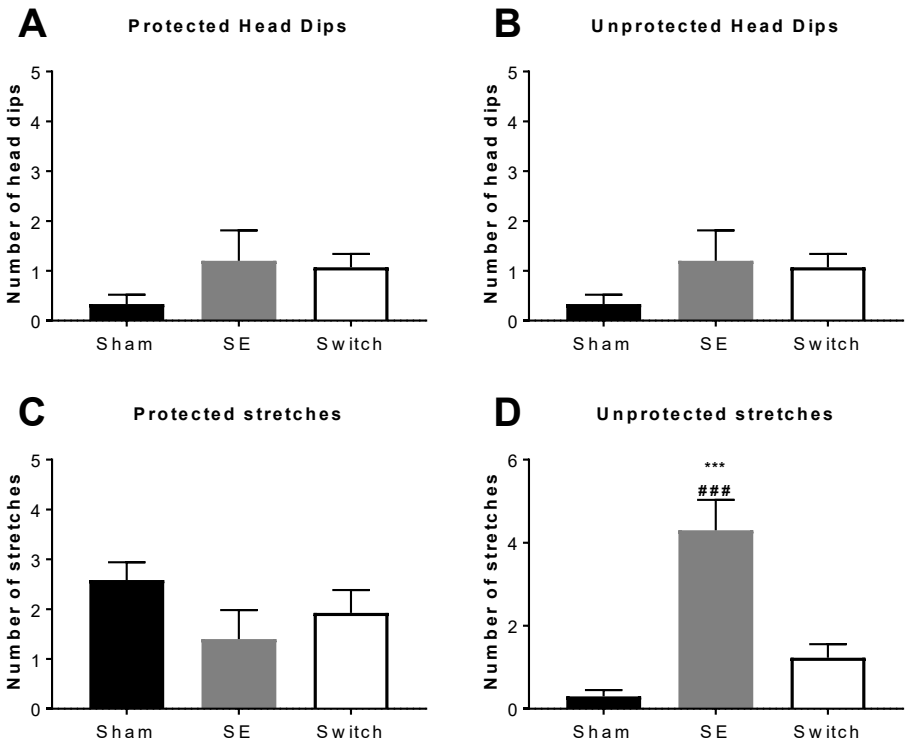

Figure 34. The total number of head dips and whole body stretches in the closed arm (protected) and open arm (unprotected) in offspring from the Sham (n = 12), Smoke Exposure (SE) (n = 10) and Switch groups (n = 14). There were no significant differences in the number of (A) protected head dips, (B) unprotected head dips and (C) protected stretches in any treatment groups. There was a significant increase in the number of unprotected stretches (D) in the SE group compared to the Sham and Switch groups. Data represented as mean ± standard deviation, one-way ANOVA with Bonferroni post-hoc test, ***$p<0.001$ vs. Sham, ###$p<0.001$ vs. Switch.

3.4.5 Maternal smoking and switching to e-cigarette aerosols during pregnancy showed an increase in global DNA methylation in offspring at all time points

Global DNA methylation was measured in the offspring whole brain at P1 and P20 as well as the hippocampus at Week 13. At the P1 brain, the average DNA methylation in the Sham group was 5.6±0.6 %. The SE group showed a significant increase in DNA methylation to 44.3±5.7 % compared to the Sham group ($p<0.001$; Figure 35A) and the Switch group ($p<0.001$). In the P20 brain, the average DNA methylation in the Sham group was 5.1±0.6 %. The SE group showed a significant increase in DNA methylation to 39.0±3.6 % compared to the Sham group ($p<0.001$) and the Switch group ($p<0.001$; Figure 35B). In the Week 13 hippocampus, the average DNA methylation in the Sham group was 9.5±1.1 %. The SE group showed a significant increase in DNA methylation to 40.8±3.2 % compared to the Sham group ($p<0.001$) and the Switch group ($p<0.001$; Figure 35C).

Importantly, in the P1 brain, DNA methylation was also significantly increased in the Switch group to 12.6±1. 2% ($p<0.001$; Figure 35A) compared to the Sham group. This result was consistent in the Switch group from the P20 brain at 12.3±2.2 % ($p<0.001$; Figure 35B) and the Switch group from the Week 13 hippocampus at 15.9±2.5 % ($p<0.001$; Figure 35C). These results suggest that e-cigarette aerosol exposure during pregnancy causes DNA hypermethylation in the offspring brain right after birth, after weaning and in adulthood, and that while this is less in the Switch group, it is still increased compared to the Sham group.

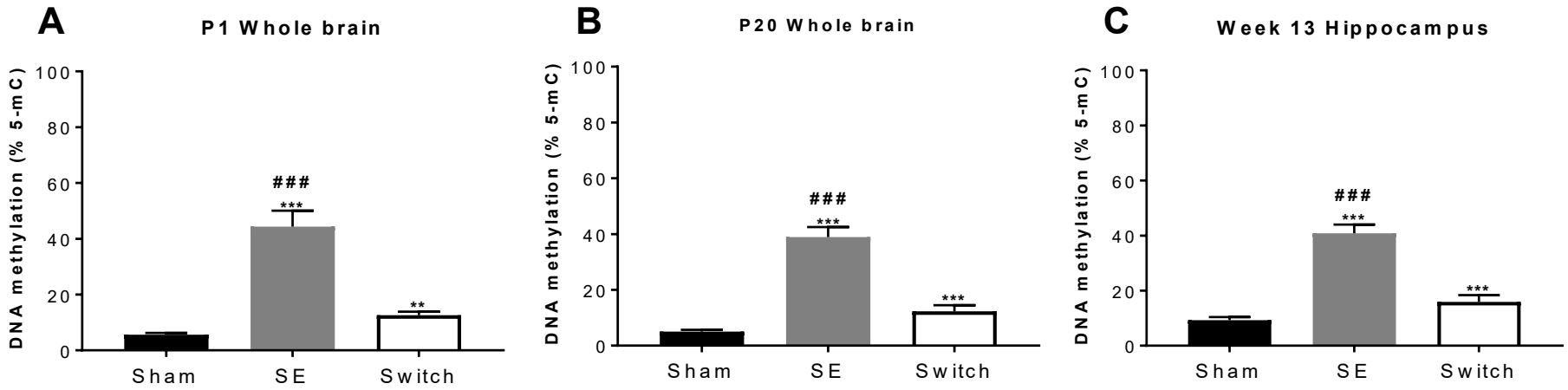

Figure 35. Global DNA methylation in offspring brains at postnatal day 1 (P1), postnatal day 20 (P20) and postnatal Week 13. From the Sham, Smoke Exposure (SE) and Switch groups (n = 4-8). DNA methylation at (A) P1 (whole brain), (B) P20 (Whole brain) and (C) Week 13 (hippocampus) showed a significant increase in DNA methylation in the SE group compared to the Sham and Switch group. Moreover, a significant increase in DNA methylation was found in the Switch group compared to the Sham group at all time points. Data represented as mean ± standard deviation, one-way ANOVA with Bonferroni post-hoc test, **p<0.01, ***p<0.001 vs. Sham, ###p<0.001 vs. Switch.

3.4.6 Maternal smoking showed decreases to Dnmt3a and Dnmt3b gene expression in offspring at P1 and Week 13

In the P1 brain, Dnmt3a gene expression in the SE group was significantly decreased to 15.0±8.8 % compared to the Sham group ($p<0.001$; Figure 36A) and the Switch group ($p<0.05$). In addition, Dnmt3b gene expression was also significantly decreased to 53.5±15.6 % in the SE group compared to the Sham group ($p<0.05$; Figure 36D). In the P20 brain, although a decreased in Dnmt3a and Dnmt3b gene expression was observed, it was not significantly different ($p<0.05$; Figure 36B&E).

In the Week 13 hippocampus, Dnmt3a gene expression was significantly decreased to 57.0±8.9 % in the SE group compared to the Sham group ($p<0.001$; Figure 36B) and the Switch group ($p<0.05$; Figure 36B). Similarly, Dnmt3b gene expression was significantly decreased to 23.7±5.0 % in the SE group compared to the Sham group ($p<0.001$; Figure 36F) and the Switch group ($p<0.001$). It is important to note that there were no significant differences observed in Dnmt3a and Dnmt3b gene expression between the Sham group and the Switch group at any time points.

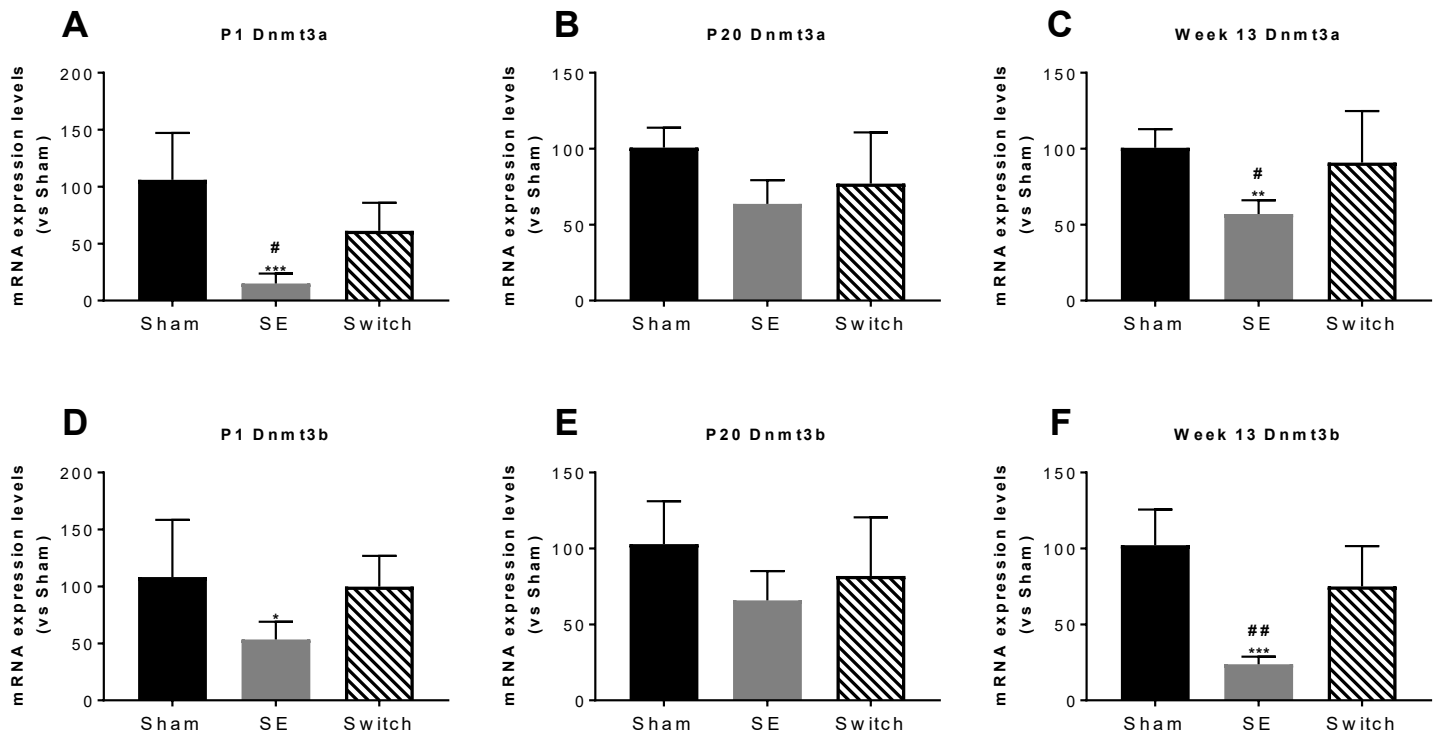

Figure 36. mRNA gene expression levels for DNA methyltransferases (Dnmt3a and Dnmt3b) in offspring at postnatal day 1 (P1) whole brain, postnatal day 20 (P20) whole brain and Week 13 hippocampus. mRNA expression levels of the SE (n = 6) group and Switch group (n = 6) were expressed as a percentage compared to the Sham group (n = 6). Data represented as mean ± standard deviation, one-way ANOVA with Bonferroni post-hoc test, *p<0.05, **p<0.01, ***p<0.001 vs. Sham, #p<0.05, ##p<0.01, ###p<0.001 vs. Switch. SE = smoke exposure.

3.4.7 Maternal smoking showed changes to Kdm5c and Kdm6b gene expression in offspring at all time points

In the P1 brain, Kdm5c gene expression was significantly increased to 841.8±327.0 % in the SE group compared to the Sham group ($p<0.001$; Figure 37A) and the Switch group ($p<0.001$). Similarly, Kdm6b gene expression was significantly increased to 4609.5±3014.6 % in the SE group compared to the Sham group ($p<0.001$; Figure 37D) and the Switch group ($p<0.001$).

In the P20 brain, Kdm5c gene expression was significantly decreased to 36.4±5.4 % in the SE group compared to the Sham group ($p<0.001$; Figure 37B) and the Switch group ($p<0.001$). Similarly, Kdm6b gene expression was also significantly decreased to 31.2±6.0 % in the SE group compared to the Sham group ($p<0.001$; Figure 37E) and the Switch group ($p<0.001$; Figure 37E).

In the Week 13 hippocampus, although there was no significant difference, Kdm5c gene expression was increased in the SE group and the Switch group to 145.2±30.7 % and 159.4±64.4 %, respectively, compared to the Sham group ($p=0.1053$; Figure 37C). However, Kdm6b gene expression was significantly increased to 161.4±31.8 % in the SE group compared to the Sham group ($p<0.05$; Figure 37F). In addition, Kdm6b gene expression was also increased to 154.2±54.3 % in the Switch group but there was no significant difference compared to the Sham group ($p=0.1159$; Figure 37F). It is important to note that there were no significant differences observed in Kdm5c and Kdm6b gene expression between the Sham group and the Switch group at any time points.

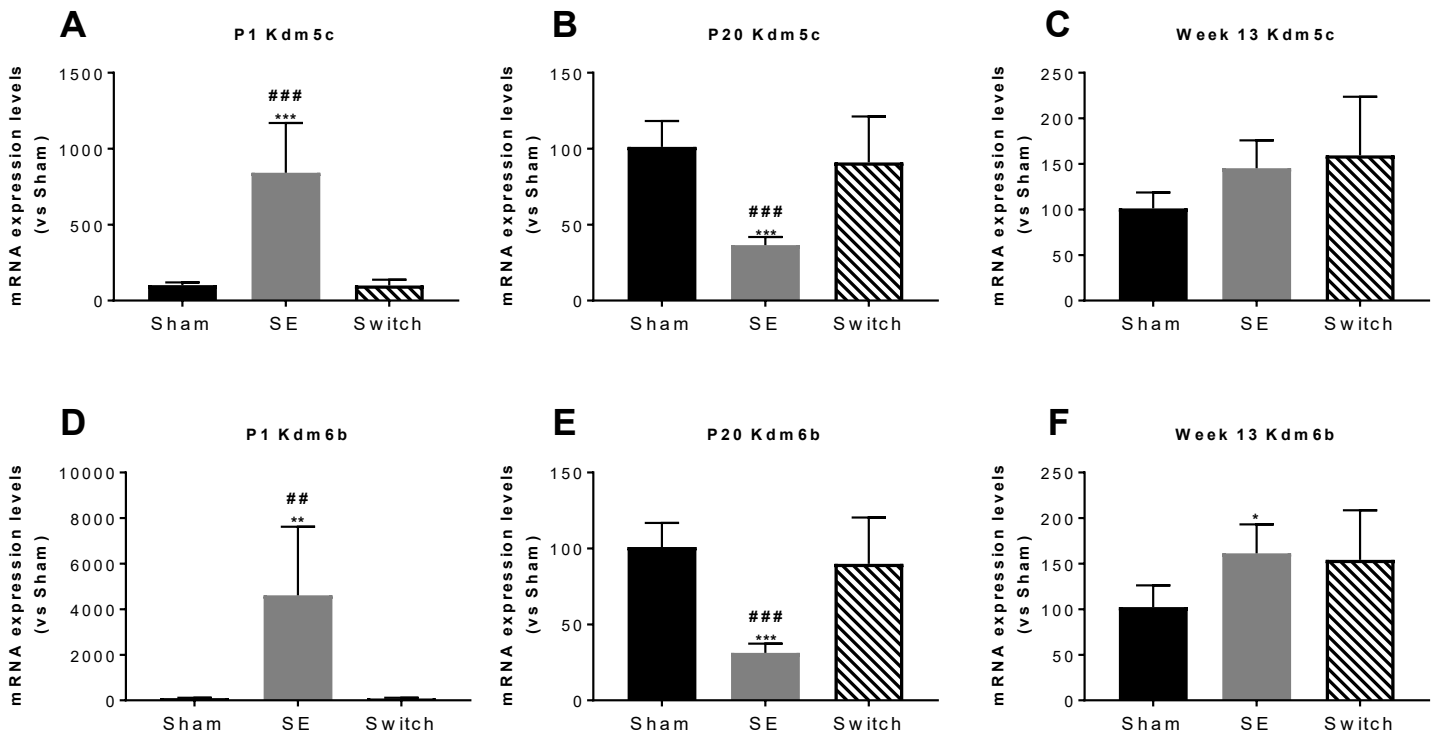

Figure 37. mRNA gene expression levels for histone methyltransferases (Kdm5c and Kdm6b) in offspring at postnatal day 1 (P1) whole brains, postnatal day 20 (P20) whole brains and Week 13 hippocampus. mRNA expression levels of the SE (n = 6) and switch group (n = 6) were expressed as a percentage compared to the Sham group (n = 6). Data represented as mean ± standard deviation, one-way ANOVA with Bonferroni post-hoc test, *p<0.05, **p<0.01, ***p<0.001 vs. Sham, ###p<0.001 vs. Switch. SE = smoke exposure.

3.4.8 Maternal smoking showed changes to Atf2 and Hdac1 gene expression in offspring at all time points

In the P1 brain tissue, Atf2 gene expression was significantly increased to 370.4±93.7 % in the SE group compared to the Sham group ($p<0.001$; Figure 38A) and the Switch group ($p<0.001$). Similarly, Hdac1 gene expression was significantly increased to 256.8±80.2 % in the SE group compared to the Sham group ($p<0.001$; Figure 38D) and the Switch group ($p<0.001$).

In the P20 brain tissue, Atf2 gene expression was significantly decreased to 36.2±5.9 % in the SE group compared to Sham group ($p<0.001$; Figure 38B) and the Switch group ($p<0.001$). Moreover, Hdac1 gene expression was significantly increased to 131.3±18.1 % in the SE group compared to the Sham group ($p<0.05$; Figure 38E).

In the Week 13 hippocampus, Atf2 gene expression was significantly increased to 152.9±17.2 % in the SE group compared to the Sham group ($p<0.001$; Figure 38C) and the Switch group ($p<0.001$). Similarly, Hdac1 gene expression was significantly increased to 126.7±9.6 % in the SE group compared to the Sham group ($p<0.001$; Figure 38F) and the Switch group ($p<0.001$). It is important to note that there were no significant differences observed in Atf2 and Hdac1 gene expression between the Sham group and the Switch group at any time points.

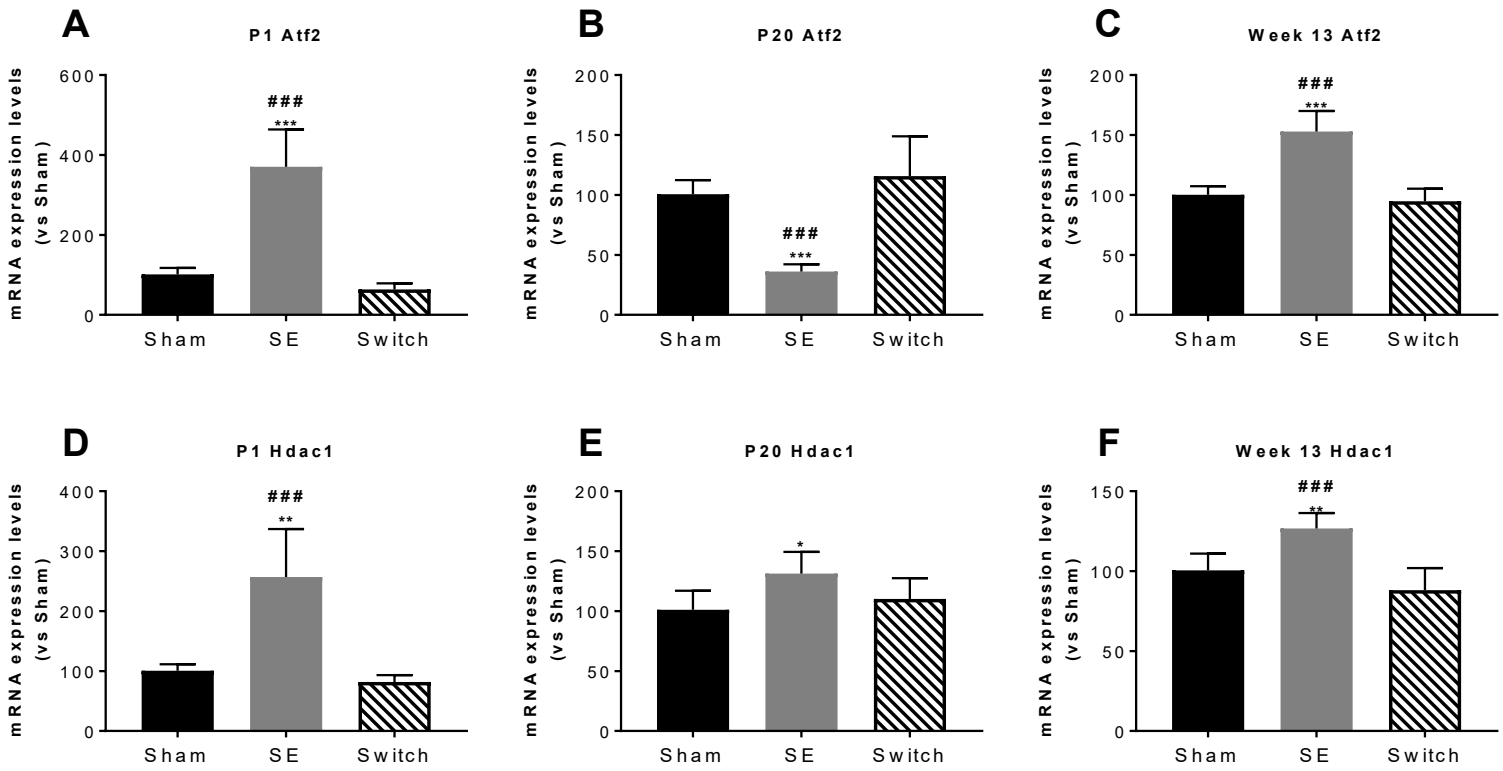

Figure 38. mRNA gene expression levels for histone acetyltransferase (Atf2) and histone deacetylase (Hdac1) in offspring at postnatal day 1 (P1) whole brains, postnatal day 20 (P20) whole brains and Week 13 hippocampus. mRNA expression levels of the SE (n = 6) and Switch group (n = 6) were expressed as a percentage compared to the Sham group (n = 6). Data represented as mean ± standard deviation, one-way ANOVA with Bonferroni post-hoc test, *p<0.05, **p<0.01, ***p<0.001 vs. Sham, ###p<0.001 vs. Switch. SE = smoke exposure.

3.4.9 Maternal smoking and switching to e-cigarette aerosols showed changes to AurkA, AurkB and AurkC gene expression in offspring at all time points

In the P1 brain, AurkA gene expression was significantly decreased to 31.7±4.4 % in the Switch group compared to the Sham group ($p<0.05$; Figure 39A) and the SE group ($p<0.001$). AurkB gene expression was significantly increased to 295.7±36.2 % in the SE group compared to the Sham group ($p<0.001$; Figure 39D) and the Switch group ($p<0.001$). However, AurkA gene expression in the Switch group was significantly decreased to 40.5±10.7 % compared to the Sham group ($p<0.001$; Figure 39D). No significant difference was found in the AurkC gene expression in the P1 brain between each treatment group (Figure 39G).

In the P20 brain, AurkA gene expression was significantly decreased to 47.1±9.1 % in the SE group compared to the Sham group ($p<0.001$; Figure 39B) and the Switch group ($p<0.001$). AurkB gene expression was significantly decreased to 49.8±14.4 % in the SE group compared to the Sham group ($p<0.001$; Figure 39E) and the Switch group ($p<0.001$; Figure 39E). AurkC gene expression was significantly decreased to 51.1±10.4 % in the SE group compared to the Switch group ($p<0.05$; Figure 39H).

In the Week 13 hippocampus, AurkA gene expression was significantly increased to 124.6±27.8 % in the SE group compared to the Switch group ($p<0.001$; Figure 39C). AurkB gene expression was significantly increased to 138.5±10.9 % in the SE group compared to the Sham group ($p<0.01$; Figure 39F) and the Switch group ($p<0.001$). However, AurkB gene expression was significantly decreased to 55.5±22.9 % in the Switch group compared to the Sham group ($p<0.001$). Finally, AurkC gene expression was significantly decreased to 34.4±10.9 % in the SE group compared to the Sham group ($p<0.01$; Figure 39I).

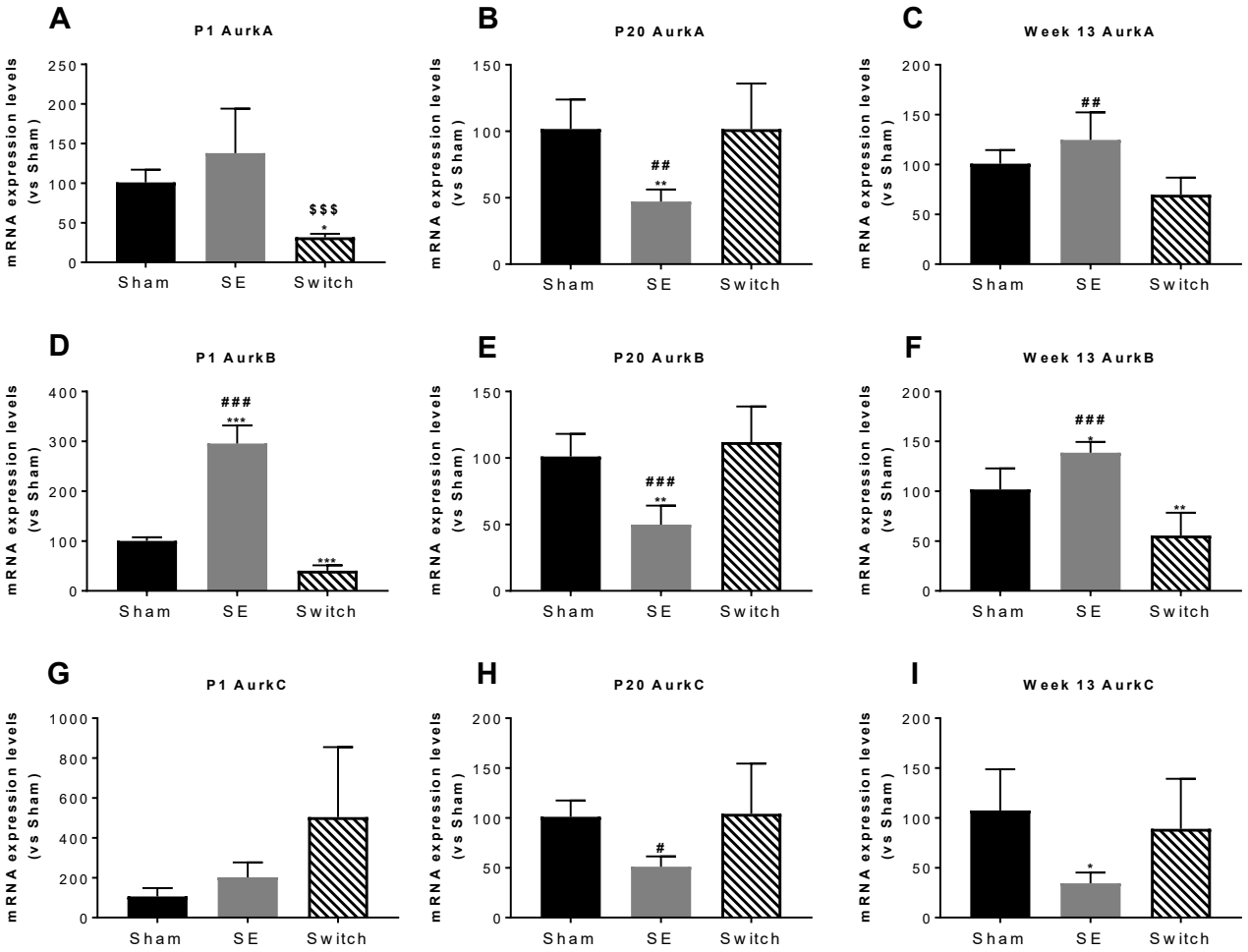

Figure 39. mRNA gene expression levels for histone phosphorylation (AurkA, AurkB and AurkC) in offspring at postnatal day 1 (P1) whole brains, postnatal day 20 (P20) whole brains and Week 13 hippocampus. mRNA expression levels in the Smoke exposure (SE) (n = 6) and Switch group (n = 6) were expressed as a percentage compared to the Sham group (n = 6). Data represented as mean ± standard deviation, one-way ANOVA with Bonferroni post-hoc test, *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. SE.

3.4.10 Maternal smoking showed a significant reduction in neuronal cell counts in the adult offspring in the hippocampus and amygdala

At P20, there were 170.43±8.83 neuronal cells/ROI in the CA1 region of the hippocampus in the Sham group (Figure 40A). This was significantly reduced in both the SE group (154.42±4.16 cells/ROI) and the Switch group (158±8.67 cells/ROI) ($p<0.05$). No significant changes in neuronal cell counts were observed at CA2 and CA3 in any treatment groups (Figure 40B&C). In the lateral amygdala there were 103.3±21.1 cells/ROI in the Sham group, 121.9±18.0 cells/ROI in the Switch group and 97.4±5.2 cells/ROI in the SE group (Figure 40D) which was significantly reduced compared to the Switch group ($p<0.05$).

At Week 13, there were 161.86±17.80 neuronal cells/ROI in the CA1 region of the hippocampus in the Sham group (Figure 40E). This was significantly decreased in the SE group to 143.00±10.56 cells/ROI compared to the Sham group ($p<0.05$) and the Switch group (164.3±12.2 cells/ROI, $p<0.05$; Figure 40E). In the CA2 region, the Sham group had a neuronal cell count of 100.00±8.74 cells/ROI. This was significantly decreased in the SE group (70.00±12.83 cells/ROI) compared to the Sham group ($p<0.05$) and the Switch group (101.3±18.0 cells/ROI) ($p<0.001$) (Figure 40F). In the CA3 region, there were 123.00±14.73 cells/ROI in the Sham group. This was significantly decreased in the SE group (103.25±8.65 cells/ROI) compared to the Sham group ($p<0.05$) and the Switch group (125.0±13.0 cells/ROI) ($p<0.05$) (Figure 40G). At the lateral amygdala nucleus, there were 84.0±14.0 cells/ROI in the Sham group. This was significantly decreased in the SE group (59.1±16.0 cells/ROI) compared to the Sham group ($p<0.05$) (Figure 40H).

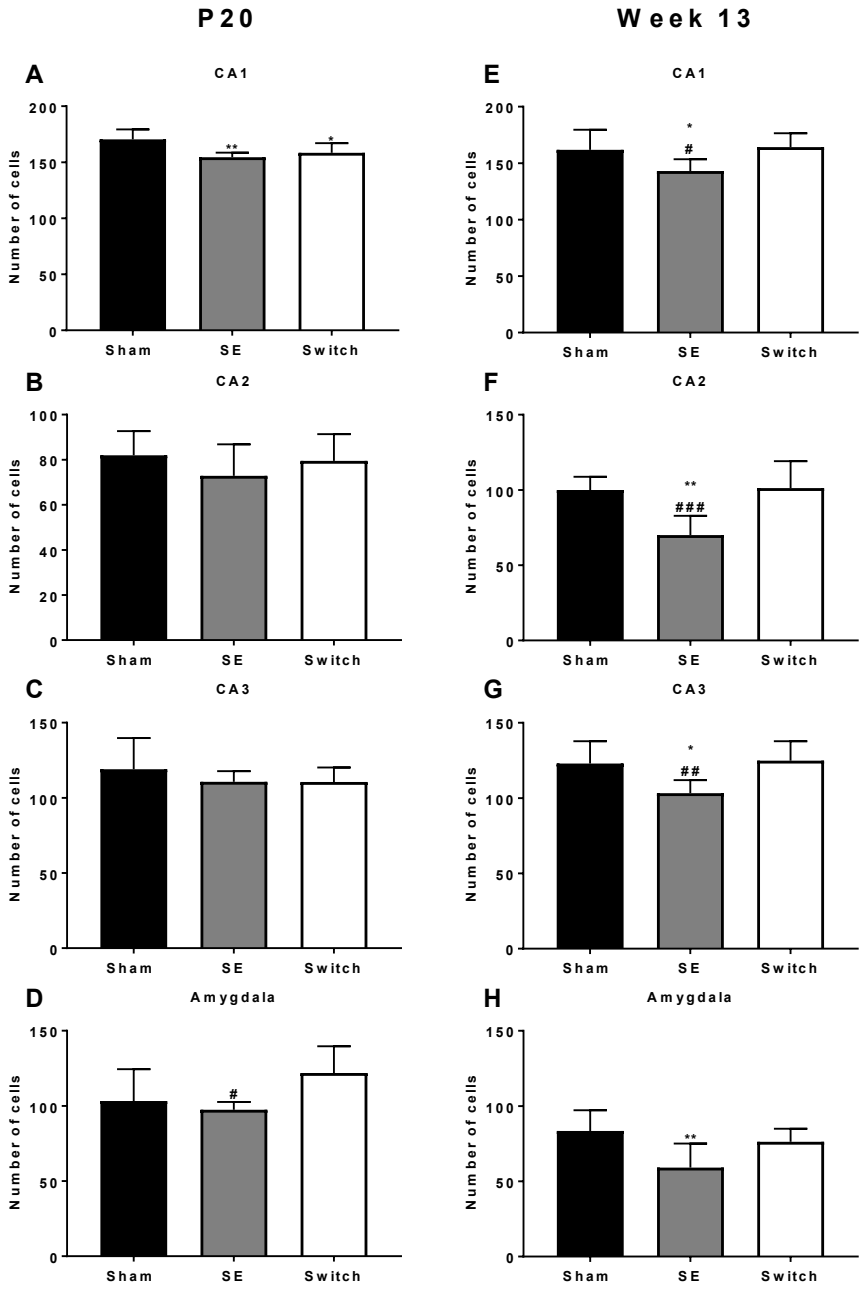

Figure 40. Neuronal cell counts of pyramidal neuronal cells in the postnatal day 20 (P20) (n = 8/group) and Week 13 offspring (n = 8/group) dorsal hippocampus and the lateral amygdala nucleus. Neuronal cells were counted at (A) CA1, (B) CA2, (C) CA3 and (D) amygdala at P20, and (D) CA1, (E) CA2, (F) CA3 and (F) amygdala at Week 13 from the Sham group, Switch group and Smoke Exposure (SE) group. Data represented as mean ± standard deviation, one-way ANOVA with Bonferroni post-hoc test. *p<0.05, **p<0.01 vs. Sham, #p<0.05, ##p<0.01, ###p<0.001 vs. Switch.

3.5 Discussion

The animal experiments outlined in **Chapter 2** observed significant changes to behaviour, global DNA methylation and epigenetic gene expression in offspring from maternal exposure to e-cigarette aerosols compared to mothers exposed to ambient air. Continuing from these experiments, the current study investigated whether switching to e-cigarette aerosols during gestation (from cigarette smoke exposure prior to gestation) alters offspring behaviour, DNA methylation and epigenetic gene expression compared to offspring from mothers exposed to cigarette smoke only.

3.5.1 Plasma cotinine levels were comparable in mothers and offspring exposed to cigarette smoke and e-cigarette aerosol from different exposure systems

In many animal studies, plasma cotinine was used as a biomarker for determining the level of nicotine absorption from different methods of nicotine delivery [48, 136, 197, 373, 374]. For the current experiments, due to changes to the smoke/aerosol exposure protocols, animals from the Switch group were exposed to treatment using a chamber system, and the animals in the smoking group were exposed to treatment using the InExpose system as requested by the Animal Care and Ethics Committee (Ethics amendment ETH15-0025). To ensure that each group was comparable to each other, plasma cotinine levels were measured in mothers and offspring right after weaning to ensure that the same amount of nicotine was being delivered by both protocols. From the results, animals treated in both systems showed comparable levels of plasma cotinine in both mothers and offspring. Therefore, the similarity in cotinine levels that was delivered from both systems permitted good comparability between each treatment group in this study. It is important to note that only the breeders were placed in the chamber during aerosol exposure, i.e. the

offspring remain in the home cage. Thus, the elevation in cotinine levels is likely to be from nicotine exposure *in utero*, breastfeeding and fur contact [375, 376].

3.5.2 Offspring from mothers exposed to cigarette smoke and e-cigarette aerosols showed a reduced body weight at birth and at weaning

From the body weight data, offspring from mothers exposed to e-cigarette aerosols containing nicotine had a reduced whole-body weight at birth and at weaning. However, no changes in body weights were observed in adulthood. These results suggest that the reduction in body weight is due to nicotine exposure from the e-cigarette aerosols and the cigarette smoke. These results have been consistent with a number of studies that showed an association with a lower birth weight and prenatal nicotine and smoke exposure [93, 196, 197, 207, 309, 377-382]. In addition, elevated cotinine levels in the serum and/or urine have also been correlated to a reduction in body weight from cigarette smoke [196, 197, 207, 381, 382] and e-cigarette aerosols with nicotine [48, 134-136].

3.5.3 Offspring from mothers exposed to e-cigarette aerosols containing nicotine showed short-term memory deficits and increased exploration and increased motor activity

Behavioural assessments using the NOR and EPM test has been widely used as a measure of memory (NOR), exploration (EPM) and anxiety (EPM) [285, 312, 383-385]. From the NOR results, offspring from mothers that were exposed to e-cigarette aerosols with nicotine, but not cigarette smoke showed short-term memory deficits. This result suggests that chemicals from the e-cigarette aerosol other than nicotine are causing memory deficits within these offspring that

persist into adulthood. Many studies have reported changes to working memory and retention in offspring from maternal smoking. Zhang and colleagues investigated memory retention from animals exposed to nicotine in drinking water and found deficits in working memory and attention [312]. In addition, Hall and colleagues investigated mice treated with tobacco smoke extract and found deficits in working memory and attention [386]. Moreover, offspring from mothers exposed to cigarette smoke showed a 15% reduction in novel object exploration compared to animals exposed to ambient air [196]. In human studies, children from mothers that smoked during pregnancy showed difficulties in completing activities that require working memory and memory retention [387-389].

Although there is sufficient evidence in human and animal studies to conclude that maternal smoking is associated with learning and memory deficits, the current study found no memory deficits in offspring from smoking mothers. This may be due to the delivery method of the nicotine, the concentration of nicotine and the experimental design of the study. Regardless, offspring from mothers exposed to either cigarette smoke or e-cigarette vapours had comparable plasma cotinine, and only offspring from the e-cigarette group showed changes to memory retention. It could be speculated that compounds other than nicotine in the e-cigarette aerosol could be causing the changes that are being observed in the NOR test. The four main ingredients that make up an e-cigarette liquid includes various concentrations of propylene glycol, glycerine, flavouring and nicotine. Although, studies are limited on the effects of glycerine inhalation on the brain, there are studies that investigated exposure to propylene glycol, e-liquid flavourants and nicotine toxicity in *in vivo* and *in vitro* experiments. In a study from Lau and colleagues, although exposure of propylene glycol was by intraperitoneal injection, they reported neuronal apoptosis at various regions in the infant mouse brain in a dose-dependent manner [143]. However, many studies have investigated the effects of various flavourings in e-cigarette liquids and found flavours like cinnamaldehyde (caramel and butterscotch flavouring), vanillin (vanilla flavouring), and 2,5-dimethylpyrazine (chocolate flavouring) were toxic to human embryonic stem cells and airway epithelial cells [170, 178, 182]. Taken together, various compounds within e-liquids might

be responsible for the memory deficits that were being observed, however, the mechanisms that drives these changes remain to be elucidated. Gas chromatography and mass spectroscopy of e-liquids are currently being analysed by other researchers at the University of Technology Sydney.

From the EPM test, an increase in offspring exploration and motor activity were observed from the time spent in the open arm and the number of centre crosses. Interestingly, offspring from mothers switching to e-cigarette aerosols showed no significant changes in anxiety behaviour compared to mothers exposed to cigarette smoke. These results indicate that offspring may have manifestations of hyperactivity from the nicotine in the cigarette smoke and e-cigarette aerosols that have been widely reported in many human and animal studies [199, 309, 390-393]. Other psychological changes that have been associated with maternal smoking also included anti-social behaviour, aggressive behaviour and depression [278, 279, 390].

Overall, switching to e-cigarettes during pregnancy showed no changes in offspring motor activity and exploration compared to offspring from mothers exposed to cigarette smoke. In addition, it appears that switching to e-cigarette during pregnancy showed minimal changes to risk-taking behaviour in the offspring compared to the sham and smoking group. Thus, replacing cigarette smoke exposure with e-cigarette aerosol exposure may have little effect on subsequent behavioural changes in the offspring.

3.5.4 Offspring from mothers exposed to e-cigarette aerosols increased levels of DNA Methylation compared to offspring exposed to ambient air

Global DNA methylation was found to be the highest in offspring from mothers exposed to tobacco cigarettes at birth, weaning and adulthood. Interestingly, switching to e-cigarettes during pregnancy reduced the percentage of DNA methylation compared to the smoking group but remains significantly high compared to offspring from mothers exposed to ambient air. Research on maternal smoking and DNA methylation has only recently been emerging, and the mechanism

that drives DNA methylation from cigarette smoke are becoming better understood. For example, exposure to tobacco cigarette smoke is now known to alter DNA methylation which can cause DNA damage, downstream changes to other chromatin modifiers, and hypoxia [394].

Switching to e-cigarettes significantly reduced DNA methylation, however, did not completely reduce methylation back to baseline compared to the sham. This is most likely due to the nicotine and other compounds being released from the e-cigarette aerosols. Nicotine has been widely known to bind to nAChR which causes the release of calcium that activates downstream pathways that are important in controlling gene expression [395]. Chemicals within tobacco cigarettes such as arsenic [396], polycyclic aromatic hydrocarbons [397], and acrolein [398] are known to cause alterations in DNA methylation and DNA damage, as well as changes to chromatin modification gene expression. Although e-cigarettes are known to release similar compounds to cigarette smoke, such as volatile organic compounds, polycyclic aromatic hydrocarbons, formaldehyde, and tobacco-specific nitrosamines [399-402], the concentration of these compounds that get released in the aerosol are minimal [101, 403].

It is important to note that the current study investigated global DNA methylation in the offspring brain, and thus, did not show which genes were methylated on the DNA. However, future studies are required to locate genes that could be affected by e-cigarette aerosol exposure. There are studies that have been done to investigate specific genes that are affected by cigarette smoke. For example, nicotine has been shown to cause changes to DNA methylation at the glutamic acid decarboxylase 67 promoter in GABAergic interneurons in the frontal cortex [404]. In addition, people who are heavy smokers showed changes to DNA methylation in tumour-related genes FHIT, RASSF1A and RUNX3 in human adenocarcinomas [405]. In tissue specific-studies of paternal nicotine exposure, McCarthy and colleagues showed changes to DNA methylation in the testes of father mice which were found to be linked with behavioural changes in two-transgenerational offspring [331]. All of these studies are important in elucidating the mechanisms underlying prenatal e-cigarette exposure and connecting DNA methylation with changes to behavioural alterations in offspring.

3.5.5 DNA methyltransferases and histone demethylase, acetyltransferase and deacetylase but not aurora kinases showed minimal changes to gene expression in offspring from mothers that switched to e-cigarette aerosols compared to offspring exposed to cigarette smoke

From the previous animal experiment, gene expression of chromatin modifiers: DNA methyltransferases (Dnmt3a, Dnmt3b), histone methyltransferases (Kdm5c, Kdm6b) and histone acetylase (Atf2), have been shown to be altered in offspring from mothers exposed to e-cigarette aerosols with and without nicotine compared to mothers exposed to ambient air. This current study investigated gene expression of the same chromatin modifiers in offspring from mothers exposed to cigarette smoke or switching to e-cigarette aerosols during pregnancy. In contrast, there were no significant changes to chromatin modifying genes Dnmt3a, Dnmt3b, Kdm5c, Kdm6b, Atf2 and Hdac1 in offspring brains from mothers switching to e-cigarette aerosols at birth, weaning and adulthood. However, aurora kinases AurkA, AurkB and AurkC showed significant changes to gene expression in offspring from mothers exposed to e-cigarette aerosols. Offspring from the smoking group showed various gene changes to the chromatin modifying genes investigated at birth, weaning and adulthood.

Dnmt3a and Dnmt3b are *de novo* enzymes that are directly involved in transferring methyl groups to the CpG islands on the DNA. Thus, DNA methyltransferases have an important role in maintaining DNA methylation within the genome. As mentioned above, nicotine has been known to cause DNA methylation within the genome [406]. It is also known that maternal smoking causes DNA hypomethylation in foetal dorsolateral prefrontal cortex [332]; however, in this study, maternal e-cigarette exposure did not seem to induce any significant changes to Dnmt3a and Dnmt3b gene expression in the offspring from the Switch group.

Similar to these results, histone demethylases (Kdm5c and Kdm6b), histone acetylase (Atf2) and histone deacetylase (Hdac1) showed no significant changes to gene expression in offspring from mothers that switched to e-cigarettes. However, significant gene changes were seen in the smoking group. Offspring from the smoking group had an increased in Kdm5c and Kdm6b gene expression right after birth followed by a significant decrease at weaning. The changes that are being observed are not well understood, however, these changes could be due to a change in environment within the womb during extensive growth changes, i.e. from significant growth that occurs during gestation and right after birth. Histone demethylase, particularly Kdm6b has been shown to be important in neuronal cell growth and differentiation *in vivo* and *in vitro* [407, 408]. In addition, the lack of Kdm6b expression resulted in impaired neuronal maturation in a mouse model and in hippocampal cell culture [409, 410]. There have been reports of Kdm5c down regulation in mouse lungs during acute exposure to cigarette smoke, however, no significant changes during chronic exposure [411].

Interestingly, Sundar and colleagues showed in the mouse lungs that Dnmt3a, Dnmt3b and AurkB gene expression was up-regulated after acute exposure of cigarette smoke, but down-regulated after chronic exposure [411]. Kdm5c gene expression was shown to be down-regulated during acute exposure (3 consecutive days) of smoke exposure, but there were no significant changes found after chronic exposure (6 months). Although Hdac1 was not seen to be changed at all in this study, other histone deacetylases such as Hdac2, Hdac4, Hdac8 and Hdac9 gene expression were shown to be variously changed after acute and chronic smoke exposure [411]. This may suggest that the length of smoke exposure affects the expression of certain epigenetic genes. It is also important to note that these changes do not completely represent the results observed in a pregnancy model, however, it provides an indication of what epigenetic changes are expected to be altered in the same gene from different lengths of direct smoke exposure. Studies on the effects of maternal e-cigarette exposure on epigenetics are still limited, therefore, more studies on epigenetic gene changes between maternal smoking and maternal ‘vaping’ are important.

Cigarette smoke exposure has widely been known to significantly affect the epigenome of an individual [412-415]. Similar toxic compounds that are found in cigarette smoke are also found in e-cigarette aerosols including aldehydes, volatile organic compounds, polyaromatic hydrocarbons, tobacco-specific nitrosamines and heavy metals (from the eroding coil). These have also been known to modify the chromatin remodelling environment [315, 399-402]. Although concentrations of different compounds vary among puff topography, e-cigarette device and e-liquid, concentrations of these compounds that were released from the e-cigarettes are generally low. Therefore, this could be the reason why gene expression of key chromatin modifiers was not overly changed in the offspring from the Switch group. This study also reinforces the severe changes that could happen to the offspring genome when mothers continue to smoke during their pregnancy.

Offspring from mothers that switched to e-cigarettes during pregnancy showed significant gene changes in AurkA and AurkB expression but not AurkC. In addition, offspring from mothers exposed to cigarette smoke also showed significant gene changes in AurkA, AurkB and AurkC. It has been shown that exposure to cigarette smoke in mice can result in AurkB gene changes in mouse lungs and human bronchial epithelial cells [411]. AurkA, AurkB and AurkC are important in the proper alignment and segregation of the chromosomes during mitotic division. Therefore, significant alterations in any of these aurora kinases could result in destabilisation and improper alignment of the chromosome which can result in neuronal dysregulation and development [416]. Overall, these results suggest that switching to e-cigarette aerosol exposure *in utero* relieved some changes to the epigenome compared to continuously being exposed to cigarette smoke.

3.5.6 Maternal smoking affects neuronal counts in regions of the dorsal hippocampus and the lateral amygdala nucleus

The neuronal cell numbers in the CA1, CA2, and CA3 of the dorsal hippocampus and lateral amygdala nucleus of the adult offspring were reduced following maternal smoke exposure. In the Switch group, however, a significant reduction in neuronal counts were only observed in the hippocampal region CA1. Unexpectedly, there were no changes in the smoking group for short-term memory in the behavioural data. Furthermore, histone deacetylases, which have been known to reduce memory retention and cognition [417], were shown to be significantly increased in the continuous smoking group but not the Switch group. As both decreased cell number and increased Hdac1 might have been expected to alter memory, these findings cannot be explained other than that there might be an underlying mechanism that is causing deficits in short-term memory after e-cigarette exposure, which may be independent of the increase in Hdac1 gene expression.

There have been studies showing the effects of nicotine on neuronal cells *in vivo*. Chronic administration of nicotine has been known to reduce neurogenesis and increase the number of apoptotic cells in the dendate gyrus [346]. Prenatal exposure to nicotine through the drinking water showed a decrease in the offspring cingulate cortex which is involved in attention and awareness [309]. In addition, prenatal nicotine exposure in rhesus monkeys have shown an increase in nAChR in the hippocampus, brain stem and frontal cortex [307]. However, there are studies that did not show any significant loss of neurons in the hippocampus after cigarette smoke exposure in animal models [418]. Future studies are needed to investigate the markers associated with apoptosis and oxidative stress since these two parameters have been shown to be elevated in the hippocampus after exposure to cigarette smoke [418].

Conversely, the e-cigarette exposure had an effect on memory but not on cell number. This highlights the difficulty of teasing out the effects of tobacco chemicals, nicotine and chemicals in the e-fluids and how they may alter neurological function at different post-natal time points. It has been known that prenatal exposure to nicotine compromises offspring neuronal number,

maturation and morphology in varying regions of the brain such as the hippocampus [306, 308, 346, 348]. This could lead to long-lasting effects that can continue in adulthood. Furthermore, stereotaxic counting is the preferred technique to count neuronal cells since it provides an absolute count within the region of interest in the brain and accounts for every individual neuron [419]. Due to the methodology used in the current study this technique was not possible and the region of interest was compared at one level of the brain only.

In a mouse model of prenatal e-cigarette exposure, Zelikoff and colleagues did not observe changes to the neuron population in the hippocampus, which is consistent with the current study's findings [51]. They did, however, find an increase in IBA-1 expression which is a known marker for microglia [51]. This could suggest that e-cigarette exposure prenatally could have an effect on brain cells other than neurons, and this needs to be further elucidated.

3.6 Conclusion

The current study shows that switching to e-cigarettes during gestation reduces some of the severe effects from maternal smoking. Switching to e-cigarette aerosols with nicotine does not improve offspring body weight, brain weight, hyperactivity or exploration within offspring compared to the smoking group, however, anxiety levels seem to be reduced to baseline. Offspring from mothers who switched to e-cigarette aerosols showed reduced DNA methylation and reduced up-regulation of selected genes, however, aurora kinases, which are important in neuronal development and maturation, were equally up-regulated compared to continuous cigarette smoke exposure.

The findings from this chapter provide evidence that switching from tobacco smoking to e-cigarette during pregnancy seems to improve offspring neurological changes compared to smoking alone, but does not from all effects. There is still further research needed to determine the mechanisms of action and contribution of individual constituents in different e-liquid products.

The results shown in this chapter have been accepted for publication:

Nguyen, T., Li, GE., Chen, H., Cranfield, CG., McGrath, KC., Gorrie, CA. *Neurological effects in the offspring after switching from tobacco cigarettes to e-cigarettes during pregnancy in a mouse model.* Toxicological Sciences, [Accepted for publication].

Chapter 4 – *In vitro* effects of e-cigarette aerosol condensate using neuronal cell culture

4.1 Introduction

4.1.1 *In vitro* blood brain barrier model

The blood brain barrier (BBB) is the network of microvascular blood vessels in the brain that forms a gateway to control the transport of glucose, amino acids, ions, and macromolecules [420]. The BBB consists of the endothelial cells with tight cell-cell contact, a basement membrane of extracellular matrix tissue, a layer of pericytes and astrocytic foot processes [420] (Figure 41). Replicating the complex interaction between cells in cell culture is deemed technically difficult [421-423]. Many studies have developed an *in vitro* BBB model that comprised of either endothelial cells only or a combination of endothelial cells, pericytes, astrocytes and neuronal cells [421, 424, 425]. These models have been widely used to examine the effects of drug delivery [426-428].

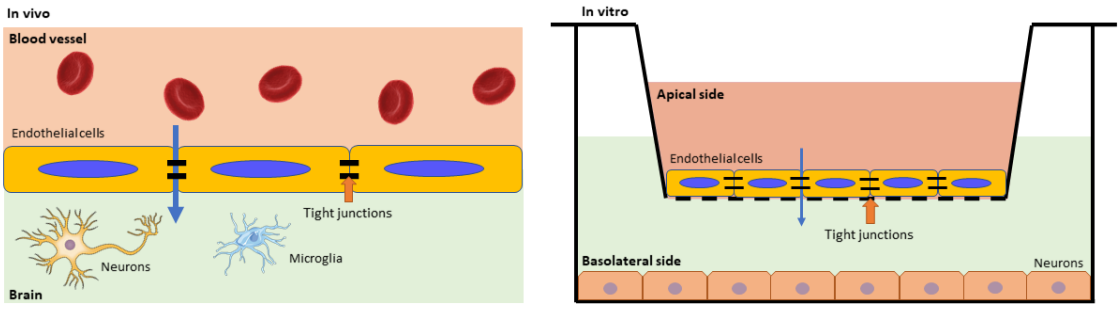

Figure 41. Schematic of *in vivo* and *in vitro* blood brain barrier illustrations. The *in vivo* blood brain barrier model shows the endothelial cells forming tight junctions. The *in vitro* blood brain barrier model uses a transwell system where endothelial cells are grown to confluency in the upper chamber and the bottom chamber consist of brain-related cells such as neurons, astrocytes and perciytes.

Currently, there are studies that examined the effects of tobacco smoke and the trans-endothelial electrical resistance (TEER) using an *in vitro* BBB model [429-433]. By using this model, Kim and colleagues have showed a reduction in endothelial membrane integrity after continuous exposure to tobacco cigarette extracts [429]. In addition, Lee and colleagues demonstrated a reduction in endothelial membrane integrity following acute tobacco cigarette exposure and a worsened membrane integrity after prolonged exposure [430]. In addition, nicotine treatment on the BBB *in vitro* using brain endothelial cells caused an increase in membrane permeability and tight junction disruption [431-433]. All of these studies have shown an effect on either tobacco cigarette smoke or nicotine, indicating that constituents released from conventional cigarettes, that might also be released from e-cigarettes, could potentially disturb the BBB.

There are limited studies examining the effects of e-cigarettes in an *in vitro* BBB model. Kaiser and colleagues investigated the effects of e-cigarette aerosols on a BBB model using mouse brain microvascular endothelial cells and found an increase in oxidative stress, a decrease in tight junction protein zonula occludens-1 (ZO-1), and an increase in vascular adhesion molecules platelet endothelial cell adhesion molecule-1 (PECAM-1), intercellular adhesion molecule 1 and vascular cell adhesion molecule 1 [434]. These results indicate that e-cigarette exposure exhibit

similar detrimental effects to tobacco cigarette smoke. Currently, there are no studies that have adapted a co-culture model containing microglial cells. However, a study has shown an increase in microglia in the hippocampus after maternal exposure to e-cigarette aerosols *in vivo* [51].

4.2 Hypothesis and aims

4.2.1 Hypothesis

The hypotheses of this study are:

1. That treatment with e-cigarette aerosol condensate (ECAC) will affect cell viability, reactive oxygen release and mitochondrial depolarisation in differentiated neuroblastoma (SHSY5Y) cells, microglial (BV2) cells and human brain endothelial cells (HBEC) in monoculture.
2. That treatment with ECAC on an endothelial membrane will alter permeability and integrity in co-culture models of HBECs, differentiated SHSY5Y cells and BV2 cells.
3. That epigenetic and inflammatory genes will be significantly changed in differentiated SHSY5Y cells and BV2 cells, respectively, in a co-culture model of HBECs, following exposure to ECAC.

4.2.2 Aims

To investigate the effects of ECAC on CNS-based cells in cell culture by:

1. Determining the effects of ECAC on cell viability (MTT assay), reactive oxygen species production (DCF assay), and mitochondrial membrane potential (JC-10 assay) in differentiated SHSY5Y cells, BV2 cells and HBECs in monoculture.
2. Determining endothelial membrane permeability and integrity following ECAC exposure using FITC-Dextran and TEER measurement using BBB co-culture cell models of HBECs, differentiated SHSY5Y cells and BV2 cells.
3. Determining epigenetic gene changes in the differentiated SHSY5Y cells following ECAC exposure using BBB co-culture model of HBECs.
4. Determining inflammatory gene changes in the BV2 cells following ECAC exposure using a BBB co-culture model.

4.3 Materials and Methodology

4.3.1 Experimental cell lines and conditions

The timeline for all the experiments is outlined in Figure 42. For *in vitro* experiments, three key cell types that forms the CNS were used to determine the effects of ECAC, namely, human neuroblastoma (SHSY5Y) cells were used to represent neuronal cells; murine microglial (BV2) cells were used for microglia; and primary human brain microvascular endothelial cells (HBEC; cAP-0002) were used for the upper layer of the BBB model.

SHSY5Y cells (kindly donated by Dr Jerran Santos, University of Technology Sydney) are neuroblastoma cells sub-cloned from SH-N-SY cells. Cells were grown in Falcon® T75 flasks and maintained in Dulbecco Modified Eagle Medium (DMEM) F12 GlutaMAX™ media supplemented with 10% foetal bovine serum (FBS) and 5 mM HEPES. Growth media was changed every 3-4 days and cells were passaged once cells reached 80%-90% confluency. Experiments were performed on SHSY5Y cells below passage number 35 to avoid cell senescence. Cells were kept in a humidified incubator with 5% CO_2 and at a temperature of 37^{o}C.

BV2 cells (kindly donated by Dr Alessander Cassorina, University of Technology Sydney) were grown in Falcon® T75 flasks and maintained in DMEM F12 GlutaMAX™ media supplemented with 10% FBS and 5 mM HEPES. Growth media was changed every 3-4 days and cells were passaged once cells reached 80-90% confluency. Experiments were performed on BV2 cells below passage number 20 to avoid cell senescence. Cells were kept in a humidified incubator with 5% CO_2 and at a temperature of 37^{o}C.

HBECs [435] (kindly donated by Associate Professor Valery Combes, University of Technology Sydney) were seeded in ultra-Low attachment T75 Flasks (Corning®, NY, USA) coated with 0.3% Type I collagen (BD Biosciences, NJ, USA) for one hour. HBEC were grown in Endothelial Cell Growth Basal Medium-2 (Lonza, Basel, CH) supplemented with 5% FBS, 1.4 µM of hydrocortisone, 5 ug/mL ascorbic acid, 5 mL chemically defined lipid concentrate, 10 mM

HEPES, and 1 ng/mL basic fibroblast growth factor. Growth media was changed every 2-3 days once cells reached 70-80% confluency. Experiments were performed on HBEC below passage number 7 to avoid cell senescence. Cells were kept in a humidified incubator with 5% CO_2 and at a temperature of 37ºC.

Mycoplasma testing was performed every three months during experimentation to ensure no mycoplasma contamination found in each cell line.

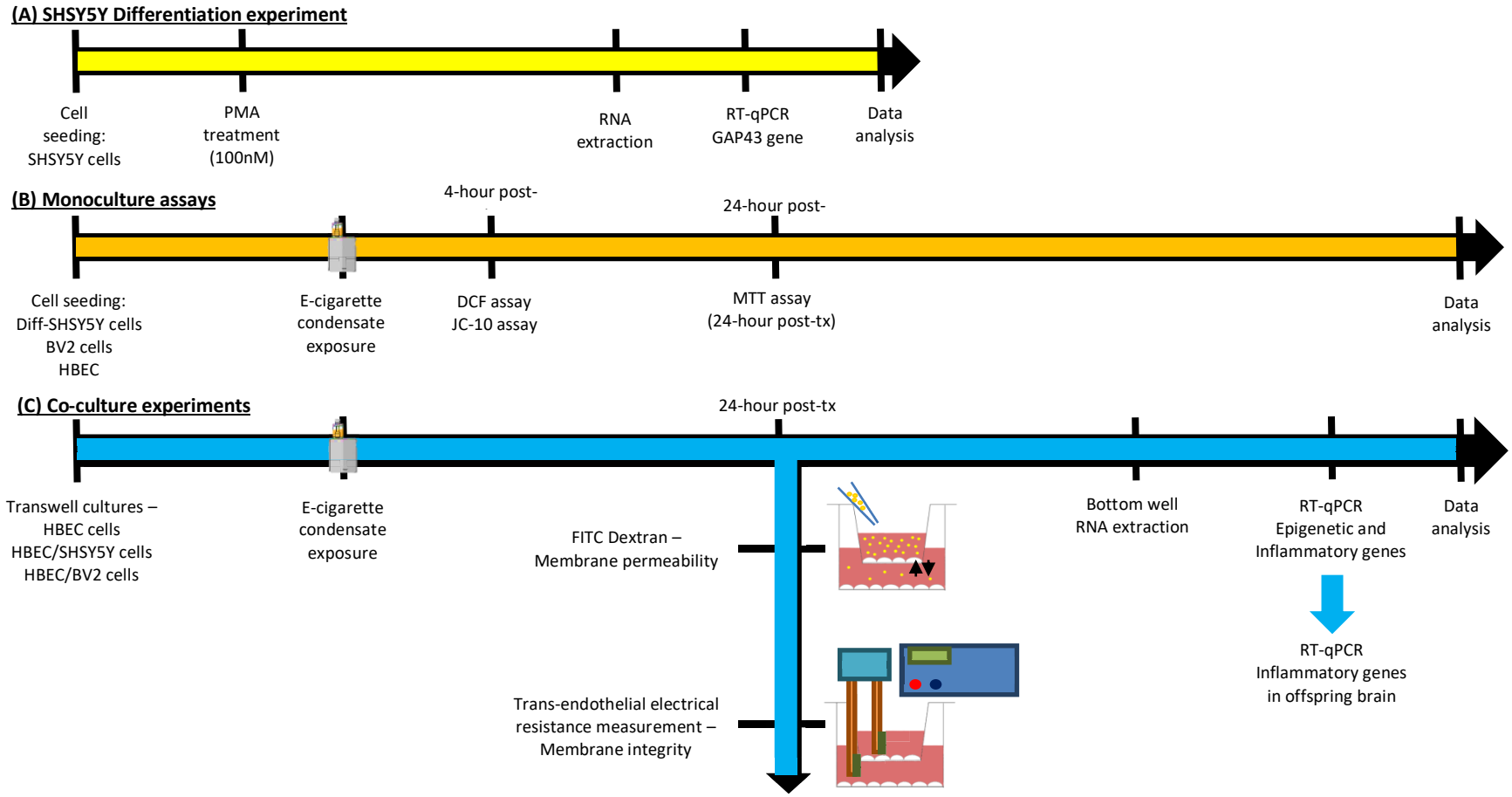

Figure 42. Timeline summary of the in vitro experiments for Chapter 4. (A) Optimisation of neuroblastoma (SHSY5Y) cells differentiation was conducted using Phorbol 12-myristate 13-acetate (PMA) and validated by growth associated protein 43 (GAP43) gene expression. (B) Monoculture assays were performed on differentiated SHSY5Y (diff-SHSY5Y) cells, microglial (BV2) cells and human brain endothelial cells (HBEC) using the methylthiazolydiphenyl-tetrazolium bromide (MTT) assay, 2' 7'-Dichlorofluorescein (DCF) assay and the JC-10 assay. (C) Co-cultures were performed using HBEC only, HBEC/diff-SHSY5Y cells, and HBEC/BV2 cells to represent a blood brain barrier model. Fluorescein isothiocyanate (FITC)-dextran and trans-endothelial electrical resistance (TEER) was measured to determine HBEC membrane integrity. RNA was extracted from diff-SHSY5Y cells and BV2 cells to analyse epigenetic and inflammatory gene expression after e-cigarette aerosol condensate treatment.

4.3.2 SHSY5Y cell differentiation – Optimisation

4.3.2.1 Differentiation with phorbol 12-myristate 13-acetate (PMA)

At 80% confluence, SHSY5Y cells were differentiated in DMEM F12/hams media (Gibco, MA, USA) supplemented with 15% FBS, 20 nM non-essential amino acids (NEAA), and 100 nM phorbol 12-myristate 13-acetate (PMA) (Sigma Aldrich, MO, USA). Cells were differentiated in a 24 well-plate at 1.3 x 10^5 cells/well with PMA for three consecutive days before the commencement of experimental treatments. Cells were starved overnight with serum-free media before experimental treatment.

4.3.2.2 Validation of PMA treated SHSY5Y cells via RT-qPCR

4.3.2.2.1 RNA extraction

To ensure that the SHSY5Y cells were differentiated using 100 mM of PMA, gene expression of GAP43 was determined based off a previous study [436]. Total RNA was extracted by the addition of 400 µl of *TRIsure*™ (Bioline, MA, USA) to the cells. The TRIsure™-cell mix was then vortexed following the addition of 1/10^{th} volume of 1-Bromo-3-Chloropropane before separation of the organic and aqueous phase by centrifugation at 12,000 g for 15 minutes at 4 °C. After centrifugation, the aqueous phase was transferred into a fresh tube before 500 µl of isopropanol was added for RNA precipitation overnight at -20 ºC. Precipitated RNA was pelleted by centrifugation at 12,000 g for 10 minutes at 4°C. Supernatant was then removed before the addition of ice-cold 75 % ethanol to wash the RNA. Tubes were spun down once more at 20,000 g for 10 minutes before supernatant was removed. RNA was left to air-dry for 10 minutes before resuspending the RNA pellet in nuclease-free water. RNA concentration was quantified using a Nanodrop®. RNA quality was checked using the *Experion RNA StdSens Analysis* Kit (Bio-Rad,

CA, USA) following the manufacturer's protocol. RNA was stored in a -80ºC freezer until ready for reverse transcription.

4.3.2.2.2 RNA quality checks

Refer to **Chapter 2 section 2.3.8** for the full protocol.

4.3.2.2.3 Reverse transcription

Refer to **Chapter 2 section 2.3.11.1** for the full protocol.

4.3.2.2.4 Real time PCR assay

Refer to **Chapter 2 section 2.3.11.2** for the full protocol. The gene that was used to determine SHSY5Y maturation is growth associated protein 43 (GAP43). GAP43 is a neuronal marker that is expressed in mature neurons and has been used as a gene to identify differentiated neuroblastoma cells in previous studies [436-439]. SHSY5Y cell differentiation was confirmed by a GAP43 upregulation of up to 3-folds compared to undifferentiated SHSY5Y cells. GAP43 gene expression were determined by the $\Delta\Delta C_T$ method [291] using GAPDH as the reference gene (Table 4).

Table 4. Details of GAP43 gene to validate PMA-treated SHSY5Y cells using GAPDH as the reference gene.

Gene ID	Description	F/R	Primer sequence (5'→3')
GAP43	Growth associated protein 43	Forward	TGATAACTCGCCGTCCTCCAAG
		Reverse	AGCAGCAGCAGTGACAGCAG
GAPDH	Glyceraldehyde 6-phosphate dehydrogenase	Forward	ACAGTTGCCATGTAGACC
		Reverse	TTGAGCACAGGGTACTTTA

4.3.3 E-cigarette aerosol condensation

For all of the *in vitro* experiments, an ECAC was used to treat each of the cell lines. It is important to note that the ECAC was used in preference to the e-liquid. This is because heating the e-liquids to an aerosol using an e-cigarette device alters their chemical composition [440], whilst testing the e-liquid without any heating may not reveal the true effects of inhaling e-cigarette aerosols. In addition, due to its viscosity and oily nature, testing e-liquids directly into culture may not provide the adequate penetration into the cell culture medium. Therefore, the ECAC was used for all of the i*n vitro* experiments. To create the ECAC, a closed system was used that incorporated an air-pump, tubing from the e-cigarette to a T25 flask, and tubing from the T25 flask to the air pump. The T25 flask was placed in an icebox filled with dry ice. The apparatus set up was illustrated as a schematic in Figure 43. The air pump was turned on and the e-cigarette device was activated simultaneously, and the e-cigarette aerosols were released from the e-cigarette device into the flask for seven seconds before the pump and e-cigarette device was switched off. This process was repeated up to 10 times with a 30 second break in between each aerosol release to avoid burning the coil inside the tank. During this process, the e-cigarette aerosol condenses into a liquid which was then collected in a T25 flask. Approximately 150 µL-300 µL condensate is needed for individual experiments. After the ECAC was collected, the entire flask was sealed with parafilm and stored at -80ºC. The ECAC was created using; PGVG, PGVG with nicotine (PGVG+nic), PGVG with tobacco flavour (PGVG+Flavour) and PGVG with tobacco flavour and nicotine (PGVG+Flavour+nic) (Table 5). Each treatment was held in separate tanks to avoid contamination. Tobacco-flavoured e-cigarettes are considered to be one of the most popular e-liquid flavours used [441]. In addition, tobacco-flavoured e-cigarettes have been rated as a popular flavour for current and former smokers [442]. Therefore, the effects of tobacco-flavoured ECAC were investigated. Propylene glycol and glycerine (vegetable glycerin) were purchased as high purity chemicals. Chemical analysis of tobacco-flavoured e-cigarette aerosols with and without nicotine are being analysed using gas chromatography and mass spectroscopy by another researcher.

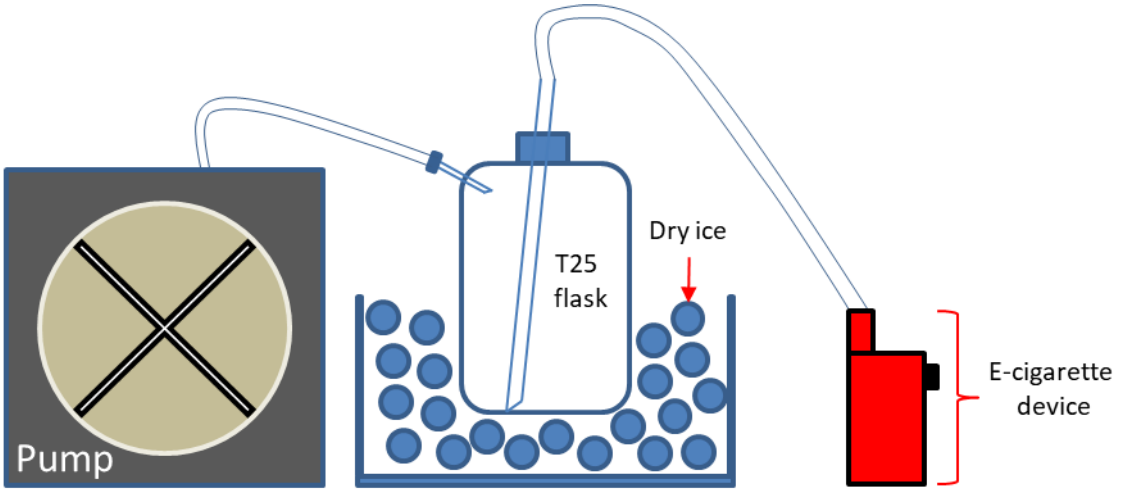

Figure 43. A schematic of the set up to create the e-cigarette aerosol condensate. E-cigarette aerosol condensate was formed when the pump draws the e-cigarette aerosols into the T25 flask that is placed on dry ice. This process allows the e-cigarette aerosols to condense to an extract.

Table 5. E-cigarette aerosol condensate treatments for the study.

Treatment groups	**Supplemented media**
Negative control	Non-treated media
PGVG	Propylene glycol and vegetable glycerin ECAC
PGVG + Nicotine	Propylene glycol, vegetable glycerin and nicotine (18mg/ml) ECAC
PGVG + Flavour	Propylene glycol, vegetable glycerin, tobacco flavouring ECAC
PGVG + Flavour + nicotine	Propylene glycol, vegetable glycerin, tobacco flavouring and nicotine (18mg/ml) = ECAC

4.3.4 Monoculture assays

For each monoculture assay, diff-SHSY5Y cells, BV2 cells and HBECs were seeded in a 96-well plate at a cell density of 30,000, 15,000 and 4000 cells/well, respectively. To control for the possible effects of media dilution from the ECAC, a subsequent experiment was performed substituting serial dilutions of ECAC for water at serial dilutions of 1 in 12.5, 1 in 25, 1 in 50 and 1 in 100 using the methylthiazolydiphenyl-tetrazolium bromide (MTT) assay. The experiment confirmed that ECAC had no effect on media dilution in monoculture (data not shown).

4.3.4.1 Methylthiazolydiphenyl-tetrazolium bromide assay

The MTT assay is a cell viability assay that determines cytotoxicity. MTT powder (Sigma, MO, USA) was dissolved at a concentration of 5 mg/mL in 0.1 M of PBS (pH 7.4). Once each cell line was seeded, cells were allowed to settle overnight before treatment with ECAC at serial dilutions (1 in 12.5, 1 in 25, 1 in 50, 1 in 100). To determine lethal concentration, serial dilutions of ECAC were diluted in media with no serum. Treatment groups were outlined as follows: **Negative control** (media only with no treatment), **PGVG**, **PGVG+nicotine**, **PGVG+Flavour**, **PGVG+Flavour+nicotine**, and a **positive control** (hydrogen peroxide treatment; H_2O_2).

All treatments were diluted in DMEM F12/hams supplemented with 5mM HEPES and NEAA with no FBS and performed in triplicates. Cells were treated for 24 hours before the addition of the MTT dye. MTT dye was added at 10 µl to 100 µl of the treatment media and incubated at 37ºC for three hours. MTT dye is utilised by the cell mitochondria, converting the dye to formazan, forming insoluble purple granules. Media and dye were completely removed before the addition of dimethyl sulfoxide (DMSO) (Sigma Aldrich, MO, USA) to solubilise formazan and turn the solution purple. The absorbance was measured at 565 nm using the infinite M1000 PRO Microplate reader (Tecan group Ltd, Männedorf, Switzerland).

4.3.4.2 2', 7' Dichlorofluorescein assay

The 2' 7'-Dichlorofluorescein (DCF) assay is a measure of ROS (Sigma-Aldrich, MO, USA). The DCF assay utilises 2' 7'-Dichlorofluorescein diacetate, a cell-permeable non-fluorescent probe that becomes highly fluorescent upon oxidation. Fluorescence was used as a measure of ROS production in the cell. The DCF powder was dissolved in DMSO at a concentration of 10 mM and stored in the dark at -20^{o}C. Each cell line was seeded and starved overnight before ECAC treatment. Each cell line was treated with ECAC with the appropriate controls at serial dilutions (1 in 50, 1 in 100, 1 in 200) and was treated for 24 hours. After treatment, cell media was removed and replaced with 100 µl DCF stock dissolved in PBS at a concentration of 10 µM. Cells were incubated for ten minutes before fluorescence was measured at 485 nm excitation and 535 nm emission. The total ROS release from cells was calculated compared to the negative control.

4.3.4.3 JC-10 Mitochondrial membrane potential assay

JC-10 mitochondrial membrane potential assay (Abcam, Cambridge, UK) was used to determine the mitochondrial membrane potential within a cell to determine whether the cell is under mitochondrial stress. In normal cells JC10 dye form aggregates when it enters the mitochondrial membrane which gives a red fluorescence emission. In apoptotic cells, the JC10 dye diffuses from the mitochondrial membrane and into the cell's cytoplasm, changing the JC10 dye to a monomeric form which gives a green fluorescence emission. By measuring the ratio of red and green fluorescent signals, the relative mitochondrial membrane integrity can be measured which then relates to the mitochondrial membrane potential.

Cells were seeded at 30,000 cells/well in a 96-well plate (black-walled, flat/clear bottom). Cells were treated with ECAC at serial dilutions of 1 in 50, 1 in 100 and 1 in 200 for 24 hours before performing the assay. The JC-10 assay was performed according to the manufacturer's protocol. Before the commencement of the assay, the JC-10 dye-loading solution was formulated by adding

50 µl of 100X JC-10 to 5 mL of Assay Buffer A. After treatment with ECAC for 4 hours, 50 µl of JC-10 dye-loading solution was added into each 96-well plate and incubated for 30 minutes at 37 ºC in the dark. After incubation, assay buffer B was added to each well before the fluorescence was monitored using the infinite M1000 PRO Microplate reader (Tecan group Ltd, Männedorf, Switzerland). Ratiometric analysis was determined by measuring the fluorescence at excitation/emission at 490/525 nm (green) and 490/590 nm (red). An increase in the fluorescent ratio indicates depolarisation of the mitochondrial membrane.

4.3.5 Differentiation of SHSY5Y cells with PMA in the presence of ECAC

To determine the impact on ECAC to developing cells, ECAC was added to developing SHSY5Y cells. SHSY5Y cells were differentiated as outlined in **section 4.3.2** with the addition of ECAC treatment. ECAC was applied at a dilution of 1 in 50 based on the results found in the monoculture experiments. A dilution of 1 in 50 allowed the optimum dilution to observe physiological changes within SHSY5Y cells without killing the cells. The optimum dilution was determined by monocultures (see **section 4.3.4**). After the third day of differentiation, total RNA was extracted from diff-SHSY5Y cells using *TRIsur*e™ (Bioline, MA, USA) outlined in **section 4.3.2.2.1**. RNA quality checks, reverse transcription and real time PCR assay was performed to determine the gene expression of GAP43 according to the methods described in **section 2.3.8, 2.3.11.1 and 2.3.11.2, respectively**. The negative control consists of SHSY5Y cells without PMA treatment and the positive control and the ECAC treatment consist of SHSY5Y cells with PMA for differentiation.

4.3.6 *In vitro* blood brain barrier model

4.3.6.1 Creating the *in vitro* blood brain barrier – Fluorescein isothiocyanate (FITC)-dextran and TEER measurements

To optimise the *in vitro* BBB model, HBEC were seeded in Corning® Transwell® polyester membrane cell culture inserts (Sigma-Aldrich, MO, USA) and the TEER was measured to determine endothelial integrity [443]. TEER is a measurement of electrical resistance of a monolayer in a transwell between the inner chamber of the transwell and the bottom chamber of the well plate. In addition, TEER measurements provide a sensitive method of determining the endothelial membrane integrity and permeability of the endothelial layer. For this study, TEER was measured using the *EVOM2 Epithelial Voltohmmeter* (World precision instruments Inc., Sarasota, FL). TEER was measured three days after seeding of HBECs. Baseline TEER measurements without cells were recorded before the commencement of each co-culture experiment and was subtracted from the TEER measurements for each treatment group. Plates were measured at exactly 10 minutes after removal from the incubator. This is to avoid temperature influences on conductance [444]. The schematic of the HBEC transwell insert setup is shown in Figure 44.

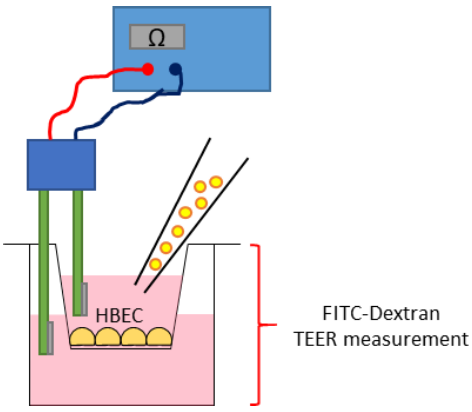

Figure 44. Fluorescein isothiocyanate (FITC) dextran and trans-endothelial electrical resistance (TEER) measurements after ECAC treatment on HBECs seeded in transwells.

After the TEER measurement of the endothelial monolayer reaches 250-300 Ω, the monolayer was treated with ECAC at a dilution of 1 in 50 and incubated for 24 hours. To control for the possible effects of media dilution from the ECAC, a subsequent experiment was performed substituting a dilution of ECAC for water at a dilution of 1 in 50 and it was confirmed that ECAC had no effects on media dilution in co-culture (data not shown). After treatment with ECAC, fluorescein isothiocyanate (FITC)-dextran (molecular weight; 70,000 kDa, Sigma Aldrich, MO, USA) was added to determine membrane permeability. The media from the upper chamber was removed and replaced with FITC-Dextran (1 mg/ml) dissolved in DMEM media without phenol red. A sample of media from the bottom chamber was collected and placed into a black-walled, clear-bottom 96 well-plate. Each time a sample of media was taken from the bottom chamber, the volume removed was replaced with fresh media. The bottom chamber media sample was then taken at 0, 5, 10, 20, 30, 40, 50 and 60 minutes. Fluorescence intensity was measured at 488 nm excitation and 525 nm emission using the infinite M1000 PRO Microplate reader (Tecan group Ltd, Männedorf, Switzerland).

4.3.6.2 Blood Brain Barrier (BBB) co-culture model with HBECs and diff-SHSY5Y cells

To determine the epigenetic effects of ECAC on mature neuronal cells in a BBB model, a co-culture was set up to mimic a BBB with HBEC seeded in the transwell insert and diff-SHSY5Y cells (HBEC/diff-SHSY5Y cells) were seeded in the bottom chamber. SHSY5Y cells were differentiated in a separate plate before the transwell inserts with HBEC were added. Once co-culture cells were combined, TEER measurements were made every three days until TEER measurements reached between 200-300 Ω indicating substantial junctions between HBEC cells by day 10. At day 11, co-culture was treated with ECAC at a dilution at 1 in 50 and incubated for 24 hours. TEER was measured at 24 hours post-treatment. Baseline TEER measurements without cells were recorded before the commencement of each co-culture experiment and was subtracted

from the TEER measurements for each treatment group. Transwell inserts were carefully removed and diff-SHSY5Y cells were collected for RNA extraction. The schematic of the co-culture set up with HBEC and diff-SHSY5Y cells is shown in Figure 45.

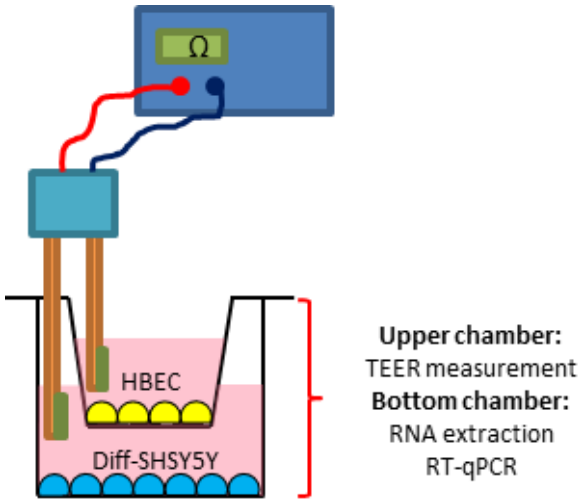

Figure 45. Experimental set-up for the co-culture of HBEC cells with diff-SHSY5Y cells. HBEC cells were seeded to confluence on the transwell insert forming a tight barrier. Diff-SHSY5Y cells are seeded to confluence onto the bottom of the well forming a neuronal cell layer. E-cigarette aerosol condensate was added to the top chamber and the effects of the e-cigarette aerosol condensate on the HBEC cell barrier is measured using TEER. The effects of the ECAC on the diff-SHSY5Y cells are measured by subsequent RNA extraction and RT-qPCR to examine epigenetic gene expression.

4.3.6.2.1 RNA extraction and quality check

For RNA extraction and RNA quality checks, refer to **section 4.3.2.2.1** and **Chapter 2 section 2.3.8**, respectively, for the full protocol.

4.3.6.2.2 Reverse transcription and real time PCR assay

For reverse transcription and real time PCR assay, refer to **Chapter 2 section 2.3.11.1-2.3.11.2**, respectively, for the full protocol. For the full list of epigenetic primer sequence, see **Table 6**.

Table 6. PCR primer sequences for the in vitro experiments.

Epigenetic chromatin modification enzymes/reference gene	Gene ID	Description	F/R	Primer sequence (5'→3')
Histone Acetyltransferase	Atf2	Activating transcription factor 2	Forward	CAGTCTTTAGAGAAGAAAGCTG
			Reverse	CTTTATCAGCAGTATGATAGCC
Histone Phosphorylation	AurkA	Aurora Kinase A	Forward	CCTACAAAAGAATATCACGGG
			Reverse	CAAGTACTTCTCTGAGCATTG
	AurkB	Aurora Kinase B	Forward	ATTGGAGTGCTTTGCTATG
			Reverse	TTTAGGTCCACCTTGACG
	AurkC	Aurora Kinase C	Forward	GAAATGATTGAGGGGAGAAC
			Reverse	AGTGGAAACCTCACATCTAC
DNA Methyltransferases	Dnmt3a	DNA methyltransferase 3a	Forward	GAAGAGAAGAATCCCTACAAAG
			Reverse	CAATAATCTCCTTGACCTTGG
	Dnmt3b	DNA methyltransferase 3b	Forward	CTTACCTTACCATCGACCTC
			Reverse	ATCCTGATACTCTGAACTGTC
Histone Demethylases	Kdm5c	Lysine (K)-specific demethylase 5c	Forward	GAAGACATTGAGAAGAATCCAG
			Reverse	CTTATCTTTCTTCCGCAGAG
	Kdm6b	Lysine (K)-specific demethylase 6b	Forward	CAGGAGAATAACAACTTCTGC
			Reverse	CACAGGAATATTGGATGCATAG
Histone Deacetylases	Hdac1	Histone deacetylase 1	Forward	GGATACGGAGATCCCTAATG
			Reverse	CGTGTTCTGGTTAGTCATATTG
Housekeeping gene	GAPDH	Glyceraldehyde 6-phosphate dehydrogenase	Forward	ACAGTTGCCATGTAGACC
			Reverse	TTGAGCACAGGGTACTTTA

4.3.6.3 BBB co-culture model with HBEC and BV2 cells

To determine the inflammatory effects of ECAC on BV2 cells in a BBB model, a co-culture was set up to mimic a BBB with HBEC seeded in the transwell insert and BV2 cells (HBEC/BV2 cells) seeded in the bottom chamber. TEER measurements were made every three days until TEER reached between 200-300 Ω indicating substantial junctions between HBEC cells by day 10. At day 11, co-culture was treated with ECAC at a dilution at 1 in 50 and incubated for 24 hours. TEER was measured at 24 hours post-treatment. Baseline TEER measurements without cells were recorded before the commencement of each co-culture experiment and was subtracted from the TEER measurements for each treatment group. Transwell inserts were carefully removed and BV2 cells were collected for RNA extraction. The schematic of the co-culture set up with HBEC and diff-SHSY5Y cells is shown in Figure 46.

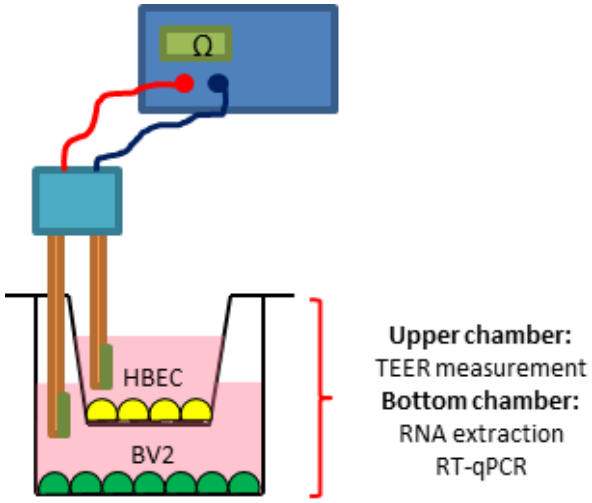

Figure 46. Experimental set-up for the co-culture of HBEC cells with differentiated BV2 cells. HBEC cells were seeded to confluence on the transwell insert forming a tight barrier.BV2 cells are seeded to confluence onto the bottom of the well, forming a microglial cell layer. ECAC was added to the top chamber and the effects of the ECAC on the HBEC membrane was measured using TEER. The effects of the ECAC on the diff-SHSY5Y cells were measured by subsequent RNA extraction and RT-qPCR to examine inflammatory gene expression.

4.3.6.3.1 RNA extraction and quality check

For RNA extraction and RNA quality checks, refer to **section 4.3.2.2.1** and **Chapter 2 section 2.3.8**, respectively, for the full protocol.

4.3.6.3.2 Reverse transcription and real time PCR assay

For reverse transcription and real time PCR assay, refer to **Chapter 2 section 2.3.11.1-2.3.11.2**, respectively, for the full protocol. The full list of inflammatory primer sequence, see Table 7.

Table 7. PCR primer sequences for the in vitro experiments.

Epigenetic chromatin modification enzymes/reference gene	Gene ID	Description	F/R	Primer sequence (5'→3')
Pro-inflammatory	IL-1α	Interleukin 1 alpha	Forward	CATAACCATGATCTGGAAG
			Reverse	ATTCATGACAAACTTCTGCC
	Il-1β	Interleukin 1 beta	Forward	GCAACAAGTGGTGTTCTC
			Reverse	CAGATTCTTTTCCTTGAGGC
	Il-6	Interleukin 6	Forward	AAGATTCCAAAGATGTAGCC
			Reverse	ACATGTCTCCTTTCTCAGG
	IL-10	Interleukin 10	Forward	GCCTTTAATAAGCTCCAAGAG
			Reverse	ATCTTCATTGTCATGTAGGC
	IL-18	Interleukin 18	Forward	TAATGCAGGTCATTCAGATG
			Reverse	CTCTCCTCTTTCCAATTCTTC
	TGFβ1	Transforming growth factor beta 1	Forward	ACCGGCCTTCCTGCTTCTCA
			Reverse	CGCCCGGGTTATGCTGGTTGT
	TNF	Tumour necrosis factor	Forward	CTCAGCCTCTTCTCCTTC
			Reverse	AGAAGATGATCTGACTGCC
Housekeeping gene	GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	Forward	ACAGTTGCCATGTAGACC
			Reverse	TTGAGCACAGGGTACTTTA

4.3.7 Conditioned media experiment

4.3.7.1 Co-culture set up and treatment

To determine the effects of ECAC on diff-SHSY5Y cells with microglial cells in the CNS, the 'conditioned' media treatment from a co-culture model with BV2 cells and HBEC was applied onto diff-SHSY5Y in a separate well plate (Figure 47). BV2 cells were seeded separately in a 24 well-plate. Once BV2 cells reached 60% confluency, HBEC seeded transwell inserts were added to the wells and TEER was measured every three days. Once TEER reached between 250-300 Ω, media from the transwell was removed and replaced with ECAC diluted in endothelial growth basal medium-2 media at a dilution of 1 in 50 and co-cultures were incubated for 24 hours. At 24 hours post-treatment, TEER measurement was conducted on the co-cultures. The media from the bottom well (conditioned media) was then transferred to diff-SHSY5Y cells and was treated for 24 hours. After each co-culture treatment, cell media was aspirated and the cells were washed with PBS before RNA extraction.

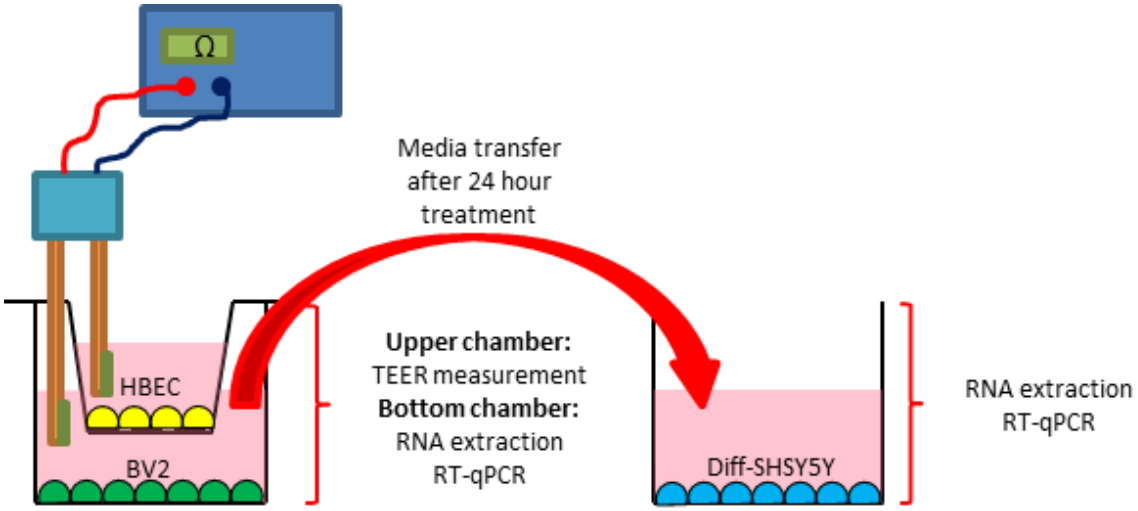

Figure 47. E-cigarette aerosol condensate treatment of a co-culture of HBEC cells with BV2 cells. Conditioned media from the bottom chamber of the co-culture was transferred to a monoculture of diff-SHSY5Y cells. TEER measurements were observed in the upper chamber of the HBEC/BV2 co-culture followed by inflammatory gene expression of BV2 cells and epigenetic gene expression of diff-SHSY5Y cells.

4.3.7.2 RNA extraction and quality checks

For RNA extraction and RNA quality checks, refer to **section 4.3.2.2.1** and **Chapter 2 section 2.3.8**, respectively, for the full protocol.

4.3.7.3 Reverse transcription and real time PCR assay

For reverse transcription and real time PCR assay, refer to **Chapter 2 section 2.3.11.1-2.3.11.2**, respectively, for the full protocol. The full list of epigenetic primer sequence, see Table 3.

4.3.8 *In vivo* experiment of e-cigarette aerosol exposure

4.3.8.1 E-cigarette device, animal experimental procedure, aerosol exposure, euthanasia and tissue collection

Brain tissues from Chapter 2 experiments were further analysed in this Chapter to investigate inflammation in the brain. For all the animal experiment, euthanasia and tissue collection procedures, refer to **Chapter 2 section 2.3.1-2.3.3** and **2.3.5** for the full protocols.

4.3.8.2 RNA extraction, quality check, reverse transcription and real time PCR assay

For the RNA extraction, quality check, reverse transcription and real time PCR assay, refer to **Chapter 2 section 2.3.7, 2.3.8, 2.3.11.1** and **2.3.11.2**, respectively, for the full protocols. The list of inflammatory primers that was analysed by RT-qPCR is found in Table 8.

Table 8. PCR primer sequences for the in vivo experiments.

Inflammatory genes	Gene ID	Description	F/R	Primer sequence (5'→3')
Inflammatory genes	IL-1α	Interleukin 1 alpha	Forward	CATAACCCATGATCTGGAAG
			Reverse	ATTCATGACAAACTTCTGCC
	Il-1β	Interleukin 1 beta	Forward	GGATGATGATGATAACCTGC
			Reverse	CATGGAGAATATCACTTGTTGG
	Il-6	Interleukin 6	Forward	GAACAACGATGATGCACTTGC
			Reverse	TCCAGGTAGCTATGGTACTCC
	IL-10	Interleukin 10	Forward	ATAACTGCACCCACTTCCCA
			Reverse	GGGCATCACTTCTACCAGGT
	IL-18	Interleukin 18	Forward	GAACAAGATCATTTCCTTTGAG
			Reverse	TGGCAAGCAAGAAAGTGTC
	TGFβ1	Transforming growth factor beta 1	Forward	TGGAGCAACATGTGGAA
			Reverse	TGCCGTACAACTCCAGTGAC
	TNF-α	Tumour necrosis factor alpha	Forward	GGATGAGAAGTTCCCAAATG
			Reverse	TGAGAAGATGATCTGAGTGTG
Reference gene	GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	Forward	GCTCACTGGCATGGCCTTCCG
			Reverse	GTAGGCCATGAGGTCCACCAC

4.3.9 Statistical analysis

For statistical analysis of the monoculture assays (MTT, DCF and JC-10 assays), FITC-Dextran, TEER measurements, and all RT-qPCR, a one-way ANOVA with *Bonferroni's* post-hoc test was used to determine statistical significance between each treatment groups. All statistical analyses were performed using Prism 7 (GraphPad, CA, USA) and the results were expressed as mean ± standard deviation. Treatment groups were considered significantly different if the *p value* was less than 0.05. Some replicates were excluded from the statistical analysis due to being clear outliers to the rest of the data points.

4.4 Results

4.4.1 SHSY5Y are differentiated with PMA

4.4.1.1 SHSY5Y differentiation optimisation

To ensure that PMA-treated SHSY5Y cells were differentiated, SHSY5Y cell morphology was examined as well as mRNA gene expression analysis of GAP43, a marker for neuronal maturity. Images taken from the differentiation showed SHSY5Y cells' morphology as stunted cell bodies with short processes on day 0, as expected (Figure 48A). By day two and three, SHSY5Y cells showed pyramidal-shaped cell bodies with long processes indicating neurite extensions (Figure 48A, red arrows). GAP43 gene expression of SHSY5Y cells treated with PMA showed an upregulation of ~400% compared to non-differentiated SHSY5Y cells (Unpaired *t*-test, $p<0.05$; Figure 48B). Overall, based on the morphological and GAP43 gene expression results, it was concluded that SHSY5Y were differentiated into mature neurons using PMA. All of the *in vitro* experiments for this study were performed using differentiated SHSY5Y cells and will now be referred to as **diff-SHSY5Ycells** for the rest of this chapter.

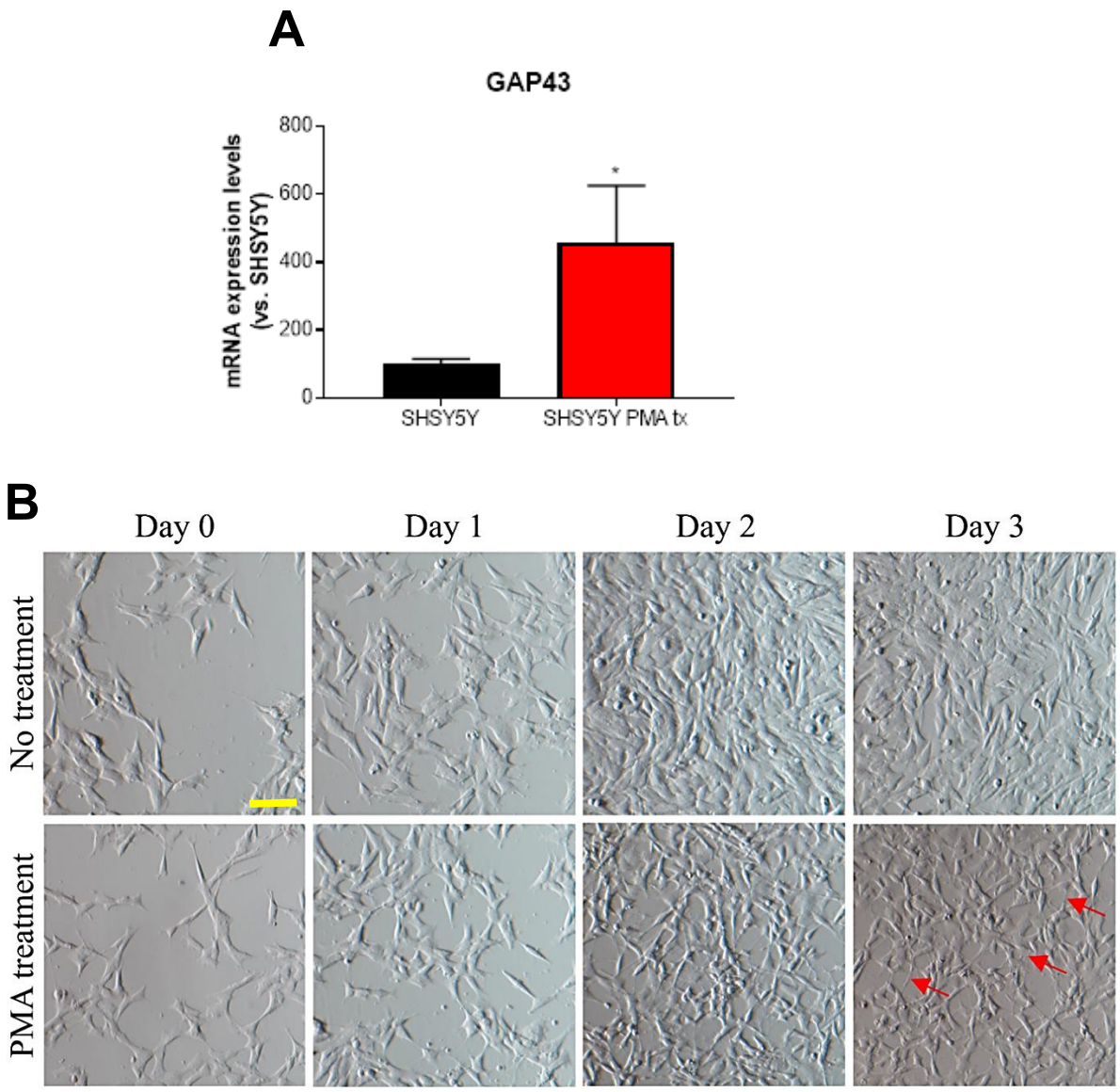

Figure 48. Validation of SHSY5Y cells treated with PMA (n = 3). (A) Representative bright field images of SHSY5Y cells treated with PMA over three consecutive days. At day three, SHSY5Y cells had pyramidal-shaped cell bodies and long processes (arrows). (B) mRNA gene expression of GAP43 on non-treated and PMA-treated SHSY5Y cells showed a 10-fold increase in GAP43 gene expression. Data is shown as a percentage compared to the negative control. Data represented as mean ± standard deviation, unpaired t-test, *$p<0.05$ vs. negative control. PMA = Phorbol 12-myristate 13-acetate, tx = treatment. Scale bar = 100µm.

4.4.1.2 Differentiation of SHSY5Y cells is reduced following treatment with e-cigarette aerosol condensate

After confirming SHSY5Y differentiation, a follow up experiment was conducted to investigate whether treating SHSY5Y cells with ECAC during differentiation with PMA stunts maturation. The justification for this experiment was to analyse whether e-cigarettes can affect normal development and differentiation that is similar to what can be observed in a developing brain. RT-qPCR results showed a significant increase in GAP43 gene expression in SHSY5Y cells treated with PMA in the PGVG (176.7%; $p<0.001$), PGVG+nic (173.7%; $p<0.01$), PGVG+Flavour (179.6%; $p<0.001$) and PGVG+Flavour+nic (189.4%; $p<0.001$) and the positive group (297.1%; $p<0.0001$) compared to the negative control. However, GAP43 gene expression of all these treatment groups were less than that of the positive control group treated with PMA only ($p<0.05$; Figure 49A). Morphologically, the non-treated SHSY5Y cells in the negative control group exhibited truncated neurological processes and a non-polarised cell body. SHSY5Y cells treated with PMA + ECAC, and PMA only, appeared to have triangular-like cell bodies with long neurological processes, however, the proportion of cells with this morphology appeared to be higher in the positive control compared to the ECAC groups (Figure 49B). Together these findings suggest that ECAC does have a negative effect on neuronal differentiation.

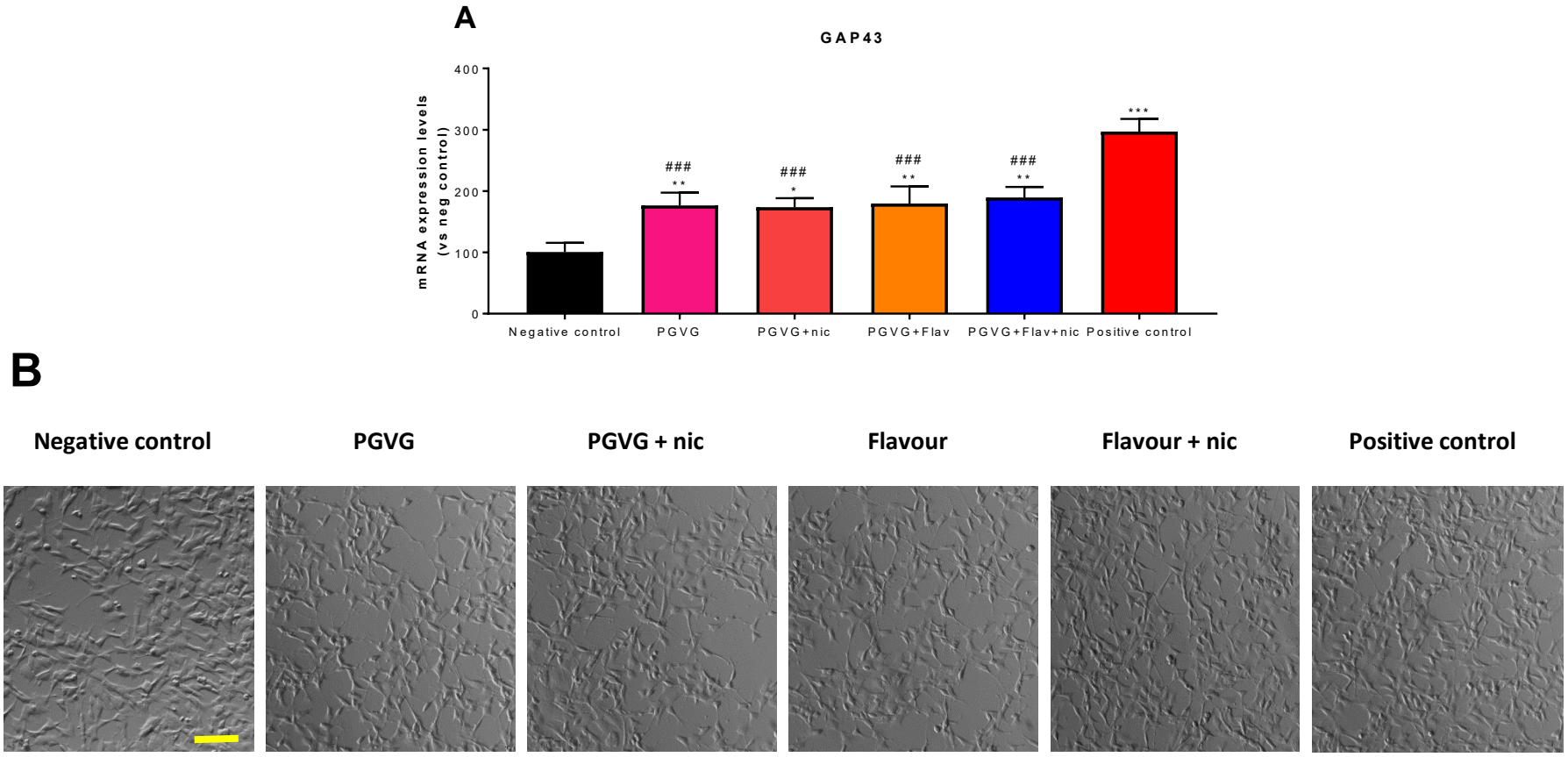

Figure 49. E-cigarette aerosol condensate treatment during SHSY5Y cell differentiation with PMA. (n = 3) (A) mRNA expression of GAP43 to determine differentiation of SHSY5Y cells treated with PMA and e-cigarette aerosol condensate at a 1 in 50 dilution. (B) Representative bright field images of SHSY5Y cells differentiation at day 3. Data represented as mean ± standard deviation, one-way ANOVA with Bonferroni post-hoc test, *p<0.05, **p<0.01, ***p<0.001 vs. negative control, ###p<0.001 vs. positive control. Neg = Negative control, PGVG = Propylene Glycol/Vegetable glycerin mixture, Nic = nicotine, Pos = Positive control. Scale bar = 100 µm.

4.4.2 Cell viability is lower in diff-SHSY5Y and BV2 cells treated with flavoured ECAC without nicotine

The effect of ECAC on diff-SHSY5Y, BV2 and HBEC cell viability were examined using the MTT assay (Figure 50). All treatment groups were standardised to the negative control which was only treated with cell media only (100%). Treatment with 600 μM H_2O_2 (positive control) was shown to have the lowest level of cell viability in all three cell lines (diff-SHSY5Y; 2.2%, BV2 cells; 9.5%, HBEC;4.4%).

In the diff-SHSY5Y cells, cell viability was significantly reduced at dilutions 1 in 12.5 (PGVG: 17.2%, PGVG+nic: 14.2%, PGVG+Flavour: 13.8%, PGVG+Flavour+nic: 15.8%) and dilution 1 in 25 (PGVG: 45.3%, PGVG+nic: 29.3%, PGVG+Flavour: 28.9%, PGVG+Flavour+nic: 46.8%) compared to the negative control ($p<0.05$) (Figure 50A). In addition, diff-SHSY5Y cells treated with PGVG+Flavour had a significantly reduced cell viability of 77.3% at a 1 in 50 dilution ($p<0.05$). There was no significant difference in diff-SHSY5Y cells viability in the PGVG, PGVG+nic, and PGVG+Flavour+nic groups at a 1 in 50 dilution. No significant difference in diff-SHSY5Y cell viability was found at a 1 in 100 dilution between all treatment groups.

In the BV2 cells, cell viability was significantly reduced at dilutions 1 in 12.5 (PGVG: 14.5%, PGVG+nic: 14.5%, PGVG+Flavour: 10.75%, PGVG+Flavour+nic: 13.5%) and 1 in 25 (PGVG: 58.5%, PGVG+nic: 54.5%, PGVG+Flavour: 37.3%, PGVG+Flavour+nic: 33.3%) compared to the negative control ($p<0.05$) (Figure 50B). In addition, BV2 cells treated with PGVG+Flavour had a significantly reduced cell viability of 80% at 1 in 50 dilution compared to the negative control ($p<0.001$). There was no significant difference in BV2 cell viability in the PGVG, PGVG+nic, and PGVG+Flavour+nic groups at dilution 1 in 50. Moreover, there was no significant difference in BV2 cell viability 1 in 100 dilution between all treatment groups.

In the HBEC, cell viability was only significantly reduced at 1 in 12.5 dilution (PGVG: 51.3%, PGVG+nic: 67.7%, PGVG+Flavour: 58.0%, PGVG+Flavour+nic: 35.0%) and 1 in 25 dilution

(PGVG: 80.1%, PGVG+nic: 71.0%, PGVG+Flavour: 79.1%, PGVG+Flavour+nic: 77.9%) compared to the negative controls ($p<0.05$) (Figure 50C). No significant decrease in cell viability was found at dilutions 1 in 50 and 1 in 100 between all treatment groups.

Overall, the MTT assay shows a significant reduction in cell viability in all three cell-lines in a dose-dependent manner. Interestingly, diff-SHSY5Y and BV2 cells were more severely affected by ECAC without nicotine. This may indicate that e-cigarette aerosols with nicotine may have a protective mechanism against cell toxicity. As a result of these experiments, a 1 in 50 dilution was selected for all co-culture experiments.

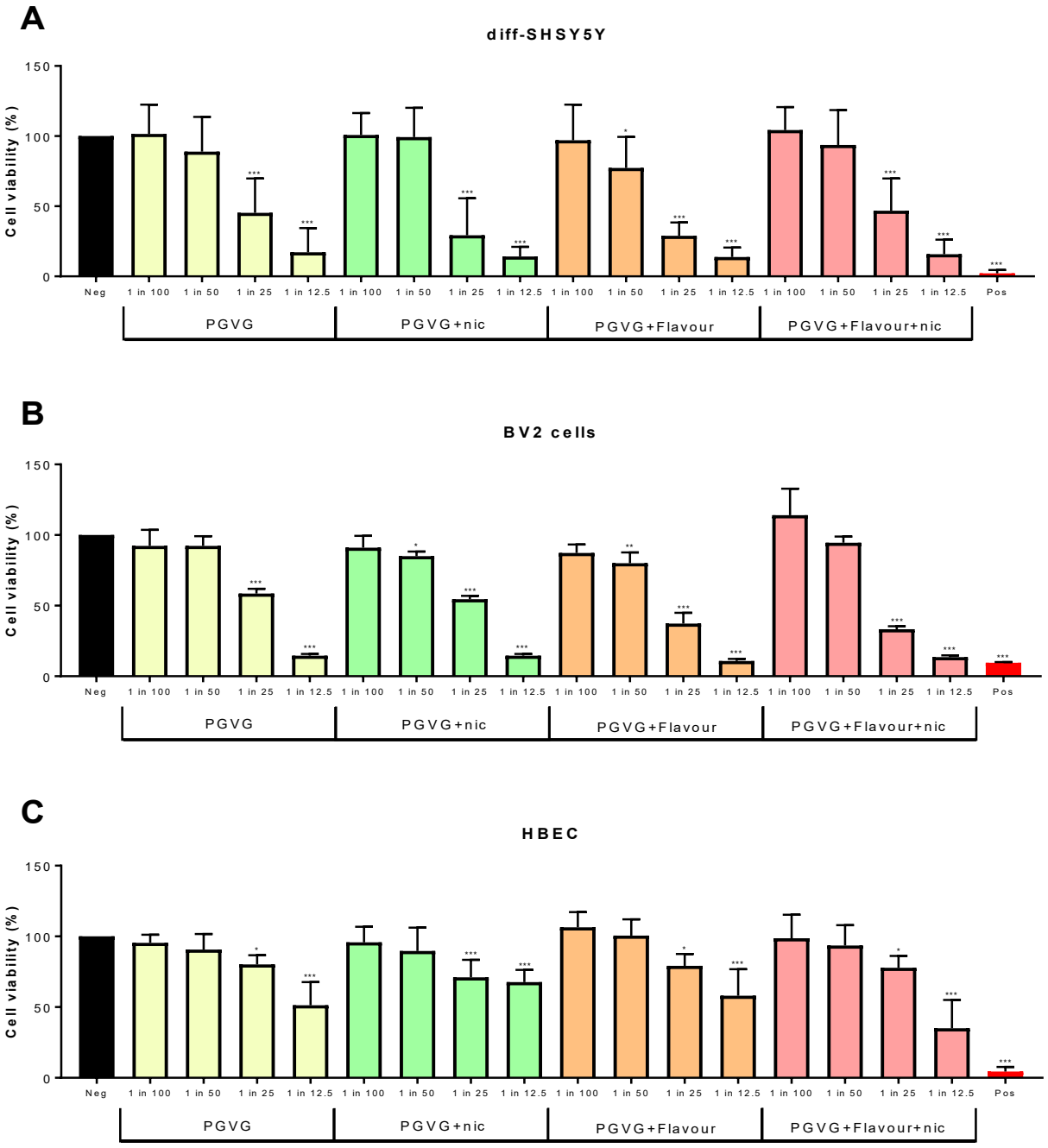

Figure 50. The MTT assay to measure cell viability of (A) diff-SHSY5Y cells, (B) BV2 cells and (C) HBEC, following exposure to e-cigarette aerosol condensate at dilutions of 1 in 12.5, 1 in 25, 1 in 50 and 1 in 100 (n = 4). Data is shown as a percentage standardised to the negative control. Data represented as mean ± standard deviation, one-way ANOVA with *Bonferroni* post-hoc test, *p<0.05, **p<0.01, ***p<0.001 vs. negative control. Neg = Negative control, PGVG = Propylene Glycol/Vegetable glycerin mixture, Nic = nicotine, Pos = Positive control.

4.4.3 Reactive oxygen species production was highest in diff-SHSY5Y, BV2 cells and HBEC treated with flavoured e-cigarette aerosol condensate without nicotine

A 1 in 200, 1 in 100 and 1 in 50 dilution was used to determine the effects of ROS production by conducting the DCF assay in diff-SHSY5Y cells, BV2 cells and HBECs (Figure 51). These dilutions provide the amount of ECAC concentration to have a sub toxic effect to each of the cell lines based on the cell viability assay. All treatment groups were standardised and normalised to the negative control.

After 4 hours of ECAC treatment, diff-SHSY5Y cells had an increase in ROS production at dilutions 1 in 50 (PGVG: 142.2%, PGVG+nic: 141.4%, PGVG+Flavour: 139.0%, PGVG+Flavour+nic: 141.0%) and 1 in 100 (PGVG: 129.8%, PGVG+nic: 127.7%, PGVG+Flavour: 136.4%, PGVG+Flavour+nic: 117.5%) compared to the negative control ($p<0.05$) (Figure 51A). No significant difference in ROS production was observed between any treatment groups at a 1 in 200 dilution.

For the BV2 cells, there was a significant increase in ROS production in all treatment groups at the dilution of 1 in 50 (PGVG: 118.5%, PGVG+nic: 117.6%, PGVG+Flavour: 121.6%, PGVG+Flavour+nic: 119.8%) compared to the negative control ($p<0.001$) (Figure 51B). At 1 in 100 dilution, there was a significant increase in ROS production in the PGVG group (113.1%, $p<0.01$), PGVG+Flavour group (114.2%, $p<0.001$) compared to the negative control. No significant changes in ROS production was observed at a 1 in 200 dilution between any treatment groups.

For the HBEC, there was a significant increase in ROS production in the PGVG (285.1%), PGVG+Flavour (284.8%) and PGVG+Flavour+nic (272.2%) groups compared to the negative control ($p<0.001$) (Figure 51C). At a 1 in 100 dilution, there was a significant increase in ROS production in the PGVG (285.7%) and PGVG+Flavour (296.1%) group compared to the negative

control ($p<0.001$). In addition, ROS production was significantly increased in the PGVG group (286.0%) at a dilution of 1 in 200 indicating that HBEC exposed to ECAC with only PGVG releases more ROS compared all other treatment groups. Overall, the results show dependent increase in ROS production in all three cell lines, indicating an increase in oxidative stress induced by exposure to ECAC.

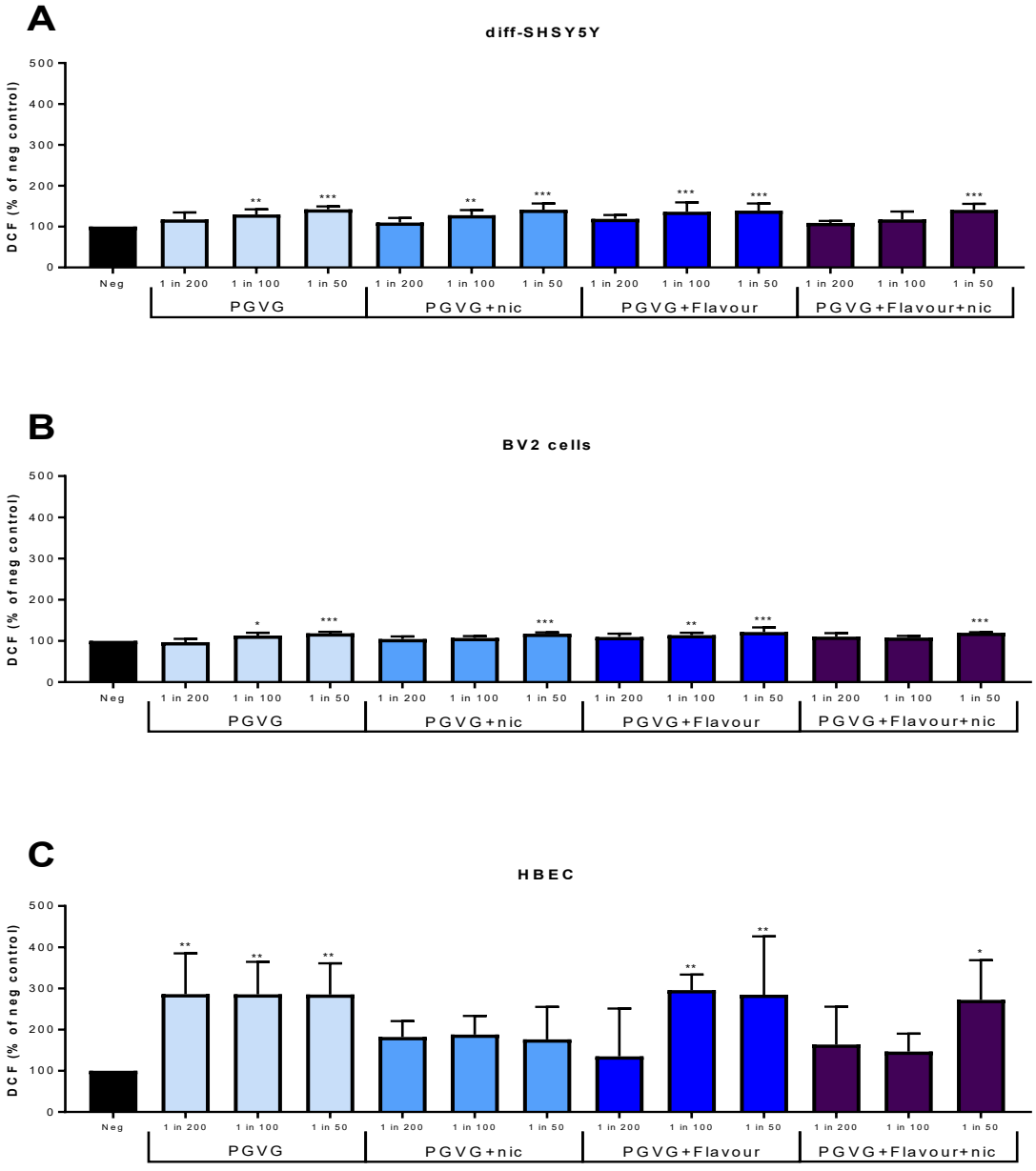

Figure 51. The DCF assay to measure reactive oxygen species released from (A) diff-SHSY5Y cells, (B) BV2 cells and (C) HBEC, following exposure to e-cigarette aerosol condensate at dilutions of 1 in 200, 1 in 100 and 1 in 50 (n = 4). Data is shown as a percentage standardised to the negative control. Data represented as mean ± standard deviation, one-way ANOVA with *Bonferroni* post-hoc test, *p<0.05, **p<0.01, ***p<0.001 vs. negative control. Neg = Negative control, PGVG = Propylene Glycol/Vegetable glycerin mixture, Nic = nicotine, Pos = Positive control.

4.4.4 Mitochondrial membrane potential depolarisation was increased in diff-SHSY5Y cells and BV2 cells treated with flavoured e-cigarette aerosol condensate without nicotine

The effect of ECAC on diff-SHSY5Y, BV2 cells and HBEC mitochondrial membrane potential was examined using the JC-10 assay. Treatment with H_2O_2 (positive control) was shown to have the highest level of membrane depolarisation in all three cell lines as expected.

In the diff-SHSY5Y cells, there was a significant increase mitochondrial membrane depolarisation at dilutions 1 in 50 (PGVG: 266.7%, PGVG+nic: 195.5%, PGVG+Flavour: 305.5%, PGVG+Flavour+nic: 132.3%) and 1 in 100 (PGVG: 231.1%, PGVG+nic: 157.6%, PGVG+Flavour: 183.6%, PGVG+Flavour+nic: 110.9%) compared to the negative control ($p<0.001$) (Figure 52A). At dilution 1 in 200, membrane depolarisation was significantly increased in the PGVG (205.5%) and PGVG+Flavour (135.5%) groups compared to the negative control ($p<0.001$). There was no significant increase in membrane depolarisation in the PGVG+nic and PGVG+Flavour+nic group compared to the negative control.

In the BV2 cells, there is a significant increase in mitochondrial membrane depolarisation at a 1 in 50 dilution (PGVG: 460.0%, PGVG+nic: 149.0%, PGVG+Flavour: 137.7%, PGVG+Flavour+nic: 199.2%) compared to the negative control ($p<0001$) (Figure 52B). At 1 in 100 dilution, significant increases in membrane depolarisation were observed in the PGVG group (195.0%) and PGVG+Flavour group (114.7%) compared to the negative control ($p<0.05$). At 1 in 200 dilution, significant increases in membrane depolarisation were observed in the PGVG group (132.0%) compared to the negative control ($p<0.001$). There were no significant differences in membrane depolarisation at dilutions of 1 in 100 and 1 in 200 in the PGVG+nic group and PGVG+Flavour+nic group.

In the HBEC, at 1 in 50 dilution, there was a significant increase in mitochondrial membrane depolarisation in the PGVG (142.2%) and PGVG+Flavour+nic group (120.7%) compared to the

negative control ($p<0.001$) (Figure 52C). At 1 in 100 dilution, membrane depolarisation was significantly increased in the PGVG group (114.8%) compared to the negative control ($p<0.01$). There were no significant changes to membrane depolarisation in the PGVG group at 1 in 200 dilution. Moreover, there were no significant changes to membrane depolarisation at any dilution in the PGVG+nic and PGVG+Flavour groups.

From the JC-10 data, there was an increase in mitochondrial membrane depolarisation in all cell lines, with the highest level of depolarisation occurring in the diff-SHSY5Y cells in a dose dependent manner. This indicates that there is an increase in apoptotic and necrotic activity in the diff-SHSY5Y cells following ECAC exposure.

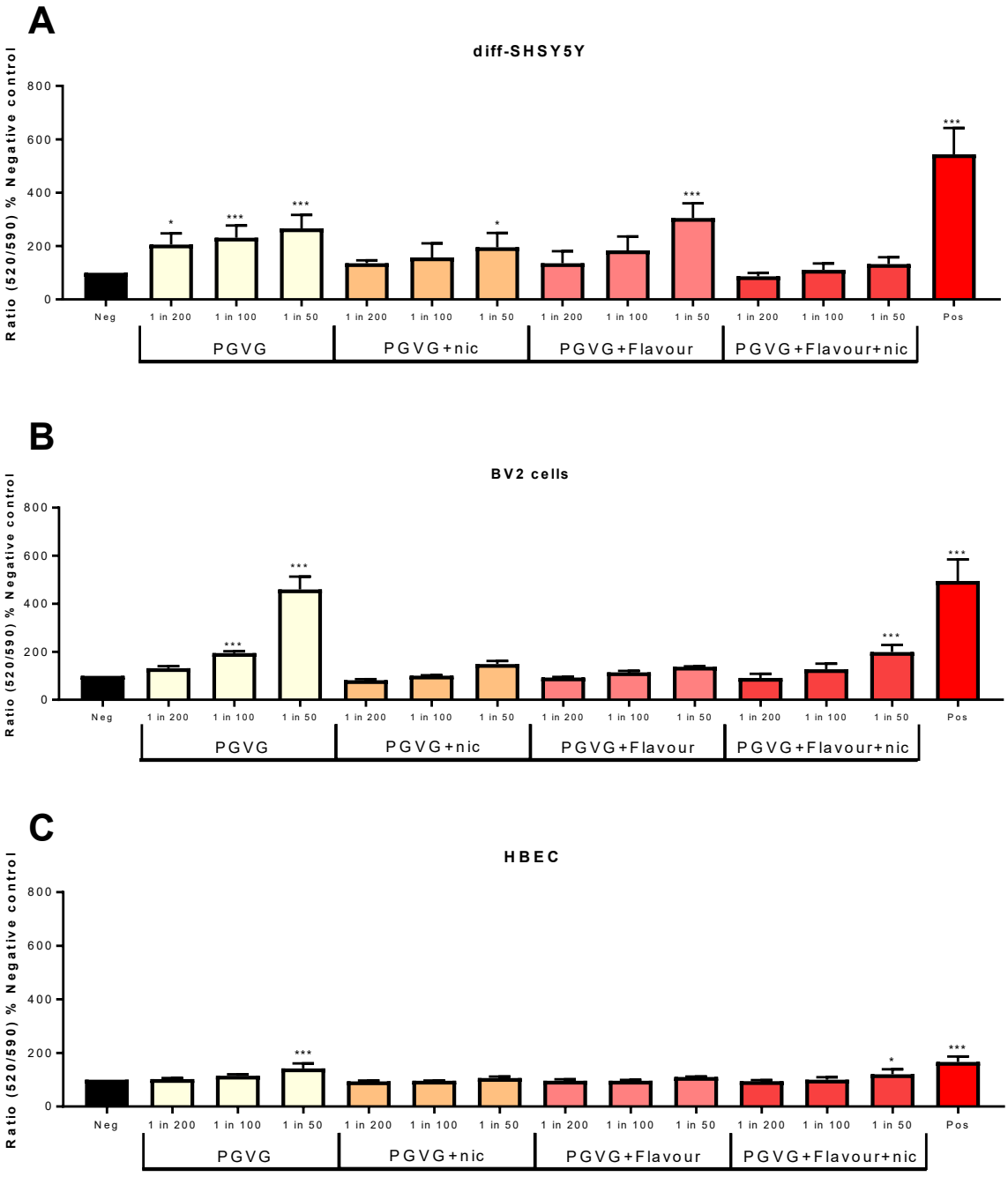

Figure 52. The JC-10 assay measures mitochondrial membrane depolarisation of (A) diff-SHSY5Y cells, (B) BV2 cells and (C) HBEC, following treatment with e-cigarette aerosol condensate at dilutions 1 in 200, 1 in 100 and 1 in 50 (n = 4). Data is shown as a percentage standardised to the negative control. Data represented as mean ± standard deviation, one-way ANOVA with *Bonferroni* post-hoc test, *p<0.05, **p<0.01, ***p<0.001 vs. negative control. Neg = Negative control, PGVG = Propylene Glycol/Vegetable glycerin mixture, Nic = nicotine, Pos = Positive control.

4.4.5 Co-culture

4.4.5.1 HBEC only co-culture experiments

4.4.5.1.1 Endothelial resistance in HBEC was decreased after treatment with e-cigarette aerosol condensate shown by FITC-Dextran permeability

FITC-Dextran is a tracer molecule that is applied into the inner chamber to determine endothelial membrane permeability by measuring the fluorescence accumulation in the bottom chamber. Sample media from the bottom chamber was collected every 5-10 minutes for 60 minutes to determine the amount of FITC-Dextran that passed through the endothelial membrane after treatment with ECAC with the appropriate controls. Real-time measurements of FITC-Dextran in the bottom chamber over 60 minutes showed that the PGVG+Flavour+nic group had the highest level of membrane permeability over time (Figure 53A). The second highest level of membrane permeability was from PGVG+Flavour. The negative control, PGVG group and PGVG+nic group had similarly low membrane permeability. 60 minutes following FITC-Dextran treatment, there was a significant increase in fluorescence in the PGVG+Flavour group and the PGVG+Flavour+nic group compared to the negative control ($p<0.001$; Figure 53B). Overall, cell-to-cell contact of HBEC was compromised by ECAC with and without nicotine.

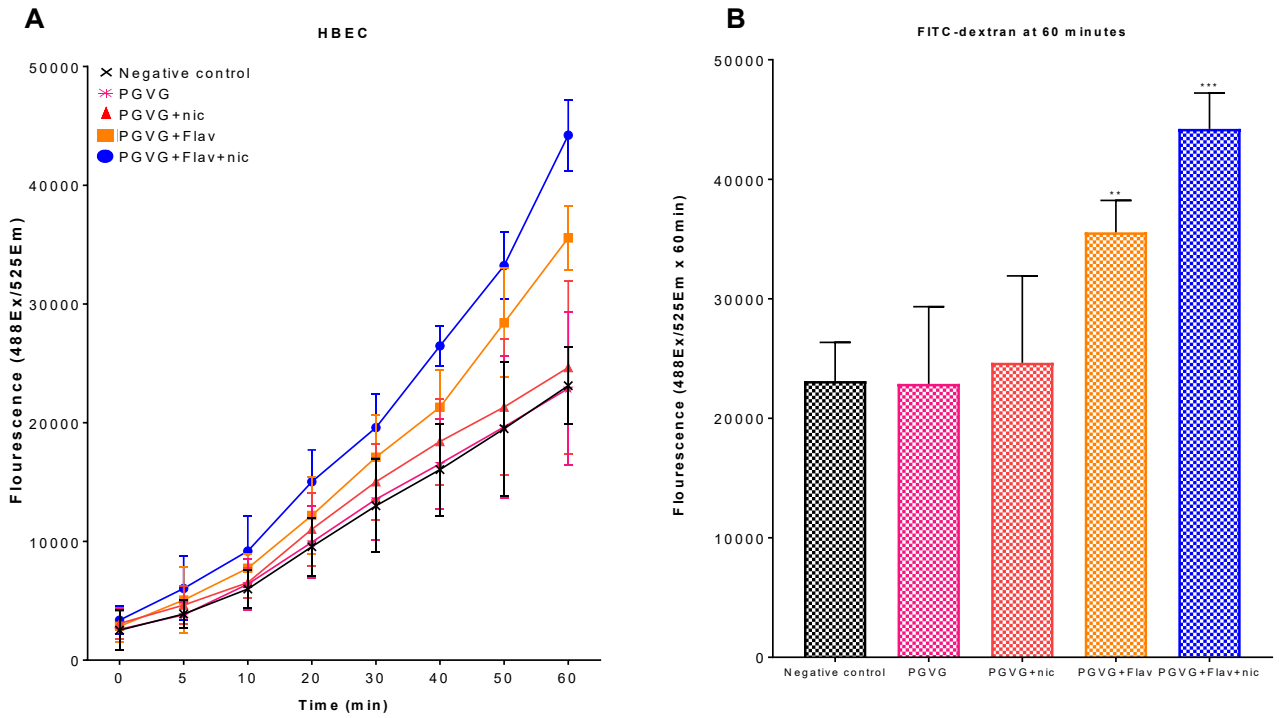

Figure 53. Permeability of tracer molecule FITC-Dextran through the human brain endothelial cell membrane treated with e-cigarette aerosol condensate with the appropriate controls (n = 3). (A) Real-time fluorescent measurements of FITC-Dextran from the bottom chamber from 0-60 minutes. (B) Bar graphs showing the significant increases in fluorescence found in the bottom chamber at 60 minutes for each treatment group. Data represented as mean ± standard deviation, one-way ANOVA with Bonferroni post-hoc test, **p<0.01, ***p<0.001 vs. negative control. Nic = nicotine, Flav = Flavour, PGVG = Propylene Glycol/Vegetable glycerin mixture.

4.4.5.1.2 Endothelial resistance in HBEC was decreased after treatment with e-cigarette aerosol condensate as shown by TEER measurements.

TEER is a measure of endothelial membrane integrity and is an important measurement to determine the health and cell-to-cell interaction of the endothelial membrane layer [445]. To create an *in vitro* model of the BBB, the endothelial cells are required to have tight junctions and cell-to-cell contact to ensure that selective movement of substances are going across the membrane [445]. From the results, HBEC TEER measurements increased over the course of 9 days and plateaued at day 10. This indicates the optimum level of endothelial cell membrane integrity (Figure 54A). The effect of ECAC on HBEC membrane integrity was, therefore, assessed at day 10. After 24 hours of treatment with ECAC with the appropriate controls, TEER was measured in each treatment group. In the negative control, the TEER after no treatment was at an average of 257.7 Ω. The TEER measurement was significantly reduced after ECAC treatment in the PGVG group (230.3 Ω), PGVG+Flavour group (231.0 Ω) and PGVG+Flavour+nic group (212.3 Ω) compared to the negative control ($p<0.05$) (Figure 54B).

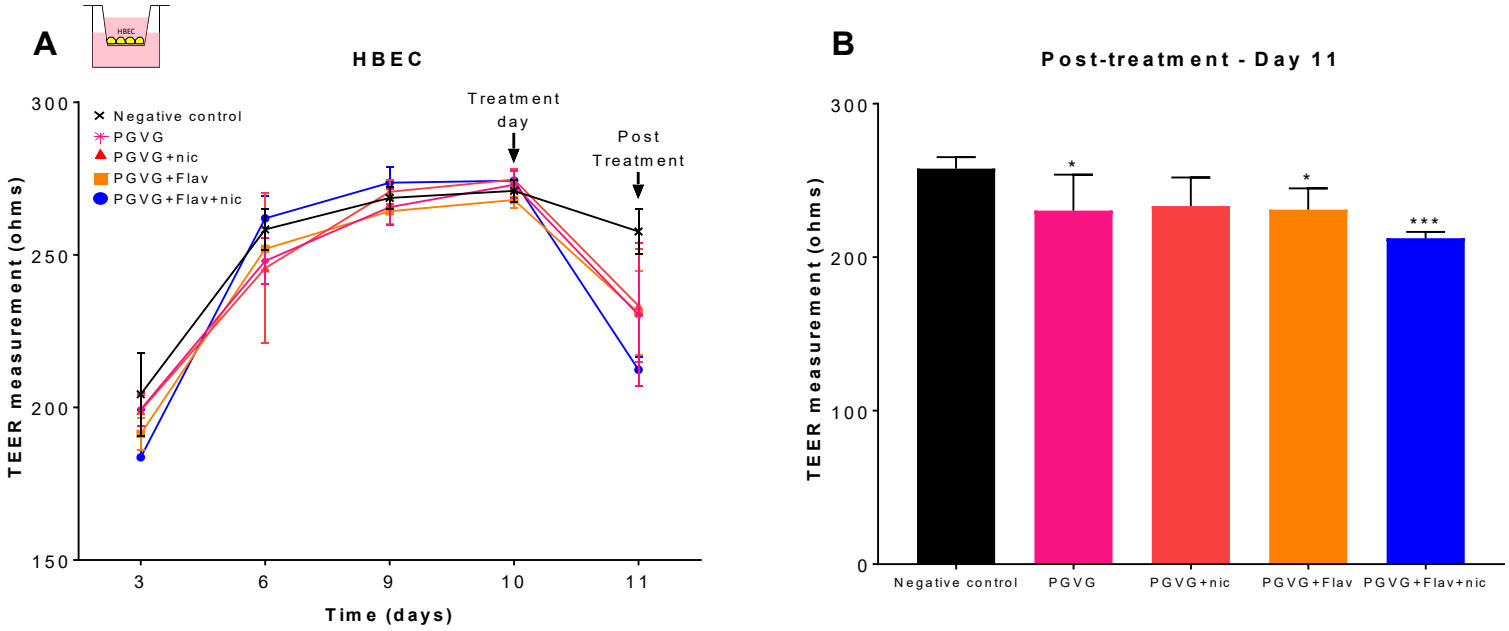

Figure 54. TEER measurements across the human brain endothelial cell (HBEC) monolayer over 11 days (n = 3). (A) TEER measurements were recorded at day 3 up to treatment with e-cigarette aerosol condensate at day 10 followed by post-treatment at day 11. (B) TEER measurements across the HBEC monolayer 24 hours after treatment with e-cigarette aerosol condensate. Decreases in HBEC TEER measurements were seen in the PGVG, PGVG+Flav group and PGVG+Flav+nic group. Data represented as mean ± standard deviation, one-way ANOVA with Bonferroni post-hoc test, *p<0.05, ***p<0.001 vs. negative control. Nic = nicotine, Flav = Flavour, PGVG = Propylene Glycol/Vegetable glycerin mixture.

4.4.5.2 HBEC co-cultured with diff-SHSY5Y cells

4.4.5.2.1 Endothelial resistance in HBEC co-culture with diff-SHSY5Y cells was lowest after treatment with flavoured e-cigarette aerosol condensate without nicotine

To determine the effects of ECAC on HBEC membrane integrity with other CNS cell lines, HBECs were co-cultured with diff-SHSY5Y cells. HBECs were grown in transwell inserts until cells become sub-confluent. Transwell inserts with HBECs were then placed into well-plates containing diff-SHSY5Y cells. TEER measurement was recorded at day three and the TEER measurement plateaued at day 10. ECAC treatment commenced on day 10 and TEER measurement was recorded at 24 hours post-treatment (Figure 55A). In the negative control, the TEER after no treatment was at 224.0 Ω. TEER measurement was significantly reduced in the PGVG+Flavour group (156.0 Ω) and PGVG+Flavour+nic group (166.7 Ω) compared to the negative control ($p<0.05$) (Figure 55B). Treatment with PGVG+Flavour and PGVG+Flavour+nic showed a greater reduction in membrane integrity in the HBEC co-cultured with diff-SHSY5Y cells.

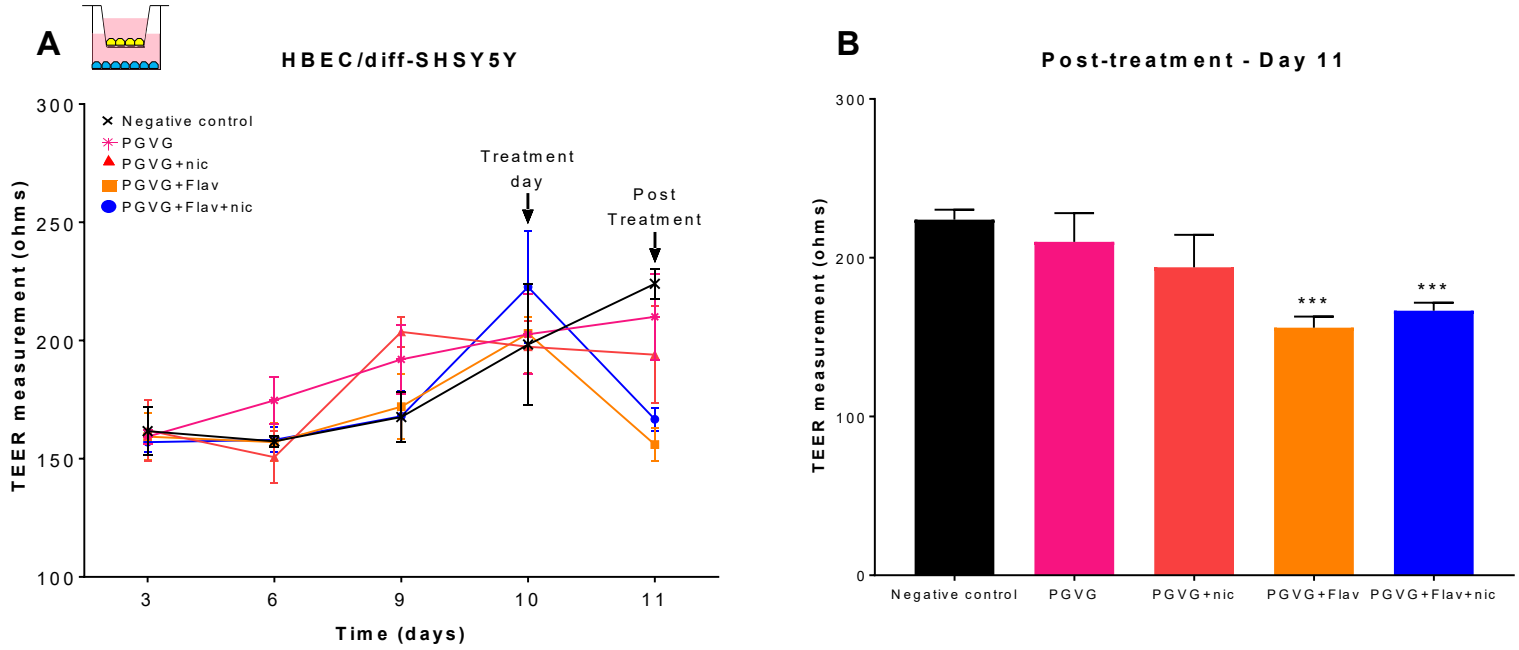

Figure 55. TEER measurements across the human brain endothelial cell (HBEC) monolayer over 11 days co-cultured with diff-SHSY5Y cells (n = 3). (A) TEER measurements were recorded at day 3, up to treatment with e-cigarette aerosol condensate at day 10, followed by post-treatment at day 11. (B) TEER measurements at 24 hours after treatment with e-cigarette aerosol condensate. There were significant decreases in TEER measurements in the PGVG+Flav group and PGVG+Flav+nic group. Data represented as mean ± standard deviation, one-way ANOVA with Bonferroni post-hoc test, ***p<0.001 vs. negative control. Nic = nicotine, Flav = Flavour, PGVG = Propylene Glycol/Vegetable glycerin mixture.

4.4.5.2.2 No significant changes to epigenetic gene expression was observed in diff-SHSY5Y cells co-cultured with HBEC

The effects of ECAC on diff-SHSY5Y cells on epigenetics were determined using a co-culture model with HBEC. There were no significant changes in epigenetic gene expression observed in any treatment groups (Table 9). In addition, inflammatory gene expression was also investigated in diff-SHSY5Y cells in co-culture with HBEC and no changes in inflammatory gene expression of IL-1β, IL-6 and TNF-α were observed in any of the treatment groups.

Table 9. mRNA expression levels of epigenetic and inflammatory genes in diff-SHSY5Y co-cultured with HBEC after e-cigarette aerosol condensate exposure (n=3).

Markers	Encoded protein	Negative control	PGVG	PGVG+nic	PGVG +Flavour	PGVG +Flavour +nic
Epigenetic markers	Dnmt3a	1.0 ±0.1	1.3 ± 0.2	0.9± 0.2	1.0 ± 0.1	1.0 ± 0.2
	Dnmt3b	1.0±0.2	1.3 ±0.1	1.0 ± 0.2	1.0 ± 0.0	1.0 ± 0.4
	Kdm5c	1.0±0.2	1.1 ± 0.3	0.8 ± 0.2	0.8 ± 0.0	0.8 ± 0.1
	Kdm6b	1.0±0.3	1.4 ± 0.2	1.0 ± 0.1	1.0 ± 0.1	0.9 ± 0.2
	Atf2	1.0±0.1	1.2 ± 0.2	1.1 ± 0.1	1.1 ± 0.0	1.1 ± 0.2
	Hdac1	1.0±0.0	0.9 ± 0.1	1.0 ± 0.0	1.0 ± 0.1	1.0 ± 0.1
	AurkA	1.0±0.1	1.1 ± 0.2	0.9 ± 0.1	1.0 ± 0.1	1.1 ± 2.0
	AurkB	1.0±0.1	1.0 ± 0.1	1.0 ± 0.0	1.0 ± 0.1	1.0 ± 0.1
	AurkC	1.0 ±0.0	1.2 ± 0.2	1.2 ± 0.3	1.1 ± 0.2	1.1 ± 0.4
Inflammatory genes	IL-1β	1.0 ± 0.1	0.7 ± 0.1	0.8 ± 0.1	0.9 ± 0.2	0.9 ± 0.1
	IL-6	1.0 ± 0.2	0.7 ± 0.6	0.9 ± 0.2	1.1 ± 0.2	1.0 ± 0.1
	TNF-α	1.0 ± 0.1	0.7 ± 0.1	0.7 ± 0.1	0.8 ± 0.0	0.9 ± 0.3

Data presented as average ± standard deviation. Nic = nicotine, Flav = Flavour, PGVG = Propylene Glycol/Vegetable glycerin mixture.

4.4.5.3 HBEC co-cultured with BV2 cells

4.4.5.2.1 Endothelial integrity of HBEC in co-culture with BV2 cells was lowest after treatment with flavoured e-cigarette aerosol condensate with nicotine

In addition to determining the effects of ECAC on HBEC membrane integrity with diff-SHSY5Y cells, microglial BV2 cells were co-cultured with HBECs. HBEC were grown on the transwell inserts until sub-confluent. Transwell inserts with HBEC were then placed into well-plates containing BV2 cells. TEER measurements were recorded at day three and TEER measurements plateaued at day 10. ECAC treatment commenced on day 10 and TEER measurement was recorded at 24 hours post-treatment (Figure 56A). In the negative control, the TEER after no treatment was at 196.0 Ω. TEER measurements were significantly reduced in the PGVG+Flavour group (143.3 Ω) and the PGVG+Flavour+nic group (134.0 Ω) compared to the negative control ($p<0.05$) (Figure 56B).

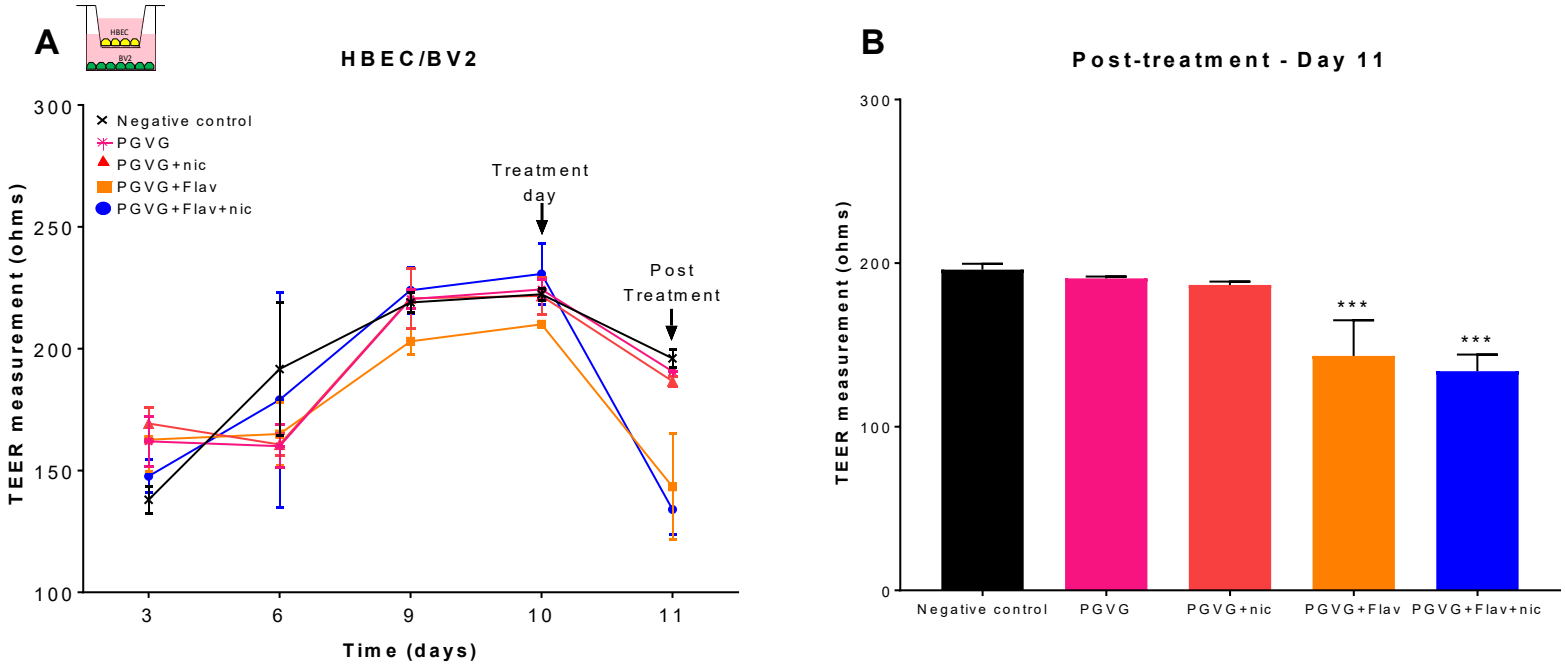

Figure 56. TEER measurements across the human brain endothelial cell (HBEC) monolayer over 11 days co-cultured with BV2 cells (n = 3). (A) TEER measurements were recorded at day 3, up to treatment with e-cigarette aerosol condensate at day 10, followed by post-treatment at day 11. (B) TEER measurements at 24 hours after treatment with e-cigarette aerosol condensate. There were significant decreases in TEER measurement in the PGVG+Flav group and PGVG+Flav+nic group. Data represented as mean ± standard deviation, one-way ANOVA with Bonferroni post-hoc test, ***p<0.001 vs. negative control. Nic = nicotine, Flav = Flavour, PGVG = Propylene Glycol/Vegetable glycerin mixture.

4.4.5.2.2 Inflammatory gene expression of IL-1α, IL-1β, IL-6, IL-10, IL-18 and TGFβ1 were significantly changed in BV2 cells treated with e-cigarette aerosol condensate

Inflammatory gene changes were investigated in BV2 cells co-cultured with HBEC after ECAC exposure. The mRNA expression levels of inflammatory markers are listed in Table 10. There was a significant increase in IL-1α in the PGVG+Flavour+nic group compared to the negative control group ($p<0.05$) and the PGVG group ($p<0.001$). In addition, IL-1β gene expression was also significantly increased in PGVG+Flavour+nic compared to all other treatment groups ($p<0.01$). IL-6 gene expression was shown to be decreased in PGVG+nic compared to the negative control group ($p<0.001$) and the PGVG+Flavour+nic group ($p<0.01$). In addition, IL-6 was also significantly decreased in the PGVG+Flavour group compared to the negative control group ($p<0.01$). ECAC with PGVG+Flavour+nic also showed a significant increase in IL-10 gene expression compared to all other treatment group ($p<0.001$). Finally, TGFβ1 gene expression was significantly increased in the PGVG+nic group compared to the negative control group ($p<0.001$) and the PGVG group ($p<0.001$). Moreover, PGVG+Flavour group also had a significant increase in TGFβ1 gene expression compared to the PGVG group ($p<0.001$). No changes in gene expression was found for IL-18 and TNF-α in any treatment groups.

Table 10. mRNA expression levels of inflammatory genes of BV2 cells co-cultured with HBEC after e-cigarette aerosol condensate exposure (n = 3).

Cell line	Encoded protein	Negative control	PGVG	PGVG +nicotine	PGVG +Flavour	PGVG +Flavour +nicotine
BV2 cells	IL-1α	1.0 ±0.2	0.9 ± 0.1	1.1 ± 0.2	1.2 ± 0.1	1.3 ± 0.2*$
	IL-1β	1.0±0.1	1.1 ± 0.2	1.1 ± 0.0	1.2 ± 0.2	1.6 ± 0.0*#$%
	IL-6	1.0±0.1	0.8 ± 0.2	0.6 ± 0.1*^	0.6 ± 0.1*	0.9 ± 0.1
	IL-10	1.0±0.3	0.7 ± 0.1	0.7 ± 0.2	1.0 ± 0.1	1.5 ± 0.2*#$%
	IL-18	1.0±0.1	0.8 ± 0.1	0.8 ± 0.2*	0.8 ± 0.1	0.9 ± 0.2
	TGFβ1	1.0±0.1	0.9 ± 0.1	1.2 ± 0.1*$	1.1 ± 0.1$	1.1 ± 0.1
	TNF-α	1.0±0.2	1.3 ± 0.7	1.2 ± 0.8	1.7 ± 0.4	1.0 ± 0.4

Data presented as average ± standard deviation, *p<0.05 vs. negative control, $p<0.05 vs. PGVG, %p<0.05 vs. PGVG+nic, #p<0.05 vs. PGVG+Flav, ^p<0.05 vs PGVG+Flavour+nic. Nic = nicotine, Flav = Flavour, PGVG = Propylene Glycol/Vegetable glycerin mixture.

4.4.5.4 Conditioned media experiment

4.4.5.4.1 Epigenetic markers but not inflammatory markers were significantly changed in diff-SHSY5Y cells treated with conditioned media from BV2 cells co-cultured with HBEC

Following from the co-culture with HBEC and diff-SHSY5Y experiment, a follow up experiment was conducted to investigate whether treating diff-SHSY5Y cells with the conditioned media from BV2 cells affects epigenetic gene expression. The mRNA expression levels of epigenetic markers are listed in

Table **11**. From the results, Kdm6b gene expression was significantly decreased in the PGVG group (p<0.05) and the PGVG+Flavour+nic group (p<0.001) compared to the negative group. In

addition, Kdm6b gene expression was also significantly decreased in the PGVG+Flavour+nic group compared to the PGVG group ($p<0.05$). For Dnmt3a gene expression, the PGVG group showed a significant decrease in Dnmt3a gene expression compared to the negative control ($p<0.001$), PGVG+nic ($p<0.001$) and PGVG+Flavour+nic ($p<0.001$). No significant difference in gene expression was found in AurkA, AurkB, AurkC, Kdm5c, Atf2, Dnmt3b and Hdac1 gene expression compared to the negative control. Inflammatory gene expression of IL-1β, IL-6 and TNF-α were not significantly different in any of the treatment groups compared to the negative control.

Table 11. mRNA expression levels of epigenetic and inflammatory genes in diff-SHSY5Y cells treated with media from BV2 cells co-cultured with HBEC after e-cigarette aerosol condensate exposure (n = 3).

Markers	Encoded protein	Negative control	PGVG	PGVG +nicotine	PGVG +Flavour	PGVG +Flavour +nicotine
Epigenetic markers	Dnmt3a	1.0 ±0.1	0.8 ± 0.0**^^$$	1.0 ± 0.0	0.9 ± 0.1	1.0 ± 0.0
	Dnmt3b	1.0±0.1	1.0 ± 0.0	1.1 ± 0.3	1.2 ± 0.1	1.1 ± 0.0
	Kdm5c	1.0±0.1	0.9 ± 0.0	1.0 ± 0.2	1.0 ± 0.1	1.0 ± 0.1
	Kdm6b	1.0±0.1	0.8 ± 0.2*	0.9 ± 0.1	0.9 ± 0.0	0.8 ± 0.0**%
	Atf2	1.0±0.2	1.0 ± 0.1	1.1 ± 0.1	1.1 ± 0.0	1.0 ± 0.1
	Hdac1	1.0±0.0	0.9 ± 0.1	1.0 ± 0.0	1.0 ± 0.1	1.0 ± 0.1
	AurkA	1.0±0.1	1.1 ± 0.2	1.0 ± 0.1	1.1 ± 0.1	1.0 ± 0.0
	AurkB	0.9 ±0.1	1.0 ± 0.0	1.0 ± 0.1	1.0 ± 0.1	1.0 ± 0.0
	AurkC	1.0 ±0.1	1.2 ± 0.0	1.3 ± 0.2	1.2 ± 0.1	1.1 ± 0.0
Inflammatory markers	IL-1β	1.0 ± 0.1	0.7 ± 0.1	0.8 ± 0.1	0.9 ± 0.2	0.9 ± 0.1
	IL-6	1.0 ± 0.2	0.7 ± 0.6	0.9 ± 0.2	1.1 ± 0.2	0.7 ± 0.1
	TNF-α	1.0 ± 0.1	0.7 ± 0.1	0.7 ± 0.1	0.8 ± 0.1	0.9 ± 0.3

Data presented as average ± standard deviation, *p<0.05 vs negative control. $p<0.05 vs PGVG+nic, %p<0.05 vs. PGVG+Flavour, ^p<0.05 vs. PGVG+Flavour+nic. Nic = nicotine, Flav = Flavour, PGVG = Propylene Glycol/Vegetable glycerin mixture.

4.4.6 *In vivo* gene expression of inflammatory markers in the offspring brain

Based off the inflammatory gene expression of BV2 cells after ECAC treatment, the same inflammatory genes were then investigated in offspring brains at P1, P20 and Week 13 to determine whether the same changes are also occurring *in vivo*. The mRNA expression levels of inflammatory markers are listed in Table 12. From the P20 offspring brain, there was a significant increase in gene expression of IL-6, IL-10 and TNF-α gene at 2040% ($p<0.05$), 1400% ($p<0.05$) and 190% ($p<0.05$), respectively, in the PGVG+Flavour+nic group compared to the Sham group. Furthermore, an increase in IL-6 (690%) and IL-10 (470%) were also observed, however, these were not statistically significant. Offspring brain at P1 and at Week 13 showed no changes to inflammatory gene expression.

Table 12. mRNA expression levels of inflammatory genes in offspring brain at Postnatal day (P) 1, P20 and Week 13 in the Sham, PGVG+Flavour and PGVG+Flavour+nic groups (n = 3).

Time points	Encoded protein	Sham	PGVG+Flavour	PGVG +Flavour +nicotine
P1 (Whole brain)	IL-1α	1.0 ±0.3	1.0 ± 0.3	1.2 ± 0.3
	IL-1β	1.0±0.2	1.0 ± 0.1	0.9 ± 0.2
	IL-6	1.3 ± 1.1	1.1 ± 0.5	0.6 ± 0.2
	IL-10	1.2 ±0.9	1.2 ± 0.7	0.5 ± 0.2
	IL-18	1.0 ±0.1	0.8 ± 0.1	0.9 ± 0.1
	TGFβ1	1.0±0.1	0.9 ± 0.1	1.1 ± 0.1
	TNF-α	1.0±0.3	0.9 ± 0.2	1.3 ± 0.3
P20 (Whole brain)	IL-1α	1.0 ±0.2	1.1 ± 0.1	1.1 ± 0.3
	IL-1β	1.0±0.1	1.4 ± 0.2	2.1 ± 0.8
	IL-6	1.6 ± 1.7	6.9 ± 8.1	20.4 ± 2.1*
	IL-10	1.1 ±0.7	4.7 ± 5.1	14.0 ± 1.4*
	IL-18	1.0±0.1	1.0 ± 0.1	1.0 ± 0.1
	TGFβ1	1.0±0.1	1.2 ± 0.1	1.2 ± 0.1
	TNF-α	1.0±0.2	0.8 ± 0.0	1.9 ± 0.4*#
Week 13 (Hippocampus)	IL-1α	1.0 ±0.4	1.0 ± 0.1	0.8 ± 0.1
	IL-1β	1.1 ±0.7	0.7 ± 0.0	0.8 ± 0.2
	IL-6	1.6 ± 1.7	2.6 ± 3.6	1.2 ± 0.5
	IL-10	1.8 ± 2.2	2.6 ± 3.3	1.5 ± 0.7
	IL-18	1.0±0.3	1.0 ± 0.3	0.9 ± 0.1
	TGFβ1	1.0±0.1	0.9 ± 0.2	0.9 ± 0.1
	TNF-α	1.0±0.3	0.7 ± 0.1	0.9 ± 0.2

Data presented as average ± standard deviation, *p<0.05 vs. Sham, #p<0.05 vs PGVG+Flavour. PGVG = Propylene Glycol/Vegetable glycerin mixture.

The summary of the results from Chapter 4 are outlined in Table 13.

Table 13. A summary table of the results from Chapter 4.

	Experiments	Diff-SHSY5Y cells	BV2 cells	HBEC
Optimisation	Differentiation with ECAC and PMA	↓ in GAP43 gene expression after treatment with ECAC and PMA in all treatment groups		
Monoculture	MTT assay	↓ Cell viability at a dose-dependent manner	↓ Cell viability at a dose-dependent manner	↓ Cell viability at a dose-dependent manner
	DCF assay	↑ ROS release at a dose-dependent manner	↑ ROS release at a dose-dependent manner	Variable increases in ROS production
	JC-10 assay	↑ Mitochondrial depolarisation at a dose-dependent manner	↑ Mitochondrial depolarisation at a dose-dependent manner	↑ Mitochondrial depolarisation at a dose-dependent manner
Co-culture	FITC-Dextran			↑ Permeability of endothelial membrane treated with PGVG+Flavour and PGVG+Flavour+nic
	TEER measurements	- Reduction in endothelial membrane integrity in HBEC only co-culture treated with PGVG, PGVG+Flavour and PGVG+Flavour+nic - Reduction in endothelial membrane integrity HBEC/diff-SHSY5Y, and HBEC/BV2 cells co-cultures treated with PGVG+Flavour and PGVG+Flavour+nic		
	mRNA Epigenetic gene expression	- HBEC/diff-SHSY5Y co-culture: no significant difference - HBEC/BV2 → diff-SHSY5Y cells (conditioned media): Significant decrease in Dmnt3a and Kdm6b gene expression after treatment with PGVG and PGVG+Flavour+nic		
	mRNA Inflammatory gene expression	- HBEC/BV2 co-culture: significant changes to IL-1α, IL-1β, IL-6, IL-10, IL-18, TGFβ1 and TNF-α gene expression after treatment with PGVG+nic, PGVG+Flavour, PGVG+Flavour+nic		
	Experiment	**P1**	**P20**	**Week 13**
In-vivo experiment	*In vivo* inflammatory gene expression	No significant difference	Significant increase in IL-6, IL-10 and TNF-α gene expression in offspring hippocampus after PGVG+Flavour+nic exposure *in utero*	No significant difference

4.5 Discussion

This current study investigated the effects of ECAC on CNS-base cell lines on monocultures and co-cultures. From the results, direct exposure of ECAC in monoculture reduces differentiation, survival and mitochondrial function in diff-SHSY5Y cells, BV2 cells and HBEC. Application of ECAC with the flavouring (with and without nicotine) disrupts the HBEC in the BBB co-culture model. Diff-SHSY5Y cells only showed epigenetic changes after being treated with conditioned media from the BV2 cells co-cultured with HBEC. BV2 cells co-cultured with HBEC showed variable changes to pro-inflammatory and anti-inflammatory gene expression following ECAC exposure with flavour and nicotine. Interestingly, the propylene glycol and vegetable glycerin mixture seem to be also having an effect on inflammatory gene expression in the BV2 cells.

4.5.1 Differentiation of SHSY5Y cells with e-cigarette aerosol condensate stunts SHSY5Y maturation

To determine the effects of ECAC on SHSY5Y cell differentiation, ECAC was added with the differentiation-inducing agent, PMA. Differentiation was confirmed by SHSY5Y cell morphology and gene expression of the mature neuronal cell marker, GAP43. These results indicate that ECAC with and without nicotine leads to stunted differentiation of SHSY5Y cells which could suggest that e-cigarette exposure may result in reduced neuronal development in the brain which can lead to delayed cognitive development. In the literature, the application of nicotine to SHSY5Y cells are shown to regulate neuronal maturation by releasing BDNF [446]. This suggest that constituents from nicotine are having an effect on neuronal differentiation, and this needs to be further investigated. However, there is a likelihood that ECAC is acting as a better solvent for PMA than the cell media, and therefore, reducing the ECAC concentration in the aqueous phase. Therefore, this could suggest that the treatment media did not effectively differentiate the SHSY5Y cells, but this is yet to be determined.

4.5.2 E-cigarette aerosol condensate effects cell viability, ROS release and mitochondrial potential in SHSY5Y cells, BV2 cell and HBEC monoculture

There are numerous studies that have shown e-cigarette fluids, extracts and flavouring constituents are cytotoxic to a number of cell lines [126, 127, 178, 179, 185]. Tobacco-flavoured e-cigarettes without nicotine had the highest level of toxicity compared to the tobacco-flavoured e-cigarettes with nicotine in diff-SHSY5Y and BV2 cells in monoculture. Although HBEC had comparable toxicity between flavour, with and without nicotine, this could suggest that nicotine may play a role as a protective agent in the diff-SHSY5Y cells and BV2 cells. This is consistent with the literature where nicotine has been shown to be neuroprotective in SHSY5Y cells and BV2 cells [447-451]. In addition, nicotine has been shown to regulate cell proliferation by interacting with the nAChR that are present on SHSY5Y cells [446].

Microglial cells are the resident macrophages of the CNS and are important in the innate immunity in response to traumatic injury [452]. In addition, environmental exposure such as manganese and diesel exhaust particles can also affect microglial cells [453]. One study that investigated the effects of a tobacco carcinogen 4-Methylnitrosamino-1-(3-pyridyl)-1-butanone (NNK) on BV2 cells showed an increase in pro-inflammatory cytokines such as IL-6, TNF-α, and monocyte chemoattractant protein 1 (MCP-1) [454]. In addition, the same study investigated the effects of NNK in a mouse model and showed microglial and astrocyte activation histologically [454]. Therefore, these results reinforce the possibility that the effects of ECAC on CNS cell lines could be due to chemical constituents other than nicotine.

In this study, treatment with ECAC showed a dose-dependent increase in cell toxicity, ROS release and mitochondrial depolarisation. This can be in combination with the release of extracellular ROS, where there was a significant increase in ROS in the flavour without nicotine in the diff-SHSY5Y cells and BV2 cells in monoculture. This suggests that cell toxicity may be due to excessive oxidative stress occurring in the mitochondria of the diff-SHSY5Y cells as well

as the BV2 cells. The mitochondria are the powerhouse of the cell where ATP is formed by oxidative phosphorylation using the electron transport chain. Overproduction of ROS in the mitochondria could result in the release of apoptotic markers such as cytochrome C and, therefore, triggering cell apoptosis. This was reflected in the JC-10 assay results where there was a dose-dependent decrease in mitochondrial depolarisation in the flavour without nicotine compared to flavour with nicotine in the SHSY5Y cells and BV2 cells.

It is also interesting to note that exposure to ECAC containing base liquids with and without nicotine had a significant effect on cell viability, ROS release and mitochondrial depolarisation in all three cell lines. There are studies in the literature that showed propylene glycol and vegetable glycerin can potentially be toxic. Sassano and colleagues have shown a reduction in human embryonic kidney cells cell viability after exposure to PGVG alone [455]. Although not in cell culture, long-term exposure to PGVG alone in a mouse model showed altered lung lipid homeostasis [456]. Ghosh and colleagues investigated the lung performance e-cigarette users and suggested that PGVG could be an underlying factor as to why these individuals have a reduction in membrane function and altered epithelial proteome [457]. This reinforces the potential harm of using propylene glycol and vegetable glycerin as base liquids when using e-cigarettes. In addition, propylene glycol has been known to form formaldehyde at high temperatures [27]. Thus, future studies are needed to elucidate the effects of different ratios of aerosolised propylene glycol and vegetable glycerin and quantify the release of formaldehyde during treatment. It is important to investigate the different ratios of base liquid used since e-cigarette users adjust the concentration of each ingredient to cater for their vaping preferences i.e. high ratio of propylene glycol provides a ‘throat hit’ and a high ratio of vegetable glycerin produces thicker aerosols [458]. This indicates that the base liquids alone had a significant effect on CNS-based cell lines and needs to be further investigated and whether treating cells with formaldehyde yields similar results.

Overall, the monoculture assays showed that diff-SHSY5Y cells, BV2 cells and HBEC had varying changes to cell viability, release of ROS and mitochondrial dysfunction after ECAC

treatment. The effects that were observed showed that ECAC treatment with flavour had a marked effect on diff-SHSY5Y cells and ECAC treatment with flavour and nicotine showed a marked effect on BV2 cells. Other combinations of e-liquid constituent such as PGVG alone and PGVG with nicotine have also showed significant effects in these cells.

4.5.3 Treatment with e-cigarette aerosol condensate causes increased permeability and decreased integrity in endothelial membrane.

Tobacco cigarette exposure has been known to cause oxidative stress, inflammation and glucose tolerance at the BBB [459-461]. BBB dysfunction can be associated with conditions such as stroke, neurodegenerative disease (Parkinson's and Alzheimer's) and type 2 diabetes [462-464]. In addition, a number of tobacco cigarette constituents such as polycyclic aromatic hydrocarbons, nicotine and heavy metals like cadmium can readily diffuse through the BBB and enter the parenchyma of the CNS [248, 465, 466]. Recently, a number of studies have investigated the effects of tobacco cigarette smoke in an *in vitro* BBB model using human and mouse brain microvascular endothelial cell lines [429, 430, 459-461].

To show the effects of e-cigarette exposure on the BBB, an *in vitro* BBB model using HBEC was designed to create a better physiological representation of the effects of e-cigarette exposure in humans. There have been a few studies that investigated the effects of tobacco cigarette exposure *in vitro*. Kim and colleagues investigated the effects of tobacco cigarette extract on monocultures of the human glioblastoma cell line T98G and co-culture of T98G and human brain microvascular endothelial cells and found that T98G cells treated with tobacco cigarette extract in co-culture were more tolerable to the treatment compared to the monoculture [429]. In addition, Lee and colleagues showed better cell viability and ROS production in T98G and human astrocytoma cell lines U-373MG co-cultured with human brain microvascular endothelial cells compared after direct application of tobacco cigarette extracts [430]. Therefore, these studies reinforce the

importance of the protective barrier of the BBB at the blood to brain interface. The BBB plays a crucial homeostatic role in selectively choosing endogenous and exogenous substances to enter and exit the CNS. Therefore, the importance of a BBB is the tight junctions between endothelial cells. These tight junctions can be characterised by quantifying tight junction proteins such as claudin-5, occludin and zonula occludin [467].

Using an *in vitro* BBB model, this current study showed that tobacco-flavoured ECAC exposure with and without nicotine disrupts the HBEC BBB model, causing the endothelial layer to become leaky. Endothelial membrane permeability was increased evident by the FITC-Dextran release into the bottom chamber of the well-plate. In addition, a decrease in membrane integrity using TEER measurements were also observed. These results were also found in the endothelial membranes treated with tobacco-flavoured ECAC with and without nicotine. In the HBEC monoculture, there was an increase ROS release in the base liquid without nicotine treatment. ROS has been linked to BBB breakdown by modification of the tight junctions [468]. TEER measurement from the base liquid only group did not show a decrease in membrane integrity. This could be because the HBEC in monoculture may not have tight cell-cell interactions and this cell-cell interaction and therefore, not representing a BBB model. This could suggest that HBEC are more resilient or protective when HBEC forms a tight membrane layer. This concept has been alluded in previous studies [429, 430].

TEER measurements in all three co-cultures (HBEC only, HBEC/diff-SHSY5Y cells, HBEC/BV2 cells) were consistent following ECAC exposure. BV2 cells co-cultured with HBEC did not show any changes to TEER measurement compared to the HBEC membrane, however, diff-SHSY5Y co-cultured with HBEC showed a more gradual increase in TEER measurement. This did not affect the endothelial membrane integrity since the TEER measurement was at 200 Ω before the treatment day, which has been determined to be a suitable baseline measurement for membrane integrity [429, 430].

One other study has investigated e-cigarette extracts on membrane integrity using mouse primary brain endothelial cells [434]. The FITC-Dextran and TEER measurement results shown in this study were consistent with Kaisar and colleagues who showed an increase in FITC-Dextran release and an decrease in TEER measurement in their BBB model using mouse brain microvascular endothelial cells following tobacco-flavoured e-cigarette extract (with nicotine) treatment [434]. Studies that have investigated the effects of e-cigarette extract on an endothelial cell transwell set up have shown an increase in ROS levels, PECAM-1, antioxidant transcription factor Nrf2 and PPAR-γ [461]. In addition, ECAC exposure on endothelial cells in transwell also showed a decrease in ZO-1 protein expression, a decrease in TEER measurement and an increase in FITC-Dextran and Rhodamine B isothiocyanate-Dextran leakiness [461]. In addition to BBB *in vitro* models, there has been studies investigating lung endothelial cells using either a co-culture or air liquid interface model of exposure. Higham and colleagues showed a reduction in TEER measurements after bronchial epithelial cells from COPD patients were exposed to e-cigarette extracts [469]. Bengalli and colleagues investigated on the TEER from human lung adenocarcinoma cells exposed to different flavoured e-cigarette aerosol extracts and found that the cinnamon and menthol flavours showed a reduction in membrane integrity [470]. All of these studies provide evidence that e-cigarette exposure may cause detrimental effects on the endothelial cells of the lungs and brain and may cause downstream affects if there was long-term exposure.

The current study found that exposure of a tight monoculture of HBECs to ECAC increased endothelial permeability following a 24-hour exposure. Increased permeability in the brain endothelial monolayer was highest in the flavour with nicotine, followed by flavour without nicotine. Accompanying these results, endothelial integrity was also compromised in the monolayer with the strongest effects observed in the flavour with nicotine and flavour without nicotine.

HBEC cells co-cultured with diff-SHSY5Y cells showed a decrease in endothelial integrity with strongest effects found in the flavour without nicotine, followed by flavour with nicotine. HBEC

cells co-cultured with BV2 cells showed a significant decrease in endothelial integrity with strongest effects found in the flavour with nicotine, followed by flavour without nicotine. It is interesting to note that the co-culture did not show significant changes to HBEC integrity regardless of the cells grown in the bottom chamber. This could suggest that the flavouring within the ECAC could be affecting the membrane integrity. Creating a tri-culture using HBEC, glioblastoma (U87MG) cells and diff-SHSY5Y was attempted during optimisation, however, this was deemed technically difficult to maintain HBEC integrity (Figure 57). Optimisation is needed to fully characterise the cell density and the period of seeding for each cell line since diff-SHSY5Y requires three days to differentiate using PMA.

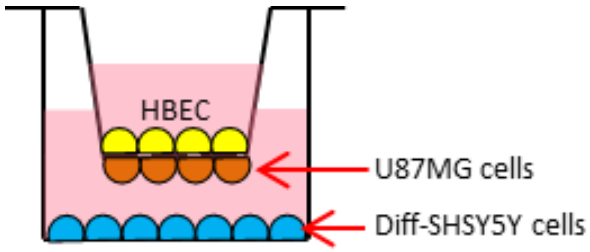

Figure 57. Schematic of the tri-culture using human brain microvascular endothelial cells (HBEC), glioblastoma (U87MG) cells, and differentiated neuroblastoma (diff-SHSY5Y) cells.

There have been studies that focused on the effects of different flavourings in e-cigarette aerosols. Muthumalage and colleagues have shown elevated cytotoxicity, IL-8 secretion and ROS release following cinnamaldehyde, diacetyl, O-vanillin and pentanedione exposure in human monocyte cell lines [176]. Clapp and colleagues found an increase in production of IL-6 and IL-8 in alveolar macrophages and neutrophils after exposure to various flavours of e-liquids [178]. Lerner and colleagues also showed an increase in IL-6 and IL-8 cytokine production from human primary lung fibroblast after exposure to e-cigarettes aerosols [146]. Higham and colleagues also found an increase in IL-6 and CXCL8 cytokine production in lung cells after exposure to e-cigarette extracts in a co-culture model [469]. In addition, the effects of ECAC on co-culture using astrocytes are needed since astrocytes have been shown to play an important role in antioxidant

defence within the brain [471]. Finally, future studies should also focus on observing changes to cytoskeleton protein (actin), cell-to-cell adhesion markers (E-adherins, beta-catenin), and tight junction proteins such as zonula occludins, claudin, cingulin and symplektin.

4.5.4 Inflammatory gene expression after e-cigarette aerosol condensate exposure of diff-SHSY5Y and BV2 cells co-cultured with HBEC.

Treatment with ECAC with flavour and nicotine in a co-culture model of BV2 cells and HBEC showed an elevation in key pro-inflammatory gene expression of IL-1α and IL-1β. In addition, IL-10, an anti-inflammatory marker, was also elevated after exposure to ECAC with flavour and nicotine. Treatment with ECAC with flavour and no nicotine showed a decrease in IL-6 gene expression.

To date, there are no studies on the effects of e-cigarette exposure on microglial cells *in vitro*. Scott and colleagues examined the effects of e-cigarette exposure (with and without nicotine) on primary alveolar macrophages from volunteers who had never smoked, and human monocyte leukaemia (THP-1) cells *in vitro.* Their study showed an increase in apoptotic cells, an increase in ROS release and pro-inflammatory cytokines secretions of IL-6, TNF-α, CXCL-8, MCP-1 and matrix metalloproteinase 9 [472]. In addition, Ween and colleagues investigated the effects of ECAC (with and without nicotine) on THP-1 macrophages, and showed a decrease in phagocytosis function and an increase in IL-1β secretion [473]. Resident macrophages in the liver which are known as Kupffer cells have also been examined following ECAC exposure and have shown reduced cell viability and increased IL-2, IL-4 and IL-6 secretions [474]. In addition to the effects of tobacco-flavoured e-cigarette use with and without nicotine, treatment with ECAC with base liquids with nicotine also showed a decrease in IL-6 and IL-18 gene expression but an increase in TGFβ1 gene expression. Again, these results could be suggesting that the base liquids

might be causing an underlying toxicity within microglial cells in an *in vitro* BBB model. Therefore, further studies are needed to determine the e-cigarette constituents that are causing these changes. Ghosh and colleagues have already tested the effects of NNK, a nitrosamine that have been shown to be present in e-cigarettes [475], on microglial cells in monoculture and have shown an increased release of IL-6, TNF-α and MCP-1 *in vitro* and microglial activation in murine brains *in vivo* [454].

4.5.5 Treatment with e-cigarette aerosol condensate with conditioned media but not HBEC alone showed changes to epigenetic gene expression in diff-SHSY5Y cells

Epigenetics are important in the control of gene expression in the genome. Epigenetic enzymes that are involved in modifying the chromatin includes DNA methyltransferases, histone demethylases, histone acetyltransferase, histone deacetylase and histone phosphorylation, all of which control the expression of genes by the addition or removal of molecular proteins such as methyl groups on the genome. This current study demonstrated no changes to epigenetic gene expression of Dnmt3a, Dnmt3b, Kdm5c, Kdm6b, Atf2, AurkA, AurkB and AurkC in diff-SHSY5Y cells after ECAC exposure in co-culture with HBEC. However, after the conditioned media from the BV2 cells co-cultured with HBEC was applied onto diff-SHSY5Y cells, epigenetic gene expression of DNA methyltransferases, Dnmt3a, and histone demethylase, Kdm5c, was significantly changed in the base liquid group and in the tobacco-flavoured with nicotine group. This goes to show the complex relationship and interaction between microglial and neuronal cells within the CNS. The effects of nicotine on these specific epigenetic genes have not been well studied in the literature. However, there have been a study that showed an increase in histone acetylation and inhibits histone deacetylases after frequent nicotine exposure in the lungs [476]. Ito and colleagues have also shown a decrease in HDAC2 gene and decrease overall

HDAC activity in smoker's lungs [477]. Wide epigenome research is important to understand the underlying mechanism in e-cigarette that can potentially affect the unborn foetus.

One study that investigated epigenetic gene expression of cerebral smooth muscle cells *in vitro* after acute exposure to cigarette smoke extract (2 hours) showed recruitment of the histone deacetylase protein, Hdac2, to promotor regions of α-actin on the smooth muscle cell, indicting a repression of transcription due to deacetylation [478].

Although there were no epigenetic changes in diff-SHSY5Y cells, there were significant changes to epigenetic gene expression when diff-SHSY5Y cells were treated with conditioned media from HBEC co-cultured with BV2 cells. Diff-SHSY5Y cells treated with conditioned media showed a decrease in histone demethylase, Kdm6b gene expression compared to the negative control. In addition, DNA methyltransferases, Dnmt3a gene expression was found to be decreased in diff-SHSY5Y cells treated with conditioned media with PGVG compared to the negative control. This suggests that PGVG condensate may cause changes to Kdm6b and Dnmt3a which are important in controlling gene expression on the genome.

There were no significant changes to IL-1β, IL-6 and TNF-α gene expression from diff-SHSY5Y cells treated with ECAC. Similar findings were found in diff-SHSY5Y cells treated with conditioned media indicating that ECAC treatment using an *in vitro* BBB model and after application of conditioned media from the BV2 cells and HBEC co-culture did not affect diff-SHSY5Y inflammatory response.

Due to the variation in methods as well as the diversity of e-cigarette devices, there are no standardisation and methodologies on investigating the effects of e-cigarettes in cell culture. More standardised tests are needed so that experiments conducted from various laboratories are comparable. Furthermore, future research is needed to examine the cytokines that were released into the media after e-cigarette exposure on BV2 cells co-cultured with HBEC to determine if there are changes to inflammation after direct application to ECAC.

4.5.6 *In vivo* treatment of e-cigarette exposure showed increase inflammatory gene expression of IL-6, IL-10 and TNF-α in offspring at postnatal day 20

To determine whether similar inflammatory responses were observed *in vivo*, inflammatory gene expression of offspring brain tissues were examined from **Chapter 2**. The results showed that following e-cigarette exposure with flavouring and nicotine, offspring brain at P20 showed a significant increase in pro-inflammatory gene expression of IL-6 and TNF-α and an increase in anti-inflammatory gene expression of IL-10. Although no significance was observed in the e-cigarette exposure with flavouring, there was an upward trend in the release of IL-6 and IL-10 at P20. This suggests that there are underlying mechanisms of inflammation that are occurring in the offspring brain after maternal exposure to e-cigarette aerosols with and without nicotine. Although gene expression was only observed in the brain, future studies are needed to determine which cells within the CNS that are releasing these cytokines. Since the microglia plays a major role in the immune response in the brain, future research is needed to identify whether microglial proteins, such as IBA-1, are elevated in the brain after e-cigarette exposure. One study has shown an increase in IBA-1 protein using immunohistochemistry in offspring after maternal exposure to e-cigarette aerosols [51]. Although not the brain, there have been studies that investigated cytokine production after e-cigarette exposure [133, 147, 178]. Clapp and colleagues and Lerner and colleagues have shown in mouse models an increase in the production of IL-6 and IL-8 after e-cigarette exposure [147, 178]. Lim and colleagues showed an increase IL-4, IL-5 and IL-13 in the lungs after chronic exposure to e-cigarette aerosols [133]. Li and colleagues showed an increase in expression of TNF-α in the liver after e-cigarette exposure [191].

4.6 Conclusion

ECAC has marked adverse effects on neuronal cells, microglial cells and human brain endothelial cells following direct application. Furthermore, ECAC has indirect adverse effects on neuronal epigenetics through possible microglia-derived inflammatory mediators using an *in vitro* BBB model. Further studies are needed to determine the release of cytokines from the microglial cells that are causing epigenetic changes in the neuronal cells and whether these inflammatory responses are reflected in an *in vivo* model of e-cigarette exposure.

Chapter 5 – Summary and conclusions

5.1 Concluding remarks

Electronic cigarettes (e-cigarettes) are battery-powered devices that convert an oily-flavoured liquid into an aerosol. E-liquid cartridges contain propylene glycol, vegetable glycerin, flavouring and may contain varying concentrations of nicotine. E-cigarettes have attracted a number of vulnerable groups such as young people and pregnant women. E-cigarettes has been perceived as safer alternative to smoking tobacco cigarettes, although there are limited studies to prove that it is actually safe. This dissertation investigates the effects of e-cigarette exposure in an animal pregnancy model and in cell culture.

Chapter 2 showed e-cigarette aerosol exposure (with and without nicotine) had significant effects on working memory, anxiety and hyperactivity in a mouse model. PCR arrays revealed eight epigenetic genes that were significantly changed right after birth, and seven epigenetic genes that were significantly changed at adulthood. Validation with RT-qPCR showed varying epigenetic changes associated with neuronal development, maturation and cell division in the offspring brain from mothers exposed to e-cigarette aerosols with and without nicotine. No changes in neuronal counts were observed in the dorsal hippocampus at CA1, CA2 and CA3 and the lateral amygdala nucleus. This chapter concludes that maternal e-cigarette exposure affects offspring behaviour and causes changes to epigenetic gene expression that can persist in adulthood.

Chapter 3 demonstrated that following a switch during pregnancy to e-cigarette aerosol exposure from tobacco cigarette smoke exposure showed deficits in short-term memory. A decrease in anxiety in both the Switch and continuous smoking group were also found, however, this finding is accompanied with an indication that both groups are hyperactive. Epigenetic gene changes showed that the continuous smoking group had a marked effect on epigenetic gene expression in the offspring compared to the Sham group. The Switch group only showed marked epigenetic

changes in histone phosphorylation which is important in mitotic division. Neuronal cell counts only showed a significant decrease in cell counts in the continuous smoking group compared to the Sham group, while the Switch group only showed minimal changes compared to Sham group. This study concludes that although switching to e-cigarettes during pregnancy reduce changes that were observed in the continuous smoking group, e-cigarette exposure did not completely change offspring behaviour and epigenetics compared to continuous cigarette smoke exposure.

Chapter 4 investigated the effects of e-cigarette condensate exposure in monoculture and co-culture models comprising of neuronal cells, microglial cells and human brain microvascular endothelial cells. In monoculture, e-cigarette condensate exposure showed a significant decrease in cell viability, an increase in ROS release and an increase in mitochondrial depolarisation in a dose-dependent manner, in all three cell lines. ECAC exposure with and without nicotine on the HBEC membrane, showed a significant decrease in TEER measurement. This is accompanied by an increase in FITC-Dextran release in the bottom chamber indicating endothelial membrane leakiness. HBEC co-cultured with diff-SHSY5Y cells or BV2 cells both showed a decrease in TEER measurement after treatment with e-cigarette condensate with and without nicotine. Epigenetic and inflammatory gene expression in diff-SHSY5Y cells from co-culture showed no significant changes. However, inflammatory gene expression of BV2 cells from co-culture showed significant changes to pro-inflammatory and anti-inflammatory genes. Conditioned media from the BV2 cells co-culture was applied to diff-SHSY5Y cells and showed a significant change in epigenetic gene expression of Dnmt3a and Kdm5c. Finally, to determine whether inflammatory genes were also being changed *in vivo*, inflammatory gene expression was investigated in offspring brain tissue after exposure to e-cigarette aerosols with and without nicotine *in utero* and showed an increase in inflammatory gene expression of IL-6, IL-10, TNF-α. This was consistent with the changes seen in the BV2 cells in co-culture. This study concludes that exposure to ECAC in monoculture of cell lines from the CNS showed reduce cell viability, increase ROS production and an increase mitochondrial membrane depolarisation. In co-culture, there was a decrease in BBB integrity, an increase in BBB permeability and various epigenetic

and inflammatory gene changes were observed in diff-SHSY5Y cells and BV2 cells. Moreover, the inflammatory gene changes observed in neuronal cells, microglial cells and human brain microvascular endothelial cells *in vitro* was consistent inflammatory gene changes that were observed in murine offspring brain *in vivo.*

In conclusion, these studies highlight the adverse neurological effects in offspring following e-cigarette use during pregnancy.

6. Bibliography

1. *Vaporizers, E-Cigarettes, and other Electronic Nicotine Delivery Systems (ENDS)*. Products, Ingredients and Components 2019 [cited 2019 15/07/19]; Available from: https://www.fda.gov/tobacco-products/products-ingredients-components/vaporizers-e-cigarettes-and-other-electronic-nicotine-delivery-systems-ends.
2. *Public Health Consequences of E-Cigarettes*, in *National Academies of Sciences, Engineering, and Medicine*, D.T.N.A.P. Washington, Editor. 2018.
3. Barrington-Trimis, J.L. and A.M. Leventhal, *Adolescents' Use of "Pod Mod" E-Cigarettes — Urgent Concerns.* New England Journal of Medicine, 2018. **379**(12): p. 1099-1102.
4. Hsu, G., J.Y. Sun, and S.H. Zhu, *Evolution of Electronic Cigarette Brands From 2013-2014 to 2016-2017: Analysis of Brand Websites.* J Med Internet Res, 2018. **20**(3): p. e80.
5. *E-cigarettes and vapor products*. Public Health - Seattle & King County 2019 21st May 2019 [cited 2019 10/07/19]; Available from: https://www.kingcounty.gov/depts/health/tobacco/data/e-cigarettes.aspx.
6. *WHO Report on the Global Tobacco Epidemic, 2019*. 2019, World Health Organization: Switzerland.
7. *Smoking and Tobacco Control*. Policy and advocacy 2006; Available from: http://www.cancer.org.au/policy-and-advocacy/position-statements/smoking-and-tobacco-control/.
8. U.S. Department of Health and Human Services, C.f.D.C.a.P., National Center for Chronic Disease Prevention and O.o.S.a.H. and Health Promotion. *The Health Consequences of Smoking—50 Years of Progress. A Report of the Surgeon General*. 2014 [cited 2016.
9. *How Tobacco Smoke Causes Disease: The Biology and Behavioral Basis for Smoking-Attributable Disease: A Report of the Surgeon General*. Pulmonary Diseases, ed. C.f.D.C.a.P.U.N.C.f.C.D.P.a.H.P.U.O.o.S.a.H. (US). 2010, Atlanta (GA): Centers for Disease Control and Prevention (US).
10. Forey, B.A., A.J. Thornton, and P.N. Lee, *Systematic review with meta-analysis of the epidemiological evidence relating smoking to COPD, chronic bronchitis and emphysema.* BMC pulmonary medicine, 2011. **11**: p. 36-36.
11. Orth, S.R. and S.I. Hallan, *Smoking: A Risk Factor for Progression of Chronic Kidney Disease and for Cardiovascular Morbidity and Mortality in Renal Patients—Absence of Evidence or Evidence of Absence?* Clinical Journal of the American Society of Nephrology, 2008. **3**(1): p. 226.
12. Roy A, R.I., Jabbour S, Prabhakaran, D. *Tobacco and Cardiovascular Disease: A Summary of Evidence.* Cardiovascular, Respiratory, and Related Disorders 2017; Available from: https://www.ncbi.nlm.nih.gov/books/NBK525170/?report=classic.
13. Xie, A., B. Croce, and D.H. Tian, *Smoking and lung cancer.* Annals of cardiothoracic surgery, 2014. **3**(2): p. 221-221.
14. Yacoub, R., et al., *Association between smoking and chronic kidney disease: a case control study.* BMC public health, 2010. **10**: p. 731-731.
15. Furrukh, M., *Tobacco Smoking and Lung Cancer: Perception-changing facts.* Sultan Qaboos University Medical Journal, 2013. **13**(3): p. 345-358.
16. Hofhuis, W., J.C. de Jongste, and P. Merkus, *Adverse health effects of prenatal and postnatal tobacco smoke exposure on children.* Archives of Disease in Childhood, 2003. **88**(12): p. 1086-1090.

17. Messner, B. and D. Bernhard, *Smoking and cardiovascular disease: mechanisms of endothelial dysfunction and early atherogenesis.* Arterioscler Thromb Vasc Biol, 2014. **34**(3): p. 509-15.
18. *Australian Health Survey: First Results, 2011-12*. General Health, Health Risks, Tobacco Smoking 2012 [cited 2018 06/06/18]; Available from: http://www.abs.gov.au/ausstats/abs@.nsf/Lookup/73963BA1EA6D6221CA257AA30014BE3E?opendocument.
19. Stevenson, R., *The quantification of drug-caused mortality and morbidity in Australia, 1998*, in *Drug statistics*. 2001, AIHW: Canberra.
20. *National Health Survey: First Results, 2017-18* Smoking 2019 [cited 2020 02/02/20]; Available from: https://www.abs.gov.au/ausstats/abs@.nsf/Lookup/by%20Subject/4364.0.55.001~2017-18~Main%20Features~Smoking~85.
21. Services, U.D.o.H.a.H., *Reducing the Health Consequences of Smoking: 25 Years of Progress. A Report of the US Surgeon General*. 1989, US Department of Health and Human Services, Office on Smoking and Health, Centers for Disease Control, Center for Chronic Disease Prevention and Health Promotion: Maryland.
22. *How Tobacco Smoke Causes Disease: The Biology and Behavioral Basis for Smoking-Attributable Disease: A Report of the Surgeon General*. Chemistry and Toxicology of Cigarette Smoke and Biomarkers of Exposure and Harm 2010 [cited 2018 04/02/18]; Available from: http://www.ncbi.nlm.nih.gov/books/NBK53014/?report=classic.
23. Zhu, S.H., et al., *Four hundred and sixty brands of e-cigarettes and counting: implications for product regulation.* Tob Control, 2014. **23 Suppl 3**: p. iii3-9.
24. Marco, E. and J.O. Grimalt, *A rapid method for the chromatographic analysis of volatile organic compounds in exhaled breath of tobacco cigarette and electronic cigarette smokers.* J Chromatogr A, 2015. **1410**: p. 51-9.
25. Goniewicz, M.L., et al., *Levels of selected carcinogens and toxicants in vapour from electronic cigarettes.* Tob Control, 2014. **23**(2): p. 133-9.
26. Schober, W., et al., *Use of electronic cigarettes (e-cigarettes) impairs indoor air quality and increases FeNO levels of e-cigarette consumers.* Int J Hyg Environ Health, 2014. **217**(6): p. 628-37.
27. Hutzler, C., et al., *Chemical hazards present in liquids and vapors of electronic cigarettes.* Arch Toxicol, 2014. **88**(7): p. 1295-308.
28. Gaur, S. and R. Agnihotri, *Health Effects of Trace Metals in Electronic Cigarette Aerosols-a Systematic Review.* Biol Trace Elem Res, 2019. **188**(2): p. 295-315.
29. *What Is the Best PG/VG Ratio for Me?* 2018 [cited 2019 10/07/19]; Available from: https://www.fivepawns.com/blog/post/what-is-the-best-pg-vg-ratio-for-me.
30. Korzun, T., et al., *E-Cigarette Airflow Rate Modulates Toxicant Profiles and Can Lead to Concerning Levels of Solvent Consumption.* ACS omega, 2018. **3**(1): p. 30-36.
31. Baassiri, M., et al., *Clouds and "throat hit": Effects of liquid composition on nicotine emissions and physical characteristics of electronic cigarette aerosols.* Aerosol Science and Technology, 2017. **51**(11): p. 1231-1239.
32. Robinson, R.J., et al., *A framework to investigate the impact of topography and product characteristics on electronic cigarette emissions.* PLOS ONE, 2018. **13**(11): p. e0206341.
33. Lee, Y.O., et al., *Examining Daily Electronic Cigarette Puff Topography Among Established and Nonestablished Cigarette Smokers in their Natural Environment.* Nicotine Tob Res, 2018. **20**(10): p. 1283-1288.
34. Robinson, R.J., et al., *Effect of e-liquid flavor on electronic cigarette topography and consumption behavior in a 2-week natural environment switching study.* PLOS ONE, 2018. **13**(5): p. e0196640.

35. Tonstad, S., et al., *Symptoms of nicotine toxicity in subjects achieving high cotinine levels during nicotine replacement therapy.* Nicotine Tob Res, 2014. **16**(9): p. 1266-71.
36. Mishra, A., et al., *Harmful effects of nicotine.* Indian Journal of Medical and Paediatric Oncology : Official Journal of Indian Society of Medical & Paediatric Oncology, 2015. **36**(1): p. 24-31.
37. Buckner, J.D., et al., *Socially anxious smokers experience greater negative affect and withdrawal during self-quit attempts.* Addict Behav, 2016. **55**: p. 46-9.
38. Baiardini, I., et al., *Smoking cessation, anxiety, mood and quality of life: reassuring evidences.* Minerva Med, 2014. **105**(5 Suppl 1): p. 15-21.
39. Stead, L.F., et al., *Nicotine replacement therapy for smoking cessation.* Cochrane Database Syst Rev, 2012. **11**: p. Cd000146.
40. Du, D., et al., *Comparison of nicotine oral soluble film and nicotine lozenge on efficacy in relief of smoking cue-provoked acute craving after a single dose of treatment in low dependence smokers.* Psychopharmacology (Berl), 2014. **231**(22): p. 4383-91.
41. Stead, L.F. and T. Lancaster, *Combined pharmacotherapy and behavioural interventions for smoking cessation.* Cochrane Database Syst Rev, 2012. **10**: p. Cd008286.
42. Mills, E.J., et al., *Adverse events associated with nicotine replacement therapy (NRT) for smoking cessation. A systematic review and meta-analysis of one hundred and twenty studies involving 177,390 individuals.* Tobacco induced diseases, 2010. **8**(1): p. 8-8.
43. Brady, B.R., et al., *Electronic cigarette use and tobacco cessation in a state-based quitline.* Journal of Smoking Cessation, 2019. **14**(3): p. 176-185.
44. Weaver, S.R., et al., *Are electronic nicotine delivery systems helping cigarette smokers quit? Evidence from a prospective cohort study of U.S. adult smokers, 2015–2016.* PLOS ONE, 2018. **13**(7): p. e0198047.
45. Farsalinos, K. and R. Niaura, *E-cigarettes and smoking cessation in the United States according to frequency of e-cigarette use and quitting duration: analysis of the 2016 and 2017 National Health Interview Surveys.* Nicotine Tob Res, 2019.
46. Chiang, S.C., et al., *E-cigarettes and smoking cessation: a prospective study of a national sample of pregnant smokers.* BMC Public Health, 2019. **19**(1): p. 964.
47. Carroll Chapman, S.L. and L.T. Wu, *E-cigarette prevalence and correlates of use among adolescents versus adults: a review and comparison.* J Psychiatr Res, 2014. **54**: p. 43-54.
48. Chen, H., et al., *Maternal E-Cigarette Exposure in Mice Alters DNA Methylation and Lung Cytokine Expression in Offspring.* Am J Respir Cell Mol Biol, 2018. **58**(3): p. 366-377.
49. Chen, H., et al., *Modulation of neural regulators of energy homeostasis, and of inflammation, in the pups of mice exposed to e-cigarettes.* Neuroscience Letters, 2018. **684**: p. 61-66.
50. Lauterstein, D.E., et al., *Frontal Cortex Transcriptome Analysis of Mice Exposed to Electronic Cigarettes During Early Life Stages.* International Journal of Environmental Research and Public Health, 2016. **13**(4): p. 417.
51. Zelikoff, J.T., et al., *Microglia Activation and Gene Expression Alteration of Neurotrophins in the Hippocampus Following Early-Life Exposure to E-Cigarette Aerosols in a Murine Model.* Toxicol Sci, 2018. **162**(1): p. 276-286.
52. Cardenia, V., et al., *The effect of electronic-cigarettes aerosol on rat brain lipid profile.* Biochimie, 2018. **153**: p. 99-108.
53. Qasim, H., et al., *Impact of Electronic Cigarettes on the Cardiovascular System.* Journal of the American Heart Association, 2017. **6**(9): p. e006353.
54. El Golli, N., et al., *Impact of e-cigarette refill liquid with or without nicotine on liver function in adult rats.* Toxicol Mech Methods, 2016. **26**(6): p. 419-26.
55. Hasan, K.M., et al., *E-cigarettes and Western Diet: Important Metabolic Risk Factors for Hepatic Diseases.* Hepatology, 2019.

56. Jamal, A., et al., *Tobacco Use Among Middle and High School Students - United States, 2011-2016.* MMWR Morb Mortal Wkly Rep, 2017. **66**(23): p. 597-603.
57. Measham, F., K. O'Brien, and G. Turnbull, *"Skittles & Red Bull is my favourite flavour": E-cigarettes, smoking, vaping and the changing landscape of nicotine consumption amongst British teenagers – implications for the normalisation debate.* Drugs: Education, Prevention and Policy, 2016. **23**(3): p. 224-237.
58. Wills, T.A., et al., *How is the effect of adolescent e-cigarette use on smoking onset mediated: A longitudinal analysis.* Psychology of addictive behaviors : journal of the Society of Psychologists in Addictive Behaviors, 2016. **30**(8): p. 876-886.
59. Adkison, S.E., et al., *Electronic nicotine delivery systems: international tobacco control four-country survey.* Am J Prev Med, 2013. **44**(3): p. 207-15.
60. Chan, G., et al., *Correlates of electronic cigarette use in the general population and among smokers in Australia - Findings from a nationally representative survey.* Addict Behav, 2019. **95**: p. 6-10.
61. Twyman, L., et al., *Electronic Cigarettes: Awareness, Recent Use, and Attitudes Within a Sample of Socioeconomically Disadvantaged Australian Smokers.* Nicotine Tob Res, 2016. **18**(5): p. 670-7.
62. Twyman, L., et al., *Electronic cigarette use in New South Wales, Australia: reasons for use, place of purchase and use in enclosed and outdoor places.* Australian and New Zealand Journal of Public Health, 2018. **42**(5): p. 491-496.
63. Kennedy, R.D., et al., *Global approaches to regulating electronic cigarettes.* Tobacco Control, 2017. **26**(4): p. 440.
64. Shapiro, H., *No Fire, No Smoke: The Global State of Tobacco Harm Reduction*, T. Burgess, Goldsmith, R., Stimson, J., Editor. 2018, Knowledge-Action-Change: London.
65. *Electronic cigarettes (e-cigarettes)*. Healthy living [cited 2020 5th March]; Available from: https://healthywa.wa.gov.au/Articles/A_E/Electronic-cigarettes-e-cigarettes.
66. *E-cigarettes*. 2020 [cited 2020 5th March]; Available from: https://www.quit.org.au/resources/policy-advocacy/policy/e-cigarettes/.
67. *Electronic cigarettes*. Tobacco and Smoking 2019 [cited 2020 5th March]; Available from: https://www.health.nsw.gov.au/tobacco/Pages/e-cigarettes.aspx.
68. *Alcohol and other drugs: health professionals*. Health professionals and NGOs 2019; Available from: https://health.nt.gov.au/professionals/alcohol-and-other-drugs-health-professionals/tobacco.
69. *New e-cigarette laws and other changes to tobacco laws in South Australia*. Tobacco laws and businesses 2019 [cited 2020 5th March]; Available from: https://www.sahealth.sa.gov.au/wps/wcm/connect/public+content/sa+health+internet/protecting+public+health/tobacco+laws+and+businesses/new+e-cigarette+laws+and+other+changes+to+tobacco+laws+in+south+australia.
70. *Electronic cigarettes*. Public Health and Well being 2019 [cited 2019 01/01/20]; Available from: https://www.health.qld.gov.au/public-health/topics/atod/tobacco-laws/electronic-cigarettes.
71. *Electronic Cigarettes*. Tobacco Control 2019 [cited 2020 01/01/20]; Available from: https://www.dhhs.tas.gov.au/publichealth/tobacco_control/electronic-cigarettes.
72. *Electronic Cigarettes*. Smoke-free environments 2019 02 JUL 2019 [cited 2020 01/01/20]; Available from: https://www.health.act.gov.au/about-our-health-system/population-health/smoke-free-environments/electronic-cigarettes.
73. *Standard for the Uniform Scheduling of Medicines and Poisons*. 2016 [cited 2016 01/09/16]; Available from: https://www.legislation.gov.au/Details/F2016L00036.
74. Douglas, H., W. Hall, and C. Gartner, *E-cigarettes and the law in Australia.* Australian Family Physician, 2015. **44**: p. 415-418.

75. *Appendix 5: Guidelines on OTC application for specific substances* in *Australia regulatory guideline for over-the-counter medicines.* 2012, Australian Government. Department of Health and Ageing. Therapeutics Goods Administration: Australian Capital Territory.
76. *Deeming Tobacco Products To Be Subject to the Federal Food, Drug, and Cosmetic Act, as Amended by the Family Smoking Prevention and Tobacco Control Act; Restrictions on the Sale and Distribution of Tobacco Products and Required Warning Statements for Tobacco Products* 2016, Food and Drug Administration, Office of the Federal Register, United States Government: United States. p. 28973-29106.
77. *FDA finalizes enforcement policy on unauthorized flavored cartridge-based e-cigarettes that appeal to children, including fruit and mint*. Press Announcements 2020 [cited 2020 15/03/20]; Available from: FDA finalizes enforcement policy on unauthorized flavored cartridge-based e-cigarettes that appeal to children, including fruit and mint.
78. *Enforcement Priorities for Electronic Nicotine Delivery Systems (ENDS) and Other Deemed Products on the Market Without Premarket Authorization*. 2020 [cited 2020 15/03/20]; Available from: https://www.fda.gov/media/133880/download.
79. Rostron, B.L., et al., *Prevalence and Reasons for Use of Flavored Cigars and ENDS among US Youth and Adults: Estimates from Wave 4 of the PATH Study, 2016-2017.* Am J Health Behav, 2020. **44**(1): p. 76-81.
80. *E-cigarettes: regulations for consumer products*. 2016 17/05/19 20/05/19]; Available from: https://www.gov.uk/guidance/e-cigarettes-regulations-for-consumer-products.
81. *Towards a Smokefree Generation - A Tobacco Control Plan for England*, G.a.P.H.P.H.H.c. centre, Editor. 2017, Crown - Department of Health: London.
82. West, R., L. Shahab, and J. Brown, *Estimating the population impact of e-cigarettes on smoking cessation in England.* Addiction, 2016. **111**(6): p. 1118-9.
83. Beard, E., et al., *Association between electronic cigarette use and changes in quit attempts, success of quit attempts, use of smoking cessation pharmacotherapy, and use of stop smoking services in England: time series analysis of population trends.* BMJ, 2016. **354**: p. i4645.
84. McNeill, A., Brose, L.S, Calder, R, Bauld, L, Robson, D., *Evidence review of e-cigarettes and heated tobacco products 2018 - A report commissioned by the Public Health England*. 2018, Public Health England: London.
85. McCarthy, K. *E-cigarette consultation*. 2016 [cited 2018 21/05/18]; Available from: https://www.health.govt.nz/news-media/media-releases/e-cigarette-consultation-opens.
86. Edwards, R., et al., *Realignment of tobacco control services-will it be sufficient to achieve the nation's Smokefree 2025 Goal?* N Z Med J, 2015. **128**(1413): p. 84-7.
87. *https://www.scoop.co.nz/stories/PO1909/S00213/ex-smoker-backlash-builds-against-vaping-flavour-ban.htm*, in *Politics*, S. media, Editor. 2019, Scoop media: New Zealand.
88. Sun, L.H. *Sixth person dies from vaping-related illness*. Health 2019 [cited 2020 15/03/20]; Available from: https://www.washingtonpost.com/health/2019/09/10/sixth-person-dies-vaping-related-illness/.
89. Hajek, P., et al., *A Randomized Trial of E-Cigarettes versus Nicotine-Replacement Therapy.* N Engl J Med, 2019. **380**(7): p. 629-637.
90. Huang, J., et al., *Vaping versus JUULing: how the extraordinary growth and marketing of JUUL transformed the US retail e-cigarette market.* Tobacco Control, 2019. **28**(2): p. 146.
91. Hilton, S., et al., *E-cigarettes, a safer alternative for teenagers? A UK focus group study of teenagers.* BMJ Open, 2016. **6**(11): p. e013271.
92. Suter, M.A., et al., *Is There Evidence for Potential Harm of Electronic Cigarette Use in Pregnancy?* Birth Defects Res A Clin Mol Teratol, 2014. **103**.

93. Suzuki, K., et al., *Is maternal smoking during early pregnancy a risk factor for all low birth weight infants?* J Epidemiol, 2008. **18**(3): p. 89-96.
94. Russell, C., T. Dickson, and N. McKeganey, *Advice From Former-Smoking E-Cigarette Users to Current Smokers on How to Use E-Cigarettes as Part of an Attempt to Quit Smoking.* Nicotine Tob Res, 2018. **20**(8): p. 977-984.
95. Bullen, C., et al., *Electronic cigarettes for smoking cessation: a randomised controlled trial.* The Lancet, 2013. **382**(9905): p. 1629-1637.
96. Halpern, S.D., et al., *A Pragmatic Trial of E-Cigarettes, Incentives, and Drugs for Smoking Cessation.* N Engl J Med, 2018. **378**(24): p. 2302-2310.
97. Sharma, R., et al., *Motivations and Limitations Associated with Vaping among People with Mental Illness: A Qualitative Analysis of Reddit Discussions.* Int J Environ Res Public Health, 2016. **14**(1).
98. Greenhalgh, E., Bayly, M., Winstanley, M. *Prevalence of smoking - young adults*. Tobacco in Australia: Facts and Issues. 2015 [cited 2018 16/6/18]; Available from: http://www.tobaccoinaustralia.org.au/chapter-1-prevalence/1-4-prevalence-of-smoking-young-adults.
99. Bao, W., et al., *Changes in Electronic Cigarette Use Among Adults in the United States, 2014-2016 Trends in e-Cigarette Use Among Adults in the United States, 2014-2016Letters.* JAMA, 2018. **319**(19): p. 2039-2041.
100. Wang, T.W., Asman, K., Gentzke, A.S., Cullen, K.A., Holder-Hayes, E., Reyes-Guzman, C., Jamal, A., Neff, L., King, B.A. *Tobacoo Product Use Among Adults - United Stated, 2017*. Morbidity and Mortality Weekly Report 2018.
101. Goniewicz, M.L., et al., *Comparison of Nicotine and Toxicant Exposure in Users of Electronic Cigarettes and Combustible Cigarettes.* JAMA Network Open, 2018. **1**(8): p. e185937-e185937.
102. Caraballo, R.S., et al., *Quit Methods Used by US Adult Cigarette Smokers, 2014-2016.* Preventing Chronic Disease, 2017. **14**: p. E32.
103. Windsor-Shellard, B., Pullin, L., Horton, M., *Adult smoking habits in the UK: 2017*, in *Statistical bulletin*, O.f.N. Statistics, Editor. 2018, Public Health England: England.
104. Fuller, E., Hawkins, V., *Smoking, drinking and drug use among young people in England in 2014*, E. Fuller, Editor. 2014, Health and Social Care Information Centre: London.
105. *Use of electronic cigarettes among children in Great Britain*. 2015, Action on Smoking and Health: London.
106. Bunnell, R.E., et al., *Intentions to smoke cigarettes among never-smoking US middle and high school electronic cigarette users: National Youth Tobacco Survey, 2011-2013.* Nicotine Tob Res, 2015. **17**(2): p. 228-35.
107. Choi, K. and J. Forster, *Characteristics associated with awareness, perceptions, and use of electronic nicotine delivery systems among young US Midwestern adults.* Am J Public Health, 2013. **103**(3): p. 556-61.
108. Jongenelis, M.I., et al., *Support for e-cigarette regulations among Australian young adults.* BMC public health, 2019. **19**(1): p. 67-67.
109. Fillon, M., *Electronic Cigarettes May Lead to Nicotine Addiction.* Journal of the National Cancer Institute, 2015. **107**(3).
110. Gentzke, A.S., et al., *Vital Signs: Tobacco Product Use Among Middle and High School Students - United States, 2011-2018.* MMWR. Morbidity and mortality weekly report, 2019. **68**(6): p. 157-164.
111. Primack, B.A., et al., *Progression to Traditional Cigarette Smoking After Electronic Cigarette Use Among US Adolescents and Young Adults.* JAMA pediatrics, 2015. **169**(11): p. 1018-1023.
112. Chaffee, B.W., S.L. Watkins, and S.A. Glantz, *Electronic Cigarette Use and Progression From Experimentation to Established Smoking.* Pediatrics, 2018. **141**(4): p. e20173594.

113. East, K., et al., *The Association Between Smoking and Electronic Cigarette Use in a Cohort of Young People.* The Journal of adolescent health : official publication of the Society for Adolescent Medicine, 2018. **62**(5): p. 539-547.
114. England, L.J., et al., *Perceptions of emerging tobacco products and nicotine replacement therapy among pregnant women and women planning a pregnancy.* Preventive Medicine Reports, 2016. **4**: p. 481-485.
115. Bowker, K., et al., *Views on and experiences of electronic cigarettes: a qualitative study of women who are pregnant or have recently given birth.* BMC Pregnancy and Childbirth, 2018. **18**(1): p. 233.
116. Cooper, S., et al., *Attitudes to E-Cigarettes and Cessation Support for Pregnant Women from English Stop Smoking Services: A Mixed Methods Study.* International Journal of Environmental Research and Public Health, 2019. **16**(1).
117. Mark, K.S., et al., *Knowledge, Attitudes, and Practice of Electronic Cigarette Use Among Pregnant Women.* Journal of Addiction Medicine, 2015. **9**.
118. Ashford, K., et al., *e-Cigarette Use and Perceived Harm Among Women of Childbearing Age Who Reported Tobacco Use During the Past Year.* Nurs Res, 2016. **65**(5): p. 408-14.
119. Whittington, J.R., et al., *The Use of Electronic Cigarettes in Pregnancy: A Review of the Literature.* Obstet Gynecol Surv, 2018. **73**(9): p. 544-549.
120. Kapaya, M., D'Angelo, D.V., Tong, V.T., England, L., Ruffo, N., Cox, S., Warner, L., Bombard, J., Guthrie, T., Lampkins, A., King, B.A., *Use of Electronic Vapor Products Before, During, and After Pregnancy Among Women with a Recent Live Birth — Oklahoma and Texas.* Morbidity and Mortality Weekly Report 2019. **68**(8): p. 189-194.
121. England, L.J., et al., *Screening practices and attitudes of obstetricians-gynecologists toward new and emerging tobacco products.* American journal of obstetrics and gynecology, 2014. **211**(6): p. 695.e1-695.e6957.
122. Smets, J., et al., *When Less is More: Vaping Low-Nicotine vs. High-Nicotine E-Liquid is Compensated by Increased Wattage and Higher Liquid Consumption.* International journal of environmental research and public health, 2019. **16**(5): p. 723.
123. Jahnke, G.D., et al., *Center for the evaluation of risks to human reproduction--the first five years.* Birth Defects Res B Dev Reprod Toxicol, 2005. **74**(1): p. 1-8.
124. Heck, J.D., et al., *Toxicologic evaluation of humectants added to cigarette tobacco: 13-week smoke inhalation study of glycerineand propylene glycol in Fischer 344 rats.* Inhal Toxicol, 2002. **14**(11): p. 1135-52.
125. *CFR - Code of Federal Regulations Title 21*. Direct Food Substances affirmed as generally recgnised as safe 2015 [cited 2016 31/07/18]; Available from: https://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/cfrsearch.cfm?fr=184.1666.
126. Farsalinos, K.E., et al., *Comparison of the cytotoxic potential of cigarette smoke and electronic cigarette vapour extract on cultured myocardial cells.* Int J Environ Res Public Health, 2013. **10**(10): p. 5146-62.
127. Bahl, V., et al., *Comparison of electronic cigarette refill fluid cytotoxicity using embryonic and adult models.* Reprod Toxicol, 2012. **34**(4): p. 529-37.
128. Ignacio, G.-S., et al., *In Vitro Systems Toxicology Assessment of Nonflavored e-Cigarette Liquids in Primary Lung Epithelial Cells.* Applied In Vitro Toxicology, 2017. **3**(1): p. 41-55.
129. Kennedy, A.E., et al., *E-cigarette aerosol exposure can cause craniofacial defects in Xenopus laevis embryos and mammalian neural crest cells.* PLOS ONE, 2017. **12**(9): p. e0185729.
130. Crotty Alexander, L.E., et al., *Chronic inhalation of e-cigarette vapor containing nicotine disrupts airway barrier function and induces systemic inflammation and multiorgan fibrosis in mice.* Am J Physiol Regul Integr Comp Physiol, 2018. **314**(6): p. R834-r847.
131. Garcia-Arcos, I., et al., *Chronic electronic cigarette exposure in mice induces features of COPD in a nicotine-dependent manner.* Thorax, 2016.

132. Lechasseur, A., et al., *Exposure to electronic cigarette vapors affects pulmonary and systemic expression of circadian molecular clock genes.* Physiological Reports, 2017. **5**(19): p. e13440.
133. Lim, H.B. and S.H. Kim, *Inhallation of e-Cigarette Cartridge Solution Aggravates Allergen-induced Airway Inflammation and Hyper-responsiveness in Mice.* Toxicological Research, 2014. **30**(1): p. 13-18.
134. McGrath-Morrow, S.A., et al., *The Effects of Electronic Cigarette Emissions on Systemic Cotinine Levels, Weight and Postnatal Lung Growth in Neonatal Mice.* PLoS ONE, 2015. **10**(2): p. e0118344.
135. Shi, H., et al., *The Effect of Electronic-Cigarette Vaping on Cardiac Function and Angiogenesis in Mice.* Scientific Reports, 2019. **9**(1): p. 4085.
136. Smith, D., et al., *Adult Behavior in Male Mice Exposed to E-Cigarette Nicotine Vapors during Late Prenatal and Early Postnatal Life.* PLoS ONE, 2015. **10**(9): p. e0137953.
137. Hua, M., M. Alfi, and P. Talbot, *Health-Related Effects Reported by Electronic Cigarette Users in Online Forums.* Journal of Medical Internet Research, 2013. **15**(4): p. e59.
138. Wieslander, G., D. Norbäck, and T. Lindgren, *Experimental exposure to propylene glycol mist in aviation emergency training: acute ocular and respiratory effects.* Occupational and Environmental Medicine, 2001. **58**(10): p. 649-655.
139. McAuley, T.R., et al., *Comparison of the effects of e-cigarette vapor and cigarette smoke on indoor air quality.* Inhal Toxicol, 2012. **24**(12): p. 850-7.
140. Panitz, D., H. Swamy, and K. Nehrke, *A C. elegans model of electronic cigarette use: Physiological effects of e-liquids in nematodes.* BMC Pharmacol Toxicol, 2015. **16**: p. 32.
141. Robertson, O.H., C.G. Loosli, and et al., *Tests for the chronic toxicity of propylene glycol and triethylene glycol on monkeys and rats by vapor inhalation and oral administration.* J Pharmacol Exp Ther, 1947. **91**(1): p. 52-76.
142. Phillips, B., et al., *Toxicity of the main electronic cigarette components, propylene glycol, glycerin, and nicotine, in Sprague-Dawley rats in a 90-day OECD inhalation study complemented by molecular endpoints.* Food Chem Toxicol, 2017. **109**(Pt 1): p. 315-332.
143. Lau, K., et al., *Propylene glycol produces excessive apoptosis in the developing mouse brain, alone and in combination with phenobarbital.* Pediatr Res, 2012. **71**(1): p. 54-62.
144. Brent, J., *Current management of ethylene glycol poisoning.* Drugs, 2001. **61**(7): p. 979-88.
145. Cervellati, F., et al., *Comparative effects between electronic and cigarette smoke in human keratinocytes and epithelial lung cells.* Toxicol In Vitro, 2014. **28**(5): p. 999-1005.
146. Lerner, C.A., et al., *Electronic cigarette aerosols and copper nanoparticles induce mitochondrial stress and promote DNA fragmentation in lung fibroblasts.* Biochem Biophys Res Commun, 2016. **477**(4): p. 620-5.
147. Lerner, C.A., et al., *Vapors Produced by Electronic Cigarettes and E-Juices with Flavorings Induce Toxicity, Oxidative Stress, and Inflammatory Response in Lung Epithelial Cells and in Mouse Lung.* PLoS ONE, 2015. **10**(2): p. e0116732.
148. Kosmider, L., et al., *Carbonyl compounds in electronic cigarette vapors: effects of nicotine solvent and battery output voltage.* Nicotine Tob Res, 2014. **16**(10): p. 1319-26.
149. Trehy, M.L., et al., *Analysis of electronic cigarette cartridges, refill solutions, and smoke for nicotine and nicotine related impurities.* Journal of Liquid Chromatography & Related Technologies, 2011. **34**(14): p. 1442-1458.
150. Westenberger, B.J. *Evaluation of e-Cigarettes*. 2009 [cited 2018 01/02/18]; Available from: http://www.fda.gov/downloads/drugs/Scienceresearch/UCM173250.pdf.
151. Kubica, P., et al., *"Dilute & shoot" approach for rapid determination of trace amounts of nicotine in zero-level e-liquids by reversed phase liquid chromatography and hydrophilic interactions liquid chromatography coupled with tandem mass spectrometry-electrospray ionization.* J Chromatogr A, 2013. **1289**: p. 13-8.

152. Hadwiger, M.E., et al., *Identification of amino-tadalafil and rimonabant in electronic cigarette products using high pressure liquid chromatography with diode array and tandem mass spectrometric detection.* J Chromatogr A, 2010. **1217**(48): p. 7547-55.
153. Davis, B., et al., *Nicotine Concentrations in Electronic Cigarette Refill and Do-It-Yourself Fluids.* Nicotine & Tobacco Research, 2014.
154. Jackson, R., M. Huskey, and S. Brown, *Labelling accuracy in low nicotine e-cigarette liquids from a sampling of US manufacturers.* Int J Pharm Pract, 2019.
155. Chun, L.F., et al., *Pulmonary toxicity of e-cigarettes.* Am J Physiol Lung Cell Mol Physiol, 2017. **313**(2): p. L193-l206.
156. Chivers, E., et al., *Nicotine and other potentially harmful compounds in "nicotine-free" e-cigarette liquids in Australia.* Med J Aust, 2019. **210**(3): p. 127-128.
157. Maina, G., et al., *Transdermal nicotine absorption handling e-cigarette refill liquids.* Regul Toxicol Pharmacol, 2016. **74**: p. 31-3.
158. Chen, B.C., et al., *Death following intentional ingestion of e-liquid.* Clin Toxicol (Phila), 2015. **53**(9): p. 914-6.
159. Cervellin, G., Luci, M., Bellini, C., Lippi, G., *Bad news about an old poison. A case of nicotine poisoning due to both ingestion and injection of the content of an electronic cigarette refill.* Emergency Care Journal, 2013. **9**(18): p. 53-54.
160. Cameron, J.M., et al., *Variable and potentially fatal amounts of nicotine in e-cigarette nicotine solutions.* Tobacco Control, 2013.
161. Wylie, C., Heffernan, A., Brown, JA., Cairns, R., Lynch, A., Robinson, J. *Exposures to e-cigarettes and their refills: calls to Australian Poisons Information Centres, 2009–2016*. 2019 [cited 2020 5th March]; Available from: https://www.mja.com.au/journal/2019/210/3/exposures-e-cigarettes-and-their-refills-calls-australian-poisons-information.
162. Cox, M. *Victorian baby dies after being poisoned by nicotine from e-cigarette*. 2019 [cited 2020 5th March]; Available from: https://www.kidspot.com.au/news/victorian-baby-dies-after-being-poisoned-by-nicotine-from-ecigarette/news-story/5ebe0240ebb1fb8ee2383c15f65e8678.
163. Christensen L.B., V.T.V., Bang J. , *Three cases of attempted suicide by ingestion of nicotine liquid used in e-cigarettes.* Clinical Toxicology, 2013. **51**(290).
164. Schipper, E.M., et al., *A new challenge: suicide attempt using nicotine fillings for electronic cigarettes.* British Journal of Clinical Pharmacology, 2014. **78**(6): p. 1469-1471.
165. *Personal importation scheme*. Consumers - Can I import it? 2015 [cited 2019 10/07/19]; Available from: https://www.tga.gov.au/node/3988.
166. Buettner-Schmidt, K., D.R. Miller, and N. Balasubramanian, *Electronic Cigarette Refill Liquids: Child-Resistant Packaging, Nicotine Content, and Sales to Minors.* Journal of pediatric nursing, 2016. **31**(4): p. 373-379.
167. Lakhan, S.E. and A. Kirchgessner, *Anti-inflammatory effects of nicotine in obesity and ulcerative colitis.* Journal of Translational Medicine, 2011. **9**: p. 129-129.
168. Kalra, R., et al., *Immunosuppressive and Anti-Inflammatory Effects of Nicotine Administered by Patch in an Animal Model.* Clinical and Diagnostic Laboratory Immunology, 2004. **11**(3): p. 563-568.
169. White, P.C., et al., *Cigarette smoke modifies neutrophil chemotaxis, neutrophil extracellular trap formation and inflammatory response-related gene expression.* J Periodontal Res, 2018. **53**(4): p. 525-535.
170. Behar, R.Z., et al., *Identification of toxicants in cinnamon-flavored electronic cigarette refill fluids.* Toxicology in Vitro, 2014. **28**(2): p. 198-208.
171. Putzhammer, R., et al., *Vapours of US and EU Market Leader Electronic Cigarette Brands and Liquids Are Cytotoxic for Human Vascular Endothelial Cells.* PLoS ONE, 2016. **11**(6): p. e0157337.

172. *Section 907 of the Federal Food, Drug, and Cosmetic Act - Tobacco Product Standards*. 2018 [cited 2019 01/02/19]; Available from: https://www.fda.gov/tobacco-products/rules-regulations-and-guidance/section-907-federal-food-drug-and-cosmetic-act-tobacco-product-standards#Menthol_Cigarettes.
173. Allen, J.G., et al., *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.* Environ Health Perspect, 2015.
174. Richtel, M., *E-cigarette makers are in an arms race for exotic vapor flavors*, in *New York Times*. 2014: New York.
175. Vasiljevic, M., D.C. Petrescu, and T.M. Marteau, *Impact of advertisements promoting candy-like flavoured e-cigarettes on appeal of tobacco smoking among children: an experimental study.* Tobacco Control, 2016.
176. Muthumalage, T., et al., *Inflammatory and Oxidative Responses Induced by Exposure to Commonly Used e-Cigarette Flavoring Chemicals and Flavored e-Liquids without Nicotine.* Frontiers in Physiology, 2018. **8**(1130).
177. van Rooy, F.G., et al., *Bronchiolitis obliterans syndrome in chemical workers producing diacetyl for food flavorings.* Am J Respir Crit Care Med, 2007. **176**(5): p. 498-504.
178. Clapp, P.W., et al., *Flavored e-cigarette liquids and cinnamaldehyde impair respiratory innate immune cell function.* American Journal of Physiology-Lung Cellular and Molecular Physiology, 2017. **313**(2): p. L278-L292.
179. Hua, M., et al., *Identification of Cytotoxic Flavor Chemicals in Top-Selling Electronic Cigarette Refill Fluids.* Scientific reports, 2019. **9**(1): p. 2782-2782.
180. Aug, A., et al., *E-Cigarette Affects the Metabolome of Primary Normal Human Bronchial Epithelial Cells.* PLoS ONE, 2015. **10**(11): p. e0142053.
181. Wu, Q., et al., *Electronic Cigarette Liquid Increases Inflammation and Virus Infection in Primary Human Airway Epithelial Cells.* PLoS ONE, 2014. **9**(9): p. e108342.
182. Sherwood, C.L. and S. Boitano, *Airway epithelial cell exposure to distinct e-cigarette liquid flavorings reveals toxicity thresholds and activation of CFTR by the chocolate flavoring 2,5-dimethypyrazine.* Respiratory Research, 2016. **17**: p. 57.
183. Sussan, T.E., et al., *Exposure to Electronic Cigarettes Impairs Pulmonary Anti-Bacterial and Anti-Viral Defenses in a Mouse Model.* PLoS ONE, 2015. **10**(2): p. e0116861.
184. Larcombe, A.N., et al., *The effects of electronic cigarette aerosol exposure on inflammation and lung function in mice.* American Journal of Physiology-Lung Cellular and Molecular Physiology, 2017. **313**(1): p. L67-L79.
185. Hwang, J.H., et al., *Electronic cigarette inhalation alters innate immunity and airway cytokines while increasing the virulence of colonizing bacteria.* J Mol Med (Berl), 2016.
186. Olfert, I.M., et al., *Chronic exposure to electronic cigarettes results in impaired cardiovascular function in mice.* J Appl Physiol (1985), 2018. **124**(3): p. 573-582.
187. Palpant, N.J., et al., *Cardiac Development in Zebrafish and Human Embryonic Stem Cells Is Inhibited by Exposure to Tobacco Cigarettes and E-Cigarettes.* PLoS ONE, 2015. **10**(5): p. e0126259.
188. Ponzoni, L., et al., *Different physiological and behavioural effects of e-cigarette vapour and cigarette smoke in mice.* Eur Neuropsychopharmacol, 2015. **25**(10): p. 1775-86.
189. Drummond, C., L.E.C. Alexander, and J. Tian, *Chronic Electronic Cigarette Vapor Inhalation Induces Renal Injury and Functional Decline in Female Mice.* The FASEB Journal, 2017. **31**(1_supplement): p. 698.1-698.1.
190. El Golli, N., et al., *Impact of e-cigarette refill liquid exposure on rat kidney.* Regul Toxicol Pharmacol, 2016. **77**: p. 109-16.
191. Li, G., et al., *A Mitochondrial Specific Antioxidant Reverses Metabolic Dysfunction and Fatty Liver Induced by Maternal Cigarette Smoke in Mice.* Nutrients, 2019. **11**(7): p. 1669.

192. El Golli, N., et al., *Impact of electronic-cigarette refill liquid on rat testis.* Toxicol Mech Methods, 2016. **26**(6): p. 427-34.
193. Miyashita, L., et al., *E-cigarette vapour enhances pneumococcal adherence to airway epithelial cells.* Eur Respir J, 2018. **51**(2).
194. Al-Odat, I., et al., *The Impact of Maternal Cigarette Smoke Exposure in a Rodent Model on Renal Development in the Offspring.* PLoS ONE, 2014. **9**(7): p. e103443.
195. Blacquière, M.J., et al., *Maternal smoking during pregnancy induces airway remodelling in mice offspring.* European Respiratory Journal, 2009. **33**(5): p. 1133.
196. Chan, Y.L., et al., *Maternal Cigarette Smoke Exposure Worsens Neurological Outcomes in Adolescent Offspring with Hypoxic-Ischemic Injury*. Vol. 10. 2017. 306.
197. Chan, Y.L., et al., *Impact of maternal cigarette smoke exposure on brain inflammation and oxidative stress in male mice offspring.* Sci Rep, 2016. **6**: p. 25881.
198. Unachukwu, U., et al., *Maternal smoke exposure decreases mesenchymal proliferation and modulates Rho-GTPase-dependent actin cytoskeletal signaling in fetal lungs.* The FASEB Journal, 2017. **31**(6): p. 2340-2351.
199. Nguyen, T., et al., *Maternal E-Cigarette Exposure Results in Cognitive and Epigenetic Alterations in Offspring in a Mouse Model.* Chem Res Toxicol, 2018. **31**(7): p. 601-611.
200. Li, G., et al., *Impact of maternal e-cigarette vapor exposure on renal health in the offspring.* Ann N Y Acad Sci, 2019.
201. Nguyen, T., et al., *Neurological effects in the offspring after switching from tobacco cigarettes to e-cigarettes during pregnancy in a mouse model.* Toxicol Sci, 2019.
202. Wetendorf, M., et al., *E-Cigarette Exposure Delays Implantation and Causes Reduced Weight Gain in Female Offspring Exposed In Utero.* Journal of the Endocrine Society, 2019. **3**(10): p. 1907-1916.
203. Wehby, G.L., et al., *The Impact of Maternal Smoking during Pregnancy on Early Child Neurodevelopment.* Journal of human capital, 2011. **5**(2): p. 207-254.
204. Anderson, T.M., et al., *Maternal Smoking Before and During Pregnancy and the Risk of Sudden Unexpected Infant Death.* Pediatrics, 2019. **143**(4).
205. Bakker, H. and V.W.V. Jaddoe, *Cardiovascular and metabolic influences of fetal smoke exposure.* European journal of epidemiology, 2011. **26**(10): p. 763-770.
206. Balte, P., et al., *Relationship between birth weight, maternal smoking during pregnancy and childhood and adolescent lung function: A path analysis.* Respir Med, 2016. **121**: p. 13-20.
207. Bernstein, I.M., et al., *Maternal smoking and its association with birth weight.* Obstet Gynecol, 2005. **106**(5 Pt 1): p. 986-91.
208. Horta, B.L., et al., *Maternal smoking during pregnancy and risk factors for cardiovascular disease in adulthood.* Atherosclerosis, 2011. **219**(2): p. 815-20.
209. Kyrklund-Blomberg, N.B., F. Granath, and S. Cnattingius, *Maternal smoking and causes of very preterm birth.* Acta Obstet Gynecol Scand, 2005. **84**(6): p. 572-7.
210. Zhang, K. and X. Wang, *Maternal smoking and increased risk of sudden infant death syndrome: a meta-analysis.* Leg Med (Tokyo), 2013. **15**(3): p. 115-21.
211. Scheffler, S., et al., *Evaluation of E-Cigarette Liquid Vapor and Mainstream Cigarette Smoke after Direct Exposure of Primary Human Bronchial Epithelial Cells.* International Journal of Environmental Research and Public Health, 2015. **12**(4): p. 3915-3925.
212. Schweitzer, K.S., et al., *Endothelial disruptive proinflammatory effects of nicotine and e-cigarette vapor exposures.* Am J Physiol Lung Cell Mol Physiol, 2015. **309**(2): p. L175-87.
213. Sancilio, S., et al., *Cytotoxicity and apoptosis induction by e-cigarette fluids in human gingival fibroblasts.* Clin Oral Investig, 2016. **20**(3): p. 477-83.
214. Higham, A., et al., *Electronic cigarette exposure triggers neutrophil inflammatory responses.* Respiratory Research, 2016. **17**(1): p. 1-11.

215. Erythropel, H.C., et al., *Formation of flavorant-propylene Glycol Adducts With Novel Toxicological Properties in Chemically Unstable E-Cigarette Liquids.* Nicotine Tob Res, 2019. **21**(9): p. 1248-1258.
216. Leslie, L.J., et al., *A comparative study of electronic cigarette vapor extracts on airway-related cell lines in vitro.* Inhalation Toxicology, 2017. **29**(3): p. 126-136.
217. Shen, Y., et al., *Transcriptome sequencing reveals e-cigarette vapor and mainstream-smoke from tobacco cigarettes activate different gene expression profiles in human bronchial epithelial cells.* Scientific Reports, 2016. **6**: p. 23984.
218. Jensen, R.P., et al., *Hidden formaldehyde in e-cigarette aerosols.* N Engl J Med, 2015. **372**(4): p. 392-4.
219. Friedman, M., N. Kozukue, and L.A. Harden, *Cinnamaldehyde content in foods determined by gas chromatography-mass spectrometry.* J Agric Food Chem, 2000. **48**(11): p. 5702-9.
220. Chen, L., et al., *Effects of cigarette smoke extract on human airway smooth muscle cells in COPD.* Eur Respir J, 2014. **44**(3): p. 634-46.
221. Li, H., et al., *Cigarette smoke extract-treated mast cells promote alveolar macrophage infiltration and polarization in experimental chronic obstructive pulmonary disease.* Inhal Toxicol, 2015. **27**(14): p. 822-31.
222. Ljungberg, L.U., et al., *Effects of nicotine, its metabolites and tobacco extracts on human platelet function in vitro.* Toxicol In Vitro, 2013. **27**(2): p. 932-8.
223. Ji, M., et al., *Nicotine Component of Cigarette Smoke Extract (CSE) Decreases the Cytotoxicity of CSE in BEAS-2B Cells Stably Expressing Human Cytochrome P450 2A13.* International journal of environmental research and public health, 2017. **14**(10): p. 1221.
224. Comer, D.M., J.S. Elborn, and M. Ennis, *Inflammatory and cytotoxic effects of acrolein, nicotine, acetylaldehyde and cigarette smoke extract on human nasal epithelial cells.* BMC pulmonary medicine, 2014. **14**: p. 32-32.
225. Thaikoottathil, J.V., et al., *Cigarette smoke extract reduces VEGF in primary human airway epithelial cells.* European Respiratory Journal, 2009. **33**(4): p. 835.
226. Neilson, L., et al., *Development of an in vitro cytotoxicity model for aerosol exposure using 3D reconstructed human airway tissue; application for assessment of e-cigarette aerosol.* Toxicol In Vitro, 2015. **29**(7): p. 1952-62.
227. Helms, H.C., et al., *In vitro models of the blood-brain barrier: An overview of commonly used brain endothelial cell culture models and guidelines for their use.* Journal of cerebral blood flow and metabolism : official journal of the International Society of Cerebral Blood Flow and Metabolism, 2016. **36**(5): p. 862-890.
228. Callahan-Lyon, P., *Electronic cigarettes: human health effects.* Tobacco Control, 2014. **23**(suppl 2): p. ii36-ii40.
229. Caponnetto, P., et al., *Impact of an electronic cigarette on smoking reduction and cessation in schizophrenic smokers: a prospective 12-month pilot study.* Int J Environ Res Public Health, 2013. **10**(2): p. 446-61.
230. Caponnetto, P., et al., *The emerging phenomenon of electronic cigarettes.* Expert Rev Respir Med, 2012. **6**(1): p. 63-74.
231. Polosa, R., et al., *Effectiveness and tolerability of electronic cigarette in real-life: a 24-month prospective observational study.* Intern Emerg Med, 2014. **9**(5): p. 537-46.
232. Vansickel, A.R. and T. Eissenberg, *Electronic Cigarettes: Effective Nicotine Delivery After Acute Administration.* Nicotine & Tobacco Research, 2013. **15**(1): p. 267-270.
233. Vardavas, C.I., et al., *Short-term pulmonary effects of using an electronic cigarette: impact on respiratory flow resistance, impedance, and exhaled nitric oxide.* Chest, 2012. **141**(6): p. 1400-6.

234. Liu, X., et al., *Efficiency and adverse events of electronic cigarettes: A systematic review and meta-analysis (PRISMA-compliant article).* Medicine, 2018. **97**(19): p. e0324-e0324.
235. Vlachopoulos, C., et al., *Electronic Cigarette Smoking Increases Aortic Stiffness and Blood Pressure in Young Smokers.* Journal of the American College of Cardiology, 2016. **67**(23): p. 2802.
236. Rhee, M.Y., et al., *Acute effects of cigarette smoking on arterial stiffness and blood pressure in male smokers with hypertension.* Am J Hypertens, 2007. **20**(6): p. 637-41.
237. Martin, E.M., et al., *E-cigarette use results in suppression of immune and inflammatory-response genes in nasal epithelial cells similar to cigarette smoke.* Am J Physiol Lung Cell Mol Physiol, 2016. **311**(1): p. L135-44.
238. Breland, A.B., et al., *Evaluating acute effects of potential reduced-exposure products for smokers: clinical laboratory methodology.* Nicotine Tob Res, 2002. **4 Suppl 2**: p. S131-40.
239. Hua, M., H. Yip, and P. Talbot, *Mining data on usage of electronic nicotine delivery systems (ENDS) from YouTube videos.* Tob Control, 2013. **22**(2): p. 103-6.
240. Dwyer, J.B., S.C. McQuown, and F.M. Leslie, *The Dynamic Effects of Nicotine on the Developing Brain.* Pharmacology & therapeutics, 2009. **122**(2): p. 125-139.
241. Ernst, M., E.T. Moolchan, and M.L. Robinson, *Behavioral and neural consequences of prenatal exposure to nicotine.* J Am Acad Child Adolesc Psychiatry, 2001. **40**(6): p. 630-41.
242. Goriounova, N.A. and H.D. Mansvelder, *Short- and Long-Term Consequences of Nicotine Exposure during Adolescence for Prefrontal Cortex Neuronal Network Function.* Cold Spring Harbor Perspectives in Medicine, 2012. **2**(12): p. a012120.
243. Yuan, M., et al., *Nicotine and the adolescent brain.* The Journal of Physiology, 2015. **593**(16): p. 3397-3412.
244. Dawkins, L., J. Turner, and E. Crowe, *Nicotine derived from the electronic cigarette improves time-based prospective memory in abstinent smokers.* Psychopharmacology (Berl), 2013. **227**(3): p. 377-84.
245. Ernst, M., et al., *Effect of nicotine on brain activation during performance of a working memory task.* Proceedings of the National Academy of Sciences of the United States of America, 2001. **98**(8): p. 4728-4733.
246. Kobiella, A., et al., *Acute and chronic nicotine effects on behaviour and brain activation during intertemporal decision making.* Addict Biol, 2014. **19**(5): p. 918-30.
247. Leao, R.M., et al., *Chronic nicotine activates stress/reward-related brain regions and facilitates the transition to compulsive alcohol drinking.* J Neurosci, 2015. **35**(15): p. 6241-53.
248. Berridge, M.S., et al., *Smoking produces rapid rise of [11C]nicotine in human brain.* Psychopharmacology (Berl), 2010. **209**(4): p. 383-94.
249. Benwell, M.E., D.J. Balfour, and J.M. Anderson, *Evidence that tobacco smoking increases the density of (-)-[3H]nicotine binding sites in human brain.* J Neurochem, 1988. **50**(4): p. 1243-7.
250. Gotti, C., M. Zoli, and F. Clementi, *Brain nicotinic acetylcholine receptors: native subtypes and their relevance.* Trends in Pharmacological Sciences, 2006. **27**(9): p. 482-491.
251. Rajmohan, V. and E. Mohandas, *The limbic system.* Indian Journal of Psychiatry, 2007. **49**(2): p. 132-139.
252. Galletti, C., et al., *Role of the medial parieto-occipital cortex in the control of reaching and grasping movements.* Exp Brain Res, 2003. **153**(2): p. 158-70.
253. Hubbs, A.F., et al., *Respiratory and olfactory cytotoxicity of inhaled 2,3-pentanedione in Sprague-Dawley rats.* Am J Pathol, 2012. **181**(3): p. 829-44.
254. Potera, C., *Still searching for better butter flavoring.* Environ Health Perspect, 2012. **120**(12): p. A457.

255. Rincon, M., *Interleukin-6: from an inflammatory marker to a target for inflammatory diseases.* Trends Immunol, 2012. **33**(11): p. 571-7.
256. Bechade, C., et al., *NOS2 expression is restricted to neurons in the healthy brain but is triggered in microglia upon inflammation.* Glia, 2014. **62**(6): p. 956-63.
257. Rosenstein, J.M., J.M. Krum, and C. Ruhrberg, *VEGF in the nervous system.* Organogenesis, 2010. **6**(2): p. 107-14.
258. Ruiz de Almodovar, C., et al., *Role and therapeutic potential of VEGF in the nervous system.* Physiol Rev, 2009. **89**(2): p. 607-48.
259. Koyama, S., et al., *Decreased level of vascular endothelial growth factor in bronchoalveolar lavage fluid of normal smokers and patients with pulmonary fibrosis.* Am J Respir Crit Care Med, 2002. **166**(3): p. 382-5.
260. Orzabal, M. and J. Ramadoss, *Impact of Electronic Cigarette Aerosols on Pregnancy and Early Development.* Current Opinion in Toxicology, 2019.
261. Bold, K.W., et al., *Reasons for Trying E-cigarettes and Risk of Continued Use.* Pediatrics, 2016. **138**(3): p. e20160895.
262. Hawkins, K.B., et al., *Adolescents' Awarenss and Perceptions of E-Cigarettes: Implications for Intervention and Tobacco Regulation.* Pediatrics, 2017. **140**(1 MeetingAbstract): p. 132.
263. Cullen, K.A., Ambrose, B.K., Gentzke, A.S., Apelberg, B.J., Jamal, A., King, B.A. *Use of Electronic Cigarettes and Any Tobacco Product Among Middle and High School Students - United States, 2011-2018*. Morbidity and Mortality Weekly Report 2018.
264. Mirbolouk, M., et al., *Prevalence and Distribution of E-Cigarette Use Among U.S. Adults: Behavioral Risk Factor Surveillance System, 2016.* Ann Intern Med, 2018. **169**(7): p. 429-438.
265. Berry, K.M., et al., *Association of Electronic Cigarette Use With Subsequent Initiation of Tobacco Cigarettes in US YouthsElectronic Cigarette Use and Subsequent Cigarette Smoking InitiationElectronic Cigarette Use and Subsequent Cigarette Smoking Initiation.* JAMA Network Open, 2019. **2**(2): p. e187794-e187794.
266. (n.d.), M.C., *Percentage of adults in the U.S. who had tried vaping or using electronic cigarettes as of 2018*, in *In Statistica - The Statistics Portal* 2018: United States.
267. Kong, G., K.E. Kuguru, and S. Krishnan-Sarin, *Gender Differences in U.S. Adolescent E-Cigarette Use.* Current addiction reports, 2017. **4**(4): p. 422-430.
268. Wagner, N.J., M. Camerota, and C. Propper, *Prevalence and Perceptions of Electronic Cigarette Use during Pregnancy.* Maternal and child health journal, 2017. **21**(8): p. 1655-1661.
269. McCubbin, A., et al., *Perceptions and use of electronic cigarettes in pregnancy.* Health education research, 2017. **32**(1): p. 22-32.
270. Glynos, C., et al., *Comparison of the effects of e-cigarette vapor vs cigarette smoke on lung function and inflammation in mice.* European Respiratory Journal, 2015. **46**(suppl 59): p. OA279.
271. Clayton, S., et al., *Vaping to Vascular Damage: The Role of E-Cigarettes on Vascular Function.* The FASEB Journal, 2017. **31**(1_supplement): p. lb651-lb651.
272. Martin, E.M. and R.C. Fry, *Environmental Influences on the Epigenome: Exposure-Associated DNA Methylation in Human Populations.* Annu Rev Public Health, 2018. **39**: p. 309-333.
273. Lim, U. and M.A. Song, *Dietary and lifestyle factors of DNA methylation.* Methods Mol Biol, 2012. **863**: p. 359-76.
274. Le Dantec, C., et al., *Chapter 21 - How the Environment Influences Epigenetics, DNA Methylation, and Autoimmune Diseases*, in *Epigenetics and Dermatology*, Q. Lu, C.C. Chang, and B.C. Richardson, Editors. 2015, Academic Press: Boston. p. 467-485.

275. Joubert, B.R., et al., *DNA Methylation in Newborns and Maternal Smoking in Pregnancy: Genome-wide Consortium Meta-analysis.* Am J Hum Genet, 2016. **98**(4): p. 680-96.
276. Suter, M., et al., *In utero tobacco exposure epigenetically modifies placental CYP1A1 expression.* Metabolism, 2010. **59**(10): p. 1481-90.
277. Su, D., et al., *Distinct Epigenetic Effects of Tobacco Smoking in Whole Blood and among Leukocyte Subtypes.* PLOS ONE, 2016. **11**(12): p. e0166486.
278. He, Y., et al., *Maternal Smoking During Pregnancy and ADHD: Results From a Systematic Review and Meta-Analysis of Prospective Cohort Studies.* J Atten Disord, 2017: p. 1087054717696766.
279. Paradis, A.D., et al., *Maternal smoking during pregnancy and offspring antisocial behaviour: findings from a longitudinal investigation of discordant siblings.* J Epidemiol Community Health, 2017. **71**(9): p. 889-896.
280. Wakschlag, L.S., et al., *Maternal smoking during pregnancy and severe antisocial behavior in offspring: a review.* American journal of public health, 2002. **92**(6): p. 966-974.
281. Chen, H., et al., *Maternal Cigarette Smoke Exposure Contributes to Glucose Intolerance and Decreased Brain Insulin Action in Mice Offspring Independent of Maternal Diet.* PLOS ONE, 2011. **6**(11): p. e27260.
282. Bailey KR, C.J., *Anxiety-Related Behaviors in Mice.* . 2nd ed. Methods of Behavior Analysis in Neuroscience, ed. B. JJ. Vol. Chapter 5. 2009, Boca Raton (FL): CRC Press/Taylor & Francis.
283. Bailey KR, C.J., *Anxiety-Related Behaviors in Mice.* . 2nd edition ed. Methods of Behavior Analysis in Neuroscience, ed. I.B. JJ. Vol. 5. 2009, Boca Raton (FL): CRC Press/Taylor & Francis.
284. Antunes, M. and G. Biala, *The novel object recognition memory: neurobiology, test procedure, and its modifications.* Cognitive Processing, 2012. **13**(2): p. 93-110.
285. Lueptow, L.M., *Novel Object Recognition Test for the Investigation of Learning and Memory in Mice.* Journal of visualized experiments : JoVE, 2017(126): p. 55718.
286. *Elevated plus maze*. 2011 [cited 2019 16/07/19]; Available from: https://en.wikipedia.org/wiki/Elevated_plus_maze.
287. Raja, M., et al., *Diagnostic Methods for Detection of Cotinine Level in Tobacco Users: A Review.* Journal of clinical and diagnostic research : JCDR, 2016. **10**(3): p. ZE04-ZE6.
288. *ISOLATE II RNA/DNA/Protein Kit product manual*, Bioline, Editor.: United State of America.
289. *RT2 Profiler PCR Array Handbook*. 2018 [cited 2018 06/06/18]; Available from: https://www.qiagen.com/us/resources/resourcedetail?id=f4b13eaa-884f-4357-abe6-1a5f9469bc32&lang=en.
290. *Data Analysis Center*. 2018 [cited 2018 06/06/18]; Available from: https://www.qiagen.com/us/shop/genes-and-pathways/data-analysis-center-overview-page/#Excel.
291. Livak, K.J. and T.D. Schmittgen, *Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method.* Methods, 2001. **25**(4): p. 402-8.
292. Paxinos, G., Franklin, K.B.J., *Paxinos and Franklin's the Mouse Brain Sterotaxic Coordinates 4th Edition*. 2012: Academic Press.
293. Okano, M., et al., *DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development.* Cell, 1999. **99**(3): p. 247-57.
294. Spannhoff, A., et al., *The emerging therapeutic potential of histone methyltransferase and demethylase inhibitors.* ChemMedChem, 2009. **4**(10): p. 1568-82.
295. Pearson, A.G., et al., *Activating transcription factor 2 expression in the adult human brain: association with both neurodegeneration and neurogenesis.* Neuroscience, 2005. **133**(2): p. 437-51.

296. Kawasaki, H., et al., *ATF-2 has intrinsic histone acetyltransferase activity which is modulated by phosphorylation.* Nature, 2000. **405**(6783): p. 195-200.
297. Willems, E., et al., *The functional diversity of Aurora kinases: a comprehensive review.* Cell Division, 2018. **13**(1): p. 7.
298. Dehmel, S., et al., *Intrauterine smoke exposure deregulates lung function, pulmonary transcriptomes, and in particular insulin-like growth factor (IGF)-1 in a sex-specific manner.* Scientific Reports, 2018. **8**(1): p. 7547.
299. Esposito, E.R., et al., *An animal model of cigarette smoke-induced in utero growth retardation.* Toxicology, 2008. **246**(2-3): p. 193-202.
300. Gandley, R.E., et al., *Cigarette exposure induces changes in maternal vascular function in a pregnant mouse model.* American journal of physiology. Regulatory, integrative and comparative physiology, 2010. **298**(5): p. R1249-R1256.
301. Kataoka, M.C., et al., *Smoking during pregnancy and harm reduction in birth weight: a cross-sectional study.* BMC pregnancy and childbirth, 2018. **18**(1): p. 67-67.
302. Zacharasiewicz, A., *Maternal smoking in pregnancy and its influence on childhood asthma.* ERJ open research, 2016. **2**(3): p. 00042-2016.
303. Spindel, E.R. and C.T. McEvoy, *The Role of Nicotine in the Effects of Maternal Smoking during Pregnancy on Lung Development and Childhood Respiratory Disease. Implications for Dangers of E-Cigarettes.* American journal of respiratory and critical care medicine, 2016. **193**(5): p. 486-494.
304. Stroud, L.R., M. McCallum, and A.L. Salisbury, *Impact of maternal prenatal smoking on fetal to infant neurobehavioral development.* Development and psychopathology, 2018. **30**(3): p. 1087-1105.
305. Lee, H., S. Chung, and J. Noh, *Maternal Nicotine Exposure During Late Gestation and Lactation Increases Anxiety-Like and Impulsive Decision-Making Behavior in Adolescent Offspring of Rat.* Toxicological research, 2016. **32**(4): p. 275-280.
306. Roy, T.S., F.J. Seidler, and T.A. Slotkin, *Prenatal nicotine exposure evokes alterations of cell structure in hippocampus and somatosensory cortex.* J Pharmacol Exp Ther, 2002. **300**(1): p. 124-33.
307. Slotkin, T.A., et al., *Effects of prenatal nicotine exposure on primate brain development and attempted amelioration with supplemental choline or vitamin C: neurotransmitter receptors, cell signaling and cell development biomarkers in fetal brain regions of rhesus monkeys.* Neuropsychopharmacology, 2005. **30**(1): p. 129-44.
308. Zhang, C., et al., *Prenatal nicotine exposure induces depressionlike behavior in adolescent female rats via modulating neurosteroid in the hippocampus.* Mol Med Rep, 2019. **19**(5): p. 4185-4194.
309. Zhu, J., et al., *Prenatal nicotine exposure mouse model showing hyperactivity, reduced cingulate cortex volume, reduced dopamine turnover, and responsiveness to oral methylphenidate treatment.* The Journal of neuroscience : the official journal of the Society for Neuroscience, 2012. **32**(27): p. 9410-9418.
310. Tian, S., S. Pan, and Y. You, *Nicotine enhances the reconsolidation of novel object recognition memory in rats.* Pharmacol Biochem Behav, 2015. **129**: p. 14-8.
311. Puma, C., et al., *Nicotine improves memory in an object recognition task in rats.* Eur Neuropsychopharmacol, 1999. **9**(4): p. 323-7.
312. Zhang, L., et al., *Attention and working memory deficits in a perinatal nicotine exposure mouse model.* PLOS ONE, 2018. **13**(5): p. e0198064.
313. Eppolito, A.K. and R.F. Smith, *Long-term behavioral and developmental consequences of pre- and perinatal nicotine.* Pharmacol Biochem Behav, 2006. **85**(4): p. 835-41.
314. Palazzolo, D.L., et al., *Trace Metals Derived from Electronic Cigarette (ECIG) Generated Aerosol: Potential Problem of ECIG Devices That Contain Nickel.* Frontiers in physiology, 2017. **7**: p. 663-663.

315. Olmedo, P., et al., *Metal Concentrations in e-Cigarette Liquid and Aerosol Samples: The Contribution of Metallic Coils.* Environ Health Perspect, 2018. **126**(2): p. 027010.
316. Lamtai, M., et al., *Effect of Chronic Administration of Nickel on Affective and Cognitive Behavior in Male and Female Rats: Possible Implication of Oxidative Stress Pathway.* Brain sciences, 2018. **8**(8): p. 141.
317. Deichmann, W., *Glycerol:—Effects upon Rabbits and Rats—.* American Industrial Hygiene Association Quarterly, 1941. **2**(1): p. 5-6.
318. Li, Y., et al., *Effects of formaldehyde exposure on anxiety-like and depression-like behavior, cognition, central levels of glucocorticoid receptor and tyrosine hydroxylase in mice.* Chemosphere, 2016. **144**: p. 2004-12.
319. Williams, M., et al., *Metal and Silicate Particles Including Nanoparticles Are Present in Electronic Cigarette Cartomizer Fluid and Aerosol.* PLOS ONE, 2013. **8**(3): p. e57987.
320. Gustin, K., et al., *Cadmium exposure and cognitive abilities and behavior at 10years of age: A prospective cohort study.* Environ Int, 2018. **113**: p. 259-268.
321. Meaney, M.J. and A.C. Ferguson-Smith, *Epigenetic regulation of the neural transcriptome: the meaning of the marks.* Nature Neuroscience, 2010. **13**: p. 1313.
322. Nafee, T.M., et al., *Epigenetic control of fetal gene expression.* Bjog, 2008. **115**(2): p. 158-68.
323. Kitsiou-Tzeli, S. and M. Tzetis, *Maternal epigenetics and fetal and neonatal growth.* Curr Opin Endocrinol Diabetes Obes, 2017. **24**(1): p. 43-46.
324. Richmond, R.C., et al., *DNA methylation as a marker for prenatal smoke exposure in adults.* International journal of epidemiology, 2018. **47**(4): p. 1120-1130.
325. Miyake, K., et al., *Association between DNA methylation in cord blood and maternal smoking: The Hokkaido Study on Environment and Children's Health.* Scientific Reports, 2018. **8**(1): p. 5654.
326. Lillycrop, K.A. and G.C. Burdge, *Epigenetic mechanisms linking early nutrition to long term health.* Best Pract Res Clin Endocrinol Metab, 2012. **26**(5): p. 667-76.
327. Liu, Y., et al., *DNA methylation at imprint regulatory regions in preterm birth and infection.* Am J Obstet Gynecol, 2013. **208**(5): p. 395.e1-7.
328. Soubry, A., et al., *Newborns of obese parents have altered DNA methylation patterns at imprinted genes.* Int J Obes (Lond), 2015. **39**(4): p. 650-7.
329. Lee, H.-S., *Impact of Maternal Diet on the Epigenome during In Utero Life and the Developmental Programming of Diseases in Childhood and Adulthood.* Nutrients, 2015. **7**(11): p. 9492-9507.
330. Ferrea, S. and G. Winterer, *Neuroprotective and neurotoxic effects of nicotine.* Pharmacopsychiatry, 2009. **42**(6): p. 255-65.
331. McCarthy, D.M., et al., *Nicotine exposure of male mice produces behavioral impairment in multiple generations of descendants.* PLOS Biology, 2018. **16**(10): p. e2006497.
332. Chatterton, Z., et al., *In utero exposure to maternal smoking is associated with DNA methylation alterations and reduced neuronal content in the developing fetal brain.* Epigenetics & Chromatin, 2017. **10**(1): p. 4.
333. Ke, J., et al., *Role of DNA methylation in perinatal nicotine-induced development of heart ischemia-sensitive phenotype in rat offspring.* Oncotarget, 2017. **8**(44): p. 76865-76880.
334. Tehranifar, P., et al., *Maternal cigarette smoking during pregnancy and offspring DNA methylation in midlife.* Epigenetics, 2018. **13**(2): p. 129-134.
335. Wiklund, P., et al., *DNA methylation links prenatal smoking exposure to later life health outcomes in offspring.* Clinical Epigenetics, 2019. **11**(1): p. 97.
336. Wu, C.-C., et al., *Paternal Tobacco Smoke Correlated to Offspring Asthma and Prenatal Epigenetic Programming.* Frontiers in Genetics, 2019. **10**(471).
337. Tatton-Brown, K., et al., *Mutations in the DNA methyltransferase gene DNMT3A cause an overgrowth syndrome with intellectual disability.* Nat Genet, 2014. **46**(4): p. 385-8.

338. Hagleitner, M.M., et al., *Clinical spectrum of immunodeficiency, centromeric instability and facial dysmorphism (ICF syndrome).* J Med Genet, 2008. **45**(2): p. 93-9.
339. Hancock, D.B., et al., *Genome-wide association study across European and African American ancestries identifies a SNP in DNMT3B contributing to nicotine dependence.* Molecular Psychiatry, 2018. **23**(9): p. 1911-1919.
340. Tahiliani, M., et al., *The histone H3K4 demethylase SMCX links REST target genes to X-linked mental retardation.* Nature, 2007. **447**(7144): p. 601-5.
341. Jensen, L.R., et al., *Mutations in the JARID1C gene, which is involved in transcriptional regulation and chromatin remodeling, cause X-linked mental retardation.* Am J Hum Genet, 2005. **76**(2): p. 227-36.
342. Iwase, S., et al., *The X-linked mental retardation gene SMCX/JARID1C defines a family of histone H3 lysine 4 demethylases.* Cell, 2007. **128**(6): p. 1077-88.
343. Yamada, T., Y. Yoshiyama, and N. Kawaguchi, *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, 1997. **749**(2): p. 329-334.
344. Huang, L.Z., L.C. Abbott, and U.H. Winzer-Serhan, *Effects of chronic neonatal nicotine exposure on nicotinic acetylcholine receptor binding, cell death and morphology in hippocampus and cerebellum.* Neuroscience, 2007. **146**(4): p. 1854-68.
345. Wielgus, J.J., et al., *Exposure to low concentrations of nicotine during cranial nerve development inhibits apoptosis and causes cellular hypertrophy in the ventral oculomotor nuclei of the chick embryo.* Brain Res, 2004. **1000**(1-2): p. 123-33.
346. Abrous, D.N., et al., *Nicotine Self-Administration Impairs Hippocampal Plasticity.* The Journal of Neuroscience, 2002. **22**(9): p. 3656-3662.
347. Li, S.P., et al., *Chronic nicotine and smoking exposure decreases GABA(B1) receptor expression in the rat hippocampus.* Neurosci Lett, 2002. **334**(2): p. 135-9.
348. Roy, T.S. and U. Sabherwal, *Effects of gestational nicotine exposure on hippocampal morphology.* Neurotoxicol Teratol, 1998. **20**(4): p. 465-73.
349. Chang, G.-Q., O. Karatayev, and S.F. Leibowitz, *Prenatal exposure to nicotine stimulates neurogenesis of orexigenic peptide-expressing neurons in hypothalamus and amygdala.* The Journal of neuroscience : the official journal of the Society for Neuroscience, 2013. **33**(34): p. 13600-13611.
350. Mahar, I., et al., *Developmental hippocampal neuroplasticity in a model of nicotine replacement therapy during pregnancy and breastfeeding.* PloS one, 2012. **7**(5): p. e37219-e37219.
351. Li, M.D., et al., *Time-dependent changes in transcriptional profiles within five rat brain regions in response to nicotine treatment.* Brain Res Mol Brain Res, 2004. **132**(2): p. 168-80.
352. Drake, P., A.K. Driscoll, and T.J. Mathews, *Cigarette Smoking During Pregnancy: United States, 2016.* NCHS Data Brief, 2018(305): p. 1-8.
353. Liu, T., et al., *Maternal smoking during pregnancy and anger temperament among adult offspring.* J Psychiatr Res, 2011. **45**(12): p. 1648-54.
354. Kovess, V., et al., *Maternal smoking and offspring inattention and hyperactivity: results from a cross-national European survey.* Eur Child Adolesc Psychiatry, 2015. **24**(8): p. 919-29.
355. Dierker, L.C., G. Canino, and K.R. Merikangas, *Association between parental and individual psychiatric/substance use disorders and smoking stages among Puerto Rican adolescents.* Drug Alcohol Depend, 2006. **84**(2): p. 144-53.
356. Hu, M.C., M. Davies, and D.B. Kandel, *Epidemiology and correlates of daily smoking and nicotine dependence among young adults in the United States.* Am J Public Health, 2006. **96**(2): p. 299-308.

357. Mays, D., et al., *Parental smoking exposure and adolescent smoking trajectories.* Pediatrics, 2014. **133**(6): p. 983-991.
358. Knopik, V.S., et al., *The Epigenetics of Maternal Cigarette Smoking During Pregnancy and Effects on Child Development.* Development and psychopathology, 2012. **24**(4): p. 1377-1390.
359. Reynolds, L.M., et al., *Tobacco exposure-related alterations in DNA methylation and gene expression in human monocytes: the Multi-Ethnic Study of Atherosclerosis (MESA).* Epigenetics, 2017. **12**(12): p. 1092-1100.
360. Ford, C., Greenhalgh, EM & Winstanley, MH. and *Pregnancy and smoking*. Tobacco in Australia: Facts and issues 2015 [cited 2019 16/01/19]; Available from: http://www.tobaccoinaustralia.org.au/3-7-pregnancy-and-smoking.
361. van der Sterren, A., Greenhalgh, EM, Knoche, D, & Winstanley, MH. *Prevalence of tobacco use among Aboriginal and Torres Strait Islander people*. Tobacco in Australia: Facts and issues 2018 [cited 2019 02/01/19]; Available from: http://www.tobaccoinaustralia.org.au/chapter-8-aptsi/8-3-prevalence-of-tobacco-use-among-aboriginal-peo.
362. Zeev, Y.B., et al., *Opportunities Missed: A Cross-Sectional Survey of the Provision of Smoking Cessation Care to Pregnant Women by Australian General Practitioners and Obstetricians.* Nicotine Tob Res, 2017. **19**(5): p. 636-641.
363. McEwen, A., R. West, and L. Owen, *General Practitioners' views on the provision of nicotine replacement therapy and bupropion.* BMC Family Practice, 2001. **2**(1): p. 6.
364. Herbert, R., T. Coleman, and J. Britton, *U.K. general practitioners' beliefs, attitudes, and reported prescribing of nicotine replacement therapy in pregnancy.* Nicotine Tob Res, 2005. **7**(4): p. 541-6.
365. Bar-Zeev, Y., et al., *Nicotine replacement therapy for smoking cessation during pregnancy.* Med J Aust, 2018. **208**(1): p. 46-51.
366. *Smoking: stopping in pregnancy and after childbirth.* Postnatal care 2010 [cited 2019 03/05/19]; Available from: https://www.nice.org.uk/guidance/ph26.
367. Griffin, C., Harding, J., Sutton, C., *Women and smoking*. 2014, Royal Australian and New Zealand College of Obstetricians and Gynaecologists: Australia.
368. Siu, A.L., *Behavioral and Pharmacotherapy Interventions for Tobacco Smoking Cessation in Adults, Including Pregnant Women: U.S. Preventive Services Task Force Recommendation Statement.* Ann Intern Med, 2015. **163**(8): p. 622-34.
369. Coleman, T., et al., *Pharmacological interventions for promoting smoking cessation during pregnancy.* Cochrane Database Syst Rev, 2012(9): p. Cd010078.
370. Coleman, T., et al., *Pharmacological interventions for promoting smoking cessation during pregnancy.* Cochrane Database of Systematic Reviews, 2015(12).
371. Oncken, C., et al., *Correlates of Electronic Cigarettes Use Before and During Pregnancy.* Nicotine Tob Res, 2017. **19**(5): p. 585-590.
372. Walf, A.A. and C.A. Frye, *The use of the elevated plus maze as an assay of anxiety-related behavior in rodents.* Nature protocols, 2007. **2**(2): p. 322-328.
373. Alasmari, F., et al., *Effects of chronic inhalation of electronic cigarettes containing nicotine on glial glutamate transporters and α-7 nicotinic acetylcholine receptor in female CD-1 mice.* Progress in Neuro-Psychopharmacology and Biological Psychiatry, 2017. **77**: p. 1-8.
374. Holloway, A.C., L.D. Kellenberger, and J.J. Petrik, *Fetal and neonatal exposure to nicotine disrupts ovarian function and fertility in adult female rats.* Endocrine, 2006. **30**(2): p. 213-6.
375. Primo, C.C., et al., *Effects of maternal nicotine on breastfeeding infants.* Revista paulista de pediatria : orgao oficial da Sociedade de Pediatria de Sao Paulo, 2013. **31**(3): p. 392-397.

376. Luck, W., et al., *Extent of nicotine and cotinine transfer to the human fetus, placenta and amniotic fluid of smoking mothers.* Dev Pharmacol Ther, 1985. **8**(6): p. 384-95.
377. Chiolero, A., P. Bovet, and F. Paccaud, *Association between maternal smoking and low birth weight in Switzerland: the EDEN study.* Swiss Med Wkly, 2005. **135**(35-36): p. 525-30.
378. Vaez, A., et al., *DNA methylation mediates the effect of maternal smoking during pregnancy on birthweight of the offspring.* International Journal of Epidemiology, 2015. **44**(4): p. 1224-1237.
379. Tweed, S., S. Bhattacharya, and P.A. Fowler, *Effects of maternal smoking on offspring reproductive outcomes: an intergenerational study in the North East of Scotland.* Human Reproduction Open, 2017. **2017**(2).
380. Wang, X., et al., *Maternal cigarette smoking, metabolic gene polymorphism, and infant birth weight.* Jama, 2002. **287**(2): p. 195-202.
381. Wang, X., et al., *Maternal smoking during pregnancy, urine cotinine concentrations, and birth outcomes. A prospective cohort study.* Int J Epidemiol, 1997. **26**(5): p. 978-88.
382. Larcombe, A.N., et al., *In utero cigarette smoke exposure impairs somatic and lung growth in BALB/c mice.* European Respiratory Journal, 2011. **38**(4): p. 932-938.
383. Bertoglio, L.J. and A.P. Carobrez, *Prior maze experience required to alter midazolam effects in rats submitted to the elevated plus-maze.* Pharmacol Biochem Behav, 2002. **72**(1-2): p. 449-55.
384. Rodgers, R.J. and A. Dalvi, *Anxiety, defence and the elevated plus-maze.* Neurosci Biobehav Rev, 1997. **21**(6): p. 801-10.
385. Bertaina-Anglade, V., et al., *The object recognition task in rats and mice: a simple and rapid model in safety pharmacology to detect amnesic properties of a new chemical entity.* J Pharmacol Toxicol Methods, 2006. **54**(2): p. 99-105.
386. Hall, B.J., et al., *Cognitive and Behavioral Impairments Evoked by Low-Level Exposure to Tobacco Smoke Components: Comparison with Nicotine Alone.* Toxicological sciences : an official journal of the Society of Toxicology, 2016. **151**(2): p. 236-244.
387. Fried, P.A., C.M. O'Connell, and B. Watkinson, *60- and 72-month follow-up of children prenatally exposed to marijuana, cigarettes, and alcohol: cognitive and language assessment.* J Dev Behav Pediatr, 1992. **13**(6): p. 383-91.
388. Ribas-Fitó, N., et al., *Maternal smoking habits and cognitive development of children at age 4 years in a population-based birth cohort.* International Journal of Epidemiology, 2007. **36**(4): p. 825-832.
389. Jacobsen, L.K., et al., *Visuospatial memory deficits emerging during nicotine withdrawal in adolescents with prenatal exposure to active maternal smoking.* Neuropsychopharmacology, 2006. **31**(7): p. 1550-61.
390. Linnet, K.M., et al., *Maternal lifestyle factors in pregnancy risk of attention deficit hyperactivity disorder and associated behaviors: review of the current evidence.* Am J Psychiatry, 2003. **160**(6): p. 1028-40.
391. Huang, L., et al., *Maternal Smoking and Attention-Deficit/Hyperactivity Disorder in Offspring: A Meta-analysis.* Pediatrics, 2018. **141**(1).
392. Max, W., H.Y. Sung, and Y. Shi, *Attention deficit hyperactivity disorder among children exposed to secondhand smoke: a logistic regression analysis of secondary data.* Int J Nurs Stud, 2013. **50**(6): p. 797-806.
393. Yochum, C., et al., *Prenatal cigarette smoke exposure causes hyperactivity and aggressive behavior: role of altered catecholamines and BDNF.* Exp Neurol, 2014. **254**: p. 145-52.
394. Lee, K.W.K. and Z. Pausova, *Cigarette smoking and DNA methylation.* Frontiers in genetics, 2013. **4**: p. 132-132.

395. Shen, J.X. and J.L. Yakel, *Nicotinic acetylcholine receptor-mediated calcium signaling in the nervous system.* Acta Pharmacol Sin, 2009. **30**(6): p. 673-80.
396. Marsit, C.J., et al., *Carcinogen exposure and gene promoter hypermethylation in bladder cancer.* Carcinogenesis, 2006. **27**(1): p. 112-6.
397. Perera, F., et al., *Relation of DNA methylation of 5'-CpG island of ACSL3 to transplacental exposure to airborne polycyclic aromatic hydrocarbons and childhood asthma.* PLoS One, 2009. **4**(2): p. e4488.
398. Tang, M.-s., et al., *Acrolein induced DNA damage, mutagenicity and effect on DNA repair.* Molecular nutrition & food research, 2011. **55**(9): p. 1291-1300.
399. Bansal, V. and K.-H. Kim, *Review on quantitation methods for hazardous pollutants released by e-cigarette (EC) smoking.* TrAC Trends in Analytical Chemistry, 2016. **78**: p. 120-133.
400. Khlystov, A. and V. Samburova, *Flavoring Compounds Dominate Toxic Aldehyde Production during E-Cigarette Vaping.* Environmental Science & Technology, 2016. **50**(23): p. 13080-13085.
401. Kim, K.-H., et al., *A critical review on the diverse preconcentration procedures on bag samples in the quantitation of volatile organic compounds from cigarette smoke and other combustion samples.* TrAC Trends in Analytical Chemistry, 2016. **85**: p. 65-74.
402. Uchiyama, S., et al., *Determination of nicotine, tar, volatile organic compounds and carbonyls in mainstream cigarette smoke using a glass filter and a sorbent cartridge followed by the two-phase/one-pot elution method with carbon disulfide and methanol.* J Chromatogr A, 2015. **1426**: p. 48-55.
403. Lestari, K.S., M.V. Humairo, and U. Agustina, *Formaldehyde Vapor Concentration in Electronic Cigarettes and Health Complaints of Electronic Cigarettes Smokers in Indonesia.* Journal of environmental and public health, 2018. **2018**: p. 9013430-9013430.
404. Satta, R., et al., *Nicotine decreases DNA methyltransferase 1 expression and glutamic acid decarboxylase 67 promoter methylation in GABAergic interneurons.* Proceedings of the National Academy of Sciences, 2008. **105**(42): p. 16356-16361.
405. Yanagawa, N., et al., *Inverse correlation between EGFR mutation and FHIT, RASSF1A and RUNX3 methylation in lung adenocarcinoma: relation with smoking status.* Anticancer Res, 2011. **31**(4): p. 1211-4.
406. Satta, R., et al., *Nicotine decreases DNA methyltransferase 1 expression and glutamic acid decarboxylase 67 promoter methylation in GABAergic interneurons.* Proc Natl Acad Sci U S A, 2008. **105**(42): p. 16356-61.
407. Park, Dae H., et al., *Activation of Neuronal Gene Expression by the JMJD3 Demethylase Is Required for Postnatal and Adult Brain Neurogenesis.* Cell Reports, 2014. **8**(5): p. 1290-1299.
408. Sherry-Lynes, M.M., et al., *Regulation of the JMJD3 (KDM6B) histone demethylase in glioblastoma stem cells by STAT3.* PLOS ONE, 2017. **12**(4): p. e0174775.
409. Wijayatunge, R., et al., *The histone demethylase Kdm6b regulates a mature gene expression program in differentiating cerebellar granule neurons.* Mol Cell Neurosci, 2018. **87**: p. 4-17.
410. Wijayatunge, R., et al., *The histone lysine demethylase Kdm6b is required for activity-dependent preconditioning of hippocampal neuronal survival.* Molecular and cellular neurosciences, 2014. **61**: p. 187-200.
411. Sundar, I.K. and I. Rahman, *Gene expression profiling of epigenetic chromatin modification enzymes and histone marks by cigarette smoke: implications for COPD and lung cancer.* American journal of physiology. Lung cellular and molecular physiology, 2016. **311**(6): p. L1245-L1258.

412. Gao, X., et al., *DNA methylation changes of whole blood cells in response to active smoking exposure in adults: a systematic review of DNA methylation studies.* Clin Epigenetics, 2015. **7**: p. 113.
413. Breitling, L.P., et al., *Tobacco-smoking-related differential DNA methylation: 27K discovery and replication.* Am J Hum Genet, 2011. **88**(4): p. 450-7.
414. Zeilinger, S., et al., *Tobacco smoking leads to extensive genome-wide changes in DNA methylation.* PLoS One, 2013. **8**(5): p. e63812.
415. Shenker, N.S., et al., *DNA methylation as a long-term biomarker of exposure to tobacco smoke.* Epidemiology, 2013. **24**(5): p. 712-6.
416. Marchal, J.A., et al., *Misregulation of mitotic chromosome segregation in a new type of autosomal recessive primary microcephaly.* Cell Cycle, 2011. **10**(17): p. 2967-77.
417. Penney, J. and L.H. Tsai, *Histone deacetylases in memory and cognition.* Sci Signal, 2014. **7**(355): p. re12.
418. Ho, Y.-S., et al., *Cigarette Smoking Accelerated Brain Aging and Induced Pre-Alzheimer-Like Neuropathology in Rats.* PLOS ONE, 2012. **7**(5): p. e36752.
419. Golub, V.M., et al., *Neurostereology protocol for unbiased quantification of neuronal injury and neurodegeneration.* Frontiers in aging neuroscience, 2015. **7**: p. 196-196.
420. Serlin, Y., et al., *Anatomy and physiology of the blood-brain barrier.* Seminars in cell & developmental biology, 2015. **38**: p. 2-6.
421. Paradis, A., D. Leblanc, and N. Dumais, *Optimization of an in vitro human blood–brain barrier model: Application to blood monocyte transmigration assays.* MethodsX, 2016. **3**: p. 25-34.
422. Ogunshola, O.O., *In vitro modeling of the blood-brain barrier: simplicity versus complexity.* Curr Pharm Des, 2011. **17**(26): p. 2755-61.
423. Thomsen, L.B., A. Burkhart, and T. Moos, *A Triple Culture Model of the Blood-Brain Barrier Using Porcine Brain Endothelial cells, Astrocytes and Pericytes.* PLOS ONE, 2015. **10**(8): p. e0134765.
424. Cecchelli, R., et al., *Modelling of the blood-brain barrier in drug discovery and development.* Nat Rev Drug Discov, 2007. **6**(8): p. 650-61.
425. Nakagawa, S., et al., *A new blood-brain barrier model using primary rat brain endothelial cells, pericytes and astrocytes.* Neurochem Int, 2009. **54**(3-4): p. 253-63.
426. Neuhaus, W., et al., *Blood-brain barrier in vitro models as tools in drug discovery: assessment of the transport ranking of antihistaminic drugs.* Pharmazie, 2012. **67**(5): p. 432-9.
427. Di Marco, A., et al., *Application of an in Vitro Blood-Brain Barrier Model in the Selection of Experimental Drug Candidates for the Treatment of Huntington's Disease.* Mol Pharm, 2019. **16**(5): p. 2069-2082.
428. Cucullo, L., et al., *Drug delivery and in vitro models of the blood-brain barrier.* Curr Opin Drug Discov Devel, 2005. **8**(1): p. 89-99.
429. Kim, J.H., et al., *Oxidative Stress Induced by Cigarette Smoke Extracts in Human Brain Cells (T98G) and Human Brain Microvascular Endothelial Cells (HBMEC) in Mono- and Co-Culture.* J Toxicol Environ Health A, 2015. **78**(15): p. 1019-27.
430. Lee, S.B., et al., *Impact of commercial cigarette smoke condensate on brain tissue co-cultured with astrocytes and blood-brain barrier endothelial cells.* J Toxicol Environ Health A, 2017. **80**(10-12): p. 533-541.
431. Hawkins, B.T., et al., *Nicotine increases in vivo blood-brain barrier permeability and alters cerebral microvascular tight junction protein distribution.* Brain Res, 2004. **1027**(1-2): p. 48-58.
432. Abbruscato, T.J., et al., *Nicotine and cotinine modulate cerebral microvascular permeability and protein expression of ZO-1 through nicotinic acetylcholine receptors expressed on brain endothelial cells.* J Pharm Sci, 2002. **91**(12): p. 2525-38.

433. Hutamekalin, P., et al., *Effect of nicotine and polyaromtic hydrocarbons on cerebral endothelial cells.* Cell Biol Int, 2008. **32**(2): p. 198-209.
434. Kaisar, M.A., et al., *Offsetting the impact of smoking and e-cigarette vaping on the cerebrovascular system and stroke injury: Is Metformin a viable countermeasure?* Redox biology, 2017. **13**: p. 353-362.
435. Wheway, J., et al., *Endothelial microparticles interact with and support the proliferation of T cells.* Journal of immunology (Baltimore, Md. : 1950), 2014. **193**(7): p. 3378-3387.
436. Ortoft, E., et al., *Human GAP-43 Gene Expression: Multiple Start Sites for Initiation of Transcription in Differentiating Human Neuroblastoma Cells.* Mol Cell Neurosci, 1993. **4**(6): p. 549-61.
437. Kume, T., et al., *Dibutyryl cyclic AMP induces differentiation of human neuroblastoma SH-SY5Y cells into a noradrenergic phenotype.* Neurosci Lett, 2008. **443**(3): p. 199-203.
438. Rayner, B.S., et al., *Protective effect of a synthetic anti-oxidant on neuronal cell apoptosis resulting from experimental hypoxia re-oxygenation injury.* J Neurochem, 2006. **97**(1): p. 211-21.
439. Sanchez, S., et al., *A cAMP-activated pathway, including PKA and PI3K, regulates neuronal differentiation.* Neurochem Int, 2004. **44**(4): p. 231-42.
440. Herrington, J.S. and C. Myers, *Electronic cigarette solutions and resultant aerosol profiles.* J Chromatogr A, 2015. **1418**: p. 192-199.
441. Farsalinos, K.E., et al., *Impact of flavour variability on electronic cigarette use experience: an internet survey.* International journal of environmental research and public health, 2013. **10**(12): p. 7272-7282.
442. Russell, C., et al., *Changing patterns of first e-cigarette flavor used and current flavors used by 20,836 adult frequent e-cigarette users in the USA.* Harm Reduction Journal, 2018. **15**(1): p. 33.
443. Srinivasan, B., et al., *TEER measurement techniques for in vitro barrier model systems.* Journal of laboratory automation, 2015. **20**(2): p. 107-126.
444. Matter, K. and M.S. Balda, *Functional analysis of tight junctions.* Methods, 2003. **30**(3): p. 228-34.
445. Srinivasan, B., et al., *TEER measurement techniques for in vitro barrier model systems.* J Lab Autom, 2015. **20**(2): p. 107-26.
446. Serres, F. and S.L. Carney, *Nicotine regulates SH-SY5Y neuroblastoma cell proliferation through the release of brain-derived neurotrophic factor.* Brain Res, 2006. **1101**(1): p. 36-42.
447. Gould, J., et al., *Nicotinic acetylcholine receptors in human neuroblastoma (SH-SY5Y) cells.* Neurosci Lett, 1992. **145**(2): p. 201-4.
448. Lukas, R.J., S.A. Norman, and L. Lucero, *Characterization of Nicotinic Acetylcholine Receptors Expressed by Cells of the SH-SY5Y Human Neuroblastoma Clonal Line.* Mol Cell Neurosci, 1993. **4**(1): p. 1-12.
449. Lutz, J.A., et al., *A nicotinic receptor-mediated anti-inflammatory effect of the flavonoid rhamnetin in BV2 microglia.* Fitoterapia, 2014. **98**: p. 11-21.
450. Sokolova, E., C. Matteoni, and A. Nistri, *Desensitization of neuronal nicotinic receptors of human neuroblastoma SH-SY5Y cells during short or long exposure to nicotine.* British journal of pharmacology, 2005. **146**(8): p. 1087-1095.
451. Riveles, K., L.Z. Huang, and M. Quik, *Cigarette smoke, nicotine and cotinine protect against 6-hydroxydopamine-induced toxicity in SH-SY5Y cells.* Neurotoxicology, 2008. **29**(3): p. 421-427.
452. Kettenmann, H., et al., *Physiology of microglia.* Physiol Rev, 2011. **91**(2): p. 461-553.
453. Kraft, A.D. and G.J. Harry, *Features of microglia and neuroinflammation relevant to environmental exposure and neurotoxicity.* International journal of environmental research and public health, 2011. **8**(7): p. 2980-3018.

454. Ghosh, D., et al., *Tobacco carcinogen induces microglial activation and subsequent neuronal damage.* Journal of Neurochemistry, 2009. **110**(3): p. 1070-1081.
455. Sassano, M.F., et al., *Evaluation of e-liquid toxicity using an open-source high-throughput screening assay.* PLOS Biology, 2018. **16**(3): p. e2003904.
456. Madison, M.C., et al., *Electronic cigarettes disrupt lung lipid homeostasis and innate immunity independent of nicotine.* The Journal of Clinical Investigation, 2019. **129**(10): p. 4290-4304.
457. Ghosh, A., et al., *Chronic E-Cigarette Exposure Alters the Human Bronchial Epithelial Proteome.* American journal of respiratory and critical care medicine, 2018. **198**(1): p. 67-76.
458. *What is the best PG/VG ratio for me?*, F. Pawns, Editor. 2018: California.
459. Naik, P., et al., *Oxidative and pro-inflammatory impact of regular and denicotinized cigarettes on blood brain barrier endothelial cells: is smoking reduced or nicotine-free products really safe?* BMC Neuroscience, 2014. **15**(1): p. 51.
460. Prasad, S., et al., *Impact of cigarette smoke extract and hyperglycemic conditions on blood–brain barrier endothelial cells.* Fluids and Barriers of the CNS, 2015. **12**(1): p. 18.
461. Sivandzade, F. and L. Cucullo, *Assessing the protective effect of rosiglitazone against electronic cigarette/tobacco smoke-induced blood-brain barrier impairment.* BMC neuroscience, 2019. **20**(1): p. 15-15.
462. Abdullahi, W., D. Tripathi, and P.T. Ronaldson, *Blood-brain barrier dysfunction in ischemic stroke: targeting tight junctions and transporters for vascular protection.* Am J Physiol Cell Physiol, 2018. **315**(3): p. C343-c356.
463. Sweeney, M.D., A.P. Sagare, and B.V. Zlokovic, *Blood-brain barrier breakdown in Alzheimer disease and other neurodegenerative disorders.* Nature reviews. Neurology, 2018. **14**(3): p. 133-150.
464. Prasad, S., et al., *Diabetes Mellitus and Blood-Brain Barrier Dysfunction: An Overview.* J Pharmacovigil, 2014. **2**(2): p. 125.
465. Piasek, M., et al., *Placental cadmium as an additional noninvasive bioindicator of active maternal tobacco smoking.* J Toxicol Environ Health A, 2016. **79**(11): p. 443-6.
466. Yan, T., et al., *Distribution of benzo[a]pyrene in discrete regions of rat brain tissue using light microscopic autoradiography and gamma counting.* Toxicological & Environmental Chemistry, 2010. **92**(7): p. 1309-1317.
467. Bauer, H.-C., et al., *"You Shall Not Pass"-tight junctions of the blood brain barrier.* Frontiers in neuroscience, 2014. **8**: p. 392-392.
468. Pun, P.B., J. Lu, and S. Moochhala, *Involvement of ROS in BBB dysfunction.* Free Radic Res, 2009. **43**(4): p. 348-64.
469. Higham, A., et al., *The effect of electronic cigarette and tobacco smoke exposure on COPD bronchial epithelial cell inflammatory responses.* International journal of chronic obstructive pulmonary disease, 2018. **13**: p. 989-1000.
470. Bengalli, R., et al., *Lung Toxicity of Condensed Aerosol from E-CIG Liquids: Influence of the Flavor and the In Vitro Model Used.* International journal of environmental research and public health, 2017. **14**(10): p. 1254.
471. Wilson, J.X., *Antioxidant defense of the brain: a role for astrocytes.* Can J Physiol Pharmacol, 1997. **75**(10-11): p. 1149-63.
472. Scott, A., et al., *Pro-inflammatory effects of e-cigarette vapour condensate on human alveolar macrophages.* Thorax, 2018. **73**(12): p. 1161.
473. Ween, M., P. Reynolds, and S. Hodge, *Hidden dangers of E-cigarettes: Airway macrophage dysfunction and altered inflammatory response.* European Respiratory Journal, 2016. **48**(suppl 60): p. OA4981.
474. Rubenstein, D.A., et al., *Tobacco and e-cigarette products initiate Kupffer cell inflammatory responses.* Mol Immunol, 2015. **67**(2 Pt B): p. 652-60.

475. Kim, H.J. and H.S. Shin, *Determination of tobacco-specific nitrosamines in replacement liquids of electronic cigarettes by liquid chromatography-tandem mass spectrometry.* J Chromatogr A, 2013. **1291**: p. 48-55.
476. Volkow, N.D., *Epigenetics of nicotine: another nail in the coughing.* Science translational medicine, 2011. **3**(107): p. 107ps43-107ps43.
477. ITO, K., et al., *Cigarette smoking reduces histone deacetylase 2 expression, enhances cytokine expression, and inhibits glucocorticoid actions in alveolar macrophages.* The FASEB Journal, 2001. **15**(6): p. 1110-1112.
478. Starke, R.M., et al., *Cigarette Smoke Modulates Vascular Smooth Muscle Phenotype: Implications for Carotid and Cerebrovascular Disease.* PLOS ONE, 2013. **8**(8): p. e71954.