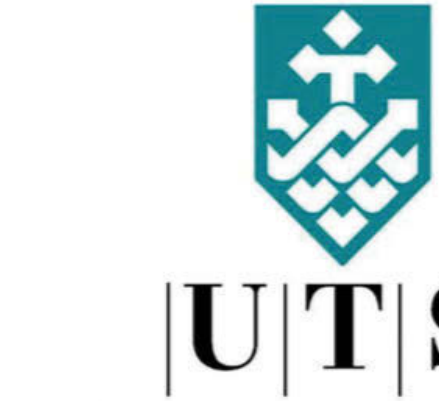
The effects of maternal cigarette smoke exposure on brain health in offspring

Yik Lung Chan

A thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy



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List of Abbreviations

(P)1	Postnatal day 1
ACEC	Animal Care & Ethics Committee
Akt	Protein kinase B
Ang II	Angiotensin II
ARNT	PER-arl hydrocarbon receptor nuclear translocator protein
Atg	Autophagy-related protein
BBB	Blood-brain barrier
CI, II, III, IV, V	Complex I, II, III, IV, V
Drp-1	Dynamin related protein-1
eNOS	Endothelial nitric oxide synthase
EPO	Erythropoietin
FADD	fas-associated death domain
Fis-1	Fission protein-1
H_2O_2	Hydrogen peroxide
HI	Hypoxic ischemic
HIE	Hypoxic ischemic encephalopathy
HIF-1α	Hypoxia inducible factor
ICAM-1	Intercellular adhesion molecule-1
IKKβ	IκB Kinase
IL	Interleukin
JNK	MAPK - c-Jun N-terminal kinases
L-C	L-Carnitine
LC3A/B-I/II	Microtubule-associated proteins light chain 1A and 1B
MAPK	Mitogen-activated protein kinase
MnSOD	Manganese superoxide dismutase
NADPH	Nicotinamide adenine dinucleotide phosphate
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NHS	Normal horse serum

NIK	NFκB - inducing kinase
Nox	NADPH oxidase
Nox	Nitric oxide
nSMase	Neuronal plasma membrane neutral sphingomyelinase
OHŸ	Hydroxyl radical
ONOOŸ	Peroxynitrate
Opa-1	Optic atrophy-1
OXPHOS	Oxidative phosphorylation
PBG	Phosphat buffered saline with goat serum
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline with Tween-20
PER	Period circadian protein
PI3-K	Phosphatidylinositol 3'-kinase
Pink-1	PTEN-induced putative kinase 1
PTEN	Phosphatase and tensin homolog
ROS	Reactive oxygen species
rtPCR	Real time polymerase chain reaction
SE	Smoke exposure
SELC	SE breeder, supplemented with L-Carnitine
SIM	Single-minded protein
src	sarcoma
Tdt	Terminal deoxynucleotidyl transferase
TNFα	Tumor Necrosis Factor α
TNFα receptor	(TNFR)1/2
TOM	Translocase of mitchondrial outer membrance proteins
TRADD	TNFR-associated dead domain
TRAF	TNFR-associated facotr
VCAM-1	Vascular cell adhesion molecule-1
VEGF	Vascular endothelial growth factor

Publications arising from this work

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Abstract

There are approximately 1 billion smokers worldwide with three million in Australia. Cigarette smoking contributes to a number of chronic diseases such as cardiovascular and cerebrovascular diseases. Although it is well known that maternal cigarette smoke exposure (SE) is detrimental to the health of offspring, more than 20% of women still smoke when they are pregnant. Previous studies only focused on brain structure, sizes and aetiology of the offspring from the smokers. However, none have investigated the impact of maternal smoking on the markers of inflammation, oxidative stress and mitochondrial wellbeing in the offspring's brain, whereas maternal smoking during pregnancy is linked to brain hypoxia-ischemic injury in the neonates and resulting cerebral palsy and associated disabilities in children. Mitochondrial integrity may play a key role, as they are the major powerhouse of the cells and vulnerable to increased oxidative stress. Mitophagy is a selective removal of damaged mitochondria by autophagy facilitated by fission and fusion. The former divides mitochondrion into healthy and damaged fragments; while the latter combines the healthy fragments to re-generate new mitochondria. Abnormal level of mitophagy markers have been observed in neurological conditions, such as stroke.

Thus, this thesis aimed to study (1) the impact of maternal cigarette smoke exposure on brain markers of inflammation, oxidative stress, and mitophagy in both dams and offspring at different ages; (2) the gender differences in response to maternal SE; (3) the impact of maternal L-Carnitine (antioxidant) supplementation during pregnancy and lactation on brain mitophagy and autophagy markers in offspring, and; (4) the impact of maternal SE on hypoxic ischemic (HI) injury in male offspring.

Virgin female Balb/c mice (6 weeks) were exposed to cigarette smoke (SE) or air (SHAM) 6 weeks prior to mating, during gestation and lactation. They were mated with male Balb/c mice (8 weeks). The pups were sacrificed at postnatal day (P) 1, P20 and 13 weeks for Aims 1-3 and P45 for Aim 4. In aim 4, hypoxic ischemic injury was induced in half of the litters via left carotid artery occlusion. Behaviour tests (novel objective recognition test, error ladder, grip test, and elevated plus maze test) were carried out in offspring with HI injury at P40 to assess motor and cognitive functions. The dams were sacrificed when the pups weaned. The brains of both dams and offspring were analysed

by western blotting, immunohistochemistry, and real-time PCR for markers of inflammation, oxidative stress and mitochondrial wellbeing.

It was found that brain inflammatory markers were increased in adult male SE offspring at 13 weeks, but not changed in female offspring by maternal SE. Brain endogenous antioxidant was reduced in male offspring, which was increased in female offspring by maternal SE. Mitochondrial oxidative phosphorylation (OXPHOS) complexes I, III and V were increased by maternal SE in male offspring but all OXPHOS complexes (I-V) were increased in female SE offspring. Brain cell damage was increased in male offspring but not in female offspring by maternal SE. Maternal L-Carnitine supplementation partially reversed the above-mentioned impacts of maternal SE in offspring's brain, including brain cell injury. HI injury reduced motor and cognitive functional outcomes in both SHAM and SE offspring but maternal SE did not worsen it. However, HI injury increased brain inflammatory markers in SE offspring, as well as mitochondrial fission markers. Autophagy and mitochondrial fusion markers were reduced by HI injury in male SE offspring. Apoptotic markers were also increased in SE offspring with HI injury.

In conclusion, maternal SE had adverse impact on the brain health in offspring with more impact on male offspring than females. Maternal L-Carnitine supplementation seems to partially reverse such maternal impact. Maternal SE can worsen the cellular outcome in the offspring's brain. Interventions to improve mitochondrial function may be plausible to mitigate the adverse impact of maternal SE.

Chapter 1
General Introduction

1.1 Smoking and maternal smoking

There are approximately 1 billion smokers worldwide (1). Although the smoking rate has decreased in Australia, in 2011 to 2012 there were still three million Australians aged 18 years and above smoking (13.3%) (2). However, this trend varies across different communities. The latest survey by the World Health Organisation in 2014 showed that the prevalence of tobacco smoke ranged from 15.8% to 34.1% in Australia, USA, UK, France, Germany and Italy (3). The smoking rate amongst the Indigenous population in Australia is even higher, with about 45% of indigenous people aged 15 years and over smoking in 2008, and this rate can be as high as 70% in some communities (4). Second-hand smoke exposure is also high in developing countries such as China, with a peak of 70% of adults exposed to second-hand smoking. About 82% of the world's population are not protected from second-hand smoking, including pregnant women (5). In combination of both first hand and second hand smokers, six million people are killed each year by smoking-related diseases (3).

Cigarette smoking is a significant risk factor for a number of chronic diseases, such as cerebrovascular and cardiovascular diseases, in addition to various respiratory disorders (6). Smoking can directly damage the endothelial cells lining blood vessels, contributing to the development of atherosclerosis. The latter can, in turn, directly lead to the onset of cardiovascular and cerebrovascular obstruction and infarction (7,8). Both cardiovascular and cerebrovascular infarction remain the top two leading causes of death in Australia, according to the latest census data from the Australian Bureau of Statistics (9).

Unfortunately, another major problem contributing to ongoing mother and baby health issues is maternal smoking. Although the number of women that smoke has been decreasing worldwide, the rate has slowed down recently and the figures vary across countries, with 15.3% in the USA (10), 16.3% in Australia, 23% in Argentina (11). Despite general education on the risk of smoking during pregnancy, it is currently estimated that approximately 20-45% women still smoke during pregnancy in Europe, Australia and South Africa; while 20% of pregnant women smoke in South America and 10% in Canada (12,13). Complete smoking cessation is difficult to achieve for at least 25% of pregnant women, especially in certain indigenous communities where the

maternal smoking rate is even higher (14,15). In Australia, among indigenous mothers, 50% reported smoking during pregnancy in 2008, and 68% of households have smokers (16,17). Cigarette smoke exposure during pregnancy is considered the leading preventable cause of adverse outcomes in newborns, such as low birth weight with small brain weight (18,19). Small brain volume is significantly correlated with lower intelligent quotient (20). However, the correlation between small brain volume and cognitive performance is still unclear. On the other hand, it has been shown that individuals with smaller hippocampus sizes are more susceptible to psychological trauma (21). A previous study also showed that verbal ability was positively correlated with cerebral volume (22).

Maternal smoking is an established risk factor for intrauterine growth restriction, perinatal morbidity and mortality, and childhood obesity (18,23). It is supported by the "foetal origin of adult disease" hypothesis proposed by Barker et al, who initially hypothesized that undernutrition *in utero* and during infancy permanently changes the physiological functions and leads to coronary heart disease and stroke in adult life (24). Barkers theory suggests that intrauterine undernutrition can permanently change energy metabolism in the body. For example, maternal smoking caused intrauterine shortage of nutrients and oxygen can result in intrauterine growth restriction and low birth weight of the offspring (25). His hypothesis also proposes that the limited nutrients are redistributed to ensure vital organ development such as the heart and the brain, on the cost of of the development of the liver and skeletal muscle, which predisposes the individual to certain chronic metabolic disease in later life, such as diabetes mellitus (26). Although the brain gets the priority for nutrient delivery, human studies have shown that maternal smoking can cause long lasting adverse effects on the structural or functional development of foetal brain, such as the reduction of brain volume and cognitive disorders (reviewed in (27)). The reduction of brain volume may be due to two mechanisms: one is the lower birthweight in such offspring (28), and the other is the direct toxic effect of nicotine, cyanide and carbon monoxide contained in cigarette smoke (28).

In human studies, it was found that there were small but significant differences in verbal and visual memory abilities in children aged between 12-18 years old from smoking mothers and non-smokers (29-31). A study also reported that smoking (>20

cigarettes/day) during pregnancy can increase the risk of internalising behaviours such as fear, anxiety and sudden change of mood in children at 18 months and 36 months of age (32). In addition, maternal smoking can increase the risk of attention deficit hyperactivity disorder in offspring, in a dose dependent manner (33). These studies reflect that maternal smoking is detrimental to the next generation. Hence smoking during pregnancy has been considered as a major and significant public health issue. However, there are conflicting findings in different studies because of the confounding factors such as socio-economic factor of the parents, maternal mental health, alcohol consumption, demographics and paternal smoking, which can often yield negative health outcomes (32). Therefore, an animal model can remove these confounding factors to determine the impact of a single factor on cognitive outcome in offspring.

Although it is understood that smoking and maternal smoking can result in various adverse outcomes, there are a limited number of studies focusing on the direct impact of cigarette smoke exposure on brain health outcomes, with even less focus on maternal smoking. A keyword search using “smoking” or “maternal smoking” or “cigarette” or “tobacco” and “brain(s)” using advanced search option on the PubMed website resulted in 282 publications with the earliest study being reported in 1961. Of these studies, only eleven publications are on the impact of maternal smoking on brain health in offspring (27,34-43). It is important to note, all the eleven studies are related to brain structure, sizes and aetiology, with none of them focusing molecular aspects of inflammation, oxidative stress and mitochondrial wellbeing in the brain.

A keyword search using “nicotine” and “brain(s)” in the title field resulted in 624 items in total (Figure 1.1). However, cigarette smoke consists of more than 4000 ingredients. Thus, nicotine alone cannot represent the collective impacts of cigarette smoke on the brain. Therefore, there is limited information on the impact of direct cigarette smoke exposure and maternal cigarette smoke exposure on different organs in offspring. This insightful analysis highlights the need to have better understanding of the molecular aspects involved in the impact of maternal smoking on offspring

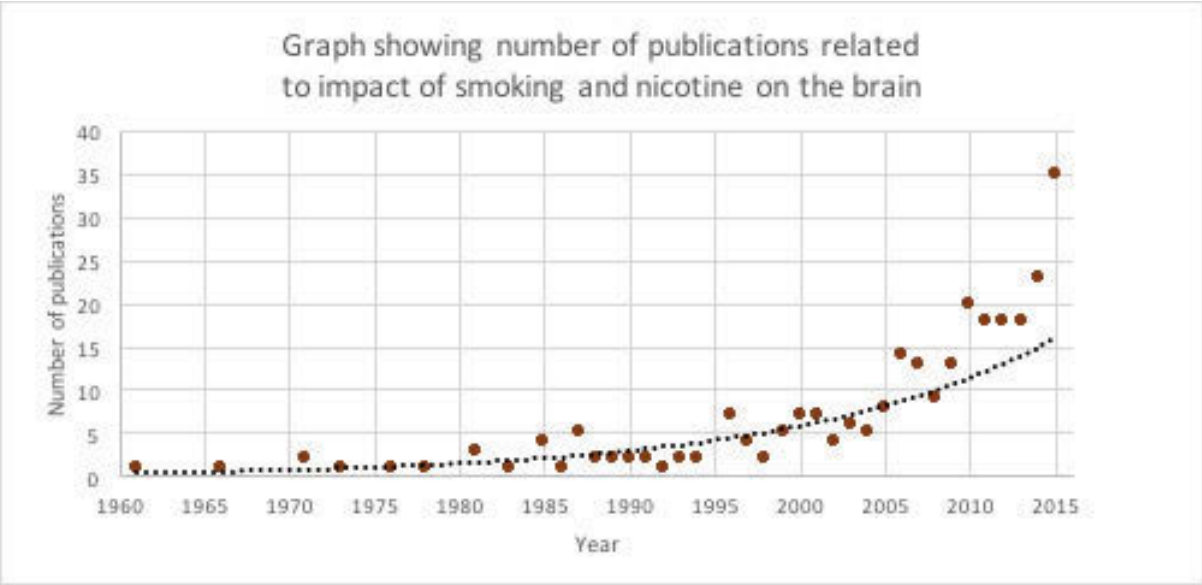

Figure 1.1 The number of publications on the impact of smoking and nicotine on brain.

1.2 Inflammation in the brain

Smoking is known to induce systemic inflammation, characterised by increased blood levels of pro-inflammatory cytokines (such as Tumor Necrosis Factor-α (TNF-α), Interleukin (IL)-1, IL-6 and IL-8) and decreased levels of anti-inflammatory cytokines (such as IL-10) (44-47). It has been suggested that chemicals inside cigarette smoke can trigger the production and release of potent pro-inflammatory cytokines and chemokines by different immune cells through a series of molecular pathways (48). Among them, nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) plays an important role. In this section, the classical pro-inflammatory cytokines induced by smoking in the brain will be discussed.

1.2.1 IL-1

IL-1 can be produced by all cell types located at the blood-brain barrier (BBB), including endothelial cells, astrocytes, microglia, oligodendrocytes, as well as neurons; with microglial cells being the primary source of locally derived IL-1 in the brain (49). The IL-1 family consists of two ligands, IL-1α and IL-1β (49). IL-1 receptor (IL-1R) is expressed by all brain cell types except for microglia (49). The neurons contain the same IL-1R signalling elements as the classical Mitogen-activated protein kinase (MAPK)/NFκB pathway as in the glial cells (Figure 2) (49). Mice lacking IL-1R have been shown to have lower levels of neural inflammation (50). However, recent studies also showed that IL-1 activates neuronal plasma membrane neutral sphingomyelinase

(nSMase), leading to the production of ceramide and activation of sarcoma (src) kinase (51,52). Ceramide can induce the signal transduction of TNF-α, which could be either neuroprotective or neurotoxic depending on the downstream signalling pathways activated (53,54). As shown in Figure 2, IL-6 and TNF-α can also be produced through this pathway.

During cerebral ischemia, there is a significant increase in the release of IL-1 from the microglia, astrocytes, and affected neurons (55-60). In the rat, intraventricular injection of recombinant human IL-1 after middle cerebral artery occlusion can enlarge brain injury which was characterized by an influx of neutrophils with high adherence in the ischemic brain regions (61,62). The deletion of IL-1α/β in mice can reduce the size of ischemic brain injury by almost 80% (63). However, the mechanism of how IL-1 propagates tissue damage remains unclear. After cigarette smoke exposure, IL-1 mRNA expression was found to be increased in the rat brain (64), which may predispose those rats to more severe tissue injury damage if cerebral ischemia occurs.

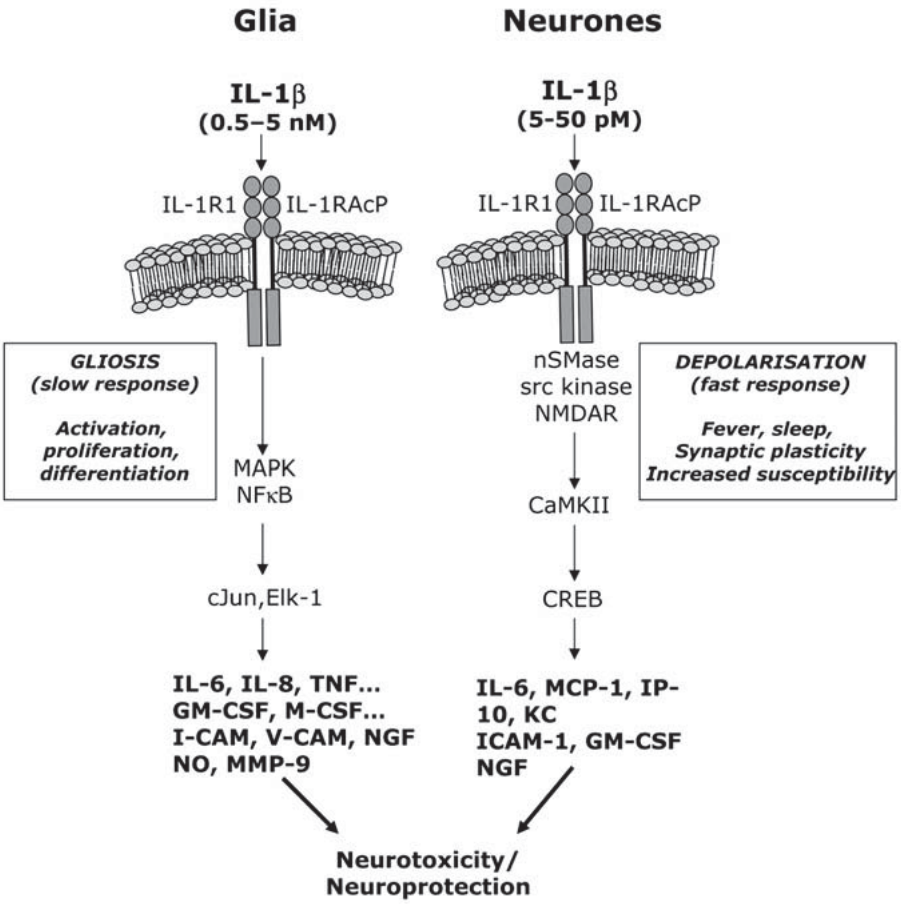

Figure 1.2. IL-1 signalling pathways in glia cells and neurons (adapted from (65)).

In glial cells, IL-1 functions through MAPK/NFκB pathway leading to the production of pro-inflammatory cytokines, such as IL-6, IL-8, TNF-α, as well as adhesion molecules, including intercellular adhesion molecule-1 (ICAM-1), and vascular cell adhesion molecule-1 (VCAM-1). In the neurons, IL-1 activates nSMase/src kinase signalling pathway to produce pro-inflammatory cytokines and adhesive molecules.

1.2.2 IL-6

Many major cell types in the brain can synthesise IL-6, which serves as a crucial molecular messenger between leucocytes, vascular endothelium and resident cells of the parenchyma. There are three major steps in the IL-6 signalling pathway. IL-6 binds to plasma membrane IL-6 receptor complexes containing signal transducing receptor chain glycoprotein 130 (Gp130) (66). Then Gp130 molecules dimerise, which leads to the activation of multiple downstream intracellular signal transduction pathways, such as Janus kinase / signal transducer and activator of transcription (JAK/STAT) (67).

In the brain, the role of IL-6 remains controversial. Several studies showed that IL-6 is either detrimental or beneficial in brain damage including stroke and traumatic brain injury (68). *In vivo* studies showed that increased levels of IL-6 can improve traumatic brain injury recovery (69,70). IL-6 has also been found to be elevated in cerebral spinal fluid and striatum in patients with Parkinson's disease (71). Temporal IL-6 elevation can promote a marked distribution of Parkinson associated proteins ubiquitin, α-synuclein and tau in cultured glial cells, as these proteins are less likely to aggregate without the stimulation of IL-6 in this cell type (72). This can act as a natural protection for the microglia cell against oxidative damage by hydrogen peroxide (73).

The contribution of IL-6 to the inflammatory response of the brain is similar to IL-1 (74). During stroke, IL-6 mRNA expression is upregulated within the first 3 hours after arterial occlusion, reaches a peak at 12 hours and persists for at least 24 hours (75). According to computed tomography and magnetic resonance imaging, a high serum level of IL-6 is closely correlated with large infarction size and poor clinical outcome in patients with stroke (76). In patients with Alzheimer's disease, IL-6 is increased around amyloid plaques and in cerebral spinal fluid in humans (77-80). Another study also showed that IL-6 can induce the production of amyloid precursor protein in primary rat

cortical neurons to enhance detrimental neurodegenerative process (81). Similar to IL-1, brain mRNA expression of IL-6 was higher in rats exposed to cigarette smoke compared with sham exposed controls (82). Increase in brain IL-6 was found to increase the risk of neurodegenerative diseases such as Alzheimer's diseases (83) with the mechanism unknown.

1.2.3 TNF–α

In the brain, TNF-α is mainly produced by the astrocytes (84), microglia (85) and neurons (86,87). Inactive TNF-α is a large trans-membrane protein (26 kDa) and through 'ectodomain shedding', TNF-α converting enzyme cleaves the extracellular domain of inactive TNF-α, which is then transformed into an active protein (17 kDa) (88).

TNF-α was initially discovered as a pro-inflammatory cytokine which can induce cell death in tumor cell lines *in vitro* and transplanted tumors *in vivo* (89). The functions of TNF-α include cell proliferation and differentiation (90,91), gene transcription (92,93), and upregulation of neuroprotective mediators (94,95). In addition, TNF-α can increase the expression of adhesion factors, such as ICAM-1 and VCAM-1 in cerebral endothelial and glial cells (96). ICAM-1 and VCAM-1 facilitate the migration and accumulation of neutrophils in the microvessels to induce further inflammatory responses (96-98).

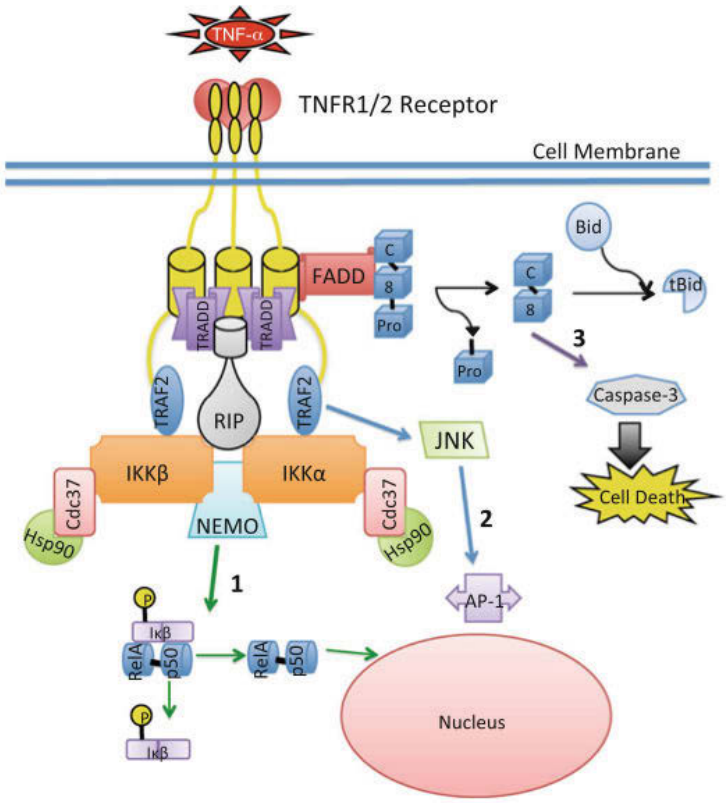

Figure 1.3. Signalling pathways of TNF-α receptor (TNFR)1/2 (adapted from O'Connor et al (99)). The Activation of TNFR1 leads to the configuration of TNFR-associated death domain (TRADD) and fas-associated death domain (FADD). TRADD then recruits TNFR-associated factor (TRAF)-2, leading to cell death and inflammation; while FADD activates the caspase-3 cascade leading to cell death.

As shown in Figure 1.3, there are two subtypes of TNF-α receptor (TNFR), low affinity TNFR1 (p55) and high affinity TNFR2 (p75) (100). TNF-α binding to TNFR leads to the activation of TNF receptor - associated death domain (TRADD), as well as fas-associated death domain (FADD) (101). This in turn initiates the apoptotic-signalling cascade and the activation of caspase-8 (102). A subsequent cleavage of effector caspases leads to cell apoptosis (101). TRADD also acts as a signal diverter with receptor-interacting protein and TNFR-associated death domain (TRAF)-2. TRAF2 recruits IκB Kinase (IKKβ), which then activates nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor alpha (IκBα), to degrade and release NFκB (103).

The other pathway involves the interaction between TRAF2 and its downstream signalling molecule NFκB - inducing kinase (NIK), which belongs to the MAPK family (104). TRAF-2 can activate the MAPK - c-Jun N-terminal kinases (JNK) cascade, which in turn leads to activation of several transcription factors, such as activator protein 1 which controls cellular processes including differentiation, proliferation, and apoptosis (105).

In humans, TNF-α concentrations in the blood are significantly higher in smokers than non-smokers, and are even higher in individuals smoking more than 1 pack/day (46). High circulating TNF-α levels and receptor signalling activities have been found in many neurological disorders, such as stroke, Alzheimer's disease, Parkinson's disease, multiple sclerosis, and traumatic brain injury (106-110). In the spontaneous hypertensive rat, brain mRNA expression of TNF-α peaked within 6-12 hours following middle cerebral artery occlusion (111). This is accompanied by an increase in IL-6 and IL-1β mRNA expression in the first several hours after ischemic injury (75). In another study performed on 23 stroke patients, serum TNF-α levels were increased within 24 hours of the onset of stroke, which was positively correlated with the volume of brain infarction (112).

1.2.4 Toll like receptor (TLR)–4

The TLR family is crucial for the innate immunity and defense mechanisms against infections (113). TLRs are classified as pathogen recognition receptors and they are initiated through pathogen-associated molecular patterns. For example, TLR4 can recognize the lipopolysaccharide moiety on the surface of gram-negative bacteria (114).

However, the innate immune response can also be initiated when there is tissue injury even in the absence of infection (115), known as sterile inflammation. During this process, endogenous molecular messengers and danger-associated molecules are released, which subsequently lead to the activation of a protein myeloid differentiation primary response gene 88 (116,117). This can also activate NFκB (118), as well as the synthesis of inflammatory mediators (including pro-inflammatory cytokines and chemokines), ICAM-1 and VCAM-1 (119). Activated NFκB pathway can also lead to

the release of pro- and anti-inflammatory cytokines, such as IL-1β, IL-6, IL-8, TNF-α, and IL-10 (118,120,121).

Studies have shown that cigarette smoking is linked to an increase in TLR4 mRNA in lung endothelial cells in patients with chronic obstructive pulmonary disease (122-124). In an experiment by Karimi et. al, cigarette smoke exposure induced IL-8 production in human monocyte-derived macrophages through a TLR4 signalling pathway dependent mechanism, which involves NFκB phosphorylation (125). It is known that an increase in TLR4 level can increase the vulnerability to neonatal hypoxic-ischemic injury through the activation of MyD88-dependent pathway leading to the production of downstream IL-1β and TNF-α in rats (126). In contrast, TLR4 can also promote the clearance of amyloid plaques by microglia suggesting a decrease in TLR4 level may also increase risk of Alzheimer's disease in mouse. However, the link between smoking and TLR4 change in the brain is unknown.

1.3 Maternal smoking and brain inflammation

Studies on the impact of smoking on brain inflammation are scarce. The majority of studies have focused on how cigarette smoke constituents impact on systemic immune response (reviewed in (44)). Reactive oxygen species (ROS) from burning cigarettes are not removed by the cigarette filters (127), which lead to the activation of inflammatory responses causing immune cell recruitment and chronic inflammation. One pathway that can be activated by ROS is the NFκB pathway as described in the previous sections. This pathway can be activated by cigarette smoke extract in various myeloid and lymphoid cells, including human T cells (128). In addition, another research focus on the impact of cigarette smoke is chronic inflammatory disorders of the airways. A study showed that cigarette smoke can cause airway inflammation even in smokers with normal lung function (129) suggesting that chronic inflammation can still occur without inducing functional abnormalities. ROS from cigarette smoke can activate inflammatory responses through stimulating macrophages which produce even more ROS than cigarette smoke itself during lung inflammation (130). This endogenous production of ROS, along with ROS from cigarette smoke might induce chronic inflammation not only in lung but also in the brain. In a more recent study by Khanna and colleagues, cigarette smoke has been shown to increase the gene expression of a number of

inflammatory cytokines, such as TNF- α, IL-1α, IL-1β and IL-6 in the brain of Lewis rats (64). Prolonged elevation of the inflammatory response in pregnant smokers may also affect the offspring as they are indirectly exposed to cigarette smoke in the womb, leading to adverse health outcomes in the long term. Chronic increase in blood inflammatory cytokines such as IL-1β, IL-6 and cerebrospinal fluid TNF-α in offspring of smoking mothers was shown to increase the severity of schizophrenia or autism spectrum disorders (131-134). Furthermore, the increase in brain inflammatory cytokines (such as IL-6) due maternal high fat diet consumption during the gestational period was found to increase anxiety-like behaviour in rat offspring (135). Intraventricular injection of adenovirus encoding IL-6 into mice can also induce autism like behaviour (136). Chronic inflammation in the brain may also contribute to the progression of neurodegenerative diseases such as Parkinson's disease and Alzheimer's disease (137). A similar stimulus to maternal smoking is air pollution as it is a confounding factor of maternal smoking according to the studies on air pollution and infant mortality (138). Indeed, long term maternal air pollutant exposure is associated with increased neuroinflammation in children (139). Air pollution contains harmful substances such as particular matter < 2.5 microns, carbon monoxide, sulphur dioxide and nitrogen dioxide (140) which are also found in cigarette smoke (141,142). This implies that maternal smoking may have similar impact on offspring as air pollution.

1.4 Hypoxia in the brain

One consequence of cigarette smoking is increased risk of cerebrovascular disease that can lead to ischemia (143). During ischemia, the delivery of oxygen and glucose is largely reduced in the area surrounding the ischemic core to form a region of brain tissue that is functionally impaired but structurally intact, known as the ischemic penumbra. In the penumbra, reduced oxygen supply occurs due to insufficient blood flow resulting in hypoxia, which can disrupt brain function, but is not severe enough to lead to a complete and irreversible reduction of cellular metabolism and tissue loss (144). Cigarette smoking *per se* can reduce blood oxygen saturation rate, thus is able to lower organ oxygen supply leading to chronic hypoxia (145).

1.4.1 Hypoxia inducible factor (HIF)–1α

Under hypoxic conditions, there are systemic and intracellular adaptations to minimise hypoxic injury and replenish oxygen supply. During hypoxia, there is an initial maximisation of ventilation and cardiac output to increase oxygen exchange and delivery (146). When oxygen levels are reduced, the transcription factor HIF-1 regulates gene expression for cellular adaptation, including those facilitating vasomotor control, angiogenesis, erythropoiesis, cell proliferation, and energy metabolism (147-149). HIF-1α was first discovered as the transcriptional activator of erythropoietin (EPO) (150). HIF-1 consists of HIF-1α and HIF-1β subunits, which belong to basic-helix-loop-helix period circadian protein (PER)-aryl hydrocarbon receptor nuclear translocator protein (ARNT) - single-minded protein (SIM) family of transcriptional factor (151). HIF-1α acts as the central regulator of hypoxia related homeostatic changes (150).

There are a few proposed mechanisms for the neuroprotective effects of HIF-1. The downstream target gene of HIF-1, EPO has been found to protect cells from hypoxic injury (152). EPO is a glycoprotein that stimulates the production of red blood cells to increase oxygen supply (153,154). *In vivo* studies showed that EPO administration can protect neurons against ischemia-induced cell death in rodents (155). Another downstream target gene of HIF-1 is vascular endothelial growth factor (VEGF), which can double the chance of cell survival in HN33 cells (hippocampal neuronal cell line) after 24 hours oxygen and glucose deprivation (156). VEGF increases endothelial cell growth to promote angiogenesis (157,158) and also has an anti-apoptotic effect through VEGF receptor-2 and the phosphatidylinositol 3'-kinase (PI3-K)/ protein kinase B (Akt) signalling pathway (159). Mice exposed to cigarette smoke showed an impaired angiogenesis following surgically induced hind limb ischemia, which was closely associated with a reduced expression of HIF-1α as well as VEGF (160). The injection of adenovirus encoding a stable form of HIF-1α can restore the expression of VEGF and completely reverse the inhibition of angiogenesis caused by cigarette smoke exposure (160).

Apoptosis starts with the decrease in mitochondrial membrane potential and the release of cytochrome c from the mitochondria (151). Cytochrome c then activates caspase-9 and its downstream signal caspase-3 leading to apoptosis. HIF-1α can inhibit the release of cytochrome c from the mitochondria to prevent apoptosis and can also activate Akt,

which inactivates caspase-9 to inhibit apoptosis (151). In a rat model of stroke, HIF-1α mRNA was up-regulated in the penumbra, together with increased expression of glucose transporter-1 and several glycolytic enzymes (161). Thus, hypoxia induced HIF-1α may increase cellular glucose uptake and metabolism in the affecting cells to increase survival rate.

However, HIF-1α may also contribute to cellular and tissue damage via promoting P53-dependent apoptosis during hypoxia or ischemia. P53 is a transcriptional factor in the central nervous system that takes part in the pathologic process of ischemic injury (162). It has been shown that p53 restrains cell growth and re-establishes the process of apoptosis (163). A study by Federoff and colleagues showed that HIF-1α can induce a delay in cortical neurons death both *in vivo* (164) and *in vitro* (165) via p53 pathway. Autophagic cell death can also be induced through HIF-1α in microglia under hypoxic conditions such as ischemic stroke (166).

1.4.2 Early growth response factor (EGR)–1

EGR1 was first discovered by Lau and Nathans in mouse fibroblasts induced by the growth factor (167). EGR1 is also known as zinc finger protein 22, nerve growth factor-induced gene A, gene containing sequences homologous to Drosophila Kr finger probe and tetradecanoyl phorbol acetate-induced sequence 8 (168). This is because that EGR1 was identified by different groups independently.

In the lung, EGR1 has been found to be a toggle switch (169). In response to ischemia, EGR1 triggers the release of pivotal regulators of inflammation, coagulation, and vascular hyperpermeability (169). It can be postulated that an upregulation of EGR1 during cerebral ischemia may exert similar functions as those in the lung. In addition, EGR1 is suggested to be an important transcriptional regulator for glial scar formation (170). At the ischemic lesion site, astrocytes accumulate in the penumbra. These astrocytes provide neuroprotection by forming a glial scar to isolate the healthy brain area from the necrotic areas caused by tissue infarction (171). The development of a glial scar involves the production of large amount of basic components and extracellular matrix (ECM) to form the scar tissues. EGR1 regulates the production of ICAM-1, coagulation protein, and ECM components (170). More importantly, EGR1 works together with HIF-1α to activate VEGF and plasminogen activator inhibitor-1 (PAI-1)

to reduce ischemic injury (169,172,173). Therefore, upregulated EGR1 plays a crucial adaptive role in post-ischemia event in the brain.

1.5 Oxidative stress in brain

When the cellular production of oxidative molecules overwhelms endogenous anti-oxidant defense systems, oxidative stress occurs (174). Excessive oxidative molecules can have a negative impact on vascular regulation leading to hypertension (174), which can further lead to serious cerebrovascular complications that increase the risks of ischemic and haemorrhagic stroke, as well as some cognitive disorders (175-177) such as Alzheimer's disease (174). Cigarette smoke has been reported to induce oxidative stress through the release of endogenous ROS upon stimulation.

1.5.1 ROS

When oxidative stress occurs, there is an overproduction of ROS, such as superoxide, hydrogen peroxide, hydroxyl radical (OH•) and peroxynitrite (ONOO•) (178,179). ROS are normally generated by four enzyme systems, including nicotinamide adenine dinucleotide phosphate (NADPH) (180), xanthine oxidase (181), uncoupled endothelial nitric oxide synthase (eNOS) (182-186), and mitochondrial electron transport chain (187).

Within the NADPH oxidase (Nox) family, mRNA expression of Nox1, Nox2, and Nox4 catalytic subunits, p22phox and its cytosolic subunits p47phox, p67phox, NoxO1, and NoxA1, are expressed in the rat basilar artery (188). These cytosolic subunits are crucial for the production of superoxide (189). Nox1, Nox2, and Nox4 are also expressed in cerebral arteries (190). Both Nox1 and Nox2 can increase the production of ROS in the cerebral arteries of mice (191-193) and rats (190), through angiotensin II (Ang II), which is a potent vasoconstrictor. A study has also shown that ROS induced by Nox1 and AngII can be attenuated in peripheral arteries through the activation of the Angiotensin II type I receptor by various mechanisms such as inhibiting tyrosine kinases or nitric oxide inactivation (194). At the same time, ROS are also powerful cerebral vasodilators which can lead to increase in blood flow (195). High level of ROS

in brain is also linked to various neurological disorders such as Parkinson's and Alzheimers disease and acute brain injury (196).

However, it seems that xanthine oxidase, mitochondrial electron transport chain, and eNOS play less of a role in ROS production, compared with NADPH (174). Xanthine oxidase donates electrons to oxygen to form O_2^- and hydrogen peroxide (197), which has been suggested to contribute to endothelial dysfunction during hypercholesterolemia (197). In the mitochondrial electron transport chain, O_2^- is formed (198). Cardiovascular diseases are closely associated with mitochondrial dysfunction and increased production of ROS during the development of atherosclerotic lesions (199). The brain is especially susceptible to ROS damage since it is a major organ to metabolise oxygen (20% of the body consumption). The increase in ROS has been linked to mitochondrial calcium uptake which can increase the permeability of mitochondrial membrane and eventually lead to cell death (200). In Lewis rat brains, cigarette smoke exposure has been shown to increase ROS triggered transcription factor, nuclear factor (erythroid-derived 2-like) 2, and inducible nitric oxide synthase indicating increased oxidative stress with reduced level of endogenous antioxidant superoxide dismutases in the brain (82). Cigarette smoke exposure can also increase the release of ROS and reactive nitrogen species in a human brain microvascular endothelial cell line (hCMEC), regardless of the concentration of nicotine level (201).

Another concern with cigarette smoke exposure is the integrity of BBB. Brain microvascular endothelial cell line is a common *in vitro* model to study BBB integrity. A study showed that when human hCMEC were exposed to cigarette smoke extract (4mg/ml), there were a significant increase of ROS (202). Co-cultured human brain cells (glioblastoma multiforme) and hCMECs also showed that cellular integrity of hCMECs was decreased from 74% to 64% by cigarette smoke extract due to increased ROS levels (202), suggesting that cigarette smoke can directly damage the BBB. When the integrity of BBB is damaged, the brain is more vulnerable to the neurodegenerative disease such as Alzheimer's diseases, as reduced BBB integrity can compromise the clearance of amyloid beta-peptide (203).

1.5.2 Manganese superoxide dismutase (MnSOD)

There is a complex antioxidant defense system to scavenge excess ROS consisting of enzymes copper-zinc SOD, MnSOD, catalase, glutathione peroxidase, as well as vitamins A, C, and E (204). This is especially important in the brain as neurons are vulnerable to oxidative stress. The first line defense against ROS are the superoxide dismutases. However, the most crucial antioxidant in the central nervous system is MnSOD which is present at a higher concentration in mitochondria than the other intracellular components. Oxidative phosphorylation (OXPHOS) complex I and III can produce ROS during normal energy metabolism (205,206); therefore, mitochondrial MnSOD is important for removal of excessive ROS (207). Interestingly, it has been demonstrated that there is a MnSOD surge during late gestation and newborn periods in mice, possibly to protect newborns against oxidative stress. Subsequently, the MnSOD level is reduced as mice reach postnatal day 4 (204). Several studies have shown that MnSOD is crucial for neuroprotection (208,209). MnSOD was able to prevent neuronal apoptosis and reduce ischemic brain injury through the suppression of peroxynitrate, lipid peroxidation as well as mitochondrial dysfunction (208).

Studies focusing on changes in brain mitochondrial MnSOD levels by smoking or maternal smoking are scarce. Most previous studies have investigated the impact of cigarette smoke exposure on MnSOD changes in peripheral tissues such as oesophagus (210), lung (211) and endothelial progenitor cells (212). MnSOD levels were found to be increased in these studies in order to scavenge overproduced ROS by long term smoking. An exhaustion of MnSOD may cause mitochondrial damage due to overproduction ROS damage in the long term.

1.5.3 Translocase of mitochondrial outer membrane proteins (TOM)

The TOM complex is the main entry portal for most cytoplasmically synthesized mitochondrial proteins. The main function of TOM complexes is to facilitate the interaction of protein transportation through the outer membrane by assembling the appropriate complexes onto the mitochondrial membrane (213). The import pathway starts from the Tom40 complex, followed by the other Tom complexes, such as Tom20, Tom22 and Tom7 (214). Among these Tom complexes, Tom20 (a peripheral subunit of the Tom40 complex) has two roles, recognising the protein precursors as well as importing these precursors (215). Tom 40 forms ion channels in lipid bilayers (216) and

interacts with polypeptide chains during transportation of protein precursors from the outer mitochondrial membrane to the inner mitochondrial membrane (217). Tom 20 is involved in the translocation of most protein precursors (218) by facilitating protein insertion at the outer mitochondrial membrane (219) (Figure 1.4) .

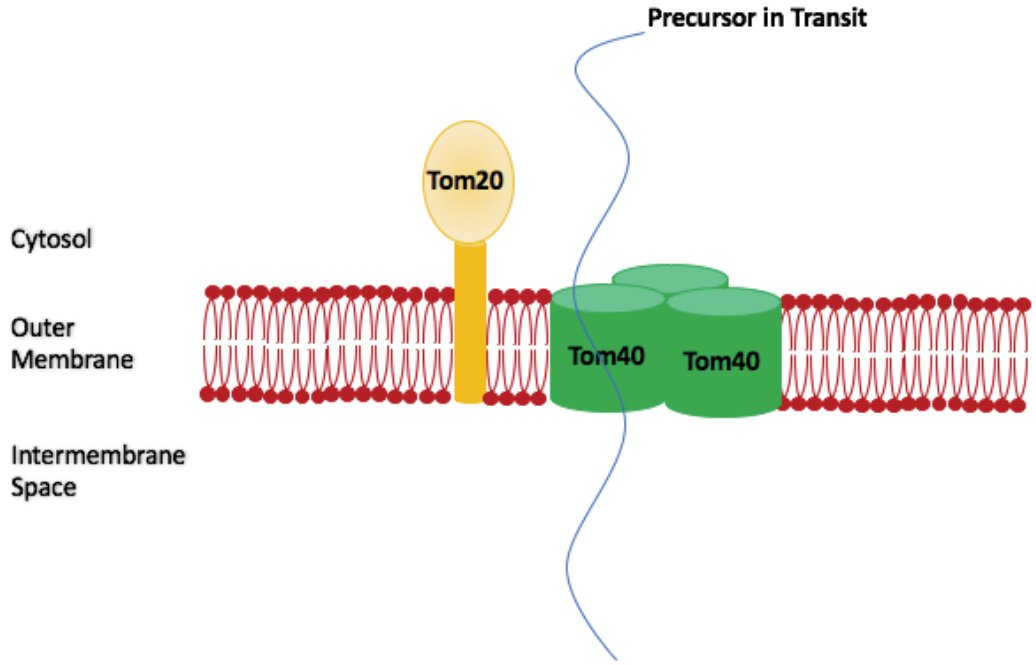

Figure 1.4. The location of Tom20 and Tom40 at the outer membrane of mitochondrion. (Modified from (220))

It has been shown that oxidative stress and impaired mitochondrial function are mediated through the decrease in Tom40 in alpha–Synuclein transgenic mice (221). An overexpression of Tom40 could thus ameliorate energy deficiency as well as oxidative stress burden in these mice (221). Tom20 is among the proteins that are degraded by the proteasomes under oxidative stress (222). However, brain changes in both Tom20 and Tom40 in response to smoking have never been reported. Understanding how maternal smoking impacts the Tom proteins can help to understand the change in brain mitochondrial functions in offspring.

1.5.4 OXPHOS complexes I – V

One main function of mitochondria is the production of ATP during oxidative phosphorylation. This process takes place at the cristae of mitochondrion and is facilitated by OXPHOS complexes I - V. Complex I is an L shaped protein that acts as the first entry point of electrons in the respiratory chain. Complex II is the second entry

for electrons to the respiratory chain. Complex III facilitates electron transfer to complex IV. The generated protons from complex I, III and IV drive ATP synthase by converting ADP to ATP (223). A significant byproduct during oxidative phosphorylation is ROS (Figure 1.5).

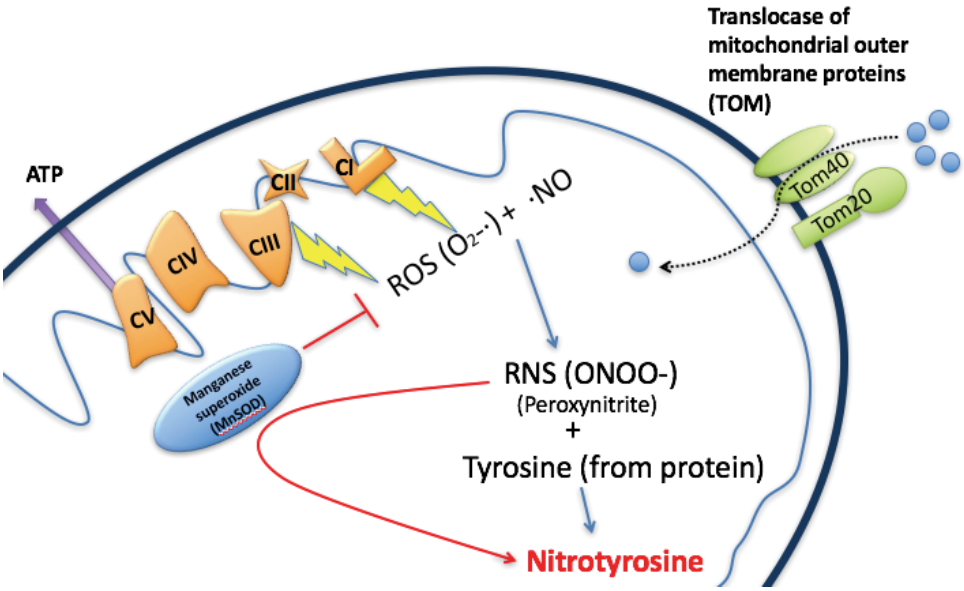

Figure 1.5. Schematic diagram showing how ROS is generated by OXPHOS proteins and interact with MnSOD. The proteins enter mitochondria through Tom20 and Tom40 complexes. OXPHOS complex V generates ATP when Complex I and Complex III produce ROS. ROS combine with nitric oxide (NO) to form peroxynitrite which can nitroxylate MnSOD to form nitrotyrosine.

It is well known that defects in different OXPHOS complexes could lead to different brain diseases. Complex I is reduced in Parkinson's disease; while mitochondrial complex III protein is reduced in human Alzheimer's brains (224). During pediatric traumatic brain injury, complex I and II protein levels are reduced at 24 hours (225). In a primate model of Huntington's disease, Complexes II and III are reduced (226), which has been linked to cognitive and motor deficits (227). The inhibition of complex I and IV has often been observed in amyotrophic lateral sclerosis leading to the degeneration of neurons (228). In patients with Down's syndrome, Complex V is significantly reduced in the frontal cortex (229).

In terms of the impact of cigarette smoke exposure, mitochondrial complex II activity is reduced in fetal mitochondria of mice exposed to cigarette smoke (230). On the other hand, prolonged cigarette smoke exposure can increase complex II, III and IV, along

with high levels of pro-inflammatory IL-6, IL-8 and IL-1β in airway epithelial cells of patients with chronic obstructive pulmonary disease (230). Cigarette smoking can also reduce mitochondrial oxidative phosphorylation in platelets (231). Rats exposed to cigarettes smoke from two cigarettes per day for two months also displayed severely damaged myocardial oxidative phosphorylation function during ischemia reperfusion injury (232). Therefore, the changes in complex I to V vary between organs in response to different insults.

ROS generated from complex I and III can form peroxynitrate with nitric oxide (Figure 1.5) (233). Peroxynitrate causes tyrosine nitration in proteins to form 3-nitrotyrosine. This type of nitration is associated with oxidative stress and increased NOS (233). MnSOD competes with nitric oxide to react with superoxide which prevents the generation of peroxynitrate and 3-nitrotyrosine. A persistent high level of 3-nitrotyrosine can cause mitochondrial damage (234). When MnSOD is reduced, mitochondria are less protected from oxidative stress in disease states. Indeed, it was found that there is an increase in brain 3- nitrotyrosine in patients with traumatic brain injury (235). Nitration itself can also cause inactivation of MnSOD, enhancing the production of peroxynitrate and secondary damage (236). It has been shown that both prenatal tobacco smoke exposure (237) and maternal nicotine exposure (238) can lead to mitochondrial damage in cardiovascular tissues with reduced SOD activity and increased oxidative stress. However, there have been no studies indicating whether maternal smoking could lead to mitochondrial damage in the brains of the offspring. How brain mitochondrial MnSOD, OXPHOS complexes, Tom20 and Tom 40 proteins are affected by smoking or maternal smoking is not yet known.

1.6 Mitochondrion, autophagy and mitophagy

Mitochondria were first observed as bean-shaped organelles in seminal electron microscopy studies performed in the 1950s (239). Mitochondria are double membrane bound organelles involved in a number of cellular processes such as ATP production, metabolite synthesis, calcium homeostasis, ROS production and even cell death (240,241). In the brain, there is a high density of mitochondria in neurons due to these cells having high metabolic and energy requirement. Mitochondrial dysfunction occurs in a significant number of brain diseases such as Amyotrophic lateral sclerosis,

Alzheimer's disease, Parkinson's disease suggesting that healthy mitochondria are of great importance in brain health (242).

There is misconception that the shape of the mitochondria remains unchanged. However, the fact is that mitochondrial structure is highly dynamic. Normally mitochondrial shape is maintained through a process called 'mitophagy' derived from the word 'autophagy'. The term autophagy means "self-eating". It is a strategy for cells to remove or repair damaged proteins. Autophagy is also the main mechanism for mitochondrial turnover during development and under pathophysiological conditions. More specifically, the term "mitophagy" is used for describing the selective removal of mitochondria by autophagy (243). Mitophagy is facilitated by fission and fusion. These two processes are balanced at steady state to maintain the overall morphology of the mitochondria. Mitochondrial fission is the separation of damaged portion of mitochondria from the healthy fragment; while mitochondrial fusion combines two healthy mitochondrial fragments to form a new mitochondria (244). In response to stress, mitochondria can change their morphology by changing their fusion and fission ratio. High fusion to fission ratio can lead to less mitochondria, with an elongated and more interconnected shape; low fusion to fission ratio leads to small spheres and short rods of mitochondria, often referred as 'fragmented mitochondria'(239). Failure to trigger mitophagy in brain has been shown to lead to neurodegenerative diseases such as Parkinson disease (245). There are different molecules responsible for mitochondrial fission, fusion and autophagy respectively. The process of mitophagy is summarised in Figure 1.6. Many of these markers have not yet been studied in the brain in response to smoking and the impact of maternal smoking on brain mitophagy has never been reported.

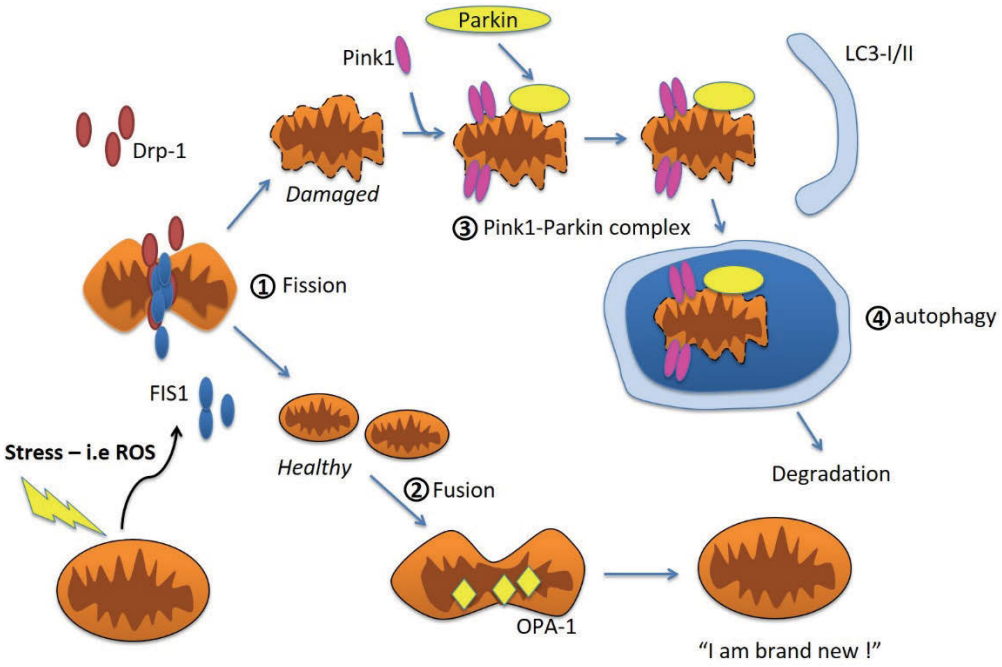

Figure 1.6. Schematic diagram of the process of autophagy and mitophagy. Damaged mitochondrial fragments are separated from the healthy part facilitated by Drp-1 and Fis-1. Damaged mitochondria attract Pink-1 and Parkin. This complex is then engulfed by LC3A/B-I/II to form autophagosome for degradation. The healthy part of a mitochondrion can bind to the healthy part of another mitochondrion through Opa -1.

1.6.1 Fission proteins – Dynamin related protein - 1 (Drp-1), fission protein (Fis-1)

Mitochondrial division was first studied in *S. cerevisiae*. Drp-1 and Fis-1 proteins are highly conserved in humans. Drp-1 is the dynamin-related guanosine triphosphatase that is essential for mitochondrial health. They are localised in a dynamic manner in the cytosol and on the mitochondrial outer membrane. Drp-1 is present at sites of mitochondrial division (246), to form a spiral structure. This spiral structure can drive the constriction of the mitochondrial outer and inner membrane to separate the damaged/older parts of mitochondria during mitochondrial division (Figure 1.6, step 1, (247). Another key mitochondrial fission protein is Fis-1. It is an 18kDa protein anchored at the outer membrane of mitochondrion. The cytosolic portion of Fis-1 consists of six α-helices that adopt a superhelical tetratricopeptide repeat (248-250), which serves as a platform to adapt Drp-1. The normal function of Drp-1 is compromised when Fis-1 is absent in yeast (251). However, this becomes unclear in

humans as an increase or decrease in Fis-1 does not necessarily affect the amount of Drp-1 in mitochondria (252). Drp-1 knockout mice have been shown to die at embryonic day 12.5 with developmental abnormalities in the forebrain (253). Neuron-specific Drp-1 knockout mice also die quickly due to neurodegeneration (253). In these Drp-1 knockout mice, mitochondria are enlarged, aggregated and sparsely distributed in neurites preventing synapse formation in primary cultured neural cells (253,254). This implies that Drp-1 mediated mitochondrial fission is essential to maintain mitochondrial health and neural development.

In comparison to Drp-1, Fis-1 has not been extensively studied in the brain. However, increased brain Fis-1 levels has been shown in Huntington's disease (255) and Alzheimer's disease (256). Smoking can induce mitochondrial fission in human airway smooth muscle (257). This increase in mitochondrial fission is similar to the changes that occur when the airway is undergoing inflammation (257). During brain inflammation, mitochondrial fission is also increased which is mediated by Drp-1 (258). Maternal cigarette smoke exposure can increase brain inflammation in rats (82), which may lead to increased mitochondrial fission due to its impact on mitochondrial integrity. This will be studied in this thesis.

1.6.2 Fusion protein – Optic atrophy 1 protein (Opa-1)

In a similar manner as mitochondrial fission, fusion protein was first identified in yeast, as a mitochondrial genome maintenance protein essential for inner membrane fusion (Figure 1.6, step 2). The mammalian orthologue in charge of fusion is Opa-1. Opa-1 has eight isoforms, with each variant being generated by alternative splicing and processing at two cleavage sites (259). Opa-1 is one of the core components of mitochondrial fusion, involved in cristae formation (260). It is ubiquitously expressed throughout the body, in the heart, skeletal muscle, liver, testis, and most abundantly in the brain and retina (261). Opa-1 deficiency leads to selective blocking of inner mitochondrial membrane fusion (244). Opa-1 knockout mice also die at embryonic day 9, showing that Opa-1 is essential for embryonic development (262). In two different mouse models of mitochondrial disorders, the overexpression of Opa-1 ameliorates mitochondrial dysfunction through the correction of cristae ultrastructure and mitochondrial respiration leading to the improvement of motor performance and prolongation of lifespan (263). Compared to mitochondrial fission, mitochondrial fusion appears to

protect cells from apoptosis, although the mechanism is not known. Prolonged cigarette smoke exposure has been shown to increase Opa-1 mRNA expression in the epithelium from patients with chronic obstructive pulmonary disease implying an increase in cristae damage (264). A reduction of brain mitochondrial fusion was observed in Alzheimer's disease (265). However, there has been no study to examine the impact of smoking or maternal smoking on brain mitochondrial fusion markers.

1.6.3 PTEN-induced putative kinase 1 (Pink-1) and Parkin protein

Both Pink-1 and Parkin function through a common pathway to regulate mitochondrial morphology, by inhibiting fusion protein and/or promoting the activation of fission proteins (266). Pink-1 accumulates on the outer membrane of damaged mitochondrion leading to the recruitment of Parkin to the dysfunctional mitochondrion (Figure 1.6, step 3) (267). Pink-1 is required for the recruitment of Parkin. Loss of either Pink-1 or Parkin leads to an early onset of Parkinson's disease (268). Pink-1 knockout can be rescued by the overexpression of Parkin, whereas Parkin knockout cannot be rescued by the overexpression of Pink-1 (269).

An increase in Pink-1 can also indicate mitochondrial stress. In primary bronchial epithelial cells from the patients with chronic obstructive pulmonary disease (264), long term cigarette smoke exposure can increase Pink-1 mRNA expression in mitochondria (264). This was accompanied by the increase in mitochondrial fission and fusion along with increased OXPHOS Complex II, III and V proteins levels (264). Although there has been no study reporting the impact of smoking on Pink-1 and Parkin changes in the brain, impact of nitric oxide from cigarette smoke on Pink-1 and Parkin has been studied (270). Nitric oxide exposure in Pink-1 deficient neuronal cells has been shown to increase Parkin expression in mitochondria (270). This suggests that Pink-1 may not be necessary for Parkin-mediated mitophagy.

1.6.4 Autophagy markers – microtubule-associated proteins light chain 1A and 1B (LC3A/B-I/II)

The major function of autophagy is to degrade cellular constituents to maintain intracellular homeostasis. Dysfunctional autophagy has been shown to be a possible mechanism for neurodegenerative diseases (271). During autophagy, autophagosomes

are formed to engulf intracellular components, such as mitochondria. LC3 was originally identified as microtubule associated protein (MAP)1A and MAP1B, and the other name of MAP1 is LC3A/B. In this thesis, LC3A/B will be used. It was later found that LC3A/B-I is involved in the formation of autophagosomal membrane with conjugation of phophatidylethanolamine to form LC3A/B-II (Figure 1.6, step 4). This is mediated by autophagy-related protein (atg) 7 and atg 3. Therefore, the conversion of LC3A/B-I to LC3A/B-II is normally used as an indicator of autophagic activity and LC3A/B-II level is correlated with autophagosome formation. Previous studies on the impact of smoking on autophagy mainly focus on the lung tissue (272-275). In particular, cigarette smoke extract or exposure has been shown to increase autophagy makers in cultured primary human bronchial epithelial cells (274) and the lung specimens from the patients with chronic obstructive pulmonary disease (273). However, there are no studies showing the changes of brain autophagy markers by smoking.

1.6.5 L-carnitine (L-C) as a therapeutic strategy

L-C is an endogenous natural quaternary ammonium compound found in all mammalian species. It is also known as one of the vital components for mitochondrial fatty acid oxidation (276). The majority of L-C is obtained from diet (75%), mostly meat products, with the remaining 25% produced endogenously, primarily in the liver and kidney (277). It is synthesized from the essential amino acids lysine and methionine (277).

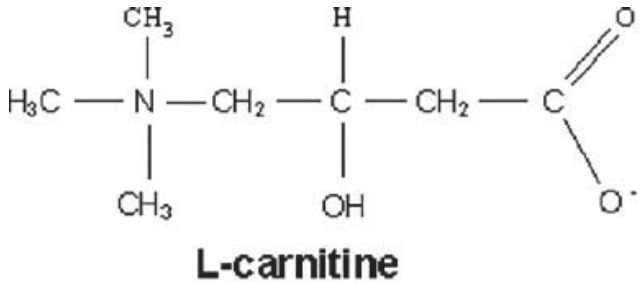

Figure 1.7. Chemical structure of L-Carnitine.

L-C has been reported to act as a free radical scavenger that protects antioxidant enzymes such as MnSOD from oxidative damage in many *in vitro* and animal studies (278-280). In a human study, it has been found that L-C can increase antioxidant

enzymes activities, suggesting that it may be useful for improving mitochondrial function in certain diseases (281). In patients with myocardial infarction, higher dose of L-C intervention has been shown to have a protective effect on cardiac muscle metabolism and function (282).

In the brain, L-C is important for several cellular functions, especially mitochondrial and peroxisome metabolism. Neuronal cells can take up L-C by a Na^+-ATP pump (283). In the mitochondrion, L-C acts as an energy carrier in the mitochondrial inner membrane, controlling the supply of acetyl-CoA and sustaining OXPHOS complex activities (283). A neuroprotective role for L-C against 3-nitropropionic acid (3-NPA) induced neurotoxicity has been reported in three month old Sprague-Dawley male rats (284). Pre-treatment with L-C before the exposure of 3-NPA can increase the activities of the endogenous free radical scavengers, catalase and SOD (284). It was suggested by Virmani and colleagues that L-C might be able to improve the symptoms of neurodegenerative disorders such as Parkinson's and Alzheimer's diseases as they are all caused by oxidative stress-induced mitochondrial dysfunction (285). The increase in brain oxidative stress in smokers (286) and possibly next generation are very similar to these neurodegenerative diseases. Thus L-C supplementation might be a good candidate to mitigate oxidative stress induced mitochondrial dysfunction in the offspring of smokers.

1.7 Gender difference in the response of offspring to maternal insult and brain disorders

Studies have shown that males are more vulnerable to the adverse impact of maternal smoking. One study showed that boys bear 1.5–2.5 time greater chance of having a cleft lip or palate due to maternal smoking (287). Another study also showed that maternal smoking can predispose male offspring to liver fibrotic cirrhosis through cellular homeostasis, inflammation and apoptosis pathways (288). Maternal smoking also increases the risk of cirrhosis in female offspring, but through the glucose metabolic pathway (288). This indicates that maternal smoking can have similar impact on the health outcomes of offspring but through different mechanisms.

The gender differences in responses to maternal insult and brain disorders are mainly driven by endogenous steroid hormones such as oestrogen (289). Oestrogen has been

shown to protect females from neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease and stroke (290). Thus oestrogen has neuroprotective effects in the brain. It has also been shown to reduce inflammatory actions in an *in vitro* model of viral infection during immunodeficiency (291). Another role of oestrogen is to act as an antioxidant to prevent lipid peroxidation (292), protein oxidation (293) and DNA damage (294). In terms of mitochondrial health, oestrogen has been shown to maintain mitochondrial membrane potential during mitochondrial toxin, 3-NPA, exposure (295).

Although the brain structures in men and women are highly similar, they have different susceptibility to specific neural diseases (296). Males are generally more vulnerable to brain disorders and mental illness. The risk for Autism Spectrum Disorders, Attention Deficit and Hyperactivity Disorders is higher in the males (297-299). Males also have a higher prevalence of Parkinson's Diseases compared to women with ratio of 1.6 : 1 (300). When females suffer from these brain disorders, they start at an older age than men (301). Astrocytes from females are more resistant to stressors such as oxygen-glucose deprivation and oxidant-induced cell death than those compared to males (302,303). The astrocytes from male and female brains also express inflammatory cytokines differently (such as IL-1ß, IL-6) in response to injury (303). Astrocytes obtained from males express higher levels of IL-1ß mRNA which can result in worse outcomes following neuronal injury (304) and multiple sclerosis (305,306). Lipopolysaccharide is also found in cigarette smoke (307) in addition to nicotine and can increase mRNA levels of IL-6, TNF-α and IL1β in astrocytes from males compared to those from the females (305). Maternal smoking can be speculated to increase brain inflammatory markers in male offspring as shown by Khanna et al. (82), but possibly not in female offspring due to the anti-inflammatory effect of oestrogen (291,308). There has been no previous report on the gender differences in brain dysfunction due to maternal insults, such as cigarette smoke exposure.

1.8 Maternal smoking and hypoxic ischemic encephalopathy (HIE)

HIE is caused by hypoxia-ischemia (HI) leading to neonatal encephalopathy within 12 to 36 hours of birth (309). HI itself is one of the causes of cerebral palsy and associated disabilities in children (309). The outcomes of HI are diminished exchange of oxygen and carbon dioxide, and severe lactic acidosis (310). About 15%-25% of newborns

affected by HIE die in the early postnatal period (311). The incidence of HIE varies between developed and developing countries. In Nigeria, a study showed that HIE occurrence was 26.2 per 1000 live births (312), and the incidence in the UK and Western Australia was 3.0 per 1000 live births (313). About 25% of newborns with HIE die during the neonatal period and about 25% of survivors live with permanent neurological disabilities (314). Patients with low grade HIE have been reported to have normal cognitive function by school age (314).

HIE is not a single event but an evolving injury. There are two phases of HIE suggested by both clinical and animal studies. The first phase involves neuronal death related to cellular hypoxia when energy failure occurs. The second phase involves delayed neuronal death which occurs at least six hours later (315). The initial inflammatory response involves activation of microglia. It is followed by the infiltration of circulating monocytes, neutrophils and T-cells. Amoeboid microglia respond rapidly to hypoxia and accumulate in injured tissues (316), where excessive amounts of inflammatory cytokines such as TNF-α and IL-1β along with ROS are produced (317). These could disrupt the development of mature BBB and brain structure in neonates (318). In addition to the inflammatory response, oxidative stress is also a common pathway recognised after HI injury. Even with mild HI, pups at postnatal day 7 were found to have more H_2O_2 accumulation in the brain compared to the adult mice (319). There are different theories suggesting the reasons why the neonatal brain is more susceptible to oxidative stress than the adult brain. One is that neonatal brain contains a higher level of unsaturated fatty acids, with higher rate of oxygen consumptions and more redox active iron molecules (320). The antioxidant glutathione peroxidase is lower in neonatal brains, where its activity falls dramatically at 2 and 24 hours after HI (321). It is also suggested that the increasing in SOD could protect neonatal brain from oxidative stress damage. However, the overexpression of SOD also exacerbates damage through increasing H_2O_2 production (322).

There has been no study on the impact of direct maternal cigarette smoke exposure on HIE in offspring. A study conducted by Li et al showed that maternal nicotine exposure increased the vulnerability of neonatal brain to HIE injury in rats (323). HIE increased the infarct size in male pups, however not in the female pups (323). This indicates that there is a gender difference in the response to HIE with males more seriously affected.

Chronic cigarette smoking has been shown to delay the cognitive recovery in patients with mild traumatic brain injury (324). Thus, maternal smoking may increase the injury severity and neurological deficits in offspring following HIE.

1.9 Hypothesis, aims and methods

Hypothesis 1: Maternal cigarette smoke exposure (SE) can increase brain inflammation and oxidative stress markers in both dams and male offspring, but not the female offspring.

Aim 1.1: To examine the impact of SE on both dams and offspring at postnatal day (P) 1, P20 and 13 weeks

Aim 1.2: To examine the gender difference in inflammatory and oxidative stress markers following maternal SE

Hypothesis 2: Brain mitochondrial dysfunction in the SE offspring is caused by the dysregulation of mitophagy and autophagy. Maternal supplementation with the antioxidant L-Carnitine supplementation can reverse the adverse impact of maternal SE on offspring.

Aim 2: To study the impact of maternal L-carnitine on brain mitophagy and autophagy markers in SE offspring.

Hypothesis 3: Maternal SE can induce more severe tissue damage in male offspring with hypoxic ischemic injury

Aim 3: To study the impact of maternal SE on brain injury caused by hypoxic ischemic injury in the male offspring

Chapter 2
Methodology

2.1 Modelling maternal cigarette smoke exposure

This study was approved by the Animal Care & Ethics Committee (#ACEC 2011-313A) at University of Technology Sydney.

Virgin female Balb/c mice (6 weeks) were obtained from Animal Resources Centre (Perth, Australia). Mice were housed at 20 ± 2°C and maintained on a 12-h light, 12-h dark cycle (lights on at 0600 h). Two groups of mice with equal average body weight were assigned to cigarette smoke exposure (SE) and sham exposure to air as control (SHAM). Both groups were fed standard laboratory chow (Gordon's Stockfeeds, Bargo, NSW) with free access to water. SE group was exposed to 2 cigarettes (Winfield Red, nicotine $\leq$ 1.2 mg, CO $\leq$ 15 mg, Philip Morris, VIC, Australia) at a time (5 minutes interval), twice a day for seven days a week for six weeks prior to mating, during gestation and lactation. SHAM group was exposed to air under the same conditions as the SE group. They were mated with male Balb/c mice (8 weeks) from the same source. Body weight of the breeders was measured weekly to determine the success of pregnancy.

After the pups were born, the litter size was adjusted to 4-6 pups per dam (sex ratio 1:1) by cross fostering within the same maternal group, in order to avoid the difference in milk availability between the litters. The excess pups were sacrificed as samples of postnatal (P)1. Pups were weaned from the dams at P20, which is when dams were culled. Half of the pups within the same litter were sacrificed at P20 and the other half were sacrificed at 13 weeks. Body weight was measured every five days during the suckling period and once every fortnight after weaning. The pups of both genders were studied at three time points (P1, P20 and 13 weeks).

2.2 Sample collection

P1 pups were sacrificed by decapitation and trunk blood was collected. The dams and pups at P20 and 13 weeks (aim 1 to 3), P45 (aim 4) were sacrificed after anesthetic overdose (Pentothal®, 0.1mg/g, i.p., Abbott Australasia Pty. Ltd., NSW, Australia). Blood was collected by cardiac puncture, and blood glucose level was measured immediately by a handhold glucose meter (Accu-Chek®, Roche Diagnostics, NJ, USA). Plasma was stored at -20°C. Then, animals were killed by decapitation and the brain

was collected and dissected into left and right half. The left half was stored at -80°C and the other half was fixed with 10% formalin and stored in 70% ethanol until further analysis. Frozen tissues were used for real-time PCR (rt-PCR) and Western Blotting analysis and the fixed tissues used for immunofluorescence analysis.

P45 pups were anesthetised by 4% isoflurane. Blood was collected by cardiac puncture. The brain was collected and dissected into two rostral and caudal halves at bregma -1 mm. the rostral half of the brain was further dissected to left and right hemispheres, which were stored at -80°C for real-time (rt-PCR) and Western Blotting analysis. The caudal half of the brain was fixed with 10% formalin for immunofluorescence analysis.

2.3 Western blotting

2.3.1 Protein extraction and quantification

Proteins of the whole tissue lysate and mitochondrial fraction were extracted by differential speed extraction method (325). Brain tissues were homogenized in 200 µl of lysis buffer (20mM HEPES buffer, pH 7.2, containing 1mM EGTA, 210mM mannitol, and 70 mM sucrose) with ceramic beads using Precelleys 24 (Geneworks, Hindmarsh SA, Australia). Samples were then centrifuged at 1,500g x g at 4 °C for five minutes. The supernatant was the whole cell protein. Half of the whole protein was further centrifuged at 10,000g at 4°C for 15 minutes. The pellet was mitochondrial fraction which was then resuspended in 50 µl of lysis buffer. Whole cell and mitochondrial proteins were quantified using Bio-Rad DC protein assay (Bio-Rad Laboratories, California, USA) according to the manufacturer's instructions. Proteins were stored at -80°C for later analysis.

2.3.2 Gel electrophoresis and transfer

Proteins samples (1µg/µl) were loaded into each well of NuPAGE® Novex 4-12% Bis-Tris protein gels (Life Technology, Carlifornia, USA). The proteins were separated on the gel at 140V for 1 hour and 20 minutes with *MES* running buffer (Life Technology, Carlifornia, USA). The separated proteins were then transferred to *PVDF* membranes using either semi-dried or wet transfer methods where applied (Thermo Scientific, Illinois, USA). The PVDF membrane was then blocked with 5% skim milk in Tris

buffered saline (1M Trizma, 3M Sodium chloride, Tween 20 (Sigma- Aldrich, Missouri, USA), TBST) for one hour at room temperature.

2.3.3 Antibody incubation and immunodetection

Primary antibodies were diluted with 2% bovine serum albumin (BSA, Sigma Aldrich, Missouri, USA) and incubated with the PVDF membrane at 4°C overnight. The PVDF membranes were then washed with TBST three times for ten minutes each. Secondary antibodies were then diluted with 2% BSA in TBST and incubated for one hour at room temperature. The membrane was then washed with TBST three times for ten minutes each before incubation with SuperSignal™ West Pico Chemiluminescent Substrate (ThermoFisher Scientific, NSW, Australia) for five minutes. The bands on the membrane were detected with LAS-3000 Imaging system (Fujifilm, Tokyo, Japan).

2.4 Real time Polymerase Chain Reaction (rt-PCR)

2.4.1 RNA extraction

Brain tissues (10-100mg) were homogenised in 1ml Trizol (Sigma Aldrich, Missouri, USA) with ceramic beads (Geneworks, Hindmarsh SA, Australia)) in a 2ml Sarstat tubes (Neptune, Preston VIC, Australia) using Precelleys 24 (Geneworks, Hindmarsh SA, Australia). The homogenized samples were then incubated for 10 minutes at room temperature. Then chloroform (200 µl) was added and shaken vigorously by hand for 15 seconds, before incubation at room temperature for 2-3 minutes. The samples were then centrifuged at 13,000g for 20 minutes at 4°C. The aqueous phase was transferred to a sterile Eppendorf tube and well mixed with 500µl isopropyl alcohol. The samples were then incubated overnight at -20°C, followed by centrifugation at 13,000g for 15 minutes at 4°C the next morning. The RNA pellets were washed with 1 ml of 75% ethanol and air-dried in the fume hood for 5-10 minutes. Then, the pellets were dissolved in DEPC-treated water. DNase was added to remove potential DNA contamination. mRNA was then quantified using Nanodrop (ND-100 spectrometer, Bio-Lab, Delaware, USA) and diluted to 200ng/µl.

2.4.2 cDNA synthesis

Random primers were added to the mRNA and incubated at 70ºC for 5 minutes (Eppendorf AG, 22331 Hamburg). The mixture was then cooled on iced for 5 minutes before dNTP (Promega, USA), M-MLV Reverse Transcriptase, RNase H Minus, Point Mutant enzyme (Promega, USA) were added. The mixture was incubated at room temperature for 10 minutes, followed by 42ºC for 1.5 hours, and then 70ºC for 15 minutes. The cDNA was then quantified using Nanodrop (ND-100 spectrometer, Bio-Lab, Delaware, USA).

2.4.3 Real-time polymerase chain reaction

mRNA expression of gene was measured using manufacture pre-optimised Taqman primers and probes (Thermofisher, New York, USA). The reaction mix was made of 5ul real-time PCR master mix (Life Tech, Platinum qPCR, Carlsbad, CA 92008 USA), 0.5µl of primers and probe for target gene labelled with FAM, 0.5µl of primers and probes for 18s labelled with VIC, 0.2µl of ROX dye (Invitrogen CA, USA), 1.3µl of DEPC-treated water and 2.5 µl of cDNA (200µm/µl). PCR plates were incubated at 50ºC for 2 minutes, 95ºC for 2 minutes, followed by 40 cycles of 95ºC for 40 seconds and 60ºC for 30 seconds (Real-plex2, Eppendorf, Hamburg, Germany). mRNA expression was calculated by using the following method. Firstly, the average of Ct FAM and Ct VIC was calculated for each sample. ΔCt was calculated by subtracting Ct VIC from Ct FAM for each sample. The average of the control group was used as the calibrator, against which ΔΔCt was calculated by subtracting the ΔCt of the calibrator from the ΔCt of each sample. Finally, the relative expression was calculated by the formula 2^-(ΔΔCt).

Table 2.1. Taqman® probe sequence (Life Technologies, CA, USA) for rt-PCR

Gene	NCBI gene references	Probe Sequence	ID
IL-1ß	NM_008361.3,M15131.1,BC011437.1	TCCTTGTGCAAGTGTCTGAAGCAGC	Mm01336189_m1
IL-6	NM_031168.1,X06203.1,X54542.1	ATGAGAAAAGAGTTGTGCAATGGCA	Mm00446190_m1
TLR4	NM_021297.2,AF095353.1,AF110133.1	CCCTGCATAGAGGTAGTTCCTAATA	Mm00445273_m1
TNF-α	NM_013693.2,X02611.1,M13049.1	CCCTCACACTCAGATCATCTTCTCA	Mm00443259_g1
iNos	NM_010927.3,AF065923.2,AF065921.2	CCTCCCACCCAGCTGCAGCTCCAC	Mm00440502_m1
EGR1	NM_007913.5,M20157.1,M19643.1	TGAGCACCTGACCACAGAGTCCTTT	Mm00656724_m1
HIF-1α	NM_010431.1,AF003695.1,X95580.1	CAGCAGGAATTGGAACATTATTGCA	Mm00468878_m1
IL-1R	NM_001123382.1,NM_008362.2,M20658.1	AGCTGACCCAGGATCAATGATACAA	Mm00434237_m1

2.5 Paraffin embedding and sectioning

Brain tissues were processed with Shandon Excelsior (ThermoFisher Scientific, NSW, Australia). The samples were dehydrated by progressively more concentrated ethanol, from 80%, 95% to 100% with 2 changes in each ethanol. Xylene was then used to remove the ethanol by washing for 3 times. Molten paraffin wax was used to replace the xylene in the samples. The processed tissues were then embedded in wax with Shandon Histocentre 3 (ThermoFisher Scientific, NSW, Australia). They were then sectioned at 7 μm with three sections on each slide (Matsunami Flass Ind, Ltd., Osaka, Japan).

2.6 Haematoxylin and Eosin staining

The slides were hydrated through xylene and decreasing graded ethanol to distilled water. They were stained with Mayer's Haematoxylin for five minutes and washed subsequently with distilled water for two minutes. The slides were then stained with

Scott's Bluing for one minute then rinsed in water for two minutes followed by staining with Eosin (1% alcohol) for two minutes. The slides were then dehydrated by 100% ethanol for two minutes twice, xylene for three minutes and lastly xylene for 5 minutes. They were then cover slipped using DPX mountant media.

2.7 Immunohistochemistry

Three sections per animal were assessed on each slide. Slides were treated with xylene and decreasing graded ethanol to distilled water for hydration. They were then incubated in PBST (Phosphate buffered saline – Triton X, pH 7.4) for 10 minutes, followed by citric acid (pH6.5) in a pressure cooker for 10 minutes. The tissues were then blocked by 5% normal goat serum in (PBST) for half an hour. Primary antibodies were then diluted with phosphate buffered saline and 5% goat serum (PBG) according to the manufacture's protocols. PBG was used to replace primary antibodies as negative controls. The tissues were incubated with either primary antibody or PBG overnight at 4ºC in a humidified chamber, and washed with PBST three times. Secondary antibodies were then diluted with PBG accordingly and incubated with the tissues for two hours at room temperature in a humidified chamber. After two PBST washes, the tissues were then stained with Biz-benzamine for cell nuclei followed by PBST wash for ten minutes. Then the slides were cover slipped in Dako fluorescent mounting medium and left for air-drying overnight in the dark room.

2.8 TUNEL and Caspase-3 staining

ApopTag®Peroxidase kits (Merck Millipore, Victoria) were used for TUNEL staining. Slides were deparaffinised and treated with xylene and decreasing graded ethanol to distilled water for hydration. The slides were the microwaved for 14 minutes in citrate buffer (pH9.0) followed by cooling in water bath for 15 minutes. The slides were then washed twice with dH_2O for two minutes each followed by PBS washing for three minutes. The slides were then quenched with peroxidase for 15 minutes at room temperature (methanol: PBS: H2O = 4: 4: 1), and incubated with PBS twice (three minutes each), before additional incubation with 50µl of equilibration buffer for 30 seconds and cover slipped. Terminal deoxynucleotidyl transferase (Tdt) (25µl) (Tdt: reaction buffer = 1:4) was added to each section, cover slipped and incubated for one

hour. Negative control was incubated with water instead of Tdt. The coverslip was then removed and stop reaction buffer was added (1.5ml stopwash buffer + 50ml H2O) at room temperature. The slides were then washed with PBS twice (three minutes each) at room temperature. Anti-Digoxigenin-Peroxidase (25µl) was added to each section, coverslipped and incubated for 40 minutes at room temperature followed by two washes with PBS for three minutes each. DAB solution (Dako) was then added and incubated for eight minutes. Slides were then washed twice with PBS for three minutes each. The slides were then blocked with 10% normal horse serum (NHS) in PBS for 30 minutes at room temperature. They were then incubated with Active Caspase-3 antibody (1:300 diluted in 1% NHS in PBS, BD Biosciences) overnight at 4˚C. The slides were then washed twice with PBS for three minutes each. ABC phosphatase was added to the slides and incubated for 45 minutes at room temperature.

Chapter 3
Impact of maternal cigarette smoke exposure on brain inflammation and oxidative stress in male mice offspring

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Impact of maternal cigarette smoke exposure on brain inflammation and oxidative stress in male mice offspring

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Maternal cigarette smoke exposure (SE) during gestation can cause lifelong adverse effects in the offspring's brain. Several factors may contribute including inflammation, oxidative stress and hypoxia, whose changes in the developing brain are unknown. Female Balb/c mice were exposed to cigarette smoke prior to mating, during gestation and lactation. Male offspring were studied at postnatal day (P) 1, P20 and 13 weeks (W13). SE dams had reduced inflammatory mediators (IL-1β, IL-6 and toll like receptor (TLR)4 mRNA), antioxidant (manganese superoxide dismutase (MnSOD)), and increased mitochondrial activities (OXPHOS-I, III and V) and protein damage marker nitrotyrosine. Brain hypoxia-inducible factor (HIF)1α and its upstream signalling molecule early growth response factor (EGR)1 were not changed in the SE dams. In the SE offspring, brain IL-1R, IL-6 and TLR4 mRNA were increased at W13. The translocase of outer mitochondrial membrane, and MnSOD were reduced at W13 with higher nitrotyrosine staining. HIF-1α was also increased at W13, although EGR1 was only reduced at P1. In conclusion, maternal SE increased markers of hypoxia and oxidative stress with mitochondrial dysfunction and cell damage in both dams and offspring, and upregulated inflammatory markers in offspring, which may render SE dams and their offspring vulnerable to additional brain insults.

Cigarette smoking is a significant risk factor for a number of chronic conditions, such as cerebrovascular and cardiovascular diseases, in addition to respiratory disorders[1], and thus remains a major cause of death worldwide[2]. Despite general education on the risks, smoking during pregnancy and passive smoking during pregnancy are still common in both developed and developing countries[3,4], and ~20–45% women smoke during pregnancy in Europe, Australia, South America, and South Africa[3–5]. Smoking and second hand smoking in pregnant women may result in placental transfer of toxic agents present in cigarettes and transmit a risk to the developing fetal brain. In addition there are increased risks of developing well-known metabolic, respiratory and behavioural disorders that are recognised in the offspring of first-hand or second-hand smoking mothers (reviewed in[6–9]). Nicotine can pass through the placenta and act as a vasoconstrictor, which can reduce uterine blood flow by up to 38%[10], leading to deprivation of oxygen and nutrients in the fetus, resulting in hypoxia and undernutrition[11]. As such, maternal smoking is a known risk factor for intrauterine growth retardation[12,13], with adaptive brain structural and functional changes occurring during fetal development[14–18]. Preterm infants from smoking mothers display significantly smaller frontal lobe and cerebellar volumes after adjustments of confounding factors such as alcohol consumption[19]. It is likely that maternal smoking alters fetal brain immune function and mitochondrial activity that make such offspring more vulnerable to brain insults.

Oxidative stress is integral to the general inflammatory response[20], which occurs due to a metabolic imbalance brought about by excess production of reactive oxygen species (ROS, such as the superoxide anion) and/or a reduced level of host antioxidant defences. Mitochondria are a major site of ROS production during oxidative phosphorylation (OXPHOS) to generate ATP[21]. During an inflammatory response, there is a high consumption of oxygen and release of the superoxide free radical (O^{-}_{2}) by the mitochondria[22], which can, in turn, impair

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Gene	NCBI gene references	Probe Sequence	ID
EGR1	NM_007913.5,M20157.1,M19643.1	TGAGCACCTGACCACAGAGTCCTTT	Mm00656724_m1
HIF-1α	NM_010431.1,AF003695.1,X95580.1	CAGCAGGAATTGGAACATTATTGCA	Mm00468878_m1
IL-1β	NM_008361.3,M15131.1,BC011437.1	TCCTTGTGCAAGTGTCTGAAGCAGC	Mm01336189_m1
IL-1R	NM_001123382.1,NM_008362.2,M20658.1	AGCTGACCCAGGATCAATGATACAA	Mm00434237_m1
IL-6	NM_031168.1,X06203.1,X54542.1	ATGAGAAAAGAGTTGTGCAATGGCA	Mm00446190_m1
TLR4	NM_021297.2,AF095353.1,AF110133.1	CCCTGCATAGAGGTAGTTCCTAATA	Mm00445273_m1
TNFα	NM_013693.2,X02611.1,M13049.1	CCCTCACACTCAGATCATCTTCTCA	Mm00443259_g1

Table 1. TaqMan probe sequence (Life Technologies, CA, USA) for rt-PCR.

mitochondrial function[23] leading to cell and organ impairment. Thus, to protect cell integrity, excessive ROS are removed by antioxidants, including mitochondrial manganese superoxide dismutase (MnSOD). Oxidative stress can also exacerbate associated inflammatory reactions by activating pathways such as c-jun N-terminal kinases and nuclear factor-κ-light-chain-enhancer of activated B cells[24]. Hence, increased antioxidant levels or activity can significantly reduce the injury size in mice following stroke[25]. However, if the brain has pre-existing oxidative stress and inflammation, both mitochondrial and cellular function can be affected especially during post-injury repair[26,27]. Cigarette smoke itself contains a substantial amount of ROS[28], which may exceed the baseline antioxidative capacity of the mitochondria to clear both endogenous and exogenous ROS. Indeed, it has been shown that smokers have decreased levels of antioxidants in their serum[29]. However, it is unclear whether smoking increases brain inflammation and oxidative stress. Therefore, we hypothesise that there may be a causal link between cigarette smoke exposure (SE), increased inflammation, oxidative stress and mitochondrial dysfunction in the brain. The aim of this study was to investigate the impact of continuous maternal cigarette smoke exposure in mice on brain inflammation, mitochondrial function and antioxidant capacity, as well as markers of hypoxia in both mothers and offspring.

Materials and Methods

Maternal cigarette smoke exposure. The animal experiments were approved by the Animal Care and Ethics Committee at the University of Technology Sydney (ACEC#2011-313A). All protocols were performed according to the Australian National Health & Medical Research Council Guide for the Care and Use of Laboratory Animals. Virgin female Balb/c mice (6 weeks, Animal Resources Centre, Perth, Australia) were housed at 20 ± 2 °C and maintained on a 12-h light, 12-h dark cycle (lights on at 06:00 h) with ad libitum access to standard laboratory chow and water. After the acclimatisation period, mice were assigned to cigarette SE or sham exposure (SHAM). The SE group was exposed to 2 cigarettes (Winfield Red, ≤16 mg tar, ≤1.2 mg nicotine, and ≤15 mg of CO; VIC, Australia) in a perspex chamber (18 L), twice daily for six weeks prior to mating, during gestation and lactation; while the SHAM group was exposed to normal air as previously described[30]. They were mated with male Balb/c mice (8 weeks) from the same source, which were not exposed to cigarette smoke. The offspring were housed 4–5/cage after weaning, and the males were studied at postnatal day (P)1, P20 (weaning), and week 13. The females will be reported separately.

Sample collection. Animals at P1 were sacrificed by decapitation, while animals older than 20 days were killed after anaesthetic overdose (Pentothal®, 0.1 mg/g, i.p., Abbott Australasia Pty. Ltd., NSW, Australia) between 9:00–12:00 h. The mothers were also culled between 9:00–12:00 h (with their last cigarette being at 15:00 h the previous day). Brains were dissected into the left and right hemispheres. The left hemisphere was stored at −80 °C for mRNA and protein analysis, while the right hemisphere was fixed with 4% formalin for immunohistochemical analysis.

Quantitative real-time PCR. Total mRNA was extracted from brain tissues using TriZol reagent (Life Technologies, CA, USA). The purified total RNA was used as a template to generate first-strand cDNA using M-MLV Reverse Transcriptase, RNase H, Point Mutant Kit (Promega, Madison, WI, USA)[31]. Genes of interest were measured using manufacturer pre-optimized and validated TaqMan primers and probes (Life Technologies, CA, USA). Only the probe sequence is provided by the manufacturer (Table 1). The probes of the target genes were labelled with FAM® dye and those for housekeeping 18 s rRNA were labelled with VIC® dye. Gene expression was standardized to 18 s RNA. The average expression of the control group was assigned as the calibrator against which all other samples are expressed as fold difference.

Western Blotting. The protein levels of early growth response factor (EGR)1, hypoxia-inducible factor (HIF)-1α, manganese superoxide dismutase (MnSOD), translocase of outer membrane (TOM)20 and OXPHOS complex proteins were measured by western blotting. The brain was homogenised using cell lysis buffers for whole protein and mitochondria protein extraction according to manufacturer's instruction[32]. Protein samples (40 μg) were separated on NuPage® Novex® 4–12% Bis-Tris gels (Life Technologies, CA, USA) and then transferred to PVDF membranes (Rockford, IL, USA), which were blocked with non-fat milk powder and incubated with the primary antibodies (EGC-1 (1:5000, Santa Cruz Biotechnology), HIF-1α (1:1000, Novus Biologicals); MnSOD (1:1000) & TOM20 (1:2000, Santa Cruz Biotechnology), Mitoprofile Total® OXPHOS complex Rodent WB antibody (1:2500, Abcam)) for overnight and then secondary antibodies (1:2000 for HIF-1α; 1:5000 for MnSOD, TOM20 and OXPHOS complex, goat anti-rabbit or rabbit anti-mouse IgG horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology)) for 1 hour. Protein expression was detected by SuperSignal

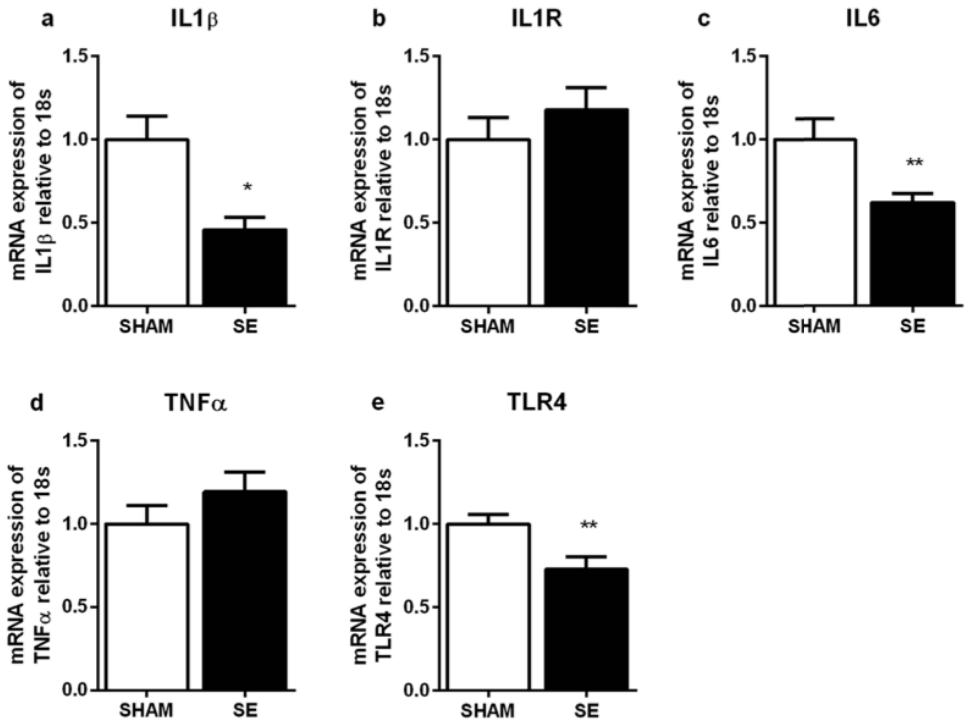

Figure 1. Brain mRNA expression of inflammatory markers in the SHAM and SE dams (n = 8). Results are expressed as mean ± S.E.M. Data were analysed by student's unpaired t-test. *P < 0.05; **P < 0.01. SE: smoke exposed.

West Pico Chemiluminescent substrate (Thermo, MA, USA) by exposure of the membrane in FujiFilm (Fujifilm, Tokyo, Japan). Protein band density was determined using Image J software (National Institute of Health, Bethesda, Maryland, USA).

Immunohistochemistry. Formalin fixed brain samples were embedded in paraffin and sectioned (4 μm). Three coronal sections were used from SHAM and SE respectively. They were incubated with primary antibodies against nitrotyrosine (1:400 dilution, Upstate Biotechnology, Temecula, CA) followed by horseradish peroxidase anti-rabbit Envision system (Dako Cytochemistry, Tokyo, Japan). The sections were then counterstained with haematoxylin. Three images of cortex from each section were captured and used for analysis. Calculation of the proportion of area stained positive for nitrotyrosine was then determined using Image J software (National Institute of Health, Bethesda, Maryland, USA). To confirm the antibody specificity, anti-Nitrotyrosine antibody was pre-incubated with 10 mM Nitrotyrosine in PBS for 1 h at room temperature before incubation on the tissue. This yielded no staining (data not shown).

Statistical methods. Results are expressed as mean ± S.E.M. The difference between groups was analysed using unpaired Student *t* tests (Statistica 9, Statsoft, USA).

Results

Effects of cigarette smoke exposure on the dams. *Body parameters.* Both SHAM and SE dams had a similar body weight at baseline (17.8 ± 0.2 vs 17.7 ± 0.2 g, n = 10). Before mating, SHAM dams were significantly heavier than the SE dams (18.7 g ± 0.3 vs 16.8 g ± 0.2 g, P < 0.05). When pups were weaned at P20, SE dams (21.9 ± 0.2 g) were 12% lighter than the SHAM dams (24.6 ± 0.4 g, P < 0.01), who also had much higher circulating levels of cotinine, which is a metabolite of nicotine (96.5 ± 33.7 vs. 1.52 ± 0.40 ng/ml in the SHAM, P < 0.05).

Brain inflammatory markers. Brain IL-1β, IL-6 and toll like receptor (TLR)4 mRNA expression were significantly decreased in the SE dams compared with the SHAM dams (P < 0.05, Fig. 1a; P < 0.01, Fig. 2c,e, respectively, n = 6 – 8). The expression of IL-1R and TNF-α mRNA were not different between the groups (Fig. 1).

Oxidative stress markers in the brain mitochondria. Brain mitochondrial MnSOD protein was reduced in the SE mothers (P < 0.01, Fig. 2a, n = 6). TOM20 protein was not different following SE. The protein levels of OXPHOS complexes CI, CIII and CV were significantly higher in the SE mothers compared to SHAM. Brain mitochondrial levels of CII and CIV were very low compared with other members of OXPHOS complexes in both SHAM and SE mothers (Fig. 2c). There was only minimal staining for nitrotyrosine in brains from SHAM mothers, and the

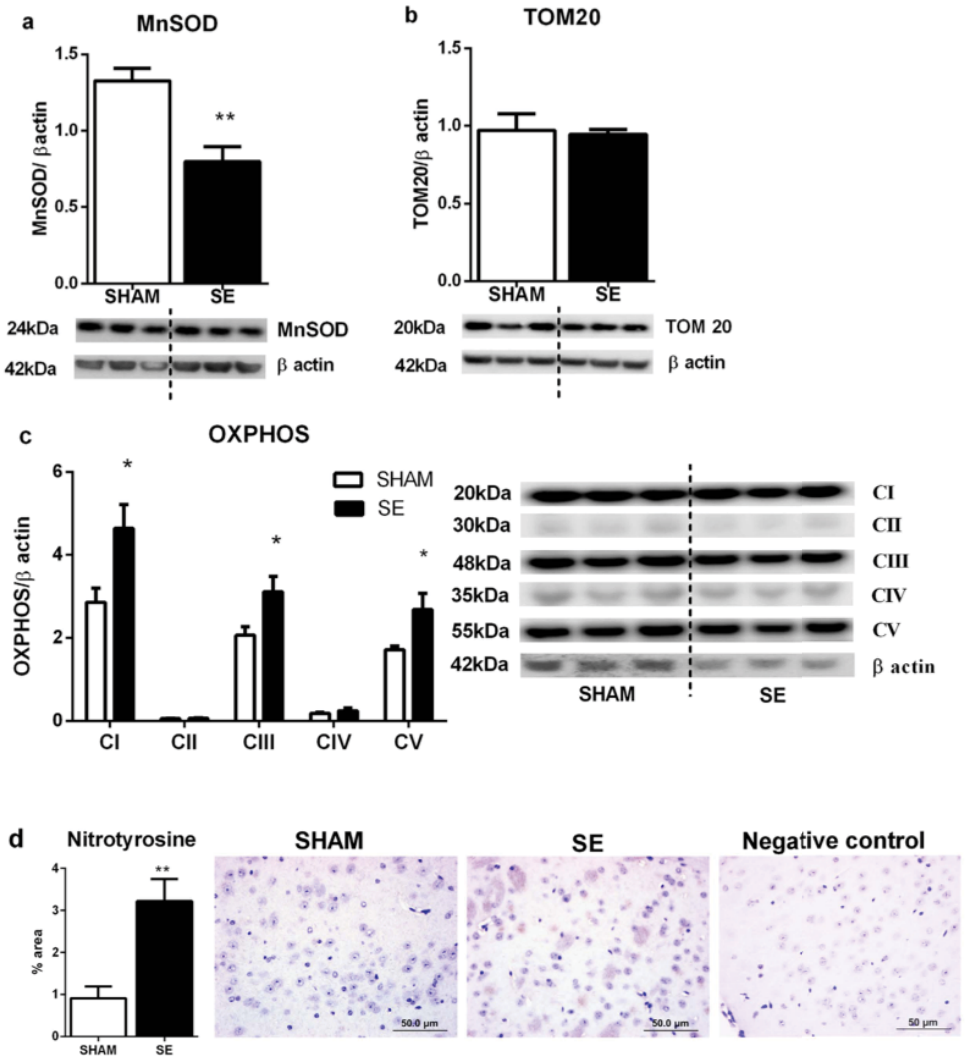

Figure 2. Brain protein levels of MnSOD (a), TOM20 (b) and OXPHOS complexes (CI, CII, CIII, CIV and CV) (c) in the SHAM and SE dams. Whole gel images of (**a**–**c**) in Supplementary Fig. 1. Immunohistochemistry for cortex nitrotyrosine in the dams (**d**) Scale bar = 50 μm (n = 4). Results are expressed as mean ± S.E.M. Data were analysed by student's unpaired t-test. *P < 0.05; **P < 0.01. MnSOD: manganese superoxide dismutase; OXPHOS: oxidative phosphorylation; SE: smoke exposed; TOM20: translocase of the mitochondrial outer membrane.

amount and intensity of staining was greater in the SE mothers. We measured the proportion of area stained positive for nitrotyrosine and this was significantly higher in the SE group (P < 0.01, Fig. 2d). Negative IgG was performed to confirm staining specificity.

Brain hypoxia markers. HIF-1α protein was reduced by 20% (P = NS) in the brains from the SE mothers (Fig. 3a); while its upstream regulator EGR-1 was simular between the groups (Fig. 3b).

Effects of maternal SE on male offspring. *Growth.* Body weights were not different between the SHAM and SE male offspring at P1 and P20 (Table 2). When the pups reached adulthood at week 13, SE offspring were significantly lighter than the SHAM offspring (P < 0.01, Table 2). Brain weights were smaller in the SE offspring at P1 and week 13 (P < 0.01), however the differences disappeared when expressed as a percentage of body weight (Table 2). Plasma cotinine levels in the SE offspring (7.60 ± 0.33 ng/ml) were double that of the SHAM offspring (3.07 ± 0.10 ng/ml, P < 0.01) at P20.

Brain inflammatory markers. Brain IL-1β mRNA expression was similar between groups at all ages (Fig. 4a–c). IL-1R mRNA expression was significantly increased in the SE offspring at all ages (Fig. 4d,e, P < 0.01; 4f, P < 0.05).

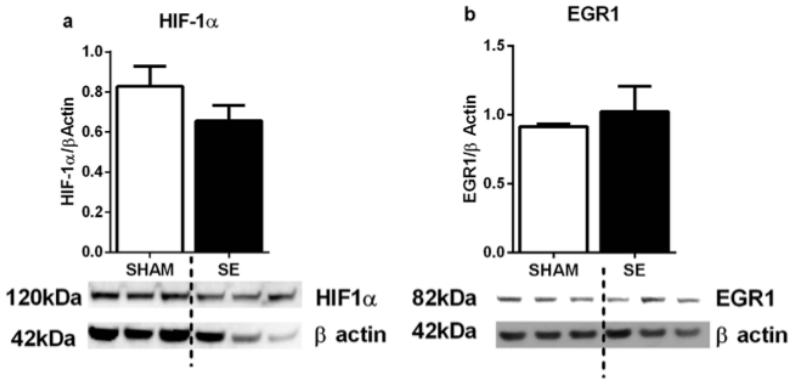

Figure 3. Brain protein levels of HIF-1α (a) and EGR1 (b) in the SHAM and SE dams (n = 3). Whole gel images of (**a**,**b**) in Supplementary Fig. 2. Results are expressed as mean ± S.E.M. Data were analysed by student's unpaired t-test. EGR1: early growth response factor: HIF-1α: hypoxia-inducible factor; SE: smoke exposed.

	Day 1		Day 20		Week 13	
Offspring	SHAM	SE	SHAM	SE	SHAM	SE
	n = 13	n = 15	n = 17	n = 18	n = 15	n = 11
Body weight (g)	1.86 ± 0.11	1.47 ± 0.04	9.9 ± 0.22	9.7 ± 0.22	26.8 ± 0.5	25.5 ± 0.3**
Brain (mg)	7.9 ± 0.19	5.8 ± 0.3**	18 ± 1.1	20 ± 1.6	30.6 ± 0.2	29.8 ± 0.2**
Brain% of body weight	4.3 ± 0.2	4 ± 0.2	1.8 ± 0.1	2.0 ± 0.1	1.1 ± 0.01	1.2 ± 0.01

Table 2. Parameters of the male offspring at different ages. Results are expressed as mean ± S.E.M. Data were analysed by student's unpaired t test. **$p < 0.01$, compared with the SHAM offspring at the same age.

IL-6 mRNA was upregulated in the SE offspring only at week 13 (Fig. 4i, $P < 0.01$). TNFα mRNA expression in the SE offspring was lower at P1 (Fig. 4j, $P < 0.05$), but not changed at P20 and week 13 (Fig. 4k,l) in comparison to SHAM controls. TLR-4 mRNA expression in the SE offspring was significantly decreased at P1 but increased at week 13 (Fig. 4m,j, $P < 0.05$) without any change at P20.

Oxidative stress markers in the brain mitochondria. At P1, mitochondrial protein levels of both MnSOD and TOM20 were similar between the SHAM and SE offspring (Fig. 5a,d). TOM20 protein was reduced at P20 in the SE offspring, but increased at week 13 (Fig. 6b, $P < 0.05$). MnSOD levels in SE offspring were reduced at week 13 (Fig. 5c,f, $P < 0.05$) compared to SHAM controls. OXPHOS complexes CI-V were not different between groups at P1 (Fig. 5f). At P20, brain OXPHOS CI and CV were significantly decreased in the SE offspring (Fig. 5g, $P < 0.05$); all the OXPHOS complexes CI–V were significantly increased in the SE offspring at week 13 (Fig. 5h, $P < 0.01$). Brain nitrotyrosine levels were increased in SE offspring at week 13 (Fig. 5d, $P < 0.01$).

Brain hypoxia markers. HIF-1α protein was significantly increased at week 13 in the brains of SE offspring ($P < 0.05$, Fig. 6c); EGR-1 was significantly reduced at P1 ($P < 0.01$, Fig. 6d), while unchanged at P20 and week13 in the brains of SE offspring in comparison to the offspring from SHAM mothers (Fig. 6e,f).

Discussion

Smoking during pregnancy is considered a major and significant public health issue. A rodent model is commonly used to study the detrimental impact of maternal tobacco exposure on offspring[19,33]. Administration of nicotine alone is insufficient to reflect the complexity of the cigarette smoke which comprises approximately 3800 constituents[33]. Here, we have investigated the impact of maternal cigarette smoke exposure on brain inflammatory markers, oxidative stress related mitochondrial activity, and markers of hypoxia in both dams and male offspring. There were similar brain changes in both SE mothers and offspring, with respect to reduced anti-oxidative capacity of the brain, which may reduce the ability of mitochondria to scavenge excess ROS generated during increased OXPHOS activity. This is reflected by increased nitrotyrosine levels, a direct product of oxidative stress, in the brains of SE mothers. However, SE mothers and adult SE offspring had quite distinct changes, in markers of brain inflammation and hypoxia, which were lower in the SE mothers, however higher in mature SE offspring. Increased brain oxidative stress and chronic inflammation are evident in certain neurodegenerative diseases, as neurons in the brain are highly sensitive to oxidative stress[34–37]. This raises the question of whether the offspring of smoking mothers may have a predisposition to neurodegeneration in adulthood.

Activation of TLRs stimulates the production of major inflammatory cytokines IL-1β and IL-6 in monocytes, which in turn enhances the expression of TLRs via a positive feedback loop[38]. In this study, TLR4 mRNA expression was downregulated in the SE mothers' brains, which is consistent with the reduced expression of

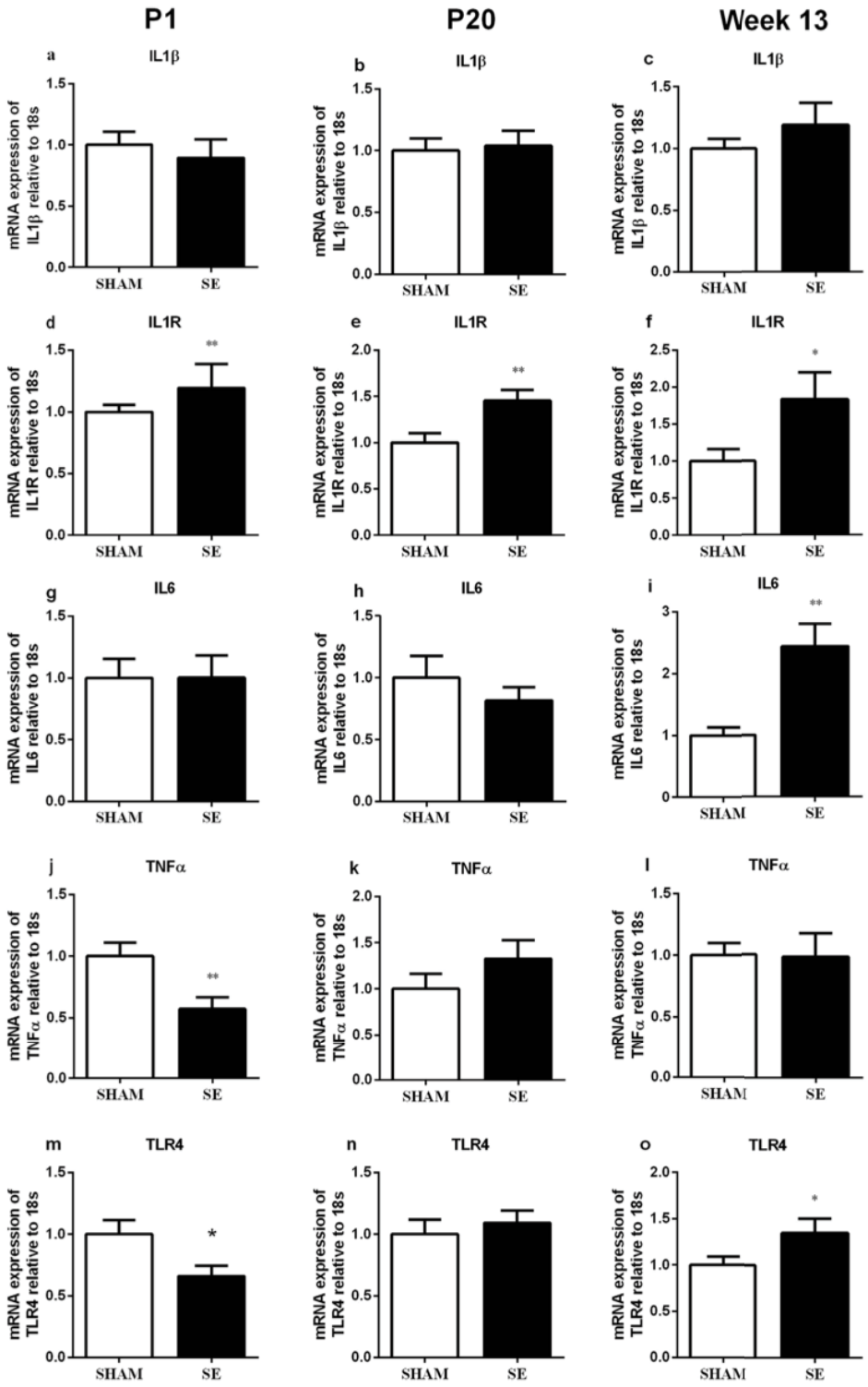

Figure 4. Brain mRNA expression of inflammatory markers in the offspring of SHAM and SE mothers at different ages (n = 8). Results are expressed as mean ± S.E.M. Data were analysed by student's unpaired t-test. *P < 0.05; **P < 0.01. SE: smoke exposed.

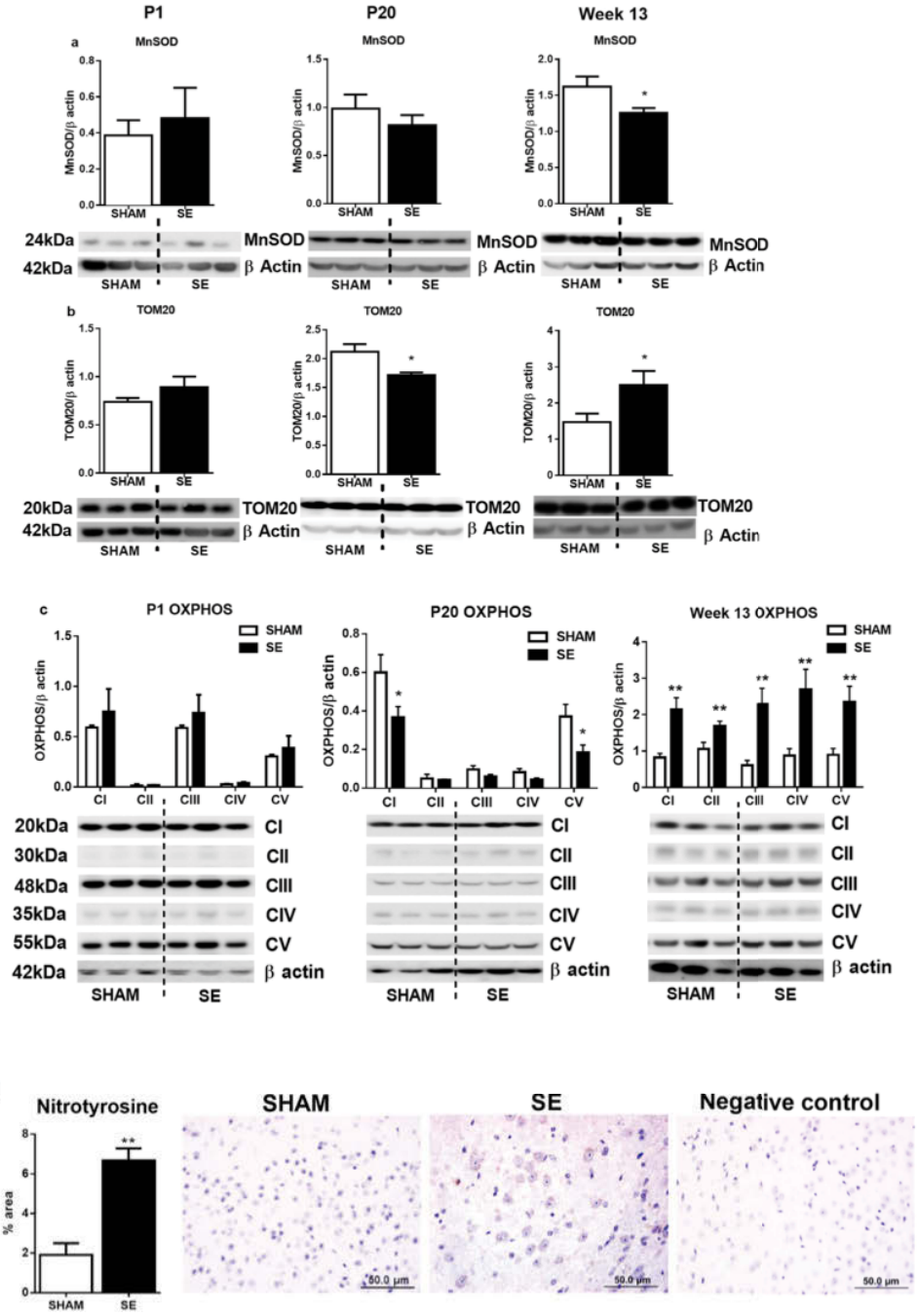

Figure 5. Protein expression of MnSOD (a), TOM20 (b) and OXPHOS complexes (CI, CII, CIII, CIV and CV) (c) in the brain mitochondria in the offspring of SHAM and SE mothers at different ages (n = 3). Whole gel images of (**a**–**c**) in Supplementary Fig. 3. Immunohistochemistry of cortex nitrotyrosine in week 13 offspring (**d**) Scale bar = 50 μm (n = 3). Results are expressed as mean ± S.E.M. Data were analysed by student's unpaired t-test. *P < 0.05; **P < 0.01. MnSOD: manganese superoxide dismutase; OXPHOS: oxidative phosphorylation; SE: smoke exposed; TOM20: translocase of the mitochondrial outer membrane.

IL-1β and IL-6 mRNA we observed. The response of TLR4 expression to cigarette smoke has been found to differ between tissues. A thirty minute exposure to cigarette smoke increased TLR4 mRNA expression in gingival epithelial cells[39]; while TLR4 mRNA expression was reduced in human macrophages and primary monocytes after

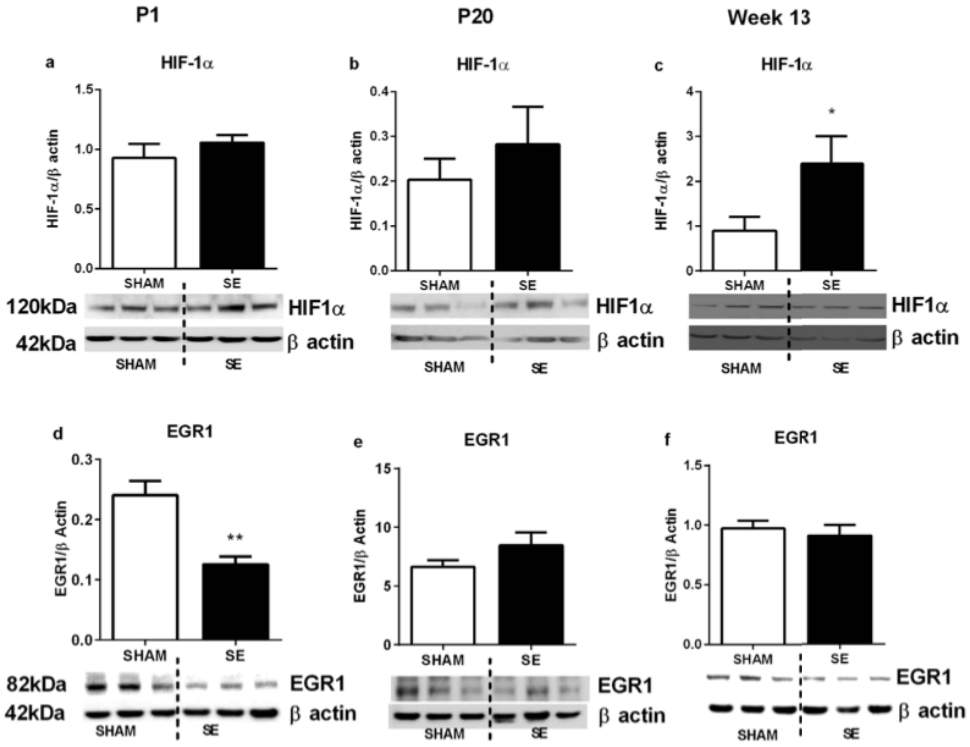

Figure 6. Brain protein levels of hypoxia markers in the offspring of SHAM and SE mothers at different ages (a–f) (n = 3). Whole gel images of (**a**–**c**) in Supplementary Fig. 4. Results are expressed as mean ± S.E.M. Data were analysed by student's unpaired t-test. **P < 0.01. EGR1: Early growth response factor; HIF-1α: hypoxia-inducible factor; SE: smoke exposed.

treatment with cigarette smoke extract[40]. However, cigarette smoking is often associated with increased inflammatory cytokines such as TNFα, IL-1β and IL-6 in the blood and organs, which are also regulated by EGR1[41]. Acute nicotine administration can increase the expression of TNFα, IL-1β and IL-6 in rat brains[42]. Khanna and colleagues found that following 30 days of exposure to 4 cigarettes/day in rats, there was a significant increase in brain inflammatory cells[43]. The difference observed in markers of brain inflammation between the Khanna study and our study may be due to two reasons. Firstly 3R4F research grade cigarettes were used in Khanna's study, which can contain different chemicals from the commercial cigarettes consumed by the humans in this study. Secondly, nicotine and cotinine clearance is known to increase during pregnancy, which may reduce the overall impact of cigarette smoke exposure[44,45], although the change in nicotine metabolism during lactation is unclear. This may affect brain inflammatory response to nicotine and most importantly other chemicals in the cigarette smoke. In addition, in another study, three weeks of treatment with low dose nicotine (<0.5 cigarette/day) was able to reduce inflammatory gene expression in the rat brain[46]. In the current study, we found that the blood cotinine levels in SE mothers were equivalent to 1–2 cigarettes/day in humans[47]. Thus, our effect may be more comparable to the low-dose nicotine treatment previously demonstrated in the literature[46], which is consistent with our observation of reduced brain expression of inflammatory markers in SE mothers. However, increased brain oxidative stress in the SE dams was also observed increased in the study by Khanna *et al.*[43], suggesting cigarette smoke is a direct cause of oxidative stress regardless of the other responses.

Cotinine levels in P20 offspring in this study are similar to those reported in human infants of continuous smokers[48], where chemicals in cigarettes were delivered through the breast milk. Interestingly, the changes of brain inflammatory markers in the SE offspring were somewhat different from their mothers. Only TLR4 mRNA expression at P1 was similar to the SE mothers, which is consistent with a previous study where reduced TLR4 in cord blood was observed in the neonates of smoking mothers[39]. However, TNF-α mRNA was reduced in P1 offspring. This suggests a differential impact of cigarette smoke exposure on mothers and chemicals delivered through the cord blood to their offspring *in utero*. This is not surprising as blood nicotine concentration in the fetus is normally higher than in the maternal blood. The different levels of nicotine and potentially other chemicals from cigarettes might be likely to account for the different inflammatory response observed in offspring versus the smoking mothers[44,45]. Although IL-1β mRNA levels were unchanged in the SE offspring, the persistent increase in IL-1R mRNA observed from birth to adulthood is likely to enhance the inflammatory activity of IL-1β. Surprisingly, at 13 weeks, expression of TLR4, IL-6, and IL-1R mRNA in brain were all increased in SE offspring, which is in contrast to pups at P1 and their SE mothers. This suggests a sustained effect of maternal cigarette smoking in the offspring to change brain inflammatory cytokine production. Microglial activation is known to

be increased by low-dose cigarette exposure SE (plasma cotinine levels of 10 ng/ml) in mice[49], which may be the reason for increased inflammatory cytokine expression that we observed in the P20 SE offspring. The increase in the inflammatory cytokines at 13 weeks may render them more susceptible to the development of neurodegenerative diseases. Neuroinflammation has been shown to plays a crucial role in the development of neurodegeneration. Rodent studies have shown that smoking can lead to pathological changes and accelerated progression of aging[50,51]. In murine cortical neurons, an increase in TLR4 can lead to β-amyloid-induced apoptosis through jun N-terminal kinase – and caspase-3-dependent mechanisms[52]. Injection of IL-1 into rat brain can lead to an elevation of β-amyloid[53], which has been shown to play a role in Alzheimer's Disease (AD). Overexpression of cytokines such as IL-6 can have a neurotoxic effect that leads to neurodegenerative disorders in some individuals[54]. The elevation of TLR4, IL-1R and IL-6 in adult SE offspring suggests that they might be more vulnerable to diseases such as AD. The incidence of AD is higher among smokers[55], which may be transmitted to the offspring due to brain changes by intrauterine SE as we have shown here. However, post-injury functional recovery of neurons is also IL-6 dependent, due to its role in neuronal and glial regeneration[56–58]. In IL-6 knockout mice there was a 60% reduction in neuronal density and decreased sensory function after injury[59]. Therefore decreased IL-6 mRNA in SE dams might also indicate a compromised ability for recovery when brain injury occurs, which requires further investigation.

Although smoking itself is not considered to be able to cause hypoxia in the brain, maternal smoking is one of the risk factors for intrauterine hypoxia, which can lead to sudden infant death after birth[60]. This is mainly due to the restriction of placental blood flow caused by nicotine, which can reduce not only nutrients, but also oxygen supply to the growing foetus[61]. Under normoxic conditions, HIF-1α protein is tightly regulated by oxygen levels. It is maintained at a low level through continuous degradation by the ubiquitin-proteosome pathway[62]. However, long-term hypoxia and the activation of various signal transduction pathways can prevent HIF-1α degradation[62]. The expression of HIF-1α protein is organ specific under systemic hypoxia[63], where HIF-1α binds to the promoter of TLR4 to upregulate TLR4 expression[64]. HIF-1α can also initiate various other hypoxia-inducible adaptations by regulating glycolysis, erythropoiesis, angiogenesis and cell proliferation[65]. Smoking itself has previously been shown to inhibit hypoxia-inducible adaptations in peripheral tissues[66]. Cigarette smoke exposure SE can also impair the production of HIF-1α as well as the stabilization of HIF-1α protein levels[66]. HIF-1 has been shown to have complex roles in the brain following injury and depending upon the stimulus and cell type being examined, can be neurotoxic or neuroprotective[67,68]. Hypoxia-induced angiogenesis is suggested to be inhibited in the smokers due to an impairment in the HIF-1 pathway[66], thus smokers are more likely to suffer from more severe injury during stroke, with a worse prognosis compared with the non-smokers[69]. Here we only observed marginal reduction in brain HIF-1α protein in the SE mothers, which may be due to the low-dose and the relatively short exposure to cigarette smoke. EGR1 regulates the expression of HIF-1α during hypoxia[70]. Although EGR1 protein was not changed in the SE mothers, it was reduced in the newborn SE offspring. This may be due to a direct suppression by chemicals in the cigarette smoke inhaled by the mothers, which are at higher levels in newly born offspring compared with the mothers. It also needs to be noted that EGR1 is not the only regulator of HIF-1α, therefore the unchanged brain HIF-1α levels we observed in P1 and P20 offspring exposed to cigarette smoke SE may be due to the actions of other factors that regulate HIF-1 function, which is beyond the scope of this study. After birth, brain oxygen is replenished, while the impact of cigarette smoke components in the breast milk on HIF-1α levels also disappeared after weaning. This may lead to higher brain HIF-1α levels at adulthood. However, HIF-1α itself can induce inflammatory responses in the brain[71] as we have observed in the adult SE offspring where TLR4 and TNFα are both upregulated. As brain EGR1 levels were unchanged at this age, it may not play a major role in increasing HIF-1α in the SE offspring. Considering the protective effect of HIF-1α, its increase in the brains of SE offspring may be an adaptation to protect against increased oxidative stress in the brain. It has been suggested that under normoxic conditions, increased oxidative stress due to excessive mitochondrial ROS production can increase HIF-1α protein levels[72]. This impact of oxidative stress is also seen in the brain of SE offspring here. Under basal conditions, 90% of ROS are produced in the mitochondria, mainly by OXPHOS complexes I and III in the electron transport chain[73]. Complex II is involved in the conversion of metabolic intermediates to complement the action of complexes I and III[74]. When the activities of both complexes I and III are inhibited, complex II will generate large amounts of superoxide[75]. Complex IV (known as cytochrome oxidase) is a crucial regulator for OXPHOS, the dysfunction of which leads to reduced ATP levels[76]; while Complex V converts ADP to ATP[74]. ROS generated during OXPHOS is both beneficial and detrimental to the cells[77]. It can activate the antioxidant defence network to prevent damage to the host itself. Thus, ROS are tightly regulated by antioxidant enzymes such as MnSOD[78]. In the SE offspring, MnSOD was unchanged at P1 and P20, possibly due to the protective effect of the antioxidant-rich breast milk[79]. Changes in mitochondrial OXPHOS complexes in brains of SE offspring mirror the changes of TOM20 levels, both at weaning and in adulthood. TOM20 imports protein into the mitochondria from the outer mitochondrial membrane[80], reflecting changes in energy needs by the mitochondria and body. Reduced OXPHOS complex and TOM20 levels at P20 may be due to a redistribution of nutrients after birth required for the catch-up growth of the other organ systems commonly seen in offspring from smokers. Similar to their mothers, brain mitochondrial complexes I–V and TOM20 in the SE offspring were all increased at 13 weeks, suggesting increased substrate metabolism, which can result in increased ROS production. Conversely, mitochondrial MnSOD levels are low and may not be sufficient to clear excess ROS, resulting in oxidative stress and related tissue damage. Here, we have observed increased levels of nitrotyrosine protein in the brains of SE offspring at 13 weeks. Elevated nitrotyrosine levels are harmful to the brain, and is one factor contributing to neurodegenerative diseases in humans[81]. However, the link between increased brain oxidative stress and any brain dysfunction in the SE offspring remains to be elucidated.

In conclusion, maternal cigarette SE differentially changed brain inflammatory and hypoxia response markers in the mother and offspring. However, oxidative stress and mitochondrial damage were changed in a similar manner in both SE mothers and their offspring, which may predispose them to neurodegeneration in later life.

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Author Contributions

H.C., N.J. and S.S. designed the study. Y.L.C., S.S., A.A.Z. and I.A. performed all the experiments. Y.L.C., S.S., C.P., B.O., I.A., A.A.Z., N.J. and H.C. contributed to the writing of the main manuscript text, and Y.L.C., S.S. and H.C. prepared Figures 1–6. Y.L.C. prepared Tables 1–2. All authors reviewed the manuscript.

Additional Information

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Chapter 4
Impact of maternal cigarette smoke exposure on brain and kidney health outcomes in female offspring

(In this chapter, Yik Lung Chan contributed to the experiments related to the brain, writing and analysis)

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Impact of maternal cigarette smoke exposure on brain and kidney health outcomes in female offspring

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Summary

Increased oxidative stress in the brain can lead to increased sympathetic tone that may further induce kidney dysfunction. Previously we have shown that maternal cigarette smoke exposure (SE) leads to significantly increased oxidative stress and inflammation in both brain and kidney, as well as reduced brain and kidney mitochondrial activity. This is closely associated with significant kidney underdevelopment and abnormal function in adulthood in the male offspring. This study aimed to investigate the impact of maternal SE on brain and kidney health in the female offspring. In this study, the mouse dams were exposed to two cigarettes, twice daily for 6 weeks prior to gestation, during pregnancy and lactation. Brains and kidneys from the female offspring were collected at 20 days (P20) and 13 weeks (W13) and were subject to further analysis. We found that mRNA expression of brain inflammatory markers interleukin-1 receptor and Toll-like receptor 4 were significantly increased in the SE offspring at both P20 and W13. Their brain mitochondrial activity markers were however increased at W13 with increased antioxidant activity. Kidney development and function in the female SE offspring were not different from the control offspring. We concluded that although brain inflammatory markers were upregulated in the SE female offspring, they were protected from some of the indicators of brain oxidative stress, such as endogenous antioxidant and mitochondrial dysfunction, as well as abnormal kidney development and function in adulthood.

KEYWORDS
brain inflammation, kidney development, mitochondrial activity, oxidative stress

1 | INTRODUCTION

There is increasing recognition of the gender difference in the physiological processes underpinning disease.[1] The impact of gender disparities on the developmental programming of adulthood diseases has been well documented. Prenatal insults can change the physiological development of the foetus and newborn leading to both brain and kidney abnormalities that may only manifest in later life. In the brain, a decrease in hippocampal volume and cortical monocyte infiltration can result in neurological disorders in a gender-dependent manner.[2] Under conditions of infection or inflammation, the microglial colonization and activation-induced neuronal loss is more severe in the male offspring compared to the female littermates.[2] In contrast, chemical toxicity in humans, such as bone and kidney damage due to cadmium exposure (as found in cigarette smoke) is less severe in males than in females.[3]

Cigarette smoke exposure (SE) during pregnancy is considered as a leading preventable cause of adverse outcomes in newborn infants, including low birth weight and reduced brain and kidney volumes.[4,5] Although the total number of women smokers has been decreasing

*These authors contributed equally to this study.

TABLE 1 Parameters of female offspring at different ages

	P20		W13	
Offspring	SHAM n=21	SE n=17	SHAM n=16	SE n=14
Body weight (g)	9.7±0.24	9.0±0.28*	22.4±0.2	20.8±0.4*
Brain (mg)	14±0.6	14±0.4	18.2±1.2	17.1±0.8
Brain %	1.4±0.1	1.6±0.1*	0.8±0.1	0.7±0.1
Kidney (mg)	70.2±6.3	63.9±2.4	128±2	118±2
Kidney %	0.73±0.06	0.72±0.02	0.57±0.01	0.57±0.01

Results are expressed as mean±SEM. Data were analysed by student's unpaired *t* test. *P<.05, compared to the SHAM offspring at the same age, n=14–21.

worldwide, the rate of decline is slowing, with recent estimates of female smoking rates being 15.3% in the USA,[6] 16.3% in Australia, and 23% in Argentina.[7] The rate of second-hand SE is high in developing countries, such as China with 70% of adults exposed to second-hand smoke.[8] The World Health Organization predicts that female smoking will reach an 'epidemic' (20% of the population) in developing countries by 2025.[9] Unfortunately, smoking is still common during pregnancy, estimated at up to 15%[10] despite targeted public health education. It has been well documented that maternal smoking is directly linked to adult obesity, type 2 diabetes, chronic kidney disease and psychopathology, as well as childhood asthma.[11–15] Our previous studies have shown that maternal cigarette SE can increase oxidative stress, mitochondrial dysfunction, and inflammation in both the brains and kidneys in male offspring.[15–17] It has been shown that increased oxidative stress in the brain can lead to increased sympathetic tone that may induce hypertension, as well as kidney dysfunction.[18,19] We have also shown that maternal smoking is closely linked to increased oxidative stress, inflammation and mitochondrial dysfunction in both brain and kidney, and renal functional disorders in male offspring at adulthood.[15,16,17,20]

There are also numerous studies on the adverse effects of maternal smoking on other organ systems.[21–23] However, these studies have mainly focused on male offspring. Such gender bias is common in animal studies. In fact, females are less susceptible to certain diseases than their male counterparts.[24] Oestrogen is known to inhibit oxidative stress and inflammation,[25,26] and able to protect female offspring against the development of hypertension due to perinatal nicotine exposure.[27] In addition, inflammation and oxidative stress also contribute to renal underdevelopment caused by maternal smoking in early life and chronic kidney disease at adulthood.[15–17,20] Furthermore, there is a gender difference in the development of renal injury where females tend to be protected from renal injury, independent of other health conditions.[28–30] Epidemiological studies confirm that the association between low birth weight and adulthood kidney disease is more evident in the males than the females[24] and that maternal smoking is a significant factor leading to low birth weight in humans.[31] However, the impact of maternal smoking on oxidative stress, the inflammatory profile and mitochondrial function in the brain and kidney, as well as renal function of female offspring in adulthood is unclear. These uncertainties formed the aims of this study.

2 | RESULTS

2.1 | Effects of maternal SE on female offspring

2.1.1 | Anthropometry

The body weights were significantly lower in the SE female offspring at both P20 and W13 (P<.05, Table 1). Although the net brain weight was not different between the groups at either P20 or W13, their percentage relative to the body weight was significantly greater than in the SHAM offspring at P20 only (P<.05, Table 1). Kidney weight was not different between the groups at both ages (Table 1). Plasma cotinine levels in the SE offspring (8.31±1.93 ng/mL) were four times that in the SHAM offspring (1.98±0.56 ng/mL, P<.05) at P20.

2.1.2 | Brain markers

At P20, brain mRNA expression of inflammatory markers, including IL1 receptor (IL1R), IL6 and toll-like receptor 4 (TLR4) were significantly upregulated in the SE offspring (P<.05); while the expression of inflammatory cytokines IL1β and tumour necrosis factor (TNF)-α was not different between the groups (Fig. 1). At W13, mRNA expression of IL1R and TLR4 remained higher in the SE offspring (P<.05), while the other markers were not different between the groups (Fig. 1).

At P20, there was no significant difference in brain protein levels of mitochondrial markers of oxidative stress, including the anti-oxidative marker manganese superoxide dismutase (MnSOD) markers of mitochondrial function. Translocase of outer membrane (TOM)20 transport proteins from outer membrane into the inner membrane of mitochondria, and oxidative phosphorylation (OXPHOS) complexes are the major sites for ATP synthesis with reactive oxygen species (ROS) as the by-products, both of which were not changed in the SE offspring (Fig. 2a–c). However at W13, the protein levels of MnSOD and the OXPHOS complexes I–IV were all significantly increased in the SE offspring compared with the SHAM offspring (P<.05, Fig. 2d–f).

Early growth response protein (EGR)1 – hypoxia induced factor (HIF)-1α pathway is an important protective mechanism during environmental hypoxia to increase cell survival rate.[32,33] At P20, HIF-1α protein levels were significantly decreased in the SE offspring (P<.05, Fig. 3a); and EGR1 mRNA expression was also significantly

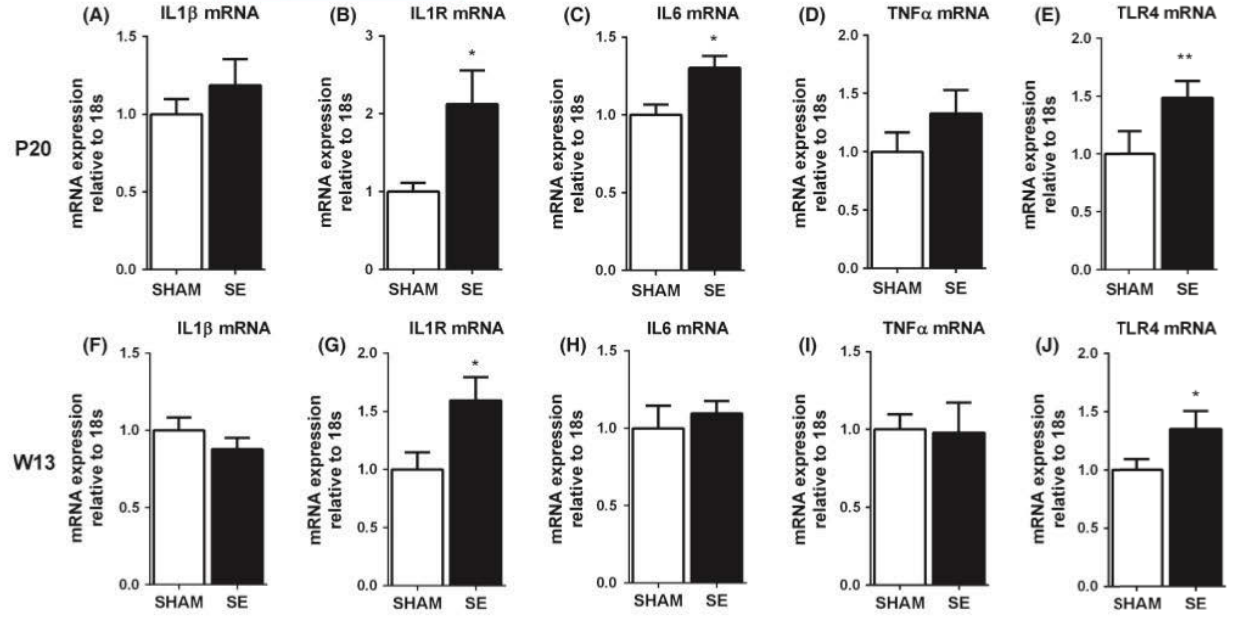

FIGURE 1 Brain mRNA expression of inflammatory markers in the SHAM and SE offspring (n=8). Results are expressed as mean±SEM. Data were analysed by student's unpaired *t* test. **P*<.05; ***P*<.01. SE, smoke exposed; TLR, toll-like receptor

downregulated at this time point (*P*<.05, Fig. 3c). At W13, neither HIF-1α protein nor EGR1 mRNA expression was changed (Fig. 3c,d).

2.1.3 | Kidney markers

mRNA levels of different growth factors involved in renal development were determined in the female offspring of SE and SHAM mothers as we have previously measured in the male offspring.[15] However, no difference was observed in the mRNA expression of specific markers associated with kidney development between the two groups at P20 and W13 (Fig. 4a,b). Interestingly, in the female offspring, there were no changes in the number of developed glomeruli and size between the groups at P20 or W13 (Fig. 5). At W13, fully developed glomeruli were present in the kidneys of both treatment groups (Fig. 5). Furthermore, at W13, the markers related to renal injury including fibronectin and collagen IV were not different between the groups (Fig. 6). Markers of renal function, such as urinary albumin/creatinine ratio and plasma creatinine, were also shown to be similar between the treatment groups at both P20 and W13 (Table 2).

3 | DISCUSSION

The major finding in this study is that female offspring appear to be protected from some of the expected detrimental effects of maternal cigarette SE, including: brain inflammation, oxidative stress and mitochondrial dysfunction, as well as renal structural and functional disorders at adulthood, which we have previously demonstrated in male offspring.[15,17]

Maternal smoking is a significant risk to public health. Nicotine, the major addictive substance contained in cigarette smoke has been commonly used to model smoking in previous studies.[25,26] However, these studies have excluded the effects of additional toxic chemicals contained in cigarette smoke[34] that may also affect foetal development and future predisposition to chronic disease. Using a model with direct cigarette SE more closely approximates the complexity of exposure to maternal cigarette smoking. Here we have evaluated the levels of cotinine, which is the major metabolic product of nicotine, and we have confirmed that it is increased in P20 female SE offspring, thereby validating the model and hence exposure to multiple toxins contained in cigarette smoke.

Our previous studies have shown that male offspring are vulnerable to maternal cigarette SE-induced underdevelopment of brain and kidney and potential dysfunction in adulthood, which are associated with increased inflammatory markers and oxidative stress.[17] In this study, at adulthood, only the receptors of the inflammatory pathway were upregulated in the brains of female offspring. In the brains of male offspring from SE mothers, in addition to those changes also demonstrated in the females, pro-inflammatory cytokine IL6 was also increased. As such, male offspring appear to have higher levels of brain inflammation than the females due to maternal SE. Increases in brain inflammatory cytokines has been shown to predispose individuals to the development of neurodegenerative diseases later in life in both genders.[35] It has been found that former male smokers have a higher risk of developing Alzheimer disease.[36] We also observed here that adult SE female offspring have normalised expression of pro-inflammatory cytokines. This may be due to potential neuroprotective effects of oestrogen which has been shown to reduce the production of pro-inflammatory cytokines, such as IL-6 and TNFα.[37]

Female offspring have been shown to be more resistant to oxidative stress as suggested by previous studies.[38-40]

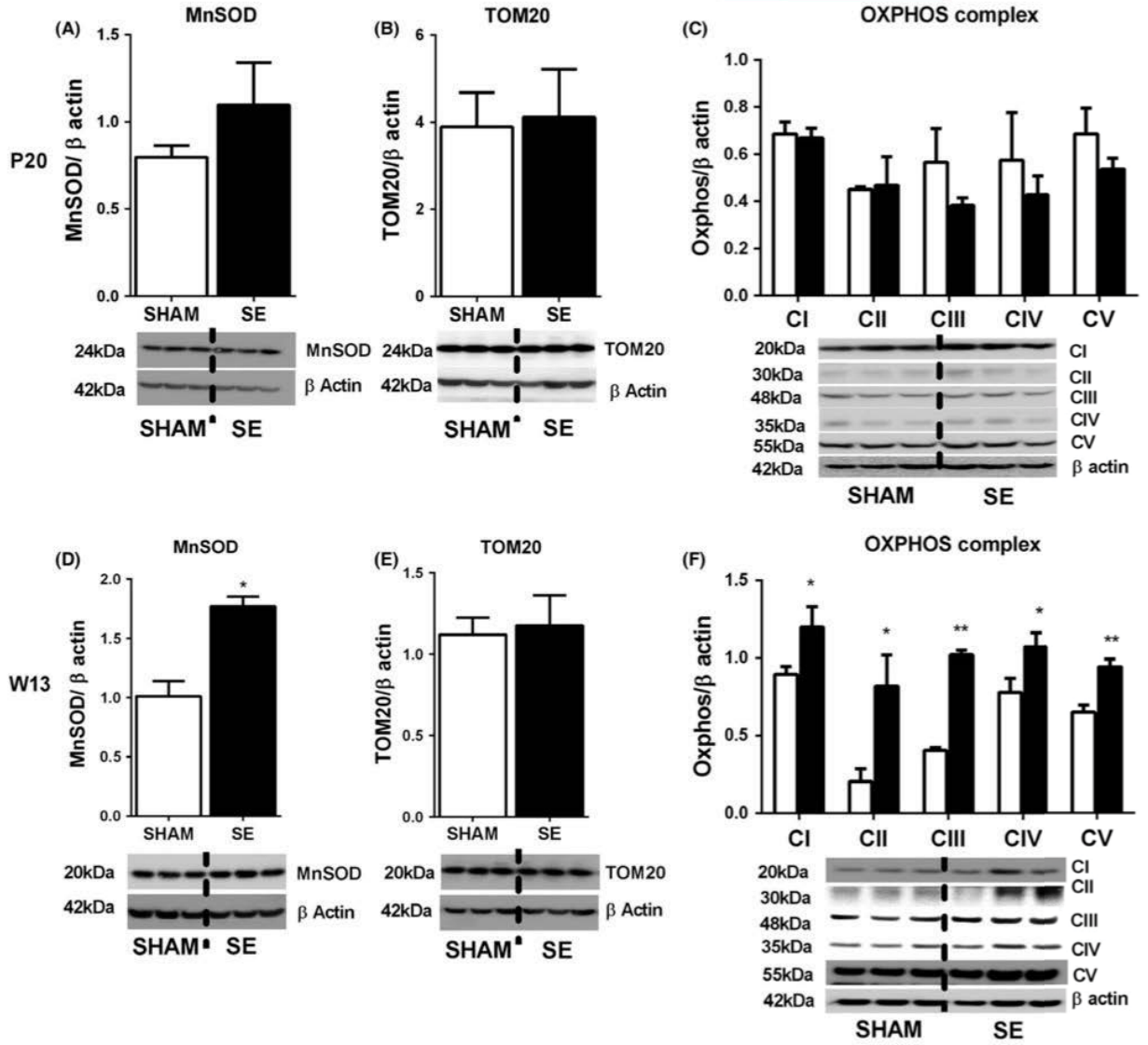

FIGURE 2 Brain mitochondrial protein levels of (a, b) MnSOD, (b, e) TOM20, and (c, f) OXPHOS complexes (CI–V) in the SHAM and SE offspring at different ages. Results are expressed as mean±SEM. Data were analysed by student's unpaired *t* test. $P<.05$; $^{**}P<.01$. MnSOD, manganese superoxide dismutase; OXPHOS, oxidative phosphorylation; SE, smoke exposed; TOM, translocase of the mitochondrial outer membrane

Mitochondria are important cellular organelles as they are involved in ATP production; while ROS is a major by-product from OXPHOS complexes I and III during the electron chain transportation during ATP production.[41] Excessive ROS accumulation leads to oxidative stress, and resultant cellular toxicity; while the antioxidant enzyme MnSOD can scavenge excessive ROS in the mitochondria to prevent such damage.[42] Excessive mitochondrial ROS characterises cerebrovascular pathophysiology.[43] As such, MnSOD has been found to reduce lipid peroxidation, protein nitration, and neuronal death after cerebral ischaemic injury.[44] In humans, maternal smoking was associated with increased oxidative stress in 3 months old babies.[45] We have recently demonstrated increased brain oxidative stress in the male SE offspring at W13, adulthood,[17] where MnSOD was significantly reduced in the face of increased both TOM20 and OXPHOS protein complexes. TOM20 transports substrates from the outer mitochondrial membrane to OXPHOS complexes for energy production,[46] which can generate more free radicals during ATP synthesis. In this study, although increased OXPHOS complexes were observed in the female offspring at W13, which suggests an increased capacity for substrate metabolism, unchanged TOM20 in the females may indicate unchanged energy metabolism in the brain. The level of brain mitochondrial MnSOD protein was increased in female SE offspring at W13, suggesting an increased ability to scavenge free radicals. As such, female offspring may be better at responding to an environment of higher oxidative stress than the males.

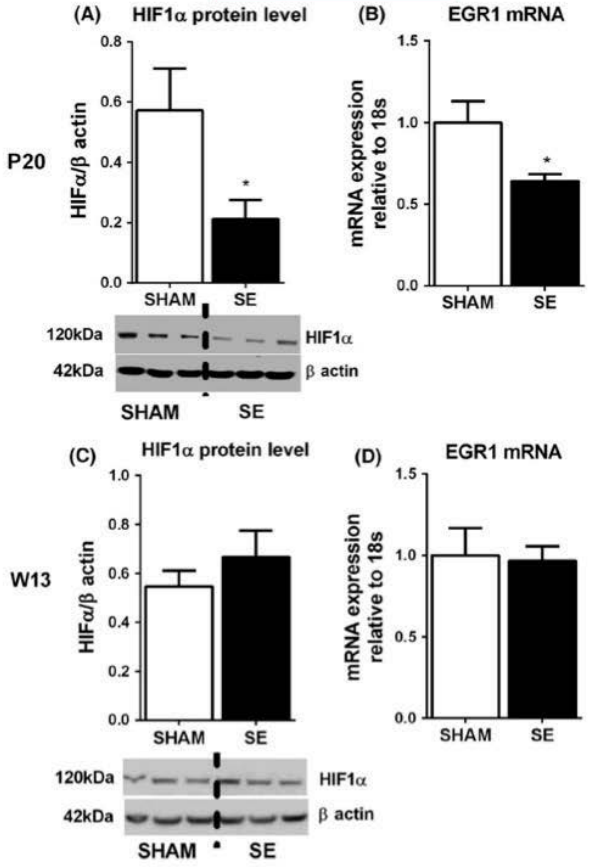

FIGURE 3 Brain HIF-1α protein and EGR1 mRNA level in the SHAM and SE offspring at P20 and W13 (n=3–8). Results are expressed as mean±SEM. Data were analysed by student's unpaired *t* test. **P*<.05. EGR1, early growth response protein; HIF, hypoxia inducible factor; SE, smoke exposed

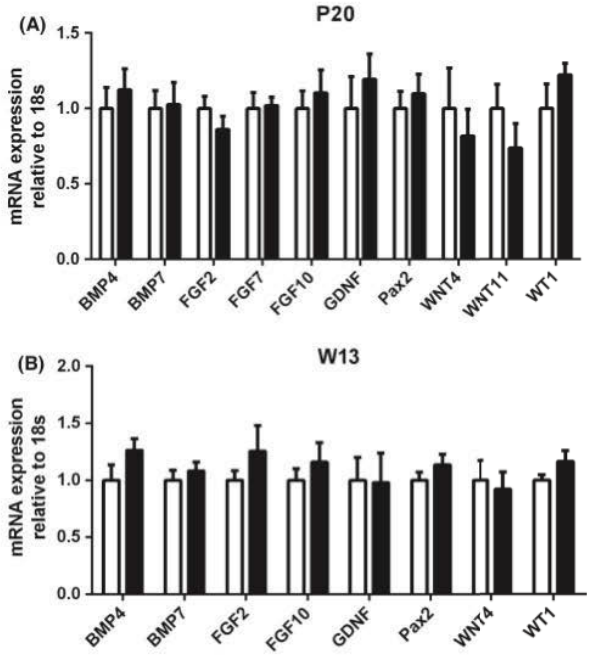

FIGURE 4 Renal mRNA expression of growth and transcription factors in the female offspring mice at (a) P20 and (b) W13. Results are expressed as mean±SEM, n=6. BMP, bone morphogenetic proteins; FGF, fibroblast growth factor; GDNF, glial cell-line derived neurotrophic factor; Pax, paired box; WNT, wingless-type MMTV integration site family member; WT, Wilms tumour inhibitory protein

In response to an hypoxic environment, EGR1 is upregulated which in turn stimulates HIF1α to protect tissue from damage and increase survival rate under conditions such as transient focal cerebral ischaemia.[32,33] HIF1α can also induce inflammatory responses in the brain to scavenge necrotic tissues.[47] EGR1 also appears to have roles in promoting synaptic transmission, plasticity, learning and long term memory.[48] Unlike the high level of HIF1α we have recently observed in the adult male offspring of SE mothers, HIF1α protein levels in the females were reduced at P20, but unchanged at W13. This indicates that there may be an adaptive mechanism in early life that protects the females from the impact of a hypoxic intrauterine environment due to maternal SE, which requires further investigation.

Although we have observed some similarities in the effects of smoking on the brain in both genders of offspring, the impact of maternal SE on kidney structure and function is considerably different between the male and female SE offspring. In the male offspring, we recently reported that changes in early growth and developmental factors lead to renal underdevelopment, with resultant renal dysfunction at adulthood following maternal SE,[15] similar to what occurs during intrauterine undernutrition where the kidney is one of the organs that are 'sacrificed', resulting in renal underdevelopment.[49] Here we demonstrate that female offspring are less susceptible to renal underdevelopment and resulting functional disorders due to maternal smoking compared to the male offspring. Either the adaptive changes in growth factors are more successful in the female, or other unknown mechanisms positively contribute. Previously, we have found that reduced mitochondrial activities and increased oxidative stress are closely related to renal underdevelopment and renal functional disorders at adulthood in the male offspring. Renal underdevelopment due to maternal smoking is an independent factor to lead to renal dysfunction in adulthood.[24]

Our findings are in agreement with previous studies showing that female offspring are less vulnerable to diseases induced by intrauterine insults.[40] It is known that gender is a risk factor for developing kidney disease due to the differences in renal structure, glomerular hemodynamics and hormonal metabolism between the males and females.[50] Sex hormones can be an important factor in affecting renal function, as oestradiol has been shown to inhibit transforming growth factor-β and transcription of downstream collagen IV, to reduce renal injury.[51]

In conclusion, our previous papers have reported upregulated inflammatory cytokine IL-6 expression, and reduced anti-oxidative capacity in the brain,[17] and renal underdevelopment and abnormal renal function in the kidney[15] of the male offspring by maternal SE. In female offspring reported in this study, we found unchanged IL-6 expression and increased anti-oxidative capacity in the brain, with normal renal development and function at adulthood. Thus, female offspring are more

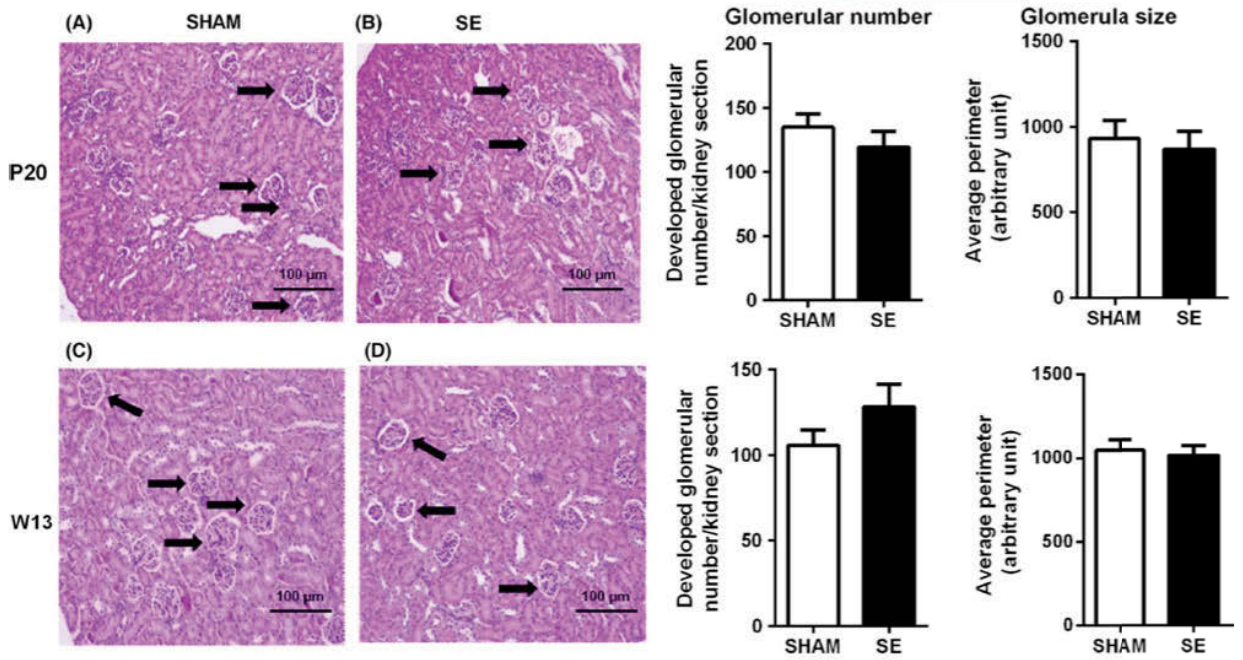

FIGURE 5 H&E stained kidney sections from the sham offspring (left panel) and SE offspring (right panel) at (a, b) P20, and (c, d) W13 and glomerular numver and size for the same age. Closed arrows indicate fully developed glomeruli. Results are expressed as mean±SEM, n=6–8. Mag. 20×

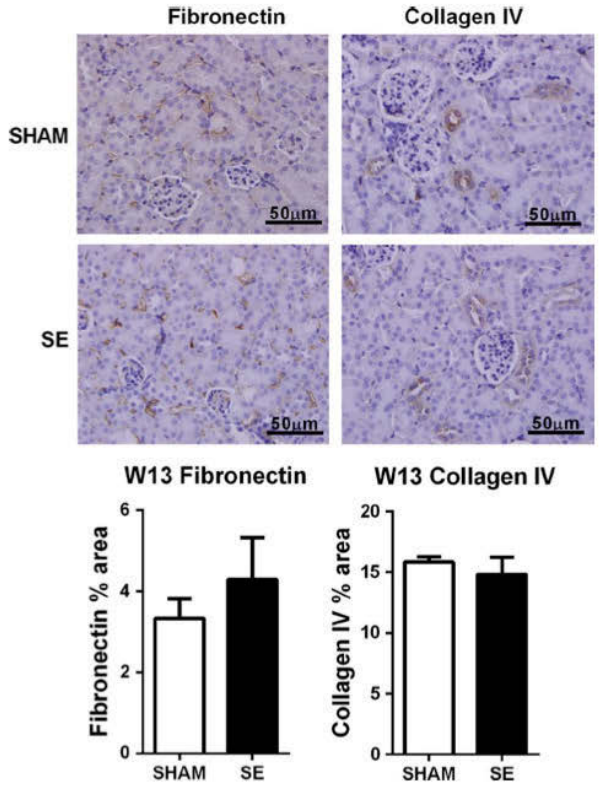

FIGURE 6 Renal fibronectin (left panel) and collagen IV (right panel) protein expression in the SHAM (top panel) and SE offspring (lower panel) at W13. Results are expressed as mean±SEM, n=6. Mag. 40×

TABLE 2 Markers of renal function at different ages

Offspring	P20 SHAM n=21	P20 SE n=17	W13 SHAM n=16	W13 SE n=14
Urinary albumin/ creatinine ratio (μg/mg)	6.4±0.9	6.2±1.6	25.3±9.8	27.7±5.1
Plasma enzymatic creatinine (μmol/L)	18.0±2.9	25.7±5.6	13.7±1.4	14.5±1.3

Results are expressed as mean±SEM. Data were analysed by student's unpaired *t* test. n=14–21.

resistant to the detrimental effects of maternal smoking on brain and kidney in comparison to male offspring. Further work is needed to determine the intrauterine factors that differentiate such gender difference.

4 | MATERIALS AND METHODS

4.1 | Maternal cigarette SE

Virgin female Balb/c mice (6 weeks, Animal Resources Centre, Perth, Australia) were housed at 20±2°C and maintained on a 12 hour / 12 hour light / dark cycle (lights on at 06.00 hours) with ad libitum access to standard laboratory chow and water. After acclimatisation, mice were assigned to SE or sham exposure (SHAM). The SE group was exposed to two cigarettes (Winfield Red, nicotine ≤1.2 mg, CO ≤15 mg; Philip Morris, Melbourne, Australia) in a perspex chamber,

twice daily for 6 weeks prior to mating, during gestation and lactation; while the SHAM group was exposed to normal air as previously described.[15] They were mated with male Balb/c mice (8 weeks) from the same source. Male breeders and suckling offspring were not exposed to cigarette smoke as we described previously.[15] The female offspring were studied at postnatal (P) day 20 (P20, weaning) and week 13 (W13, mature age).

4.2 | Sample collection

Female offspring were killed after anaesthetic overdose (Pentothal, 0.1 mg/g, i.p.; Abbott Australasia, Macquarie Park, Australia) as previously described.[15] Blood was collected via cardiac puncture and plasma was stored at −20°C. Plasma cotinine concentrations were measured by enzyme-linked immunosorbent assay (ELISA; Abnova, Taipei, Taiwan). Urine was collected directly from the bladder. The brain was dissected into the left and right hemisphere. The left brain hemisphere and left kidney were stored at −80°C for mRNA and protein analysis, while the right kidney was fixed with 10% formalin for histological analysis.

4.3 | Quantitative real-time (rt)-PCR

Brain inflammatory markers and renal developmental markers were measured by rt-PCR. Total mRNA was extracted from the brain tissues using TriZol reagent (Life Technologies, Foster City, CA, USA). The purified total RNA was used as a template to generate first-strand cDNA using M-MLV Reverse Transcriptase, RNase H, Point Mutant Kit (Promega, Madison, WI, USA) as previously described.[52] Genes of interest were measured using manufacturer pre-optimized and validated Taqman primers and probes (Life Technologies; EGR1, probe TGAGCACCTGACCACAGAGTCCTTT, NCBI references: NM_007913.5, M20157.1, M19643.1, ID Mm00656724_m1; IL-1β, probe TCCTTGTGCAAGTGTCTGAAGCAGC, NCBI references: NM_008361.3, M15131.1, BC011437.1, ID Mm01336189_m1; IL-1R, probe AGCTGACCCAGGATCAATGATACAA, NCBI references: NM_001123382.1, NM_008362.2, M20658.1, ID Mm00434237_m1; IL-6, probe ATGAGAAAAGAGTTGTGCAATGGCA, NCBI references: NM_031168.1, X06203.1, X54542.1, ID Mm00446190_m1; TLR4, probe CCCTGCATAGAGGTAGTTCCTAATA, NCBI references: NM_021297.2, ID Mm00445273_m1; TNFα, probe CCCTCACACTCAGATCATCTTCTCA, NCBI references: NM_013693.2, X02611.1, M13049.1, ID Mm00443259_g1) or pre-optimized SYBR Green primers as previously published.[15] For the Taqman probes, target genes were labelled with FAM and the housekeeping 18s rRNA was labelled with VIC. Gene expression was standardized to 18s RNA. Then the average expression of the control group was assigned as the calibrator against which all other samples are expressed as fold difference.

4.4 | Western blotting

The protein levels of HIF-1α, MnSOD, TOM20 and OXPHOS complexes proteins were measured. The brain was homogenised using cell lysis buffers for whole protein and mitochondrial protein extraction. Protein samples of 40 µg were separated on NuPage Novex 4–12% Bis-Tris gels (Life Technologies) and then transferred to polyvinylidene fluoride (PVDF) membranes (Thermo Fisher Scientific, Waltham, MA, USA). Membranes were then blocked with non-fat milk powder and incubated with primary antibodies HIF-1α (1:1000; Novus Biologicals, Littleton, CO, USA), MnSOD (1:1000) & TOM20 (1:2000; Santa Cruz Biotechnology, Dallas, TX, USA) and Mitoprofile Total OXPHOS complex rodent WB antibody (1:2500; Abcam, Cambridge, UK) for overnight and then goat anti-rabbit or rabbit anti-mouse IgG horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology, 1:2000 for HIF-1α; 1:5000 for MnSOD, TOM20 and OXPHOS complex). Protein expression was detected by SuperSignal West Pico Chemiluminescent substrate (Thermo Fisher, Waltham, MA, USA) by exposure of the membrane in FujiFlim (Fujifilm, Tokyo, Japan). Protein band density was determined with Image J software (NIH, Bethesda, MD, USA).

4.5 | Kidney histology

Fixed kidney samples were embedded in paraffin and renal structure was examined using haemotoxylin and eosin (H&E) staining. Glomerular number was estimated by counting the developed glomeruli in three to four non-consecutive kidney sections from the same animal, and six to eight animals were used from each group. Glomerular size for each animal was measured using Image J (Image J; NIH) in an average of six different images for the same kidney section then averaged.[15] For IHC staining, kidney sections were incubated with rabbit anti-mouse primary antibodies against fibronectin (1:500) and collagen IV (1:500) (Abcam, Cambridge, UK), and Envision & HRP-labelled polymer secondary anti-rabbit antibodies (Dako, Carpinteria, CA, USA), followed by horseradish peroxidase enzyme and DAB for colour detection (Dako). On average, six different non-overlapping fields of the same kidney section were captured. Quantitation of the positive signals (the intensity of the brown colour) in the captured images was performed using Image J software (NIH) and the percentage of the brown colour of the whole field was determined and averaged.

4.6 | Albumin and creatinine assays

Urine albumin and creatinine were measured using a Murine Microalbuminuria ELISA kit (Albuwell M; Exocell Inc, Philadelphia, PA, USA) and a Creatinine Companion kit (Exocell Inc) respectively as previously described.[15] Serum enzymatic creatinine levels were measured by an automated analyser (ARCHITECT; Abbott Australasia).[15]

4.7 | Statistical methods

Results are expressed as mean±SEM. Data were analysed for parametric distribution. The difference between groups was analysed using unpaired student's *t* test (Statistica 9; Statsoft, Tulsa, OK, USA).

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DISCLOSURE

The authors have nothing to disclose.

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Chapter 5
Maternal L-Carnitine supplementation improves brain health in offspring from cigarette smoke exposed mothers

frontiers in Molecular Neuroscience

ORIGINAL RESEARCH
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Maternal L-Carnitine Supplementation Improves Brain Health in Offspring from Cigarette Smoke Exposed Mothers

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Maternal cigarette smoke exposure (SE) causes detrimental changes associated with the development of chronic neurological diseases in the offspring as a result of oxidative mitochondrial damage. Maternal L-Carnitine administration has been shown to reduce renal oxidative stress in SE offspring, but its effect in the brain is unknown. Here, we investigated the effects of maternal L-Carnitine supplementation on brain markers of oxidative stress, autophagy, mitophagy and mitochondrial energy producing oxidative phosphorylation (OXPHOS) complexes in SE offspring. Female Balb/c mice (8 weeks) were exposed to cigarette smoke prior to mating, during gestation and lactation with or without L-Carnitine supplementation (1.5 mM in drinking water). In 1 day old male SE offspring, brain mitochondrial damage was suggested by increased mitochondrial fusion and reduced autophagosome markers; whereas at 13 weeks, enhanced brain cell damage was suggested by reduced fission and autophagosome markers, as well as increased apoptosis and DNA fragmentation markers, which were partially reversed by maternal L-Carnitine supplementation. In female SE offspring, enhanced mitochondrial regeneration was suggested by decreased fission and increased fusion markers at day 1. At 13 weeks, there was an increase in brain energy demand, oxidative stress and mitochondrial turnover, reflected by the protein changes of OXPHOS complex, fission and autophagosome markers, as well as the endogenous antioxidant, which were also partially normalized by maternal L-Carnitine supplementation. However, markers of apoptosis and DNA fragmentation were not significantly changed. Thus L-Carnitine supplementation may benefit the brain health of the offspring from smoking mothers.

Keywords: maternal smoking, autophagy, mitophagy, oxidative stress, gender difference

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Abbreviations: Cox, cytochrome c oxidase subunit; Drp-1, dynamin-related protein-1; Fis-1, fission protein; LC3A/B, light chain 3 microtubule associated protein A/B; MnSOD, manganese superoxide dismutase; Opa-1, optic atrophy 1; OXPHOS, oxidative phosphorylation; P, postnatal; Pink-1, phosphatase and tensin homolog induced putative kinase; ROS, reactive oxygen species; SE, cigarette smoke exposed; SELC, SE breeders supplied with L-Carnitine; Tom-20, translocase of outer membrane-20.

INTRODUCTION

Cigarette smoking is a leading cause of death and morbidity worldwide. Despite increased public education and government policies to ban smoking in public places (Balmford et al., 2016), smoking among women of childbearing age and during pregnancy is still common (Mendelsohn et al., 2014). This is partially due to low success rates of smoking cessation during pregnancy (Glover et al., 2016). The adverse impact of maternal smoking on health outcomes in the next generation has been well studied, including increased risk of type 2 diabetes mellitus, impaired renal function and structure, and sudden infant death (Jaakkola and Gissler, 2004; Shah et al., 2006; Al-Odat et al., 2014; Fang et al., 2015). In addition, we have shown increased inflammation, abnormal mitochondrial metabolic markers and oxidative stress-related cell injury in the brains of offspring from cigarette smoke exposed (SE) mothers (Chen et al., 2011; Chan et al., 2016b).

Autophagy removes damaged or junk organelles in cells (Ashrafi and Schwarz, 2013). Mitophagy is when autophagy occurs in the mitochondria, which is an important quality control mechanism to remove damaged mitochondria (Ashrafi and Schwarz, 2013). This process recycles intact mitochondrial fragments to generate new healthy mitochondria through fission and fusion (Bereiter-Hahn, 1990; Westermann, 2010) to maintain mitochondrial integrity. In stroke, reduced mitophagy and autophagy may hinder the prompt clearance of damaged mitochondria in the brain, leading to a loss of protection from increased reactive oxygen species (ROS) and reduced mitochondrial ATP supply (Frugier et al., 2016). A reduction in autophagy to clear aggregated proteins appears to underlie the development of neurodegeneration in Parkinson's disease (Zhang et al., 2015). Our previous data also suggests that maternal SE impairs brain mitochondrial levels of oxidative phosphorylation (OXPHOS) complexes in male offspring with reduced endogenous antioxidant capacity from birth (Chan et al., 2016b). This is due to the changes of mitochondrial dynamics in the brain from birth to adulthood (Hagberg et al., 2014). However, the changes in markers of autophagy and mitophagy in this process are unclear. This formed the first aim of this study.

Furthermore, as SE offspring have reduced brain antioxidants and increased cellular oxidative damage (Chan et al., 2016b), boosting the antioxidant capacity during early life may ameliorate the impact of maternal SE on brain outcomes. The antioxidant L-Carnitine has been shown to improve white matter lesion after chronic hypoperfusion in rats (Ueno et al., 2015) and can provide neuroprotection by elevating brain antioxidant capacity in aging rats (Juliet et al., 2005). We have shown that maternal L-Carnitine supplementation during pregnancy and lactation can alleviate oxidative stress, as well as mitochondrial and renal dysfunction in offspring from SE mothers (Nguyen et al., 2015). As such, this approach may also ameliorate the impact of maternal SE on the brain by affecting mitophagy and autophagy markers. In this study, we investigated the impact of maternal L-Carnitine supplementation during gestation and lactation on brain markers of mitophagy, autophagy, mitochondrial antioxidant and OXPHOS complexes I–V in SE offspring of both genders.

MATERIALS AND METHODS

Animals

The animal experiments were approved by the Animal Care and Ethics Committee at the University of Technology Sydney (ACEC#2011-313A). All protocols were performed according to the Australian National Health and Medical Research Council Guide for the Care and Use of Laboratory Animals. Female Balb/c mice (8 weeks, Animal Resources Centre, Perth, WA, Australia) were housed at 20 ± 2°C and maintained on a 12 h light, 12 h dark cycle (lights on at 06:00 h) with *ad libitum* access to standard rodent chow and water. After the acclimatization period, mice were assigned to sham exposure (SHAM), and SE groups. The SE group was exposed to two cigarettes (Winfield Red, ≤1.2 mg nicotine; VIC, Australia) in a perspex chamber (15L), twice daily for 6 weeks prior to mating, during gestation and lactation; while the SHAM group was exposed to air during the same period of time as previously described (Al-Odat et al., 2014). For each session, the mice were exposed the smoke from one cigarette for 15 min with a 5-min interval between two cigarettes. Female breeders were mated with males (8 weeks) from the same source, which were not exposed to cigarette smoke. Half of the SE breeders were continuously supplied with L-Carnitine (SE breeders supplied with L-Carnitine [SELC], 1.5 mM directly dissolved in drinking water) during gestation and lactation periods as previously described (Nguyen et al., 2015). L-Carnitine dose was determined according to a previous publication (Ratnakumari et al., 1995). Normal drinking water was provided to the SHAM and SE dams. Brains from offspring of both genders were collected at postnatal (P) day 1 (male = 17; female = 20), P20 (male = 14; female = 10) and 13 weeks (male = 10; female = 8). P1 mice were sacrificed by decapitation, while animals older than 20 days were sacrificed by anesthetic overdose (Pentothal®, 0.1 mg/g, i.p., Abbott Australasia Pty. Ltd., Macquarie Park, NSW, Australia) between 9:00–12:00 h. The brains were stored at −80°C for protein analysis.

Western Blotting

The protein levels of dynamin-related protein (Drp)-1, fission protein (Fis)-1, phosphatase and tensin homolog induced putative kinase (Pink)-1, Parkin, optic atrophy (Opa)-1, light chain 3 microtubule-associated protein A/B (LC3A/B), manganese superoxide dismutase (MnSOD), translocase of outer membrane (Tom)-20 and OXPHOS complexes were measured by western blotting. Brains were homogenized using lysis buffer for whole protein and mitochondrial protein extraction as previously described (Nguyen et al., 2015). Protein samples (20 μg) were separated on NuPage® Novex® 4%–12% Bis-Tris gels (Life Technologies, Carlsbad, CA, USA), then transferred to PVDF membranes (Rockford, IL, USA), which were blocked with non-fat milk and incubated with primary

antibodies (OXPHOS complexes; 1:2500, Abcam, Cambridge, UK), Drp-1 (1:2000, Novus Biotechnology, Littleton, CO, USA), Opa-1 (1:2000, Novus Biotechnology, Littleton, CO, USA), LC3A/B (1:2000, Cell Signaling Technology, Danvers, MA, USA), Tom-20 (1:2000, Santa Cruz Biotechnology, Dallas, TX, USA) and MnSOD (1:2000, Millipore, Billerica, MA, USA), Pink-1 (1:1000, BioVision Incorporated, Milpitas, CA, USA), Fis-1 (1:500, Santa Cruz Biotechnology, Dallas, TX, USA) and Parkin (1:500, Cell Signaling Technology, Danvers, MA, USA) for overnight. Membranes were then incubated in secondary antibodies (goat anti-rabbit or rabbit anti-mouse IgG horseradish peroxidase-conjugated secondary antibodies, 1:5000 for OPA-1, MnSOD, Tom-20, OXPHOS complexes; 1:2000 for Drp-1, Pink-1, LC3A/B; 1:500 for Parkin; Santa Cruz Biotechnology, Dallas, TX, USA) for 1 h. Protein expression was detected by SuperSignal West Pico Chemiluminescent substrate (Thermo, Waltham, MA, USA) and Fujifilm LAS-3000 (Fujifilm, Tokyo, Japan). Protein band density was determined using IMAGEJ software (National Institute of Health, Bethesda, MD, USA). Results are expressed as a ratio of the individual marker intensity relative to β-actin or cytochrome c oxidase subunit (Cox) IV band intensity.

Immunohistochemistry

As this study aimed to investigate the long-term impact of maternal SE and L-Carnitine supplement on the offspring, the brains from the offspring at 13 weeks, representing adulthood, were accessed for apoptosis and DNA damage. Brain sections at bregma—1 mm (n = 4 per group) were deparaffinized and treated with xylene and decreasing graded ethanol to distilled water for hydration. The sections were then microwaved for 17 min in citrate buffer (pH 9.0) followed by cooling in water bath for 15 min for heat-induced epitope retrieval. The slides were then quenched with peroxidase (methanol: PBS: H_2O = 5:5:2) for 15 min at room temperature.

For Caspase-3 staining, the tissues were blocked with 10% normal horse serum for 30 min then incubated with Caspase-3 antibody (1:300, BD Biosciences, Macquarie Park, NSW, Australia) overnight, followed by secondary antibodies (Goat anti-rabbit HRP, 1:200, Vector Laboratories, Burlingame, CA, USA) for 45 min. Diaminobenzidine solution (K346811, DAKO, USA) was then added and incubated for 8 min, followed by counterstaining with Harri's Hematoxylin, dehydration through graded ethanol to xylene, and coverslipped. Quantification was performed on three slides from each brain blinded to the study groups. Positive cells (brown staining) were counted and the results are represented as the percentage of all cells within a given area.

ApopTag®Peroxidase kits (Merck Millipore, Bayswater, VIC, Australia) were used for TUNEL staining. For TUNEL staining, 40 μl of equilibration buffer was added on each section for 30 s after the hydrogen peroxidase quenching step. Terminal deoxynucleotidyl transferase (Tdt, Tdt: reaction buffer = 1:4) was added to each section, coverslipped and incubated for 1 h. Negative control was incubated with water instead of Tdt. Coverslip was then removed and the stop reaction buffer was added. Anti-Digoxigenin-Peroxidase was added to each slide, coverslipped and incubated for 40 min at 37°C. Diaminobenzidine solution (K346811, DAKO, USA) was then added and incubated for 8 min, followed by counterstaining with Harri's Hematoxylin, dehydration through graded ethanol to xylene, and coverslipped. Quantification was performed on three slides from each brain blinded to study group. Positive cells (brown staining) were counted and the results are represented as the percentage of all cells within a given area.

Statistical Methods

Results are expressed as mean ± SEM. Normality was tested prior to statistical analysis. If the data were not normally distributed, they were log transformed to research normality. The differences between groups were analyzed using one-way ANOVA followed with Fisher's LSD test (Statistica 9, Statsoft, Tulsa, OK, USA). $P < 0.05$ was considered significant.

RESULTS

Male Offspring

Body Parameters

The body weight of the SE offspring was only significantly smaller than SHAM offspring at P1 ($P < 0.01$, **Table 1**); whereas SELC offspring were heavier than the SE offspring at P1 ($P < 0.05$, **Table 1**). There were no significant differences in body weights of the male offspring at P20 and 13 weeks among the groups. At P1, SE offspring showed a smaller percentage of brain weight than SHAM offspring ($P < 0.05$, **Table 1**), which was normalized by maternal L-Carnitine treatment ($P < 0.01$, **Table 1**). There was no difference in brain weight among the groups at P20 and 13 week (**Table 1**).

Mitophagy Markers

At P1, mitochondrial Drp-1 was increased by 50% in SE offspring compared to SHAM ($P < 0.05$, **Figure 1A**); while Fis-1, Pink-1, and Parkin and Opa-1 levels were similar between the SHAM and SE offspring (**Figures 1D,G,J,M**). Maternal L-Carnitine supplementation significantly normalized mitochondrial Drp-1 and reduced Fis-1 levels in the SE offspring ($P < 0.05$, **Figures 1A,D**), but had no impact on Pink-1, Parkin and Opa-1 proteins at P1 (**Figures 1G,J,M**). At P20, Fis-1 protein level was increased in the SE offspring, but was not affected by maternal L-Carnitine supplementation ($P < 0.05$, **Figure 1E**). The other mitochondrial fission and fusion markers were not different among the three experimental groups (**Figures 1B,H,K,N**). At 13 weeks, brain mitochondrial levels of Drp-1, Fis-1, and Opa-1 were decreased in the SE offspring ($P < 0.05$, **Figures 1C,F,O**). With L-Carnitine supplementation, mitochondrial Drp-1 and Pink-1 protein level was significantly increased in SELC compared with the SE offspring ($P < 0.01$, **Figure 1C**); Opa-1 levels were normalized ($P < 0.05$, **Figures 1C,I,O**). However, Parkin was reduced in the SELC offspring ($P < 0.01$, **Figure 1L**).

Autophagy Markers

At P1, LC3A/B-I was significantly reduced in the SE offspring ($P < 0.05$, **Figure 2A**). The LC3A/B-II to LC3A/B-I ratio was

TABLE 1 | Parameters of the male offspring at different ages.

Offspring	Day 1			Day 20			13 weeks		
	SHAM $n = 15$	SE $n = 18$	SELC $n = 17$	SHAM $n = 14$	SE $n = 8$	SELC $n = 9$	SHAM $n = 10$	SE $n = 8$	SELC $n = 8$
Body weight (g)	1.51 ± 0.06	1.30 ± 0.04**	1.61 ± 0.05†	9.97 ± 0.16	9.61 ± 0.14	9.74 ± 0.43	25.6 ± 0.3	24.8 ± 0.3	25.7 ± 0.4
Brain (mg)	10.5 ± 0.3	10.9 ± 0.2	11.1 ± 0.3	26.0 ± 0.3	25.5 ± 0.4	26.2 ± 0.4	29.4 ± 0.4	29.0 ± 0.3	29.5 ± 0.2
Brain%	6.52 ± 0.26	5.84 ± 0.14*	6.92 ± 0.15††	2.61 ± 0.04	2.65 ± 0.04	2.73 ± 0.11	1.15 ± 0.02	1.17 ± 0.02	1.15 ± 0.02

*Results are expressed as mean ± SEM. Data were analyzed by one way ANOVA. *$P < 0.05$; **$P < 0.01$, compared with the SHAM offspring at the same age. †$P < 0.05$; ††$P < 0.01$, compared with the smoke exposed (SE) offspring at the same age.*

increased in the SE offspring ($P < 0.05$, **Figure 2G**), which was normalized in SELC offspring (**Figure 2G**). At P20, the LC3A/B-II level was significantly lower in SE offspring ($P < 0.05$, **Figure 2E**), which was normalized by maternal L-Carnitine supplementation ($P < 0.05$ vs. SE offspring, **Figure 3E**). At 13 weeks, LC3A/B-II level and LC3A/B-II to LC3A/B-I ratio were decreased in SE offspring (LC3A/B-II, $P < 0.05$, **Figure 2F**; LC3A/B-II/I ratio, $P < 0.01$, **Figure 2I**), while LC3A/B-II/I ratio was significantly increased in SELC, compared to the SE offspring ($P < 0.05$, **Figure 2I**). Only LC3A/B-I level was decreased by maternal L-Carnitine treatment ($P < 0.05$, **Figure 2C**).

Mitochondrial Functional Markers

At P1, the mitochondrial Tom-20 level was nearly doubled in SE offspring, although without statistical significance (**Figure 3D**). MnSOD and mitochondrial OXPHOS complexes were not significantly altered by maternal SE (**Figures 3A,D,G**). In contrast, L-Carnitine doubled and tripled mitochondrial MnSOD and Tom-20 levels, respectively in the SELC offspring ($P < 0.05$, **Figures 3A,D**), without having a significant impact on OXPHOS complexes (**Figure 3G**). At P20, the mitochondrial MnSOD level was increased in the SE compared with SHAM offspring (**Figure 3B**), while maternal L-Carnitine supplementation only reduced OXPHOS complex III levels ($P < 0.05$, **Figure 3H**) without affecting the other complex subunits. At 13 weeks, mitochondrial MnSOD was decreased ($P < 0.05$, **Figure 3C**), while OXPHOS Complex III was significantly increased in the SE offspring ($P < 0.05$ vs. SHAM offspring, **Figure 3I**). Maternal L-Carnitine had no significant impact on MnSOD, TOM20 and OXPHOS complexes.

Cell Apoptosis and DNA Fragmentation

At 13 weeks, there was significant increase in caspase-3 and TUNEL positive cell numbers in the cortex of male SE offspring compared with the SHAM offspring ($P < 0.05$, **Figures 4D,H**). Maternal L-Carnitine treatment normalized caspase-3 level ($P < 0.05$, **Figure 4D**). Maternal L-Carnitine treatment nearly normalized TUNEL levels although without statistical significance (**Figure 4H**).

Female Offspring

Body Parameters

The body weight of the SE females was significantly smaller than SHAM offspring at P1, P20 ($P < 0.05$ vs. SHAM, **Table 2**) and 13 weeks ($P < 0.01$, **Table 2**). L-Carnitine treatment increased the body weight in SELC offspring at P1 ($P < 0.01$, **Table 2**), but not at P20 and 13 weeks. Net brain weight was not different between the SHAM and SE offspring, while it was only increased in SELC offspring at P1 ($P < 0.05$ vs. SE offspring, **Table 2**). The percentage of brain weight was similar among the three groups at P1, P20 and 13 weeks.

Mitophagy Markers

At P1, mitochondrial fission markers Drp-1, Fis-1 and Parkin were significantly decreased in the SE compared to SHAM offspring ($P < 0.05$, Drp-1 and Parkin; $P < 0.01$ Fis-1; **Figures 5A,D,J**). Mitochondrial Opa-1 was significantly higher in the SE offspring ($P < 0.05$, **Figure 5M**). L-Carnitine normalized Drp-1, Fis-1 and Opal-1 levels ($P < 0.05$, **Figures 5A,D,M**), and tripled the level of Parkin ($P < 0.01$, **Figure 5J**) in the SELC compared to the SE offspring. At P20, mitochondrial Drp-1 and Parkin levels were still decreased in the SE offspring ($P < 0.05$, **Figures 5B,K**), which were not affected by maternal L-Carnitine treatment during gestation and lactation (**Figures 5B,E,H,K,N**). At 13 weeks, mitochondrial Drp-1 and Fis-1 levels were higher in the SE offspring ($P < 0.05$, **Figures 5C,F**), while only Fis-1 levels were normalized by maternal L-Carnitine treatment ($P < 0.05$, **Figure 5F**). Pink-1 protein level was significantly reduced by maternal L-Carnitine treatment, compared to SE offspring ($P < 0.05$, **Figure 5I**).

Autophagy Markers

At P1, there was a small, but not significant increase in LC3A/B-II level (**Figure 6D**) and significantly increased LC3A/B-II/I ratio in the SE offspring, which were both normalized by maternal L-Carnitine treatment ($P < 0.01$, **Figure 6G**). At P20, LC3A/B-II and LC3A/B-II/I ratios were increased in the SE offspring ($P < 0.05$, **Figures 6E,H**), however only LC3A/B-II level was normalized in the SELC offspring ($P < 0.05$, **Figure 6E**). At 13 weeks, LC3A/B-I and LC3A/B-II protein levels were increased in the SE offspring ($P < 0.05$, **Figures 6C,F**) and normalized by maternal L-Carnitine treatment ($P < 0.01$, **Figures 6C,F**).

Mitochondrial Functional Markers

There was no significant difference in MnSOD, Tom-20 and OXPHOS complexes among the three groups at P1 (**Figures 7A,D,G**). At P20, only OXPHOS Complex I

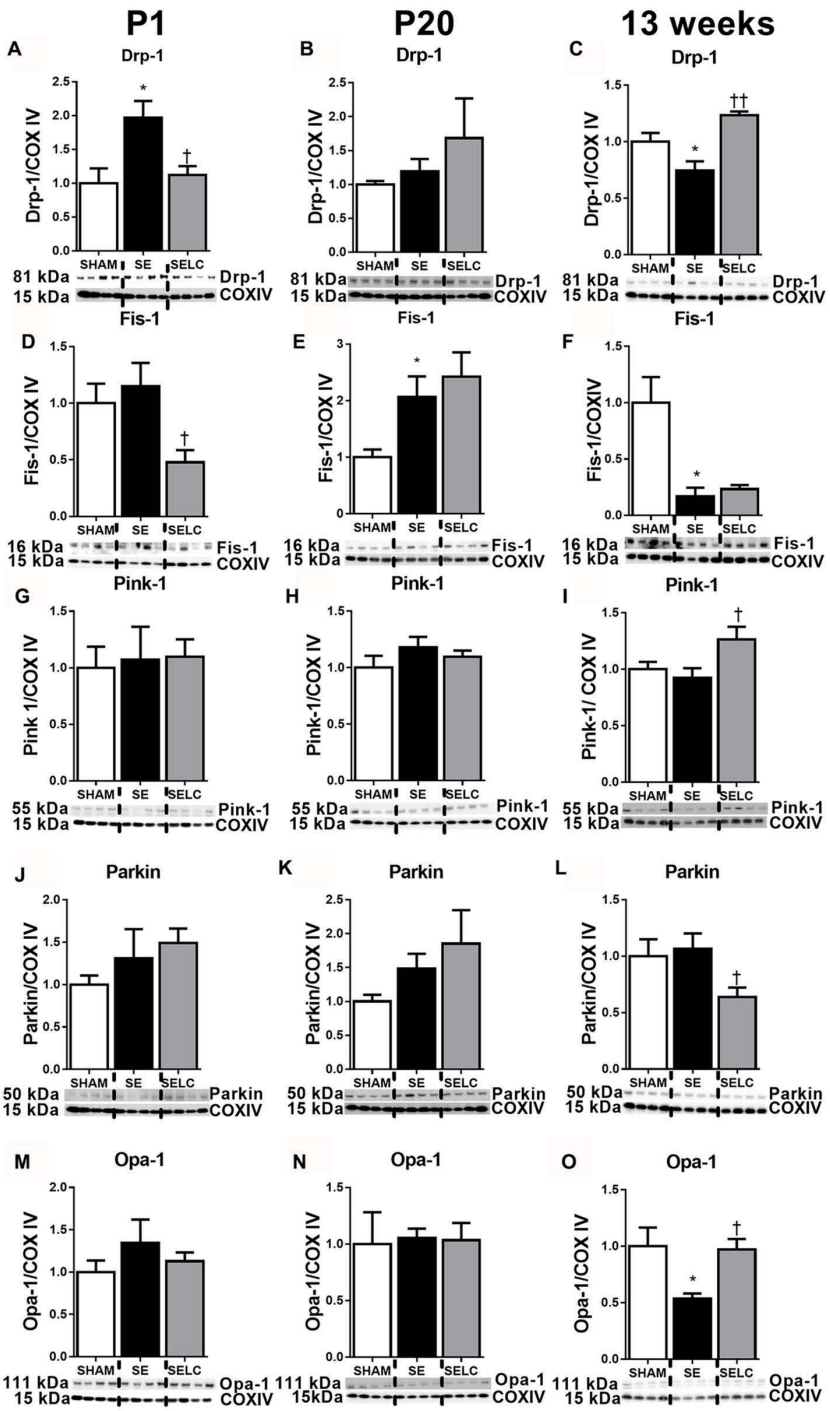

FIGURE 1 | Brain mitochondria protein levels of Drp-1 (A–C), Fis-1 **(D–F)**, Pink-1 **(G–I)**, Parkin **(J–L)** and Opa-1 **(M–O)** in the male SHAM, SE and SELC dams ($n = 4$) at different ages. Results are expressed as mean ± SEM. Data was analyzed by one-way ANOVA with Fisher's LSD test. $^{*}P < 0.05$, compared to SHAM; $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$, compared to SE. Drp-1, dynamin-related protein-1; Fis-1, fission protein-1; Pink-1, phosphatase and tensin homolog (PTEN)-induced putative kinase-1; Opa-1, Optic atrophy-1; Cox IV, cytochrome c oxidase; SE, smoke exposed; SELC, SE with L-Carnitine.

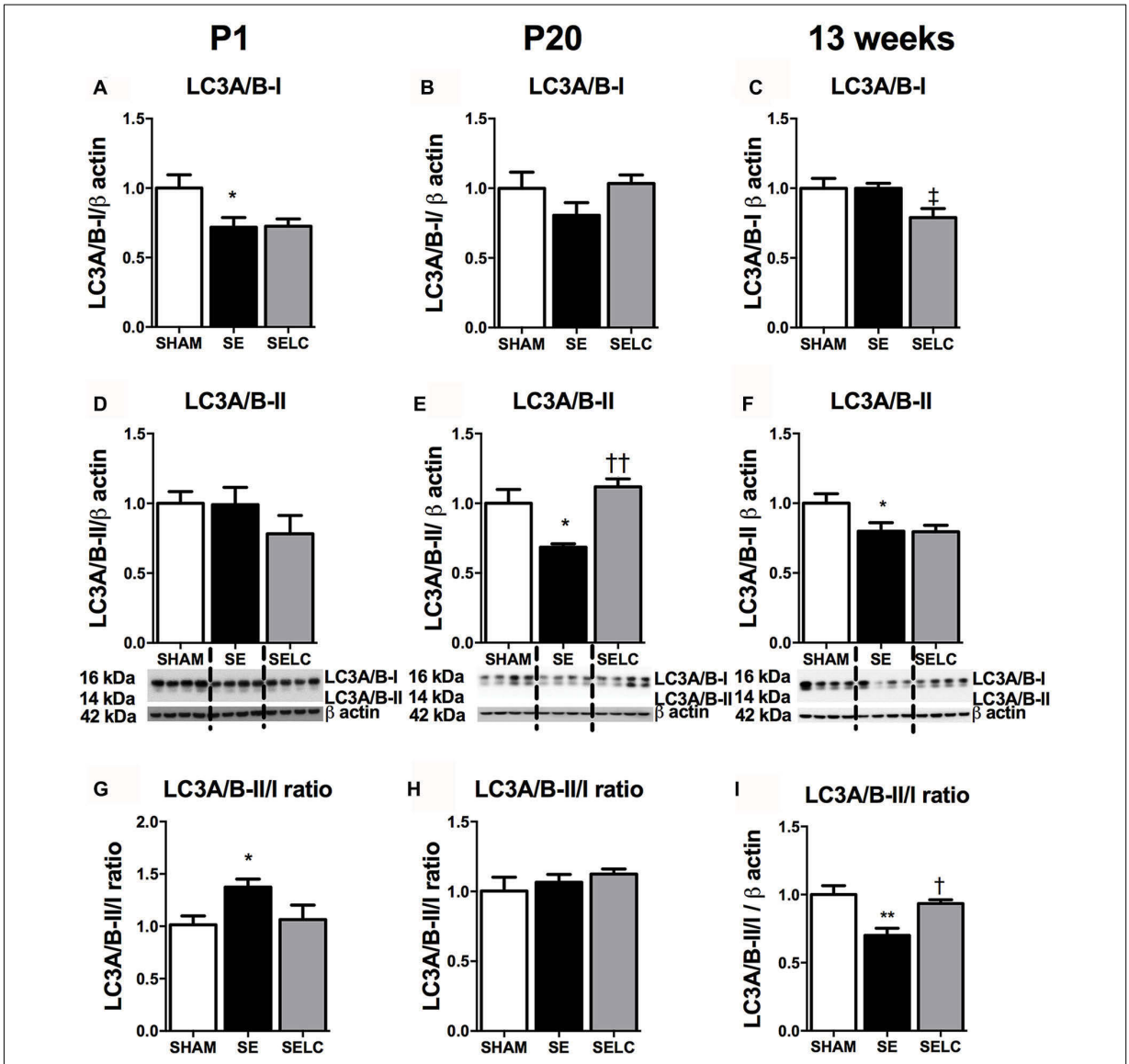

FIGURE 2 | Brain protein levels of LC3A/B-I (A–C), LC3A/B-II **(D–F)**, LC3A/B-II/LC3A/B-I ratio **(G–I)** in the male SHAM, SE and SELC dams (n = 4) at different ages. Results are expressed as mean ± SEM. Data was analyzed by one-way ANOVA with Fisher's LSD test. $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$, compared to SE. $^{\ddagger}P < 0.05$, compared to SHAM; $^{*}P < 0.05$, $^{**}P < 0.01$ compared to SHAM. LC3A/B, Light chain 3 microtubule-associated protein A/B; SE, smoke exposed; SELC, SE with L-Carnitine.

protein was tripled in the SE offspring, which was reduced by maternal L-Carnitine treatment ($P < 0.05$, **Figure 7H**) in the face of a 20% reduction in Tom-20. At 13 weeks, MnSOD level was doubled in the SE offspring, which was normalized by maternal L-Carnitine treatment ($P < 0.05$, **Figure 7C**). There was a 50% increase in Tom-20 in the SE offspring, although without statistical significance (**Figure 7F**). OXPHOS complex I level was also increased in the SE offspring ($P < 0.05$, **Figure 7I**); maternal L-Carnitine treatment significantly reduced OXPHOS complex I ($P < 0.01$, **Figure 7I**) and III ($P < 0.05$, **Figure 7I**) protein levels.

Cell Apoptosis and DNA Fragmentation

At 13 weeks, there were no significant changes in capase-3 and TUNEL positive cell numbers among the three female experimental groups, although cortex capase-3 and TUNEL positive cells in the SE offspring

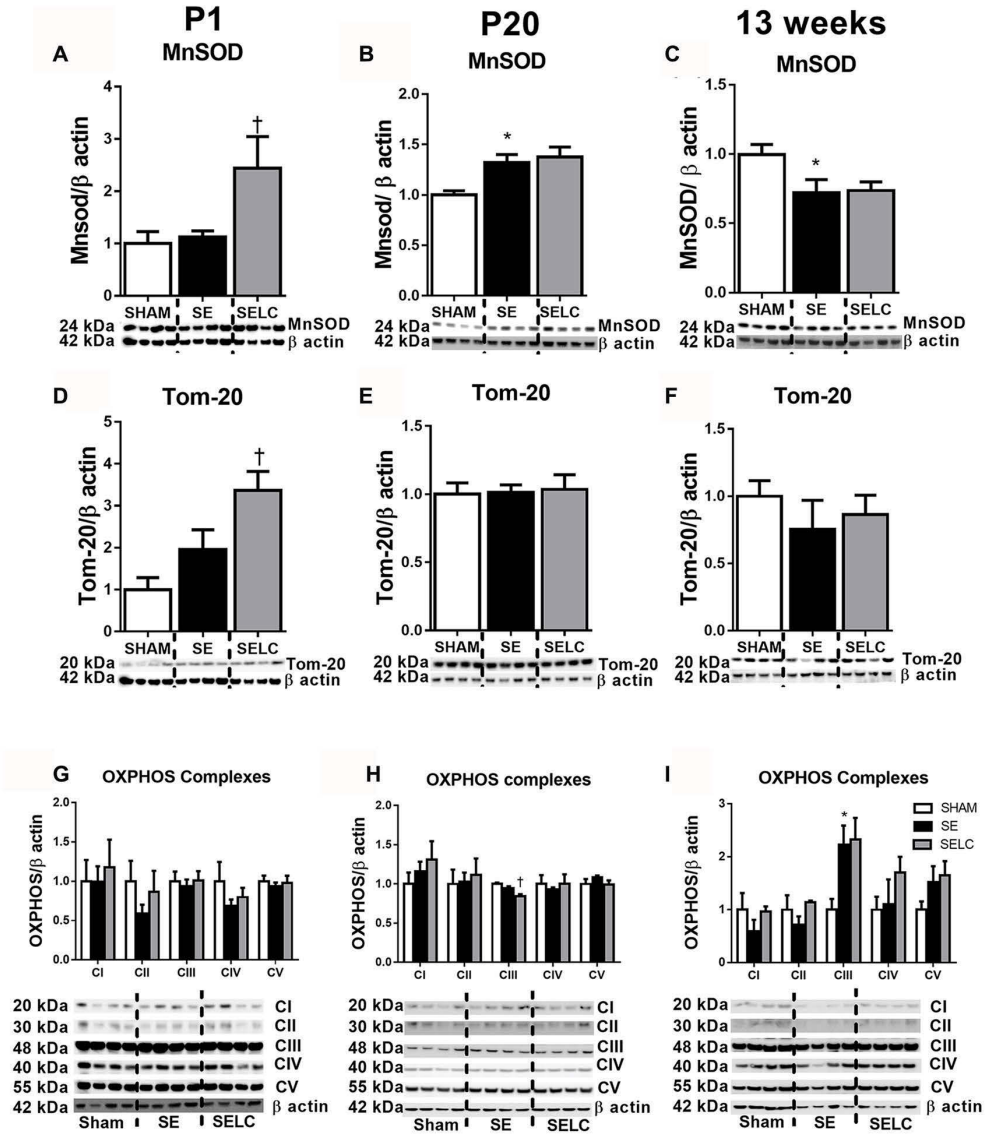

FIGURE 3 | Brain mitochondria protein levels of MnSOD (A–C), Tom-20 **(D–F)**, OXPHOS Complexes I-IV **(G–I)** in the male SHAM, SE and SELC dams ($n = 4$) at different ages. Results are expressed as mean ± SEM. Data was analyzed by one-way ANOVA with Fisher's LSD test. $^{*}P < 0.05$, compared to SHAM; $^{\dagger}P < 0.05$, compared SE. MnSOD, manganese superoxide dismutase; Tom-20, translocase of the mitochondrial outer membrane-20; OXPHOS, oxidative phosphorylation; SE, smoke exposed; SELC, SE with L-Carnitine.

were more than doubled than the SHAM offspring (**Figures 8D,H**).

DISCUSSION

In the current study, there was a gender difference on the impacts of maternal SE on brain markers of mitophagy, the autophagosome and mitochondrial energy metabolism in offspring. Developmental changes of these markers from birth to maturity were also observed. In the male offspring, increased fission markers and reduced autophagosome markers at P1 suggesting an increase in mitochondrial damage and thereby overconsumption of the autophagosome, while data from adult offspring suggest reduced mitophagy but increased cellular damage. In the female offspring, mitochondrial fusion markers on P1 suggest increased

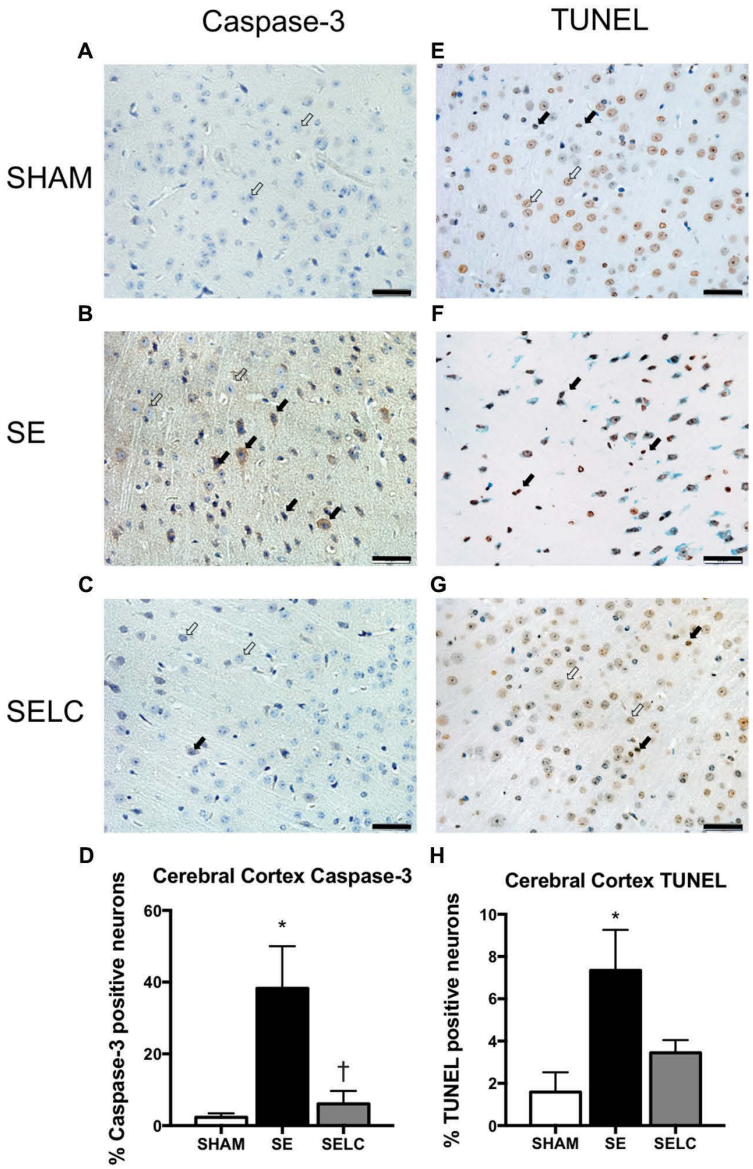

FIGURE 4 | Immunostaining for Caspase-3 staining in cerebral cortex in the male offspring at 13 weeks (*n* = 4, A–D). Caspase-3 positive (close arrow) and caspase-3 negative (open arrow). TUNEL staining in cerebral cortex in the male offspring at 13 weeks (*n* = 4, **E–H**). TUNEL positive (close arrow) and TUNEL negative (open arrow). Scale bar = 20 μm. 40× magnification. Results are expressed as mean ± SEM. Data was analyzed by one-way ANOVA with Fisher's LSD test. $^{*}P < 0.05$, compared SHAM; $^{\dagger}P < 0.05$, compared to SE. SE, smoke exposed; SELC, SE with L-Carnitine.

mitochondrial regeneration, while in adults increased mitochondrial fission and autophagosome markers were observed, with high levels of MnSOD and OXPHOS complex I suggesting an increase in energy demand and oxidative stress, thereby more mitochondrial turnover (Twig et al., 2008; Lee et al., 2012). Maternal L-Carnitine supplementation during gestation and lactation partially normalized the above-mentioned changes in both male and female offspring, suggesting a possible benefit on brain health of SE offspring.

In the developing brain, substantially high energy demand increases the need for glucose, oxygen and cerebral blood flow (Hagberg et al., 2014). Mitochondria are the cellular power house and thus play important roles during brain development which is a highly energy-dependent process (Benard and Karbowski, 2009). Intrauterine environmental stress due to

TABLE 2 | Parameters of the female offspring at different ages.

Offspring	Day 1			Day 20			13 weeks		
	SHAM *n* = 11	SE *n* = 20	SELC *n* = 18	SHAM *n* = 8	SE *n* = 11	SELC *n* = 10	SHAM *n* = 8	SE *n* = 8	SELC *n* = 8
Body weight (g)	1.45 ± 0.05	1.40 ± 0.06*	1.63 ± 0.05††	10.4 ± 0.5	9.49 ± 0.16*	9.74 ± 0.25	22.0 ± 0.4	21.3 ± 0.5**	21.0 ± 0.2
Brain (mg)	10.0 ± 0.5	10.1 ± 0.1	10.9 ± 0.3†	26.4 ± 0.5	25.5 ± 0.2	25.8 ± 0.3	26.9 ± 2.5	29.6 ± 0.4	29.3 ± 0.2
Brain %	6.88 ± 0.29	6.71 ± 0.30	6.60 ± 0.18	2.57 ± 0.11	2.68 ± 0.04	2.68 ± 0.05	1.22 ± 0.12	1.40 ± 0.02	1.40 ± 0.02

*Results are expressed as mean ± SEM. Data were analyzed by one way ANOVA. *P < 0.05; **P < 0.01, compared with the SHAM offspring at the same age. †P < 0.05; ††P < 0.01, compared to SE offspring of the same age.*

maternal smoking can cause adverse birth outcomes that have been well-documented (Ekblad et al., 2015). In the current study, body weight and percentage of brain weight were reduced in male SE offspring at P1 only; whereas female SE offspring remained small from birth to adulthood, without affecting percentage brain weight. This suggests that brains of the female SE offspring may be protected. This seems to be consistent with our previous observation that maternal SE enhanced risk of renal disorders in male offspring at adulthood (Al-Odat et al., 2014), but not in the female offspring (Chan et al., 2016a).

Mitophagy is a crucial process to maintain mitochondrial integrity, where damaged mitochondria can be degraded and the intact components can be recycled to generate new functional mitochondria (Benard and Karbowski, 2009). This process is achieved through fission and fusion (Westermann, 2010). Fission can fragment damaged mitochondrial parts to remove the dysfunctional components in the autophagosome by autophagy. During this process, Fis-1 is located in the outer mitochondrial membrane to recruit Drp-1 (Onoue et al., 2013), which forms a spiral to slice both the inner and outer mitochondrial membranes (Elgass et al., 2013). The damaged mitochondrial fragments are then tagged by Pink-1, followed by the recruitment of Parkin which ubiquitinates outer membrane proteins (Narendra et al., 2008). Through Pink-1 and Parkin, damaged mitochondrial fragments can be eliminated (Narendra et al., 2008) by autophagy. Autophagy activity is normally reflected by the levels of LC3A/B-I, LC3A/B-II and their ratios. LC3A/B-I is converted to LC3A/B-II (Kouno et al., 2005); which then forms autophagosomes to contain damaged organelles. LC3A/B knockout mice die shortly after birth, due to the lack of autophagy (Komatsu et al., 2005). Thus, the ratio between LC3A/B-II and LC3A/B-I or LC3A/B-II level itself can reflect autophagosome accumulation. On the other hand, the process of fusion, which is mediated by Opa-1 can facilitate healthy mitochondrial fragments to form new functional mitochondria (Kanazawa et al., 2008), which includes the exchange of mitochondrial DNA (Youle and van der Bliek, 2012). When there is increased energy demand or presence of stressor such as smoking, fusion is increased, thereby energy synthesis can be maintained (Westermann, 2012). Thus, fusion is considered as a protective mechanism during stress when energy demands are increased. Indeed, it has been found that increased mitophagy activity can improve neural survival in traumatically injured brain (Wei et al., 2015).

Here in the newborn male SE offspring, both fission and autophagosome markers were increased without changes in the fusion marker, suggesting the mitochondrial damage by maternal SE is irreparable. In the adult male SE offspring, reduced mitochondrial MnSOD suggests increased oxidative stress which may be linked to increased OXPHOS complex III, which is the major site of ROS production. This can lead to direct damage to cellular organelles including mitochondria. However, autophagosome markers are decreased which may indicate there was a defect in the removal of mitochondria fragments, while reduced mitophagy markers may indicate that there were less healthy mitochondria fragments to be recycled. Mitophagy defects have been found in neurodegenerative diseases such as Hungtington's disease, Alzheimer's disease and Parkinson's disease (Schapira and Gegg, 2011; Banerjee et al., 2015), suggesting roles in maintaining neuronal integrity. Autophagy is known to block caspase-3 dependent apoptosis, a marker for cell injury (Mariño et al., 2014). As such, offspring at 13 weeks, representing adulthood, were investigated for the long term impact of maternal SE and L-carnitine supplementation. Here, markers of cell apoptosis and DNA damage were higher in adult male SE offspring, suggesting cell damage. Increase in DNA fragmentation can also increase the risk of neurodegenerative diseases (Lenardo et al., 2009). Indeed, the risk of cognitive and behavioral disorders is higher in offspring of smokers (Chen and Morris, 2007; Knopik et al., 2016; Palmer et al., 2016). Therefore, changes in brain mitophagy in the male SE offspring may predict impaired brain function later in life.

In the newborn female SE offspring, mitochondrial fission was reduced, however markers of fusion and the autophagosome were increased, which may preserve the function of mitochondria. In fact, following nutrient deprivation, healthy mitochondria elongate and fuse together (Rambold et al., 2011) to prevent degradation by autophagy (Gomes et al., 2011). A reduction in fission with increased fusion can promote the formation of elongated mitochondrial networks in order to preserve mitochondrial function (Mishra and Chan, 2014). In adulthood, both mitochondrial fission and autophagosome markers, and OXPHOS complex I were increased, suggestive of increased energy demand and mitochondrial turnover. However, the endogenous antioxidant MnSOD was increased, which may be an adaptation to counteract increased free radicals during ATP synthesis in the OXPHOS complexes. This pattern is very different from that of the male SE offspring. Opposite to what was found in male SE offspring, the apoptotic marker caspase-3 level was not changed in female's brain

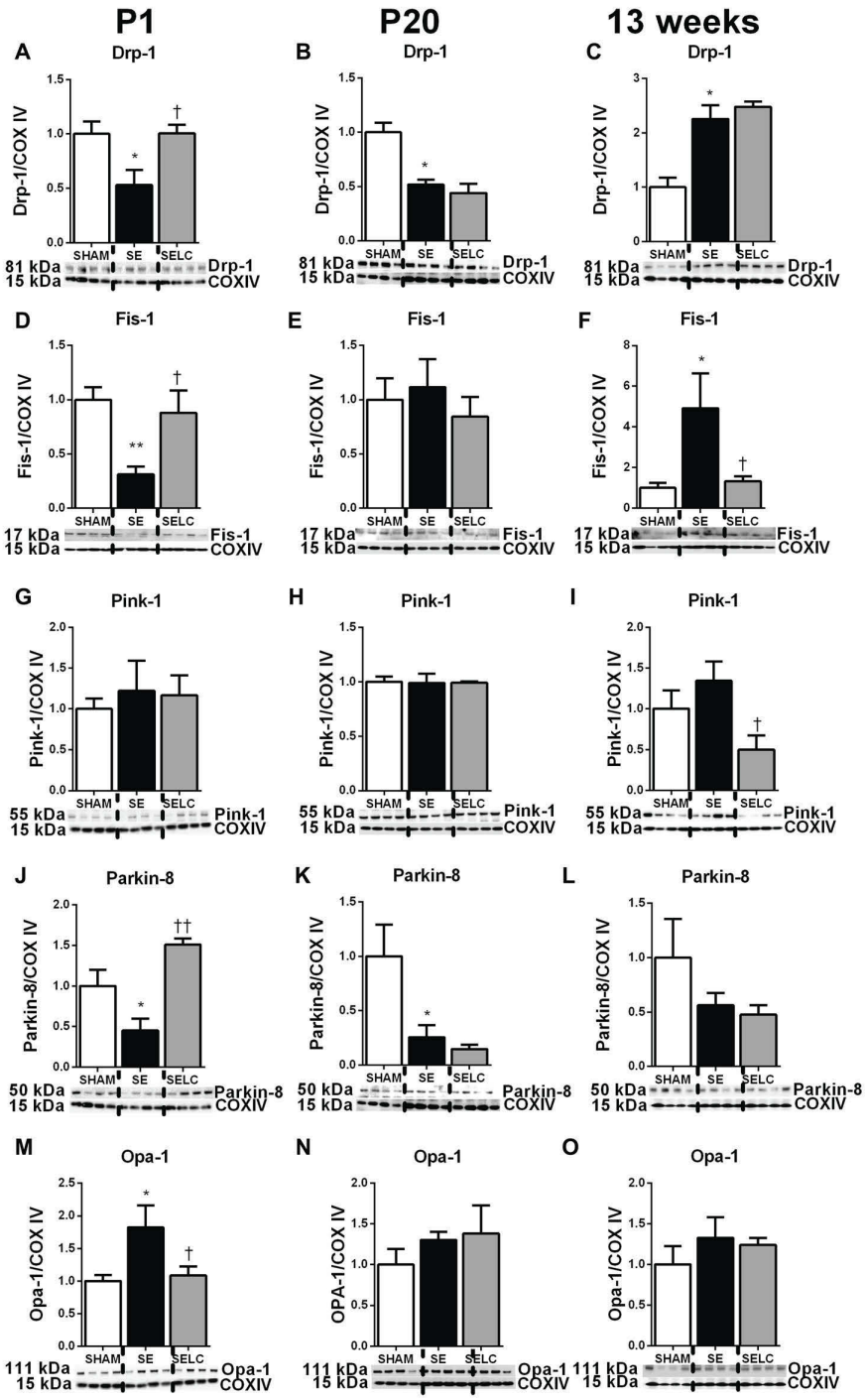

FIGURE 5 | Brain mitochondria protein levels of Drp-1 (A–C), Fis-1 **(D–F)**, Pink-1 **(G–I)**, Parkin **(J–L)** and Opa-1 **(M–O)** in the female SHAM, SE and SELC dams ($n = 4$) at different ages. Results are expressed as mean ± SEM. Data was analyzed by one-way ANOVA with Fisher's LSD test. $^{*}P < 0.05$, $^{**}P < 0.01$, compared to SHAM; $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$, compared to SE. Drp-1, dynamin-related protein-1; Fis-1, fission protein-1; Pink-1, phosphatase and tensin homolog (PTEN)-induced putative kinase-1; Opa-1, optic atrophy-1; Cox IV, cytochrome c oxidase; SE, smoke exposed; SELC, SE with L-Carnitine.

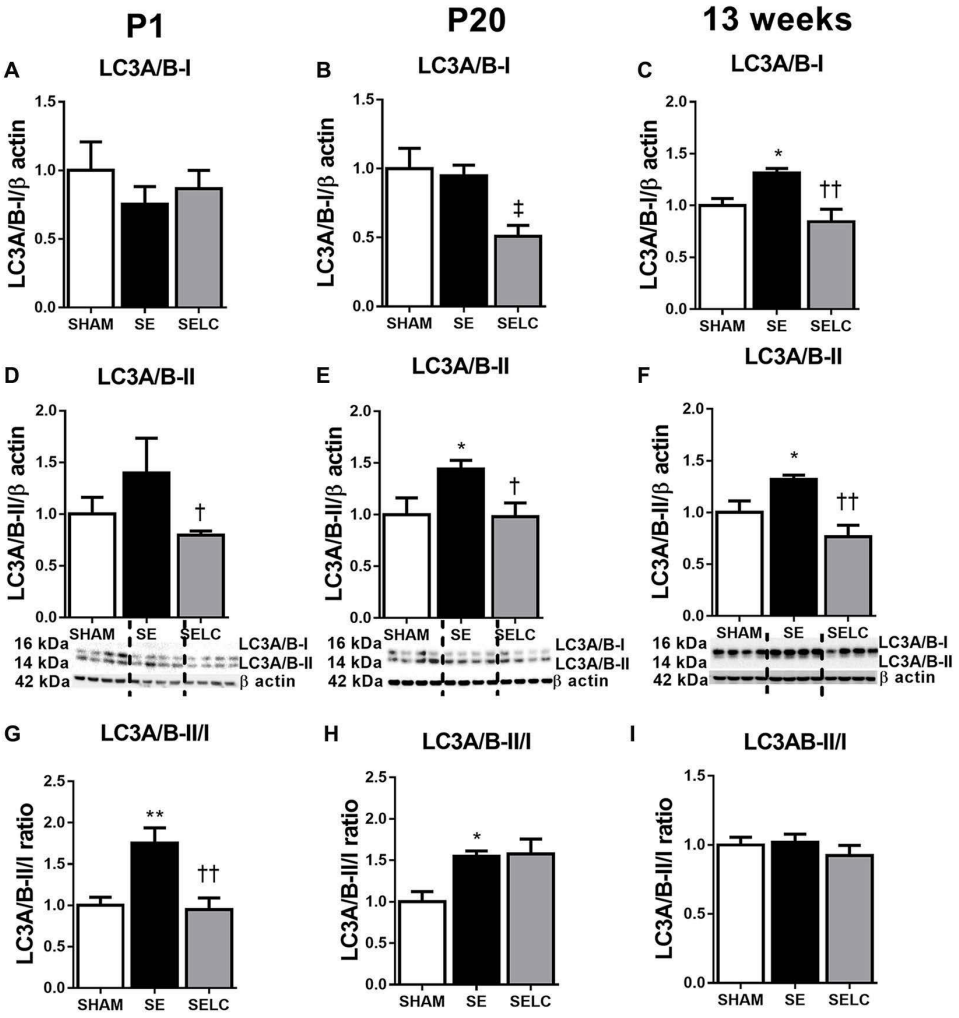

FIGURE 6 | Brain protein levels of LC3A/B-I (A–C), LC3A/B-II **(D–F)**, LC3A/B-II to LC3A/B-I ratio **(G–I)** in the female SHAM, SE and SELC dams (n = 4) at different ages. Results are expressed as mean ± SEM. Data was analyzed by one-way ANOVA with Fisher's LSD test. $^{*}P < 0.05$, $^{**}P < 0.01$, compared to SHAM; $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$, compared to SE. $^{\ddagger}P < 0.05$, compared to SHAM. LC3A/B, light chain 3 microtubule-associated protein A/B; SE, smoke exposed; SELC, SE with L-Carnitine.

suggesting better persevered cell integrity (Chandler et al., 1998). This is consistent with unchanged brain inflammatory and oxidative stress markers in our previous study using the same model (Chan et al., 2016a). The marginal change in TUNEL staining somewhat mirrors the changes in mitophagy markers in the female's brain, suggesting the mitophagy regulation may play a critical role in cell integrity.

There is a significant gender difference in response to maternal SE as shown by us previously (Chan et al., 2016a,b), where female offspring seem to be more protected from increased brain inflammatory and oxidative stress markers by maternal SE. Here in addition to mitophagy and autophagy markers, cell injury seems to be more pronounced in the male offspring. One potential mechanism is the sex hormone estrogen. Estrogen has been shown to bear neuroprotective property (Brann et al., 2007). During cerebral ischemia, the blockage of estrogen receptors can increase infarction size following carotid artery occlusion in female rats (Sawada et al., 2000); whereas the increase in estrogen was shown to increase the expression of the anti-apoptotic gene (B-cell lymphoma 2) in mice with cerebral

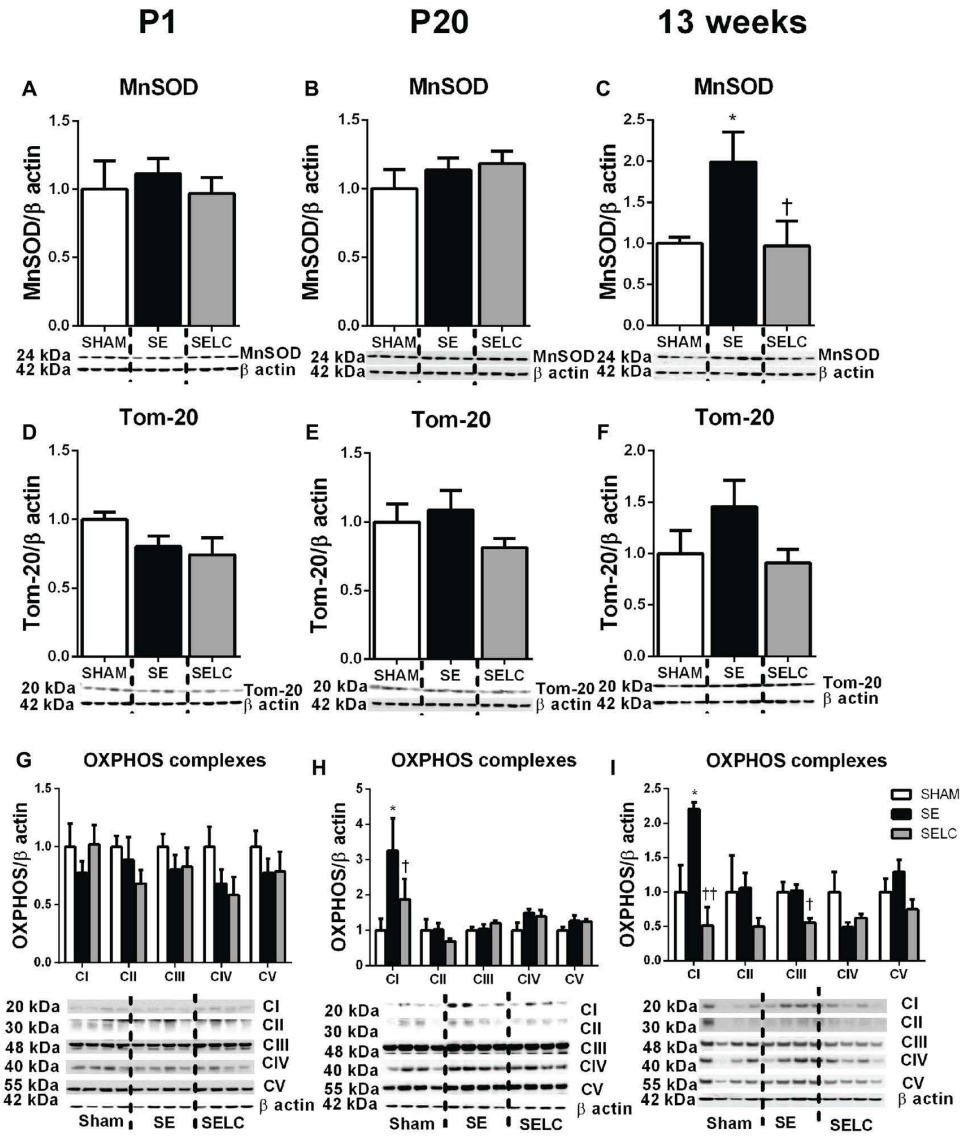

FIGURE 7 | Brain mitochondria protein levels of MnSOD (A–C), Tom-20 **(D–F)**, OXPHOS Complexes I-IV **(G–I)** in the female SHAM, SE and SELC dams ($n = 4$) at different ages. Results are expressed as mean ± SEM. Data was analyzed by one-way ANOVA with Fisher's LSD test. $^{*}P < 0.05$, compared to SHAM; $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$, compared to SE. MnSOD, manganese superoxide dismutase; Tom-20, translocase of the mitochondrial outer membrane-20; OXPHOS, oxidative phosphorylation; SE, smoke exposed; SELC, SE with L-Carnitine.

ischemia (Dubal et al., 1999). Thus it is explainable why the cell injury markers were less changed in female SE offspring.

Complete smoking cessation is difficult to achieve during pregnancy, especially in certain communities where maternal smoking rates are particularly high (Chertok and Archer, 2015; Glover et al., 2016). A pregnancy supplement is more feasible approach, in comparison to difficulties introducing behavioral changes (i.e., smoking cessation) to improve fetal health outcomes in this population. The antioxidant L-Carnitine, is approved by the US Food and Drug Administration to treat Carnitine deficiency in dialysis patients (Guarnieri et al., 2001). It is also an over-the-counter supplement for athletes and bodybuilders and also shown to help weight loss in adults (Pooyandjoo et al., 2016). We have previously shown that L-Carnitine supplementation during gestation and lactation can reverse the detrimental impact of maternal SE on renal development and function in offspring (Nguyen et al., 2015).

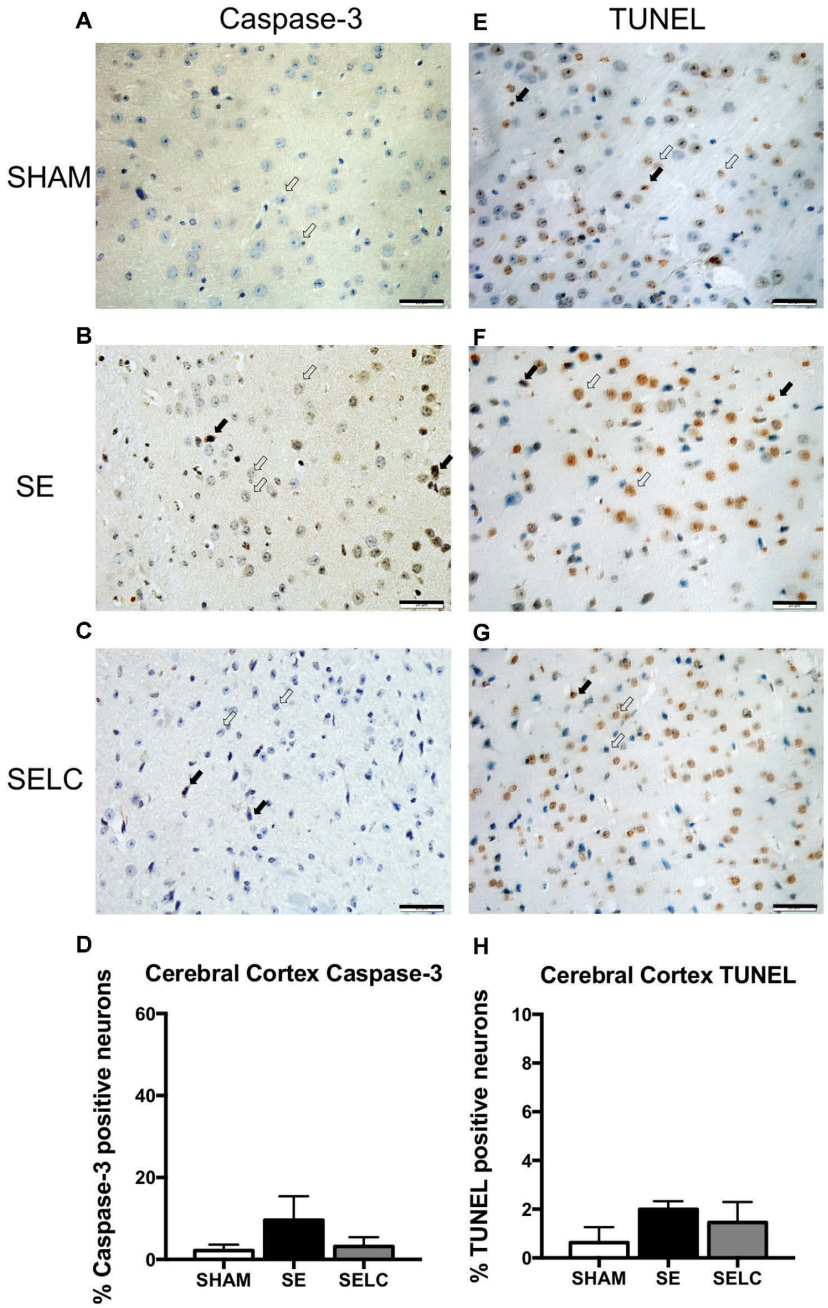

FIGURE 8 | Immunostaining for Caspase-3 staining in cerebral cortex in the female offspring at 13 weeks (*n* = 4, A–D). Caspase-3 positive (close arrow) and caspase-3 negative (open arrow). TUNEL staining in cerebral cortex in the female offspring at 13 weeks (*n* = 4, **E–H**). TUNEL positive (close arrow) and TUNEL negative (open arrow). Scale bar = 20 μm. 40X magnification. Results are expressed as mean ± SEM. Data was analyzed by one-way ANOVA with Fisher's LSD test. SE, smoke exposed; SELC, SE with L-Carnitine.

In the current study, SE offspring displayed smaller birth weight and body weight at adulthood, which is consistent with our previous observations (Chan et al., 2016a,b). Maternal L-Carnitine supplementation normalized body weight at birth and thereafter that at adulthood in the SE offspring of both genders, as well as small brain weight at birth in the

males. This suggests that the protection in the offspring is most likely due to the maternal intrauterine effect on fetal growth, which further resulted in normal postnatal growth. However, the exact mechanism requires further investigation. Interestingly, the changes in mitochondrial fission and fusion makers, as well as OXPHOS complex and MnSOD were partially normalized in the SE offspring of both genders, as well as cell injury markers Caspase-3 and TUNEL. There is consistent with the observations in kidney development and function in the same cohort of SE offspring (Nguyen et al., 2015). L-Carnitine has been shown to regulate both mitochondria and autophagy processes (Hagen et al., 1998; Long et al., 2009; Shenk et al., 2009; Marcovina et al., 2013; Zhu et al., 2015). However, it is difficult to separate these two aspects in the current study design. Thus, maternal L-Carnitine supplementation during gestation and lactation may improve the health outcomes in SE offspring in the long term.

In conclusion, maternal SE impaired brain mitochondrial markers of fission and fusion and increased oxidative stress in both genders, with the effect delayed in the females. Our study confirms the benefits of L-Carnitine use in high-risk pregnancies to improve potential health outcomes in offspring by replenishing mitophagy function in brains of offspring.

AUTHOR CONTRIBUTIONS

HC, NMJ and SS designed the study. YLC, HC, and IA-O performed all the experiments. YLC, BGO, SS, NMJ and HC contributed to the writing of the main manuscript text, and YLC prepared **Figures 1–8** and **Tables 1**, **2**. All authors reviewed the manuscript.

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Chapter 6
Impact of maternal smoking on hypoxic ischemic injury in offspring

6.1 Introduction

Oxygen deprivation before and around the time of birth can result in hypoxic-ischemic (HI) brain damage. Perinatal HI encephalopathy is one of the major causes of neonatal death and long-term disability, which occurs in 1-6 per 1000 term newborns (326), leading to major neurological consequences including cerebral palsy, seizures, visual and learning impairment (327). In humans, HI encephalopathy causes a decrease in blood oxygen saturation rate and blood flow, interrupting normal fetal brain development (328). The cerebral cortex, hippocampus and sub-ventricular regions are the brain regions most vulnerable to HI damage in rats (329). Maternal smoking has been shown to cause hypoxia in the fetus of rhesus monkey with the mechanisms not completely understood (330). It has been proposed that nicotine in cigarette smoke can reduce blood flow to the placenta due to its vasoconstrictor properties (331). In addition, cigarette smoking can increase carboxyhaemoglobin levels during pregnancy which can reduce the oxygen carrying capacity of both fetal and maternal red blood cells (332,333). In Chapter 3, the metabolite of nicotine, cotinine, was found to be increased in the plasma of 20 day old offspring from SE dams, suggesting that nicotine can be transmitted through breast milk to offspring. Incomplete combustion of tobacco leaves can also produce carbon monoxide that can bind to hemoglobin causing temporary hypoxia (334). One study also found that maternal smoking can increase the risk of placental infarction and microinfarctions (335). Such infarctions can lead to the interruption of blood supply to part of the placenta causing placental ischemia, which is one of the most common mechanism underlying HI injury in the fetus (336).

Normal brain function requires a large amount of oxygen for oxidative phosphorylation to produce ATP. HI encephalopathy can rapidly reduce ATP production which is crucial for brain development during the fetal period, due to the impairment of cerebral blood

flow and oxygen delivery to the brain (337). Autophagy and mitophagy may be induced by HI injury as increased brain autophagosomes have also been found in neonatal rats with HI injury (338).

Smoking ten or more cigarettes per day during pregnancy has also been shown to increase the risk of cerebral palsy which is a well-known outcome of HI encephalopathy (339). Data in Chapter 4 showed that maternal SE reduced the markers of mitochondrial fission and autophagy in brains of adult offspring which may lead to an accumulation of damaged mitochondria (Chapter 4). This was accompanied by reduced levels of the mitochondrial antioxidant MnSOD. Male offspring were shown to be more vulnerable to the impact of maternal SE than females, as reflected by reduced mitochondrial MnSOD levels and increased mitochondrial ROS producing OXPHOS complexes I and III (Chapter 4)(340). Data from Chapter 3 showed that maternal SE increased markers of hypoxia, along with oxidative cellular damage in brains of offspring. Thus the damage to brain cells might be due to increased oxidative stress and subsequently impaired brain mitochondrial markers of fission and fusion that affect mitochondrial integrity (Chapter 5).

Perinatal nicotine exposure has been shown to enhance the vulnerability to HI brain injury in neonatal rats (323). In addition, it has been shown that insufficient removal of damaged mitochondria or excessive degradation of mitochondria can increase cell death during cerebral ischemia (341,342). Therefore, the change in mitophagy markers by maternal SE may worsen the neurological outcomes following HI brain injury. Thus, this study aimed to investigate the impact of maternal SE on markers of tissue injury, mitophagy, autophagy and inflammation in the brains of male offspring with HI injury induced on postnatal day(P) 10. Brain size, motor and cognitive function (assessed by novel objective recognition test, grip traction test, foot fault test, and elevated plus maze

test) was assessed in offspring at P40. Brain markers of inflammation, mitophagy, autophagy, mitochondrial antioxidants, apoptosis and DNA damage were also examined at the experimental end point.

6.2 Methodology

6.2.1 Maternal cigarette smoke exposure

The animal experiments were approved by Animal Care and Ethics Committee at the University of Technology Sydney (ACEC# 2014-029). All protocols were performed according to the Australian National Health & Medical Research Council Guide for the Care and Use of Laboratory Animals. Thirty virgin female Balb/c mice (6 weeks, Animal Resources Centre, Perth, Australia) were housed at 20 ± 2 °C and maintained on a 12-h light, 12-h dark cycle (lights on at 06:00 h) with *ad libitum* access to standard laboratory chow and drinking water. Cigarette smoke exposure and breeding were performed as per the description in Chapter 2, section 2.1. The male offspring were housed 4–5/cage after weaning, and were harvested at P45.

6.2.2 Modelling HI encephalopathy in the offspring

At P10, half of the pups from each litter were anesthetised by 2.5% isoflurane (*1% O_2*, Veterinary companies of Australia, Kings Park, NSW) and underwent left carotid artery occlusion as previously published (343). All equipment was sterilized before surgery. An incision was made on the left side of the neck. Tissues and fat were teased carefully to expose the left carotid artery. Two surgical knots were tied on the artery with forceps (Figure 6.1). In the pups with sham surgery, the left carotid artery was exposed without any ligation. The wound was closed by Vetbond^{TM} glue (3M, MN, USA).

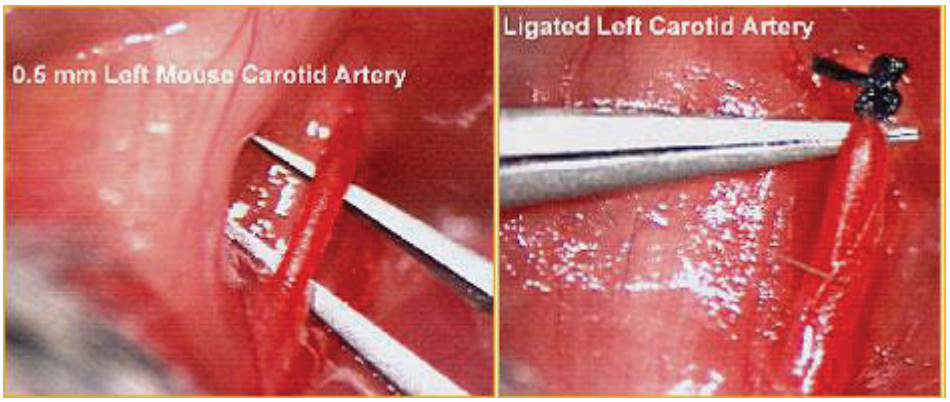

Figure 6.1 Image of left carotid artery occlusion

Sixty minutes after the surgery, the pups were exposed to 8% oxygen with 92% nitrogen in a humid chamber for half an hour in a 37°C water bath to induce HI injury. The other half of the litter underwent the same procedure without occlusion and was exposed to normal room air in a humidified chamber. This resulted in four experimental groups (SHAM: maternally exposed to air with no injury; HI: maternal exposed to air with HI injury; SE: maternally exposed to cigarette smoke with no injury; SEHI: maternally exposed to cigarette smoke with HI injury, n=12 per group).

6.2.3 Behavioral tests at P40

Novel objective recognition test. This test evaluates short term recognition memory retention. It is the nature of a mouse to explore a new object over an old one. A mouse with a cognitive deficit will not be able to remember the old object, therefore will spend a similar amount of time or even more time exploring it. Each mouse was placed in a dark-colored box containing two identical green square blocks (shown in figure 6.2) and was allowed to explore the box for two 5-minute sessions. During the second session, one of the objects was replaced with a new orange triangular shaped object. The time spent exploring each object was recorded as previously published (344). The results are presented as the percentage of the total time spent on the new object out of the total time spent exploring both objects.

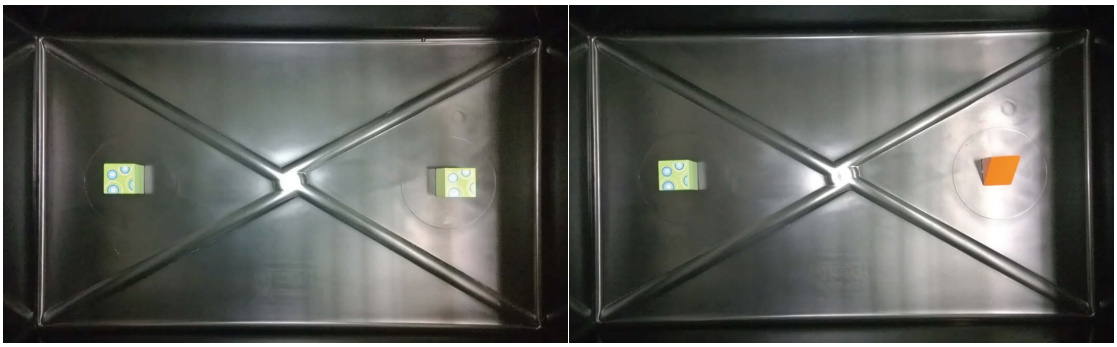

Figure 6.2. Image of the setting for novel object recognition test showing the same objects on the left and different objects on the right

Grip traction test. Forelimb muscle strength was tested by the ability of a mouse to hang on to a plastic rod (0.6cm in diameter, 50 cm above the ground horizontally) by the front limbs as described in a previous study (Figure 6.3) (345). The test lasted a maximum of 2 minutes and was stopped when the mouse fell off or when the hind limbs were placed onto the rod. A foam pad was laid under the rod to prevent injury due to falling.

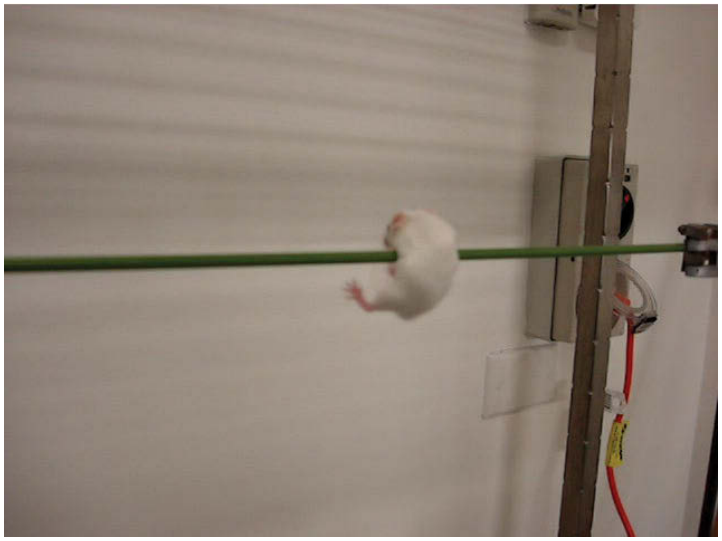

Figure 6.3. A mouse undergoing the grip traction test

Foot fault test. The mouse was placed on a horizontal grid (20 cm x 20 cm, square 1 x 1 cm) (Figure 6.4). When a mouse misplaced a forelimb or hindlimb, the foot fell between the grid squares. The number of foot faults and total number of footsteps taken within 2 minutes were recorded. The results were expressed as the percentage of foot fault out of

the total number of steps.

Figure 6.4. A mouse walking on the grid

Elevated plus maze. Each mouse was placed in the cross section of the elevated plus maze for ten minutes (Figure 6.5) (346). The time spent in the closed and open arms were recorded. The mouse will choose the closed arms if it is anxious. The results are expressed as the percentage of time spent in open arms out of total time spent moving in the maze**.**

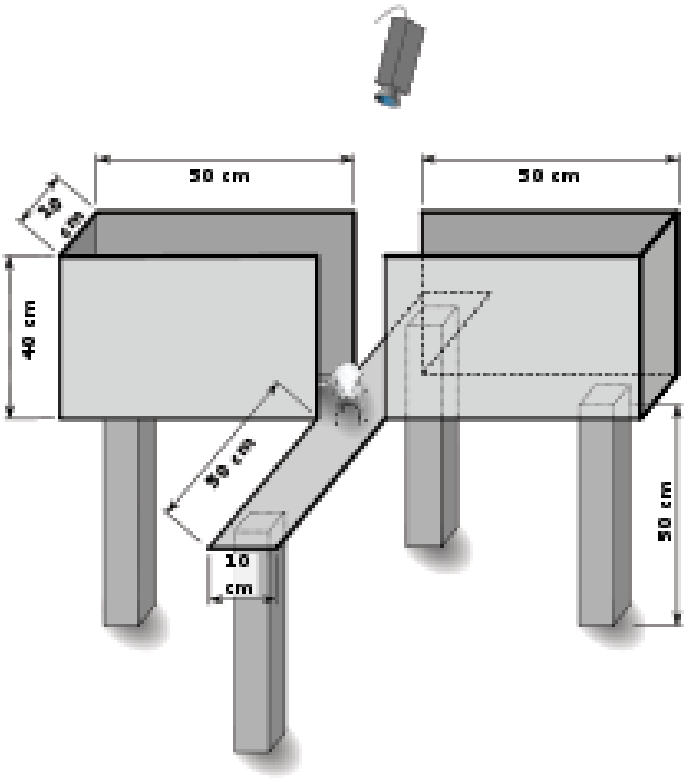

Figure 6.5. The setup of elevated plus maze

6.2.4 Western blotting

The protein levels of mitophagy markers (dynamin-related protein (Drp)-1, fission –1 protein, Phosphatase and tensin homolog induced putative kinase (Pink)-1, Parkin, and optic atrophy (Opa)-1), autophagy marker (light chain 3 microtubule-associated protein A/B (LC3A/B)), and antioxidant MnSOD), and mitochondrial function markers (translocase of outer membrane (Tom)-20, Tom-40 and OXPHOS complexes I-V) were measured by western blotting. The brain hemisphere ipsilateral to the occluded carotid artery was homogenized using lysis buffer for whole protein and mitochondrial protein extraction as described in Chapter 2. Protein samples (20μg) were separated using NuPage Novex 4-12% Bis-Tris gels (Life Technologies, CA, USA) and transferred to PVDF membranes (Rockford, IL, USA), which were blocked with non-fat milk and incubated with primary antibodies. The concentrations of primary and secondary antibodies are shown in Table 6.1. Protein expression was detected by SuperSignal

West Pico Chemiluminescent substrate (Thermo, MA, USA) and Fujifilm LAS-3000 (Fujifilm, Tokyo, Japan). The density of the protein bands was determined using ImageJ software (National Institute of Health, Bethesda, Maryland, USA). Results are expressed as a ratio of the intensity of the protein of interest relative to the band intensity of β-actin or cytochrome c oxidase subunit (Cox) IV.

6.2.5 rt-PCR

Total mRNA was extracted from ipsilateral brain tissues using Trizol reagent (Life Technologies, CA, USA). The purified total RNA was used as a template to generate first-strand cDNA using M-MLV Reverse Transcriptase, RNase H, Point Mutant Kit (Promega, Madison, WI, USA) as described in Chapter 2. Genes of interest were measured using manufacturer pre-optimized and validated TaqMan® primers and probes (Life Technologies, CA, USA). The probe sequence of the inflammatory markers tested provided by the manufacturer are listed in Table 1 in Chapter 2. The probes of the target genes were labelled with FAM and those for housekeeping 18s rRNA were labelled with VIC. Gene expression was standardized to 18s RNA. The average expression of the control group was assigned as the calibrator, against which all other samples are expressed as fold differences.

Table 6.1. Information of primary and secondary antibodies for Western Blotting

Primary Antibodies	Host	Brand	Primary antibody dilution	Secondary antibody dilution
OXPHOS Complex	Mouse	Abcam, Cambridge, UK	1:2500	1:5000
Drp-1	Rabbit	Novus Biotechnology, Littleton, USA	1:2000	1:2000
Opa-1	Rabbit	Novus Biotechnology, Littleton, USA	1:2000	1:5000
LC3A/B	Rabbit	Cell Signaling Technology, MA, USA	1:2000	1:2000
Tom-20	Rabbit	Santa Cruz Biotechnology, Texas, USA	1:2000	1:5000
Tom-40	Rabbit	Santa Cruz biotechnology, Texas, USA	1:2000	1:5000
MnSOD	Rabbit	Millipore, MA, USA	1:2000	1:5000
Pink-1	Rabbit	BioVision Incorporated, CA, USA	1:2000	1:2000
Fission-1	Rabbit	Santa Cruz Biotechnology, Texas, USA	1:500	1:500
Parkin	Mouse	Cell Signaling Technology, MA, USA	1:500	1:500
β actin	Mouse	Santa Cruz Biotechnology, Texas, USA	1:5000	1:5000
COXIV	Rabbit	Novus Biologicals, Colorado, USA	1:5000	1:5000
Goat Anti-mouse HRP	NA	Santa Cruz Biotechnology, Texas, USA	NA	NA
Goat Anti-Rabbit HRP	NA	Santa Cruz Biotechnology, Texas, USA	NA	NA

6.2.6 Histology

Brain tissues were processed with Shandon Excelsior (ThermoFisher Scientific, MA, USA) and embedded in wax as described in Chapter 2. They were sectioned at 7 μm with three sections collected on each slide. The slides were hydrated through xylene and a decreasing gradient of ethanol. They were stained with Mayer's Haematoxylin and Eosin staining as described in Chapter 2. The slides were then dehydrated through

100% ethanol (2 x 2 minutes), and xylene (3 minutes and 5 minutes). They were then cover slipped using DPX mounting media. The size difference between the left and right hemispheres from each animal was measured with ImageJ (National Institute of Health, Bethesda, Maryland, USA). Three sections that were a distance of 70 μm apart at bregma level of approximately – 1mm was used to measure injury.

6.2.8 Caspase-3 staining

The sections were hydrated through xylene, gradient ethanol with reduced concentrations and distilled water. They were then incubated in phosphate buffered saline (PBS) at pH 7.4 for 10 minutes, before heat-induced epitope retrieval in the microwave (Homemaker; EM926ENV;900W) for 14 minutes using 10% TRIS-EDTA antigen retrieval buffer (1 mM EDTA, 1 mM sodium citrate, 2 mM Tris, pH9.0) followed by cooling in a water bath for 15 minutes. The tissues were then blocked with hydrogen peroxidase (10% Hydrogen peroxide, 25ml Methanol, 25ml PBS) for 25 minutes, followed by 10% normal horse serum (NHS) for 30 minutes. The tissues were then incubated with Caspase-3 antibody (1:300, BD Biosciences, Australia) overnight and washed with PBS. The sections were incubated with secondary antibodies (Goat anti-rabbit HRP) for 45 minutes, followed by DAB staining for 8 minutes. Then the slides were counterstained with haematoxylin. The number of Caspase-3 positive stained cells were counted in the cerebral cortex, hippocampus and hypothalamus of the left brain hemisphere.

6.2.9 TUNEL staining

ApopTag®Peroxidase kit (Merck Millipore, Victoria) was used for TUNEL staining. Sections were deparaffinised and treated with xylene and gradient ethanol in decreasing concentrations. The slides were then microwaved (Homemaker; EM925ENV; 900W) for 14 minutes in 10% TRIS-EDTA antigen retrieval buffer (1 mM EDTA, 1mM

sodium citrate, 2mM Tris, pH9.0) for heat-induced epitope retrieval followed by cooling in a water bath for 15 minutes. The slides were then washed with dH_2O (2 x 2 minutes) followed by PBS for three minutes. The slides were then quenched with peroxidase for 15 minutes at room temperature (methanol:PBS:H_2O = 4:4:1). The slides were incubated with PBS (2 x 3 minutes), followed by 50µl of equilibration buffer on each section for 30 seconds, and coverslipped. Terminal deoxynucleotidyl transferase (Tdt, 25µl, Tdt: reaction buffer = 1:4) was added to each section, coverslipped and incubated for one hour. Negative control was incubated with water instead of Tdt. The coverslip was then removed and stop reaction buffer was added (1.5ml stopwash buffer + 50ml H_2O) at room temperature. The slides were then washed with PBS (2 x three minutes). Anti-Digoxigenin-Peroxidase (25µl) was added to each section, coverslipped and incubated for 40 minutes at room temperature followed by PBS wash (2 x 3 minutes). Slides were incubated with DAB solution (Dako) for eight minutes and then washed with PBS (2 x 3 minutes), followed by counterstaining and dehydrating. Three sections (70 µm apart from each other) from bregma – 1mm were examined. The number of TUNEL positive stained and cells was counted in the cerebral cortex, hippocampus and hypothalamus of the left brain hemisphere.

6.2.10 MitoTracker Orange staining

Mitochondrial density was evaluated by labeling formalin-fixed, paraffin-embedded tissue sections with MitoTracker Orange dye as previously published (Thermofisher Scientific, MA, USA). After deparaffinization and rehydration through graded ethanol solutions, the sections were incubated with MitoTracker Orange (1:5000 in PBS) for 30 minutes at room temperature. The sections were rinsed with PBS for 3 times, cover-slipped with Vectashield mounting medium (Vector Laboratories, CA, USA), and examined by fluorescence microscopy using a fluorescein filter. All the sections were

exposed to the same duration of incubation time and a negative control was used to offset the background fluorescent staining. ImageJ software (National Institute of Health, Bethesda, Maryland, USA) was used to assess the density of the MitoTracker staining (mean grey scale value).

6.2.11 Statistical analysis

Results are expressed as mean ± S.E.M. The differences between groups were analysed using two-way ANOVA followed by Bonferroni post-hoc tests. Data in the same group were analysed by Student's t test. $P<0.05$ was considered significant (Prism 7.0, Graphpad, USA).

6.3 Results

6.3.1 Body Parameters

At P45, body weight and brain weight of SE offspring were significantly smaller than the SHAM offspring ($P<0.01$, Table 6.2); however, the percentage of brain weight was not different between the groups. HI injury did not significantly affect the anthropometric parameters of the littermates (Table 6.2).

Table 6.2. Parameters of the male offspring at P45

Offspring	SHAM n = 12	HI n = 12	SE n = 12	SEHI n = 12
Body weight (g)	20.2 ± 0.2	20.0 ± 0.3	19.1 ± 0.3**	19.3 ± 0.3
Brain (mg)	31.1 ± 0.8	30.1 ± 0.2	29.3 ± 0.3*	29.0 ± 0.3
Brain %	1.54 ± 0.04	1.51 ± 0.02	1.53 ± 0.03	1.50 ± 0.02

Results are expressed as mean ± S.E.M. Data were analysed by two-way ANOVA followed by Bonferroni post-hoc tests. * $P < 0.05$; ** $P < 0.01$, compared with the SHAM offspring of the same age.

Maternal SE did not affect the size of left hemisphere (SE vs SHAM, Figure 6.6). However, HI injury reduced the size of the left hemisphere in SHAM and SE offspring with HI injury by 5.37% (± 1.22%) and 6.54% (± 0.73%), respectively compared to non-injured littermates (both $P<0.01$, Figure 6.5). There was no significant difference in the size of the left hemisphere between the HI and SEHI offspring.

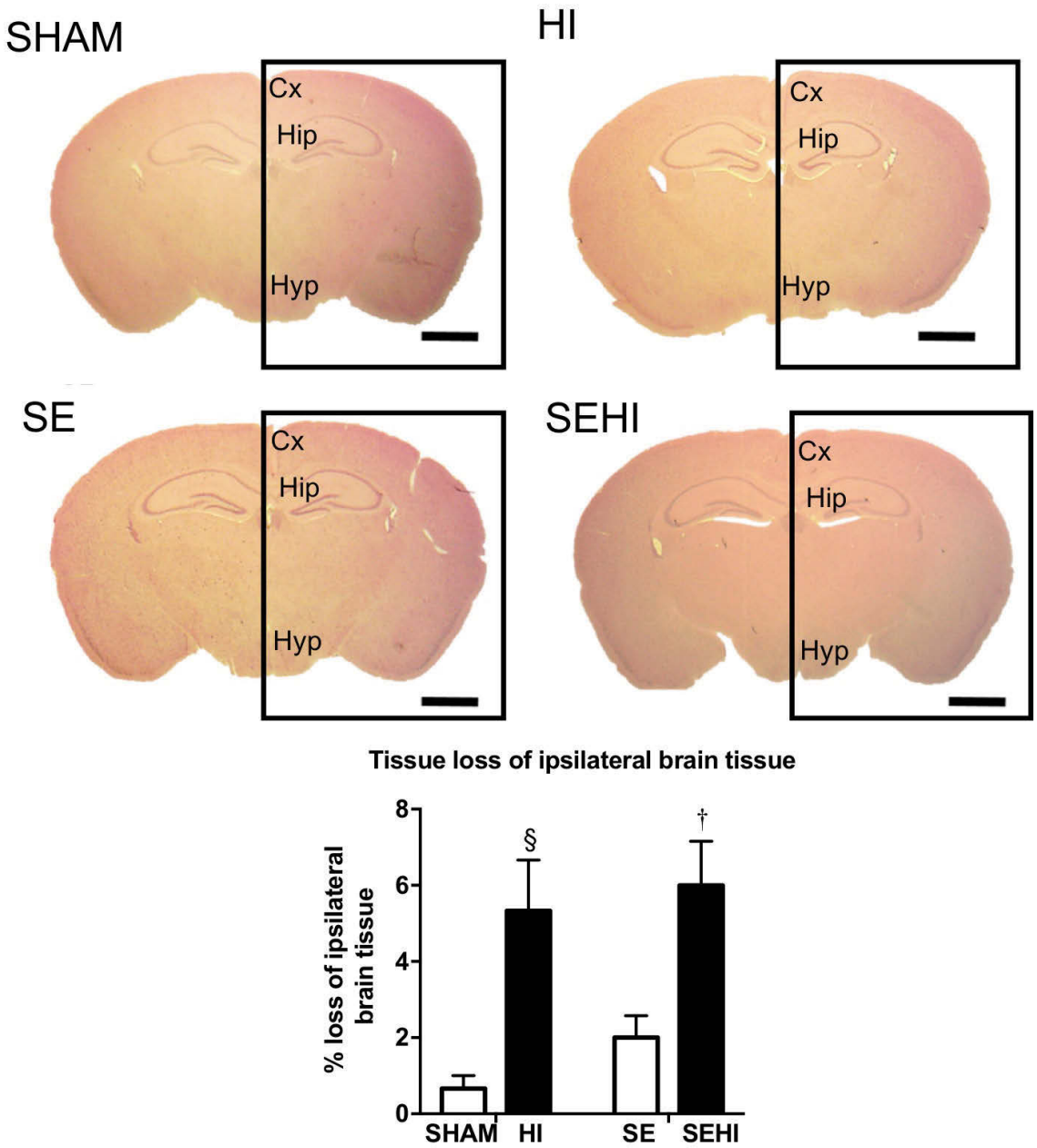

Figure 6.6. Representative images and quantification of brain size at Bregma -1mm in P45 male offspring (n=5). Scale bar = 80 µm. Results are expressed as the percentage

loss of ipsilateral brain hemisphere (black rectangle enclosed area) ± SEM. P<0.05 by conditional t test, § HI vs SHAM; † SEHI vs HI. Cx: Cerebral cortex; HI: hypoxic-ischemic injury; Hip: hippocampus; Hyp: Hypothalamus; SE: smoke exposed; SEHI: smoke exposed with hypoxic-ischemic injury.

6.3.3 Behavioral Tests

In the novel objective recognition test, maternal SE marginally reduced the percentage of total time spent on the new objects in SE offspring by 15% without statistical significance (Figure 6.7a). HI offspring spent 8% less time exploring the novel object than the SHAM offspring, whereas SEHI spent 13% less time than their non-injured littermates (Figure 6.7a). In the grip traction test, SE offspring spent less time holding onto the rod (P<0.05 vs SHAM, Figure 6.7b). Offspring with HI injury spent similarly less time on the rod compared to their uninjured littermates regardless of maternal group (P<0.01, Figure 6.7b). SE offspring made more foot faults even without injury in the foot fault test (P<0.05 vs SHAM, Figure 6.7c). HI injury increased the number of foot faults in the HI offspring (P<0.01 vs SHAM, Figure 6.7c) but not in the SEHI offspring (Figure 6.7c). In the elevated plus maze test, SE offspring spent 87% less time on the open arm compared to the SHAM offspring (P<0.05, Figure 6.7d). HI injury did not affect the anxiety level in the SHAM offspring, however it normalised the anxiety level in the SE offspring (P<0.05 vs SE, Figure 6.7d).

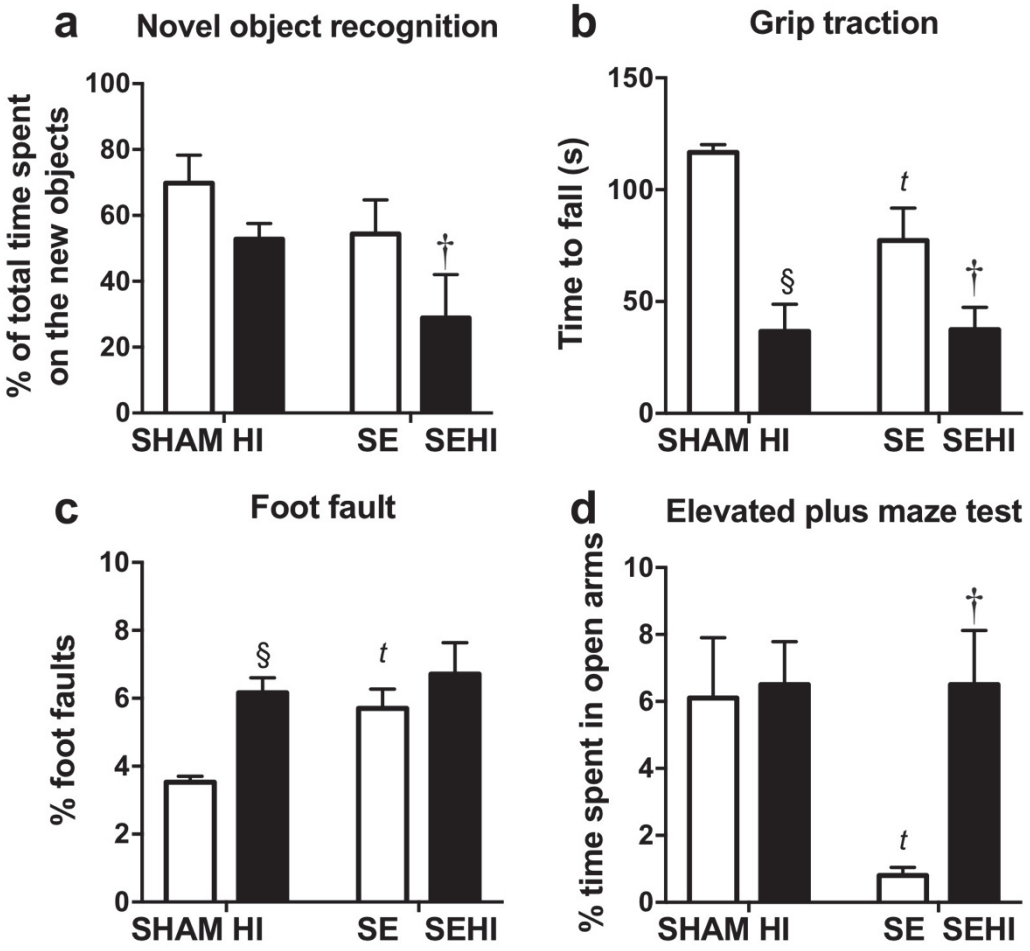

Figure 6.7. The results of Novel object recognition test (a), grip traction test (b), elevated plus maze (c) and foot fault test (d) in male offspring at P45 (n=12). Results are expressed as mean ± SEM. P<0.05 by conditional t test, §, P<0.05, HI vs SHAM; *t*, P<0.05, SE vs SHAM; †, P<0.05, SEHI vs SE. HI: hypoxic-ischemic injury; SE: smoke exposed; SEHI: smoke exposed and hypoxic-ischemic injury.

6.3.4 Brain inflammatory and oxidative stress markers

Brain mRNA expression of TLR-4 and IL-1β was significantly reduced by maternal SE (P<0.05, Figure 6.8a,c); only IL-1β in the SEHI group was increased compared with non-injured littermates (P<0.05 vs SE, Figure 6.8c). Maternal SE increased IL-6 mRNA expression (P<0.05 SE vs SHAM, Figure 6.8b), which was not significantly affect by

HI injury. Maternal SE did not affect IL-1R1, TNF-α and iNOS mRNA expression at P45 (Figure 6.8d,e,f). HI injury only increased iNOS mRNA expression in the SEHI group (P<0.05 vs SE, Figure 6.8f).

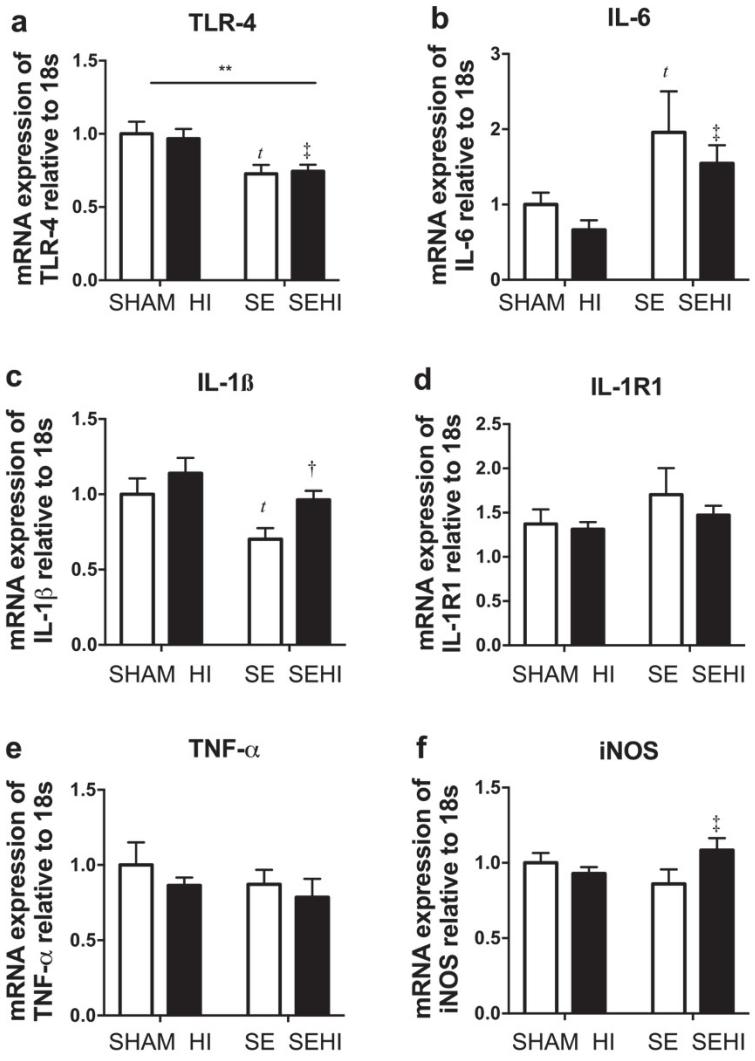

Figure 6.8. Brain mRNA expression of inflammatory markers in the male offspring at P45 (n=6). Results are expressed as mean ± S.E.M. **, P<0.01 overall maternal SE effect. *t* P<0.05 by conditional t test, SE vs SHAM; † SEHI vs SE; ‡ SEHI vs HI. HI: hypoxic-ischemic injury; SE: smoke exposed; SEHI: smoke exposed and hypoxic-ischemic injury.

6.3.5 Brain autophagy and mitophagy markers

Maternal SE decreased Parkin but increased LC3AB-I protein levels in the SE compared to SHAM offspring ($P<0.01$, $P<0.05$ respectively, Figure 6.9d,g). HI injury significantly increased Fission-1 in both HI and SEHI groups ($P<0.01$ vs their non-injured littermates); while it only increased Parkin in SEHI group ($P<0.01$ vs SE, Figure 6.9d). HI injury reduced Pink-1, Opa-1, LC3AB-I and II protein levels only in the SEHI group ($P<0.05$ vs SE, Figure 6.9c, e, g, h).

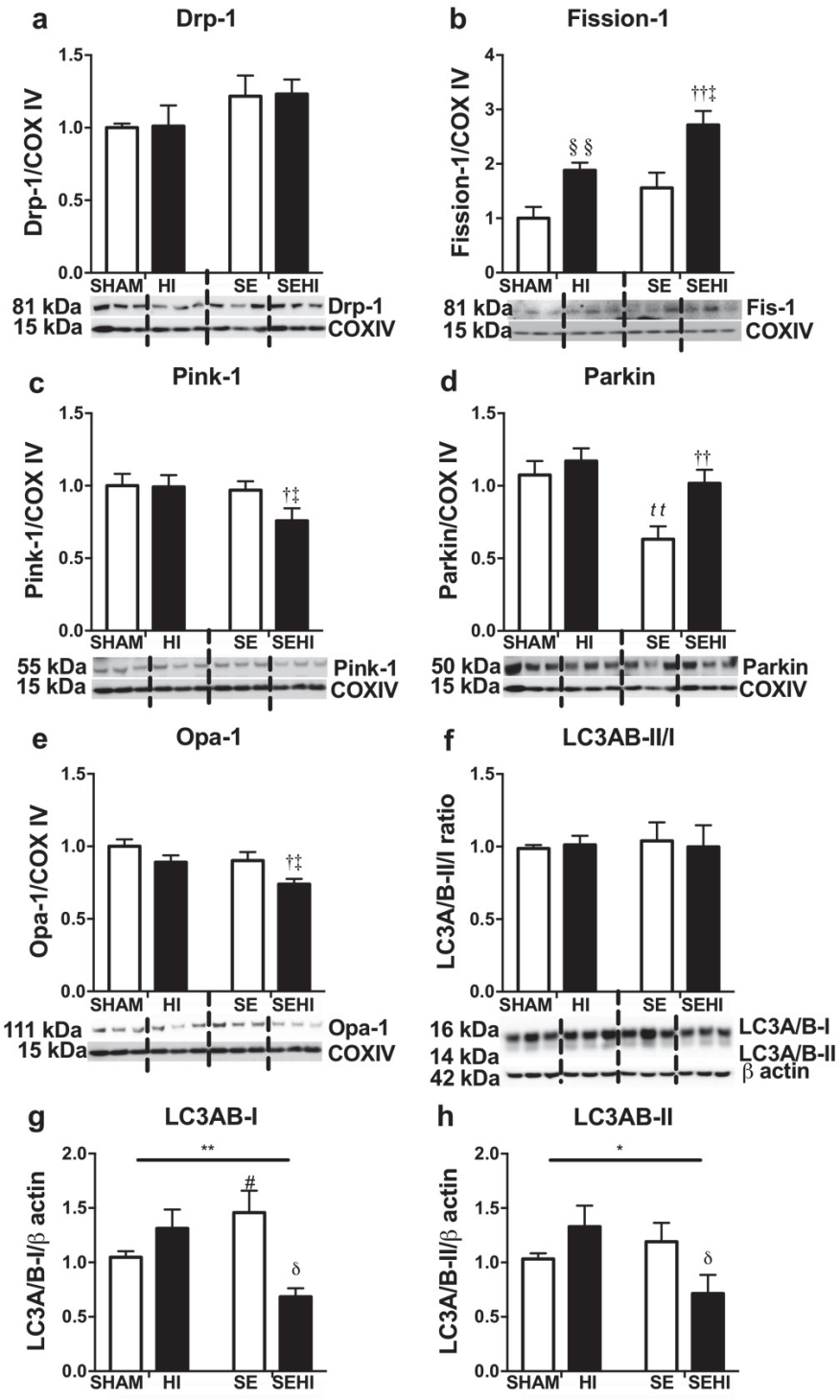

Figure 6.9. Brain mitochondrial levels of Drp-1, Fission-1, Pink-1, Parkin, Opa-1 (a-e) and brain LC3AB-II/I ratio, LC3AB-I and LC3AB-II protein levels (f-h) in the male offspring at P45 (n=6). Results are expressed as mean ± S.E.M. *, P<0.05 overall maternal SE effect. # P<0.05 vs SHAM. δ P<0.05, vs SE. P<0.05 by conditional t test, §§, P<0.01, HI vs SHAM; *tt,* P<0.01, SE vs SHAM; ††, P<0.01; †, P<0.05, SEHI vs SE; ‡, P<0.05, SEHI vs HI. Drp-1: Dynamin-related protein-1; Pink-1:Phosphatase and tensin homolog (PTEN)-induced putative kinase -1; Opa-1: Optic atrophy-1 ; LC3AB:

Light chain 3 microtubule-associated protein AB; Cox IV: Cytochrome c oxidase; HI: hypoxic-ischemic injury; SE: smoke exposed; SEHI: smoke exposed and hypoxic-ischemic injury

6.3.6 Brain markers of mitochondrial function

Maternal SE reduced brain OXPHOS complex III, IV and V protein levels ($P<0.05$ vs SHAM, Figure 6.10d). HI injury reduced MnSOD ($P<0.05$, Figure 6.10a) and OXPHOS complex III levels in offspring from the SHAM dams only ($P<0.05$ HI vs SHAM, Figure 6.10d). In contrast, the levels of OXPHOS complex I, II and III were increased in SEHI offspring by HI ($P<0.05$ vs SE, Figure 6.10d). Tom-20 and Tom-40 were not affected by maternal SE (Figure 6.10b and c) or HI injury (Figure 6.10b and c).

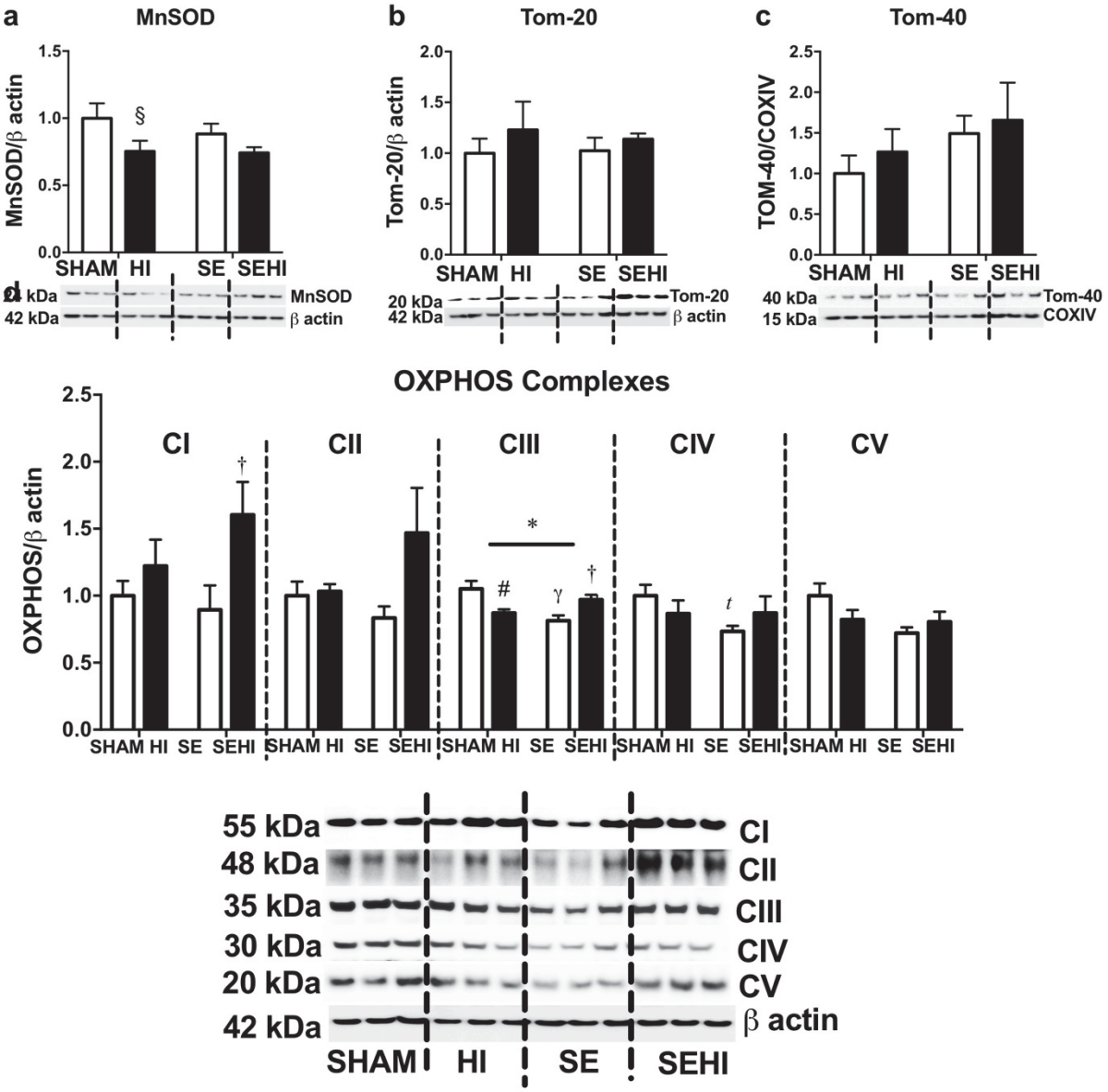

Figure 6.10. Brain mitochondria MnSOD, Tom-20, Tom-40 and OXPHOS complexes I – V levels (a-d) in male offspring at P45 (n=6). Results are expressed as mean ± S.E.M. * P<0.05, overall maternal SE effect. # P<0.05, vs SHAM. γ P<0.05, vs SHAM. P<0.05 by conditional t test. §, P<0.05, HI vs SHAM; †, P<0.05, SEHI vs SE. MnSOD: manganese superoxide dismutase; Tom: translocase of the mitochondrial outer membrane; OXPHOS: oxidative phosphorylation; Cox IV: Cytochrome c oxidase; HI: hypoxic-ischemic injury; SE: smoke exposed; SEHI: smoke exposed and hypoxic-ischemic injury.

6.3.7 Brain mitochondrial density

Maternal SE did not affect mitochondrial density in the cerebral cortex, hippocampus and hypothalamus (Figure 6.11). However, HI injury reduced mitochondrial density in the cerebral cortex of both HI and SEHI offspring ($P<0.05$ vs non-injured littermates, Figure 6.11a). Mitochondrial density in the hippocampus was also reduced in the SEHI offspring ($P<0.01$ vs SE, Figure 6.11c). There were no changes in mitochondrial density observed in the hypothalamus (Figure 6.11d).

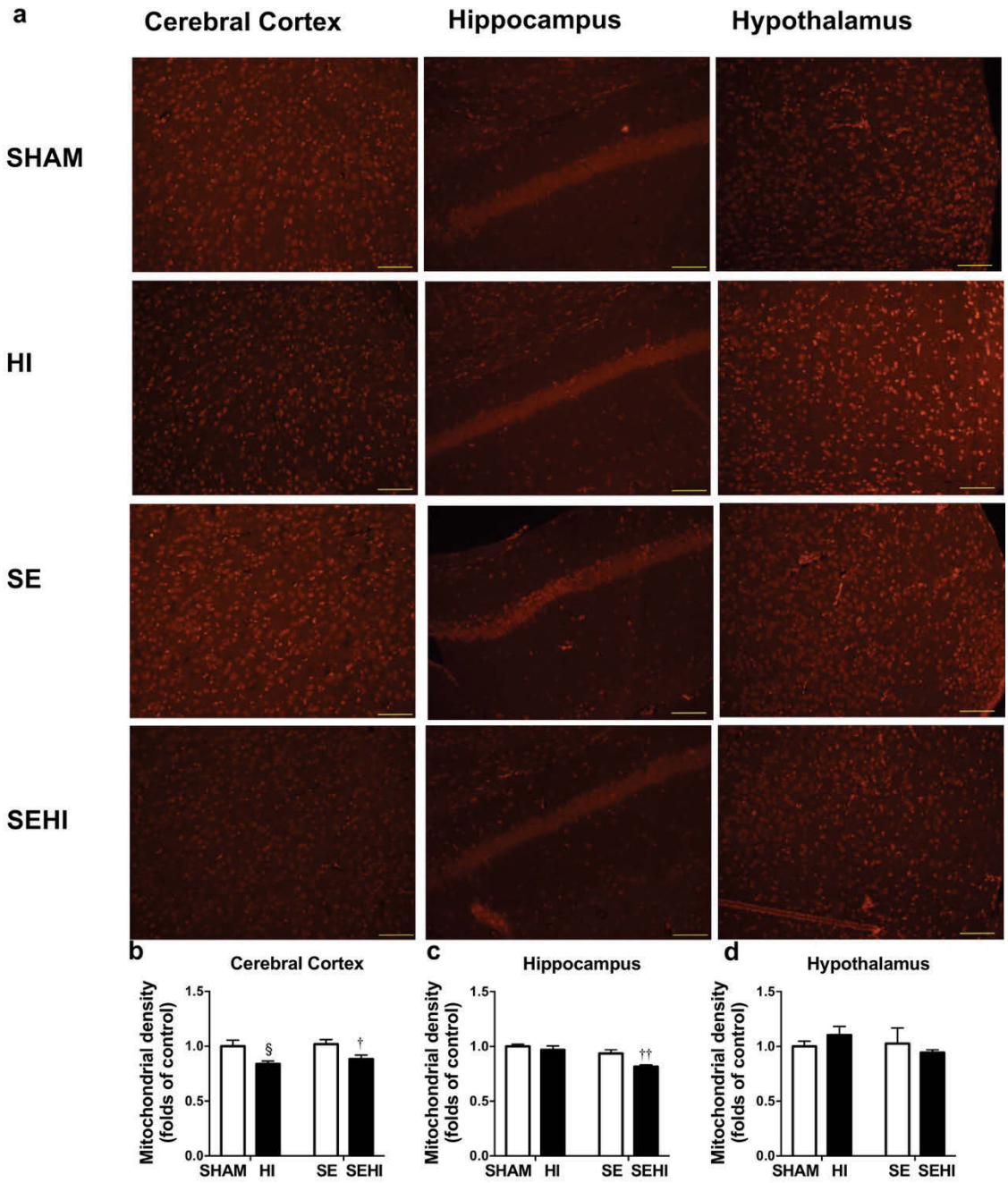

Figure 6.11. Mitochondrial density in the cerebral cortex, hippocampus and hypothalamus in the male offspring at P45 (n=5). Results are expressed as mean ± S.E.M. P<0.05 by conditional t test, §, P<0.05, HI vs SHAM; ††, P<0.01; †, P<0.05, SEHI vs SE; ‡, P<0.05, SEHI vs HI. Scale bar = 40 μm. Magnification = 20X. HI: hypoxic-ischemic injury; SE: smoke exposed; SEHI: smoke exposed and hypoxic-ischemic injury.

6.3.8 Cell apoptosis markers

Maternal SE increased Capase-3 level in the cerebral cortex of the SE offspring ($P<0.05$ vs SHAM offspring, Figure 6.12b). HI injury increased Caspase-3 levels in the cerebral cortex in both HI and SEHI offspring ($P<0.05$ vs non-injured littermates, Figure 6.12b). In the cortex, Caspase-3 levels in the SEHI offspring was higher than that in the HI offspring ($P<0.01$, Figure 6.12b). The level of Caspase-3 in the hypothalamus was also increased by HI in offspring from the SHAM dams only ($P<0.05$ HI vs SHAM, Figure 6.12d). Capase-3 levels in the hippocampus were not changed between the groups (Figure 6.12c).

Maternal SE increased the number of TUNEL positive cells in the cerebral cortex and hypothalamus in non-injured offspring ($P<0.05$ SE vs SHAM, Figure 6.13b, d). HI injury increased TUNEL positive cells in the cerebral cortex and hypothalamus in offspring from SHAM dams ($P<0.01$, Figure 6.13b; $P<0.05$, Figure 6.13d,). There were no changes in TUNEL positive cells in SEHI compared to their non-injured littermates (Figure 6.12). There were no significant changes in TUNEL positive cells in the hippocampus between the groups (Figure 6.13).

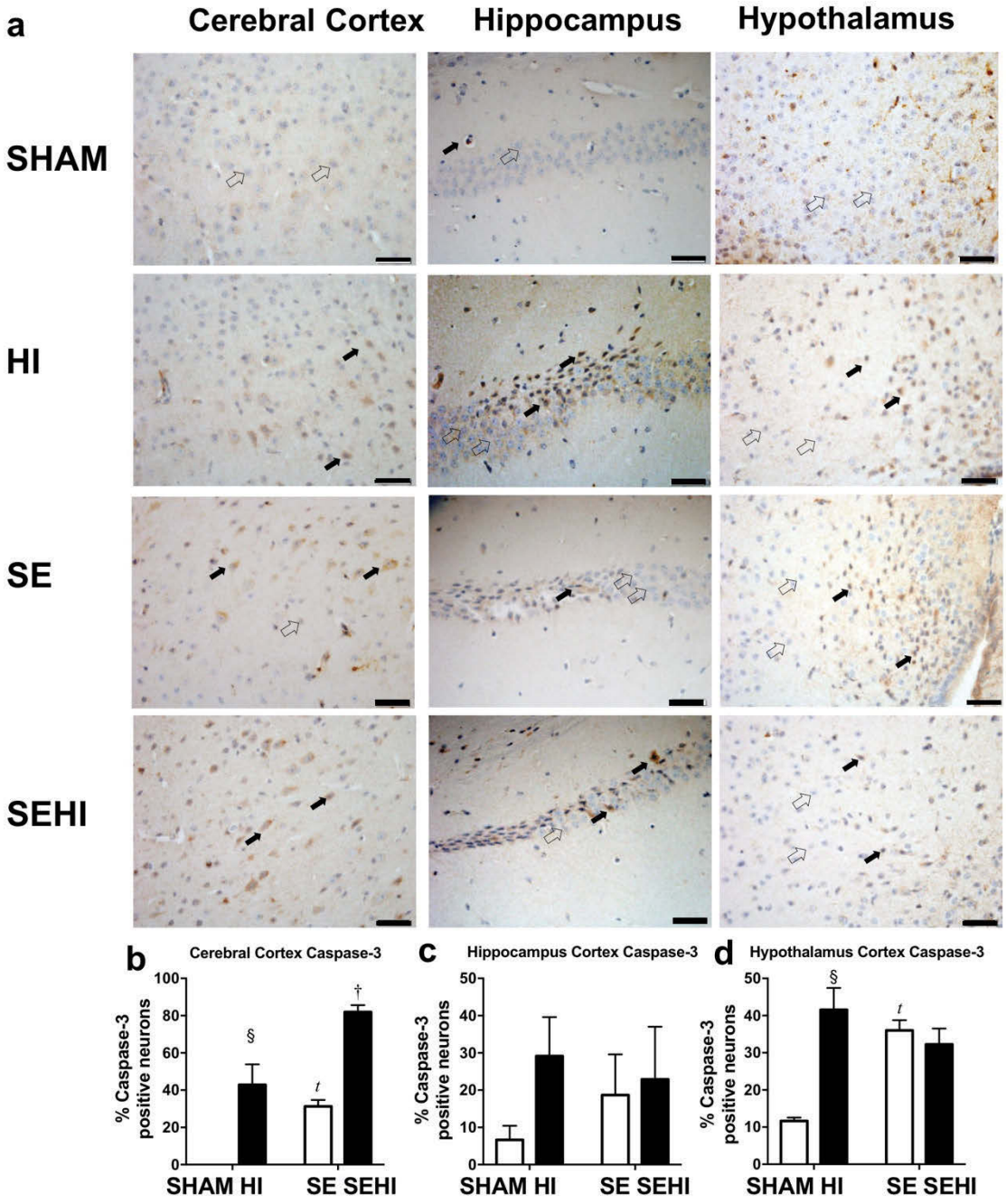

Figure 6.12. Immunostaining of Caspase-3 in cerebral cortex, hippocampus, and hypothalamus in the male offspring at P45 (n=5). Casp-3 positive (closed arrow) and Casp-3 negative (open arrow). Scale bar = 20 μm. Results were expressed as mean ± S.E.M. P<0.05 by conditional t test, *t*, P<0.05, SE vs SHAM; §, P<0.05, HI vs SHAM; †, P<0.05, SEHI vs SE. HI: hypoxic-ischemic injury; SE: smoke exposed; SEHI: smoke exposed and hypoxic-ischemic injury.

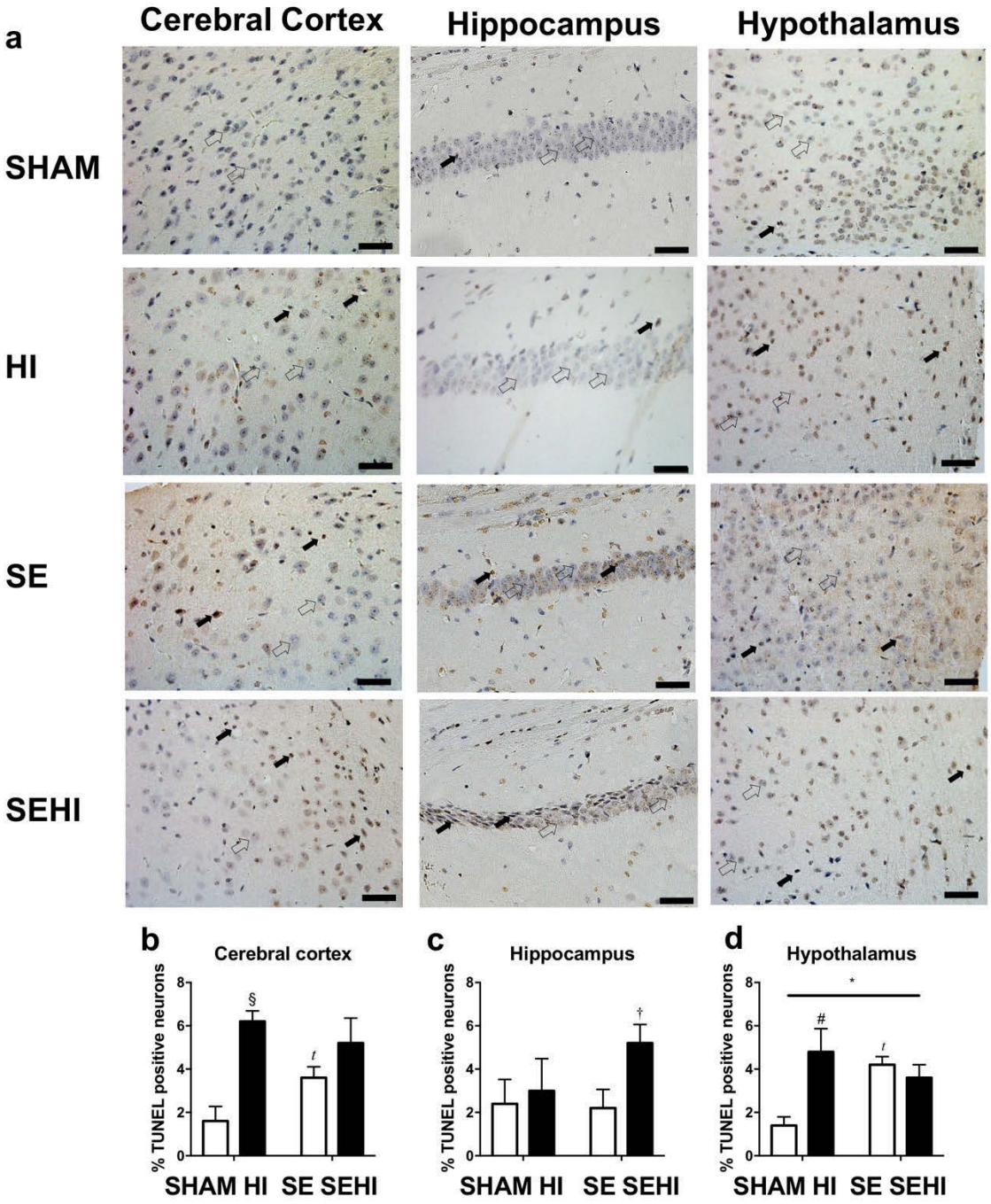

Figure 6.13. TUNEL staining in cerebral cortex, hippocampus and hypothalamus in the male offspring at P45 (n=5). TUNEL positive (closed arrow) and TUNEL negative (open arrow). Scale bar = 20 μm. Results are expressed as mean % TUNEL positive neurons ± S.E.M. * overall maternal SE effect. #, P<0.05 vs SHAM offspring. P<0.05 by conditional t test; *t*, P<0.05, SE vs SHAM; §, P<0.05, HI vs SHAM; †, P<0.05, SEHI vs SE; HI: hypoxic- ischemic injury; SE: smoke exposed; SEHI: smoke exposed and hypoxic-ischemic injury.

6.4 Discussion

It has been shown in humans, that maternal SE can increase the risk of HI injury in newborn infants which can lead to disabilities such as cerebral palsy (347). This study further examined the impact of maternal SE on the histological and functional outcomes of brain HI injury performed in early postnatal life in male offspring. In this study, HI injury did not affect the total brain weight in any of the groups; however, it reduced the size of left hemisphere, whereas maternal SE did not have additional effect. A previous study by Yong et al showed that perinatal nicotine exposure increased infarct size in male Sprague-Dawley rats 48 hours after HI injury (348). The difference might be due to the difference in HI modelling. In this study, the mice were exposed to 8% oxygen for 30 mins, whereas in the rat study, a 2.5-hour hypoxia session was used. In comparison with Yong's study, HI injury is much milder in our study therefore only the size of the left hemisphere was affected. Even though, evidence of increased apoptosis and DNA damage markers were observed by HI injury, maternal SE increased apoptosis and DNA damage in selected brain regions following HI injury, in the face of increased markers of inflammation, mitochondrial fission but reduced mitochondrial fusion and autophagy. This may explain why some of the outcomes following HI injury are worse in the SE offspring.

Here, both body weight and brain weight were reduced in SE offspring which is consistent with the results obtained in Chapters 3 and 4. This is accompanied by certain deficits in functional outcomes. SE offspring displayed increased levels of anxiety reflected by less time spent in the open arms during elevated plus maze test. Such anxiety can be interpreted as fear against the open environment. In humans, nicotine administration can lead to anxiolytic effect in a dose-dependent manner with significant

gender differences. The effect is more striking in rats at low doses (0.05 and 0.10 mg/kg) in adolescent females and at a high dose (0.25 mg/kg) in adolescent males (349). Although smoking itself does not directly lead to antisocial behavior in humans, it has been shown that maternal smoking during pregnancy in humans is a risk factor for antisocial behavior and anxiety and depression in both male and female offspring (350,351). This is in line with an animal study which showed that prenatal nicotine exposure can increase anxiety levels (352). HI encephalopathy regardless of its severity, was found to reduce anxiety-like behaviour in Sprague-Dawley rats due to decreased activity of tyrosine hydroxylase in the substantia nigra (353). In this study, HI injury did not affect the anxiety level after HI but reduced it in SE offspring, perhaps due to a higher baseline anxiety level in SE offspring without injury.

Although there was a reduction in the brain size and increase in cell damage in both HI and SEHI offspring, this only marginally affected their short term memory function as reflected by their performance in the novel object recognition test. It needs to be noted that there were no significant hippocampal damage in terms of cell apoptosis markers between the injury and non-injured groups. DNA damage observed in the brains of SEHI offspring does not seem to affect memory function. This may be due to the compensation by the healthy contralateral side. A study from Barbara et al. showed that there was a reduction of memory function in novel object recognition test at P32 which was recovered at P46 in mice with HI injury (354). Our result is consistent with this study where maternal SE did not worsen recognition memory function under HI injured conditions, suggesting that neural plasticity is more resilient to neonatal HI injury.

Grip test and foot fault test objectively demonstrate deficits in motor function (355). Maternal SE reduced grip strength and increased foot faults of offspring even without injury, suggesting intrauterine environment may play a major role in this deficit, such as

chronic hypoxia. This is in line with increased cortical markers of apoptosis and DNA fragmentation. Postnatal HI injury had a similar impact on forelimb grip strength and mistakes made during walking on the grid in offspring regardless of the maternal group. This suggests that HI injury is more potent stressor than maternal impact to damage motor function. This aligns with increased markers of cell apoptosis in cerebral cortex. The observation in the current model of HI injury is similar to what has been shown in mice with mild traumatic brain injury (356). While the HI group showed significantly worse impairment in coordination of forelimbs and hindlimbs, the changes are marginal in the SEHI group compared with their non-injured littermates, suggesting resilience of the brain to maintain basic coordination function. The behavioural test results are consistent with different human retrospective studies which correlated maternal SE to impaired locomotor function in offspring. A study in Denmark, Norway and Sweden indicated that maternal SE has a weak correlation with balance in 5 years old children (357). On the other hand, it has also been shown that maternal SE only marginally reduced motor competence in 11 year old offspring, particularly on the non-dominant side (358). Thus maternal SE led to some baseline motor and cognitive changes in the male offspring. However, it did not cause further reduction in memory retention, grip strength or limb coordination when combined with HI injury. Thus a follow-up study into late adulthood is needed to examine the longer term outcomes of these stressors.

Deficits in motor coordination have been shown to be mediated by increased IL-6 levels in mice with mild traumatic brain injury (356). Thus, here the motor defects in SE offspring may be mediated through the upregulated brain IL-6 mRNA expression. Activation of TLRs can induce the production of pro-inflammatory cytokines IL-1β and IL-6 (359). During brain ischemia-reperfusion injury, the TLR-4 pathway is activated to produce a large amount of pro-inflammatory cytokines to elicit the systemic

inflammatory response (360). In this study, TLR-4 mRNA was decreased in SE offspring along with reduced IL-1β mRNA. Compared with Chapter 2, there are age-related differences in inflammatory cytokine expression. An increase in IL-6 normally stimulates the expression of TLR (119). In Chapter 3, both IL-6 and TLR-4 mRNA expression was increased at 13 weeks in SE offspring with increased. In this study, TLR-4 mRNA expression was lower in SE offspring at P45 albeit increased IL-6 expression which may suggest that the persistent high level of IL-6 increases TLR-4 mRNA level at an older age due to the positive feedback, but not at younger age (361). In a mouse model of middle cerebral artery occlusion, TLR-4 deficient mice had lower levels of inflammatory cytokines and smaller infarct volumes (362). One of the early responses to HI injury in human newborns is an increase in blood IL-1β and TNF-α levels, which may also be attributed to increased release from brain cells such as microglia, astrocytes and neuron (363). Increased levels of plasma IL-1β in umbilical cord correlate with enhanced severity of HI injury in human infants (364). In this study the effect of HI injury in both HI and SEHI offspring is not that extensive, reflected by unchanged brain weight and mildly impaired functional outcomes. However, IL-1β mRNA was still increased in SEHI offspring compared with the non-injured littermates, with increased levels of iNOS, markers of cortical cell apoptosis and DNA fragmentation in the hippocampus. This indicates that maternal SE increased cellular injury after HI. Whether this can lead to long term consequences, such as early onset neurodegeneration and worsened neurological outcomes in adulthood, requires further investigation.

During HI, there are substantial changes in energy needs in the brain, and mitochondria play a vital role in post injury brain adaptions. Autophagy is a process where cells autodigest proteins, lipids and organelles in the cytoplasm for removal and turnover

(365). The process of autophagy in mitochondria is called mitophagy (365) which can be further divided into fusion and fission (366,367). Mitochondrial fragmentation is facilitated by fission proteins Drp-1 and Fission-1 (368) which are responsible for slicing the inner and outer mitochondrial membranes, respectively (369). Healthy portions of the mitochondria are fused through fusion process facilitated by Opa-1 proteins (370). Mitochondrial fragmentation is associated with increased neuronal cell death caused by a variety of experimental stressors (371). Among different proteins associated with mitophagy, Parkin labels damaged mitochondria for autophagic degradation (269). Maternal SE has been shown to reduce Parkin protein levels which might compromise the removal of damaged mitochondria (371). Mitochondria are particularly susceptible to oxidative damage as 2-4% of the oxygen consumed by mitochondria (372) is converted to superoxide anions through the OXPHOS complexes (373). Damaged mitochondria tend to produce more free radicals than healthy ones during physiological stress, which can further damage proteins, lipids and DNA and consequently yield more free radicals. Mitochondria are not well protected from ROS as there is limited resource of the endogenous antioxidant enzyme MnSOD (374). Once the mitochondria are damaged, the production of antioxidants is also reduced. As shown in Chapter 3, nitrotyrosine staining was increased in the brains of SE offspring, suggesting increased oxidative cell damage (375). Reduced Parkin was found to be causally related to changes in mitochondrial morphology and neuronal apoptosis (371). In the current study, there was an increase in the apoptotic marker caspase-3 in the cerebral cortex of SE offspring. The LC3A/B-I protein level was increased in SE offspring, but its conversion to LC3A/B-II remained unchanged. This may indicate that although damaged mitochondria can bind to Parkin, they may not be able to be engulfed by the autophagosome for recycling. As a result, while maternal SE caused increased

mitochondrial fission and reduced fusion in brain tissue of offspring, it did not lead to a significant change in the mitochondrial density in the brain regions examined in this study. This may be due to the limitation of mitotracker staining, which can't distinguish healthy and damaged mitochondria. Reduced OXPHOS complex III, IV and V, may well reflect impaired mitochondrial function and ability to synthesize sufficient ATP, although the mitochondrial density itself was not unchanged by maternal SE.

HI injury increased markers of mitochondrial fission in HI offspring. This is not surprising as mitochondrial fission aids the removal of damaged mitochondrial portions for degradation (368). Fission-1 levels were higher in SE offspring with HI injury suggesting that HI injury caused more severe mitochondrial damage in the SE offspring. Both Parkin and Pink-1 function to promote mitochondrial fission through Drp-1. Pink-1 is also recruited to depolarized mitochondria and phosphorylates Parkin to ubiquitinate and degrade damaged mitochondria (376,377). Pink-1 has been shown to protect different cell types from various stressors, including oxidative stress, mitochondrial blockers and apoptosis (378). In the current study, Pink-1 levels were not affected by maternal SE nor HI alone, but only by the combination of both, suggesting compromised neuroprotective function in SE offspring following HI injury. Pink-1 also initiates the translocation of Parkin onto damaged mitochondria; however, the increase in Parkin and Fission-1 can still activate the process of mitophagy without Pink-1 (379), such as what was observed here in the SEHI offspring. The removal of damaged mitochondria through Fission-1 and Parkin will still use up the autophagosome (379), to compromise autophagy procedure leading to cell death (380). Indeed, reduced LC3A/B-II and increased markers of apoptosis were observed in the cerebral cortex of the SEHI offspring. A reduction in Opa-1 levels in SEHI offspring also indicates that there are less healthy mitochondrial fragments to regenerate new mitochondria.

Mitochondrial density in both cerebral cortex and hippocampus was decreased in SEHI offspring due to injury; whereas OXPHOS complex I and III proteins were adaptively increased in SEHI offspring suggesting that there is increased demand for ATP to manage damage repair. This can also increase the production of free radicals (205,206), leading to upregulated iNOS in SEHI offspring. As such, the endogenous antioxidant MnSOD is needed in HI injured animals to counteract increased levels of oxidative stress. Although iNOS was not significantly changed in SE offspring, increased MnSOD still indicates increased oxidative stress, which is expected during HI injury.

In conclusion, maternal SE alone impaired several functional outcomes in offspring the additional HI injury did not worsen these deficits. However, indicators of apoptosis, inflammation, oxidative stress and mitophagy were worsened by maternal SE in the setting of HI in SE offspring. Further study is needed to follow up SE offspring into adulthood and geriatric age to examine long term cell fate and functional decline.

Chapter 7

General discussion and future perspectives

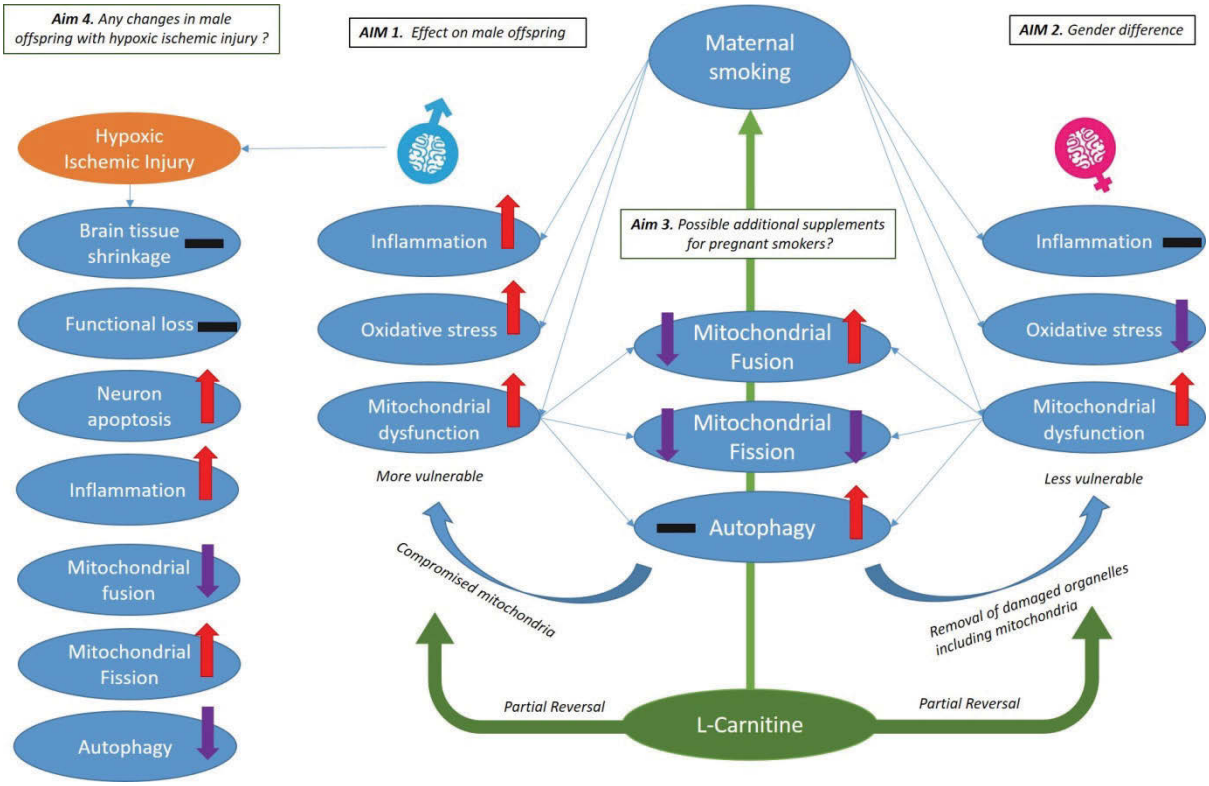

Figure 7.1. Diagram depicting the novel findings from different studies

Cigarette smoking is a known modifiable risk factor for numerous chronic diseases, such as cerebrovascular and cardiovascular diseases (7,44,381,382), as well as a range of respiratory disorders (383-385). Cigarette smoking is considered a harmful habit by the general public. Despite extensive public education, plain cigarette packaging and increase in taxation against cigarettes smoking still kills ~15,000 Australians per annum and costs Australia $31.5 billion in social and economic costs (386). 14.7% of Australians smoked daily in 2014 (387).

In Australia, since the adoption of new packaging regulations in 2012, 12% of women continue to smoke (386). Out of this population, 1 in 4 women smoked during

pregnancy and this percentage was much higher in the Aboriginal community at alarming 50% (386). The majority of retrospective studies focused on the impact of maternal smoking on anthropometry parameters in the offspring which are not adequate to represent all the pathophysiological changes in the body (18,36-38,40,388,389). Furthermore, majority of animal studies used nicotine administration to mimic cigarette smoking (47,238,323,388,390-394) which provides limited information as cigarette smoke contains more than 4000 chemical constituents including more than 40 carcinogens (395). Some poisonous substances such as tar, nitric oxides, carbon monoxide, hydrogen cyanide and metals have detrimental impacts on brain development or function. In addition, the combustion of tobacco leaves releases very high levels of superoxide and other reactive oxygen species (such as hydroxyl radical, hydrogen peroxide, and peroxynitrite) (396-399). The studies of the impact of maternal smoking on the brain health of offspring is scarce, as discussed in the introduction chapter of this thesis. Therefore, it was essential to examine the impact of maternal smoking on brain health in offspring.

During my PhD research, I found that maternal smoking increased inflammatory and oxidative stress markers and mitochondrial dysfunction in brain of the offspring in adulthood (Figure 7.1, Aim 1). These changes may predispose the offspring to pathophysiological changes such as increase in amyloid accumulation, and inflammation leading to Parkinson and Alzheimer's disease through changing inflammatory profile in brain (83,400,401). These findings are not surprising as maternal smoking increases the risk of other developing diseases (for example COPD) in offspring (402).

Interestingly, the outcome of maternal smoking was also different between the genders. In the second study in my thesis (Figure 7.1, Aim 2), it was found that female offspring had a different response to maternal smoking. They had no changes in inflammatory markers perhaps due to higher mitochondrial antioxidant levels. The increased antioxidant level might be able to remove excessive reactive oxygen species and reduce further damage from maternal smoking.

However, abnormal mitochondrial functional markers were found in offspring of both genders. This raised the question on the regulation of mitochondrial health in these offspring. Mitochondria biogenesis and health is regulated by mitophagy and autophagy (365,403). The process of mitophagy can remove fragments of damaged mitochondria through degradation by autophagy and combining the healthy fragments to generate new mitochondria. In Study 3 (Figure 7.1, Aim 3), it was found that male offspring from SE mothers has a reduction of mitochondrial fusion and fission markers with no changes in autophagy markers. This is also different from female offspring when there was an increase in mitochondrial fusion and autophagy markers but reduced fission markers. These changes may explain the findings in Study 1 where there were increase in both inflammation and oxidative stress in male offspring. This is because that increased autophagy in female offspring can remove damaged organelles in the brain such as mitochondria, which can reduce the production of endogenous ROS (404) to make the female offspring resistant to the effect of maternal smoking. Mitochondrial DNA is inherited maternally (405), both male and female offspring have the same mitochondria with same exposures. However, female offspring was less vulnerable to maternal smoking exposure. This might be due to oestrogen level differences between male and female in adulthood, which was suggested to play a role of neuroprotection (290).

During pregnancy, physicians and gynaecologist often prescribe supplements such Elevit to prevent complications such as spina bifida on the fetus (406), additional supplements can be prescribed to smokers when they are pregnant. Although smoking is a self-inflicted action, smokers don't often quit by self-control. The success rate of quitting smoke through cold turkey method is low (407). Therefore, it is important to investigate what the available options are to alleviate the impact of maternal smoking on offspring. In Study 3, maternal L-Carnitine supplementation was used to reverse the impact of maternal smoking on offspring (Figure 7.1, Aim 3). This supplement was found to partially reverse the impact of maternal smoking, possibly due to its anti-oxidative nature. L-carnitine can also promote the production of endogenous antioxidant manganese superoxide dismutase (408), and thus protect mitochondria from excessive ROS induced damage by maternal smoking.

It was found that maternal smoking is one of the risk factors for hypoxic-ischemic (HI) injury in offspring (330,409), which may be related to increased inflammatory markers and reduced mitochondrial antioxidant, specifically in male offspring. In the last study of this thesis, a model of maternal smoking in addition to HI injury in the offspring was investigated (Figure 7.1, Aim 4). The results indicated that there was only marginal reduction of neural function in male offspring as well as brain atrophy which is consistent to findings in human studies (410). Maternal smoking also increased mitochondrial fission but reduced mitochondrial fusion markers in offspring with HI injury. This indicates that there is a change of mitochondrial dynamics by maternal smoking. The change in mitochondrial fission and fusion profile was accompanied by the reduction of autophagy markers. This combination resulted in increased neuron apoptosis in cerebral cortex of SE offspring with HI injury.

Future studies can investigate other therapeutic targets for early intervention of maternal smoking during pregnancy. As L-Carnitine was found to partially normalise the impact of maternal smoking on offspring to the level of offspring from non-smokers other antioxidants options such as Acetyl-L-Carnitine or nitroxide-containing antioxidants such as Tempol (411)could also be considered. Interestingly, Acetyl-L-Carnitine was found to protect neuronal function from alcohol-induced oxidative damage in brain (412) and L-Carnitine can reduce brain injury after hypoxia-ischemia in newborn rats (413). Thus, it would also be interesting to examine if L-Carnitine can reverse the impact of maternal smoking on HI brain injury in offspring. Although L-Carnitine is an overcounter supplement, it is unknown whether it has safety issues during pregnancy. The safety and dosage of L-Carnitine will need to be further investigated.

On the other hand, more work can be done to investigate the impact of alternative nicotine delivery options, such as electronic cigarettes. These might be used as cigarette replacement during pregnancy. Nicotine replacement therapy during pregnancy is controversial as maternal nicotine exposure can also induce a series of adverse events such as hypoxic ischemic brain injury in the offspring (238,323,391,392). However, the impact of maternal e-vaping is largely unknown due to the combination of flavoring substances which have been shown to be toxic by inhaling.

As offspring's mitochondria are inherited from the mothers (405), maternal smoking can change mitochondrial proteins in offspring due to modification on mitochondrial DNA during the gestation period. In a previous study, it was found that mitochondrial membrane potential was reduced in the ovulated oocytes in obese mice as well as

increased autophagy compared to oocytes from the lean mice (414). Such changes in the SE offspring are unknown. As mitochondria has a central role in many of the cellular processes such as apoptosis, aging and oxidative metabolism as well as ATP synthesis, it is crucial to understand changes in the mitochondria (415). Indeed, it was found that maternal smoking during pregnancy can affect the region of transcription and replication control in placental mitochondrial chromosome (416). Maternal smoking might induce a range of epigenetic modifications including genomic DNA methylation, post translational modification of histone tails and regulation of gene expression by non-coding RNAs (417). Studying these changes may aid in discovering the mechanism of brain mitophagy change in the SE offspring.

This study focused on the overall changes in markers of inflammation, oxidative stress and mitochondrial wellbeing in the whole brain. The limitation was the inability to investigate the impact on specific cell types and regions of the brain. Future study can investigate the outcome in different cell types such as microglia, neurons and astrocytes when technique allows.

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