Modulation of neural regulators of energy homeostasis, and of inflammation, in the pups of mice exposed to e-cigarettes

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Abstract

Background: Maternal smoking can lead to perturbations in central metabolic regulators such as neuropeptide Y (NPY) and pro-opiomelanocortin (POMC) signalling components in offspring. With the growing interest in e-cigarettes as a tobacco replacement, this short report assessed central metabolic regulation in offspring of mouse dams exposed to e-cigarettes. We examined the impact of continuous use of e-cigarettes, and e-cigarette replacement of tobacco cigarettes during pregnancy. Supplementation of an antioxidant L-carnitine was also co-used with tobacco cigarette in the mother to determine whether the impact of maternal tobacco smoking was oxidative stress driven.

Methods: Balb/c mice were exposed to either nicotine-containing (E-cig18) or nicotine-free (E-cig0) e-cigarette aerosols or tobacco smoke (SE) prior to mating and until their pups were weaned. After mating, two SE sub-groups were changed to E-cig18 exposure (Replacement), or supplementation L-carnitine while SE was continued. Male offspring were studied at weaning age.

Results: The offspring of E-cig0 dams were the heaviest with the most body fat. Replacing SE with E-cig18 during pregnancy resulted in offspring with significantly less body fat. E-cig0 offspring had significantly increased mRNA expression of brain NPY and iNOS. Maternal SE upregulated mRNA expression of NPY, NPY Y1 receptor, POMC downstream components, and iNOS expression, which were normalised in Replacement offspring, but only partially normalised with maternal L-carnitine supplementation during gestation and lactation.

Conclusions: Maternal exposure to either tobacco and nicotine-free e-cigarettes lead to disturbances in the level of central homeostatic control markers in offspring, suggesting that maternal exposure to e-cigarettes is not without risks.

Highlights

- Maternal exposure to nicotine-free e-vapor disturbs brain metabolic markers
- Maternal tobacco smoke exposure disturbs brain metabolic regulators
- Nicotine-containing e-cigarette replacement reverses the effect of maternal smoking

- Increased oxidative stress may be a mechanism underlies these perturbations

Keywords: maternal smoking, e-cigarette, neuropeptide Y, MC4R, iNOS

Abbreviations

α-MSH	α-melanocyte-stimulating hormone
E-cig18	e-vapour from nicotine containing e-fluid
E-cig0	nicotine-free e-vapour
iNOS	inducible nitric oxide synthases
MCR	melanocortin receptors
NPY	neuropeptide Y
Y1R	NPY Y1 receptor
POMC	proopiomelanocortin
SE	cigarette smoke exposure
SE+LC	SE dams was administered with L–carnitine
Sim	single-minded gene

Introduction

The brain plays an important role in energy metabolic homeostasis, while studies have demonstrated that maternal smoking can disturb such regulation in the offspring. Specifically, children born to smoking mothers have increased risk of childhood obesity and a preference for 'junk' food, where the potent appetite stimulator neuropeptide Y (NPY) may play an important role [16]. NPY is a potent stimulator of feeding and principally produced in the arcuate nucleus of the hypothalamus. NPY production is suppressed by cigarette smoke exposure (SE) as well as postsynaptic NPY Y1 receptor levels leading to anorexia and weight loss [11, 24]; whereas smoking cessation was shown to increase NPY production resulting in overeating and weight gain [3, 20].

In addition to NPY, the melanocortin system is known to serve a central role in the regulation of food intake whereby the cleavage products of proopiomelanocortin (POMC), including α-melanocyte-stimulating hormone (α-MSH), act through binding to the melanocortin receptors (MCRs) to counteract the NPY system [16, 31]. Of the MCRs, MC4R, and its downstream molecule single-minded gene (Sim)1, have been the most extensively studied [2]. The melanocortin system is one of the most important pathways involved in food intake and energy regulation, with mutations contributing to ~4% of genetic obesity in humans [23]. The anorexigenic neurons respond synchronously with orexigenic neurons to maintain the balance between orexigenic and anorexigenic neuropeptides [27].

Significant levels of nicotine are detectable in the breast milk of nursing mothers who smoke. Towards weaning, the offspring of such mothers may undergo nicotine withdrawal by reducing milk intake and increasing solid food consumption, which can have an impact upon feeding behaviour and energy homeostasis, resulting in increased risk of obesity later in life [16].

Quitting cigarette smoking during pregnancy is desirable to optimise the future health of the unborn child. However, smoking cessation is difficult to achieve due to nicotine addiction. There is a widely held belief that the availability of electronic cigarettes (e-cigarettes) provides the opportunity to quit smoking without the associated issues of nicotine withdrawal. E-cigarette vaporises liquids composed of a mixture of vegetable oils, propylene glycol and/or glycerine and flavourings, in the presence or absence of nicotine [30]. While the vapour from e-cigarettes contains fewer toxins than cigarette smoke, it is not toxin free[18]. As the success of smoking cessation during pregnancy is lower than the general population [4], e-cigarettes are an attractive option to be used as a replacement for tobacco cigarettes by pregnant women, despite the lack of efficacy and safety evidence. Furthermore, e-cigarette usage (“e-vaping” or “vaping”) among women of reproductive age is increasing [35]. Indeed, recent evidence suggests that most pregnant women perceive e-cigarettes as a safer alternative to tobacco cigarettes [37]. Currently, the rate of e-cigarettes usage during pregnancy appears similar to tobacco cigarette smoking, with up to 15%

of pregnant women using e-cigarettes [37]. The evidence of the long-term impacts of e-vaping either nicotine containing and nicotine-free e-fluids is limited. Therefore, this study aimed to investigate the impact of prolonged maternal e-vapour exposure and replacing cigarette smoking with nicotine containing e-vapour on brain metabolic regulators in the offspring using a mouse model. As brain metabolic regulators at weaning age can predict future risk of obesity [15, 17], male offspring (which are more susceptible to such changes) were examined at this age.

In this study, we compared the impact of e-cigarettes (using both nicotine-containing and nicotine-free fluids) to SE with regard to the expression of brain metabolic regulators in the offspring of exposed murine dams. Exposure to e-vapour instead of SE, during gestation and lactation, was also assessed as we hypothesise that such a replacement regime may mitigate the dysregulation of brain metabolic regulators wrought by maternal smoking. As any effects may be mediated through elevated brain oxidative stress and/or inflammation [32], brain inducible nitric oxide synthases (iNOS) expression was also examined. Previously Chan *et al.* demonstrated supplementation of SE mothers with anti-oxidant L-carnitine ameliorated brain pathology in their offspring [5]. In the current study, any benefits of e-cigarette replacement were compared with those of L-carnitine supplementation.

Methods and materials

Tobacco cigarette smoke exposure and e-cigarette exposure

The animal experiments were approved by the Animal Care and Ethics Committee of the University of Technology Sydney (ACEC#2011-313A, ETH15-0025), and performed as per Australian National Health & Medical Research Council Guide for the Care and Use of Laboratory Animals. Female BALB/c mice (7 weeks, Animal Resource Centre, WA, Australia), housed in standard conditions [5], were randomised into, ambient air (Sham), e-vapour from nicotine-containing e-fluid (18 mg/mL, tobacco flavour, Vaper Empire, Australia. E-cig18), nicotine-free e-fluid (tobacco

flavour, Vaper Empire, Australia. E-cig0) groups (n=8). The e-liquids are all used by human consumers. E-vapour was generated from an e-cigarette device (KangerTech NEBOX, KangerTech, Shenzen, China) as we have published [13]. The nicotine dosage per treatment was equivalent to 2 cigarettes (2.4 mg) as determined by our previous studies using cigarette smoke exposure [5]. As we have published, plasma cotinine levels in the E-cig18 offspring [13] were similar as the SE offspring at weaning age [36] suggesting similar nicotine exposure level of the mothers exposed to e-vapour and tobacco smoke. E-cig18 and E-cig0 mice were exposed for 30 min, twice daily for 6 weeks prior to mating, during gestation and up until the resultant pups were weaned [13].

At the same time, an additional group of mice were subjected to cigarette smoke (SE) exposure during the same period as the above e-vapor exposure. SE group was exposed to cigarette smoke (2 cigarettes (Winfield Red, VIC, Australia) for 30 min twice daily) [5]. In two sub-groups, SE exposure was replaced with nicotine-containing e-vapor (Replacement), or accompanied by antioxidant L-carnitine supplement (SE+LC), during gestation and lactation. For the Replacement group, SE exposure was switched to E-cig18 vapor exposure upon mating until the pups were weaned. Previously, the benefits of supplementation of SE mice with the antioxidant, L-carnitine were demonstrated with regard to the brain health of their offspring [5]. To determine whether any beneficial effect of e-cigarette replacement was comparable to antioxidant supplementation, a sub-group of SE dams was administered with L–carnitine (1.5mM in drinking water [5]) during gestation and lactation.

The male breeders and offspring stayed in their home cage during exposure. As female offspring tend to be protected from the adverse impact of maternal SE [6], only one male offspring from each litter were sacrificed at 20 days (normal weaning age). Body weight was recorded. The epidydimal fat of both sides was micro-dissected from the epididymis and weighed immediately.

Real-time PCR

Total mRNA was extracted from the whole brain using TriZol reagent (Thermo Fisher Scientific,

MA, USA) and first-strand cDNA generated as we have described previously [9, 12]. The genes of interest (NPY, NPY Y1 receptor (Y1R), POMC and its downstream MC4R and single-minded gene (Sim)1, the hormone regulator of NPY and POMC leptin receptor (Ob-Rb), and oxidative stress marker iNOS) were measured using manufacturer (Thermo Fisher Scientific, MA, USA) pre-optimised and validated TaqMan® primers and probes published previously [9, 12]. The probes of the target genes were labelled with carboxyfluorescein (FAM) and housekeeping gene 18s rRNA was labelled with VIC. Values are expressed as fold change from the control.

Statistical analysis

Results are expressed as mean ± SEM and analysed by one-way ANOVA followed by Tukey *post hoc* tests (Statistica 10, Statsoft Inc. OK, USA). $P<0.05$ is considered statistically significant.

Results

As shown in Table 1, at 20 days, E-cig0 offspring were much heavier than the Sham and E-cig18 offspring ($P<0.05$), with heavier abdominal fat ($P<0.05$). Maternal exposure to nicotine-containing e-vapour did not affect mRNA expression of NPY (Figure 1a) nor NPY Y1 receptor (Y1R, Figure 1b), the appetite inhibiting gene pro-opiomelanocortin (POMC, Figure 1c) nor its downstream, melanocortin 4 receptor (MC4R, Figure 1d) and single-minded gene (Sim)1 (Figure 1e), and only marginally reduced leptin receptor Ob-Rb mRNA expression in the offspring (Figure 1f). However, maternal exposure to nicotine free e-vapour significantly increased brain mRNA expression of NPY ($P<0.01$ vs Sham, Figure 1a) and iNOS ($P<0.05$ vs Sham, Figure 1g). Although POMC expression was upregulated by 50% compared with the Sham offspring, it did not reach statistical significance (Figure 1c). Compared with E-cig18 offspring, brain MC4R and Ob-Rb expression were higher in the E-cig0 group (both $P<0.01$, E-cig18 vs E-cig0, Figure 1d, f).

Although SE offspring had similar body weight and fat mass as the Sham offspring, the Replacement offspring had much smaller fat mass than the Sham group ($P<0.05$, Table 2). Maternal

L-Carnitine supplementation had no impact on body weight and fat mass at this age. Maternal SE significantly increased brain NPY ($P<0.05$, Figure 2a), NPY Y1R ($P<0.01$, Figure 2b), MC4R ($P<0.01$, Figure 2c), Ob-Rb ($P<0.01$, Figure 2f) and iNOS expression ($P<0.05$, Figure 2g) compared with the Sham offspring. Although Sim1 level in the SE offspring doubled the level in the Sham group, it did not reach statistical significance. Maternal e-vapour replacement during gestation and lactation normalised the changes in NPY, NPY Y1R, MC4R, Sim1, Ob-Rb and iNOS in the Replacement group compared with the SE offspring ($P<0.05$ for NPY, Sim1 and iNOS; $P<0.01$ for NPY Y1R, MC4R, and Ob-RB, Figure 2a,b,d,e,g). The effect of Replacement to normalise brain markers was more potent than maternal L-carnitine supplementation.

Discussion

The first major finding of this study is that maternal exposure to nicotine-free e-vapor induces significant adiposity, dysregulation of brain metabolic regulatory pathways and increased iNOS expression suggesting that maternal e-vapor exposure is not necessarily risk-free. The second major finding is that replacement of tobacco smoke with (nicotine-containing) e-vapour normalised the effects of tobacco smoke upon the same regulatory pathways, which is comparable and superior to the supplementation with the antioxidant L-carnitine during gestation and lactation.

In this short report, we did not attempt to carry out mechanistic studies and were unable to measure protein levels due to a lack of sample availability. Whilst this is a limitation of the current study, our previous research has concomitant increases in mRNA and protein as expected [7, 8, 10]. We used two types of e-cigarettes fluids that were identical apart from the presence and absence of nicotine. In our other study [14], we have shown that inflammatory responses are not dependent upon the presence of nicotine, in fact some responses were greater in the absence of nicotine, reinforcing the notion that others chemicals produced in the heated e-vapor are potent to induce inflammatory responses. In the current study, we also observed similar hyper-activation

caused by the nicotine free e-fluids. It has been well established that nicotine processes anti-inflammatory property [25]. Although there was no significant change in the other metabolic regulators in the offspring from the mothers with continuous exposure to nicotine-containing e-vapor, the expression of POMC, MC4R, and Ob-Rb in the E-cig0 offspring was also higher than the E-cig18 offspring. This may also suggest that nicotine-free may be more harmful than the nicotine-containing ones if the same amount is inhaled.

Although the solvents in the e-fluids have been demonstrated to be safe for oral intake, they can be converted to toxic by-products during heating, inducing an inflammatory response and oxidative stress [26]. It is alarming to observe increased adiposity and a moderate but significant increase in a marker of inflammation/oxidative stress, iNOS, in the offspring from dams exposed to nicotine-free e-vapor. This was accompanied by some level of dysregulation of brain metabolic markers, with the upregulation of NPY a potential contributor to increased adiposity in E-cig0 offspring. This raises concerns about the solvents used in the e-fluid. Since the components of e-cigarette fluid can also produce a harmful effect on the unborn child even without the presence of nicotine, this warrants further investigation as to what components in the e-vapour were responsible as vaping nicotine-free e-fluid, especially during pregnancy, may not be risk-free.

That no differences in the anthropometric data were observed between the offspring of the Sham dams and SE dams may reflect the relatively low dosage of the tobacco smoke dose used (equivalent to light smokers), consistent with our previous study [1]. However, what is notable is that the replacement of tobacco smoke with nicotine-containing e-vapor after mating resulted in male pups with significantly lower fat mass when compared to offspring of dams continuously exposed to tobacco smoke.

Nicotine can have a profound impact on the foetal and suckling offspring's brain [16]. Intrauterine nicotine exposure is clearly linked to downregulated hypothalamic NPY and POMC gene expression in the newborn primates [16]. The speculation is that when the pups of nicotine-exposed dams are weaned from breastmilk [33], they will go through nicotine withdrawal [16].

Without the continuing inhibition of nicotine and other chemicals in the tobacco smoke, NPY and POMC gene expression can rebound [16]. In this study, such rebound seems to occur, where with the exception of POMC. All the signaling elements of NPY and POMC pathways examined here were upregulated in the SE offspring at 20 days (the time of weaning). The adipocyte-derived hormone leptin inhibits brain NPY expression and promotes POMC transcription to αMSH to inhibit feeding. In primates, serum leptin levels are reduced by ~50% in newborns from nicotine-treated mothers compared with those from control mothers [21]. Such reduction may upregulate its receptor in the brain, as shown in the SE offspring.

The absolute amount of chemicals in the e-cigarette derived e-vapor are much less than in cigarette smoke, therefore e-vaping is speculated to be safer than smoking [19]. Encouragingly, such speculation was supported by the observation in the Replacement offspring, where persistent administration of nicotine (albeit through e-vapour exposure) normalised the abovementioned changes in the SE offspring. The findings also highlight the potential role of the cigarette smoke components other than nicotine, in the maternal programming of metabolic regulation in the offspring. As such switching to nicotine-containing e-vapor during pregnancy led to no significant changes in the Replacement offspring relative to the offspring of sham-exposed dams.

Another notable observation was that changes in the metabolic markers mirrored changes in iNOS expression. Therefore, inflammation or oxidative stress may be involved in the dysregulation of these brain regulatory pathways wrought by maternal smoking or e-vaping. Mitochondria may be the vehicle to transfer the redox status from the mother to the offspring, as mitochondrial DNA is only inherited via the maternal lineage [28], and mitochondrial DNA can be epigenetically programmed [34]. Further support for a role for redox modulation was the observation that maternal antioxidant supplementation using L-carnitine [22] partially reversed the observed changes in the brains of SE offspring. Whether the action of L-carnitine was due to direct scavenging, or direct effects upon iNOS expression, or was linked to changes in fatty acid metabolism (the latter which can re-model brain metabolic regulators as suggested previously

[29]) is yet to be determined.

In conclusion, maternal exposure to the vapor from nicotine-free e-cigarettes can induce adiposity and dysregulation of brain metabolic regulatory pathways in their offspring. Such dysregulation may involve inflammation and/or oxidative stress. However, changing from cigarette smoking to vaping may be beneficial with regard to post-natal energy homeostasis. However, since our data suggest that e-cigarettes alone cause adverse effects, their use is unlikely to be without risk. Future studies are required to identify the active components in e-vapor. Based on the current findings, caution should still be taken before recommending e-cigarette use as an alternative to tobacco during pregnancy.

Declaration of interests

The authors have no conflict of interest to declare.

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Figure captions

Figure 1: mRNA expression of metabolic homeostatic regulars and iNOS in the brain of male offspring at 20 days. Comparisons were made between the offspring of dams exposed to either: ambient air (Sham, n=6), vapor from nicotine-containing (E-cig18, n=6) or nicotine-free (E-cig0, n=7) e-cigarette fluid. Results are expressed as mean ± SE. **P<0.01; †P<0.05, ††P<0.01 vs all the other groups.

Figure 2: mRNA expression of metabolic homeostatic regulars and iNOS in the brain of male offspring at 20 days. Comparisons were made between the offspring of dams exposed to either: ambient air (Sham, n=6); or tobacco cigarette smoke (SE, n=6), respectively, for 6 weeks prior to mating and during gestation and lactation; and tobacco cigarette smoke-exposed dams switched after mating to either nicotine-containing vapor (Replacement, n=7) or accompanied by L-carnitine supplementation in their drinking water (SE+LC, n=5). Results are expressed as mean ± SE. *P<0.05, **P<0.01; †P<0.05, ††P<0.01 vs all the other groups.

TABLE 1. Anthropometry data in Sham, E-cig18, and E-cig0 groups at 20 days

	Sham (n=6)	**E-cig18 (n=6)**	**E-cig0 (n=7)**
Body weight (g)	9.71±0.13	9.80±0.16	11.1±0.2†
Fat mass (g)	0.060±0.004	0.070±0.03	0.12±0.01†
Fat%	0.61±0.04	0.72±0.03	1.07±0.07†

Results are expressed as mean ± SE. † P<0.05 vs all the other groups.

TABLE 2. Anthropometry data in Sham, SE, Replacement, and SE+LC groups at 20 days

	Sham (n=6)	**SE (n=6)**	**Replacement (n=7)**	**SE+LC (n=5)**
Body weight (g)	9.71±0.13	9.71±0.14	9.57±0.18	9.74±0.43
Fat mass (g)	0.060±0.004	0.077±0.007	0.052±0.005#	0.073±0.010
Fat%	0.61±0.04	0.79±0.07	0.54±0.05#	0.72±0.08

Results are expressed as mean ± SE. #P<0.05, vs SE.

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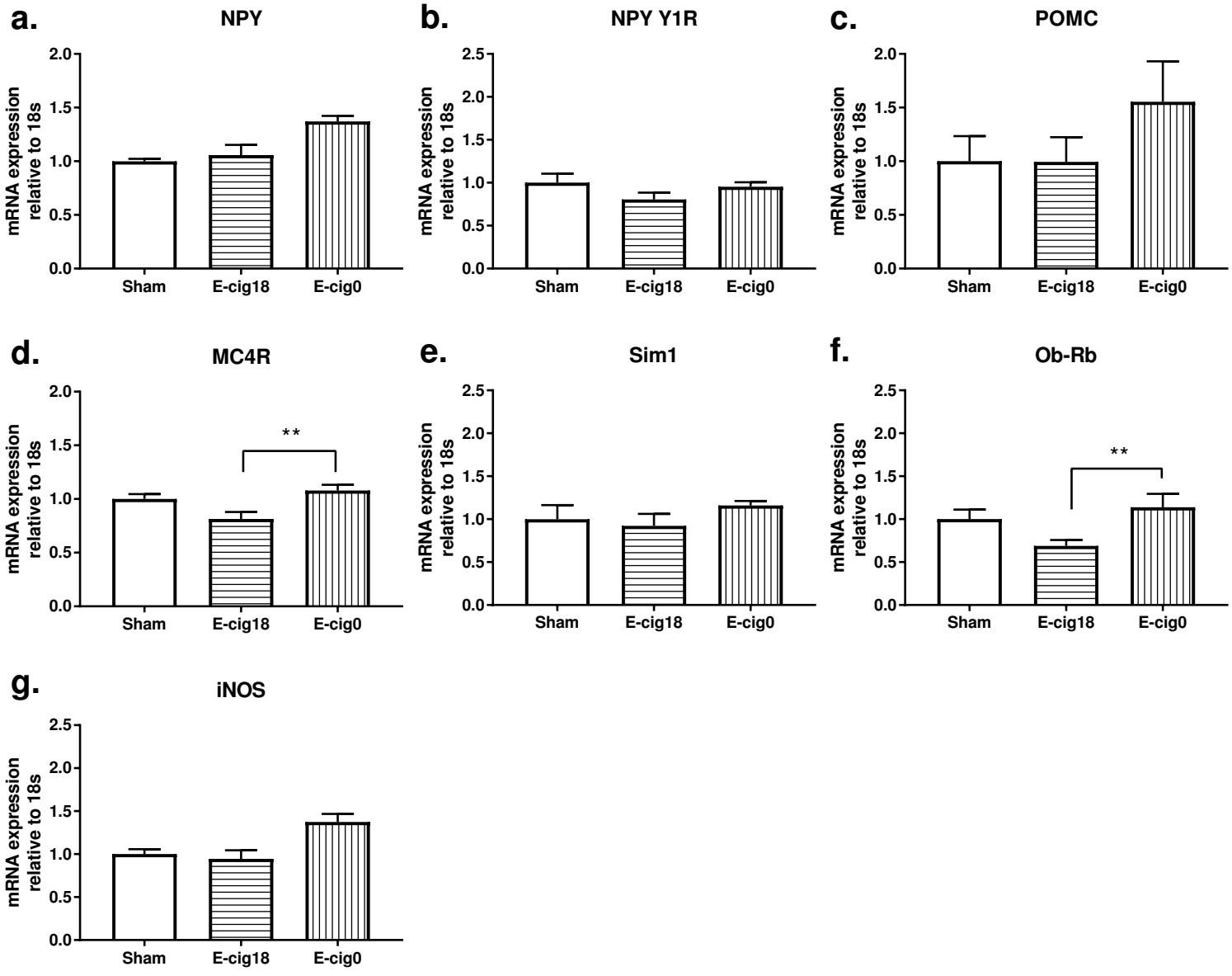
Figure 1


a.
NPY
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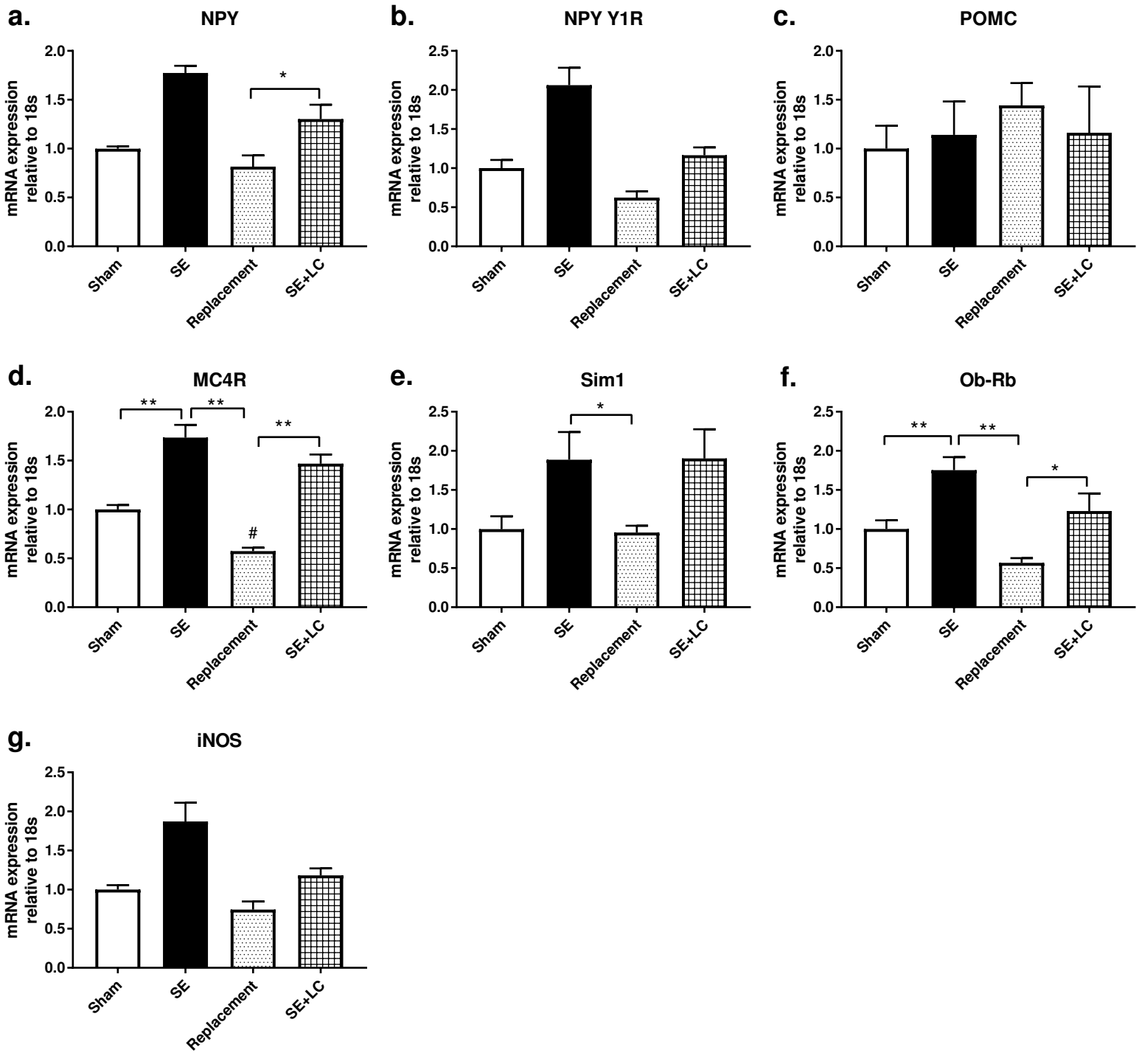
g.
iNOS
mRNA expression relative to 18s
Sham
E-cig18
E-cig0
**

Figure 2


a.
NPY
b.
NPY Y1R
c.
POMC
d.
MC4R
e.
Sim1
f.
Ob-Rb
g.
iNOS
mRNA expression relative to 18s
Sham
SE
Replacement
SE+LC
*
**
#