Brain Behavior and Immunity

# Brain health is independently impaired by E-vaping and high-fat diet

--Manuscript Draft--

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| **Abstract:** | Tobacco smoking and high-fat diet (HFD) independently impair short-term memory. E-cigarettes produce e-vapour containing flavourings and nicotine. Here, we investigated whether e-vapour inhalation interacts with HFD to affect short-term memory and neural integrity. Balb/c mice (7 weeks, male) were fed a HFD (43% fat, 20kJ/g) for 16 weeks. In the last 6 weeks, half of the mice were exposed to tobacco-flavoured e-vapour from nicotine-containing (18mg/L) or nicotine-free (0mg/L)) e-fluids twice daily. Short-term memory function was measured in week 15. HFD alone did not impair memory function, but increased brain phosphorylated (p)-Tau and astrogliosis marker, while neuron and microglia levels were decreased. E-vapour exposure significantly impaired short-term memory function independent of diet and nicotine. Nicotine free e-vapour induced greater changes compared to the nicotine e-vapour and included, increased systemic cytokines, increased brain p-Tau and decreased postsynaptic density protein (PSD)-95 levels in chow-fed mice, and decreased astrogliosis marker, increased microglia and increased glycogen synthase kinase levels in HFD-fed mice. Increased hippocampal apoptosis was also differentially observed in chow and HFD mice. In conclusion, E-vapour exposure impaired short-term memory independent of diet and nicotine, and was correlated to increased systemic inflammation, reduced PSD-95 level and increased astrogliosis in chow-fed mice, but decreased gliosis and increased microglia in HFD-fed mice, indicating the inflammatory nature of e-vapour leading to short term memory. |
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| **Opposed Reviewers:** | |
| **Response to Reviewers:** | |

Dear editor,

Thank you for allowing us to revise our manuscript. Please find enclosed a revised manuscript entitled " Brain health is independently impaired by E-vaping and high-fat diet" [authors: Hui Chen, Baoming Wang, Gerard Li, Joel R Steele, Sandy Stayte, Bryce Vissel, Yik Lung Chan, Chenju Yi, Sonia Saad, Rita Machaalani, Brian G Oliver] for consideration of publication.

We would like to thank both reviewers for their positive comments and constructive suggestions. We have addressed the comments raised by the reviewers, and amended the manuscript accordingly.

We hope that our revised manuscript will meet with your approval and be suitable for publication in your journal. We are looking forward to hearing about your decision.

Yours sincerely,

Professor Brian Oliver, PhD

## Response to the reviewers

Reviewer #1:

1. Please clarify the immunohistochemistry result of caspase 3.

Response: We apologise for the missing X-axis information. This has now been corrected and the figure legend clarified accordingly. We have also clarified the discussion paragraph pertaining to this data to make it more specific.

2. Could the authors explain the discrepancy between the immunohistochemistry result and western blotting results for caspase 3?

Response: We apologise if we lead the reviewer to perceive we undertook western blotting for Caspase-3. We do not have this data in the manuscript. Although we did attempt it, the signal was faint and we ran out of antibody, so we were not able to troubleshoot it further. We believe the immunohistochemistry data for caspase-3 is superior given it allows for specific region analysis of the hippocampus important for correlation with the memory data as we have presented it.

3. Please be consistent with whether capital Synapsin throughout the manuscript. For Tau statistics, P=0.05 was reported; whereas for GFAP, P=0.052 was used. Please be consistent with how you report such close to significant value.

Response: We have revised the manuscript accordingly.

Reviewer #2:

1. The title as it stands poses the question "Is there a synergic effect of e-vaping and high fat diet consumption on brain health?" I wonder whether a title that encapsulates the main message of the paper would be more appropriate.

Response: Thank you for this suggestion, we have revised the title to "Brain health is independently impaired by E-vaping and high-fat diet".

2. The graphical abstract has E-vporing. Please change to E-vapour.

Response: We have corrected the typo.

3. Why were Balb/c mice used in the study? Can the authors also justify why male mice were used? Did the authors assess lung inflammation in the current study? I am wondering whether there is lung inflammation in the e-cigarette models and whether this is spilling over into the blood to cause the observed neuroinflammation. Or, is it happening independent of any effects on the lung?

Response: Balb/c mice were used due to their susceptibility to the effects of cigarette smoke exposure / e-vapour exposure, and we have now clarified these in the Methods section (Page 3 line 86-87). We used only males as we have found males and females had similar responses to direct environmental toxin exposure (Chan et al. Sci Rep 2016;6:25881).

We did assess lung inflammation, which will be reported separately. We agree with the reviewer that lung-derived inflammatory mediators are likely to in-part drive some of the changes we have seen. However, we did see some discrepancies in the inflammatory response between the brain and the lung, which may be caused by the selection of blood brain barrier. In future studies we would like to address this specific research question as in the present study we would only be able to do this by correlation, which is perhaps a little too superficial. We now allude to this by adding a sentence in the discussion (page 8 line 327-329).

# ~~Is there a synergic effect of e~~Brain health is independently impaired by E-vaping and high-fat diet ~~consumption on brain health?~~

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**Keywords: e-cigarette, nicotine, memory, synapse, neuron, glia**

## Abstract

Tobacco smoking and high-fat diet (HFD) independently impair short-term memory. E-cigarettes produce e-vapour containing flavourings and nicotine. Here, we investigated whether e-vapour inhalation interacts with HFD to affect short-term memory and neural integrity. Balb/c mice (7 weeks, male) were fed a HFD (43% fat, 20kJ/g) for 16 weeks. In the last 6 weeks, half of the mice were exposed to tobacco-flavoured e-vapour from nicotine-containing (18mg/L) or nicotine-free (0mg/L)) e-fluids twice daily. Short-term memory function was measured in week 15. HFD alone did not impair memory function, but increased brain phosphorylated (p)-Tau and astrogliosis marker, while neuron and microglia levels were decreased. E-vapour exposure significantly impaired short-term memory function independent of diet and nicotine. Nicotine free e-vapour induced greater changes compared to the nicotine e-vapour and included, increased systemic cytokines, increased brain p-Tau and decreased postsynaptic density protein (PSD)-95 levels in chow-fed mice, and decreased astrogliosis marker, increased microglia and increased glycogen synthase kinase levels in HFD-fed mice. Increased hippocampal apoptosis was also differentially observed in chow and HFD mice. In conclusion, E-vapour exposure impaired short-term memory independent of diet and nicotine, and was correlated to increased systemic inflammation, reduced PSD-95 level and increased astrogliosis in chow-fed mice, but decreased gliosis and increased microglia in HFD-fed mice, indicating the inflammatory nature of e-vapour leading to short term memory.

## Introduction

Tobacco smoking remains the leading cause of preventable death and disability worldwide. E-cigarettes (electronic nicotine delivery devices) are controversial, being marketed as quit-~~-~~smoking aids despite many users having never smoked conventional cigarettes (Knight-West and Bullen, 2016; Layden et al., 2019). E-cigarettes are popular amongst younger people, partially as a gadget and as a 'safe cigarette' due to fewer toxicants produced compared with ~~the~~ conventional tobacco cigarettes (Li et al., 2018; Ruszkiewicz et al., 2020).

Tobacco smoking is well-known to affect cognitive function, especially short-term memory (Sabia et al., 2012). Synaptic plasticity plays a key role in memory formation during learning, although whether the memory is stored in the ~~synapase~~synapse is unclear (Martin et al., 2000). Nicotine may affect memory retention by altering presynaptic neurotransmitter release and synaptic potentiation (Sabia et al., 2012). Human research on e-vaping is still lacking, given the short appearance of this product on the market and its potential to cause permanent cognitive impacts during neuronal development. Interestingly, recent publications on the influence of e-vaping on neurocognitive function in animal models focus on *in utero* exposure, which is critical for early life neural development (Ruszkiewicz et al., 2020). These studies on direct exposure focus on neurotoxic effects and lung injury (Ruszkiewicz et al., 2020). While *in utero* exposure can impair short-term memory function (Church et al., 2020; Nguyen et al., 2018), it is unknown whether direct long-term inhalation of e-vapour can influence memory function.

Obesity/overweight is a global health issue, affecting 39% of the population worldwide in 2016, with morbidity continuing to increase (WHO, 2018). Obesity is a low grade inflammatory disease, due to the recruitment and accumulation of tissue macrophages in response to lipid influx in metabolic tissues (Lumeng et al., 2008). High-fat diet (HFD) consumption is well-known to cause insulin resistance leading to the development of type 2 diabetes. Such insulin resistance does not just occur in the glucose metabolic organs, but also in the brain, where glucose uptake is normally insulin-independent (Arnold et al., 2014). This phenomenon correlates with spatial memory change and reduced synaptic plasticity (Arnold et al., 2014). However, it is not known if smoking accelerates the cognitive functional decline associated with obesity. Body mass index (BMI) positively correlates with e-cigarette use (Lanza et al., 2017), making it essential to investigate their combined impacts.

As shown by us and others, in mice exposed directly or *in utero* to e-vapour, some of the adverse effects are nicotine independent (Chen et al., 2018a; Chen et al., 2018b; Church et al., 2020; Li et al., 2019). This may be because the heated solvent (mainly lipid-based) produces neurotoxic chemicals (Ruszkiewicz et al., 2020). Of the thousands of e-cigarette flavours aiming to attract younger users, several are toxic after heating (Farsalinos et al., 2013; Kosmider et al., 2016; Lee et al., 2019). Despite the variety of flavours, tobacco-flavoured e-fluids are the most popular option. In Australia, nicotine-containing e-fluids are banned from sale. Therefore, using our well-established mouse models of e-vapour exposure and HFD consumption (Chen et al., 2007; Chen et al., 2018a), we used tobacco flavoured e-fluids with and without nicotine, aiming to investigate how e-vapour exposure alone affects brain markers related to cognition and in the presence of long-term HFD consumption. We examined the independent and combined effects of these two risk factors- representing an inhaled (e-vapour) versus an ingested (HFD) form of lipids on memory behaviour, cell integrity, brain cell levels, synaptic protein markers, brain insulin signaling, inflammation, apoptosis, and oxidative stress responses.

## Materials and Methods

### *Animal experiments*

All animal experiments were approved by the Animal Ethics and Care Committee at Northern Sydney

Health District (RESP17/93) and all experiments were performed according to the Australian National Health & Medical Research Council Guide for the Care and Use of Laboratory Animals. Male Balb/c mice (7 weeks) were housed at standard ~~condition~~conditions (room temperature 20±2°C with a 12h light, 12h dark cycle, *ad libitum* access to standard rodent chow and water). Balb/c mice were used due to their susceptibility to the influence of cigarette smoke exposure.

Half of the mice were fed a HFD (43% fat, 20kJ/g, Specialty Feeds, WA, Australia) for 10 weeks to induce obesity with the other half fed standard chow as control (14% fat, 14kJ/g, Gordon's Specialty Stockfeeds, NSW, Australia). From weeks 11-16, two sub-groups of mice (n=10) in each dietary group were exposed to nicotine-containing e-vapour (18mg/mL (regular strength) nicotine, tobacco flavor, 50% Propylene Glycol (PG)/50%Vegetable Glycerin (VG), Vaper Empire, VIC) or nicotine-free e-vapour (0mg/mL, tobacco flavour, 50% PG/50% VG, Vaper Empire, VIC) for 30 minutes, twice daily in a 19L chamber as we have previously published (Chen et al., 2018a); while the same diets were maintained. E-vapour was generated from a 3rd generation e-cigarette device (KangerTech NEBOX, KangerTech, Shenzen, China). The amount of nicotine produced by the nicotine-containing e-fluid is equivalent to 2 cigarettes (2.4 mg nicotine) per exposure, adopted from our previous model representing light smokers (Chan et al., 2016; Vivekanandarajah et al., 2016). The treatment chart is illustrated in Figure 1.

At the endpoint, the mice were weighed and deeply anaesthetised with isoflurane (2%) before cardiac puncture for blood collection. The left hemisphere of the brain was snap-frozen and kept at -80°C, and the right hemisphere was fixed in formalin. Retroperitoneal fat pads were dissected and weighed to evaluate adiposity. Cotinine level was measured using a commercial ELISA kit (Abnova, Taipei, Taiwan) as per the manufacturer's instructions. Serum proinflammatory cytokines IL-1β and TNFα were measured by a Bio-Plex Pro™ Mouse Chemokine Panel 33-Plex kit (Bio-Rad, CA, USA) according to the manufacturer's instruction.

***Behavioural test***

The novel objective recognition (NOR) test was used to evaluate short term memory retention. At week 15 of the experiment, mice were placed in a dark-coloured box containing two identical square blocks for familiarisation and test phases (5-minute sessions, 1-hour interval), as we have published (Chen et al., 2016). During the test phase, one of the objects was replaced with a triangular shaped object. The results represent the percentage of the total time spent with the new object out of the total time spent with both objects, as previously published (Quinn et al., 2008; Thanos et al., 2015). It is the nature of a mouse to explore a novel object; thus spending a similar amount of time with each object (old and new) is a sign of impaired short term memory.

***Western Blotting***

Western blotting was performed for markers of neural integrity (phosphorylated (p)-Tau), inflammation (IL-1β), oxidative stress (manganese superoxide dismutase (MnSOD), and insulin signaling elements (phosphorylated and total 5' AMP-activated protein kinase (AMPK), Protein kinase B (Akt), and Glycogen synthase kinase 3 (GSK)). We also measured the number of neurons (via Neuronal Nuclei, NeuN), glia (Glial fibrillary acidic protein, GFAP), and microglia (Ionized calcium-binding adaptor protein-1, Iba-1). The left hemisphere of the brain (excluding the cerebellum and brainstem) was homogenised using cell lysis buffers for the whole protein. Protein samples (40µg) 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 nonfat milk powder and incubated with the primary antibodies IL-1β (1:2,000; Cat #NBP1-19775, NOVUS), AMPK (1:1000; Cat #5831, Cell Signaling Technology), p-AMPK (1:1000; Cat #50081, Cell Signaling Technology), Akt (1:1000; Cat #4685 Cell Signaling Technology), p-Akt (1:1000; Cat #13038, Cell Signaling Technology), GSK (1:1000; 12456, Cell Signaling Technology), MnSOD (1:2000; Cat

#2651886MERCK), PSD-95 (1:1000; Cat #3409S, Cell Signaling Technology), Synapsin (1:1000; Cat #5297S, Cell Signaling Technology), NeuN (1:2000; Cat #MAB377, MilliporeSigma), GFAP (1:1000; Cat #Z0334, Dako), Iba-1 (1:2500; Cat # 019-19741 Wako Chemicals) overnight and then secondary antibodies (goat anti-rabbit (cat #STAR124P, BIO-RAD) or rabbit anti-mouse; (Cat #ab 6789, Abcam) IgG horseradish peroxidase-conjugated secondary antibodies, Santa Cruz Biotechnology, Texas, USA) for 1 hour. Protein expression was detected by SuperSignal West Pico Chemiluminescent substrate (Thermo, MA, USA) by exposure of the membrane in Chemidoc MP (Bio-Rad, California, USA). Protein band density measured using ImageJ software (National Institute of Health, Bethesda, Maryland, USA) with the results expressed as a ratio of the individual marker intensity relative to β-actin (1:1000, Cat #AHP2417, Bio-Rad) band intensity.

***Immunostaining***

For neuron staining, frozen brain hemispheres were sectioned for the hippocampus, and the slides were incubated with mouse monoclonal neuronal nuclei (NeuN 1:500, MAB377, Merck Millipore, Australia) antibody for 72h at 4°C followed by biotin-labelled secondary antibody (ab6813, Abcam, UK) overnight at 4°C. All sections were then incubated in avidin-biotin complex (Vector Laboratories) at room temperature 1 hr before NeuN immunolabelling detected with 3,3'-Diaminobenzidine (DAB, Abacus) until desired staining levels achieved. Sections were mounted and coverslipped with 50% glycerol solution (Sigma-Aldrich, MS, USA). Quantification of NeuN-labelled cells was performed using the optical fractionator method *(*Zeiss Axio Scan.Z1, Zeiss, Germany*)* and the use of Stereo Investigator 7 software (MBF Bioscience). Every $6^{th}$ section was quantified, with a total of five sections sampled per animal. For estimations of NeuN positive populations a counting frame of 40µm x 40µm and a grid size of 84µm x 60µm was used in conjunction with a guard zone height of 5µm and dissector height of 10µm. The coefficient of error attributable to the sampling was calculated according to Gundersen and Jensen (Gundersen and Jensen, 1987) with errors ≤0.10 regarded as acceptable.

Formalin fixed paraffin embedded hippocampal tissue sections, 100 µm apart for each case (n=5 per group), were stained for apoptosis (active caspase-3) and cell death (Terminal deoxynucleotidyl transferase end labelling method; TUNEL) following our previously described methods (Chan et al., 2017a; Chan et al., 2017b). In brief, sections were deparaffinised in xylene, hydrated by being taken through decreased gradient ethanol concentrations to distilled water. They were then antigen retrieved by microwaving followed by cooling and proceeded with active Caspase-3 or TUNEL staining. For active caspase-3, sections were incubated with the antibody (1:300, 559565, BD Biosciences, Australia) overnight, followed by the secondary antibody (horse anti-rabbit HRP, 1:200, Vector Laboratories, USA), avidin–biotin complex (ABC) (VEPH4000, Vector Laboratories Inc., California, USA) and then colour developed using the diaminobenzidine (DAB) chromogen (K346811, Dako). For TUNEL, this was via the ApopTag®Peroxidase kit (S7100, Merck Millipore, Victoria). Sections were briefly incubated with 50µl of equilibration buffer and then terminal deoxynucleotidyl transferase (tdt; 1:4 with reaction buffer) was added to each section, coverslipped and incubated for 1 hour at 37°C. Negative controls were incubated with water instead of Tdt. The reaction was stopped by incubating the sections in stopwash buffer. Anti-Digoxigenin-Peroxidase was added, followed by colour development in DAB. All sections were counterstained in haematoxylin, dehydrated through increasing concentrations of ethanol and coverslipped in DPX. Quantification was performed for the dorsal hippocampus with reference to the brain atlas by Paxinos and Franklin (Franklin and Paxinos, 2019) ensuring only sections that spanned -1.34 to -2.3mm relative to bregma were quantified given it was along this stretch that the 3 CA regions (CA1-3) were present. Only the CA1 and CA3 regions were quantified given their role in memory (Dimsdale-Zucker et al., 2018; Farovik et al., 2010). Sections were scanned (Zeiss Axio Scan.Z1, Zeiss, Germany) and quantified using the NDP.view2 program (Hamamatsu Photonics), as a percentage of positive staining (% positive).

***Statistical method***

The results are expressed as mean ± standard error of the mean (SEM) and analysed by two-way ANOVA followed by Fisher's least significant difference (LSD) post hoc tests (GraphPad Prism 8.3, GraphPad, CA, USA). P< 0.05 was considered statistically significant.

## Results

***Body weight and adiposity***

Plasma cotinine levels reflect the level of nicotine exposure. Sham exposed mice and nicotine-free e-vapour exposed mice had similar cotinine levels regardless of diet (Table 1), similar to our previous observations (Chan et al., 2016; Vivekanandarajah et al., 2016). Nicotine-containing e-vapour exposed mice had nearly 4 times the cotinine levels in their blood (P<0.01 vs other groups fed the same diet, Table 1).

At week 0, all groups started at a similar body weight (Table 1). HFD consumption induced significant weight gain in the HFD+sham mice (P<0.05 vs Chow+sham, Table 1), with more than doubled fat mass (P<0.01 vs Chow+sham for both net fat mass and standardised by body weight). In chow-fed mice, e-vapour exposure reduced body weight regardless of nicotine, while in HFD-fed mice, this is only obvious in mice exposed to nicotine-free e-vapour (P<0.05, HFD+e-cig0 vs HFD+sham) with smaller fat pads (P<0.01 HFD+e-cig0 vs HFD+sham for both net fat mass and as a percentage of body weight, Table 1).

***Short term memory***

HFD consumption itself did not affect the ability to recognise a new object; however, e-vapour exposure impaired recognition. In chow-fed mice, both Chow+e-cig18 and Chow+e-cig0 groups spent less time on the new object compared with the Chow+sham group (P<0.05, 0.01 respectively, Figure 2a). In the HFD mice, a similar effect of e-vapour exposure was observed albeit without statistical significance (P=0.06 HFD+e-cig18 vs HFD+Sham, P= 0.07 HFD+e-cig0 vs HFD+Sham, Figure 2a).

***Neural integrity markers and cell type levels in the whole brain***

In non-genetically modified mice, p-Tau can be detected in degenerated neurons (Baker and Götz, 2016). HFD consumption significantly increased p-Tau level (P<0.05 HFD+sham vs Chow+sham, Figure 2b). In chow-fed mice, the protein level of p-Tau was nearly doubled by e-vapour exposure regardless of nicotine but did not reach statistical significance (P=0.054 Chow+e-cig18 vs Chow+sham, P=0.08 Chow+e-cig0 vs Chow+sham, Figure 2b). In HFD mice, additional e-vapour exposure did not have a significant impact on p-Tau levels.

The number of neurons was semi-quantitatively assessed by NeuN which produced 2 protein bands at 46kDa and 48kDa. Based on the literature, the 46kDA predominates in the nucleus and 48kDA predominates in the cytoplasm of neurons (Maxeiner et al., 2014). We found HFD decreased overall NeuN protein levels (P<0.01 HFD effect for both 46 kDa and 48 kDa, Figure 2e), while nicotine-containing e-vapour only reduced 46kDa- NeuN in chow-fed mice (P<0.05 Chow+e-cig18 vs Chow+Sham, Figure 2c). Microglial marker Iba-1 was reduced in HFD-fed mice (P<0.05 HFD+sham vs Choow+sham, Figure 2d), but was increased by exposure to e-vapour (P<0.05 HFD+e-cg0 vs HFD+sham, Figure 2d). Astrocyte marker GFAP was increased by by nicotine-free e-vapour exposure alone (P<0.01 Chow+e-cig0 vs Chow+sham, Figure 2e) and HFD consumption (P=0.052 HFD+sham vs Chow+sham, Figure 2e), but was decreased by additional e-vapour exposure in the HFD groups (P<0.05 HFD+e-cig18 vs HFD+Sham, P< 0.01 HFD+e-cig0 vs HFD+Sham, Figure 2e).

***Hippocampus specific neuronal and apoptosis markers***

At the specific hippocampal level focusing on CA1 and CA3 pyramidal layers, NeuN staining was significantly reduced in the CA1 of Chow+e-cig0 and HFD+e-cig18 groups compared with sham-exposed mice fed the same diet (P<0.05 Chow+e-cig0 vs Chow+sham, P<0.05 HFD+e-cig18 vs HFD+sham, Figure 3a), while no significant difference was found in CA3 region (Figure 3b).

For the apoptosis markers, in the CA1 region, TUNEL was increased due to HFD in the e-vapour groups (P<0.05, overall diet effect, Figure 3c), while in the CA3 region, TUNEL positive cells were increased in the Chow+e-cig18 group (P<0.05 vs Chow+sham, Figure 3d) and HFD+e-cig0 group (P<0.05 vs Chow+e-cig0, Figure 3d). For active Caspase-3, no statistically significant changes were evident ~~but~~except for an overall increase in the CA1 region was evident in the HFD groups compared to their chow counterparts (P< 0.05 overall HFD effect, Figure 3e).

***Inflammation and oxidative stress in the whole brain***

In the serum, HFD consumption doubled the amount of the pro-inflammatory cytokine IL-1β (P=0.~~079~~08 vs Chow+sham, Table 1) and increased TNFα level by 2.5 times (P<0.05 vs Chow+sham, Table 1). In Chow-fed mice, exposure to nicotine e-vapour doubled serum IL-1β and TNFα levels albeit without statistical significance, whereas exposure to nicotine-free e-vapour led to significantly increased IL-1β and TNFα (both P<0.05 vs Chow+sham, Table 1), which was not observed in HFD-fed mice. However, there was no such change in brain IL-1β protein level (data not shown) and TNFα protein was not measurable. The endogenous antioxidant MnSOD level can reflect the oxidative stress level. In the brain, MnSOD level was marginally lower in e-vapour exposed mice consuming chow diet, however neither HFD consumption nor e-vapour exposure significantly changed MnSOD level (Figure 4a).

***Synaptic protein markers in the whole brain***

HFD alone did not significantly change the postsynaptic density protein PSD-95 level, while e-vapour exposure significantly reduced the PSD-95 protein level in chow-fed mice (P<0.01, Chow+e-cig18 and Chow+e-cig0 vs Chow+sham, Figure 3b). However, no significant impact of e-vapour was found in HFD-fed mice (Figure 4b). Neither HFD consumption nor e-vapour exposure affected ~~synapsin~~the Synapsin level (Figure 4c).

***~~Brain insulin~~Insulin signaling pathway markers in the whole brain***

Neither HFD nor e-vapour exposure changed p-AMPK and total AMPK levels (Figure 5a). There was a significant increase in the ratio of p-AMPK/total AMPK in HFD+e-cig0 mice compared to HFD+sham group (P<0.05, Figure 5a). While p-Akt was not significantly changed by either HFD or e-vapour, total Akt was significantly reduced in Chow+e-cig0 and HFD+Sham groups (both P<0.05 vs Chow+Sham, Figure 5b). The ratio of p-Akt/total Akt was significantly increased in Chow+e-cig0 and HFD+Sham groups due to the reduced total level (P<0.05 vs Chow+Sham, Figure 5b). GSK level was significantly increased by e-vapour exposure among the HFD mice regardless of nicotine level (P< 0.01, HFD+e-cig18, HFD+e-cig0 vs HFD+Sham, Figure 5c).

## Discussion

In this study, we examined the effects of two types of lipids, inhaled via e-vapour exposure and ingested via HFD feeding. The major finding is that e-vapour exposure impaired short-term memory function, independent of both diet and nicotine. However, there was no clear pattern of how HFD consumption and e-vapour exposure regulate neurodegenerative and apoptotic markers. Although synaptic plasticity seems to correlate to the functional memory decline in chow-fed mice and altered gliosis to the memory decline in HFD-fed mice, we did not see strong evidence of the influence of nicotine.

In our model, synaptic change may contribute to cognitive impairment. PSD-95 is a structural protein critical to maintaining the excitatory postsynaptic density PSD and synaptic function (Chen et al., 2011). Nicotine has been shown to slow down PSD-95 degradation after short-term treatment and it is considered to protect synapses, especially in the setting of dementia (Inestrosa et al., 2013; Rezvani et al., 2007). In fact, smoking can exacerbate the development of Alzheimer's disease, suggesting chemicals other than nicotine could be the major player (Moreno-Gonzalez et al., 2013). This theory is well presented in our mice exposed to e-vapour, whose short-term memory was impaired in the presence of reduced PSD-95 level. HFD consumption, on the other hand, was linked to reduced PSD-95 without affecting other synaptic markers in mice (Arnold et al., 2014). Here, long-term HFD consumption alone did not change PSD-95 level, in line with normal memory performance. The Synapsin level was unaffected in our model. Nevertheless, e-vapour exposure and HFD does not seem to interact regarding their influence on the synaptic marker and memory in our model.

The memory impairment in HFD-fed mice seems to be driven by altered gliosis and involves the GSK insulin signalling pathway. Recently, astrocytes have been closely related to learning and memory function (Alberini et al., 2018; Kol et al., 2020; Moraga-Amaro et al., 2014), while microglia play an important role in obesity-related cognitive decline before metabolic disorders are developed (Cope et al., 2018). Here, we saw in the whole brain, an increase in GFAP as an astrogliosis marker and a decrease in Iba-1 as a microglia marker in HFD-fed mice, which may be an adaptive response to preserve their short-term memory function. The decrease in whole-brain microglia number is consistent with a previous study (Milanova et al., 2019) yet opposite to the literature where increased Iba-1 was observed in the hypothalamus regions (Baufeld et al., 2016; Huang et al., 2019). This may be due to the involvement of the hypothalamus in energy homeostatic regulation in response to HFD feeding. Nevertheless, additional e-vapour exposure impaired such adaptation to decrease GFAP reflecting astrogliosis and increase microglia in HFD-fed mice, which correlate with their declined memory function.

Human studies have linked type 2 diabetes to the development of dementia, especially in those without familial history (Yarchoan and Arnold, 2014). While blood glucose has been suggested to be the independent factor even without the diagnosis of diabetes, the underlying connection has been suggested to be insulin resistance in the brain, or reduced insulin signaling activity (de Matos et al., 2018; Sandoval and Sisley, 2015; Yarchoan and Arnold, 2014). Insulin resistance is due to increased Akt phosphorylation activated by AMPK, which can no longer suppress GSK (de Matos et al., 2018; Yarchoan and Arnold, 2014). The latter induces Tau hyperphosphorylation and β-amyloid accumulation leading to neurodegeneration (de Matos et al., 2018; Yarchoan and Arnold, 2014). Smoking has also been shown to interrupt brain insulin signaling, possibly through inducing AMPK (Deochand et al., 2016; Martínez de Morentin et al., 2012). However, it is unknown how smoking and type 2 diabetes interact during the development of dementia. In non-genetically modified mice, it is observed that β-amyloid accumulation was not occurring despite Tau still being hyperphosphorylated (Baker and Götz, 2016). In this study, HFD consumption and e-vapour exposure differentially affected each element of the AMPK-Akt-GSK-Tau pathway, with no distinct pattern, suggesting other unknown pathways involved.

~~Nevertheless, Tau phosphorylation was increased by e-vapour exposure alone and HFD consumption alone, as expected, which seems not to be consistent with~~At the specific hippocampal regional level, our data indicate active apoptotic mechanisms ~~given the overall HFD effect to increase Caspase-3 and TUNEL~~ present due to HFD in the CA1 region ~~and~~, while that for the effect of nicotine-containing e-vapour ~~exposure~~was only apparent in chow-fed mice in CA3 regions. ~~The changes in p-Tau in Chow+e-cig18m and Chow+e-cig0 mice correlates with their cognitive change, however not in HFD-fed mice~~This was partly mirrored by the NeuN counts within these regions. Our attention was given to

CA1 and CA3, because CA1 plays a role in consolidating memories of different events (Daumas et al., 2005), whereas CA3 plays a role in the rapid encoding of spatial information related to different objects and pattern separation, namely episodic memory processing (Rolls, 2013). ~~A similar mismatch was also observed between the number of apoptotic cells and NeuN counts in the hippocampus, and cognitive behavior. In the whole brain, overall NeuN was reduced by HFD consumption but not e-vapour exposure, which may be related to regions involed in energy homeostasis instead of neurocognition. Overall,~~Thus, our data infers that HFD affects memory consolidation, while nicotine-containing e-vapour affects the rapid encoding of spatial information related to different objects and in spatial pattern separation but only in chow mice, perhaps due to the potent effect of HFD alone on Caspase-3 in CA3 region. In the whole brain, overall NeuN was reduced by HFD consumption but not e-vapour exposure, which may be related to regions involved in energy homeostasis instead of neurocognition. Future studies will need to adopt different behavioural tests and methods to examine ~~neural integrity~~these separate memory pathways in such a model.

While the industry claims the ingredients within the e-fluids are safe, heat can break down compounds in the e-fluids to produce toxins. In addition to the nicotine, e-fluids contain flavourings and solvents (propylene glycol and vegetable glycerin). Heated e-cigarette fluids produced inflammatory responses in the airway of mice even at a low dose which is also nicotine independent (Chen et al., 2018a), suggesting the solvent and ~~flavouring~~flavourings may be toxic when superheated during vaping, inducing inflammatory responses (Behar et al., 2014; Lerner et al., 2015). Indeed in this study, serum pro-inflammatory cytokines were increased in chow-fed mice exposed to e-vapour, especially in the nicotine-free group. However, when HFD and e-vapour exposure were combined, there was no additive effect, suggesting e-vapour exposure may dominate the systemic inflammatory response. Furthermore, systemic inflammation did not correlate with brain inflammation in this study, evident by the lack of change for IL-1β and microglia. This was unexpected, given several studies reporting the neurotoxic effects due to direct exposure (reviewed (Ruszkiewicz et al., 2020). Yet, those studies found this to be brain region-specific, so it remains to be determined at the cellular level whether that is also true for our model. Moreover, consideration of lung-derived inflammatory mediators which are likely to in-part drive some of the changes we have seen, should be made and are currently being investigated to help tease out the effects.

Each puff of heated e-cigarette liquids regardless of nicotine concentration contains $10^{11}$ free radicals (Sussan et al., 2015), and this oxidative challenge results in oxidative stress both *in vitro and in vivo*, and even *in utero* (Barrington-Trimis and Leventhal, 2018; Chen et al., 2018a; Ganapathy et al., 2017; Muthumalage et al., 2018; Ruszkiewicz et al., 2020). However, in this study, e-vapour exposure seems to induce marginal oxidative stress in the brain, which may be related to the relatively low dose of exposure adopted. Higher doses may yield a stronger oxidative response in the brain.

There are inevitably limitations in this study. Firstly, we did not use an obese prone mouse strain, but one prone to the impact of smoking, which may affect the development of memory deficit in mice with HFD-feeding only. In addition, we only used the novel objective recognition test as the initial screening test due to its high sensitivity to early memory function decline. Other cognitive tests such as Morris Water maze and T maze need to be assessed in future studies on the impact of e-cigarette.

## Conclusion

E-vapour exposure impairs short term memory retention function in mice and appears to be independent of the presence of nicotine. Different mechanisms are involved in ~~differentt~~different dietary conditions leading to the same cognitive dysfunction, essentially being that e-vapour exposure involves systemic inflammation, reduced synaptic activity and increased astrogliosis in chow-fed mice,

but for HFD-fed mice, this is via decreased gliosis and increased microglia.

## Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Author Contributions

HC, CY, SSaad, RM, BGO: conceptualisation; HC, GL: animal experiments and tissue collection; BW, GL, JRS, YLC, RM, SStayte, BV: tissue analysis; HC, BW, RM: preparing figures and table; HC, RM, BGO: original draft; all authors have read, edited, and approved the final submission.

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## Data Availability Statement

All data are provided in this paper.

## References

Alberini, C.M., Cruz, E., Descalzi, G., Bessières, B., Gao, V., 2018. Astrocyte glycogen and lactate: New insights into learning and memory mechanisms. Glia 66, 1244-1262.

Arnold, S.E., Lucki, I., Brookshire, B.R., Carlson, G.C., Browne, C.A., Kazi, H., Bang, S., Choi, B.-R., Chen, Y., McMullen, M.F., Kim, S.F., 2014. High fat diet produces brain insulin resistance, synaptodendritic abnormalities and altered behavior in mice. Neurobiology of disease 67, 79-87.

Baker, S., Götz, J., 2016. A local insult of okadaic acid in wild-type mice induces tau phosphorylation and protein aggregation in anatomically distinct brain regions. Acta Neuropathologica Communications 4, 32.

Barrington-Trimis, J.L., Leventhal, A.M., 2018. Adolescents' Use of "Pod Mod" E-Cigarettes - Urgent Concerns. N Engl J Med 379, 1099-1102.

Baufeld, C., Osterloh, A., Prokop, S., Miller, K.R., Heppner, F.L., 2016. High-fat diet-induced brain region-specific phenotypic spectrum of CNS resident microglia. Acta neuropathologica 132, 361-375.

Behar, R.Z., Davis, B., Wang, Y., Bahl, V., Lin, S., Talbot, P., 2014. Identification of toxicants in cinnamon-flavored electronic cigarette refill fluids. Toxicology in vitro : an international journal published in association with BIBRA 28, 198-208.

Chan, Y.L., Saad, S., Al-Odat, I., Oliver, B., Pollock, C., M. Jones, N., Chen, H., 2017a. Maternal L-Carnitine supplementation improves brain health in offspring from cigarette smoke exposed mothers. Frontiers in Molecular Neuroscience 10, 33.

Chan, Y.L., Saad, S., Machaalani, R., Oliver, B.G., Vissel, B., Pollock, C., Jones, N.M., Chen, H., 2017b. Maternal Cigarette Smoke Exposure Worsens Neurological Outcomes in Adolescent Offspring with Hypoxic-Ischemic Injury. Frontiers in Molecular Neuroscience 10, 306.

Chan, Y.L., Saad, S., Pollock, C., Oliver, B., Al-Odat, I., Zaky, A.A., Jones, N., Chen, H., 2016. Impact of maternal cigarette smoke exposure on brain inflammation and oxidative stress in male mice offspring. Sci Rep 6, 25881.

Chen, H., Chan, Y.L., Nguyen, L.T., Mao, Y., De Rosa, A., Beh, I.T., Chee, C., Oliver, B., Herok, G., Saad, S., Gorrie, C., 2016. Moderate traumatic brain injury is linked to acute behaviour deficits and long term mitochondrial alterations. Clinical and experimental pharmacology & physiology.

Chen, H., Hansen, M.J., Jones, J.E., Vlahos, R., Anderson, G., Morris, M.J., 2007. Detrimental metabolic effects of combining long term cigarette smoke exposure and high-fat diet in mice. Am J Physiol Endocrinol Metab 293, E1564-1571.

Chen, H., Li, G., Chan, Y.L., Chapman, D.G., Sukjamnong, S., Nguyen, T., Annissa, T., McGrath, K.C., Sharma, P., Oliver, B.G., 2018a. Maternal E-cigarette Exposure in Mice Alters DNA Methylation and Lung Cytokine Expression in Offspring. American Journal of Respiratory Cell and Molecular Biology 58, 366-377.

Chen, H., Li, G., Chan, Y.L., Nguyen, T., van Reyk, D., Saad, S., Oliver, B.G., 2018b. Modulation of neural regulators of energy homeostasis, and of inflammation, in the pups of mice exposed to e-cigarettes. Neuroscience Letters 684, 61-66.

Chen, X., Nelson, C.D., Li, X., Winters, C.A., Azzam, R., Sousa, A.A., Leapman, R.D., Gainer, H., Sheng, M., Reese, T.S., 2011. PSD-95 is required to sustain the molecular organization of the postsynaptic density. Journal of Neuroscience 31, 6329-6338.

Church, J.S., Chace-Donahue, F., Blum, J.L., Ratner, J.R., Zelikoff, J.T., Schwartzer, J.J., 2020. Neuroinflammatory and Behavioral Outcomes Measured in Adult Offspring of Mice Exposed Prenatally to E-Cigarette Aerosols. Environmental health perspectives 128, 047006.

Cope, E.C., LaMarca, E.A., Monari, P.K., Olson, L.B., Martinez, S., Zych, A.D., Katchur, N.J., Gould, E., 2018. Microglia Play an Active Role in Obesity-Associated Cognitive Decline. Journal of Neuroscience 38, 8889-8904.

Daumas, S., Halley, H., Francés, B., Lassalle, J.-M., 2005. Encoding, consolidation, and retrieval of contextual memory: differential involvement of dorsal CA3 and CA1 hippocampal subregions. Learn Mem 12, 375-382.

de Matos, A.M., de Macedo, M.P., Rauter, A.P., 2018. Bridging Type 2 Diabetes and Alzheimer's Disease: Assembling the Puzzle Pieces in the Quest for the Molecules With Therapeutic and Preventive Potential. Medicinal Research Reviews 38, 261-324.

Deochand, C., Tong, M., Agarwal, A.R., Cadenas, E., de la Monte, S.M., 2016. Tobacco Smoke Exposure Impairs Brain Insulin/IGF Signaling: Potential Co-Factor Role in Neurodegeneration. Journal of Alzheimer's Disease 50, 373-386.

Dimsdale-Zucker, H.R., Ritchey, M., Ekstrom, A.D., Yonelinas, A.P., Ranganath, C., 2018. CA1 and CA3 differentially support spontaneous retrieval of episodic contexts within human hippocampal subfields. Nature Communications 9, 294.

Farovik, A., Dupont, L.M., Eichenbaum, H., 2010. Distinct roles for dorsal CA3 and CA1 in memory for sequential nonspatial events. Learning & Memory 17, 12-17.

Farsalinos, K.E., Romagna, G., Tsiapras, D., Kyrzopoulos, S., Spyrou, A., Voudris, V., 2013. Impact of Flavour Variability on Electronic Cigarette Use Experience: An Internet Survey. International Journal of Environmental Research and Public Health 10, 7272-7282.

449 Franklin, K., Paxinos, G., 2019. Paxinos and Franklin's the Mouse Brain in Stereotaxic Coordinates,
450 Compact. 5th Edition. Elsevier Academic Press.
451 Ganapathy, V., Manyanga, J., Brame, L., McGuire, D., Sadhasivam, B., Floyd, E., Rubenstein, D.A.,
452 Ramachandran, I., Wagener, T., Queimado, L., 2017. Electronic cigarette aerosols suppress cellular
453 antioxidant defenses and induce significant oxidative DNA damage. PLoS ONE 12, e0177780-
454 e0177780.
455 Gundersen, H.J., Jensen, E.B., 1987. The efficiency of systematic sampling in stereology and its
456 prediction. J Microsc 147, 229-263.
457 Huang, H.-T., Tsai, S.-F., Wu, H.-T., Huang, H.-Y., Hsieh, H.-H., Kuo, Y.-M., Chen, P.-S., Yang,
458 C.-S., Tzeng, S.-F., 2019. Chronic exposure to high fat diet triggers myelin disruption and
459 interleukin-33 upregulation in hypothalamus. BMC Neuroscience 20, 33.
460 Inestrosa, N.C., Godoy, J.A., Vargas, J.Y., Arrazola, M.S., Rios, J.A., Carvajal, F.J., Serrano, F.G.,
461 Farias, G.G., 2013. Nicotine Prevents Synaptic Impairment Induced by Amyloid-β Oligomers
462 Through α7-Nicotinic Acetylcholine Receptor Activation. NeuroMolecular Medicine 15, 549-569.
463 Knight-West, O., Bullen, C., 2016. E-cigarettes for the management of nicotine addiction. Subst
464 Abuse Rehabil 7, 111-118.
465 Kol, A., Adamsky, A., Groysman, M., Kreisel, T., London, M., Goshen, I., 2020. Astrocytes
466 contribute to remote memory formation by modulating hippocampal–cortical communication during
467 learning. Nature Neuroscience.
468 Kosmider, L., Sobczak, A., Prokopowicz, A., Kurek, J., Zaciera, M., Knysak, J., Smith, D.,
469 Goniewicz, M.L., 2016. Cherry-flavoured electronic cigarettes expose users to the inhalation irritant,
470 benzaldehyde. Thorax 71, 376-377.
471 Lanza, H.I., Pittman, P., Batshoun, J., 2017. Obesity and Cigarette Smoking: Extending the Link to
472 E-cigarette/Vaping Use. Am J Health Behav 41, 338-347.
473 Layden, J.E., Ghinai, I., Pray, I., Kimball, A., Layer, M., Tenforde, M., Navon, L., Hoots, B.,
474 Salvatore, P.P., Elderbrook, M., Haupt, T., Kanne, J., Patel, M.T., Saathoff-Huber, L., King, B.A.,
475 Schier, J.G., Mikosz, C.A., Meiman, J., 2019. Pulmonary Illness Related to E-Cigarette Use in
476 Illinois and Wisconsin — Preliminary Report. New England Journal of Medicine, doi:
477 10.1056/NEJMoa1911614. [Epub ahead of print].
478 Lee, W.H., Ong, S.-G., Zhou, Y., Tian, L., Bae, H.R., Baker, N., Whitlatch, A., Mohammadi, L.,
479 Guo, H., Nadeau, K.C., Springer, M.L., Schick, S.F., Bhatnagar, A., Wu, J.C., 2019. Modeling
480 Cardiovascular Risks of E-Cigarettes With Human-Induced Pluripotent Stem Cell–Derived
481 Endothelial Cells. Journal of the American College of Cardiology 73, 2722-2737.
482 Lerner, C.A., Sundar, I.K., Yao, H., Gerloff, J., Ossip, D.J., McIntosh, S., Robinson, R., Rahman, I.,
483 2015. Vapors produced by electronic cigarettes and e-juices with flavorings induce toxicity, oxidative
484 stress, and inflammatory response in lung epithelial cells and in mouse lung. PloS one 10, e0116732.
485 Li, G., Chan, Y.L., Nguyen, L.T., Mak, C., Zaky, A., Anwer, A.G., Shi, Y., Nguyen, T., Pollock,
486 C.A., Oliver, B.G., Saad, S., Chen, H., 2019. Impact of maternal e-cigarette vapor exposure on renal
487 health in the offspring. Annals of the New York Academy of Sciences 1452, 65-77.
488 Li, G., Saad, S., Oliver, B.G., Chen, H., 2018. Heat or Burn? Impacts of Intrauterine Tobacco Smoke
489 and E-Cigarette Vapor Exposure on the Offspring's Health Outcome. Toxics 6.
490 Lumeng, C.N., DelProposto, J.B., Westcott, D.J., Saltiel, A.R., 2008. Phenotypic switching of
491 adipose tissue macrophages with obesity is generated by spatiotemporal differences in macrophage
492 subtypes. Diabetes 57, 3239-3246.
493 Martin, S.J., Grimwood, P.D., Morris, R.G.M., 2000. Synaptic Plasticity and Memory: An Evaluation
494 of the Hypothesis. Annual Review of Neuroscience 23, 649-711.
495 Martínez de Morentin, P.B., Whittle, A.J., Fernø, J., Nogueiras, R., Diéguez, C., Vidal-Puig, A.,
496 López, M., 2012. Nicotine induces negative energy balance through hypothalamic AMP-activated
497 protein kinase. Diabetes 61, 807-817.

Maxeiner, S., Glassmann, A., Kao, H.T., Schilling, K., 2014. The molecular basis of the specificity and cross-reactivity of the NeuN epitope of the neuron-specific splicing regulator, Rbfox3. Histochem Cell Biol 141, 43-55.
Milanova, I.V., Kalsbeek, M.J.T., Wang, X.-L., Korpel, N.L., Stenvers, D.J., Wolff, S.E.C., de Goede, P., Heijboer, A.C., Fliers, E., la Fleur, S.E., Kalsbeek, A., Yi, C.-X., 2019. Diet-Induced Obesity Disturbs Microglial Immunometabolism in a Time-of-Day Manner. Frontiers in Endocrinology 10.
Moraga-Amaro, R., Jerez-Baraona, J.M., Simon, F., Stehberg, J., 2014. Role of astrocytes in memory and psychiatric disorders. Journal of Physiology-Paris 108, 240-251.
Moreno-Gonzalez, I., Estrada, L.D., Sanchez-Mejias, E., Soto, C., 2013. Smoking exacerbates amyloid pathology in a mouse model of Alzheimer's disease. Nature Communications 4, 1495.
Muthumalage, T., Prinz, M., Ansah, K.O., Gerloff, J., Sundar, I.K., Rahman, I., 2018. Inflammatory and Oxidative Responses Induced by Exposure to Commonly Used e-Cigarette Flavoring Chemicals and Flavored e-Liquids without Nicotine. Frontiers in Physiology 8.
Nguyen, T., Li, G.E., Chen, H., Cranfield, C.G., McGrath, K.C., Gorrie, C.A., 2018. Maternal E-Cigarette Exposure Results in Cognitive and Epigenetic Alterations in Offspring in a Mouse Model. Chem Res Toxicol 31, 601-611.
Quinn, H.R., Matsumoto, I., Callaghan, P.D., Long, L.E., Arnold, J.C., Gunasekaran, N., Thompson, M.R., Dawson, B., Mallet, P.E., Kashem, M.A., 2008. Adolescent rats find repeated Δ9-THC less aversive than adult rats but display greater residual cognitive deficits and changes in hippocampal protein expression following exposure. Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology 33, 1113-1126.
Rezvani, K., Teng, Y., Shim, D., De Biasi, M., 2007. Nicotine Regulates Multiple Synaptic Proteins by Inhibiting Proteasomal Activity. Journal of Neuroscience 27, 10508-10519.
Rolls, E.T., 2013. The mechanisms for pattern completion and pattern separation in the hippocampus. Frontiers in systems neuroscience 7, 74-74.
Ruszkiewicz, J.A., Zhang, Z., Gonçalves, F.M., Tizabi, Y., Zelikoff, J.T., Aschner, M., 2020. Neurotoxicity of e-cigarettes. Food and Chemical Toxicology 138, 111245.
Sabia, S., Elbaz, A., Dugravot, A., Head, J., Shipley, M., Hagger-Johnson, G., Kivimaki, M., Singh-Manoux, A., 2012. Impact of smoking on cognitive decline in early old age: the Whitehall II cohort study. Archives of general psychiatry 69, 627-635.
Sandoval, D., Sisley, S.R., 2015. Brain GLP-1 and insulin sensitivity. Molecular and cellular endocrinology 418 Pt 1, 27-32.
Sussan, T.E., Gajghate, S., Thimmulappa, R.K., Ma, J., Kim, J.-H., Sudini, K., Consolini, N., Cormier, S.A., Lomnicki, S., Hasan, F., Pekosz, A., Biswal, S., 2015. Exposure to Electronic Cigarettes Impairs Pulmonary Anti-Bacterial and Anti-Viral Defenses in a Mouse Model. PLOS ONE 10, e0116861.
Thanos, P.K., Roushdy, K., Sarwar, Z., Rice, O., Ashby, C.R., Jr., Grandy, D.K., 2015. The effect of dopamine D4 receptor density on novelty seeking, activity, social interaction, and alcohol binge drinking in adult mice. Synapse (New York, N.Y.) 69, 356-364.
Vivekanandarajah, A., Chan, Y.L., Chen, H., Machaalani, R., 2016. Prenatal cigarette smoke exposure effects on apoptotic and nicotinic acetylcholine receptor expression in the infant mouse brainstem. NeuroToxicology 53, 53-63.
WHO, 2018. Fact sheet: obesity and overweight. Available from: https://www.who.int/news-room/fact-sheets/detail/obesity-and-overweight 16 Feb 2018.
Yarchoan, M., Arnold, S.E., 2014. Repurposing Diabetes Drugs for Brain Insulin Resistance in Alzheimer Disease. Diabetes 63, 2253-2261.

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**Table 1. Bodyweight, fat mass, and plasma cotinine level in the mice at 16 weeks**

| | Chow+sham | Chow+e-cig18 | Chow+e-cig0 | HFD+sham | HFD+e-cig18 | HFD+e-cig0 |
|---|---|---|---|---|---|---|
| Body weight at week 0 (g) | 20.1± 0.3 | 20.1±0.3 | 20.3±0.3 | 20.0±0.3 | 20.2±0.6 | 20.3±0.4 |
| Body weight at week 16 (g) | 27.4±0.2 | 25.3±0.5** | 25.6±0.4* | 29.1±0.6* | 28.7±0.8 †† | 27.4±0.6‡ # |
| Fat mass (g) | 0.18±0.02 | 0.10±0.03 | 0.13±0.02 | 0.40±0.07** | 0.31±0.06†† | 0.20±0.01## |
| Fat % | 0.67±0.87 | 0.41±0.10 | 0.53±0.08 | 1.36±0.20** | 1.05±0.17†† | 0.75±0.05## |
| Serum cotinine (ng/ml) ϕ | 4.21±0.26 | 16.26 ± 1.82** | 4.44 ± 0.56†† | 4.04 ± 0.47 | 15.74 ± 1.84## | 3.39 ± 0.65δδ |
| Serum IL-1β (pg/ml) ϕ | 1358 ± 150 | 2566 ± 743 | 3565 ± 248* | 2869 ± 539 | 2388 ± 596 | 3395 ± 824 |
| Serum TNFα (pg/ml) ϕ | 401 ± 53 | 827 ± 295 | 1145 ± 193* | 1009 ± 204* | 729 ± 226 | 892 ±183 |

The results are expressed as mean ± SEM, n = 10 (ϕ n = 5-7), * P<0.05, ** P<0.01 vs Chow+Sham, # P<0.05, ##P<0.01 vs HFD+Sham, †† P<0.01 vs Chow+e-cig18mg, ‡ P<0.05 vs Chow+e-cig0, δδ P < 0.01 vs HFD+e-cig18mg.



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**Figure legends**

Figure 1. Treatment timeline and grouping. The population was divided into normal chow or High Fat Diet. The three conditions were ambient air (control/sham), E-vapour + nicotine (vehicle + nicotine), and the E-vapour exclusively (vehicle itself).

Figure 2. Novel Object Recognition test result (a, n=10), phosphorylated (p)-Tau protein levels (b, n=6), protein levels of NeuN (c), Iba-1 (d) and GFAP (e) in the whole brain. The results are expressed as mean ± SEM, n = 5-6. * P<0.05, ** P<0.01 vs Chow-Sham, # P<0.05 vs HFD-Sham. Representative western blotting bands are from the same gel and the groups are divided by solid lines in line with the bar figure.

Figure 3. NeuN positive cell counts in the CA1 (a) and CA3 regions (b), percentage of neurons expressing apoptosis markers, TUNEL and Caspase-3 positive counts in the CA1 (c,e) and (d,f) of the hippocampus. Representative images of groups with significant change (g). The results are expressed as mean ± SEM, n = 5.

Figure 4. Protein levels of the endogenous antioxidant manganese superoxide dismutase (MnSOD, a) and synaptic plasticity markers, postsynaptic density protein (PSD)-95 (b) and Synapsin (c). The results are expressed as mean ± SEM, n =6. **P<0.01 vs Chow-Sham. Representative western blotting bands are from the same gel and the groups are divided by solid lines in line with the bar figure.

Figure 5. Protein levels of insulin signaling elements, (a) phosphorylated (p)-5' AMP-activated protein kinase (AMPK), total AMPK, and the ratio between p-AMPK and total AMPK, (b) p-protein kinase B (Akt), total Akt, and the ratio between p-Akt/Akt, and (c) Glycogen Synthase Kinase-3 (GSK). The results are expressed as mean ± SEM, n = 6. * P< 0.05 vs Chow-Sham, #P< 0.05, ## P<0.01 vs HFD-Sham. Representative western blotting bands are from the same gel and the groups are divided by solid lines in line with the bar figure.

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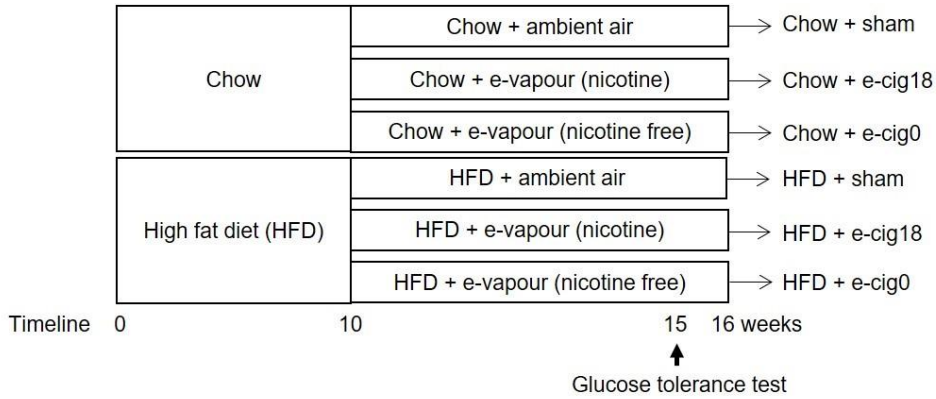


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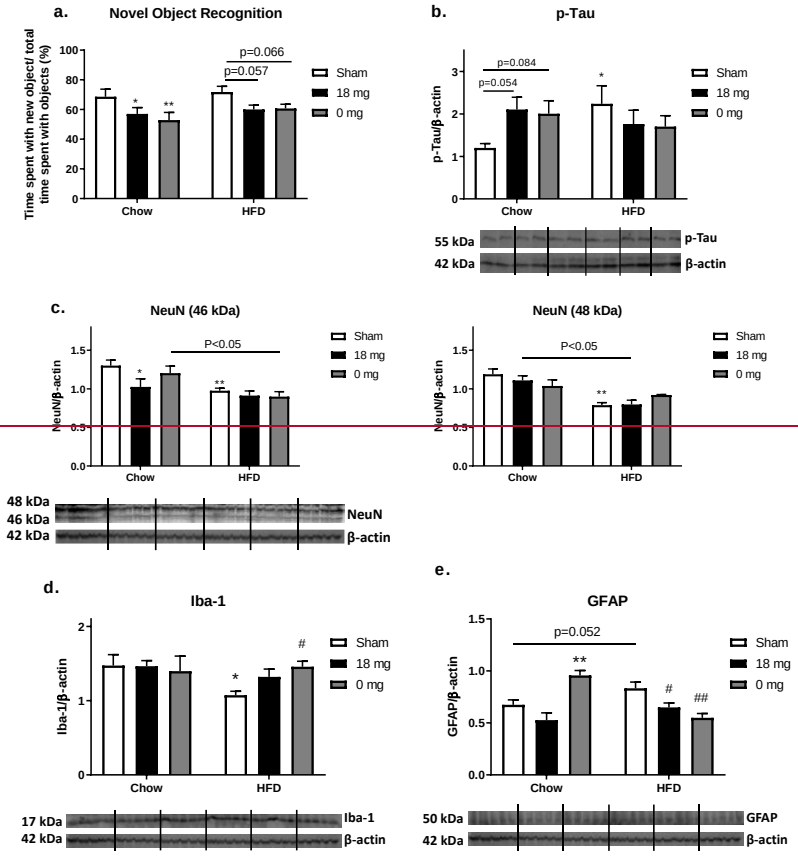
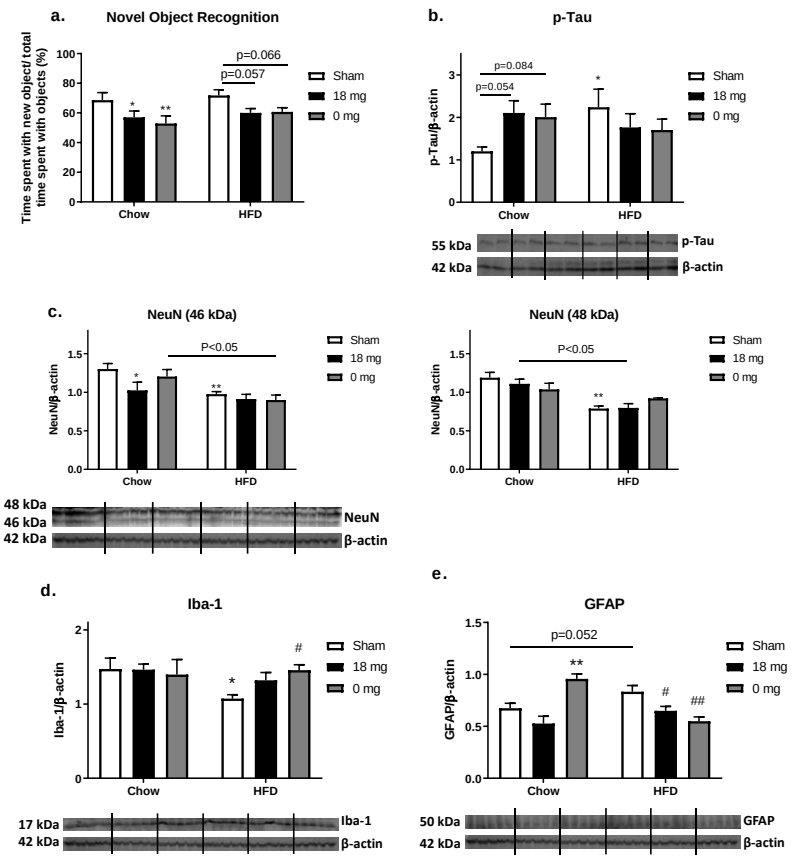
573 Figure 1


a.
Novel Object Recognition
Time spent with new object/ total time spent with objects (%)
p=0.066
p=0.057
Sham
18 mg
0 mg
Chow
HFD
b.
p-Tau
p=0.084
p=0.054
p-Tau/β-actin
55 kDa
42 kDa
p-Tau
β-actin
c.
NeuN (46 kDa)
NeuN (48 kDa)
P<0.05
NeuN/β-actin
48 kDa
46 kDa
42 kDa
NeuN
β-actin
d.
Iba-1
Iba-1/β-actin
17 kDa
Iba-1
e.
GFAP
p=0.052
GFAP/β-actin
50 kDa
GFAP

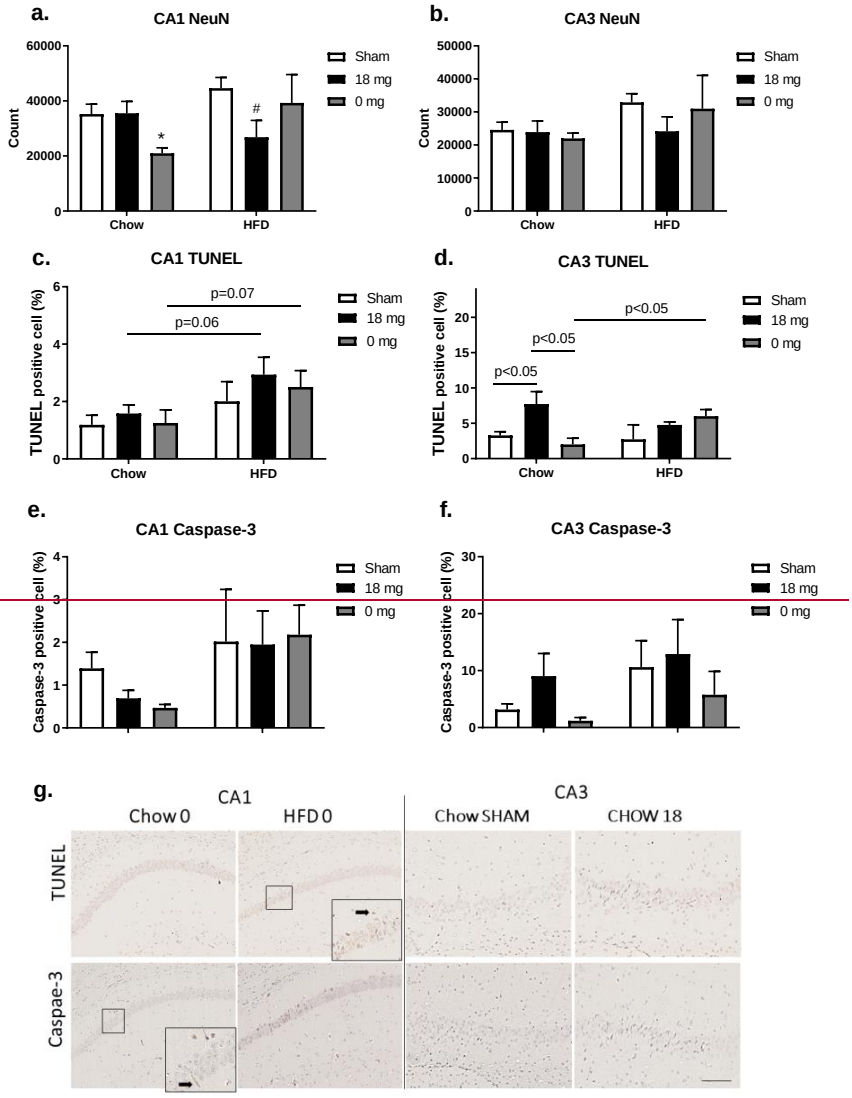
574

Field Code Changed



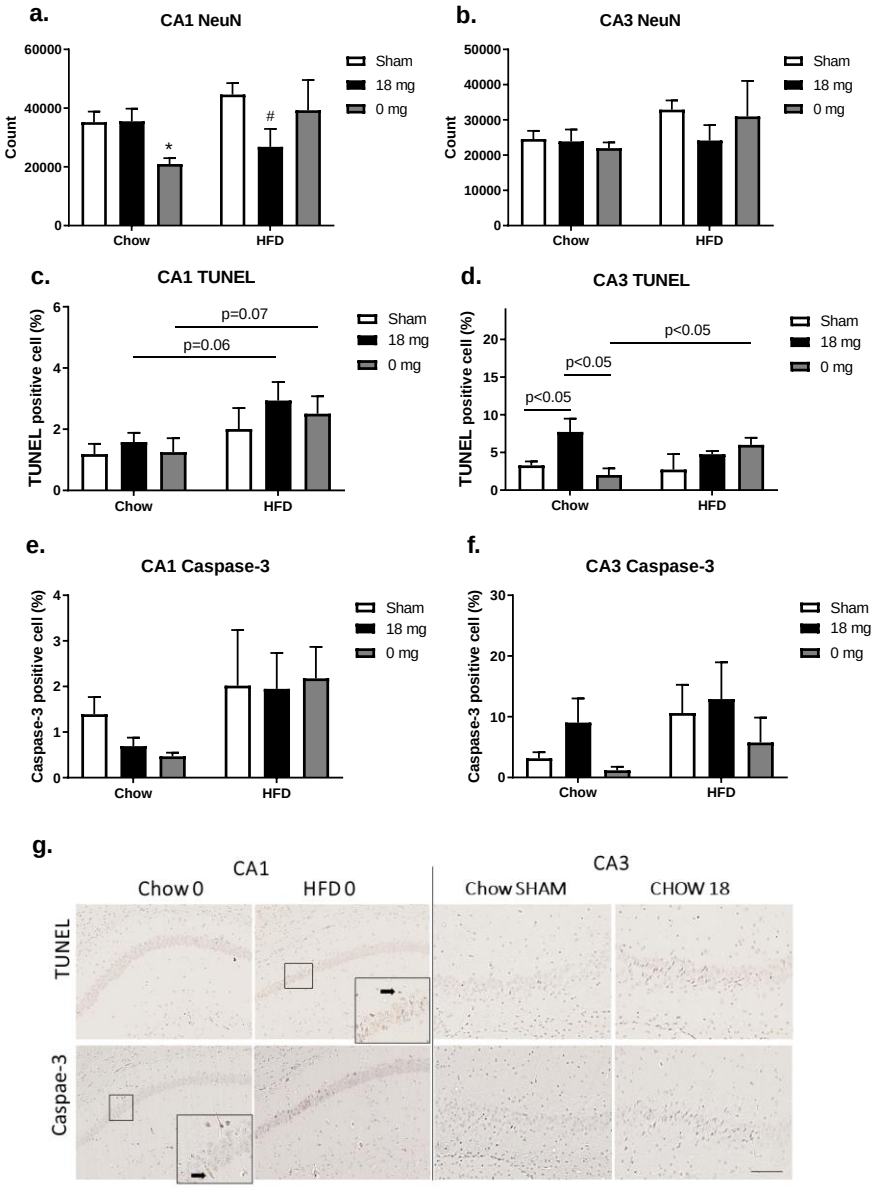

575

576 Figure 2


a.
CA1 NeuN
Count
Sham
18 mg
0 mg
Chow
HFD
b.
CA3 NeuN
c.
CA1 TUNEL
TUNEL positive cell (%)
p=0.07
p=0.06
d.
CA3 TUNEL
p<0.05
e.
CA1 Caspase-3
Caspase-3 positive cell (%)
f.
CA3 Caspase-3
g.
CA1
CA3
Chow 0
HFD 0
Chow SHAM
CHOW 18
TUNEL
Caspae-3

577

Field Code Changed




578

579 Figure 3

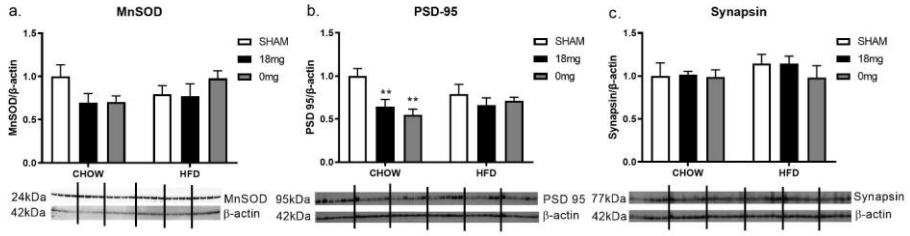



Figure 4

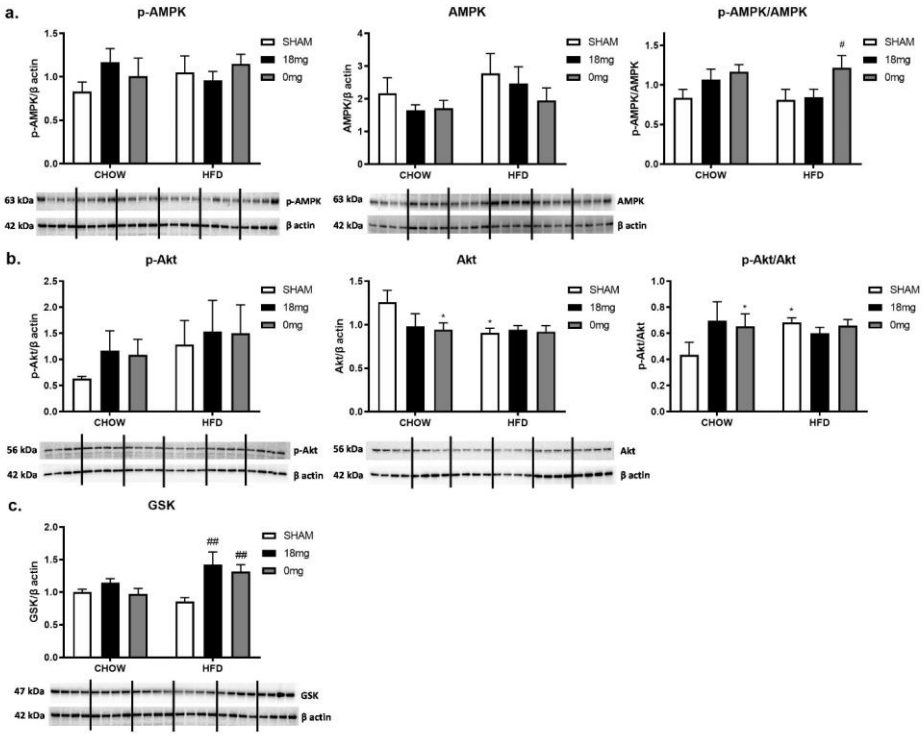


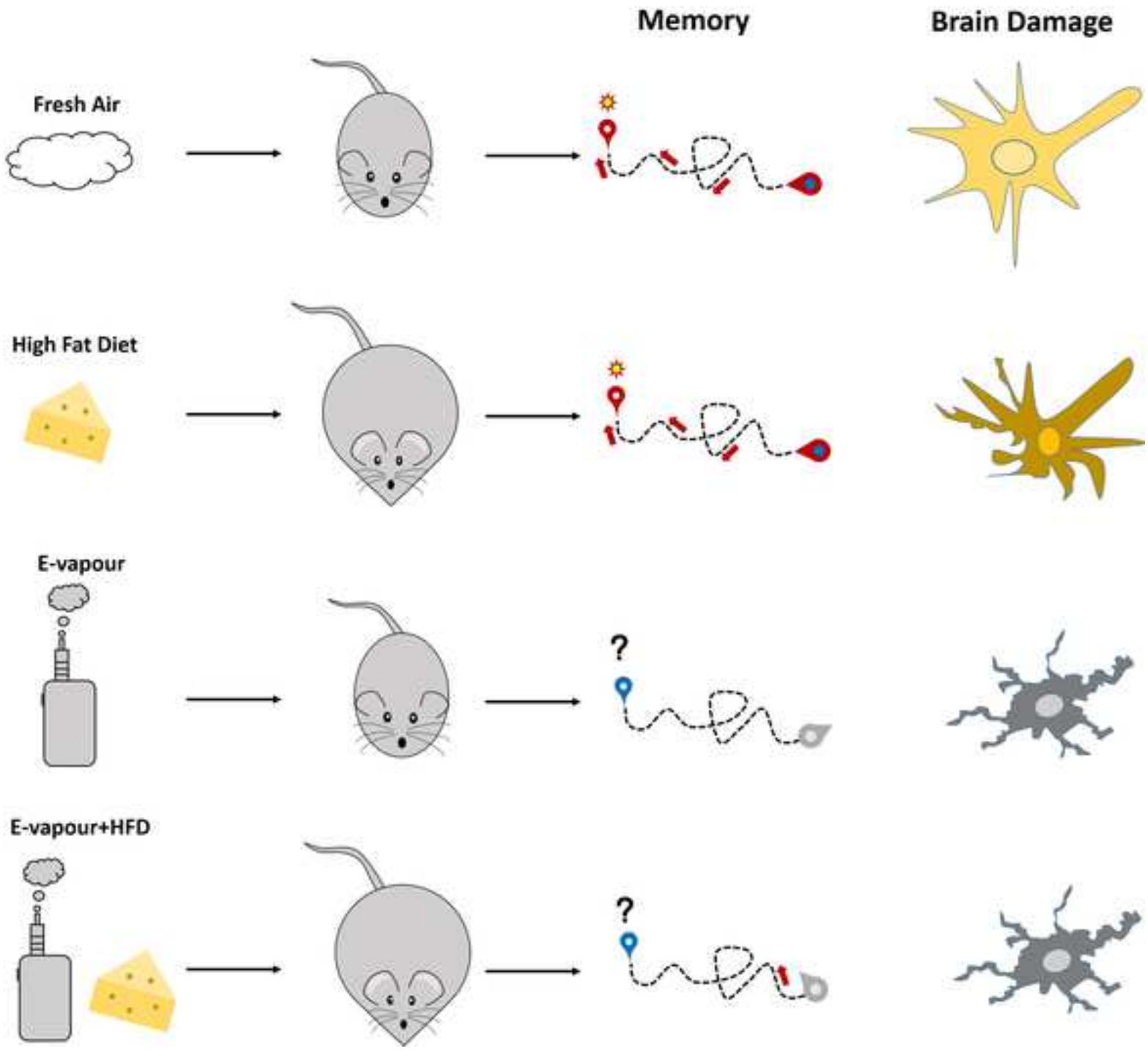

Figure 5

## Highlights

- E-vapour exposure impairs short term memory, independent of nicotine
- E-vapour exposure increased brain p-Tau levels, independent of nicotine
- E-vapour exposure decreased brain postsynaptic density protein PSD-95 levels in low fat diet fed mice
- E-vapour exposure changed gliosis in high fat diet fed mice
- High fat diet consumption does not interact with E-vapour exposure on cognition

Graphical Abstract

Click here to access/download;Graphical Abstract;E-cig HFD Graphic abstract.jpg



# Brain health is independently impaired by E-vaping and high-fat diet

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**Keywords: e-cigarette, nicotine, memory, synapse, neuron, glia**

## Abstract

Tobacco smoking and high-fat diet (HFD) independently impair short-term memory. E-cigarettes produce e-vapour containing flavourings and nicotine. Here, we investigated whether e-vapour inhalation interacts with HFD to affect short-term memory and neural integrity. Balb/c mice (7 weeks, male) were fed a HFD (43% fat, 20kJ/g) for 16 weeks. In the last 6 weeks, half of the mice were exposed to tobacco-flavoured e-vapour from nicotine-containing (18mg/L) or nicotine-free (0mg/L)) e-fluids twice daily. Short-term memory function was measured in week 15. HFD alone did not impair memory function, but increased brain phosphorylated (p)-Tau and astrogliosis marker, while neuron and microglia levels were decreased. E-vapour exposure significantly impaired short-term memory function independent of diet and nicotine. Nicotine free e-vapour induced greater changes compared to the nicotine e-vapour and included, increased systemic cytokines, increased brain p-Tau and decreased postsynaptic density protein (PSD)-95 levels in chow-fed mice, and decreased astrogliosis marker, increased microglia and increased glycogen synthase kinase levels in HFD-fed mice. Increased hippocampal apoptosis was also differentially observed in chow and HFD mice. In conclusion, E-vapour exposure impaired short-term memory independent of diet and nicotine, and was correlated to increased systemic inflammation, reduced PSD-95 level and increased astrogliosis in chow-fed mice, but decreased gliosis and increased microglia in HFD-fed mice, indicating the inflammatory nature of e-vapour leading to short term memory.

## Introduction

Tobacco smoking remains the leading cause of preventable death and disability worldwide. E-cigarettes (electronic nicotine delivery devices) are controversial, being marketed as quit-smoking aids despite many users having never smoked conventional cigarettes (Knight-West and Bullen, 2016; Layden et al., 2019). E-cigarettes are popular amongst younger people, partially as a gadget and as a 'safe cigarette' due to fewer toxicants produced compared with conventional tobacco cigarettes (Li et al., 2018; Ruszkiewicz et al., 2020).

Tobacco smoking is well-known to affect cognitive function, especially short-term memory (Sabia et al., 2012). Synaptic plasticity plays a key role in memory formation during learning, although whether the memory is stored in the synapse is unclear (Martin et al., 2000). Nicotine may affect memory retention by altering presynaptic neurotransmitter release and synaptic potentiation (Sabia et al., 2012). Human research on e-vaping is still lacking, given the short appearance of this product on the market and its potential to cause permanent cognitive impacts during neuronal development. Interestingly, recent publications on the influence of e-vaping on neurocognitive function in animal models focus on *in utero* exposure, which is critical for early life neural development (Ruszkiewicz et al., 2020). These studies on direct exposure focus on neurotoxic effects and lung injury (Ruszkiewicz et al., 2020). While *in utero* exposure can impair short-term memory function (Church et al., 2020; Nguyen et al., 2018), it is unknown whether direct long-term inhalation of e-vapour can influence memory function.

Obesity/overweight is a global health issue, affecting 39% of the population worldwide in 2016, with morbidity continuing to increase (WHO, 2018). Obesity is a low grade inflammatory disease, due to the recruitment and accumulation of tissue macrophages in response to lipid influx in metabolic tissues (Lumeng et al., 2008). High-fat diet (HFD) consumption is well-known to cause insulin resistance leading to the development of type 2 diabetes. Such insulin resistance does not just occur in the glucose metabolic organs, but also in the brain, where glucose uptake is normally insulin-independent (Arnold et al., 2014). This phenomenon correlates with spatial memory change and reduced synaptic plasticity (Arnold et al., 2014). However, it is not known if smoking accelerates the cognitive functional decline associated with obesity. Body mass index (BMI) positively correlates with e-cigarette use (Lanza et al., 2017), making it essential to investigate their combined impacts.

As shown by us and others, in mice exposed directly or *in utero* to e-vapour, some of the adverse effects are nicotine independent (Chen et al., 2018a; Chen et al., 2018b; Church et al., 2020; Li et al., 2019). This may be because the heated solvent (mainly lipid-based) produces neurotoxic chemicals (Ruszkiewicz et al., 2020). Of the thousands of e-cigarette flavours aiming to attract younger users, several are toxic after heating (Farsalinos et al., 2013; Kosmider et al., 2016; Lee et al., 2019). Despite the variety of flavours, tobacco-flavoured e-fluids are the most popular option. In Australia, nicotine-containing e-fluids are banned from sale. Therefore, using our well-established mouse models of e-vapour exposure and HFD consumption (Chen et al., 2007; Chen et al., 2018a), we used tobacco flavoured e-fluids with and without nicotine, aiming to investigate how e-vapour exposure alone affects brain markers related to cognition and in the presence of long-term HFD consumption. We examined the independent and combined effects of these two risk factors- representing an inhaled (e-vapour) versus an ingested (HFD) form of lipids on memory behaviour, cell integrity, brain cell levels, synaptic protein markers, brain insulin signaling, inflammation, apoptosis, and oxidative stress responses.

## Materials and Methods

### *Animal experiments*

All animal experiments were approved by the Animal Ethics and Care Committee at Northern Sydney Health District (RESP17/93) and all experiments were performed according to the Australian National

Health & Medical Research Council Guide for the Care and Use of Laboratory Animals. Male Balb/c mice (7 weeks) were housed at standard conditions (room temperature 20±2°C with a 12h light, 12h dark cycle, *ad libitum* access to standard rodent chow and water). Balb/c mice were used due to their susceptibility to the influence of cigarette smoke exposure.

Half of the mice were fed a HFD (43% fat, 20kJ/g, Specialty Feeds, WA, Australia) for 10 weeks to induce obesity with the other half fed standard chow as control (14% fat, 14kJ/g, Gordon's Specialty Stockfeeds, NSW, Australia). From weeks 11-16, two sub-groups of mice (n=10) in each dietary group were exposed to nicotine-containing e-vapour (18mg/mL (regular strength) nicotine, tobacco flavor, 50% Propylene Glycol (PG)/50%Vegetable Glycerin (VG), Vaper Empire, VIC) or nicotine-free e-vapour (0mg/mL, tobacco flavour, 50% PG/50% VG, Vaper Empire, VIC) for 30 minutes, twice daily in a 19L chamber as we have previously published (Chen et al., 2018a); while the same diets were maintained. E-vapour was generated from a 3rd generation e-cigarette device (KangerTech NEBOX, KangerTech, Shenzen, China). The amount of nicotine produced by the nicotine-containing e-fluid is equivalent to 2 cigarettes (2.4 mg nicotine) per exposure, adopted from our previous model representing light smokers (Chan et al., 2016; Vivekanandarajah et al., 2016). The treatment chart is illustrated in Figure 1.

At the endpoint, the mice were weighed and deeply anaesthetised with isoflurane (2%) before cardiac puncture for blood collection. The left hemisphere of the brain was snap-frozen and kept at -80°C, and the right hemisphere was fixed in formalin. Retroperitoneal fat pads were dissected and weighed to evaluate adiposity. Cotinine level was measured using a commercial ELISA kit (Abnova, Taipei, Taiwan) as per the manufacturer's instructions. Serum proinflammatory cytokines IL-1β and TNFα were measured by a Bio-Plex Pro™ Mouse Chemokine Panel 33-Plex kit (Bio-Rad, CA, USA) according to the manufacturer's instruction.

***Behavioural test***

The novel objective recognition (NOR) test was used to evaluate short term memory retention. At week 15 of the experiment, mice were placed in a dark-coloured box containing two identical square blocks for familiarisation and test phases (5-minute sessions, 1-hour interval), as we have published (Chen et al., 2016). During the test phase, one of the objects was replaced with a triangular shaped object. The results represent the percentage of the total time spent with the new object out of the total time spent with both objects, as previously published (Quinn et al., 2008; Thanos et al., 2015). It is the nature of a mouse to explore a novel object; thus spending a similar amount of time with each object (old and new) is a sign of impaired short term memory.

***Western Blotting***

Western blotting was performed for markers of neural integrity (phosphorylated (p)-Tau), inflammation (IL-1β), oxidative stress (manganese superoxide dismutase (MnSOD), and insulin signaling elements (phosphorylated and total 5' AMP-activated protein kinase (AMPK), Protein kinase B (Akt), and Glycogen synthase kinase 3 (GSK)). We also measured the number of neurons (via Neuronal Nuclei, NeuN), glia (Glial fibrillary acidic protein, GFAP), and microglia (Ionized calcium-binding adaptor protein-1, Iba-1). The left hemisphere of the brain (excluding the cerebellum and brainstem) was homogenised using cell lysis buffers for the whole protein. Protein samples (40µg) 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 nonfat milk powder and incubated with the primary antibodies IL-1β (1:2,000; Cat #NBP1-19775, NOVUS), AMPK (1:1000; Cat #5831, Cell Signaling Technology), p-AMPK (1:1000; Cat #50081, Cell Signaling Technology), Akt (1:1000; Cat #4685 Cell Signaling Technology), p-Akt (1:1000; Cat #13038, Cell Signaling Technology), GSK (1:1000; 12456, Cell Signaling Technology), MnSOD (1:2000; Cat #2651886MERCK), PSD-95 (1:1000; Cat #3409S, Cell Signaling Technology), Synapsin (1:1000; Cat

#5297S, Cell Signaling Technology), NeuN (1:2000; Cat #MAB377, MilliporeSigma), GFAP (1:1000; Cat #Z0334, Dako), Iba-1 (1:2500; Cat # 019-19741 Wako Chemicals) overnight and then secondary antibodies (goat anti-rabbit (cat #STAR124P, BIO-RAD) or rabbit anti-mouse; (Cat #ab 6789, Abcam) IgG horseradish peroxidase-conjugated secondary antibodies, Santa Cruz Biotechnology, Texas, USA) for 1 hour. Protein expression was detected by SuperSignal West Pico Chemiluminescent substrate (Thermo, MA, USA) by exposure of the membrane in Chemidoc MP (Bio-Rad, California, USA). Protein band density measured using ImageJ software (National Institute of Health, Bethesda, Maryland, USA) with the results expressed as a ratio of the individual marker intensity relative to β-actin (1:1000, Cat #AHP2417, Bio-Rad) band intensity.

***Immunostaining***

For neuron staining, frozen brain hemispheres were sectioned for the hippocampus, and the slides were incubated with mouse monoclonal neuronal nuclei (NeuN 1:500, MAB377, Merck Millipore, Australia) antibody for 72h at 4°C followed by biotin-labelled secondary antibody (ab6813, Abcam, UK) overnight at 4°C. All sections were then incubated in avidin-biotin complex (Vector Laboratories) at room temperature 1 hr before NeuN immunolabelling detected with 3,3'-Diaminobenzidine (DAB, Abacus) until desired staining levels achieved. Sections were mounted and coverslipped with 50% glycerol solution (Sigma-Aldrich, MS, USA). Quantification of NeuN-labelled cells was performed using the optical fractionator method *(*Zeiss Axio Scan.Z1, Zeiss, Germany*)* and the use of Stereo Investigator 7 software (MBF Bioscience). Every $6^{th}$ section was quantified, with a total of five sections sampled per animal. For estimations of NeuN positive populations a counting frame of 40μm x 40μm and a grid size of 84μm x 60μm was used in conjunction with a guard zone height of 5μm and dissector height of 10μm. The coefficient of error attributable to the sampling was calculated according to Gundersen and Jensen (Gundersen and Jensen, 1987) with errors ≤0.10 regarded as acceptable.

Formalin fixed paraffin embedded hippocampal tissue sections, 100 μm apart for each case (n=5 per group), were stained for apoptosis (active caspase-3) and cell death (Terminal deoxynucleotidyl transferase end labelling method; TUNEL) following our previously described methods (Chan et al., 2017a; Chan et al., 2017b). In brief, sections were deparaffinised in xylene, hydrated by being taken through decreased gradient ethanol concentrations to distilled water. They were then antigen retrieved by microwaving followed by cooling and proceeded with active Caspase-3 or TUNEL staining. For active caspase-3, sections were incubated with the antibody (1:300, 559565, BD Biosciences, Australia) overnight, followed by the secondary antibody (horse anti-rabbit HRP, 1:200, Vector Laboratories, USA), avidin–biotin complex (ABC) (VEPH4000, Vector Laboratories Inc., California, USA) and then colour developed using the diaminobenzidine (DAB) chromogen (K346811, Dako). For TUNEL, this was via the ApopTag®Peroxidase kit (S7100, Merck Millipore, Victoria). Sections were briefly incubated with 50μl of equilibration buffer and then terminal deoxynucleotidyl transferase (tdt; 1:4 with reaction buffer) was added to each section, coverslipped and incubated for 1 hour at 37$^{o}$C. Negative controls were incubated with water instead of Tdt. The reaction was stopped by incubating the sections in stopwash buffer. Anti-Digoxigenin-Peroxidase was added, followed by colour development in DAB. All sections were counterstained in haematoxylin, dehydrated through increasing concentrations of ethanol and coverslipped in DPX. Quantification was performed for the dorsal hippocampus with reference to the brain atlas by Paxinos and Franklin (Franklin and Paxinos, 2019) ensuring only sections that spanned -1.34 to -2.3mm relative to bregma were quantified given it was along this stretch that the 3 CA regions (CA1-3) were present. Only the CA1 and CA3 regions were quantified given their role in memory (Dimsdale-Zucker et al., 2018; Farovik et al., 2010). Sections were scanned (Zeiss Axio Scan.Z1, Zeiss, Germany) and quantified using the NDP.view2 program (Hamamatsu Photonics), as a percentage of positive staining (% positive).

***Statistical method***

The results are expressed as mean ± standard error of the mean (SEM) and analysed by two-way ANOVA followed by Fisher's least significant difference (LSD) post hoc tests (GraphPad Prism 8.3, GraphPad, CA, USA). $P < 0.05$ was considered statistically significant.

## Results

### *Body weight and adiposity*

Plasma cotinine levels reflect the level of nicotine exposure. Sham exposed mice and nicotine-free e-vapour exposed mice had similar cotinine levels regardless of diet (Table 1), similar to our previous observations (Chan et al., 2016; Vivekanandarajah et al., 2016). Nicotine-containing e-vapour exposed mice had nearly 4 times the cotinine levels in their blood ($P<0.01$ vs other groups fed the same diet, Table 1).

At week 0, all groups started at a similar body weight (Table 1). HFD consumption induced significant weight gain in the HFD+sham mice ($P<0.05$ vs Chow+sham, Table 1), with more than doubled fat mass ($P<0.01$ vs Chow+sham for both net fat mass and standardised by body weight). In chow-fed mice, e-vapour exposure reduced body weight regardless of nicotine, while in HFD-fed mice, this is only obvious in mice exposed to nicotine-free e-vapour ($P<0.05$, HFD+e-cig0 vs HFD+sham) with smaller fat pads ($P<0.01$ HFD+e-cig0 vs HFD+sham for both net fat mass and as a percentage of body weight, Table 1).

### *Short term memory*

HFD consumption itself did not affect the ability to recognise a new object; however, e-vapour exposure impaired recognition. In chow-fed mice, both Chow+e-cig18 and Chow+e-cig0 groups spent less time on the new object compared with the Chow+sham group ($P<0.05$, 0.01 respectively, Figure 2a). In the HFD mice, a similar effect of e-vapour exposure was observed albeit without statistical significance ($P=0.06$ HFD+e-cig18 vs HFD+Sham, $P= 0.07$ HFD+e-cig0 vs HFD+Sham, Figure 2a).

### *Neural integrity markers and cell type levels in the whole brain*

In non-genetically modified mice, p-Tau can be detected in degenerated neurons (Baker and Götz, 2016). HFD consumption significantly increased p-Tau level ($P<0.05$ HFD+sham vs Chow+sham, Figure 2b). In chow-fed mice, the protein level of p-Tau was nearly doubled by e-vapour exposure regardless of nicotine but did not reach statistical significance ($P=0.054$ Chow+e-cig18 vs Chow+sham, $P=0.08$ Chow+e-cig0 vs Chow+sham, Figure 2b). In HFD mice, additional e-vapour exposure did not have a significant impact on p-Tau levels.

The number of neurons was semi-quantitatively assessed by NeuN which produced 2 protein bands at 46kDa and 48kDa. Based on the literature, the 46kDA predominates in the nucleus and 48kDA predominates in the cytoplasm of neurons (Maxeiner et al., 2014). We found HFD decreased overall NeuN protein levels ($P<0.01$ HFD effect for both 46 kDa and 48 kDa, Figure 2e), while nicotine-containing e-vapour only reduced 46kDa- NeuN in chow-fed mice ($P<0.05$ Chow+e-cig18 vs Chow+Sham, Figure 2c). Microglial marker Iba-1 was reduced in HFD-fed mice ($P<0.05$ HFD+sham vs Choow+sham, Figure 2d), but was increased by exposure to e-vapour ($P<0.05$ HFD+e-cg0 vs HFD+sham, Figure 2d). Astrocyte marker GFAP was increased by by nicotine-free e-vapour exposure alone ($P<0.01$ Chow+e-cig0 vs Chow+sham, Figure 2e) and HFD consumption ($P=0.052$ HFD+sham vs Chow+sham, Figure 2e), but was decreased by additional e-vapour exposure in the HFD groups ($P<0.05$ HFD+e-cig18 vs HFD+Sham, $P< 0.01$ HFD+e-cig0 vs HFD+Sham, Figure 2e).

### *Hippocampus specific neuronal and apoptosis markers*

At the specific hippocampal level focusing on CA1 and CA3 pyramidal layers, NeuN staining was

significantly reduced in the CA1 of Chow+e-cig0 and HFD+e-cig18 groups compared with sham-exposed mice fed the same diet (P<0.05 Chow+e-cig0 vs Chow+sham, P<0.05 HFD+e-cig18 vs HFD+sham, Figure 3a), while no significant difference was found in CA3 region (Figure 3b).

For the apoptosis markers, in the CA1 region, TUNEL was increased due to HFD in the e-vapour groups (P<0.05, overall diet effect, Figure 3c), while in the CA3 region, TUNEL positive cells were increased in the Chow+e-cig18 group (P<0.05 vs Chow+sham, Figure 3d) and HFD+e-cig0 group (P<0.05 vs Chow+e-cig0, Figure 3d). For active Caspase-3, no statistically significant changes were evident except for an overall increase in the CA1 region was evident in the HFD groups compared to their chow counterparts (P< 0.05 overall HFD effect, Figure 3e).

***Inflammation and oxidative stress in the whole brain***

In the serum, HFD consumption doubled the amount of the pro-inflammatory cytokine IL-1β (P=0.08 vs Chow+sham, Table 1) and increased TNFα level by 2.5 times (P<0.05 vs Chow+sham, Table 1). In Chow-fed mice, exposure to nicotine e-vapour doubled serum IL-1β and TNFα levels albeit without statistical significance, whereas exposure to nicotine-free e-vapour led to significantly increased IL-1β and TNFα (both P<0.05 vs Chow+sham, Table 1), which was not observed in HFD-fed mice. However, there was no such change in brain IL-1β protein level (data not shown) and TNFα protein was not measurable. The endogenous antioxidant MnSOD level can reflect the oxidative stress level. In the brain, MnSOD level was marginally lower in e-vapour exposed mice consuming chow diet, however neither HFD consumption nor e-vapour exposure significantly changed MnSOD level (Figure 4a).

***Synaptic protein markers in the whole brain***

HFD alone did not significantly change the postsynaptic density protein PSD-95 level, while e-vapour exposure significantly reduced the PSD-95 protein level in chow-fed mice (P<0.01, Chow+e-cig18 and Chow+e-cig0 vs Chow+sham, Figure 3b). However, no significant impact of e-vapour was found in HFD-fed mice (Figure 4b). Neither HFD consumption nor e-vapour exposure affected the Synapsin level (Figure 4c).

***Insulin signaling pathway markers in the whole brain***

Neither HFD nor e-vapour exposure changed p-AMPK and total AMPK levels (Figure 5a). There was a significant increase in the ratio of p-AMPK/total AMPK in HFD+e-cig0 mice compared to HFD+sham group (P<0.05, Figure 5a). While p-Akt was not significantly changed by either HFD or e-vapour, total Akt was significantly reduced in Chow+e-cig0 and HFD+Sham groups (both P<0.05 vs Chow+Sham, Figure 5b). The ratio of p-Akt/total Akt was significantly increased in Chow+e-cig0 and HFD+Sham groups due to the reduced total level (P<0.05 vs Chow+Sham, Figure 5b). GSK level was significantly increased by e-vapour exposure among the HFD mice regardless of nicotine level (P< 0.01, HFD+e-cig18, HFD+e-cig0 vs HFD+Sham, Figure 5c).

## Discussion

In this study, we examined the effects of two types of lipids, inhaled via e-vapour exposure and ingested via HFD feeding. The major finding is that e-vapour exposure impaired short-term memory function, independent of both diet and nicotine. However, there was no clear pattern of how HFD consumption and e-vapour exposure regulate neurodegenerative and apoptotic markers. Although synaptic plasticity seems to correlate to the functional memory decline in chow-fed mice and altered gliosis to the memory decline in HFD-fed mice, we did not see strong evidence of the influence of nicotine.

In our model, synaptic change may contribute to cognitive impairment. PSD-95 is a structural protein critical to maintaining the excitatory postsynaptic density PSD and synaptic function (Chen et al.,

2011). Nicotine has been shown to slow down PSD-95 degradation after short-term treatment and it is considered to protect synapses, especially in the setting of dementia (Inestrosa et al., 2013; Rezvani et al., 2007). In fact, smoking can exacerbate the development of Alzheimer's disease, suggesting chemicals other than nicotine could be the major player (Moreno-Gonzalez et al., 2013). This theory is well presented in our mice exposed to e-vapour, whose short-term memory was impaired in the presence of reduced PSD-95 level. HFD consumption, on the other hand, was linked to reduced PSD-95 without affecting other synaptic markers in mice (Arnold et al., 2014). Here, long-term HFD consumption alone did not change PSD-95 level, in line with normal memory performance. The Synapsin level was unaffected in our model. Nevertheless, e-vapour exposure and HFD does not seem to interact regarding their influence on the synaptic marker and memory in our model.

The memory impairment in HFD-fed mice seems to be driven by altered gliosis and involves the GSK insulin signalling pathway. Recently, astrocytes have been closely related to learning and memory function (Alberini et al., 2018; Kol et al., 2020; Moraga-Amaro et al., 2014), while microglia play an important role in obesity-related cognitive decline before metabolic disorders are developed (Cope et al., 2018). Here, we saw in the whole brain, an increase in GFAP as an astrogliosis marker and a decrease in Iba-1 as a microglia marker in HFD-fed mice, which may be an adaptive response to preserve their short-term memory function. The decrease in whole-brain microglia number is consistent with a previous study (Milanova et al., 2019) yet opposite to the literature where increased Iba-1 was observed in the hypothalamus regions (Baufeld et al., 2016; Huang et al., 2019). This may be due to the involvement of the hypothalamus in energy homeostatic regulation in response to HFD feeding. Nevertheless, additional e-vapour exposure impaired such adaptation to decrease GFAP reflecting astrogliosis and increase microglia in HFD-fed mice, which correlate with their declined memory function.

Human studies have linked type 2 diabetes to the development of dementia, especially in those without familial history (Yarchoan and Arnold, 2014). While blood glucose has been suggested to be the independent factor even without the diagnosis of diabetes, the underlying connection has been suggested to be insulin resistance in the brain, or reduced insulin signaling activity (de Matos et al., 2018; Sandoval and Sisley, 2015; Yarchoan and Arnold, 2014). Insulin resistance is due to increased Akt phosphorylation activated by AMPK, which can no longer suppress GSK (de Matos et al., 2018; Yarchoan and Arnold, 2014). The latter induces Tau hyperphosphorylation and β-amyloid accumulation leading to neurodegeneration (de Matos et al., 2018; Yarchoan and Arnold, 2014). Smoking has also been shown to interrupt brain insulin signaling, possibly through inducing AMPK (Deochand et al., 2016; Martínez de Morentin et al., 2012). However, it is unknown how smoking and type 2 diabetes interact during the development of dementia. In non-genetically modified mice, it is observed that β-amyloid accumulation was not occurring despite Tau still being hyperphosphorylated (Baker and Götz, 2016). In this study, HFD consumption and e-vapour exposure differentially affected each element of the AMPK-Akt-GSK-Tau pathway, with no distinct pattern, suggesting other unknown pathways involved.

At the specific hippocampal regional level, our data indicate active apoptotic mechanisms present due to HFD in the CA1 region, while that for the effect of nicotine-containing e-vapour was only apparent in chow-fed mice in CA3 regions. This was partly mirrored by the NeuN counts within these regions. Our attention was given to CA1 and CA3, because CA1 plays a role in consolidating memories of different events (Daumas et al., 2005), whereas CA3 plays a role in the rapid encoding of spatial information related to different objects and pattern separation, namely episodic memory processing (Rolls, 2013). Thus, our data infers that HFD affects memory consolidation, while nicotine-containing e-vapour affects the rapid encoding of spatial information related to different objects and in spatial pattern separation but only in chow mice, perhaps due to the potent effect of HFD alone on Caspase-3

in CA3 region. In the whole brain, overall NeuN was reduced by HFD consumption but not e-vapour exposure, which may be related to regions involved in energy homeostasis instead of neurocognition. Future studies will need to adopt different behavioural tests and methods to examine these separate memory pathways in such a model.

While the industry claims the ingredients within the e-fluids are safe, heat can break down compounds in the e-fluids to produce toxins. In addition to the nicotine, e-fluids contain flavourings and solvents (propylene glycol and vegetable glycerin). Heated e-cigarette fluids produced inflammatory responses in the airway of mice even at a low dose which is also nicotine independent (Chen et al., 2018a), suggesting the solvent and flavourings may be toxic when superheated during vaping, inducing inflammatory responses (Behar et al., 2014; Lerner et al., 2015). Indeed in this study, serum pro-inflammatory cytokines were increased in chow-fed mice exposed to e-vapour, especially in the nicotine-free group. However, when HFD and e-vapour exposure were combined, there was no additive effect, suggesting e-vapour exposure may dominate the systemic inflammatory response. Furthermore, systemic inflammation did not correlate with brain inflammation in this study, evident by the lack of change for IL-1β and microglia. This was unexpected, given several studies reporting the neurotoxic effects due to direct exposure (reviewed (Ruszkiewicz et al., 2020). Yet, those studies found this to be brain region-specific, so it remains to be determined at the cellular level whether that is also true for our model. Moreover, consideration of lung-derived inflammatory mediators which are likely to in-part drive some of the changes we have seen, should be made and are currently being investigated to help tease out the effects.

Each puff of heated e-cigarette liquids regardless of nicotine concentration contains $10^{11}$ free radicals (Sussan et al., 2015), and this oxidative challenge results in oxidative stress both *in vitro and in vivo*, and even *in utero* (Barrington-Trimis and Leventhal, 2018; Chen et al., 2018a; Ganapathy et al., 2017; Muthumalage et al., 2018; Ruszkiewicz et al., 2020). However, in this study, e-vapour exposure seems to induce marginal oxidative stress in the brain, which may be related to the relatively low dose of exposure adopted. Higher doses may yield a stronger oxidative response in the brain.

There are inevitably limitations in this study. Firstly, we did not use an obese prone mouse strain, but one prone to the impact of smoking, which may affect the development of memory deficit in mice with HFD-feeding only. In addition, we only used the novel objective recognition test as the initial screening test due to its high sensitivity to early memory function decline. Other cognitive tests such as Morris Water maze and T maze need to be assessed in future studies on the impact of e-cigarette.

## Conclusion

E-vapour exposure impairs short term memory retention function in mice and appears to be independent of the presence of nicotine. Different mechanisms are involved in different dietary conditions leading to the same cognitive dysfunction, essentially being that e-vapour exposure involves systemic inflammation, reduced synaptic activity and increased astrogliosis in chow-fed mice, but for HFD-fed mice, this is via decreased gliosis and increased microglia.

## Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Author Contributions

HC, CY, SSaad, RM, BGO: conceptualisation; HC, GL: animal experiments and tissue collection; BW,

GL, JRS, YLC, RM, SStayte, BV: tissue analysis; HC, BW, RM: preparing figures and table; HC, RM, BGO: original draft; all authors have read, edited, and approved the final submission.

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**Data Availability Statement**

All data are provided in this paper.

**References**

Alberini, C.M., Cruz, E., Descalzi, G., Bessières, B., Gao, V., 2018. Astrocyte glycogen and lactate: New insights into learning and memory mechanisms. Glia 66, 1244-1262.

Arnold, S.E., Lucki, I., Brookshire, B.R., Carlson, G.C., Browne, C.A., Kazi, H., Bang, S., Choi, B.-R., Chen, Y., McMullen, M.F., Kim, S.F., 2014. High fat diet produces brain insulin resistance, synaptodendritic abnormalities and altered behavior in mice. Neurobiology of disease 67, 79-87.

Baker, S., Götz, J., 2016. A local insult of okadaic acid in wild-type mice induces tau phosphorylation and protein aggregation in anatomically distinct brain regions. Acta Neuropathologica Communications 4, 32.

Barrington-Trimis, J.L., Leventhal, A.M., 2018. Adolescents' Use of "Pod Mod" E-Cigarettes - Urgent Concerns. N Engl J Med 379, 1099-1102.

Baufeld, C., Osterloh, A., Prokop, S., Miller, K.R., Heppner, F.L., 2016. High-fat diet-induced brain region-specific phenotypic spectrum of CNS resident microglia. Acta neuropathologica 132, 361-375.

Behar, R.Z., Davis, B., Wang, Y., Bahl, V., Lin, S., Talbot, P., 2014. Identification of toxicants in cinnamon-flavored electronic cigarette refill fluids. Toxicology in vitro : an international journal published in association with BIBRA 28, 198-208.

Chan, Y.L., Saad, S., Al-Odat, I., Oliver, B., Pollock, C., M. Jones, N., Chen, H., 2017a. Maternal L-Carnitine supplementation improves brain health in offspring from cigarette smoke exposed mothers. Frontiers in Molecular Neuroscience 10, 33.

Chan, Y.L., Saad, S., Machaalani, R., Oliver, B.G., Vissel, B., Pollock, C., Jones, N.M., Chen, H., 2017b. Maternal Cigarette Smoke Exposure Worsens Neurological Outcomes in Adolescent Offspring with Hypoxic-Ischemic Injury. Frontiers in Molecular Neuroscience 10, 306.

Chan, Y.L., Saad, S., Pollock, C., Oliver, B., Al-Odat, I., Zaky, A.A., Jones, N., Chen, H., 2016. Impact of maternal cigarette smoke exposure on brain inflammation and oxidative stress in male mice offspring. Sci Rep 6, 25881.

Chen, H., Chan, Y.L., Nguyen, L.T., Mao, Y., De Rosa, A., Beh, I.T., Chee, C., Oliver, B., Herok, G., Saad, S., Gorrie, C., 2016. Moderate traumatic brain injury is linked to acute behaviour deficits and long term mitochondrial alterations. Clinical and experimental pharmacology & physiology.

Chen, H., Hansen, M.J., Jones, J.E., Vlahos, R., Anderson, G., Morris, M.J., 2007. Detrimental metabolic effects of combining long term cigarette smoke exposure and high-fat diet in mice. Am J Physiol Endocrinol Metab 293, E1564-1571.

Chen, H., Li, G., Chan, Y.L., Chapman, D.G., Sukjamnong, S., Nguyen, T., Annissa, T., McGrath, K.C., Sharma, P., Oliver, B.G., 2018a. Maternal E-cigarette Exposure in Mice Alters DNA Methylation and Lung Cytokine Expression in Offspring. American Journal of Respiratory Cell and Molecular Biology 58, 366-377.

Chen, H., Li, G., Chan, Y.L., Nguyen, T., van Reyk, D., Saad, S., Oliver, B.G., 2018b. Modulation of neural regulators of energy homeostasis, and of inflammation, in the pups of mice exposed to e-cigarettes. Neuroscience Letters 684, 61-66.

Chen, X., Nelson, C.D., Li, X., Winters, C.A., Azzam, R., Sousa, A.A., Leapman, R.D., Gainer, H., Sheng, M., Reese, T.S., 2011. PSD-95 is required to sustain the molecular organization of the postsynaptic density. Journal of Neuroscience 31, 6329-6338.

Church, J.S., Chace-Donahue, F., Blum, J.L., Ratner, J.R., Zelikoff, J.T., Schwartzer, J.J., 2020. Neuroinflammatory and Behavioral Outcomes Measured in Adult Offspring of Mice Exposed Prenatally to E-Cigarette Aerosols. Environmental health perspectives 128, 047006.

Cope, E.C., LaMarca, E.A., Monari, P.K., Olson, L.B., Martinez, S., Zych, A.D., Katchur, N.J., Gould, E., 2018. Microglia Play an Active Role in Obesity-Associated Cognitive Decline. Journal of Neuroscience 38, 8889-8904.

Daumas, S., Halley, H., Francés, B., Lassalle, J.-M., 2005. Encoding, consolidation, and retrieval of contextual memory: differential involvement of dorsal CA3 and CA1 hippocampal subregions. Learn Mem 12, 375-382.

de Matos, A.M., de Macedo, M.P., Rauter, A.P., 2018. Bridging Type 2 Diabetes and Alzheimer's Disease: Assembling the Puzzle Pieces in the Quest for the Molecules With Therapeutic and Preventive Potential. Medicinal Research Reviews 38, 261-324.

Deochand, C., Tong, M., Agarwal, A.R., Cadenas, E., de la Monte, S.M., 2016. Tobacco Smoke Exposure Impairs Brain Insulin/IGF Signaling: Potential Co-Factor Role in Neurodegeneration. Journal of Alzheimer's Disease 50, 373-386.

Dimsdale-Zucker, H.R., Ritchey, M., Ekstrom, A.D., Yonelinas, A.P., Ranganath, C., 2018. CA1 and CA3 differentially support spontaneous retrieval of episodic contexts within human hippocampal subfields. Nature Communications 9, 294.

Farovik, A., Dupont, L.M., Eichenbaum, H., 2010. Distinct roles for dorsal CA3 and CA1 in memory for sequential nonspatial events. Learning & Memory 17, 12-17.

Farsalinos, K.E., Romagna, G., Tsiapras, D., Kyrzopoulos, S., Spyrou, A., Voudris, V., 2013. Impact of Flavour Variability on Electronic Cigarette Use Experience: An Internet Survey. International Journal of Environmental Research and Public Health 10, 7272-7282.

Franklin, K., Paxinos, G., 2019. Paxinos and Franklin's the Mouse Brain in Stereotaxic Coordinates, Compact. 5th Edition. Elsevier Academic Press.

Ganapathy, V., Manyanga, J., Brame, L., McGuire, D., Sadhasivam, B., Floyd, E., Rubenstein, D.A., Ramachandran, I., Wagener, T., Queimado, L., 2017. Electronic cigarette aerosols suppress cellular antioxidant defenses and induce significant oxidative DNA damage. PLoS ONE 12, e0177780-e0177780.

Gundersen, H.J., Jensen, E.B., 1987. The efficiency of systematic sampling in stereology and its prediction. J Microsc 147, 229-263.

Huang, H.-T., Tsai, S.-F., Wu, H.-T., Huang, H.-Y., Hsieh, H.-H., Kuo, Y.-M., Chen, P.-S., Yang, C.-S., Tzeng, S.-F., 2019. Chronic exposure to high fat diet triggers myelin disruption and interleukin-33 upregulation in hypothalamus. BMC Neuroscience 20, 33.
Inestrosa, N.C., Godoy, J.A., Vargas, J.Y., Arrazola, M.S., Rios, J.A., Carvajal, F.J., Serrano, F.G., Farias, G.G., 2013. Nicotine Prevents Synaptic Impairment Induced by Amyloid-β Oligomers Through α7-Nicotinic Acetylcholine Receptor Activation. NeuroMolecular Medicine 15, 549-569.
Knight-West, O., Bullen, C., 2016. E-cigarettes for the management of nicotine addiction. Subst Abuse Rehabil 7, 111-118.
Kol, A., Adamsky, A., Groysman, M., Kreisel, T., London, M., Goshen, I., 2020. Astrocytes contribute to remote memory formation by modulating hippocampal–cortical communication during learning. Nature Neuroscience.
Kosmider, L., Sobczak, A., Prokopowicz, A., Kurek, J., Zaciera, M., Knysak, J., Smith, D., Goniewicz, M.L., 2016. Cherry-flavoured electronic cigarettes expose users to the inhalation irritant, benzaldehyde. Thorax 71, 376-377.
Lanza, H.I., Pittman, P., Batshoun, J., 2017. Obesity and Cigarette Smoking: Extending the Link to E-cigarette/Vaping Use. Am J Health Behav 41, 338-347.
Layden, J.E., Ghinai, I., Pray, I., Kimball, A., Layer, M., Tenforde, M., Navon, L., Hoots, B., Salvatore, P.P., Elderbrook, M., Haupt, T., Kanne, J., Patel, M.T., Saathoff-Huber, L., King, B.A., Schier, J.G., Mikosz, C.A., Meiman, J., 2019. Pulmonary Illness Related to E-Cigarette Use in Illinois and Wisconsin — Preliminary Report. New England Journal of Medicine, doi: 10.1056/NEJMoa1911614. [Epub ahead of print].
Lee, W.H., Ong, S.-G., Zhou, Y., Tian, L., Bae, H.R., Baker, N., Whitlatch, A., Mohammadi, L., Guo, H., Nadeau, K.C., Springer, M.L., Schick, S.F., Bhatnagar, A., Wu, J.C., 2019. Modeling Cardiovascular Risks of E-Cigarettes With Human-Induced Pluripotent Stem Cell–Derived Endothelial Cells. Journal of the American College of Cardiology 73, 2722-2737.
Lerner, C.A., Sundar, I.K., Yao, H., Gerloff, J., Ossip, D.J., McIntosh, S., Robinson, R., Rahman, I., 2015. Vapors produced by electronic cigarettes and e-juices with flavorings induce toxicity, oxidative stress, and inflammatory response in lung epithelial cells and in mouse lung. PloS one 10, e0116732.
Li, G., Chan, Y.L., Nguyen, L.T., Mak, C., Zaky, A., Anwer, A.G., Shi, Y., Nguyen, T., Pollock, C.A., Oliver, B.G., Saad, S., Chen, H., 2019. Impact of maternal e-cigarette vapor exposure on renal health in the offspring. Annals of the New York Academy of Sciences 1452, 65-77.
Li, G., Saad, S., Oliver, B.G., Chen, H., 2018. Heat or Burn? Impacts of Intrauterine Tobacco Smoke and E-Cigarette Vapor Exposure on the Offspring's Health Outcome. Toxics 6.
Lumeng, C.N., DelProposto, J.B., Westcott, D.J., Saltiel, A.R., 2008. Phenotypic switching of adipose tissue macrophages with obesity is generated by spatiotemporal differences in macrophage subtypes. Diabetes 57, 3239-3246.
Martin, S.J., Grimwood, P.D., Morris, R.G.M., 2000. Synaptic Plasticity and Memory: An Evaluation of the Hypothesis. Annual Review of Neuroscience 23, 649-711.
Martínez de Morentin, P.B., Whittle, A.J., Fernø, J., Nogueiras, R., Diéguez, C., Vidal-Puig, A., López, M., 2012. Nicotine induces negative energy balance through hypothalamic AMP-activated protein kinase. Diabetes 61, 807-817.
Maxeiner, S., Glassmann, A., Kao, H.T., Schilling, K., 2014. The molecular basis of the specificity and cross-reactivity of the NeuN epitope of the neuron-specific splicing regulator, Rbfox3. Histochem Cell Biol 141, 43-55.
Milanova, I.V., Kalsbeek, M.J.T., Wang, X.-L., Korpel, N.L., Stenvers, D.J., Wolff, S.E.C., de Goede, P., Heijboer, A.C., Fliers, E., la Fleur, S.E., Kalsbeek, A., Yi, C.-X., 2019. Diet-Induced Obesity Disturbs Microglial Immunometabolism in a Time-of-Day Manner. Frontiers in Endocrinology 10.

Moraga-Amaro, R., Jerez-Baraona, J.M., Simon, F., Stehberg, J., 2014. Role of astrocytes in memory and psychiatric disorders. Journal of Physiology-Paris 108, 240-251.
Moreno-Gonzalez, I., Estrada, L.D., Sanchez-Mejias, E., Soto, C., 2013. Smoking exacerbates amyloid pathology in a mouse model of Alzheimer's disease. Nature Communications 4, 1495.
Muthumalage, T., Prinz, M., Ansah, K.O., Gerloff, J., Sundar, I.K., Rahman, I., 2018. Inflammatory and Oxidative Responses Induced by Exposure to Commonly Used e-Cigarette Flavoring Chemicals and Flavored e-Liquids without Nicotine. Frontiers in Physiology 8.
Nguyen, T., Li, G.E., Chen, H., Cranfield, C.G., McGrath, K.C., Gorrie, C.A., 2018. Maternal E-Cigarette Exposure Results in Cognitive and Epigenetic Alterations in Offspring in a Mouse Model. Chem Res Toxicol 31, 601-611.
Quinn, H.R., Matsumoto, I., Callaghan, P.D., Long, L.E., Arnold, J.C., Gunasekaran, N., Thompson, M.R., Dawson, B., Mallet, P.E., Kashem, M.A., 2008. Adolescent rats find repeated Δ9-THC less aversive than adult rats but display greater residual cognitive deficits and changes in hippocampal protein expression following exposure. Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology 33, 1113-1126.
Rezvani, K., Teng, Y., Shim, D., De Biasi, M., 2007. Nicotine Regulates Multiple Synaptic Proteins by Inhibiting Proteasomal Activity. Journal of Neuroscience 27, 10508-10519.
Rolls, E.T., 2013. The mechanisms for pattern completion and pattern separation in the hippocampus. Frontiers in systems neuroscience 7, 74-74.
Ruszkiewicz, J.A., Zhang, Z., Gonçalves, F.M., Tizabi, Y., Zelikoff, J.T., Aschner, M., 2020. Neurotoxicity of e-cigarettes. Food and Chemical Toxicology 138, 111245.
Sabia, S., Elbaz, A., Dugravot, A., Head, J., Shipley, M., Hagger-Johnson, G., Kivimaki, M., Singh-Manoux, A., 2012. Impact of smoking on cognitive decline in early old age: the Whitehall II cohort study. Archives of general psychiatry 69, 627-635.
Sandoval, D., Sisley, S.R., 2015. Brain GLP-1 and insulin sensitivity. Molecular and cellular endocrinology 418 Pt 1, 27-32.
Sussan, T.E., Gajghate, S., Thimmulappa, R.K., Ma, J., Kim, J.-H., Sudini, K., Consolini, N., Cormier, S.A., Lomnicki, S., Hasan, F., Pekosz, A., Biswal, S., 2015. Exposure to Electronic Cigarettes Impairs Pulmonary Anti-Bacterial and Anti-Viral Defenses in a Mouse Model. PLOS ONE 10, e0116861.
Thanos, P.K., Roushdy, K., Sarwar, Z., Rice, O., Ashby, C.R., Jr., Grandy, D.K., 2015. The effect of dopamine D4 receptor density on novelty seeking, activity, social interaction, and alcohol binge drinking in adult mice. Synapse (New York, N.Y.) 69, 356-364.
Vivekanandarajah, A., Chan, Y.L., Chen, H., Machaalani, R., 2016. Prenatal cigarette smoke exposure effects on apoptotic and nicotinic acetylcholine receptor expression in the infant mouse brainstem. NeuroToxicology 53, 53-63.
WHO, 2018. Fact sheet: obesity and overweight. Available from: https://www.who.int/news-room/fact-sheets/detail/obesity-and-overweight 16 Feb 2018.
Yarchoan, M., Arnold, S.E., 2014. Repurposing Diabetes Drugs for Brain Insulin Resistance in Alzheimer Disease. Diabetes 63, 2253-2261.

**Table 1. Bodyweight, fat mass, and plasma cotinine level in the mice at 16 weeks**

| | Chow+sham | Chow+e-cig18 | Chow+e-cig0 | HFD+sham | HFD+e-cig18 | HFD+e-cig0 |
|---|---|---|---|---|---|---|
| Body weight at week 0 (g) | 20.1± 0.3 | 20.1±0.3 | 20.3±0.3 | 20.0±0.3 | 20.2±0.6 | 20.3±0.4 |
| Body weight at week 16 (g) | 27.4±0.2 | 25.3±0.5** | 25.6±0.4* | 29.1±0.6* | 28.7±0.8 †† | 27.4±0.6‡ # |
| Fat mass (g) | 0.18±0.02 | 0.10±0.03 | 0.13±0.02 | 0.40±0.07** | 0.31±0.06†† | 0.20±0.01## |
| Fat % | 0.67±0.87 | 0.41±0.10 | 0.53±0.08 | 1.36±0.20** | 1.05±0.17†† | 0.75±0.05## |
| Serum cotinine (ng/ml) ϕ | 4.21±0.26 | 16.26 ± 1.82** | 4.44 ± 0.56†† | 4.04 ± 0.47 | 15.74 ± 1.84## | 3.39 ± 0.65δδ |
| Serum IL-1β (pg/ml) ϕ | 1358 ± 150 | 2566 ± 743 | 3565 ± 248* | 2869 ± 539 | 2388 ± 596 | 3395 ± 824 |
| Serum TNFα (pg/ml) ϕ | 401 ± 53 | 827 ± 295 | 1145 ± 193* | 1009 ± 204* | 729 ± 226 | 892 ±183 |

The results are expressed as mean ± SEM, n = 10 (ϕ n = 5-7), * P<0.05, ** P<0.01 vs Chow+Sham, # P<0.05, ##P<0.01 vs HFD+Sham, †† P<0.01 vs Chow+e-cig18mg, ‡ P<0.05 vs Chow+e-cig0, δδ P < 0.01 vs HFD+e-cig18mg.

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## Figure legends

Figure 1. Treatment timeline and grouping. The population was divided into normal chow or High Fat Diet. The three conditions were ambient air (control/sham), E-vapour + nicotine (vehicle + nicotine), and the E-vapour exclusively (vehicle itself).

Figure 2. Novel Object Recognition test result (a, n=10), phosphorylated (p)-Tau protein levels (b, n=6), protein levels of NeuN (c), Iba-1 (d) and GFAP (e) in the whole brain. The results are expressed as mean ± SEM, n = 5-6. * $P<0.05$, ** $P<0.01$ vs Chow-Sham, # $P<0.05$ vs HFD-Sham. Representative western blotting bands are from the same gel and the groups are divided by solid lines in line with the bar figure.

Figure 3. NeuN positive cell counts in the CA1 (a) and CA3 regions (b), percentage of neurons expressing apoptosis markers, TUNEL and Caspase-3 positive counts in the CA1 (c,e) and (d,f) of the hippocampus. Representative images of groups with significant change (g). The results are expressed as mean ± SEM, n = 5.

Figure 4. Protein levels of the endogenous antioxidant manganese superoxide dismutase (MnSOD, a) and synaptic plasticity markers, postsynaptic density protein (PSD)-95 (b) and Synapsin (c). The results are expressed as mean ± SEM, n =6. **$P<0.01$ vs Chow-Sham. Representative western blotting bands are from the same gel and the groups are divided by solid lines in line with the bar figure.

Figure 5. Protein levels of insulin signaling elements, (a) phosphorylated (p)-5' AMP-activated protein kinase (AMPK), total AMPK, and the ratio between p-AMPK and total AMPK, (b) p-protein kinase B (Akt), total Akt, and the ratio between p-Akt/Akt, and (c) Glycogen Synthase Kinase-3 (GSK). The results are expressed as mean ± SEM, n = 6. * $P< 0.05$ vs Chow-Sham, #$P< 0.05$, ## $P<0.01$ vs HFD-Sham. Representative western blotting bands are from the same gel and the groups are divided by solid lines in line with the bar figure.

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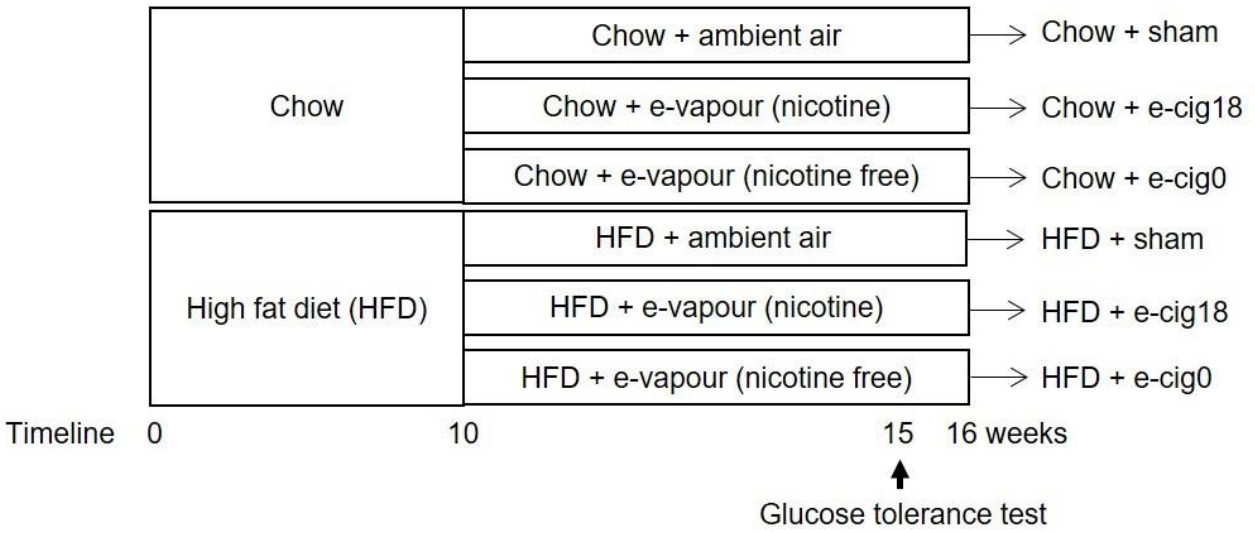


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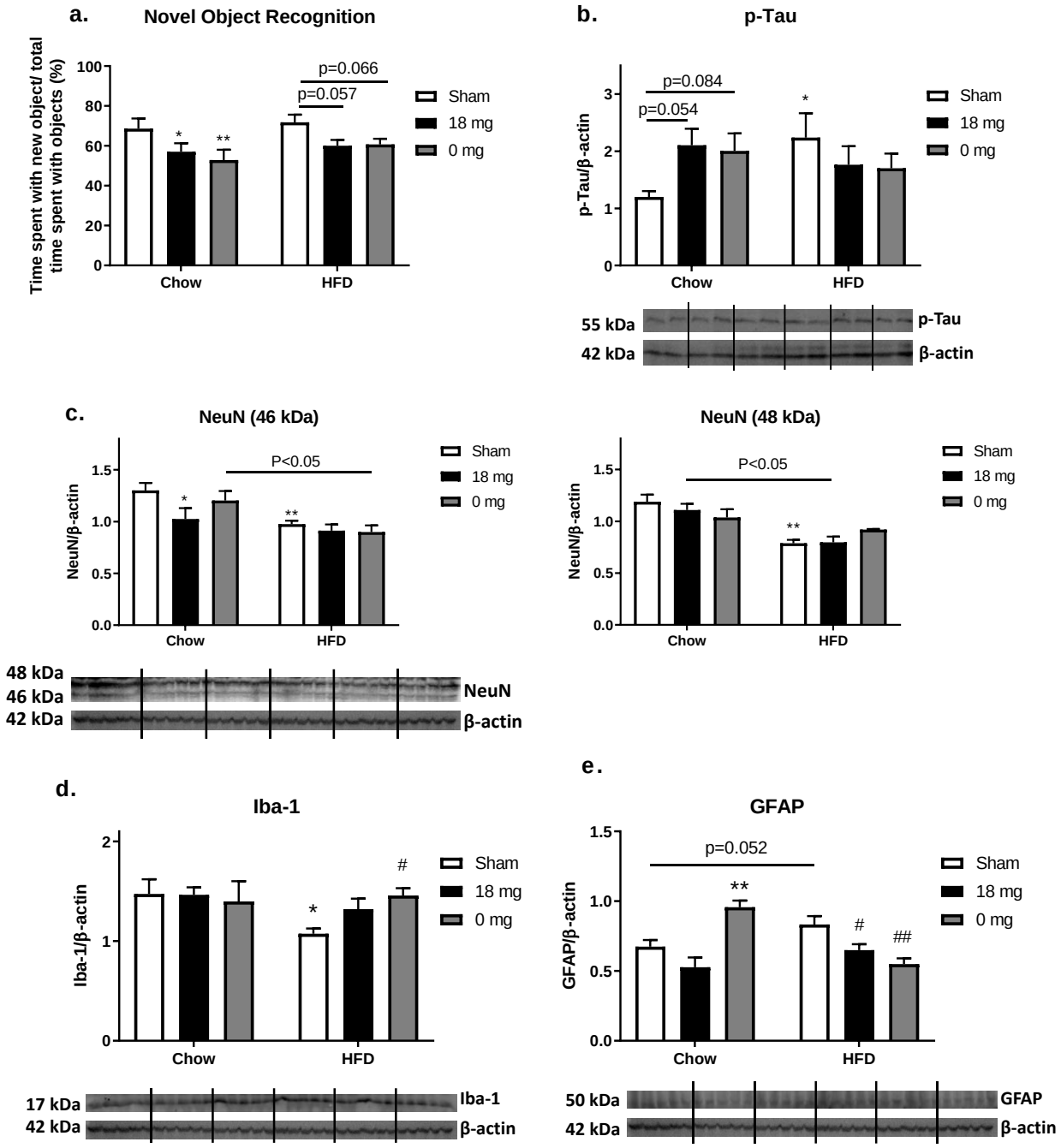
564 Figure 1

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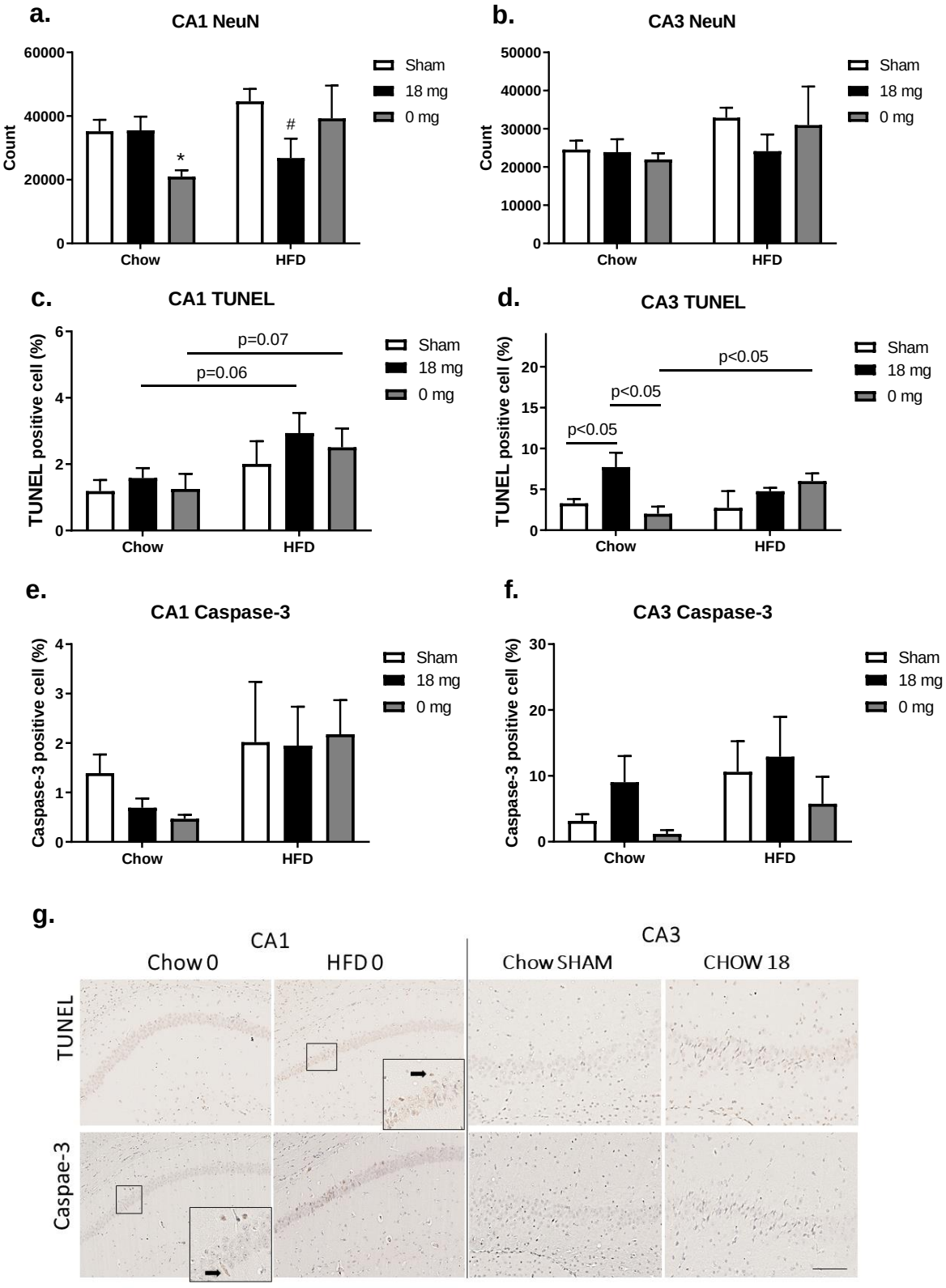
566 Figure 2

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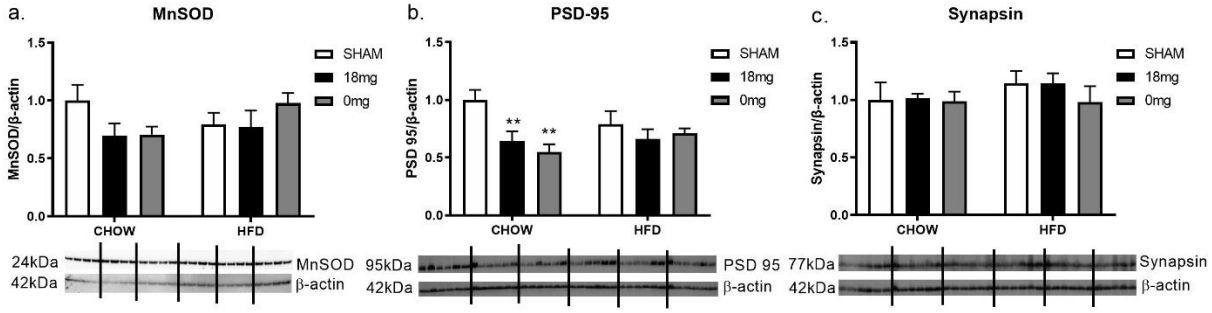
568 Figure 3

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570 Figure 4

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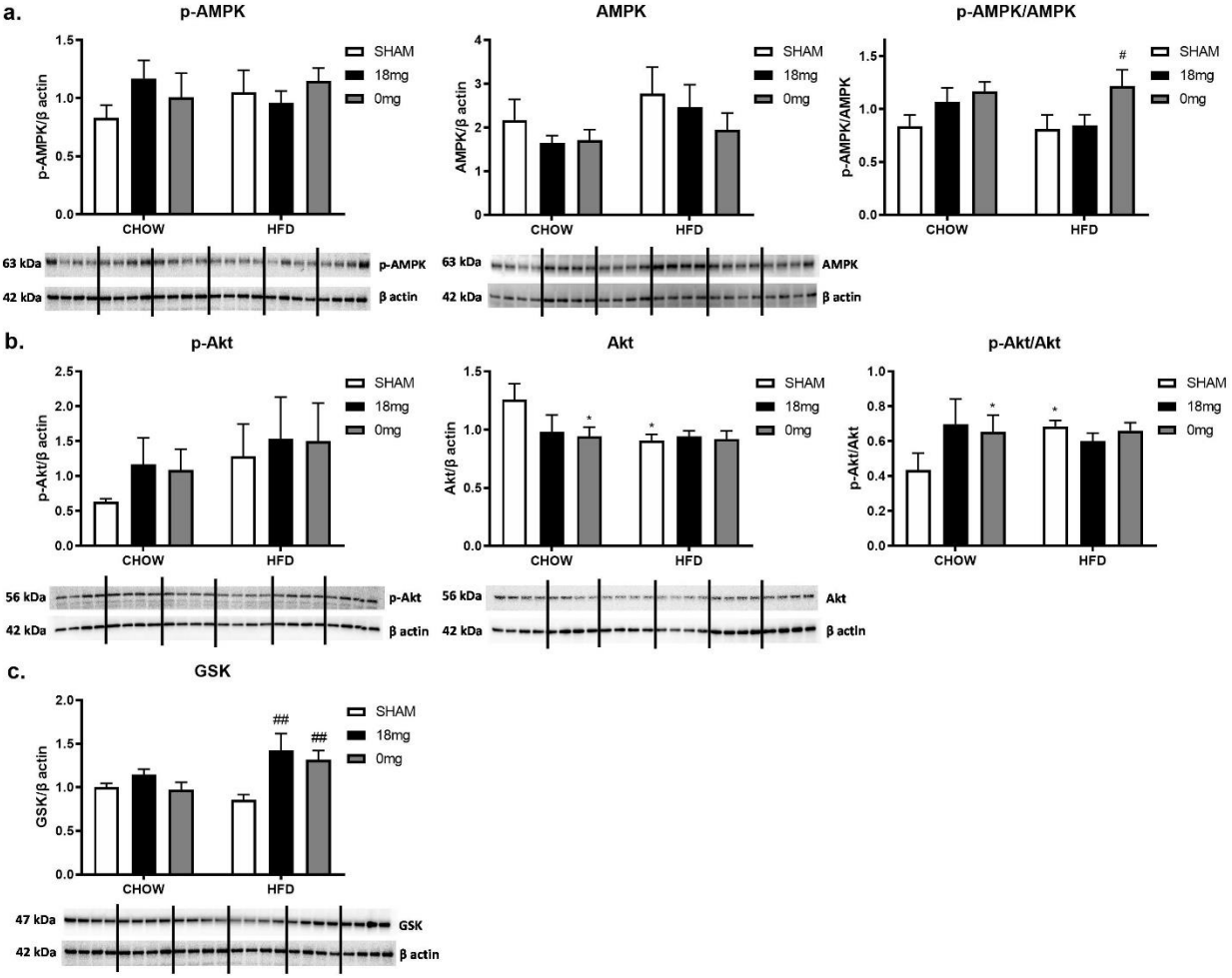


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573 Figure 5