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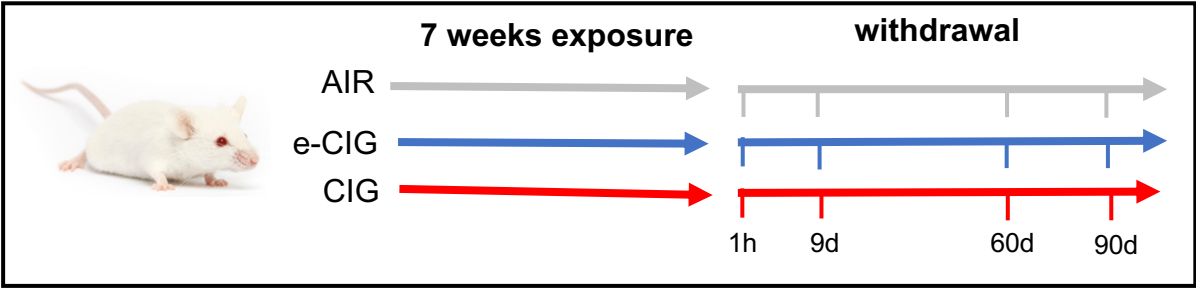
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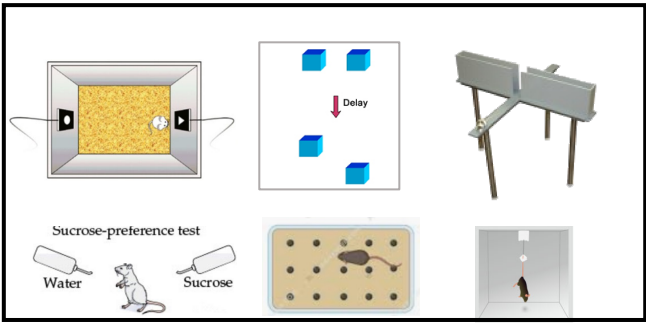
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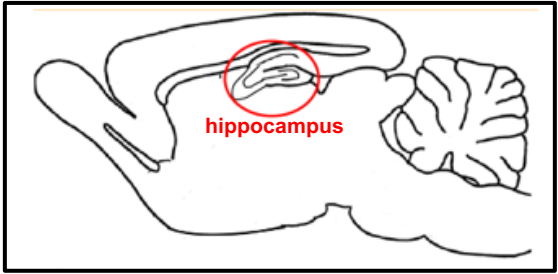


**Behavioural Analysis**



Virtual object recognition  
Spatial object recognition  
Elevated plus maze  
Sucrose preference  
Marble burying  
Tail suspension

**Molecular and Biochemical Analysis**



Crf, CrfR1  
PSD  
AMPArs,  
NMDARs

**Persistent cognitive and affective alterations at late withdrawal stages after long-term intermittent exposure to tobacco smoke or electronic cigarette vapour: behavioural changes and their neurochemical correlates.**

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## Abstract

Smoking cessation induces a withdrawal syndrome associated with anxiety, depression, and impaired neurocognitive functions, but much less is known about the withdrawal of e-cigarettes (e-CIG).

We investigated in Balb/c mice the behavioural and neurochemical effects of withdrawal for up to 90 days after seven weeks' intermittent exposure to e-CIG vapour or cigarette smoke (CIG).

The withdrawal of e-CIG and CIG induced early behavioural alterations such as spatial memory deficits (spatial object recognition task), increased anxiety (elevated plus maze test) and compulsive-like behaviour (marble burying test) that persisted for 60-90 days. Notably, attention-related (virtual object recognition task) and depression-like behaviours (tail suspension and sucrose preference tests) appeared only 15-30 days after withdrawal and persisted for as long as up to 90 days.

At hippocampal level, the withdrawal-induced changes in the levels of AMPA receptor GluA1 and GluA2/3 subunits, PSD 95 protein, corticotropin-releasing factor (Crf) and Crf receptor 1 (CrfR1) mRNA were biphasic: AMPA receptor subunit and PSD95 protein levels initially remained unchanged and decreased after 60-90 days, whereas Crf/CrfR1 mRNA levels initially increased and then markedly decreased after 60 days. These late reductions correlated with the behavioural impairments, particularly the appearance of depression-like behaviours.

Our findings show that major behavioural and neurochemical alterations persist or even first appear late after the withdrawal of chronic CIG smoke or e-CIG vapour exposure, and underline importance of conducting similar studies of humans, including e-CIG vapers.

**Kew words: Cigarette smoke; e-cigarette vapour; withdrawal; behaviour; Crf; Crf receptors; NMDA, AMPA glutamate receptors.**

## 1. Introduction

Chronic nicotine abuse in the form of smoking increases the risk of developing many diseases and leads to physical dependence characterised by the withdrawal (WDW) syndrome [1] which is associated with various symptoms such as anxiety, depression, obsessive/compulsive behaviour and impaired neurocognitive functions, including attention, working memory and loss of concentration for review see [2]. Though much less studied, a WDW syndrome similar to, or perhaps milder than, that induced by smoking cessation may be induced also by e-CIG vaping [3, 4].

Little is known about the persistence of WDW syndrome in smokers other than the fact that it is characterised by a wide range of heterogeneous symptoms, the severity of which may increase after 30 days of abstinence [5]. However, dysfunctional activation in the orbito- and pre-frontal cortex, areas essential for attention [7], working memory [8], and dependence [9], was observed in one of the very few studies that have tracked WDW for longer periods (approximately 11 years) [6]. Furthermore, it has been observed that current and former smokers (who had stopped smoking for at least six months) experienced depression, anhedonia, and somatic symptoms such as decreased appetite, lack of motivation, and impaired sleep [10].

Findings from animal studies indicate that prolonged exposure to nicotine delivered by means of minipumps, drinking water, systemic injections, intravenous self-administration procedures, or electronic cigarettes (e-CIGs) leads to a typical WDW syndrome [2] characterised by somatic signs [11], anxiety [12, 13], anhedonia as assessed in terms of an increased reward threshold [13] or decreased preference for sucrose [14], decreased attention as assessed using a 5-choice serial reaction time task (5-CSRTT) [15], and impaired spatial memory as assessed using a spatial object recognition task [16]. However, the WDW syndrome in rodents has so far only been observed for a maximum of 30 days after the cessation of tobacco smoke [17].

On the basis of the promising results of a previous one-month study of anxiety and memory impairment in mice previously exposed to e-CIG vapour or tobacco cigarette (CIG) smoke [16], we monitored the effects of the same exposure on affective behaviour and memory impairment for 90 days after cessation, a condition more similar to that encountered by human smokers. To this end, we tested murine anxiety-like-behaviour using the elevated plus maze (EPM) task, which is based on rodents' innate drive to explore new environments [18], murine compulsive-like behaviour, using the marble burying test [19], and depressive-like behaviour, using the tail suspension test [20], and the (an)hedonic levels using the sucrose preference test [21]. Cognitive deficits were evaluated using the virtual object recognition task (VORT), which assesses visual attention [22], and the spatial object recognition task, which assesses spatial working memory and was previously shown to be sensitive to the effects of nicotine WDW [23].

Several neurochemical modifications are known to occur in neurotransmitter pathways during nicotine addiction and WDW. Among them changes in corticotropin-releasing factor (CrF), its peptides and their receptors (CrFRs), are involved in controlling stress responses and, particularly,

acute and long-term drug WDW syndrome [24-27]. Glutamate receptors, in particular ionotropic AMPA receptors, are critical for nicotine dependence [28]. We studied whether possible alterations in both transmitter systems in the hippocampal formation could correlate with the affective (stress, depression) and cognitive (spatial memory) behaviours assessed during long-term nicotine WDW.

## 2. Experimental procedures

### 2.1 Animals

Three month-old male BALB/cJ mice (Charles River, Calco, Como) were group housed (five mice per cage) in a constant humidity and 21°C temperature-controlled animal facility on a 12 h/12 h light/dark cycle (lights on at 8:00 a.m.), with ad libitum access to food and water. The cob-bedding was changed weekly. This strain was chosen on the basis of our previous findings demonstrating WDW alterations after smoke/vapour cessation for at least one month [16]. Animals were assigned randomly to different groups. **Experiments were performed during the light phase between 9.00 a.m. and 6 p.m.** All of the experimental procedures respected the guidelines established by the Italian Council on Animal Care, and were approved by Italian Government Decree No. 947/2017-PR. Every effort was made to minimise the number of mice used and their suffering. All the behavioural experiments followed the ARRIVE guidelines. A total of 180 divided in 60 animals for each condition (AIR, e-CIG vapour/CIG smoke) was included in the present study. For each condition animals were divided in 6 groups (A,B,C,D,E,F) of 10 animals each and submitted, to every time interval, to different behavioural tests as follows:

Group A: VORT, Day 1; Elevated plus maze, Day 60; Tail suspension, Day 90;

Group B: Tail suspension, Day 30; Marble burying, Day 60; Sucrose preference; **VORT**, Day 90;

Group C: Tail suspension, Day 15; VORT, Day 60; Marble burying, Day 90;

Group D: Sucrose preference, Day 15, Spatial object recognition, Day 60; Elevated plus maze, Day 90;

Group E: VORT, Day 30; Sucrose preference, Day 60; Spatial object recognition, Day 90;

Group F: VORT, Day 15; Sucrose preference, Day 30; Tail suspension, Day 60.

A further group of 120 mice was used for biochemical and neurochemical analysis. These mice were euthanized one h, 9, 60 or 90 days after their last exposure to AIR, e-CIG vapour or CIG smoke.

### 2.2. Exposure to cigarette smoke and e-cigarette vapour

One week after their arrival, the mice were divided into treatment groups of 30 mice each and transferred to plexiglass inhalation chambers (22 cm wide 40 cm long 20 cm high) connected to a mechanical ventilator (Rodent Ventilator, Model 7025, Ugo Basile, Biological Research Instruments, Varese, Italy) as previously described [16]. The ventilator delivered puffs of e\_CIG vapour, CIG smoke or air, for three 30-min sessions/day for seven weeks. The sessions began at 8.00 a.m., 1.00

p.m. and 6.00 p.m. The flow rate was 200 ml/min, the frequency 25 puffs/min and the volume of each puff 8 ml. During each session, the animals in the CIG group were exposed to the smoke of 7 commercial CIG containing 0.8 mg of nicotine/CIG (for a total of 16.8 mg/day), 10 mg of tar and 10 mg of carbon monoxide. The animals of e-CIG group were exposed to e-CIG vapour containing 5.6 mg of nicotine/session (for a total of 16.8 mg/day) dissolved in an aqueous solution that also contained other compounds (propylene glycol (55%), glycerin (35%) flavour and fragrance agents [4,7,9-Megastigmatrien-3-one (0.1 mg/ml), 3-ethyl-2-hydroxycyclopent-2-en-1-one (0.7 mg/ml)] Ethyl Maltol (0.67 mg/ml), vanilline (0.15 mg/ml), neryl acetate (<0.1 mg/ml) as previously reported [16].

Control (AIR) animals were exposed only to air in three 30-min sessions/day for seven weeks.

1h after 7 weeks exposure to AIR, eCIG or CIG the content of nicotine and cotinine in brain and cotinine in the urine of exposed mice were determined [16].

1, 15, 30, 60 and 90 days after the last session of exposure to AIR, e-GIG or CIG mice underwent behavioural testing and then were euthanized by means of cervical dislocation, and their brains were rapidly removed, dissected and stored at -80 °C.

### 2.3. Behavioural studies

All behavioural experiments were performed by three experimenters blinded to treatment. To reduce the number of animals (3R) each animal was subjected to a maximum of 3 different tests spaced by at least 15 days (see 2.1).

#### 2.3.1 Virtual object recognition test

Virtual object recognition test (VORT) was conducted over a two-day period according to [22]. After habituation to the test apparatus (an open plastic arena of 60 cm × 50 cm × 30 cm) for 10 min on Day 1, animals were subjected to a familiarization ( $T_1$ ) and recognition trial ( $T_2$ ) on day 2.  $T_1$  consisted of a 10-min session during which two 2D identical shapes on a black background were shown on two 3.5-inch widescreen iPod displays as previously described [22]. Shapes were simple geometrical shapes (square, triangle, circle, cross, etc.) with equal surface (2.5 cm<sup>2</sup>). Each shape was shown on a 3rd generation iPod Touch (Apple) through iTunes for the duration of the experiment (320 pixels horizontal axis and 480 pixels vertical axis). The luminosity of the screens was constant across the two screens and testing sessions. The two iPods were attached to the middle of the opposite walls of the arena at 2 cm from the floor and inserted in a plastic transparent container (one for each iPod). Attention was paid to the choice of the shapes within each treatment group that is that the pairing of shapes was randomly chosen. A score of shape recognition was carried out when the animal was within 0.5 cm of a shape with its nose towards the shape. During  $T_2$  each mouse was subjected to



two delays (24 and 48 h) changing the shapes across the experiments. Total time spent close to the shapes during  $T_2$  was also calculated.

### 2.3.2 Spatial object recognition test

The test was carried out according to [23] with slight modifications. Two visual cues were placed on two adjacent walls of an opaque white Plexiglas cage (58 x 50 x 43 cm) and dimly lit from above (27 lx) the cage: a black and white striped pattern (21x 19.5 cm) was affixed to the centre of the northern wall and a black and grey checked pattern (26.5x 20 cm) was placed at the centre of the western wall. Two sets of identical objects were used: one set of objects consisted of an inverted 50 ml falcon tube (Fisher Scientific, Pittsburgh, PA) filled with clean mouse bedding, and the other set consisted of a 10 cm high tower made of yellow and green plastic interlocking blocks. The positions of the objects were randomly counterbalanced between the various positions. The objects were counterbalanced across locations. The cage and all objects were cleaned with acetic acid 0.1% before and after each behavioural procedure. A camera mounted above the cage recorded the experiment. Exploration to the object was defined as a mouse having its nose directed toward the object within approximately 1 cm [29]. Climbing or sitting on objects was not considered object exploration. 30 s were allowed to explore the objects. Mice that did not spend such time during training or testing were excluded from analysis. On day one, the mice were pre-exposed to the cage for 10 min. After one day, the mice returned to the cage and the time spent exploring the two objects was recorded. Forty-eight hours later the mice were re-exposed to the cage where the object that had been previously more explored was moved to a different position.

### 2.3.3 Tail suspension test

The tail suspension test is a mouse behavioural test useful to evaluate depression-related behaviours. The test was conducted according to [30]. Mice were moved from the colony to the testing room and allowed to adapt to the new environment for at least 1 h before testing. Then they were individually suspended, on a suspension bar, on a paper adhesive tape, 35 cm above the table top. The tape was placed approximately 1 cm from the tip of the tail. Animals were suspended for 6 min, and the duration of immobility was recorded by a video camera. Once the experiment time (6 min) was elapsed, the mouse returned to the home cage and a trained observer, unaware of the treatments, measured the time spent in immobility off line. Mice were considered immobile only when they hung passively and completely motionless. Approximately 10% of mice climbed their tails during these tests, and were excluded from data analysis.

### 2.3.4 Sucrose preference test

A two-bottle choice procedure was used to test for differences between the groups for their relative preference for sucrose over water according to [31] for the procedure and to [32] for the concentration of sucrose. In this test, the animals were singly housed for three days and tested with a free choice

between two bottles, one with sucrose (3% in tap water) and another with tap water available for 24 h. The position of bottles was switched every 24 h to eliminate potential side preferences. Each day, bottles were weighed to determine consumption levels (three consecutive days). Sucrose preference was defined as having average sucrose consumption level (averaged across the three day period) of 75% or higher. The preference for sucrose was calculated as a percentage of the consumed sucrose solution to the total volume of liquid consumed. A decrease of sucrose preference to a level at/or below 65%, was taken as a criterion for anhedonia [33]. This criterion was based on the fact that none of the control animals exhibited <65% preference for sucrose. On the basis of the chosen criterion of 65% of sucrose preference, mice were assigned to the anhedonic or non-anhedonic group.

### 2.3.5 Elevated plus maze test

Anxiety was evaluated using the elevated plus maze test as previously described [16]. The apparatus consisted of two opposite open arms (35 cm x 10 cm) and two enclosed arms (35 cm x 10 cm) extending from a common central platform (10 cm x 10 cm). The animals were moved to the plus maze room in order to facilitate their adaptation to the novel surroundings for 20 min, and were then individually placed onto the centre of the apparatus facing an open arm. The maze was wiped clean with water and dried after each trial. An arm entry was recorded when all four paws of the mouse were in the arm. The number of open- and closed-arm entries and the time spent in the open arms were recorded and expressed as percentages (open entries/total entries x 100; open time/total time x 100). The percentage of time spent in the open arms and the percentage of open-arm entries were used as measures of anxiety [34]. The total closed-arm entries were analysed as measure of non-specific changes in locomotor activity.

### 2.3.6 Marble burying test

The marble burying test utilizes spontaneous digging behaviour, characteristic of rodents, to assess anxiety-like/compulsive behaviour [35]. After acclimation (1h), each mouse was placed in a cage (26 cm x 20 cm x 14 cm), where 20 marbles had been equally distributed on top of mouse bedding (5 cm in depth). The number of marbles buried in 15 min and the latency to the first marble burying were measured.

## 2.4 Neurochemical studies

### 2.4.1 Brain tissue dissection

One h, 9, 60 or 90 days after their last exposure to AIR or nicotine through e-CIG or CIG, mice were euthanized by means of cervical dislocation, their brains were rapidly removed, and the hippocampi were dissected, placed in 1.5 mL Eppendorf tubes, and quickly frozen on dry ice before being stored at -80 °C.

#### 2.4.2 Tissue homogenates and membrane preparation

After thawing the hippocampal tissues of each mouse were separately homogenised manually in 20 volumes (w/v) of ice-cold 50 mM Tris HCl, 5 mM KCl, 2.5 mM MgCl<sub>2</sub>, pH 7. The homogenates were then diluted 1:1 with sample buffer and loaded, separated on 9% SDS-PAGE and electrophoretically transferred.

#### 2.4.3 Immunoblotting and densitometric quantification of Western blot bands

The AMPA receptor subunits were analysed by means of Western blotting as previously described [36]. For AMPA receptor subunit detection we used anti-GluA2-3 and anti-GluA1 antibodies (Abs) produced and characterised by us as described in [36] (see supplementary figure 2). For NMDA receptor subunit detection we used anti-GluN2A (clone A3-2D10, Life technologies, Waltham, MA, USA), anti-GluN2B (clone N59/20; Antibodies Incorporated, Davis, CA, USA), anti-NR1 subunit Ab produced by us and analysed in [37]; We also used anti-PSD95 Ab (clone K28/43, Antibodies Incorporated, Davis, CA, USA) and anti-actin Ab (clone AC-40; Sigma-Aldrich, St. Louis, MO, USA). For AMPA and NMDA receptor subunits and PSD the signal was normalised to the actin content and the values were normalised by taking the mean values of the AIR exposed mice as one.

#### 2.4.4 qPCR

qPCR experiments were performed as in [38], with modifications. Briefly, RNA was extracted from hippocampus with the Arum total RNA fatty and fibrous tissue kit (Bio-Rad, Hercules, CA, USA) and quantified by absorbance in a NanoDrop 2000c UV-Vis spectrophotometer (ThermoFisher Scientific). RNA purity was confirmed by a ratio value of absorbance 260/280  $\geq$  2. RNA integrity was verified by 1% agarose electrophoresis. cDNA was synthesized by using the iScript Advanced cDNA synthesis Kit (Bio-Rad) following manufacturer's instructions. qPCR was performed in real-time PCR reactions by Sybr Green technology in a 7900HT Fast Real-Time PCR System (Applied Biosystems, Thermofisher Scientific, Waltham, MA, USA) with 30 ng cDNA and Sso Advanced Universal SYBR Green Supermix (Bio-Rad) at the following conditions: stage 1: 95°C, 20s; stage 2: 40x(95°C, 3s; 60°C, 30s). The primers were: Crf Forward 5'-GGAGCCGCCCATCTCTCT-3'; Crf Reverse 5'-TCCTGTTGCTGTGAGCTTGCT-3'; CrfR1 Forward 5'-GATCAGCAGTGTGAGAGCCT-3'; CrfR1 Reverse 5'-GTTGTAGCGGACACCGTAG-3'; Ywhaz Forward 5'-TAGGTCATCGTGGAGGGTCG-3'; Ywhaz Reverse 5'-GAAGCATTGGGGATCAAGAACTT-3', purchased from Eurofins, Italy. To provide quantification, a threshold cycle (Ct) number was defined in the early logarithmic phase of the amplification plot and the relative expression of gene transcripts was calculated by the Delta-Delta Ct (DDCt) method and converted to relative expression ratio (2-DDCt) for statistical analysis [39] by normalizing to the endogenous reference gene Ywhaz. A dissociation curve was built in the 60-95°C range to evaluate amplification product specificity.

## 2.5 Statistical analysis

The data are given as mean values  $\pm$  SEM.

Two-way ANOVA (followed by Bonferroni's *post hoc* test, when applicable), was used to compare animal performance in the behavioural experiments (Graph Pad Prism 6 software).

For PCR studies responses were analysed using a 1-way ANOVA approach with Treatment (AIR, CIG and e-CIG) as the factor of interest. An additional blocking factor Plate was also included in the model to account for any plate-to plate variability as samples were analysed in different plates using a complete block design [40]. The 1-way ANOVA analysis was followed by Planned Comparisons of the predicted means to compare the mean of the CIG and e-CIG groups to the mean of the control (AIR) group. The analysis was performed using the InVivoStat software [41]. Data were log-transformed where appropriate to stabilize the variance and satisfy the parametric assumptions.

The data from the Western blotting studies were analysed for normal distribution using the Kolmogorov-Smirnov test and when the normal distribution was not met data were analysed by Kruskal–Wallis test followed by Dunn's *post hoc* test (non-parametric data). A P value  $<0.05$  was considered statistically significant.

## 3. Results

### 3.1 Behavioural studies

#### 3.1.1 Virtual object recognition test

VORT is a novel variant of a well-established spontaneous object recognition task used to assess recognition memory for 3D objects, based on visual attention [22]. When a 24-hour delay was applied in VORT from 1 to 90 days after smoke/vapour cessation, visual attention deficits were detected starting at 30 day interval in both CIG and e-CIG groups compared to AIR group (time:  $F_{4,135} = 8.35$ ,  $P < 0.0001$ ; treatment:  $F_{2,135} = 21.54$ ,  $P < 0.0001$ ; time x treatment:  $F_{8,135} = 2.20$ ,  $P = 0.03$ ; two-way ANOVA followed by Bonferroni post-hoc test) (Figure 1A). When a 48-hour delay was applied, significant deficits in CIG and e-CIG were detected since the earliest time interval (time:  $F_{4,135} = 13.52$ ,  $P < 0.0001$ ; treatment:  $F_{2,135} = 76.84$ ,  $P < 0.0001$ ; time x treatment:  $F_{8,135} = 11.48$ ,  $P < 0.0001$ ; two-way ANOVA followed by Bonferroni post-hoc test), and progressively worsened through the days (Figure 1B). CIG and e-CIG groups did not significantly differ from AIR group in total exploration time after 24 h (time:  $F_{5,162} = 2.09$ ,  $P = 0.06$ ; treatment:  $F_{2,162} = 0.97$ ,  $P = 0.38$ ; time x treatment:  $F_{10,162} = 11.48$ ,  $P = 0.60$ ) or 48 h (time:  $F_{4,135} = 2.39$ ,  $P = 0.06$ ; treatment:  $F_{2,135} = 0.03$ ,  $P = 0.97$ ; time x treatment:  $F_{8,135} = 0.12$ ,  $P = 0.99$ ; two-way ANOVA followed by Bonferroni post-hoc test) (Figures 1C and D).

#### 3.1.2 Spatial object recognition test

This task relies on a rodent's innate preference for novelty. Animals that remember the original training experience will preferentially explore the displaced object relative to the non-displaced object.

Since our previous studies revealed a spatial memory deficit 30 days after nicotine withdrawal [16], we submitted two groups of animals to the spatial object recognition task at longer time points (60 and 90 days) after smoke/vapour cessation. A spatial memory deficit as revealed by a decrease in the discrimination index was detected in both CIG and e-CIG groups at both 60 and 90 days of WDW (time:  $F_{1,54} = 1.01$ ,  $P = 0.32$ ; treatment:  $F_{2,54} = 25.41$ ,  $P < 0.0001$ ; time x treatment:  $F_{2,54} = 0.66$ ,  $P = 0.52$ ; two-way ANOVA followed by Bonferroni post-hoc test) (Figure 2A). Conversely, no difference between groups was found in the total exploration time (time:  $F_{2,54} = 0.40$ ,  $P = 0.66$ ; treatment:  $F_{2,54} = 1.07$ ,  $P = 0.34$ ; time x treatment:  $F_{4,54} = 1.41$ ,  $P = 0.23$ ; two-way ANOVA followed by Bonferroni post-hoc test) (Figure 2B).

### 3.1.3 Tail suspension test

Tail suspension test is based on the observation that after initial escape-oriented movements, rodents develop an immobile posture when placed in an escapable stressful situation, which can be rescued by antidepressant treatment. The elicited immobility duration, index of depressive-like behaviour, started to be significantly increased in e-CIG group at 30 days of WDW and recovered at 90 days whereas started to be significantly increased at 60 days of WDW and remained altered at 90 days in CIG group (time:  $F_{3,108} = 4.73$ ,  $P = 0.04$ ; treatment:  $F_{2,108} = 14.03$ ,  $P < 0.0001$ ; time x treatment:  $F_{6,108} = 4.71$ ,  $P = 0.0003$ ; two-way ANOVA followed by Bonferroni post-hoc test) (Figure 3A).

### 3.1.4 Sucrose preference test

Rodents are born with an interest in sweet foods or solutions. The sucrose preference test is a reward-based test, used as an indicator of anhedonia, based on the decreased ability to experience pleasure that represents one of the core symptoms of depression. Significant decrease in sucrose preference was detected in e-CIG group starting from 30 days of WDW and up to 90 days, and in CIG group starting from 60 days of WDW and up to 90 days (time:  $F_{3,108} = 6.88$ ,  $P < 0.0001$ ; treatment:  $F_{2,108} = 11.01$ ,  $P < 0.0001$ ; time x treatment:  $F_{6,108} = 2.15$ ,  $P = 0.04$ ; two-way ANOVA followed by Bonferroni post-hoc test) (Figure 3B). Conversely, no difference between groups was shown in the total liquid consumption (time:  $F_{3,108} = 1.5$ ,  $P = 0.21$ ; treatment:  $F_{2,108} = 1.88$ ,  $P = 0.15$ ; time x treatment:  $F_{6,108} = 1.40$ ,  $P = 0.22$ ; two-way ANOVA followed by Bonferroni post-hoc test) (Figure 3C). Notably, during WDW, there was a progressive increase in the percentage of anhedonic animals in both CIG and e-CIG groups compared to the AIR group, more pronounced in e-CIG group (Fisher exact probability test) (Figure 3D).

### 3.1.5 Elevated plus maze test

Elevated plus maze test relies upon rodents' proclivity toward dark, enclosed spaces and an unconditioned fear of heights/open spaces. The preference for being in open arms over closed arms is a measure of anxiety-like behaviour. Since we previously found an anxiety-like behaviour at the elevated plus maze from one to 30 days after smoke/vapour cessation [16], we submitted CIG, e-CIG and AIR exposed mice to this test at 60 and 90 days of WDW. CIG group showed an anxiety-like behaviour in terms of significant decrease of open arm entries and time at 60 days that recovered at 90 days, while e-CIG group decrease did not reach significance (open arm entries: time:  $F_{2,54} = 13.84$ ,  $P = 0.0005$ ; treatment:  $F_{2,54} = 3.24$ ,  $P = 0.05$ ; time x treatment:  $F_{2,54} = 1.65$ ,  $P = 0.2$ ; open arm time: time:  $F_{1,54} = 23.34$ ,  $P < 0.0001$ ; treatment:  $F_{1,54} = 4.35$ ,  $P = 0.01$ ; time x treatment:  $F_{2,54} = 0.84$ ,  $P = 0.43$ ; two-way ANOVA followed by Bonferroni post-hoc test) (Figure 4A). Locomotion index (total arm entries) did not differ between the groups (time:  $F_{1,54} = 0.07$ ,  $P = 0.78$ ; treatment:  $F_{2,54} = 2.04$ ,  $P = 0.15$ ; time x treatment:  $F_{2,54} = 0.79$ ,  $P = 0.46$ ; two-way ANOVA followed by Bonferroni post-hoc test) (Figure 4C).

### 3.1.6 Marble burying test

Marble burying is an animal model test used to depict anxiety or obsessive-compulsive disorder behaviour. It is based on the observation that rats and mice will bury either harmful or harmless objects in their bedding. As already observed at earlier times of WDW, the number of buried marbles, was significantly increased in CIG and e-CIG groups compared to AIR group at both 60 and 90 days after smoke/vapour cessation, being e-CIG value significantly increased also with respect to CIG at 60 days of WDW (time:  $F_{1,54} = 1.30$ ,  $P = 0.25$ ; treatment:  $F_{2,54} = 14.68$ ,  $P < 0.0001$ ; time x treatment:  $F_{2,54} = 4.99$ ,  $P = 0.01$ ; two-way ANOVA followed by Bonferroni post-hoc test) (Figure 4D). No significant difference in latency to bury the first marble was observed between groups (time:  $F_{1,54} = 0.03$ ,  $P = 0.86$ ; treatment:  $F_{2,54} = 0.20$ ,  $P = 0.81$ ; time x treatment:  $F_{2,54} = 0.54$ ,  $P = 0.59$ ; two-way ANOVA followed by Bonferroni post-hoc test) (Figure 4E).

## 3.2 Neurochemical studies

### 3.2.1. AMPA and NMDA receptor subunits and PSD95 protein levels

Previous studies have shown that nicotine use leads to enduring neuroadaptations in the corticostriatal and cortico-VTA glutamatergic brain circuitry in smoking experienced subjects even after long periods of WDW (reviewed in [28, 42]. We, therefore, analysed the expression of AMPA and NMDA receptor subunits and of scaffold protein PSD95 in the hippocampus of mice exposed to e-CIG or CIG 1h (8 mice), and 9 (8 mice), 60 (7 mice) or 90 (7 mice) days after smoke/vapour cessation. As shown in figure 5A, in the hippocampus the comparison of mice exposed to AIR to those exposed to e-CIG or CIG showed that there is no change in the level of GluA2/3 subunits in the hippocampus 1 h after last smoke/vapour exposure or after 9 days of WDW. However, at 60 ( $\chi^2 = 9.1$   $p = 0.002$ ) and 90 ( $\chi^2 = 12.0$   $p = 0.002$ ) days of WDW there was a decreased level of the GluA2/3

subunit. Also the level of the GluA1 subunit was unchanged between AIR and e-CIG or CIG exposed mice 1h or 9 days after exposure cessation but it was decreased at 60 ( $\chi^2=6.0$   $p=0.041$ ) and 90 ( $\chi^2=9.3$   $p=0.004$ ) days of WDW (Figure 5B). The decrease in AMPA receptor subunit levels was paralleled by a decrease in the scaffold protein PSD95 levels at 60 ( $\chi^2=11.1$   $p=0.0001$ ) and 90 ( $\chi^2=12.9$   $p=0.0001$ ) days of WDW (Figure 5C). No change in the level of NMDA subunit (NR1, NR2A and NR2B) levels between AIR and nicotine-exposed groups was determined at any time of WDW (Supplementary Figure 1).

### 3.2.2 *Crf* and *CrfR1* gene expression

One hour after the end of a seven-week CIG or e-CIG exposure, the expression of *Crf* and its receptor *CrfR1* were evaluated by qPCR. For *Crf* a significant treatment effect was detected by ANOVA ( $F_{2,15}=4.25$ ,  $p=0.035$ ). Planned comparisons between AIR, CIG and e-CIG groups showed that *Crf* mRNA in the e-CIG group was significantly increased with respect to AIR ( $p=0.011$ , Fig. 6A). The *CrfR1* receptor displayed a similar trend, although no significant alteration was detected ( $p=0.085$  in the e-CIG group, Fig. 6B).

At 9 days of WDW, *Crf* levels in CIG and e-CIG groups were still higher than in AIR group. Indeed, ANOVA tests suggested a treatment effect ( $F_{2,38}=3.19$ ,  $p=0.052$ ), while post-hoc analysis showed that both e-CIG and CIG exposure ( $p=0.029$  and  $p=0.041$ , respectively; Fig. 6A) induced significant *Crf* mRNA increase. In contrast, *CrfR1* levels showed a trend towards reduction ( $p=0.066$  in the e-CIG group, Fig. 6B).

Remarkably, at 60 days of WDW, both *Crf* and *CrfR1* mRNA were dramatically reduced in the CIG and e-CIG groups. Indeed, *Crf* levels showed a significant Treatment effect in ANOVA tests ( $F_{2,25}=3.45$ ,  $p=0.048$ ), with both e-CIG and CIG groups displaying significant decreases ( $p=0.038$  and  $p=0.024$ , respectively; Fig. 6A). Likewise, a treatment effect was detected in the analysis of *CrfR1* levels ( $F_{2,25}= 5.24$ ,  $p=0.013$ ) and e-CIG and CIG displayed lower levels in comparisons with controls ( $p=0.0046$  and  $p=0.027$ , respectively; Fig. 6B).

## 4. Discussion

The principal findings of this study indicate that mice exposed to e-CIG or CIG for seven weeks are still affected by marked deficits in cognitive and affective-like behaviour, and alterations in the hippocampal glutamatergic and *Crf* systems 2-3 months after WDW.

The behavioural effects of nicotine deprivation and the related neurochemical modifications have been widely studied in nicotine-exposed animals, but usually over relatively short periods of time (generally five days for the induction of nicotine dependence and no more than 30 days for nicotine WDW) [17]. The initial interest of this study, therefore, lies in the fact that the mice inhaled the nicotine in CIG smoke or e-CIG vapour during three 30-minute sessions/day for seven weeks, and were

monitored for up to 90 days after cessation. This intermittent experimental design more closely resembles human exposure than the continuous administration frequently used in the past insofar as nicotine dependence is obtained over a relatively long time and the post-WDW behavioural and neurochemical alterations are investigated over a period of WDW that represents a greater proportion of a mouse natural life span [16]. Furthermore, we compared the effects of conventional CIGs and e-CIGs during WDW because, although nicotine is the main psychoactive compound in both, CIGs also contain other products that can interfere with the results [43].

It is important to underline that after 7 weeks of exposure, 1 h after the last exposure, the levels of nicotine and cotinine in the brain were very similar between e-CIG and CIG mice [16] and in line with the levels found by many authors in rodents that received nicotine via different administration ways (reviewed by [44]), tobacco smoking [45] or in human smokers [46].

Nicotine WDW in humans is associated with symptoms such as anxiety, depression, compulsive behaviour and deficits in cognitive functions [2], and it is particularly interesting that our mice showed increased anxiety/depression, compulsive-like behaviours and cognitive deficits for up to 90 days after the cessation of smoke/vapour. Attention deficits appeared as early as 15 days after cessation, and persisted for at least 90 days; furthermore, when the delay in the VORT was increased to 48 hours, there were significant deficits after just one day of WDW, possibly because of the increased difficulty of the test. This suggests that the initial deficit is less pronounced, and worsens after a longer period of WDW. Since it is known that mood disorders are associated with cognitive deficits, including attentional, executive and memory impairments [47], it cannot be excluded that memory deficit could be due to a dysfunction in visual attention and emotion.

Only a few studies have investigated attention deficits during nicotine WDW, none of which did so for an extended period. However, one study of the 5-CSRTT in rats that had received nicotine 3.16 mg/kg/day for seven days found an increase in the percentage of omissions after 10 and 16 h of nicotine abstinence that progressively recovered within 106 h of WDW [15], and a similar attention deficit has been found in mice after four but not 52 h of nicotine WDW using the same task [48]. The main differences between these studies and our model are the lower nicotine dose and the much shorter period of nicotine exposure, which suggests that persistent attentional alterations during WDW require neuro-adaptations that develop over a longer period of exposure that is more similar to that experienced by human smokers or vapers.

Impaired attention was accompanied by a memory deficit revealed by the spatial object recognition test that lasted at least 90 days in both groups of mice, which is in line with previous findings obtained using the Morris water maze, the radial-arm maze and spatial object recognition tasks in rodents during early nicotine WDW [23, 49]. Human data concerning cognitive deficits are discordant; the effects of nicotine WDW on attention are generally small [50,51], but its effects on working memory range from small [51] to large [52]. As pointed out in the review by [52], these differences may be due to differences in the duration of abstinence as well as in specific task parameters.



1 The increased anxiety-like behaviour measured using the elevated plus maze started one day after  
2 WDW [16] and lasted for up to 30 days in the e-CIG-exposed mice and for up to 60 days in the CIG-  
3 exposed mice. Increased anxiety-like behaviour after extended nicotine WDW is in line with the  
4 findings of [53], who observed it after three months of nicotine WDW in rats that had received  
5 subcutaneously administered nicotine 0.36 mg/kg/day for three weeks. The modulatory effect of  
6 nicotine on emotional status has also been demonstrated in human studies: nicotine acutely induces  
7 anxiolytic effects in chronic users, whereas acute WDW increases anxiety [54, 55]; however, little if  
8 anything is known about the effect of prolonged nicotine WDW.

9 Compulsiveness is considered a core feature of drug dependence [56], but has not been investigated  
10 during nicotine WDW except by [57]). We measured it using the marble burying test. Marble burying  
11 is a natural behaviour that has been used to test anxiety-related and compulsive behaviour-related  
12 drugs [19] and, as it is thought to be a valid model of obsessive-compulsive disorders [58], it is  
13 currently used to test rodent models [59]. In accordance with our previous findings obtained using  
14 15-day periods of WDW [16], the number of marbles buried by the mice in both groups was  
15 significantly larger 60 days post-WDW than at baseline, and significantly higher in the mice exposed  
16 to e-CIG. The behaviour tended to recover after 90 days, as did the difference between the two  
17 groups. These findings suggest that compulsiveness is important during nicotine WDW and warrant  
18 further investigation in nicotine WDW models.

19 Depression-like behaviour and anhedonia were evaluated by means of the tail suspension and  
20 sucrose preference tests, respectively, appeared after 30 days of WDW in the e-CIG group and 60  
21 days in the CIG group, and persisted until day 90. This is in line with the findings of previous studies  
22 that recorded an increase in the duration of immobility in mice after two hours [60] and 15 days of  
23 nicotine WDW [61]; similarly, the forced swimming test has revealed depression-like behaviour in  
24 rodents 1-60 days after nicotine WDW [62-66].

25 There are no published data concerning the onset of anhedonia in Balb/c mice during nicotine WDW.  
26 However, one study has found that high nicotine doses of 40 mg/kg/day for 28 days increased the  
27 intracranial self-stimulation threshold for four days during spontaneous nicotine WDW in C57BL/6J  
28 mice but not Balb/c mice [67], and another study of C57BL/6J mice found a reduction in sucrose  
29 preference on the first day after the removal of minipumps delivering 12 or 24 mg/kg/day of nicotine  
30 that continued for four days [14]. The differences in the time of onset and the course of the depressive  
31 symptoms are probably due to the different routes of administration used (osmotic pumps or  
32 parenteral administration) and doses (12, 24 or 40 mg/kg/day).

33 In conclusion, our previous study [16] and this more extensive study of mice chronically exposed to  
34 CIG smoke or e-CIG vapour confirms the presence of cognitive and affective alterations at the end  
35 of the treatment period and after smoke/vapour WDW that are substantially consistent with those  
36 experienced by humans. More specifically, our current findings demonstrate that the behavioural  
37 alterations are markedly persistent, being detected up to 90 days after smoke/vapour WDW, but not  
38

stable as some of them decline (e.g., anxiety at the elevated plus test), some worsen over time (e.g., attention in the VORT), and some only become apparent long after WDW (depression-like behaviours in the tail suspension and anhedonia tests).

It is particularly interesting to correlate these last behavioural alterations with the neurochemical changes observed in the hippocampal region, which also only became apparent long after WDW. Our analysis showed that long-term exposure to e-CIG vapour or CIG smoke increased Crf/CrfR1 levels in the hippocampus in a similar manner to that reported by [38] and other groups using shorter nicotine exposure periods. Furthermore, interesting new findings were that the mice in both groups still had very high hippocampal Crf levels but greatly reduced CrfR1 levels after nine days of WDW in comparison with those exposed to AIR, and, surprisingly, markedly lower levels of both Crf and CrfR1 than the AIR-exposed mice after 60 days of WDW.

These findings integrate and expand previously published data indicating that Crf/CrfR1 sustain and maintain an anxiogenic and stressful state after nicotine WDW [68, 69]. In the case of acute experiments, this stressful response is probably due to the activation of CrfR1 [70,71], and can be counteracted by the administration of CrfR1 antagonists [72]. On the contrary, [73] have reported that the over-expression of Crf in the amygdala decreases the dysphoric-like state associated with nicotine WDW. As previously pointed out, it is difficult to compare these studies with ours and with each other because most of the experimental designs are acute and involved different nicotine doses, administration periods and durations of WDW, and the neurochemical modifications were observed in different brain areas.

Crf is locally produced by GABAergic interneurons of the hippocampal fields CA1 and CA3 [74], and acts on CrfR1 expressed by pyramidal neurons [75]. The activity of this system is stimulated by stressful conditions and other physiological states, and promotes synaptic plasticity in dendritic pyramidal cells and their conversion from a thin to mushroom-like morphology. This change is typical of synaptic learning and is accompanied by the insertion of AMPA receptors into the synapse and long-term potentiation (LTP) [76]. Activation of the hippocampal Crf system (for example, by means of a local injection of Crf) supports hippocampal functions including memory formation [75], and the selective inactivation of Crf interneurons impairs object recognition memory [77]. An acute increase in Crf release induces CrfR1-dependent hippocampal plasticity, whereas the prolonged activation of Crf transmission leads to dendritic retraction, loss of LTP, and memory impairment [75]. In parallel with the late decline in hippocampal Crf and CrfR1 mRNA levels, we also observed a significant decrease in the levels of AMPA receptor subunits and scaffold protein PSD 95 that was not apparent at the end of CIG or e-CIG exposure or nine days after cessation, but only developed after 60 and 90 days of nicotine WDW. The changes in AMPA receptors were not due to a general neuronal damage as NMDA receptor levels were in the control range.

The alterations in the markers of both Crf and AMPA hippocampal transmission paralleled the development of depression-like behaviours. Interestingly, it is known that chronic stress is a major

determinant of depression and affects learning and memory by altering the structure of hippocampal neurons, and that its most consistently observed effects on the hippocampus related to the development of depression-like behaviour are a reduction in the branching of pyramidal cell dendrites (reviewed in [78], a reduction in AMPA receptors and synaptic proteins, and a reduction in AMPA transmission and AMPA-mediated plasticity [79,80]. Accordingly, post-mortem studies of major depressive disorder patients have revealed alterations in the expression of synapse- and glutamate-related genes [81].

Our findings of the late down-regulation of the hippocampal Crf system and a reduction in AMPA subunit receptors and PSD95 scaffold proteins indicate that a loss of neuroplasticity in hippocampal circuitry is a major determinant of late nicotine WDW syndrome, and may support the cognitive and affective impairments detected in mice exposed to e-CIG and CIG several months after WDW. On the basis of these findings, future experiments will investigate the hypothesis that dendritic spines and structural plasticity in hippocampal synapses are profoundly altered in the late stage of nicotine WDW.

Finally, our study confirms that CIG smoking and e-CIG vaping induce dependence and WDW symptoms whose time course and severity are similar in terms of behavioural alterations and neurochemical changes. Although it would deserve much attention from researchers in the field, there is still a lack of studies of humans who have stopped e-CIG vaping. This is important because the sale of e-CIGs is increasing in a number of countries especially among adolescents who consider them a less toxic form of nicotine administration than tobacco smoking, even though the safety of long-term e-CIG abuse has not been completely established and its toxicological effects are still unclear [82]. However, it is known that they include cardiovascular effects mainly due to nicotine [83] and pulmonary effects [82] due to the numerous chemicals that are added with the aim of improving flavour and adsorption kinetics [43]. The vapour of e-CIGs also contains compounds that can increase the production of reactive oxygen species, and trace metals that may increase inflammation, cytotoxicity and genotoxicity [83]. Moreover, e-CIGs are considered a gateway to other drugs of abuse such as cocaine and tetrahydrocannabinol [84], and our neurochemical findings support this view. It is not surprising that we found e-CIG WDW alterations similar to those induced by CIGs because e-CIG vaping is the nicotine delivery system that is the most similar to tobacco smoking in terms of nicotine pharmacokinetics. The toxicological effects induced by e-CIGs raise concern because their consumption is greatly supported by the tobacco industry and some tobacco control experts. Our findings stress the importance of further studies aimed at better clarifying the pharmacological and toxicological properties of e-CIGs.

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#### **Declaration of Competing Interest**

The authors declare no competing financial interest

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## 6. Figure legends

**Figure 1.** Visual attention evaluated in VORT during WDW. Performance was evaluated in terms of: mean discrimination index when 2D geometric shapes were presented stationary using 24h (A) or 48 h(B) delay; total exploration time using 24h (C) or 48h (D) delay. Data are expressed as mean  $\pm$  SEM of 10 mice/group. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , \*\*\*\* $P < 0.0001$  vs corresponding AIR group. # $P < 0.05$ , ## $P < 0.01$ , #### $P < 0.0001$  vs corresponding Day 1 (Bonferroni *post hoc* test).

**Figure 2.** Spatial object recognition in terms of (A) discrimination index and (B) total exploration time evaluated in CIG and e-CIG mice compared to AIR group 60 and 90 days after smoke/vapour cessation, using 48-h delay. Data are expressed as mean  $\pm$  SEM of 10 mice/group. \*\*\* $P < 0.001$ , \*\*\*\* $P < 0.0001$  vs corresponding AIR group (Bonferroni *post hoc* test).

**Figure 3.** Depressive-like behaviour evaluated in CIG and e-CIG mice compared to AIR group during abstinence at different time intervals. (A) immobility time evaluated for 6 min in tail suspension test; (B-D) Sucrose preference test, evaluated in terms of percentage of the consumed sucrose solution to the total volume of liquid consumed (B); Total daily (mean of three days) intake (C); Percentage of anhedonic animals (those consuming less than 65% of sucrose solution) (D). Data are expressed as mean  $\pm$  SEM of 10 mice/group. \* $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\* $P < 0.001$ , \*\*\*\* $P < 0.0001$  vs corresponding AIR group; \$  $P < 0.05$  vs same treatment at 15 days).  $^{\&}P < 0.05$ , vs corresponding CIG group (Bonferroni or Fisher exact probability test).

**Figure 4.** Evaluation of anxiety-like behaviour in CIG and e-CIG mice compared to AIR group during abstinence at different time intervals. (A-C) elevated plus maze task in terms of percentage of open arm entries (A) and time (B), total number of entries (C). (D-E) Total number of buried marbles (D) and latency to the first burial (E) evaluated within 15 min, in the marble burying task. Data are expressed as mean  $\pm$  SEM of 10 mice/group. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\*\* $P < 0.0001$  vs corresponding AIR group; \$ $P < 0.05$  \$ $P < 0.01$  vs same treatment at 60 days.  $^{\&}P < 0.05$  vs corresponding CIG group (Bonferroni *post hoc* test).

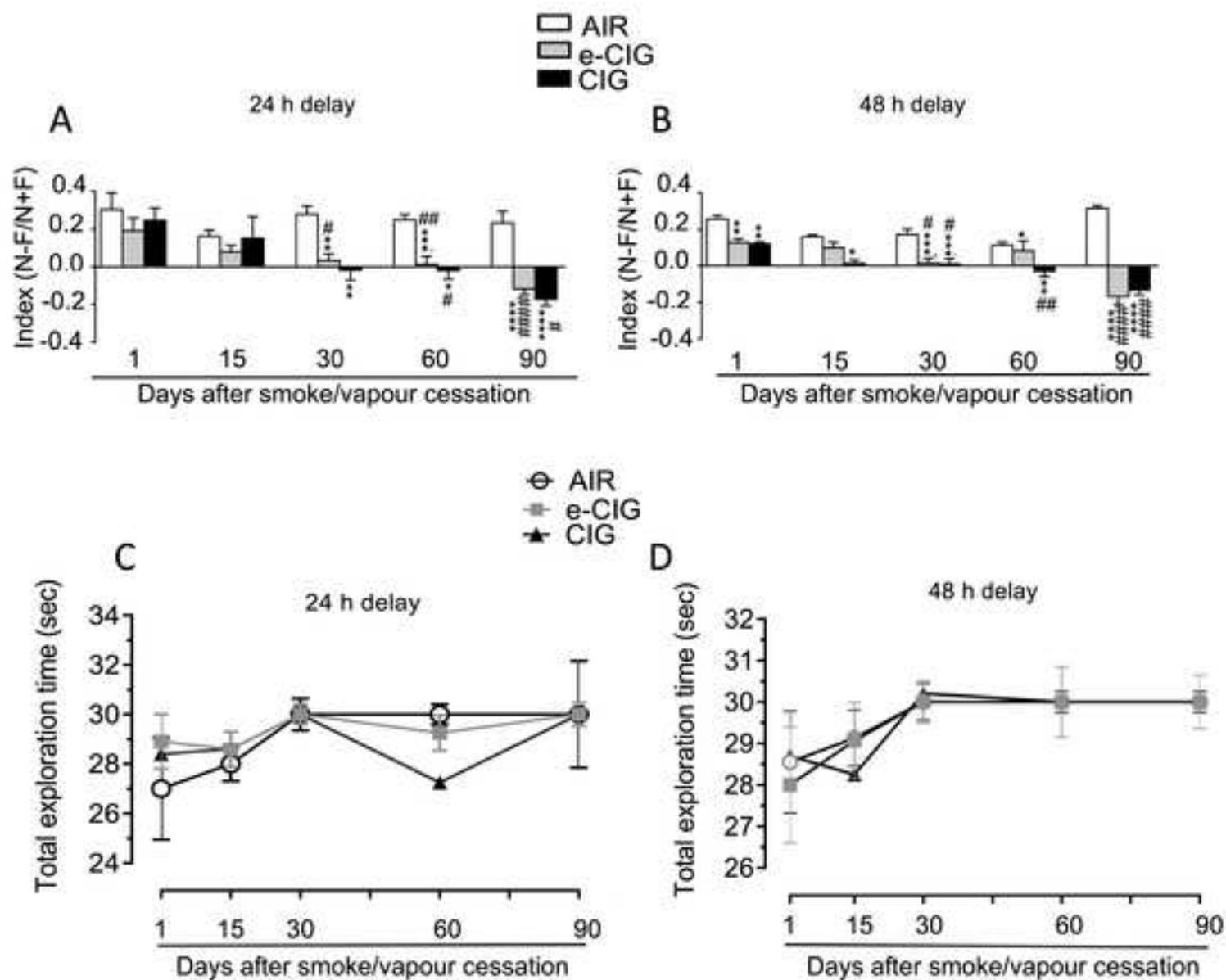
**Figure 5.** Analysis of AMPAR subunit and PSD 95 protein levels in the hippocampus of mice exposed to AIR, CIG or e-CIG after one hour ( $n=8$ ), nine ( $n=8$ ), 60( $n=7$ ) and 90( $n=7$ ) days. Proteins were separated on 7.5 % acrylamide SDS gels, electrotransferred to nitrocellulose, and probed with antibodies as described in Methods. Identical amounts of proteins were always loaded to the same gel, and the loading was further verified by actin staining. The Western blot analysis is expressed as the ratio between the e-CIG- or CIG- and the average of AIR-exposed mice. Each bar shows the mean values  $\pm$  SEM obtained by analysing samples from six-seven mice tested in four different experiments. The Western blotting data were statistically analysed using Kruskal–Wallis test

1 followed by Dunn post hoc test (non-parametric data) (\* $p < 0.05$ , \*\* $p < 0.01$ ), and were significantly  
2 different from those of the AIR-exposed mice under the same condition.  
3

4 **Figure 6.** Relative mRNA expression of Crf (A) or CrfR1 (B) in mouse hippocampus after e-CIG or  
5 CIG exposure. Mice were exposed to e-CIG, CIG or AIR for 7 weeks and euthanised after 1h or after  
6 a 9-day or 60-day WDW. \*\*:  $p < 0.01$ , \*:  $p < 0.05$ , #:  $0.05 < p < 0.09$  in the Planned Comparison of e-CIG  
7 or CIG vs. AIR.  
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## VIRTUAL OBJECT RECOGNITION

Figure 1



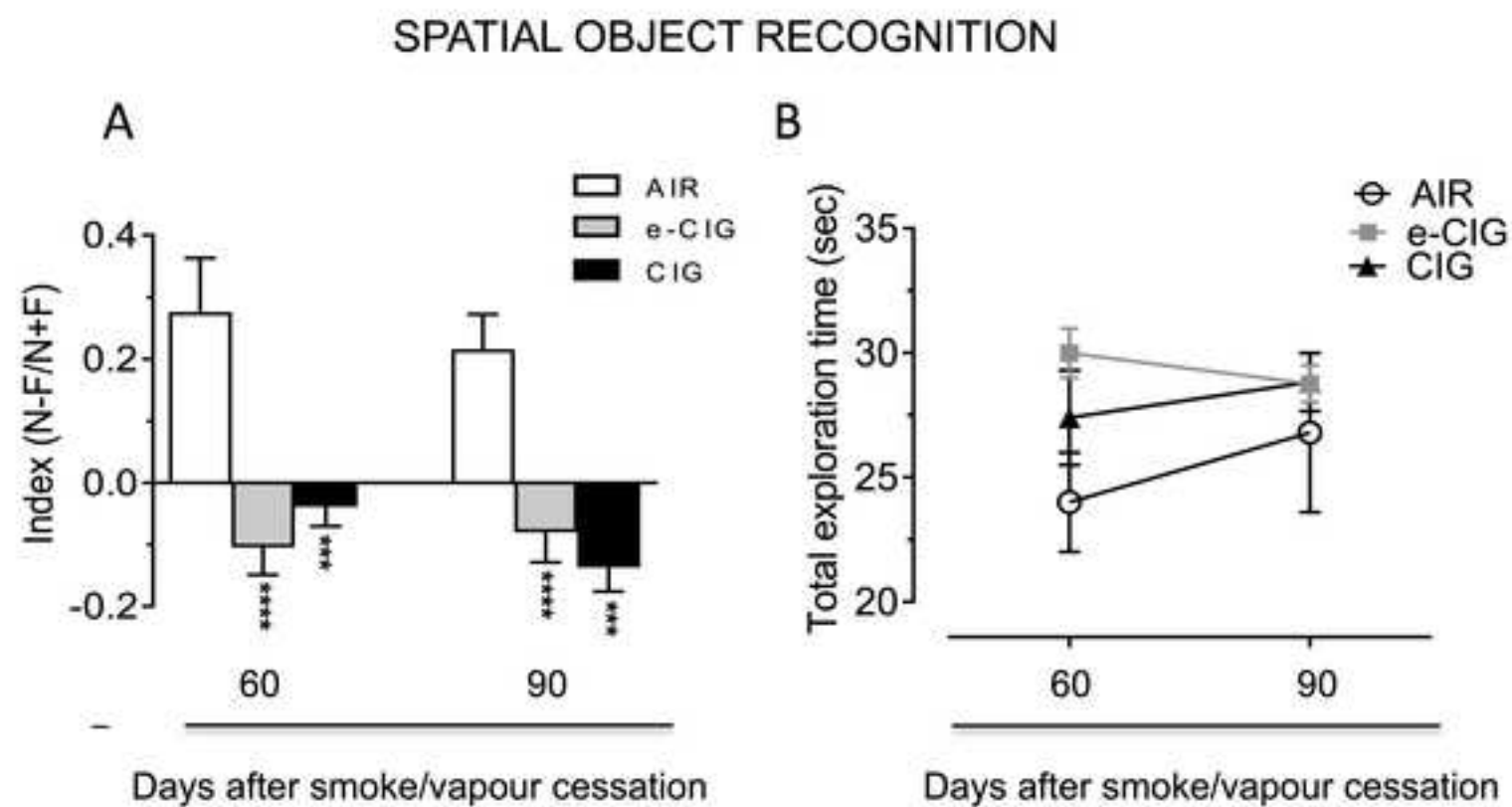


Figure 3

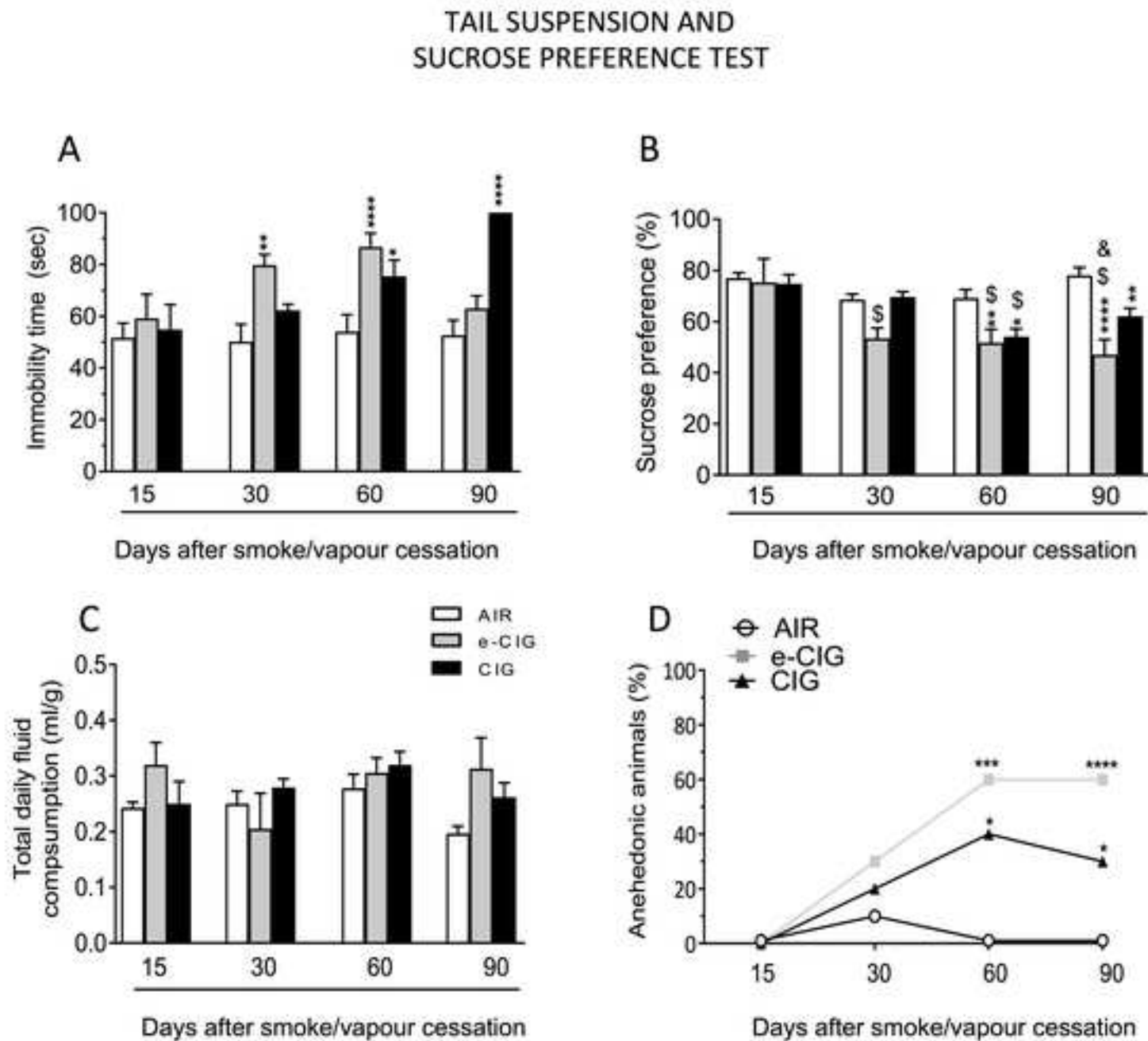


Figure 4

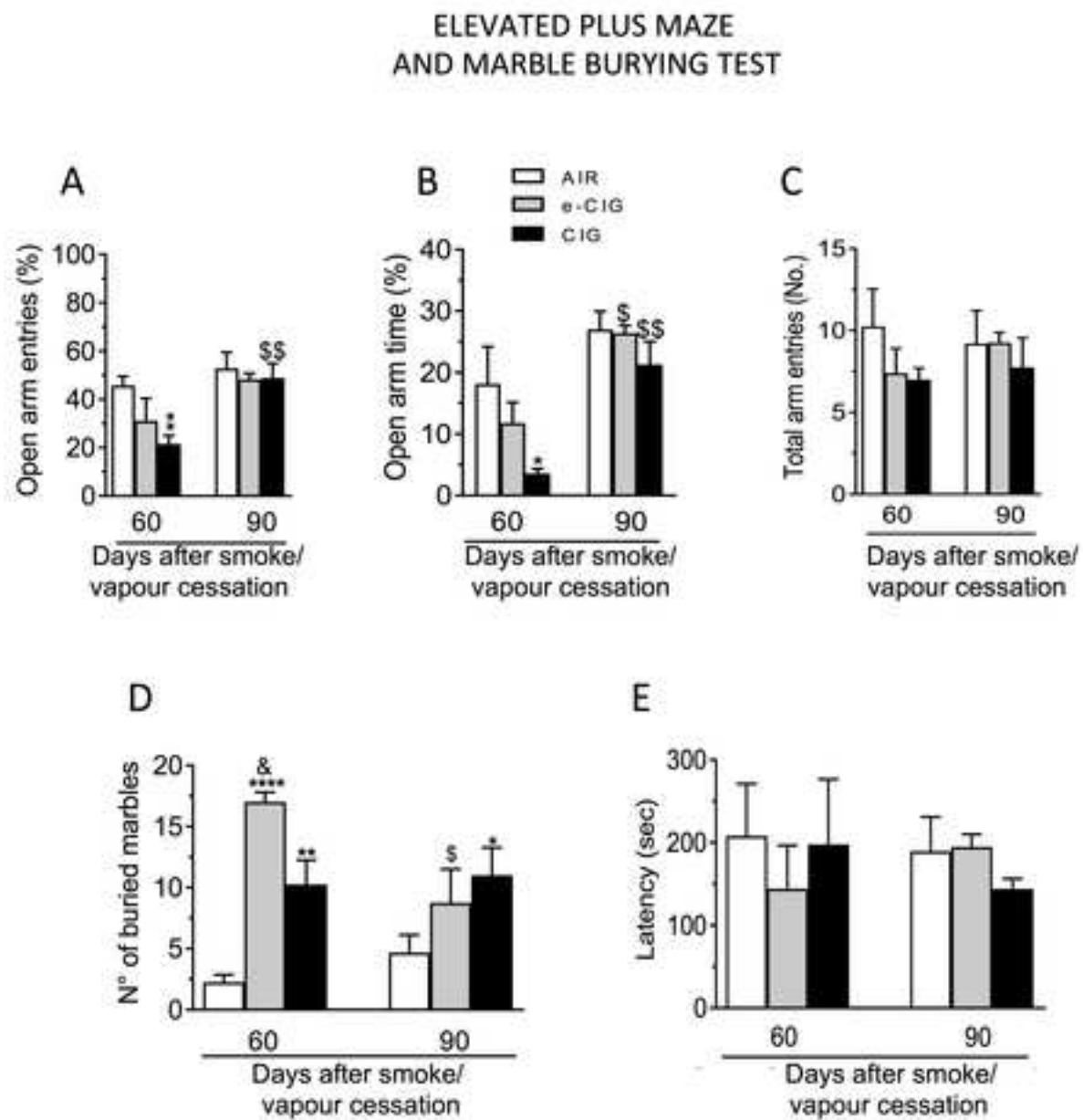
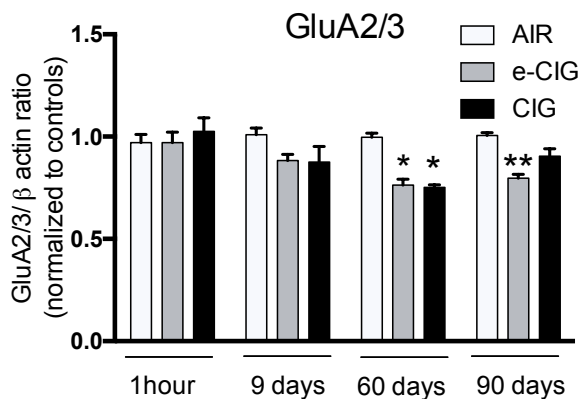


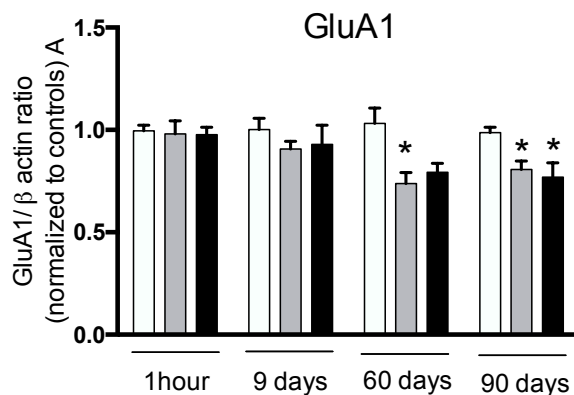


Figure 5

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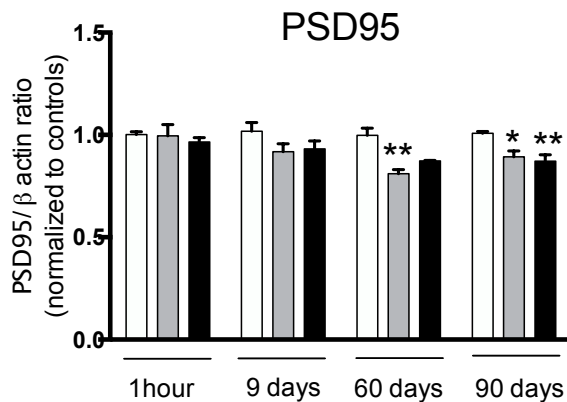
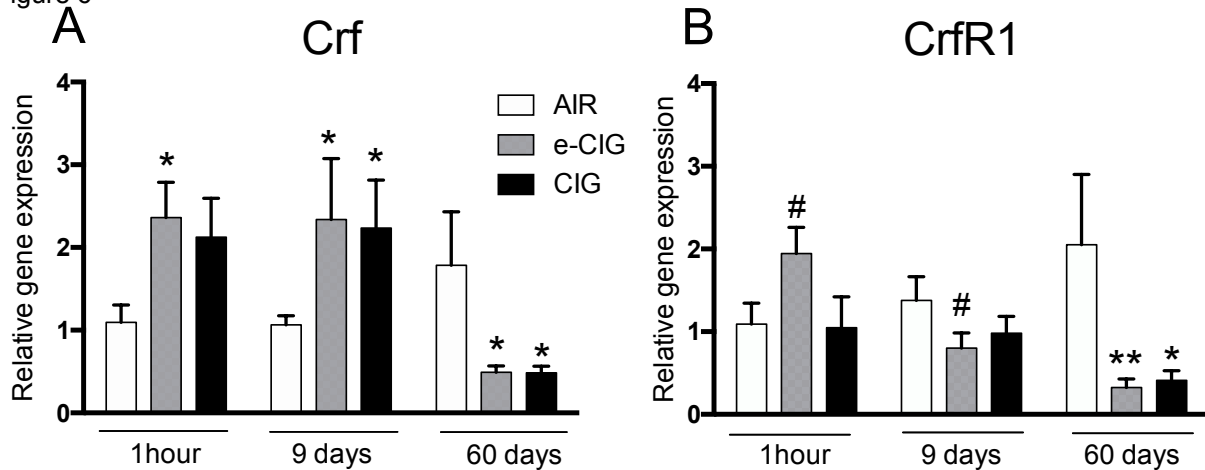


Figure 6



All authors declare that do not have conflict of interest , with no actual or potential conflict of interest including any financial, personal or other relationships with other people or organizations within three years of beginning the work submitted.