

This is the peer reviewed version of the following article:

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 / Ponzoni, L.; Braidà, D.; Carboni, L.; Moretti, M.; Viani, P.; Clementi, F.; Zoli, M.; Gotti, C.; Sala, M.. - In: PHARMACOLOGICAL RESEARCH. - ISSN 1043-6618. - 158:(2020), pp. 104941-104941. [10.1016/j.phrs.2020.104941]

Terms of use:

The terms and conditions for the reuse of this version of the manuscript are specified in the publishing policy. For all terms of use and more information see the publisher's website.

06/05/2026 01:29

(Article begins on next page)

Pharmacological Research

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.

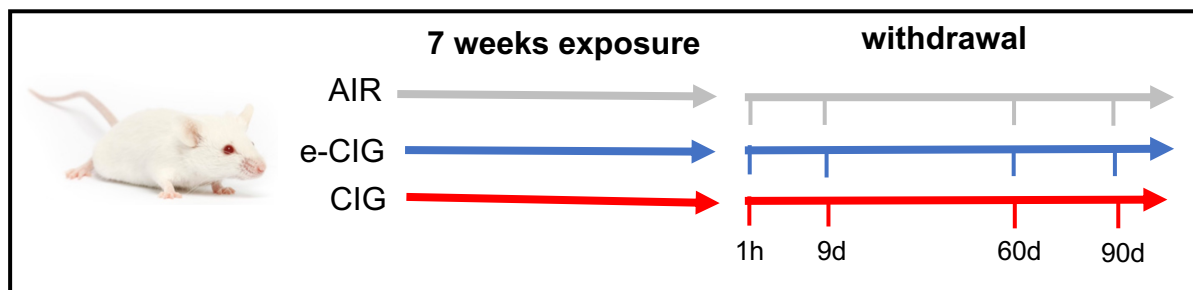
--Manuscript Draft--

| | |
|------------------------------|--|
| Manuscript Number: | YPHRS-D-20-00451R1 |
| Article Type: | Research Paper |
| Keywords: | Cigarette smoke; e-cigarette vapour; withdrawal; Behaviour; Crf; NMDA, AMPA receptors |
| Corresponding Author: | Cecilia Gotti ITALY |
| First Author: | Luisa Ponzoni, PhD |
| Order of Authors: | Luisa Ponzoni, PhD Daniela Braidà, Dr Lucia Carboni, Dr Milena Moretti, Dr Paola Viani, Prof Francesco Clementi, Prof Michele Zoli, Prof Cecilia Gotti, Dr Mariaelvina Sala, Dr |
| Abstract: | <p>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).</p> <p>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).</p> <p>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.</p> <p>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.</p> <p>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.</p> |
| Suggested Reviewers: | Georgianna G Gould gouldg@uthscsa.edu expert in the field Imad M Damaj Instituto de Medicina Molecular m.damaj@vcuhealth.org |

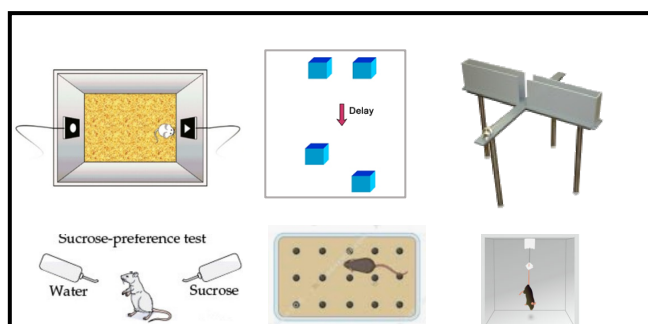
| | |
|--|---|
| | Expert in the field |
| | Mohammed Shoaib mohammed.shoaib@ncl.ac.uk Expert in the field |
| | Cristian Chiamulera cristiano.chiamulera@univr.it |

Highlights

- Balb/c mice exposed to e-CIG or CIG for seven weeks show marked long-term deficits in cognitive and affective-like behaviour at late withdrawal times.
- In comparison with mice exposed to AIR, Crf/CrfR1 levels in the hippocampi of mice exposed to e-CIG vapour or CIG smoke are increased at the end of exposure and greatly decreased after 60 days of withdrawal.
- During late withdrawal, there is a significant decrease in the levels of AMPA receptor subunits and scaffold protein PSD 95 in the hippocampus of mice exposed to e-CIG vapour or CIG smoke, but no change in NMDA subunit levels.
- e-CIG vapour and CIG smoke induce similar, in terms of time course and severity, behavioural and neurochemical alterations during both dependence and withdrawal phases.



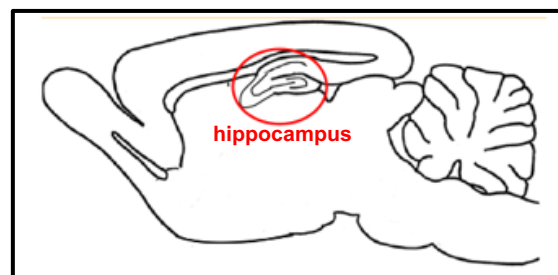
Behavioural Analysis



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



Molecular and Biochemical Analysis



- Crf, CrfR1
- PSD
- AMPArs,
- NMDARs

1 **Persistent cognitive and affective alterations at late withdrawal stages after long-term**
2 **intermittent exposure to tobacco smoke or electronic cigarette vapour: behavioural changes**
3 **and their neurochemical correlates.**
4
5
6
7
8
9

10 Luisa Ponzoni^{1,2,3°}, Daniela Braida^{3°}, Lucia Carboni⁴, Milena Moretti^{1,3}, Paola Viani^{1,3}, Francesco
11 Clementi^{1,3}, Michele Zoli⁵, Cecilia Gotti^{1,3*} and Mariaelvina Sala^{1,3*}

12 ¹CNR, Institute of Neuroscience, Milan, Italy; ²Fondazione Zardi-Gori, Milan, Italy; ³Dept. of Medical
13 Biotechnology and Translational Medicine, Università degli Studi di Milano, Milan, Italy; ⁴Department
14 of Pharmacy and Biotechnology, Alma Mater Studiorum - Università di Bologna, Bologna, Italy;
15 ⁵Department of Biomedical, Metabolic and Neural Sciences, Center for Neuroscience and
16 Neurotechnology (CfNN), University of Modena and Reggio Emilia, 41125 Modena, Italy.
17
18
19
20
21
22
23
24

25 °These authors contributed equally to this work

26 *Co-corresponding authors
27
28
29
30

31 Corresponding author: Dr Cecilia Gotti, CNR, Institute of Neuroscience, Via Vanvitelli 32, 20129
32 Milano, Italy. Phone +39-02-50316974; Fax +39-02-50217132; E-mail: cecilia.gotti@in.cnr.it
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

Abstract

1 Smoking cessation induces a withdrawal syndrome associated with anxiety, depression, and
2 impaired neurocognitive functions, but much less is known about the withdrawal of e-cigarettes (e-
3 CIG).
4
5

6
7 We investigated in Balb/c mice the behavioural and neurochemical effects of withdrawal for up to 90
8 days after seven weeks' intermittent exposure to e-CIG vapour or cigarette smoke (CIG).
9

10
11 The withdrawal of e-CIG and CIG induced early behavioural alterations such as spatial memory
12 deficits (spatial object recognition task), increased anxiety (elevated plus maze test) and compulsive-
13 like behaviour (marble burying test) that persisted for 60-90 days. Notably, attention-related (virtual
14 object recognition task) and depression-like behaviours (tail suspension and sucrose preference
15 tests) appeared only 15-30 days after withdrawal and persisted for as long as up to 90 days.
16
17
18

19
20 At hippocampal level, the withdrawal-induced changes in the levels of AMPA receptor GluA1 and
21 GluA2/3 subunits, PSD 95 protein, corticotropin-releasing factor (Crf) and Crf receptor 1 (CrfR1)
22 mRNA were biphasic: AMPA receptor subunit and PSD95 protein levels initially remained
23 unchanged and decreased after 60-90 days, whereas Crf/CrfR1 mRNA levels initially increased and
24 then markedly decreased after 60 days. These late reductions correlated with the behavioural
25 impairments, particularly the appearance of depression-like behaviours.
26
27
28
29

30
31 Our findings show that major behavioural and neurochemical alterations persist or even first appear
32 late after the withdrawal of chronic CIG smoke or e-CIG vapour exposure, and underline importance
33 of conducting similar studies of humans, including e-CIG vapers.
34
35
36
37
38
39
40

41 **Kew words: Cigarette smoke; e-cigarette vapour; withdrawal; behaviour; Crf; Crf receptors;**
42 **NMDA, AMPA glutamate receptors.**
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

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,

1 acute and long-term drug WDW syndrome [24-27]. Glutamate receptors, in particular ionotropic
2 AMPA receptors, are critical for nicotine dependence [28]. We studied whether possible alterations
3 in both transmitter systems in the hippocampal formation could correlate with the affective (stress,
4 depression) and cognitive (spatial memory) behaviours assessed during long-term nicotine WDW.
5
6
7

8 **2. Experimental procedures**

9 *2.1 Animals*

10 Three month-old male BALB/cJ mice (Charles River, Calco, Como) were group housed (five mice
11 per cage) in a constant humidity and 21°C temperature-controlled animal facility on a 12 h/12 h
12 light/dark cycle (lights on at 8:00 a.m.), with ad libitum access to food and water. The cob-bedding
13 was changed weekly. This strain was chosen on the basis of our previous findings demonstrating
14 WDW alterations after smoke/vapour cessation for at least one month [16]. Animals were assigned
15 randomly to different groups. **Experiments were performed during the light phase between 9.00 a.m.**
16 **and 6 p.m.** All of the experimental procedures respected the guidelines established by the Italian
17 Council on Animal Care, and were approved by Italian Government Decree No. 947/2017-PR. Every
18 effort was made to minimise the number of mice used and their suffering. All the behavioural
19 experiments followed the ARRIVE guidelines. A total of 180 divided in 60 animals for each condition
20 (AIR, e-CIG vapour/CIG smoke) was included in the present study. For each condition animals were
21 divided in 6 groups (A,B,C,D,E,F) of 10 animals each and submitted, to every time interval, to
22 different behavioural tests as follows:
23
24
25
26
27
28
29
30
31
32

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

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

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

36 Group D: Sucrose preference, Day 15, Spatial object recognition, Day 60; Elevated plus maze, Day
37 90;

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

39 Group F: VORT, Day 15; Sucrose preference, Day 30; Tail suspension, Day 60.
40
41
42
43
44
45

46 A further group of 120 mice was used for biochemical and neurochemical analysis. These mice were
47 euthanized one h, 9, 60 or 90 days after their last exposure to AIR, e-CIG vapour or CIG smoke.
48
49
50
51
52

53 *2.2. Exposure to cigarette smoke and e-cigarette vapour*

54 One week after their arrival, the mice were divided into treatment groups of 30 mice each and
55 transferred to plexiglass inhalation chambers (22 cm wide 40 cm long 20 cm high) connected to a
56 mechanical ventilator (Rodent Ventilator, Model 7025, Ugo Basile, Biological Research Instruments,
57 Varese, Italy) as previously described [16]. The ventilator delivered puffs of e_CIG vapour, CIG
58 smoke or air, for three 30-min sessions/day for seven weeks. The sessions began at 8.00 a.m., 1.00
59
60
61
62
63
64
65

1 p.m. and 6.00 p.m. The flow rate was 200 ml/min, the frequency 25 puffs/min and the volume of each
2 puff 8 ml. During each session, the animals in the CIG group were exposed to the smoke of 7
3 commercial CIG containing 0.8 mg of nicotine/CIG (for a total of 16.8 mg/day), 10 mg of tar and 10
4 mg of carbon monoxide. The animals of e-CIG group were exposed to e-CIG vapour containing 5.6
5 mg of nicotine/session (for a total of 16.8 mg/day) dissolved in an aqueous solution that also
6 contained other compounds (propylene glycol (55%), glycerin (35%) flavour and fragrance agents
7 [4,7,9-Megastigmatrien-3-one (0.1 mg/ml), 3-ethyl-2-hydroxycyclopent-2-en-1-one (0.7 mg/ml)]
8 Ethyl Maltol (0.67 mg/ml), vanilline (0.15 mg/ml), neryl acetate (<0.1 mg/ml) as previously reported
9 [16].
10
11
12
13
14

15 Control (AIR) animals were exposed only to air in three 30-min sessions/day for seven weeks.
16

17
18 1h after 7 weeks exposure to AIR, eCIG or CIG the content of nicotine and cotinine in brain and
19 cotinine in the urine of exposed mice were determined [16].
20
21

22 1, 15, 30, 60 and 90 days after the last session of exposure to AIR, e-GIG or CIG mice underwent
23 behavioural testing and then were euthanized by means of cervical dislocation, and their brains were
24 rapidly removed, dissected and stored at -80 °C.
25
26
27

28 29 30 2.3. Behavioural studies

31 All behavioural experiments were performed by three experimenters blinded to treatment. To reduce
32 the number of animals (3R) each animal was subjected to a maximum of 3 different tests spaced by
33 at least 15 days (see 2.1).
34
35
36
37

38 2.3.1 Virtual object recognition test

39 Virtual object recognition test (VORT) was conducted over a two-day period according to [22]. After
40 habituation to the test apparatus (an open plastic arena of 60 cm × 50 cm × 30 cm) for 10 min on
41 Day 1, animals were subjected to a familiarization (T₁) and recognition trial (T₂) on day 2. T₁ consisted
42 of a 10-min session during which two 2D identical shapes on a black background were shown on
43 two 3.5-inch widescreen iPod displays as previously described [22]. Shapes were simple geometrical
44 shapes (square, triangle, circle, cross, etc.) with equal surface (2.5 cm²). Each shape was shown on
45 a 3rd generation iPod Touch (Apple) through iTunes for the duration of the experiment (320 pixels
46 horizontal axis and 480 pixels vertical axis). The luminosity of the screens was constant across the
47 two screens and testing sessions. The two iPods were attached to the middle of the opposite walls
48 of the arena at 2 cm from the floor and inserted in a plastic transparent container (one for each iPod).
49 Attention was paid to the choice of the shapes within each treatment group that is that the pairing of
50 shapes was randomly chosen. A score of shape recognition was carried out when the animal was
51 within 0.5 cm of a shape with its nose towards the shape. During T₂ each mouse was subjected to
52
53
54
55
56
57
58
59
60
61
62
63
64
65

1 two delays (24 and 48 h) changing the shapes across the experiments. Total time spent close to the
2 shapes during T₂ was also calculated.
3

4 *2.3.2 Spatial object recognition test*

5 The test was carried out according to [23] with slight modifications. Two visual cues were placed on
6 two adjacent walls of an opaque white Plexiglas cage (58 x 50 x 43 cm) and dimly lit from above (27
7 lx) the cage: a black and white striped pattern (21x 19.5 cm) was affixed to the centre of the northern
8 wall and a black and grey checked pattern (26.5x 20 cm) was placed at the centre of the western
9 wall. Two sets of identical objects were used: one set of objects consisted of an inverted 50 ml falcon
10 tube (Fisher Scientific, Pittsburgh, PA) filled with clean mouse bedding, and the other set consisted
11 of a 10 cm high tower made of yellow and green plastic interlocking blocks. The positions of the
12 objects were randomly counterbalanced between the various positions. The objects were
13 counterbalanced across locations. The cage and all objects were cleaned with acetic acid 0.1%
14 before and after each behavioural procedure. A camera mounted above the cage recorded the
15 experiment. Exploration to the object was defined as a mouse having its nose directed toward the
16 object within approximately 1 cm [29]. Climbing or sitting on objects was not considered object
17 exploration. 30 s were allowed to explore the objects. Mice that did not spend such time during
18 training or testing were excluded from analysis. On day one, the mice were pre-exposed to the cage
19 for 10 min. After one day, the mice returned to the cage and the time spent exploring the two objects
20 was recorded. Forty-eight hours later the mice were re-exposed to the cage where the object that
21 had been previously more explored was moved to a different position.
22
23
24
25
26
27
28
29
30
31
32
33
34
35

36 *2.3.3 Tail suspension test*

37 The tail suspension test is a mouse behavioural test useful to evaluate depression-related
38 behaviours. The test was conducted according to [30]. Mice were moved from the colony to the
39 testing room and allowed to adapt to the new environment for at least 1 h before testing. Then they
40 were individually suspended, on a suspension bar, on a paper adhesive tape, 35 cm above the table
41 top. The tape was placed approximately 1 cm from the tip of the tail. Animals were suspended for 6
42 min, and the duration of immobility was recorded by a video camera. Once the experiment time (6
43 min) was elapsed, the mouse returned to the home cage and a trained observer, unaware of the
44 treatments, measured the time spent in immobility off line. Mice were considered immobile only when
45 they hung passively and completely motionless. Approximately 10% of mice climbed their tails during
46 these tests, and were excluded from data analysis.
47
48
49
50
51
52
53
54
55

56 *2.3.4 Sucrose preference test*

57 A two-bottle choice procedure was used to test for differences between the groups for their relative
58 preference for sucrose over water according to [31] for the procedure and to [32] for the concentration
59 of sucrose. In this test, the animals were singly housed for three days and tested with a free choice
60 of sucrose. In this test, the animals were singly housed for three days and tested with a free choice
61
62
63
64
65

1 between two bottles, one with sucrose (3% in tap water) and another with tap water available for 24
2 h. The position of bottles was switched every 24 h to eliminate potential side preferences. Each day,
3 bottles were weighed to determine consumption levels (three consecutive days). Sucrose preference
4 was defined as having average sucrose consumption level (averaged across the three day period)
5 of 75% or higher. The preference for sucrose was calculated as a percentage of the consumed
6 sucrose solution to the total volume of liquid consumed. A decrease of sucrose preference to a level
7 at/or below 65%, was taken as a criterion for anhedonia [33]. This criterion was based on the fact
8 that none of the control animals exhibited <65% preference for sucrose. On the basis of the chosen
9 criterion of 65% of sucrose preference, mice were assigned to the anhedonic or non-anhedonic
10 group.
11
12
13
14
15
16

17 *2.3.5 Elevated plus maze test*

18 Anxiety was evaluated using the elevated plus maze test as previously described [16]. The apparatus
19 consisted of two opposite open arms (35 cm x 10 cm) and two enclosed arms (35 cm x 10 cm)
20 extending from a common central platform (10 cm x 10 cm). The animals were moved to the plus
21 maze room in order to facilitate their adaptation to the novel surroundings for 20 min, and were then
22 individually placed onto the centre of the apparatus facing an open arm. The maze was wiped clean
23 with water and dried after each trial. An arm entry was recorded when all four paws of the mouse
24 were in the arm. The number of open- and closed-arm entries and the time spent in the open arms
25 were recorded and expressed as percentages (open entries/total entries x 100; open time/total time
26 x 100). The percentage of time spent in the open arms and the percentage of open-arm entries were
27 used as measures of anxiety [34]. The total closed-arm entries were analysed as measure of non-
28 specific changes in locomotor activity.
29
30
31
32
33
34
35
36
37
38
39

40 *2.3.6 Marble burying test*

41 The marble burying test utilizes spontaneous digging behaviour, characteristic of rodents, to assess
42 anxiety-like/compulsive behaviour [35]. After acclimation (1h), each mouse was placed in a cage (26
43 cm x 20 cm x 14 cm), where 20 marbles had been equally distributed on top of mouse bedding (5 cm
44 in depth). The number of marbles buried in 15 min and the latency to the first marble burying were
45 measured.
46
47
48
49
50

51 *2.4 Neurochemical studies*

52 *2.4.1 Brain tissue dissection*

53 One h, 9, 60 or 90 days after their last exposure to AIR or nicotine through e-CIG or CIG, mice were
54 euthanized by means of cervical dislocation, their brains were rapidly removed, and the hippocampi
55 were dissected, placed in 1.5 mL Eppendorf tubes, and quickly frozen on dry ice before being stored
56 at -80 °C.
57
58
59
60
61
62
63
64
65

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 MgCl₂, 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'-GGAGCCGCCATCTCTCT-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

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

4 Since our previous studies revealed a spatial memory deficit 30 days after nicotine withdrawal [16],
5 we submitted two groups of animals to the spatial object recognition task at longer time points (60
6 and 90 days) after smoke/vapour cessation. A spatial memory deficit as revealed by a decrease in
7 the discrimination index was detected in both CIG and e-CIG groups at both 60 and 90 days of WDW
8 (time: $F_{1,54} = 1.01$, $P = 0.32$; treatment: $F_{2,54} = 25.41$, $P < 0.0001$; time x treatment: $F_{2,54} = 0.66$, $P =$
9 0.52 ; two-way ANOVA followed by Bonferroni post-hoc test) (Figure 2A). Conversely, no difference
10 between groups was found in the total exploration time (time: $F_{2,54} = 0.40$, $P = 0.66$; treatment: $F_{2,54}$
11 $= 1.07$, $P = 0.34$; time x treatment: $F_{4,54} = 1.41$, $P = 0.23$; two-way ANOVA followed by Bonferroni
12 post-hoc test) (Figure 2B).

21 3.1.3 Tail suspension test

22 Tail suspension test is based on the observation that after initial escape-oriented movements,
23 rodents develop an immobile posture when placed in an escapable stressful situation, which can be
24 rescued by antidepressant treatment. The elicited immobility duration, index of depressive-like
25 behaviour, started to be significantly increased in e-CIG group at 30 days of WDW and recovered at
26 90 days whereas started to be significantly increased at 60 days of WDW and remained altered at
27 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
28 treatment: $F_{6,108} = 4.71$, $P = 0.0003$; two-way ANOVA followed by Bonferroni post-hoc test) (Figure
29 3A).

38 3.1.4 Sucrose preference test

39 Rodents are born with an interest in sweet foods or solutions. The sucrose preference test is a
40 reward-based test, used as an indicator of anhedonia, based on the decreased ability to experience
41 pleasure that represents one of the core symptoms of depression. Significant decrease in sucrose
42 preference was detected in e-CIG group starting from 30 days of WDW and up to 90 days, and in
43 CIG group starting from 60 days of WDW and up to 90 days (time: $F_{3,108} = 6.88$, $P < 0.0001$;
44 treatment: $F_{2,108} = 11.01$, $P < 0.0001$; time x treatment: $F_{6,108} = 2.15$, $P = 0.04$; two-way ANOVA
45 followed by Bonferroni post-hoc test) (Figure 3B). Conversely, no difference between groups was
46 shown in the total liquid consumption (time: $F_{3,108} = 1.5$, $P = 0.21$; treatment: $F_{2,108} = 1.88$, $P = 0.15$;
47 time x treatment: $F_{6,108} = 1.40$, $P = 0.22$; two-way ANOVA followed by Bonferroni post-hoc test)
48 (Figure 3C). Notably, during WDW, there was a progressive increase in the percentage of anhedonic
49 animals in both CIG and e-CIG groups compared to the AIR group, more pronounced in e-CIG group
50 (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

1 subunit. Also the level of the GluA1 subunit was unchanged between AIR and e-CIG or CIG exposed
2 mice 1h or 9 days after exposure cessation but it was decreased at 60 ($\chi^2=6.0$ $p=0.041$) and 90
3 ($\chi^2=9.3$ $p=0.004$) days of WDW (Figure 5B). The decrease in AMPA receptor subunit levels was
4 paralleled by a decrease in the scaffold protein PSD95 levels at 60 ($\chi^2=11.1$ $p=0.0001$) and 90
5 ($\chi^2=12.9$ $p=0.0001$) days of WDW (Figure 5C). No change in the level of NMDA subunit (NR1, NR2A
6 and NR2B) levels between AIR and nicotine-exposed groups was determined at any time of WDW
7 (Supplementary Figure 1).
8
9

10 11 12 13 3.2.2 *Crf* and *CrfR1* gene expression

14 One hour after the end of a seven-week CIG or e-CIG exposure, the expression of *Crf* and its
15 receptor *CrfR1* were evaluated by qPCR. For *Crf* a significant treatment effect was detected by
16 ANOVA ($F_{2,15}=4.25$, $p=0.035$). Planned comparisons between AIR, CIG and e-CIG groups showed
17 that *Crf* mRNA in the e-CIG group was significantly increased with respect to AIR ($p=0.011$, Fig. 6A).
18 The *CrfR1* receptor displayed a similar trend, although no significant alteration was detected
19 ($p=0.085$ in the e-CIG group, Fig. 6B).
20
21

22 At 9 days of WDW, *Crf* levels in CIG and e-CIG groups were still higher than in AIR group. Indeed,
23 ANOVA tests suggested a treatment effect ($F_{2,38}=3.19$, $p=0.052$), while post-hoc analysis showed
24 that both e-CIG and CIG exposure ($p=0.029$ and $p=0.041$, respectively; Fig. 6A) induced significant
25 *Crf* mRNA increase. In contrast, *CrfR1* levels showed a trend towards reduction ($p=0.066$ in the e-
26 CIG group, Fig. 6B).
27
28

29 Remarkably, at 60 days of WDW, both *Crf* and *CrfR1* mRNA were dramatically reduced in the CIG
30 and e-CIG groups. Indeed, *Crf* levels showed a significant Treatment effect in ANOVA tests
31 ($F_{2,25}=3.45$, $p=0.048$), with both e-CIG and CIG groups displaying significant decreases ($p=0.038$
32 and $p=0.024$, respectively; Fig. 6A). Likewise, a treatment effect was detected in the analysis of
33 *CrfR1* levels ($F_{2,25}= 5.24$, $p=0.013$) and e-CIG and CIG displayed lower levels in comparisons with
34 controls ($p=0.0046$ and $p=0.027$, respectively; Fig. 6B).
35
36
37
38
39
40
41
42
43
44
45

46 47 4. Discussion

48 The principal findings of this study indicate that mice exposed to e-CIG or CIG for seven weeks are
49 still affected by marked deficits in cognitive and affective-like behaviour, and alterations in the
50 hippocampal glutamatergic and *Crf* systems 2-3 months after WDW.
51
52

53 The behavioural effects of nicotine deprivation and the related neurochemical modifications have
54 been widely studied in nicotine-exposed animals, but usually over relatively short periods of time
55 (generally five days for the induction of nicotine dependence and no more than 30 days for nicotine
56 WDW) [17]. The initial interest of this study, therefore, lies in the fact that the mice inhaled the nicotine
57 in CIG smoke or e-CIG vapour during three 30-minute sessions/day for seven weeks, and were
58
59
60
61
62
63
64
65

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

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

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

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

31 Impaired attention was accompanied by a memory deficit revealed by the spatial object recognition
32 test that lasted at least 90 days in both groups of mice, which is in line with previous findings obtained
33 using the Morris water maze, the radial-arm maze and spatial object recognition tasks in rodents
34 during early nicotine WDW [23, 49]. Human data concerning cognitive deficits are discordant; the
35 effects of nicotine WDW on attention are generally small [50,51], but its effects on working memory
36 range from small [51] to large [52]. As pointed out in the review by [52], these differences may be
37 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
10
11
12
13 Compulsiveness is considered a core feature of drug dependence [56], but has not been investigated
14 during nicotine WDW except by [57]). We measured it using the marble burying test. Marble burying
15 is a natural behaviour that has been used to test anxiety-related and compulsive behaviour-related
16 drugs [19] and, as it is thought to be a valid model of obsessive-compulsive disorders [58], it is
17 currently used to test rodent models [59]. In accordance with our previous findings obtained using
18 15-day periods of WDW [16], the number of marbles buried by the mice in both groups was
19 significantly larger 60 days post-WDW than at baseline, and significantly higher in the mice exposed
20 to e-CIG. The behaviour tended to recover after 90 days, as did the difference between the two
21 groups. These findings suggest that compulsiveness is important during nicotine WDW and warrant
22 further investigation in nicotine WDW models.

23
24
25
26
27
28
29
30 Depression-like behaviour and anhedonia were evaluated by means of the tail suspension and
31 sucrose preference tests, respectively, appeared after 30 days of WDW in the e-CIG group and 60
32 days in the CIG group, and persisted until day 90. This is in line with the findings of previous studies
33 that recorded an increase in the duration of immobility in mice after two hours [60] and 15 days of
34 nicotine WDW [61]; similarly, the forced swimming test has revealed depression-like behaviour in
35 rodents 1-60 days after nicotine WDW [62-66].

36
37
38
39
40 There are no published data concerning the onset of anhedonia in Balb/c mice during nicotine WDW.
41 However, one study has found that high nicotine doses of 40 mg/kg/day for 28 days increased the
42 intracranial self-stimulation threshold for four days during spontaneous nicotine WDW in C57BL/6J
43 mice but not Balb/c mice [67], and another study of C57BL/6J mice found a reduction in sucrose
44 preference on the first day after the removal of minipumps delivering 12 or 24 mg/kg/day of nicotine
45 that continued for four days [14]. The differences in the time of onset and the course of the depressive
46 symptoms are probably due to the different routes of administration used (osmotic pumps or
47 parenteral administration) and doses (12, 24 or 40 mg/kg/day).

48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65
66
67
68
69
70
71
72
73
74
75
76
77
78
79
80
81
82
83
84
85
86
87
88
89
90
91
92
93
94
95
96
97
98
99
100
101
102
103
104
105
106
107
108
109
110
111
112
113
114
115
116
117
118
119
120
121
122
123
124
125
126
127
128
129
130
131
132
133
134
135
136
137
138
139
140
141
142
143
144
145
146
147
148
149
150
151
152
153
154
155
156
157
158
159
160
161
162
163
164
165
166
167
168
169
170
171
172
173
174
175
176
177
178
179
180
181
182
183
184
185
186
187
188
189
190
191
192
193
194
195
196
197
198
199
200
201
202
203
204
205
206
207
208
209
210
211
212
213
214
215
216
217
218
219
220
221
222
223
224
225
226
227
228
229
230
231
232
233
234
235
236
237
238
239
240
241
242
243
244
245
246
247
248
249
250
251
252
253
254
255
256
257
258
259
260
261
262
263
264
265
266
267
268
269
270
271
272
273
274
275
276
277
278
279
280
281
282
283
284
285
286
287
288
289
290
291
292
293
294
295
296
297
298
299
300
301
302
303
304
305
306
307
308
309
310
311
312
313
314
315
316
317
318
319
320
321
322
323
324
325
326
327
328
329
330
331
332
333
334
335
336
337
338
339
340
341
342
343
344
345
346
347
348
349
350
351
352
353
354
355
356
357
358
359
360
361
362
363
364
365
366
367
368
369
370
371
372
373
374
375
376
377
378
379
380
381
382
383
384
385
386
387
388
389
390
391
392
393
394
395
396
397
398
399
400
401
402
403
404
405
406
407
408
409
410
411
412
413
414
415
416
417
418
419
420
421
422
423
424
425
426
427
428
429
430
431
432
433
434
435
436
437
438
439
440
441
442
443
444
445
446
447
448
449
450
451
452
453
454
455
456
457
458
459
460
461
462
463
464
465
466
467
468
469
470
471
472
473
474
475
476
477
478
479
480
481
482
483
484
485
486
487
488
489
490
491
492
493
494
495
496
497
498
499
500
501
502
503
504
505
506
507
508
509
510
511
512
513
514
515
516
517
518
519
520
521
522
523
524
525
526
527
528
529
530
531
532
533
534
535
536
537
538
539
540
541
542
543
544
545
546
547
548
549
550
551
552
553
554
555
556
557
558
559
560
561
562
563
564
565
566
567
568
569
570
571
572
573
574
575
576
577
578
579
580
581
582
583
584
585
586
587
588
589
590
591
592
593
594
595
596
597
598
599
600
601
602
603
604
605
606
607
608
609
610
611
612
613
614
615
616
617
618
619
620
621
622
623
624
625
626
627
628
629
630
631
632
633
634
635
636
637
638
639
640
641
642
643
644
645
646
647
648
649
650
651
652
653
654
655
656
657
658
659
660
661
662
663
664
665
666
667
668
669
670
671
672
673
674
675
676
677
678
679
680
681
682
683
684
685
686
687
688
689
690
691
692
693
694
695
696
697
698
699
700
701
702
703
704
705
706
707
708
709
710
711
712
713
714
715
716
717
718
719
720
721
722
723
724
725
726
727
728
729
730
731
732
733
734
735
736
737
738
739
740
741
742
743
744
745
746
747
748
749
750
751
752
753
754
755
756
757
758
759
760
761
762
763
764
765
766
767
768
769
770
771
772
773
774
775
776
777
778
779
780
781
782
783
784
785
786
787
788
789
790
791
792
793
794
795
796
797
798
799
800
801
802
803
804
805
806
807
808
809
810
811
812
813
814
815
816
817
818
819
820
821
822
823
824
825
826
827
828
829
830
831
832
833
834
835
836
837
838
839
840
841
842
843
844
845
846
847
848
849
850
851
852
853
854
855
856
857
858
859
860
861
862
863
864
865
866
867
868
869
870
871
872
873
874
875
876
877
878
879
880
881
882
883
884
885
886
887
888
889
890
891
892
893
894
895
896
897
898
899
900
901
902
903
904
905
906
907
908
909
910
911
912
913
914
915
916
917
918
919
920
921
922
923
924
925
926
927
928
929
930
931
932
933
934
935
936
937
938
939
940
941
942
943
944
945
946
947
948
949
950
951
952
953
954
955
956
957
958
959
960
961
962
963
964
965
966
967
968
969
970
971
972
973
974
975
976
977
978
979
980
981
982
983
984
985
986
987
988
989
990
991
992
993
994
995
996
997
998
999
1000

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

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

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

21 Crf is locally produced by GABAergic interneurons of the hippocampal fields CA1 and CA3 [74], and
22 acts on CrfR1 expressed by pyramidal neurons [75]. The activity of this system is stimulated by
23 stressful conditions and other physiological states, and promotes synaptic plasticity in dendritic
24 pyramidal cells and their conversion from a thin to mushroom-like morphology. This change is typical
25 of synaptic learning and is accompanied by the insertion of AMPA receptors into the synapse and
26 long-term potentiation (LTP) [76]. Activation of the hippocampal Crf system (for example, by means
27 of a local injection of Crf) supports hippocampal functions including memory formation [75], and the
28 selective inactivation of Crf interneurons impairs object recognition memory [77]. An acute increase
29 in Crf release induces CrfR1-dependent hippocampal plasticity, whereas the prolonged activation of
30 Crf transmission leads to dendritic retraction, loss of LTP, and memory impairment [75]. In parallel
31 with the late decline in hippocampal Crf and CrfR1 mRNA levels, we also observed a significant
32 decrease in the levels of AMPA receptor subunits and scaffold protein PSD 95 that was not apparent
33 at the end of CIG or e-CIG exposure or nine days after cessation, but only developed after 60 and
34 90 days of nicotine WDW. The changes in AMPA receptors were not due to a general neuronal
35 damage as NMDA receptor levels were in the control range.

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

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

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.

5. References

- [1] S.M. Colby, S.T. Tiffany, S. Shiffman, R.S. Niaura, Are adolescent smokers dependent on nicotine? A review of the evidence, *Drug Alcohol Depend* 59 Suppl 1 (2000) S83-95.
- [2] I. McLaughlin, J.A. Dani, M. De Biasi, Nicotine withdrawal, *Curr Top Behav Neurosci* 24 (2015) 99-123.
- [3] J.R. Hughes, P.W. Callas, Prevalence of withdrawal symptoms from electronic cigarette cessation: A cross-sectional analysis of the US Population Assessment of Tobacco and Health, *Addict Behav* 91 (2019) 234-237.
- [4] G. St Helen, N. Nardone, N. Addo, D. Dempsey, C. Havel, P. Jacob, 3rd, N.L. Benowitz, Differences in nicotine intake and effects from electronic and combustible cigarettes among dual users, *Addiction* 115(4) (2020) 757-767.
- [5] T.M. Piasecki, M.C. Fiore, T.B. Baker, Profiles in discouragement: two studies of variability in the time course of smoking withdrawal symptoms, *J Abnorm Psychol* 107(2) (1998) 238-51.
- [6] A. Neuhaus, M. Bajbouj, T. Kienast, P. Kalus, D. von Haebler, G. Winterer, J. Gallinat, Persistent dysfunctional frontal lobe activation in former smokers, *Psychopharmacology (Berl)* 186(2) (2006) 191-200.
- [7] A.J. Fallgatter, W.K. Strik, Right frontal activation during the continuous performance test assessed with near-infrared spectroscopy in healthy subjects, *Neurosci Lett* 223(2) (1997) 89-92.
- [8] G. McCarthy, A. Puce, R.T. Constable, J.H. Krystal, J.C. Gore, P. Goldman-Rakic, Activation of human prefrontal cortex during spatial and nonspatial working memory tasks measured by functional MRI, *Cereb Cortex* 6(4) (1996) 600-11.
- [9] R.Z. Goldstein, N.D. Volkow, Drug addiction and its underlying neurobiological basis: neuroimaging evidence for the involvement of the frontal cortex, *Am J Psychiatry* 159(10) (2002) 1642-52.
- [10] T.M. Piasecki, D.E. Jorenby, S.S. Smith, M.C. Fiore, T.B. Baker, Smoking withdrawal dynamics: III. Correlates of withdrawal heterogeneity, *Exp Clin Psychopharmacol* 11(4) (2003) 276-85.
- [11] D.H. Malin, J.R. Lake, P. Newlin-Maultsby, L.K. Roberts, J.G. Lanier, V.A. Carter, J.S. Cunningham, O.B. Wilson, Rodent model of nicotine abstinence syndrome, *Pharmacol Biochem Behav* 43(3) (1992) 779-84.
- [12] S.D. Grabus, B.R. Martin, S.E. Brown, M.I. Damaj, Nicotine place preference in the mouse: influences of prior handling, dose and strain and attenuation by nicotinic receptor antagonists, *Psychopharmacology (Berl)* 184(3-4) (2006) 456-63.
- [13] E.D. Holliday, T.J. Gould, Chronic Nicotine Treatment During Adolescence Attenuates the Effects of Acute Nicotine in Adult Contextual Fear Learning, *Nicotine Tob Res* 19(1) (2017) 87-93.
- [14] Y. Alkhlaif, D. Bagdas, A. Jackson, A.J. Park, I.M. Damaj, Assessment of nicotine withdrawal-induced changes in sucrose preference in mice, *Pharmacol Biochem Behav* 161 (2017) 47-52.

- 1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65
- [15] M. Shoaib, L. Bizarro, Deficits in a sustained attention task following nicotine withdrawal in rats, *Psychopharmacology (Berl)* 178(2-3) (2005) 211-22.
- [16] L. Ponzoni, M. Moretti, M. Sala, F. Fasoli, V. Mucchietto, V. Lucini, G. Cannazza, G. Gallesi, C.N. Castellana, F. Clementi, M. Zoli, C. Gotti, D. Braidà, Different physiological and behavioural effects of e-cigarette vapour and cigarette smoke in mice, *Eur Neuropsychopharmacol* 25(10) (2015) 1775-86.
- [17] Y. Abreu-Villaca, V.M.S. Guimaraes, A. Nunes-Freitas, A.C. Dutra-Tavares, A.C. Manhaes, C.C. Filgueiras, A. Ribeiro-Carvalho, Tobacco smoke and ethanol during adolescence: Both combined- and single-drug exposures lead to short- and long-term disruption of the serotonergic system in the mouse brain, *Brain Res Bull* 146 (2019) 94-103.
- [18] R. Arantes, J. Tejada, G.G. Bosco, S. Morato, A.C. Roque, Mathematical methods to model rodent behavior in the elevated plus-maze, *J Neurosci Methods* 220(2) (2013) 141-8.
- [19] G. de Brouwer, A. Fick, B.H. Harvey, W. Wolmarans, A critical inquiry into marble-burying as a preclinical screening paradigm of relevance for anxiety and obsessive-compulsive disorder: Mapping the way forward, *Cogn Affect Behav Neurosci* 19(1) (2019) 1-39.
- [20] J.A. Bouwknecht, Behavioral studies on anxiety and depression in a drug discovery environment: keys to a successful future, *Eur J Pharmacol* 753 (2015) 158-76.
- [21] H. Anisman, K. Matheson, Stress, depression, and anhedonia: caveats concerning animal models, *Neurosci Biobehav Rev* 29(4-5) (2005) 525-46.
- [22] D. Braidà, A. Donzelli, R. Martucci, L. Ponzoni, A. Pauletti, A. Langus, M. Sala, Mice discriminate between stationary and moving 2D shapes: application to the object recognition task to increase attention, *Behav Brain Res* 242 (2013) 95-101.
- [23] J.W. Kenney, M.D. Adoff, D.S. Wilkinson, T.J. Gould, The effects of acute, chronic, and withdrawal from chronic nicotine on novel and spatial object recognition in male C57BL/6J mice, *Psychopharmacology (Berl)* 217(3) (2011) 353-65.
- [24] A.W. Bruijnzeel, M.S. Gold, The role of corticotropin-releasing factor-like peptides in cannabis, nicotine, and alcohol dependence, *Brain Res Brain Res Rev* 49(3) (2005) 505-28.
- [25] J.M. Deussing, A. Chen, The Corticotropin-Releasing Factor Family: Physiology of the Stress Response, *Physiol Rev* 98(4) (2018) 2225-2286.
- [26] M.J. Henckens, J.M. Deussing, A. Chen, Region-specific roles of the corticotropin-releasing factor-urocortin system in stress, *Nat Rev Neurosci* 17(10) (2016) 636-51.
- [27] N. Dedic, A. Chen, J.M. Deussing, The CRF Family of Neuropeptides and their Receptors - Mediators of the Central Stress Response, *Curr Mol Pharmacol* 11(1) (2018) 4-31.
- [28] F. Pistillo, F. Clementi, M. Zoli, C. Gotti, Nicotinic, glutamatergic and dopaminergic synaptic transmission and plasticity in the mesocorticolimbic system: focus on nicotine effects, *Prog Neurobiol* 124 (2015) 1-27.

- 1 [29] R.A. Bevins, J. Besheer, Object recognition in rats and mice: a one-trial non-matching-to-sample
2 learning task to study 'recognition memory', *Nat Protoc* 1(3) (2006) 1306-11.
- 3 [30] L. Steru, R. Chermat, B. Thierry, P. Simon, The tail suspension test: a new method for screening
4 antidepressants in mice, *Psychopharmacology (Berl)* 85(3) (1985) 367-70.
- 5 [31] M. Kundakovic, S. Lim, K. Gudsnuk, F.A. Champagne, Sex-specific and strain-dependent
6 effects of early life adversity on behavioral and epigenetic outcomes, *Front Psychiatry* 4 (2013)
7 78.
- 8 [32] S.R. Lewis, S. Ahmed, C. Dym, E. Khaimova, B. Kest, R.J. Bodnar, Inbred mouse strain survey
9 of sucrose intake, *Physiol Behav* 85(5) (2005) 546-56.
- 10 [33] T. Strekalova, N. Gorenkova, E. Schunk, O. Dolgov, D. Bartsch, Selective effects of citalopram
11 in a mouse model of stress-induced anhedonia with a control for chronic stress, *Behav Pharmacol*
12 17(3) (2006) 271-87.
- 13 [34] S. Hogg, A review of the validity and variability of the elevated plus-maze as an animal model
14 of anxiety, *Pharmacol Biochem Behav* 54(1) (1996) 21-30.
- 15 [35] J.R. Turner, L.M. Castellano, J.A. Blendy, Nicotinic partial agonists varenicline and sazetidine-
16 A have differential effects on affective behavior, *J Pharmacol Exp Ther* 334(2) (2010) 665-72.
- 17 [36] F. Pistillo, F. Fasoli, M. Moretti, T. McClure-Begley, M. Zoli, M.J. Marks, C. Gotti, Chronic nicotine
18 and withdrawal affect glutamatergic but not nicotinic receptor expression in the mesocorticolimbic
19 pathway in a region-specific manner, *Pharmacol Res* 103 (2016) 167-76.
- 20 [37] J. Mapelli, D. Gandolfi, A. Vilella, M. Zoli, A. Bigiani, Heterosynaptic GABAergic plasticity
21 bidirectionally driven by the activity of pre- and postsynaptic NMDA receptors, *Proc Natl Acad Sci*
22 U S A 113(35) (2016) 9898-903.
- 23 [38] L. Carboni, B. Romoli, S.T. Bate, P. Romualdi, M. Zoli, Increased expression of CRF and CRF-
24 receptors in dorsal striatum, hippocampus, and prefrontal cortex after the development of nicotine
25 sensitization in rats, *Drug Alcohol Depend* 189 (2018) 12-20.
- 26 [39] K.J. Livak, T.D. Schmittgen, Analysis of relative gene expression data using real-time
27 quantitative PCR and the 2(-Delta Delta C(T)) Method, *Methods* 25(4) (2001) 402-8.
- 28 [40] S.T. Bate, R.A. Clark, *The design and statistical analysis of animal experiments*. Cambridge
29 University Press (2014).
- 30 [41] R.A. Clark, M. Shoab, K.N. Hewitt, S.C. Stanford, S.T. Bate, A comparison of InVivoStat with
31 other statistical software packages for analysis of data generated from animal experiments, *J*
32 *Psychopharmacol* 26(8) (2012) 1136-42.
- 33 [42] R.A. Wise, G.F. Koob, The development and maintenance of drug addiction,
34 *Neuropsychopharmacology* 39(2) (2014) 254-62.
- 35 [43] J. Margham, K. McAdam, M. Forster, C. Liu, C. Wright, D. Mariner, C. Proctor, Chemical
36 Composition of Aerosol from an E-Cigarette: A Quantitative Comparison with Cigarette Smoke,
37 *Chem Res Toxicol* 29(10) (2016) 1662-1678.

- 1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65
- [44] S.G. Matta, D.J. Balfour, N.L. Benowitz, R.T. Boyd, J.J. Buccafusco, A.R. Caggiula, C.R. Craig, A.C. Collins, M.I. Damaj, E.C. Donny, P.S. Gardiner, S.R. Grady, U. Heberlein, S.S. Leonard, E.D. Levin, R.J. Lukas, A. Markou, M.J. Marks, S.E. McCallum, N. Parameswaran, K.A. Perkins, M.R. Picciotto, M. Quik, J.E. Rose, A. Rothenfluh, W.R. Schafer, I.P. Stolerman, R.F. Tyndale, J.M. Wehner, J.M. Zirger, Guidelines on nicotine dose selection for in vivo research, *Psychopharmacology (Berl)* 190(3) (2007) 269-319.
- [45] M.A. Kaiser, R.R. Kallem, R.K. Sajja, A.E. Sifat, L. Cucullo, A convenient UHPLC-MS/MS method for routine monitoring of plasma and brain levels of nicotine and cotinine as a tool to validate newly developed preclinical smoking model in mouse, *BMC Neurosci* 18(1) (2017) 71.
- [46] M.A. Russell, M. Jarvis, R. Iyer, C. Feyerabend, Relation of nicotine yield of cigarettes to blood nicotine concentrations in smokers, *Br Med J* 280(6219) (1980) 972-6.
- [47] L. Schock, M. Schwenzer, W. Sturm, K. Mathiak, Alertness and visuospatial attention in clinical depression, *BMC Psychiatry* 11 (2011) 78.
- [48] K.K. Higa, A. Grim, M.E. Kamenski, J. van Enkhuizen, X. Zhou, K. Li, J.C. Naviaux, L. Wang, R.K. Naviaux, M.A. Geyer, A. Markou, J.W. Young, Nicotine withdrawal-induced inattention is absent in alpha7 nAChR knockout mice, *Psychopharmacology (Berl)* 234(9-10) (2017) 1573-1586.
- [49] E.D. Levin, F.J. McClernon, A.H. Rezvani, Nicotinic effects on cognitive function: behavioral characterization, pharmacological specification, and anatomic localization, *Psychopharmacology (Berl)* 184(3-4) (2006) 523-39.
- [50] C.S. Myers, R.C. Taylor, E.T. Moolchan, S.J. Heishman, Dose-related enhancement of mood and cognition in smokers administered nicotine nasal spray, *Neuropsychopharmacology* 33(3) (2008) 588-98.
- [51] F. Patterson, C. Jepson, A.A. Strasser, J. Loughhead, K.A. Perkins, R.C. Gur, J.M. Frey, S. Siegel, C. Lerman, Varenicline improves mood and cognition during smoking abstinence, *Biol Psychiatry* 65(2) (2009) 144-9.
- [52] R.L. Ashare, M. Falcone, C. Lerman, Cognitive function during nicotine withdrawal: Implications for nicotine dependence treatment, *Neuropharmacology* 76 Pt B (2014) 581-91.
- [53] J. Morud, J. Strandberg, A. Andren, M. Ericson, B. Soderpalm, L. Adermark, Progressive modulation of accumbal neurotransmission and anxiety-like behavior following protracted nicotine withdrawal, *Neuropharmacology* 128 (2018) 86-95.
- [54] C.S. Pomerleau, O.F. Pomerleau, The effects of a psychological stressor on cigarette smoking and subsequent behavioral and physiological responses, *Psychophysiology* 24(3) (1987) 278-85.
- [55] R. West, P. Hajek, What happens to anxiety levels on giving up smoking?, *Am J Psychiatry* 154(11) (1997) 1589-92.

- 1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65
- [56] G.F. Koob, Antireward, compulsivity, and addiction: seminal contributions of Dr. Athina Markou to motivational dysregulation in addiction, *Psychopharmacology (Berl)* 234(9-10) (2017) 1315-1332.
- [57] M.S. Bello, R.D. Pang, G.S. Chasson, L.A. Ray, A.M. Leventhal, Obsessive-compulsive symptoms and negative affect during tobacco withdrawal in a non-clinical sample of African American smokers, *J Anxiety Disord* 48 (2017) 78-86.
- [58] P. Alonso, C. Lopez-Sola, E. Real, C. Segalas, J.M. Menchon, Animal models of obsessive-compulsive disorder: utility and limitations, *Neuropsychiatr Dis Treat* 11 (2015) 1939-55.
- [59] A.K. Kraeuter, P.C. Guest, Z. Sarnyai, Object Burying Test for Assessment of Obsessive Compulsive Behaviors in Mice, *Methods Mol Biol* 1916 (2019) 81-85.
- [60] T. Hayase, Differential effects of TRPV1 receptor ligands against nicotine-induced depression-like behaviors, *BMC Pharmacol* 11 (2011) 6.
- [61] M. Motaghinejad, S. Fatima, M. Karimian, S. Ganji, Protective effects of forced exercise against nicotine-induced anxiety, depression and cognition impairment in rat, *J Basic Clin Physiol Pharmacol* 27(1) (2016) 19-27.
- [62] Z. Bagosi, M. Palotai, B. Simon, P. Bokor, A. Buzas, B. Balango, D. Pinter, M. Jaszberenyi, K. Csabafi, G. Szabo, Selective CRF2 receptor agonists ameliorate the anxiety- and depression-like state developed during chronic nicotine treatment and consequent acute withdrawal in mice, *Brain Res* 1652 (2016) 21-29.
- [63] G. Biala, P. Polak, A. Michalak, M. Kruk-Slomka, B. Budzynska, Influence of calcium channel antagonists on nonsomatic signs of nicotine and D-amphetamine withdrawal in mice, *Pharmacol Rep* 66(2) (2014) 212-22.
- [64] R. Chellian, V. Pandey, Protective effect of alpha-asarone against nicotine-induced seizures in mice, but not by its interaction with nicotinic acetylcholine receptors, *Biomed Pharmacother* 108 (2018) 1591-1595.
- [65] N.R. Kotagale, C.T. Chopde, M.J. Umekar, B.G. Taksande, Chronic agmatine treatment prevents behavioral manifestations of nicotine withdrawal in mice, *Eur J Pharmacol* 754 (2015) 190-8.
- [66] M.A. Roni, S. Rahman, The effects of lobeline on nicotine withdrawal-induced depression-like behavior in mice, *Psychopharmacology (Berl)* 231(15) (2014) 2989-98.
- [67] A.K. Stoker, S. Semenova, A. Markou, Affective and somatic aspects of spontaneous and precipitated nicotine withdrawal in C57BL/6J and BALB/cByJ mice, *Neuropharmacology* 54(8) (2008) 1223-32.
- [68] A. Cohen, J. Treweek, S. Edwards, R.M. Leao, G. Schulteis, G.F. Koob, O. George, Extended access to nicotine leads to a CRF1 receptor dependent increase in anxiety-like behavior and hyperalgesia in rats, *Addict Biol* 20(1) (2015) 56-68.

- 1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65
- [69] A. Buzas, P. Bokor, B. Balango, D. Pinter, M. Palotai, B. Simon, K. Csabafi, G. Telegdy, G. Szabo, Z. Bagosi, Changes in striatal dopamine release and locomotor activity following acute withdrawal from chronic nicotine are mediated by CRF1, but not CRF2, receptors, *Brain Res* 1706 (2019) 41-47.
- [70] T.E. Grieder, M.A. Herman, C. Contet, L.A. Tan, H. Vargas-Perez, A. Cohen, M. Chwalek, G. Maal-Bared, J. Freiling, J.E. Schlosburg, L. Clarke, E. Crawford, P. Koebel, V. Repunte-Canonigo, P.P. Sanna, A.R. Tapper, M. Roberto, B.L. Kieffer, P.E. Sawchenko, G.F. Koob, D. van der Kooy, O. George, VTA CRF neurons mediate the aversive effects of nicotine withdrawal and promote intake escalation, *Nat Neurosci* 17(12) (2014) 1751-8.
- [71] R. Zhao-Shea, S.R. DeGroot, L. Liu, M. Vallaster, X. Pang, Q. Su, G. Gao, O.J. Rando, G.E. Martin, O. George, P.D. Gardner, A.R. Tapper, Increased CRF signalling in a ventral tegmental area-interpeduncular nucleus-medial habenula circuit induces anxiety during nicotine withdrawal, *Nat Commun* 6 (2015) 6770.
- [72] A.W. Bruijnzeel, G. Zislis, C. Wilson, M.S. Gold, Antagonism of CRF receptors prevents the deficit in brain reward function associated with precipitated nicotine withdrawal in rats, *Neuropsychopharmacology* 32(4) (2007) 955-63.
- [73] X. Qi, Z. Shan, Y. Ji, V. Guerra, J.C. Alexander, B.K. Ormerod, A.W. Bruijnzeel, Sustained AAV-mediated overexpression of CRF in the central amygdala diminishes the depressive-like state associated with nicotine withdrawal, *Transl Psychiatry* 4 (2014) e385.
- [74] A. Hooper, J. Maguire, Characterization of a novel subtype of hippocampal interneurons that express corticotropin-releasing hormone, *Hippocampus* 26(1) (2016) 41-53.
- [75] Y. Chen, A.L. Andres, M. Frotscher, T.Z. Baram, Tuning synaptic transmission in the hippocampus by stress: the CRH system, *Front Cell Neurosci* 6 (2012) 13.
- [76] J.N. Bourne, K.M. Harris, Balancing structure and function at hippocampal dendritic spines, *Annu Rev Neurosci* 31 (2008) 47-67.
- [77] A. Hooper, P.M. Fuller, J. Maguire, Hippocampal corticotropin-releasing hormone neurons support recognition memory and modulate hippocampal excitability, *PLoS One* 13(1) (2018) e0191363.
- [78] P.M. Maras, T.Z. Baram, Sculpting the hippocampus from within: stress, spines, and CRH, *Trends Neurosci* 35(5) (2012) 315-24.
- [79] N. Li, R.J. Liu, J.M. Dwyer, M. Banasr, B. Lee, H. Son, X.Y. Li, G. Aghajanian, R.S. Duman, Glutamate N-methyl-D-aspartate receptor antagonists rapidly reverse behavioral and synaptic deficits caused by chronic stress exposure, *Biol Psychiatry* 69(8) (2011) 754-61.
- [80] A.J. Kallarackal, M.D. Kvarata, E. Cammarata, L. Jaber, X. Cai, A.M. Bailey, S.M. Thompson, Chronic stress induces a selective decrease in AMPA receptor-mediated synaptic excitation at hippocampal temporoammonic-CA1 synapses, *J Neurosci* 33(40) (2013) 15669-74.

- 1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
- [81] V. Duric, M. Banasr, C.A. Stockmeier, A.A. Simen, S.S. Newton, J.C. Overholser, G.J. Jurjus, L. Dieter, R.S. Duman, Altered expression of synapse and glutamate related genes in post-mortem hippocampus of depressed subjects, *Int J Neuropsychopharmacol* 16(1) (2013) 69-82.
- [82] L.F. Chun, F. Moazed, C.S. Calfee, M.A. Matthay, J.E. Gotts, Pulmonary toxicity of e-cigarettes, *Am J Physiol Lung Cell Mol Physiol* 313(2) (2017) L193-L206.
- [83] A. Merecz-Sadowska, P. Sitarek, H. Zielinska-Blizniewska, K. Malinowska, K. Zajdel, L. Zakonnik, R. Zajdel, A Summary of In Vitro and In Vivo Studies Evaluating the Impact of E-Cigarette Exposure on Living Organisms and the Environment, *Int J Mol Sci* 21(2) (2020).
- [84] L. Ponzoni, M. Moretti, D. Braidà, M. Zoli, F. Clementi, P. Viani, M. Sala, C. Gotti, Increased sensitivity to Delta(9)-THC-induced rewarding effects after seven-week exposure to electronic and tobacco cigarettes in mice, *Eur Neuropsychopharmacol* 29(4) (2019) 566-576.

34
35
36
37
38

Declaration of Competing Interest

The authors declare no competing financial interest

39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

Funding

Grant by the University of Bologna (RFO 2015) to LC. Grant “Dipartimenti di eccellenza 2018-2022, MIUR, Italy” to the Department of Biomedical, Metabolic and Neural Sciences”.

D.B. was recipient of Fondazione Zardi-Gori fellowship

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<0.05vs corresponding CIG group (Bonferroni *post hoc* test).

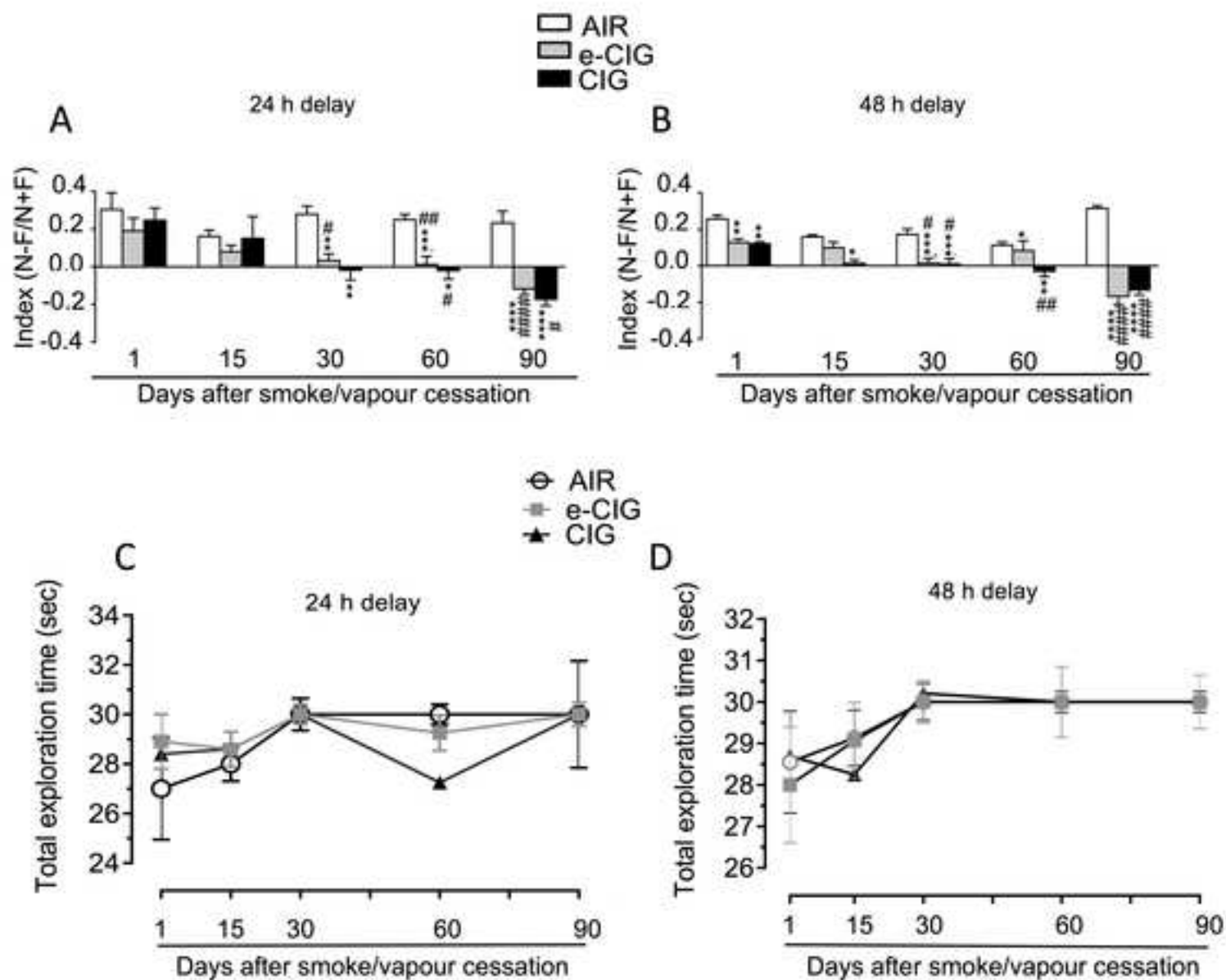
Figure 5. Analysis of AMPAR subunit and PSD 95 protein levels in the hippocampus of mice exposed to AIR, CIG or e-CIG after one hour (n=8), nine (n=8), 60(n=7) and 90(n=7) days. Proteins were separated on 7.5 % acrylamide SDS gels, electrotransferred to nitrocellulose, and probed with antibodies as described 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

5 **Figure 6.** Relative mRNA expression of Crf (A) or CrfR1 (B) in mouse hippocampus after e-CIG or
6 CIG exposure. Mice were exposed to e-CIG, CIG or AIR for 7 weeks and euthanised after 1h or after
7 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
8 or CIG vs. AIR.
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

VIRTUAL OBJECT RECOGNITION

Figure 1



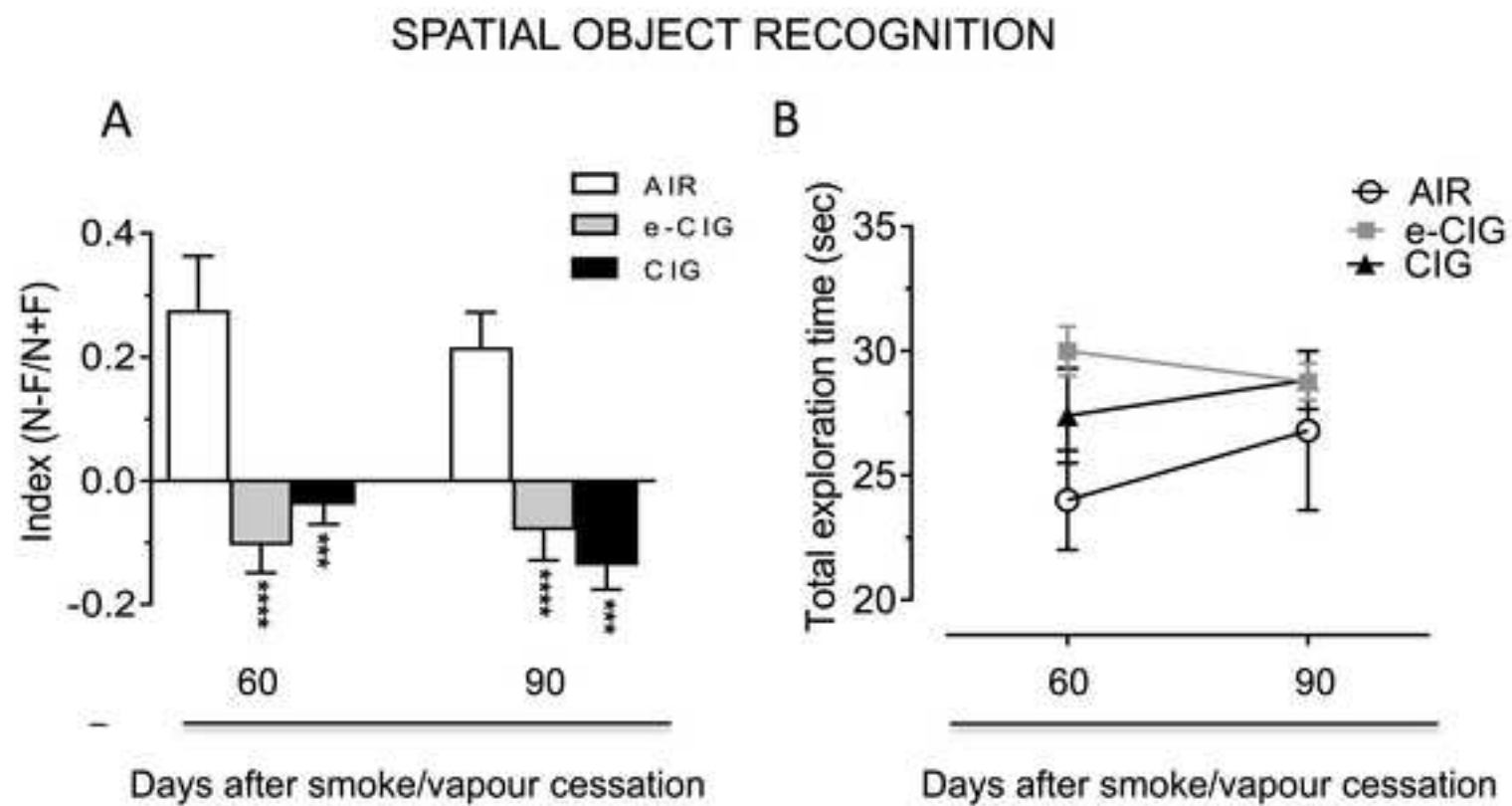


Figure 3

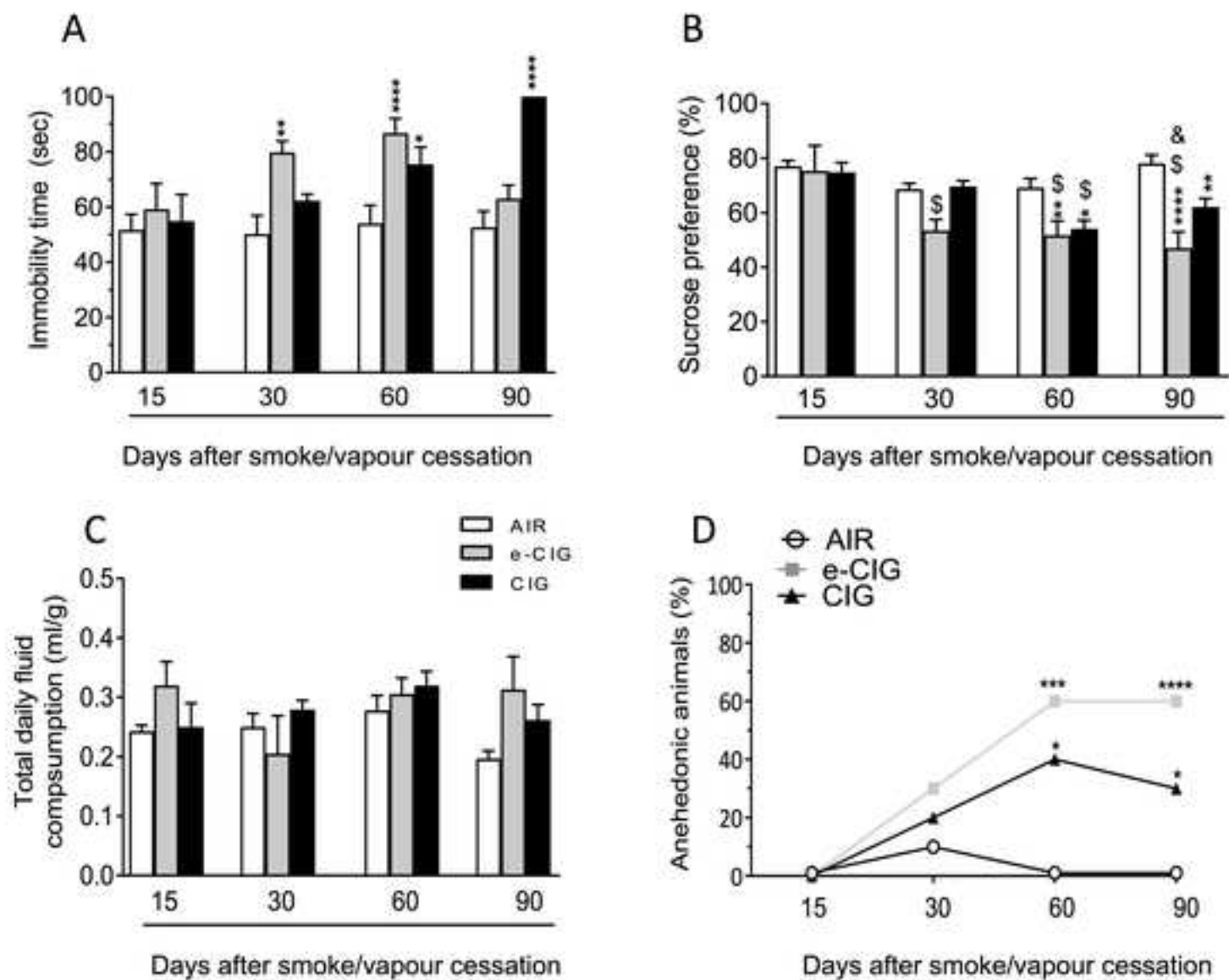
TAIL SUSPENSION AND
SUCROSE PREFERENCE TEST

Figure 4

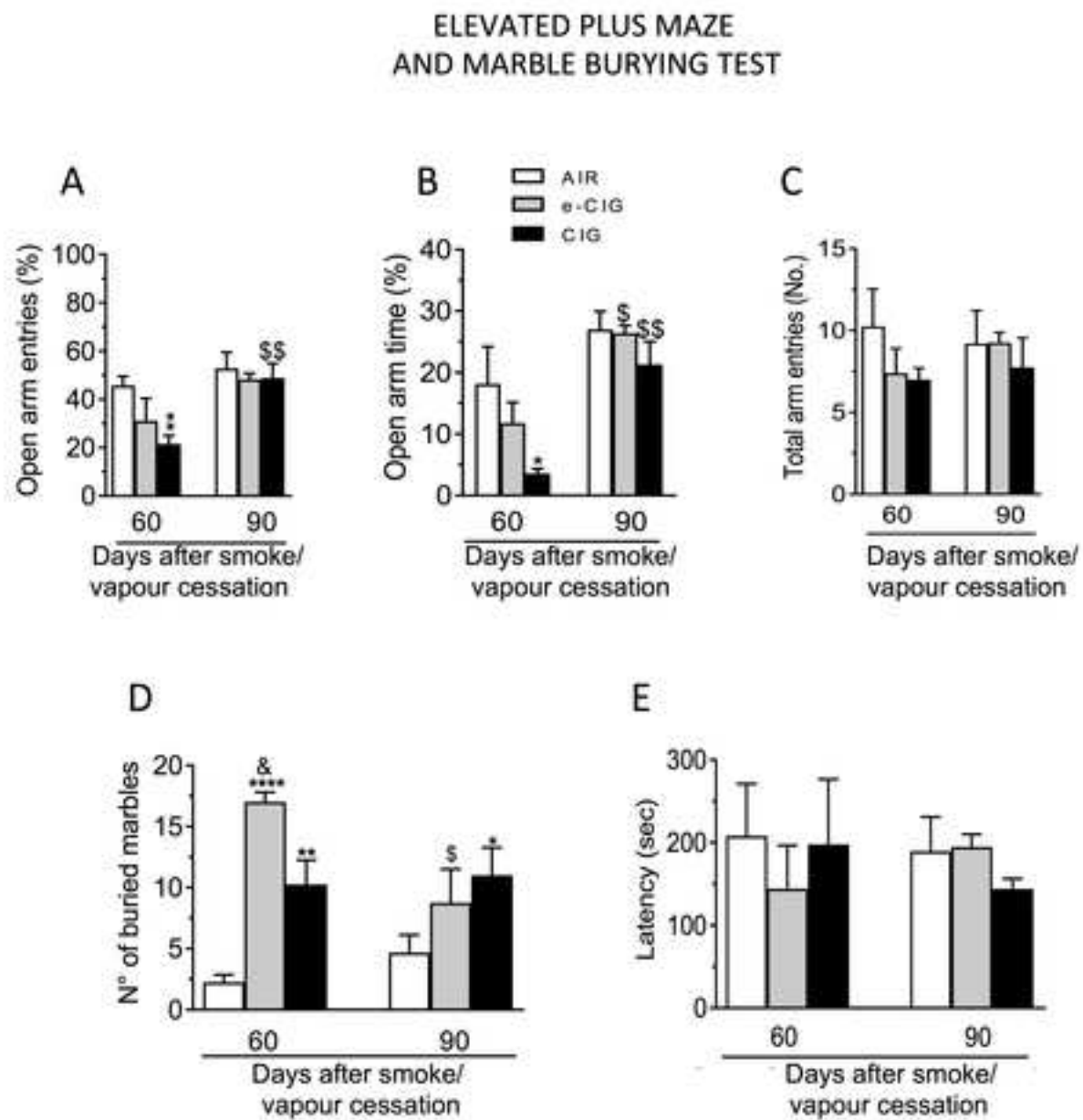


Figure 5

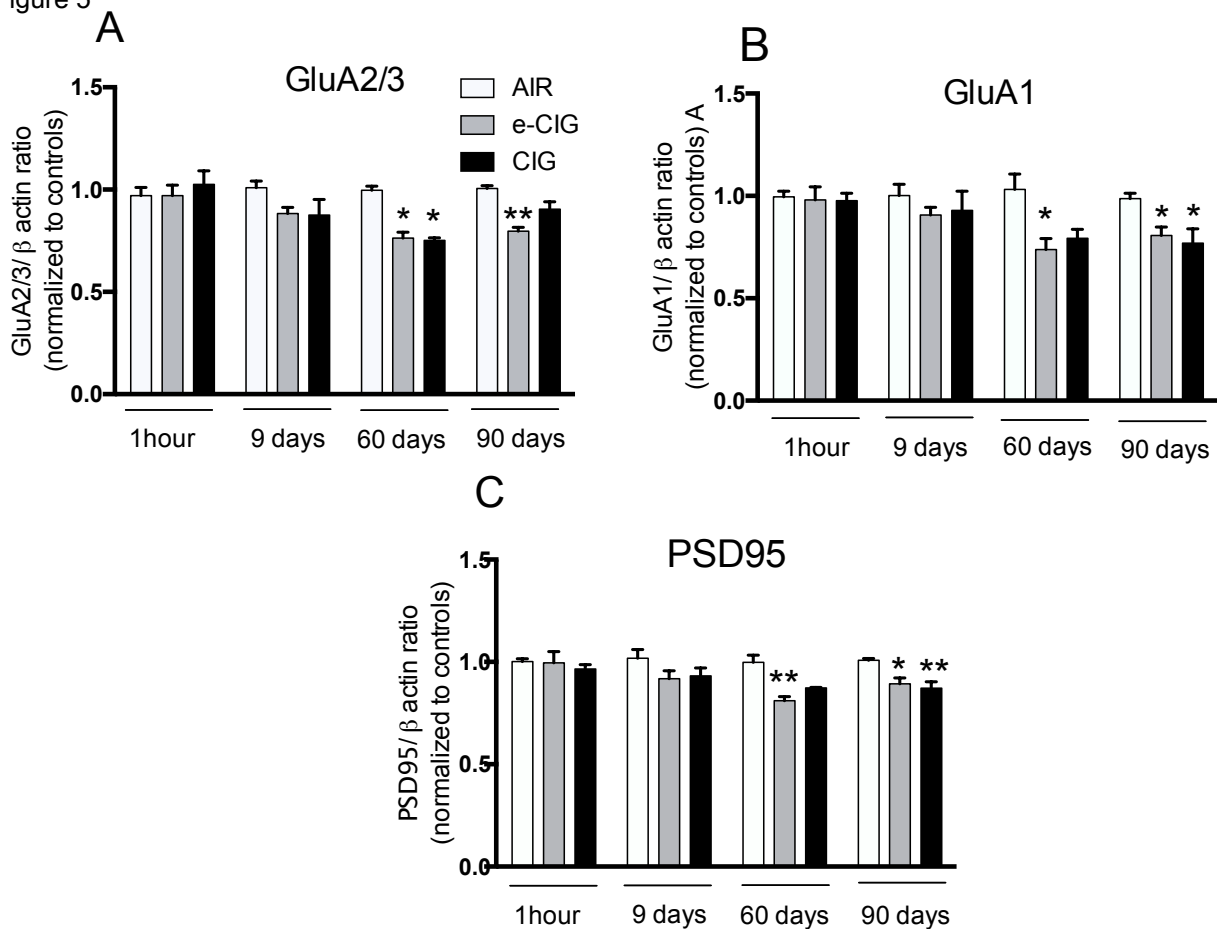
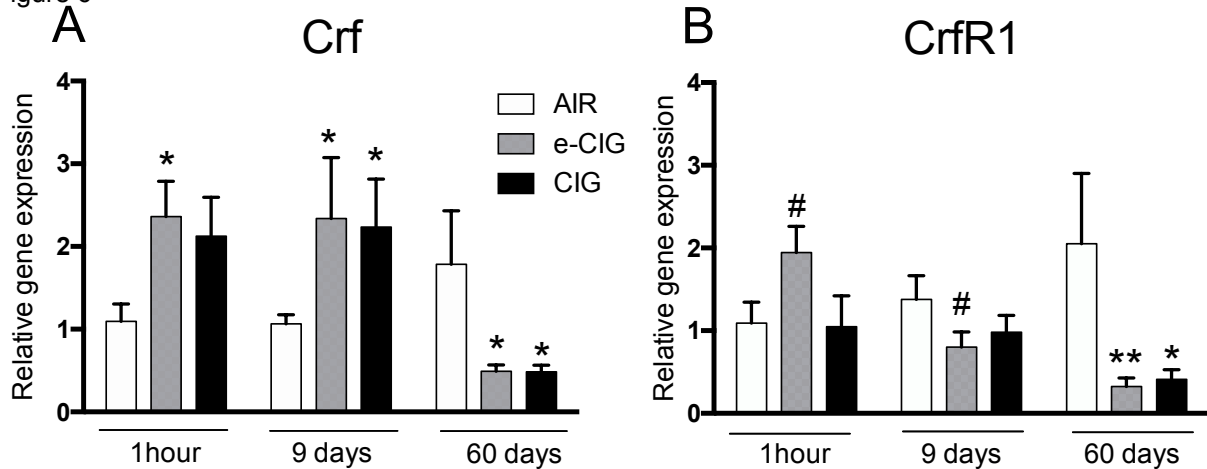


Figure 6



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.



Click here to access/download
Supplementary Material
Supplementary Ponzoni et al.docx

