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Different physiological and behavioural effects of e-cigarette vapour and cigarette smoke in mice / Ponzoni, L; Moretti, M; Sala, M; Fasoli, F; Mucchietto, V; Lucini, V; Cannazza, Giuseppe; Gallesi, G; Castellana, Carmela Nives; Clementi, F; Zoli, Michele; Gotti, C; Braidà, D.. - In: EUROPEAN NEUROPSYCHOPHARMACOLOGY. - ISSN 0924-977X. - STAMPA. - 25:10(2015), pp. 1775-1786. [10.1016/j.euroneuro.2015.06.010]

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19/10/2024 15:39

Manuscript Number: ENP-15-66R1

Title: Different physiological and behavioural effects of e-cigarette vapour and cigarette smoke in mice

Article Type: Research article

Section/Category: BS - Basic Science

Keywords: nicotine; nicotinic receptors; cigarette smoke; electronic cigarettes; withdrawal

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Abstract: Nicotine is the primary addictive substance in tobacco smoke and electronic cigarette (e-cig) vapour. Methodological limitations have made it difficult to compare the role of the nicotine and non-nicotine constituents of tobacco smoke. The aim of this study was to compare the effects of traditional cigarette smoke and e-cig vapour containing the same amount of nicotine in male BALB/c mice exposed to the smoke of 21 cigarettes or e-cig vapour containing 16.8 mg of nicotine delivered by means of a mechanical ventilator for three 30-minutes sessions/day for seven weeks. One hour after the last session, half of the animals were sacrificed for neurochemical analysis, and the others underwent mecamylamine-precipitated or spontaneous withdrawal for the purposes of behavioural analysis. Chronic intermittent non-contingent, second-hand exposure to cigarette smoke or e-cig vapour led to similar brain cotinine and nicotine levels, similar urine cotinine levels and the similar up-regulation of $\alpha 4\beta 2$ nicotinic acetylcholine receptors in different brain areas, but had different effects on body weight, food intake, and the signs of mecamylamine-precipitated and spontaneous withdrawal episodic memory and emotional responses. The findings of this study demonstrate for the first time that e-cig vapour induces addiction-related neurochemical, physiological and behavioural alterations. The fact that inhaled cigarette smoke and e-cig vapour have partially different dependence-related effects indicates that compounds other than nicotine contribute to tobacco dependence.



National Research Council of Italy



NEUROSCIENCE INSTITUTE

Pisa Padova Milano Cagliari

Milano

Prof. Andreas Meyer-Lindenberg

Editor-in-Chief

European Neuropsychopharmacology

Milan, May 25, 2015

Dear Prof. Andreas Meyer-Lindenberg

,

Thank you for considering acceptable, after a minor revision, our manuscript entitled "Different physiological and behavioural effects of e-cigarette vapour and cigarette smoke in mice." by Ponzoni L. , et al. We have taken the Reviewer#1 further comments into account in revising the final version of the manuscript.

In the manuscript you will find, in red our corrections.

We do hope that you will now find the new version of the paper suitable for publication in the European Neuropsychopharmacology and look forward to hearing from you in due course.

Yours faithfully,

Dr Cecilia Gotti



NEUROSCIENCE INSTITUTE

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Comments of reviewer

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Different physiological and behavioural effects of e-cigarette vapour and cigarette smoke in mice.

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[°] Recipients of fellowships from the Fondazione Fratelli Confalonieri, Milan

RUNNING TITLE: Electronic/standard cigarette smoking and withdrawal

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Abstract

Nicotine is the primary addictive substance in tobacco smoke and electronic cigarette (e-cig) vapour. Methodological limitations have made it difficult to compare the role of the nicotine and non-nicotine constituents of tobacco smoke. The aim of this study was to compare the effects of traditional cigarette smoke and e-cig vapour containing the same amount of nicotine in male BALB/c mice exposed to the smoke of 21 cigarettes or e-cig vapour containing 16.8 mg of nicotine delivered by means of a mechanical ventilator for three 30-minutes sessions/day for seven weeks. One hour after the last session, half of the animals were sacrificed for neurochemical analysis, and the others underwent mecamylamine-precipitated or spontaneous withdrawal for the purposes of behavioural analysis. Chronic intermittent non-contingent, second-hand exposure to cigarette smoke or e-cig vapour led to similar brain cotinine and nicotine levels, similar urine cotinine levels and the similar up-regulation of $\alpha 4\beta 2$ nicotinic acetylcholine receptors in different brain areas, but had different effects on body weight, food intake, and the signs of mecamylamine-precipitated and spontaneous withdrawal episodic memory and emotional responses. The findings of this study demonstrate for the first time that e-cig vapour induces addiction-related neurochemical, physiological and behavioural alterations. The fact that inhaled cigarette smoke and e-cig vapour have partially different dependence-related effects indicates that compounds other than nicotine contribute to tobacco dependence.

Key words: nicotine; nicotinic receptors; cigarette smoke; electronic cigarettes, mecamylamine-precipitated withdrawal; spontaneous withdrawal

1. Introduction

Tobacco smoking is responsible for more than five million deaths a year throughout the world. **Prolonged exposure** to inhaled tobacco smoke is associated with various types of cancer, bronchitis, emphysema and cardiovascular diseases, and the leading preventable cause of premature deaths, in western countries. Among the more than 5000 components of tobacco smoke (Rodgman et al. 2000), nicotine is thought to be primarily responsible for the development and maintenance of tobacco smoking, the withdrawal (WDW) symptoms associated with its discontinuation (Grabus et al. 2006, Hughes 2007), and the relapses that frequently occur during attempts at cessation (Piasecki et al. 2001). However, non-nicotine factors also seem to contribute to smoking reward and reinforcement (Rose, 2006). Many smokers have recently switched to e-cigarettes (e-cig) as an alternative means of nicotine delivery because they look, feel and taste like traditional cigarettes. Dawkins et al. (2012, 2013) found that smokers using e-cig had less desire to smoke, fewer symptoms associated with abstinence from tobacco, and improved prospective and working memory. Furthermore, e-cig are increasingly used as a means of reducing or stopping smoking despite the contrary recommendation of the World Health Organization (World Health Organization, 2008).

The most widely used means of examining the effects of chronic nicotine administration on animals are osmotic mini-pumps, repeated peripheral injections, i.v. self-administration and, more recently, cigarette smoke exposure (Matta et al. 2007). It has also been shown that administering nicotine vapour similar to that inhaled when smoking e-cig leads to blood nicotine levels in rats that are equivalent to those observed in smokers, and makes rats nicotine dependent (George et al. 2010). Passive exposure to cigarette smoke or aqueous tobacco extracts induces addiction in rodents although methodological limitations have made it difficult to assess the respective roles of nicotine and non-nicotine constituents (Brennan et al. 2014).

The vast majority of the behavioural effects of nicotine are due to its interaction with nicotinic acetylcholine receptors (nAChRs), a heterogeneous class of ligand-gated cation channels that are widely expressed in the brain (Gotti et al., 2009). High nicotine concentrations activate nAChRs, but the lower concentrations observed in the blood of regular smokers desensitise most

heteromeric nAChRs and induce a long-term increase in their number known as up-regulation (reviewed in Picciotto et al., 2003; Colombo et al., 2013). Chronic nicotine use leads to neuroadaptations in various brain areas that are thought to promote and sustain the use of tobacco, whereas nicotine WDW (or smoking cessation in humans), disrupts the equilibrium reached in the presence of nicotine and induces unpleasant sensation (reviewed in Paolini and De Biasi, 2011; Pistillo et al., 2014). Humans experience a WDW syndrome whose severity closely correlates to smoking relapse, that emerges within hours the cessation of cigarette smoking. Nicotine WDW in rodents is characterised by similar somatic signs and affective changes (including increased anxiety, anhedonia and irritability) (reviewed in Paolini and De Biasi, 2011).

The aim of this study was to validate a rodent model of e-cig exposure that induces high urinary levels of cotinine (the major nicotine metabolite) and mimics the intermittence and route of nicotine administration in humans. In order to compare the effects of inhaled cigarette smoke and e-cig vapour containing the same amount of nicotine, we developed a system based on previous nicotine inhalation research (George et al., 2010) that allows the vaporised liquid contained in commercial e-cigs to be introduced into a smoke chamber and compared its neurochemical (nicotine and cotinine brain levels, nAChR up-regulation), physiological (body weight and food intake) and behavioural (memory function and emotional profile after mecamylamine precipitated and spontaneous WDW) effects with those induced by cigarette smoke.

2. Experimental procedures

2.1. Animals

One hundred and eighty three month-old male BALB/cJ mice (Charles River, Calco, Como) were group housed (five mice per cage) in a humidity and constant 21°C temperature-controlled animal facility on a 12h/12h light/dark cycle (lights on at at 8:00 a.m.), with *ad libitum* access to food and water. The cob-bedding was changed weekly.

This strain was selected on the basis of recent findings in our Department (Dr Lucini unpublished data) showing that these animals develop respiratory diseases after the same smoke exposure as that used in this study.

All of the experimental procedures respected the guidelines established by the Italian Council on Animal Care, and were approved by Italian Government Decree No. 28/2013. Every effort was made to minimise the number of mice used and their suffering. Figure 1a shows a flow chart of the study.

2.2.Exposure to cigarette and e-cig

One week after their arrival, the mice were divided into three groups of 30 mice each and transferred to perspex inhalation chambers (22 cm wide x 40 cm long x 20 cm high) connected to a mechanical ventilator (Rodent Ventilator, Model 7025, Ugo Basile, Biological Research Instruments, Varese, Italy) delivering the smoke or air, for three 30-minute 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 cigarette smoke group were exposed to the smoke of 21 commercial cigarettes containing 0.8 mg of nicotine/cigarette (for a total of 16.8 mg/day), 10 mg of tar and 10 mg of carbon monoxide; the animal in the 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 others compounds (see above); and the animal in the control group were exposed only to air alone. The assessed physiological parameters were the animal body weight (g) and food intake (g/mouse/day). Food intake was calculated as total food consumption divided by the number of mice in each cage. At the end of each exposure session, the animals were removed and the inhalation chamber remained opened in order to eliminate any residual smoke or vapour through the fume hood.

2.3. Biochemical studies

2.3.1. Brain tissue dissection. One hour after the last session, mice were sacrificed, their brains were quickly removed, and different areas brain areas were carefully dissected on ice.

2.3.2. Brain and urinary cotinine/nicotine. Urine samples were collected from animals housed overnight in metabolic cages, in groups of five at the end of the first, fourth and seventh week. The urine samples were centrifuged, frozen at -20°C until assay. Urinary cotinine concentration was measured using an enzyme immunoassay method DRI® Cotinine Assay on CDx90 analyser (ThermoFisher Scientific, distributed by Tema Ricerca, BO, Italy). The detection limit was 34 ng/ml. and the measurement range 34-2000 ng/ml.

Brain nicotine and cotinine levels were quantitatively determined using LC-MS/MS as previously described (Vieira-Brock et al, 2011), with minor modifications. The Liquid chromatography was performed using an HP 1200 system (Agilent Technologies, Germany), equipped with a Discovery HS-F5 column (150 mm \times 2.1 mm, 3 μm) (Supelco, Milan, Italy). The LC system was coupled by an electrospray ion source operated in positive mode to an Agilent 6410 triple quadrupole-mass spectrometer for quantitative analysis and to an Agilent 6520 Accurate-Mass Q-TOF for qualitative analysis. The measurements were made separately using samples of ventral tegmental area, prefrontal cortex and olfactory bulb.

2.3.3. Antibody production and characterization. The subunit-specific polyclonal antibodies (Abs) used have been previously described Grady et al. (2009). For the immunoprecipitation experiments the affinity purified Abs (4mg/ ml of wet resin) were cross-linked to Protein A SepharoseTM CL-4B (GE Healthcare) by means of 20 mM dimethyl pimelidate (Thermo Scientific) according to manufacturer instructions.

2.3.4. Binding studies. Membranes and 2% Triton X-100 extracts from: hippocampus, nucleus accumbens, caudate-putamen, habenula and interpeduncular nucleus were prepared as previously described (Moretti et al. 2010).

In each experiment, the areas from five mice from each experimental group were pooled and homogenized. ^{125}I - α Bungarotoxin (α Bgtx) and ^3H -epibatidine (Epi) binding to total brain homogenate was performed as previously described (Moretti et al. 2010). ^3H -Epi binding to nAChR present in 2% Triton X-100 extracts obtained from brain areas was assessed using ^3H -Epi that binds to multiple heteromeric nAChR subtypes with pM affinity and to $\alpha 7$ -nAChR with nM affinity. In order to ensure that the $\alpha 7$ -containing subtypes did not contribute to ^3H -Epi binding solubilised receptors (present in the extract and immunoprecipitation experiments) were first incubated for 3 hours with 1 μM cold α Bgtx (Tocris, Bristol, UK) which specifically binds to $\alpha 7$ -nAChR (and thus prevents ^3H -Epi binding to these sites). Extracts were labelled with 2 nM [^3H]-Epi at 4°C and following overnight incubation receptors were captured using DEAE-SepharoseTM Fast flow (GE Healthcare, Uppsala, Sweden). The bound receptors were eluted with 1N NaOH and after addition of the scintillation mixture (filter count, GE Healthcare, Uppsala, Sweden) counted in a beta counter. Non-specific binding (averaging 5-10% of total binding) was determined in parallel samples containing 100 nM unlabelled Epi.

^3H -Epi (s.a. 50-66 Ci/mmol) and ^{125}I - α Bgtx, (s.a. 200 Ci/mmol) were purchased from PerkinElmer (Boston, USA) and non-radioactive ligands from Tocris (Bristol, UK)

2.3.5. Immunoprecipitation of ^3H -Epibatidine-labelled receptors by subunit-specific antibodies. The tissue extracts, were preincubated with 2 μM α Bgtx, labelled with 2 nM ^3H -Epi, and incubated overnight with a saturating concentration of affinity purified anti-subunit IgG (10 μg) bound to Sepharose-ProteinA (GE Healthcare, Italy). The immunoprecipitation was recovered by centrifugation. The level of Ab immunoprecipitation was expressed as as fmol of immunoprecipitated receptors/mg of protein and is the mean \pm SEM of 3-5 experiments for each treatment (air, e-cig, cigarette).

2.4. Behavioural studies

2.4.1. Mecamylamine-precipitated nicotine WDW. Immediately after the last air, smoke or e-cig exposure, all the animals received mecamylamine hydrochloride s.c. (1 mg/kg) (Sigma-Aldrich, MO, USA), and were individually placed inside a circular clear plastic observation cylinder according to Castanê et al. (2002) in order to observe the signs and symptoms of abstinence for 30 min after injection, after 10 min acclimatisation. The evaluated abstinence signs were: locomotor activity, wet dog shakes, front paw tremors, sniffing and scratches and the symptoms were: body tremor, ptosis, wet dog shakes, teeth chattering, paw tremor, scratching, genital licks, sniffing and piloerection . The number of wet dog shakes, front paw tremors, sniffs and scratches was counted. Ptosis, genital licks, body tremor, piloerection and teeth chattering were scored 1 for appearance or 0 for non-appearance within each period of 5 min. A global withdrawal score was calculated for each animal by giving each individual sign a relative weight of 0.5 for each episode of wet dog shaking, front paw tremor, sniffing and scratching, and 1 for the presence of ptosis, genital lick, tremor, piloerection and teeth chattering during each 5 min observation. In order to verify whether mecamylamine *per se* has any effect on mice unexposed to smoke or e-cig, two groups of five of treatment naïve mice were injected with mecamylamine or vehicle following the protocol described above.

Motor activity (Sala et al. 2013) was evaluated in an automated activity cage for 15 min immediately after treatment in different groups of animals.

2.4.2. Spontaneous WDW. 24 hours, 15 and 30 days after the last air, smoke or e-cig exposure, different groups of mice underwent the following behavioural tasks:

2.4.2.1. Spontaneous motor activity. Horizontal movements were evaluated for 15 min as described above.

2.4.2.2. Spatial object recognition. Object location was carried in an arena. The test was carried out according to Kenney et al. (2011) with slight modifications. Two visual cues were placed on two adjacent walls of an opaque white Plexiglas cage (58×50×43 cm) and dimly lit from above (27 lx) the cage: a black and white striped pattern (21×19.5 cm) was affixed to the centre of the northern

wall and a black and gray checkered pattern (26.5×20 cm) was placed at the center of the western wall. Two sets of identical objects were used in the experiments: 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 objects were counterbalanced across locations. The cage and all objects were thoroughly wiped down with acetic acid 0.1% before and after all of the behavioural procedures which were observed and recorded by means of a camera mounted above the cage. Exploration was defined as a mouse having its nose directed toward the object within approximately 1 cm (Bevins and Besheer, 2006). Climbing or sitting on objects was not scored as object exploration. Mice that did not spend more than a total of 30 s exploring the objects on training or testing were excluded from analysis.

During the last treatment day, the mice were pre-exposed to the cage for 10 min. Twenty four hours later, returned to the cage and allowed to explore the two different objects placed in the NE and NW corners; the time spent exploring the objects was recorded. Forty eight hours later the mice were re-exposed to the cage with the object that has been previously more explored, moved to the SW corner.

2.4.2.3. Elevated Plus Maze. Anxiety was evaluated using the elevated plus maze test as previously described (Baird et al. 2007). The apparatus consisted of two opposite open arms (35 x 10 cm) and two enclosed arms (35 x 10 cm) extending from a common central platform (10 x 10 cm). The animals were moved to the plus maze laboratory 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 by a trained observer unaware of the treatments, 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 (Hogg, 1996). The total closed-arm entries were analysed as measures of non-specific changes in locomotor activity.

2.4.2.4. Marble Burying Test. The marble burying test utilizes spontaneous digging behaviour, characteristic of rodents, to assess anxiety-like/compulsive behaviour (Turner et al. 2010). After acclimation (1 h), each mouse was placed in a cage (26 x 20 x14 cm), in which 20 marbles had been equally distributed on top of mouse bedding (5 cm in depth). After 15 min, the number of buried marbles and the latency to the first marble burying were counted.

2.5. Statistical Analyses

Data were expressed as mean \pm SEM. Different groups were assessed by one or two-way analyses of variance (ANOVAs) for repeated measures or Kruskal Wallis test for multiple non parametric comparisons followed by Tukey's, Bonferroni or Dunn's post-hoc test. The accepted value for significance was $p < 0.05$. All statistical analyses were done using the software Prism, version 6 (GraphPad, San Diego, CA, USA).

3. Results

3.1. E-cigarette vapour does not affect body weight and food intake

Smoking lowers body weight, which makes many people reluctant to quit smoking (Chiolero et al. 2008). Weight gain and food intake were evaluated weekly during the 7-week of exposure to cigarette smoke or e-cig vapour and for 1 month after their cessation (Figure 1b and 1c). For body weight there was an effect of treatment ($F_{(2, 216)} = 29.09$, $p < 0.0001$), an effect of time ($F_{(7, 216)} = 112.80$, $p < 0.0001$), and a treatment x time interaction ($F_{(14, 216)} = 1.92$, $p < 0.05$). A significant reduction of body weight in mice exposed to cigarette smoke starting from day 14, was shown. During cessation there was a difference in body weight gain for treatment ($F_{(2, 216)} = 5.33$, $p < 0.0001$), time ($F_{(7, 216)} = 72.4$, $p < 0.0001$), and treatment x time interaction ($F_{(14, 216)} = 2.46$, $p = 0.025$). A difference of body weight was found only during the first day after WDW. For food intake there was an effect of treatment ($F_{(2, 216)} = 12.79$, $p = 0.0002$), time ($F_{(7, 216)} = 11.48$, $p < 0.0001$), but not treatment x time interaction ($F_{(14, 216)} = 1.68$, $p = 0.12$) during smoke/vapour exposure. A

significant reduction of food intake in mice exposed to cigarette smoke, was shown starting from day 14 to day 35. After WDW there was a difference among groups. Two-way ANOVA revealed time ($F_{(2, 216)} = 6.32$, $p < 0.003$), but not treatment ($F_{(7, 216)} = 1.61$, $p < 0.12$), and no treatment x time interaction effect ($F_{(14, 216)} = 2.88$, $p = 0.48$).

3.2. Both e-cigarette vapour and cigarette smoke increase brain cotinine and nicotine levels and urinary cotinine levels

Brain nicotine (pg/mg of tissue) and cotinine (pg/mg of tissue concentrations) were evaluated at the end of 7-week exposure, and found to be very similar between e-cig vapour and cigarette smoke exposed mice and significantly different from those of mice exposed to air (nicotine: $F_{(2,6)} = 17.18$, $p = 0.0023$; cotinine: $F_{(2,6)} = 14.19$, $p = 0.0053$;) (Fig 1d and 1e). Urinary cotinine levels have been widely used to assess active or passive exposure to cigarette smoke (Matta et al. 2007). Exposure to e-cig led to an increase of cotinine levels (pg/ml) across the 7 weeks (Figure 1f). There was an effect of treatment ($F_{(2, 81)} = 6.72$, $p = 0.003$), but not of time ($F_{(7, 81)} = 2.81$, $p = 0.07$), and of treatment x time interaction ($F_{(4, 81)} = 0.74$, $p = 0.56$). *Post-hoc* analysis revealed that across the weeks cotinine levels were significantly higher in both e-cig and cigarette exposed groups compared to air group.

3.3. Both e-cigarette and cigarette selectively up regulate $\alpha 4\beta 2$ -containing nicotinic receptors in brain areas

We performed preliminary studies to check the time course of treatment effects on the expression of ^3H -Epi labelled heteromeric receptors and ^{125}I - α Bgtx labelled homomeric receptors in total brain homogenates, and found no difference after three weeks and significant differences after seven weeks (data not shown), that was chosen for further studies.

We found that in cortex, hippocampus, nucleus accumbens and caudate-putamen there was a

significant difference in ^3H -Epi binding to the heteromeric receptors between the experimental and control groups (Figure 2 and Table1). Immunoprecipitation experiments showed that this difference was due to a significant increase in $\alpha 4\beta 2$ -containing receptors in e-cig and cigarette groups with no effects on $\alpha 6$ -containing receptor expressed in all four areas or on the $\alpha 6$ -containing receptors selectively expressed in the nucleus accumbens and caudate-putamen. We finally analysed whether exposure to e-cig or cigarette had any effects on the expression of the receptors in habenula and interpeduncular nucleus two areas that express a very high level of $\alpha 3\beta 4$ -containing receptors. As shown in Figure 2e and 2f we did not detect any significant effect.

3.4. Mecamylamine-precipitated withdrawal after e-cigarette is less severe compared to that of cigarette

The effect of 7-week exposure to e-cig or cigarette on mecamylamine-precipitated WDW is reported in Figure 3. A significant treatment effect on the development of signs ($F_{(2,27)} = 8.15$, $p = 0.001$, one-way ANOVA) and symptoms ($\chi^2 = 8.45$, $p = 0.01$) (Figure 3a), was shown. There was a significant increase in the mean score of signs and symptoms in mice exposed to cigarette, while only in the mean score of symptoms in mice exposed to e-cig. There was a different severity of nicotine-WDW syndrome ($F_{(2,27)} = 5.89$, $p = 0.01$). Global score was higher in cigarette exposed mice compared to e-cig exposed mice (Figure 3b) ($\chi^2 = 10.15$, $p = 0.006$). The severity of WDW led us to examine spontaneous motor activity. There was a difference between groups in horizontal ($F_{(2,27)} = 14.82$, $p < 0.001$) (Figure 3c) and vertical counts (Figure 3d) ($F_{(2,27)} = 10.49$, $p = 0.0001$) where only mice exposed to cigarettes showed a significant decrease. To test whether mecamylamine *per se* caused WDW-like effects we also administered mecamylamine to naïve mice. No difference was found either in the WDW global scores (saline: 1.0 ± 0.1 , mecamylamine: 1.16 ± 0.66) or in horizontal (saline: 939 ± 172 , mecamylamine: 1200 ± 74) and vertical counts (saline: 74 ± 9 , mecamylamine: 92 ± 26) between saline and mecamylamine treated naïve mice.

3.5. Spatial working memory and emotional profile are differentially impaired after spontaneous withdrawal from cigarette and e-cigarette.

Spatial working memory and emotional profile are affected during smoking or nicotine WDW both in humans (George et al. 2010; Kenny and Markou, 2001; Camfield et al. 2013) and rodents (Kenney et al. 2011; Yohn et al. 2014). Thus, we investigated whether exposure to cigarette /e-cig produced changes in spatial object recognition task, marble burying and elevated plus maze test (Figure 4) accompanied by motor impairment. ANOVA showed no difference between groups on spontaneous motor activity (treatment : $F_{(2,81)} = 0.2, p = 0.81$), time: $F_{(2,81)} = 0.13, p = 0.94$) and time x treatment: $F_{(4,81)} = 0.55, p = 0.77$) (Figure 4a). Evaluation of discrimination index (Figure 4b) revealed a treatment ($F_{(2,81)} = 71.69, p < 0.0001$) and a time x treatment effect ($F_{(4,81)} = 2.97, P = 0.02$), but no significant effect was found for time ($F_{(2,81)} = 2.81, p = 0.07$). At 24 h, mice exposed to cigarette were more impaired than those exposed to e-cig. Significant differences between groups in the number of arm entries and time in the elevated plus maze were observed (open arm entries: treatment $F_{(2,81)} = 554.30, p < 0.0001$, time $F_{(2,81)} = 6.22, p = 0.003$ and time x treatment $F_{(4,81)} = 7.29, p < 0.0001$; open arm time: treatment $F_{(2,81)} = 116.10, p < 0.0001$, time $F_{(2,81)} = 117.60, p < 0.0001$) and time x treatment effect ($F_{(4,81)} = 5.71, p = 0.0004$), (closed arm: maze entries: treatment $F_{(2,81)} = 30.50, p < 0.0001$, time $F_{(2,81)} = 1.67, p = 0.36$ and time x treatment effect $F_{(4,81)} = 1.55, p = 0.75$). No differences were found in the mean number of total arm entries (entries: treatment $F_{(2,81)} = 0.22, p = 0.90$, time $F_{(2,81)} = 0.49, p = 0.81$ and time x treatment effect $F_{(4,81)} = 1.18, p = 0.91$). The number of open entries and time in cigarette and e-cig groups were significantly lower compared to controls while the number of closed arm entries is higher (Figure 4c). Notably, in cigarette-exposed group the number of entries was always less than in e-cig group suggesting a more anxious profile.

In the marble burying test there was a difference between groups in the number of buried marbles (Figure 4d) (treatment: $F_{(2,81)} = 45.03, p < 0.0001$), time: $F_{(2,81)} = 18.82, p < 0.0001$), treatment x time interaction: $F_{(2,81)} = 45.03, p < 0.0001$). Cigarette and e-cig groups buried significant more marbles compared to air group. Notably, e-cig group showed a greater increase from 24 h to 30 days.

There was a significant main effect of treatment on the latency to the first burying ($F_{(2,81)} = 224.0$, $p < 0.0001$), a significant effect of time ($F_{(2,81)} = 6.31$, $p < 0.0001$), and a significant treatment by time interaction ($F_{(2,81)} = 12.43$, $p < 0.0001$). *Post-hoc* test revealed a significant decrease in the latency for e-cig and cigarette groups when compared to controls. Interestingly, at 30 days the e-cig group showed a significant reduction of this parameter in comparison with the cigarette group.

4. Discussion

This is the first study comparing cigarette smoke and e-cig vapour administered to mice by means of inhalation, using the same dose of nicotine for both treatments and its findings provide the first evidence that exposure to e-cig vapour has a number of effects during the period of exposure and for a long time, after exposure cessation. Previous studies (reviewed in Brennan et al. 2014) have attempted to assess possible differences in the dependence-related effects of nicotine and cigarette smoke by comparing nicotine directly administered by means of injections or osmotic minipumps with inhaled smoke or injected aqueous tobacco extracts, but their main limitations are the different methods of administration (directly administered nicotine vs. inhaled smoke) and the different compositions of aqueous tobacco extracts and inhaled smoke.

The body weight of human smokers is less than that of non-smokers (Albanes et al. 1987) and increases when they stop smoking (Pistelli et al. 2009). The mice exposed to cigarette smoke (but not those exposed to e-cig vapour) showed significantly reduced food intake and body weight over time. The effects of smoking on body weight are attributed to the nicotine in tobacco because nicotine decreases feeding in animal models (Hussmann et al. 2014), possibly as a result of its interaction with nAChRs expressed in critical hypothalamic circuits (Mineur et al. 2011). However, the fact that there was not a significant reduction in food intake and body weight in the e-cig group suggests that compounds other than the nicotine in cigarettes are involved.

Cotinine is the most useful biological marker of nicotine exposure, and its levels in the blood, urine and other tissues of smokers remain stable for long periods (Matta et al. 2007). We found very similar brain nicotine and cotinine levels and urinary cotinine levels, which were in line with those previously found in mice (Catanzaro et al. 2007) or human smokers (Jung et al. 2012).

Like cigarette smoke, e-cig vapour increased the number of $\alpha 4\beta 2$ -containing receptors in the cortex, hippocampus, nucleus accumbens and caudate putamen, but had no effect on the other subtypes expressed in these areas. This selective effect on $\alpha 4\beta 2$ receptors is probably due to the higher affinity and/or nicotine functional desensitisation of this subtype, which may be an important initial step in the up-regulation process. Experimental evidence shows that by acting as a protein

chaperone of this subtype, nicotine favours the maturation and assembly of the $\alpha\beta 2$ subtype in the endoplasmic reticulum, but other mechanisms such as decreased $\alpha\beta 2$ degradation and / or turnover (reviewed in Colombo et al. 2013) may also play a role. In agreement with the findings of other studies of animal models of chronic nicotine administration (Marks et al. 1992; Nguyen et al. 2004), as also previously reported (Marks et al. 2011) we found that neither treatments affected the expression of the $\beta 2$ -containing receptors and $\alpha 3 \beta 4$ receptors in the habenulo–interpeduncular pathway, which are critical for the signs of nicotine WDW, probably because the brain nicotine concentrations of the exposed mice were much lower than the affinity of nicotine for this subtype (Benowitz, 2009).

Withdrawal from cigarette smoke induces a wide range of signs and symptoms in human smokers such as irritability, anxiety, headache, fatigue and insomnia (Le Foll and Golberg, 2009). Accordingly, our mice exposed to cigarette smoke showed a wide range of behavioural alterations but those exposed to e-cig vapour showed less severe somatic signs of abstinence and were not affected by reduced motor function. It has also been found that rats exposed to nicotine vapour for eight hours /day for seven days show significant somatic signs of WDW 30 min after the administration of mecamylamine (George et al. 2010). The signs associated with mecamylamine precipitated WDW of e-cig exposed mice in our study were different from those observed in other studies in which CD or BALB/c mice were implanted with osmotic minipumps delivering nicotine (Castane et al. 2002; Varani et al. 2012), but this may have been due to the different route of administration and dosage.

Nicotine WDW leads to learning and memory deficits (Kenney et al. 2011) in both animals and humans (Gould and Leach, 2014). Nicotine affects spatial but not novel object recognition memory in mice (Kenney et al. 2011). Spatial working memory can be impaired in abstinent smokers (Camfield et al. 2013; Wing et al. 2011) but Domier *et al* (2007) found that the deleterious effects of nicotine on cognitive performance in nicotine-dependent subjects did not include selective attentional deficit. The worsened discrimination index which was not accompanied by motor deficit in either group, suggests a selective spatial memory impairment. Nicotine WDW disrupts hippocampus-dependent but not hippocampus-independent learning in mice (Gould and Leach,

2014). Previous studies have demonstrated that nicotine acts on $\alpha 4\beta 2$ but not $\alpha 7$ nAChRs in the dorsal hippocampus and disrupts learning during WDW (Gould and Leach, 2014) and this may be worsened by the $\alpha 4\beta 2$ up-regulation observed in both of our treatment groups.

Increased anxiety is associated with smoking cessation in humans (Hughes, 2007) and mice (Stoker et al., 2008). Increased anxiety-like behaviour in comparison with controls (air group) was found during spontaneous WDW in both treatment groups (fewer entries and shorter times in the open arms) but the effect was significantly greater in the mice exposed to cigarette smoke. The compulsion to smoke is considered to be a central feature of cigarette smoke dependence and it has been found that smokers are more likely to have obsessive compulsive disorders and phobias than non-smokers (Bruijnzeel, 2012). The marble burying test is considered a test of compulsive activity (Albelda and Joel 2012), and so the increased number of marbles buried in the e-cig group indicates that e-cig WDW increases highly repetitive/perseverative responses (Thomas et al. 2009) more than cigarette.

In conclusion, the findings of this study show that chronic intermittent non-contingent exposure to e-cig vapour or cigarette smoke has the same effects on brain nicotine and cotinine concentrations and nAChR up-regulation, but cigarette smoke leads to more severe mecamylamine-precipitated WDW and more evident cognitive deficit 24 hours after cessation, whereas e-cig vapour elicits more severe anxiety and compulsive behaviour. This different profile may be attributable to the presence of compounds and/or metabolites that are present in cigarette smoke but not in e-cig vapour and may affect brain function. Further studies will be necessary to investigate the effect of 7-week exposure of non-nicotinic constituents of standard cigarettes and e-cigs, testing denicotinized cigarettes and e-cigs without nicotine, respectively

Although e-cigs are rapidly gaining acceptance as smoking cessation aids it is necessary to consider their possible long-term effects on anxiety, and their differential addictive liability in subjects with specific psychological traits (e.g., compulsiveness). It is worth noting that a recent clinical trial did not show any significant improvement in the cigarette smoking cessation using e-cigs in comparison with other less addictive methods of nicotine delivery (Bullen et al. 2013).

Role of funding source

Funding for this study was provided by Fondazione Zardi Gori (“Drug Abuse and Related diseases” Project, 2014) and the CNR Research Project on Aging, Regione Lombardia Projects NUTEC ID 30263049 and MbMM-convenzione n°18099/RCC (CG), by grant from the Italian Ministry of Health RF2009-1549619 (MZ). The Fondazione Zardi Gori, the CNR and Ministry of Health had no further role in study design, in the collection, analysis and interpretation of data, in the writing of the report

Disclosure

Participated in research design: Braida D., Sala M., Zoli M., Clementi F. and Gotti C.

Conducted experiments: Moretti M., Lucini V., Fasoli F., Muchietto V. and Ponzoni L

Performed data analysis: Moretti M., Lucini V., Fasoli F., Muchietto V. and Ponzoni L

Cotinine and nicotine analysis: Gallesi G., Castellana CN., and Cannazza G.

Wrote or contributed to writing of the manuscript: Braida D., Sala M., M Zoli M., Clementi F. and Gotti C.

All authors contributed to and have approved the final manuscript.

Conflict of interest

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.

Acknowledgements

We thank Dr. Massimo Bruno for the chemical identification of e-cig components.

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Figure legends

Figure 1. a) Experimental design. The animals were exposed for seven weeks to e-cig vapour, cigarette smoke or air. One hour after the last exposure during the third and seventh week of treatment, animals from the different groups of animals were sacrificed for binding studies. At the end of the seventh week, one hour after the last exposure, different groups (air, e-cig, cigarette) of animals were injected with mecamylamine and immediately tested for precipitated withdrawal syndrome. Further groups were submitted to memory and emotional tasks from 1 to 30 days after smoke/vapour exposure cessation.

b-f) Exposure for 7 weeks to nicotine differentially altered body weight, food intake and cotinine/nicotine concentrations in brain and urines. Body weight (b) and food intake (c) were measured 3 times a week. Values represent mean \pm SEM of 3 weekly recordings (N = 10 for each group). (d-e) Brain cotinine (d) and nicotine (e) levels evaluated at the end of the seventh week. (N = 6 for each group). Urine cotinine levels (f) evaluated during the first, the fourth and the seventh week in mice exposed to smoke of e-cig, cigarette, or air. (N = 5 for each group). Results are expressed as mean \pm SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ compared to corresponding air groups; \$ $p < 0.05$, \$\$\$ $p < 0.001$ compared to corresponding air and e-cig groups; # compared to corresponding e-cig and cigarette groups (Bonferroni or Tukey's test).

Figure 2. Immunoprecipitation analysis of the subunit content of extracts prepared from different brain areas of air, e-cig or cigarette exposed mice. Extracts of the cortex (a), hippocampus (b), nucleus accumbens (c), caudate-putamen (d), habenula (e) or interpeduncular nucleus (f) prepared from air, e-cig and cigarette exposed mice were labelled with 2 nM ^3H -Epi and immunoprecipitated by prot A-antibody (using saturating concentrations (10 μg) of anti-subunit antibodies as described in Experimental procedures . Antibodies directed against two separate peptides of the same subunits were used in the case of the $\alpha 4$, $\alpha 5$, $\alpha 6$ and $\beta 2$ subunits (see Grady et al., 2009). The amount immunoprecipitated by each antibody was subtracted from the value obtained in control samples containing an identical concentration of normal rabbit IgG, and the results are expressed as fmol of immunoprecipitated labelled ^3H -Epi nAChR/mg of protein. Mean

values \pm SEM of 3-4 experiments. For statistical comparison, data were analysed using one way ANOVA followed by Bonferroni's post-hoc * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ compared to corresponding air groups.

Figure 3. Mecamylamine (MEC) precipitated withdrawal syndrome from nicotine is milder in mice exposed to e-cig vapour than in those exposed to cigarette smoke. **(a)** Abstinence signs (wet dog shakes, front paw tremors, sniffing and scratches) and symptoms (body tremor, ptosis, wet dog shakes, teeth chattering, paw tremor, scratching, genital licks, sniffing and piloerection) were evaluated every 5 min for 30 min after injection. **(b)** Global withdrawal score was calculated by giving to each individual sign a relative weight of 0.5 and 1 for the presence of each symptom during each observation. **(c)** Horizontal and (d) vertical motor activity were evaluated in a different group of animals for 15 min immediately after MEC treatment using an automated activity cage and expressed in terms of number of horizontal counts. * $p < 0.05$, ** $p < 0.01$ vs corresponding air group; \$\$ $p < 0.01$, \$\$\$ $p < 0.001$ vs corresponding air and e-cig group (Tukey's test). Values represent mean \pm SEM.

Figure 4. During spontaneous withdrawal syndrome evaluated at 24 h, 15 and 30 days, animals exposed to e-cig vapour showed cognitive deficit and anxiety-like behaviour after spontaneous withdrawal. **(a)** Motor activity, in terms of total number of horizontal counts was evaluated for 30 min. **(b)** Spatial object recognition was carried out interposing a delay of 48 hours between T_1 and T_2 and expressed in terms of discrimination index. **** $p < 0.0001$ compared to air group; ° $p < 0.05$ compared to corresponding cigarette group. **(c)** Percentage of open arm (top left) and closed arm (bottom, left) entries, open arm time (top right) and total number of entries (bottom right) evaluated in the elevated plus maze task. * $p < 0.05$, ** $p < 0.01$ **** $p < 0.0001$ compared to corresponding air group; ° $p < 0.05$, °° $p < 0.01$ compared with corresponding cigarette group; § $p < 0.0001$ compared to corresponding 15 and 30 days; & $p < 0.05$ compared to corresponding 15 days. **(d)** Number of marbles buried (left) and latency to the first burial (right) evaluated within 15 min, in the marble burying test. *** $p < 0.001$, **** $p < 0.0001$ compared with corresponding e-cig

and cigarette group; °°°° $p < 0.0001$ compared with corresponding cigarette group; && $p < 0.01$ compared with corresponding 15 days; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$, #### $p < 0.0001$ vs corresponding 24 hours group (Bonferroni *post-hoc* test). Values represent mean \pm SEM. N=10 for each group.

Different physiological and behavioural effects of e-cigarette vapour and cigarette smoke in mice.

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Abstract

Nicotine is the primary addictive substance in tobacco smoke and electronic cigarette (e-cig) vapour. Methodological limitations have made it difficult to compare the role of the nicotine and non-nicotine constituents of tobacco smoke. The aim of this study was to compare the effects of traditional cigarette smoke and e-cig vapour containing the same amount of nicotine in male BALB/c mice exposed to the smoke of 21 cigarettes or e-cig vapour containing 16.8 mg of nicotine delivered by means of a mechanical ventilator for three 30-minutes sessions/day for seven weeks. One hour after the last session, half of the animals were sacrificed for neurochemical analysis, and the others underwent mecamylamine-precipitated or spontaneous withdrawal for the purposes of behavioural analysis. Chronic intermittent non-contingent, second-hand exposure to cigarette smoke or e-cig vapour led to similar brain cotinine and nicotine levels, similar urine cotinine levels and the similar up-regulation of $\alpha 4\beta 2$ nicotinic acetylcholine receptors in different brain areas, but had different effects on body weight, food intake, and the signs of mecamylamine-precipitated and spontaneous withdrawal episodic memory and emotional responses. The findings of this study demonstrate for the first time that e-cig vapour induces addiction-related neurochemical, physiological and behavioural alterations. The fact that inhaled cigarette smoke and e-cig vapour have partially different dependence-related effects indicates that compounds other than nicotine contribute to tobacco dependence.

Key words: nicotine; nicotinic receptors; cigarette smoke; electronic cigarettes, mecamylamine-precipitated withdrawal; spontaneous withdrawal

1. Introduction

Tobacco smoking is responsible for more than five million deaths a year throughout the world. **Prolonged exposure** to inhaled tobacco smoke is associated with various types of cancer, bronchitis, emphysema and cardiovascular diseases, and the leading preventable cause of premature deaths, in western countries. Among the more than 5000 components of tobacco smoke (Rodgman et al. 2000), nicotine is thought to be primarily responsible for the development and maintenance of tobacco smoking, the withdrawal (WDW) symptoms associated with its discontinuation (Grabus et al. 2006, Hughes 2007), and the relapses that frequently occur during attempts at cessation (Piasecki et al. 2001). However, non-nicotine factors also seem to contribute to smoking reward and reinforcement (Rose, 2006). Many smokers have recently switched to e-cigarettes (e-cig) as an alternative means of nicotine delivery because they look, feel and taste like traditional cigarettes. Dawkins et al. (2012, 2013) found that smokers using e-cig had less desire to smoke, fewer symptoms associated with abstinence from tobacco, and improved prospective and working memory. Furthermore, e-cig are increasingly used as a means of reducing or stopping smoking despite the contrary recommendation of the World Health Organization (World Health Organization, 2008).

The most widely used means of examining the effects of chronic nicotine administration on animals are osmotic mini-pumps, repeated peripheral injections, i.v. self-administration and, more recently, cigarette smoke exposure (Matta et al. 2007). It has also been shown that administering nicotine vapour similar to that inhaled when smoking e-cig leads to blood nicotine levels in rats that are equivalent to those observed in smokers, and makes rats nicotine dependent (George et al. 2010). Passive exposure to cigarette smoke or aqueous tobacco extracts induces addiction in rodents although methodological limitations have made it difficult to assess the respective roles of nicotine and non-nicotine constituents (Brennan et al. 2014).

The vast majority of the behavioural effects of nicotine are due to its interaction with nicotinic acetylcholine receptors (nAChRs), a heterogeneous class of ligand-gated cation channels that are widely expressed in the brain (Gotti et al., 2009). High nicotine concentrations activate nAChRs, but the lower concentrations observed in the blood of regular smokers desensitise most

heteromeric nAChRs and induce a long-term increase in their number known as up-regulation (reviewed in Picciotto et al., 2003; Colombo et al., 2013). Chronic nicotine use leads to neuroadaptations in various brain areas that are thought to promote and sustain the use of tobacco, whereas nicotine WDW (or smoking cessation in humans), disrupts the equilibrium reached in the presence of nicotine and induces unpleasant sensation (reviewed in Paolini and De Biasi, 2011; Pistillo et al., 2014). Humans experience a WDW syndrome whose severity closely correlates to smoking relapse, that emerges within hours the cessation of cigarette smoking. Nicotine WDW in rodents is characterised by similar somatic signs and affective changes (including increased anxiety, anhedonia and irritability) (reviewed in Paolini and De Biasi, 2011).

The aim of this study was to validate a rodent model of e-cig exposure that induces high urinary levels of cotinine (the major nicotine metabolite) and mimics the intermittence and route of nicotine administration in humans. In order to compare the effects of inhaled cigarette smoke and e-cig vapour containing the same amount of nicotine, we developed a system based on previous nicotine inhalation research (George et al., 2010) that allows the vaporised liquid contained in commercial e-cigs to be introduced into a smoke chamber and compared its neurochemical (nicotine and cotinine brain levels, nAChR up-regulation), physiological (body weight and food intake) and behavioural (memory function and emotional profile after mecamylamine precipitated and spontaneous WDW) effects with those induced by cigarette smoke.

2. Experimental procedures

2.1. Animals

One hundred and eighty three month-old male BALB/cJ mice (Charles River, Calco, Como) were group housed (five mice per cage) in a humidity and constant 21°C temperature-controlled animal facility on a 12h/12h light/dark cycle (lights on at at 8:00 a.m.), with *ad libitum* access to food and water. The cob-bedding was changed weekly.

This strain was selected on the basis of recent findings in our Department (Dr Lucini unpublished data) showing that these animals develop respiratory diseases after the same smoke exposure as that used in this study.

All of the experimental procedures respected the guidelines established by the Italian Council on Animal Care, and were approved by Italian Government Decree No. 28/2013. Every effort was made to minimise the number of mice used and their suffering. Figure 1a shows a flow chart of the study.

2.2.Exposure to cigarette and e-cig

One week after their arrival, the mice were divided into three groups of 30 mice each and transferred to perspex inhalation chambers (22 cm wide x 40 cm long x 20 cm high) connected to a mechanical ventilator (Rodent Ventilator, Model 7025, Ugo Basile, Biological Research Instruments, Varese, Italy) delivering the smoke or air, for three 30-minute 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 cigarette smoke group were exposed to the smoke of 21 commercial cigarettes containing 0.8 mg of nicotine/cigarette (for a total of 16.8 mg/day), 10 mg of tar and 10 mg of carbon monoxide; the animal in the 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 others compounds (see above); and the animal in the control group were exposed only to air alone. The assessed physiological parameters were the animal body weight (g) and food intake (g/mouse/day). Food intake was calculated as total food consumption divided by the number of mice in each cage. At the end of each exposure session, the animals were removed and the inhalation chamber remained opened in order to eliminate any residual smoke or vapour through the fume hood.

2.3. Biochemical studies

2.3.1. Brain tissue dissection. One hour after the last session, mice were sacrificed, their brains were quickly removed, and different areas brain areas were carefully dissected on ice.

2.3.2. Brain and urinary cotinine/nicotine. Urine samples were collected from animals housed overnight in metabolic cages, in groups of five at the end of the first, fourth and seventh week. The urine samples were centrifuged, frozen at -20°C until assay. Urinary cotinine concentration was measured using an enzyme immunoassay method DRI® Cotinine Assay on CDx90 analyser (ThermoFisher Scientific, distributed by Tema Ricerca, BO, Italy). The detection limit was 34 ng/ml. and the measurement range 34-2000 ng/ml.

Brain nicotine and cotinine levels were quantitatively determined using LC-MS/MS as previously described (Vieira-Brock et al, 2011), with minor modifications. The Liquid chromatography was performed using an HP 1200 system (Agilent Technologies, Germany), equipped with a Discovery HS-F5 column (150 mm \times 2.1 mm, 3 μm) (Supelco, Milan, Italy). The LC system was coupled by an electrospray ion source operated in positive mode to an Agilent 6410 triple quadrupole-mass spectrometer for quantitative analysis and to an Agilent 6520 Accurate-Mass Q-TOF for qualitative analysis. The measurements were made separately using samples of ventral tegmental area, prefrontal cortex and olfactory bulb.

2.3.3. Antibody production and characterization. The subunit-specific polyclonal antibodies (Abs) used have been previously described Grady et al. (2009). For the immunoprecipitation experiments the affinity purified Abs (4mg/ ml of wet resin) were cross-linked to Protein A Sepharose™ CL-4B (GE Healthcare) by means of 20 mM dimethyl pimelidate (Thermo Scientific) according to manufacturer instructions.

2.3.4. Binding studies. Membranes and 2% Triton X-100 extracts from: hippocampus, nucleus accumbens, caudate-putamen, habenula and interpeduncular nucleus were prepared as previously described (Moretti et al. 2010).

In each experiment, the areas from five mice from each experimental group were pooled and homogenized. ^{125}I - α Bungarotoxin (α Bgtx) and ^3H -epibatidine (Epi) binding to total brain homogenate was performed as previously described (Moretti et al. 2010). ^3H -Epi binding to nAChR present in 2% Triton X-100 extracts obtained from brain areas was assessed using ^3H -Epi that binds to multiple heteromeric nAChR subtypes with pM affinity and to $\alpha 7$ -nAChR with nM affinity. In order to ensure that the $\alpha 7$ -containing subtypes did not contribute to ^3H -Epi binding solubilised receptors (present in the extract and immunoprecipitation experiments) were first incubated for 3 hours with 1 μM cold α Bgtx (Tocris, Bristol, UK) which specifically binds to $\alpha 7$ -nAChR (and thus prevents ^3H -Epi binding to these sites). Extracts were labelled with 2 nM [^3H]-Epi at 4°C and following overnight incubation receptors were captured using DEAE-SepharoseTM Fast flow (GE Healthcare, Uppsala, Sweden). The bound receptors were eluted with 1N NaOH and after addition of the scintillation mixture (filter count, GE Healthcare, Uppsala, Sweden) counted in a beta counter. Non-specific binding (averaging 5-10% of total binding) was determined in parallel samples containing 100 nM unlabelled Epi.

^3H -Epi (s.a. 50-66 Ci/mmol) and ^{125}I - α Bgtx, (s.a. 200 Ci/mmol) were purchased from PerkinElmer (Boston, USA) and non-radioactive ligands from Tocris (Bristol, UK)

2.3.5. Immunoprecipitation of ^3H -Epibatidine-labelled receptors by subunit-specific antibodies. The tissue extracts, were preincubated with 2 μM α Bgtx, labelled with 2 nM ^3H -Epi, and incubated overnight with a saturating concentration of affinity purified anti-subunit IgG (10 μg) bound to Sepharose-ProteinA (GE Healthcare, Italy). The immunoprecipitation was recovered by centrifugation. The level of Ab immunoprecipitation was expressed as as fmol of immunoprecipitated receptors/mg of protein and is the mean \pm SEM of 3-5 experiments for each treatment (air, e-cig, cigarette).

2.4. Behavioural studies

2.4.1. Mecamylamine-precipitated nicotine WDW. Immediately after the last air, smoke or e-cig exposure, all the animals received mecamylamine hydrochloride s.c. (1 mg/kg) (Sigma-Aldrich, MO, USA), and were individually placed inside a circular clear plastic observation cylinder according to Castanê et al. (2002) in order to observe the signs and symptoms of abstinence for 30 min after injection, after 10 min acclimatisation. The evaluated abstinence signs were: locomotor activity, wet dog shakes, front paw tremors, sniffing and scratches and the symptoms were: body tremor, ptosis, wet dog shakes, teeth chattering, paw tremor, scratching, genital licks, sniffing and piloerection . The number of wet dog shakes, front paw tremors, sniffs and scratches was counted. Ptosis, genital licks, body tremor, piloerection and teeth chattering were scored 1 for appearance or 0 for non-appearance within each period of 5 min. A global withdrawal score was calculated for each animal by giving each individual sign a relative weight of 0.5 for each episode of wet dog shaking, front paw tremor, sniffing and scratching, and 1 for the presence of ptosis, genital lick, tremor, piloerection and teeth chattering during each 5 min observation. In order to verify whether mecamylamine *per se* has any effect on mice unexposed to smoke or e-cig, two groups of five of treatment naïve mice were injected with mecamylamine or vehicle following the protocol described above.

Motor activity (Sala et al. 2013) was evaluated in an automated activity cage for 15 min immediately after treatment in different groups of animals.

2.4.2. Spontaneous WDW. 24 hours, 15 and 30 days after the last air, smoke or e-cig exposure, different groups of mice underwent the following behavioural tasks:

2.4.2.1. Spontaneous motor activity. Horizontal movements were evaluated for 15 min as described above.

2.4.2.2. Spatial object recognition. Object location was carried in an arena. The test was carried out according to Kenney et al. (2011) with slight modifications. Two visual cues were placed on two adjacent walls of an opaque white Plexiglas cage (58×50×43 cm) and dimly lit from above (27 lx) the cage: a black and white striped pattern (21×19.5 cm) was affixed to the centre of the northern

wall and a black and gray checkered pattern (26.5×20 cm) was placed at the center of the western wall. Two sets of identical objects were used in the experiments: 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 objects were counterbalanced across locations. The cage and all objects were thoroughly wiped down with acetic acid 0.1% before and after all of the behavioural procedures which were observed and recorded by means of a camera mounted above the cage. Exploration was defined as a mouse having its nose directed toward the object within approximately 1 cm (Bevins and Besheer, 2006). Climbing or sitting on objects was not scored as object exploration. Mice that did not spend more than a total of 30 s exploring the objects on training or testing were excluded from analysis.

During the last treatment day, the mice were pre-exposed to the cage for 10 min. Twenty four hours later, returned to the cage and allowed to explore the two different objects placed in the NE and NW corners; the time spent exploring the objects was recorded. Forty eight hours later the mice were re-exposed to the cage with the object that has been previously more explored, moved to the SW corner.

2.4.2.3. Elevated Plus Maze. Anxiety was evaluated using the elevated plus maze test as previously described (Baird et al. 2007). The apparatus consisted of two opposite open arms (35 x 10 cm) and two enclosed arms (35 x 10 cm) extending from a common central platform (10 x 10 cm). The animals were moved to the plus maze laboratory 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 by a trained observer unaware of the treatments, 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 (Hogg, 1996). The total closed-arm entries were analysed as measures of non-specific changes in locomotor activity.

2.4.2.4. Marble Burying Test. The marble burying test utilizes spontaneous digging behaviour, characteristic of rodents, to assess anxiety-like/compulsive behaviour (Turner et al. 2010). After acclimation (1 h), each mouse was placed in a cage (26 x 20 x14 cm), in which 20 marbles had been equally distributed on top of mouse bedding (5 cm in depth). After 15 min, the number of buried marbles and the latency to the first marble burying were counted.

2.5. Statistical Analyses

Data were expressed as mean \pm SEM. Different groups were assessed by one or two-way analyses of variance (ANOVAs) for repeated measures or Kruskal Wallis test for multiple non parametric comparisons followed by Tukey's, Bonferroni or Dunn's post-hoc test. The accepted value for significance was $p < 0.05$. All statistical analyses were done using the software Prism, version 6 (GraphPad, San Diego, CA, USA).

3. Results

3.1. E-cigarette vapour does not affect body weight and food intake

Smoking lowers body weight, which makes many people reluctant to quit smoking (Chiolero et al. 2008). Weight gain and food intake were evaluated weekly during the 7-week of exposure to cigarette smoke or e-cig vapour and for 1 month after their cessation (Figure 1b and 1c). For body weight there was an effect of treatment ($F_{(2, 216)} = 29.09$, $p < 0.0001$), an effect of time ($F_{(7, 216)} = 112.80$, $p < 0.0001$), and a treatment x time interaction ($F_{(14, 216)} = 1.92$, $p < 0.05$). A significant reduction of body weight in mice exposed to cigarette smoke starting from day 14, was shown. During cessation there was a difference in body weight gain for treatment ($F_{(2, 216)} = 5.33$, $p < 0.0001$), time ($F_{(7, 216)} = 72.4$, $p < 0.0001$), and treatment x time interaction ($F_{(14, 216)} = 2.46$, $p = 0.025$). A difference of body weight was found only during the first day after WDW. For food intake there was an effect of treatment ($F_{(2, 216)} = 12.79$, $p = 0.0002$), time ($F_{(7, 216)} = 11.48$, $p < 0.0001$), but not treatment x time interaction ($F_{(14, 216)} = 1.68$, $p = 0.12$) during smoke/vapour exposure. A

significant reduction of food intake in mice exposed to cigarette smoke, was shown starting from day 14 to day 35. After WDW there was a difference among groups. Two-way ANOVA revealed time ($F_{(2, 216)} = 6.32$, $p < 0.003$), but not treatment ($F_{(7, 216)} = 1.61$, $p < 0.12$), and no treatment x time interaction effect ($F_{(14, 216)} = 2.88$, $p = 0.48$).

3.2. Both e-cigarette vapour and cigarette smoke increase brain cotinine and nicotine levels and urinary cotinine levels

Brain nicotine (pg/mg of tissue) and cotinine (pg/mg of tissue concentrations) were evaluated at the end of 7-week exposure, and found to be very similar between e-cig vapour and cigarette smoke exposed mice and significantly different from those of mice exposed to air (nicotine: $F_{(2,6)} = 17.18$, $p = 0.0023$; cotinine: $F_{(2,6)} = 14.19$, $p = 0.0053$;) (Fig 1d and 1e). Urinary cotinine levels have been widely used to assess active or passive exposure to cigarette smoke (Matta et al. 2007). Exposure to e-cig led to an increase of cotinine levels (pg/ml) across the 7 weeks (Figure 1f). There was an effect of treatment ($F_{(2, 81)} = 6.72$, $p = 0.003$), but not of time ($F_{(7, 81)} = 2.81$, $p = 0.07$), and of treatment x time interaction ($F_{(4, 81)} = 0.74$, $p = 0.56$). *Post-hoc* analysis revealed that across the weeks cotinine levels were significantly higher in both e-cig and cigarette exposed groups compared to air group.

3.3. Both e-cigarette and cigarette selectively up regulate $\alpha 4\beta 2$ -containing nicotinic receptors in brain areas

We performed preliminary studies to check the time course of treatment effects on the expression of ^3H -Epi labelled heteromeric receptors and ^{125}I - α Bgtx labelled homomeric receptors in total brain homogenates, and found no difference after three weeks and significant differences after seven weeks (data not shown), that was chosen for further studies.

We found that in cortex, hippocampus, nucleus accumbens and caudate-putamen there was a

significant difference in ^3H -Epi binding to the heteromeric receptors between the experimental and control groups (Figure 2 and Table1). Immunoprecipitation experiments showed that this difference was due to a significant increase in $\alpha 4\beta 2$ -containing receptors in e-cig and cigarette groups with no effects on $\alpha 6$ -containing receptor expressed in all four areas or on the $\alpha 6$ -containing receptors selectively expressed in the nucleus accumbens and caudate-putamen. We finally analysed whether exposure to e-cig or cigarette had any effects on the expression of the receptors in habenula and interpeduncular nucleus two areas that express a very high level of $\alpha 3\beta 4$ -containing receptors. As shown in Figure 2e and 2f we did not detect any significant effect.

3.4. Mecamylamine-precipitated withdrawal after e-cigarette is less severe compared to that of cigarette

The effect of 7-week exposure to e-cig or cigarette on mecamylamine-precipitated WDW is reported in Figure 3. A significant treatment effect on the development of signs ($F_{(2,27)} = 8.15$, $p = 0.001$, one-way ANOVA) and symptoms ($\chi^2 = 8.45$, $p = 0.01$) (Figure 3a), was shown. There was a significant increase in the mean score of signs and symptoms in mice exposed to cigarette, while only in the mean score of symptoms in mice exposed to e-cig. There was a different severity of nicotine-WDW syndrome ($F_{(2,27)} = 5.89$, $p = 0.01$). Global score was higher in cigarette exposed mice compared to e-cig exposed mice (Figure 3b) ($\chi^2 = 10.15$, $p = 0.006$). The severity of WDW led us to examine spontaneous motor activity. There was a difference between groups in horizontal ($F_{(2,27)} = 14.82$, $p < 0.001$) (Figure 3c) and vertical counts (Figure 3d) ($F_{(2,27)} = 10.49$, $p = 0.0001$) where only mice exposed to cigarettes showed a significant decrease. To test whether mecamylamine *per se* caused WDW-like effects we also administered mecamylamine to naïve mice. No difference was found either in the WDW global scores (saline: 1.0 ± 0.1 , mecamylamine: 1.16 ± 0.66) or in horizontal (saline: 939 ± 172 , mecamylamine: 1200 ± 74) and vertical counts (saline: 74 ± 9 , mecamylamine: 92 ± 26) between saline and mecamylamine treated naïve mice.

3.5. Spatial working memory and emotional profile are differentially impaired after spontaneous withdrawal from cigarette and e-cigarette.

Spatial working memory and emotional profile are affected during smoking or nicotine WDW both in humans (George et al. 2010; Kenny and Markou, 2001; Camfield et al. 2013) and rodents (Kenney et al. 2011; Yohn et al. 2014). Thus, we investigated whether exposure to cigarette /e-cig produced changes in spatial object recognition task, marble burying and elevated plus maze test (Figure 4) accompanied by motor impairment. ANOVA showed no difference between groups on spontaneous motor activity (treatment : $F_{(2,81)} = 0.2, p = 0.81$), time: $F_{(2,81)} = 0.13, p = 0.94$) and time x treatment: $F_{(4,81)} = 0.55, p = 0.77$) (Figure 4a). Evaluation of discrimination index (Figure 4b) revealed a treatment ($F_{(2,81)} = 71.69, p < 0.0001$) and a time x treatment effect ($F_{(4,81)} = 2.97, P = 0.02$), but no significant effect was found for time ($F_{(2,81)} = 2.81, p = 0.07$). At 24 h, mice exposed to cigarette were more impaired than those exposed to e-cig. Significant differences between groups in the number of arm entries and time in the elevated plus maze were observed (open arm entries: treatment $F_{(2,81)} = 554.30, p < 0.0001$, time $F_{(2,81)} = 6.22, p = 0.003$ and time x treatment $F_{(4,81)} = 7.29, p < 0.0001$; open arm time: treatment $F_{(2,81)} = 116.10, p < 0.0001$, time $F_{(2,81)} = 117.60, p < 0.0001$) and time x treatment effect ($F_{(4,81)} = 5.71, p = 0.0004$), (closed arm: maze entries: treatment $F_{(2,81)} = 30.50, p < 0.0001$, time $F_{(2,81)} = 1.67, p = 0.36$ and time x treatment effect $F_{(4,81)} = 1.55, p = 0.75$). No differences were found in the mean number of total arm entries (entries: treatment $F_{(2,81)} = 0.22, p = 0.90$, time $F_{(2,81)} = 0.49, p = 0.81$ and time x treatment effect $F_{(4,81)} = 1.18, p = 0.91$). The number of open entries and time in cigarette and e-cig groups were significantly lower compared to controls while the number of closed arm entries is higher (Figure 4c). Notably, in cigarette-exposed group the number of entries was always less than in e-cig group suggesting a more anxious profile.

In the marble burying test there was a difference between groups in the number of buried marbles (Figure 4d) (treatment: $F_{(2,81)} = 45.03, p < 0.0001$), time: $F_{(2,81)} = 18.82, p < 0.0001$), treatment x time interaction: $F_{(2,81)} = 45.03, p < 0.0001$). Cigarette and e-cig groups buried significant more marbles compared to air group. Notably, e-cig group showed a greater increase from 24 h to 30 days.

There was a significant main effect of treatment on the latency to the first burying ($F_{(2,81)} = 224.0$, $p < 0.0001$), a significant effect of time ($F_{(2,81)} = 6.31$, $p < 0.0001$), and a significant treatment by time interaction ($F_{(2,81)} = 12.43$, $p < 0.0001$). *Post-hoc* test revealed a significant decrease in the latency for e-cig and cigarette groups when compared to controls. Interestingly, at 30 days the e-cig group showed a significant reduction of this parameter in comparison with the cigarette group.

4. Discussion

This is the first study comparing cigarette smoke and e-cig vapour administered to mice by means of inhalation, using the same dose of nicotine for both treatments and its findings provide the first evidence that exposure to e-cig vapour has a number of effects during the period of exposure and for a long time, after exposure cessation. Previous studies (reviewed in Brennan et al. 2014) have attempted to assess possible differences in the dependence-related effects of nicotine and cigarette smoke by comparing nicotine directly administered by means of injections or osmotic minipumps with inhaled smoke or injected aqueous tobacco extracts, but their main limitations are the different methods of administration (directly administered nicotine vs. inhaled smoke) and the different compositions of aqueous tobacco extracts and inhaled smoke.

The body weight of human smokers is less than that of non-smokers (Albanes et al. 1987) and increases when they stop smoking (Pistelli et al. 2009). The mice exposed to cigarette smoke (but not those exposed to e-cig vapour) showed significantly reduced food intake and body weight over time. The effects of smoking on body weight are attributed to the nicotine in tobacco because nicotine decreases feeding in animal models (Husmann et al. 2014), possibly as a result of its interaction with nAChRs expressed in critical hypothalamic circuits (Mineur et al. 2011). However, the fact that there was not a significant reduction in food intake and body weight in the e-cig group suggests that compounds other than the nicotine in cigarettes are involved.

Cotinine is the most useful biological marker of nicotine exposure, and its levels in the blood, urine and other tissues of smokers remain stable for long periods (Matta et al. 2007). We found very similar brain nicotine and cotinine levels and urinary cotinine levels, which were in line with those previously found in mice (Catanzaro et al. 2007) or human smokers (Jung et al. 2012).

Like cigarette smoke, e-cig vapour increased the number of $\alpha 4\beta 2$ -containing receptors in the cortex, hippocampus, nucleus accumbens and caudate putamen, but had no effect on the other subtypes expressed in these areas. This selective effect on $\alpha 4\beta 2$ receptors is probably due to the higher affinity and/or nicotine functional desensitisation of this subtype, which may be an important initial step in the up-regulation process. Experimental evidence shows that by acting as a protein

chaperone of this subtype, nicotine favours the maturation and assembly of the $\alpha\beta 2$ subtype in the endoplasmic reticulum, but other mechanisms such as decreased $\alpha\beta 2$ degradation and / or turnover (reviewed in Colombo et al. 2013) may also play a role. In agreement with the findings of other studies of animal models of chronic nicotine administration (Marks et al. 1992; Nguyen et al. 2004), as also previously reported (Marks et al. 2011) we found that neither treatments affected the expression of the $\beta 2$ -containing receptors and $\alpha 3 \beta 4$ receptors in the habenulo–interpeduncular pathway, which are critical for the signs of nicotine WDW, probably because the brain nicotine concentrations of the exposed mice were much lower than the affinity of nicotine for this subtype (Benowitz, 2009).

Withdrawal from cigarette smoke induces a wide range of signs and symptoms in human smokers such as irritability, anxiety, headache, fatigue and insomnia (Le Foll and Golberg, 2009). Accordingly, our mice exposed to cigarette smoke showed a wide range of behavioural alterations but those exposed to e-cig vapour showed less severe somatic signs of abstinence and were not affected by reduced motor function. It has also been found that rats exposed to nicotine vapour for eight hours /day for seven days show significant somatic signs of WDW 30 min after the administration of mecamylamine (George et al. 2010). The signs associated with mecamylamine precipitated WDW of e-cig exposed mice in our study were different from those observed in other studies in which CD or BALB/c mice were implanted with osmotic minipumps delivering nicotine (Castane et al. 2002; Varani et al. 2012), but this may have been due to the different route of administration and dosage.

Nicotine WDW leads to learning and memory deficits (Kenney et al. 2011) in both animals and humans (Gould and Leach, 2014). Nicotine affects spatial but not novel object recognition memory in mice (Kenney et al. 2011). Spatial working memory can be impaired in abstinent smokers (Camfield et al. 2013; Wing et al. 2011) but Domier *et al* (2007) found that the deleterious effects of nicotine on cognitive performance in nicotine-dependent subjects did not include selective attentional deficit. The worsened discrimination index which was not accompanied by motor deficit in either group, suggests a selective spatial memory impairment. Nicotine WDW disrupts hippocampus-dependent but not hippocampus-independent learning in mice (Gould and Leach,

2014). Previous studies have demonstrated that nicotine acts on $\alpha 4\beta 2$ but not $\alpha 7$ nAChRs in the dorsal hippocampus and disrupts learning during WDW (Gould and Leach, 2014) and this may be worsened by the $\alpha 4\beta 2$ up-regulation observed in both of our treatment groups.

Increased anxiety is associated with smoking cessation in humans (Hughes, 2007) and mice (Stoker et al., 2008). Increased anxiety-like behaviour in comparison with controls (air group) was found during spontaneous WDW in both treatment groups (fewer entries and shorter times in the open arms) but the effect was significantly greater in the mice exposed to cigarette smoke. The compulsion to smoke is considered to be a central feature of cigarette smoke dependence and it has been found that smokers are more likely to have obsessive compulsive disorders and phobias than non-smokers (Bruijnzeel, 2012). The marble burying test is considered a test of compulsive activity (Albelda and Joel 2012), and so the increased number of marbles buried in the e-cig group indicates that e-cig WDW increases highly repetitive/perseverative responses (Thomas et al. 2009) more than cigarette.

In conclusion, the findings of this study show that chronic intermittent non-contingent exposure to e-cig vapour or cigarette smoke has the same effects on brain nicotine and cotinine concentrations and nAChR up-regulation, but cigarette smoke leads to more severe mecamylamine-precipitated WDW and more evident cognitive deficit 24 hours after cessation, whereas e-cig vapour elicits more severe anxiety and compulsive behaviour. This different profile may be attributable to the presence of compounds and/or metabolites that are present in cigarette smoke but not in e-cig vapour and may affect brain function. Further studies will be necessary to investigate the effect of 7-week exposure of non-nicotinic constituents of standard cigarettes and e-cigs, testing denicotinized cigarettes and e-cigs without nicotine, respectively

Although e-cigs are rapidly gaining acceptance as smoking cessation aids it is necessary to consider their possible long-term effects on anxiety, and their differential addictive liability in subjects with specific psychological traits (e.g., compulsiveness). It is worth noting that a recent clinical trial did not show any significant improvement in the cigarette smoking cessation using e-cigs in comparison with other less addictive methods of nicotine delivery (Bullen et al. 2013).

Role of funding source

Funding for this study was provided by Fondazione Zardi Gori (“Drug Abuse and Related diseases” Project, 2014) and the CNR Research Project on Aging, Regione Lombardia Projects NUTEC ID 30263049 and MbMM-convenzione n°18099/RCC (CG), by grant from the Italian Ministry of Health RF2009-1549619 (MZ). The Fondazione Zardi Gori, the CNR and Ministry of Health had no further role in study design, in the collection, analysis and interpretation of data, in the writing of the report

Disclosure

Participated in research design: Braida D., Sala M., Zoli M., Clementi F. and Gotti C.

Conducted experiments: Moretti M., Lucini V., Fasoli F., Muchietto V. and Ponzoni L

Performed data analysis: Moretti M., Lucini V., Fasoli F., Muchietto V. and Ponzoni L

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All authors contributed to and have approved the final manuscript.

Conflict of interest

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.

Acknowledgements

We thank Dr. Massimo Bruno for the chemical identification of e-cig components.

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Figure legends

Figure 1. a) Experimental design. The animals were exposed for seven weeks to e-cig vapour, cigarette smoke or air. One hour after the last exposure during the third and seventh week of treatment, animals from the different groups of animals were sacrificed for binding studies. At the end of the seventh week, one hour after the last exposure, different groups (air, e-cig, cigarette) of animals were injected with mecamylamine and immediately tested for precipitated withdrawal syndrome. Further groups were submitted to memory and emotional tasks from 1 to 30 days after smoke/vapour exposure cessation.

b-f) Exposure for 7 weeks to nicotine differentially altered body weight, food intake and cotinine/nicotine concentrations in brain and urines. Body weight (b) and food intake (c) were measured 3 times a week. Values represent mean \pm SEM of 3 weekly recordings (N = 10 for each group). (d-e) Brain cotinine (d) and nicotine (e) levels evaluated at the end of the seventh week. (N = 6 for each group). Urine cotinine levels (f) evaluated during the first, the fourth and the seventh week in mice exposed to smoke of e-cig, cigarette, or air. (N = 5 for each group). Results are expressed as mean \pm SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ compared to corresponding air groups; \$ $p < 0.05$, \$\$\$ $p < 0.001$ compared to corresponding air and e-cig groups; # compared to corresponding e-cig and cigarette groups (Bonferroni or Tukey's test).

Figure 2. Immunoprecipitation analysis of the subunit content of extracts prepared from different brain areas of air, e-cig or cigarette exposed mice. Extracts of the cortex (a), hippocampus (b), nucleus accumbens (c), caudate-putamen (d), habenula (e) or interpeduncular nucleus (f) prepared from air, e-cig and cigarette exposed mice were labelled with 2 nM ^3H -Epi and immunoprecipitated by prot A-antibody (using saturating concentrations (10 μg) of anti-subunit antibodies as described in Experimental procedures . Antibodies directed against two separate peptides of the same subunits were used in the case of the $\alpha 4$, $\alpha 5$, $\alpha 6$ and $\beta 2$ subunits (see Grady et al., 2009). The amount immunoprecipitated by each antibody was subtracted from the value obtained in control samples containing an identical concentration of normal rabbit IgG, and the results are expressed as fmol of immunoprecipitated labelled ^3H -Epi nAChR/mg of protein. Mean

values \pm SEM of 3-4 experiments. For statistical comparison, data were analysed using one way ANOVA followed by Bonferroni's post-hoc * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ compared to corresponding air groups.

Figure 3. Mecamylamine (MEC) precipitated withdrawal syndrome from nicotine is milder in mice exposed to e-cig vapour than in those exposed to cigarette smoke. **(a)** Abstinence signs (wet dog shakes, front paw tremors, sniffing and scratches) and symptoms (body tremor, ptosis, wet dog shakes, teeth chattering, paw tremor, scratching, genital licks, sniffing and piloerection) were evaluated every 5 min for 30 min after injection. **(b)** Global withdrawal score was calculated by giving to each individual sign a relative weight of 0.5 and 1 for the presence of each symptom during each observation. **(c)** Horizontal and (d) vertical motor activity were evaluated in a different group of animals for 15 min immediately after MEC treatment using an automated activity cage and expressed in terms of number of horizontal counts. * $p < 0.05$, ** $p < 0.01$ vs corresponding air group; \$\$ $p < 0.01$, \$\$\$ $p < 0.001$ vs corresponding air and e-cig group (Tukey's test). Values represent mean \pm SEM.

Figure 4. During spontaneous withdrawal syndrome evaluated at 24 h, 15 and 30 days, animals exposed to e-cig vapour showed cognitive deficit and anxiety-like behaviour after spontaneous withdrawal. **(a)** Motor activity, in terms of total number of horizontal counts was evaluated for 30 min. **(b)** Spatial object recognition was carried out interposing a delay of 48 hours between T_1 and T_2 and expressed in terms of discrimination index. **** $p < 0.0001$ compared to air group; ° $p < 0.05$ compared to corresponding cigarette group. **(c)** Percentage of open arm (top left) and closed arm (bottom, left) entries, open arm time (top right) and total number of entries (bottom right) evaluated in the elevated plus maze task. * $p < 0.05$, ** $p < 0.01$ **** $p < 0.0001$ compared to corresponding air group; ° $p < 0.05$, °° $p < 0.01$ compared with corresponding cigarette group; § $p < 0.0001$ compared to corresponding 15 and 30 days; & $p < 0.05$ compared to corresponding 15 days. **(d)** Number of marbles buried (left) and latency to the first burial (right) evaluated within 15 min, in the marble burying test. *** $p < 0.001$, **** $p < 0.0001$ compared with corresponding e-cig

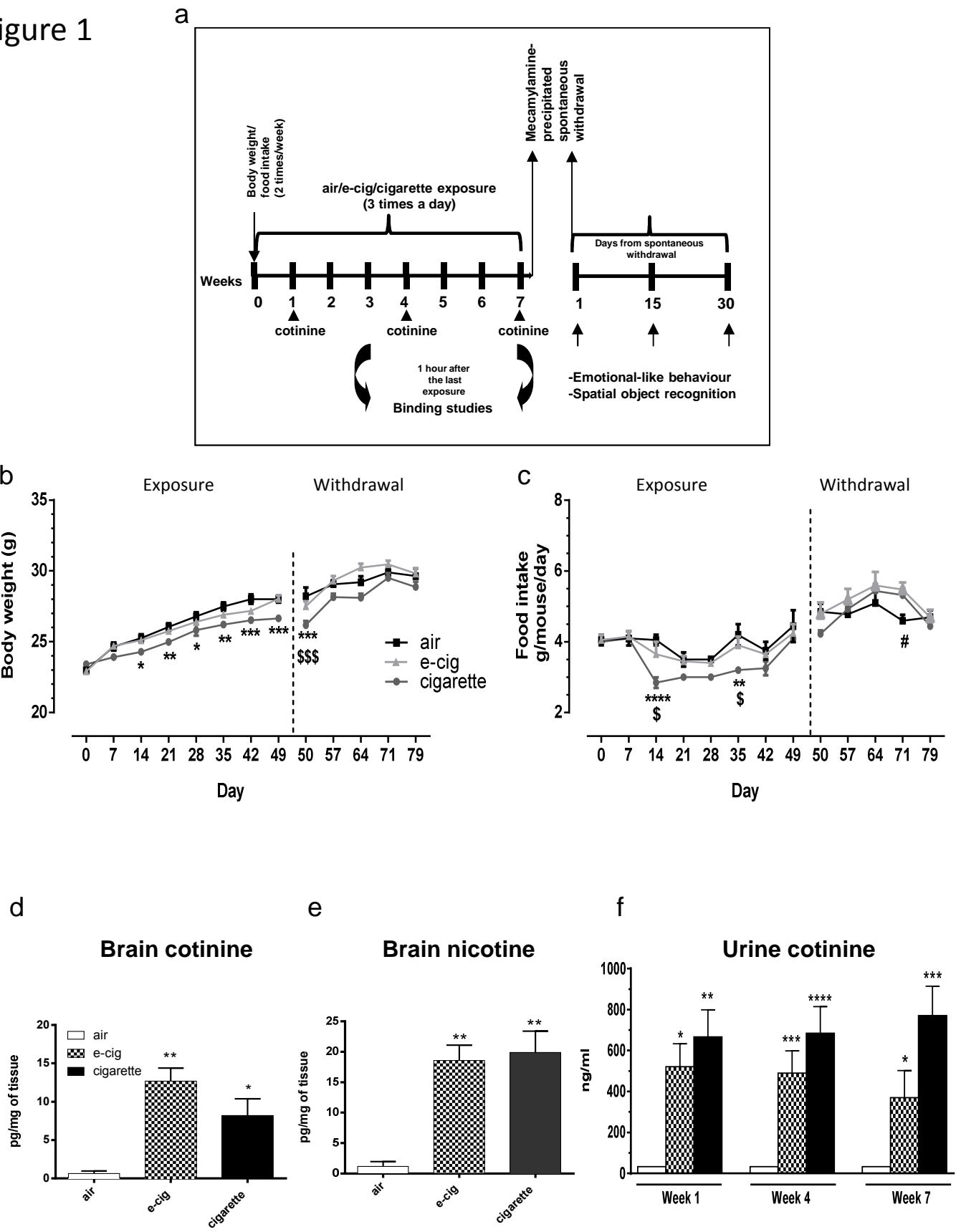
and cigarette group; °°°° $p < 0.0001$ compared with corresponding cigarette group; && $p < 0.01$ compared with corresponding 15 days; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$, #### $p < 0.0001$ vs corresponding 24 hours group (Bonferroni *post-hoc* test). Values represent mean \pm SEM. N=10 for each group.

Table 1: Quantitative immunoprecipitation analysis of the subunit composition of the nicotinic subtypes expressed in the brain areas of air, e-cig and cigarette exposed mice.

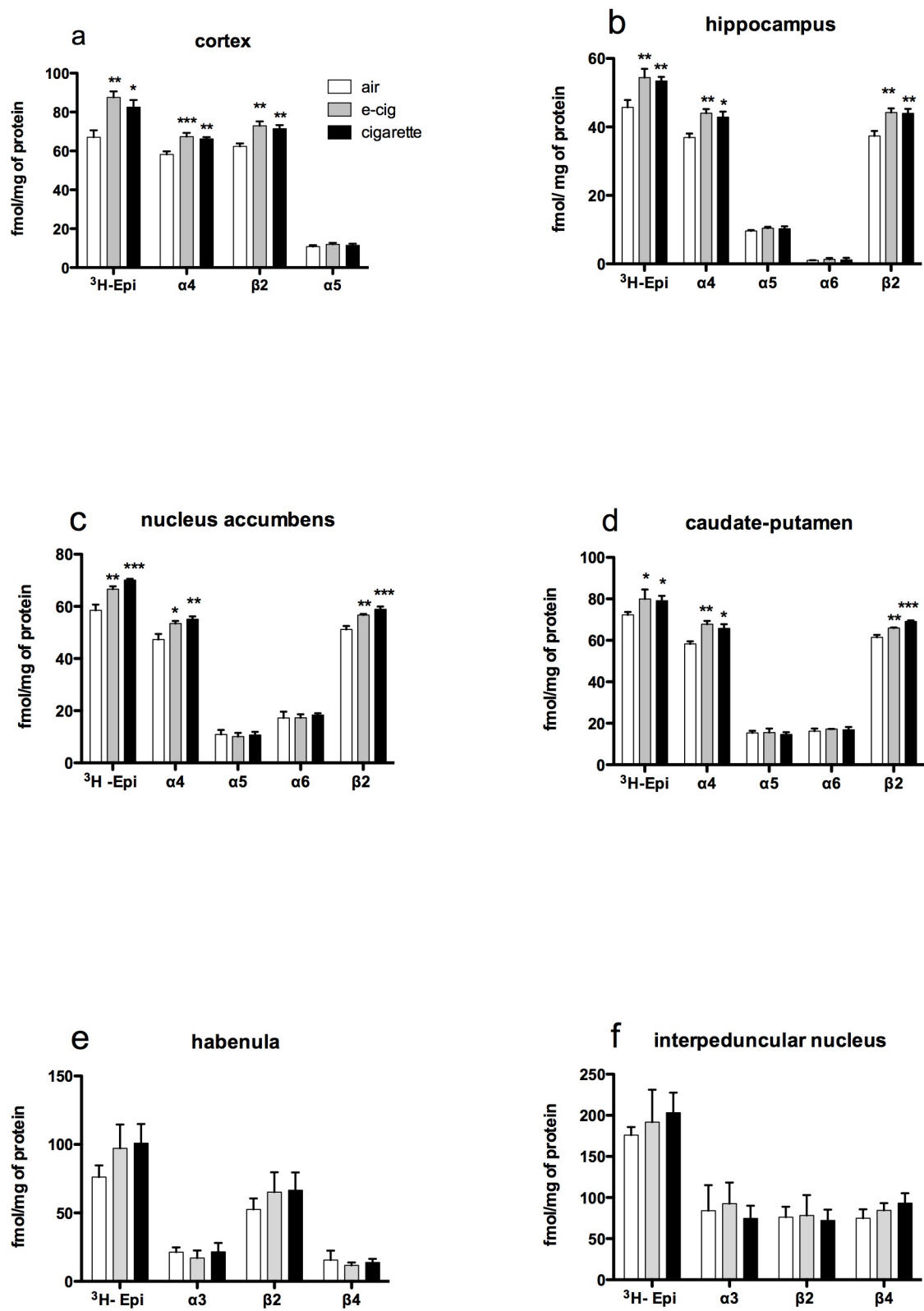
Area and subunit expression	Air	E-cig	Cigarette	One way Anova
Cortex				
³ H-Epi receptors	67.0±3.6	87.36±3.1	82.5±3.6	F_(2, 27) = 6.89; p= 0.004
α4	58.2±1.6	67.4±1.9	66.2±0.9	F_(2,33) =10.78; p =0.0002***
α5	10.8±0.8	11.9±0.8	11.5±0.8	Ns p=0.61
β2	62.4±1.1	73.0±2.2	71.4±1.9	F_(2,33) =8.9; p=0.0008***
Hippocampus				
³ H-Epi receptors	42.7±2.2	52.45±2.5	53.4±1.2	F_(2,15) =27.7; p=0.0032
α4	36.9±1.1	44.0±1.2	42.9±1.6	F_(2,15) =7.25; p=0.0069**
α5	9.6±0.2	10.4±0.4	10.3±0.3	Ns p=0.49
α6	1.04±0.1	1.3±0.4	1.19±0.5	Ns p=0.89
β2	37.4±1.4	44.2±1.2	43.9±1.3	F_(2,15) =8.16; p = 0.0045**
Nucleus accumbens				
³ H-Epi receptors	58.5± 2.1	66.6±1.0	70.0± 0.5	F_(2,9) = 15.44; p 0.0012**
α4	47.3±2.1	53.4±1.0	55.0±1.0	F_(2,15) =7.5; p= 0.0068**
α5	10.9 ±1.7	10.0±1.4	10.7±1.1	Ns p=0.91
α6	17.2±2.4	17.3±1.3	18.3±0.7	Ns p=0.86
β2	51.1±1.3	56.7±0.3	58.9±1.1	F_(2,15) =15.8; p=0.0002***
Caudate-putamen				
³ H-Epi receptors	72.3±1.4	79.9±4.6	79.1±2.3	F_(2,9) =8.0; p 0.010**
α4	58.3±1.2	67.8±1.6	65.8 ±2.0	F_(2,12) =10.33; p= 0.0030**
α5	15.3 ±1.0	15.5 ±1.9	14.6±1.1	Ns p=0.89
α6	16.2±1.3	17.2±0.3	16.9±1.2	Ns p=0.88
β2	61.5±1.1	65.9±0.3	69.2±0.4	F_(2,12) =7.14, p=0.0090**

The subunit content of nAChRs is given as fmol of immunoprecipitated receptors/mg of protein and is the mean ± SEM of 3-5 experiments for each treatment (air , e-cig, cigarette). Comparision between groups was performed using one way Anova followed by Bonferroni post hoc test

Figure 1



Figure(s)



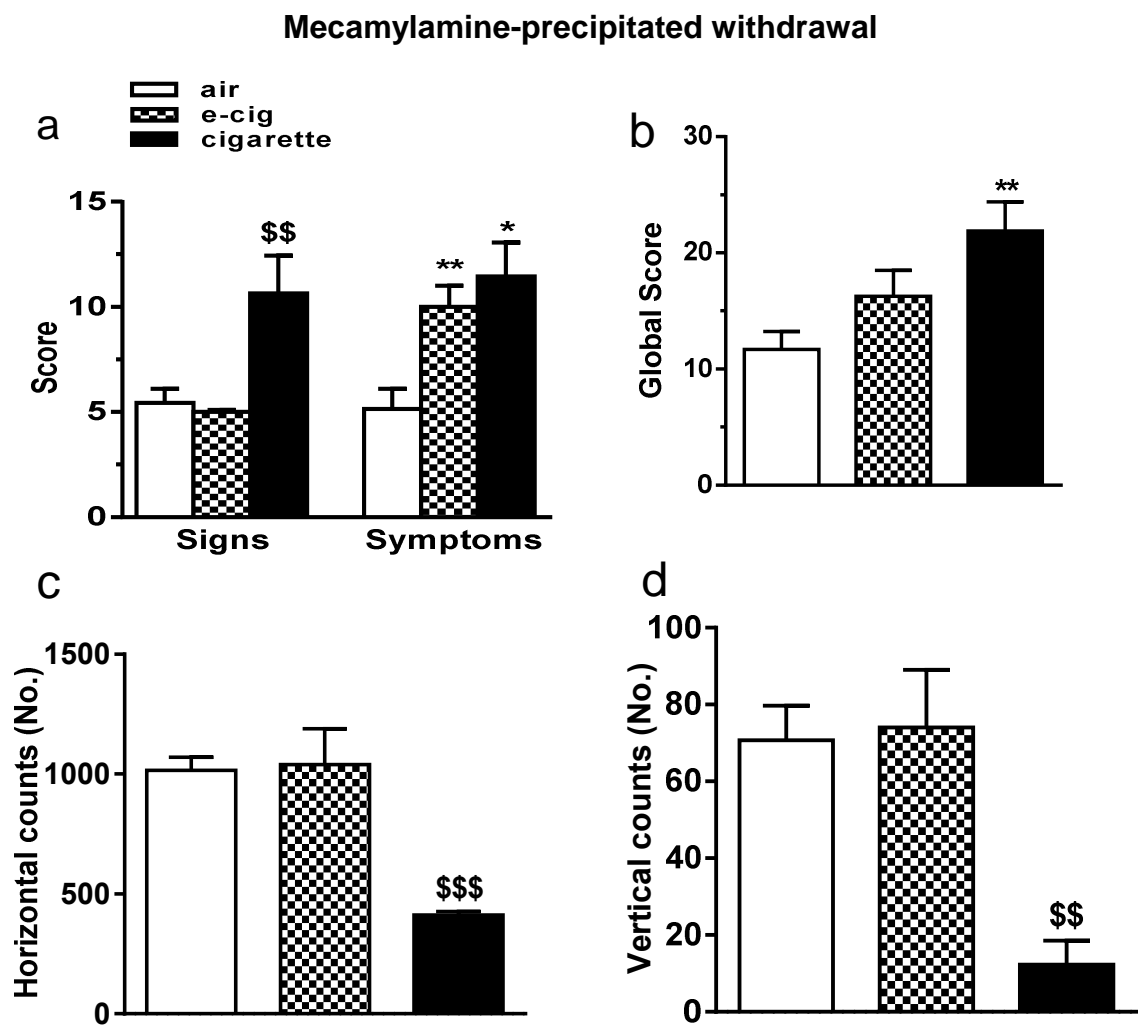
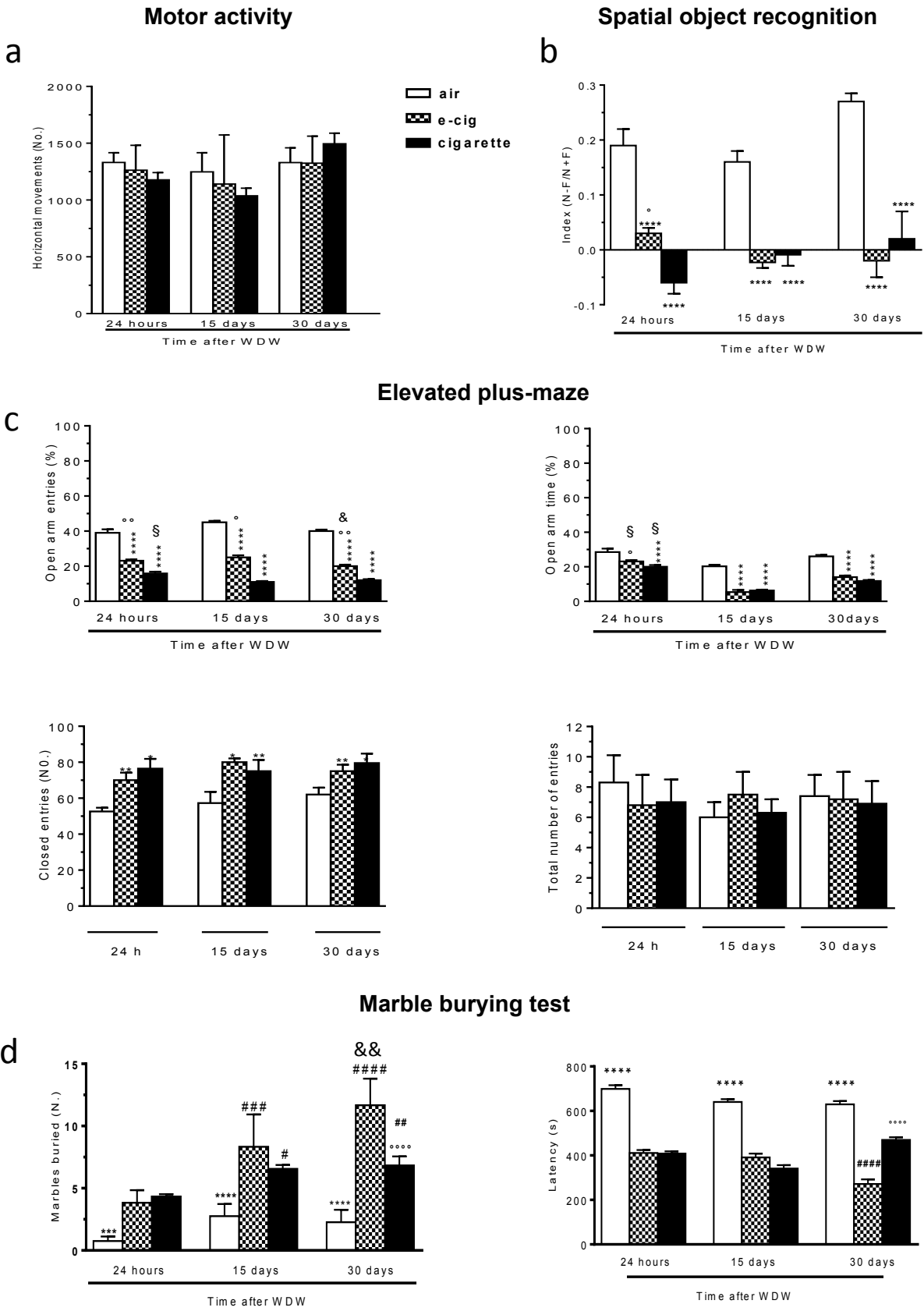


Figure 3

Figure(s)
Figure 4



Role of funding source

Funding for this study was provided by Fondazione Zardi Gori (“Drug Abuse and Related diseases” Project, 2014) and the CNR Research Project on Aging, Regione Lombardia Projects NUTEC ID 30263049 and MbMM-convenzione n°18099/RCC (CG), by grant from the Italian Ministry of Health RF2009-1549619 (MZ). The Fondazione Zardi Gori, the CNR and Ministry of Health had no further role in study design, in the collection, analysis and interpretation of data, in the writing of the report

Contributors

Participated in research design: Braida D., Sala M., Zoli M., Clementi F. and Gotti C.

Conducted experiments: Moretti M., Lucini V., Fasoli F., Muchietto V. and Ponzoni L

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Wrote or contributed to writing of the manuscript: Braida D., Sala M., M Zoli M.,
Clementi F. and Gotti C.

All authors contributed to and have approved the final manuscript.

*Conflict of Interest

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.

Acknowledgements

We thank Dr. Massimo Bruno for the chemical identification of e-cig components.

Supplementary Material

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