Increased sensitivity to Δ9-THC-induced rewarding effects after seven-week exposure to electronic and tobacco cigarettes in mice

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Running title

Δ^9-THC-induced CPP in e-CIG and CIG pre-exposed mice

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Abstract

Cigarette (CIG) smoking often precedes the use of illegal drugs. Electronic-cigarettes (e-CIGs) have been promoted as a means of stopping smoking and reducing the harmful effects of CIGs on the population. However, although e-CIGs eliminate some of the morbidity associated with combustible tobacco, they are still nicotine-delivery devices. In order to study whether the nicotine delivered via e-CIG acts as "a gateway drug" to the use of cannabis, we analysed the behavioural and molecular effects of seven weeks' pre-exposure to air (AIR), e-CIGs or CIGs on addiction-related conditioned place preference (CPP) in mice using a sub-threshold (0.01 mg/kg) dose of delta-9-tetrahydrocannabinol (Δ^9-THC), the principal psychoactive constituent of cannabis. After eight and 66 days of withdrawal, this Δ^9-THC dose was ineffective in inducing CPP in mice pre-exposed to pump-driven AIR, but very effective in mice pre-exposed to e-CIGs or CIGs.

Exposure to e-CIGs or CIGs increases the expression of ΔFosB in the nucleus accumbens (NAc), which remains high during short-term e-CIG or CIG withdrawal and long-term CIG withdrawal and is not influenced by treatment with Δ^9-THC. At the end of e-CIG or CIG exposure and during withdrawal, the mice also had a higher AMPA receptors GluA1/GluA2-3 ratio in the NAc.

Chronic nicotine exposure increases sensitivity to rewarding effects of Δ^9-THC in mice and produces long-lasting neurobiological changes regardless of the delivery method (CIG vs. e-CIG). The exposure to passive tobacco smoke or e-CIG vapours can similarly increase vulnerability to the effects of cannabis and possibly other drugs of abuse.

Key words:

Electronic cigarette vapour; cigarette smoke; Δ^9-THC ; conditioned place preference; withdrawal; CB1 receptors; AMPA receptors

1. Introduction

Tobacco use remains a major public health problem and there is still an urgent need for better pharmacotherapies to treat nicotine dependence. The use of electronic cigarettes (e-CIGs) has attracted considerable and controversial attention: some authors show that they are safer than tobacco cigarettes (CIGs) and effective as smoking cessation aids (Dawkins et al. 2013a, 2013b), whereas others express concerns about their potential health hazards (Czogala et al. 2014; Sears et al. 2017). Nicotine the main addictive agent delivered via CIG smoke or e-CIG vapour, is rewarding and reduces stress and anxiety (Benowitz, 2010).

The endogenous molecular targets of nicotine are neuronal nicotinic receptors (nAChRs) (Pistillo et al. 2015) and nicotine abuse-related effects involve the activation of various brain circuits including the mesolimbic dopaminergic (DAergic) system which is involved in reinforcement, motivation, self-regulation (Volkow et al. 2017) and habit formation (Maskos et al. 2005; Pistillo et al. 2015). Furthermore, the rewarding and addictive properties of nicotine require the integrity of the opioid (Kishioka et al. 2016), cannabinoid (Gamaleddin et al. 2015) and ghrelin neuromodulatory systems (Zallar et al. 2017).

Smoking cessation leads to an aversive state that acts as a negative reinforcer of tobacco consumption (Paolini and De Biasi, 2011), and the emergence of negative symptoms during abstinence is one of the main reasons for continued smoking (reviewed in Pistillo et al. 2015). Chronic nicotine exposure leads to neuro-adaptations in various brain circuits that promote and sustain dependence. One important step in the sequence of molecular events leading to abuse-related behaviour is the increased expression of FosB and its ΔFosB isoform in the ventral striatum (Kandel and Kandel, Nestler, 2008; Nestler et al. 2001; 2014; Pich et al. 1997).

ΔFosB is a transcription factor whose unusually long half-life allows it to accumulate and remain for weeks in chronically active cells. In the nucleus accumbens (NAc), ΔFosB is induced by drugs of abuse, natural rewards (Pitchers et al. 2010, 2013) and several types of stress (Nestler, 2008; Perrotti et al. 2004; Vialou et al. 2010).

CIG smoking is a gateway for illegal drugs such as cocaine and heroin (Kandel and Kandel, 2014; Lai et al. 2000). The term “gateway” is used to describe a sequential progression in the use of addictive substances from tobacco and alcohol to cannabis,

and then to other illicit drugs (Kandel et al. 1992; Kandel and Kandel, 2015; Kelley and Middaugh, 1999; Linskey et al. 2003).

When administered to animals, nicotine and delta-9-tetrahydrocannabinol (**Δ**9-THC), (the primary psychoactive costituent of cannabis) produce several common pharmacological effects (reviewed in Scherma et al., 2016). In particular, their reinforcing and anxiolytic effects are synergistic when their sub-threshold doses are administered together (Valjent et al 2002; Balerio et al, 2006; Scherma et al. 2016).

Marijuana smoking is frequent among adolescent tobacco smokers (Rabin et al. 2015; Rubistein et al. 2014), and even more frequent among heavy smokers. Although no direct association has been found between the early onset of smoking and later cannabis use, early nicotine use may increase the risk of developing a cannabis use disorder (Rubistein et al. 2014). Cannabis use is often associated with tobacco use (Agrawal et al. 2009) and nicotine dependence (Okoli et al. 2008), and decreases the likelihood of smoking cessation (Amos et al. 2004).

Despite the close correlation between early nicotine use and cannabis abuse, there are few preclinical data indicating whether nicotine alters the rewarding properties of cannabis or the mechanisms involved. Furthermore, it is not known whether nicotine delivery via CIG or e-CIG is relevant in establishing a gateway to Δ^9-THC.

We have recently developed a technique that allows conventional CIG smoke or e-CIG vapour to be chronically and intermittently introduced into a smoke chamber containing mice, thus mimicking the human route of passsive smoking and simulating the pharmacokinetic characteristics associated with CIG smoking or e-CIG inhalation. We have shown that such chronic exposure to e-CIG vapour or CIG smoke containing the same amount of nicotine gives rise to a number of similarities and some differences (Ponzoni et al. 2015). Mice exposed to CIG smoke and e-CIG vapour have high levels of nicotine in brain and cotinine (the major nicotine metabolite) in urine that are comparable with those found in human smokers. Both types of exposure cause nAChR up-regulation, decreased food intake and body weight, and typical withdrawal behaviours (Ponzoni et al. 2015) that are similar to the behavioural and psychological alterations found in smokers and abstinent humans (Koob and Volkow, 2010).

The aims of this study were to test whether there is an alteration in sensitivity to Δ^9-THC reinforcement in chronically e-CIG- or CIG-exposed mice during short- (eight-day) and long-term (66-day) CIG or e-CIG withdrawal, identify the abuse-related

neurochemical alterations occurring in these mice at the end of exposure and during withdrawal, and analyse the possible differences between CIG and e-CIG exposure.

2. Experimental procedures

2.1. Animals

A total of 284 eight-week-old male Balb/c mice (Charles River, Calco, Como, Italy) were housed five per cage in a climatically controlled colony room with a 12 h light/dark cycle (lights on at 7.00 am) with food and water *ad libitum*. The experiments were performed with the experimenter blinded to treatment.

All of the experimental procedures were carried out in accordance with the European Community Council Directive No. 86/609/EEC and the subsequent Italian law on the 'Protection of animals used for experimental and other scientific reasons'. Every effort was made to minimise the number of animals used and their discomfort. The experimental schedule is shown in Figure 1.

2.2. Exposure to e-CIGs and CIGs

One week after their arrival, the mice were divided into three groups of 30 mice each and exposed to pump driven e-CIG, CIG or AIR as previously described for three 30-minute sessions/day, for seven weeks.

The level of brain nicotine and cotinine evaluated at the end of 7-week exposure, were very similar between e-CIG vapour and CIG smoke exposed mice and significantly different from those of mice exposed to air (Ponzoni et al. 2015). (See Supplementary Data for details of the apparatus).

2.3 Behavioural studies

2.3.1. Elevated Plus Maze test

Fifty nine days after the last session of exposure and 24 h before the CPP test began, the mice underwent elevated plus maze task to assess anxiety (see Supplementary Data for further details).

2.3.2. Conditioned place preference (CPP) test

Two (condition B), 30 (condition C) or 60 (condition D) days after the last session of exposure, mice underwent CPP testing (see scheme on Figure 1).

Apparatus. CPP was tested in a shuttle box as described elsewhere (Braida et al. 2008). Briefly, the apparatus was divided into two equally sized compartments separated by a guillotine door. The compartments had different visual and textured cues in the form of brown and white horizontal lines or circles, and a rough or smooth wooden floor. The visual and tactile cues were balanced so that no evident preference was shown before conditioning.

Procedure. The task consisted of three phases: preconditioning, conditioning and post-conditioning.

Pre-conditioning (day 1). To check for any initial place preference bias, each mouse was allowed to explore the two compartments for 15 min and the time spent by each animal in the two compartments was recorded.

Conditioning (*days 2-6*). Conditioning sessions (five for Δ^9-THC and five for vehicle) were carried out twice a day at 9 am and 4 pm. In the morning session five minutes after the i.p. injection of Δ^9-THC each animal was confined to the drug-paired compartment for 30 min with the door closed. In the afternoon session they received vehicle i.p. and were confined to the vehicle-paired compartment for 30 min. All of the experiments were counterbalanced between drug and vehicle chamber pairing. Control animals always received vehicle in the morning and in the afternoon.

Post-conditioning (day 7). On the test day, neither drug nor vehicle was injected. Each mouse was put in one of -the two compartments, with access to both sides, and the time spent in each of the two compartments was measured over a 15-min period as an indicator of rewarding properties. Preference was expressed as the difference (Δ) in the amount of time they spent in the conditioning chamber during post and pre-conditioning.

2.4. Biochemical studies

2.4.1. Brain tissue dissection

One h (condition A) or two (condition B) or 60 (condition D) days after their last exposure to nicotine through e-CIG or CIG , the mice were euthanized by means of cervical dislocation, their brains were rapidly removed, and the areas of interest were

dissected, placed in 1.5 mL Eppendorf tubes, and quickly frozen on dry ice before being stored at −80 °C.

2.4.2. Antibody production and characterisation

For AMPA receptor (AMPAR) subunit detection we used anti-GluA2-3 and anti-GluA1 antibodies (Abs) produced and characterised by us as described in (Pistillo et al. 2016).

2.4.3. Tissue homogenates and membrane preparation

After thawing, the NAc, prefrontal cortex (PFC) or ventral tegmental area (VTA) tissues of each mouse were separately homogenised manually in 20 volumes (w/v) of ice-cold TME buffer (50 mM Tris HCl, 1 mM EDTA, and 3 mM $MgCl_2$, pH 7.4) for binding and GTP□S stimulation. The homogenates were centrifuged at 20000xg for 30 min at 4°C, and the resulting pellets were resuspended in assay buffer (50 mM Tris HCl, 3 mM $MgCl_2$, 0.2 mM EGTA, 100 mM NaCl, pH 7.4), homogenised, and diluted to a concentration of ~2 mg/mL with assay buffer.

For ΔFosB analysis, the dissected NAc tissues were homogenised in 20 mM HEPES buffer (pH 7.8) with 0.4 M NaCl, 20% glycerol, 5 mM MgCl, 0.5 mM EDTA, 0.1mM EGTA, 1% Nonidet P-40 containing 500 □M dithiothreitol and 1% phosphatase inhibitor cocktail 3 (Sigma-Aldrich). The samples were loaded, separated on 10% SDS-PAGE and electrophoretically transferred. Protein concentrations were measured using the Pierce™ BCA Protein Assay Kit.

2.4.4. Immunoblotting and densitometric quantification of Western blot bands

The AMPAR subunits were analysed by means of Western blotting as previously described (Pistillo et al. 2016). ΔFosB was detected using an anti-FosB Ab (clone 5G4, 1:1000, Cell Signalling), and the signal was normalized to the actin content determined in the same Western blot using an anti-actin Ab (clone AC-40, 1:3000, Sigma-Aldrich). Under condition A, B and D, the ΔFosB values in the e-CIG and CIG exposed mice were normalised by taking the values in the AIR exposed mice as one. For AMPAR subunits the signal was normalised to the actin content and the values were normalised by taking the values in the AIR exposed mice as one. The GluA1/GluA2-3 ratio was

calculated by dividing the normalised values of GluA1 by the normalised values of GluA2-3 in each sample.

2.4.5. [^{3}H]-CP-55940 binding assay

NAc membranes (20 and 40 □g of membrane protein) were incubated for 2 h at 32°C with 1 nM [^{3}H]-CP-55940 (175 Ci/mmol, Perkin Elmer, Boston) in a final volume of 0.1 mL of TME containing 10 mg/mL of BSA in siliconized tubes. Non-specific binding was determined in the presence of 30 □M CP-55940. Incubation was terminated by rapid filtration through Whatman GF/C filters pretreated with 0.5% polyethyleneimine (PEI). The filters were washed three times with ice-cold Tris-HCl buffer (pH 7.4) containing 1 mg/mL BSA. Filter bound radioactivity was counted in a liquid scintillation □ counter. At each time point (one hour, two or 60 days after last exposure) at least four separate experiments were performed in triplicate and in each experiment the binding data (CPM specific binding/□g of protein) were normalized by taking as 1 the value of AIR exposed mice.

2.4.6. Agonist –stimulated [^{35}S]GTP□S binding

Agonist–stimulated [^{35}S]GTP□S binding was carried out as previously described (Breivogel et al. 1997) with some modifications (see Supplementary Data).

The data of concentration-effect parameters for activation of [^{35}S]GTP□S binding to NAc membranes were statistically analysed using Graph Pad Prism 6 software and were obtained by analysing four-five independent experiments performed in duplicate using a three-parameters logistic model for monotonic curves in which the Hill coefficient was fixed equal to one.

2.5. Statistical analysis

The data are given as mean values ± SEM, and were statistically analysed using Graph Pad Prism 6 software. Two-way ANOVA (followed by Bonferroni's *post hoc* test, when applicable), was used to compare animal performance in the behavioural experiments. The data from the binding and 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. Seven-week exposure to e-CIGs or CIGs increases the rewarding properties of Δ^9-THC during withdrawal.

To determine whether previous exposure to e-CIGs or CIGs alters the sensitivity to the rewarding properties of Δ^9-THC, Balb/c mice underwent a CPP test that measures the rewarding properties of drugs of abuse (Carr et al., 1989). As no data are available concerning the effect of Δ^9-THC on Balb/c mice undergoing a CPP test, we preliminarily determined a Δ^9-THC dose-response curve in naive mice. One-way ANOVA revealed a difference in the time spent in the Δ^9-THC-paired chamber on the test day among the groups ($F_{3, 36}$=6.90, p=0.001; Figure 2a). *Post hoc* analysis showed a significant increase in the time spent in the drug-associated chamber starting from a dose of 0.03 mg/kg, and so we used a 0.01 mg/kg sub-threshold dose of Δ^9-THC for the subsequent experiments. As shown in Figure 2b-d, when the animals underwent the CPP test after vehicle or Δ^9-THC injection, there was a main effect of treatment ($F_{5, 166}$=24.25, p=0.0001) and a treatment x time interaction ($F_{10, 166}$= 2.52, p=0.007), but no main effect of time ($F_{2, 166}$=1.49, p=0.22) (two-way ANOVA (Figure 2). *Post hoc* analysis confirmed that a threshold dose of 0.01 mg/kg Δ^9-THC did not induce any rewarding effect when the animals were pre-exposed to AIR, but 7-week pre-exposure to e-CIGs or CIGs significantly increased the time spent in the drug-paired compartment at all the tested times (eight, 36 or 66 days after CIG or e-CIG exposure cessation), even if there was a progressive decrease in the rewarding .

These findings suggest that pre-exposure to e-CIGs or CIGs provoke a long-lasting increased sensitivity to Δ^9-THC-induced rewarding effects.

We have previously found (Ponzoni et al. 2015) that exposure to e-CIGs/CIGs affects emotional profile from 24 h to 30 days after nicotine withdrawal. We verified whether this alteration persisted for 60 days by evaluating anxiety-like behaviour in the elevated plus maze test. We observed significant between-groups differences in the number of open arm entries and the time spent in the open arm (open arm entries: χ^2 =6.42, p=0.04; open arm time: χ^2 =7.65, p=0.02). The number of open arm entries and the time spent in the open arm were lower in the CIG or e-CIG exposed groups than in the AIR group,

but the difference was statistically significant only in CIG group (Figure S1). No differences were found in the mean number of total arm entries ($F_{2, 27}$=2.25, p=0.12), indicating that motor function was not altered.

3.2. CB1 receptor levels and agonist stimulated [^{35}S]GTP□S binding are decreased after 66 days of withdrawal but not one hour after exposure or after eight days of withdrawal in mice previously exposed to CIGs or e-CIGs for seven weeks.

We investigated whether the increased rewarding properties of Δ^9-THC during withdrawal were due to changes in CB1 receptor levels by means of binding studies using the CB1 receptor ligand [^{3}H]-CP-55940. Preliminary saturation binding curves showed that the affinity (Kd) of [^{3}H]-CP-55940 to NAc membrane was 0.3 nM, and so the number of receptors was determined using a saturating concentration of 1 nM and NAc membranes obtained from mice one hour (condition A), eight days (condition B) or 66 days (condition D) after seven weeks exposure to AIR, e-CIG or CIG. The eight- and 66-day samples were those obtained from mice that underwent the CPP test two or 60 days after AIR, e-CIG or CIG exposure.

There was no difference in receptor levels between any of the groups of condition A or condition B (Figure 3). In condition D, Kruskal-Wallis analysis showed no difference between the mice exposed to AIR that received vehicle or Δ^9-THC, but a significant decrease in receptor levels in the e-CIG, e-CIG+ Δ^9-THC, CIG+ Δ^9-THC mice compared to AIR mice (χ^2=17.83 p =0.0032) (Figure 3).

We also verified whether there was a change in the activation of CB1 receptors after vehicle or Δ^9-THC treatment at different times of withdrawal in the mice exposed to AIR, CIG or e-CIG mice by measuring CP-55940-stimulated [^{35}S]GTP□S binding to NAc membranes. Non-linear regression analysis of the activation curves of the condition A mice showed no differences in Emax or EC_{50} between the groups (Table 1). There was also no difference in the Emax and EC_{50} values between the mice of condition B groups that received vehicle or 0.01 mg/kg Δ^9-THC. On the contrary, analysis of the activation curves of the samples obtained from mice in the condition D (66 days) showed no difference in EC_{50} and basal values, but the Emax values were significantly different ($F_{5,169}$=12.3, p<0.0001). Table 1 shows the Emax values at 66 days of the groups treated with vehicle ($F_{2,84}$ 4.2 p=0.017) or Δ^9-THC ($F_{2,85}$ 6.9 p=0.0017), which indicate that the CIG and e-CIG mice treated with vehicle or 0.01

mg/kg Δ^9-THC had significantly lower E_{max} values than AIR mice treated with vehicle or vehicle+ Δ^9-THC (Table 1).

In conclusion, after 66 days withdrawal, the e-CIG and CIG mice that received vehicle or Δ^9-THC had fewer CB1 receptors and a lower functional response to the CB1 agonist CP-55940.

3.3. Seven-week exposure to e-CIGs or CIGs increases NAc ΔFosB levels, which remain high during long-term withdrawal

Chronic exposure to nicotine leads to neuroadaptations in the various brain circuits that promote and sustain nicotine dependence. An important step in the sequence of molecular events leading to abuse-related behaviour is the increased expression of FosB and its ΔFosB isoform in the ventral striatum (Nestler, 2008).

In preliminary experiments, we analysed the expression of ΔFosB in the NAc of naïve mice treated with vehicle or 0.01 mg/kg Δ^9-THC using the CPP schedule, and found no difference between groups (Kruskal Wallis analysis p=0.8) (Figure S2). We also found no significant changes in basal ΔFosB levels in the NAc of the mice exposed to AIR in conditions A, B and D (data not shown). However when we compared the e-CIG, CIG- and AIR-exposed mice one hour, eight or 66 days after the end of eCIG or CIG exposure, we found a significant increase in NAc ΔFosB levels in the mice exposed to e-CIG and CIG (Fig. 4a). One hour (condition A: χ^2 =10.2 p=0.003) or eight days (condition B: χ^2=14.0 p=0.0009) after e-CIG and CIG cessation, ΔFosB levels were significantly higher in the e-CIG and CIG groups than in the AIR group. After 66 days (condition D: χ^2=25.2 p=0.0001), only the CIG group showed a significant increase, and treatment with Δ^9-THC did not further increase ΔFosB levels (Fig. 4a).

3.4 Seven-week exposure to e-CIGs or CIGs alters the ratio between GluA1 and GluA2-3 AMPA receptor subunits in the mesocorticolimbic system during withdrawal

It is known that addictive drugs remodel the mesocorticolimbic reinforcement circuitry by inducing the widespread adaptation of glutamatergic synapses (van Huijstee and Mansvelder, 2014). A typical alteration in glutamatergic transmission during addictive drug withdrawal is an increase in the ratio between the AMPAR GluA1 and GluA2

subunit expression, which leads to the formation of AMPARs with greater Ca^{2+} permeability (Scofield et al. 2016). This and the fact that ΔFosB up-regulation alters glutamate transmission (Kelz et al. 1999; van Huijstee and Mansvelder, 2014) prompted us to analyse the NAc expression of AMPAR GluA1 and GluA2-3 subunits under conditions A, B and D. Under condition A, the GluA1/GluA2-3 ratio was significantly higher in the CIG or e-CIG groups than in the AIR group (Kruskal-Wallis test χ^2=14.30, p<0.0001) (Fig. 4b). Under condition B, the GluA1/GluA2-3 ratio was significantly increased only in the e-CIG group (Kruskal-Wallis test χ^2=7.14, p=0.0187). Finally, under condition D, the ratio was significantly higher in the CIG, e-CIG+ Δ^9-THC and CIG+ Δ^9-THC groups than in the AIR group (Kruskal-Wallis test χ^2=19.63, p=0.0006). The changes in the levels of GluA1 and GluA2-3 subunits under the different conditions are shown in supplementary Figure S3.

We also evaluated GluA1 and GluA2-3 expression in the VTA and PFC areas of the mesocorticolimbic pathway (Fig. S4). There was no change in the GluA1/GluA2-3 ratio at any time in the VTA, but the ratio was increased in the PFC in the CIG group under condition B (χ^2=7.60, p=0.0107) and significantly increased in the CIG or e-CIG groups under condition D (χ^2=8.87, p=0.0150).

4. Discussion

The main findings of this study are that a sub-threshold dose of Δ^9-THC is ineffective in inducing CPP in mice pre-exposed to AIR, but very effective after eight, 36 and 66 days of e-CIG or CIG withdrawal in mice pre-exposed to e-CIG or CIG for seven weeks. CPP involves demanding learning processes to ensure that mice associate drug experience with a spatial context. However, it is unlikely that the Δ^9-THC increased CPP is related to possible improvement in nicotine-induced cognitive function because learning and memory impairments are among the most frequently reported behavioural effects of nicotine-withdrawal in humans and experimental animals (Kutlu and Gould, 2016) during withdrawal. In addition, mice exposed to CIG smoke or CIG vapour using the same schedule as that used in the present study showed spatial object recognition impairment from 24 hours to at least 30 days after withdrawal (Ponzoni et al. 2015).

Our results are in line with those of Li et al. (2014) who found that one week's pretreatment with nicotine dramatically increased the CPP induced by a sub-threshold dose of cocaine. This effect was accompanied by changes in some specific metabolites

in NAc and striatum, thus creating a favourable metabolic environment for enhancing the conditioned rewarding effect of cocaine.

Exposure to e-CIG vapour or CIG smoke increases the expression of ΔFosB in the NAc, and this increase persists after short-term e-CIG or CIG withdrawal and long-term CIG withdrawal.

Chronic exposure to e-CIG vapour or CIG smoke does not change the number or function of CB1 receptors in the NAc, which only decrease during long-term e-CIG or CIG withdrawal. Upon cessation and after short- and long-term withdrawal, there is an increase in the AMPAR GluA1/GluA2-3 ratio in the NAc of e-CIG- and CIG-exposed mice.

The primary mediators of the psychoactive properties of Δ^9-THC are presynaptic CB1 receptors which, after Δ^9-THC binding, can modify the synaptic efficacy of the neuronal circuits involved in reinforcement. The first hypothesis we tested was that the increased sensitivity to the rewarding effect of Δ^9-THC was due to the up-regulation of CB1 receptors. When we analysed the expression and function of these receptors one hour after e-CIG or CIG exposure or eight days after withdrawal, we did not find any difference between the AIR-, e-CIG- or CIG-exposed mice that received vehicle or Δ^9-THC. Howeve, after a very long withdrawal period, there was a decrease in CB1 levels and function after vehicle or Δ^9-THC treatments. It has previously been reported that repeated Δ^9-THC administrations can regulate CB1 receptor desensitisation and down-regulation in specific brain areas (Lazenka et al. 2014a, 2014b). However, as these adaptations were obtained using a Δ^9-THC dose that was 1000 times higher and given for longer times than those used in our study the down-regulation of CB1 receptors we observed is probably due to long-term adaptations in NAc circuitry that are unrelated to pharmacological CB1 receptor stimulation. Overall, the data relating to CB1 receptors do not point to their involvement in the increased sensitivity to rewarding effect of Δ^9-THC observed in mice chronically exposed to CIG or e-CIG. ΔFosB up-regulation in the NAc is critical for the enhanced rewarding effect of many drugs of abuse (Pitchers et al. 2013) and so we hypothesized that it may also be involved in the observed increased sensitivity to Δ^9-THC. The sub-threshold dose of Δ^9-THC used in our CPP test was not sufficient to increase ΔFosB expression in naïve mice, but, there was a long-lasting accumulation of ΔFosB in the NAc of mice exposed to CIGs or e-CIGs for seven-weeks. The ΔFosB up-regulation induced by chronic CIG or e-CIG

exposure may modulate the signalling of Δ^9-THC at receptor /effector level and lower the threshold for Δ^9-THC rewarding effect. The effects of ΔFosB may extend beyond the regulation of drug sensitivity *per se* to the more complex behaviours related to addiction processes. One study (Vialou et al. 2010) has shown that basal NAc concentrations of ΔFosB determine an individual's initial vulnerability to the stress of social defeat, and the degree of ΔFosB induction in response to chronic stress determines the individual's susceptibility or resilience (Vialou et al. 2010). The authors found that susceptible mice have higher GluA1 levels in the NAc than controls or resilient mice and increased medium spiny neuron excitability in response to glutamate. Our findings indicate that e-CIG- and CIG- exposed mice show greater anxiety-like behaviour after 30 (Ponzoni et al. 2015) and 60 days' withdrawal (Fig. S1), and, a larger number of NAc GluA1 receptors after 60 days than controls, which suggests that their anxiety-like behaviour may have been driven by a similar mechanism. The gene encoding the GluA2 subunit in AMPARs is one of the target genes of ΔFosB and this, together with the functionally relevant changes in NAc glutamatergic transmission induced by many psychostimulants (reviewed in van Huijstee and Mansvelder, 2014; Wolf and Tseng, 2012) prompted us to evaluate the expression of AMPAR subunits in the mesocorticolimbic circuit. In agreement with previously published data concerning motivated and addictive behaviour (Gipson et al., 2013; Kenny et al. 2009) we found that chronic CIG or e-CIG exposure and its withdrawal for different times induce an increase in the GluA1/GluA2-3 ratio in the NAc.

Ninety-five percent of the nerve cells in the NAc are GABAergic medium spiny neurons that highly express the calcium-impermeable AMPARs (CI-AMPARs) containing the GluA2 subunit (Reimers et al. 2011) whereas AMPARs lacking the GluA2 subunit (also called Ca^{2+} -permeable CP-AMPARs) account for less than 10% of all AMPARs (Conrad et al. 2008; Pistillo et al. 2015; Reimers et al. 2011). GluA1-containing CP-AMPARs accumulate in NAc synapses after the withdrawal of extended access cocaine self-administration (Conrad et al. 2008) and mediate cue-induced cocaine seeking on withdrawal day 45. This CP-AMPAR-dependent enhancement of drug seeking behaviour is due to an increase in the reactivity of NAc medium spiny neurons to glutamate as a result of the greater conductance of CP-AMPARs in comparison with the CI-AMPARs that normally dominate in NAc synapses. The increased ratio between GluA1-containing CP-AMPARs and GluA2-3-containing CI-

AMPARs could significantly increase synaptic strength (Guire et al. 2008) thus altering the sensitivity of NAc synapses to the stimulating action of Δ^9-THC.

In conclusion, our data show that in a model system in which e-CIG vapour or CIG smoke are chronically and intermittently inhaled by mice (thus leading to similar brain nicotine and urine cotinine levels), e-CIG exposure has addiction-related neurochemical and behavioural effects that are very similar to those of chronic CIG exposure. In particular, chronic e-CIG and CIG exposure both induce increased sensitivity to a sub-threshold dose of Δ^9-THC, thus suggesting that they share the gateway effect that has been attributed to chronic nicotine exposure (Kandel and Kandel, 2014). In both cases, the gateway effect is very persistent as it was observed two months after CIG or e-CIG withdrawal. We hypothesize that this persistent effect may be due to long-lasting neurochemical effects in the NAc that include a persistent increase in ΔFosB expression and the AMPAR GluA1/GluA2-3 subunit ratio. Future studies will be needed to address whether nicotine exposure via different means causes changes in sensitivity to rewarding effects of other drugs of abuse.

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Competing financial interests

The authors declare no competing financial interest.

Authors contributions

CG, MM and MZ designed, performed and interpreted biochemical experiments and contributed to the design and interpretation of behavioural experiments. LP, BD and

MS designed, performed and interpreted behavioural experiments. CG, FC, PV, BD, M. and MZ supervised the entire work and wrote the manuscript with the input of all co-authors.

Conflict of interest

All other authors declare no potential conflict of interest.

Supplementary materials

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FIGURE LEGENDS

Figure 1 Timeline of experimental treatments and behavioural measurements. The animals were divided into four groups of 30 mice each and exposed (3 times a day) for 7 weeks to AIR or e-CIG or CIG. One hour after the last exposure, at the end of the seventh week, the first group of animals (Condition A) was euthanized for biochemical and molecular evaluation. The remaining groups (Conditions B,C and D) were tested for CPP after a 5-day conditioning regimen consisting of Δ^9-THC or vehicle injections respectively two , 30 or 60 days after the last exposure.The mice under Condition D underwent the elevated plus maze task (EPM) test one day before beginning the conditioning regimen so that their emotional profile could be evaluated. One hour after the CPP test, the animals under Conditions B and D were sacrificed for biochemical and molecular evaluation. Δ^9THC : Δ^9Tetrahydrocannabinol, VEH: Vehicle

Figure 2 Pre-exposure for seven weeks to e-CIG or CIG increases the rewarding properties of Δ^9-THC during withdrawal. (a) Δ9-THC given i.p., dose-dependently induces conditioned place preference in naïve mice. §p<0.05, §§ p<0.01 vs vehicle group; $$p<0.01 vs 0.01mg/kg (Tukey's *post hoc* test). After eight (b), 36 (c) and 66 (d) days of withdrawal, there was no significant change from the pre-conditioning preference in e-CIG, CIG and AIR groups treated with vehicle, whereas a sub-threshold dose of Δ^9-THC (0.01 mg/kg, i.p.) induced a greater place preference on test day in the e-CIG and CIG groups. *p<0.05, **p<0.01, ****p<0.0001 in comparison with AIR +Δ^9-THC; $$ p<0.05, $$$$ p<0.0001 in comparison with the corresponding group +vehicle; #p<0.05, ### p<0.001 in comparison with the corresponding treatment after eight days; &p<0.05 in comparison with CIG + Δ^9-THC at the same withdrawal intervals (each bar n = 10-12).

Figure 3 The density of CB1 receptors in the NAc membranes of mice was estimated under condition A (one hour withdrawal,WDW), B (eight days WDW) and D (66 days WDW) using a saturating concentration of 1nM [^{3}H]-CP-55940 in the presence or not of an excess of cold CP-55940, and was expressed as CPM-specific binding /□g of protein and normalized by taking the value in the AIR exposed mice as one (each bar n=7-8).

Kruskal-Wallis analysis of group D (66 days WDW) and Dunn's post hoc test showed that there was a significant decrease in receptor levels in the e-CIG, e-CIG+ Δ^9-THC and CIG+ Δ^9-THC mice *p<0.05 vs AIR.

Figure 4 ΔFosB and AMPAR subunit ratio in the NAc of mice under conditions A, B or D.

NAc proteins of the mice exposed to AIR (n=7), eCIG (n=7) or CIG (n=7) under conditions A, B and D, were separated on 10% (ΔFosB) or 7.5 % acrylamide SDS gels (GluA1 and GluA2-3), electrotransferred to nitrocellulose, and probed with antibodies as described in the Methods. Identical amounts of proteins were always loaded on the same gel, and the loading was further verified by actin staining.

a) The Western blot analysis is expressed as a ratio between the e-CIG, CIG and AIR-exposed mice. Each bar shows the mean values ± SEM obtained by analyzing the samples obtained from seven mice tested in five different experiments. Underneath each graph is a representative blot of the corresponding conditions. The Western blotting data were statistically analyzed using one-way ANOVA followed by Dunn's post hoc test (*p< 0.05, ** p< 0.01 and ***p< 0.001), and were found to be significantly different from the AIR-exposed mice under the same condition.

b) The Western blot values of the e-CIG or CIG exposed mice were normalised by taking the values of the AIR-exposed mice as one. The GluA1/GluA2-3 ratio was obtained by dividing the normalised GluA1values by the normalised GluA2-3 values.

Table 1 Concentration-effect parameters for activation of [^{35}S]GTP□S binding to NAc membranes

Withdrawal interval	Exposure and Treatment	E_{max} % stimulation	EC_{50}, nM (CI)
1 hour	AIR	188±6	75(32-128)
	e-CIG	178±6	15(4-57)
	CIG	194±6	58(28-123)
8 days	AIR+vehicle	167±6	30(8-116)
	e-CIG+vehicle	170±5	46(18-119)
	CIG+vehicle	175±6	95(36-251)
	AIR+Δ^9THC	177±6	35(13-90)
	e-CIG+Δ^9THC	173±5	32(14-75)
	CIG+Δ^9THC	170±5	55(21-140)
66 days	AIR+vehicle	214±7*	13(4-45)
	e-CIG+vehicle	191±4	11(3-32)
	CIG+vehicle	202±5	15(7-34)
	AIR+Δ^9THC	187±6**	5 (2-20)
	e-CIG+Δ^9THC	166±4	10(3-27)
	CIG+Δ^9THC	169±5	17 (6-49)

Concentration-effect parameters for activation of [^{35}S]GTP□S binding to NAc membranes. The maximal theoretical effect for stimulating [^{35}S]GTP□S binding (E_{max}), and concentrations that produce a half-maximal response (EC_{50}) are given. CI = confidence interval.

Figure 1

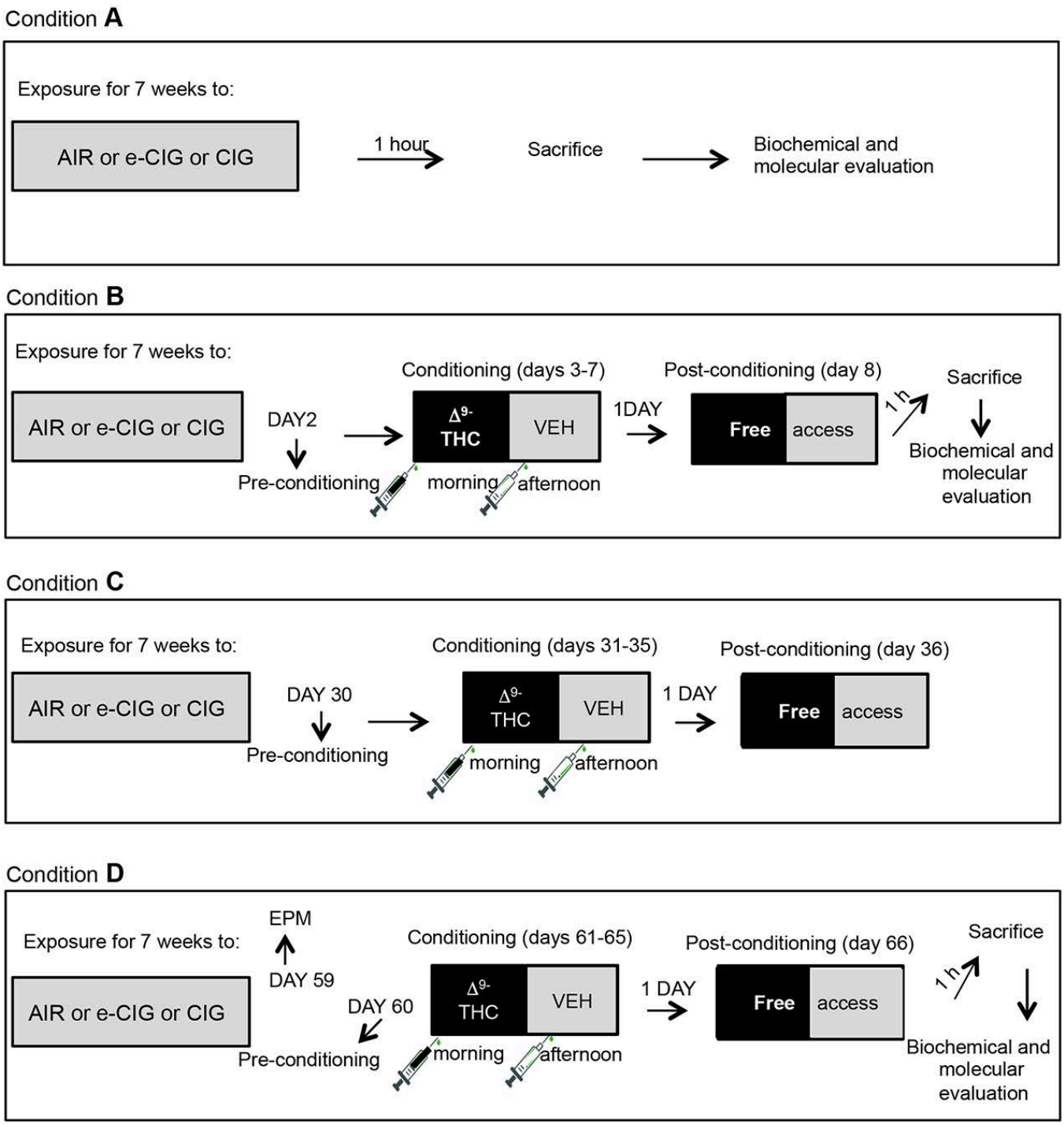

Condition A
Exposure for 7 weeks to:
AIR or e-CIG or CIG
1 hour
Sacrifice
Biochemical and molecular evaluation
Condition B
Exposure for 7 weeks to:
AIR or e-CIG or CIG
DAY2
Pre-conditioning
Conditioning (days 3-7)
Δ9-THC
VEH
morning
afternoon
1DAY
Post-conditioning (day 8)
Free
access
1 h
Sacrifice
Biochemical and molecular evaluation
Condition C
Exposure for 7 weeks to:
AIR or e-CIG or CIG
DAY 30
Pre-conditioning
Conditioning (days 31-35)
Δ9-THC
VEH
morning
afternoon
1 DAY
Post-conditioning (day 36)
Free
access
Condition D
Exposure for 7 weeks to:
AIR or e-CIG or CIG
EPM
DAY 59
DAY 60
Pre-conditioning
Conditioning (days 61-65)
Δ9-THC
VEH
morning
afternoon
1 DAY
Post-conditioning (day 66)
Free
access
1 h
Sacrifice
Biochemical and molecular evaluation

Figure 2

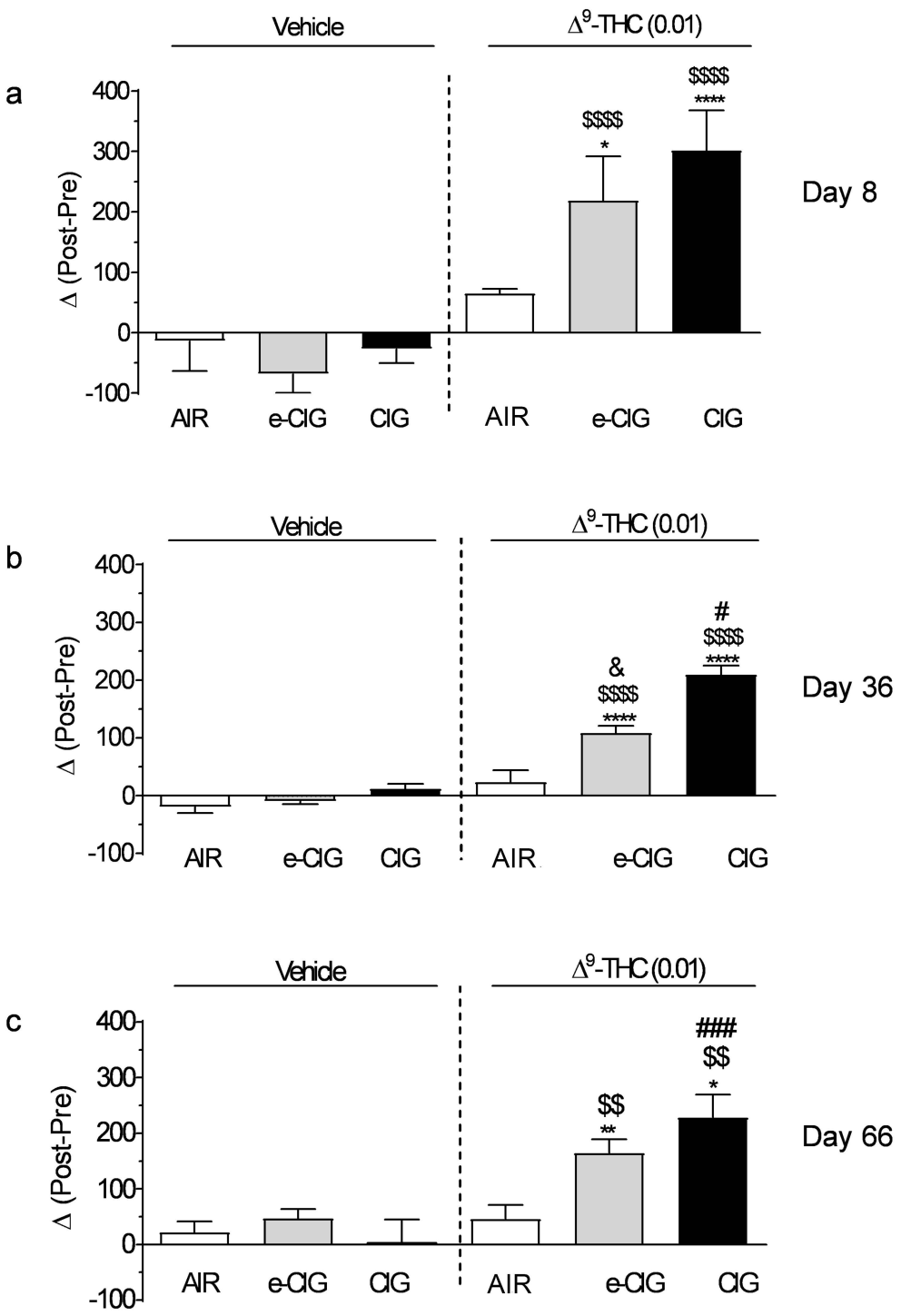

a
Vehicle
Δ9-THC (0.01)
Δ (Post-Pre)
400
300
200
100
0
-100
AIR
e-CIG
CIG
$$$$
*
$$$$
****
Day 8
b
Vehicle
Δ9-THC (0.01)
Δ (Post-Pre)
AIR
e-CIG
CIG
&
$$$$
****
#
$$$$
****
Day 36
c
Vehicle
Δ9-THC (0.01)
Δ (Post-Pre)
AIR
e-CIG
CIG
$$
**
###
$$
*
Day 66

Figure 3

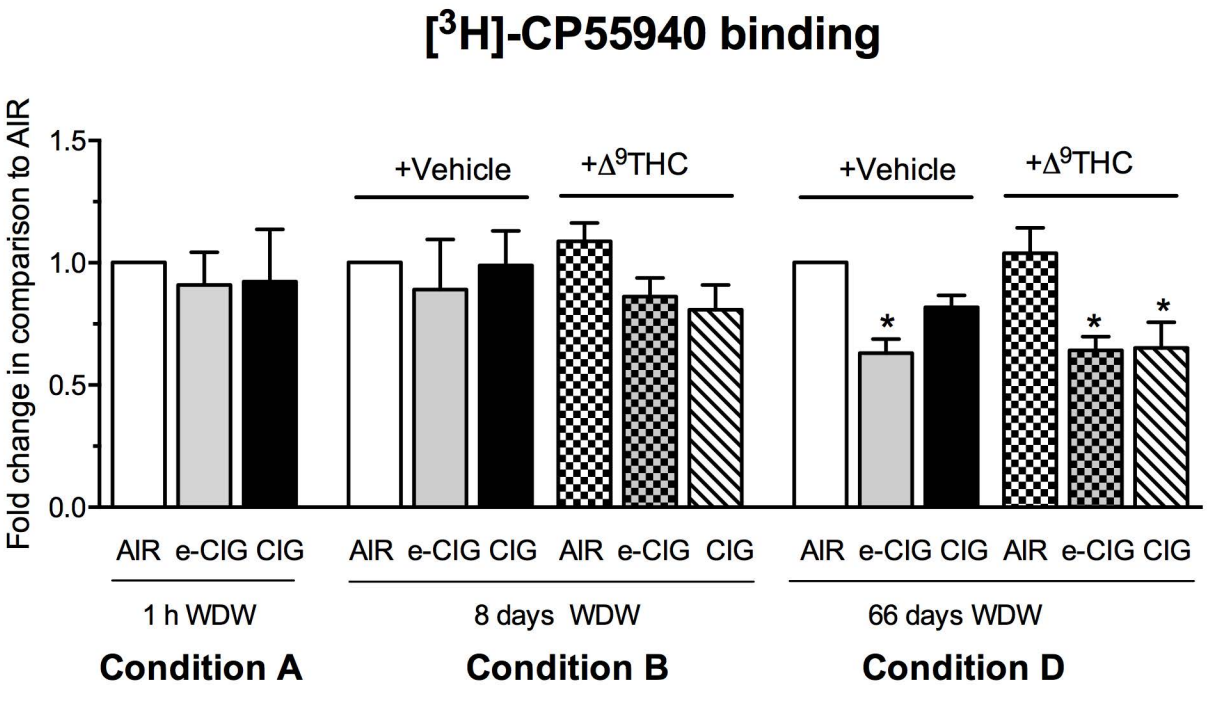
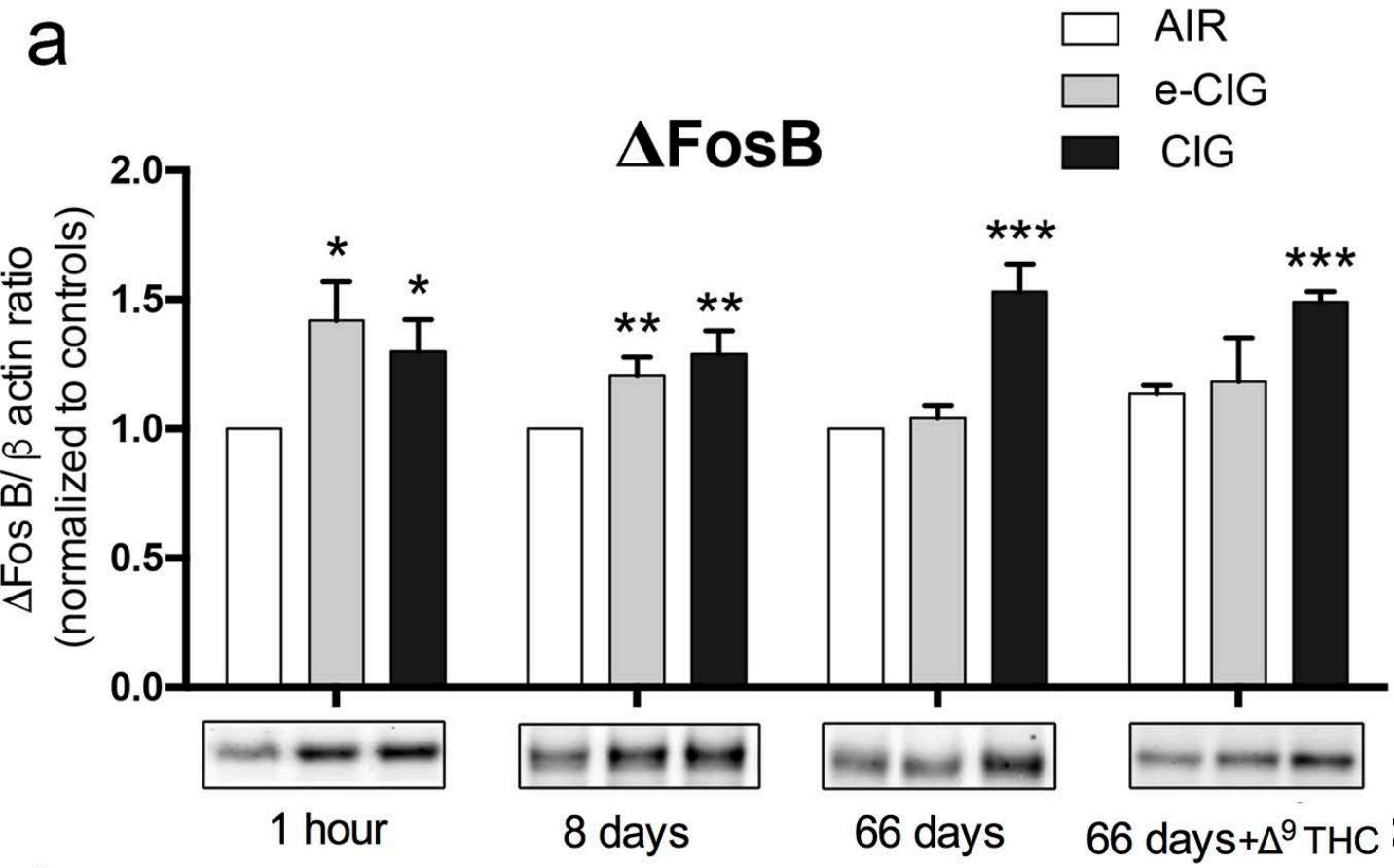
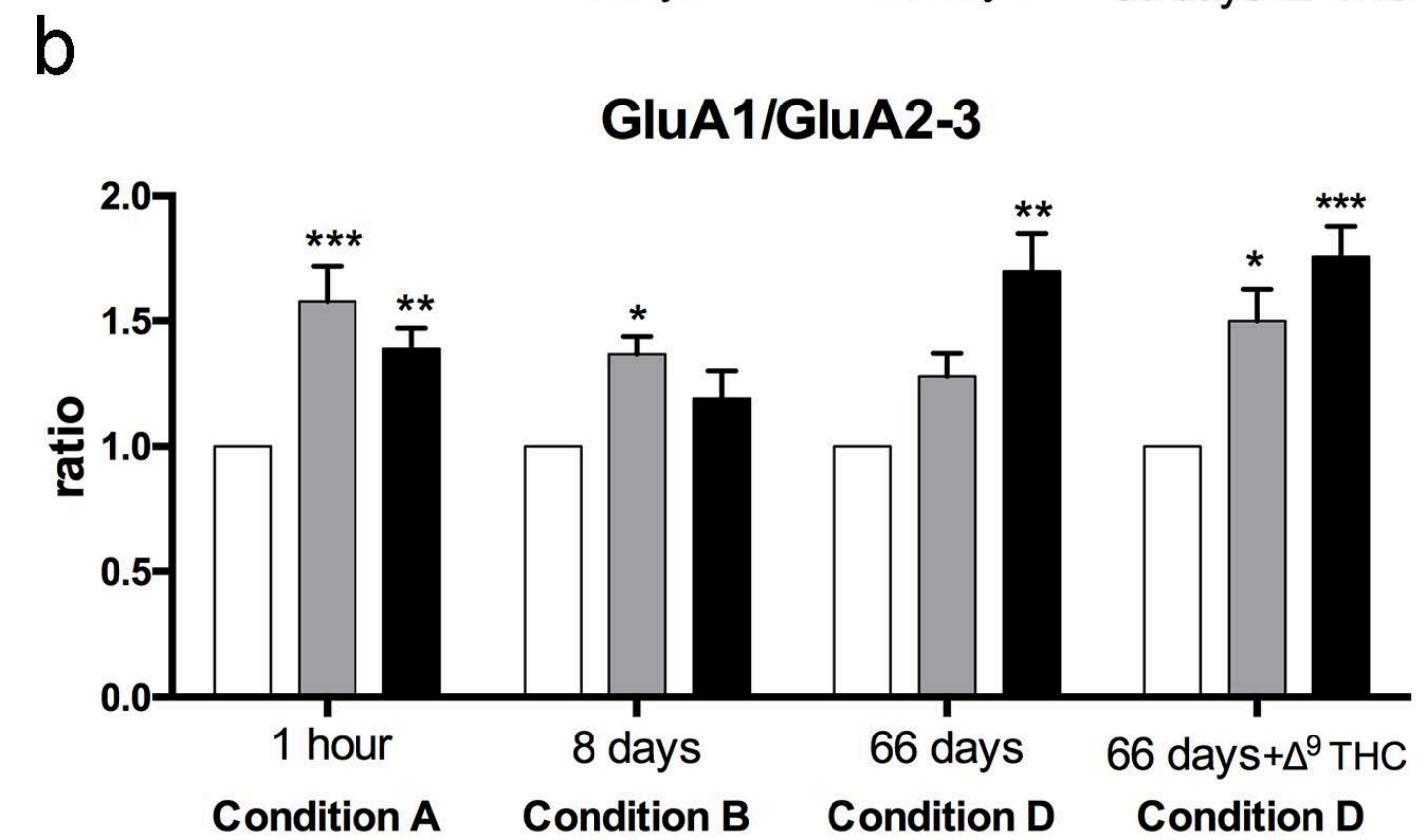

[3H]-CP55940 binding
Fold change in comparison to AIR
1.5
1.0
0.5
0.0
+Vehicle
+Δ9THC
+Vehicle
+Δ9THC
*
*
*
AIR e-CIG CIG AIR e-CIG CIG AIR e-CIG CIG AIR e-CIG CIG
1 h WDW
8 days WDW
66 days WDW
Condition A
Condition B
Condition D

Figure 4


a
AIR
e-CIG
CIG
ΔFosB
ΔFos B/ β actin ratio
(normalized to controls)
2.0
1.5
1.0
0.5
0.0
*
*
**
**
***
***
1 hour
8 days
66 days
66 days+Δ9 THC


b
GluA1/GluA2-3
ratio
2.0
1.5
1.0
0.5
0.0
***
**
*
**
*
***
1 hour
8 days
66 days
66 days+Δ9 THC
Condition A
Condition B
Condition D
Condition D