Original Paper

In utero Δ9-tetrahydrocannabinol exposure confers vulnerability towards cognitive impairments and alcohol drinking in the adolescent offspring: Is there a role for neuropeptide Y?

Journal of Psychopharmacology
2020, Vol. 34(6) 663–679

DOI: 10.1177/0269881120916135
journals.sagepub.com/home/jop
SAGE

Anna Brancato[1], Valentina Castelli[1,2], Gianluca Lavanco[3,4,5], Rosa Anna Maria Marino[6] and Carla Cannizzaro[1]

Abstract

Background: Cannabinoid consumption during pregnancy has been increasing on the wave of the broad-based legalisation of cannabis in Western countries, raising concern about the putative detrimental outcomes on foetal neurodevelopment. Indeed, since the endocannabinoid system regulates synaptic plasticity, emotional and cognitive processes from early stages of life interfering with it and other excitability endogenous modulators, such as neuropeptide Y (NPY), might contribute to the occurrence of a vulnerable phenotype later in life.

Aims: This research investigated whether in utero exposure to Δ9-tetrahydrocannabinol (THC) may induce deficits in emotional/cognitive processes and alcohol vulnerability in adolescent offspring. NPY and excitatory postsynaptic density (PSD) machinery were measured as markers of neurobiological vulnerability.

Methods: Following in utero THC exposure (2 mg/kg delivered subcutaneously), preadolescent male rat offspring were assessed for: behavioural reactivity in the open field test, neutral declarative memory and aversive limbic memory in the Novel Object and Emotional Object Recognition tests, immunofluorescence for NPY neurons and the PSD proteins Homer-1, 1b/c and 2 in the prefrontal cortex, amygdala and nucleus accumbens at adolescence (cohort 1); and instrumental learning, alcohol taking, relapse and conflict behaviour in the operant chamber throughout adolescence until early adulthood (cohort 2).

Results: In utero THC-exposed adolescent rats showed: (a) increased locomotor activity; (b) no alteration in neutral declarative memory; (c) impaired aversive limbic memory; (d) decreased NPY-positive neurons in limbic regions; (e) region-specific variations in Homer-1, 1b/c and 2 immunoreactivity; (f) decreased instrumental learning and increased alcohol drinking, relapse and conflict behaviour in the operant chamber.

Conclusion: Gestational THC impaired the formation of memory traces when integration between environmental encoding and emotional/motivational processing was required and promoted the development of alcohol-addictive behaviours. The abnormalities in NPY signalling and PSD make-up may represent the common neurobiological background, suggesting new targets for future research.

Keywords
Prenatal THC, adolescence, limbic memory, instrumental learning, alcohol vulnerability, neuropeptide Y

Introduction

The endocannabinoid signalling pathway (ECS) is widely expressed in the central nervous system (CNS), where it has major roles in regulating synaptic plasticity through a balancing activity mainly played on excitatory and inhibitory transmissions (Cannizzaro et al., 2006; Wenzel and Cheer, 2018). Its components are expressed and are functional from early developmental stages, when they start regulating neural stem-cell proliferation, differentiation, migration and survival, neuronal connectivity and synaptogenesis, until complete maturation of the CNS (Fernández-Ruiz et al., 2000; Fride et al., 2009; Grant et al., 2018). This poses a relevant issue, since early exposure to exogenous cannabinoids such as Δ9-tetrahydrocannabinol (THC) during neurodevelopment may interfere with the homeostatic role of endocannabinoids and might potentially confer increased vulnerability to adverse neuropsychiatric outcomes (Schonhofen et al., 2018).

Indeed, the broad-based legalisation of cannabis has created a strong need to understand the risks that may be associated with

[1]Department of Health Promotion, Mother-Child Care, Internal Medicine and Medical Specialties of Excellence 'G. D'Alessandro', University of Palermo, Palermo, Italy
[2]Department of Biomedicine, Neuroscience and Advanced Diagnostics, University of Palermo, Palermo, Italy
[3]INSERM U1215, NeuroCentre Magendie, Bordeaux, France
[4]University of Bordeaux, Bordeaux, France
[5]Department of Biomedical and Biotechnological Sciences, Section of Pharmacology, University of Catania, Catania, Italy
[6]Department of Anatomy and Neurobiology, School of Medicine, University of Maryland, Baltimore, USA

Corresponding author:
Carla Cannizzaro, Laboratory of Neuropsychopharmacology, Department of Health Promotion, Mother-Child Care, Internal Medicine and Medical Specialties of Excellence 'G. D'Alessandro', University of Palermo, Piazza delle Cliniche 2, Palermo, Sicilia 90127, Italy.
Email: carla.cannizzaro@unipa.it

cannabis use, particularly for sensitive subgroups such as pregnant women (Grant et al., 2018) and adolescents. Indeed, cannabis is perceived as a natural and harmless substance, as well as an efficient self-medicating strategy for a vast array of medical conditions (Fitzcharles and Eisenberg, 2018; Hasin et al., 2017; Klumpers and Thacker, 2018; Martins et al., 2016), including morning sickness and vomiting during pregnancy (Chang et al., 2019; Metz and Borgelt, 2018). THC, the main psychoactive ingredient of cannabis derivatives, readily crosses the foeto-placental barrier and, due to slower foetal clearance (Harbison and Mantilla-Plata, 1972; Hutchings et al., 1989), results in long-lasting prenatal exposure beyond maternal consumption. It interacts with foetal CB1 receptors, which are detected from the earliest stage of embryo development and throughout prenatal and postnatal development in many areas of the brain (Berrendero et al., 1998; Mato et al., 2003).

Whereas it has been proved that cannabis use during pregnancy is associated with an increased risk of adverse obstetrical outcomes (Gunn et al., 2016), detrimental neuro-behavioural consequences in the progeny are still poorly explored. On the contrary, abnormal activation of the ECS plays a de facto role in emotional dysregulation and cognitive deficits observed in adolescent heavy users of cannabis (Maij et al., 2017). Emerging preclinical evidence demonstrates that adolescent exposure to THC selectively targets molecular and neuropharmacological signalling pathways in both cortical and subcortical regions, including the prefrontal cortex (PFC) and the mesolimbic dopamine (DA) pathway, comprising the ventral tegmental area (VTA) and the nucleus accumbens (NAc; Wenzel and Cheer, 2018).

Adolescent neurodevelopment represents a critical period wherein regulatory connectivity between higher-order cortical regions and subcortical emotional processing circuits is established (Gogtay et al., 2004). THC-induced perturbation of neurodevelopmental trajectories poses a risk for mood disorders, vulnerability to alcohol and drug consumption during adolescence (Renard et al., 2017, 2018). Significant evidence shows that the neuropeptide Y (NPY) system is crucial for emotional control and resilience to stress in accordance with its wide expression in key regions for neuro-excitability and plasticity (Plescia et al., 2014a; Reichmann and Holzer, 2016; Robinson and Thiele, 2017). NPY protects against the negative behavioural consequences of chronic exposure to stressors, including anxiety and depression, and promotes robust reductions in alcohol craving and taking through the activation of NPY Y1 receptors in the extended amygdala (Amy) and medial PFC (mPFC) (Cippitelli et al., 2010; Heilig and Thorsell, 2002; Pandey et al., 2003; Robinson et al., 2019; Robinson and Thiele, 2017). NPY is also able to modulate synaptic strength and plasticity by modulating presynaptic calcium entry and the rearrangement of the architecture of the postsynaptic density (PSD), including the trafficking of ion channel receptors and the recruitment of scaffold proteins functional to synaptic plasticity such as the Homer isoforms (Bacci et al., 2002; Goyal and Chaudhury, 2013; Molosh et al., 2013). Hence, it has been suggested that NPY may produce modifications in cognitive performance (Bennett et al., 1997; Maeda et al., 1993). Indeed, neurochemical analyses have revealed that its overexpression in both the hypothalamus and Amy drives cognitive resiliency and ameliorates negative effects of stress on memory (Sweis et al., 2013). Moreover, robust fear-inhibiting effects of intra-cerebroventricular injection of NPY were demonstrated on contextual fear conditioning in rats (Lach and de Lima, 2013), whereas inactivation of Y1R in mice produces an impairment in reversal learning (Longo et al., 2018).

A potential role for NPY in cannabinoid effects on neurotransmission and synaptic functioning was shown by the association of cannabis use with increased Y1 mRNA expression levels in the PFC (Caberlotto and Hurd, 2001), where cannabinoid receptors are highly localised (Glass et al., 1997). A complex interaction between ECS and NPY signalling has been suggested to contribute to the emotional imbalance induced by chronic stress in mice (Lomazzo et al., 2017), and to anxiety-like behaviour and cognitive deficits observed in adulthood following adolescent exposure to intermittent alcohol (Sanchez-Marin et al., 2017). On these bases, we hypothesised that manipulation of ECS modulatory signalling by THC during critical periods of neurodevelopment such as pregnancy may lead to aberrant plasticity in discrete areas of the brain such as the PFC, the NAc and the Amy, where alterations in NPY signal together with modifications in PSD protein make-up can represent the background of a vulnerable phenotype for cognitive dysregulation and excessive alcohol intake.

To assess this, we analysed different behavioural patterns in adolescent offspring prenatally exposed to THC: behavioural reactivity, neutral declarative and aversive limbic memory, instrumental learning, and alcohol vulnerability in terms of discrete alcohol-related behaviours, such as alcohol taking, alcohol seeking following a forced abstinence and alcohol use despite negative consequences. Moreover, NPY-ergic expression was assessed in key areas of the brain, along with the expression of PSD Homer isoforms, as markers of the postsynaptic functional machinery.

Methods

Animals and treatment

Twelve adult female nulliparous Wistar rats (200–220 g; Envigo, Milan, Italy) were housed in pairs in standard rat cages (40 cm × 60 cm × 20 cm), with ad libitum access to water and food in a temperature- and humidity-controlled room (22 ± 2°C and 55 ± 5%, respectively) on a 12-hour light/dark cycle. Timed pregnancy was performed by housing each female rat with a single breeding male rat. The day on which sperm was detected in the vaginal smear (Cannizzaro et al., 2008; Plescia et al., 2014b) was designed as gestational day (GD) 0. Pregnant females were then given daily subcutaneous injections with THC (2 mg/kg) or vehicle from GD 5 to GD 20. This dose was chosen because it corresponds to the average content of THC in a mild cannabis cigarette (5%; Frau et al., 2019). After weaning, male rats were housed in pairs, and each experimental group for the behavioural and operant drinking paradigms included one or two independent male rats per each litter of vehicle- or THC-treated dams. All experiments were conducted in accordance with animal protocols approved by the Committee for the Protection and Use of Animals of the University of Palermo in accordance with the current Italian legislation on animal experimentation (D.L. 26/2014) and the European directives (2010/63/EU) on care and use of laboratory animals. Every effort was made to minimise the number of animals used and their sorrow.

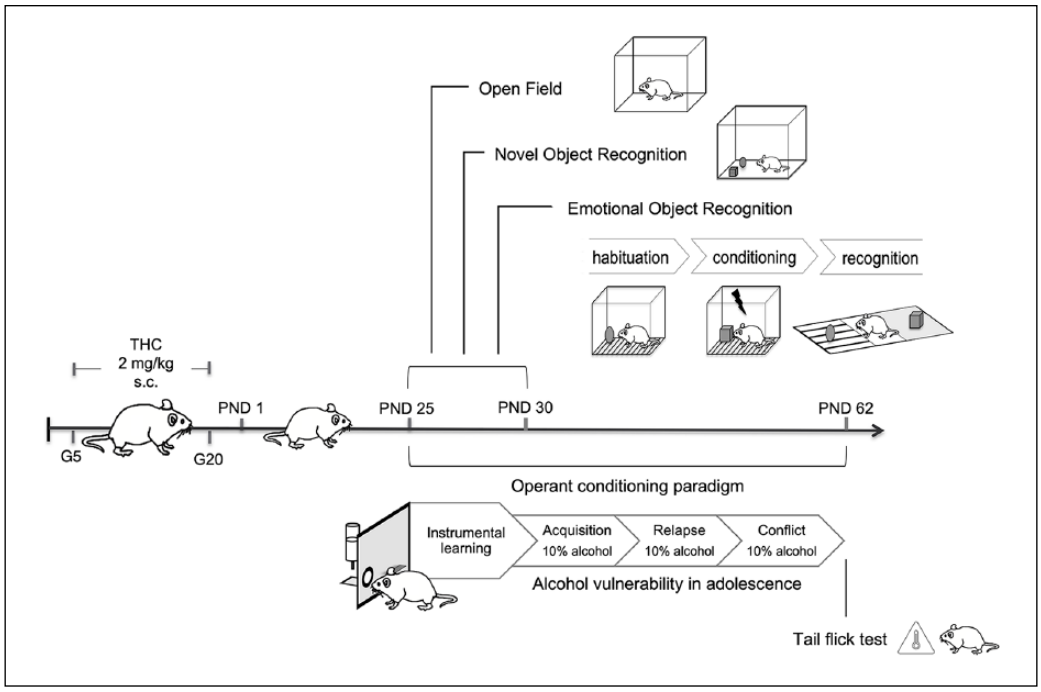

Figure 1. Behavioural procedures. Male rat offspring, prenatally exposed to either vehicle (CTRL) or Δ9-tetrahydrocannabinol (THC; prenatal cannabinoid exposure (PCE)) were tested from postnatal day (PND) 25 onwards for behavioural reactivity in a novel environment by the open field test (OFT); for declarative and limbic learning and memory by the Novel Object Recognition test and the Emotional Object Recognition test, respectively; for instrumental learning, by operant conditioning; for vulnerability to alcohol in an operant paradigm by alcohol taking in the acquisition phase, alcohol seeking following forced abstinence in the relapse phase and alcohol use despite negative consequences (i.e. resistance to punishment) in the conflict phase; for nociception by the tail-flick test.

Drugs

THC (THC Pharm GmbH, Frankfurt, Germany) was suspended in a vehicle of 1% ethanol + 1% Tween 80 in saline or vehicle. Alcohol (96%; Carlo Erba Reagenti, Milan, Italy) was dissolved in tap water at 10% v/v.

Behavioural procedures

Male rat offspring, prenatally exposed to either vehicle (CTRL) or THC (prenatal cannabinoid exposure (PCE)) were tested during pre-adolescence and adolescence, starting from postnatal day (PND) 25 onwards, during the light phase.

Offspring underwent a behavioural battery tailored to explore behavioural reactivity, neutral and aversive limbic memory and alcohol vulnerability (Figure 1). In detail, behavioural reactivity in a novel environment was assessed by the open field test (OFT; Brancato et al., 2018a; Plescia et al., 2015); neutral declarative and aversive limbic learning and memory were explored with the Novel Object Recognition test and the Emotional Object Recognition test, respectively (Brancato et al., 2016; Cannizzaro et al., 2016, 2019). In a different cohort of offspring, instrumental learning, that is the ability to acquire a simple instrumental response in order to gain access to water, was investigated by using an operant-conditioning chamber as previously described, in water-restricted conditions (modified from Oakeshott et al., 2011). The offspring vulnerability to alcohol motivational properties was then tested in an operant paradigm designed to explore discrete alcohol-related behaviours, such as alcohol taking in the acquisition phase, alcohol seeking following a forced abstinence in the relapse phase and alcohol use despite negative consequences, that is resistance to punishment, in the conflict phase (Brancato et al., 2014; Cacace et al., 2012; Deroche-Gamonet et al., 2004; Plescia et al., 2013; Ripley and Stephens, 2011). At the end of the conflict session, rats were tested for nociception in the tail-flick test (Cannizzaro et al., 2016).

OFT

Locomotor activity and behavioural reactivity in a novel environment were measured in an open field. The apparatus was a Plexiglas square box, 44 cm long, 44 cm wide and 20 cm high. The behaviour of the rats was monitored and quantified by an automatic video-tracking system, AnyMaze (Stoelting Europe, Dublin, Ireland), in a mean light intensity (100 lx) illuminated chamber. The test provides quali-quantitative mapping of the motor pattern, measuring total distance travelled (TDT) as a measure of locomotor activity, and number of central transitions (NCT) from the peripheral to the central area of the arena as measures of explorative behaviour (Cacace et al., 2011). Each experimental session lasted five minutes.

Novel Object Recognition test

Offspring were tested for declarative learning and memory employing the Novel Object Recognition test, as previously described (Cannizzaro et al., 2016). On day 1, a five-minute

habituation session was performed at 10.00 am in order to let the animals freely explore the arena, which was the same as in the OFT. Twenty-four hours after the habituation session, rats underwent a five-minute training session when they were presented with two identical, non-toxic objects (i.e. two red metal cans) which were placed against a wall in the open field arena. To prevent coercion to explore the objects, rats were released against the centre of the opposite wall with their backs to the objects. The time spent exploring each object was recorded using the AnyMaze video-tracking system (Stoelting Europe); a 2 cm^2 area surrounding the objects was defined such that nose entries were recorded as time for object exploration. After the training session, animals were placed in their home cage for a 24-hour retention interval. Then, the animals were returned to the arena where they found two objects: one was identical to the familiar one but previously unused (to prevent olfactory cues and the necessity to wash objects during experimentation), and the other was a novel object (a yellow hard plastic cup). Time spent exploring the novel (TN) and familiar (TF) objects was recorded during a five-minute session. Objects were randomised and counterbalanced across animals. The objects and the arena were thoroughly cleaned at the end of each experimental session. The recognition index (RI %), that is, the percentage of time spent investigating the novel object out of the total object investigation time (RI % = TN/(TN + TF)%), is a measure of novel object recognition and the main index of retention (Botton et al., 2010; Mumby et al., 2002). A RI % of >50% indicated that the rat spent more time exploring the novel object, thus recalling the memory of the familiar object; a RI % of <50% indicated that time was mainly spent exploring the familiar object, as it was a novel one.

Emotional Object Recognition test

The Emotional Object Recognition test was used for the assessment of aversive limbic memory (Brancato et al., 2016; Cannizzaro et al., 2019). It employed two distinct contexts (A and B) placed in different rooms. The context A chamber was a rectangular arena with white floor (100 cm long × 30 cm wide × 43 cm high). Rat behaviour was recorded and analysed using the AnyMaze video-tracking system (Stoelting Europe). A left and a right zone (40 cm × 30 cm) on both ends of the context A chamber, as well as a neutral zone in the centre of the box (20 cm), represented the arena settings (modified from Ramirez et al., 2015). The context A chamber was customised with two different non-toxic objects (i.e. a plastic ball, 3.5 cm diameter, and a plastic pepper, 3 cm × 3 cm × 4 cm) that were placed against the end walls of the left and the right zones of the arena, according to the procedure described below. The objects and their position were counterbalanced within the experimental groups.

The context B chamber was a rectangular chamber (45 cm × 22 cm × 22 cm) equipped with a grid floor, opaque ceiling and dark walls. Rats were allowed to explore the context B chamber for habituation and conditioned/cued learning; they were then tested in the context A chamber for emotional recognition. The floors and walls of the chambers were cleaned thoroughly with 70% isopropanol, dried with tissue paper and rinsed again with water 10 minutes before animals entered the chambers. Rats were transported to and from the experimental room in their home cages using a wheeled cart. The cart and cages remained in an anteroom to the experimental rooms during all behavioural experiments.

Experimental design

Habituation. Habituation took place in the context B chamber and consisted of two separate sessions: environmental exploration, during which rats were put in the arena and left undisturbed to explore the chamber for five minutes; neutral-object exploration, in which an object (plastic ball or pepper) was placed in the opposite corner with respect to the rat's entry and presented to the animals for 10 minutes. Between the two sessions, rats were taken and returned to their home cages for 10 minutes.

Cued fear-conditioned learning. One hour after neutral-object exploration, rats were re-placed in the context B chamber, exposed to a novel object (emotional object) and trained for fear conditioning. The session lasted 560 seconds, and six two-second 0.3 mA shocks were delivered at second 120, 200, 280, 360, 440 and 520. At the end of the session, animals were returned in their home cages for a four-hour retention interval.

Emotional object recognition: context A chamber. Four hours after cued fear-conditioned learning, rats were put into the context A chamber and tested for emotional object discrimination and object place aversion in the neutral context A chamber. They were allowed to explore the new context freely for five minutes. For each rat, the favourite zone, between the left and right ones, was recorded at epoch baseline (BSL). Afterwards, the object experienced during fear conditioning (the emotional object) was placed in the favourite zone; the object experienced during habituation (the neutral object) was placed in the less preferred zone. Rats explored objects and zones during minutes 5–8 (epoch ON-1). During minutes 8–11, objects were removed from the arena (epoch OFF). During minutes 11–14, the objects were reintroduced in the same positions as during minutes 5–8 (epoch ON-2). Finally, rats were placed in their home cages and returned to the holding room. At the end of each experimental session, both the objects and the arena were cleaned with a 70% solution of isopropanol. Time spent exploring the objects and zones was recorded along the epochs. Emotional object discrimination was measured by the percentage of emotional object avoidance, which was calculated as: 100 − (time spent on the emotional object/time spent on neutral + emotional object)%). Object place conditioning was measured by the percentage of target zone preference, which was calculated as the percentage of time spent in the target zone during BSL, ON and OFF epochs.

Operant tasks

Apparatus. The experimental sessions were carried out in custom-built operant-conditioning chambers (30 cm × 28 cm × 37 cm) located within a dimly lit, sound-attenuating shell, with a fan mounted at one end of the cubicle that was active throughout the session for white noise. Each chamber was equipped with two levers located on opposite walls of the chamber, 3.5 cm above the grid floor, controlling the delivery of 0.05 mL of liquid reinforcement for each lever press into two separate dippers and a light stimulus above the lever. The grid floor was connected to

electric-shock generator to deliver a footshock (0.2 mA) during the punished period (see conflict experiment). The hardware was controlled by an Arduino-based control unit (patent pending, application n. 102019000022341), which allowed to record all the events during the experimental sessions. The chamber was thoroughly cleaned before the introduction of each animal to ensure that the rat's behaviour was not affected by the detection of another rat's scent.

Instrumental learning. During the instrumental learning experiment, rats were trained to press a lever via a simple free operant procedure where each lever press was reinforced with 0.05 mL of tap water throughout a 20-minute session. No reinforcement was delivered without a lever press. The instrumental learning experiment was carried out once daily along five days. Rats' access to water was restricted, since they were allowed to drink for one hour per day at the end of the experimental session. The number of lever presses and water intake were recorded. Animals were trained to a learning criterion, requiring them to obtain 20 reinforcements across two consecutive experimental sessions (modified from Oakeshott et al., 2011).

Alcohol vulnerability. The offspring that reached the learning criterion within five days underwent an alcohol vulnerability assessment in terms of motivation for alcohol in the operant chamber. In order to match the sample size of the CTRL and PCE groups, and to control for litter effect, we used six rats per group, composed of one pup per litter. The operant paradigm included acquisition, relapse following forced abstinence and conflict experiments.

During the acquisition experiment, the rats would lever press via a Fixed Ratio 1, so each lever press was reinforced with 0.05 mL of alcohol 10% (lever 1) or water (lever 2) throughout a 20-minute session. The acquisition experiment was carried out daily for 21 sessions. Rats were not water-restricted. The number of lever presses and fluid intake was recorded. Weekly and daily mean numbers of lever presses and alcohol intake (g/kg) were calculated.

Following the 21-day acquisition experiment, alcohol operant administration was suspended to achieve a forced abstinence. Thus, rats were left undisturbed in their home cages for one week and received water and food ad libitum.

Afterwards, in the relapse experiment, rats were exposed to alcohol following the forced abstinence period and allowed to lever press for either 10% alcohol or water in the operant chamber, in the same experimental conditions as for acquisition, for five days. The number of lever presses and fluid intake was recorded. Daily mean numbers of lever presses and alcohol intake (g/kg) were calculated.

The difference between the mean daily alcohol intake, in terms of g/kg, during the relapse session and the mean alcohol intake during the last five days of the acquisition session was calculated as a measure of the alcohol deprivation effect, that is the marked increase in alcohol intake that follows periods of withdrawal.

In the conflict experiment, the delivery of the reinforcement (both water and alcohol) was paired with the delivery of a mild footshock, according to a within-session schedule. In detail, each session started with a non-punished interval (three minutes in duration), when each lever press was reinforced as described above (Fixed Ratio 1, water or alcohol 10% according to the lever). During the punished response interval (one minute long), the light stimulus was on, and each lever press controlled the delivery of the reinforcement (either alcohol or water, according to the lever), along with a mild footshock (0.2 mA; Cacace et al., 2012). Non-punished and punished periods alternated according to the three-minute–one-minute schedule for one 20-minute session. The number of unpunished and punished lever presses was automatically recorded. The percentage of punished responses, over the total number of non-punished and punished operant responses, emitted in each of the five time-bins of the conflict session was calculated as a measure of punishment resistance.

Tail-flick test. At the end of the conflict session, nociception was explored by measuring tail-flick latency in the hot-water immersion tail-flick test (Cannizzaro et al., 2016). Briefly, 2 cm of the rat tail was immersed in a water-bath apparatus (MPM Instruments Srl, Bernareggio, Italy) maintained at 52 ± 0.5°C. Latency to response was determined by a vigorous tail flick. A cut-off time of 10 seconds was imposed to minimise tissue damage.

Immunofluorescence experiments. At PND 35, one offspring per litter (n=6 per group) from the first cohort of rats was anaesthetised (chloral hydrate, 300 mg/kg) and trans-cardially perfused with cold phosphate-buffered saline (PBS; pH 7.4), followed by 4% paraformaldehyde in PBS. Brains were dissected and post fixed overnight in the same fixative used for perfusion. Fixed brains were coronally sectioned at a thickness of 40 µm using a microtome (Campden Instruments, Loughborough, UK; Carletti et al., 2013). Serial sections were collected through the rostral-caudal dimensions (every sixth slice) and stored at 4°C in 0.02% sodium azide in PBS until the immunofluorescence staining (Brancato et al., 2017).

Sections containing mPFC (from the bregma 3.20 mm to 2.20 mm), NAc (from the bregma 2.20 mm to 1.60 mm) and Amy (from the bregma −1.80 mm to −2.30 mm) were selected (Di Liberto et al., 2017; Paxinos and Watson, 1998), washed in PBS for 30 minutes and incubated in blocking solution (3% Normal Goat Serum (NGS), 0.3% Triton X-100 in PBS) for two hours at room temperature under gentle shacking. Afterwards, sections were incubated in primary antibody solution (3% NGS, 0.3% Tween-20 in PBS) with either rabbit anti-NPY (T-4070 1:1000; Peninsula Laboratories International, Inc., San Carlos, CA), mouse monoclonal anti-Homer 1 (#sc136358; 1:250; Santa Cruz Biotechnology, Dallas, TX), mouse monoclonal anti-Homer 1b/c (#sc25271; 1:250; Santa Cruz Biotechnology), rabbit polyclonal anti-Homer 2 (#160 203, 1:650; Synaptic Systems, Goettingen, Germany) for 72 hours. Subsequently, sections were washed in PBS solution for one hour, incubated in secondary antibody solution (Alexa Fluor® 488 AffiniPure Goat Anti-Rabbit IgG or Alexa Fluor® 594 AffiniPure Goat Anti-Mouse IgG; 1:200; Jackson ImmunoResearch, West Grove, PA) for two hours under gentle shacking. After washing for one hour, slices were briefly incubated with DAPI (1 µg/mL). Sections were mounted onto adhesive slides (Superfrost® Plus; Thermo Fisher Scientific, Waltham, MA) and coverslipped using Vectashield® HardSetTM Antifade mounting medium. Images were acquired at 20×, 40× and 100× magnification using an epifluorescence microscope (Meji Techno, Saitama, Japan) and Deltapix Insight software (Figure 2). NPY-positive (NPY+) neurons (from 20× images) and Homer proteins immunofluorescence (from 40× images) were quantified using Image J and verified by a trained experimenter.

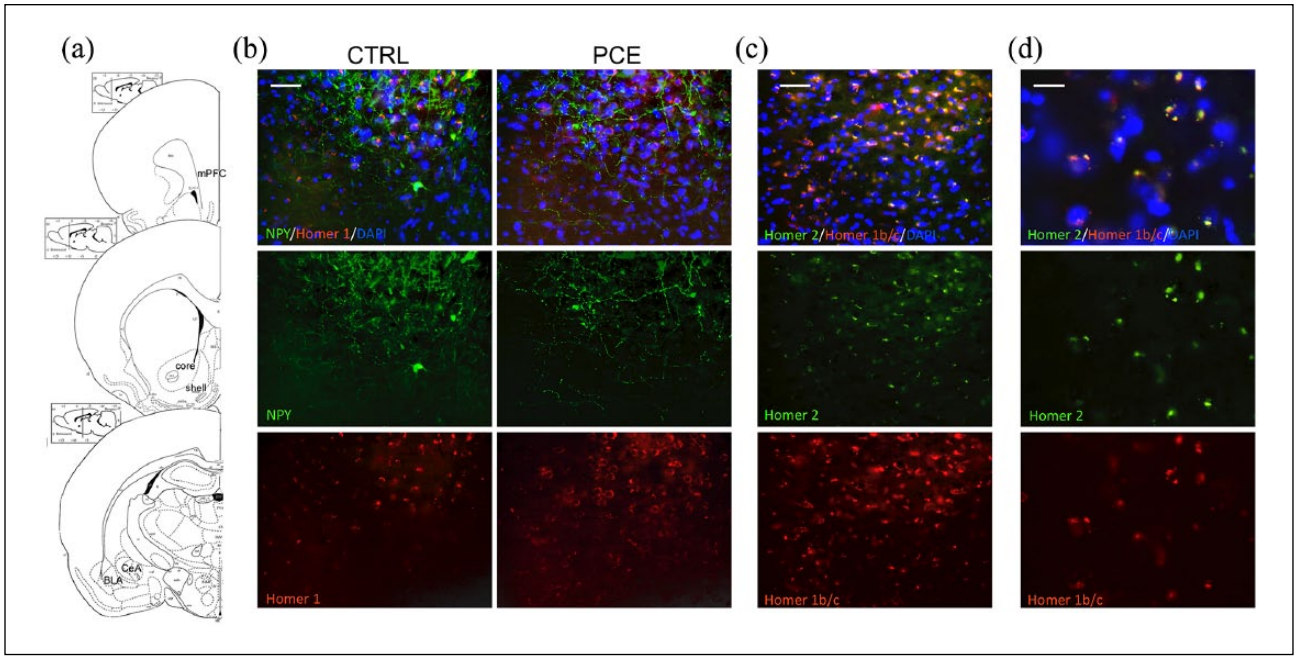

Figure 2. Immunofluorescent experiments. (a) Neuropeptide Y positive (NPY+) neurons and Homer proteins were separately quantified in the medial prefrontal cortex (mPFC), shell and core subregions of the nucleus accumbens (NAc), basolateral amygdala (BLA) and central amygdala (CeA) in the two experimental groups. Representative photomicrographs of immunofluorescent staining for (b) NPY and Homer 1 in the two experimental groups (magnification 40×, scale bar=40 µm for all images) and for Homer 1b/c and 2 at (c) magnification 40×, scale bar=40 µm for all images and (d) magnification 100×, scale bar=10 µm for all images.
CTRL: control; PCE: prenatal cannabinoid exposure.

Statistical analysis

All data were tested for normality and equal variances. When data exhibited normality and equal variances, differences between groups were determined using either Student's *t*-test or a two-way analysis of variance (ANOVA) either regular or for repeated measures, followed by a Bonferroni post hoc test. Data that did not display equal variances were analysed using the non-parametric Mann–Whitney *U*-test. The performance of rats to reach the learning criterion was analysed using Kaplan–Meier event analysis over the instrumental learning period, and the resulting curves were compared by employing the log-rank Mantel–Cox test. Statistical analysis was carried out using GraphPad Prism v6.1 (GraphPad Software, Inc., San Diego, CA). All values represent the mean ± standard error of the mean. An alpha level of 0.05 was adopted throughout.

Results

Reproduction parameters

Gestational cannabinoid exposure did not significantly affect reproduction parameters such as number of dams giving birth, length of pregnancy, litter size at birth, postnatal mortality (the number of pups that died before weaning) and male:female ratio (Table 1). Unpaired Student's *t*-test analysis on data from body weight at birth showed a small but significant decrease in PCE pups compared to CTRL rats, which disappeared at the time of the first behavioural assessment (PND 25; Table 1).

Behavioural assessment

OFT. At PND 25, male offspring were tested in the open field arena to evaluate the effects of PCE on locomotor activity and exploratory behaviour. Statistical analysis highlights that PCE rats significantly increased TDT (two-tailed Student's *t*-test, $t=4.925$, $df=22$, $p<0.001$; Figure 3(a)) compared to the CTRL group. No significant differences were observed in NCT (two-tailed Mann–Whitney *U*-test, $U=41.00$, $p=0.0708$; data not shown).

Novel Object Recognition test. Offspring underwent the Novel Object Recognition test in order to evaluate the effect of PCE on declarative learning and memory. The results of Student's *t*-test on the RI% show no significant differences in PCE offspring when compared to CTRL counterparts ($t=1.007$, $df=22$, $p=0.3249$; Figure 3(b)).

Emotional Object Recognition test. Offspring were tested in the Emotional Object Recognition test for assessing the effects of PCE on the acquisition of fear-associated declarative memory. When data from the emotional object avoidance (%) were considered, CTRL rats clearly avoided the emotional object, previously associated with the aversive experience in the context B chamber, and displayed 69 ± 4% emotional object avoidance, whereas PCE offspring's emotional object avoidance reached 54 ± 3%. The results of the two-tailed Mann–Whitney test highlight that PCE rats showed a significant decrease in the avoidance of the fear-associated object compared to CTRL ($U=28.50$; $p=0.0105$; Figure 3(c)).

Table 1. Effects of gestational cannabinoid exposure on reproduction parameters.

Reproduction parameters	CTRL	PCE	Statistics
Percentage of dams giving birth	100%	100%	
Length of pregnancy (days), median (IQR)	21 (1.8)	21 (1.0)	n.s. (Mann–Whitney *U*-test, $p=0.394$, $U=11.0$)
Litter size at birth, median (IQR)	10.5 (1.5)	10 (3.5)	n.s. (Mann–Whitney *U*-test, $p=0.632$, $U=14.5$)
Body weight of pups (g), mean ± *SD* per litter			
At birth	6.3 ± 0.4	5.7 ± 0.4	* (Student's *t*-test, $p=0.028$, $t=2.57$, $df=10$)
At weaning time	31.4 ± 3.1	29.8 ± 4.4	n.s. (Student's *t*-test, $p=0.480$, $t=0.733$, $df=10$)
Postnatal mortality, median (IQR) per litter	0.00 (0.02)	0.06 (0.43)	n.s. (Mann–Whitney *U*-test, $p=0.182$, $U=10.5$)
Male:female ratio, median (IQR) per litter	1.3 (0.4)	1.4 (1.6)	n.s. (Mann–Whitney *U*-test, $p=0.972$, $U=17.5$)

CTRL: control; IQR: interquartile range; PCE: prenatal cannabinoid exposure; *SD*: standard deviation; n.s.: not significant.
*Statistically significant.

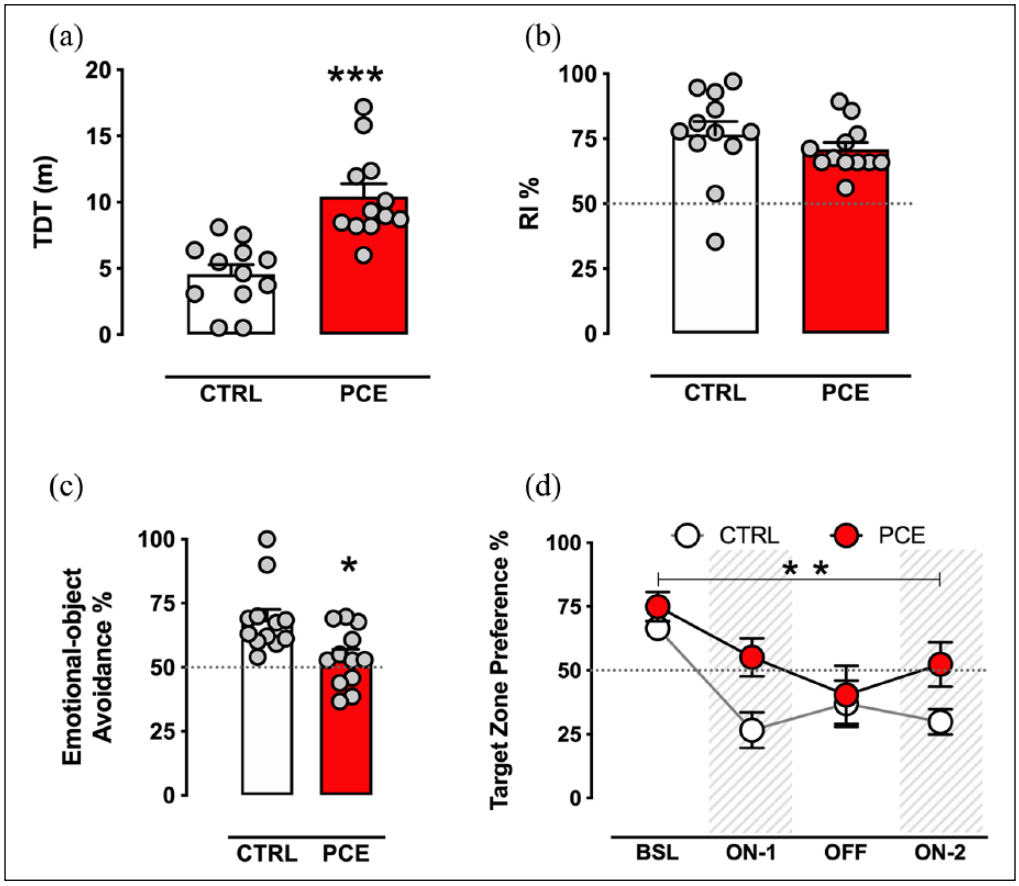

Figure 3. Effects of PCE on behavioural reactivity and declarative learning and memory in preadolescent offspring. PCE increased (a) locomotor activity in terms of total distance travelled (TDT) and (b) induced no significant effects in neutral declarative learning and memory in the Novel Object Recognition test in terms of recognition index % (RI%) compared to the CTRL group. On the other hand, PCE decreased limbic learning and memory in the Emotional Object Recognition test compared to the CTRL group in terms of (c) emotional object avoidance and (d) target zone preference (here, circles indicate the mean ± standard error of the mean (SEM) of $n=12$ rats).
*$p<0.05$; **$p<0.01$; ***$p<0.001$ vs. CTRL group.
CTRL: control; PCE: prenatal cannabinoid exposure.

In the Emotional Object Recognition test, the preferred zone of the context A chamber is determined during BSL and paired to the emotional object afterwards. When the target zone preference (%) along the test epochs was analysed, a repeated-measures two-way ANOVA showed a significant effect of test epoch ($F(3,66)=7.874$; $p=0.0001$) and PCE ($F(1,22)=9.367$; $p=0.0057$; Figure 3(d)).

Operant task

Instrumental learning. The effects of PCE on the offspring's instrumental learning were assessed by evaluating their ability to acquire a simple instrumental task. The offspring performance was measured in terms of days elapsed to reach the learning criterion, and the Kaplan–Meier analysis showed that median

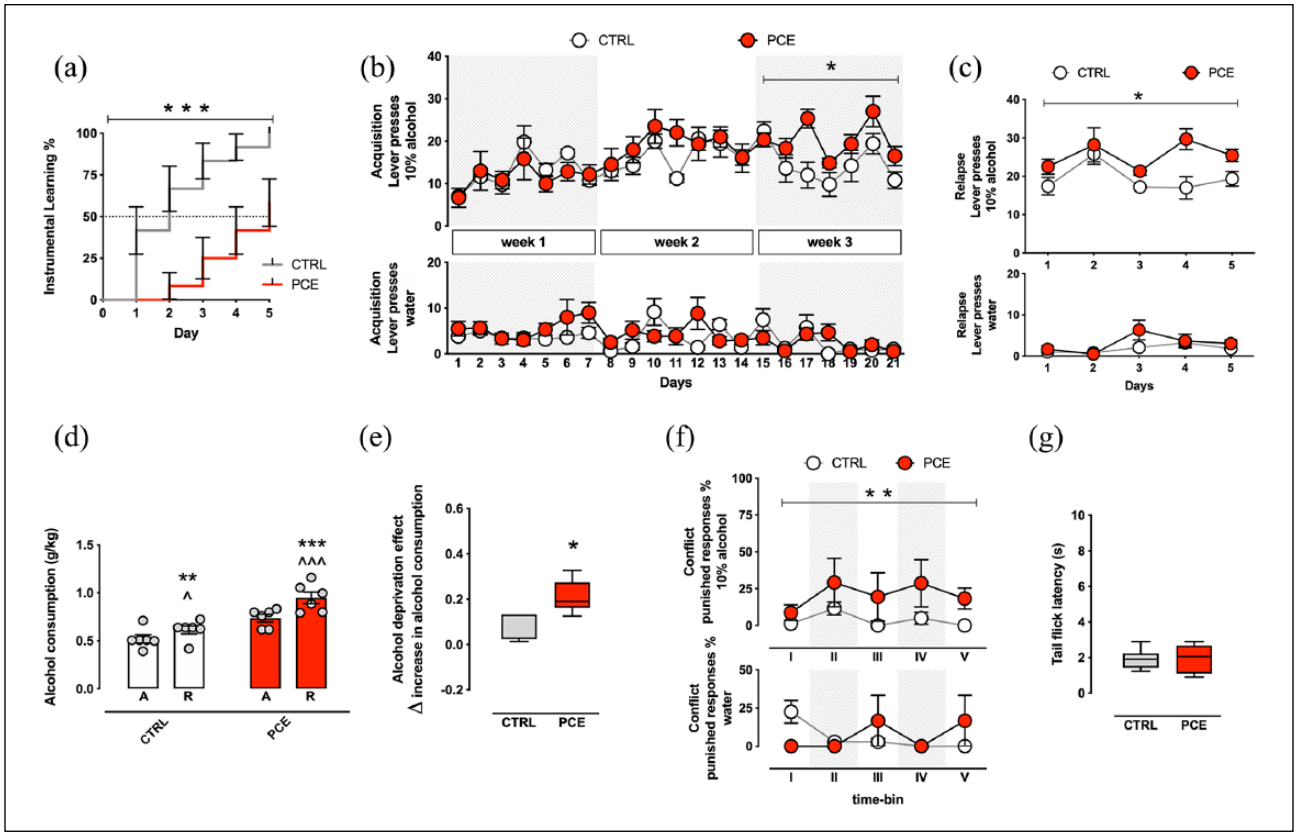

Figure 4. Effects of PCE on instrumental learning and motivation for alcohol in adolescent offspring. PCE decreased (a) instrumental learning in a simple operant task compared to the CTRL group, (b) PCE increased motivation for alcohol in the last week of the acquisition phase of the operant paradigm and (c) in the relapse session following forced abstinence compared to the CTRL group. (d) PCE offspring displayed a higher mean daily alcohol intake (in terms of g/kg) during relapse compared to the CTRL group and when compared to their own mean daily alcohol intake during the previous five days of acquisition, (e) showing a higher deprivation effect than the CTRL group in terms of difference (Δ) in alcohol consumption. Moreover, (f) PCE progeny showed a higher resistance to punishment for alcohol than the CTRL group along the five time-bins of the conflict session. (g) No differences in nociception were observed in the tail-flick test. Circles indicate the mean ± SEM of $n=6$ rats.
$^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$ vs. CTRL group; $^{\wedge}p<0.05$; $^{\wedge\wedge}p<0.01$ Relapse (R) vs. Acquisition (A) phases.
CTRL: control; PCE: prenatal cannabinoid exposure.

learning times were two days for CTRL rats and five days for PCE offspring. In addition, 0.0% of CTRL rats and 41.7% of PCE rats failed to reach the criterion within five days, and thus were censored and withdrawn from the alcohol vulnerability experiment. The log-rank Mantel–Cox test for comparison of survival curves indicated that learning performance was significantly decreased in PCE offspring compared to CTRL rats ($\chi^2=12.10$, $df=1$, $p=0.0005$; Figure 4(a)).

Alcohol vulnerability. In order to assess the effects of PCE on the vulnerability to the motivational properties of alcohol, six offspring per experimental group were tested in an operant paradigm that models discrete alcohol-related behaviours, such as alcohol taking in the acquisition phase, alcohol seeking following a forced abstinence in the relapse phase and alcohol use despite negative consequences, that is, resistance to punishment, in the conflict phase.

When rats underwent the acquisition phase, the number of lever presses for 10% alcohol ($M \pm SD$) increased along the three-week paradigm (week 1: CTRL = 13 ± 3; PCE = 12 ± 5; week 2: CTRL = 16 ± 4; PCE = 19 ± 2; week 3: CTRL = 15 ± 3; PCE = 20 ± 2; Figure 4(b)). PCE offspring performed a higher number of lever presses (average per week) than CTRL rats. A repeated-measures two-way ANOVA that included PCE as the between-subject factor and week as the repeated-measure factor showed a significant main effect of PCE ($F(1,10)=5.102$, $p=0.0475$), weeks ($F(2,20)=11.70$, $p=0.0004$) and their interaction ($F(2,20)=3.535$, $p=0.0485$) on the number of responses emitted throughout the 21 days. In particular, a Bonferroni post hoc test indicated no significant differences between the two groups during the first two weeks of the acquisition period ($t=0.6329$, $df=30$, $p>0.05$; $t=1.564$, $df=30$, $p>0.05$), while PCE rats displayed a significant increase in the number of lever presses compared to CTRL animals on the last week of the paradigm ($t=3.065$, $df=30$, $p<0.05$; Figure 4(b), top panel). No significant effect of PCE was observed on number of lever presses for water ($F(1,10)=1.145$, $p=0.3097$; Figure 4(b), lower panel).

Following the acquisition experiment, the adolescent rats were tested for alcohol-seeking behaviour after forced abstinence in the relapse paradigm. PCE offspring emitted a higher number of lever presses for 10% alcohol compared to CTRL rats ($M \pm SD$: CTRL = 19 ± 4; PCE = 24 ± 4; Figure 4(c)). In

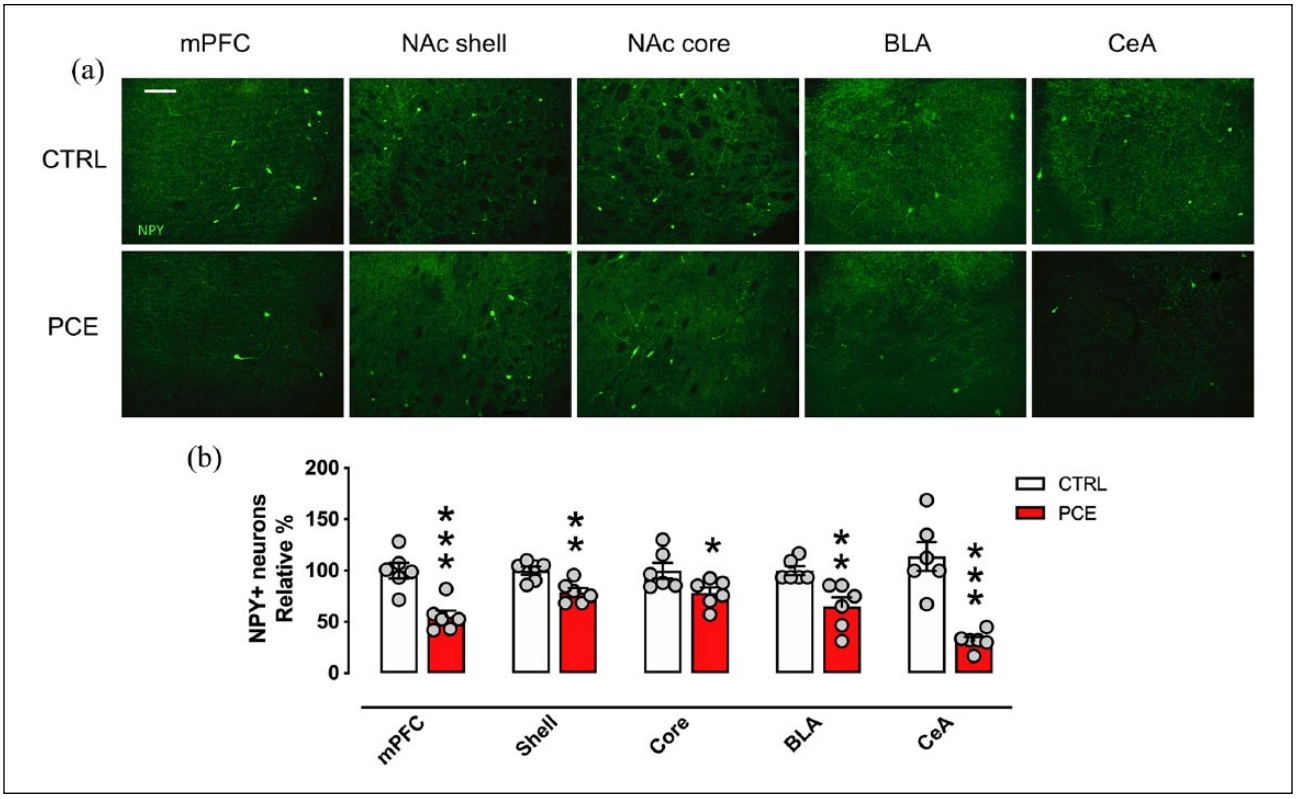

Figure 5. Effects of PCE on NPY+ neurons in the limbic brain regions. (a) Representative photomicrographs of NPY immunofluorescent staining in the mPFC, shell and core subregions of the NAc and BLA and CeA in the two experimental groups. 20×, scale bar = 100 µm for all images. (b) PCE significantly decreased the relative % of NPY+ neurons in the mPFC and in the shell and core of the NAc, BLA and CeA with respect to the CTRL group. Each bar represents the mean ± SEM of $n = 6$ rats. Each circle indicates the mean of five slices of each experimental subject.
*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ vs. CTRL group.
CTRL: control; PCE: prenatal cannabinoid exposure.

particular, a repeated-measures two-way ANOVA showed a significant main effect of PCE ($F(1,10) = 5.841$, $p = 0.0363$) and days ($F(4,40) = 5.096$, $p = 0.0021$) on the number of lever presses throughout the five-day relapse period (Figure 4(c), top panel). No significant effects of PCE were observed on number of lever presses for water ($F(1,10) = 1.811$, $p = 0.2081$; Figure 4(c), lower panel).

In addition, in order to assess the effects of PCE on the occurrence of the deprivation effect, we compared the mean alcohol intake during the relapse session with the mean amount of alcohol consumed during the last five days of training. The results of a repeated-measured two-way ANOVA that included PCE as the between-subject factor and deprivation as the within-subject factor showed a significant effect of PCE ($F(1,10) = 18.84$, $p = 0.0015$), deprivation ($F(1,10) = 67.12$, $p < 0.0001$) and their interaction ($F(1,10) = 9.818$, $p = 0.0106$; Figure 4(d)). A Bonferroni post hoc test indicated that CRTL offspring displayed a significant increase in mean alcohol consumption during relapse compared to their own levels during the last five acquisition days (CTRL: $t = 3.577$, $df = 10$, $p = 0.0101$). PCE offspring showed increased alcohol consumption following the deprivation compared to their own levels during the last five acquisition days (PCE: $t = 8.009$, $df = 10$, $p < 0.001$), and they consumed more alcohol than CTRL rats during the last five days of acquisition ($t = 3.284$, $df = 20$, $p = 0.0074$) and during relapse ($t = 5.046$, $df = 20$, $p = 0.0001$; Figure 4(d)). In particular, PCE progeny showed a higher deprivation effect than CTRL rats in terms of difference between alcohol intake during relapse and the last five days of acquisition (two-tailed Student's t-test, $t = 3.133$, $df = 10$, $p = 0.0106$, $\eta^2 = 0.4954$; Figure 4(e)).

Eventually, when the adolescent offspring were tested for punishment resistance in the conflict session, the repeated-measures two-way ANOVA on the percentages of punished responses for alcohol, over total responses – punished plus non-punished alcohol lever presses – throughout the five time-bins, showed that PCE offspring emitted an increased percentage of punished responses for alcohol compared to CTRL rats (PCE: $F(1,10) = 11.57$, $p = 0.0067$; Figure 4(f), top panel). No significant differences were observed on the percentage of punished lever presses for water (PCE: $F(1,10) = 0.05257$, $p = 0.8233$; Figure 4(f), lower panel).

Tail-flick test. The results of Student's t-test on tail-flick latency showed no significant difference in nociception in PCE offspring compared to CTRL progeny ($t = 0.100$, $df = 10$, $p = 0.9223$; Figure 4(g)).

Effects of PCE on NPY+ neurons

The effects of PCE on NPY+ neurons were assessed by immunofluorescence and are reported as percentages of the mean values of the CTRL group (Figure 5(a) and (b)).

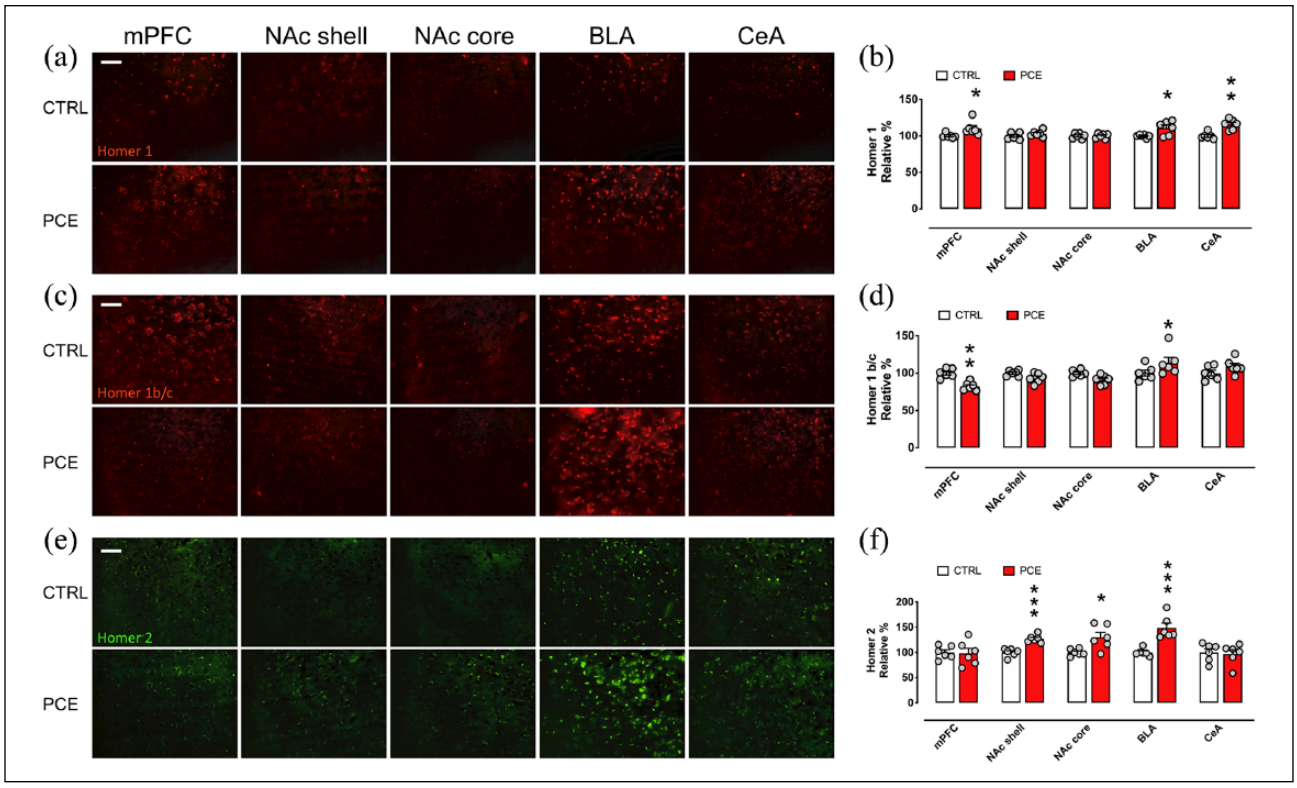

Figure 6. Effects of PCE on Homer protein expression in the limbic brain regions. (a) Representative photomicrographs of Homer 1 immunofluorescent staining in the mPFC, NAc and amygdala (Amy) of the two experimental groups. (b) PCE increased Homer 1 in the mPFC and BLA and CeA compared to the CTRL group. (c) Representative photomicrographs of Homer 1b/c immunofluorescent staining in the mPFC, NAc and Amy of the two experimental groups. (d) In PCE rats, we observed decreased Homer 1b/c expression in the mPFC and increased Homer 1b/c expression in the BLA compared to the CTRL group. (e) Representative photomicrographs of Homer 2 immunofluorescent staining in the mPFC, NAc and Amy of the two experimental groups. (f) PCE increased Homer 2 immunofluorescence in the shell and core of the NAc and BLA compared to the CTRL group. Each bar represents the mean ± SEM of $n=6$ rats. Each circle indicates the mean of five slices of each experimental subject.
$^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$ vs. CTRL group. Magnification 40×, scale bar=40 µm for all images.
CTRL: control; PCE: prenatal cannabinoid exposure.

The analysis of data from mPFC indicates that PCE significantly decreased the number of NPY+ neurons ($t=4.686$, $p<0.001$, $df=10$) compared to the CTRL group.

The results of Student's *t*-test on the number of NPY+ neurons in PCE rats in shell and core subregions of the NAc indicate a significant decrease in the number of NPY+ neurons both in shell ($t=3.668$, $p=0.004$, $df=10$) and core ($t=2.330$, $p=0.042$, $df=10$) compared to CTRL rats.

NPY+ neurons were also measured in basolateral Amy (BLA) and central Amy (CeA): Student's *t*-test highlighted a significant decrease in NPY+ neurons in BLA ($t=3.529$, $p=0.0054$, $df=10$) and CeA ($t=5.646$, $p<0.001$, $df=10$) of PCE rats compared to CTRL rats.

Effects of PCE on Homer proteins

Expression levels of the PSD proteins Homer 1, 1b/c and 2 in the mPFC, NAc and Amy were assessed by immunofluorescence and are reported as relative immunofluorescence to the mean values of the CTRL group.

When the effects of PCE on Homer 1 protein were analysed (Figure 6(a) and (b)), the results of Student's *t*-test indicated that PCE increased the expression of Homer 1 in mPFC ($t=2.430$, $p=0.035$, $df=10$) and in both BLA ($t=2.670$, $p=0.023$, $df=10$) and CeA ($t=4.659$, $p=0.0010$, $df=10$) compared to the CTRL group.

Moreover, when the expression of the C–C isoform Homer 1b/c was assessed (Figure 6(c) and (d)), the results of Student's *t*-test indicated that PCE rats showed a significant decrease in Homer 1b/c in mPFC ($t=5.255$, $p<0.001$, $df=10$) and an increase in BLA ($t=2.829$, $p=0.018$, $df=10$) compared to CTRL rats.

In addition, in the limbic regions of PCE and CTRL rats (Figure 6(e) and (f)), the results of Student's *t*-test indicated that PCE rats displayed a significant increase in Homer 2 immunofluorescence in NAc shell ($t=5.963$, $p<0.001$, $df=10$) and core ($t=2.967$, $p=0.014$, $df=10$) and BLA ($t=4.949$, $p<0.001$, $df=10$) compared to CTRL rats.

Discussion

The present research investigated the effects of in utero THC exposure on behavioural reactivity, neutral and limbic memory, instrumental learning and alcohol vulnerability in adolescent offspring. Our results expand previous and recent observations on

the existence of enduring behavioural sequelae in the offspring of mothers exposed to cannabinoids during pregnancy (Bara et al., 2018; Calvigioni et al., 2014; Frau et al., 2019; Scheyer et al., 2019), and they highlight how the manipulation of the endocannabinoid signal at critical developmental stages impacts cognitive functions when these are strongly dependent on emotional processing, such as limbic memory. Moreover, for the first time, our results show that PCE confers vulnerability to alcohol motivational properties and contributes to the development of alcohol addictive–like behaviours in adolescent offspring. The occurrence of this multifaceted phenotype is associated with a reduction in NPY+ neurons and disarrangement in the PSD Homer make-up in relevant function-related brain regions.

Although a significant amount of literature exists on the postnatal consequences of drinking alcohol during pregnancy in humans, data on cannabis use are not always consistent and persuasive. Pregnant women may use cannabis as a natural substitute for prescribed medications to manage mood, stress and morning sickness, as well as the well-known recreational effects (Metz and Borgelt, 2018). Recent data show almost doubled levels of self-reported cannabis use in the year before pregnancy and during pregnancy, with daily use increasing from 1.17% to 3.05% (Young-Wolff et al., 2019). Unfortunately, clinical assessment of developmental outcomes of prenatal cannabis exposure is limited by poor control of timing and dosage, disclosure, underreporting and underestimation. Therefore, although mimicking human developmental cannabis exposure presents some limitations, the animal model of prenatal THC exposure is essential in systematically exploring specific neurobiological mechanisms that underlie putative abnormalities in adolescent brain and behaviour, and allows the intervening confounding variables to be controlled (DiNieri and Hurd, 2012). Under our experimental conditions, adolescent offspring that were exposed in utero to THC displayed increased locomotor activity, but no differences in the exploratory pattern of the arena compared to CTRL rats. Preclinical data on developmental effects of cannabinoid exposure on the ontogeny of motor behaviour have shown different and sometimes conflicting results (Brake et al. 1987; Campolongo et al., 2011; Fride and Mechoulam, 1996; Fried, 1976; Navarro et al., 1995; Trezza et al., 2008). However, our results are in line with human data showing that children and adolescents prenatally exposed to cannabis are hyperactive as well as impulsive (Fried and Smith, 2001; Fried and Watkinson, 1988; Goldschmidt et al., 2004). Under the neutral conditions of the open field arena, prenatal THC exposure did not induce signs of anxiety-like behaviour. Thus, the augmentation in locomotion could be the result of a decreased habituation to a novel environment compared to the CTRL group. Since habituation per se represents a learning process of the environmental setting, one could speculate that the hyper-locomotor effect observed in this study may underlie a delay in simple learning (Breit et al., 2019) rather than a prominent effect on emotionality. Further investigation of spatial learning abilities (Cannizzaro et al., 2007) of PCE progeny will clarify this point.

The endocannabinoid system critically modulates attention and memory processing due to high CB1 receptor's expression in brain areas involved in the modulation of cognitive functions (Ameri, 1999; Davies et al., 2002; Lafourcade et al., 2007; Trezza et al., 2012). Clinical studies have consistently shown that cannabis use during pregnancy can induce selective, deleterious effects on children's sleep, cognitive functions (memory and scholastic performance), as well as executive functions (reasoning, attention, impulsivity and motivation) and emotional processing (Schreiber and Pick, 2019). However, in our preclinical model of prenatal exposure to THC, we did not observe changes of neutral explicit memory in adolescent offspring, in line with previous reports (Bara et al., 2018; Macúchová et al., 2017). Interestingly, when we tested adolescent offspring for aversive limbic memory, that is, explicit memory traces of an emotionally salient experience (Brancato et al., 2016), we observed that while CTRL animals recognised and avoided the emotional object paired to the aversive emotional experience and the area of the maze where it was placed, PCE offspring decreased both avoidance of the emotional object and conditioned place aversion, indicating that PCE exerted a detrimental effect on the formation of emotionally aversive memory traces. The recognition of the emotional object as fearful – and its avoidance – makes the Emotional Object Recognition task a discriminative paradigm based on active responses of rats – rather than on implicit non-specific freezing behaviour – that enables the researcher to correlate animal cognitive performance with critical aspects of human emotional memory.

The evidence of a specific limbic memory impairment was not unexpected. Indeed, the endocannabinoid system has emerged as an important regulator of the stress response and emotional behaviour (Hill et al., 2011; Lutz et al., 2015). Consistently, prenatal exposure to cannabinoids induces subtle yet persisting changes in cognitive functions that emerge when cognitive abilities require emotional integration (Bara et al., 2018; Mereu et al., 2003; Morena et al., 2015). Indeed, whereas activation of CB1 receptors within the mPFC strongly potentiates normally non-salient emotional memory formation (Brancato et al., 2018b; Laviolette and Grace, 2006; Tan et al., 2014), THC administration alters facial recognition (Hindocha et al., 2015), decreases, in the Amy, blood oxygen level–dependent response to threatening faces (Phan et al., 2008) and preferentially impairs memory for emotional events in humans (Ballard et al., 2013). The mechanisms by which CB1 transmission regulates emotional memory formation are not entirely understood, but a single systemic stimulation of the cannabinoid transmission by the CB1 agonist WIN 55, 212-2 decreases limbic memory in the same Emotional Object Recognition task in adult rats (Brancato et al., 2016). Besides directly modulating excitatory/inhibitory signalling, the endocannabinoid system can biphasically modulate both rewarding and aversive emotional information by functionally interacting with the DA system (Laviolette and Grace, 2006; Rey et al., 2012), which is of exceptional importance for gating attention and facilitating conditioned stimulus associations during fear conditioning (Bromberg-Martin et al., 2010; Pezze and Feldon, 2004). Therefore, we can hypothesise that prenatal THC exposure may unbalance the integration between the abilities to form explicit memories and the emotional valence paired to that memory by dysregulating mesocorticolimbic transmission and the processing of emotionally salient information (Cannizzaro et al., 2019).

Prenatal THC-induced perturbation of neurodevelopmental trajectories in the mesocorticolimbic system may also pose a risk for vulnerability to alcohol and drug consumption early in adolescence. Surprisingly, no data on adolescents' vulnerability to alcohol – the most abused drug among teenagers (UNODC, 2018) – as a result of early exposure to THC are currently available.

To test that, we designed an operant paradigm and recorded alcohol taking, alcohol relapse and alcohol use despite negative consequences in both PCE and CTRL adolescent offspring. The first result concerned the rate of acquisition of the operant paradigm before the alcohol vulnerability study had started: while all CTRL rats acquired the instrumental responding for water in five days, only about half of the preadolescent PCE rats learnt the same operant task in the same time interval and joined the CTRL rats in the operant task for alcohol; the remaining offspring were then excluded from the experiment. The delay in the acquisition of the operant task observed in PCE rats is consistent with data showing an acquisition deficit in heroin self-administration in THC-exposed rats (Spano et al., 2007), and since a motor deficit can be ruled out by the open field outcome, it might result from impaired effort-related decision making as a consequence of the prenatal THC exposure. Clinical data also support this idea, since acute THC reduces the emission of high-effort choices (Lawn et al., 2016), and chronic cannabis users exhibit a poorer effort lavished in a memory recall test (Hirst et al., 2017). Further investigation on specific molecular targets is certainly recommended. On the other hand, our data show that interference in the cannabinoid system during gestation promoted alcohol vulnerability in adolescent rats. Indeed, whether during the first weeks of alcohol presentation no differences in the operant behaviour appeared between the two groups, during the last week of the acquisition window, PCE rats increased the number of lever presses compared to CTRL animals, likely as a result of an increased motivation for alcohol. Moreover, after a week of forced abstinence, both PCE and CTRL rats elevated their responses for alcohol compared to the acquisition phase, but PCE rats displayed a greater increase than CTRL animals. Indeed, re-presentation of alcohol after a period of forced abstinence usually leads to a robust but temporary increase in alcohol intake over baseline drinking – a relapse-like behaviour referred to as the alcohol deprivation effect (Vengeliene et al., 2014), which reflects increased craving or increased reinforcing value of alcohol in humans (Söderpalm et al., 2019). Preclinical and clinical findings have consistently reported that alcohol taking and craving are modulated, at least in part, by endocannabinoid transmission (De Vries and Schoffelmeer, 2005; Serra et al., 2002). CB1-receptor function is required for alcohol-mediated activation of VTA DA neurons (Cheer et al., 2007), supporting the hypothesis that alcohol-rewarding properties implicate ECS-mediated reduction of GABA inhibition onto VTA DA neurons (Barrot et al., 2012; Lupica and Riegel, 2005).

The voluntary administration of alcohol in the presence of response-contingent shock punishment reliably models compulsive drug use despite adverse consequences (Cacace et al., 2012; Deroche-Gamonet et al., 2004; Plescia et al., 2013), thus capturing a core feature of the vulnerable phenotype to addiction (Vanderschuren and Ahmed, 2013). In the operant conflict procedure used here, responses were alternatively paired to a foot-shock, signalled by a light cue. This led to a suppression of conditioned responses for the reinforcement in CTRL rats. Instead, the contingent punishment was less effective in inhibiting the operant responding for alcohol in PCE adolescent rats, which displayed a higher number of punished responses than CTRL animals, indicating either an increase in the motivation for alcohol or a lesser sensitivity to the punishment. However, PCE rats did not show differences in tail-flick latency compared to CTRL rats, ruling out a non-specific effect on nociception.

As far as we know, this is the first report showing that prenatal THC exposure may induce a susceptible phenotype to alcohol-addictive properties in adolescent offspring; the inconsistency with previous research that did not highlight a facilitatory effect of perinatal THC on alcohol operant behaviour (Economidou et al., 2007) shows that doses, time of exposure and experimental design may represent fundamental variables in the identification of behavioural read-outs of neurodevelopmental vulnerability. Indeed, in our experimental conditions, THC exposure from the early gestational period to delivery (a time window equivalent to the first and second trimesters in humans) was able to induce alcohol vulnerability in adolescent offspring, whereas in the study by Economidou et al. (2007), higher THC doses (5 mg/kg) administered within the GD 15–PND 9 period (corresponding to the second and third trimester in humans) did not exert such an effect. It is worth mentioning that further methodological issues (i.e. the inclusion of a five-second time-out after receiving a reinforcer) may be responsible for the lack of effects in the former study, which, however, did address the issue for the first time.

Previous studies have demonstrated that developmental exposure to cannabinoids enhances sensitivity to heroin and morphine self-administration and heroin seeking following a mild food deprivation stress (Spano et al., 2007; (Vela et al. 2008)) likely through alterations in DA receptor 2 gene expression in the NAc of the offspring exposed in utero to cannabis (DiNieri et al., 2011). Our previous results showed that the systemic administration of a D2 autoreceptor agonist and a specific CB1 antagonist were able to decrease drug seeking, relapse after forced abstinence and resistance to punishment for alcohol's neuroactive metabolite acetaldehyde (Brancato et al., 2014; Plescia et al., 2013). Altogether, abnormal DA and EC signalling may result in modifications in the motivational properties of alcohol, thus highlighting that THC in utero exposure may pose a risk for increased vulnerability to alcohol-addictive behaviours.

On the other hand, one could parallel the data from the conflict session with the results of the Emotional Object Recognition test, interpreting the abnormal resistance to the punishment in the operant chamber as a weaker learning of the pairing between the punishment and the lever pressing.

Furthermore, since dysfunctions of the synaptic triad amongst glutamate, GABA and DA in the mesocorticolimbic regions have long been implicated in the underlying pathogenesis of alcohol use disorders (Cannizzaro et al., 2019; Spiga et al., 2014), it is reasonable to suggest that the augmented operant behaviour for alcohol observed in this study can result from prenatal THC-induced aberrant plasticity in specific areas of the brain that are functionally associated with reward, motivation and salience attribution (Brancato et al., 2014, 2018b; Cannizzaro et al., 2019).

Indeed, PCE is able to ablate endocannabinoid long-term depression and heighten excitability of PFC pyramidal neurons (Bara et al., 2018), suggesting that a persistent impairment in synaptic strength (Ma et al., 2018) may underlie the behavioural phenotype observed in adolescent PCE offspring in this study.

Overall, from this first set of data, it appears that aversive and conflicting environmental challenges can be particularly helpful in unveiling the early phenotypic consequences of PCE. Indeed, prenatal THC in the adolescent progeny did not affect neutral declarative memory, whereas it significantly impaired aversive limbic memory. Accordingly, alcohol vulnerability emerged massively in stress-related conditions, such as following forced

abstinence or in a conflict paradigm. Based on these observations, we may hypothesise that prenatal THC exposure can exert a detrimental effect on coping abilities when integration between environmental stimuli's encoding and emotional control is required, making male offspring more vulnerable towards dysfunction in emotionally salient learning and memory and sensitivity to alcohol-addictive properties. Whether female offspring display the same characteristics is not known at the moment. However, male offspring seem to be particularly vulnerable to detrimental effects of in utero THC, since prenatal THC exposure altered sensorimotor gating functions in a THC challenge in preadolescent male offspring, whereas female offspring were resilient (Frau et al., 2019).

Among the players that contribute to adaptive coping with stress, NPY plays a major role. A considerable amount of literature on NPY supports an anxiolytic and antidepressant-like activity (Bowers et al., 2012; Heilig, 2004; Wu et al., 2011) and its association with decreased stress responses and the expression of resilience in rodents and humans (Morgan et al., 2000; Sajdyk et al., 2008). Furthermore, NPY signalling controls alcohol-related vulnerability, since the activation of Y1 and Y2 receptors in the extended Amy and mPFC decreased alcohol consumption and self-administration (Robinson et al., 2019; Robinson and Thiele, 2017). In the current study, we measured NPY+ neurons in brain regions crucial to cognitive functions, motivation and emotional regulation, such as mPFC, NAc and Amy. Despite limited evidence on the interplay between endocannabinoid system and NPY signalling, previous work from this group observed an inverse relation between the two systems (Plescia et al., 2014a), since the administration of AM281, a selective CB1 antagonist, significantly increased NPY+ neurons both in the hippocampus and in the NAc of control and acetaldehyde-withdrawn rats.

Now, we report that THC interference in the endocannabinoid signalling during pregnancy is associated with a decreased number of NPY+ cells in the mPFC. NPY is abundantly expressed in cortical areas where it exerts a potent inhibitory effect on neuronal excitability of projection neurons (Bacci et al., 2002). As a matter of fact, the decrease in the NPY-ergic tone observed in the PFC of PCE adolescent offspring was associated with dampened limbic memory and instrumental learning, both functions dependent on the contribution of the mPFC (Caballero et al., 2019). Therefore, on the basis of the existing evidence, it is reasonable to speculate that the decrease in NPY signalling would significantly alter the functional activity of the projecting neurons (Vollmer et al., 2016), contributing to the cognitive impairment observed in PCE offspring. A future deeper investigation on the functionality of mPFC will ascertain our hypothesis. Besides its contribution to the expression of fear-related implicit memory, NPY is reported to enhance memory retention in T-maze footshock avoidance and step-down passive avoidance training in mice (Flood et al., 1987). Recently, blunted DA signalling and altered glutamate connectivity in the NAc have been proven to be a major neural underpinning of impaired aversive limbic memory (Cannizzaro et al., 2019). In this regard, we examined NPY+ neurons in the NAc and observed a significant decrease in NPY+ cells in the shell and core subregions of PCE rats compared to controls. Clinical and preclinical reports indicate that NPY in the NAc may modulate salience attribution, regardless of the stimulus valence (Brown et al., 2000; Josselyn and Beninger, 1993; Warthen et al., 2019), suggesting that the observed reduction in NPY levels in NAc GABAergic interneurons might contribute to the dampened cognitive functions displayed by PCE rats in this study. Glutamatergic networks are critically involved in synaptic plasticity during learning and memory (Burgos-Robles et al., 2007; Sotres-Bayon et al., 2006). Primarily, synaptic plasticity occurs in the PSD where scaffold proteins modulate the signalling cascade starting from membrane receptors and ultimately regulate dendritic structure and function in the glutamatergic synapse (Iasevoli et al., 2013; Tomasetti et al., 2017; Vessey and Karra, 2007). Previous evidence reports that perinatal THC exposure induces presynaptic disarrangement of discrete components of glutamatergic transmission (Campolongo et al., 2007; Suárez et al., 2004). Thus, in order to verify the occurrence of abnormal neuroplasticity at the excitatory postsynaptic site, we investigated the expression of different isoforms of the PSD proteins of the Homer family – Homer 1, 1 b/c and 2 – following prenatal THC exposure. As a matter of fact, along with decreased NPY expression, we found a general increase in different Homer isoforms in the corticolimbic brain regions. In more detail, in the mPFC of PCE rats, the increased expression of Homer 1 was paralleled by a decrease in the constitutively expressed long isoform Homer 1 b/c, which normally provides the structural scaffold of the excitatory signalosome (Castelli et al., 2017). This suggests that a Homer 1 increase is related to the overexpression of the activity-induced, short isoform Homer 1a that serves as transient disruptor of optimal postsynaptic glutamate signalling (Clifton et al., 2019). Again, besides the decrease in the NPY-ergic tone, increased recruitment of the long isoform Homer 2 was measured in both the shell and core subregions of the NAc, suggesting an abnormal excitatory postsynaptic plasticity.

Enhanced activity of the mPFC could in turn suppress aversive limbic memory by activating inhibitory Amy microcircuits (Maren and Quirk, 2004; Paré et al., 2004). At this level, PCE offspring displayed decreased NPY+ cells in the BLA and CeA, and increased Homer expression – namely, Homer 1 in both the BLA and CeA and the long functional isoforms Homer 1b/c and 2 in the BLA compared to controls. This abnormal PSD make-up might contribute to alterations in the local modulatory mechanism of negative limbic memory (Quirk and Mueller, 2008). Intriguingly, alterations of discrete functional components of the PSD at the glutamatergic synapse within the NAc and Amy underlie the development of the acute and chronic alcohol-induced behavioural plasticity (Castelli et al., 2017). In particular, preclinical and clinical data suggest that Homer 2 is an essential and active player in the expression of alcohol-induced behavioural and cellular plasticity and, in turn, is crucial in promoting alcohol consumption. Virus-mediated Homer 2 overexpression in the NAc enhanced alcohol motivational properties in an operant self-administration paradigm and facilitated the expression of alcohol-conditioned place preference (Haider et al., 2015; Szumlinski et al., 2008). Therefore, here, we suggest that the reduced NPY expression – and the spread disarrangement of the PSD make-up – in the limbic brain regions observed during adolescence, may represent pieces of the intricate puzzle – the neurobiological substrate – that underpins the escalation in alcohol consumption of PCE male rats. Accordingly, several reports indicate that reduced NPY levels in the NAc contribute to increased sensitivity to alcohol (Barkley-Levenson et al., 2016; Borkar et al., 2016; Plescia et al., 2014a), whereas infusions of

NPY into the CeA normalise alcohol intake in alcohol-preferring rats (Zhang et al., 2010). However, an interplay between the disarrangement in NPY and PSD proteins and aberrant plasticity due to the higher alcohol intake during the last week of acquisition in PCE male rats cannot be ruled out, thus contributing to the occurrence of the alcohol-prone phenotype observed during the relapse and conflict sessions.

Overall, these findings provide the first evidence of deficient NPY signalling and Homer-mediated dysfunctional synaptic plasticity as a common background of limbic memory dysfunction, impaired instrumental learning and the onset of vulnerability to alcohol in PCE adolescent male offspring

At this point in our research, it is difficult to interpret the current data on the basis of a mechanistic interaction between early endocannabinoid manipulation and NPY dysregulation. One hypothesis may involve CB1 receptors on GABAergic interneurons: during prenatal development, alterations in cannabinoid signalling at NPY+ GABAergic interneurons may lead to impaired neuronal migrations in cortical brain regions (Berghuis et al., 2007; Saez et al., 2014), positioning defects and excitatory/inhibitory neurotransmission imbalance.

On the other hand, more recent insight into the developmental effects of THC directly refers to epigenetic alterations. Besides enduring alterations in the expression of the proenkephalin and DA receptor 2 genes in the NAc of subjects exposed to THC during either prenatal or adolescent developmental periods (DiNieri et al., 2011; Tomasiewicz et al., 2012), increased levels of dimethylation of lysine 9 on histone H3, a transcriptionally repressive mark that controls *npy* gene expression in limbic brain regions (Berkel et al., 2019), have also been described following prenatal THC exposure (DiNieri et al., 2011), providing a putative epigenetic substrate for the modifications in NPY expression observed in this study.

In conclusion, in utero exposure to THC can involve enduring consequences on the neurodevelopmental trajectories towards adolescence, resulting in a vulnerable phenotype for impaired emotional/cognitive functions and alcohol addictive-like behaviour in male rat offspring. This is associated with dysregulation in NPY expression and signalling and PSD make-up in mesocorticolimbic regions. Further investigations on the persistence of the alcohol-vulnerable phenotype in later stages of life, the occurrence in female offspring and a putative causal relation between the molecular and the behavioural features observed as a consequence of the gestational THC exposure will address the need for clarifications on the neuro-teratogenic effects of THC.

Declaration of conflicting interests

The authors declared no potential conflicts of interest with respect to the research, authorship and/or publication of this article.

Funding

The authors disclosed receipt of the following financial support for the research, authorship and/or publication of this article: This study was supported by funding no. 008629 to C.C.

ORCID iDs

Anna Brancato https://orcid.org/0000-0002-8635-4146
Carla Cannizzaro https://orcid.org/0000-0002-7351-1712

References

Ameri A (1999) The effects of cannabinoids on the brain. *Prog Neurobiol* 58: 315–348.
Bacci A, Huguenard JR and Prince DA (2002) Differential modulation of synaptic transmission by neuropeptide Y in rat neocortical neurons. *Proc Natl Acad Sci U S A* 99: 17125–17130.
Ballard ME, Gallo DA and De Wit H (2013) Pre-encoding administration of amphetamine or THC preferentially modulates emotional memory in humans. *Psychopharmacology (Berl)* 226: 515–529.
Bara A, Manduca A, Bernabeu A, et al. (2018) Sex-dependent effects of in utero cannabinoid exposure on cortical function. *Elife* 7.
Barkley-Levenson AM, Ryabinin AE and Crabbe JC (2016) Neuropeptide Y response to alcohol is altered in nucleus accumbens of mice selectively bred for drinking to intoxication. *Behav Brain Res* 302: 160–170.
Barrot M, Sesack SR, Georges F, et al. (2012) Braking dopamine systems: a new GABA master structure for mesolimbic and nigrostriatal functions. *J Neurosci* 32: 14094–14101.
Bennett GW, Ballard TM, Watson CD, et al. (1997) Effect of neuropeptides on cognitive function. *Exp Gerontol* 32: 451–469.
Berghuis P, Rajnicek AM, Morozov YM, et al. (2007) Hardwiring the brain: endocannabinoids shape neuronal connectivity. *Science* 316: 1212–1216.
Berkel TDM, Zhang H, Teppen T, et al. (2019) Essential role of histone methyltransferase G9a in rapid tolerance to the anxiolytic effects of ethanol. *Int J Neuropsychopharmacol* 22: 292–302.
Berrendero F, García-Gil L, Hernández ML, et al. (1998) Localization of mRNA expression and activation of signal transduction mechanisms for cannabinoid receptor in rat brain during fetal development. *Development* 125: 3179–3188.
Borkar CD, Upadhya MA, Shelkar GP, et al. (2016) Neuropeptide Y system in accumbens shell mediates ethanol self-administration in posterior ventral tegmental area. *Addict Biol* 21: 766–775.
Botton PH, Costa MS, Ardais AP, et al. (2010) Caffeine prevents disruption of memory consolidation in the inhibitory avoidance and novel object recognition tasks by scopolamine in adult mice. *Behav Brain Res* 214: 254–259.
Bowers ME, Choi DC and Ressler KJ (2012) Neuropeptide regulation of fear and anxiety: implications of cholecystokinin, endogenous opioids, and neuropeptide Y. *Physiol Behav* 107: 699–710.
Brake SC, Hutchings DE, Morgan B, et al. (1987) Delta-9-tetrahydrocannabinol during pregnancy in the rat: II. Effects on ontogeny of locomotor activity and nipple attachment in the offspring. *Neurotoxicol Teratol* 9: 45–49.
Brancato A, Bregman D, Ahn HF, et al. (2017) Sub-chronic variable stress induces sex-specific effects on glutamatergic synapses in the nucleus accumbens. *Neuroscience* 350: 180–189.
Brancato A, Castelli V, Cavallaro A, et al. (2018a) Pre-conceptional and peri-gestational maternal binge alcohol drinking produces inheritance of mood disturbances and alcohol vulnerability in the adolescent offspring. *Front Psychiatry* 9: 150.
Brancato A, Cavallaro A, Lavanco G, et al. (2018b) Reward-related limbic memory and stimulation of the cannabinoid system: an upgrade in value attribution? *J Psychopharmacol* 32: 204–214.
Brancato A, Lavanco G, Cavallaro A, et al. (2016) The use of the Emotional-Object Recognition as an assay to assess learning and memory associated to an aversive stimulus in rodents. *J Neurosci Methods* 274: 106–115.
Brancato A, Plescia F, Marino RA, et al. (2014) Involvement of dopamine D2 receptors in addictive-like behaviour for acetaldehyde. *PLoS One* 9: e99454.
Breit KR, Zamudio B and Thomas JD (2019) The effects of alcohol and cannabinoid exposure during the brain growth spurt on behavioral development in rats. *Birth Defects Res* 111: 760–774.
Bromberg-Martin ES, Matsumoto M and Hikosaka O (2010) Dopamine in motivational control: rewarding, aversive, and alerting. *Neuron* 68: 815–834.

Brown CM, Coscina DV and Fletcher PJ (2000) The rewarding properties of neuropeptide Y in perifornical hypothalamus vs. nucleus accumbens. *Peptides* 21: 1279–1287.

Burgos-Robles A, Vidal-Gonzalez I, Santini E, et al. (2007) Consolidation of fear extinction requires NMDA receptor-dependent bursting in the ventromedial prefrontal cortex. *Neuron* 53: 871–880.

Caballero JP, Scarpa GB, Remage-Healey L, et al. (2019) Differential effects of dorsal and ventral medial prefrontal cortex inactivation during natural reward seeking, extinction, and cue-induced reinstatement. *eNeuro* 6.

Caberlotto L and Hurd YL (2001) Neuropeptide Y Y1 and Y2 receptor mRNA expression in the prefrontal cortex of psychiatric subjects: relationship of Y2 subtype to suicidal behavior. *Neuropsychopharmacology* 25: 91–97.

Cacace S, Plescia F, Barberi I, et al. (2012) Acetaldehyde oral self-administration: evidence from the operant-conflict paradigm. *Alcohol Clin Exp Res* 36: 1278–1287.

Cacace S, Plescia F, La Barbera M, et al. (2011) Evaluation of chronic alcohol self-administration by a 3-bottle choice paradigm in adult male rats. Effects on behavioural reactivity, spatial learning and reference memory. *Behav Brain Res* 219: 213–220.

Calvigioni D, Hurd YL, Harkany T, et al. (2014) Neuronal substrates and functional consequences of prenatal cannabis exposure. *Eur Child Adolesc Psychiatry* 23: 931–941.

Campolongo P, Trezza V, Cassano T, et al. (2007) Perinatal exposure to delta-9-tetrahydrocannabinol causes enduring cognitive deficits associated with alteration of cortical gene expression and neurotransmission in rats. *Addict Biol* 12(3–4): 485–495.

Campolongo P, Trezza V, Ratano P, et al. (2011) Developmental consequences of perinatal cannabis exposure: behavioral and neuroendocrine effects in adult rodents. *Psychopharmacology (Berl)* 214: 5–15.

Cannizzaro C, D'Amico M, Preziosi P, et al. (2006) Presynaptic effects of anandamide and WIN55,212-2 on glutamatergic nerve endings isolated from rat hippocampus. *Neurochem Int* 48: 159–165.

Cannizzaro C, Malta G, Argo A, et al. (2016) Behavioural and pharmacological characterization of a novel cannabinomimetic adamantane-derived indole, APICA, and considerations on the possible misuse as a psychotropic spice abuse, in C57bl/6J mice. *Forensic Sci Int* 265: 6–12.

Cannizzaro C, Plescia F, Gagliano M, et al. (2007) Effects of pre- and postnatal exposure to 5-methoxytryptamine and early handling on an object-place association learning task in adolescent rat offspring. *Neurosci Res* 59: 74–80.

Cannizzaro C, Plescia F, Gagliano M, et al. (2008) Perinatal exposure to 5-methoxytryptamine, behavioural-stress reactivity and functional response of 5-HT1A receptors in the adolescent rat. *Behav Brain Res* 186: 98–106.

Cannizzaro C, Talani G, Brancato A, et al. (2019) Dopamine restores limbic memory loss, dendritic spine structure, and NMDAR-dependent LTD in the nucleus accumbens of alcohol-withdrawn rats. *J Neurosci* 39: 929–943.

Carletti F, Ferraro G, Rizzo V, et al. (2013) Antiepileptic effect of dimethyl sulfoxide in a rat model of temporal lobe epilepsy. *Neurosci Lett* 546: 31–35.

Castelli V, Brancato A, Cavallaro A, et al. (2017) Homer2 and alcohol: a mutual interaction. *Front Psychiatry* 8: 268.

Chang JC, Tarr JA, Holland CL, et al. (2019) Beliefs and attitudes regarding prenatal marijuana use: perspectives of pregnant women who report use. *Drug Alcohol Depend* 196: 14–20.

Cheer JF, Wassum KM, Sombers LA, et al. (2007) Phasic dopamine release evoked by abused substances requires cannabinoid receptor activation. *J Neurosci* 27: 791–795.

Cippitelli A, Damadzic R, Hansson AC, et al. (2010) Neuropeptide Y (NPY) suppresses yohimbine-induced reinstatement of alcohol seeking. *Psychopharmacology (Berl)* 208: 417–426.

Clifton NE, Trent S, Thomas KL, et al. (2019) Regulation and function of activity-dependent homer in synaptic plasticity. *Mol Neuropsychiatry* 5: 147–161.

Davies SN, Pertwee RG and Riedel G (2002) Functions of cannabinoid receptors in the hippocampus. *Neuropharmacology* 42: 993–1007.

Deroche-Gamonet V, Belin D and Piazza PV (2004) Evidence for addiction-like behavior in the rat. *Science* 305: 1014–1017.

De Vries TJ and Schoffelmeer AN (2005) Cannabinoid CB1 receptors control conditioned drug seeking. *Trends Pharmacol Sci* 26: 420–426.

Di Liberto V, Frinchi M, Verdi V, et al. (2017) Anxiolytic effects of muscarinic acetylcholine receptors agonist oxotremorine in chronically stressed rats and related changes in BDNF and FGF2 levels in the hippocampus and prefrontal cortex. *Psychopharmacology (Berl)* 234: 559–573.

DiNieri JA and Hurd YL (2012) Rat models of prenatal and adolescent cannabis exposure. *Methods Mol Biol* 829: 231–242.

DiNieri JA, Wang X, Szutorisz H, et al. (2011) Maternal cannabis use alters ventral striatal dopamine D2 gene regulation in the offspring. *Biol Psychiatry* 70: 763–769.

Economidou D, Mattioli L, Ubaldi M, et al. (2007) Role of cannabinoidergic mechanisms in ethanol self-administration and ethanol seeking in rat adult offspring following perinatal exposure to Delta9-tetrahydrocannabinol. *Toxicol Appl Pharmacol* 223: 73–85.

Fernández-Ruiz J, Berrendero F, Hernández ML, et al. (2000) The endogenous cannabinoid system and brain development. *Trends Neurosci* 23: 14–20.

Fitzcharles MA and Eisenberg E (2018) Medical cannabis: a forward vision for the clinician. *Eur J Pain* 22: 485–491.

Flood JF, Hernandez EN and Morley JE (1987) Modulation of memory processing by neuropeptide Y. *Brain Res* 421: 280–290.

Frau R, Miczán V, Traccis F, et al. (2019) Prenatal THC exposure produces a hyperdopaminergic phenotype rescued by pregnenolone. *Nat Neurosci* 22: 1975–1985.

Fride E and Mechoulam R (1996) Developmental aspects of anandamide: ontogeny of response and prenatal exposure. *Psychoneuroendocrinology* 21: 157–172.

Fride E, Gobshtis N, Dahan H, et al. (2009) The endocannabinoid system during development: emphasis on perinatal events and delayed effects. *Vitam Horm* 81: 139–158.

Fried PA (1976) Short and long-term effects of pre-natal cannabis inhalation upon rat offspring. *Psychopharmacology (Berl)* 50: 285–291.

Fried PA and Smith AM (2001) A literature review of the consequences of prenatal marihuana exposure: an emerging theme of a deficiency in aspects of executive function. *Neurotoxicol Teratol* 23: 1–11.

Fried PA and Watkinson B (1988) 12- and 24-month neurobehavioural follow-up of children prenatally exposed to marihuana, cigarettes and alcohol. *Neurotoxicol Teratol* 10: 305–313.

Glass M, Dragunow M and Faull RL (1997) Cannabinoid receptors in the human brain: a detailed anatomical and quantitative autoradiographic study in the fetal, neonatal and adult human brain. *Neuroscience* 77: 299–318.

Gogtay N, Giedd JN, Lusk L, et al. (2004) Dynamic mapping of human cortical development during childhood through early adulthood. *Proc Natl Acad Sci U S A* 101: 8174–8179.

Goldschmidt L, Richardson GA, Cornelius MD, et al. (2004) Prenatal marijuana and alcohol exposure and academic achievement at age 10. *Neurotoxicol Teratol* 26: 521–532.

Goyal RK and Chaudhury A (2013) Structure activity relationship of synaptic and junctional neurotransmission. *Auton Neurosci* 176: 11–31.

Grant KS, Petroffa R, Isoherranend N, et al. (2018) Cannabis use during pregnancy: pharmacokinetics and effects on child development. *Pharmacol Ther* 182: 133–151.

Gunn JKL, Rosales CB, Center KE, et al. (2016) Prenatal exposure to cannabis and maternal and child health outcomes: a systematic review and meta-analysis. *BMJ Open* 6: e009986.

Haider A, Woodward NC, Lominac KD, et al. (2015) Homer2 within the nucleus accumbens core bidirectionally regulates alcohol intake by both P and Wistar rats. *Alcohol* 49: 533–542.

Harbison RD and Mantilla-Plata B (1972) Prenatal toxicity, maternal distribution and placental transfer of tetrahydrocannabinol. *J Pharmacol Exp Ther* 180: 446–453.

Hasin D, Sarvet A, Cerdá M, et al. (2017) US adult illicit cannabis use, cannabis use disorder, and medical marijuana laws: 1991–1992 to 2012–2013. *JAMA Psychiatry* 74: 579–588.

Heilig M (2004) The NPY system in stress, anxiety and depression. *Neuropeptides* 38: 213–224.

Heilig M and Thorsell A (2002) Brain neuropeptide Y (NPY) in stress and alcohol dependence. *Rev Neurosci* 13: 85–94.

Hill MN, McLaughlin RJ, Pan B, et al. (2011) Recruitment of prefrontal cortical endocannabinoid signaling by glucocorticoids contributes to termination of the stress response. *J Neurosci* 31: 10506–10515.

Hindocha C, Freeman TP, Schafer G, et al. (2015) Acute effects of delta-9-tetrahydrocannabinol, cannabidiol and their combination on facial emotion recognition: a randomised, double-blind, placebo-controlled study in cannabis users. *Eur Neuropsychopharmacol* 25: 325–334.

Hirst RB, Young KR, Sodos LM, et al. (2017) Trying to remember: effort mediates the relationship between frequency of cannabis use and memory performance. *J Clin Exp Neuropsychol* 39: 502–512.

Hutchings DE, Martin BR, Gamagaris Z, et al. (1989) Plasma concentrations of delta-9-tetrahydrocannabinol in dams and fetuses following acute or multiple prenatal dosing in rats. *Life Sci* 44: 697–701.

Iasevoli F, Tomasetti C and de Bartolomeis A (2013) Scaffolding proteins of the post-synaptic density contribute to synaptic plasticity by regulating receptor localization and distribution: relevance for neuropsychiatric diseases. *Neurochem Res* 38: 1–22.

Josselyn SA and Beninger RJ (1993) Neuropeptide Y: intraaccumbens injections produce a place preference that is blocked by cis-flupenthixol. *Pharmacol Biochem Behav* 46: 543–552.

Klumpers LE and Thacker DL (2019) A brief background on cannabis: from plant to medical indications. *J AOAC Int* 102: 412–420.

Lach G and De Lima TC (2013) Role of NPY Y1 receptor on acquisition, consolidation and extinction on contextual fear conditioning: dissociation between anxiety, locomotion and non-emotional memory behavior. *Neurobiol Learn Mem* 103: 26–33.

Lafourcade M, Elezgarai I, Mato S, et al. (2007) Molecular components and functions of the endocannabinoid system in mouse prefrontal cortex. *PLoS One* 2: e709.

Laviolette SR and Grace AA (2006) The roles of cannabinoid and dopamine receptor systems in neural emotional learning circuits: implications for schizophrenia and addiction. *Cell Mol Life Sci* 63: 1597–1613.

Lawn W, Freeman TP, Pope RA, et al. (2016) Acute and chronic effects of cannabinoids on effort-related decision-making and reward learning: an evaluation of the cannabis 'amotivational' hypotheses. *Psychopharmacology (Berl)* 233: 3537–3552.

Lomazzo E, König F, Abassi L, et al. (2017) Chronic stress leads to epigenetic dysregulation in the neuropeptide-Y and cannabinoid CB1 receptor genes in the mouse cingulate cortex. *Neuropharmacology* 113: 301–313.

Longo A, Fadda M, Brasso C, et al. (2018) Conditional inactivation of Npy1r gene in mice induces behavioural inflexibility and orbitofrontal cortex hyperactivity that are reversed by escitalopram. *Neuropharmacology* 133: 12–22.

Lupica CR and Riegel AC (2005) Endocannabinoid release from midbrain dopamine neurons: a potential substrate for cannabinoid receptor antagonist treatment of addiction. *Neuropharmacology* 48: 1105–1116.

Lutz B, Marsicano G, Maldonado R, et al. (2015) The endocannabinoid system in guarding against fear, anxiety and stress. *Nat Rev Neurosci* 16: 705–718.

Ma T, Cheng Y, Roltsch Hellard E, et al. (2018) Bidirectional and long-lasting control of alcohol-seeking behavior by corticostriatal LTP and LTD. *Nat Neurosci* 21: 373–383.

Macúchová E, Nohejlová K, Ševčíková M, et al. (2017) Sex differences in the strategies of spatial learning in prenatally-exposed rats treated with various drugs in adulthood. *Behav Brain Res* 327: 83–93.

Maeda K, Kawata E, Sakai K, et al. (1993) Effects of putative cognitive function-enhancing drugs and dopaminergic agents on somatostatin and neuropeptide Y in rat brain. *Eur J Pharmacol* 233: 227–235.

Maij DL, Van De Wetering BJ and Franken IH (2017) Cognitive control in young adults with cannabis use disorder: an event-related brain potential study. *J Psychopharmacol* 31: 1015–1026.

Maren S and Quirk GJ (2004) Neuronal signaling of fear memory. *Nat Rev Neurosci* 5: 844–852.

Martins SS, Mauro CM, Santaella-Tenorio J, et al. (2016). State-level medical marijuana laws, marijuana use and perceived availability of marijuana among the general U.S. population. *Drug Alcohol Depend* 169: 26–32.

Mato S, Del Olmo E and Pazos A (2003) Ontogenetic development of cannabinoid receptor expression and signal transduction functionality in the human brain. *Eur J Neurosci* 17: 1747–1754.

Mereu G, Fà M, Ferraro L, et al. (2003) Prenatal exposure to a cannabinoid agonist produces memory deficits linked to dysfunction in hippocampal long-term potentiation and glutamate release. *Proc Natl Acad Sci U S A* 100: 4915–4920.

Metz TD and Borgelt LM (2018) Marijuana use in pregnancy and while breastfeeding. *Obstet Gynecol* 132: 1198–1210.

Molosh AI, Sajdyk TJ, Truitt WA, et al. (2013) NPY Y1 receptors differentially modulate GABAA and NMDA receptors via divergent signal-transduction pathways to reduce excitability of amygdala neurons. *Neuropsychopharmacology* 38: 1352–1364.

Morena M, De Castro V, Gray JM, et al. (2015) Training-associated emotional arousal shapes endocannabinoid modulation of spatial memory retrieval in rats. *J Neurosci* 35: 13962–13974.

Morgan CA 3rd, Wang S, Southwick SM, et al. (2000) Plasma neuropeptide-Y concentrations in humans exposed to military survival training. *Biol Psychiatry* 47: 902–909.

Mumby DG, Gaskin S, Glenn MJ, et al. (2002) Hippocampal damage and exploratory preferences in rats: memory for objects, places, and contexts. *Learn Mem* 9: 49–57.

Navarro M, Rubio P and Fonseca FR (1995) Behavioural consequences of maternal exposure to natural cannabinoids in rats. *Psychopharmacology (Berl)* 122: 1–14.

Oakeshott S, Port RG, Cummins-Sutphen J, et al. (2011) HD mouse models reveal clear deficits in learning to perform a simple instrumental response. *PLoS Curr* 3: RRN1282.

Pandey SC, Carr LG, Heilig M, et al. (2003) Neuropeptide y and alcoholism: genetic, molecular, and pharmacological evidence. *Alcohol Clin Exp Res* 27: 149–154.

Paré D, Quirk GJ and Ledoux JE (2004) New vistas on amygdala networks in conditioned fear. *J Neurophysiol* 92: 1–9.

Paxinos G and Watson C (1998) *The Rat Brain in Stereotaxic Coordinates*. San Diego, CA: Academic Press.

Pezze MA and Feldon J (2004) Mesolimbic dopaminergic pathways in fear conditioning. *Prog Neurobiol* 74: 301–320.

Phan KL, Angstadt M, Golden J, et al. (2008) Cannabinoid modulation of amygdala reactivity to social signals of threat in humans. *J Neurosci* 28: 2313–2319.

Plescia F, Brancato A, Marino RA, et al. (2013) Acetaldehyde as a drug of abuse: insight into AM281 administration on operant-conflict paradigm in rats. *Front Behav Neurosci* 7: 64.

Plescia F, Brancato A, Marino RAM, et al. (2014a) Effect of acetaldehyde intoxication and withdrawal on NPY expression: focus on endocannabinoidergic system involvement. *Front Psychiatry* 5: 138.

Plescia F, Brancato A, Venniro M, et al. (2015) Acetaldehyde self-administration by a two-bottle choice paradigm: consequences on emotional reactivity, spatial learning, and memory. *Alcohol* 49: 139–148.

Plescia F, Marino RA, Navarra M, et al. (2014b) Early handling effect on female rat spatial and non-spatial learning and memory. *Behav Processes* 103: 9–16.

Quirk GJ and Mueller D (2008) Neural mechanisms of extinction learning and retrieval. *Neuropsychopharmacology* 33: 56–72.

Ramirez S, Liu X, MacDonald CJ, et al. (2015) Activating positive memory engrams suppresses depression-like behaviour. *Nature* 522: 335–339.

Reichmann F and Holzer P (2016) Neuropeptide Y: a stressful review. *Neuropeptides* 55: 99–109.

Renard J, Rosen LG, Loureiro M, et al. (2017) Adolescent cannabinoid exposure induces a persistent sub-cortical hyper-dopaminergic state and associated molecular adaptations in the prefrontal cortex. *Cereb Cortex* 27: 1297–1310.

Renard J, Rushlow WJ and Laviolette SR (2018) Effects of adolescent THC exposure on the prefrontal GABAergic system: implications for schizophrenia-related psychopathology. *Front Psychiatry* 9: 281.

Rey AA, Purrio M, Viveros MP, et al. (2012) Biphasic effects of cannabinoids in anxiety responses: CB1 and GABA(B) receptors in the balance of GABAergic and glutamatergic neurotransmission. *Neuropsychopharmacology* 37: 2624–2634.

Ripley TL and Stephens DN (2011) Critical thoughts on current rodent models for evaluating potential treatments of alcohol addiction and withdrawal. *Br J Pharmacol* 164: 1335–1356.

Robinson SL and Thiele TE (2017) The role of neuropeptide Y (NPY) in alcohol and drug abuse disorders. *Int Rev Neurobiol* 136: 177–197.

Robinson SL, Marrero IM, Perez-Heydrich CA, et al. (2019) Medial prefrontal cortex neuropeptide Y modulates binge-like ethanol consumption in C57BL/6J mice. *Neuropsychopharmacology* 44: 1132–1140.

Saez TM, Aronne MP, Caltana L, et al. (2014) Prenatal exposure to the CB1 and CB2 cannabinoid receptor agonist WIN 55,212-2 alters migration of early-born glutamatergic neurons and GABAergic interneurons in the rat cerebral cortex. *J Neurochem* 129: 637–648.

Sajdyk TJ, Johnson PL, Leitermann RJ, et al. (2008) Neuropeptide Y in the amygdala induces long-term resilience to stress-induced reductions in social responses but not hypothalamic–adrenal–pituitary axis activity or hyperthermia. *J Neurosci* 28: 893–903.

Sanchez-Marin L, Pavon FJ, Decara J, et al. (2017) Effects of intermittent alcohol exposure on emotion and cognition: a potential role for the endogenous cannabinoid system and neuroinflammation. *Front Behav Neurosci* 11: 15.

Scheyer AF, Melis M, Trezza V, et al. (2019) Consequences of perinatal cannabis exposure. *Trends Neurosci* 42: 871–884.

Schonhofen P, Bristot IJ, Crippa JA, et al. (2018) Cannabinoid-based therapies and brain development: potential harmful effect of early modulation of the endocannabinoid system. *CNS Drugs* 32: 697–712.

Schreiber S and Pick CG (2019) Cannabis use during pregnancy: are we at the verge of defining a 'fetal cannabis spectrum disorder'? *Med Hypotheses* 124: 53–55.

Serra S, Brunetti G, Pani M, et al. (2002) Blockade by the cannabinoid CB(1) receptor antagonist, SR 141716, of alcohol deprivation effect in alcohol-preferring rats. *Eur J Pharmacol* 443: 95–97.

Söderpalm B, Danielsson K, de Bejczy A, et al. (2019) Combined administration of varenicline and bupropion produces additive effects on accumbal dopamine and abolishes the alcohol deprivation effect in rats. *Addict Biol* 10: e12807.

Sotres-Bayon F, Cain CK and LeDoux JE (2006) Brain mechanisms of fear extinction: historical perspectives on the contribution of prefrontal cortex. *Biol Psychiatry* 60: 329–336.

Spano MS, Ellgren M, Wang X, et al. (2007) Prenatal cannabis exposure increases heroin seeking with allostatic changes in limbic enkephalin systems in adulthood. *Biol Psychiatry* 61: 554–563.

Spiga S, Talani G, Mulas G, et al. (2014) Hampered long-term depression and thin spine loss in the nucleus accumbens of ethanol-dependent rats. *Proc Natl Acad Sci U S A* 111: E3745–E3754.

Suárez I, Bodega G, Rubio M, et al. (2004) Prenatal cannabinoid exposure down- regulates glutamate transporter expressions (GLAST and EAAC1) in the rat cerebellum. *Dev Neurosci* 26(1): 45–53.

Sweis BM, Veverka KK, Dhillon ES, et al. (2013) Individual differences in the effects of chronic stress on memory: behavioral and neurochemical correlates of resiliency. *Neuroscience* 246: 142–159.

Szumlinski KK, Ary AW, Lominac KD, et al. (2008) Accumbens Homer2 overexpression facilitates alcohol-induced neuroplasticity in C57BL/6J mice. *Neuropsychopharmacology* 33: 1365–1378.

Tan H, Ahmad T, Loureiro M, et al. (2014) The role of cannabinoid transmission in emotional memory formation: implications for addiction and schizophrenia. *Front Psychiatry* 5: 73.

Tomasetti C, Iasevoli F, Buonaguro EF, et al. (2017) Treating the synapse in major psychiatric disorders: the role of postsynaptic density network in dopamine–glutamate interplay and psychopharmacologic drugs molecular actions. *Int J Mol Sci* 18 .

Tomasiewicz HC, Jacobs MM, Wilkinson MB, et al. (2012) Proenkephalin mediates the enduring effects of adolescent cannabis exposure associated with adult opiate vulnerability. *Biol Psychiatry* 72: 803–810.

Trezza V, Campolongo P, Cassano T, et al. (2008) Effects of perinatal exposure to delta-9-tetrahydrocannabinol on the emotional reactivity of the offspring: a longitudinal behavioral study in Wistar rats. *Psychopharmacology (Berl)* 198: 529–537.

Trezza V, Campolongo P, Manduca A, et al. (2012) Altering endocannabinoid neurotransmission at critical developmental ages: impact on rodent emotionality and cognitive performance. *Front Behav Neurosci* 6: 2.

United Nations on Drugs and Crime (UNODC) (2018) *World Drug Report 2018*. Vienna, Austria: United Nations on Drugs and Crime.

Vanderschuren LJ and Ahmed SH (2013) Animal studies of addictive behavior. *Cold Spring Harb Perspect Med* 3: a011932.

Vela G, Martín S, García-Gil L, et al. (2008) Maternal exposure to delta9-tetrahydrocannabinol facilitates morphine self-administration behavior and changes regional binding to central mu opioid receptors in adult offspring female rats. *Brain Res* 807: 101–109.

Vengeliene V, Bilbao A and Spanagel R (2014) The alcohol deprivation effect model for studying relapse behavior: a comparison between rats and mice. *Alcohol* 48: 313–320.

Vessey JP and Karra D (2007) More than just synaptic building blocks: scaffolding proteins of the post-synaptic density regulate dendritic patterning. *J Neurochem* 102: 324–332.

Vollmer LL, Schmeltzer S, Schurdak J, et al. (2016) Neuropeptide Y impairs retrieval of extinguished fear and modulates excitability of neurons in the infralimbic prefrontal cortex. *J Neurosci* 36: 1306–1315.

Warthen KG, Sanford B, Walker K, et al. (2019) Neuropeptide Y and representation of salience in human nucleus accumbens. *Neuropsychopharmacology* 44: 495–502.

Wenzel JM and Cheer JF (2018) Endocannabinoid regulation of reward and reinforcement through interaction with dopamine and endogenous opioid signaling. *Neuropsychopharmacology* 43: 103–115.

Wu G, Feder A, Wegener G, et al. (2011) Central functions of neuropeptide Y in mood and anxiety disorders. *Expert Opin Ther Targets* 15: 1317–1331.

Young-Wolff KC, Sarovar V, Tucker LY, et al. (2019) Self-reported daily, weekly, and monthly cannabis use among women before and during pregnancy. *JAMA Netw Open* 2: e196471.

Zhang H, Sakharkar AJ, Shi G, et al. (2010) Neuropeptide Y signaling in the central nucleus of amygdala regulates alcohol-drinking and anxiety-like behaviors of alcohol-preferring rats. *Alcohol Clin Exp Res* 34: 451–461.