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Mesolimbic dopamine dysregulation as a signature of information processing deficits imposed by prenatal THC exposure

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Abstract:	Cannabis is the illicit drug most widely used by pregnant women worldwide. Its growing acceptance and legalization have markedly increased the risks of child psychopathology, including psychotic-like experiences, which lowers the age of onset for a first psychotic episode. As the majority of patients with schizophrenia go through a premorbid condition long before this occurs, understanding neurobiological underpinnings of the prodromal stage of the disease is critical to improving illness trajectories and therapeutic outcomes. We have previously shown that male rat offspring prenatally exposed to Δ 9 -tetrahydrocannabinol (THC), a rat model of prenatal cannabis exposure (PCE), exhibit extensive molecular and synaptic changes in dopaminergic neurons of the ventral tegmental area (VTA), converging on a hyperdopaminergic state. This leads to a silent psychotic-like endophenotype that is unmasked by a single exposure to THC. Here, we further characterized the VTA dopamine neuron and sensorimotor gating functions of PCE rats exposed to an acute stress or a challenge of the D2 receptor agonist apomorphine, by using in vivo single-unit recordings correlated with Prepulse Inhibition (PPI) analyses. At pre-puberty, PCE male rat offspring display a reduced population activity of VTA dopamine neurons in vivo , the majority of which are tonically active. PCE male progeny also exhibit enhanced sensitivity to dopamine D2 (DAD2) receptor activation by apomorphine and a vulnerability to acute stress, which is associated with compromised sensorimotor gating functions. This data extends our knowledge of the multifaceted sequelae imposed by PCE in the mesolimbic dopamine system of male rats, which renders a neural substrate highly susceptible to subsequent challenges that may trigger psychotic-like outcomes.
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Cover Letter

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Cagliari, June 15th, 2020

Dear Drs Oleson and Khokhar,

Please, find attached our manuscript entitled *" Mesolimbic dopamine dysregulation as a signature of information processing deficits imposed by prenatal THC exposure "* for your consideration for publication in your Special Issue on "Cannabinoids and the brain" at *Progress in Neuro-Psychopharmacology & Biological Psychiatry* as a Research Paper.

Accumulating evidence about the potential dangers of using cannabis during pregnancy is gaining traction, propelled by a reduction of the stigma associated to its consumption. In fact, upon its expanding legalization and liberalization, physicians and health-care providers are noticing that a growing number of women are willing to discuss their use of cannabis during pregnancy to ease morning sickness, aches, pains, and other challenges of pregnancy. To complicate this issue, physicians do not sufficiently caution pregnant women, or women of childbearing age, about the potential dangers of cannabis use during pregnancy. Hence, cannabis is the most used illicit drug used by pregnant women worldwide.

Epidemiological studies have shown that exposure to cannabis in the womb predisposes offspring to a wide array of behavioral and cognitive deficits by interfering with endocannabinoid signaling during development, especially in those brain regions involved in psychiatric disorders. Aberrant reward system function is believed to play a role in many psychiatric disorders. In particular, imbalances in dopamine, a neuromodulator that encodes salience to internal and external stimuli, is critically involved in reward-related behaviors and cognitive functions and numerous psychiatric and neurological conditions. Within this framework, we have recently shown the impact of prenatal cannabis exposure (PCE) on ventral tegmental area (VTA) dopamine neuron activity in acute brain slices and their behavioural readout (Frau et al. , 2019). By combining electrophysiological and behavioral analyses in vivo, we confirmed and extended a robust psychotic-like phenotype induced by PCE, which is consistent with both the DAD2 super-sensitivity and stress hypotheses of schizophrenia. Our data suggest an aberrant attribution of salience given that deficits in PPI are not stimulus-

specific but stimulus-driven and that cannabis impacts the developing brain similarly to alcohol and cocaine. Hence, we believe that, in addition to its importance for the cannabis field, this manuscript will be of interest to your broad readership.

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Therefore, we would appreciate if you chose other reviewers for our manuscript.
Finally, we confirm that the paper is submitted with the full consent of all co-authors and the paper has not been published and is not under consideration in other journals.
The authors also declare no competing financial interests.
We look forward to the comments of the editors and referees. Thank you very much in advance for consideration.

Sincerely yours (on behalf of all co-authors)

Miriam Melis

Mesolimbic dopamine dysregulation as a signature of information processing deficits imposed by prenatal THC exposure

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KEY WORDS: cannabis, development, dopamine, dopamine D2 receptor, neuropsychiatric disorders, stress, vulnerability

Highlights

- Prenatal THC induces an aberrant dopaminergic function in vivo
- PCE male offspring manifest a DAD2 receptor sensitivity
- PCE male progeny display maladptive responses to an acute unescapable stress

Abbreviations: THC, Δ9-tetrahydrocannabinol

Abstract

Cannabis is the illicit drug most widely used by pregnant women worldwide. Its growing acceptance and legalization have markedly increased the risks of child psychopathology, including psychotic-like experiences, which lowers the age of onset for a first psychotic episode. As the majority of patients with schizophrenia go through a premorbid condition long before this occurs, understanding neurobiological underpinnings of the prodromal stage of the disease is critical to improving illness trajectories and therapeutic outcomes. We have previously shown that male rat offspring prenatally exposed to Δ^9-tetrahydrocannabinol (THC), a rat model of prenatal cannabis exposure (PCE), exhibit extensive molecular and synaptic changes in dopaminergic neurons of the ventral tegmental area (VTA), converging on a hyperdopaminergic state. This leads to a silent psychotic-like endophenotype that is unmasked by a single exposure to THC. Here, we further characterized the VTA dopamine neuron and sensorimotor gating functions of PCE rats exposed to an acute stress or a challenge of the D2 receptor agonist apomorphine, by using *in vivo* single-unit recordings correlated with Prepulse Inhibition (PPI) analyses. At pre-puberty, PCE male rat offspring display a reduced population activity of VTA dopamine neurons *in vivo*, the majority of which are tonically active. PCE male progeny also exhibit enhanced sensitivity to dopamine D2 (DAD2) receptor activation by apomorphine and a vulnerability to acute stress, which is associated with compromised sensorimotor gating functions. This data extends our knowledge of the multifaceted sequelae imposed by PCE in the mesolimbic dopamine system of male rats, which renders a neural substrate highly susceptible to subsequent challenges that may trigger psychotic-like outcomes.

1. INTRODUCTION

Psychosis and schizophrenia affect 20 million people worldwide (WHO, 2019)(2018). Although it is typically diagnosed in late adolescence, it emerges earlier on in life, especially in males (Hollis and Rapoport, 2008). Clinical evidence indicates that prenatal cannabis exposure (PCE) increases the risk for child psychopathology, including psychotic-like experiences (Bolhuis et al. , 2018, Fine et al. , 2019, Paul et al. , submitted, Singh et al. , 2020). Of note, these studies also suggest that PCE contributes to a significantly lower age of typical onset of first psychotic episode. However, the research on the mechanisms of PCE-induced psychotic-like experiences and the underlying loss of sensorimotor gating functions before puberty is still in its infancy .(Frau et al. , 2019)

Although an association between PCE and diverse psychiatric disorders has been established, in high-income countries cannabis use among pregnant women has been on an alarming sharp rise, with the greatest use in the first trimester (Brown et al. , 2017, Singh, Filion, 2020, Volkow et al. , 2019). Indeed, this increasing cannabis legal availability has led to a common misconception that it is a safe natural remedy even during vulnerable periods such as pregnancy. Consequently, offspring neurodevelopment is at risk due to the interference of cannabis' ingredients with the important functions played by the endocannabinoid system during this period of vulnerability to insult (Alpar et al. , 2016, Richardson et al. , 2016, Scheyer et al. , 2019).

According to the "two-hit" hypothesis of psychiatric disorders, PCE acts as a "first hit" by deranging offspring neurodevelopment towards the manifestation of psychiatric symptoms upon a "second hit" (e.g., early life adversity, drug abuse) (Richardson, Hester, 2016). Evidence shows that PCE affects the development of many brain regions involved in processing the salience of stimuli to support adaptive behaviors (Calvigioni et al. , 2014, Hurd et al. , 2019,

Richardson, Hester, 2016, Scheyer, Melis, 2019, Szutorisz and Hurd, 2018) where dopamine is key (Bromberg-Martin et al. , 2010). The mesolimbic dopamine pathway, projecting from the ventral tegmental area (VTA) to subcortical regions, including the nucleus accumbens (NAc), signals motivational and incentive salience(Berridge, 2012, Kapur, 2003) and is closely related to psychosis and positive symptoms in schizophrenia (Ziauddeen and Murray, 2010). This signaling system is sensitized by PCE, which endows it with 'silent' functional aberrations, such as impaired sensorimotor gating, which manifest when acutely exposed to THC before puberty (Frau, Miczan, 2019). In particular, PCE increases the probability of dopamine release upon an acute THC challenge exemplified by *ex vivo* firing activity of VTA dopamine neurons, *in vivo* extracellular dopamine levels in the NAc shell (NAcS), deficits of gating functions measured by pre-pulse inhibition (PPI) of startle reflex and psychomotor agitation (Frau, Miczan, 2019).

Ergo, we further tested the hypothesis that PCE induces a multifaceted dysregulation of the mesolimbic dopamine system conferring the offspring with a psychotic-like endophenotype vulnerable to acute challenges, including stress. By using *in vivo* electrophysiological and behavioral analysis, we find that PCE reduced the population activity of VTA dopamine neurons while increasing the percentage of those neurons tonically active in male offspring before puberty. Enhanced sensitivity of these cells to dopamine D2 (DAD2) receptor activation by apomorphine and to its PPI-disruptive effects accompany this PCE-endophenotype at prepuberty in male offspring. Furthermore, acute inescapable stress deteriorates gating functions only in PCE male pre-adolescent rats. This data extends our understanding of the multifaceted developmental deviations of the male rat mesolimbic dopamine system imposed by PCE and beginning early in life. We propose that this animal model potentially recapitulates for THC-induced psychosis (Compton et al. , 2009, McGrath et al. , 2010) but it might also elucidate the mechanisms underlying the vulnerability displayed by PCE children towards

psychotic-like experiences (Bolhuis, Kushner, 2018, Fine, Moreau, 2019, Paul, Hatoum, submitted).

2. MATERIALS AND METHODS

2.1 Subjects

All procedures were performed in accordance with the European legislation EU Directive 2010/63 and were approved by the Animal Ethics Committees of the University of Cagliari and by Italian Ministry of Health (auth. n. 659/2015-PR). We made all efforts to minimize pain and suffering and to reduce the number of animals used. Primiparous female Sprague Dawley (Envigo) rats were used as mothers and single housed during pregnancy. Offspring were weaned at ~PND21 and maintained without any further manipulation in standard conditions of temperature (21 ± 1°C) and humidity (60%) on normal 12-h light/dark cycle with ad libitum access to food and water until the experimental day (PND 24-28). Because we previously observed an effect that was sex-dependent and specific for this developmental milestone (Frau, Miczan, 2019), all experiments hereafter were carried out in male pre-adolescent rats. We did not use more than two males from each litter for the same experiment, to control for litter effect. All the additional male pups in each litter were used for different experiments, in order to minimize the total number of animals.

2.2 Drugs and treatments

Δ^9-Tetrahydrocannabinol (THC) was purchased from THC PHARM GmbH (Frankfurt, Germany). THC resin was dissolved in ethanol at 20% final concentration, and then sonicated for 30 minutes. THC was emulsified in 1-2% Tween 80, and then dissolved in sterile physiological saline. Rat dams were subcutaneously (s.c.) administered THC or vehicle 2 mg

kg^{-1} in a volume of 2 ml kg^{-1} from gestational day 5 (GD5) until GD20. Apomorphine (APO) was purchased from Merck Sigma-Aldrich. APO was dissolved in a solution containing 0.9% NaCl with 0.1 mg/ml ascorbic acid. For electrophysiological experiments, 0.2 to 0.8 µg kg^{-1} ml cumulative APO was intravenously (i.v.) administered. For behavioral experiments, 62.5, 125.0, 187.5 or 250.0 µg kg^{-1} ml APO was administered (s.c.) to different rat groups.

2.3 In vivo single unit extracellular recordings

Male rats were anesthetized with 400 mg kg^{-1} intraperitoneal (i.p.) chloral hydrate. For i.v. administration of pharmacological agents a cannula was inserted into their femoral vein. Rats were placed in a stereotaxic apparatus (Kopf, Tujunga, CA, USA) with their body temperature maintained at 37 ± 1 °C by a heating pad. Extracellular activity of neurons was recorded (bandpass filter 0.1–10.000 Hz) with glass micropipettes filled with 2% Pontamine sky blue dissolved in 0.5 M sodium acetate. Individual action potentials were isolated and amplified by means of a window discriminator (Neurolog System, Digitimer, Hertfordshire, UK) and displayed on a digital storage oscilloscope (TDS 3012, Tektronics, Marlow, UK). Experiments were sampled on line with Spike2 software by a computer connected to CED1401 interface (Cambridge Electronic Design, Cambridge, UK). Single-unit activity of neurons located in the lateral posterior ventral tegmental area (VTA, AP: − 5.0 mm from bregma; L: ± 0.4–0.6 mm from the midline; V: 6.5–7.5 mm from the cortical surface) was recorded extracellularly (bandpass filter 0.1–10,000 Hz). Putative dopamine neurons were isolated and identified according to published criteria (Grace and Bunney, 1984a, b), i.e., a firing rate < 10 Hz and > 2.5 ms duration of the action potential. Bursts were defined as the occurrence of two spikes at an interspike interval of < 80 ms and terminated when the interspike interval exceeded 160 ms. To estimate the cell population spontaneous activity, the electrode was passed in predetermined tracks

separated by 100 µm and the total number of active cells divided by the number of tracks (cells/track). At the end of recording sessions, a 15 mA current has been passed for 15 min through the micropipette in order to mark the recording site. The position of the electrodes in the brain has been microscopically identified on serial 60 µm sections stained with Neutral Red.

2.4 Behavioral test

2.4.1 Forced swim test

Acute stress was triggered by a modified Porsolt forced swim test (FST) (Huber et al. , 2001). Male rats were placed into a graduated and transparent cylinder (50 x 20 x 20 cm) containing 2l of cold water for 10 mins. During the task, animals were recorded and behaviors were scored. Two different kind of behaviors were analyzed: passive coping (measured as duration of time spent floating with the absence of any movement except those necessary for keeping the nose above water) and active coping behaviors (time spent swimming and climbing/straggling). Following the FST, rats were immediately dried out and then placed in a startle cage for the PPI testing.

2.4.2 Startle reflex and Pre-pulse Inhibition.

Startle reflex and Pre-pulse Inhibition (PPI) were tested as previously described (Frau et al. , 2017). Briefly, the apparatus (Med Associates) consisted of four standard cages placed in sound-attenuated chambers with fan ventilation. Each cage consisted of a Plexiglas cylinder of 5 cm diameter, mounted on a piezoelectric accelerometric platform connected to an analog-digital converter. Two separate speakers conveyed background noise and acoustic bursts, each one properly placed so as to produce a variation of sound within 1 dB across the startle cage. Both speakers and startle cages were connected to a main PC, which detected and analyzed all chamber variables with specific software. Before each testing session, acoustic

stimuli and mechanical responses were calibrated via specific devices supplied by Med Associates. The testing session featured a background noise of 70 dB and consisted of an acclimatization period of 5 min, followed by three consecutive sequences of trials (blocks). Unlike the first and the third block, during which rats were presented with only five pulse-alone trials of 115 dB, the second block consisted of a pseudorandom sequence of 50 trials, including 12 pulse-alone trials, 30 trials of pulse preceded by 74, 78, or 86 dB pre-pulses (10 for each level of pre-pulse loudness), and eight no-stimulus trials, where only the background noise was delivered. Inter-trial intervals were selected randomly between 10 and 15 s. The % PPI value was calculated using the following formula: 100 − [(mean startle amplitude for pre-pulse pulse trials/mean startle amplitude for pulse alone trials)*100]. PPI values related to different prepulse levels were collapsed, given that no interactions were found between pre-pulse levels throughout the study.

2.5 Data analysis

Rats were randomly assigned to each group. Statistical analysis was performed with GraphPad Prism 6 (San Diego CA, USA) software). Statistical outliers were identified with the Grubb's test (α=0.05) and excluded from the analysis.

For dose-curve electrophysiological experiments, drug-induced changes in firing activity were calculated by averaging the effects of the drug for the 2-min period following drug administration. Statistical significance was then assessed using repeated measures two-way ANOVA followed by Bonferroni's multiple comparisons test.

In vivo action potential durations were measured from the start of action potential to the negative trough, and statistical difference between groups was tested using Student's t-test. Action potentials were normalized defining 0% the smallest value and 100% the largest value in each

data set to study the rising phase and the after-hyperpolarization period. $T_{1/2}$ (time at which voltage is halfway between bottom and top, expressed as ms) and slope (steepness of the curve, with a larger value denoting a shallow curve, expressed as V) were calculated with logistic function. Statistical significance was then assessed using Student's t-test.

For behavioral experiments, PPI%, startle amplitude and activity or immobility during the FST were analyzed using Student's t-test or two-way ANOVA when appropriated, followed by Tukey's post hoc test. For dose-curve in behavioral experiments with apomorphine, drug-induced changes in normalized PPI% was calculated using non-linear regression model for each dose of apomorphine tested and compared for IC50 values. Significance level was set at $p<0.05$.

3. RESULTS

3.1 Impact of PCE on VTA putative dopamine neurons in vivo

Aberrant reward learning is common in symptomatic schizophrenic patients and in individuals at risk of developing psychosis (Roiser et al. , 2013, Roiser et al. , 2009). To further investigate whether PCE impacts electrophysiological properties of dopamine neurons, we recorded the extracellular spontaneous activity of 133 putative dopamine neurons located in the lateral posterior VTA (**Figure 1a**) of *in vivo* anesthetized male pre-adolescent rats. In fact, we previously found that PCE effect was sex-dependent and specific for this developmental milestone (Frau, Miczan, 2019). Dopamine neurons located in the parabrachial pigmented nucleus (PBP) of the VTA project to the lateral NAcS (Farassat et al. , 2019, Lammel et al. , 2008), and encode reward and salience (Lammel et al. , 2012). *In vivo*, dopamine neurons display different firing modes depending on both their intrinsic state and afferent input. They show a regular, irregular pattern or a bursting mode, which have been associated to tonic and

phasic dopamine release (Floresco et al. , 2003). We find that PCE does not affect the average spontaneous firing frequency of putative dopamine neurons in the VTA *in vivo* (**Figure 1b**,**c**; unpaired t-test, $t_{(131)}$ =0.504; PCE n_{cells}=59, n_{rats}=10; CTRL n_{cells}=74, n_{rats}=9). However, acute administration of THC increased firing frequency only in PCE offspring (**Figure 1 d**,**e**; p = 0.016, RM two-way ANOVA, $F_{(1,10)}$ = 8.41; PCEn_{cells} =8, CTRL n_{cells} =4) without affecting their burst firing (**Figure 1f**; p = 0.11, RM two-way ANOVA, $F_{(1,10)}$ =3.101). This supports that PCE increases vulnerability to acute effects of THC in male offspring (Frau, Miczan, 2019).

An analysis of VTA population activity reveals a reduced number of spontaneously active putative dopamine cells in PCE offspring (n_{rats}=10, n_{cells}=59) when compared to controls (CTRL, n_{rats}=9, n_{cells}=74) (**Figure 2a**, p = 0.041, unpaired t-test, $t_{(17)}$=2.21). Since PCE does not affect the total number of TH-positive cells within the VTA (Frau, Miczan, 2019), one possible explanation could be a shift in the percentage of cells exhibiting tonic and phasic activity. Accordingly, PCE decreased phasic activity of putative dopamine cells as measured as percentage of spikes in burst (**Figure 2b**,**c**, p = 0.049, unpaired t-test, $t_{(129)}$ = 1.66) and burst rate (**Figure 2d**, p = 0.019, unpaired t-test, $t_{(111.8)}$ = 2.09). Autocorrelogram analysis substantiates that PCE diminishes the percentage of putative dopamine cells showing phasic activity (20% vs 32% in PCE and CTRL, respectively) while increasing the percentage of tonically active cells (46 vs 23 % in PCE and CTRL, respectively) displaying a single spiking mode (**Figure 2e**, p = 0.018, Chi-square test).

Frequency and pattern of discharge of VTA dopamine cells, which depend on, among other factors, the after-hyperpolarization period (AHP), also affect action potential (AP) shape and duration. We found that PCE decreases AP duration of putative dopamine neurons (**Figure 3a**,**b**, p= 0.003, unpaired t-test, $t_{(87)}$= 3.009; 1.77 ± 0.03 ms, n_{cells} = 38) as compared to CTRL (1.96 ± 0.05 ms, n_{cells}= 51) as well as AHP (**Figure 3c,d**) when measured as $T_{1/2}$ (**Figure 3e,**

p= 0.031, unpaired t-test, $t_{(85)}$= 1.883; PCE 0.518 ± 0.048 ms, n_{cells} = 38; CTRL 0.679 ± 0.065 ms, n_{cells} = 49) and slope (**Figure 3f,** p< 0.0001, unpaired t-test, $t_{(85)}$= 4.332; PCE 0.311 ± 0.008 V, n_{cells} = 38; CTRL 0.241 ± 0.011 V, n_{cells} = 49), features of regularly spiking cells. Notably, PCE did not affect the rising phase (**Figure 3c,g**) when measured both $T_{1/2}$ (**Figure 3h,** p= 0.689, unpaired t-test, $t_{(77)}$= 0.041; PCE 0.363 ± 0.015 ms, n_{cells} = 36; CTRL 0.375 ± 0.023 ms, n_{cells} = 43) and slope (**Figure 3i,** p= 0.636, unpaired t-test, $t_{(79)}$= 0.476; PCE 0.125 ± 0.007 V, n_{cells} = 36; CTRL 0.133 ± 0.014 V, n_{cells} = 45). Altogether, these results suggest that PCE reduces population activity, the percentage of putative dopamine cells exhibiting phasic activity, and modifies mechanisms regulating their intrinsic excitability, such as ion conductance underlying the AHP.

3.2 PCE increases responsiveness to dopamine D2 receptor agonist apomorphine.

In midbrain dopamine neurons, DAD2 receptors located on the soma and the dendrites contribute to the potassium conductance underlying the AHP (Ford, 2014). DAD2 autoreceptors trigger G-protein activated inwardly rectifying potassium channels (GIRK) resulting in the modulation of intrinsic excitability of dopamine neurons and dopamine release in projection areas (Ford, 2014). To assess whether or not an enhanced DAD2/GIRK signal is related to the shorter AHP observed in PCE offspring putative dopamine neurons, we built a dose-response relationship for the effects of the DAD2 receptor agonist apomorphine (APO). APO (20-80 μg kg^{-1} i.v.) was more potent in male PCE offspring putative dopamine neurons (**Figure 4a,b;** p= 0.0075, unpaired t-test, $t_{(8)}$ = 3.086; IC50: 35.7 μg kg^{-1}) than in CTRL (IC50: 82.3 μg kg^{-1}). This “super-sensitivity” to DAD2 receptor agonist APO prompted us to investigate its effects on sensorimotor gating functions, which are impaired in patients with psychotic disorders (Braff et al. , 1995). Indeed, measures of sensorimotor gating as deficits in the PPI of startle reflex are

among the most widely studied physiological markers used in animal models of schizophrenia and psychosis (Braff and Geyer, 1990). We confirmed that PCE does not affect basal PPI values (Frau, Miczan, 2019) (**Figure 4c**; PCE-VEH vs CTRL-VEH, p = 0.855; two-way ANOVA and Tukey's post-hoc test), but it endows male offspring with a susceptibility to the lowest dose of APO tested, which disrupts PPI (**Figure 4c**; PCE-APO vs CTRL-APO, p = 0.032; PCE-APO vs CTRL-VEH, p = 0.025; p = 0.011, APO x PCE interaction, $F_{(1,32)} = 7.269$; two-way ANOVA and Tukey's post-hoc test). As a result, APO is more potent in inducing PPI deficits (**Figure 4d**; p= 0.036, nonlinear regression for PCE and CTRL dose-response relationships, $F_{(1,29)} = 4.797$) in PCE male offspring (IC50: 150 μg kg^{-1}) than in CTRL (IC50: 200 μg kg^{-1}). Notably, at the lowest dose tested, APO does not modify startle amplitude in either group (p= 0.104, APO x PCE interaction, two-way ANOVA and Tukey's post-hoc test, $F_{(1,29)} = 2.806$, data not shown). Taken together, our results suggest that PCE sensitizes male offspring to the effect of DAD2 receptor activation.

3.3 Acute stress impairs gating functions in PCE male offspring

PCE acts as a "first hit" bestowing on male offspring an endophenotype of a dopamine system function susceptible to subsequent insults (Frau, Miczan, 2019). Stress is one of the major determinants in the onset of severe dopamine-related psychiatric conditions (Fallon and Dursun, 2011, Kahn et al. , 2015, Otte et al. , 2016) with the mesolimbic dopamine system playing a role in the adaptation to environmental stressors (Cabib et al. , 2012, Douma and de Kloet, 2020, Gil and Armario, 1998). To test whether PCE interferes with behavioral adaptation to acute stress, male offspring were subjected to a widely used acute stressor (Huber, Darling, 2001) that is the forced swim test (FST, **Figure 5a**). PCE offspring display increased active behaviors (i.e. swimming and climbing) during the FST session (**Figure 5b**, PCE-FST vs CTRL-FST, p= 0.019, unpaired t-test,

$t_{(10)}$ = 2.796), thus suggesting a failure in adopting coping/adaptive strategies in response to an acute inescapable stressor (Commons et al. , 2017). Such an acute inescapable stressor (FST) also induces deficits in PPI only in PCE offspring (**Figure 5c;** PCE-FST vs CTRL-FST, p= 0.042, unpaired t-test, $t_{(14)}$ =2.238), whereas startle response is not affected (PCE-FST vs CTRL-FST, p = 0.736, unpaired t-test, $t_{(14)}$ = 0.343). Collectively, these data support that PCE acts as first hit (Richardson, Hester, 2016) and endows the male offspring with an aberrant salient attribution, key for the development of a psychotic-like phenotype.

4. DISCUSSION

The major finding of the present study is that maternal THC exposure induces a multifaceted dysregulation of dopamine cell activity *in vivo* in male rat offspring at prepuberty. This not only promotes an endophenotype susceptible to an otherwise ineffective dose of THC (present data and (Frau, Miczan, 2019)) but also to acute stress, which deteriorates gating functions, one of the best established translational endophenotypes of schizophrenia, which might contribute to the onset of psychotic experiences observed in children of mothers using cannabis during pregnancy (Bolhuis, Kushner, 2018, Fine, Moreau, 2019, Paul, Hatoum, submitted). Our findings support and extend previous preclinical studies showing that *in utero* exposure to cocaine and alcohol reduces *in vivo* the number of spontaneously active dopamine neurons in juvenile anesthetized rats without affecting their discharge rate (Choong and Shen, 2004, Wang and Pitts, 1994). Particularly, the observation that these effects following prenatal alcohol exposure persist across the entire lifespan (Shen et al. , 1999) warrants further investigations into the effects on PCE offspring and raises concerns on whether these alterations in population activity do subside with development.

The observed decreased number of spontaneously active dopamine neurons does not result from a PCE-induced loss of dopamine neurons of the VTA per se, as confocal microscopy reveals that neither affects tyrosine hydroxylase (TH)-positive dopamine neuron density nor TH levels measured in individual cells (Frau, Miczan, 2019). Rather, this reduction in population activity might reflect a depolarization inactivation. This phenomenon is a state of silence occurring when membrane potentials are more depolarized than those supporting the generation of action potentials (APs). Because PCE affects dopamine cell passive properties, increases their overall excitability, and reduces the probability of GABA released in the VTA (Frau, Miczan, 2019) , this might indeed represent a possible scenario. In fact, *ex vivo* dopamine neurons exhibit both depolarized resting membrane potentials and voltage thresholds along with a lower brake exerted by GABA afferents that might lead to their disinhibition first (Frau, Miczan, 2019), followed by a depolarization inactivation upon moderate stimulation *in vivo*. Alternatively, PCE-induced decreased population activity might reflect the actions of somato-dendritically released dopamine. Indeed, hyperactive PCE dopamine cells of the VTA (Frau, Miczan, 2019) might silence the neighboring cells through a dopamine-mediated chemical transmission described between pairs of these neurons that occurr through activation of DAD2 receptors (Vandecasteele et al. , 2008). Notably, the coexistence of chemical and electrical transmission described for subsets of dopamine cells (Grace and Bunney, 1983, Vandecasteele et al. , 2005) promotes a highly precise synchronization of spiking activity that cannot be observed *ex vivo* (Vandecasteele, Glowinski, 2005) but it does occur *in vivo* (Grace and Bunney, 1983, Morris et al. , 2004). This phenomenon would explain similar dopamine levels measured in the NAcS in the absence of relevant stimuli (i.e., acute THC) (Frau, Miczan, 2019) along with a similar basal discharge rate. Whether this represents a compensatory mechanism to allow a maximal stimulus-dependent excitation of the remaining spontaneously

active dopamine cells to overcome the increased percentage of silent neurons or metaplastic changes triggered by the interference of THC (or its metabolites) with the endocannabinoid system in the womb is to be established. Nonetheless, this gain of function of dopamine circuits at prepuberty indicates that PCE acts as a “first hit” leading to susceptibility system to subsequent challenges (i.e., acute THC or stress). This is important because elevated dopamine release properties have been implicated in the pathophysiology of schizophrenia (Grace, 2016, Hietala et al. , 1995, Howes et al. , 2011) and are a risk factor for vulnerability to diverse neuropsychiatric disorders. Accordingly, here we report that acute THC at prepuberty selectively increases the firing rate of PCE dopamine neurons *in vivo,* which extends our previous *ex vivo* findings showing that THC enhances both their spontaneous and evoked activity and their spike fidelity (Frau, Miczan, 2019). Importantly, this effect requires activation of type-1 cannabinoid (CB1) receptors, the molecular target of THC, whose presynaptic control on GABA release probability is enhanced in the VTA of PCE offspring (Frau, Miczan, 2019), another ultrastructural facet that might contribute to metaplastic changes within the VTA circuit and explain the observed larger prevalence of single spiking dopamine neurons.

We reported that PCE shifts the percentage of dopamine cells exhibiting tonic and phasic activity, with a net decrease of those exhibiting phasic (i.e., bursting, ~20%) activity versus an increase of those tonically active and displaying a single spiking mode (~46%). This is in agreement with the notion that a reduction of GABAergic tone from the ventral pallidum increases the number of tonically firing dopamine neurons in the VTA (Floresco, West, 2003), and with the role played by prefrontal cortex (PFC) afferents in the control of burst firing of VTA dopamine cells (Gariano and Groves, 1988, Tong et al. , 1996). Accordingly, PCE markedly reduces (~45%) PFC input (i.e., vGluT1) density impinging on dopamine cells (Frau, Miczan, 2019), causes a profound rewiring of cortical circuitry (Alpar et al. , 2014, Tortoriello et al. ,

2014) and plasticity (Bara et al. , 2018, de Salas-Quiroga et al. , 2015). Notably, PCE effects resemble a damage of PFC, which has long been associated with deteriorations of gating functions and an enhanced DAD2 receptor sensitivity (Swerdlow et al. , 1995). Our indirect evidence for an increased "supersensitivity" of DAD2 receptors in PCE male offspring is consistent with the one reported *in vivo* in VTA dopamine cells, but not NAc principal neurons, of rats prenatally exposed to alcohol (Shen et al. , 1995). The similar observations of an increased potency of DAD2 receptor agonist in silencing dopamine cell firing activity in animals prenatally exposed to alcohol warrant further investigations to disentangle the mechanisms underpinning PCE-induced *in vivo* "supersensitivity". At this stage, we can only speculate an increased expression of DAD2 receptors and/or more receptors in a high affinity state for dopamine, which appears to be common in animal models of schizophrenia (e.g., brain lesions, drug sensitization, social isolation, birth injury) (Seeman, 2011) and an enhanced coupling to downstream intracellular pathways involving a loss of control of these receptors through discrete mechanisms (e.g., desensitization, internalization, dimer formation, GTP regulation) (Seeman, 2013). This is important because DAD2 supersensitivity is implicated in the pathophysiology of schizophrenia, is associated to aberrant dopamine transmission (Seeman et al. , 2002) and to its abnormal downstream signaling cascade, including glycogen synthase kinase-3β (GSK-3β) (Lovestone et al. , 2007, Oda et al. , 2015). Accordingly, we previously reported that GSK-3β inhibitor pregnenolone can reprogram VTA dopamine circuit in PCE male offspring, restores dopamine cell function and its behavioral readout (Frau, Miczan, 2019). Notably, these actions are ascribed to pregnenolone itself and not its well-known downstream metabolites (e.g., progesterone, allopregnanolone), thus suggesting GSK-3β, among the others, as a possible molecular target candidate. Our findings, though not definitive of a DAD2 supersensitivity, provide an interpretative framework for other preclinical studies pointing to

dopamine supersensitivity as a common basis for psychosis and schizophrenia (Seeman, 2013).

We propose that stress-induced disruption of PPI represents a hyperdopaminergic response likely to be stimulus-driven but not stimulus-specific, and as such it does not necessarily mirror baseline measures of mesolimbic dopamine system function (i.e., average discharge rate, extracellular dopamine levels in NAcS). Our current results are consistent with the stress-vulnerability model of schizophrenia (Zubin and Spring, 1977), which posits that a susceptibility arising from the interaction among multiple factors (e.g., genetic, environmental, biological) confers poor stress-coping skills and an increased risk to manifest the disorder (Howes et al. , 2017). The observation that PCE male offspring at prepuberty exhibit poor adaptive strategies in response to an unavoidable acute stressor, which compromises gating functions, also corroborates the hypothesis that such biased dopamine system, as a part of brain-wide adaptations (Alpar, Di Marzo, 2016, Calvigioni, Hurd, 2014, Hurd, Manzoni, 2019, Morris et al. , 2011, Richardson, Hester, 2016, Scheyer, Melis, 2019, Szutorisz and Hurd, 2018, Tirado Munoz et al. , 2020) altering information processing in multiple domains, may affect the threshold for tolerating an unavoidable stressor to support adaptive behavior. Because dopamine neurons play a critical role in processing the salience of stimuli (Bromberg-Martin and Hikosaka, 2009, Ungless, 2004), such dysregulation might attribute incentive salience to otherwise irrelevant environmental stimuli (Howes, McCutcheon, 2017, Kapur, 2003). Accordingly, PCE-induced aberrant dopaminergic function might contribute to a multifaceted over-attribution of salience involving both rewarding (Brancato et al. , 2020, Spano et al. , 2007, Szutorisz et al. , 2014) and aversive signaling (present data and (Bolhuis, Kushner, 2018, Fine, Moreau, 2019, Paul, Hatoum, submitted)) and results in discrete neuropsychiatric disorders (Alpar, Di Marzo, 2016, Calvigioni, Hurd, 2014, Hurd, Manzoni, 2019, Morris, DiNieri, 2011,

Richardson, Hester, 2016, Scheyer, Melis, 2019, Szutorisz and Hurd, 2018, Tirado Munoz, Belen Lopez-Rodriguez, 2020).

Finally, as the debate on the pros and cons of cannabis legalization and liberalization occurs in social media, public meetings, and legislative assemblies, cannabis use among pregnant and child-bearing age women has been steadily increasing, unlike that of alcohol and tobacco (Administration, 2011, Agrawal et al. , 2019, Brown, Sarvet, 2017, Volkow, Han, 2019). Considering how the risks of neurodevelopmental adverse effects associated with maternal cannabis use are underestimated (Jansson et al. , 2018, Volkow et al. , 2017), especially during the first trimester (Volkow, Han, 2019), we emphasize that PCE dysregulates dopamine system function *in vivo* similarly to prenatal cocaine and alcohol (Choong and Shen, 2004, Wang and Pitts, 1994). Since PCE is a relevant modifiable predictor of neuropsychiatric disorders, preventative strategies have to be implemented as public health interventions regarding the awareness of the harm associated with maternal cannabis use. Of similar importance, as early staging and intervention may be effective treatment strategies for PCE children, our findings warrant further investigations on the extensive neurobiological maladaptations induced by exposure to prenatal cannabis ingredients to uncover age- and gender-specific therapeutic approaches.

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7. AUTHOR CONTRIBUTIONS

Claudia Sagheddu: Investigation, Visualization, Formal analysis; **Francesco Traccis:** Investigation, Visualization, Formal analysis; **Valeria Serra:** Investigation; **Mauro Congiu:**. Investigation; **Roberto Frau:** Methodology, Formal analysis, Writing - Review & Editing; **Joseph Francois Cheer:** Funding acquisition, Writing -Review & Editing; **Miriam Melis:** Conceptualization, Methodology, Resources, Supervision, Project administration, Funding acquisition, Writing -Original Draft.

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8. FIGURE LEGENDS

Figure 1. *In vivo,* acute THC increases firing frequency of putative VTA dopamine cell in male PCE offspring at prepuberty.

(**a**) Histological brain section showing the recording site (black triangle) in the ventral tegmental area of male SD rat at PND 27. Abbreviations: Aq, aqueduct; RN, red nucleus; SN, substantia nigra, VTA, ventral tegmental area. Scale bar 1.0 mm. (**b**) Representative firing rate histograms of VTA dopamine cells from CTRL and PCE male rats. (**c**) Average firing frequency of VTA dopamine cells did not vary between CTRL (n_{cells}=74) and PCE (n_{cells}=59) rats (unpaired t-test, t_{131}=0.504; data are represented as means s.e.m. with single values). (**d**) Representative rate histograms of the effect of THC (0.5 mg/kg THC i.v.) on a VTA dopamine neuron from CTRL and PCE male rats. Arrows indicate the time of THC injection. (**e**) Time course of the effect of acute THC on firing frequency of VTA dopamine neurons from PCE (n_{cells} =8) and controls (n_{cells}=4). (**f**) Time course of the effect of THC on burst firing (% difference) of VTA dopamine neurons of PCE (n_{cells} =8) and controls (n_{cells}=4). All data represented as average ± s.e.m. *p<0.05.

Figure 2. PCE affects *in vivo* spontaneous firing mode of putative VTA dopamine neurons.

(**a**) Scatter plot showing the average number of spontaneously active VTA dopamine neurons encountered per track (i.e., population activity) per animal. Population activity (i.e., cell/track) is reduced in PCE (n_{rats}=10) male offspring as compared with CTRL (n_{rats}=9) (**b**) Representative traces of spontaneous firing activity of dopamine neurons from offspring of CTRL and PCE rats at pre-adolescence. (**c**) Percentage of spikes in burst is reduced in PCE (n_{cells}=59) when

compared to CTRL offspring (n_{cells}=74). Data are represented as box and whisker plot with single values –min to max. (**d**) Burst rate is reduced in PCE (n_{cells}=53) when compared to CTRL (n_{cells}=63) offspring. Data are represented as box and whisker plot with single values –min to max. (**e**) Stack bars represent the percentage of dopamine cells displaying different firing mode from PCE (regular, n_{cells}=27; irregular, n_{cells}=20; bursting, n_{cells}=12) as compared with CTRL (regular, n_{cells}=17; irregular, n_{cells}=32; bursting, n_{cells}=25) offspring. R=regular; I=irregular; B=bursty. Data are represented as means ± s.e.m. with single values. *p<0.05.

Figure 3. PCE impacts intrinsic properties of *in vivo* VTA dopamine cells from male rats.

(**a**) Plot of averaged action potentials recorded from VTA dopamine neuron from PCE (n_{cells}=38) and CTRL (n_{cells}=51) male rats. (**b**) The duration of dopamine neuron action potentials is reduced in PCE (n_{cells}=38) when compared to CTRL. (**c**) Normalized average action potential amplitude of VTA dopamine neuron from PCE (n_{cells}=38) and CTRL (n_{cells}=51) male rats. (**d**) Sigmoidal fitting of normalized after hyperpolarization (AHP) phase of average action potentials from PCE (n_{cells} = 38) and CTRL (n_{cells} = 49) rats. (**e**) PCE shortens AHP $T_{1/2}$ while making AHP slope steeper (**f**). (**g**) Sigmoidal fitting of normalized rising phase of average action potentials from PCE (n_{cells} = 36) and CTRL (n_{cells} = 43) rats. (**h**) $T_{1/2}$ and steepness (**i**) of rising phase of dopamine action potential is not affected in PCE male rats. Data are represented as box and whisker plot with single values –min to max. *p<0.05, **p<0.01, ****p<0.0001.

Figure 4. PCE increases responsiveness to the dopamine D2 receptor agonist apomorphine.

(**a**) Representative firing rate histograms of the effect of apomorphine (APO) on dopamine neuron spontaneous activity recorded from the VTA of CTRL and PCE male rats. Arrows

indicate time of drug cumulative administration while the numbers indicate the doses (μg kg^{-1}, i.v.). (**b**) PCE potentiated the effects of APO on *in vivo* dopamine cell firing rate (n_{cells}= 5 per group). Dotted lines represent IC50 values (PCE = 35.6 μg kg^{-1}; CTRL = 82.8 μg kg^{-1}). Symbols and bars represent means ± s.e.m. (**c**) Acute APO (62.5 μg kg^{-1}; s.c.) selectively disrupts sensorimotor gating functions (measured as pre-pulse inhibition, PPI%) in PCE rat male offspring. Data are represented with box-and-whisker plots with single values. (**d**) Dose-response curve for APO-induced PPI deficits in rat male offspring (n_{rats}=7-11/group). Dotted lines represent IC50 values (PCE = 150μg kg^{-1}; CTRL = 200 μg kg^{-1}). Symbols and bars represent means ± s.e.m. *p<0.05.

Figure 5. PCE male rat offspring are more sensitive to the effects of acute stress.

(**a**) Schematic representation of the stress protocol, i.e., forced swim test (FST). (**b**) Effect of FST on coping behaviors in male rats: active behaviors are increased in PCE animals as compared to CTRL (n_{rats}=6 per group) (**c**) FST disrupts sensorimotor gating functions only in PCE male rats (n_{rats}= 8 per group). All data are represented with box-and-whisker plots with single values. *p< 0.05

Figure 1

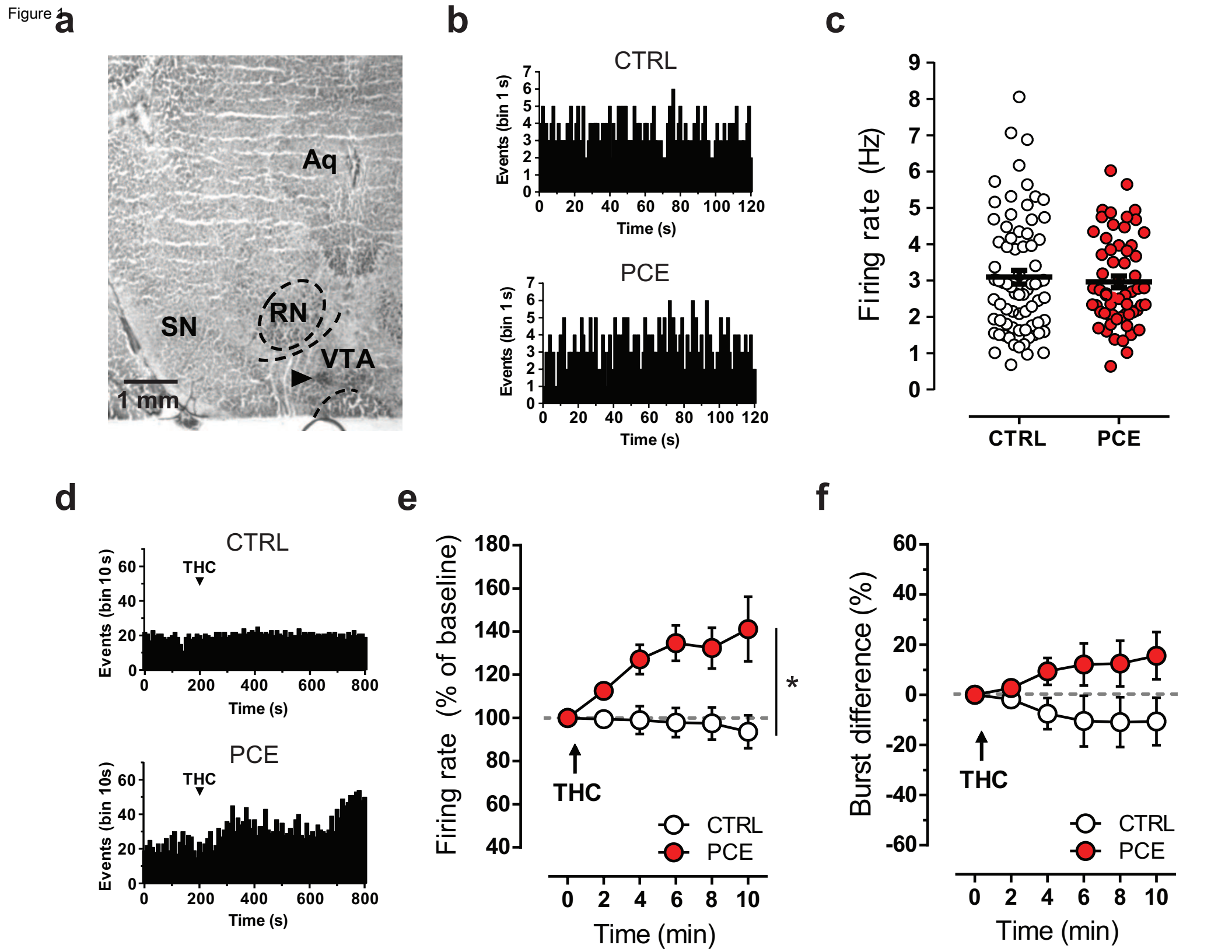

a
Aq
SN
RN
VTA
1 mm
b
CTRL
Events (bin 1 s)
Time (s)
PCE
c
Firing rate (Hz)
CTRL
PCE
d
CTRL
THC
Events (bin 10 s)
Time (s)
PCE
e
Firing rate (% of baseline)
THC
CTRL
PCE
*
Time (min)
f
Burst difference (%)
THC
CTRL
PCE
Time (min)

Figure 2

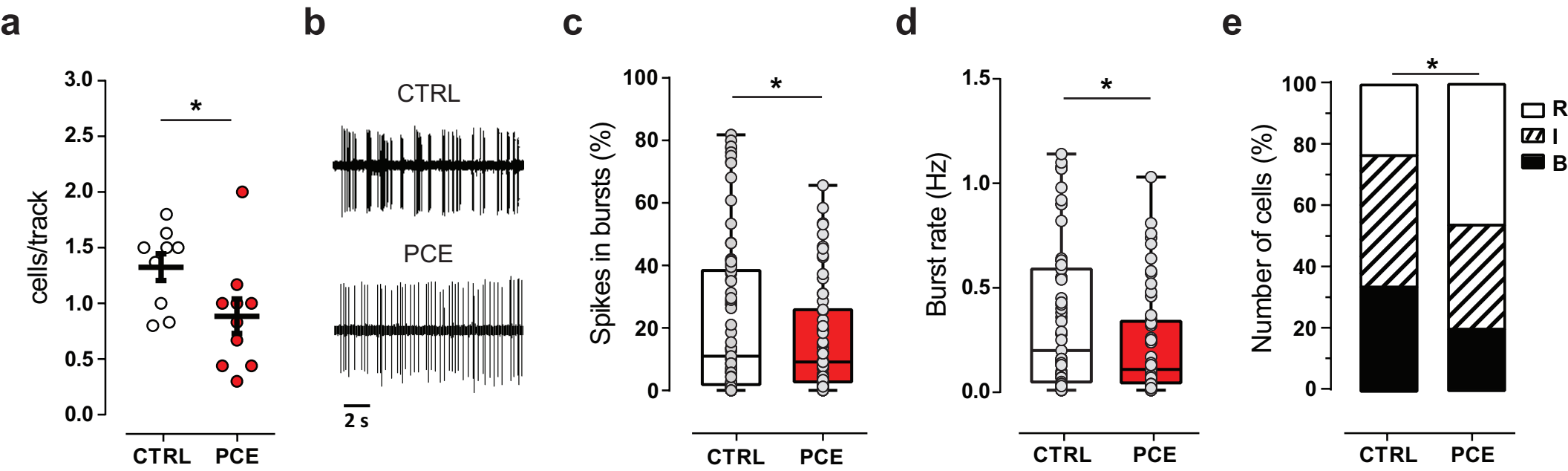

a
b
c
d
e
cells/track
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
CTRL
PCE
CTRL
PCE
2 s
Spikes in bursts (%)
100
80
60
40
20
0
*
CTRL
PCE
Burst rate (Hz)
1.5
1.0
0.5
0.0
*
CTRL
PCE
Number of cells (%)
100
80
60
40
20
0
*
R
I
B
CTRL
PCE

Figure 3

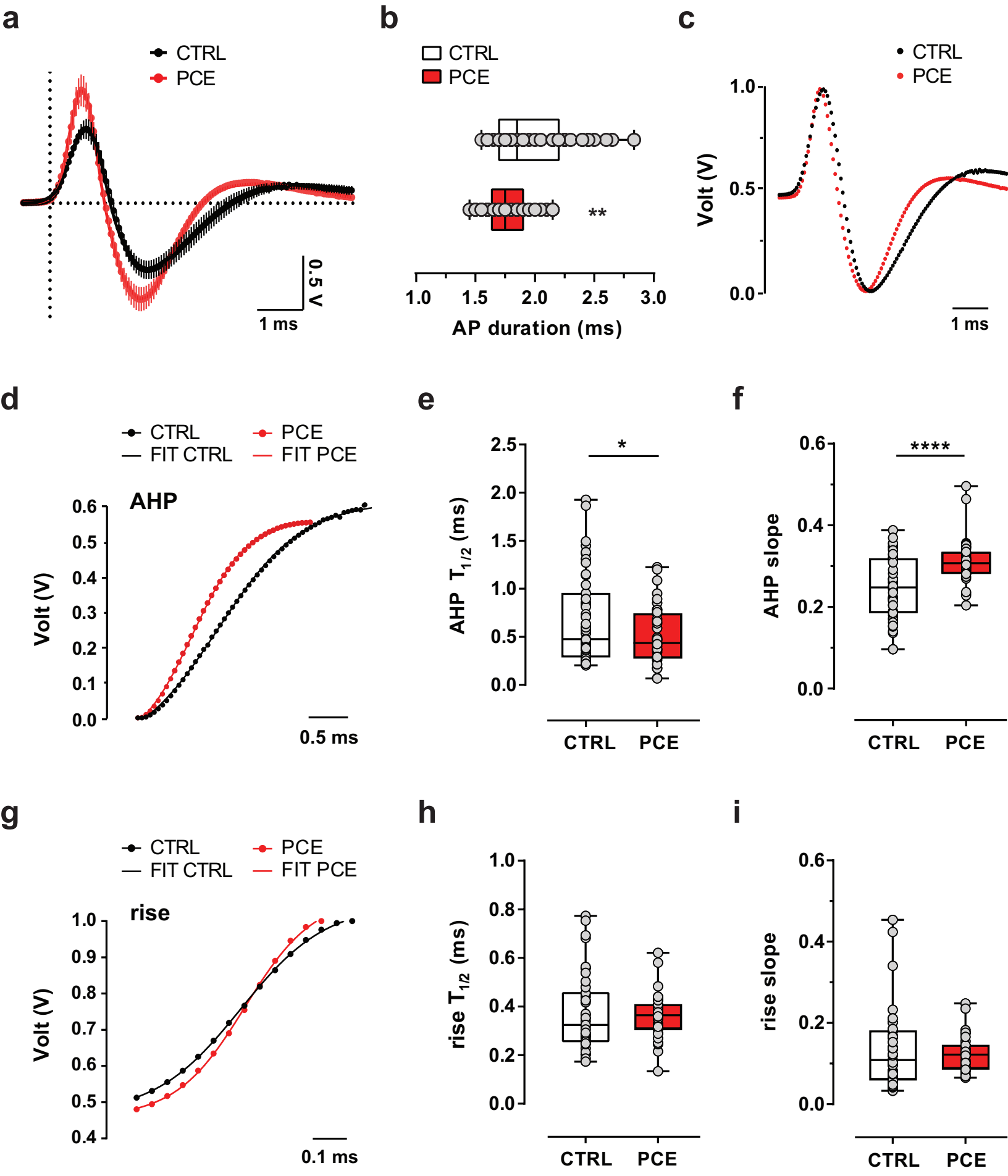

a
CTRL
PCE
0.5 V
1 ms
b
CTRL
PCE
**
1.0
1.5
2.0
2.5
3.0
AP duration (ms)
c
CTRL
PCE
Volt (V)
1.0
0.5
0.0
1 ms
d
CTRL
PCE
FIT CTRL
FIT PCE
AHP
Volt (V)
0.5 ms
e
AHP T1/2 (ms)
*
CTRL
PCE
f
AHP slope
****
CTRL
PCE
g
CTRL
PCE
FIT CTRL
FIT PCE
rise
Volt (V)
0.1 ms
h
rise T1/2 (ms)
CTRL
PCE
i
rise slope
CTRL
PCE

Figure 4

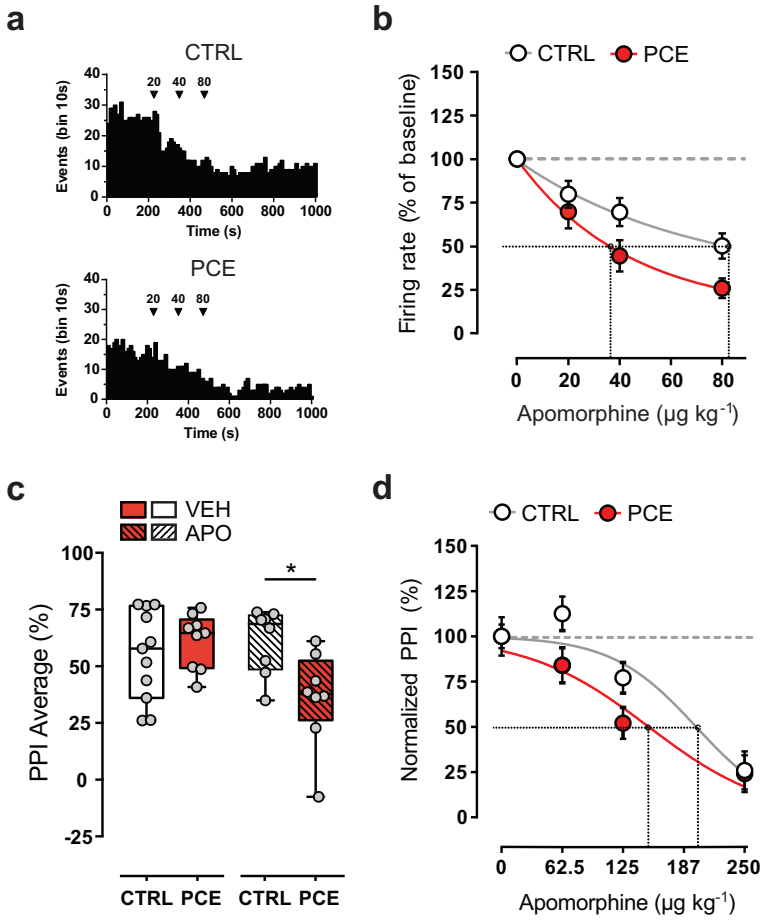
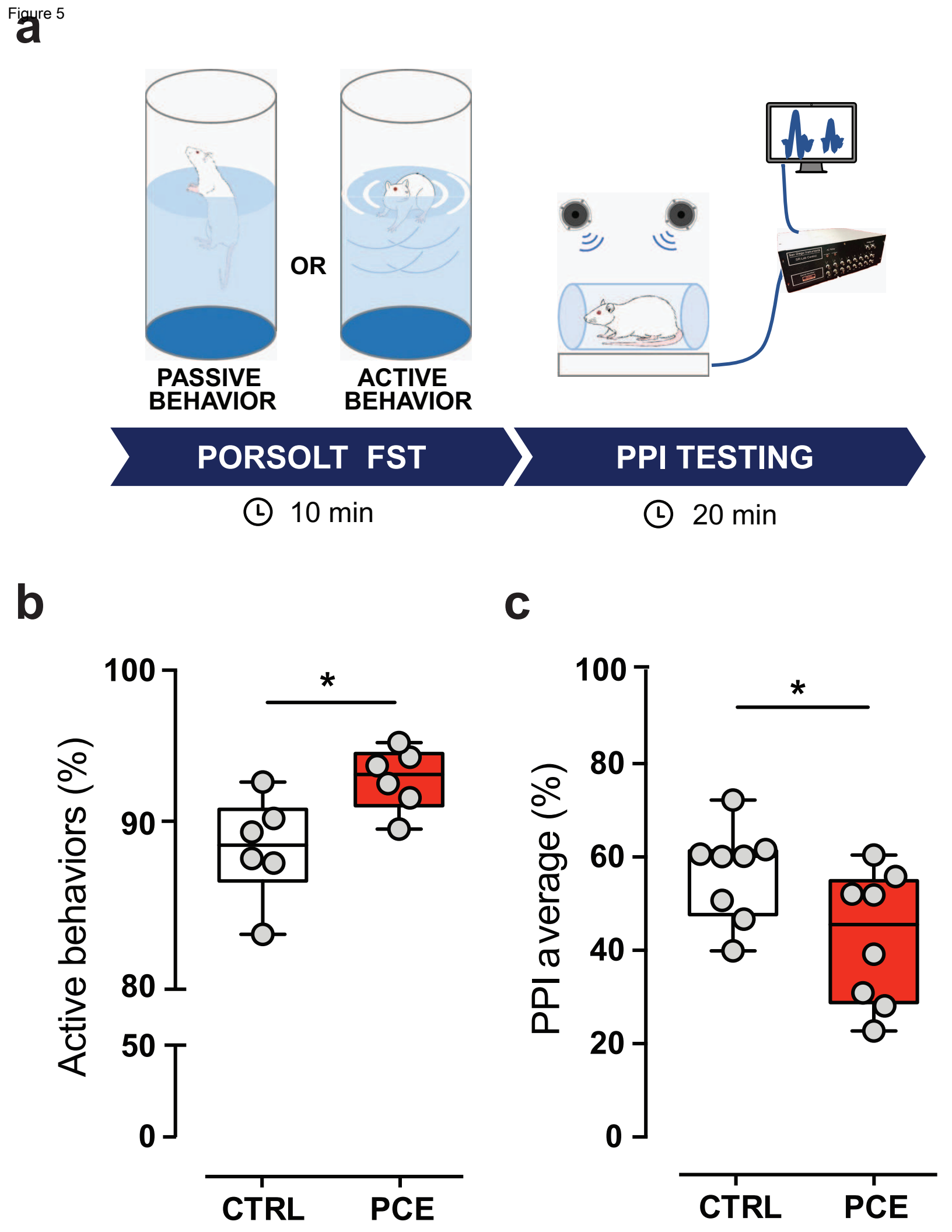

a
CTRL
20 40 80
Events (bin 10s)
Time (s)
PCE
b
CTRL PCE
Firing rate (% of baseline)
Apomorphine (µg kg-1)
c
VEH
APO
PPI Average (%)
CTRL PCE CTRL PCE
d
CTRL PCE
Normalized PPI (%)
Apomorphine (µg kg-1)

Figure 5


a
OR
PASSIVE BEHAVIOR
ACTIVE BEHAVIOR
PORSOLT FST
10 min
PPI TESTING
20 min
b
Active behaviors (%)
100
90
80
50
0
*
CTRL
PCE
c
PPI average (%)
100
80
60
40
20
0
*
CTRL
PCE

ETHICAL STATEMENT

The authors outline their individual contributions to the paper by using the relevant CRediT roles as it follows:

Claudia Sagheddu: Investigation, Visualization, Formal analysis; **Francesco Traccis:** Investigation, Visualization, Formal analysis; **Valeria Serra:** Investigation; **Mauro Congiu:**. Investigation; **Roberto Frau:** Methodology, Formal analysis, Writing - Review & Editing; **Joseph Francois Cheer:** Funding acquisition, Writing -Review & Editing; **Miriam Melis:** Conceptualization, Methodology, Resources, Supervision, Project administration, Funding acquisition, Writing -Original Draft.