Doctoral Thesis

Long-term effects of adolescent Δ9-THC exposure in Substance Use Disorder liability assessed through behavioural, neuroimaging and transcriptome studies

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Tampoco necesitan agradecimientos, pero sí creo necesario recordar a los migrantes y desposeídos que despiertan todo tipo de preguntas mientras tratan de resolver su propia subsistencia. La historia reciente de nuestra relación con el cannabis está marcada por su prohibición y por la criminalización de su uso alrededor del mundo. Esta circunstancia ha afectado de manera desigual a ciertos sectores de la población y por ello la historia moderna del cannabis es también la historia de la persecución, represión y control de los sufís y de los campesinos *fellahin* en el mundo árabe y Egipto, de los inmigrantes indios en Sudáfrica, de los *manges* en Grecia, de los esclavos africanos en Brasil o de las migraciones de mexicanos hacia su vecino del norte. Fueron estos últimos, a principios del siglo XX, los acusados de introducir la planta y extender su uso por los Estados Unidos en el marco de una cruzada prohibicionista que enarbolaba ideas pseudocientíficas como la relación inconcusa entre el consumo de cannabis y la progresión hacia la locura, la delincuencia y el abuso de otras drogas. Especulaciones que, no obstante, calaron en el imaginario popular, se emplearon para modificar la legislación y que ciertamente forman parte del contexto cultural en el que se planteó esta investigación. Durante estos años he tenido la suerte de ser acogido tanto por Brasil como por Estados Unidos, dos países en los que el impacto negativo de la prohibición del cannabis y su criminalización ha sido tristemente notable; y en los que paradójicamente la reciente ola de legalización parece que también excluirá de los beneficios a los mismos que padecieron su prohibición. La ciencia es fundamentalmente un método empleado por personas que no somos, ni debemos ser, impermeables al contexto social y las injusticias con las que coexistimos. De la misma manera, no podemos permitirnos ignorar o minimizar la influencia de nuestro trabajo y del conocimiento que obtenemos cuando lo hacemos público y es devuelto a la sociedad. No es la primera vez que se expresan ideas similares, pero nunca está de más recordarlas.

Ahora que ya habéis leído mis proclamas, empiezan los agradecimientos. Pero no sin antes hacer otra pequeña reflexión. Si bien parece que heredamos muchos problemas, no solo científicos, de nuestros predecesores y de las sociedades en las que nos toca vivir, no debemos heredar pasivamente las soluciones empleadas sin cuestionarlas ni mejorarlas. La ciencia es una herramienta que requiere ser utilizada de cierta manera, con cierta actitud. No hay ciencia si caemos en elitismos o clasismos, cuando no hay diálogo, ni cuando anteponemos nuestros criterios personales o buscamos un beneficio individual. No hay ciencia si escondemos debilidades y fallos, ni cuando nos excusamos o nos acusamos. No hay ciencia sin asumir responsabilidades; y si la hay, no merece la pena. Gracias a mis padres por construir en mí esta manera de entender nuestras acciones y nuestras relaciones. Todos sabemos que no siempre es cómodo vivir de esta manera, pero con vosotros ha sido más fácil entender su valor.

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Javier.

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LIST OF ABBREVIATIONS

µm	Micrometre
1H MR	Proton Hydrogen 1 Magnetic Resonance
2-AG	2-Arachidonoylglycerol
2-CSRTT	Two-Choice Serial Reaction Time Task
5-CSRTT	Five-Choice Serial Reaction Time Task
5-HIAA	5-hydroxyindoleacetic acid
5-HT	Serotonin (5-hydroxytryptamine)
ABC	Avidin-Biotin Complex
Abcc9	ATP Binding Cassette Subfamily C Member 9
AC	Anterior Comissure
ACC	Anterior Cingulate Cortex
ACE	Adolescent Cannabis Exposure
ACs	Adenylyl Cyclases
AEA	Anandamide
Agt	Angiotensinogen
Akt / PKB	Protein kinase B
ALP	Active Lever Press
ANOVA	analysis of variance
A-O	Action-Outcome
ATP	Adenosine triphosphate
BL	Baseline
BLA	Basolateral Amygdala
BP	Breaking point
Bpm	Beats per minute
BVV	Brain Ventricle Volume / Volumetry
Ca^{2+}	Calcium ion
Calb1	Calbindin 1
cAMP	Cyclic adenosine monophosphate
Cartpt	Cocaine- and amphetamine-regulated transcript Prepropeptide
Cb	Cerebellum
CB	Cannabinoid
CB_1	Cannabinoid Receptor 1
CB_2	Cannabinoid Receptor 2
CBUD	Cannabis Use Disorder
CC	Corpus Callosum
Cck	Cholecystokinin
CD74	Cluster of Differentiation 74 Molecule
cDNA	Complementary DNA
CeA	Central Amygdala
Cl^-	Chloride ion
CLP	Correct lever presses
cm	Centimetres
CPu	Caudate Putamen / Dorsal Striatum
Cr	Creatine
CREB	cAMP response element-binding protein
CRTC1	CREB-regulated transcription coactivator 1
CS	Conditioned Stimulus
CS^-	Conditioned Stimulus to neutral or negative outcomes
CS^+	Conditioned Stimulus to a positive outcome
CSA	Cocaine Self-Administration
CSF	Cerebrospinal fluid

CSPT	Cortico-Striato-Pallido-Thalamic
CS-US	Conditioned Stimulus-Unconditioned Stimulus
CUD	Cocaine Use Disorder
Cx	Cortex
D_1	Dopamine Receptor 1
D_2	Dopamine Receptor 2
DA	Dopamine
DAB	3,3′-Diaminobenzidine
DAergic	Dopaminergic
DE	Differential expression / Differentially expressed
DEG	Differentially expressed gene(s)
DH	Dorsal Hippocampus
DLS	Dorsolateral Striatum
DMS	Dorsomedial Striatum
DNA	Deoxyribonucleic acid
DS	Discriminative Stimulus
dSTR	Dorsal Striatum
Dus2	Dihydrouridine Synthase 2
eCB	Endocannabinoid
eCBS	Endocannabinoid System
FA	Fractional Anisotropy
FAAH	Fatty acid amide hydrolase
FDR	False Discovery Rate
Flt1	Vascular endothelial growth factor receptor
FR	Fixed Ratio
G / cm	Gauss per centimetre (G/cm
GABA	Gamma-Aminobutyric acid
Gabra4	GABA receptor protein 4
Gabre	GABA(A) Subunit Epsilon
Gal	Galanin
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GO	Gene Ontology
GP	Globus Pallidus
GPC+PCh	Glycerophosphorylcholine + Phosphorylcholine
GPCRs	G-Protein Coupled Receptors
Grin2d	Glutamate Receptor Ionotropic NMDA 2D
Grip1	Glutamate receptor-interacting protein
Grk4	G protein-coupled receptor kinase 4
GSK	Glycogen synthase kinase
GT	Goal Track
H	Height
h	Hour
HC	Hippocampal Commissure
HE	Head entry
Hipk2	Homeodomain-interacting protein kinase 2
HIPP	Hippocampus
Hsf4	Heat shock transcription factor 4
HSP	Heat shock protein
i.d.	Inside Diameter
i.p.	Intraperitoneally
IC	Internal Capsule
ILP	Inactive / Incorrect Lever Presses
inj	Injection
Irs4	Insulin Receptor Substrate 4
ITI	Inter-trial-interval
IU/mL	International units per millilitre

K+	Potassium ion
KCC2	Potassium-chloride transporter member 5
KCNAB2	Voltage-gated potassium channel subunit beta-2
Kcnj10	ATP-sensitive inward rectifier potassium channel 10
Kcnt1	Sodium-activated potassium channel
KEEG	Kyoto Encyclopaedia of Genes and Genomes
Kif21a	Kinesin family member 21a
KP	KEGG pathway
L	Length
LP	Lever Press
LPTO	Lever Press during Time-Out
LS	Lateral Septal nucleus
MAG	Magazine time
MAP / MAPK	Mitogen-activated protein kinase
Mapk10	Mitogen-Activated Protein Kinase 10
MD	Mean Diffusivity
mg	Milligram
min	Minute(s)
mL	Millilitre
mm	Millimetre
mPFC	Medial Prefrontal Cortex
MRI	Magnetic Resonance Imaging
mRNA	Messenger RNA
MS	Medial septal nucleus
ms	Millisecond
mTOR	mechanistic target of rapamycin
Myo9a	Myosin 9a
Na+	Sodium ion
NAA+NAAG	N-acetyl-aspartate + N-acetyl-aspartyl-glutamate
NAc	Nucleus Accumbens
NaCl	Sodium Chloride
NCAM	Neural cell adhesion molecule
Nek10	Never In Mitosis A-Related Kinase 10
NMDA	N-Methyl-D-aspartic acid
NOTCH3	Neurogenic locus notch homolog protein 3
Nov	Nephroblastoma overexpressed
Nr4a2	Nuclear receptor subfamily 4 group A member 2
NSI	No stimuli interval
ºC	Celsius degrees
OR	Omission Response
PANTHER	Protein Analysis Through Evolutionary Relationships
PAS	Period circadian protein - Aryl hydrocarbon receptor nuclear translocator protein – Single minded protein
PB	Phosphate Buffer
PBS	Phosphate-buffered saline
PCA	Pavlovian conditioned Approach
PCr	Phosphocreatine
PCR	Polymerase Chain Reaction
PEACE	Protracted Effects of Adolescent Cannabis Exposure
PerR	Perseverative Responses
PFA	Paraformaldehyde
PFC	Prefrontal Cortex
PIT	Pavlovian-to-instrumental transfer
PKB / Akt	Protein kinase B
PKC	Protein kinase C
Plxna3	Plexin 3

PND	Post-Natal Day
PPAR	Peroxisome Proliferator-Activated Receptors
PreR	Premature Responses
Ptprb	Tyrosine Phosphatase Receptor Type B
RARE	Rapid Acquisition with Relaxation Enhancement
RF	Radio Frequency
Rgs5	Regulator of G Protein Signalling 5
Rims2	Regulating Synaptic Membrane Exocytosis 2
RNA	Ribonucleic acid
RNAseq	Ribonucleic acid sequencing
ROI	Region of interest
s	Second
SC1 / SPARCL1	Secreted protein acidic and rich in cysteine-like protein 1
Scn8a	Sodium channel protein type 8 subunit alpha
Shc3	SHC Adaptor Protein 3
Slc	Solute carrier
Slc12a5	Potassium-chloride transporter member 5
Slc17a6	Vesicular glutamate transporter 2
SNu	Septal Nuclei
S-R	Stimulus-Response
ST	Sign Track
STR	Striatum
SUD	Substance Use Disorder
Sypl1	Synaptophysin-like protein 1
Syt17	Synaptogamin 17
T1-W	Spin–Lattice Relaxation Time-Weighted
T2-W	Spin-Spin Relaxation Time-Weighted
Tenm4	Teneurin Transmembrane Protein 4
TGFβ	Transforming Growth Factor beta
THA	Thalamus
TOALP	Time Out Active Lever Press
TOR	Time-out responses
TPRV4	Transient Receptor Potential Cation Channel Subfamily V member 4
TR/TE	Repetition Time and Echo Time
Trh	Thyrotropin-releasing hormone
Trk	Tyrosine kinase
TRP	Transient Receptor Potential ion channels
Ttr	Transthyretin
VAPOR	Variable Power Radiofrequency Pulses with Optimized Relaxation delays
VEH	Vehicle
VGLUT2	Vesicular glutamate transporter 2
VR	Variable Ratio
vSTR	Ventral Striatum
VTA	Ventral Tegmental Area
Vwf	Von Willebrand factor
WDR	World Drug Report
Zfhx3	Zinc Finger Homeobox 3
Δ^9-THC / THC	Delta-9-tetrahydrocannabinol / Tetrahydrocannabinol

ABSTRACT

Different lines of evidence have associated cannabis exposure during adolescence with an enhanced risk of developing psychiatric disorders. Furthermore, the idea that cannabis use may be a gateway to other drugs has been investigated by several approaches. However, despite decades of research, it is not clear if cannabis consumption could enhance the liability towards substance use disorders (SUDs). In this work we aim to increase our understanding of the protracted effects of adolescent cannabinoid exposure, exploring the diverse causal mechanisms involved in the modulation of SUD liability.

As such, male and female adolescent Wistar rats were administered 9 injections of THC (3 mg/kg) or the vehicle alone every other day from postnatal day (PND) 28 to 42 and they were then left undisturbed until adulthood PND90. Different sets of rats were then subjected to five different experimental regimes: (1) studies of the structural (MRI) and metabolic ([1H]-spectroscopy) changes produced in the brain *in vivo*. Behavioural experiments aimed at (2) measuring the ability of conditioned cues to influence instrumental responses (Pavlovian to instrumental transfer, PIT) and motor impulsivity (two-choice serial reaction time task, 2-CSRTT), or (3) to assess the propensity of individuals to engage with a conditioned stimulus (Pavlovian conditioned approach) and their habit formation propensity. (4) A multi-component cocaine self-administration (CSA) protocol was used to evaluate alterations to cocaine addiction-like behaviours. Moreover, (5) a Ribonucleic acid sequencing (RNA-seq) study was undertaken to explore the protracted effects on gene expression in the NAc Shell after adolescent THC exposure.

Adult THC-treated animals displayed volumetric and microstructural alterations to subcortical regions (1), and complimentary brain ventricle volumetry showed reductions in the size of their lateral ventricles. A white matter analysis found a reduced fractional anisotropy in several tracts due to THC administration, prominently in rostral sections, while in vivo 1HR spectroscopy identified lower levels of cortical choline compounds in these animals. In males that received THC there was enhanced PIT and weaker motor impulsivity (2), whereas females that received THC displayed enhanced motor impulsivity. (3) THC-treated animals were more goal-directed but showed no differences in habit formation compared to the control rats. (4) Cocaine addiction-like behaviours were mostly unaltered, although significantly, males administered THC showed a higher intake under progressive ratio and females a higher rebound of cocaine intake after re-establishing low-effort conditions. (5) RNAseq revealed THC-induced alterations in gene expression with a marked sex-specific character. The differentially expressed genes highlighted changes to glutamatergic synapses, and in ion binding, axonal growth and hormonal activity, among other categories.

These results show that mild THC exposure during adolescence leaves a lingering mark on brain structure and function, reflected in adult behaviour, and that is relevant to the motivational aspects of behaviour and SUDs even after prolonged drug-free periods. Some of the changes found mimic those evident in human epidemiology and they highlight the importance of sex-specific effects in cannabis research. Adolescent THC exposure changes the reactivity to reward-related cues and affects the expression of impulsive behaviours, protracted effects that also influence drug administration patterns in a sex dependent manner. However, despite the evident alterations to brain development and the impact on adult psychological traits, a deterministic direction towards increased vulnerability to substance use disorders cannot be inferred from these changes.

INTRODUCTION

1. EPIDEMIOLOGY OF SUBSTANCE USE DISORDERS

Despite the growing understanding of substance use disorders (SUDs), they are far from being controlled and in fact, drug use and drug-associated problems are currently showing an upward trend (World Drug Report, 2020). In recent years, the number of drug users (people who consumed substances controlled under the international drug control conventions and their non-medical use) reached 269 million people, 5.3 % of the population worldwide. However, if we only consider individuals suffering from SUDs, this estimated number drops to 35.6 million, 0.45% of the population.

Box 1: Substance Use Disorders

The fifth edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-5) defines Substance Use Disorders as:
"A cluster of cognitive, behavioural, and physiological symptoms indicating that the individual continues using the substance despite significant substance-related problems" .

This definition is widely accepted, but a shift towards dimensional approaches of psychopathologies, rather than outcome based manuals (as used by the different DSM editions) is progressively gaining support. In 2010, the National Institute of Mental Health proposed the Research Domains Criterion as new a research framework for mental illnesses, integrating information across distinct domains of observable neurobiological and behavioural measures (Insel et al., 2010). A dimensional approach to SUDs presumes that neuroadaptations in dissociable brain circuits make distinct contributions to each stage of the drug-use cycle, and that they influence drug reward, withdrawal, craving and relapse. Thus, SUDs are better viewed as a multicomponent pathology.

Moreover, SUDs are not the result of just one factor, they are rather the result of the interaction of an individual within a specific ecological context. Genetic factors, environmental factors and repeated exposure to drugs of abuse are the main contributors to the etiology of SUDs.

Drug abuse is not usually restricted to one but several types of drugs, and individuals frequently undergo a sequential initiation into and exhibit some degree of polyconsumption. Remarkably, drug use often starts (although not exclusively) with legal and attainable drugs. Notably, and perhaps not surprisingly, the number of illegal SUD-related problems are dwarfed by those associated with legal substances like alcohol and nicotine. Alcohol use disorders affect around 1.4% of the global population, more than three times the number of all other SUDs, and it is a problem that is responsible for 3 million deaths every year. There are also 1.3 billion tobacco users and this is directly responsible for half of the deaths reported among its users (World Health Organization, 2019). In this sense, the progressive shift in many jurisdictions towards consenting cannabis products to be sold and marketed for recreational use might increase the pervasive health outcomes associated with cannabis. Indeed, concerns have been raised about the effects of cannabis on several cognitive functions, and that it may also alter drug use patterns and the development of SUDs. This hypothetical link is still under debate and hopefully, the experiments carried out as part of this thesis will shed some light over this issue. The surge in this idea and the epidemiological connection between the use of different drugs in the development of SUDs is briefly presented in Box 6. The Gate Way Hypothesis. The main experimental results will be reviewed in this introduction.

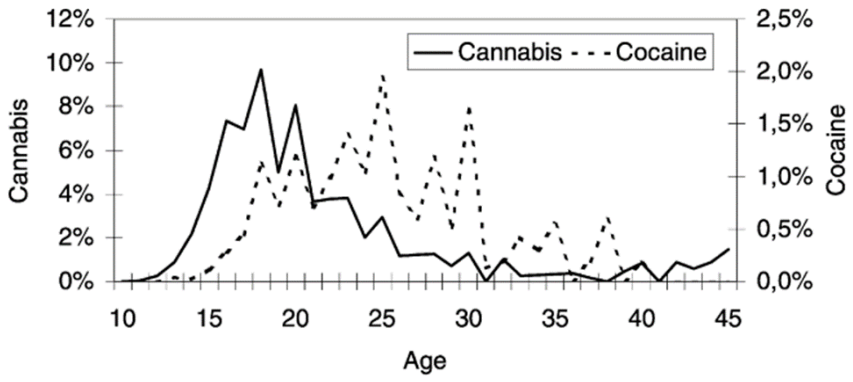


Figure 1. Starting ages of cannabis and cocaine use. Image adapted from Van Ours, 2003 " *Is cannabis a stepping-Stone for cocaine?"* A dataset of inhabitants of Amsterdam was used and it was concluded that despite some evidence of cannabis being a "stepping-stone" for cocaine, unobserved personal characteristics may better respond to this correlation over a causal link between cannabis and cocaine use.

In addition, The World Drug Report, 2020 emphasizes that drug use trends and SUDs are not equally widespread worldwide. Instead, there are marked age and gender particularities, and overarching economic biases, which must be contemplated when elaborating and implementing prevention policies. Consequently, SUD research must address these variables whenever possible to attain accurate knowledge about drug use and SUDs.

Box 2: Cannabis

Cannabis is a popular herbaceous flowering plant that has been present in human settlements for thousands of years, widely used as a drug in modern societies around the world. Humans have taken advantage of its fibers for textile purposes, but we have also consumed it as medicine, and experienced its psychotropic properties in religious contexts and/or with recreational motivations. In the mid-20th century we started to discover what is it in the diverse strains of cannabis that interacts with our brain and body, known as (phyto)cannabinoids (Mechoulam & Gaoni, 1967), and soon after, what is in our brain and body that interacts with these cannabinoids, the endocannabinoid system (Devane et al., 1988; William A. Devane et al., 1992; Joshi & Onaivi, 2019).

Even with these regional differences, cannabis and cocaine are among the most popular drugs worldwide. The most highly consumed drug under international drug control is cannabis, with up to 192 million users in 2018, and this is the case in most countries around the world. However, cocaine is the most popular stimulant in America, Europe and Oceania, and its production is growing unceasingly, reaching all-time highs in recent years (World Drug Report, 2020). The 2019 European Drug Report showed that cannabis and cocaine are the two major illicit drugs consumed in the continent. Cannabis is, without doubt the most established drug, with up to 27.4% (91.2 million people) declaring use at some time in their life, and 7.4% (24.7 million people) in the last year. Meanwhile, 1.2% (3.9 million people) declared to have used cocaine in the previous year. In Spain, 1.6 million people suffer SUDs, 5.1% of the population (7.1% if alcohol consumption is included), presenting risky drug consumption patterns in recent surveys.

1.1. THE EPIDEMIOLOGY OF CANNABIS USE

Studies of cannabis use mainly focus on adolescents. Cannabis derivatives are the most widely used illegal drug with a prevalence five times higher than that of other illegal substances, although the legal status is changing rapidly in many jurisprudences (World Drug Report, 2020). Remarkably, cannabis consumption is particularly intense among adolescents and young adults. In the age range between 15 and 34 years, 14.6 million people (11.7% of European citizens) have used cannabis in the last year. This percentage increases to 15.2% (8.8 million people) if we focus on citizens aged 15 to 24 years (European Monitoring Centre for Drugs and Drug Addiction, 2020). Moreover, the European School Survey Project on Alcohol and Other Drugs (ESPAD), which focuses on trends of substance use among students aged 15 to 16 years, found that cannabis use has gone up from 11% in 1995 to 16% in the last survey. Regarding sex-dependent differences in the patterns of use among European adolescents, boys were more likely to have used cannabis at age 13 or younger. Also, boys had a more elevated frequency of high-risk cannabis use (4.7 % of boys vs. 3.3 % of girls: ESPAD, 2019). In the case of Spain, similar trends of use are found, with a higher relative use of cannabis by adolescents than among adults (12.6% aged between 15 and 17 and 5.5% aged over 35). Notably, the mean age of onset is 14.9 years of age for infrequent users (last month), and there is an upward trend in the number of problematic users (at least three marihuana joints a day: Plan Nacional sobre Drogas, 2017; Observatorio Español de las Drogas y las Adicciones, 2020).

Social contexts have a determinant influence on drug initiation, including cannabis use, and social motives and peer involvement in cannabis play a role in patterns of adolescent cannabis use. Moreover, previous substance use, especially tobacco, and the presence of premorbid or comorbid psychopathologies, especially mood disorders, are commonly identified as risk factors for cannabis use disorders (CBUDs: Courtney, Mejia, & Jacobus, 2017). Moreover, CBUDs are increasingly common and they remain largely undertreated, presenting a high comorbidity with these psychophysiological alterations (Hasin et al., 2016). Indeed, there has been an increase in cannabis related health problems that have paralleled the increasing potency of the cannabis consumed. In Spain, the number of cannabis-related emergencies increased from 1,589 (25% of all drug-related emergencies) in 2008 to 1,980 (33%) in 2011 and 49.4% in 2018. Most of these cases are related to cannabis-induced acute adverse effects after intoxication: tachycardia, sensation of vertigo and fainting possibly associated with a decrease in blood pressure. These physical manifestations are usually accompanied by

episodes of acute anxiety (Cone, 1993; Observatorio Español de las Drogas y las Adicciones, 2020). This trend is related to the presence and popularity of high-potency cannabinoid products, directly associated with the relative content of delta-9-tetrahydrocannabinol (Δ^9-THC or just THC), that have increased their market share in recent years.

Apart from the problems associated with acute cannabis use, long-lasting noxious effects after repeated use are undoubtedly more worrying for consumers' health, and more compromising at the social level. Notably, cannabis use in late adolescence and early adulthood is associated with future unemployment, lower income and greater welfare dependence, and less overall satisfaction in relationships and life (Fergusson & Boden, 2008). Moreover, prolonged cannabis use has a facilitating effect on the onset of psychiatric problems in some individuals, generating symptoms such as confusion and psychotic disorders (reviewed by Curran et al., 2016; Patel & Marwaha, 2020; National Academies of Sciences, Engineering, and Medicine, 2017; Quiroga, 2000; Volkow et al., 2014). Thus, interventions focused on early consumption seem to be an epidemiological need, although more research is needed to thoroughly understand the changes produced by adolescent cannabis use and how these consequences arise.

Box 3: Cannabinoids and their acute effects

Phytocannabinoids are a plant-derived terpenoid molecules, members of a large class of unsaturated hydrocarbons produced by the cannabis plant, although they are also present in other plants (Gertsch, Pertwee & Di Marzo, 2010). More than one hundred different (phyto)cannabinoids are secreted by the characteristic glandular trichomes (from the Greek **τρίχωμα**, hair) on the seed, stalk and leaves of the plant but in particular, in the flowers where they form viscous resin agglomerations that may protect the plant against insect predation and other environmental sources of stress.

Although many types of phytocannabinoids have captured the attention of researchers (CBD, CBG, CBC, CBN...), tetrahydrocannabinol (including its precursor THCA and the derivate Δ^9-THC) is the main psychoactive compound and the most abundant in some strains. The physiological effects of THC are usually dose-dependent, although it can show a bi-phasic effect, and they in part rely on individual differences, the current status of the organism and some contextual variables.

THC consumption produces changes in perception and cognition that ranges from a reduction in anxiety, enhanced well-being, heightened sensory experience and/or sexual arousal, and euphoria, to drowsiness, fragmented thinking, disturbed memory, dysphoria, anxiety, alterations to time perception, depersonalization, hallucinations, and aggravations of psychotic states. At a peripheral nervous system and psychomotor level, users may experience an amelioration of motor coordination, muscle relaxation, analgesia, appetite stimulation, antiemetic effects, reduced bowel movements and delayed gastric emptying but also, weaknesses, unsteady gait, slurred speech, ataxia, hypoxia, hyposalivation and vomiting. THC and other cannabinoids also affect the respiratory/cardiovascular system, producing vasodilation, enhanced heart activity, increase in oxygen demand, orthostatic hypotension, hypertension in a horizontal position and bronchodilation.

Moreover, although probably less salient, cannabinoids like THC also interact with several hormones and glucose metabolism, they modulate the activity of the immune system with anti-inflammatory and antiallergic effects, among others, and they influence gene expression in a variety of tissues and cells.

At the neuropsychological level, prolonged consumption is related to alterations in cognitive functioning, which include deficits in organizational capacity, attention and the filtering of irrelevant information, memory and learning. Other frequently reported consequences of cannabis use are motivational deficits, including the inhibition of sexual appetite, which may be related to the effect of cannabinoids on the endocrine system and the alteration of hormones related to stress. Significantly, the detrimental consequences on memory and cognition resulting from cannabis use in teenage years have also been associated with structural changes in the brain structures responsible for pleasure and reward (Worley, 2019: See Box 8. Reinforcement, Reward-processes, and Reward system). Therefore, there is a possible biological basis for the effect of cannabis use in modulating the response towards other drugs, its impact on SUD liability and probably, its influence on the progression towards the use of other drugs. Notwithstanding, early experiences and adolescent drug exposure to other drugs such as alcohol, nicotine, psychostimulants, or opioids do also entail long-lasting behavioural and neurobiological consequences that affect cognition, socio-emotional processing, and the reward systems (Salmanzadeh et al., 2020).

1.2. THE EPIDEMIOLOGY OF COCAINE USE

It is estimated that there are 18,070,000 cocaine users worldwide (having consumed cocaine in the last year), although cocaine use is especially prevalent in wealthy economies: North America with around 2.1% (6,800,000 people), Oceania and New Zealand 2.2% 420,000 and Western Europe 1.3% (4,240,000 people). Indeed, the percentage drops to 0.9% (2,740,000 people) in South America, and it remains at around 0.6% in Central America and the Caribbean. Consumption is even lower in Africa, with the exception of South Africa that has rates around 1%, while Asia registers around 1,670,000 people, 0.06% (World Drug Report, 2020). In Europe, cocaine is the most commonly used illegal drug after cannabis and the most often used stimulant. Up to 2.1% of the European citizens aged between 15 to 34 years have consumed cocaine in the last year (European Drug Report, 2019), and the results in Spain are similar to the European mean, with a 2.2% rate of consumption within the same age group in the last year (Observatorio Español de las Drogas y las Adicciones, 2020).

The illegal market for cocaine is still on the rise. In the past few years the estimated global production of cocaine reached an all-time high, and global seizures have also increased marginally, reaching the largest quantities reported to date (World Drug Report, 2020). The price of cocaine has remained relatively stable worldwide, although other more harmful alternatives like crack, a smokable form of cocaine, are cheaper and potentially more addictive, and its use is again spreading from 2014 in various European countries. In line with the general European trend, since 2015 Spain also registered an increase in consumption. The last official report registered a prevalence of 2.2% (around 600,000 people) in cocaine use within the last year among people aged between 15-64 years of age, although this number halves to 1.1% when consumption within the last 30 days is considered. Cocaine dependence usually peaks at around 23 years of age (in Europe and the USA). Compared to cannabis and alcohol, cocaine dependence develops faster. Within the first year of use, around 5% of cocaine users develop dependence (Wagner & Anthony, 2002). Cocaine also has a marked gender bias and in Europe, 12.1 million males and 5.8 million females have consumed cocaine within their lifetime. In Spain, 3.2% of men declared having consumed cocaine in the past year, as opposed to only 0.8% of women (Encuesta Sobre Alcohol y Drogas en España, 2019).

Box 4: Cocaine

Cocaine is one of the numerous alkaloids naturally occurring in Erythroxylum coca, an indigenous plant of the foothills of the Amazonian Andes. Approximately 1% by weight of the plant is cocaine and even today, most of the world's current cocaine comes from South American countries such as Peru, Colombia, Ecuador or Bolivia.

Cocaine is a strong stimulant that can exert a powerful psychoactive effect, which varies from person to person, the dose consumed, and the tolerance developed after repeated use. At low doses cocaine usually causes an intense stimulation of the central nervous system, euphoria, sexual arousal and a loss of appetite. Intoxication can lead to intense agitation and a loss of contact with reality. Overdoses produce elevations in body temperature, high blood pressure and abnormal heart rhythms, which may even lead to death. The "crash" or comedown when the effects of cocaine wear off can produce restlessness, anxiety and paranoia. Chronic administration is associated with sleep disturbances, anhedonia, dysphoria and depression.

The cocaine produced in the plant is thought to have a protective effect against insects due to its toxic effects and interactions with chemical neurotransmission (Nathanson et al., 1993). Indigenous people of South America consumed coca leaves in religious rituals, and they took advantage of its effects as an anesthetic and to increase stamina. Different methods for the extraction, isolation and synthesis of this alkaloid were defined by German chemists in the 19th century, and it soon becomes a popular substance in western pharmacopeias, and it was even introduced into food products and beverages. The abuse and widespread use of cocaine has led to the control and regulation of this substance in the 20th century. Nonetheless, it is still today the most popular illegal stimulant.

Cocaine-related health problems are on the rise, as are abuse-related disorders. Cocaine use produces tremendous deleterious effects in personal and social spheres. Undoubtedly, one of the most serious concerns about cocaine is its abuse and the development of a cocaine use disorder (CUD), which is associated with motivational alterations and disruptions of normal hedonic response, as well as and persistent detrimental alterations of cognitive and executive functions. From 2008 to 2017, the global burden of disease in terms of disability-adjusted life years due to SUDs has increased by 24% and CUDs by 17%. Indeed, the increase in CUDs lies only behind that in opioid use disorders that have increased by 28% (World Drug Report, 2020). Data

from treatment services, emergency presentations and drug-related deaths suggest cocaine's role in Europe's drug problem is growing. In Spain, 48.4% of patients seen at emergency services for drugs of abuse ingested cocaine. In comparison, cannabis and cannabinoid derivatives represented 24.2% of the patients (Miró et al., 2018), and it is estimated that high-risk cocaine use prevalence, although difficult to gauge accurately, is around 0.3 % in people between 15-64 years of age (Observatorio Español de las Drogas y las Adicciones, 2020).

Drug polyconsumption is a frequent phenomenon and cocaine in particular is strongly linked to the use of other licit and illicit substances, a situation that directly influences the progression and severity of CUD. The association between cannabis and cocaine remains unclear (see Box 6: The Gate Way Hypothesis) and causal links are hard to establish, especially in when related to the long-term effects of adolescent cannabinoid use in humans (see section 2.1.). However, besides the concomitant use of cigarettes and alcohol in patients suffering CUDs (John & Wu, 2017), it was found that the use of cannabis after treatment for CUD increased the odds of relapsing to cocaine use after sustained remission (Aharonovich et al., 2005). Moreover, prior cannabis consumption has been associated with more severe cocaine withdrawal symptoms, increased craving and higher rehospitalization rates among in-patients with CUDs (Viola et al., 2014). The existing clinical and preclinical evidence linking cannabis use with the use of other drugs will be reviewed in the following sections, with an emphasis on cocaine.

Box 5: Cocaine pharmacodynamics and pharmacokinetics.

The usual routes of cocaine administration permit rapid delivery into the bloodstream and therefore, they ensure the prompt onset of its effects, enhancing the abuse liability of this drug. The preferred route of administration is intranasal insufflation (snorted: 83.6%), with smoking inhalation far less frequent (13.3%), albeit preferential in the case of crack cocaine, and only rarely is cocaine administered intravenously (0.4%). The addictive potential of cocaine is facilitated by its rapid and widespread distribution, being a weak base (pKa 8.6) cocaine is a very water-soluble, and it rapidly crosses body membranes including the blood-brain barrier. Moreover, cocaine is also rapidly metabolized, it has a short half-life, approximately 0.7 to 1.5 hours, which favors repetitive binge-like self-administration. In some consumers this behaviour is further enhanced since its rapid metabolization may lead to an unpleasant feeling known as the "cocaine crash". Most of the cocaine administered is eliminated within a few hours.

Cocaine interacts with diverse elements of the central nervous system. The anesthetic effects of cocaine are related to its ability to block voltage-gated sodium channels in nerve cells, which inhibits depolarization and blocks the initiation and the conduction of nerve impulses. When cocaine crosses the blood-brain barrier it reaches multiple sites of action, although the main effects of cocaine are attributed to its ability to block the dopamine transporter, a protein responsible for the reuptake of dopamine (see Box 7. Dopaminergic system involvement with reward-related behaviours and SUDs). However, cocaine also blocks the reuptake of other monoamine neurotransmitters, such as noradrenaline and serotonin, allowing cocaine to interfere with a wide variety of psychomotor and cognitive functions. Moreover, cocaine affects brain structures like the Nucleus Accumbens (modulating motivational and hedonic aspects of the drug), hippocampus and amygdala (implicated in the formation and recall of drug associated memories), and the prefrontal cortex (determinant for the integration of information for decision making). CUDs progress with pervasive long-lasting neurobiological effects in dopaminergic signaling and nerve cell structure, also affecting transcriptional activity in these regions (Nestler, 2005).

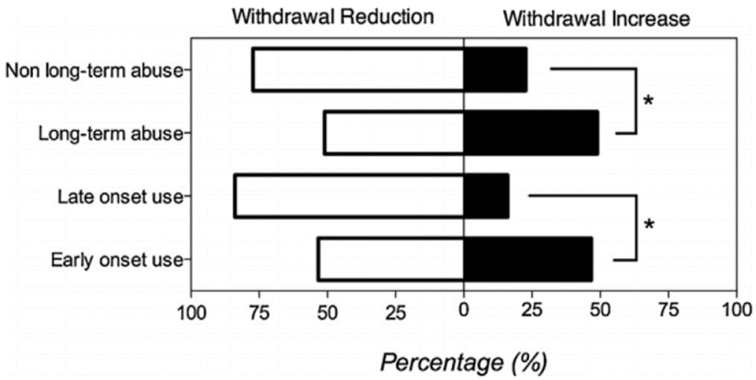


Figure 2. The severity of cocaine withdrawal symptoms after two weeks of detoxification. Data obtained from cocaine-dependent in-patients who participated in a drug rehabilitation program at a public hospital in Southern Brazil. Although there were no differences regarding *cannabis use in the last 30 days prior to enrolment, long-term abuse of cannabis and early onset were associated with stronger cocaine withdrawal symptoms. However, this study could not conclude if individual differences predate cannabis abuse and age at onset (*Modified from Viola et al., 2014).

2. PROTRACTED EFFECTS OF ADOLESCENT CANNABIS EXPOSURE ON DRUG USE

Cannabis-gateway effects in humans are understood to represent relatively stable changes induced by cannabis use that predispose the individual to further progress into other drug use, such as cocaine. Human research must control many confounding factors to isolate such gateway effects, for example cannabis-driven alterations in drug progression and abuse). Among these are all the influences that should be assigned to a common liability model of drug progression, such as genetic inheritance and personality traits present before any drug use (e.g. impulsiveness, openness to experience and neuroticism), in addition to social and economic contextual factors (e.g. family, friends, economic resources, life history and stressors). Significantly, when these confounding variables are neglected, the effect of cannabis might be overestimated (Beck et al., 2009; Fergusson, Boden, & Horwood, 2006)

The study of the causal factors of cannabis-gateway effects requires examining the long-term effects of adolescent cannabis exposure (ACE - not derived from acute intoxication or withdrawal) on different psychobiological factors. However, clinical cannabis research must deal with many confounding variables, and a wide diversity of methodological criteria and research tools. Thus, among all the evidence available, information that specifically covers the protracted effects of adolescent cannabis exposure (PEACE) in response to other drugs of abuse is relatively scarce (Higuera-Matas, Ucha, & Ambrosio, 2015; Hurd et al., 2019; Stringfield & Torregrossa, 2021b).

Box 6. The Gate Way Hypothesis

Even though cannabis was accessible and coexisted with other drugs in many cultures, the first claims of a cannabis-induced gateway-effect occurred under a global effort to control drug trafficking and drug use. During the Second Opium Conference celebrated in Geneva in 1925, the Egyptian delegate Mohamed El Guindy recklessly fought for the inclusion of cannabis on the list of controlled substances together with opium and cocaine (Kendell, 2003). Cannabis, according to El Guindy not only led to *"insanity"* but it also served as a gateway to other drugs, and vice versa, and if it was not controlled cannabis would replace other drugs and *"become a terrible menace to the whole world"*. Despite the lack of formal evidence El Guindy's strong statements found support in other delegates and were not disputed by most of the attendants who lack objective scientific knowledge about cannabis (Dave Bewley-Taylor, Tom Blickman, & Martin Jelsma, 2014). This theoretical idea became popular in the USA where it was called the Steppingstone Theory (Anthony, 2012; McWilliams, 1990) and it was frequently used as an argument for prohibition. However, not everyone shared that alarmist vision, and many epidemiological studies and drug researchers debated this deterministic idea.

During the 70s the scientific focus fell onto the individual pattern of use and drug sequences (Cohen, 1972), and a series of studies by Denise Kandel and her colleagues brought a renewed interest to this topic. Originally limited to the epidemiological study of the patterns of drug use progression (Kandel, 1975; Kandel & Faust, 1975; Single, Kandel, & Faust, 1974), this topic evolved over the following years into an increasingly complex cluster of research approaches and statements sheltered under the umbrella term Gateway hypothesis (Kleinig, 2015).

In her book "Stages and pathways of drug involvement" (Kandel, 2002), Denise Kandel tried to synthesize the different branches of research and define the criteria that could validate the gateway hypothesis. Essentially, the existence of a gateway effect relies on proving a (1) sequence, an (2) association or a (3) causation. Notwithstanding, research results offer conflicting data regarding each of these three propositions. Critical voices claim that sequences of drug use initiation and progression are and highly influenced by culture and drug-market contingencies, thus they are usually merely descriptive. Associations between cannabis use and cocaine use or abuse are highly controversial. A recent review conclude that there seems to be *"limited evidence of a statistical association between cannabis use and changes in the rates and use patterns of other licit and illicit substances and the development of substance dependence and/or a substance abuse disorder"* (National Academies of Sciences, Division, Practice, & Agenda, 2017). Due to these difficulties some researchers, including Denise Kandel, have turned their focus onto causal studies in an attempt to determine whether cannabis use is an independent risk factor for, or causally contributes to the initiation, use and dependence on other drugs of abuse later in life.

Nevertheless, different lines of research have explored SUD (or the more general term addiction) related traits. These have frequently taken advantage of imaging techniques that open the possibility to compare the cannabis-induced changes with others produced by SUDs, or that could entail an increased risk for SUDs. Moreover, results obtained through these approaches help design animal models, scrutinize features unreachable in human research, and compare and validate the results obtained.

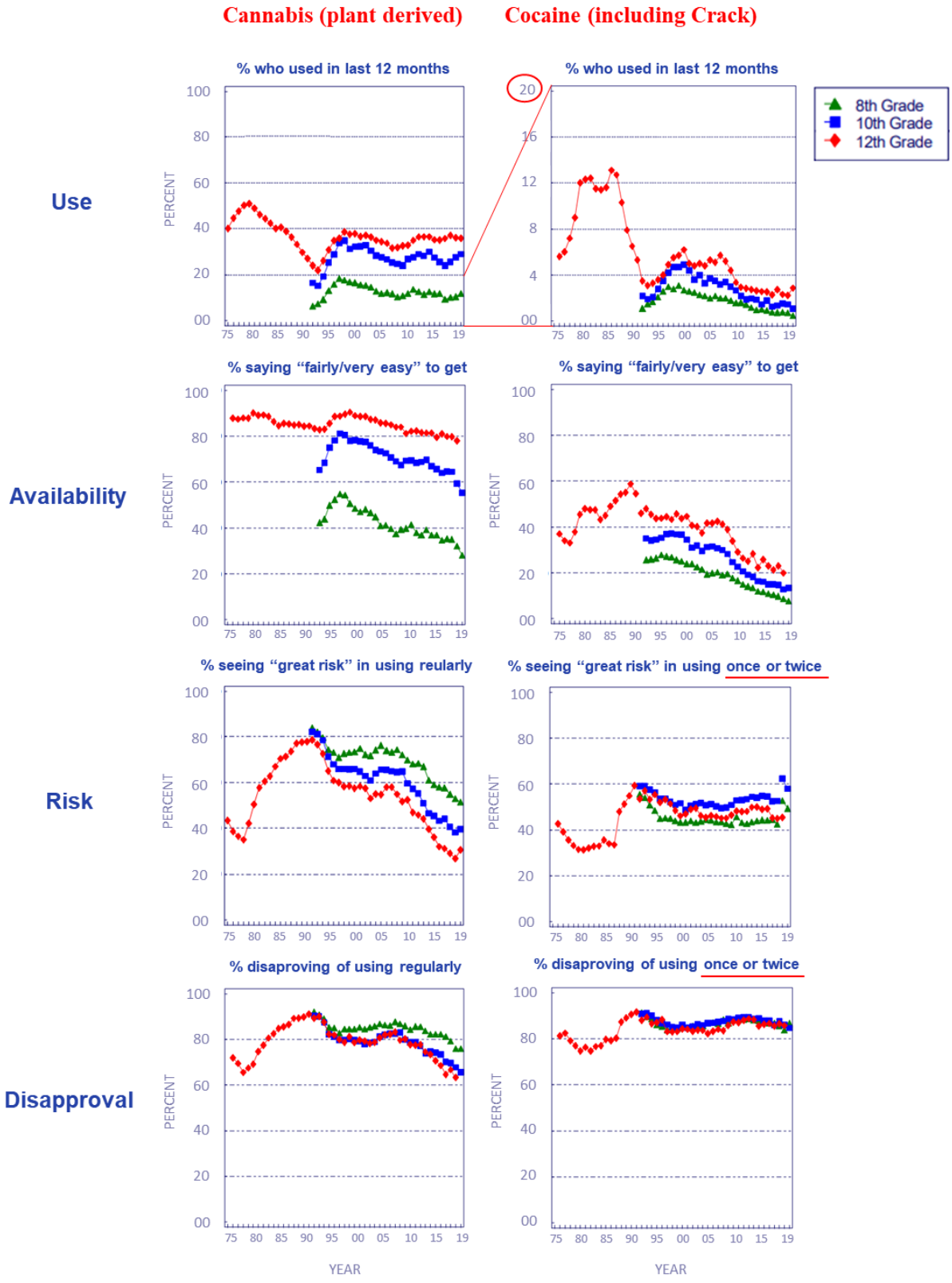


Figure 3. Trends in Annual Use, Risk, Disapproval and Availability. Data from Monitoring Future: National Survey Results On Drug Use 1975–2020 a long-term study of substance use and related factors among U.S. adolescents, college students and adult high school graduates, conducted annually and supported by the National Institute on Drug Abuse. Note that trends in cannabis (little change) and cocaine (decline) use do not necessarily overlap, and other psychosocial variables may influence the initiation and continuation in the use of both drugs. However, a differential interaction with cocaine after cannabis use cannot be inferred by this data. 8th grade (13-14 years) 10th grade (15-16 years) and 12th grade (17-18 years).

2.1. CONFOUNDING FACTORS

There is extensive literature on the neurological and cognitive alterations associate with cannabis use, and its effects during brain development (Gorey et al., 2019; Lubman, Cheetham, & Yücel, 2015). However, the wide variety of research methodologies and distinct demographic characteristics of the samples are a hindrance to reaching general conclusions. Studies addressing the effects of cannabis do not always share the same methodological criteria and thus, generalizing results is usually complex and tentative. For example, differences in the ages in the samples, the consumption volume between the subjects recruited and the heterogeneity of the periods of abstinence (when addressing non-acute effects), and the exclusion of individuals of both sexes, are among the most relevant sources of disparity.

The effects of cannabinoid consumption (acute or habitual, mild or intense) can have short-term or enduring long-term consequences, the long-term outcomes more likely to occur after habitual (rather than acute exposure) and/or intense (rather than mild) consumption, and following an early (rather than late) initiation. A key feature to understand cannabis-induced neurological and cognitive alterations is to distinguish the acute and residual effects from the long-lasting alterations. Acute and brief effects of cannabis intoxication may sustain part of the gateway effects (e.g. acute intoxication or a concomitant use), yet long-term changes, the effects that remain after clearance and withdrawal effects, are more likely to sustain gateway effects in the long-term. However, both human and preclinical studies have employed a wide variety of abstinence and clearance periods.

In this sense, periods of abstinence of less than a week are not usually considered sufficient to avoid the effects of withdrawal as a confounding factor due to discrepancies with studies that use more extended periods of abstinence (Jager et al., 2007). A sufficient period of abstinence is relevant since we are looking for changes that are not due to acute or chronic exposure, and not related to a period of incomplete washout nor to withdrawal. There seem to be persistent neuroanatomical and neuropsychological alterations that depend on previously mentioned factors that could persist to some degree (Gonzalez et al., 2017), e.g. hippocampal morphology and episodic memory impairments among adults (Meier et al., 2012; Smith et al., 2015). However, these effects are considered mild for some authors, or at least they may not outweigh the beneficial outcomes of therapeutic cannabis use. That was the conclusion of a meta-analysis of long term neurocognitive effects referred to as residual or non-acute (Grant et al., 2003). Although these authors included studies that only required participants to be drug-free on the day of neuropsychological testing, they found only small detrimental effects of cannabis and small side effects in the domains that showed some differences, failing to find substantial effects on neurocognitive functioning. Nonetheless, in a more recent systematic review and meta-analysis of neurocognitive effects (Ganzer et al., 2016), including 38 studies published between 2004 and 2015 that included abstinence periods of at least 14 days, there was evidence of protracted effects. Moreover, periods of abstinence must also consider withdrawal from other drugs. Sequential progression is better viewed as an ever-expanding repertoire of drug use behaviours, whereas concurrent use, rather than stepping out of the previous stages, is common (Mills & Noyes, 1984). Thus, any research into cannabis gateway effects has to also deal with hypothetical preceding gateway effects of other drugs that potentially led to cannabis use, and that may further influence initiation and the patterns of drugs use.

Finally, Cannabis gateway effects must consider sex-dependent influences. Epidemiologically, more males consume cannabis and they tend to meet more criteria of CBUDs, although females progress faster from initial use to CBUDs (Khan et al., 2013). Moreover, the sex differences in response to cannabis have a special relevance during adolescence (Patton et al., 2002), and different neuropsychological vulnerabilities modulate the initiation and impact of cannabis (Crane, Schuster, Mermelstein, & Gonzalez, 2015). The extent to which biological sexual dimorphism or sociocultural variables are responsible for these effects is an interesting issue that not every research study deals with (Ketcherside, Baine, & Filbey, 2016). Even though not all the studies that included sex as a factor detected sex-related differences, those that did usually found enhanced vulnerability to several deleterious effects in women (Cooper & Craft, 2018; Khan et al., 2013). Whenever data is available regarding sex-specific differences in behavioural, cognitive or neuroimaging parameters in the studies reviewed here, this will be noted.

Box 7. The involvement of the dopaminergic system in reward-related behaviours and SUDs.

Dopamine (DA) has and still plays a central role in SUD research, and in the broader field of behavioural neuroscience. The earliest evidence of its involvement in reward-related behaviours came in 1954 from the studies of Olds and Milner, which revealed that intracranial electrical stimulation of certain brain areas reinforced behaviours contingently associated with the onset of the current. Importantly these brain areas were in part comprised of DAergic neurons (Crow, 1972). From the 1960s and throughout the 80s, different research lines linked drug-induced locomotor responses and operant drug self-administration with the activity of midbrain DA neurons (Wise and Bozarth, 1987). Remarkably a series of brain microdialysis studies initiated by Di Chiara & Imperato at the University of Cagliari, located on the beautiful Mediterranean island of Sardinia, showed that different drugs of abuse (e.g. opiates, ethanol, nicotine, amphetamine, and cocaine) increased DA release in the Striatum, and particularly in the Nucleus Accumbens (Di Chiara & Imperato, 1988). Moreover, radiotracer imaging studies in the 90s confirmed the increases in striatal DA in response to different amphetamine challenges in humans (Laruelle et al., 1995; Volkow et al., 1994, 1999). As a result, a DA-based theory of addiction emerged, linking this to an enhanced potential risk for drug abuse, which even became popular outside of scientific contexts. DA was frequently characterized as a neurotransmitter of 'pleasure' and that which produces reward (Nash, 1997).

Nevertheless, conflicting results have challenged the existence of a simple linear relationship between striatal DA signaling and enhanced wanting or liking in the context of SUDs, and other behaviours. Some drugs (e.g. alcohol, tobacco, ketamine and cannabis) do not inevitably induce a strong striatal DA release in humans even though they were abused epidemiologically, whereas other drugs (e.g. modafinil) do induce DA release without having shown abuse potential in clinical settings (Jasinski, 2000; Volkow et al., 2009). Moreover, other studies showed that pharmacological DA blockade did not dampen the rewarding actions of opiates or block the rewarding effects of stimulants in clinical trials (Rothman, 1994; Van Ree & Ramsey, 1987). Although DAergic alterations like DA receptor availability and changes in striatal DA release, are frequently linked to a history of drug-abuse or associated with substance abuse liability, there is also some degree of variability due to the specific drugs involved, the animal model and the testing protocol employed (Nutt et al., 2015).

The DAergic system projection neurons that synthesize and release the neurotransmitter DA, and the set of neurons that express the DA receptors and channels that provides the signals, play distinct roles in normal brain function, although to some extent they all modulate reward, approach and exploratory/exploiting behaviours that influence SUDs. There are five different DAergic pathways: (1) the mesocorticolimbic DAergic pathway, the best studied in terms of reinforcement learning and motivational salience, which encompasses the mesolimbic pathway from Ventral Tegmental Area to Ventral striatum and amygdala, and the mesocortical pathway from the Ventral Tegmental Area to the prefrontal cortex; (2) the nigrostriatal pathway from the Substantia Nigra pars compacta to the dorsal striatum, involved in basal ganglia motor loops and that is necessary for fluent motor function, but can also influence cognition and reward (Wise, 2009); (3) the Incertohypothalamic pathway from the subthalamic zona incerta and brainstem locomotor centers that regulates locomotion and motivated behaviour (e.g. feeding); (4) the Tuberoinfundibular pathway from the hypothalamic arcuate nucleus to the pituitary gland, associated with neuroendocrine activity; and (5) the Hypothalamospinal pathway from the hypothalamus to the brainstem and spinal cord networks, also associated with neuroendocrine activity.

DA is released from DAergic neurons in a tonic or phasic manner through calcium-mediated mechanisms, each with different implications for reward learning (Liu & Kaeser, 2019). Phasic midbrain DA release is rapid and transiently increases the extracellular DA as evident in the early studies of DA involvement in SUDs. It is thought that this transmission encodes reward prediction errors, a quantitative value of reward that transfers to the reward-predictive cue after learning, thereby preceding and initiating desire/approach behaviours (Nasser et al., 2017; Schultz & Dickinson, 2000). Tonic midbrain DA release involves a steady action potential firing at a constant frequency, and it is thought to regulate energy expenditure. Increased tonic DA favours exploration, while decreased tonic DA activity favours energy conservation and resource exploitation (Beeler et al., 2010).

Once released DA exerts its actions on two distinct families of GPCR receptors, D1 and D2-like. D1-like receptors (D1 and D5) mediate excitatory neurotransmission primarily by increasing adenylyl cyclase activity when the Gs unit is uncoupled and by enhancing PKA. Conversely, D2-like receptors (D2, D3 and D4) inhibit adenylyl cyclase activity via a Gi protein (Surmeier et al., 2007). Dopamine receptors can form oligomers with other receptors (e.g. histamine, adenosine and NMDR) but also between themselves in certain cell types. D1-D2 dopamine receptor heteromers are more frequent in regions of the basal ganglia like the globus pallidus and the Nucleus Accumbens Shell (Hasbi et al., 2011).

2.2. NEUROBIOLOGICAL EVIDENCE

2.2.1. NEUROIMAGING STUDIES

Imaging techniques provide an excellent non-invasive way to study the structure and function of the brain. These techniques have already been broadly used to assess reward-related behaviours and to characterize behavioural endophenotypes of drug addiction (Jupp & Dalley, 2014). Brain-imaging has also extensively addressed the effects of cannabinoids, frequently focusing on adolescent and/or long-term effects

(Ganzer et al., 2016). However, studies focusing on all these features are logically scarcer, although some insights can be gained from these and conclusions can be drawn.

Reward related changes in brain structures and activity

Although there is no conclusive data, the long-term effects of cannabis use, especially during developmental periods, has been linked to white matter abnormalities. This is particularly relevant since proper white matter integrity is crucial for efficient communication between brain regions, shaping cognitive and behavioural performance. Moreover, structural integrity has been linked to substance use and risk taking in adolescence (Jacobus et al., 2013). A longitudinal study (Becker et al., 2015) found that cannabis users displayed deviations from normal fractional anisotropy (FA) signal growth during development (See Box 17. Diffusion tensor imaging). Reduced FA was evident in several white matter tracts, including sections of the superior longitudinal fasciculus, superior frontal gyrus, corticospinal tract and corpus callosum (CC), together with a less longitudinal reduction of radial diffusion (water diffusion perpendicular to the tract) in sections of the superior longitudinal fasciculus, corticospinal tract, and posterior cingulum. Importantly, higher cannabis intake correlated with reduced longitudinal growth in FA and functional impairment in measures of verbal learning. Similarly, earlier age of cannabis use onset was associated with lower white matter coherence (Orr, Paschall, & Banich, 2016). Notably, this study found volumetric alterations within structures of the reward system, specifically, changes in Nucleus Accumbens (NAc) shape linked to early age of onset, and changes in the shape of the amygdala and hippocampus associated with consumption levels. Remarkably cortical volumes remain unaffected. also showed that Cannabis use may also affect the integrity of white matter fibre tracts in prefrontal regions, notably increasing trace, a measure of overall isotropic diffusivity (Gruber & Yurgelun-Todd, 2005). Lastly, these changes do not seem to affect white matter volume in cannabis users asked to remain abstinent on the day of the study (integrity measurements not reported: Gilman et al., 2014). However, cannabis may also affect grey matter density in the NAc, hypothalamus, and amygdala structures, also producing volumetric and shape alterations.

In terms of reward-related activity, 12 hours abstinent users displayed a significant inverse correlation between apathy (measured with an Apathy Evaluation Scale) and dopamine (DA) synthesis in the entire striatum (STR) and its associative subdivisions, indexed using PET as the influx rate constant of [^{18}F]-DOPA uptake (Bloomfield et al., 2014). Cannabis abusers do not differ in baseline striatal DA receptor availability but rather, they show significantly blunted responses when challenged with methylphenidate (Volkow et al., 2014). Moreover, female but not male cannabis abusers showed hypofrontality, and an attenuated regional brain metabolic response in response to methylphenidate, particularly in the putamen (or dorsal lateral striatum, DLS) and caudate nucleus (dorsomedial striatum, DMS), midbrain, thalamus (THA), and cerebellum (Cb).

Using an fMRI approach, cannabis use was associated with reduced reward anticipation in the caudate and putamen (both structures can be referred to as the dorsal striatum -dSTR), but increased reward outcome-related activity in the putamen. This effect may even be underestimated, dampening subsequent motivational processes and failing to predict upcoming rewards due to the hyperactivity during effective outcome activity reflecting an unexpected reward (Van Hell et al., 2010). Dampened activation during reward anticipation in the NAc has also been observed in association with greater cannabis use. Remarkably, enhanced NAc activity is associated with earlier onset of cannabis use, suggesting that greater activation of these areas may be a risk factor for substance use rather than a consequence of cannabis use (Martz et al., 2016). Indeed, dependent cannabis users also displayed significantly attenuated (social) reward experience linked to decreased striatal activation relative to control subjects (Zimmermann et al., 2019), and this effect was more salient as the lifetime exposure to cannabis increased.

Conversely, similar studies showed opposite effects, with a greater BOLD response registered in the right ventral striatum (vSTR) of cannabis users during instrumental response anticipation for non-drug rewards, irrespective of the period of abstinence (Nestor et al., 2010), and striatal hyperactivity was evident during the anticipatory stages of reward, curiously more pronounced during non-rewarding events (Jager et al., 2013). Other studies failed to detect differences in NAc activation during reward processing, even when activity was altered in users of alcohol and tobacco (Karoly et al., 2015). This apparent discrepancy might be the result of differences in sample recruitment, confounding variables not taken into account (e.g. concomitant drug use), differences in the control groups employed, or methodological differences in the task design and the measurements obtained. Be that as it may, neuroimaging studies show that cannabis use alters the mechanism

of reward response and anticipation. Moreover, there seems to be a differential sensitivity and processing of reward versus loss outcomes in monetary incentive delay tasks. Cannabis users show greater sensitivity to reward and reduced sensitivity to loss, as evident by the enhanced activation of the orbitofrontal cortex and cingulate gyrus, and the lower orbitofrontal cortex activation (Filbey, Dunlop, & Myers, 2013) and left insular cortex hypoactivity in response to loss (Nestor et al., 2010). In addition, cannabis use has also been related to increased activation of the caudate nucleus under neutral conditions and following punishment (Enzi et al., 2015).

Cannabis use may also modify the response to drug and non-drug-related cues. Differences in cannabis cue reactivity in regular users seem to be associated with CUD severity rather than to cannabis use *per se* (Cousijn et al., 2013). Furthermore, cannabis dependence may be associated with an enhanced BOLD response to appetitive cues like sex in several areas (left striatum, anterior insula, right hippocampus, amygdala and anterior cingulate cortex: Wetherill et al., 2014). Two other studies found no difference in cue-reactivity to rewards but an enhanced response in areas of the mesocorticolimbic reward system specific to cannabis cues, raising doubts as to the existence of a generalized hypersensitivity to reward cues (Cousijn et al., 2013; Filbey et al., 2016). Significantly, salience attribution, the prominence and allocation of cognitive resources due to the presence of a given stimulus compared to others around it, have been recently reviewed (Wijayendran, O'neill, & Bhattacharyya, 2018), concluding that although long-term users may not differ in performance, the underlying neural processes diverge from control subjects.

Box 8. Reinforcement, reward-processes, and reward system.

For the American Psychological Association, "reward" is a lay word that is nearly synonymous with reinforcement (APA Dictionary of Psychology). Ivan Pavlov used the term reinforcement for the first time to describe the strengthening of the association between an unconditioned and a conditioned stimulus during a process of associative learning. However, reinforcement was later used in reference to the process by which the frequency or probability of a response is increased by a dependent relationship or contingency with a stimulus or circumstance during instrumental learning (these stimulus or circumstances usually being defined as reinforcers). In these settings reward is often interchangeable with reinforcers and reinforcement. Although there is no consensus, reward as a process is generally used in reference to the strengthening of an action by means of appetitive (positive reinforcement) or the termination of aversive (negative reinforcement) consequences for an organism. Thus, reward is conceived in opposition to punishment, the latter entailing the termination of an appetitive stimulus or circumstance (negative punishment), or the experience of an aversive one (positive punishment).

In this sense reward related processes are understood as those governing the ability of an organism to perceive, seek and exploit beneficial outcomes, and escape from the aversive and detrimental ones. Consequently, they are a central feature of adaptive fitness and survival rooted in basic molecular and cellular mechanisms. In complex organisms, the neural basis responsible for these processes are usually referred to by the overarching term, brain reward system. These systems involve several brain areas connected thorough different neural pathways that integrate different types of learning and memory processes, serving to select and organize approach and avoidance behaviours, and integrating emotions to efficiently orchestrate motivated behaviours (Schultz, 2015).

Neuroanatomically, the brain reward system involves much of the brain: cortical regions including the prefrontal cortex, anterior cingulate cortex and insular cortex; temporal lobe structures like the hippocampus and amygdala; a large group of subcortical nuclei known as the basal ganglia (located at the base of the forebrain), integrated by the ventral (NAc Core and shell subdivisions, and the olfactory tubercle), the dorsal striatum (dorsomedial striatum or caudate nucleus and dorsal lateral striatum or Putamen), the ventral pallidum, the Globus pallidus (external and internal region), the substantia nigra, and the subthalamic nucleus; within the diencephalon, several nuclei of the thalamus and hypothalamus; close to the thalamus, two other structures the substantia innominata and the bed nucleus of stria terminalis; and two other important structures in the brainstem, the ventral tegmental area in the midbrain and the parabrachial nuclei in the dorsolateral pons. Most of these structures are integrated within a system of neural circuits known as the cortico-basal ganglia-thalamo-cortical loop. Neurochemically, the brain's reward system is mainly connected by Glutamatergic interneurons (primarily coming from cortical regions), DAergic pathways (nigrostriatal and mesocorticolimbic) and GABAergic medium spiny neurons (connecting striatal regions). Although the system receives inputs from other neurotransmitter systems or it is present within it.

Reward related processes are not unitary processes and while no definitive taxonomy exists, distinct parts and circuits of the brain reward system may respond to different stimuli, such as: Pavlovian learning (associative learning and motivational salience), instrumental habit learning or goal-directed behaviours (Zald & Treadway, 2017).

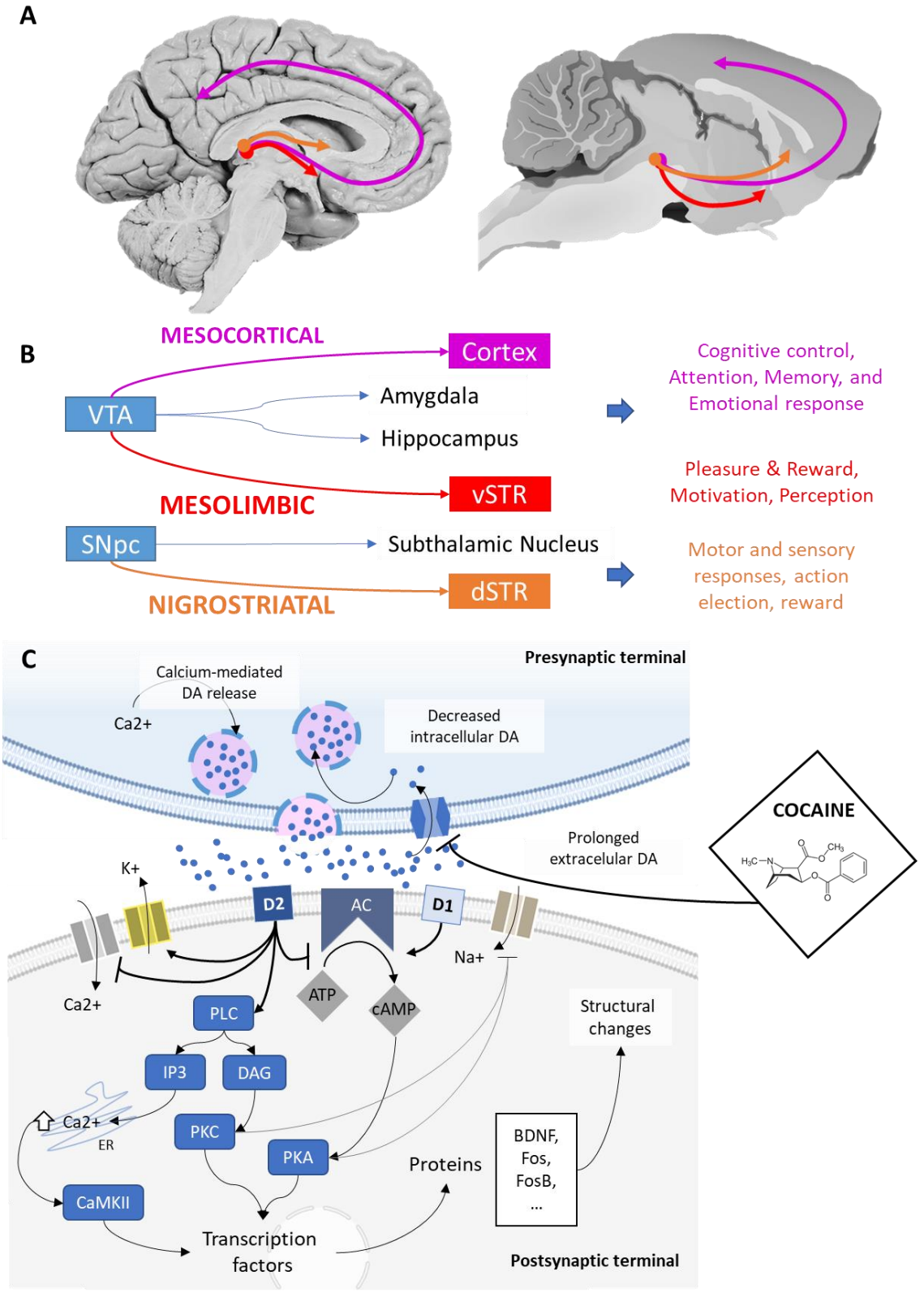


Figure 4. Dopaminergic pathways and the impact of cocaine on dopamine signalling. **A**. Sagittal representation of the human and rat brain and a simplified illustration of three major dopaminergic pathways. **B.** Two dopaminergic nuclei (VTA, Ventral Tegmental Area, and SNpc, Substantia Nigra pars compacta), their principal afferents to other brain areas (vSTR and dSTR, ventral and dorsal Striatum) and the main functions ascribed to them are shown. **C.** Main molecular mechanism associated to dopaminergic signalling and the impact of cocaine on dopaminergic transmission. AC, Adenyl cyclase; ATP, Adenosine triphosphate; cAMP, Cyclic adenosine monophosphate; PLC, phospholipase C; IP3, inositol trisphosphate; DAG 1,2-diacylglycerol; PKC, protein kinase C; PKA , protein kinase A; CaMKII, Ca^{2+}/calmodulin-dependent protein kinase II; BDNF, Brain-derived neurotrophic factor.

Effects after long-term abstinence periods

While to some extent some studies may have captured non-acute effects of cannabis when short periods of abstinence are assessed, residual effects and withdrawal cannot be completely ruled out and thus, one must be careful when extrapolating the changes observed to long-term abstinent users. However, there are fewer studies in which protracted periods of abstinence of at least several weeks have been examined. In one study, cannabinoid levels in urine were screened before including participants (Urban et al., 2012), which can take about a month to clear in regular users, yet this study did not find significant differences in the behavioural and physiological effects of amphetamine injection, unlike other studies in which shorter periods of abstinence were assessed (Volkow et al., 2014). However, participants that can remain abstinent without problems may not be a representative sample. Nevertheless, a relative amelioration of the different biological and behavioural features affected by cannabis was observed in different studies. For example, combining fMRI measurements and performance in a Stroop task (to measure cognitive-control) showed that during an abstinence period of one-year, activity in the anterior cingulate cortex (ACC), dorsolateral prefrontal cortex (PFC), and vSTR (key brain regions for SUDs) progressively ameliorated (increased activity) in participants who used less cannabis (Koberet al., 2014).

Other critical issues in the studies reviewed are that they do not always include individuals of both sexes and although the participants are usually young, the studies do not always cover specific differences related to early or adolescent use. The existent literature regarding residual effects of cannabis use in both adolescent and adult brains has been reviewed (Blest-Hopley, Giampietro, & Bhattacharyya, 2018) and although a wide variety of abstinent periods from hours to weeks or months were considered, common effects following cannabinoid challenge were excluded. The metanalysis confirmed the existence of different patterns of brain activity in adult and adolescent cannabis users, employing a range of cognitive activation tasks combined with fMRI. Compared to healthy controls, adult cannabis users displayed greater activation in the superior and posterior transverse temporal and inferior frontal gyri, and weaker activation of the striatum, insula and middle temporal gyrus. By contrast, adolescent cannabis users displayed stronger activity in the inferior parietal gyrus and putamen.

As noted previously, abstinent periods can overcome cannabis-induced changes in brain activity. In this sense, 69 studies of cognitive functioning in adolescent and young adult cannabis consumers were reviewed (Scott et al., 2018), identifying hints of reduced cognitive functioning, but only in regular and heavy cannabis users, and emphasizing the overall small effect size. Remarkably, the effect size was no different from zero when the threshold of abstinence of at least 72 hours was applied. Consequently, it was concluded that poorer cognitive functioning associated with cannabis might not be clinically relevant, and major deficits are associated with acute and withdrawal effects. However, in a review of the long-term neurocognitive effects, including morphological studies there appeared to be sufficient evidence (9 out of 10 studies) of structural differences in the brain after prolonged periods of abstinence, mainly in cortical areas, in the orbitofrontal region and in the hippocampus (Ganzer et al., 2016). Functional imaging also provided clear evidence of long-term changes (16 out of 17 studies), in the activity of prefrontal and hippocampal areas but also, in the cerebellum area.

Thus, cannabis-driven alterations could be transient and/or generally mild or inexistent with after periods of abstinence. But there is evidence for cannabis-driven causal effects that could affect progression to other drugs and the expression of SUDs. Specifically, the long-term disruption of cortical areas and the possible modification of normal processing of reward-related mechanisms rely on alterations within the structures of the brain reward system.

Animal research with neuroimaging techniques

Results on humans is often subject to significant heterogeneity and must deal with many confounding variables. Moreover, these studies may raise questions that are difficult to respond based on epidemiological data and that are too costly to follow-up with more clinical studies. Thus, animal models may represent useful tools to shed light on some aspects of the impact of adolescent cannabis use on neurobiological changes. Remarkably, despite their strong translational relevance to human studies, animal models of adolescent cannabis exposure have not often taken advantage of imaging studies. However, two imaging studies were performed using a fluorodeoxyglucose ([^{18}F]FDG) PET scan to explore changes in brain metabolism of two similar models of the long-term effects of periadolescent exposure to the synthetic CB_1 and CB_2 cannabinoid

receptor agonist CP 55,940 (Higuera-Matas et al., 2008, 2011) (See Box 9. The components and functions of the endocannabinoid system). Clear sex-dependent outcomes were detected, with females more strongly affected. Adult CP 55,940-treated females showed basal hyperactivation of the frontal cortex and septal nuclei, and hypoactivation of the amygdalo-entorhinal cortex. Conversely, females showed a lower metabolic demand for glucose in the septal nuclei in response to an acute dose of cocaine, while males reduced FDG uptake in the dSTR.

Box 9. The components and functions of the endocannabinoid system

The **endocannabinoid system** (eCBS) is a biological signaling system comprised of three main elements: enzymes, ligands, and receptors. These elements are distributed in different cell types found in distinct body tissues, organs and glands that constitute the immune system, and the central and peripheral nervous system. This widespread distribution allows the eCBS to participate in many different physiological and psychobiological events (Joshi & Onaivi, 2019).

The main eCB ligands that act as biochemical signals are the fatty acids Arachidonoylethanolamine (Anandamide or AEA) and 2-Arachidonoylglycerol (2-AG). In addition, other endogenous lipids have affinity towards CB receptors like 2-Arachidonyl glyceryl ether (noladin ether or 2-AG), Lysophosphatidylinositol (LPI), N-arachidonoyl dopamine (NADA), and virodhamine or O-arachidonoyl-ethanolamine (OAE: Reggio, 2010). The synthesis and release of these eCB ligands occur in response to increases in intracellular Ca^{2+} and after activation of specific G-protein coupled receptors (GPCRs) like the dopamine receptors (Alger, 2004). After their synthesis, these lipidic signaling molecules can be freely diffused or bind to carrier proteins and transported, although the lipid carriers for these ligands and alternative transport mechanisms are yet to be fully elucidated (Nicolussi & Gertsch, 2015).

The eCBS is an ancient phylogenetic system and the associated metabolic apparatus, the enzymes involved in the synthesis and degradation of these lipidic molecules, date back to unicellular common ancestors of animals and plants (Elphick, 2012). AEA is mainly catalyzed from N-acyl-phosphatidylethanolamine (NAPE) by the enzyme NAPE-specific phospholipase D (NAPE-PLD: Liu et al., 2008; Okamoto et al., 2004), whereas 2-AG is primarily catalyzed from diacylglycerol (DAG) by the enzyme DAG lipase α (DAGL α) (Reisenberg et al., 2012). Remarkably, the major rate-limiting step to produce AEA and 2-AG is the conversion of NAPE from phosphatidylethanolamine by N-acyltransferase, and DAG from phosphoinositides by phospholipase C, both $Ca2^{+}$-sensitive process. Although alternative routes of degradation are known, Fatty acid amide hydrolase (FAAH) is considered the main degradation enzyme for AEA, and Monoacylglycerol lipase (MAGL) for 2-AG (Di Marzo, 2006). Importantly, these enzymes are not exclusive to eCBS-related ligands and they participate in the synthesis and degradation of other molecules.

The functions ascribed to these ligands are mostly determined by their activity on their main target proteins, the CB_1 and CB_2 cannabinoid receptors, of which AEA is a partial agonist and 2-AG a full agonist. In addition, phytocannabinoids (e.g. THC, CBD) and manufactured synthetic cannabinoids (e.g. WIN55,212-2, CP 55,940) also exert their effects primarily via the rhodopsin-like CB_1 and CB_2 GPCRs. Notwithstanding, the eCBS is not limited to these receptors and the rhodopsin-like receptor family also includes serotonin and opioid receptors, and both endogenous and exogenous cannabinoids also interact with these receptors to some degree. Another three GPCRs are frequently identified as possible members of the eCBS due to their structural similarity to CB_1 and CB_2, and their affinity to endogenous and exogenous cannabinoids, namely GPR18, GPR55 and GPR119. Cannabinoids also interact with transient receptor potential ion channels (TRP) and nuclear receptors of the family of peroxisome proliferator-activated receptors (PPAR: Pertwee et al., 2010; Sun & Bennett, 2007). Moreover, endocannabinoids and exogenous cannabinoids can modulate the activity of different ion channels (e.g. calcium, sodium and potassium channels, and glycine receptors: Al Kury et al., 2014; Watkins, 2019).

Within the central nervous system, the eCBS intervenes in emotional states and motivational processes that regulate behaviour and cognitive processes related to foraging and the exploitation of natural rewards, such as food, exercise, sex, social interactions and also substances like drugs of abuse (Parsons & Hurd, 2015). Notably, the eCBS is an essential element during development and CB receptors influence the expression of genes encoding proteins involved in cell proliferation, neuronal migration and axon elongation, as well as neuron-glia cell adhesion molecules (Fernandez-Ruiz et al., 2004). In particular, CB_1 seems to be critically involved in the transition from synaptogenesis to synaptic communication and it helps shape the precise topography of neuronal circuits (Deshmukh et al., 2007; Harkany et al., 2007).

2.2.2. EFFECTS ON NEUROBIOLOGICAL SYSTEMS

Several studies have employed a targeted approach to explore the neurobiological changes induced by exposure to cannabis. A considerable number of these studies were performed during adolescence and some of them on animal models aimed to assess long-term effects. Although not every study has focused directly on the possible implications for SUD liability, most of them discussed hypothetical modifications to reward and drug-

related responses after ACE. These will be briefly reviewed here, focusing on some of the long-term alterations documented in the key neurobiological systems.

GABAergic and Glutamatergic systems

The GABAergic and Glutamatergic systems are the major neurotransmitter systems in the mammalian brain, fine-tuning the brain's overall level of excitation and thus, inevitably affecting SUD (D'Souza, 2015; Malcolm, 2003). Several studies have revealed that in these systems, PEACE disrupts the balance between inhibitory and excitatory communication among brain areas, and in a sex-dependent manner. In the hippocampus of both male and females rats, an increase in GABA release following K^+-induced depolarization was documented, probably due to the decrease in GABA transporter 1 (GAT1) meseenger RNA (mRNA) that presumably prolong the presence of GABA in the synaptic cleft and an increased the density of the inhibitory metabotropic $GABA_B$ receptor (Higuera-Matas et al., 2012). Recently, a protocol of THC self-administration revealed weaker GABAergic tone in both male and female adult rats, reduced GAB_AbR2 and $GABA_AR1_\alpha$ (trend) expression in the prelimbic cortex (PrL), and reduced $GABA_AR1_\alpha$ in the dorsal hippocampus (DH) (Stringfield & Torregrossa, 2021a). No changes were found in the infralimbic cortex (IL), VTA, NAc and basolateral amygdala (BLA). Interestingly, limited access to WIN 55,512-2 self-administration during adolescence partially produced the opposite profile of changes immediately after interruption of cannabis use: increased GAB_AbR2 in the IL and PrL, and increased GAT1 in the PrL (Kirschmann et al., 2017). Notably, some of the results obtained underline the special sensibility of females to ACE. Specifically, females but not males showed a decline in K^+ evoked Glu levels and a decrease $GABA_A$ receptor density in the hippocampus (Higuera-Matas et al., 2012; Zamberletti et al., 2012a). Studies including only males linked ACE to reduced transmission of GABAergic interneurons in the mPFC, which may result in a long-term increase in prefrontal excitability (Cass et al., 2014a). By contrast, an increase in the soma size of parvalbumin-positive cells (GABAergic interneurons) was found in the PFC of male mice (Behan et al., 2012). Studies including only females also reported several GABAergic anomalies in the PFC of adult females after ACE. Decreased level of the GABAergic neuronal marker GAD_{67} (Glutamate decarboxylase-67) were evident in the PFC, in addition to other GABAergic containing GAD_{67}+/Parvalbumin+ cells and GAD_{67}+/Cholecystokinin cell populations (Zamberletti et al., 2014). Finally, it is important to note that different time windows may have a distinct impact on GABAergic maturation. In this sense, repeated CB_1 receptor stimulation during early (PND35 to 40) or mid-adolescence (PND40 to 45) generated a frequency-dependent prefrontal disinhibition state during adulthood that was not evident when the treatment occurred from PND50 to 55 or PND75 to 80 (Cass et al., 2014).

Regarding glutamatergic alterations, a decreased density of the NMDA receptor (NMDAR) was found in the hippocampal tissue of male rats (Rubino et al., 2009), a key element for synaptic plasticity and LTP, as well as decreased levels of the postsynaptic density protein 95 (PSD-95), a scaffolding protein in excitatory neurons needed to maintain synaptic strength. A reduction in PSD-95 in the adult PFC of male rats treated with CP 55,940 during adolescence was later confirmed (Renard et al., 2016). Mouse models of PEACE also showed hippocampal downregulation of Metabotropic glutamate receptor 5 (mGlu5: Gleason et al., 2012) and interestingly, mGlu5 has been associated with SUDs and it is required for drug-related instrumental self-administration without altering conditioned associations (Chiamulera et al., 2001; Fowler, Varnell, & Cooper, 2011). However, no differences were found in the rat hippocampal expression of mGlu5 (Higuera-Matas et al., 2012), while enhanced expression of the NMDAR variants GluN2B and GluA1 in the PFC of young adult (P75) rats were only evident in females (Rubino et al., 2015). Moreover, ACE produced a temporary increase in GluN2A during withdrawal and prevented the natural transient decrease in PSD-95 in the PFC from PND46 to 60. These changes could have developmental consequences and affect the pruning of glutamatergic synapses, such as the elimination of asymmetric excitatory synapses in the PFC.

Adult male and female rats also showed a reduction of GluR2/3 in the PrL, but not in the NAc, BLA, DH or VTA (Stringfield & Torregrossa, 2021a). However, sex-dependent alterations in glutamatergic receptor expression have been documented previously (Higuera-Matas et al., 2012). In that study, female but not male adult rats had a lower NMDAR density in the hippocampus, and less Activity-Regulated Cytoskeleton-Associated Protein (Arc). Interestingly, Arc is an immediate early gene activated in a NMDAR-dependent manner that is also downregulated in the hippocampus and PFC after ACE (Llorente-Berzal et al., 2013). Notably, Arc plays a relevant role in synaptic plasticity and it requires MAPK activation, part of the signalling cascades activated by CB_1. Moreover, postsynaptic levels of Arc are increased by the brain-derived neurotrophic factor (BDNF), among others signals that have been seen to be downregulated in the hippocampal CA3 region of female but not male

rats (López-Gallardo et al., 2012). Additionally, recent research found that females, but not males, increase the BDNF levels in the PFC after adolescent cannabinoid exposure (Poulia et al., 2019) and C57BL/6 male mice (females not included) do also increase BDNF-TrkB signaling and synaptogenesis in the NAc after repeated WIN55,212-2 within the NAc, although this last study showed no changes in the PFC, DG, the hippocampal regions CA1 and CA3 (Dong et al., 2019).

However, polysialylated neural cell adhesion molecule (PSA-NCAM) is upregulated in the hippocampus of females but not males indicating enhanced cell self-renewal or neural plasticity (Higuera-Matas et al., 2009). In this regard, chronic CP 55,940 treatment during adolescence altered the morphology of layer II/III pyramidal neurons in the adult PFC (reducing length, number and complexity) and impaired long-term potentiation (LTP) in the hippocampus–PFC circuit at adulthood (Renard et al., 2016). This findings were later confirmed (Miller et al., 2018) when alterations to the developmental trajectory of dendritic arbors (including reduced complexity) and premature pruning of dendritic spines of layer III pyramidal neurons was reported.

Monoaminergic systems

The influence of cannabinoids and their modulation of monoaminergic systems have been frequently explored. Remarkably monoaminergic systems (including DA, norepinephrine and serotonin) are involved in psychomotor responses, emotion, arousal and certain types of memory, activities that reflect the intimate relationship of these systems with SUDs. Overall, evidence regarding DAergic alterations resulting from chronic cannabis use seem to point to reduced presynaptic DAergic function or altered DAergic responses to different stimuli (Bloomfield, et al., 2016). However, these changes may or may not have a presence after prolonged periods of abstinence (See introduction section 2.2.1. Effects of long-term abstinence periods), and animal models of PEACE become a useful tool to control and equate these variables.

Several PEACE studies reported changes in DAergic receptor expression, although these changes may vary across brain areas, be sex-dependent in nature, and discrepancies between studies are habitual. Be as it may, changes in DA receptors in striatal areas may be more pronounced after heavier treatments and more evident with shorter periods of abstinence. This may be the case for the decreased D_2 receptor density in the NAc (but not in the PFC or CPu) found in both males and females by Zamberletti et al., 2012 but not by Higuera-Matas et al., 2011. In the latter study, D_2 receptor density measured on P121, was decreased exclusively in the CA1 hippocampal area. Zamberletti et al., 2012 also reported an increased D_2 receptor density in the PFC. Moreover, while D_1 receptor density was increased in the NAc shell of males, but not females, in Higuera-Matas et al., 2011, it was increased in the NAc of females, but not males, in Zamberletti et al., 2012. This apparent discrepancy could be due to an effect of the differential inclusion of the NAc subdivisions in the analysis, although sex differences in striatal areas are a common output. Irrespective of receptor density, there seems to be enhanced DA uptake in the dSTR of females but not males, as inferred by an increased expression of the Dopamine Active Transporter (DAT: Higuera-Matas et al., 2011).

An increase in DA turnover was inferred by a higher 3,4-dihydroxyphenylacetic acid (DOPAC)/DA ratio in the dSTR and vSTR of male mice (females not included: Behan et al., 2012), in addition to reduced density of tyrosine hydroxylase positive cells in the VTA. Notably, hints of mesocortical pathway hyper-DAergic status were reported (Renard et al., 2017), with increased VTA DA neuronal firing and other molecular changes with known DAergic relationships in rats treated with THC during adolescence and tested on PDN75 than in rats exposed to THC during adulthood. Thus, there seems to be evidence of widespread dysregulation of DA activity and DAergic neurons, and the modulation of the relative weight of D_1 and D_2 receptor expression and activity in different brain areas and with a marked sex-dependent nature.

Serotoninergic and cholinergic systems

In terms of serotoninergic signalling, adolescent THC increased the number of Serotonin Transporter (SERT) positive fibres in the parietal cortex of adult male but not female rats (Lopez-Rodriguez et al., 2014). Moreover, THC treatment also led to significant increases of the serotonin (5-HT) metabolite 5-hydroxyindoleacetic acid (5-HIAA) and 5-HIAA/5-HT ratio in the PFC and within the hippocamus increased SERT and 5-HT levels and decreased 5-HIAA/5-HT ratio in male but not females. (Poulia et al., 2019).Relevantly, neural activity in the dorsal raphe nucleus, the largest serotonergic nucleus, was weakened after ACE in adult male rats at least (Bambico et al., 2010). Indeed, these authors also observed that a high dose of WIN 55,212-2 during adolescence caused hyperactivity of Locus Coeruleus noradrenergic neurons, yet not when

administered in adulthood. This neurochemical profile, together with a behavioural assessment, led to the suggestion that PEACE induces anxiety-like and depressive-like behaviours in adulthood.

Despite the evidence that cannabinoids modulate cholinergic systems (Scherma et al., 2016), there is relative little data on research about the long-term effects of cannabis on this signalling system. Acetylcholine (ACh) and cholinergic receptors (AChR), like the monoaminergic systems, affect and regulate various CNS functions closely related to SUDs, including motivation and reward, attention, arousal, stress response, mood, memory, sensory and motor processing, sleep, and nociception (Sofuoglu & Mooney, 2009). However, when nicotinic receptor density was measured in the PFC of male and female adult rats after ACE, no significant alterations were found (Mateos et al., 2011). The PEACE on the cholinergic system remains poorly characterized.

Opioidergic system

The opioid system is involved in various physiological and pathophysiological activities but also it is well known for regulating sensorial and cognitive processes related to pain, pleasure and reward. Studies of PEACE performed on subjects of both sexes have shown some degree of diversity. The μ opioid receptor (MOR) density increased in the subcallosal streak of both males and females, although contrasting regulation was seen in the Cingulate Cortex, hippocampus (CA2 and CA3), and several thalamic nuclei: downregulation in males and upregulation in females (Biscaia et al., 2008). Studies of PEACE performed on males alone also found an increased in MOR activity in the substantia nigra and VTA, but there were no significant alterations in MOR density among limbic regions (Ellgren et al., 2007). Males and females also differed in other parameters. ACE produced a decreased MOR function in the NAc Shell exclusively in males (Biscaia et al., 2008), and ACE enhanced NAc levels of Dynorphin A exclusively in females (Rubino et al., 2008), an endogenous opioid peptide associated with the adverse effects of withdrawal in the NAc (Muschamp & Carlezon, 2013). The mRNA levels of Proenkephalin (PENK), an endogenous opioid polypeptide hormone, have also been analysed in other PEACE studies. Tomasiewicz et al., 2012 and Ellgren et al., 2007 found increased PENK mRNA in the NAc Shell of adult male rats; however, Morel et al., 2009, using a longer abstinence period, reported a reduction of PENK mRNA in the NAc Shell and the dSTR of adult male rats. Noteworthy increased levels NAc levels of PENK are associated with increased drug self-administration (Cadet et al., 2016; Tomasiewicz et al., 2012).

Endocannabinoid system

When male and female subjects were studied long-term downregulation of CB_1 receptor density was evident in the PFC, dSTR, vSTR, hypothalamus, hippocampus, Thalamus, amygdala, globus pallidus, substantia nigra, VTA and Cb (Zamberletti et al., 2012). In addition to a loss in density, the study of Zamberletti et al., 2012 a general downregulation of CB_1 receptor function was also found in the same areas in both males and females. CB_1 receptor downregulation has also been reported in the PrL, VTA, and IL but not in the NAc, IL, DH, or BLA (Stringfield & Torregrossa, 2021a). By contrast, other studies found an increase in CB_1 receptor function in the hippocampus and dentate gyrus (Higuera-Matas et al., 2012), and sex-specific differences in CB_1 receptor density and function have been reported in the hippocampus (Mateos et al., 2011; López-Gallardo et al., 2012; Lopez-Rodriguez et al., 2014). Recently, voluntary oral consumption of THC during adolescence was seen to reduce CB_1 expression in the VTA of adult male but not female rats, with no changes in the NAc (Kruse et al., 2019). Moreover, while CB_1 expression was reduced in VTA glutamatergic terminals it was preserved in GABAergic terminals. Studies only including males or females also produced conflicting results. In studies only on males, CB_1 receptor density across several key brain areas that express the receptor do not seem to be significantly altered after moderate (Ellgren et al., 2007) or long periods of abstinence (Morel et al., 2009; Behan et al., 2012). Studies only performed on females also failed to find significant alterations to CB_1 receptor density (Chadwick et al., 2011), although a sustained decrease of CB_1 receptor density was described elsewhere in the PFC of female rats (Rubino et al., 2015). In addition, CB_1 receptor function was downregulated after treatment and it transiently increased during withdrawal before reaching control levels. Recently a complementary study showed that deficits in eCB-mediated neuronal plasticity associated with the decrease of CB_1 receptor density in the adult PFC of female rats can be rescued with a URB597 treatment; a FAAH inhibitor that subsequently increases anandamide (AEA) levels (Cuccurazzu et al., 2018).

There is also evidence of PEACE on endoligand expression and activity, for example MAGL and FAAH were upregulated in the hippocampus of adult male mice after WIN 55,512-2 adolescent treatment (Gleason et al., 2012). In female rats MAGL activity was downregulated in the PFC right after the chronic THC treatment, although this recovered and reached control levels with protracted withdrawal (Rubino et al., 2015). However, downregulation of anandamide (AEA) was still evident in the PFC in young adults (<PDN75) and basal measurements may not reflect the spectrum of changes induced by ACE. In this regard, food-restricted adult male rats with ACE showed increased levels of AEA and oleoylethanolamide (OAE) in the vSTR, an AEA analogue that functions as an endogenous ligand of the PPAR-α (Schoch et al., 2018). In addition to decreased AEA in the Cb of unrestricted animals, no change in AEA or OAE levels was detected in the mPFC under any condition.

Box 10. CB receptor activity and SUDs

The best studied effects of cannabinoids in the organism are those derived from their interaction with CB_1 and CB_2 receptors. Both CB_1 and CB_2 are expressed in neural and non-neuronal cells and although not as prominently as CB_2, there is some CB_1 in immune cells, with both inhibiting cytokine release (Howlett et al., 2002). There are also cannabinoid receptors on glial cells, with CB_1 predominantly expressed in astrocytes and CB_2 in microglial cells, modulating inflammatory process, cell support and synaptic transmission (Gutierrez-Rodriguez et al., 2018; Komorowska-Muller & Schmole, 2021; Scheller & Kirchhoff, 2016). Yet it is in neuronal cells that CB_1 is more commonly expressed. Notably, CB_1 is the most abundant GPCR in the mammalian brain (Tsou et al., 1998), while CB_2 is mostly expressed in peripheral immune cells (Chen et al., 2017). Usually CB_1 is expressed presynaptically in the cell membrane of terminal axons of central and peripheral neurons, thereby permitting retrograde communication that originates in the postsynaptic terminal. The activation of these cannabinoid receptors initially provokes the inhibition of neurotransmitter release from the presynaptic terminal (Pertwee, 2006). In addition, intracellular CB_1 can be found attached to the endosome as a result of receptor internalization after agonist activity as part of the endocytic cycle (Leterrier et al., 2004), although it is also expressed in organelles like lysosomes and mitochondria, modulating essential cell metabolic processes (B é nard et al., 2012; Brailoiu, Oprea, Zhao, Abood, & Brailoiu, 2011; Thibault et al., 2013; Zou & Kumar, 2018).

CB_1, and to a lesser extent CB_2, is especially abundant in the basal ganglia, amygdala, hippocampus, cortex and cerebellum, while CB_2 is also expressed in the striatum, amygdala, hippocampus and the VTA. This distribution allows the eCBS to modulate, psychomotor activity, emotional and motivational behaviour, along with learning and memory functions like spatial and declarative memories. There is also extensive evidence that the eCBS participates in SUDs and reward-related processes (Mackie, 2008; Manzanares et al., 2018).

Although CB_1 expression and activity is remarkably variable in different types of neurons at different locations (Busquets-Garcia, Bains, & Marsicano, 2018; Pertwee, 2008), CB_1 is predominantly localized in GABAergic neurons, and to a lesser extent in glutamatergic neurons (Bonilla-Del Río et al., 2019; Gutierrez-Rodriguez et al., 2018). The inhibition of GABA release leads to depolarization-induced suppression of inhibition, and conversely, the inhibition of glutamate release depolarization-induced suppression of excitation. Moreover, eCB-modulation of long-term forms of synaptic plasticity, lasting minutes, hours or longer, is also a widespread phenomenon in the brain that can occur in both excitatory and inhibitory synapses (Heifets & Castillo, 2009). Due to the regulation of inhibitory and excitatory outputs, the eCBS modulates the activity of other types of cells, and thus, it is relevant for the development and expression of SUDs. For example, GABAergic transmission can inhibit dopamine neurons of the VTA, thereby restraining mesocorticolimbic dopaminergic pathway activity. CB_1 activation in GABAergic terminals reduces the inhibitory effect of GABA, subsequently facilitating the release of tonic and phasic dopamine that shapes reward-related processes. Conversely, glutamatergic transmission from cortical areas reaches and excites neuronal populations in the striatum that are innervated by dopaminergic axons. CB_1 activation of these glutamatergic terminals can diminish their excitatory inputs and modulate functions ascribed to this area, like movement and reinforcement learning. Thus, exogenous cannabinoids with CB receptor affinity like THC can affect the brain reward system and it is generally considered that acute THC administration can cause an increase in presynaptic DA synthesis and release, whereas sustained consumption or administration of THC may blunt DA signaling (Bloomfield et al., 2016b).

Endocrine system

Finally, the eCBS is known to affect the hypothalamic-pituitary-adrenal (HPA) and hypothalamic-pituitary-gonadal (HPG) axes, affecting hormones, for example suppressing gonadal steroids, growth hormones, prolactin and thyroid hormone, while activating the HPA. However, this is little evidence of long-term effects in the expression of different hormones, even though cannabis exposure during adolescence seems to at least delay hormone-dependent maturation (Brown & Dobs, 2002; Sims et al., 2018). Indeed, adolescent cannabis exposure diminishes testosterone, dihydrotestosterone, luteinizing hormone (LH) and follicle-stimulating hormone (FSH) in male rats and while cannabis withdrawal seems to ameliorate testosterone levels, albeit not completely, it does allow dihydrotestosterone levels to recover. LH levels may also recover better than FSH after cannabis withdrawal (Gupta & Elbracht, 1983). Interactions between hormones and adolescent cannabis exposure have already been documented (Winsauer et al., 2011), with adolescent THC not significantly altering

adult female CB_1 expression in the STR, although THC-dependent elevation of CB_1 in the hippocampus is absent in ovariectomized rats. Moreover, CB_1 binding efficacy was altered by THC in the Globus Pallidus (GP). Other studies showed a differential response to stress conditions after ACE but failed to find differences in glucocorticoid receptor levels (Abush & Akirav, 2013).

2.2.3. CANNABINOID INDUCED EPIGENETIC ALTERATIONS

Cannabis-induced epigenetic alterations are progressively gaining interest, bringing a more comprehensive and detailed view of the effect of eCB signaling modifications and their functional implications (Szutorisz & Hurd, 2016, 2018). The study of gene expression and epigenetics after cannabis use has been carried out using techniques like gene microarrays, CHIP and more recently, RNA-seq, along with other classic methods to identify and quantify protein and gene expression (e.g. PCR or Western Blotting). The areas currently mapped include the PFC, hippocampus and basal ganglia nuclei. However, the number of studies performed is still limited and they involve the use of distinct animal models, cannabinoids and regimes of administration over different developmental periods, not always including sex as an independent variable. However, even depite these differences, some similarities and patterns of alterations may be extracted.

CB_1 signalling machinery and the acute effects of cannabis on gene expression

The CB_1 signalling machinery modulates synaptic activity and participates in synaptic plasticity. CB_1 is coupled to $G_{i/o}$ proteins, although under certain circumstances it can bind to G_s proteins, albeit with lower efficacy than to $G_{i/o}$ (Finlay et al., 2017). Activation of the G_i proteins lead to the inhibition of adenylyl cyclases (ACs), in turn reducing intracellular cAMP concentrations and hence reducing the activity of the cAMP/cAMP-dependent kinase (PKA) pathway. CB_1-$Gi_{/o}$ activation also reduces Ca^{2+} entry into the cell in two distinct ways: through direct G-protein mediated inhibition, although the mechanism involved is not yet fully described; and through a cAMP-dependent mechanism as PKAs positively influence Ca^{2+} channels. Among other interactions, the cAMP/PKA pathway phosphorylates numerous metabolic enzymes and transcription factors that regulate gene expression. In fact, cAMP/PKA signalling participates in the transcriptomic changes necessary for presynaptic long-term plasticity, a long-lasting increase or decrease in neurotransmitter release (Yang & Calakos, 2013). However, the precise molecular mechanisms involved in long term synaptic plasticity may vary across brain regions and involve different cell types. The establishment of these changes depends on Ca^{2+} signalling and the differential activation of the cAMP/PKA and PI3K/Akt pathways, and the subsequent effects on downstream target proteins and transcriptional activity (Piette et al., 2020).

Together with the modulation of the cAMP/PKA pathway, CB_1 Gi/o protein activity can shape metabolic processes and gene expression in the cell through the concurrent activation of the mitogen-activated protein kinase (MAP kinase) and the phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt) pathways (reviewed in Howlett et al., 2010; Pertwee et al., 2010; Zou & Kumar, 2018). Notably, the precise cascade of events also varies within each cell type (Howlett et al., 2010). While the MAP/ERK pathway activates genes associated with neural growth, proliferation, differentiation and inflammation, the PI3K/Akt pathway participates in the regulation of Ca^{2+} signalling and glucose metabolism, apoptosis, cell proliferation, transcription and cell migration. Importantly, both the MAP/ERK and PI3K/Akt pathways influence synaptic plasticity. Like CB_1, CB_2 is also frequently coupled to Gi/Goα subunits and thus, it can also inhibit the activity of ACs and activate the MAPK-ERK pathway through the Gβγ subunit.

Starting from a simple model (acute dosing and early expression), transcriptional changes in biological processes related to cell proliferation and cell survival were seen in hippocampal tissue from male CD1 mice by RNAseq 2 hours after a single dose of THC (3 mg/Kg) at PDN35 or PDN120 (Leishman et al., 2018). The transcriptional regulation induced by THC was more extensive in the adult (189) as opposed to the adolescent mice (31), with all the genes differentially expressed in the adolescents also being differentially expressed in the adults in the same direction. Although these acute changes might not persist or they may even provoke allostatic compensation, the picture obtained is useful to see where and how THC begins to exert its epigenetic actions. Using cell culture techniques, RNA-seq analysis was applied to super antigen-activated lymph node cells and $CD4^+$T cells exposed to THC (Yang et al., 2016). A functional analysis suggested that THC altered elements in networks that affected cell proliferation, survival and death. Another study performed in neurons derived from human-induced pluripotent stem cells (hIPSCs) exposed to THC found alterations to synaptic function,

demethylation and ion channel components, and displaying significant alterations to synapses, mitochondria and glutamate signalling (Guennewig et al., 2018).

Chronic effects of cannabis exposure

Chronic effects of cannabis exposure on the transcriptome have been explored in a gene microarray (24,000 cDNA clones: Kittler et al., 2000), also employing hippocampal RNA from adult male Sprague-Dawley rats treated with THC (10 mg/kg i.p.) for 1, 7 or 21 days. Among other categories, genes related to metabolism (e.g. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), Cytochrome oxidase), cell adhesion (e.g. Neural cell adhesion molecule (NCAM)), myelination/glial differentiation (e.g. Myelin basic protein), Receptors/signal transduction (e.g. Angiotensin A1 Receptor, Calmodulin, Calreticulin), and protein folding (e.g. HSP70, Ubiquitin-conjugate enzyme) were altered differentially. Curiously some of these genes were biphasically or triphasically altered, being differentially up/down-regulated at different time points. Moreover, genes that remained altered for the entire duration of the chronic treatment were predominantly associated with membrane repair and synapse structure. These results were compared to those from hippocampal cells treated with WIN55212-2 prior to achieving neurotoxic levels of NMDA exposure (Grigorenko et al., 2002) and assessing RNA expression using spotted cDNA microarrays (1,200 cDNA clones). Cannabinoid treatment showed effective attenuation of NMDA neurotoxicity, reversing the regulation of several genes closely related to CB_1 receptor-linked signalling, calcium-binding proteins and structural proteins in the synapse (e.g. somatostatin, c-k-ras proto-oncogene, GABA-A receptor gamma-2 subunit, cAMP response element-binding protein (CREB)1, Protein kinase C (PKC)I-alpha, cAMP protein kinase inhibitor, dipeptidyl aminopeptidase related protein, ezrin, PKC1-alpha, neuromodulin, mitochondrial Adenosine triphosphate (ATP) synthase D-subunit, syntaxin binding protein Sec1, microsomal glutathionine S-transferase, MAP kinase 1, cathepsin B). Importantly, there were genes commonly altered in both studies (e.g. calcium-transporting plasma membrane ATPase, Sec1-syntaxin binding protein, GABAA receptor-beta 3 subunits and p27kip1-microtubule related protein), and genes differentially expressed in the *in vitro* WIN-only condition and in several of the *in vivo* treatment conditions also closely related to cannabinoid receptor-coupled signalling pathways, membrane and synapse structure, motility and neuron growth (e.g. Transferrin, Calmodulin, Myelin proteolipid protein, Myelin basic protein, β-tubulin, Peptide elongation factor, Polyubiquitin, NCAM, Growth-associated protein ST2, protein, Regulator of PKC, Cytochrome oxidase, Heat shock protein (HSP) 70, Secreted protein acidic and rich in cysteine)-like protein 1, Fructose-bisphosphate aldolase, Proteosomal ATPase). These early studies provided a remarkable initial overview of the transcriptomic changes induced by cannabinoid, and later approaches have addressed more specific issues: assessing these changes after extended periods of clearance and withdrawal, and/or checking the chronicity of the patterns of expression observed. Moreover, subsequent studies expanded the analysis to other brain regions and different cell types, importantly assessing age-related differences, interactions with specific developmental periods and sex-specific differences.

Cannabinoid exposure may also produce region- and age-specific alterations to an epigenetic mechanism like histone modifications. Chronic exposure of female rats to increasing doses of THC for 11 days affected histone modifications in different brain areas (hippocampus, NAc, and amygdala), leading to transcriptional repression in adolescents and transcriptional activation in adults (Prini et al., 2017a). Interestingly, the primary cannabinoid effect was followed by a homeostatic response to counterbalance the transcriptional repression only in the adolescent hippocampus and NAc. Furthermore, this adolescent cannabinoid treatment alters the expression of genes associated with synaptic plasticity in the PFC (41 selected genes were assessed using a RT2 Profile PCR Array Custom Rat Synaptic Plasticity kit) mainly through H3K9me3 modifications, an effect that was involved in cognitive deficits since pharmacological blockade of H3K9me3 during adolescence prevented the THC-induced cognitive deficits (Prini et al., 2017b).

Protracted effects of adolescent cannabis exposure

As these transcriptomic studies may have been limited to a subset of genes, only performed on females and after short withdrawal periods (48 hours maximum: Prini et al., 2017a, 2017b), some of these caveats were explored recently. As such, a decrease in the phosphorylated form of several proteins (Glycogen synthase kinase (GSK)-3α/β, Protein kinase B (Akt) Threonine (Thr)308, mechanistic target of rapamycin (mTOR), p70S6 Kinase, and β-Catenin) was detected when quantified in western blots of the adult male rat PFC 30 days after adolescent THC exposure (Renard et al., 2017). Significantly, these were linked to enhanced DAergic signalling within the mesocortical circuits. Using

RNA-seq developmental alterations to the transcriptome of layer III Prelimbic pyramidal neurons and non-pyramidal cells in the PFC were recorded after adolescent treatment quite similar to that employed here, although exclusively in male long-Evans Rats (Miller et al., 2018). After two weeks of abstinence, genes related to actin, cytoskeleton, and dendritic regulation were altered in THC-treated animals, and although similar gene ontologies were altered in control-treated animals, genes involved in chromatin modification and histone methylation were only altered following THC administration. Notably, there was an enhanced cytoskeletal organization and formation after receiving THC, and a suppression of neurite branching. Although the study didn't include sex as a factor, the combination of genome sequencing and morphological approaches provides valuable evidence of cannabis-induced adolescent changes in premature pruning and protracted atrophy of distal apical trees, remarkably similar to chronic stress-mediated atrophy.

Box 11. Neurobiological mechanism of THC

The effects of cannabinoids on the organism are thought to be primarily driven by its activation of the CB_1 and CB_2 receptors. THC exhibits partial agonist activity at CB_1 and CB_2 receptors but also, it interacts with other elements of the eCBS. THC acts as an agonist of GPR55 (Howlett et al., 2002) and it inhibits lysophosphatidylinositol (LPI), an endogenous GPR55 ligand (Anavi-Goffer et al., 2012). THC is also an agonist of GPR18, where it is even more potent than at GPR55, CB_1 or CB_2 (Ashton, 2012), and there is even evidence of THC agonist activity at GPR119 (Morales, 2017). Moreover, THC act as an antagonist of TRPV2 and TRPM8 (Qin et al., 2008), and it exerts moderate agonist activity at TRPV3, TRPV4 and TRPA1(De Petrocellis et al., 2011; Shibasaki, 2016). THC does no bind directly to PPARα but it can upregulate this receptor in a dose-dependent manner, and enhance the activity of the fatty acid 2 hydroxylase, an enzyme involved in cell differentiation under certain conditions (Takeda et al., 2014). THC and its metabolites can also drive anti-inflammatory effects and time-dependent vasorelaxation *in vivo* through PPARγ (O' Sullivan, 2007).

THC can also interact with other neurobiological systems outside the eCBS. It has antagonist-related activity at the serotonin $5HT_{3A}$ receptor (Barann et al., 2002; Shi et al., 2012), an ion channel permeable to Na^+, K^+ and Ca^{2+}, with excitatory effects in neurons. THC also acts as an allosteric modulator, changing the receptor's response to stimulus by two opioid receptors (OPRs): μOPR and δOPR. The effects of activating these receptors may include the anticonvulsant and analgesic effects of cannabis, and at a cognitive level, a modulation of the euphoric and hedonic feelings, stress perception and antidepressant effects reported by cannabis consumers (Kathmann et al., 2006). Similarly, THC binds to and acts like a positive allosteric modulator at glycine receptors (GLyR), a widely distributed family of ionotropic receptors of the amino acid glycine that once activated, allows chloride (Cl^-) into the cell, thereby polarizing the neuron and serving as an inhibitory input. THC interacts with GLyRα_1 and GLyRα_3, present throughout the CNS but profusely expressed in the hippocampus, spinal cord and brainstem, and consequently, GLyRs might participate in the THC-driven sedative effects on pain perception and locomotion (Xiong et al., 2011). Finally, there are interactions between cannabinoids and the hypothalamic-pituitary-adrenal system. Among the better documented effects, it is known that THC can start a cascade of hormonal effects that include modulating the release of several hormones, including: cortical hormones, gonadotropin-releasing hormone, luteinizing hormone, follicle-stimulating hormone, prolactin, thyroid hormones, growth hormones, and gonadal hormones (Borowska et al., 2018; Harclerode, 1984).

It is noteworthy that the results obtained regarding the interruption of the normal PFC maturation have been considered in the context of vulnerabilities to psychiatric disorders like SUDs, aligning these results with causal biological variables behind cannabis-gateway effects (Miller et al., 2018). Recently a model of cross-sensitization between the WIN 55,212-2 and cocaine in adolescent and adult male rats was used to assess the molecular events in the PFC and NAc underpinning treatments and the interaction with the age-related differences (Scherma et al., 2020). Cross-sensitization was tested after 9 days withdrawal and the tissue brain was obtained the following day. Adolescent but not adult animals showed cross-sensitization to cocaine after chronic WIN55,212-2 treatment but not the other way around, which was associated with histone hyperacetylation in the PFC. Moreover RNA-seq analysis of the adolescent PFC showed differential expression of 7 genes, including ribosomal protein L19, keratin 2 and acetyl-CoA acyltransferase 2. An analysis of genes with significant skipped exon events showed an enrichment of genes related to neurotransmitter receptor transport and protein localization to postsynaptic membrane. Among these genes were transcription factors (e.g. neuronal PAS domain protein 2, E74 Like ETS Transcription Factor 1, Heat shock factor 1, coiled-coils domains and several zinc finger proteins), genes relevant for signal transduction (e.g. *Mapk10*, *Nek10*), genes related to neurite outgrow, axon guidance and myelinisation (e.g. *Myo9a*, *Kif21a*, *Plxna3*, *Tenm4*, *Tenm3*), genes encoding proteins involved in synaptic activity and different receptor components (e.g. *Grk4*, *Grip1*, *Gabra4*, *Rims2*, *Kcnt1*, *Sypl1*). A subsequent analysis of mRNA expression in the NAc between the cocaine and WIN–cocaine groups found no significant changes. The studies presented here take these approaches a step further by including sex as a variable and interrogating the NAc transcriptome after a longer period of abstinence after use of the phytocannabinoid THC.

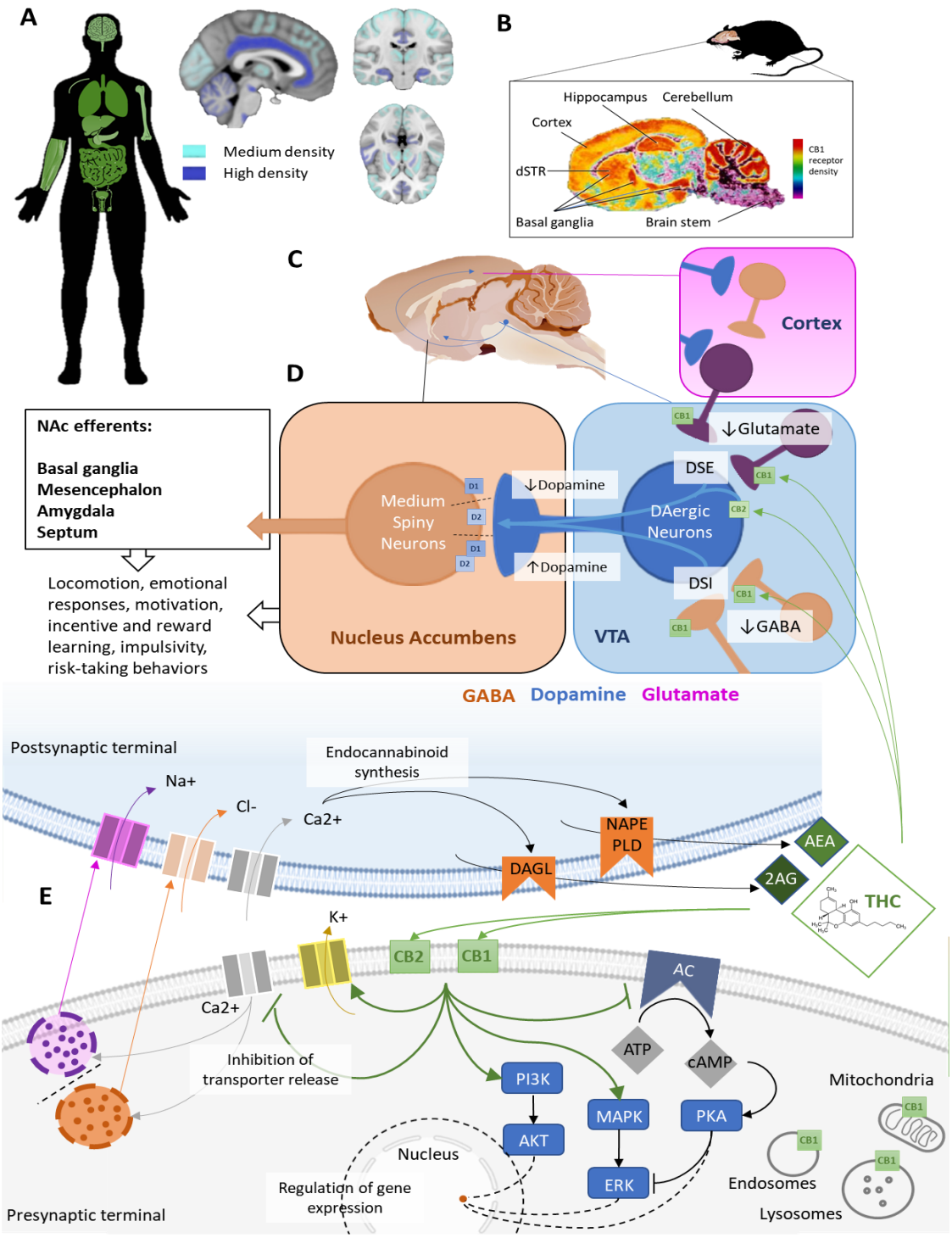


Figure 5. The endocannabinoid system and the impact of THC on endocannabinoid signalling. A. Representation of the cannabinoid receptor distribution in the human body and the CB1 expression in the human brain (adapted from Bloomfield et al., 2019). **B.** Autoradiograph of cannabinoid receptors in a sagittal view of the rat brain (adapted from Thomas, 2009): dSTR, dorsal Striatum. **C.** Simplified diagram of the mesocorticolimbic dopaminergic circuit. **D.** Representation of the main acute effects on GABAergic, Dopaminergic and glutamatergic neurons derived from the activation of distinct populations of CB1 and CB2 receptors by cannabinoids: VTA, Ventral Tegmental Area; DSI, Depolarization-induced suppression of inhibition; DSE, Depolarization-induced suppression of excitation. Some elements are intentionally excluded for clarity. Dashed lines separate distinct neuronal populations of Medium Spiny neurons expressing D1, D2 or D1-D2 receptors. **E.** Molecular mechanism within the synaptic communication responsible for the inhibition of transporter release, regulation of gene expression and endocannabinoid synthesis. Dashed line represents a gap to include process and cell parts outside the presynaptic terminal. Endocannabinoid degradation and THC metabolism are excluded from the graph: NAPE PLD, N-acyl phosphatidylethanolamine phospholipase D; DAGL, Diacylglycerol lipase; MAPK, mitogen-activated protein kinase extracellular signal-regulated kinase; PKA, Protein kinase A; AKT, Protein kinase B; PI3K, Phosphoinositide 3'-kinase; ATP, Adenosine triphosphate; cAMP, Cyclic adenosine monophosphate.

2.3. SUD-RELATED TRAITS

2.3.1. REWARD RELATED PROCESSES ALTERED BY CANNABINOID EXPOSURE IN HUMANS

Adolescent cannabis use may influence and alter several traits relevant to SUDs. Among them, cannabis-induced alterations of motivational and reward processes may have a direct impact on the development of SUDs. Reduced motivation (increased apathy, reduced effort and or reward sensitivity) is often described as a consequence of cannabis use, and longitudinal studies have offered partial support for a causal link (Pacheco-Colón, Limia, & Gonzalez, 2018). Heavy users report that cannabis impaired their motivation (Kouri et al., 1995), and dependent users show lower levels of motivation relative to non-dependent users (Looby & Earleywine, 2007), and longitudinally, cannabis use but not alcohol or nicotine, predicts less persistence and initiation of different activities (Lac & Luk, 2018). Moreover, performance-based measures associate lower motivation with greater cannabis use (Lane et al., 2005), and cannabis users show greater motivational deficits and worse mood compared to tobacco smokers and non-smokers (Martin-Soelch et al., 2009). However, all these conclusions are extracted from actual cannabis users, and the extent of these changes after prolonged periods of abstinence is not covered.

Other studies failed to see differences between users and non-users in self-reports of motivation and life satisfaction (Barnwell, Earleywine, & Wilcox, 2006). Additionally, motivational deficits in cannabis users frequently present some degree of comorbidity with depressive-like symptoms, and it may be an underlying cause contributing to the decreased sensitivity to reward (Musty & Kaback, 1995; Wright et al., 2016; Onaemo, Fawehinmi, & D'Arcy, 2021). Moreover, when controlling for confounding variables, such as depression, the effects on motivation, effort-related decision-making and reward learning often disappear (Lane et al., 2005; Lawn et al., 2016). Although the causal relationships and associations between depression and cannabis use are also debatable, adolescent cannabis use in particular seems to put a large number of young people at risk of developing depression (Gobbi et al., 2019).

Closely related to motivation and reward, cannabis has been involved in the modulation of affection and emotionality, and differential processing of aversive stressful events. Regarding affective salience, current cannabis users exposed to unpleasant stimuli showed lower arousal and higher pleasantness than control subjects, accompanied by hyperactivity of the hypothalamus-pituitary-adrenal (HPA) axis in cannabis users. Moreover, these effects seemed to be only partially recovered after six months of abstinence (Somaini et al., 2012). Since altered HPA stress reactivity is usually associated with SUDs, these results might be considered a risk for the latter (Lovallo, 2006).

2.3.2. COGNITIVE CONTROL AND IMPULSIVITY

Another frequently reported feature in SUDs is the alteration of cognitive control and executive functions. These processes are associated with cortical areas that govern cognitive-behavioural control, which is affected by stress and ultimately affects reward-related processes. There was early evidence of significant long-term impairments in selective attention and concentration in cannabis users abstinent from 6 weeks to 2 years (Solowij, 1995). Remarkably, the alteration of several neurocognitive functions, including attention and concentration deficits, is dose-dependent (Bolla et al., 2002). Nonetheless, other studies failed to find differences in these domains (Lyons et al., 2004; Pope et al., 2001, 2002, 2003). When assessing executive functioning with a battery of tests (Verdejo-García et al., 2005), drug use was significantly correlated with working memory, cognitive flexibility and analogical reasoning. Moreover, the severity of cannabis was the best predictor of poor performance in the cognitive flexibility, a task that required finding the correct transformation in a sequence of geometric figures. However, experimental groups only included abstinent cannabis and polysubstance abusers, making it difficult to gauge the severity of the change. No changes were found in a verbal fluency task and there was no significant evidence of long-term deficits (Pope et al., 2001; 2002), although in a subsequent study, early-onset cannabis users showed impaired verbal fluency compared to late-onset users (Pope et al., 2003). However, other non-pharmacological factors may also correspond to this effect.

Impulsivity, the tendency to perform impulsive actions, is an inability to inhibit behavioural responses and/or the tendency to make impulsive choices, a distorted decision-making process when choosing between different outcomes, is frequently associated or described as a main feature of SUDs but it is also as a risk factor in the progression towards this pathology (Jentsch et al., 2014). Several studies failed to find long-term effects using the Stroop test, which demands the inhibition of some aspects of attention to prevent incorrect actions (Lyons et al., 2004; Pope et al., 2001; 2002; 2003). Similarly, while another study failed to detect performance deficits in a Stroop task, brain activity in prefrontal brain regions differed in abstinent cannabis users from controls (Eldreth et al., 2004). By contrast, several studies using a more demanding cognitive impulsivity task (Wisconsin Card Sort Test) reported a pervasive effect of cannabis (Bolla et al., 2002; Pope et al., 2001, 2002, 2003). Even after long periods of abstinence (28 days), former heavy cannabis users are biased towards risky options associated with higher reward opportunities (Bolla et al., 2002). However, elsewhere no differences in this task between male monozygotic twins were found irrespective of the amounts of cannabis consumed (Lyons et al., 2004). In addition, in a Frontal Systems Behaviour Scale there was a trend towards a significant long-term impairment in decision-making and risk-taking behaviours in cannabis users compared with non-cannabis using controls using (Verdejo-García et al., 2006).

However, impulsivity does not always predict cocaine abuse in humans. Impulsivity and gender were not significant predictors of cocaine dependence (Butelman et al., 2020) despite increasing self-exposure to cannabis, which did predict earlier onset of the heaviest use of cocaine. Together with impulsivity, sensation-seeking (or novelty-seeking) is considered another endophenotype related to SUDs, the development of compulsive drug administration and in facilitating relapse (Jupp & Dalley, 2014). Recently, long-term abstinent cannabis-dependent patients were shown to have greater impulsiveness and sensation-seeking but not decision-making deficits (Delibaş et al., 2018). However, there was no longitudinal data to determine whether the effect of cannabis on these features might be a premorbid characteristic of the sample. Thus, sensation-seekers and highly impulsive people were more likely to initiate cannabis use in the past, and at least after a sufficient period of abstinence, deficits in decision-making may be no longer detectable.

2.3.3. ALTERATIONS TO REWARD RELATED BEHAVIOUR AND COGNITIVE CONTROL IN ANIMAL MODELS

ACE has differential effects on reward sensitivity and preference. A shift in preference and/or the consumption of natural rewards can reflect a general alteration of emotional and motivational processes related to hedonic responses and reward learning that may affect the expression of SUDs. Changes in preference and consumption of natural rewards like sucrose or palatable food after cannabinoid exposure are mixed and show a high degree of sex-dependent effects. Exploring some of the short- and long-term cognitive effects of late-adolescence cannabinoid (WIN 55,212-2) exposure showed that sucrose consumption was unaltered by chronic administration 24 h, 10 or 30 days after the last drug injection (Abush & Akirav, 2012). Similarly, no significant effects in sucrose preference were seen in adult female rats pretreated with CP 55,940 during adolescence (Chadwick et al., 2011). However, acute THC treatment produces selective enhancement of the incentive value of sucrose in adult female rats (Olarte-Sánchez et al., 2015). Recently, adolescent WIN 55,212-2 exposure was shown to increase sucrose consumption in male mice but to decrease it in females (Pushkin et al., 2019). Remarkably nicotine co-exposure had the same effect in males but ameliorated sucrose consumption in females. Similarly, male adult male rats exposed to WIN 55,212-2 during adolescence showed an increase in palatable food intake when rats were allowed to freely consume familiar or novel palatable food pellets (Schoch et al., 2018). However, this effect was specific to the first day and moreover, food restriction elicited an increased intake of a sucrose solution in control rats but not in cannabinoid exposed rats. In this sense, a decrease in sucrose preference was documented in male and female adult rats treated with THC during adolescence (Rubino et al., 2008). Moreover, increased anhedonia associated with a decrease in sucrose preference and palatable food consumption was reported in female rats treated with THC (P35 to P45) and tested during adulthood (PND98 to 104: Realini et al., 2011). It seems that the effect of cannabinoids on food-intake and food-reward may be specific to the set-up, with choice settings producing a decrease in preference and forced-choice settings leading to transient increases of palatable food consumption. Choice and instrumental paradigms are necessary to extract conclusions over the real impact in the motivation of cannabinoid treatments. Thus, the

observed effects may be specific to the experimental settings and moreover, they could not be generalized to any other reinforcer, including different types of drugs.

The rewarding value of natural reinforcers has also been tested under instrumental paradigms. Adolescent CP 55,940 had no impact on low demand schedules (Fixed-ratio 1) of food-reinforced behaviour (food pellets), even if animals had *ad libitum* access to chow food or they were food-deprived (Higuera-Matas et al., 2008). Using a more complex food - motivated task that involved the learning and repetition of response sequences, chronic adolescent THC produced low response rates during acquisition and performance (Winsauer et al., 2011). Remarkably this effect was only seen in female rats that did not undergo adolescent ovariectomy (males not included). However, when adult male rats were exposed to THC during adolescence, no changes in response acquisition for sugar pellets were seen using up to FR5 schedules (Friedman et al., 2019). Thus, adolescent cannabinoid exposure may affect the ability to learn and perform complex but not simple operant tasks in a sex-dependent manner.

Reward seeking can become a habit-like action, which for some authors is a distinct feature of SUDs. As seen previously, some of the results observed in food-reward settings regarding consumption and preference could be interpreted as different reactivities to devaluation. However, cannabis-gateway animal models did not directly address this feature with instrumental paradigms. The study best addressing this topic showed that adult rats chronically exposed to WIN 55,212-2 showed delays at the beginning of a reversal-learning task (indicative of S-R learning) while exposure during adolescence had no effect (Johnson et al., 2019). Nonetheless, other studies did report impaired learning or behavioural flexibility in male and female rats exposed to cannabinoid agents during adolescence (Harte & Dow-Edwards, 2010).

Habits are thought to be automated or irreflexive actions triggered by cues. However, even if it is the case in some situations, drug-use patterns cannot always be constrained by this definition. Nonetheless, instrumental performance (and motivation) is highly influenced by Pavlovian cues associated with rewarding (or aversive) events (Campese et al., 2020; Cartoni, Balleine, & Baldassarre, 2016). Learned cues do indeed code and provide information about the rewards available in a situation and their value. Within the framework of the incentive-sensitization theory, reward-associated cues can trigger an excessive wanting that overrides other alternative pathways of actions, for example, leading to increased rates of relapse. There are no studies specifically addressing this issue in animal models of cannabis-gateway effects. However, there are reasons to believe that adolescent cannabis might modulate Pavlovian learning and the weight of reward-associated cues in instrumental behaviour. Paradigms like Pavlovian to instrumental transfer (PIT) can help explore this phenomenon.

Moreover, incentive salience attribution resulting from pavlovian learning can render certain cues attractive and elicit approach toward them. This feature is differentially expressed by animals, and it is considered a feature of two distinct Cognitive-Motivational Styles (Sarter & Phillips, 2018). This trait can be easily captured by Pavlovian conditioned approach tasks where animals can be classified into goal-trackers (GTs) and sign-trackers (STs), STs being more prone to attribute higher incentive salience to reward-associated cues. The effects of chronic adolescent (PND30 to 43) cannabinoid treatment (WIN55-212,2) were evaluated in young adult male rats (>PND60), showing the emergence of a mixed phenotype characterized by increased lever and food cup approaches than by control rats that developed a clearer bias toward ST (Schoch et al., 2018). More recently, adult male but not female rats exhibited more conditioned responses to the reward-predicting lever during the acquisition of the task, yet not during maintenance (Kruse et al., 2019.)- The experiments performed in this thesis will help to replicate and extend these recent findings.

Some animal studies have explored changes in cognitive control and other closely related features, like attentional deficits and impulsivity (Nigg, 2016). Recently, after chronic adolescent exposure to WIN 55,212-2 adult mice become more impulsive in a delay-discounting procedure compared to mice exposed only during adulthood (Johnson et al., 2019). Using male and females rats exposed to WIN 55, 212-2 (from PND30 to 60) and tested as young adults (PND70), only slight effects on risky choice (highest levels of risk-preference) were observed in WIN-treated animals in the 67% sessions in a probabilistic reward task, although the animals' ability to flexibly respond to changes in reward contingencies was not impaired (Jacobs-Brichford et al., 2019). Neuronal activity of these animals in the mPFC was also explored, finding an overall reduction in task-dependent mPFC activity in WIN-treated animals that was discussed in the context of the known maturational impairments of the excitatory-inhibitory signal balance and maturation of the PFC (Cass et al., 2014; Renard et al., 2017;

Zamberletti et al., 2014). Previously, it was shown that adult male rats (>PND85) treated with WIN 55,212-2 during late adolescence (PND40 to 65) had impaired behavioural flexibility in an attentional Set-Shifting Task (Gomes et al., 2015). Similarly, male and female rats exposed to THC during early adolescence showed impaired learning flexibility in the reversal trial of an active avoidance test (Harte & Dow-Edwards, 2010).

2.3.4. ATTENTIONAL, EMOTIONAL AND MEMORY DEFICITS IN ANIMAL MODELS

Importantly, long-lasting alterations to basic attentional processes have been observed after chronic adolescent cannabinoid treatment. There are several reports of disruptions to pre-pulse inhibition (PPI) (Wegener & Koch, 2009; Abela et al., 2019; Gleason et al., 2012), usually interpreted as an index of the inability to filter out the unnecessary information that is present in some psychiatric disorders (Kohl et al., 2013). Significantly, the regulation of PPI and SUDs share some neural structures (Arenas et al., 2019; Volkow & Morales, 2015) and a decrease in PPI has been considered a vulnerability towards developing locomotor sensitization to cocaine (Arenas et al., 2020). However, sensorimotor gating deficits are not always a feature of all forms of impulsivity (Feja et al., 2015). Thus, cannabis is able to induce changes in several cognitive processes, producing a specific profile independent of naturally occurring phenotypes. Remarkably, these sensorimotor gating deficits appear to decrease over time (Abela et al., 2019).

A well-characterized long-term impact of cannabis during adolescence on emotional regulation might also affect SUDs (Koob, 2015), in this regard some apparently conflicting results have been produced. In the elevated plus arms maze, a classic anxiety task, the common trend is towards no effect in both males and females (Rubino et al., 2008; O'Tuathaigh et al., 2010; Bortolato et al., 2014; Higuera-Matas et al., 2009, Llorente-Berzal et al., 2013, Mateos et al., 2011; Schoch et al., 2018; Sestan-Pesa et al., 2020). Only one study documented an increase in anxiety (Stopponi et al., 2014) and decreases have been more frequently being reported when using CP 55,940, WIN 55,212-2 or THC (Biscaia et al., 2003; Wegener & Koch, 2009; Cadoni et al., 2015).

In the open field test, again the common trend is towards a lack of significant differences (Alejandro Higuera-Matas et al., 2015; Sestan-Pesa et al., 2020). Only, in one study were increased anxiety-like (less time spent in the centre) behaviours found with THC, but no changes in locomotion (Llorente-Berzal et al., 2013). Conversely, CP 55,940 treated females increased the immobility time in the open field, and both males and females treated with CP 55,940 showed more internal ambulation in the open field, two indexes of low anxiety, and additionally CP 55,940 treated females were also hypoactive in the hole-board (and index of low anxiety) (Biscaia et al., 2003). No differences were evident in male mice tested 90 days after the end of a chronic adolescent THC exposure (Tantra et al., 2014). WIN 55,212-2 not only increased motor activity and rearings in the open field but more time was spent in the centre (Wegener & Koch, 2009). A high degree of locomotor activity in a novel environment is related to novelty-seeking and high-responder phenotypes. Interestingly, high-responders are thought to facilitate drug intake acquisition and increase cocaine self-administration (SA) (Davis et al., 2008; Kuhn et al., 2019), although adolescent cannabinoid exposure does not seem to enhance this behaviour and probably not this phenotype. Moreover, WIN55-212,2 treated rats spent more time exploring a novel environment but showed no differences in locomotor response to novelty (Schoch et al., 2018).

This latter finding is interesting in the light of the influence of oxytocin mediating social behaviour, novelty-seeking and SUDs (Tops et al., 2014). Moreover, THC treatment disrupted social novelty preference, dampening the interest towards an unfamiliar mouse relative to a familiar mouse (O'Tuathaigh et al., 2010). Although two studies failed to find significant effects in the social interaction test (Gleason et al., 2012; Zamberletti et al., 2012), other studies have repeatedly shown and confirmed deficits in male and female rats exposed to adolescent cannabinoid treatment (O'Shea et al., 2006; Quinn et al., 2008; Realini et al., 2011). Interestingly, this effect is also sensitive to the age at exposure, since increased social anxiety is seen in rats treated during adolescence but not during adulthood (O'Shea et al., 2004). More recently, THC exposure during adolescence induced in young adult male rats (>PND75) was seen to produce weaker social motivation and a lower social cognition index in a social interaction task (Renard et al., 2017).

The elevated plus maze and the open field take advantage of the natural aversion of rodents to open spaces and heights, usually preferring dark rather than highly illuminated areas. Using the light and dark test, a long-term increase of anxiety was reported in young adult male rats exposed to THC during adolescence (Renard et al., 2017). However, in similar tasks (Emergence test) no significant effects were detected in male

rats after adolescent CP 55,940 exposure (O'Shea et al., 2006). Actual aversive stimulus has been tested in different tasks. In terms of aversion related learning, impairment was evident in a fear conditioning task in male mice treated with WIN 55,212-2 (Gleason et al., 2012), although other authors showed no deficits in the active and passive place avoidance tasks (Rubino et al., 2009; Harte & Dow-Edwards, 2010; Abboussi et al., 2014).

Although the results obtained in these tasks are usually interpreted in the context of anxiety, other traits may be influencing these behavioural outputs. The effect of cannabinoid treatment has been considered as decreased emotionality rather than a simple anxiolytic effect (Biscaia et al., 2003). In fact, decreased emotionality has been related to anhedonia and depression (Gorwood, 2008). Notably, depressive-like phenotypes are linked to a differential expression of SUD features (Rappeneau & Bérod, 2017). The forced swimming test, a popular task to identify depressive-like phenotypes and assess antidepressant properties of pharmacological drugs (Slattery & Cryan, 2012), consistently showed depressive-like symptoms in adult male and female rats after adolescent (Rubino et al., 2008; Realini et al., 2011; Zamberletti et al., 2012; Cuccurazzu et al., 2018) and adult (Bambico et al., 2010) THC treatment, although in one study no significant effects were evident after adolescent WIN55-212,2 administration (Abush & Akirav, 2013). This blunted emotionality may contrast with some of the results in sucrose consumption/preference tests, although the complexity of reward processing impairment observed in subjects with depression-like symptoms may be considerably underestimated and these tests may not differentiate adequately between motivational and consummatory types of anhedonia (Thomsen, 2015).

Lastly, some lines of research have underlined the role of many different memory systems (not just reward and aversion-related learning) in SUDs and compulsive behaviours (Goodman & Packard, 2016). In this respect, adolescent cannabinoid treatments have generally seen to produce working memory deficits, assessed in the spatial working memory of the Y maze paradigm, after protracted periods of withdrawal in both males and females (O'Tuathaigh et al., 2010; Mateos et al., 2011; Rubino et al., 2015; and see Cadoni et al., 2015 for negative results). Deficits in spatial working memory were also witnessed in the Morris water maze (Rubino et al., 2009) and the radial maze (Abboussi et al., 2014). However, these effects may be transient (Abush & Akirav, 2012), which might explain why other studies found no deficits using this latter protocol (Cha et al., 2007; Cha et al., 2006; Higuera-Matas et al., 2009). Interestingly, after adolescent self-administration of WIN 55,212-2, young adult male rats showed better working memory relative to rats that underwent sucrose SA in a delay-Match-to-Sample task (Kirschmann et al., 2017a). Recently, this enhanced working memory after adolescent THC self-administration was confirmed (Stringfield & Torregrossa, 2021a). There is also a large amount of evidence of impairments in short-term memory tasks and it is noteworthy that short-term memory and working memory are closely related terms with the boundaries of which are disputed by some authors (Aben et al., 2012). Still, deficits in the novel object recognition task, usually associated with short-term memory dependent on hippocampal structures, are commonly reported in male and female rodents after different cannabinoid treatments (Higuera-Matas et al., 2015; Renard et al., 2017). Only two studies with a relatively late adolescent cannabinoid treatment (starting >PND40) failed to produce deficits in this task (Cadoni et al., 2015; Schulzet al., 2013), which may indicate a special sensitivity to suffer these alterations in early developmental windows. Similarly, novel place recognition tasks, usually interpreted as spatial memory, showed deficits in male and female rats that underwent adolescent cannabis treatment (Mateos et al., 2011; Renard et al., 2013; Zamberletti et al., 2014; Abela et al., 2019) but results in object location tasks have also shown decreased discrimination index in both males and females (Poulia et al., 2019). However, late adolescent cannabinoid treatments (starting >PND45) produced no deficits (Abush & Akirav, 2013), or progressive recovery of performance with sufficient withdrawal times (Abush & Akirav, 2012). Interestingly, in another study deficits in short-term and working memory were produced exclusively by experimenter administration of WIN 55,212-2, but not if the cannabinoid was self-administered (Kirschmann et al., 2017a and 2017b). The generalization of this effect to other traits presumably affected in animal models has not been extensively explored.

Box 12. eCBS, adolescence and SUDs

Adolescence is an ontogenic process that involves a series of behavioural, physiological and morphological changes that shape the transition from juvenility into youth and adulthood. Although there are evident interspecies differences, adolescence is a common developmental stage among mammalian species (Spear, 2004). During this period, animals reallocate energy resources to enhance survival, sexual selection, and fertility fitness to fully function as adult individuals. Adolescence includes pubertal development, the activation of the neuroendocrine hypothalamic-pituitary-gonadal axis and gonadal maturation, but also a growth spurt, and cognitive and brain maturation (Hochberg & Belsky, 2013). Importantly, some features of eCB signaling make it a key element during development from early embryonic periods all the way to adulthood (Harkany et al., 2007). CB receptors influence the expression of genes encoding proteins involved in cell proliferation, neuronal migration and axon elongation, in addition to neuron-glia cell adhesion molecules (Fernandez-Ruiz et al., 2004). Moreover, CB_1 seems to be critically involved in the transition from synaptogenesis to synaptic communication and it shapes the precise topographic development of neuronal circuits (Harkany et al., 2007; Deshmukh et al., 2007).

The eCBS itself has marked developmental features. After birth, the density of CB_1 receptors doubles before reaching adolescence in most of the brain regions where it is present, with slight regional differences. Binding efficiency also increases until reaching its maximum level during adolescence, thereafter decreasing gradually, first within the prefrontal and limbic regions, and later in sensorimotor areas. This pattern of expression is necessary for the fine-tuning of neural connections. Aging involves a decrease in the expression of CB_1 in the hippocampus, caudate nucleus, substantia nigra and globus pallidus (Belue et al., 1995; Rodriguez de Fonseca et al., 1994; Heng et al., 2011; McLaughlin et al., 1994; Verdurand et al., 2011). Besides the presence of eCB receptors, enzymes and ligands in the gray matter (neuron cell bodies and glia, dendrites and unmyelinated axons, synapses and capillaries), the CB_1 receptor is also expressed profusely in the white-matter, long-range myelinated axons, especially in early stages of development, although some expression persists in the adult mammalian brain (Romero et al., 1997; Harkany et al., 2007).

There are also marked sexual differences in the expression of CB receptors. During adolescence, levels of CB_1 receptors peak around PND40 in males and PND30 in female rats. Adolescent male CB_1 receptors are less efficient compared to adolescent females, and moreover, females seem to have significantly higher CB_1 and CB_2 mRNA levels in all brain regions (Craft et al., 2013; Rubino & Parolaro, 2011). However, at least within the hippocampus, Marco et al., 2007 obtained a lower CB_1 immunoreactivity signal, and Reich et al., 2009 lower CB_1 density in females compared to males. Noteworthy CB_1 receptor expression and function are also affected by variations in sex hormones, thyroid and growth hormones, and the hypothalamic-pituitary-adrenal (HPA) axis hormones (corticotrophin-releasing hormone and glucocorticoids (Rodriguez de Fonseca et al., 1994; Hillard, 2015; Riebe et al., 2010; Wagner, 2016). Moreover, it is also clear that the presence of CB_1 receptors in the hypothalamus can modulate the hormonal tone of the brain and body. These features are present throughout development but are of special relevance in critical neurodevelopmental periods like adolescence, with several implications for the individual expression of sex differences and disruptions that might entail neuropsychiatric implications, including the modulation of SUDs liability (Viveros et al., 2012).

Endocannabinoid ligands and enzymatic activity undergo changes during development, with higher levels of 2-AG than of AEA, both increasing progressively until birth. After birth, there is a gradual increase in AEA in corticolimbic areas until early adolescence (around PND35 in rats) when it reduces to reach a minimum (around PND45) before subsequently increasing to reach adult levels (PND70). This change is also accompanied by a shift in the opposite direction of the degrading enzyme FAAH (Lee et al., 2013), while NAPE- PLD activity increases drastically between PND15 and PND20, and then increases progressively until reaching adult levels (Morishita et al., 2005). In rats, fetal 2-AG has a remarkably distinct transient peak in the first postnatal days, then increasing until adolescence albeit with a notable attenuation in mid-adolescence (around PND30), and decreasing again to adult levels (after PND50: Fernandez-Ruiz et al., 2000; Meyer et al., 2017; Harkany et al., 2007). The developmental trajectories of DAGL and MAGL are less well-known, but they should follow and influence the levels of 2-AG, and there is a more rapid decrease in MAGL expression after the onset of adolescence (Long et al., 2012). Remarkably, DAGL regulates axon growth and guidance during development, and it is required for the generation and migration of new neurons in the adult brain, presumably in part through its action on CB_1 receptors via 2-AG synthesis (Reisenberg et al., 2012). There are also some sex-specific differences in the metabolism of endogenous cannabinoids that are, to some extent, age-dependent and enhanced during adolescence. For example, adolescent female rats have lower levels of MAGL in the vSTR and amygdala, and higher levels of FAAH in the frontal cortex. Moreover, exposure to stressors during development (maternal deprivation) interacts with the baseline sex differences of cannabinoid enzyme expression (Marco et al., 2014).

2.4. RESEARCH WITH DRUGS OF ABUSE

2.4.1. METHODOLOGICAL ISSUES OF THE GATEWAY EFFECT IN ANIMAL MODELS

Animal models were first introduced to test the gateway hypothesis in 1997, long after the first claims of a gateway hypothesis, the first human studies exploring the gateway effects of cannabis, and the first animal models of addiction and self-administration. A model of nicotine to opioid (fentanyl) gating was developed in 1997 by Klein but never published, although the methods and results are known (Grunberg & Faraday, 2002). In this initial model, passive nicotine treatment (6 mg/kg/day) during adolescence (from PND41 to PND60) led to increased adult fentanyl self-administration in male Wistar rats. This effect disappeared when the nicotine dose was higher (12 mg/kg/day) and surprisingly, this effect was attenuated in a subgroup of stressed males (20 min immobilization per day during fentanyl SA). Female Wistar rats did not change their SA behaviour as an effect of nicotine or stress. Klein argued that pharmacological exposure was sufficient to increase opioid consumption in males but in females, other biological and environmental variables might be necessary to explain human gateway effects.

This approach generates several questions and issues that are worth highlighting to understand the main characteristics of the gateway experimental design with animal models: Are sex differences determinant in the effects of a drug? Does the effect of previous treatment depend on the age of onset? What is an adequate dose? Is an increased SA *per se* an indication of a gateway effect? How do early experiences and personality traits shape the response to a drug? Are Gateway effects generalizable to all drugs or are they drug-specific relationships?

Table 1. Methodological issues

GATEWAY DRUG AGE:	YOUTH	ADULT
GATEWAY DRUG REGIME:	CHRONIC	ACUTE
GATEWAY EFFECT LAPSE:	LONG TERM	SHORT TERM
GATEWAY EFFECT TEST AGE:	ADULT	ADOLESCENT
DRUG ADMINISTRATION	PASSIVE	ACTIVE
DRUG COMBINATIONS:	SEQUENTIAL	OVERLAPPING
DRUG PROGRESSION:	FORCED	VOLUNTARY
INDIVIDUAL DIFFERENCES:	Effect of the GATEWAY drug (IV)	Modulates GATEWAY effect (DV)

*IV, Independent variable; DV, Dependent Variable.

Epidemiological characteristics of human drug progression studies are methodological features that animal models must cover. Especially because further from being independent features with known and stable effects, there seems to be a high degree of interaction between them. Table 1 Summarize a list of the most relevant methodological features in animal models that are also present in the current experimental design.

Treatment effects, and thus the possible gateway effects, can vary in relation to the age of onset, and usually but not exclusively, gateway effects have been studied in adolescent animals. This overrepresentation of early ages aims to represent the human epidemiological evidence showing that first contact with drugs often occurs during this period, and that there age-dependent drug effects with adolescence usually linked to more detrimental outcomes and interference with important developmental processes. Furthermore, this increased vulnerability in humans exists in the most common animal models (Whyte et al., 2018). In this sense, alcohol, nicotine and cannabinoids, three classic gateway drugs, have a higher potential to induce changes in rodent models when drug exposure is during adolescence rather than in adults (Spear, 2016). Similarly, the effects of the gated drug might differ if tested during adolescence or during adulthood (Dow-Edwards & Izenwasser, 2011). Adolescence *per se* may represent a differential risk for SUDs and potentially, both drugs can be tested during the same developmental stage or sufficiently well spaced to be tested in different developmental stages.

In this sense, if we are able to find a long-term gating effect in adults after adolescent exposure, this effect should presumably, although not conclusively, also be present or even enhanced in adolescence. However, more importantly, if we didn´t find a gating effect in adults we could not affirm that there is no gateway effect because it may be present in adolescence. Thus, the gateway effect lapse, i.e. the time elapsed between exposure to the gateway and the following drug, is another major source of variation that can help discriminate

how long-lasting are the changes produced by gateway drug exposure. Gateway effect lapse can be roughly and loosely divided into short and long-term effects. Long-term effects are aimed to ensure a total clearance of the drug and the recovery from its residual or transient effects after exposure. Short-term effects involve testing that can be potentially performed in parallel with the administration of the gateway drug, immediately after or after a short period of clearance. Importantly some cannabis-induced effects may be transient and the potential gateway effects might diminish over time.

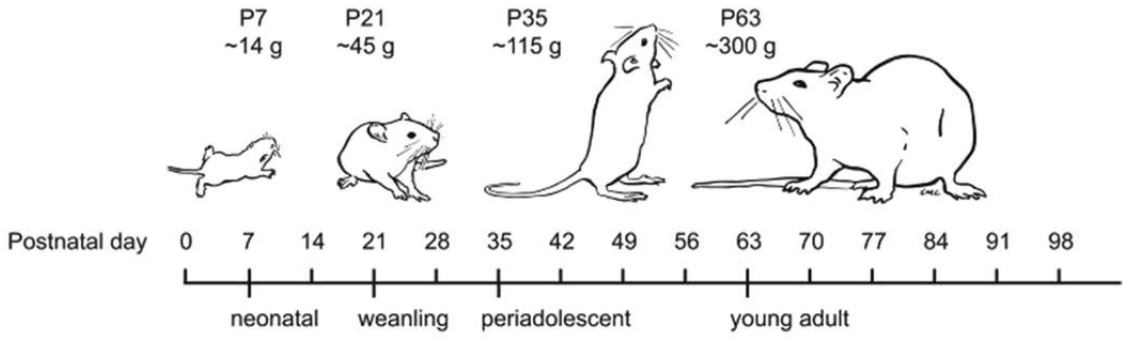


Figure 6. Correlation of body weight with different phases of postnatal days (taken form (Sengupta, 2013). Although there is some discrepancy in terms of the existence of adolescence itself and the exact age boundaries of this developmental period in rodents or other animal models, it is generally accepted that the rat peri-adolescence period begins at approximately postnatal day 28 and ends after postnatal day 40 (Spear & Brake, 1983).

Another important variable is how the progression from one drug to another occurs. In a human context, initiation of the use of one drug usually leads to a change in the exposure to and availability of other drugs, and sources of reward. In gateway animal models, the progression from one drug to another is usually more restricted in diverse ways. In gateway animal models to date, the experimenter's control and select one gateway drug the effects of which are tested with another selected drug, according to the research questions to be addressed and the methodological instruments available. Thus, progression is always imposed. Future gateway animal models could explore this issue by applying different drug-choice paradigms in the selection and progression of drugs and rewards. Moreover, humans usually combine the use of drugs and frequently, there is an accumulation of drug use patterns or at least some degree of overlap in the progression of drug use. By contrast, animal models tend to clearly separate the exposure/consumption of drugs. This clear separation of both drugs helps isolate the effect of the gateway drug. However, future gateway animal models could explore this concurrent use and interactive effects of drugs that are missed when drug exposure is sequential. In this sense, overlapping use of two different drugs, simultaneously or successively, can have a combined effect that can be even more rewarding than each one of them in isolation. For example. human drug users combine alcohol and cocaine, which generates coca-ethylene, a psychoactive metabolite with an effect on reward systems (Pennings et al., 2002), or alternatively, they may consume cannabis after stimulants to offset some of the possible unpleasant subjective effects, such as anxiety and paranoia (Tambaro & Bortolato, 2015).

The administration of both the gateway drug and the gated drug can be achieved in a passive or active fashion. Active administration entails a higher face validity but it is not always possible or necessary to answer a specific experimental question. However, active self-administration is best suited to study changes in motivation towards a drug by examining drug-seeking and drug-taking behaviours. Finally, gateway animal models have to deal with individual differences, which can first be considered as an independent variable, determining its influence on the response to drugs and the gateway drug effect. Sex-specific differences are one of the most important variables in this sense. Both animal and human evidence shows a high degree of variability in response to cannabis (Higuera-Matas et al., 2015) and thus, it is important to address this issue in experiments when possible and interpret the results accordingly, being cautious to draw intersex generalizations from the data obtained. Moreover, other differences with a genetic basis, previous psycho-behavioural traits and previous experimental manipulations can be tested as independent variables (Cadoni et al., 2015). In this sense, gateway animal models should also test the weight of environmental exposure to stressors since the human gateway hypothesis revealed an undoubtedly weight of these strains that might even override any correlation between cannabis and subsequent progression and abuse of other drugs (McCutcheon & Watts, 2018). Secondly, individual differences can arise from the procedures as dependent variables and thus, the aim is to measure the

effect of a gateway drug on these variables (with relevance for a possible gateway effect). In this sense, using cannabis as a gateway drug can modify the abuse liability for other drugs through altered neurobiological changes (alterations to the organization and activity of CNS cells), including changes in the gene expression (epigenetic alterations of transcriptional activity), and the expression of psycho-behavioural traits (e.g. reward-learning, impulsivity or stress reactivity).

The model employed in these experiments uses chronic passive exposition to THC during the peri-adolescent period (from PND28 to PND44). The regime of administration was set as 3 mg/Kg, administered intraperitoneally (i.p.) on alternate days (9 injections over 17days), achieving an accumulated dose of 27 mg/Kg in the period of administration. To study the long-term effects of adolescent administration, experiments were performed after a clearance period of around 46 days, approximately around PND90. The cannabis gateway effect was tested in adulthood with a multicomponent cocaine self-administration session. Drug exposure did not overlap at any moment and the shift to the gated drug was imposed for every subject, and other dependent variables were explored. Neurobiological changes were measured with whole-brain magnetic resonance imaging techniques and NAc Shell RNA-seq, and behavioural changes in SUD-related traits related with reward-learning and impulsivity were measured. Besides cannabis exposure, sex was also included as an independent variable to measure and control for the expected sex-specific differences in basal values and its interaction with adolescent THC exposure. See the Materials and Methods section for more details.

The following sections will review the more recent preclinical studies of cannabis gateway effects, placing special interest in those studies aimed at exploring the long-term effects of adolescent exposure.

2.4.2. CANNABIS CROSS-SENSITIZATION WITH OTHER DRUGS OF ABUSE

Several drugs can induce changes in locomotor activity (Meyer et al., 2009; Valjent et al., 2010) and provoke different stereotypies (Sakharov et al., 1989). These responses can be sensitized due to drug exposure, meaning that repeated exposure to the drug leads to an enhanced expression of these outcomes. Far from being an isolated phenomenon, psychomotor sensitization involves neural mechanism, long-term plasticity in DAergic circuits that are involved in motivational and reinforcement processes, potentially influencing SUDs and addictive features (Perrine et al., 2015; Robinson et al., 1982; Robinson & Berridge, 1993). Cannabinoids can induce sensitization of these pathways (De Vries et al., 2002; Ginovart et al., 2012), although the extent and stability of this phenomenon could be weaker and limited compared to other drugs of abuse (Varvel et al., 2007). Nevertheless, it is cross-sensitization, the effect of acquiring a sensitized response to a different stimulus than the one that induced sensitization in the first place, which is an interesting phenomenon from the point of view of the gateway hypothesis. Animal models using cannabinoids as gateway drugs can easily exploit this phenomenon to assess whether this exposure modulates future response to another drug and other psychological processes, such as responses to contextual stimuli like reward-paired cues or stressful conditions, to name some of the most relevant sensitization processes related to SUDs.

Employing adult rats, chronic or acute THC exposure was seen to affect amphetamine sensitization differentially (Gorriti et al., 1999). Acute exposure antagonized amphetamine-induced locomotor responses, while chronic exposure generated tolerance to this effect, although during cannabis withdrawal (only 24 hours) rats showed enhanced locomotor cross-sensitization. Adult rats also showed that THC pre-exposure produced cross-tolerance to the motor-depressant effects of heroin and it did not increase cocaine-induced locomotion but rather, it seemed to enhance the anxiogenic effects of cocaine (Panlilio et al., 2007). This latter effect was also related to the reduced reinforcing value of cocaine observed in THC-exposed rats in a previous experiment. In terms of adolescent exposure, drug-induced locomotor activity could depend on the age of testing (Dow-Edwards & Izenwasser, 2011). Early adolescent (PND34 to 42) cannabis exposure had little effect on locomotor activity but it enhanced cocaine-induced locomotor activity (tested at PND46), while in young adults (PND66 to 74) THC exposure blunted locomotor activity but it had no effect on cocaine-induced locomotor activity (tested at PND78).

Notably, cross-sensitization was tested in these experiments after a short post-treatment period, and thus, residual effects could be contributing to this effect that may not be generalizable to adult individuals. However, cross-sensitization studies employing stimulants have produced contradictory results. Indeed, WIN 55,212-2 or THC treatment (PND28 to 32) did not produce relevant changes in amphetamine-induced

locomotor activity or stereotypic behaviour during late adolescence (PND40) or early adulthood (around PND70: Ellgren et al., 2004). Moreover, reported no change in locomotor stimulation of an acute or repeated cocaine exposure (1, 3.2, 10, 18 mg/kg ip) to adult (PND90) male rats was seen after adolescent THC treatment (1mg/kg ip, PND28 to 45: Friedman et al., 2019). These experiments show that cross-sensitization effects may disappear with enough clearance. However, a stronger locomotor response to amphetamine was recorded in male rats exposed to the synthetic cannabinoid WIN 55,212-2 during late adolescence (PND40 to 65) and tested in adulthood (after PND85: Gomes et al., 2015). Similarly, cross-sensitization to cocaine was present in adolescents but not in adults, pre-exposed to WIN 55,212-2 (from PND42 to 52) after one week of abstinence, and interestingly there was a lack of cross-sensitization from cocaine to cannabinoids (Kononoff et al., 2018; Scherma et al., 2020).

Adolescent cannabis-induced cross-sensitization also interacts with individual differences and genetic backgrounds (Cadoni et al., 2015) as evident when using the addict-prone Lewis rat strain and the resilient Fischer 344 rat strain (Cadoni, 2016). In these animals, a brief 3-day treatment of THC starting around PND40 was associated with an increased DA response to heroin in the NAc Core in Fischer 344 and Lewis rats30 days after the last THC injection, but only in the NAc Shell of Lewis rats. Moreover, all the previous studies were only performed on male rats, even though sex-specific differences had been previously identified (Lee et al., 2014) with females but not males displaying increased amphetamine-induced stereotypies after adolescent treatment with the CB_1 agonist HU-210. Indeed, it remains unresolved whether there is a cross-sensitization effect in females in previous studies that obtained negative results. Thus, there are cross-sensitization effects between cannabis and other drugs that vary as a result of the regime of cannabinoid administration, the clearance period, age and sex.

Drug-induced locomotor activity and stereotypic behavioural studies have some limitations, and they provide little information about the rewarding properties of a drug and of the motivational disposition of the animal. As indicated previously, locomotor activity and stereotypic sensitization share common substrates and mechanistic processes, yet they are not equivalent to other forms of sensitization, like incentive sensitization that is more relevant to SUDs and addictions (Robinson & Berridge, 2008). Other experimental tools and set-ups, possibly entailing a more complex analysis of the behaviour will be addressed below.

2.4.3. DRUG PREFERENCE

Animals exposed to cannabinoids may also show a shift in preferences and hedonic responses to drugs of abuse like opiates and cocaine. In a series of experiments, adolescent THC chronic treatment of mice provoked an increase in cocaine Conditioned Place Preference (CPP) two weeks after the end of the treatment (unpublished data). As for the cross-sensitization effects, the age of cannabis exposure determines the final output and for example, increased cocaine CPP was evident in Zebra finches after adolescent THC treatment but not when the treatment was administered during adulthood (Aldhafiri et al., 2019). Strain differences were also detected in heroin CPP (Cadoni et al., 2015), with Lewis rats expressing more heroin-CPP than Fischer 344 rats, but when exposed to THC during adolescence they were also more vulnerable to priming. Fischer 344 rats also enhanced their heroin CPP, which also made them resistant to extinction. Moreover, early life experiences like maternal deprivation suppress morphine CPP in rats exposed to THC during adolescence, while non-deprived rats with an equivalent adolescent exposure were more sensitive to morphine conditioning (Morel et al., 2009). Shifts in alcohol preference using a two-bottle choice procedure have also shown that CD1 male mice previously habituated to alcohol and co-exposed to WIN 55,212-2 during early adolescence increase alcohol intake after ACE in a sustained way that is still significant during adulthood (Frontera et al., 2018). Recently, it was shown that male and female Long-Evans and Wistar rats chronically administered THC after the onset of puberty did not exhibit a clear enhanced preference to subthreshold doses of d-amphetamine (0.5, 0.7 and 1 mg/Kg: Keeley et al., 2018). In addition, the authors saw no differences in NAc and DH cFos expression, which led them to conclude that THC might not necessarily sensitize the response to all drugs of abuse.

Table 2. The influence of PEACE on cross-sensitization and drug preference. The days of cannabinoid treatment are indicated by vertical green lines, and the test days are indicated by a vertical lines with different colours associate to the results obtained: blue (decreased liability towards Substance Use Disorders (SUDs), black (No Significant Effects / NSE) or red lines (enhanced liability towards SUDs). SD, Sprague-Dawley rats; LE, Lewis rats; MD, Maternal deprivation; F344, Fischer344 rats; PND, Post Natal Day.

Although drug CPP is usually understood as a result of drug reward, the paradigm also has some limitations, and it has been subjected to different interpretations that make a straightforward translation to the study of SUDs in humans difficult (Tzschentke, 2007). Tools that allow the animal to actively choose the drug can provide a more natural understanding of the behavioural and motivational effects of a drug.

Box 13. Place Preference Conditioning

In 1953, somewhat accidentally, James Olds and Peter Milner observed that when activating an electrode located in the septal area of a rat, the animal started to spend more time in the area where the experimenters had triggered the current. This preference phenomenon occurred in an open field, but it was the reasoning behind the modern place conditioning test: animals seem to spend more time in environments associated with positive experiences and less in environments associated with aversive consequences.

Modern Conditioned Place Preference (CPP) apparatus are divided into different compartments with different perceptual cues. Protocols usually expose the animal several times to a stimulus (e.g. cocaine, THC, social interaction) in one of the compartments (using the other as a control) and then test preference by letting the animal walk freely between the compartments. After repeated free exploration sessions in the absence of the previous stimulus, animals can extinguish the preference. However, re-exposition to the original stimulus can prime the preference (or aversion) for one of the compartments again. Although drugs can induce CPP, the phenomenon entails different confounding factors and a distinct interpretation. Thus, it is not seen as a valid model of addiction and its better viewed as a test. Nonetheless, preference is usually interpreted as a reinforcement index, and it is a useful test to examine neural circuits involved in drug-related responses (Bardo & Bevins, 2000).

2.4.4. DRUG SELF-ADMINISTRATION

Animal models including drug SA of the cannabinoid itself or of the gated drug increase the face validity dramatically, although SA is usually limited to the gated drug. Several studies have shown that exposure to cannabinoids during adolescence may increase opioid SA. In male rats treated with THC during adolescence (PND28 to 49) and tested in late adolescence (PND57), higher intake with low doses of heroin, and enhanced acquisition and maintenance of heroin SA, was reported (Ellgren et al., 2007). These were reproduced and extended showing increased heroin SA in male rats one month after the last THC injection (Tomasiewicz et al., 2012). However, there are also negative data as elsewhere no difference in heroin acquisition after adolescent THC treatment (PND35 to 46) was detected when tested one month after the last THC dose (Stopponi et al., 2014). Moreover, no preference shift in oral morphine consumption was detected elsewhere (Biscaia et al., 2008; Morel et al., 2009; Nguyen et al., 2020). Regarding psychostimulants, acquisition of cocaine self-administration was seen to be weaker in rats after adolescent WIN 55,212-2 exposure than in controls, yet no other differences were seen in subsequent phases (Kononoff et al., 2018). Differences were evident in the acquisition of cocaine SA in adult animals that also underwent an adolescent WIN 55,212-2 treatment (Friedman et al., 2019), however the effect was only present with the lower dose of cocaine (0.1 mg/kg/infusion) whereas a four-fold higher dose (0.5 mg/kg/0.1 ml) had been used in the earlier study (Kononoff et al., 2018). Thus, it seems that adolescent THC exposure can render animals more sensitive to variations in the dose of cocaine.

Again, individual differences related to sex have been determinant in the studies that used the drug SA. In two different experiments carried out using CP 55,940 during adolescent treatment, sex-dependent responses towards two different drugs were described. In one males but not females with adolescent CP-55,940 had higher morphine SA (Biscaia et al., 2008), while in the other females but not males previously treated with CP-55,940 showed facilitation for early cocaine SA acquisition (Higuera-Matas et al., 2008), although differences in SA disappeared in the maintenance phase. Other sex-specific differences were seen when adolescent exposure to THC in vapor chambers had no impact in oxycodone SA in either sex, yet females but not males had increased fentanyl SA (Nguyen et al., 2020). Interestingly, females also developed tolerance to some THC-induced effects more rapidly.

The acquisition and maintenance of drug SA, especially under low demanding fixed-ratio schedules, does not portrait the key features of SUDs, although it might be associated with other manifestations like increased motivation to seek and consume drugs, or higher susceptibility to relapse. Protocols of drug SA can be modified to identify and quantify the expression of different components of SUDs. These measures exceed the aims of the original formulation of the gateway hypothesis, which does not attempt to assess abuse liability but simply, the passage from one drug to another. However, human research has progressively included

enhanced abuse liability within the gateway hypothesis (in addition to the sequential evolution and the probabilistic association) and causality is hard to identify in epidemiological studies. Thus, animal models inspired by the cannabis-gateway hypothesis have also incorporated these features.

Box 14. Drug Self-Administration

The introduction of active administration, namely self-administration, enhanced the ecological and construct validity of the animal models and tests for studying SUDs. Behaviourally self-administration can be further divided into operant and non-operant. In the non-operant procedures the experimental subject is given access to the drug without the need for a previous response. As early as 1926, Carl P. Richter investigated the non-operant oral consumption of alcohol by rats in the context of caloric intake studies. Some years later, Richter & Campbell (1940) presented studies of solution preference in the free-choice bottle model, which was later incorporated in formal addiction studies (Sala et al., 1993). Conversely, operant procedures imply an active response or a chain of different responses (second-order schedule) to enable drug access or consumption. James Olds pioneered another key advance in SUD animal models of research, operant drug self-administration procedures, presenting the first set-up that allowed a rat to self-administer drugs directly into the nervous system (Olds & Olds, 1958). Nonetheless, the most successful and extended method of operant drug self-administration was developed by James Weeks (1962), intravenous (iv) self-administration, which was used to serially test a diverse spectrum of common drugs. This approach showed that drugs of abuse induced (with few exceptions) self-administration behaviour in animals, indicating that these types of experiments can be used for the early assessment of abuse liability (Collins et al., 1983).

Operant conditioning procedures, especially those performed in Skinner boxes, employ a wide variety of reinforcement schedules and set-ups. The simplest and straightforward programs use Fixed Ratio (FR) schedules in which an outcome is issued after a pre-selected (fixed) number (ratio) of responses are completed. Using drugs as reinforcers, these schedules are sufficient to assess the potential abuse liability of a drug because of its unconditioned psychopharmacological effects and individual differences on the acquisition and maintenance of drug self-administration. Operant conditioning set-ups usually count on distinct manipulandum. For example, in a first-order schedule, one manipulandum can be associated contingently with the drug (active), while the other may remain without consequences (inactive), which allow the experimenter to assess discrimination, preference, and inspecific motor behaviours. Notably, there is usually a time-out period after the completion of the schedule to prevent overdosing and incidental activation of the manipulandum, although it could also serve as an index of drug-seeking as a consequence of prevented drug access. In addition, the presence of different cues during operant conditioning sessions can provoke the establishment of associative learning if and when they are contingently linked with delivery (conditioned stimulus) or availability (discriminative stimulus).

2.4.5. ADDICTION LIKE BEHAVIOURS

Cannabinoid exposure during adolescence does not appear to increase motivation for drug consumption when measured by Progressive ratio (PR) schedules. Adolescent exposure to WIN55,212-2 did not generate differences in cocaine PR schedules (Kononoff et al., 2018) and likewise, no differences in PR responses to cocaine were evident after adolescent THC treatment (Friedman et al., 2019). Moreover, this lack of effect seemed to extent to other drugs as adolescent cannabinoid treatment had no effect in PR responding for morphine (Biscaia et al., 2008). Recently, no differences in PR responses to oxycodone or fentanyl were observed after adolescent THC consumption (Nguyen et al., 2020), although punished drug intake, and other types of compulsive drug-seeking and intake, have not yet been addressed in gateway studies. Regarding the escalation of drug intake, adolescent cannabinoid exposure does not seem to produce changes, as addressed for the escalation of cocaine (Kononoff et al., 2018), oxycodone and fentanyl intake (Nguyen et al., 2020). Moreover, no difference in extinction learning was found between experimental groups when heroin infusions were prevented (Stopponi et al., 2014). Finally, some forms of relapse may be altered after ACE, with no clear differences in stress-induced heroin reinstatement but increased sensitivity to cue-induced reinstatement reported (Tomasiewicz et al., 2012). However, the stressor used was food deprivation (Tomasiewicz et al., 2012), whereas elsewhere yohimbine was used (an α-2 adrenoceptor antagonist that increases noradrenaline release and induces anxiety), which did produce increased reinstatement of heroin-seeking (Stopponi et al., 2014). The interaction between ACE and response to stressors is highly relevant since stressful environments may enhance SUD vulnerability (Fouyssac et al., 2020), and this may inspire new research approaches to assess the gateway effect of ACE. Current evidence on PEACE points to a THC-mediated modulation of the process involved in reinstatement of opioid seeking in a stressor-specific manner, yet no studies to date have addressed cocaine-seeking in conditions of abstinence. Nonetheless, the anxiogenic effects of cocaine abstinence were prevented in mice exposed to WIN55,212-2 in adolescence, whilst they displayed enhanced depressive-like symptoms in adulthood (Aguilar et al., 2017).

During the development of SUDs, individuals may have differential access to drug and non-drug reinforcers, which demand decision-making processes to select different paths of action. New methods have been developed in the last decades to address this ecological reality in the field of addiction, such as choice procedures (Ahmed, 2012) and voluntary abstinence (Venniro et al., 2020). Moreover, SUDs are a phenomenon that cannot be detached from social interaction (Skog, 2005; Venniro et al., 2018). Interestingly, eCBS signalling modulates social behaviour (Wei et al., 2017) and there is evidence that PEACE is involved in social anxiety (O'Shea et al., 2004, 2006; Quinn et al., 2008; Realini et al., 2011). However, approaches that allow the study of volitional preference shifts toward drugs of abuse over non-drug rewards, a central feature underlying the gateway hypothesis, remain unexplored in gateway animal models.

Box 15. Addiction-like behaviours in animal models

The reinforcing value, the amount that individuals are willing to work for a reinforcer, may not be fully assessed on fixed-ratio schedules of drug intake, especially those of low demand. Thus, the core feature of a SUD, the perseverance in substance use despite significant substance-related problems, can only be guessed. Distinct motivational aspects of drug-reinforced behaviour can be inferred by changing the reinforcement schedules and the associated contingencies. For example, the implementation of second order-schedules, where the completion of an initial reinforcement schedule starts a second schedule (usually signaled by conditioned cues) that ultimately leads to drug consumption, are useful to differentiate pharmacological effects from drug-paired stimuli (Everitt & Robbins, 2000). However, despite the value of these protocols, other manipulations are more popular in SUD research. Elevated and increasingly demanding ratios of reinforcement are better suited to measure motivation for consumption than fixed-ratio schedules (Bentzley et al., 2013). Remarkably the maximum effort in these settings is usually equivalent to the breaking point (maximum response requirement achieved) in progressive ratio schedules, where the effort to obtain a reinforcer is arithmetic or augments exponentially after each reinforcement (Kuhn et al., 2019). In addition, drug-taking may have pervasive consequences for humans, although SUDs overcome the co-occurrence or probability of those, a feature that may not be completely modeled by positive reinforcement. Consequently, the inclusion and pairing of aversive consequences (e.g. foot-shocks) with drug-seeking or taking can be used to test the compulsive aspects of SUDs (Vanderschuren et al., 2017).

The conditions or patterns of access and the time spent consuming a drug of abuse are risk factors for developing SUDs. Consequently, manipulating these variables also helps to increase ecological validity and remarkably, to generate SUD-like phenotypes. Long-term exposure (between 10 and 30 days but up to 3 months), extended access sessions (e.g. 6 hours or more) and intermittent access (intercalating drug-available and no-drug-available periods within a session) are known to increase motivation for drug-taking, producing an escalation of drug intake and generating compulsive-like behaviours (Ahmed, Walker, & Koob, 2000; Edwards & Koob, 2013; Vanderschuren & Everitt, 2004; Zimmer et al., 2012). It is worth mentioning, despite the value of these models over recent years, that operant choice procedures allow subjects to explore and exploit alternative reinforcers and they are becoming useful tools to assess resource misallocation in translationally relevant scenarios (Banks & Negus, 2017).

A key feature of human SUD is the high rate of relapse and the resumption of high-risk patterns of drug abuse after a drug-free period. Several animal models have tried to capture this phenomenon and study the incubation of drug craving, a time-dependent increased desire for drug consumption over a period of protracted abstinence from drug-taking. As interoceptive features cannot be easily assessed in animal models, the term incubation of drug-seeking is preferred. Reinstatement in a drug-seeking model implies using several extinction training sessions where instrumental responses are no longer reinforced, to later test different forms of reinstatement of drug-seeking (with drug-taking prevented) by drug-paired cues, stress preexposure, drug priming and in withdrawal states. Similarly, reacquisition models implement a period of extinction but during the test session, the outcome is made available again. Alternatively, relapse models assess changes in drug-seeking (also under drug-free conditions) after or across a period of forced abstinence that allows the time-dependent assessment of the evolution of drug-seeking. Moreover, relapse models have incorporated phases of voluntary abstinence induced by alternative reinforcers or imposed by pairing negative consequences with drug-seeking to force abstinence (Venniro et al., 2016).

reference	Subjects / Strain	Adolescent Cannabionoid Exposure		Results		
		Agent	Regime of administration	PND	Test	Cannabinoid effect
ACQUISITION OF DRUG SELF-ADMINISTRATION			20 25 30 35 40 45 50 55 60 65 70 75 80 85 90 +			
Ellgren et al., 2007	LE ♂	THC 1.5 mg/kg i.p. THC 1.5 mg/kg i.p.		PND57 PND57	Heroin SA (30 µg/kg), FR-1 Heroin SA maintenance (30 and 60 µg/kg)	↑Acquisition ↑ Responding and intake at different doses
Biscaia et al., 2008	WR ♀/♂	CP-55,940 0.4 mg/kg, (2 ml/kg) i.p.		PND 70	Morphine SA (1 mg/kg); FR-1	↑♂ Fixed Ratio
Higuera-Matas et al., 2008	WR ♀/♂	CP-55,940 0.4 mg/kg i.p. CP-55,940 0.4 mg/kg i.p.		PND75 PND75	Cocaine SA (1 mg/kg); FR-1; 30min session Cocaine SA (1 mg/kg); FR-1; 120min session	↑♀ on acquisition NSE on maintenance
Tomasiewicz et al., 2012	LE ♂	THC 1.5 mg/kg i.p.		PND77	Heroin SA (30 g/kg); FR-1	↑Acquisition
Stopponi et al., 2014	Wistar ♂	THC 2'5, 5 & 10 mg/kg i.p. twice		PND75	Heroin SA (20µg/0.1ml); FR-1	NSE
Scherma et al., 2016	LH ♂	THC 2.5, 5, 10mg/Kg i.p.		PND 70	WIN55,212-2 SA (12.5 µg/100µl); FR-1	↑ Intake
Kononoff et al., 2018	SD ♂	WIN55,212-2 2, 4 and 8 mg/kg i.p.		PND 71	Cocaine SA (0.5 mg/kg i.v.); Short Access	↓ Acquisition (delayed)
Friedman et al., 2019	SD ♂	THC 1mg/kg i.p. THC 1mg/kg i.p.		PND90 PND90	Cocaine SA (0.32 mg/kg) ; FR-1 to 5 Cocaine SA (0.1 mg/kg)	NSE on aquisition ↑ Responding
DOSE RESPONSE			20 25 30 35 40 45 50 55 60 65 70 75 80 85 90 +			
Ellgren et al., 2007	LE ♂	THC 1.5 mg/kg i.p.		PND57	Heroin SA; Dose response curve (7.5 to 100µg/kg)	↑ Response and intake at different doses
Tomasiewicz et al., 2012	LE ♂	THC 1.5 mg/kg i.p.		PND77	Heroin SA; Dose response curve (7.5 to 100 µg/kg)	↑ Response and intake at different doses
Friedman et al., 2019	SD ♂	THC 1mg/kg i.p.		PND90	Cocaine SA; Dose-effect curves (32 µg/kg to 1.0 mg/kg)	↑ Responding for lower doses
Nguyen et al., 2019	Wistar ♂/♀ Wistar ♀	THC 100mg/mL v.i. 30min/2*day THC 100mg/mL v.i. 30min/2*day		PND112 PND112	Oxycodone SA; Dose substitution (0.006 to 0.15mg/kg) Fentanyl SA; Dose substitution (0.625 to 10 µg/kg)	NSE ↑(♀) especially at the lowest dose
EXTENDED ACCESS			20 25 30 35 40 45 50 55 60 65 70 75 80 85 90 +			
Kononoff et al., 2018	SD ♂	WIN55,212-2 2, 4 and 8 mg/kg i.p.		PND91	Cocaine SA (0.5 mg/kg) Escalation (6h Sessions)	NSE
Nguyen et al., 2019	Wistar ♂/♀	THC 100mg/mL v.i. 30min/2*day		PND112	Oxycodone SA (0.15 mg/kg); FR-1; 8h Sessions	NSE
MOTIVATION FOR DRUG SEEKING AND EXTINCTION			20 25 30 35 40 45 50 55 60 65 70 75 80 85 90 +			
Biscaia et al., 2008	Wistar ♀/♂	CP-55,940 0.4 mg/kg, (2 ml/kg) i.p.		PND 70	Morphine SA (1 mg/kg); Progressive Ratio (1 mg/kg)	NSE on Progressive Ratio
Stopponi et al., 2014	Wistar ♂	THC 2'5, 5 & 10 mg/kg i.p. twice		PND75	Heroin Seeking extinction	NSE
Kononoff et al., 2018	SD ♂	WIN55,212-2 2, 4 and 8 mg/kg i.p. WIN55,212-2 2, 4 and 8 mg/kg i.p.		PND90 PND114	Cocaine SA (0.5 mg/kg) Progressive Ratio Cocaine SA (0.5 mg/kg) Progressive Ratio (after escalat	NSE NSE
Nguyen et al., 2019	Wistar ♂/♀	THC 100mg/mL v.i. 30min/2*day		PND112	Oxycodone SA (0.15 mg/kg); Progressive Ratio	NSE
Friedman et al., 2019	SD ♂	THC 1mg/kg i.p.		PND90	Cocaine SA (0.1 or 0.32 mg/kg) Progressive Ratio	NSE
RELAPSE AND REINSTATEMENT OF DRUG SEEKING			20 25 30 35 40 45 50 55 60 65 70 75 80 85 90 +			
Tomasiewicz et al., 2012	LE ♂	THC 1.5 mg/kg i.p. THC 1.5 mg/kg i.p.		PND77 PND77	Cue-induced heroin seeking Stress-induced heroin seeking	↑ Seeking responses NSE on Seeking responses
Stopponi et al., 2014	Wistar ♂	THC 2'5, 5 & 10 mg/kg i.p. twice		PND75	Yohimbine-induced heroin reinstatement	↑ Seeking responses

Table 3. Results of PEACE drug self-administration and addiction-like behaviours. The days of cannabinoid treatment are indicated by vertical green lines, and the test days are indicated by a vertical lines with different colours associate to the results obtained: blue (decreased liability towards Substance Use Disorders (SUDs), black (No Significant Effects / NSE) or red lines (enhanced liability towards SUDs). SD, Sprague-Dawley rats; LE, Lewis rats; MD, Maternal deprivation; F344, Fischer344 rats; PND, Post Natal Day.

HYPOTHESIS AND GOALS

In the present work, we have carried out a wide-ranging and multiparametric evaluation of the protracted effects of cannabinoid exposure in the developing adolescent brain, with particular emphasis on reward processing and the potential of cannabis to act as a gateway drug leading to cocaine addiction later in life.

Four main objectives were established:

1. To explore changes induced by chronic THC treatment in the developing adolescent brain with neuroimaging techniques.

   (see introduction section 2.2.1)

2. To explore how chronic THC treatment during adolescence affects reward-processing behavioural outcomes and behavioural traits related to addiction.

   (see introduction section 2.3.3 and 2.3.4)

3. To apply a cocaine self-administration program that discriminates different prototypic behavioural patterns of drug addiction.

   (see introduction section 2.4.4 and 2.4.5)

4. To carry out a survey of transcriptomic changes triggered by adolescent THC treatment within the reward system.

   (see introduction section 2.2.2 and 2.2.3.)

The working hypotheses are:

1. THC treatment during adolescence will reproduce changes observed in human MRIs, such as impaired myelinisation and to a lesser extent, volumetric alterations to the temporal lobe and subcortical structures.

2. The protracted effects of adolescent cannabis exposure will interfere with reward-processing

   2.1. The protracted effects of adolescent cannabis exposure will increase instrumental actions under the influence of reward-predictive stimulus in a Pavlovian to instrumental transfer protocol.

   2.2. The protracted effects of adolescent cannabis exposure will increase impulsivity-related measurements in a waiting impulsivity task.

   2.3. The protracted effects of adolescent cannabis exposure will increase the incentive salience of a reward conditioned stimulus in a Pavlovian-conditioned approach task.

   2.4. The protracted effects of adolescent cannabis exposure will increase stimulus-response learning in a habit-forming instrumental protocol.

3. The protracted effects of adolescent cannabis exposure will modulate cocaine addiction-like features with an enhanced impact on adult females exposed to THC during adolescence.

4. The protracted effects of adolescent cannabis exposure will affect the transcriptomic profile in the NAc shell. We expect changes in genes and gene ontologies that affect: components of nervous system cells, especially cytoskeletal elements, dendrites and axons; biological processes involved in development and signalling; and molecular functions related to transcriptional activity. We expect these changes to be related to the neurobiological basis of substance use disorder, including modulation of the DAergic system.

MATERIALS AND METHODS

1. ANIMALS

These studies were carried out on Wistar albino rats obtained from Charles-River S.A. (Saint-Germain-sur-l'Arbresle, France) were mated (one male per one female) at the university bioterium 2 weeks after their arrival. A total of 32 litters from different progenitors were used to establish the experimental groups. After birth, the litters were sex-balanced (between PND0 and PND1) and culled to a litter size of 10 ± 2 pups per dam. The animals were weaned at PND22 and placed in different cages of 2 or 3 sibling animals for each experimental group (sex and treatment). All animals were maintained at a constant temperature (20±2 ºC) under a reverse 12 h/12 h light/dark cycle (lights on at 20:00 h), with free access to food and water (commercial diet for rodents A04/A03; Panlab, Barcelona, Spain), unless otherwise specified at the beginning of some of the experimental procedures. Importantly, all efforts were made to minimize the pain and discomfort of the experimental animals, and all the procedures were conducted in accordance with the European Union legislation on the protection of animals used for scientific purposes (2010/63/EU Directive) and they were approved by the Ethics Board of the Universidad Nacional de Educación a Distancia.

2. ADOLESCENT THC TREATMENT

Δ9-Tetrahydrocannabinol (THC) was purchased from THCPharm (Frankfurt, Germany) as resin and dissolved in pure ethanol (Merck). The THC-Ethanol solutions were aliquoted into opaque vials, which were filled with nitrogen to avoid oxidation and stored at -20 ºC. Pure ethanol was similarly aliquoted in other vials and stored. On the treatment days the final solution was prepared by adding kolliphor (PEG-35 castor oil; Merck) and saline (0.9% NaCl solution; Vitulia, Spain) in a 1:1:18 proportion. The adolescent chronic THC treatment was performed every other day from PND28 to PND44. Animals received an intraperitoneal injection (2mL/kg), which delivered a dose of 3 mg/kg THC to the treatment groups. This THC dose is considered mild and non-aversive, although it can produce neurochemical changes in synaptic plasticity in brain regions involved in reward learning (Mato et al., 2004, 2005). The equivalency of this dose in human patterns of consumption would be similar to smoking one or two marihuana cigarettes (assuming a THC concentration around 8%). Regarding the ethanol exposure in both the THC and vehicle solutions, the concentration in the total volume is 5%, thus animals received a dose of approximately 0.0789 gr/kg that does not induce significant behavioural effects.

3. OVERVIEW OF EXPERIMENTS

After the chronic adolescent treatment, all rats were left undisturbed in their home cages with food and water available ad libitum. Animals from different litters were assigned to 5 different experiments to increase genetic variability within each experiment. All experiments began around PND90: experiment 1 refers to MRI studies; experiment 2 started with PIT and ended with the 2CSRTT; experiment 3 started with PCA and ended with an operant-conditioning protocol to study habit formation; experiment 4 consisted of a multicomponent cocaine self-administration protocol; and in experiment 5, we obtained NAc Shell samples to conduct a RNA-seq study.

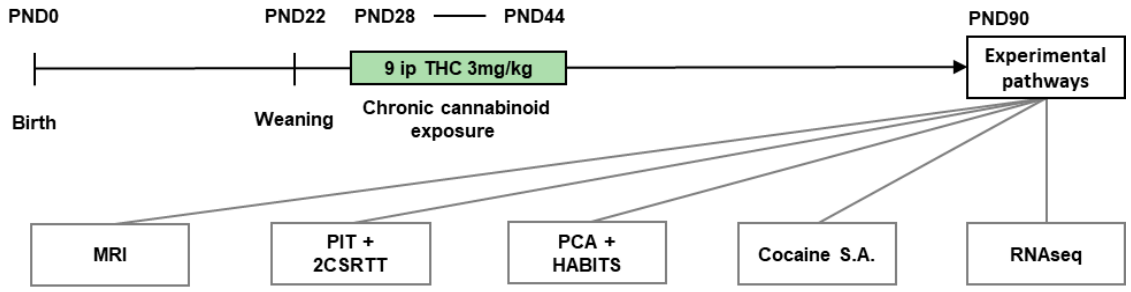


Figure 7. Timeline of Adolescent THC treatment and subsequent experiments during adulthood.

4. EXPERIMENT 1: MAGNETIC RESONANCE IMAGING

At PND80 a total of 12 VEH animals (5 males and 7 females, controls treated with the vehicle alone) 16 THC animals (9 males and 7 females) were transferred to another bioterium in the facilities of the Instituto de Investigaciones Biomédicas. The rats were kept in a room isolated from other animals for at least one week to acclimatize and reduce the stress provoked bythe novel environment and transportation. After this acclimatizing period rats underwent the MRI and spectroscopy studies. The MRI experiments were performed using a Bruker Pharmascan system (Bruker Medical Gmbh, Ettlingen, Germany) using a 7.0-T horizontal-bore superconducting magnet, equipped with a 1H selective quadrature 40mm coil and a Bruker gradient insert with a 90 mm diameter (maximum intensity 36 G/cm). All data were acquired using a Hewlett-Packard console running Paravision 5.1 software (Bruker Medical Gmbh) operating on a Linux platform.

Box 16. Magnetic Resonance

Magnetic Resonance (MR) is the phenomenon by which particles respond to the application of magnetic fields by absorbing and emitting electromagnetic radiation. The discovery and application of this phenomenon dates back to 1944 when the soviet physicist, Y.K. Zavoysky first observed electron-spin resonance, and subsequently, a group of USA physicists observed proton magnetic resonance (also known as Nuclear Magnetic Resonance) for the first time in 1946. Magnetic-resonance devices apply a strong magnetic field that superimposes the weak magnetic field intrinsically produced by the spin of the particles, causing the particle to align with the strong magnetic field. When this synchronization happens a steady force acts in a unified direction. This is called resonance. A radiofrequency pulse, a rapid change in the amplitude of the magnetic radiofrequency, is then introduced to make the particle spin out and when the pulse disappears, the particles release energy and return to the equilibrium (resonance state).

Applied to organisms, MR permits the non-invasive exploration of tissues. In this sense, the most common MR Imaging (MRI) set-up takes advantage of the changes in energy released by hydrogen atoms in MR devices, which are simultaneously scanned by antennas and digitalized to reconstruct an image. Since hydrogen atoms are very abundant and at the same time differentially expressed, the signal obtained provides valuable spatial information of the structures (composition and shapes) of the areas explored. MRI protocols use different configurations or MRI sequences, particular sets of pulse sequences and gradients that provoke know variations in the amount of energy released by the protons or the time they take to realign as a function of the specific chemical composition of the molecules within a given tissue. Using specific setups, MR devices can also be employed to measure levels of different metabolites, small molecules of biological relevance. This application is known as Magnetic Resonance Spectroscopy (MRS).

Within 5 days of counterbalancing the groups all the rats were tested. Animals were anesthetized with a 2% isoflurane-oxygen mixture in an induction chamber and the flow of anaesthetic gas was constantly regulated to maintain a heart rate of 50 ± 20 bpm. The animals were placed into the centre of the volume radio frequency (RF) coil and positioned in the magnet under continuous inhalation anaesthesia via a nose cone. A respiratory sensor connected to a monitoring system (SA Instruments, Stony Brook, NY) was placed under the abdomen to monitor the rate and depth of respiration.

T2-weighted (T2-W) spin-echo anatomical images were acquired with a rapid acquisition with relaxation enhancement (RARE) sequence in axial and coronal orientations applying the following parameters: TR, 3000 ms; TE, 44 ms; RARE factor, 8; Av, 3; FOV, 3.5 cm; acquisition matrix, 256 × 256 corresponding to an in-plane resolution of 136 × 136 μm^2; slice thickness, 1.50 mm which produced a total of 18 slices for axial and 8 for coronal images. Volumetric analyses were made by manually selecting the region of interest (ROI) of each anatomical image and then calculating the area with Image J software. All measurements were obtained blind to the animal's experimental group to avoid possible bias. For statistical analysis, total brain volume and he relative regional area or volume were calculated for the: Striatum (STR), Nucleus Accumbens (NAc), Hippocampus (HIPP), Cortex (Cx), Globus pallidus (GP), Thalamus (THA), Amygdala, Septal Nuclei (SNu), and Cerebellum (Ce). In addition, the volume occupied by each ventricle and the total ventricular volume, relative to both the total and regional brain volume, were also calculated.

Diffusion-weighted images were acquired with a spin-echo single-shot echo-planar imaging (EPI) pulse sequence using the following parameters: Repetition Time and Echo Time (TR/TE) 3500/40ms; averages 1; diffusion gradient duration 3.5 ms; diffusion gradient separation 20 ms; gradient directions 7; two b values (100 and 1400 s/mm^2); slices thickness 1.5 mm without a gap. All the EPI data were acquired with a single-shot EPI sequence, a 96 x 96 matrix and a zero-filled in k space to construct a 128 x 128 image matrix corresponding to an in-plane resolution of 273 x 273 μm^2. Fractional anisotropy (FA), mean diffusivity (MD), trace, the eigenvalues, and eigenvector maps were calculated with a in house software application written in Matlab (R2007a). The values of these indices were extracted using the Image J software in the maps obtained by manually selecting ROIs in each slice, and using the corresponding T2-W anatomical image and the Paxinos-Watson brain atlas as

a reference. Grey matter values of FA and MD were extracted from the Cingulate Cortex (CCG), STR, NAc, HIPP, GP, THA and SNu, and the white matter FA signal was extracted for the Corpus callosum (CC), Internal capsule (IC) and Hippocampal Comissure (HC) tracts.

Box 17. Diffusion Tensor Imaging

Differences in the diffusion of water molecules can be captured and inferred by applying determined MR sequences, and using computer analysis to reconstruct and generate a coherent maps of the signal. Within the brain, the movement of water molecules is determined by the distinct characteristic of the tissues (gray and white matter), its relative levels of different constituents and the microstructure of those (macromolecules, fibers and membranes). Diffusion tensor imaging (DTI) refers to a specific procedure that measures the diffusion of water molecules and describes their directionality. Two main measures are extracted with this technique, Mean diffusivity (MD) and FA.

MD is a measure of the amount of diffusion in a space, where higher values are associated with more diffusion, i.e. more water molecules passing from one point to adjacent spaces. In neural tissue, the MD is interpreted as a change in the barriers and obstacles. For example, higher MD will be associated with lower macromolecule density, fiber degradation of membrane breakdown in extreme cases.

FA is a measure of the directionality of diffusion in a space. The measure obtained lies between 0 1 (totally isotropic) and 1 (totally anisotropic). In neural tissue, FA is usually interpreted as an index of myelinization since myelin sheaths in the axons facilitate the diffusion in one direction, thereby increasing the anisotropic diffusion. Parallel to changes in FA, white matter usually shows changes in MD in the opposite direction. In the gray matter, changes in FA could entail other interpretation, such as changes in gliosis, astrocytic alterations or apoptosis (Stebbins, 2010).

After obtaining the T2W images a ^{1}H MR *in vivo* spectroscopy study of two brain regions was performed: cortex and striatum. The spectroscopy protocol used a Point-Resolved Spatially Spectroscopy, combined with VAriable Power radiofrequency pulses with Optimized Relaxation delays (VAPOR) water suppression, applying the following parameters: TR 3000 ms; TE 35 ms; Av 128; voxel volume 3 mm^3. First and Second-order shims were automatically adjusted using the FASTMP application in a large voxel (4 mm^3). All ^{1}H spectra were automatically analysed using LCModel version 6.2-OR (Stephen Provencher, Oakville, ON; Canada). Statistical analysis was performed with the concentration values of each metabolite relative to creatine (Cr) + phosphocreatine (PCr) for those with a standard deviation under 20%.

5. APPARATUS AND PROCEDURES FOR THE BEHAVIOURAL EXPERIMENTS

Two days before starting the behavioural testing described in the experimental pathways 2 and 3, the ad libitum access to food was stopped and the rat's weight was maintained between 90-95% of their initial weight. Additionally, weight gain produced by the normal development of these animals without dietary restrictions was taken as the reference to calculation of the food regime and the weight range in which they had to be maintained. No food restriction was imposed on the animals employed for drug self-administration, neuroimaging studies or RNA-seq. Animals received their daily food after the experimental sessions. All the procedures were performed during the rat's dark cycle and the testing rooms remained in the dark.

The behavioural tests used in experimental pathways 2 and 3 were performed in operant boxes (29.53 L x 24.84 W x 18.67 H cm: Med Associates), which were placed inside sound-attenuating chambers equipped with a fan to ventilate the space and reduce the animal's discomfort during the sessions. Each box was equipped with a clicker placed outside of the box that was employed as a cue in some procedures. A magazine hole was placed in one of the walls and connected to a feeder located outside the box that dispensed the food pellets as required (Noyes pellets; Testdiets). All conditioning boxes were equipped with two retractile levers on the right and left sides of the food magazine. Above each lever there were two lamps and another lamp was placed 2 cm from the top lid in the centre of the same wall. The cage had a stainless-steel grid floor with a bedding tray underneath it.

Experimental pathway 4: "Cocaine self-administration" was performed in Coulburn Instruments operant boxes (30 L x 25, 4 W x 30 H cm). In one of the walls there two levers with cue lights above them. The stainless-Steel Grid floor was connected to a shock generator to study compulsive consumption. Each box was placed inside a sound-attenuating chamber and it was equipped with a fan to help ventilate and maintain the correct temperature inside. A pumping system (Harvard apparatus) was situated outside the chamber. In the self-administration session, the activation of the pump pushed a syringe placed on it and deliver the cocaine solution through plastic tubing to a swivel tethering system attached to the centre of the lid.

6. EXPERIMENT 2: BEHAVIOURAL TRAITS

6.1. PAVLOVIAN-TO-INSTRUMENTAL TRANSFER (PIT)

The PIT protocol was designed according to previously used protocols, addressing factors that modulate the expression of this phenomenon (Hall et al., 2001; Holmes, Marchand, & Coutureau, 2010) and based on preliminary tests conducted in the laboratory. A total of 24 adult male rats (male-VEH n=12; Male-THC n=12) and 22 adult female rats (Female-VEH n=11; Female-THC n=11) were included in the final procedure. The PIT protocol involved four consecutive phases:

(1) PAVLOVIAN TRAINING → (2) INSTRUMENTAL TRAINING → (3) EXTINCTION → (4) PIT TEST

Box 18. Pavlovian-to-Instrumental transfer

Associative learning (instrumental and Pavlovian) accounts for the recognition and integration of information about the relationship between different stimuli and events. This relational information generates predictions about the subsequent events that might occur when a stimulus is present or absent, and can determine whether an action is convenient or not in a specific context. Learning, by means of neurophysiological changes, allow some stimuli to exert condition reflexes and/or to modulate the vigor and/or direction of instrumental actions. One way to explore the influence of predictive conditioned stimulus (CS) in some behaviours is to use Pavlovian-to-Instrumental Transference (PIT) protocols. This paradigm measures the change in instrumental responses in the presence or absence of a learned CS.

PIT protocols in animal models are usually carried out in operant chambers, following a basic protocol with three main parts: (1) Pavlovian training that involves exposing the animal to a stimulus (e.g. a clicker, light or tone), so-called CS^+ which is paired with a positive outcome, usually the delivery of a reinforcer (e.g. a food pellet or sucrose solution). However, Pavlovian training can include presentations of a non-rewarded stimulus (CS^-); (2) Instrumental training where animals can press a lever under different ratios of reinforcement to obtain reinforcers without any CS; and finally, (3) the test session where transfer occurs. The PIT test consists of several presentations of the CS^+ (and/or CS^-) that provokes changes in instrumental behaviour. Importantly lever pressing remains unrewarded to isolate the effects of the CS. Usually, the test session is preceded by one or more extinction sessions, or at least one extinction period before the test starts. During extinction training, the lever associated with the reinforcer remains protracted but pressing is unrewarded, reducing the instrumental behaviour and facilitating the measuring of the changes linked to CS^+ reexposure.

The principal variants of this protocol include aversive outcomes (aversive PIT) instead of reinforcers (appetitive PIT), and the inclusion of different CS+ and reinforcers (specific PIT) instead of one (general PIT). Many factors influence PIT expression, such as: the order, duration, and the number of pavlovian and instrumental sessions; and the number and nature of the $CS^{+/-}$ chosen (Holmes et al., 2010). The advantage of PIT relies upon the independent establishment of Pavlovian and instrumental learning, which is hard to elucidate in more natural conditions and learning contexts.

Pavlovian training sessions aimed to create associations for two conditioned stimuli (CS) with different outcomes. One stimulus was associated with the delivery of a food pellet into the magazine (CS^+) in a VI30 ratio. The other stimulus was presented but remained unrewarded (CS^-). A clicker sound or turning on the home box light were used as the CS^+ or CS^-, counterbalanced within the groups. Each CS was presented for 2 minutes and separated by passage through a 1 min No stimuli interval (NSI). The subjects performed a total of 10 sessions, consisting of four complete cycles of the following sequence:

$$[\text{NSI} \rightarrow \text{CS}^{+/-} \rightarrow \text{NSI} \rightarrow \text{CS}^{-/+}] \text{ x } 4$$

During the Pavlovian training sessions, the number of head entries (HE) into the magazine was recorded under each condition (CS^+ HE and CS^- HE), and a CS^+ HE ratio was calculated as:

$$\text{CS}^+ \text{ HE ratio } = \frac{\text{CS}^+\text{HEs}}{\text{CS}^+\text{HEs } + \text{ CS}^-\text{HEs}}$$

After Pavlovian training animals performed 7 sessions of instrumental training under different ratios. At the beginning of the session, two levers were protracted into the box on both sides of the magazine. The pressing of one of the levers was rewarded under different reinforcement schedules (active lever press, ALP), while pressing the other lever had no programmed contingencies (inactive lever press, ILP). Reward delivery activated a Time Out of 5 seconds in which ALP pressure remained unrewarded (Time Out Active Lever Press, TOALP). The sessions ended after 30 minutes or when the animal reached 50 rewards. Time to complete the session

was also registered and analysed. The first Instrumental Training session was held in fixed ratio 1 (FR1), which was followed by three sessions with variable ratio (VR)-5 and three more sessions with VR10. No CS was associated with ALP or ILP, or presented during the session. To assess learning and proper discrimination between the rewarded and the unrewarded lever, an ALP ratio was calculated as:

$$\text{ALP ratio} = \frac{\text{ALPs}}{\text{ALPs} + \text{ILPs}}$$

After instrumental sessions, in order to decrease response rate before the test, two extinction sessions of 20 minutes each were carried out. The sessions started with the protraction of the levers into the box but the levers were not associated with CS presentation or reinforcement contingencies, and neither of the levers was rewarded. Lever presses performed on the former active or inactive levers were recorded and the Total LP (ALP+ILP) was calculated.

The day after the second extinction session animals underwent the PIT test in a single session. The PIT test session began with an extinction period of 20 minutes after which four cycles of $CS^{+/-}$ presentations were initiated following the same sequence used in the Pavlovian sessions, although each CS and No stimuli interval lasted 2 min. ALP remained unrewarded and the percentage of ALPs on CS^{+} was used as the main index of PIT. We also measured the Pavlovian approach in contrast to the instrumental transfer, for which we calculated the percentage of HEs on CS+:

$$\%\text{ALP on } CS^{+} = \frac{CS^{+}\text{ALPs}}{CS^{+}\text{ALPs} + CS^{-}\text{ALPs}} * 100 \qquad \%\text{HE on } CS^{+} = \frac{CS^{+}\text{HE}}{CS^{+}\text{HE} + CS^{-}\text{HE}} * 100$$

Box 19. PIT neural basis and relevance for SUDs

PIT has been extensively used in preclinical SUD research, frequently linked to the influence of drug-associated cues in triggering drug-seeking and relapse (Everitt et al., 2001; O'Brien et al., 1998). While some researchers have discussed their results with PIT protocols as SUD liability (see Lamb, Schindler, & Pinkston, 2016), other studies have included actual drugs of abuse as reinforcers associated with the instrumental and Pavlovian training (see Cartoni et al., 2016), and recently individual differences in the expression of PIT have been weighted in drug self-administration protocols (Takahashi et al., 2019).

The studies carried out within this paradigm have identified some neurobiological correspondences (reviewed by Corbit & Balleine, 2016). The NAc Core and the central amygdala are responsible for the general PIT, whereas the NAc Shell and the basolateral amygdala are necessary for specific PIT to occur (Corbit & Balleine, 2005; 2011). However, PIT is not restricted to these nuclei, and the activity of closely related areas like the dorsal striatum has also been implicated. Lesions to the dorsolateral striatum prevent any type of transfer, while lesions of the dorsomedial striatum only affect the specific PIT (Corbit & Janak, 2007). Moreover, transfer expression relies on dopaminergic signaling. For example, the inactivation of projections from the ventral tegmental area to the dorsal striatum prevents PIT even when the Pavlovian and instrumental associations have already been acquired (Murschall & Hauber, 2006). Notably, the paradigm has been applied to humans with similar results regarding neural substrates and the behavioural factors involved (Bray et al., 2008; Talmi et al., 2008; see Cartoni et al., 2016 for a review).

6.2. THE TWO-CHOICE SERIAL REACTION-TIME TASK (CSRTT)

In this work, we used an version of the five-choice serial reaction-time task (5-CSRTT) in which the apparatus and protocol (Bari, Dalley, & Robbins, 2008) was adapted to operant boxes with only two response options (2-CSRTT), as already used successfully elsewhere (Hoang, 2010). The 2-CSRTT design has the advantage of not being so demanding at a procedural and attentional level, allowing a more focused assessment of impulsivity and inhibitory control.

Ten days after the end of the PIT animals underwent food-restriction again and the 2-CSRTT protocol commenced. First, the rats went through two sessions of cue-lever training, one for each lever. During these sessions, both levers were protracted and the cue light over one of the levers (right or left) remained switched on, with only presses of this lever rewarded under FR1. Cue-lever training was limited to 30 pellets. Following cue-lever training, the animals began daily 2-CSRTT training sessions. In each session, the animals underwent 100 trials where cue lights were switched on randomly, signalling which lever press option was contingently associated with reward. Twelve phases with increasing demands were implemented and in each phase, the duration of a light stimulus over a lever that signalled the availability of a pellet was progressively shortened (30 s in phase 1, to 0.5 s in phase 12). The response time that the rats had to press the lever to obtain the reward was also shortened progressively (from 30 s in phase 1 to 5 s in phase 12). The Inter-trial-interval (ITI) also

increased across sessions (from 2 s in phase 1 to 5 s in phase 12). Rats progressed to the next phase if they managed to perform at least 80% of the correct lever presses (CLP). Further lever presses after a CLP during the response time were registered as perseverative responses (PerR), while responses at any of the levers during the ITI were counted as premature responses (PreR). PreR and lever presses in the not-signalled lever, namely incorrect lever presses (ILP), or failure to respond in a trial (Omission Response-OR) were punished with a time out (TO) of 5 s. During this TO, responses were considered as time-out responses (TOR) and caused the TO period to be reinitiated. Animals quickly learned to avoid responses before cues were present to obtain a new pellet. Once phase 12 was reached, 6 more consecutive sessions with the same requirements but with only 75% of the correct responses required were implemented to serve as the baseline (BL) before testing.

Test sessions were similar to phase 12 sessions but the ITIs had a longer duration of 9 s. A total of three tests were carried out with two retraining sessions (equal to phase 12 training sessions) after the first and second tests. As an effect of the prolonged waiting time, animals tend to increment the number of PreR. A stable increase in PreR across the tests is considered a stable motor impulsivity trait, whereas an increase restricted to the first test session is considered a state-dependent impulsive response to a new context. We calculated the percentage of PreR in each BL or long-ITI test session as follows:

$$\%\ \mathrm{PreR}\ = \frac{\mathrm{PreR}}{(\mathrm{PreR}) + (\mathrm{CLP}) + (\mathrm{ILP}) + (\mathrm{OR})} * 100$$

In addition, we calculated the percentage increase of PreR for each of the test sessions as follows:

$$\%\ \mathrm{Increase}\ = \frac{\mathrm{PreR\ on\ test}\ -\ \mathrm{Mean\ PreR\ on\ the\ previous\ 2\ BL\ sessions}}{\mathrm{PreR\ on\ test}} * 100$$

Box 20: Motor Impulsivity & Serial Reaction Time Tasks

Impulsivity can be defined as a tendency to act prematurely without foresight (Dalley, Everitt, & Robbins, 2011), although this behaviour can be manifested in several domains or manners, and under slightly different situations. One classic way of dividing impulsivity is between cognitive impulsivity and motor impulsivity. Cognitive impulsivity involves biased decision making, which leads to a preference for immediate rewards at the expense of greater delayed rewards. By contrast, motor impulsivity involves difficulties inhibiting responses and refraining from actions. Each type of impulsivity has its own psychobiological features, nonetheless, they also show some degree of overlap, both between them and in relation to other psychological functions, such as motivational processes (Voon, 2014).

The Serial Reaction Time Tasks (SRTTs) are a well-known paradigm initially designed to investigate attentional processes in the context of human pathologies, such as attention deficit hyperactivity disorder (Navarra et al., 2008; Robinson et al., 2008), although the animal version has been profusely used to study impulsivity and impulse control (Bari et al., 2008). SRTT protocols measure the inability to inhibit well-established motor responses, sometimes referred to as waiting-impulsivity, although it is considered a particular form of motor impulsivity.

The main objective of the protocols used in the SRTT is to provoke and quantify the number of premature responses before the presentation of a discriminative stimulus (light pilot on one of the signs) which indicates the availability of a reward. These premature responses are considered a form of impulsive behaviour and/or a failure to control impulses (see Dalley, Everitt, & Robbins, 2011). Animals are trained in boxes with different manipulandum (usually nose-pokes or levers), each of them provided with light cues over the manipulandum indicating that an instrumental response (nose poking or lever press) will be rewarded. Across sessions, rats are trained to wait for the light cue and during the test sessions, the time that elapses during the trials until the presentation of a cue is extended unexpectedly, causing a relative increase in the emission of premature responses. These responses are penalized with a Time Out period in which the animal does not get the reward and is forced to wait for a new trial to begin. Protocols usually include several test sessions that can differentiate between context-induced impulsivity due to the change in the expected waiting periods, and trait impulsivity defined as a stable increase or decrease across these test sessions. Finally, the population of animals is divided between high/low impulsivity based on the distribution obtained in the test.

7. EXPERIMENT 3: BEHAVIOURAL TRAITS

7.1. PAVLOVIAN CONDITIONED APPROACH (PCA)

The Pavlovian conditioned approach (PCA) protocol was designed in accordance with Fitzpatrick & Morrow, 2016, a procedure designed to evaluate the contribution of incentive salience to reward conditioned stimulus, and to phenotype the subjects into sign trackers (ST) and goal trackers (GT). A total of 20 adult male rats (Male-VEH n=10; Male-THC n=10) and adult 20 female rats (Female-VEH n=10; Female-THC n=10) were included in the final procedure.

Box 21: Neural basis of impulsivity and relevance for SUDs

Impulsivity has been frequently related to SUDs, to the point that it plays a vital role in some general theories proposed to explain this phenomenon (Koob & Le Moal, 2008). Impulsivity has been viewed both as a result of drug use and as a risk factor to develop a SUD. Extensive research has related the different types of impulsivity with addiction to different drugs of abuse. It is relevant to highlight here that there seems to be a strong correspondence between high motor impulsivity and an greater acquisition of cocaine use and cocaine abuse behaviours (Dalley et al., 2008; Molander et al., 2011), the development of compulsive use (Belin et al., 2008), and susceptibility to relapse (Economidou et al., 2009; Moeller et al., 2001).

At a neurobiological level, impulsivity measured by 5-CSRTT has been related to the activity and interplay between cortical regions (e.g. the ventral and dorsal striatum, the ventral tegmental area and the Locus Coeruleus: Dalley et al., 2008), yet not all cortical regions exert the same influence. For example, an area frequently related to impulsivity control, the orbitofrontal cortex, has a prominent role in cognitive impulsivity tasks and decision making but a minor role in inhibitory control tasks related to motor impulsivity (Chudasama et al., 2003). Other cortical regions like the infraralimbic cortex and the anterior cingulate cortex play a more determinant role in waiting-impulsivity. In this sense, increases in premature responses have been described using NMDA receptor antagonists in the infraralimbic cortex or after producing lesions in this area (Chudasama et al., 2003, Murphy, et al., 2005; 2012). Lesions in the anterior cingulate cortex that influence the ability to discriminate visual stimuli (Chudasama et al., 2003) also increase impulsivity (Muir, Everitt, & Robbins, 1996). These cortical areas have direct and indirect influences on basal ganglia regions such as the Nucleus Accumbens Core and Shell, and the dorsomedial striatum through afferents from the Locus Coeruleus and the Ventral Tegmental Area (Voon, 2014). The dorsomedial striatum seems to have a particular relationship with the prominence of premature responses and lesions in this area are known to increase impulsivity (Christakou et al., 2001; Rogers et al., 2001) Inactivation of the NAc Shell (with the GABAergic agonist Muscimol) also produces an increase in premature responses in the 5-CSRTT, although the same procedure applied to the NAc Core produced a general unspecific alteration in performance. Besides GABA-ergic mechanisms, motor impulsivity is sensitive to the neurotransmitter systems that interacts with these brain areas and thus, while it is especially reliant on dopaminergic activity, there is also an influence of glutamatergic, noradrenergic and serotonergic signaling (Voon, 2014; Economidou et al., 2012).

Animals performed a single magazine training session in which the feeder dispensed 25 food pellets into the magazine under a Variable Interval of 90 seconds (VI90). On the following day, the animals began the PCA protocol, consisting of 8 daily sessions. Each session was composed of 25 trials in which the feeder dispensed pellets into the magazine on a VI60 regime. A lever on one side of the magazine (right or left, counterbalanced) was extended 8 s before the reward and this lever was retracted at the moment of reward presentation. The other lever was present throughout the whole session and served as a measure of general locomotor activity. Neither of the levers had programmed contingencies. Interaction with the CS^+ lever (CS^+ LPs), inactive lever presses in between each CS^+ (ILPs) or during the CS^+ (CS^+ ILPs), the HE and time spent in the magazine (MAG), both in between each CS^+ presentation and during the CS+ (CS^+ HE and CS^+ MAG, respectively) was all recorded. The main PCA index used, suggestive of a bias to GT or ST was calculated each day as the mean of three other indexes:

i) Response bias, i.e., the ratio of the total number of lever presses and magazine entries for a session during the CS+ presentations:

$$\text{Response bias} = \frac{CS^+\text{LPs} - CS^+\text{HEs}}{CS^+\text{LPs} + CS^+\text{HEs}}$$

ii) Latency score, i.e., average latency to perform a lever press or magazine entry during the 8 s of CS+ presentation:

$$\text{Latency score} = \frac{\text{Mean LPs latency} - \text{Mean HE latency}}{8}$$

iii) Probability difference, i.e., the probability of performing a HE during the CS+ presentations minus the probability of performing a LP during the CS+ presentation:

$$\text{Probability difference} = \text{P(HE)} - \text{P(LP)}$$

Every index score ranged from 1 (absolute GT) to -1 (absolute ST), with 0 representing no preference bias. Animals with a PCA score higher than 0.5 were classified as GTs, whereas animals with a PCA lower than -0.5 were categorized as STs.

Box 22: Pavlovian Conditioned Approach

Organisms associate environmental cues with other co-occurring events that further guide behaviour, especially when they are aversive or rewarding. Interestingly, instead of providing information, under some circumstances reward-related cues can become attractive on their own and elicit approach behaviours. Individual differences in cue-reward learning can be captured by Pavlovian conditioned approach (PCA) protocols in a clear and straightforward way.

The PCA protocol in operant chambers takes advantage of this phenomenon by pairing the extension of a lever into the box (reward-related cue) with the delivery of a food pellet (rewarding event: Tomie, 1996). After repeated exposure to this chain of events, the lever becomes a conditioned stimulus (CS) and some animals starts to direct attention towards the CS and interact with it, a conditioned response denominated sign tracking (ST). By contrast, others use the CS primarily as a reward-predicting cue and direct their attention towards the reward magazine, a conditioned response denominated goal-tracking (GT).

Notably, these endophenotypes maintain a close relationship with other psychological traits. STs compared to GTs have been related to increased deficits in attentional control (reviewed by Colaizzi et al., 2020; Kucinski, et al., 2018) and impulsive action (Lovic et al., 2011). Furthermore STs may be more prone to develop rigid habit-like behaviours and the ST behaviour relies highly on stimulus-response association (Morrison et al., 2015). ST and GT learning styles have been extensively studied in rodents but there is also evidence for this bias in humans (see Colaizzi et al., 2020).

We also calculated another additional measure: response probability (similar to probability difference but only for the first response, being a CS+ LP or a CS+ HE), performed in each trial:

$$\text{First response probability} = \frac{\text{1st Response HE}}{25} - \frac{\text{1st Response LP}}{25}$$

Box 23: Neurobiological bases Sign/Goal tracking trade-off & SUD liability

A bias towards ST has been considered an endophenotype that entails an increased risk for different neurobiological disorders, among them SUDs (Tomie, 2018). Attentional deficits, motor impulsivity or insensitivity to outcome devaluation are traits linked to ST, and they have also been independently associated with SUD liability, while ST is usually interpreted within the incentive sensitization theory of addiction. Indeed, ST is considered a risk factor toward increased drug-seeking and relapse (Saunders & Robinson, 2010), and interestingly, it can bias the preference of a drug over food (Tunstall & Kearns, 2015). Nonetheless, other approaches suggested that the GT-ST bias can determine differential pathways in the progression of SUD-related behaviours, implying different vulnerabilities but not necessarily an increased risk *per se*. For example, GT and ST bias may bias sensitivity to different relapse "triggers", with contextual cues more GT and STs more prone to discrete cues (Saunders et al., 2014).

Neurobiological characterization of the ST/GT model has defined two distinct profiles, sometimes depicted as bottom-up or cue-driven attention in one extreme and top-down or goal-driven attentional control. One of the most well documented neurobiological features arise when animals are exposed to reward-related cues. STs rather than GTs are characterized by enhanced dopaminergic tone in subcortical areas and a relatively unresponsive cholinergic activity in the cortex (see (Sarter & Phillips, 2018).

7.2. HABIT FORMATION

After the final PCA session the animals were left undisturbed for 10 days in their home cages with food and water ad libitum before starting the habit formation protocol. Animals performed a non-habit-forming brief training and a habit-forming extended training to assess possible bias induced by ACE in S-R/A-O learning (see Box 24).

The brief training consisted of five consecutive daily sessions, with one active lever (left or right counterbalanced) present during the whole session. Training started with a FR1 session and followed with two sessions using a variable interval of reinforcement of 30 s (VI30) and two VI60 sessions. All sessions were limited to 30 pellets or 30 minutes, but all animals consumed the 30 pellets before reaching this time limit. The day after the last VI60 training session the devaluation test sessions were performed. Devaluation was achieved by sensory-specific satiety, animals were allowed to freely eat pellets (devalued condition) or chow food (non-devalued) for one hour. Pre-feeding conditions were counterbalanced between groups and after feeding, the animals underwent a brief extinction session of 5 minutes in the operant boxes. During the test session, the two levers present were present in order to discriminate reinforcer seeking from general locomotor activity. None of the levers had programmed contingencies. The day after the first test animals were retrained in a regular VI60 session and the next day performed a second complimentary test with the food condition switched. The difference between LP performed in the devalued and non-devalued conditions was calculated (LPs difference):

$$\text{LPs Difference: } \mathrm{LP}_{\mathrm{Non-Devalued}} - \mathrm{LP}_{\mathrm{Devalued}}$$

A habit formation index was calculated taking as a reference the:

$$\text{Habit formation Index: } \frac{\mathrm{LP}_{\mathrm{Devalued}}}{\mathrm{LP}_{\mathrm{Devalued}} + \mathrm{LP}_{\mathrm{NonDevalued}}} * 100$$

A day after the second devaluation test animals were again retrained with a VI60 session. Finally, the next day a contingency degradation test was performed. Contingency degradation was achieved by an omission test in which response refrainment was rewarded in a VI30 and lever pressing started a new VI30 period. For extended training, animals performed another 10 sessions with a VI60 ratio. Next animals underwent the same counterbalanced devaluation tests and the final contingency degradation test. HEs and MAG during all sessions and tests was measured, and the time spent to complete the training sessions and contingency degradation sessions were also registered for analysis (Session Time).

Box 24: S-R Habits vs A-O Goal directed behaviours

Habits are an efficient mean of processing information, and they guide behaviour in familiar environments and repetitive conditions. However, if the contingencies and contextual demands change, persistence in learned responses may be detrimental. Habits are performed even when an action is no longer rewarded, and it could impede the exploration of alternative courses of actions and the exploitation of other resources.

Stimulus-response (S-R) or 'habit' learning can be attained through operant conditioning training. An instrumental action that leads to a reinforcer potentially involves the presence of certain stimuli during training. With training, all these stimuli (S) can become associated with the response (R) independently of the goal, and eventually, its presence can prompt the onset of the instrumental action (S-R) associated independently of the state of the organism and not linked to a specific goal. By contrast, action-outcome behaviours (A-O) are goal-directed actions that rely on the value ascribed to the outcome and that can vary according to the situation and the state of the organism, as well as the probability of obtaining it (Everitt & Robbins, 2005). Two basic tests have been commonly used to assess S-R as opposed to A-O learning: outcome devaluation and contingency degradation. In outcome devaluation, pre-feeding or pairing food ingestion with illness reduces the motivation to obtain the reinforcer, thereby decreasing actions aimed to obtain the goal if the behaviour is A-O driven, or still prompting instrumental actions when possible if they are S-R driven. For contingency degradation testing, the contingent relationships between the different elements (stimulus, actions and outcomes) are changed or degraded (e.g. to obtain a reward an animal has to hold a response instead of performing the previously learned action): if the behaviour is A-O driven the animal will modify its behaviour but if it is S-R driven, there will be enhanced resistance to change.

8. EXPERIMENT 4: COCAINE SELF-ADMINISTRATION

At PND90, animals underwent a single FR1 food-reinforced instrumental training session limited to 10 reinforcers in the operant boxes (Med Associates). If one animal did not achieve the 10 reinforcers it was food-restricted and submitted to another session the following day. All animals complete this brief training within 2 sessions, the vast majority in the first session.

The day after finishing the food self-administration training a catheter was implanted surgically into the jugular vein to allow intravenous cocaine self-administration. Animals were anesthetized with 5% isoflurane and this state was maintained with 1.5-2% isoflurane during the surgical procedures. An incision was made to implant a polyvinyl chloride catheter (0.064 d.i.) into the jugular vein at the height of the atrium. This catheter arrives subcutaneously until it exits through the middle scapular region. Another incision was made in the animal's back

and a mesh was attached to a pedestal made with dental cement, to which a screw was attached (Plastics One). After surgery the animals were placed individually in independent home boxes and they were left undisturbed to recover for 8 to 10 days before starting the Cocaine Self-Administration (CSA) protocol. From this moment and after every self-administration session, they were infused daily through the catheter with 0.1 ml of a saline solution containing heparin (1.5 IU/ml) and gentamicin (40 mg/ml) to keep the catheter patent and drug-free. Thiopental (Braun) sodium was used to verify correct functionality of the catheter a day before the beginning of the self-administration procedure and once again after the first day or second day of force abstinence.

The CSA sessions were all performed in the Coulburn boxes described earlier and inside the operant box, an active lever (AL) with different programmed contingencies and an inactive lever (IL) without programmed contingencies, remained protracted during the sessions. Over the AL there was a cue light that was turned on at the beginning of the session and turned off for 10 s (time out) at the beginning of an infusion that lasted for 7 s (or a foot shock in compulsive -punished- seeking), indicating cocaine availability. Cocaine (Alcaliber, Spain) infusions (0.5 mg/kg in 100 µL of sterile saline solution) were administered by an electronic pump (Harvard Apparatus, USA). To ensure an equal dose of cocaine among the subjects the infusion rate of the pumping system was adjusted to each animal's weight. Lever presses during time out (TOLP) did not have any programmed contingencies. The protocol consisted of 6 consecutive phases: (1) acquisition,12 daily sessions lasting 2 hours each under an FR1 schedule; (2) motivation for consumption, 6 sessions of 2 hours under a progressive ratio (PR) schedule (Sánchez-Cardoso et al., 2007); (3) re-baseline, 3 sessions of 2 hours under FR1; (4) compulsive (punished) seeking, a single 1 hour session under an FR3 schedule in which the animal randomly received an infusion or a 0.5 mA plant shock for 0.5 s. (5) extended access, 10 sessions of 6 hours each under FR1; (6) cue-induced relapse, 4 sessions of 1 hour each with cues as in the acquisition sessions but without drug delivery, occurring after 1, 30, 60 and 100 days of forced abstinence.

The self-administration sessions were composed of 6 consecutive phases: (1) Acquisition, 12 sessions of 2 hours on FR1; (2) Motivation for Consumption, 6 sessions of 2 hours on Progressive Ratio (PR) using the following sequence of requirements: 1, 2, 3, 4, 5, 6, 7, 8, 10, 12, 14, 16, 18, 20, 22, 24, 28, 32, 36, 40, 44, 48, 52, 56, 64, 72, 80, 88, 96, 104, 112, 120, 128, 136, 144, etc… (Richardson & Roberts, 1996); (3) Reestablishment, 3 sessions of 2 hours on FR1; (4) Compulsive (punished) seeking, a 1 hour session under FR3 where the animal randomly received an infusion or a 0.5 second 0.5 mA plant shock; (5) Extended Access, 10 sessions of 6 hours on FR1, after which the animals started a forced abstinence period; (6) Incubation of Drug Seeking, 4 sessions held on days 1, 30, 60 and 100 of forced abstinence for 1 hour with cues as in FR1 but without drug delivery.

Box 25: SR/A-O learning influence in SUDs and its neurobiological bases

The hypothesis that the evolution towards SUDs can be a form of habit-based learning where the subject progressively loses his ability to exert control over drug use (frequently described as a habit-like pattern of action) has produced an extensive amount of research in the past decades (Everitt & Robbins, 2005, 2016; Robbins & Everitt, 1999). The rationale behind the interaction of habits and SUDs posits that a predisposition to develop faster and more rigid S-R behaviour is a vulnerability factor to develop further drug-seeking habits and compulsive drug-intake (Everitt & Robbins, 2013, 2016). Moreover, food reinforcers can generate habitual responses but self-administration of different drugs of abuse enhance this bias (Dickinson, Wood, & Smith, 2002; Miles, Everitt, & Dickinson, 2003) a result that matches with the classic view of drugs as habit-forming agents. However, drug use is not an exclusively S-R driven response, early evidence showed that drug-seeking in rats (as well as sucrose seeking) is a goal-directed behaviour that entails a representation of the outcome (Olmstead et al., 2001) but on the history of self-administration rats can shift from goal-directed cocaine-seeking to habit like S-R respondent type of seeking (Pelloux, Everitt, & Dickinson, 2007).

The exploration of the neural basis of the evolution from goal-directed to habit-like drug use has been roughly depicted as a transition, in the effective activation and control over behaviour, from the vSTR to dSTR and within the dSTR from DMS to DLS (Everitt & Robbins, 2005, 2013, 2016). The goal-directed system has been related to the interactions between mPFC and posterior DMS (Shiflett, Brown, & Balleine, 2010; Yin, Ostlund, Knowlton, & Balleine, 2005). In fact, the habit system relies on the anterior DLS and motor areas (Balleine & O'Doherty, 2010; Yin, Knowlton, & Balleine, 2004). Dopamine release in the different striatal areas is a key feature of its participation in the actual behaviour. Well trained behaviours, among them drug-seeking, show enhanced dopamine activity in the DLS. Moreover blocking dopamine transmission in this area can decrease these behaviours and render them sensitive to devaluation (Ito, Dalley, Robbins, & Everitt, 2002; Vanderschuren, Di Ciano, & Everitt, 2005; Zapata, Minney, & Shippenberg, 2010), yet it is ineffective in impeding learning (e.g. the acquisition of cocaine self-administration) presumably when the goal-directed component has a stronger relative weight (Murray, Everitt, & Belin, 2012). Notably, the specular phenomenon is also true. Blocking DA transmission in DMS can impede learning but it is ineffective to block well-trained behaviours, including cocaine seeking (Murray et al., 2012).

In the Acquisition, re-baseline and Extended Access phases, the main measurements were ALPs/infusions, TOLP, ILPs.

To check for escalation of drug intake during the extended access sessions we compared the infusions achieved during the first hour of the first extended access session to the infusions achieved during the first hour of the last extended access session.

To address the Motivation for Consumption we also registered the breaking point reached and calculated a specific motivation index:

$$Motivation\ Index\ = \frac{\text{Infusions PR Session}}{\text{Infusions } on\ 12th\ Acquisition\ session}$$

After PR sessions animals underwent three re-baseline sessions. A rebound index was calculated as follows:

$$Rebound\ index\ = \frac{\text{Mean Infusions } (10-12th)\ Acquisition\ Sessions}{\text{Mean Infusions } \ Re-baseline\ Sessions}$$

On Compulsive Consumption, we also calculated a specific compulsivity index as:

$$Compulsivity\ Index\ = \frac{\text{Events (shock or reward) on Compulsive Consumption}}{\text{Infusions } \ on\ the\ 1st\ hour\ of\ 3rd\ RA}$$

9. EXPERIMENT 5: NUCLEUS ACCUMBENS SHELL RNA-seq

At PND90, animals were deeply anaesthetised with isoflurane and sacrificed by decapitation. After extracting their brain, coronal slices (1 mm thick) were obtained with the help of a brain matrix between approximately 2.28 mm and 1.08 mm anterior from Bregma. With the two dissecting lancet-shaped needles, the NAc (shell division) was dissected out according to the Paxinos and Watson atlas (9). All the surfaces and tools used for dissection were sterilised and treated with RNAseZap® (Ambion), and all the steps were carried out with caution to ensure RNA integrity was maintained. The tissue samples were snap-frozen on dry ice and stored at -70 ºC. The tissue samples were homogenised and the RNA extracted using the RNeasy Mini Kit (Qiagen). Libraries were prepared following the instructions for the NEBNext Ultra Directional RNA Library Prep kit for Illumina (New England Biolabs), as detailed in "Chapter 1: Protocol for use with NEBNext Poly(A) mRNA Magnetic Isolation Module". The total RNA (1 µg) used as the input to start the protocol was quantified with an Agilent 2100 Bioanalyzer using an RNA 6000 nano LabChip kit. We performed the library amplification included in the cited protocol with 14 PCR cycles, and the libraries obtained were validated and quantified with an Agilent 2100 Bioanalyzer using a DNA7500 LabChip kit. An equimolecular pool of libraries were titrated by quantitative PCR using the "Kapa-SYBR FAST qPCR kit forLightCycler480" (Kapa BioSystems) and a reference standard for quantification. The pool of libraries was denatured prior to being seeded on a flow-cell at a density of 2.2 pM, where clusters were formed and sequenced using a "NextSeq™ 500 High Output Kit" in a 1x75 single read sequencing run on a NextSeq500 sequencer.

The Chipster analysis suite (Kallio et al., 2011) was used to conduct the data processing and analysis. Briefly, the quality of the raw data obtained (singleEnd, stranded) was analysed on FASTQC and PRINSEQ and no low-quality bases, or very few, were detected at the end of the reads. Thus, no trimming was performed (Williams et al., 2016). Reads were aligned to the Rattus_norvergicus. Rnor_6.0.87 reference genome using TOPHAPT and the alignment of the counts per read was performed using HTSeq. Differential gene expression analysis was performed using CUFFDIFF, with replicates analysed to explore the differences in transcriptomic profiles between factor levels. All RNA-seq data sets generated and/or analysed during the current study were added to the Gene Expression Omnibus (GEO) under the accession number GSE158188.

Gene ontologies (GO) enrichment and overrepresentation were calculated with the online tools and databases of the PANTHER Classification System (Mi et al., 2019) for each gene subset obtained in the differential analysis. The GO analysis allows a comprehensive scrutiny of large data sets of genes highlighting its significant association with different subcategories belonging to three main domains: molecular function, biological process, and cellular component. In addition, PANTHER pathway overrepresentation analysis were also run.

10. STATISTICAL ANALYSIS

Statistical analysis of the data from the imaging and behavioural studies was performed using the IBM SPSS Statistics package v.24. A descriptive analysis of the dependent variables obtained in the experiments was performed and used to select the appropriate statistical tools. Basic analysis included checking for normality, homoscedasticity and the presence of outliers. Extreme outliers (above the 3rd quartile+1.5*interquartile range or below the 1st quartile-1.5*interquartile range) were eliminated from the statistics and transformations of the variables performed to archive homoscedasticity.

All experiments include two between-subject independent variables with two levels each: Sex (Male/Female) and Treatment (THC/VEH). To compare the mean differences in a single dependent variable between the groups two-way ANOVAs were used. Significant interactions between Sex and Treatment were further explored and determined using a Simple Effects analysis syntaxis for SPSS. When previous descriptive analysis detected deviations from normality, violations of homoscedasticity and different size groups non-parametric tests were used: Mann-Whitney and Kruskal-Wallis. Repeated Measures (RM) ANOVA was employed to analyse within-subject variation across *sessions* or tests in any variable. Deviations from the sphericity in the RM analysis were checked and a Greenhouse-Geisser correction applied if violated.

The level of significance p was established at $\alpha = 0.05$, and p values between 0.05 and 0.06 were considered as statistical trends. For RNAseq adjusted p-values (p values corrected for multiple comparisons) were considered in the differential gene expression analysis, and the False discovery Rate (FDR: expected proportion of true null hypotheses rejected out of the total number of null hypotheses rejected) was calculated in the GO analysis. Only adjusted p-values and a FDR of <0.05 were considered significant.

RESULTS

1. EXPERIMENT 1: MAGNETIC RESONANCE IMAGING

1.1. VOLUMETRY

The MRI data showed that exposure to THC during adolescence provokes structural alterations evident in adult animals. Specifically, there was a reduction in the volume of the dSTR in adult females that had been exposed to chronic THC treatment during adolescence (Figure 9). The GP was also smaller in the THC exposed animals, although this effect was only significant in the right hemisphere and the effect was only considered a trend when both hemispheres were analysed globally. Sex-specific differences in the volumetric analysis were patent in the total brain, Cx (total volume and left side), Cb and Hippocampus with a m>f (male larger than female) pattern in each of those areas. No differences were detected between males and females in the areas of the brain slices corrected for body weight, or as an effect of treatment (see Table 4).

Brain ventricle volumetry (BVV) revealed a global reduction in the ventricular space in adult animals treated with THC. Taking a closer look, a global effect of THC was evident in the lateral ventricles. However, THC did not produce a significant effect in the third ventricle and there was a smaller aqueduct of Silvius volume in THC males compared to control males. The fourth ventricle presented a clear and significant m>f pattern but no treatment effect was found (see below Figure 8).

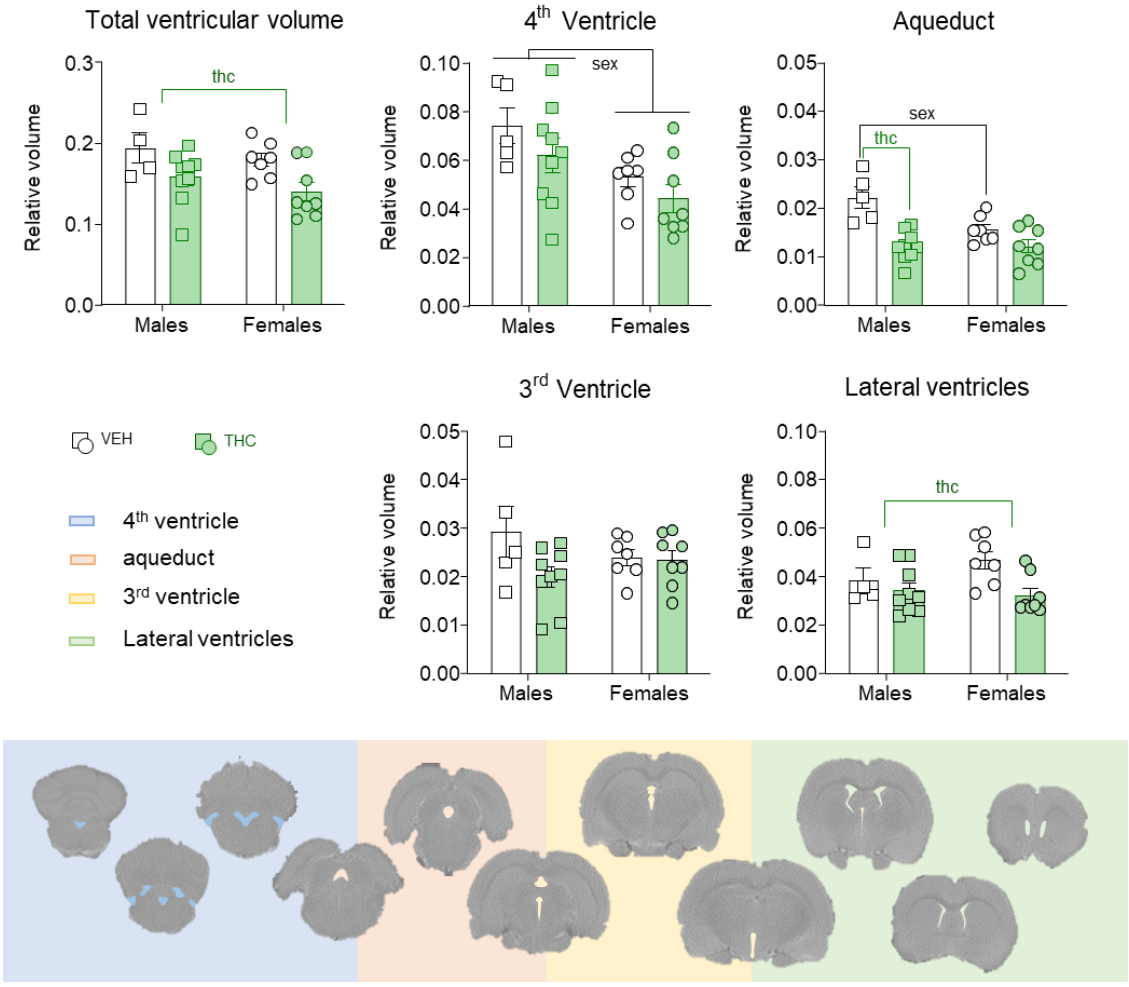


Figure 8. Brain ventricle volumetry. The graphs show the individual values (dots) and Mean ± SEM (bars). **A)** The whole ventricular volume decreased in THC-treated animals ($F_{1,23}$=8.961; p=0.006; ηp^2=0.28). In order to explore the source of this effect, we analysed the different sections of the brain ventricular system. **B)** In the fourth ventricle we only observed a male>female sexual dimorphism ($F_{1,25}$=9.053; p=0.006; ηp^2=0.26). **C)** A significant Sex x Adolescent Treatment interaction ($F_{1,25}$= 5.575; p= 0.026; ηp^2=0.18) appeared in the brain aqueduct, and our follow-up analysis showed a male>female sexual dimorphism in VEH animals ($F_{1,25}$= 9.598; p=.005; ηp^2=0.27) and significant differences between within the males. More specifically, THC-exposed male rats had a smaller volume ($F_{1,25}$= 24.51; p<0.000; ηp^2= 0.49). **D)** In the third ventricle there was a trend towards a smaller volume in THC animals ($F_{1,25}$= 3.408; p= 0.077; ηp^2= 0,12). **E)** In the lateral ventricles the volume was smaller in THC animals ($F_{1,25}$= 6.341; p= 0.019; ηp^2= 0.19). **F)** The different fill colours represent the ventricle area used to obtain the values represented in each corresponding graph. From caudal (left) to rostral (right): IV ventricle, aqueduct, III ventricle and lateral ventricles. The full results can be seen in Table 5

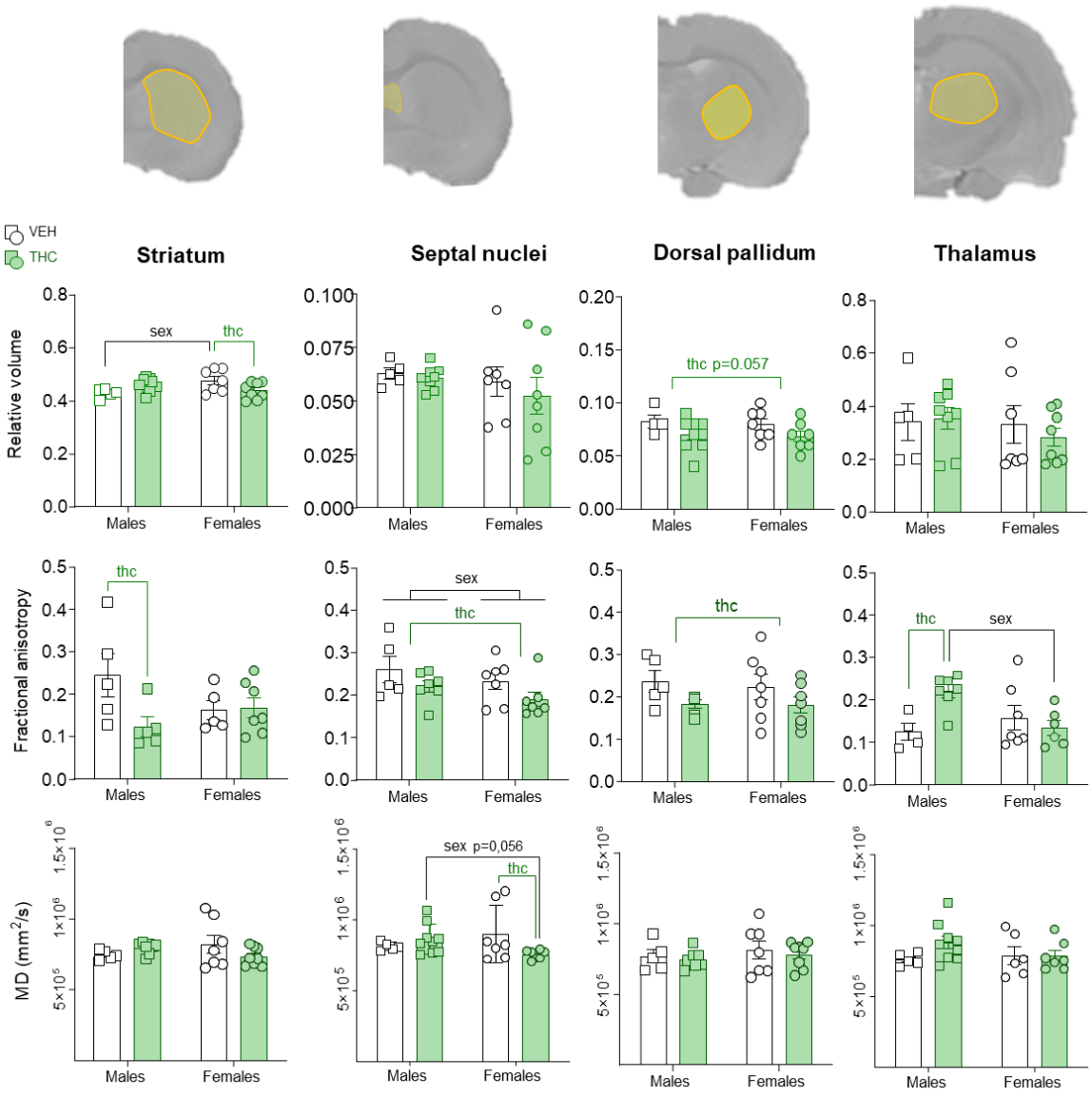


Figure 9. MRI Grey matter analysis. Male VEH n=5; Male THC n=9; Female VEH n=7; Female THC n=8. The most representative effects are depicted. Graphs represent the individual values (dots) and the mean ± SEM (bars). Within each graph, the green lines and "thc" represent a significant effect of the THC treatment (Adolescent Treatment), while the black lines and "sex" represent statistically significant effects of the factor Sex. The columns from left to right represent the volumetric analysis, calculated as the relative volume of the structure within the sections it was contained in, the DTI values obtained for mean diffusivity (MD) and fractional anisotropy (FA) in each of the four different structures depicted in each row; from top to bottom: Striatum (STR), Septal Nuclei (SNu), Globus Pallidus (GP) and Thalamus. **First row / volumetric analysis across brain areas**. In the STR we found a significant Sex x Adolescent Treatment interaction ($F_{1,25}$=8.806; p=0.007; η_p^2=0.26), with females VEH having an overall larger relative volume over males VEH ($F_{1,25}$=5.783; p=0.024; η_p^2=0.19), yet females THC had a smaller volume than females VEH ($F_{1,25}$=7.161; p=0.013; η_p^2=0.22). In the GP, THC animals showed an upward trend (F1,23=4.022; p=0.057; η_p^2=0.15) that was only significant in the right GP (F1,22=4.494; p=0.046; η_p^2=0.17). Other significant male>female volumetric alterations were found in left Hippocampus (HIPP), Total Cortex (Cx) and Cerebellum (see Table 4). **Second row / FA values across brain areas.** In the anterior section of the STR, there was a Sex x Adolescent Treatment interaction ($F_{1,17}$=6.364; p=0.022; η_p^2=0.27); the analysis of the simple effects showed decreased FA values in male THC rats compared to male VEH animals. We also detected another Sex x Adolescent Treatment interaction ($F_{1,20}$=7.057; p=0.015; η_p^2=0.26) in the Thalamus, this time suggesting an increased FA in THC males compared to their VH controls ($F_{1,20}$=8.144; p=0.001; η_p^2=0.28) and their female counterparts (F1,20=8.346; p=0.009; η_p^2=0.29). In the caudal SNu we observed a general Sex effect ($F_{1,21}$=4.850; p=0.039; ηp2=0.18) and also a lower FA in THC rats (F1,21=6.999; p=0.015; η_p^2=2.250). The decreased FA in THC animals was also statistically significant in the GP ($F_{1,21}$=4.309; p=0.05; η_p^2=0.17). **Third row / MD values across brain areas.** A Sex x Adolescent Treatment interaction was detected in the rostral SNu (H=9.284; p=0.026;^2_R=0.33), with a lower MD in the THC females than the VEH females (U=9; p=0.028; η^2=0.34), with a lower MD also in THC females than males (U=10; p=0.012; η^2=0.39). See additional data in Table 7.

1.2. DIFFUSION TENSOR IMAGING

The DTI analysis revealed a reduced FA in the rostral STR of male-THC rats, whereas the opposite effect was found on the thalamic FA signal, enhanced in THC males relative to their controls. The FA in the GP and caudal SNu was reduced by THC in both sexes (See Figure 9 and Table 6). In the grey matter the MD most evident alteration was a reduced MD in the rostral section of the SNu in THC females relative to their controls, with no other significant effects (See Table 7).

In rostral sections the FA signal in the CC and AC was weaker in THC treated animals of both sexes. By contrast, in the posterior sections of these tracts and in the IC no significant effects were detected. Moreover, the FA signal in the HC appeared to have been significantly dampened in females that received THC than in those that received the vehicle alone (see Figure 10 and Table 8).

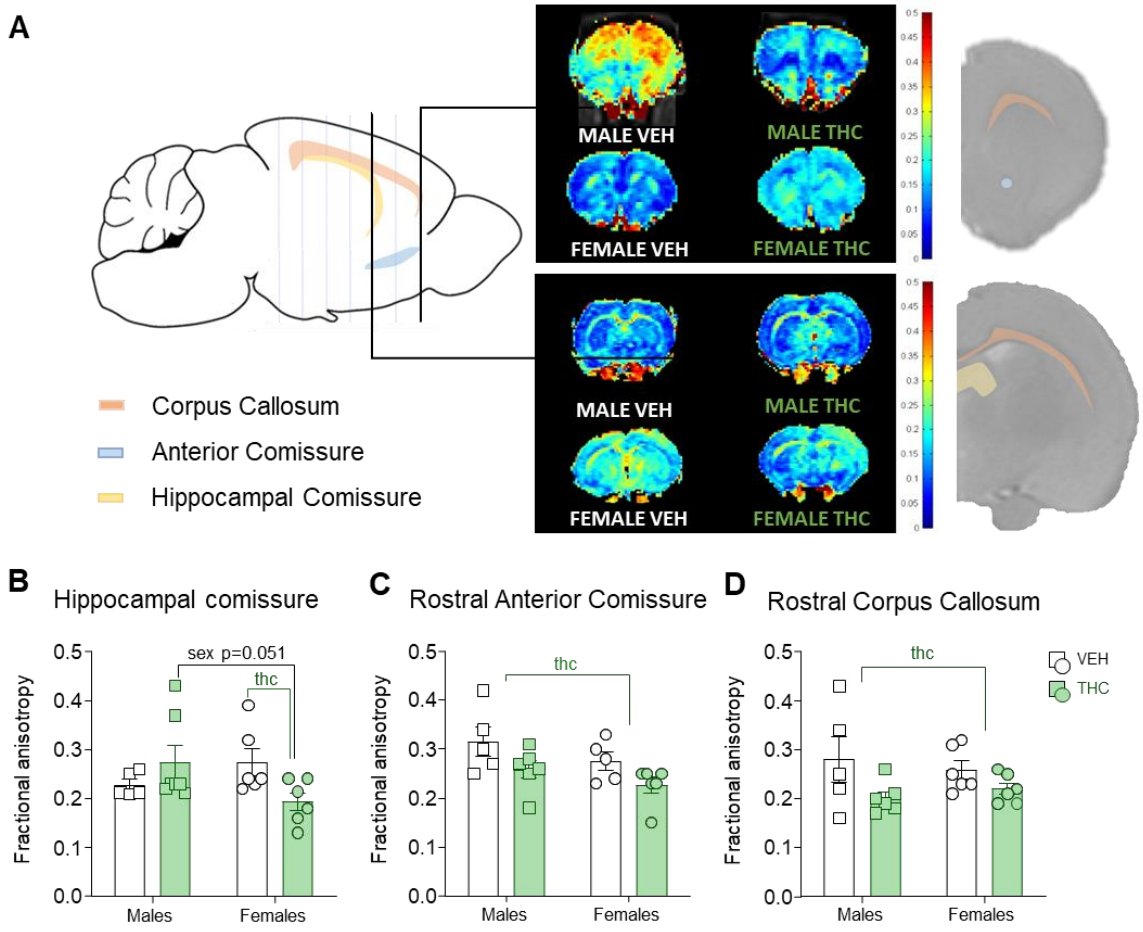


Figure 10. DTI FA Analysis White matter tracts. The graphs show the individual values (dots) and the mean ± SEM (bars). Within each graph, the green lines and "THC" represent a significant effect of the factor Treatment. The black lines and "sex" represent statistically significant effects of the factor Sex. **A)** Representation of the three major white matter tracts and the corresponding DTI FA maps where we detected significant changes in the signal. **B)** Graphs of the FA values obtained in the tracts mentioned above. The FA signal in the hippocampal commissure showed a Sex x Adolescent Treatment interaction ($F_{1,18}$=5.537; p= 0.030; η_p^2= 0.23), which upon further analysis indicated a reduced FA in females treated with THC than in control females ($F_{1,18}$=5,693; p=0,028; η_p^2=0,240) and their male counterparts ($F_{1,18}$=4.359; p=0.051; η_p^2= 0.19). In the rostral regions, adult animals of both sexes subjected to a chronic adolescent THC treatment had a reduced FA. **C)** anterior commissure ($F_{1,17}$=5.322; p=0.034; η_p^2=0.23) and **D)** corpus callosum ($F_{1,19}$= 5.298; p= 0.034; η_p^2= 0.23). No significant effects of the Adolescent THC Treatment were observed in the internal capsule (data not shown). See Table 8 for more details concerning statistical tests results.

1.3. ^{1}HR SPECTROSCOPY

A weaker ^{1}HR spectroscopy signal for choline compounds (GPCP+Ch) was detected in adult subjects treated with THC in the voxel employed for cortical measurement, yet no other metabolite changes were found in the cortex or in the STR voxel employed (see Figure 11 and Table 9).

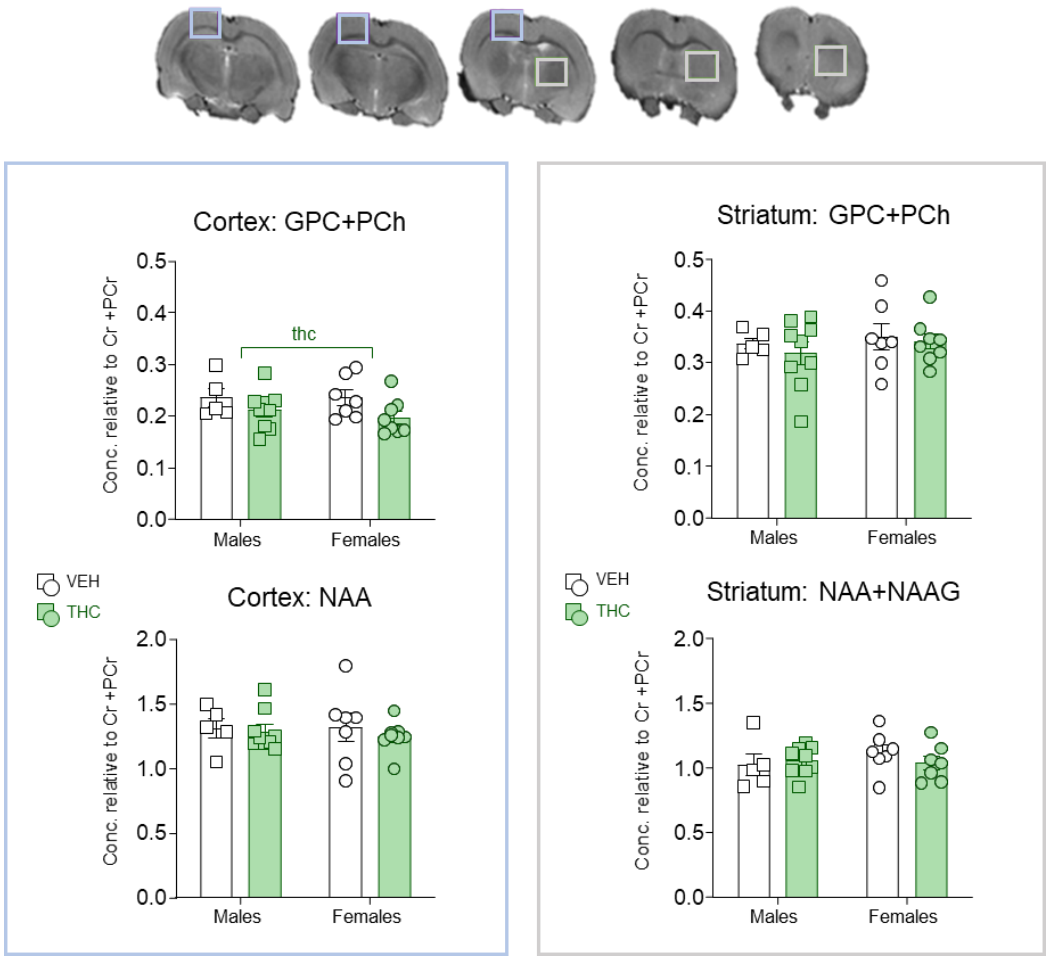


Figure 11: 1H NMR Spectroscopy. The graphs represent the individual values (dots) and the mean ± SEM (bars). Green lines and "THC" represent a significant effect of the factor Treatment. **A)** The 3 mm^3 voxel located in the cortex (in blue) or the striatum (in grey) used to obtain the spectra. **B)** The cortical GPC+PCh signal (glycerophosphorylcholine, phosphorylcholine, choline) was weaker in THC-treated animals ($F_{1,25}$=4.629; p=0.041; η_p^2=0.15), while the NAA ($F_{3,25}$= 0.941; p= 0.436; η_p^2= 0.10) and NAA+NAAG values ($F_{3,25}$= 0.298; p= 0.826; η_p^2= 0.03 ; graph not shown) remained unchanged. **C)** In the striatum, neither the GPC+PCh ($F_{3,25}$=0.493; p=0.690; ηp2=0.056) nor NAA ($F_{3,25}$=0.298; p=0.826; η_p^2=0.03) were altered by THC treatment, nor were sex specific difference detected. See Table 9 for further details.

Table 4. Gray Matter Volumetry

Area		p	Effect	Statistic value	*df* 1,e		Effect size	1-β	MALE VEH			MALE THC			FEMALE VEH			FEMALE THC		
									MEAN	SEM	N	MEAN	SEM	N	MEAN	SEM	N	MEAN	SEM	N
Total Brain Volume	**	0.000	SEX	F= 43.27	1	25	0.63	1.00	1.36E+06	3.02E+04	5	1.34E+06	1.40E+04	9	1.24E+06	1.69E+04	7	1.21E+06	1.90E+04	8
Cortex	*	0.031	SEX	F= 5.248	1	25	0.17	0.60	3.924	0.284	5	3.530	0.096	9	3.263	0.123	7	3.277	0.266	8
Left	*	0.027	SEX	F= 5.637	1	22	0.20	0.62	1.559	0.033	4	1.723	0.059	7	1.799	0.063	7	1.814	0.076	8
Right	ns	0.251	Corrected Model	F= 1.466	3	22	0.17	0.33	1.554	0.027	4	1.686	0.049	7	1.729	0.060	7	1.687	0.051	8
Hippocampus	ns	0.300	Corrected Model	F= 1.288	3	25	0.13	0.30	0.501	0.043	5	0.503	0.023	9	0.452	0.030	7	0.446	0.019	8
Left	*	0.029	SEX	F= 5.389	1	25	0.18	0.61	0.257	0.018	5	0.253	0.010	9	0.226	0.015	7	0.226	0.007	8
Right	ns	0.467	Corrected Model	F= 0.875	3	25	0.10	0.21	0.244	0.026	5	0.250	0.013	9	0.226	0.015	7	0.220	0.013	8
Cerebellum	**	0.015	SEX	F= 6.896	1	25	0.22	0.71	2.550	0.040	5	2.521	0.027	9	2.206	0.130	7	2.380	0.111	8
	****	**0.007**	**SEX * TMT**	**F= 8.806**	**1**	**25**	**0.26**	**0.81**												
	ns	0.081	THC effects in MALE	F= 3.301	1	25	0.12	0.42												
Dorsal Striatum	**	0.024	THC effects in FEMALE	F= 5.783	1	25	0.19	0.64	0.428	0.007	5	0.460	0.009	9	0.477	0.016	7	0.438	0.011	8
	**	0.013	SEX effects in VEH	F= 7.161	1	25	0.22	0.73												
	ns	0.167	SEX effects in THC	F= 2.022	1	25	0.08	0.28												
	*	**0.050**	**SEX * TMT**	**F= 4.260**	**1**	**25**	**0.15**	**0.51**												
	ns	0.149	THC effects in MALE	F= 2.218	1	25	0.08	0.30												
Left	ns	0.165	THC effects in FEMALE	F= 2.043	1	25	0.08	0.28	0.222	0.006	5	0.237	0.005	9	0.241	0.008	7	0.227	0.007	8
	ns	0.085	SEX effects in VEH	F= 3.226	1	25	0.11	0.41												
	ns	0.296	SEX effects in THC	F= 1.140	1	25	0.04	0.18												
	**	**0.004**	**SEX * TMT**	**F= 10.38**	**1**	**25**	**0.29**	**0.87**												
	ns	0.170	THC effects in MALE	F= 2.000	1	25	0.07	0.28												
Right	**	0.004	THC effects in FEMALE	F= 10.32	1	25	0.29	0.87	0.207	0.005	5	0.219	0.003	9	0.235	0.009	7	0.211	0.004	8
	**	0.003	SEX effects in VEH	F= 10.98	1	25	0.31	0.89												
	ns	0.302	SEX effects in THC	F= 1.110	1	25	0.04	0.17												
Amygdala	ns	0.626	Corrected Model	F= 0.592	3	25	0.07	0.15	0.088	0.007	5	0.085	0.009	9	0.083	0.012	7	0.071	0.009	8
Left	ns	0.367	Corrected Model	F= 1.102	3	25	0.12	0.26	0.042	0.004	5	0.045	0.004	9	0.036	0.006	7	0.036	0.004	8
Right	ns	0.723	Corrected Model	F= 0.445	3	0	0.05	0.13	0.046	0.003	5	0.045	0.004	9	0.044	0.007	7	0.039	0.004	8
Globus Pallidus	t	0.057	TMT	F= 4.022	1	23	0.15	0.48	0.082	0.005	4	0.071	0.005	9	0.079	0.005	7	0.068	0.004	7
Left	ns	0.780	Corrected Model	F= 0.364	3	23	0.05	0.11	0.040	0.004	5	0.038	0.002	9	0.039	0.002	6	0.036	0.002	7
Right	*	0.046	TMT	F= 4.494	1	22	0.17	0.53	0.038	0.002	4	0.033	0.003	9	0.037	0.003	7	0.030	0.001	6
Nucleus Accumbens	ns	0.839	Corrected Model	F= 0.280	3	25	0.03	0.10	0.094	0.015	5	0.083	0.010	9	0.091	0.009	7	0.082	0.007	8
Left	ns	0.750	Corrected Model	F= 0.407	3	25	0.05	0.12	0.047	0.008	5	0.040	0.005	9	0.045	0.005	7	0.041	0.003	8
Right	ns	0.899	Corrected Model	F= 0.195	3	25	0.02	0.08	0.047	0.007	5	0.043	0.005	9	0.046	0.005	7	0.042	0.004	8
Thalamus	ns	0.832	Corrected Model	F= 0.290	3	25	0.03	0.10	0.340	0.070	5	0.338	0.040	9	0.331	0.071	7	0.282	0.034	8
Left	ns	0.876	Corrected Model	F= 0.228	3	25	0.03	0.09	0.171	0.035	5	0.169	0.019	9	0.167	0.038	7	0.144	0.019	8
Right	ns	0.783	Corrected Model	F= 0.359	3	25	0.04	0.11	0.169	0.036	5	0.168	0.021	9	0.164	0.033	7	0.138	0.016	8
Septal Nuclei	ns	0.920	Corrected Model	F= 0.164	3	24	0.02	0.08	0.063	0.002	5	0.061	0.002	8	0.059	0.007	7	0.057	0.008	8

The main test performed are two-ANOVA with Sex (Male/Female) and Treatment (THC/VEH) as within subject factors. Interactions are analysed with a simple effect analysis. Corrected model associated values are reported when factor effects or interactions have associated p values over 0.1.

Table 5. Brain Ventricle Volumetry

Area		p	Effect	Statistic value	*df* 1	*df* e	Effect size	1-β	MALE VEH			MALE THC			FEMALE VEH			FEMALE THC		
									MEAN	SEM	N	MEAN	SEM	N	MEAN	SEM	N	MEAN	SEM	N
Brain ventricles	**	0.006	TMT	F= 8.961	1	23	0.28	0.82	0.194	0.019	4	0.167	0.007	8	0.180	0.008	7	0.140	0.012	8
4th ventricle	**	0.006	SEX	F= 9.053	1	25	0.27	0.82	0.074	0.007	5	0.062	0.007	9	0.053	0.004	7	0.044	0.006	8
	*	0.024	SEX	F= 5.794	1	25	0.19	0.64												
	**	0.000	TMT	F= 23.98	1	25	0.49	1.00												
	*****	**0.026**	**SEX * TMT**	**F= 5.575**	**1**	**25**	**0.18**	**0.62**												
Aqueduct	*	0.000	THC effects in MALE	F= 24.51	1	25	0.50	1.00	0.022	0.002	5	0.012	0.001	9	0.016	0.001	7	0.012	0.001	8
	ns	0.074	THC effects in FEMALE	F= 3.474	1	25	0.12	0.43												
	**	0.005	SEX effects in VEH	F= 9.598	1	25	0.28	0.85												
	ns	0.972	SEX effects in THC	F= 0.001	1	25	0.00	0.05												
3rd ventricle	ns	0.150	Corrected Model	F= 1.934	3	25	0.19	0.44	0.029	0.005	5	0.020	0.002	9	0.024	0.002	7	0.024	0.002	8
Lateral Ventricles	*	0.019	TMT	F= 6.341	1	24	0.19	0.63	0.038	0.005	4	0.034	0.003	9	0.047	0.004	7	0.032	0.003	8
	*****	**0.029**	**SEX * TMT**	**F= 5.394**	**1**	**24**	**0.00**	**0.06**												
	ns	0.781	THC effects in MALE	F= 0.079	1	24	0.30	0.87												
Left	**	0.004	THC effects in FEMALE	F= 10.45	1	24	0.21	0.67	0.015	0.001	4	0.016	0.001	9	0.021	0.002	7	0.014	0.001	8
	*	0.020	SEX effects in VEH	F= 6.197	1	24	0.01	0.09												
	ns	0.568	SEX effects in THC	F= 0.336	1	24	0.21	0.68												
Right	*	0.025	TMT	F= 5.705	1	24	0.13	0.44	0.018	0.004	4	0.014	0.002	9	0.019	0.001	7	0.014	0.001	8

The main test performed are two-ANOVA with Sex (Male/Female) and Treatment (THC/VEH) as within subject factors. Interactions are analysed with a simple effect analysis. Corrected model associated values are reported when factor effects or interactions have associated p values over 0.1.

Table 6. FA Gray matter

Area	p		Effect	Statistic value	*df* 1	*df* e	Effect size	1-β	MALE VEH			MALE THC			FEMALE VEH			FEMALE THC		
									MEAN	SEM	N	MEAN	SEM	N	MEAN	SEM	N	MEAN	SEM	N
Hippocampus	ns	0.381	Corrected Model	F= 1.068	3	24	0.12	0.25	0.175	0.028	5	0.249	0.035	9	0.194	0.030	6	0.193	0.027	8
Bregma -5mm	ns	0.746	Corrected Model	F= 0.411	3	24	0.05	0.12	0.192	0.041	5	0.255	0.038	9	0.225	0.041	6	0.233	0.034	8
Bregma -3,5mm	ns	0.360	Corrected Model	F= 1.124	3	23	0.13	0.26	0.159	0.021	5	0.215	0.032	8	0.162	0.028	6	0.153	0.026	8
Substancia Nigra	t	0.056	SEX	F= 4.067	1	23	0.15	0.49	0.226	0.015	5	0.292	0.025	9	0.214	0.010	6	0.219	0.016	7
Septal Nuclei	*	0.047	SEX	F= 4.397	1	21	0.15	0.52	0.243	0.018	5	0.270	0.024	9	0.239	0.017	7	0.189	0.013	7
Bregma -0,5mm	*	0.039	SEX	F= 4.850	1	21	0.19	0.56	0.260	0.031	5	0.221	0.013	7	0.233	0.019	7	0.174	0.006	6
	*	0.015	TMT	F= 6.999	1	21	0.25	0.71												
Bregma +1mm	ns	0.384	Corrected Model	F= 1.063	3	23	0.12	0.25	0.225	0.008	5	0.260	0.032	7	0.245	0.019	7	0.207	0.022	8
	*****	**0.015**	**SEX * TMT**	**F= 7.057**	**1**	**20**	**0.26**	**0.71**												
	*	0.010	THC effects in MALE	F= 8.144	1	20	0.29	0.78												
Thalamus	ns	0.441	THC effects in FEMALE	F= 0.619	1	20	0.03	0.12	0.125	0.021	4	0.221	0.015	7	0.159	0.028	7	0.135	0.017	6
	ns	0.335	SEX effects in VEH	F= 0.976	1	20	0.05	0.16												
	*	0.009	SEX effects in THC	F= 8.346	1	20	0.29	0.79												
Globus Pallidus	*	0.050	TMT	F= 4.309	1	21	0.17	0.51	0.237	0.025	5	0.183	0.010	6	0.223	0.030	7	0.181	0.019	7
Dorsal Striatum	ns	0.900	Corrected Model	F= 0.193	3	25	0.02	0.08	0.199	0.040	5	0.179	0.023	9	0.207	0.036	7	0.186	0.024	8
Bregma -0,5mm	ns	0.789	Corrected Model	F= 0.350	3	22	0.05	0.11	0.160	0.025	5	0.207	0.039	7	0.204	0.042	6	0.191	0.023	8
Bregma +1mm	ns	0.974	Corrected Model	F= 0.072	3	22	0.01	0.06	0.189	0.050	5	0.171	0.020	7	0.189	0.037	6	0.192	0.038	8
	*	0.030	TMT	F= 5.603	1	17	0.25	0.61												
	*****	**0.022**	**SEX * TMT**	**F= 6.364**	**1**	**17**	**0.27**	**0.66**												
	**	0.005	THC effects in MALE	F= 10.53	1	17	0.38	0.86												
Bregma +2,5mm	ns	0.907	THC effects in FEMALE	F= 0.014	1	17	0.00	0.05	0.246	0.051	5	0.102	0.008	4	0.163	0.022	5	0.169	0.023	7
	ns	0.095	SEX effects in VEH	F= 3.116	1	17	0.16	0.38												
	ns	0.089	SEX effects in THC	F= 3.250	1	17	0.16	0.40												
Cingulate Cortex	ns	0.701	Corrected Model	F= 0.477	3	25	0.05	0.13	0.199	0.030	5	0.186	0.017	9	0.212	0.034	7	0.172	0.021	8
Bregma -0,5mm	ns	0.666	Corrected Model	F= 0.531	3	21	0.07	0.14	0.167	0.039	4	0.223	0.043	7	0.205	0.040	6	0.173	0.021	8
Bregma +1mm	ns	0.915	Corrected Model	F= 0.170	3	22	0.02	0.08	0.203	0.041	5	0.171	0.016	7	0.199	0.042	6	0.187	0.035	8
Bregma +2,5mm	ns	0.172	Corrected Model	F= 1.851	3	19	0.23	0.40	0.231	0.041	5	0.168	0.017	7	0.165	0.024	5	0.150	0.020	6
Motor Cortex	ns	0.670	Corrected Model	F= 0.525	3	23	0.06	0.14	0.240	0.051	4	0.204	0.015	9	0.215	0.029	6	0.193	0.018	8
Bregma -0,5mm	ns	0.669	Corrected Model	F= 0.527	3	21	0.07	0.14	0.193	0.035	4	0.254	0.044	7	0.218	0.038	6	0.210	0.017	8
Bregma +1mm	ns	0.835	Corrected Model	F= 0.286	3	21	0.04	0.10	0.248	0.055	4	0.208	0.019	7	0.218	0.035	6	0.204	0.031	8
Bregma +2,5mm	*	0.026	SEX	F= 6.081	1	15	0.29	0.64	0.258	0.057	3	0.207	0.018	7	0.184	0.020	4	0.162	0.004	5
Nucleus Accumbens	ns	0.901	Corrected Model	F= 0.192	3	24	0.02	0.08	0.216	0.041	5	0.189	0.012	8	0.206	0.022	7	0.201	0.026	8
Bregma +1mm	ns	0.941	Corrected Model	F= 0.130	3	22	0.02	0.07	0.198	0.041	5	0.188	0.014	7	0.203	0.030	6	0.213	0.035	8
Bregma +2,5mm	ns	0.444	Corrected Model	F= 0.932	3	19	0.13	0.22	0.235	0.044	5	0.202	0.012	6	0.200	0.022	6	0.174	0.020	6

The main test performed are two-ANOVA with Sex (Male/Female) and Treatment (THC/VEH) as within subject factors. Interactions are analysed with a simple effect analysis. Corrected model associated values are reported when factor effects or interactions have associated p values over 0.1.

Table 7. MD Grey matter

Area		p	Effect	Statistic value	*df* 1	*df* e	Effect size	1-β	MALE VEH Mean	MALE VEH SEM	MALE VEH N	MALE THC Mean	MALE THC SEM	MALE THC N	FEMALE VEH Mean	FEMALE VEH SEM	FEMALE VEH N	FEMALE THC Mean	FEMALE THC SEM	FEMALE THC N
Hippocampus	ns	0.313	Corrected Model	F= 1.252	3	24	0.14	0.29	7.86E+05	1.38E+04	5	8.70E+05	2.39E+04	9	8.00E+05	5.72E+04	6	8.17E+05	2.92E+04	8
Bregma -5mm	t	0.057	TMT	F= 4.045	1	22	0.16	0.49	7.81E+05	1.82E+04	4	8.69E+05	2.54E+04	9	7.61E+05	1.79E+04	5	8.04E+05	3.80E+04	8
	ns	0.289	Corrected Model	F= 1.331	3	23	0.15	0.31												
Bregma -3,5	ns	0.102	Corrected Model	F= 2.357	1	20	0.26	0.51	8.06E+05	2.94E+04	5	8.36E+05	2.31E+04	8	7.22E+05	2.62E+04	4	8.04E+05	3.20E+04	7
Bregma -2mm	ns	0.181	Corrected Model	F= 1.778	3	22	0.20	0.40	7.80E+05	2.23E+04	4	9.00E+05	5.25E+04	9	7.59E+05	5.38E+04	6	8.05E+05	4.10E+04	7
Substancia Nigra	ns	0.422	Corrected Model	F= 0.976	3	22	0.12	0.23	8.03E+05	1.26E+04	5	8.46E+05	2.36E+04	8	8.53E+05	7.68E+04	6	7.68E+05	2.68E+04	7
Septal Nuclei	ns	0.583	Corrected Model	F= 0.662	3	24	0.08	0.17	8.10E+05	1.02E+04	4	8.55E+05	3.01E+04	9	8.66E+05	7.98E+04	7	7.90E+05	1.80E+04	8
Bregma -0,5mm	ns	0.922	Corrected Model	F= 0.160	3	25	0.02	0.08	8.71E+05	6.32E+04	5	8.47E+05	3.88E+04	9	8.69E+05	6.82E+04	7	8.28E+05	3.19E+04	8
	*	0.026	**GROUPS**	H= 9.284	3	25	0.33													
	ns	0.518	THC effects in MALE	U= 17	1	14	0.04													
Bregma +1mm	*	0.028	THC effects in FEMALE	U= 9	1	15	0.35		8.12E+05	2.97E+04	5	8.64E+05	1.06E+05	9	9.05E+05	1.96E+05	7	7.52E+05	3.18E+04	8
	ns	0.808	SEX effects in VEH	U= 16	1	12	0.01													
	*	0.012	SEX effects in THC	U= 10	1	17	0.39													
Thalamus	ns	0.246	Corrected Model	F= 1.481	3	23	0.16	0.34	7.60E+05	1.92E+04	5	8.79E+05	4.57E+04	9	7.86E+05	6.03E+04	6	7.85E+05	3.83E+04	7
Globus Pallidus	ns	0.719	Corrected Model	F= 0.450	3	24	0.05	0.13	7.67E+05	4.61E+04	5	7.44E+05	2.11E+04	8	8.10E+05	6.39E+04	7	7.80E+05	3.25E+04	8
Dorsal Striatum	t	0.055	SEX * TMT	F= 4.091	1	23	0.15	0.49	7.48E+05	1.43E+04	5	8.29E+05	3.34E+04	9	8.25E+05	6.34E+04	7	7.36E+05	2.28E+04	8
Bregma -0,5mm	t	0.052	SEX * TMT	F= 4.157	1	25	0.14	0.50	7.40E+05	1.12E+04	5	8.06E+05	3.78E+04	9	8.24E+05	7.43E+04	7	7.07E+05	1.29E+04	8
Bregma +1mm	ns	0.661	Corrected Model	F= 0.538	3	24	0.06	0.14	7.73E+05	2.87E+04	4	7.95E+05	3.69E+04	9	8.34E+05	9.39E+04	7	7.37E+05	3.43E+04	8
Bregma +2,5mm	ns	0.388	Corrected Model	F= 1.057	3	23	0.13	0.25	7.35E+05	8.09E+03	4	8.24E+05	2.99E+04	8	8.10E+05	4.15E+04	7	7.77E+05	3.37E+04	7
Cingulate Cortex	ns	0.554	Corrected Model	F= 0.713	3	23	0.09	0.18	8.03E+05	2.14E+04	5	8.43E+05	1.66E+04	8	8.13E+05	6.55E+04	6	7.78E+05	2.33E+04	8
Bregma -0,5mm	ns	0.330	Corrected Model	F= 1.201	3	25	0.13	0.28	7.87E+05	2.92E+04	5	8.57E+05	4.16E+04	9	8.92E+05	9.29E+04	7	7.67E+05	1.32E+04	8
Bregma +1mm	ns	0.624	Corrected Model	F= 0.596	3	24	0.07	0.16	8.33E+05	3.08E+04	4	8.48E+05	3.70E+04	9	9.13E+05	1.27E+05	7	7.82E+05	3.63E+04	8
Bregma +2,5mm	ns	0.523	Corrected Model	F= 0.770	3	23	0.09	0.19	8.00E+05	2.06E+04	5	8.60E+05	3.36E+04	8	8.12E+05	4.25E+04	7	7.94E+05	3.44E+04	7
Motor Cortex	ns	0.377	Corrected Model	F= 1.079	3	24	0.12	0.25	7.47E+05	2.55E+04	5	7.90E+05	3.03E+04	8	8.46E+05	7.04E+04	7	7.53E+05	2.44E+04	8
	*****	**0.037**	**SEX * TMT**	**F= 4.858**	**1**	**25**	**0.16**	**0.56**												
	ns	0.231	THC effects in MALE	F= 1.506	1	25	0.06	0.22												
Bregma -0,5mm	ns	0.067	THC effects in FEMALE	F= 3.678	1	25	0.13	0.45	7.36E+05	1.89E+04	5	8.23E+05	4.19E+04	9	8.56E+05	7.98E+04	7	7.29E+05	1.27E+04	8
	ns	0.121	SEX effects in VEH	F= 2.577	1	25	0.09	0.34												
	ns	0.142	SEX effects in THC	F= 2.301	1	25	0.08	0.31												
Bregma +1mm	ns	0.610	Corrected Model	F= 0.619	3	24	0.07	0.16	7.74E+05	4.22E+04	4	8.05E+05	3.90E+04	9	8.83E+05	1.07E+05	7	7.71E+05	4.14E+04	8
Bregma +2,5mm	ns	0.325	Corrected Model	F= 1.222	3	22	0.14	0.28	7.47E+05	2.51E+04	5	8.37E+05	3.38E+04	7	7.98E+05	3.85E+04	7	7.67E+05	3.53E+04	7
Nucleus Accumbens	ns	0.429	Corrected Model	F= 0.957	3	23	0.11	0.23	7.64E+05	1.78E+04	5	8.09E+05	1.71E+04	8	8.13E+05	5.19E+04	7	7.50E+05	2.85E+04	8
Bregma +1mm	ns	0.586	Corrected Model	F= 0.658	3	23	0.08	0.17	7.85E+05	3.04E+04	4	8.12E+05	3.65E+04	9	7.67E+05	5.70E+04	6	7.40E+05	3.13E+04	8
Bregma +2,5mm	ns	0.345	Corrected Model	F= 1.164	3	23	0.13	0.27	7.58E+05	1.29E+04	5	8.37E+05	2.16E+04	8	8.04E+05	4.72E+04	7	7.73E+05	3.17E+04	7

The main test performed are two-ANOVA with Sex (Male/Female) and Treatment (THC/VEH) as within subject factors. Interactions are analysed with a simple effect analysis. Corrected model associated values are reported when factor effects or interactions have associated p values over 0.1.

Table 8. FA White matter

Area	p	Effect	Statistic value	*df* 1	*df* e	Effect size	1-β	MALE VEH MEAN	MALE VEH SEM	MALE VEH N	MALE THC MEAN	MALE THC SEM	MALE THC N	FEMALE VEH MEAN	FEMALE VEH SEM	FEMALE VEH N	FEMALE THC MEAN	FEMALE THC SEM	FEMALE THC N
Corpus Callosum	ns 0.330	Corrected Model	F= 1.22	3	19	0.161	0.274	0.298	0.014	5	0.305	0.011	6	0.307	0.016	6	0.275	0.013	6
Bregma -6,8mm	ns 0.864	Corrected Model	F= 0.245	3	19	0.037	0.088	0.364	0.025	5	0.356	0.030	7	0.382	0.031	6	0.352	0.010	5
Bregma -5mm	ns 0.102	Corrected Model	F= 2.357	3	20	0.261	0.505	0.304	0.011	5	0.346	0.016	7	0.305	0.013	6	0.305	0.013	6
Bregma -3,5mm	ns 0.370	Corrected Model	F= 1.11	3	19	0.149	0.252	0.260	0.013	5	0.313	0.036	6	0.285	0.018	6	0.265	0.014	6
Bregma -2mm	ns 0.129	Corrected Model	F= 2.155	1	18	0.264	0.458	0.304	0.044	5	0.277	0.015	6	0.290	0.020	6	0.218	0.013	5
Bregma -0,5mm	ns 0.607	Corrected Model	F= 0.626	3	20	0.086	0.157	0.270	0.005	5	0.307	0.021	7	0.298	0.026	6	0.282	0.021	6
Bregma +1mm	ns 0.719	Corrected Model	F= 0.452	3	20	0.064	0.125	0.292	0.025	5	0.289	0.019	7	0.325	0.043	6	0.287	0.013	6
Bregma +2,5mm	* 0.034	TMT	F= 5.298	1	19	0.238	0.583	0.280	0.047	5	0.202	0.013	6	0.258	0.019	6	0.220	0.012	6
Hipocampal Comissure	*** 0.030**	**SEX * TMT**	**F= 5.537**	**1**	**18**	**0.235**	**0.605**												
	ns 0.320	THC effects in MALE	F= 1.046	1	18	0.055	0.163												
	* 0.028	THC effects in FEMALE	F= 5.694	1	18	0.240	0.617	0.288	0.008	4	0.307	0.011	6	0.312	0.012	6	0.272	0.014	6
	ns 0.214	SEX effects in VEH	F= 1.663	1	18	0.085	0.231												
	t 0.051	SEX effects in THC	F= 4.359	1	18	0.195	0.506												
Internal Capsule	ns 0.498	Corrected Model	F= 0.822	3	19	0.115	0.194	0.264	0.024	5	0.272	0.023	6	0.265	0.012	6	0.237	0.005	6
Bregma -3,5mm	* 0.015	SEX	F= 7.28	1	18	0.288	0.723	0.328	0.009	4	0.335	0.027	6	0.302	0.005	6	0.273	0.007	6
Bregma -2mm	ns 0.197	Corrected Model	F= 1.729	3	18	0.224	0.375	0.318	0.038	5	0.246	0.022	5	0.285	0.014	6	0.278	0.007	6
	*** 0.027**	**SEX * TMT**	**F= 5.739**	**1**	**19**	**0.232**	**0.623**												
	ns 0.072	THC effects in MALE	F= 3.64	1	19	0.161	0.441												
Bregma -0,5mm	ns 0.159	THC effects in FEMALE	F= 2.145	1	19	0.101	0.285	0.195	0.010	4	0.266	0.032	7	0.248	0.027	6	0.198	0.007	6
	ns 0.178	SEX effects in VEH	F= 1.952	1	19	0.093	0.264												
	t 0.055	SEX effects in THC	F= 4.195	1	19	0.181	0.494												
Anterior Comissure	ns 0.253	Corrected Model	F= 1.482	3	18	0.198	0.325	0.220	0.009	4	0.252	0.020	6	0.253	0.031	6	0.202	0.011	6
Bregma +1mm	ns 0.456	Corrected Model	F= 0.912	3	17	0.139	0.208	0.235	0.016	4	0.228	0.016	6	0.250	0.016	5	0.217	0.011	6
Bregma +2,5mm	* 0.034	TMT	F= 5.323	1	17	0.238	0.585	0.280	0.047	5	0.202	0.013	6	0.258	0.019	6	0.220	0.012	6

Table 9. ^{1}HR MR spectroscopy

Metabolite	p	Effect	Statistic value	*df* 1	*df* e	Effect size	1-β	MALE VEH MEAN	MALE VEH SEM	MALE VEH N	MALE THC MEAN	MALE THC SEM	MALE THC N	FEMALE VEH MEAN	FEMALE VEH SEM	FEMALE VEH N	FEMALE THC MEAN	FEMALE THC SEM	FEMALE THC N
Cortex: GPC PCh	* 0.041	TMT	F= 4.629	1	25	0.16	0.543	0.236	0.018	5	0.212	0.013	9	0.236	0.015	7	0.198	0.012	8
Cortex: NAA	ns 0.436	Corrected Model	F= 0.941	3	25	0.10	0.227	1.053	0.155	5	1.205	0.066	9	1.258	0.086	7	1.147	0.046	8
Cortex: NAA+NAAG	ns 0.826	Corrected Model	F= 0.298	3	22	0.04	0.098	1.314	0.075	5	1.249	0.034	8	1.320	0.109	7	1.258	0.010	6
STR: GPC PCh	ns 0.690	Corrected Model	F= 0.493	3	25	0.06	0.136	0.337	0.011	5	0.318	0.022	9	0.350	0.025	7	0.341	0.015	8
STR: NAA+NAAG	ns 0.699	Corrected Model	F= 0.480	3	25	0.05	0.133	1.021	0.087	5	1.056	0.037	9	1.121	0.059	7	1.156	0.128	8

The main test performed are two-ANOVA with Sex (Male/Female) and Treatment (THC/VEH) as within subject factors. Interactions are analysed with a simple effect analysis. Corrected model associated values are reported when no factor effects or interactions have associated p values over 0.1.

2. EXPERIMENT 2: BEHAVIOURAL TRAITS

2.1. PAVLOVIAN TO INSTRUMENTAL TRANSFER

Across Pavlovian training sessions, the CS^+ HE ratio showed a significant Sex x Treatment interaction, although there were no significant simple effects. All groups progressively increased their bias to perform more HE during CS^+ than during CS^- and ended the Pavlovian training with ratios over 0.7. Females performed more HE during the Pavlovian training during both CS^+ and CS^-. There was also a trend for females to make more HE during the NSI. No differences between groups were detected in the last Pavlovian training session before instrumental training.

Over the time course of the instrumental training sessions, animals rapidly learned instrumental contingencies associated with both levers. The lever press ratio at the end of the training was over 0.8. Females took more time to achieve the limit of reinforcers in each session and they performed less TOALP during the instrumental training. No effect in the number of head entries was found across the sessions. There was a decrease in the number of ALP across the extinction sessions. The repeated measures ANOVA showed a Sex x Treatment interaction for ILP but no significant simple effects were evident (see Figure 12).

The majority of VEH animals (80% of males, 70% of females) expressed PIT showing that the procedure designed and employed was able to reproduce this phenomenon, and a similar proportion of animals treated with THC expressed PIT effectively (66.67% of males, 80% of females). Nonetheless, PIT was generally enhanced in THC animals (a bigger proportion of animals expressed a PIT over 75%: see Figure 13).

During the PIT testing session, the two-way ANOVA of the subjects that expressed %ALP on CS^+ above 50%, resulted in a global effect of THC and a Sex x Treatment interaction (Table 11). The simple effect analysis showed that THC-exposed males had a higher %CS+ ALP compared to their controls. This effect mainly resulted from a lower CS^- ALPs in the group of THC-exposed males with no differences in the rate of CS^+ ALPs as a result of THC (see Figure 14). The CS^+ HE ratio also showed a significant Sex x Treatment interaction resulting from the increased ratio in THC females relative to the VEH females (Figure 14).

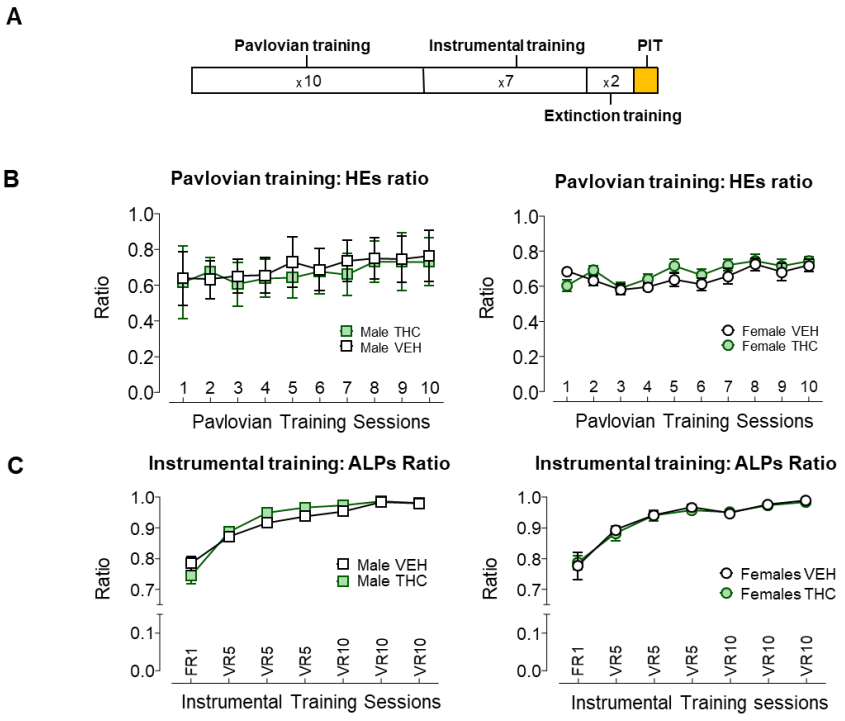


Figure 12: Pavlovian to Instrumental Transfer (PIT) training. A) Timeline of the experimental phases. **B)** Head entries (HEs) ratio across Pavlovian training sessions. Males and females are plotted separately for the sake of visual clarity. There was a general increase in the number of HEs during CS^+ across the sessions ($F_{5.7,341.6}$=9.657; p=0.000; η_p^2=0.14). Also, there was a Sex x Treatment interaction ($F_{5.7,341.6}$ =2.338; p=0.033; η_p^2=0.03), although no differences were detected in the analysis of the simple effects. **C)** Active Lever Press ratio across training sessions, with males and females plotted separately for the sake of visual clarity. There was a general increase in the discrimination and preference for the AL over the IL across the sessions ($F_{2.09,396}$=136.664; p=0.000; η_p^2=0.67). The graphs show the means ± SEM.

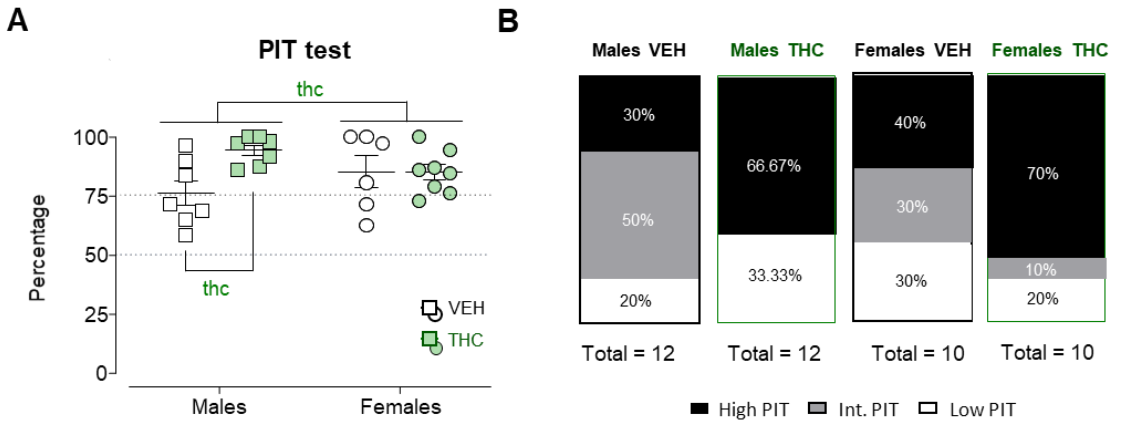


Figure 13: Pavlovian to Instrumental Transfer (PIT) test. A) The %ALP on CS+ on Pavlovian to Instrumental Transfer (PIT). A general Treatment effect was found (F1,25=4.685; p=0.040; ηp^2=0.15) suggesting increased PIT in THC animals, however, the significant Sex x Adolescent Treatment interaction (F1,25=4.996; p=0.035; ηp^2=0.16) revealed that the increase in %ALPs on CS+ was only significant in THC males compared to VEH males (F1,25=10.108; p=0.004;ηp^2=0.28). Animals who did not express PIT (more than 50% ALPs on CS+) were excluded from this analysis. The graph exclusively shows the values from animals that actually showed PIT (more than 50% of the ALP during the CS+) (see Figure 14 and Table 11 for additional data). The graph shows the individual values (dots) and the mean ± SEM. Green lines and "THC" represent a significant effect of the factor Treatment. **B)** Distribution of the intensity of PIT expression. Individuals classified as High PIT expressed a %ALP on CS+ above 75%, intermediate between 75% and 50%, and low PIT below 50%.

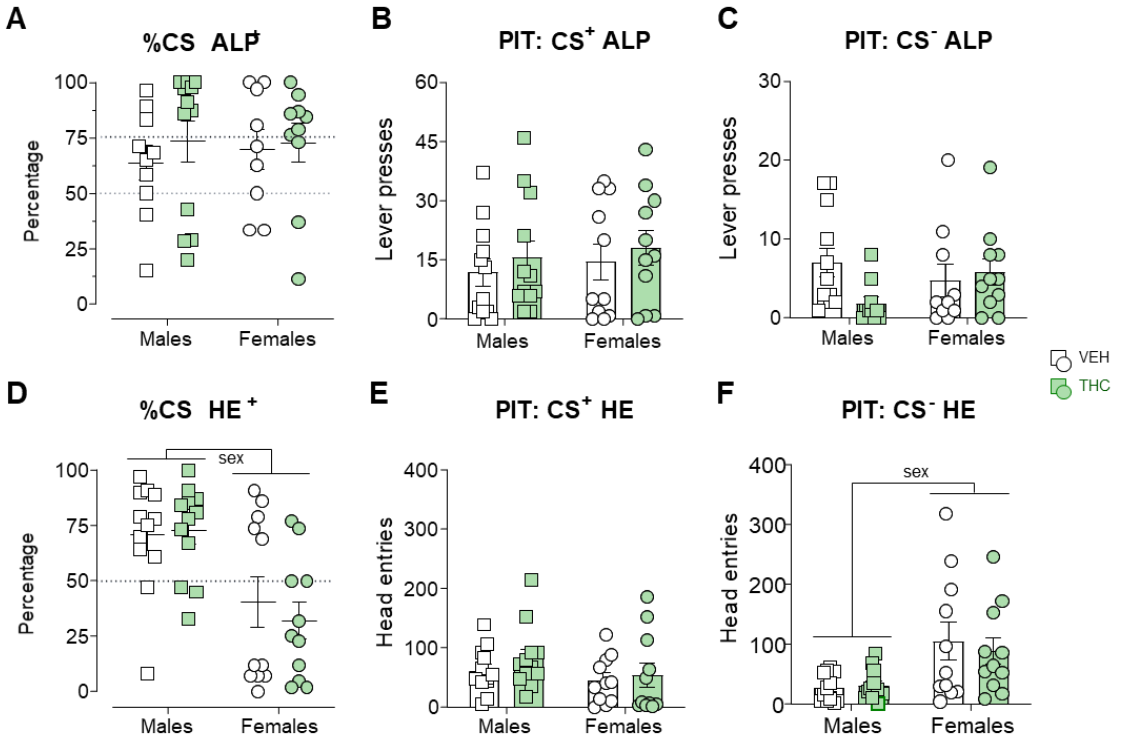


Figure 14: Pavlovian to Instrumental Transfer (PIT) test. The graphs represent the individual values (dots) and the mean ± SEM (bars). Within each graph, the green lines and "thc" represent a significant effect of the factor Treatment. Black lines and "sex" represent statistically significant effects of the factor Sex. **A)** Percentage of lever presses during the CS+ in the transfer test. No differences between the groups were detected, including animals that did not express PIT ($F_{3,43}$=1.031; p=0.389; ηp^2=0.07). **B)** Total Lever Presses during the CS+ in the transfer test. No differences between groups were detected ($F_{3,42}$=0.385; p=0.764; ηp^2=0.02). **C)** Total Lever Presses during the CS- in the transfer test. **D)** Percentage of HEs during the CS+ in the transfer test. Females made relatively fewer HEs during the CS+ as compared to the CS- than males ($F_{1,45}$=18.095; p=0.000; ηp^2=0.301). The mean percentage of HEs during the CS+ was clearly above 50% in the males, indicating clear conditioning of the CS+, while in the females this value was below 50%. **E)** Total HEs during the CS+ in the transfer test. No differences were detected between the groups ($F_{3,42}$ =1.085; p=0.366; ηp^2=0.07). **F)** Total head entries during the CS- in the transfer test. Females made more HEs during the CS- presentations than the males ($F_{1,42}$=12.460; p=0.001; ηp^2=0.22).

2.2. TWO-CHOICE SERIAL REACTION TIME TASK

Animals exposed to THC required fewer training sessions to reach a stable baseline (p=0.055: see Figure 15). During the six baseline sessions there was a lower CR rate in THC animals and conversely, THC-treated animals performed more IR. PreR, PerR, OR, TOR and HE varied across the sessions but there were no differences between the distinct groups (see Table 12). However, a multivariate analysis showed a Sessions x Treatment interaction, indicating that THC treatment induced variations in the raw PreR during some sessions. A repeated measures analysis of the last three session did actually show a significant interaction with Treatment (p=0.002) (see Table 13). However, as the baseline sessions were meant to stabilize the behaviour and establish a starting point, we calculated the relative increase in PreR in the subsequent long ITI sessions in order to measure relative changes in motor impulsivity within each group in addition to the percentage of PreR (% PreR).

In the first long-ITI session, the relative increase in PreR was higher in female rats exposed to THC than the female-VEH rats. Also, THC-exposed rats (regardless of their sex) showed a heightened relative increase in PreR in the second long-ITI sessions. However, this effect disappeared once the rats were already accustomed to the ITI challenge in the third long-ITI session, suggesting that these effects also interact with the novelty of the task and that they reflect state-like impulsivity more than a stable trait. In addition, during the baseline training sessions (phase 12 of training), THC-exposed rats performed worse (i.e. they made fewer correct responses: see Table 12), although the size of this effect was small. Moreover, this poorer performance was transient and no longer evident on the last day of training, and it was absent during the tests (see Figure 15, and Table 12 for further details).Regarding the first long ITI session, males treated with THC performed fewer PreR than their controls, revealing a state dependent reduction in motor impulsivity. Nonetheless, stable softening of a motor impulsivity trait was not found, as the mean PreR in the subsequent tests performed didn't reach any treatment nor sex significant effect. The First ITI session PreR (raw counts) and %PreR showed interactive differences. In males that received THC, the PreR and %PreR were significantly lower than in the male controls that received the vehicle alone. Also, a sex specific difference arose, with the fewer raw PreR and a lower %PreR in females than in males that received the vehicle alone. A repeated measures analysis across the three ITI sessions identified a Sex x Treatment interaction that pointed to the same statistical effects between male groups due to THC treatment and sex differences within control animals in both the PreR and %PreR parameters. Nonetheless, no group differences were found in single ANOVA analysis of the 2nd or 3rd long ITI test (see Table 13).

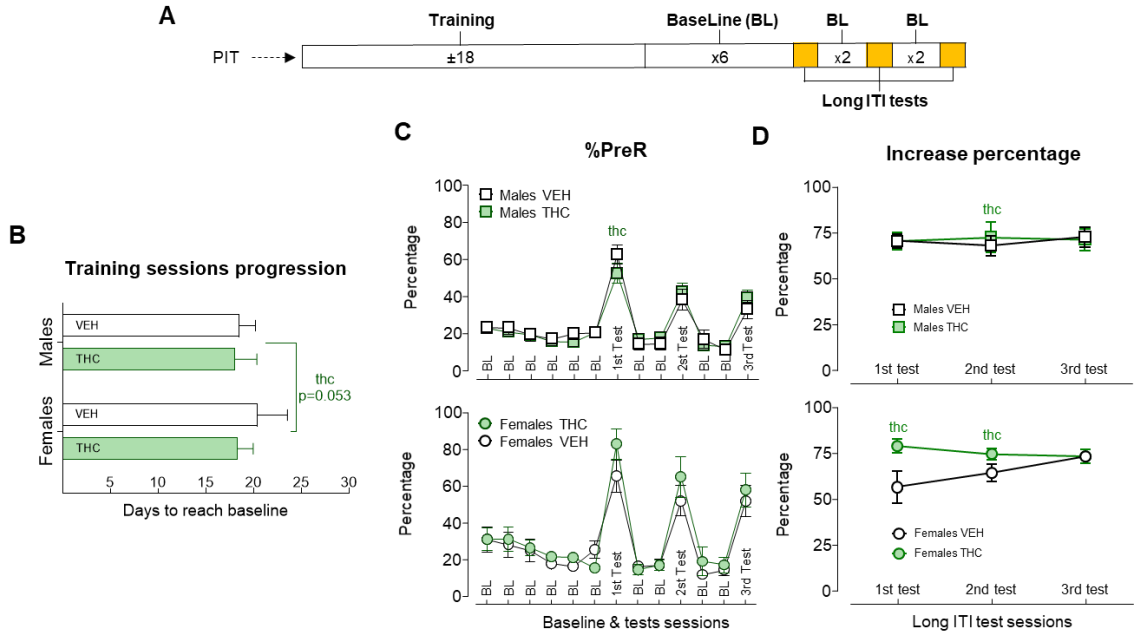


Figure 15: 2-Choice Serial Reaction Time Task. **A)** Timeline of the experimental phases. **B)** Number of training days required to reach the values in the baseline sessions. THC animals showed a trend ($F_{1,44}$=3.888; p=0.055; ηp^2=0.08) towards requiring fewer days to reach baseline sessions than VEH rats. **C)** Percentage of premature responses in the final experimental stages. In the first long-ITI test, a Sex x Treatment interaction ($F_{1,44}$=7.483; p=0.009; ηp^2=0.14) and further simple effects analysis showed an effect of sex due to an increase in the PreR in females VEH compared to males VEH ($F_{1,44}$=7.630; p=0.008; ηp^2=0.14) and a decrease in PreR in Male THC compared to males VEH ($F_{1,44}$=5.740; p=0.021; ηp^2=0.11). **D)** Percentage of premature responses increased in the test relative to the previous baseline sessions. The relative increase showed a Sex x Treatment interaction in the first long ITI session ($F_{1,44}$= 4.034; p= 0.051; ηp^2= 0.08), the simple effects analysis showed a stronger increase in THC females compared to their VEH counterparts ($F_{1,44}$= 7.892; p= 0.007; ηp^2= 0.15) and a general Treatment effect in the second long ITI session ($F_{1,44}$= 5.240; p= 0.027; ηp^2=0.10).

Table 10. PIT Training results

		**	0.000	Sessions	R.M.	within-subjects (GG)	F= 11.33	4.95	202.9	0.22	1.00											
		*****	**0.041**	**Sessions*SEX*TMT**	**R.M.**	**within-subjects (GG)**	**F= 2.375**	**4.95**	**202.9**	**0.06**	**0.75**											
		ns	0.115	(SESSIONS*TMT) in MALES	R.M.	within-subjects (GG)	F= 1.896	4.18	91.98	0.08	0.57											
	CS+ HE ratio	ns	0.112	(SESSIONS*TMT) in FEMALES	R.M.	within-subjects (GG)	F= 1.838	5.05	95.96	0.09	0.61	0.704	0.005	12	0.665	0.005	12	0.671	0.006	11	0.712	0.007	10
		ns	0.261	(SESSIONS*SEX) in VEH	R.M.	within-subjects (GG)	F= 1.324	4.82	101.1	0.06	0.44											
		ns	0.103	(SESSIONS*SEX) in THC	R.M.	within-subjects (GG)	F= 1.965	4.24	84.75	0.09	0.59											
		t	0.051	SEX * TMT	R.M.	between subjects	F= 4.032	1.00	41	0.09	0.50											
Pavlovian Training	CS+ HE	**	0.000	Sessions	R.M.	within-subjects (GG)	F= 33.58	2.74	112.3	0.45	1.00	150.5	5.624	12	179.98	5.624	12	193.34	6.136	11	222.95	6.749	10
		**	0.000	Sessions	R.M.	within-subjects	F= 10.04	9	369	0.20	1.00											
		*****	**0.040**	**SEX * TMT**	**R.M.**	**between subjects**	**F= 4.504**	**1**	**41**	**0.10**	**0.55**											
		t	0.056	(SESSIONS*TMT) in MALES	R.M.	between subjects	F= 4.086	1	22	0.16	0.49											
	CS- HE	ns	0.344	(SESSIONS*TMT) in FEMALES	R.M.	between subjects	F= 0.941	1	19	0.05	0.15	61.4	2.322	12	85.967	2.322	12	91.591	2.533	11	80.79	2.787	10
		*	0.024	(SESSIONS*SEX) in VEH	R.M.	between subjects	F= 5.956	1	21	0.22	0.64											
		ns	0.645	(SESSIONS*SEX) in THC	R.M.	between subjects	F= 0.218	1	20	0.01	0.07											
	ITI HE	**	0.000	Sessions	R.M.	within-subjects (GG)	F= 13.22	5.09	208.7	0.24	1.00	60.83	2.155	12	82.65	2.155	12	83.727	2.351	11	78.24	2.586	10
	CS+ HEs ratio	ns	0.731	Corrected model	ANOVA	between subjects	F= 0.432	3	42	0.03	0.13	0.790	0.04	12	0.7601	0.043	12	0.7362	0.039	11	0.7859	0.025	11
Pavlovian Training Session 10	CS+ HE	ns	0.324	Corrected model	ANOVA	between subjects	F= 1.192	3	42	0.08	0.30	198.7	23.81	12	252.25	25	12	278.73	39.50	11	308.73	71.33	11
	CS- HE	ns	0.668	Corrected model	ANOVA	between subjects	F= 0.524	3	41	0.04	0.15	56.67	14.37	12	74.75	12.85	12	74.9	8.134	10	77.364	16.28	11
	ISI HE	ns	0.288	Group	KW		H= 3.765	3		0.02		52.88	9.766	12	69.292	13.87	12	114	25.39	11	54.5	7.492	10
		******	**0.001**	**SEX * TMT**	**R.M.**	**within-subjects**	**F= 4.162**	**6**	**252**	**0.09**	**0.98**											
		*	0.043	(SESSIONS*TMT) in MALES	R.M.	within-subjects (GG)	F= 3.233	2.22	48.82	0.13	0.62											
	LP ratio	ns	0.252	(SESSIONS*TMT) in FEMALES	R.M.	within-subjects (GG)	F= 1.426	1.96	39.23	0.07	0.28	0.907	0.003	12	0.913	0.003	12	0.914	0.003	11	0.919	0.003	11
		ns	0.237	(SESSIONS*SEX) in VEH	R.M.	within-subjects (GG)	F= 1.490	1.98	41.48	0.07	0.30											
		*	0.030	(SESSIONS*SEX) in THC	R.M.	within-subjects (GG)	F= 3.811	2.00	41.98	0.15	0.66											
Instrumental training	ALPs	**	0.000	Sessions	R.M.	within-subjects (GG)	F= 52994	6	252	1.00	1.00	203.6	0.123	12	203.26	0.123	12	202.94	0.134	11	203.49	0.134	11
	ILPs	**	0.000	Sessions	R.M.	within-subjects	F= 22.64	6	252	0.35	1.00	14.32	0.498	12	11.333	0.498	12	12.286	0.543	11	13.247	0.543	11
	LPs	**	0.000	Sessions*Lever	R.M.	within-subjects	F= 6153	6	252	0.99	1.00	109	0.234	12	107.3	0.234	12	107.61	0.256	11	108.37	0.256	11
	TOALP	**	0.020	Sessions	R.M.	within-subjects (GG)	F= 4.049	2.05	86.03	0.09	0.71	3.452	0.157	12	3.8929	0.157	12	2.0909	0.171	11	2.5195	0.171	11
	HE	**	0.000	Sessions	R.M.	within-subjects	F= 46.83	6	252	0.53	1.00	121.3	2.04	12	109.18	2.04	12	129.88	2.225	11	116.99	2.225	11
	Time to complete	*	0.042	Sessions	R.M.	within-subjects	F= 2.222	6	252	0.05	0.78	633.3	10.25	12	477.2	10.25	12	615.35	11.18	11	534.73	11.18	11
	ALPs	**	0.000	Sessions	R.M.	Within-subjects	F= 226.2	2	84	0.84	1.00	156.3	2.889	12	133.6	2.889	12	143.3	3.152	11	147.9	3.152	11
		******	**0.006**	**Sessions*SEX*TMT**	**R.M.**	**Within-subjects (GG)**	**F= 6.110**	**1.63**	**68.6**	**0.13**	**0.82**											
		ns	0.064	THC effects in MALE	R.M.	Within-subjects	F= 2.928	2	44	0.12	0.54											
Extinction training	ILPs	t	0.052	THC effects in FEMALE	R.M.	Within-subjects (GG)	F= 3.611	1.48	29.6	0.15	0.54	29.97	0.851	12	29.08	0.851	12	28.82	0.928	11	25.64	0.928	11
		ns	0.067	SEX effects in VEH	R.M.	Within-subjects	F= 2.879	2	42	0.12	0.53											
		ns	0.064	SEX effects in THC	R.M.	Within-subjects (GG)	F= 3.283	1.49	31.2	0.14	0.51											
	Total LPs	**	0.000	Sessions	R.M.	Within-subjects	F= 240.0	2	84	0.85	1.00	186.3	3.150	12	162.6	3.150	12	172.2	3.437	11	173.5	3.437	11
	HE	*	0.043	Sessions	R.M.	Within-subjects (GG)	F= 3.742	2.00	60.723	0.07	0.50	113.9	4.205	12	131.9	4.205	12	133.5	4.587	11	100.3	4.587	11

Test performed are two-ANOVA with Sex (Male/Female) and Treatment (VEH/THC) as within subject factors or Repeated Measures (RM) ANOVA using the between-factor Session. Corrected model values are reported when factor effects and interactions have associated p values over 0.1.

Table 11. PIT Test results

Phase	Measure	p		Effect	Test	Statistic value	*df* 1,e		Effect size	1-β	Male VEH Mean	Male VEH SEM	Male VEH N	Male THC Mean	Male THC SEM	Male THC N	Female VEH Mean	Female VEH SEM	Female VEH N	Female THC Mean	Female THC SEM	Female THC N
Pavlovian to Instrumental Transfer Test	CS+ ALP	ns	0.764	Corrected model	ANOVA	F= 0.385	3	42	0.03	0.12	11.83	3.464	12	15.583	4.206	12	14.55	4.479	11	18.00	4.342	11
	CS- ALP	ns	0.073	SEX * TMT	ANOVA	F= 3.392	1	39	0.08	0.44	7.000	1.784	12	1.900	0.823	10	4.800	2.037	10	5.818	1.639	11
	%ALP on CS+	ns	0.389	Corrected model	ANOVA	F= 1.031	3	43	0.07	0.26	53.130	9.586	12	73.320	9.432	12	62.810	10.632	10	72.730	8.736	10
		*	0.040	TMT	ANOVA	F= 4.685	1	25	0.16	0.55												
		****	**0.035**	**SEX*TMT**	**ANOVA**	**F= 4.996**	**1**	**25**	**0.17**	**0.57**												
	%ALP CS+	**	0.004	THC effects in MALE	ANOVA	F= 10.11	1	25	0.29	0.86												
	>50%	ns	0.961	THC effects in FEMALE	ANOVA	F= 0.002	1	25	0.00	0.05	76.074	5.261	7	94.950	2.089	8	85.250	6.598	6	84.940	3.212	8
		ns	0.163	SEX effects in VEH	ANOVA	F= 2.065	1	25	0.08	0.28												
		ns	0.093	SEX effects in THC	ANOVA	F= 3.045	1	25	0.11	0.39												
	CS+ HE	ns	0.366	Corrected model	ANOVA	F= 1.085	3	42	0.07	0.27	81.08	14.43	12	75.25	16.25	12	58.54	26.00	13	112.917	16.469	12
	CS- HE	**	0.001	SEX	ANOVA	F= 12.46	1	42	0.23	0.93	27.25	6.799	12	30.83	7.348	12	105.3	31.85	11	88.546	22.032	11
	%HE on CS+	**	0.000	SEX	ANOVA	F= 18.10	1	45	0.30	0.99	70.63	7.062	12	72.49	5.948	12	40.32	11.58	11	31.945	8.222	11
		**	0.003	SEX	ANOVA	F= 10.11	1	45	0.18	0.88												
		*	0.014	TMT	ANOVA	F= 6.530	1	45	0.13	0.71												
		****	**0.000**	**SEX*TMT**	**ANOVA**	**F= 26.32**	**1**	**45**	**0.37**	**1.00**												
	%HE on CS+	ns	0.068	THC effects in MALE	ANOVA	F= 3.688	1	22	0.14	0.45	76.54	5.64	12	59.82	6.63	12	22.56	8.21	13	72.48	4.52	12
	>50%	**	0.000	THC effects in FEMALE	ANOVA	F= 27.06	1	23	0.54	1.00												
		**	0.000	SEX effects in VEH	ANOVA	F= 28.41	1	23	0.55	1.00												
		ns	0.129	SEX effects in THC	ANOVA	F= 2.489	1	22	0.10	0.33												
	CS+ ILP	ns	0.296	Corrected model	ANOVA	F= 1.273	3	42	0.08	0.32	0.750	0.372	12	0.667	0.225	12	2.818	1.678	11	2.182	0.932	11
	CS- ILP	ns	0.479	Corrected Model	ANOVA	F= 0.840	3	42	0.06	0.22	2.750	0.854	12	2.750	0.954	12	4.909	1.734	11	2.364	1.337	11
	ISI ILP	ns	0.285	Corrected Model	ANOVA	F= 1.305	3	42	0.09	0.32	1.500	0.754	12	4.917	1.469	12	7.455	2.893	11	6.091	3.226	11

Test performed are two-ANOVA with Sex (Male/Female) and Treatment (VEH/THC) as within subject factors or Repeated Measures (RM) ANOVA using the between-factor Session. Corrected model values are reported when factor effects and interactions have associated p values over 0.1.

Table 12. 2- CSRTT (I)

Phase	Measure	p	Effect	Test	Contrast	Statistic	value	*df*1	*df* e	Effect size	1-β	Male VEH Mean	Male VEH SEM	Male VEH N	Male THC Mean	Male THC SEM	Male THC N	Female VEH Mean	Female VEH SEM	Female VEH N	Female THC Mean	Female THC SEM	Female THC N
Training	Days to reach baseline	t	0.055 TMT	ANOVA	Between subjects	F=	3.888	1	44	0.08	0.49	18.50	0.485	12	18.00	0.674	12	20.42	0.900	12	18.33	0.466	12.0
	HE	*	0.040 Sessions	R.M.	Within subjects	F=	2.371	5	215	0.05	0.75	443.11	15.472	12	402.264	14.813	12	369.653	14.813	12	371.806	14.813	12
	Correct responses	**	0.002 Sessions*TMT	R.M.	Within subjects	F=	3.975	5	220	0.08	0.95	87.28	0.324	12	86.167	0.324	12	87.764	0.324	12	87.875	0.324	12
	Omissions responses	ns	0.103 Sessions	R.M.	Within subjects (GG)	F=	1.960	3.98	220	0.04	0.58	6.042	0.243	12	4.694	0.243	12	5.292	0.243	12	4.986	0.243	12
BL sessions	Incorrect responses	t	0.064 Sessions*TMT	R.M.	Within subjects (GG)	F=	2.120	5	190.6	0.05	0.69	6.681	0.359	12	9.139	0.359	12	6.944	0.359	12	7.139	0.359	12
	Perseverative responses	**	0.006 Sessions	R.M.	Within subjects (GG)	F=	5.996	1.70	74.98	0.12	0.83	1.528	0.106	12	1.319	0.106	12	1.833	0.106	12	1.264	0.106	12
	Time-Out responses	**	0.008 Sessions	R.M.	Within subjects (GG)	F=	4.735	2.27	99.96	0.10	0.82	12.81	0.610	12	9.250	0.610	12	11.708	0.610	12	11.708	0.610	12
	Premature responses	**	0.000 Sessions	R.M.	Within subjects (GG)	F=	5.862	3.57	157.1	0.12	0.97	27.82	1.259	12	30.292	1.259	12	24.000	1.259	12	24.431	1.259	12
		**	0.002 Sessions*TMT	R.M.	MANOVA	F=	4.718	5	40	0.37	0.96												
	Premature responses	ns	0.209 Corrected model	ANOVA		F=	1.575 ,		44	0.10	0.39	26.67	3.642	12	21.833	5.209	12	25.500	4.633	12	15.417	1.798	12
	HE	ns	0.279 Corrected model	ANOVA		F=	1.324	3	44	0.08	0.33	457.2	68.805	12	398.750	46.782	12	356.667	41.027	12	323.833	37.403	12
	Correct responses	ns	0.945 Corrected model	ANOVA		F=	0.125	3	44	0.01	0.07	88.25	1.737	12	88.833	1.787	12	89.417	1.340	12	89.250	0.818	12
6th BL	Omissions responses	ns	0.741 Corrected model	ANOVA		F=	0.417	3	44	0.03	0.13	5.167	1.167	12	3.917	0.529	12	5.250	1.388	12	4.417	0.557	12
	Incorrect responses	ns	0.855 Corrected model	ANOVA		F=	0.258	3	44	0.02	0.10	6.583	1.956	12	7.250	1.697	12	5.333	1.281	12	6.333	1.208	12
	Perseverative responses	ns	0.666 Corrected model	ANOVA		F=	0.527	3	44	0.04	0.15	1.583	0.633	12	0.750	0.392	12	1.083	0.417	12	1.167	0.405	12
	Time-Out responses	ns	0.590 Corrected model	ANOVA		F=	0.646	3	44	0.04	0.17	12.50	2.737	12	9.750	1.767	12	11.083	2.148	12	8.667	1.350	12
		*****	**0.048 Sessions*SEX*TMT**	**R.M.**	**Within subjects (GG)**	**F=**	**3.481**	**1.55**	**68.2**	**0.07**	**0.56**												
		**	0.002 (Sessions*TMT) in MALES	R.M.	Within subjects	F=	6.933	2	44	0.24	0.91												
	Premature responses	ns	0.618 (Sessions*TMT) in FEMALES	R.M.	Within subjects (GG)	F=	0.331	1.24	27.4	0.01	0.09	67.111	2.047	12	59.861	2.047	12	56.389	2.047	12	68.500	2.047	12
		*	0.015 (Sessions*SEX) in VEH	R.M.	Within subjects (GG)	F=	6.049	1.26	27.8	0.22	0.72												
		ns	0.724 (Sessions*SEX) in THC	R.M.	Within subjects	F=	0.325	2	44	0.01	0.10												
		*****	**0.028 Sessions*SEX*TMT**	**R.M.**	**Within subjects**	**F=**	**3.713**	**2**	**88**	**0.08**	**0.67**												
		**	0.003 (Sessions*TMT) in MALES	R.M.	Within subjects	F=	6.738	2	44	0.23	0.90												
	% Premature responses	ns	0.673 (Sessions*TMT) in FEMALES	R.M.	Within subjects	F=	0.399	2	44	0.02	0.11	0.447	0.041	12	0.376	0.041	12	0.404	0.041	12	0.451	0.041	12
		**	0.002 (Sessions*SEX) in VEH	R.M.	Within subjects	F=	7.042	2	44	0.24	0.91												
		ns	0.723 (Sessions*SEX) in THC	R.M.	Within subjects	F=	0.326	2	44	0.02	0.10												
Long ITIs	% Increase	ns	0.455 Sessions	R.M.	Within subjects	F=	0.794	2	84	0.02	0.18	0.702	0.011	12	0.715	0.011	12	0.646	0.011	12	0.754	0.011	12
sessions	Mean Pre.Resp, ITI	ns	0.576 Corrected model	ANOVA		F=	0.668	3	48	0.04	0.18	67.111	7.903	12	59.861	6.602	12	56.389	6.822	12	68.500	6.961	12
	HE	ns	0.104 Sessions	R.M.	Within subjects (GG)	F=	2.413	1.73	71.1	0.06	0.44	601.306	80.517	12	636.543	81.225	12	461.471	57.030	12	620.278	106.078	12
	Correct responses	*	0.004 Corrected model	R.M.	Within subjects (GG)	F=	7.444	1.34	59.1	0.15	0.94	86.823	2.282	12	89.589	2.219	12	89.284	2.147	12	87.751	4.238	12
		*****	**0.011 Sessions*SEX*TMT**	**R.M.**	**Within subjects**	**F=**	**4.715**	**2**	**86**	**0.10**	**0.78**												
		**	0.007 (Sessions*TMT) in MALES	R.M.	Within subjects	F=	5.513	2	42	0.21	0.83												
	Omision responses	ns	0.500 (Sessions*TMT) in FEMALES	R.M.	Within subjects	F=	0.704	2	44	0.03	0.16	6.994	1.747	12	4.877	1.529	12	6.655	1.414	12	5.588	1.552	12
		ns	0.246 (Sessions*SEX) in VEH	R.M.	Within subjects	F=	1.449	2	44	0.06	0.29												
		*	0.042 (Sessions*SEX) in THC	R.M.	Within subjects	F=	3.434	2	42	0.14	0.61												
	Incorrect responses	**	0.009 TMT	R.M.	Within subjects	F=	4.973	2	88	0.10	0.80	3.489	1.069	12	3.827	1.247	12	3.217	0.825	12	4.691	1.637	12
	Perseverative responses	ns	0.897 Corrected model	R.M.	Within subjects	F=	0.108	2	86	0.00	0.07	0.722	0.298	12	0.657	0.328	12	1.278	0.376	12	1.722	1.476	12
	Time-Out responses	**	0.000 Sessions	R.M.	Within subjects	F=	13.339	2	88	0.23	1.00	26.861	6.086	12	15.222	3.209	12	20.167	4.926	12	23.167	4.154	12

Test performed are two-ANOVA with Sex (Male/Female) and Treatment (VEH/THC) as within subject factors or Repeated Measures (RM) ANOVA using the between-factor Session. Corrected model values are reported when factor effects and interactions have associated p values over 0.1.

Table 13. 2-CSRTT (II)

Phase	Measure	p	Effect	Test	Contrast	Statistic value	df1	df e	Effect size	1-β	Male VEH Mean	Male VEH SEM	Male VEH N	Male THC Mean	Male THC SEM	Male THC N	Female VEH Mean	Female VEH SEM	Female VEH N	Female THC Mean	Female THC SEM	Female THC N
1st long ITI session	Pre.Resp. (BL pre test)	ns	0.349 Corrected model	ANOVA		F= 1.125	3	44	0.07	0.28	27.2	4.3	12	24.7	4.9	12	21.0	3.1	12	18.3	1.8	12
		**	**0.009 SEX* TMT**	ANOVA		**F= 7.483**	**1**	**44**	**0.15**	**0.76**												
		*	0.021 THC effects in MALE	ANOVA	Simple effects	F= 5.740	1	44	0.12	0.65												
	Premature responses	ns	0.148 THC effects in FEMALE	ANOVA	Simple effects	F= 2.169	1	44	0.05	0.30	98.1	32.0	12	69.8	24.7	12	65.4	30.3	12	82.8	28.3	12
		**	0.008 SEX effects in VEH	ANOVA	Simple effects	F= 7.630	1	44	0.15	0.77												
		ns	0.275 SEX effects in THC	ANOVA	Simple effects	F= 1.224	1	44	0.03	0.19												
		**	**0.009 SEX * TMT**	ANOVA		**F= 7.459**	**1**	**44**	**0.14**	**0.76**												
		**	0.008 THC effects in MALE	ANOVA	Simple effects	F= 7.790	1	44	0.15	0.78												
	% Premature responses	ns	0.290 THC effects in FEMALE	ANOVA	Simple effects	F= 1.148	1	44	0.03	0.18	0.6	0.2	12	0.4	0.2	12	0.5	0.2	12	0.5	0.2	12
		*	0.020 SEX effects in VEH	ANOVA	Simple effects	F= 5.804	1	44	0.12	0.65												
		ns	0.153 SEX effects in THC	ANOVA	Simple effects	F= 2.112	1	44	0.05	0.30												
		t	**0.051 SEX * TMT**	ANOVA		**F= 4.034**	**1**	**44**	**0.08**	**0.50**												
		ns	0.975 THC effects in MALE	ANOVA	Simple effects	F= 0.001	1	44	0.00	0.05												
	% Increase	**	0.007 THC effects in FEMALE	ANOVA	Simple effects	F= 7.892	1	44	0.15	0.79	0.7	0.0	12	0.7	0.0	12	0.6	0.1	12	0.8	0.0	12
		ns	0.082 SEX effects in VEH	ANOVA	Simple effects	F= 3.167	1	44	0.07	0.41												
		ns	0.294 SEX effects in THC	ANOVA	Simple effects	F= 1.126	1	44	0.03	0.18												
	Correct responses	ns	0.309 Corrected model	ANOVA		F= 1.234	3	44	0.08	0.31	82.5	2.9	12	86.8	7.6	12	89.1	5.9	12	79.4	23.3	12
	Incorrect responses	ns	0.355 Corrected model	ANOVA		F= 1.112	3	42	0.07	0.28	2.2	0.5	12	4.6	4.0	11	3.2	1.5	12	7.4	8.9	11
	Omisions responses	ns	0.45 Corrected model	ANOVA		F= 0.897	3	44	0.06	0.23	9.7	2.3	12	6.9	5.3	12	5.4	5.6	12	8.1	7.2	12
	Perseverative	ns	0.419 Corrected model	ANOVA		F= 0.963	3	44	0.06	0.25	0.7	0.3	12	0.8	0.8	12	0.5	0.7	12	0.6	1.0	11
	TimeOut	ns	0.18 Corrected model	ANOVA		F= 1.704	3	44	0.10	0.42	38.0	6.9	12	22.3	22.2	12	23.8	10.3	12	28.8	15.5	12
	HE	t	0.057 SEX	ANOVA		F= 3.823	1	44	0.08	0.48	710.7	25.1	12	634.4	86.8	12	490.2	86.8	12	515.3	86.8	12
2nd Long ITI session	Pre.Resp (BL pre test)	ns	0.933 Corrected model	ANOVA		F= 0.144	3	44	0.01	0.07	38.8	11.8	12	31.8	11.2	12	33.5	4.7	12	31.4	5.3	12
	Prematures reponses	ns	0.769 Corrected model	ANOVA		F= 0.378	3	44	0.03	0.12	56.5	32.6	12	62.2	31.0	12	51.9	28.8	12	64.9	37.8	12
	% Premature responses	ns	0.687 Corrected model	ANOVA		F= 0.496	3	44	0.03	0.14	0.4	0.2	12	0.3	0.1	12	0.4	0.2	12	0.4	0.2	12
	% Increase	*	0.027 TMT	ANOVA		F= 5.240	1	44	0.11	0.61	0.7	0.1	12	0.8	0.1	11	0.6	0.0	12	0.7	0.0	12
	Correct responses	ns	0.788 Corrected model	ANOVA		F= 0.352	3	44	0.02	0.11	89.1	1.4	12	90.5	9.9	12	90.7	7.2	12	92.0	3.5	12
	Incorrect responses	ns	0.75 Corrected model	ANOVA		F= 0.406	3	43	0.04	0.12	2.0	0.4	11	3.4	5.8	12	3.0	3.0	12	3.0	1.7	12
	Omisions responses	ns	0.426 Corrected model	ANOVA		F= 0.948	3	44	0.06	0.24	6.8	1.2	12	5.9	5.6	12	4.1	3.3	12	4.6	4.3	12
	Perseverative	ns	0.437 Corrected model	ANOVA		F= 0.924	3	44	0.06	0.24	0.6	0.3	12	1.5	1.8	11	1.0	1.1	12	1.1	1.1	12
	TimeOut	ns	0.282 Corrected model	ANOVA		F= 1.313	3	44	0.82	0.33	22.1	4.5	12	13.8	12.4	12	17.3	14.8	12	24.0	13.2	12
	HE	t	0.052 SEX	ANOVA		F= 3.994	1	41	0.09	0.50	740.1	118.3	12	669.3	285.3	9	489.8	230.9	12	554.5	253.2	12
3rd Long ITI session	Pre.Resp. (BL pre test)	ns	0.774 Corrected model	ANOVA		F= 0.372	3	44	0.02	0.12	40.1	12.8	12	33.4	9.4	12	26.4	5.4	12	36.2	8.6	12
	Prematures responses	ns	0.770 Corrected model	ANOVA		F= 0.377	3	44	0.03	0.12	46.8	27.4	12	47.7	24.0	12	51.8	29.3	12	57.8	31.8	12
	% Prematures responses	ns	0.806 Corrected model	ANOVA		F= 0.327	3	44	0.02	0.11	0.3	0.2	12	0.3	0.1	12	0.3	0.1	12	0.4	0.2	12
	% Increase	ns	0.988 Corrected model	ANOVA		F= 0.057	3	44	0.13	0.06	0.7	0.1	12	0.7	0.2	12	0.7	0.1	12	0.7	0.1	12
	Correct responses	ns	0.483 Corrected model	ANOVA		F= 0.832	3	43	0.05	0.22	88.9	1.8	12	88.4	9.2	12	91.2	4.7	12	91.9	4.4	12
	Incorrect responses	ns	0.410 Corrected model	ANOVA		F= 0.983	3	44	0.06	0.25	5.3	1.3	12	2.8	3.0	12	4.1	3.8	12	3.7	2.7	12
	Omisions responses	ns	0.109 Corrected model	ANOVA		F= 2.136	3	44	0.13	0.51	1.7	0.4	12	2.5	1.4	12	1.8	0.9	12	1.8	0.9	12
	Perseverative	ns	0.226 Corrected model	ANOVA		F= 1.506	3	44	0.09	0.37	0.9	0.3	12	0.6	1.2	12	1.6	1.5	12	0.9	0.9	12

The tests performed were two way ANOVAs with Sex (Male/Female) and Treatment (VEH/THC) as within subject factors or Repeated Measures (RM) ANOVA using the between-factor Session. Corrected model values are reported when the factor effects and interactions have associated p values over 0.1.

3. EXPERIMENT 3: BEHAVIOURAL TRAITS

3.1. PAVLOVIAN CONDITIONED APPROACH

The repeated measures ANOVA showed a significant progression in the PCA index across the training session (see Figure 16). In the last training session, we observed a significant Treatment effect, which cannot be attributed to Treatment effects in terms of the amount of CS^+ LPs, ILPs, HE, CS^+HE or MAG and the CS^+MAG in that session (see Table 14). Instead, this was mainly due to the effects of THC on the main variables that constitute the PCA index. Indeed, there was a significant Treatment effect in the probability difference and latency score, and there was a trend towards statistical significance in the response bias. Another complementary variable was the first response probability which showed the same significant Treatment effect, whereby animals exposed to THC tended to first explore the magazine instead of interacting with the CS^+ once the later appeared (Figure 16 and Table 14).

This effect was also significant along the training sessions, although the components of the PCA index did not show any Treatment effect in the repeated measures analysis. With regards to the sex differences, independent of the adolescent treatment, females spent more time seeking the reinforcer in the food magazine than their counterparts, as reflected by the Sex effect on CS^+HE, MAG and CS^+MAG over the training sessions (See Table 14).

The classification of animals into GTs or STs also revealed, as expected, that more animals fell into the former category in the groups exposed to THC during adolescence. This was further confirmed by a crosstabs analysis that highlighted an effect of Treatment ($\chi^2(2,N=40)=6.667$, $p=0.036$) but not Sex ($\chi^2(2,N=40)=0.267$, $p=0.875$: see Figure 16). In the control groups, the majority of animals had an intermediate phenotype.

3.2. HABIT FORMATION

During the short training instrumental training sessions, animals successfully acquired lever-pressing behaviour as suggested by the significant interaction with the Sessions factor. However, there were no significant effects of Sex or Treatment. In addition, we found no effects along with short training sessions as a result of the Sex or Treatment factors in any of the measurements analysed (see Table 15).

During the outcome devaluation test after the short training session, female rats that had received THC performed more lever presses in both the devalued and non-devalued conditions relative to their controls and their male counterparts (see Figure 17 and Table 15). However, there were no differences in LPs difference or the Habit index among groups and all successfully decrease their lever pressing behaviour in the devalued condition. In the contingency degradation test there were also no differences between the groups.

Across the extended training sessions all groups progressively increased their lever pressing behaviour seeking for the reinforcer under the successive VI60 sessions. During the tests after extended training there were no differences in LPs in the devalued as opposed to the non-devalued condition, indicative of increased S-R behaviour (see Figure 17 and Table 16). There were also no group differences in the LPs difference or the Habit index among groups. Although, the time spent in the magazine showed a significant Sex x Adolescent Treatment interaction with THC-males spending more time inspecting the magazine than VEH-males. This effect was not evident in the females that received THC. Moreover, there was a Treatment effect in the time spent in the magazine during the contingency degradation test, with THC-exposed animals spending more time interacting with the food magazine, an effect reminiscent of the increased GT behaviour seen in the PCA experiment (see Table 16)

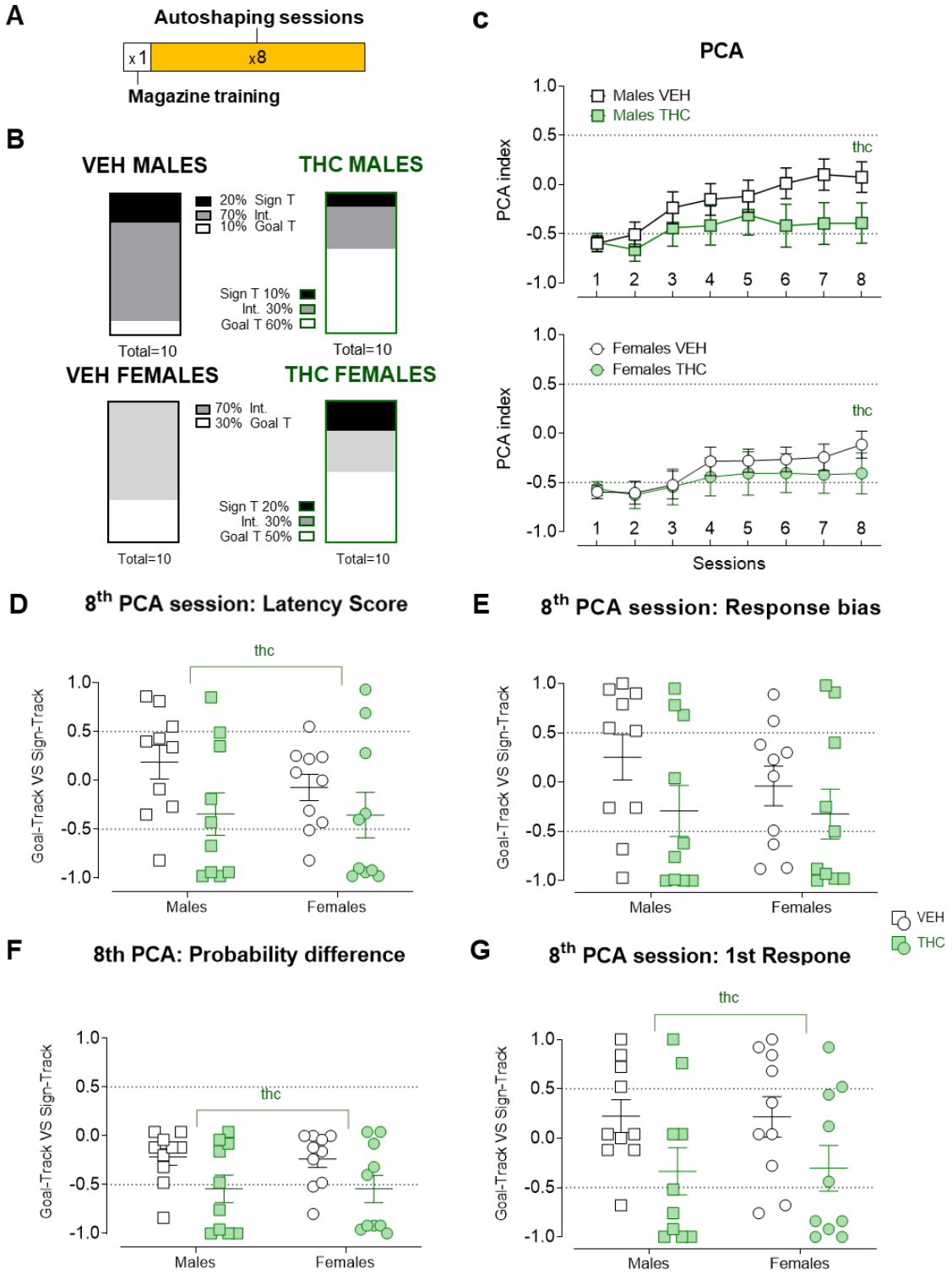


Figure 16: Pavlovian Conditioned Approach (Male VEH n=10; Male THC n=10; Female VEH n=10; and Female THC n=10). **A)** Timeline of the experimental phases. **B)** Relative distribution of the three different PCA clusters in each group. Animals were classified as Sign trackers if the PCA index > 0.5; intermediate if PCA index < 0.5 and > - 0.5; goal-trackers if PCA index < -0.5. **C)** PCA index across the eight auto-shaping sessions. Treatment biased the index towards negative values, indicating increased goal-tracking in the 8th PCA session ($F_{1,36}$=4.539; p=0.040; ηp^2=0.11). **D), E)** and **F)** distribution of each PCA index component in the 8th session. An increase in goal-tracking behaviour in THC animals was significant in the Probability difference ($F_{1,36}$=7.397; p=0.010; ηp^2=0.17) and Latency score ($F_{1,36}$= 4.408; p= 0.043; ηp^2=0.11) indices, although it did not reach statistical significance in the Response bias ($F_{1,36}$=3.058; p=0.089; ηp^2=0.08). **G)** Another additional proxy not included in the general index was also calculated, namely First response, that was also significantly affected in the direction by same THC ($F_{1,36}$= 6.447; p= 0.016; ηp^2= 0.15). The graphs represent individual values (dots) and the mean ± SEM (lines).

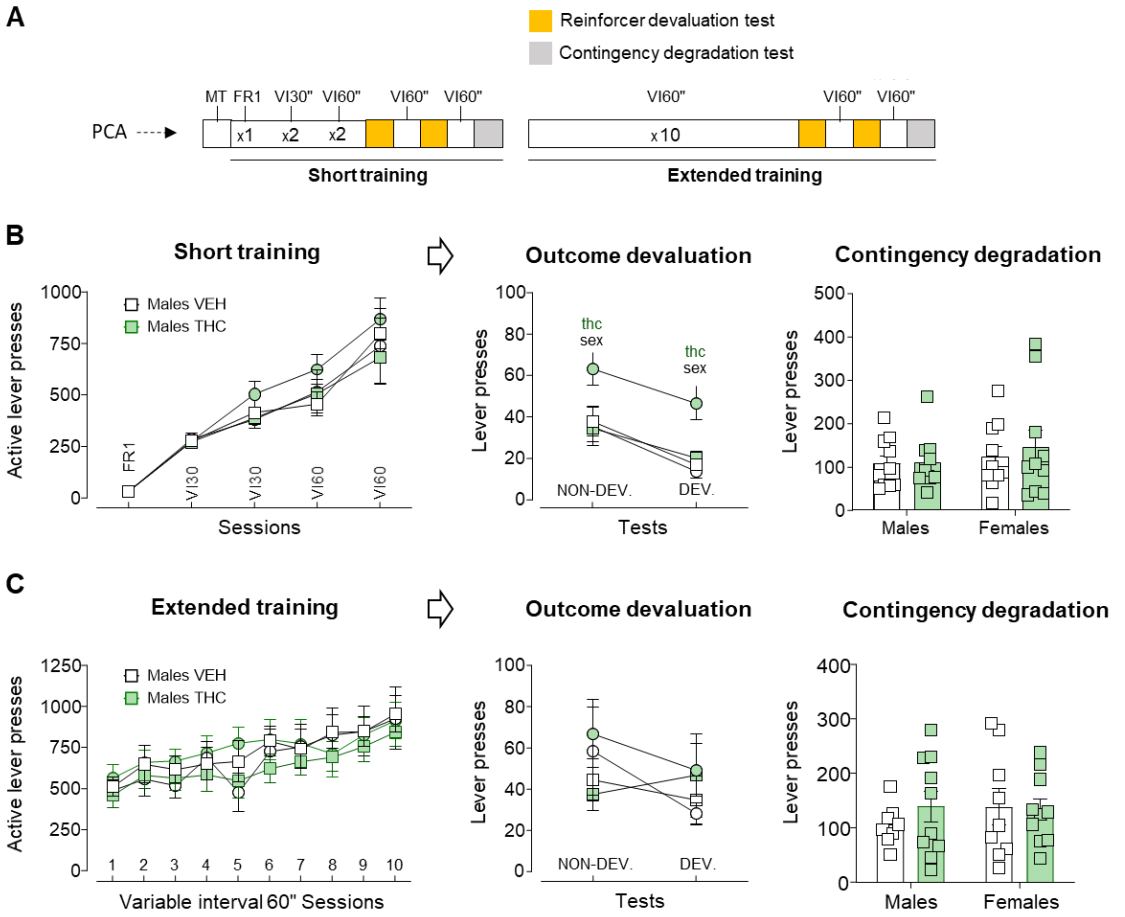


Figure 17: Habit formation. A) Timeline of the experimental phases. **B)** Lever presses during short training sessions and sensory-specific satiety outcome devaluation test. No effect of Sex or Adolescent Treatment was observed across the training sessions. In the test sessions, there was increased lever pressing in THC females in both conditions compared to the VEH females ($F_{1,18}$= 10.740; p= 0.004; ηp^2= 0.37) and THC males ($F_{1,18}$= 9.526; p= 0.006; ηp^2= 0.35). All the animals decreased their response in the devalued condition, suggesting goal-directed behaviour and the absence of habitual responding ($F_{1,36}$= 30.976; p<0.000; ηp^2= 0.37) **C)** The extended training sessions and sensory-specific satiety outcome devaluation test. All groups progressively increased their responses as the training sessions progressed ($F_{4.48,147.72}$ = 21.575; p<0.000; ηp^2= 0.39). There were no session effects on LPs in the tests, indicating the absence of devaluation and probably, the development of a stimulus-response guided behaviour compatible with a habit. There were no Sex or Adolescent Treatment effects ($F_{1,35}$=1.294; p=0.263; ηp^2= 0.03) and no effects were detected during the contingency degradation tests.

Table 14. PCA Results

	PCA index	~~*~~	~~0.002~~	~~Sessions~~	R.M.	~~Within subjects (GG)~~	~~F= 5.827~~	~~2.6~~	~~107.6~~	~~0.14~~	~~0.92~~	~~0.177~~	~~0.043~~	~~10~~	~~0.452~~	~~0.043~~	~~10~~	~~0.363~~	~~0.043~~	~~10~~	~~0.476~~	~~0.043~~	~~10~~
	Probability difference	*	0.042	Sessions	R.M.	Within subjects (GG)	F= 2.653	3.6	127.9	0.07	0.69	0.328	0.034	10	0.523	0.034	10	0.397	0.034	10	0.545	0.034	10
	Response bias	**	0.000	Sessions	R.M.	Within subjects (GG)	F= 14.98	2.8	99.7	0.29	1.00	0.082	0.053	10	0.415	0.053	10	0.359	0.053	10	0.429	0.053	10
	Latency score	**	0.000	Sessions	R.M.	Within subjects (GG)	F= 11.59	3.0	108.5	0.24	1.00	0.107	0.045	10	0.410	0.045	10	0.349	0.045	10	0.426	0.045	10
	First respose	**	0.007	Sessions	R.M.	Within subjects	F= 3.686	4	117.8	0.09	0.87	-0.121	0.062	10	0.325	0.062	10	0.038	0.062	10	0.387	0.062	10
		*	0.025	Sessions*SEX	R.M.	MANOVA (Pillai's Trace)	F= 2.767	7	29	0.40	0.83												
	LPs	ns	0.393	Sessions	R.M.	Within subjects (GG)	F= 1.034	4.2	146.7	0.03	0.33	16.96	1.27	10	23.72	1.41	9	30.40	1.27	10	22.14	1.27	10
		ns	0.114	Sessions*SEX	R.M.	Within subjects (GG)	F= 1.877	4.2	146.7	0.05	0.57												
Sessions 1-8	CS+ LPs	**	0.000	Sessions	R.M.	Within subjects (GG)	F= 10.04	3.8	132.0	0.22	1.00	0.313	0.037	10	0.653	0.041	9	0.600	0.037	10	0.450	0.037	10
		*	0.047	SEX * TMT	R.M.	Between subjects	F= 4.224	1	35	0.11	0.52												
	HE	**	0.008	Sessions	R.M.	Within subjects (GG)	F= 4.114	3.1	35	0.11	0.85	287.5	10.41	10	278.639	11.569	9	415.8	10.412	10	333.4	10.41	10
		*	0.010	SEX	R.M.	Between subjects	F= 7.523	1	35	0.18	0.76												
	CS+ HE	t	0.058	Sessions	R.M.	Within subjects (GG)	F= 2.505	3.3	114.1	0.07	0.63	62.89	4.452	10	66.26	4.947	9	90.14	4.452	10	74.58	4.452	10
	MAG	ns	0.204	Sessions	R.M.	Within subjects (GG)	F= 1.638	1.9	65.1	0.04	0.32	7731.8	455.6	10	8109.2	506.2	9	11379.8	455.6	10	10932.2	455.6	10
		*	0.033	SEX	R.M.	Between subjects	F= 4.906	1	35	0.12	0.58												
	CS+MAG	**	0.001	Sessions	R.M.	Within subjects (GG)	F= 5.026	3.8	133.8	0.13	0.95	2448.3	271.3	10	3115.6	301.5	9	5170.6	271.3	10	4961.0	271.3	10
		*	0.013	SEX	R.M.	Between subjects	F= 6.895	1	35	0.16	0.72												
	PCA index	*	0.040	TMT	ANOVA		F= 4.539	1	36	0.11	0.55	0.074	0.154	10	-0.393	0.204	10	-0.115	0.137	10	-0.407	0.207	10
	Probability difference	*	0.010	TMT	ANOVA		F= 7.397	1	36	0.17	0.75	-0.216	0.086	10	-0.544	0.142	10	-0.236	-0.087	10	-0.544	0.140	10
	Response bias	ns	0.089	TMT	ANOVA		F= 3.058	1	36	0.78	0.40	0.253	0.730	10	-0.291	0.822	10	0.037	0.634	10	-0.322	0.801	10
	Latency score	*	0.043	TMT	ANOVA		F= 4.408	1	36	0.11	0.53	0.186	0.173	10	-0.343	-0.217	10	-0.073	0.134	10	-0.356	0.233	10
	First respose	*	0.016	TMT	ANOVA		F= 6.447	1	36	0.15	0.70	0.224	0.166	10	-0.336	-0.239	10	-0.216	0.205	10	-0.304	0.232	10
Session 8	CS+ LPs	ns	0.585	Corrected model	ANOVA		F= 0.654	3	36	0.05	0.17	55.20	11.369	10	43.20	16.696	10	67.400	18.301	10	38.900	16.22	10
	lLPs	ns	0.445	Corrected model	ANOVA		F= 0.912	3	36	0.07	0.23	18.30	6.300	10	36.00	14.281	10	21.600	3.813	10	20.800	4.791	10
	HE	ns	0.156	Corrected model	ANOVA		F= 1.847	3	36	0.13	0.44	321.4	64.483	10	265.5	37.775	10	494.7	109.310	10	330.0	59.95	10
	CS+ HE	ns	0.995	Corrected model	ANOVA		F= 0.023	3	36	0.00	0.05	67.10	32.804	10	75.20	15.649	10	71.40	19.257	10	72.40	17.56	10
	MAG	ns	0.594	Corrected model	ANOVA		F= 0.641	3	36	0.05	0.17	8981.1	2206.4	10	7492.3	1805.4	10	12534.9	3639.6	10	10574.2	2801.8	10
	CS+ MAG	ns	0.180	Corrected model	ANOVA		F= 1.722	3	36	0.13	0.41	2657.3	3424.4	10	5036.9	4515.2	10	4743.8	4129.5	10	7500.5	6500.7	10

The tests performed were two-ANOVAs with Sex (Male/Female) and Treatment (VEH/THC) as the within subject factors or a Repeated Measures (RM) ANOVA using the between-factor Session. Corrected model values are reported when factor effects and interactions have associated p values above 0.1.

Table 15. Short habit training results

Phase	Measure		p	Effect	Test	Contrast	Statistic value	*df* 1	*df* e	Effect size	1-β	Male VEH Mean	Male VEH SEM	Male VEH N	Male THC Mean	Male THC SEM	Male THC N	Female VEH Mean	Female VEH SEM	Female VEH N	Female THC Mean	Female THC SEM	Female THC N
Short training	ALP	**	0.000	Sessions	R.M.	within subjects	F= 97.12	1.63	58.81	0.73	1.00	390.02	15.09	10	370.40	15.09	10	384.98	15.09	10	454.92	15.09	10
	Session time	**	0.000	Sessions	R.M.	within subjects	F= 288.7	2.36	77.94	0.90	1.00	23.70	0.23	10	24.63	0.28	8	25.00	0.253	9	23.96	0.23	10
	HEs	**	0.004	Sessions	R.M.	within subjects	F= 8.003	1.28	46.03	0.18	0.85	333.40	18.33	10	495.06	18.33	10	392.98	18.33	10	350.20	18.33	10
	MAG	**	0.000	Sessions	R.M.	within subjects	F= 26.13	2.39	86.00	0.42	1.00	7902.1	349.3	10	10154.4	349.3	10	10166.9	349.3	10	9042.3	349.34	10
		*	0.019	SEX	ANOVA		F= 6.020	1	36	0.14	0.67												
		**	0.001	TMT	ANOVA		F= 14.13	1	36	0.28	0.96												
		****	**0.003**	**SEX * TMT**	**ANOVA**		**F= 9.782**	**1**	**36**	**0.21**	**0.86**												
	LPs (devalued)	ns	0.658	THC effects in MALE	ANOVA		F= 0.200	1	36	0.01	0.07	17.00	2.56	10	20.00	3.51	10	13.80	2.48	10	46.50	8.07	10
		**	0.000	THC effects in FEMALE	ANOVA		F= 23.72	1	36	0.40	1.00												
		ns	0.637	SEX effects in VEH	ANOVA		F= 0.227	1	36	0.01	0.08												
		**	0.000	SEX effects in THC	ANOVA		F= 15.58	1	36	0.30	0.97												
Short training Outcome Devaluation Test		*	0.037	Corrected model	ANOVA		F= 3.139	3	36	0.21	0.68												
		t	**0.052**	**SEX * TMT**	**ANOVA**		**F= 4.051**	**1**	**36**	**0.10**	**0.50**												
		ns	0.750	THC effects in MALE	ANOVA		F= 0.103	1	36	0.00	0.06												
	LPs (non devalued)	*	0.016	THC effects in FEMALE	ANOVA		F= 6.376	1	36	0.15	0.69	37.70	6.80	10	34.20	6.25	10	35.40	9.54	10	62.90	7.80	10
		ns	0.834	SEX effects in VEH	ANOVA		F= 0.045	1	36	0.00	0.06												
		*	0.012	SEX effects in THC	ANOVA		F= 6.944	1	36	0.16	0.73												
	LPs difference	ns	0.834	Corrected model	ANOVA		F= 0.288	3	36	0.02	0.10	20.70	1.98	10	14.20	1.54	10	21.60	3.12	10	16.40	1.07	10
	Habit Formation index	ns	0.740	Corrected model	ANOVA		F= 0.42	3	36	0.03	0.13	0.37	0.07	10	0.41	0.06	10	0.33	0.05	10	0.40	0.03	10
	HEs (devalued)	ns	0.089	Corrected model	ANOVA		F= 2.350	3	36	0.16	0.54	11.70	1.59	10	12.50	2.91	10	11.10	2.24	10	21.00	4.56	10
	HEs (non devalued)	ns	0.327	Corrected model	ANOVA		F= 1.192	3	36	0.09	0.29	21.60	4.20	10	21.80	4.42	10	19.50	2.43	10	29.00	3.84	10
	MAG (devalued)	ns	0.756	Corrected model	ANOVA		F= 0.397	3	0	0.03	0.12	182.20	39.60	10	332.80	157.13	10	288.70	117.86	10	328.60	97.11	10
	MAG (non devalued)	ns	0.544	Corrected model	ANOVA		F= 0.724	3	36	0.06	0.19	1354.80	1051.59	10	593.10	207.83	10	333.10	65.05	10	460.80	96.94	10
Short training Contingency Degradation Test	LPs	ns	0.305	Corrected model	ANOVA		F= 1.253	3	36	0.09	0.31	122.80	14.79	10	118.40	23.44	10	233.10	78.46	10	164.70	45.82	10
	Session time	ns	0.985	Corrected model	ANOVA		F= 0.050	3	36	0.00	0.06	29.00	2.113	10	29.70	1.874	10	29.80	2.195	10	30.10	2.089	10
	HEs	ns	0.462	Corrected model	ANOVA		F= 0.878	3	36	0.07	0.22	480.40	71.75	10	621.10	100.23	10	441.00	84.96	10	504.9	70.15	10
	MAG	ns	0.212	Corrected model	ANOVA		F= 1.577	3	36	0.12	0.38	14022.0	2463.90	10	23667.3	5449.88	10	13158.6	3668.1	10	15955.0	2993.5	10

The tests performed were two-ANOVA with Sex (Male/Female) and Treatment (VEH/THC) as the within subject factors or Repeated Measures (RM) ANOVA using the between-factor Session. The corrected model values are reported when the factor effects and interactions have associated p values above 0.1.

Table 16. Extended habit training results

Phase	Measure		p	Effect	Test	Contrast	Statistic value	*df*1	*df* e	Effect size	1-β	Male VEH Mean	Male VEH SEM	Male VEH N	Male THC Mean	Male THC SEM	Male THC N	Female VEH Mean	Female VEH SEM	Female VEH N	Female THC Mean	Female THC SEM	Female THC N
	ALPs	**	0.000	Sessions	R.M.	within subjects	F= 21.58	4.48	147.72	0.40	1.00	726.78	29.23	10	633.02	29.23	10	721.23	36.53	8	758.03	32.47	9
	Session time	**	0.000	Sessions	R.M.	within subjects	F= 4.464	5.45	185.31	0.12	0.98	29.69	0.12	10	30.02	0.12	10	30.05	0.15	8	29.89	0.12	10
		*	0.024	Sessions * SEX	R.M.	within subjects	F= 2.706	4.80	167.88	0.07	0.80												
		*****	**0.015**	**Sessions * SEX * TMT**	**R.M.**	**within subjects**	**F= 2.965**	**4.80**	**168.88**	**0.08**	**0.84**												
Long trainning	HEs	ns	0.115	(Sessions*TMT) in MALE	R.M.	within subjects	F= 1.962	3.71	66.87	0.10	0.54	423.70	14.08	10	529.88	14.08	10	367.56	15.64	9	343.85	14.08	10
		ns	0.240	(Sessions*TMT) in FEMALE	R.M.	within subjects	F= 1.406	4.14	70.45	0.08	0.42												
		ns	0.756	(Sessions*SEX) in VEH	R.M.	within subjects	F= 0.647	9.00	153.00	0.04	0.31												
		**	0.007	(Sessions*SEX) in THC	R.M.	within subjects	F= 3.925	3.91	70.43	0.18	0.88												
	MAG	*	0.023	Sessions	R.M.	within subjects	F= 4.301	1.69	58.98	0.11	0.68	12495.5	579.33	10	16770.8	579.33	10	11629.9	643.70	9	11061.6	579.33	10
	LPs (devalued)	ns	0.683	Corrected model	ANOVA		F= 0.503	3	35	0.04	0.14	34.70	11.71	10	46.60	20.27	10	28.11	5.37	9	49.30	12.80	10
	LPs (non devalued)	ns	0.480	Corrected model	ANOVA		F= 0.843	3	35	0.07	0.21	44.50	10.01	10	37.50	7.91	10	58.44	21.41	9	66.90	16.69	10
	Habit Formation index	ns	0.974	Corrected model	ANOVA		F= 0.073	3	35	0.01	0.06	0.43	0.09	10	0.46	0.10	10	0.40	0.07	9	0.44	0.10	10
	LPs difference	ns	0.627	Corrected model	ANOVA		F= 0.587	3	35	0.05	0.16	9.80	14.82	10	-9.10	22.07	10	30.33	18.81	9	17.60	27.19	10
Long trainig	HEs (devalued)	ns	0.784	Corrected model	ANOVA		F= 0.357	3	35	0.03	0.11	17.90	3.33	10	17.90	2.64	10	15.67	1.70	9	20.20	3.88	10
Outcome	HEs (non devalued)	ns	0.318	Corrected model	ANOVA		F= 1.217	3	35	0.09	0.30	19.10	2.95	10	27.60	5.39	10	30.44	3.74	9	26.70	4.88	10
Devaluation	MAG (devalued)	ns	0.863	Corrected model	ANOVA		F= 0.246	3	35	0.02	0.09	283.20	87.74	10	323.90	77.59	10	367.56	94.73	9	371.70	77.20	10
Test		*****	**0.033**	**SEX * TMT**	**ANOVA**		**F= 4.902**	**1**	**0**	**0.12**	**0.58**												
		*	0.036	THC effects in MALE	ANOVA		F= 4.741	1	0	0.12	0.56												
	MAG (non devalued)	ns	0.338	THC effects in FEMALE	ANOVA		F= 0.942	1	0	0.03	0.16	339.40	72.51	10	781.80	154.89	10	795.00	177.35	9	592.40	160.82	10
		*	0.036	SEX effects in VEH	ANOVA		F= 4.763	1	0	0.12	0.57												
		ns	0.358	SEX effects in THC	ANOVA		F= 0.869	1	0	0.02	0.15												
	LPs	ns	0.709	Corrected model	ANOVA		F= 0.464	3	35	0.04	0.13	104.8	10.44	10	139.2	28.71	10	138.6	32.95	9	133.3	20.10	10
Long training	Session time	t	0.06	SEX	ANOVA		F= 3.858	1	35	0.10	0.5	29.80	0.998	10	32.9	3.44	10	40.89	6.147	9	35.90	2.373	10
Contingency	HEs	ns	0.286	Corrected model	ANOVA		F= 1.313	3	35	0.10	0.32	592.60	97.25	10	833.20	143.92	10	560.3	77.54	9	630.5	93.61	10
degradation test	MAG	*	0.026	TMT	ANOVA		F= 5.421	1	0	0.13	0.62	3.13	0.60	10.00	4.43	0.72	10.00	2.39	0.35	9.00	3.78	0.53	10.00

The tests performed were two-ANOVA with Sex (Male/Female) and Treatment (VEH/THC) as the within subject factors or Repeated Measures (RM) ANOVA using the between-factor Session. The corrected model values are reported when the factor effects and interactions have associated p values above 0.1.

4. EXPERIMENT 4: COCAINE SELF-ADMINISTATION

Our study of cocaine addiction-like behaviours showed that the acquisition of cocaine self-administration under continuous access (fixed-ratio 1 -FR1- schedule of reinforcement) was not modified by THC exposure during adolescence. On the first day of the progressive ratio (PR) schedule, there was a significant Sex x Treatment interaction and the follow up of this interaction showed that females that received the vehicle alone consumed significantly more cocaine infusions than the male-VEH rats (see Table 17). No effects on were detected in any of the other measurements.

Females VEH obtained more infusions during the first session and across all six consecutive sessions under progressive ratio. We also observed higher cocaine intake under high-effort conditions (PR) in the THC exposed male rats compared to male VEH rats (see Figure 18 and Table 17). The analysis of the ALPs or the BPs across the six PR sessions only showed a significant effect of the Sessions factor (see Table 17), whereas the number of ALPs and the motivation index remained stable (See Table 17, and Appendix B for graphical representation of ALP and motivation index during PR).

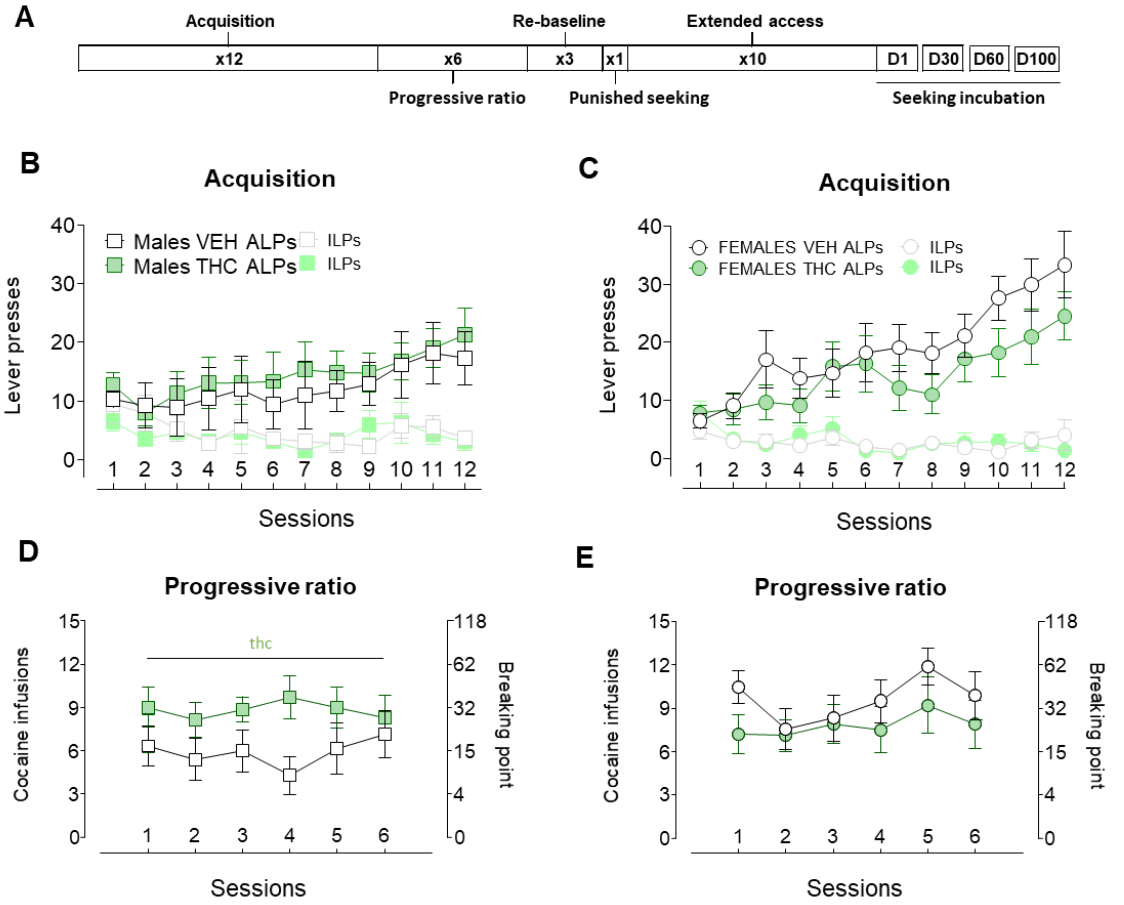


Figure 18: Cocaine self-administration (I). The mean values are depicted with circles or squares joined by lines in the repeated measures graphs. Circles and squares represent individual values in those graphs showing a single index. Error lines reflect the SEM. The initial sample sizes: male VEH n=15; Male THC n=18; Female VEH n=15; Female THC n=15. **A)** Timeline of the experimental phases. **B)** Active (ALPs) and inactive lever presses (ILPs) across the twelve acquisition sessions (2h). All groups acquired a preference for the active lever (Lever: $F_{1,43}$=39.218; p<0.000; ηp^2=0.48) and increased their self-administration behaviour (Lever x Session: $F_{4,175.3}$=7.259; p<0.000; ηp^2=0.14) with no differences due to Sex or Adolescent Treatment. **C)** Cocaine infusions across and breaking points (BP) the six progressive ratio sessions (2h). There was no Session effect in the number of cocaine infusions ($F_{5,25}$=2.021; p=0.080; ηp^2=0.07) but a significant Sex x Adolescent Treatment interaction was observed ($F_{1,25}$=5.215; p=0.031; ηp^2=0.17). A follow-up analysis showed an increase in the number of cocaine infusions in THC males compared to VEH males ($F_{1,25}$=6.197; p=0.032; ηp^2=0.38), an effect that was absent in the females. In addition, VEH females achieve a higher number of infusions compared to VEH males ($F_{1,25}$=7.717; p=0.018; ηp^2=0.41).

We returned the rats to an FR1 schedule for three days and at this stage. The intake incremented during these sessions (See appendix B for graphical representation of during re-baseline after the PR sessions) probably as an effect of the involuntary limited intake during PR sessions, but there were no differences related to Sex or Treatment. However, female-THC rats and male-VEH rats showed a stronger rebound in their cocaine consumption than the female-VEH rats (see Figure 18, Table 17). Notably, the mean consumption of the female

THC rats (25.1) during these sessions was still below the mean consumption of Female VEH rats (35.5), although these were not significant differences when assessed by ANOVA. After these sessions, we evaluated if there was a compulsive component in the cocaine-seeking behaviour of the rats. In the punished seeking test, all the rats reduced the number of infusions obtained relative to the last reacquisition session and interestingly, there were no effects due to Sex or Treatment (see Figure 19, and Table 18) (See Appendix B for a graphical representation of raw number of infusions obtained).

Subsequently we allowed the rats to self-administer cocaine for 6 hours a day under a FR1 schedule of reinforcement for 10 days. We did not observe any significant escalation in our animals (see Table 16) yet the behaviour of THC-females differed somewhat from that of THC-males and that of the VEH controls. (Figure 19). There was a surge in the responses from session 5 that seemed to stabilize from session 6 to session 10. After the extended access sessions, we withdrew the rats from cocaine and analysed seeking responses 1, 30, 60 and 100 days after forced withdrawal. Rats had no access to the drug during these test sessions, in which there were more seeking responses after 30 days than after one day of withdrawal -incubation of seeking phenomenon. Indeed, there was a significant effect of Session and interestingly, the female rats showed more robust seeking behaviour an indicated by a significant effect of Sex (See Table 18).

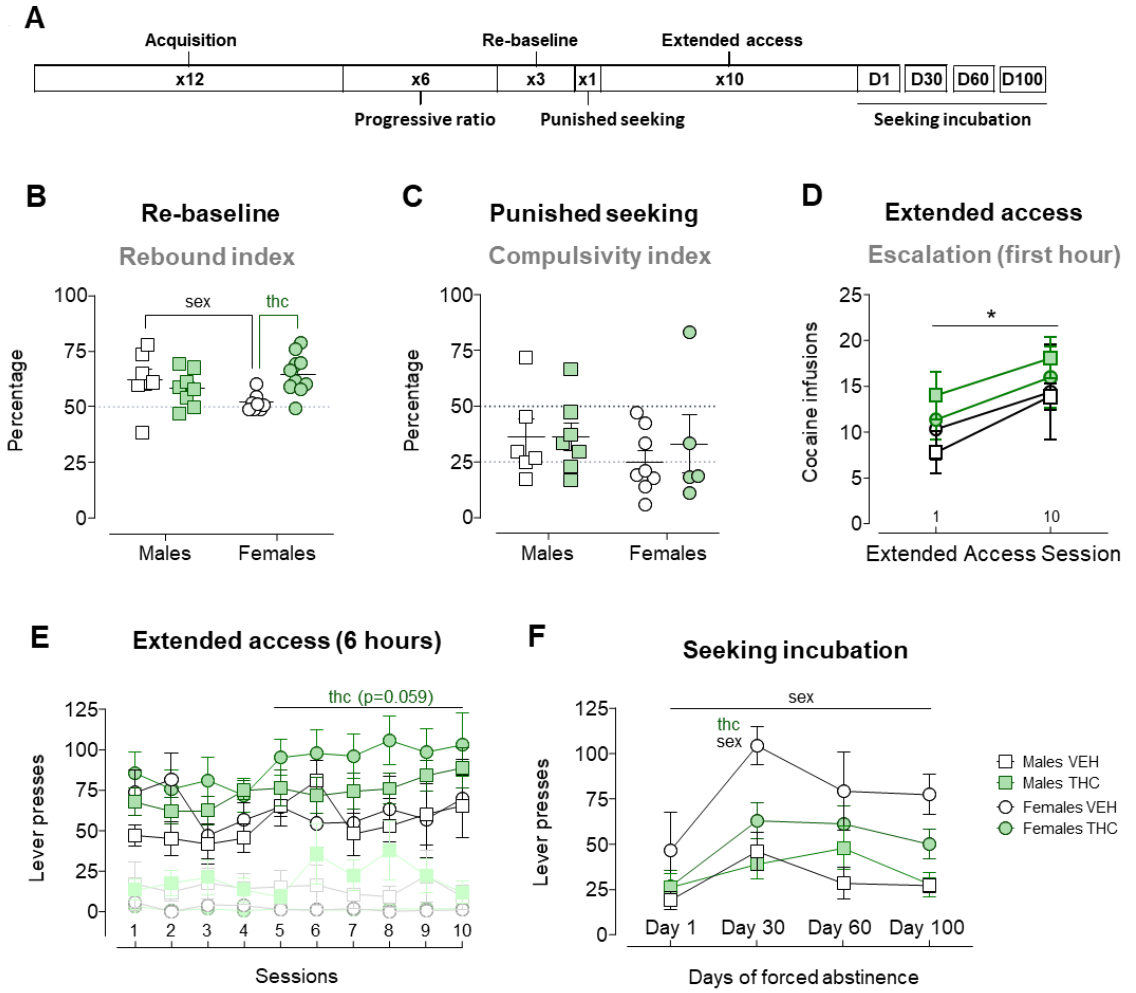


Figure 19: Cocaine self-administration (I). The mean values are depicted with circles or squares joined by lines in the repeated measures graphs. Circles and squares represent individual values in those graphs showing a single index. Error lines reflect the SEM. **A)** Timeline of the experimental phases..**B)** Rebound index in during re-baseline FR1 sessions (2h). There was a Sex x Adolescent Treatment interaction ($F_{1,29}$=7.507; p=0.010; ηp^2=0.20) in the rebound of the self-administration behaviour after the high effort conditions imposed by the progressive ratio (PR). The simple effect analysis showed that cocaine intake in VEH females remained roughly equal (around 50%) but lower than in VEH males (62±1.8%: $F_{1,29}$=5.165; p=0.031; ηp^2=0.15), while there was a stronger increase in THC females than VEH females in FR1 responses relative to the last acquisition sessions ($F_{1,29}$=9.497; p=0.004; ηp^2=0.24). However, no such effect was evident in the male groups. **C)** The compulsivity index from the punished seeking phase (1h). Percentage of events achieved (shocks or cocaine infusions) normalized to the infusions achieved during the first hour of the last reacquisition session. There were no differences due to Sex or Adolescent Treatment. **D)** Infusions achieved during the first hour of the first and last extended access sessions. All groups escalate their intake (Session: $F_{1,20}$=4.349; p=0.05; ηp^2=0.179). **E)** ALPs on the FR1 regime and ILPs across the ten sessions of extended access (6h). Self-administration was stable across the sessions ($F_{3.9,82.7}$=1.395; p=0.243; ηp^2=0.06). We observed a trend towards an effect of Adolescent Treatment in the average cocaine infusions during the second half of the phase (sessions 6 to 10: $F_{1,21}$=3.977; p=0.059; ηp^2=0.16). **F)** Lever pressing in the four extinction sessions as an index of seeking incubation during forced abstinence. Lever pressing behaviour was found to vary across Sessions ($F_{2.05,43.05}$=6.618; p=0.003; ηp^2=0.24) and, probably driven by the higher lever pressing of female VEH

rats, a Sex effect (male<female) was also detected ($F_{1,22}$=11.607; p=0.003; ηp^2=0.36). The ad hoc analysis of the withdrawal day 30 session showed a lower seeking in VEH males ($F_{1,22}$=17.751; p<0.000; ηp^2=0.45) and THC females ($F_{1,22=}$11.924; p=0.002; ηp^2=0.35) compared to VEH females.

Table17. Cocaine Self-Administration (I)

Phase	Measure		p	Effect	Test	Contrast	Statistic value	*df* 1	*df* e	Effect size	1-β	Male VEH Mean	Male VEH SEM	Male VEH N	Male THC Mean	Male THC SEM	Male THC N	Female VEH Mean	Female VEH SEM	Female VEH N	Female THC Mean	Female THC SEM	Female THC N
	Infusions	**	0.000	Sessions	R.M.	Within subjects (GG)	F= 12.541	5.98	257.32	0.23	1.00	12.48	3.23	11	15.48	2.86	14	19.13	3.38	10	14.26	3.09	12
	ILPs	ns	0.209	Sessions	R.M.	Within subjects (GG)	F= 1.582	2.10	124.00	0.03	0.34	4.39	1.31	15	4.04	1.20	18	4.47	1.31	15	3.12	1.31	15
	ALPs & ILPs	**	0.000	Lever	R.M.	Within subjects	F= 39.218	1.00	43.00	0.48	1.00												
Acquisition	ALPs & ILPs	**	0.000	Lever*Sessions	R.M.	Within subjects (GG)	F= 7.259	4.08	175.39	0.14	1.00												
	ALPs & ILPs	**	0.315	Lever*SEX	R.M.	Within subjects	F= 1.034	1	43	0.02	0.17												
	ALPs Mean s10 to 12	ns	0.432	Corrected model	ANOVA		F= 0.931	3	54	0.05	0.24	14.14	15.39	14	17.79	13.28	16	23.51	16.90	13	17.49	13.61	15
	LPTOs	ns	0.131	Sessions	R.M.	Within subjects (GG)	F= 1.752	4.56	227.99	0.03	0.57	2.94	1.57	13	3.63	1.46	15	6.60	1.57	13	4.68	1.57	13
	ALPs	ns	0.200	Corrected model	ANOVA		F= 1.627	3	36	0.12	0.39	65.86	79.15	7	142.18	147.93	11	254.60	202.69	10	158.00	216.77	12
		*****	**0.039**	**SEX * TMT**	**ANOVA**		**F= 4.708**	**1**	**28**	**0.14**	**0.55**												
		ns	0.215	THC effects in MALE	ANOVA	Simple effects	F= 1.612	1	28	0.05	0.23												
Progressive Ratio 1st Session	Infusions	ns	0.072	THC effects in FEMALE	ANOVA	Simple effects	F= 3.498	1	28	0.11	0.44	6.33	3.44	6	9.00	3.65	7	10.44	3.50	9	7.20	4.24	10
		*	0.048	Sex effects in VEH	ANOVA	Simple effects	F= 4.268	1	28	0.13	0.51												
		ns	0.342	Sex effects in THC	ANOVA	Simple effects	F= 0.936	1	28	0.03	0.15												
	Breaking point	ns	0.157	Corrected model	ANOVA		F= 1.863	3	30	0.16	0.43	21.29	16.91	7	31.22	31.07	9	60.50	33.94	8	33.10	44.58	10
	Motivation index	ns	0.729	Corrected model	ANOVA		F= 0.436	3	28	0.05	0.13	30.57	7.14	6	24.90	3.47	7	29.50	3.62	9	31.87	4.13	10
	ALPs	**	0.003	Sessions	R.M.	Within subjects	F= 3.857	5	145	0.12	0.94	88.62	27.11	7	182.19	27.11	7	259.82	21.09	9	188.88	60.01	10
		*****	**0.031**	**SEX * TMT**	**R.M.**	**Between subjects**	**F= 5.215**	**1**	**25**	**0.17**	**0.59**												
		*	0.032	THC effects in MALE	R.M.	Between subjects	F= 6.197	1	25	0.38	0.61												
Progressive Ratio	Infusions	ns	0.290	THC effects in FEMALE	R.M.	Between subjects	F= 1.204	1	25	0.07	0.18	4.63	1.65	5	8.83	1.39	7	9.85	1.30	8	7.65	1.23	9
		*	0.018	Sex effects in VEH	R.M.	Between subjects	F= 7.717	1	25	0.41	0.72												
		ns	0.563	Sex effects in THC	R.M.	Between subjects	F= 0.352	1	25	0.02	0.09												
	Breaking point	**	0.005	Sessions	R.M.	Within subjects (GG)	F= 4.763	2.77	80.28	0.14	0.87	21.29	13.17	7	38.28	11.62	9	62.50	13.17	7	40.37	11.02	10
	Motivation index	ns	0.130	Sessions	R.M.	Within subjects	F= 1.739	5	140	0.06	0.59	24.70	1.63	6	24.90	1.40	7	26.10	1.07	9	31.20	0.98	10

The **tests** performed are two-ANOVAs with Sex (Male/Female) and Treatment (VEH/THC) as the within subject factors or Repeated Measures (RM) ANOVA using the between-factor Session. The corrected model values are reported when the factor effects and interactions have associated p values above 0.1

Table18. Cocaine Self-Administration (II)

Phase	Measure		p	Effect	Test	Contrast	Statistic value	*df* 1	*df* e	Effect size	1-β	Male VEH Mean	Male VEH SEM	Male VEH N	Male THC Mean	Male THC SEM	Male THC N	Female VEH Mean	Female VEH SEM	Female VEH N	Female THC Mean	Female THC SEM	Female THC N
	ALPs	ns	0.011	Sessions	R.M.	Within subjects	F= 4.835	2	62	0.13	0.78	22.95	2.09	7	34.07	1.46	10	35.50	1.83	8	28.17	1.46	10
	Mean ALPs	ns	0.846	Corrected model	ANOVA		F= 0.271	3	29	0.03	0.10	34.10	9.74	7	34.25	4.48	8	35.50	5.21	8	29.17	3.55	10
		*****	**0.010**	**SEX * TMT**	**ANOVA**		**F= 7.507**	**1**	**29**	**0.21**	**0.75**												
Rebaseline		ns	0.380	THC effects in MALE	ANOVA	Simple effects	F= 0.796	1	29	0.03	0.14												
	Rebound index	**	0.004	THC effects in FEMALE	ANOVA	Simple effects	F= 9.497	1	29	0.25	0.85	62.13	1.79	7	51.63	2.30	9	51.86	0.49	8	64.62	2.83	10
		*	0.031	Sex effects in VEH	ANOVA	Simple effects	F= 5.165	1	29	0.15	0.59												
		ns	0.126	Sex effects in THC	ANOVA	Simple effects	F= 2.483	1	29	0.08	0.33												
Rebaseline 3rd	ALPs	ns	0.443	Corrected model	ANOVA		F= 0.920	3	31	0.08	0.23	24.43	7.04	7	33.50	6.12	10	41.50	7.89	8	32.50	6.13	10
Session	RB3.1sthour	ns	0.328	Corrected model	ANOVA		F= 1.195	3	30	0.11	0.29	14.63	4.36	8	17.13	1.86	8	24.50	5.95	8	16.30	2.67	10
	ALPs	ns	0.945	Corrected model	ANOVA		F= 0.124	3	30	0.01	0.07	29.71	12.66	7	32.88	8.19	8	26.00	7.96	9	27.00	7.03	10
	Events (inf+shocks)	ns	0.842	Corrected model	ANOVA		F= 0.276	3	29	0.03	0.10	7.71	2.74	7	9.88	2.58	8	8.00	2.82	8	7.00	1.53	10
Punished Seeking	Infusions (inf)	ns	0.787	Corrected model	ANOVA		F= 0.353	3	27	0.04	0.11	5.83	2.12	6	6.71	1.90	7	4.88	1.95	8	4.50	1.05	10
	Shocks	ns	0.578	Corrected model	ANOVA		F= 0.67	3	27	0.07	0.17	3.00	0.86	6	4.00	0.93	7	3.13	0.87	8	2.50	0.52	10
	Compulsivity index	ns	0.632	Corrected model	ANOVA		F= 0.582	3	27	0.06	0.15	35.99	8.11	6	36.23	6.30	7	25.09	5.09	8	32.29	6.88	10
	ALPs Sessions 1- 10	ns	0.243	Sessions	R.M.	Within subjects (GG)	F= 1.395	3.94	82.73	0.06	0.41	58.12	5.45	5	74.45	3.41	8	64.18	6.81	4	90.53	3.41	8
Extended Access	ALPs Sessions 6-10	ns	0.665	Sessions	R.M.	Within subjects (GG)	F= 0.599	4	84	0.03	0.19	60.00	6.77	5	79.65	4.23	8	63.35	8.46	4	100.18	4.23	8
		t	0.059	TMT	R.M.	Between subjects	F= 3.977	1	21	0.16	0.48												
	Escalation of drug intake	*	0.050	Sessions	R.M.	Between subjects	F= 4.349	1	20	0.18	0.51	8.60	2.62	5	16.75	16.75	8	12.25	2.93	4	14.00	2.21	7
Seeking	ALPs 1- 4	**	0.003	Sessions	R.M.	Within subjects (GG)	F= 6.618	2.05	43.05	0.24	0.90	36.60	4.47	4	66.78	1.73	9	56.81	3.62	4	44.95	1.63	8
Incubation		**	0.003	SEX	R.M.	Within subjects	F= 11.607	1	21	0.36	0.90												
		**	0.001	Corrected model	ANOVA		F= 7.402	3	22	0.50	0.96												
		**	0.000	SEX	ANOVA		F= 21.369	1	22	0.49	0.99												
Seeking		**	0.008	TMT	ANOVA		F= 8.434	1	22	0.28	0.79												
Incubation	ALPs SI2	*****	**0.038**	**SEX* TMT**	**ANOVA**		**F= 4.847**	**1**	**22**	**0.18**	**0.56**	46.00	10.50	5	39.00	8.04	9	114.00	4.42	4	63.13	10.04	8
Day 30		ns	0.607	THC effects in the males	Simple effects		F= 0.272	1	22	0.01	0.08												
		**	0.002	THC effects in the females	Simple effects		F= 11.924	1	22	0.35	0.91												
		**	0.000	Sex effects in VEH rats	Simple effects		F= 17.751	1	22	0.45	0.98												
		t	0.051	Sex effects in THC rats	Simple effects		F= 4.258	1	22	0.16	0.51												

The **tests** performed are two-ANOVAs with Sex (Male/Female) and Treatment (VEH/THC) as the within subject factors or Repeated Measures (RM) ANOVA using the between-factor Session. The corrected model values are reported when the factor effects and interactions have associated p values above 0.1

5. EXPERIMENT 5: RNAseq ANALYSIS - NAc Shell

A total of 96 differentially expressed (DE) genes were identified in the analysis of the male groups and 87 DE genes between the female groups. A selection of the significantly associated GO terms with a more consistent representation (in terms of a higher log fold enrichment, less FDR) was identified (as highlighted in figure 19). These two distinct collections of DE genes only had 9 DE genes in common. Notably, there were only 20 DE genes between Male-VEH and Female-VEH controls. These findings indicate that the NAc Shell does not present a marked sexodimorphic transcriptional activity in adult animals, although the changes induced by adolescent THC were strongly determined by sex. A Cuffdiff differential expression analysis performed between Male-THC vs Female-THC revealed up to 612 DE genes, 506 of which were exclusively altered in this comparison. Thus, the THC-induced changes entailed a regulation of some genes in opposite directions, which may be subtle relative to the VEH-controls and that were not easily detected in the VEH vs THC comparison and the factorial analysis. A general overall picture of the findings will be summarized below. The functions and related GO descriptions for each DE gene was consulted in the GO consortium database (http://geneontology.org/), Uniprot (https://www.uniprot.org/) and the NCBI Reference Sequence Database (https://www.ncbi.nlm.nih.gov/refseq/). The discussion will delve further into the relationships of these findings with SUDs.

5.1. SHARED TRANSCRIPTOMIC ALTERATIONS

Only 9 genes appeared to be DE in both males and females as an effect of THC administration. Two were upregulated by THC, independent of the sex, and both these genes play a role in the regulation of glutamatergic synaptic activity: *Slc17a6* (Solute Carrier Family 17 Member 6) and *Calb1* (Calbindin 1). Another two genes were downregulated by THC in both sexes: *RGD1310819,* the function of which is unknown; and *Dus2* (Dihydrouridine Synthase 2), that has been related to translational regulation of gene expression. The other five genes were modified by THC in opposite directions in each sex: *Nov* (Nephroblastoma overexpressed), associated with the GO codes of cell adhesion, cell migration, proliferation, differentiation and survival, and anti-inflammatory processes; *Ttr* (Transthyretin), associated with neurogenesis, neuronal survival and synaptic plasticity; *Cck*, neuropeptide involved in hormonal activity; *Tenm4* (Teneurin Transmembrane Protein 4), that mediates neuronal developmental, neural connectivity and regulates oligodendrogenesis and myelinisation processes; and *Zfhx3* (Zinc Finger Homeobox 3) a transcription factor involved in transcriptional regulation.

5.2. MALE TRANCRIPTOME ALTERATIONS

A total of 96 DE (adjusted $p < 0.05$) were found in the comparison between the male groups as an effect of adolescent THC exposure (See appendix C for the complete list of genes). The GO analysis found a significant enrichment of genes related to neural activity at the cellular level and regarding genes implicated in developmental processes, neurogenesis and behaviour. Looking at the most strongly up-regulated genes in males, adolescent THC exposure had an effect of genes related to transcriptional activity (*Satb2, Bhlhe22, Nr4a2*), genes involved in genome repair and stability (*Ercc8, Mgmt*), and genes involved in ribosomal activity (*Polr3k, RGD1359290, Rpl30*) and hence, protein synthesis. Among the most strongly downregulated genes we also found transcripts related to gene expression, replication (*Mcm7, Ccdc77, Nek5*) and protein metabolism (*Adgrf5*). The gene most strongly down-regulated by chronic adolescent THC administration in males was *Greb1*, a pan steroid-responsive gene involved in cell growth and proliferation. Likewise, among the most strongly down-regulated genes, we found other transcripts involved in cell growth and cell differentiation, such as the *Shc3* (SHC Adaptor Protein 3), *Flt1* (Vascular endothelial growth factor receptor) and *Notch3* (notch receptor 3) genes.

Shc3 is known to interact with RICS (Rho GTPase-activating protein 32), which in turn regulates dendrite spine morphology and *Trk* receptors, thereby affecting neuronal differentiation and survival. Other genes related to Tyrosine phosphorylation were also downregulated like *Ptprb* (Tyrosine Phosphatase Receptor Type B). *Ptprb* also plays a role in angiogenesis and interestingly, other genes related to correct vascularization were also downregulated: *Flt1* encodes a vascular endothelial growth factor that is also tyrosine kinase (Trk) receptor; the NOTCH3 protein is involved in the maintenance of blood vessels; *Rgs5* (Regulator of G Protein Signalling 5) is involved in the induction of endothelial apoptosis; *Vwf* (von Willebrand factor) has been implicated in blood vessel formation; and *Abcc9* (ATP Binding Cassette Subfamily C Member 9) is associated with ATP-sensitive potassium channels and expressed in vascular tissue. Finally, several neural synthesized haemoglobins (*Hba-a1, Hba-a2* and *Hbb)* were downregulated in males after adolescent THC exposure.

A

96 MALE VEH vs MALE THC

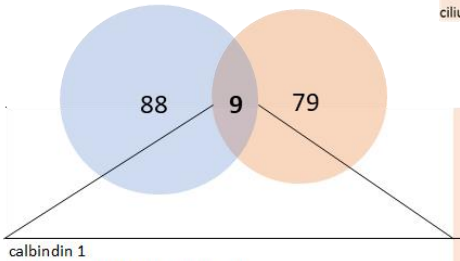
CELLULAR COMPONENT	Count	Fold Enrichment	FDR
neuron part	21	2,9	0,028
BIOLOGICAL PROCESS	**Count**	**Fold Enrichment**	**FDR**
single-organism behavior	13	5,6	0,0014
behavior	15	4,6	0,0019
neurogenesis	23	2,9	0,0013
generation of neurons	21	2,9	0,0035
nervous system development	28	2,5	0,0014
system development	43	2	0,0021

B

87 FEMALE VEH vs FEMALE THC

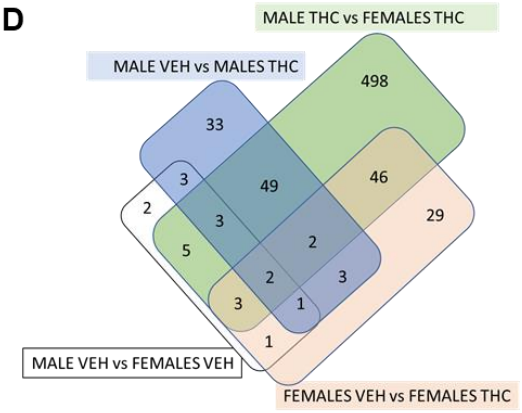
CELLULAR COMPONENT	Count	Fold Enrichment	FDR
dynein complex	4	43.81	1.54E-03
microtubule associated complex	5	18.25	2.36E-03
axonemal dynein complex	3	65.71	4.34E-02
cytoskeletal part	15	3.06	4.50E-02
MOLECULAR FUNCTION	**Count**	**Fold Enrichment**	**FDR**
hormone activity	6	15.17	1.64E-02
BIOLOGICAL PROCESS	**Count**	**Fold Enrichment**	**FDR**
nucleobase metabolic process	3	50.59	2.54E-02
microtubule-based movement	5	18.52	1.75E-02
microtubule-based process	6	9.96	3.23E-02
cilium-dependent cell motility	4	45.77	4.60E-02
cilium or flagellum-dependent cell motility	4	45.77	2.30E-02

C



	F VEH vs F THC	M VEH vs M THC	M VEH vs F VEH	M THC vs F THC
calbindin 1	0.50	0.64		
solute carrier family 17 member 6	0.62	0.57		0.67
dihydrouridine synthase 2	-0.55	-0.50		
similar to putative protein (5S487)	-0.53	-0.52		
transthyretin	-1.26	2.95	3.47	
nephroblastoma overexpressed	-0.95	1.91	1.14	-1.72
cholecystokinin	-0.59	1.01	0.78	-0.82
zinc finger homeobox 3	0.76	-0.77		1.13
teneurin transmembrane protein 4	0.52	-0.50		0.34
	FOLD CHANGE			

D



E

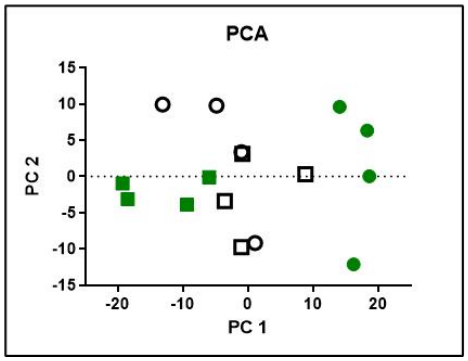


Figure 20. RNAseq. A) GO terms overrepresented in the subset of 96 DE genes between Male VEH and Male THC. **B)** The GO terms overrepresented in the subset of 96 DE genes found between Male VEH and Males THC. **C)** Venn diagram of the DE genes in these previous comparisons, common genes altered by THC in both comparisons. Each row represents the gene and the Log Fold Change for each comparison when FDR < 0.05. **D)** Venn diagram of the four main subsets of DE found in the CUFFDIFF analysis**. E)** Principal component analysis (PCA) performed on DESeq2. PC1 accounts for 71% of the variance, while PC2 accounts for a 13% of the variance.

Table 19. Upregulated Male transcripts

symbol	MALE VEH				MALE THC				FEMALE VEH				FEMALE THC				name	M VEH vs M THC	F VEH vs F THC	M VEH vs F VEH	M THC vs F THC
Satb2	31	15	49	18	61	22	157	321	17	30	185	21	60	15	8	158	SATB homeobox 2	2.26			-1.13
Ifnlr1	83	17	12	12	50	149	162	101	100	94	125	50	116	22	68	24	interferon lambda receptor 1	1.83		1.52	-0.85
Rspo2	6	22	29	3	7	13	40	135	12	11	45	24	7	12	16	56	R-spondin 2	1.66			-1.08
RGD1359290	3	119	92	1	4	120	121	235	91	97	135	175	84	139	93	118	Ribosomal_L22 domain containing protein RGD	1.21			
LOC102548695	93	84	295	77	449	532	312	54	262	73	552	610	458	587	425	315	zinc finger protein 45-like	1.16		1.27	0.54
Alb	40	116	112	23	131	115	208	131	28	226	29	424	120	124	105	128	albumin	1.01			
Mgmt	39	40	57	99	63	108	174	145	54	53	144	65	88	54	56	52	O-6-methylguanine-DNA methyltransferase	0.99			-0.82
C1ql3	113	63	163	99	108	106	121	522	218	114	94	77	75	72	105	130	complement C1q like 3	0.94			-1.01
Nxph3	195	153	252	147	202	165	404	699	177	167	457	180	256	130	170	255	neurexophilin 3	0.93			-0.73
Nr4a2	89	98	199	109	156	138	363	257	168	107	481	137	245	76	130	539	nuclear receptor subfamily 4 group A member 2	0.85			
LOC100360791	666	1731	0	50	35	855	1726	1749	2	845	1069	1073	677	854	1747	3	tumor protein, translationally-controlled 1	0.79			
Bhlhe22	93	98	162	107	85	88	105	499	146	169	96	122	98	63	80	133	basic helix-loop-helix family member e22	0.69			-0.89
Rpl30	441	629	562	865	1301	694	903	1109	600	720	767	801	622	748	758	843	ribosomal protein L30	0.64			
Syt17	371	386	475	577	402	376	684	1276	475	666	685	572	523	423	440	564	synaptotagmin 17	0.62			-0.46
Pter	106	114	104	173	166	163	181	131	101	200	118	223	113	176	96	158	phosphotriesterase related	0.59			-0.51
Alg11	426	429	478	354	399	582	773	758	481	693	554	869	532	736	654	656	ALG11 alpha-1,2-mannosyltransferase	0.51			
Tmem178a	344	275	594	400	384	403	558	995	339	488	748	469	416	514	438	518	transmembrane protein 178A	0.49			
Polr3k	1425	1039	1648	1161	1801	2022	1894	1704	1143	1596	2408	1539	1605	1132	1613	1207	RNA polymerase III subunit K	0.46			
Ercc8	1048	784	1742	1406	1729	1689	1990	1631	1470	1206	1897	1367	1472	1537	1520	1715	ERCC excision repair 8, CSA ubiquitin ligase complex subunit	0.43			
Dkk3	2109	1366	2014	1382	2504	2177	1956	2852	1893	1710	1766	2578	1158	1386	1568	1852	dickkopf WNT signaling pathway inhibitor 3	0.42			-0.58
Bloc1s2	814	757	929	863	1170	1078	1158	1154	969	799	1393	1200	815	1027	792	840	biogenesis of lysosomal organelles complex 1 subunit 2	0.40			
	RAW COUNTS																	FOLD CHANGE			

Associated Fold Change in the expression of genes in Male-THC relative to Males-VEH rats (M VEH vs M THC); Female-THC relative to Female -VEH rats (F VEH vs F THC); Female -VEH relative to Male -VEH rats (M VEH vs F VEH); and Male-THC relative to Female-THC rats (F THC vs M THC)

Table 20. Downregulated Male transcripts

symbol	MALE VEH				MALE THC				FEMALE VEH				FEMALE THC				name	M VEH vs M THC	F VEH vs F THC	M VEH vs F VEH	M THC vs F THC
Hba-a2	574	1700	679	1207	717	790	607	631	748	646	624	1227	775	699	563	990	hemoglobin alpha, adult chain 2	-0.63			
Mcm7	386	488	425	551	335	269	309	310	413	470	357	801	313	414	388	546	minichromosome maintenance complex component 7	-0.63			0.51
Abcc9	178	377	223	286	193	159	165	186	214	186	211	243	166	238	182	253	ATP binding cassette subfamily C member 9	-0.65			
AABR07006030.1	71	163	82	79	65	67	86	96	197	77	82	77	117	185	103	127	chordin	-0.66			0.88
Flt1	365	687	346	551	289	329	352	276	289	262	330	483	347	351	303	633	fms related tyrosine kinase 1	-0.67			0.45
Hba-a1	1100	3383	1384	2503	1374	1523	1170	1313	1412	1155	1150	2234	1539	1293	1056	2179	hemoglobin alpha, adult chain 2	-0.67		-0.57	
Adcy1	523	1054	383	284	200	320	387	563	207	659	391	644	502	513	115	870	adenylate cyclase 1	-0.68			0.53
Adgrf5	381	733	401	549	360	322	324	338	367	268	342	566	356	353	365	606	adhesion G protein-coupled receptor F5	-0.68			0.43
Vwf	410	586	293	416	323	280	239	257	250	217	272	592	336	299	307	504	von Willebrand factor	-0.69			0.49
Hbb	1449	3433	2081	2631	1955	1362	1040	1592	969	1877	1210	2340	1650	1588	1149	2979	hemoglobin subunit beta	-0.71		-0.66	0.37
Soga1	366	424	271	156	137	212	217	181	134	376	214	304	278	295	155	493	suppressor of glucose, autophagy associated 1	-0.73			0.76
Tns1	152	349	196	152	98	160	106	145	96	219	159	303	190	188	122	304	tensin 1	-0.74			0.72
Rgs5	253	510	266	377	205	186	239	267	190	248	277	407	214	275	191	383	regulator of G protein signaling 5	-0.74			
Shc3	408	736	230	223	179	246	265	261	150	367	221	435	296	317	120	617	SHC adaptor protein 3	-0.79			0.56
Gal3st4	163	209	170	222	111	113	112	104	93	179	113	286	132	165	156	237	galactose-3-O-sulfotransferase 4	-0.81			0.69
Ptprb	150	249	128	154	80	84	116	125	70	90	88	178	103	125	76	222	protein tyrosine phosphatase receptor type B	-0.86			
Notch3	346	674	297	254	201	272	173	222	256	264	191	298	229	271	195	374	notch receptor 3	-0.88		-0.72	
Nek5	257	246	248	239	235	196	26	32	210	99	315	75	177	37	26	195	NIMA related kinase 5	-0.98			
Ccdc77	278	291	71	556	305	85	71	80	529	296	67	397	56	267	602	471	coiled-coil domain containing 77	-1.15			1.38
Greb1	339	187	86	264	82	73	63	80	199	179	152	279	157	296	169	207	growth regulating estrogen receptor binding 1	-1.52			1.48
	RAW COUNTS																	FOLD CHANGE			

Associated Fold Change in the expression of genes in Male-THC relative to Males-VEH rats (M VEH vs M THC); Female-THC relative to Female -VEH rats (F VEH vs F THC); Female -VEH relative to Male -VEH rats (M VEH vs F VEH); and Male-THC relative to Female-THC rats (F THC vs M THC).

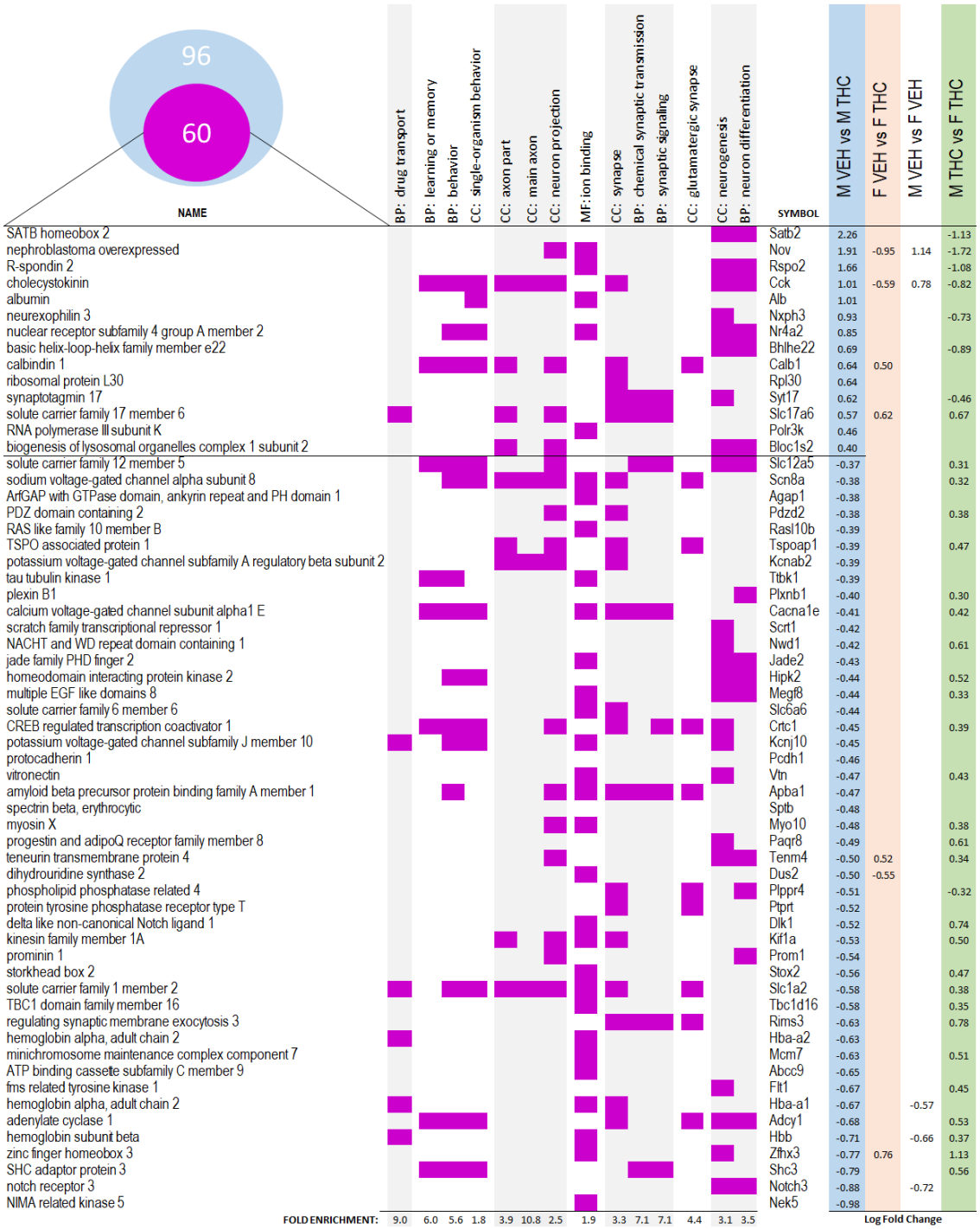


Figure 21. Principal GO terms and DE genes identified when comparing THC and VEH males. In total there were 96 DE genes in the Male-VEH vs Male-THC comparison. The Venn diagram represents the 60 DE genes (out of the total of 96) that were associated with the most representative GO terms depicted in the graph. Each row represents a gene and their associated symbol, their presence in one of the GO terms (BP stands for Biological process; CC for cellular component and MF for Molecular Function) highlighted with purple square blocks, and the value of the LogFoldChange if differentially expressed (FDR adjusted p-value < 0.05) in any of the Cuffdiff pairwise comparison. The rows are ordered by the LogFoldChange of the genes in the male comparisons.

5.3. FEMALE TRANCRIPTOME ALTERATIONS

A total of 87 DE genes were found in the comparison between females as an effect of adolescent THC exposure (See appendix C for the complete list of genes). Biological process terms overrepresented in the GO analysis pointed to significant alterations of microtubule-based movement and cilium or flagellum-dependent cell motility. At the cellular component level, the GO terms associated with the overrepresentation of genes were mostly associated with dynein and axonemal dynein complex, microtubule associated complex and cytoskeletal part. THC treatment also protractedly altered genes involved in hormone activity. This set of DE genes included the upregulation of *Gal* or *Trh*, and the downregulation of *Cck* and *Cartpt*. Nonetheless, other transcripts like *Zfhx3* with known hormonal interactions were also considered to be DE genes.

In the case of females, as in males, cell growth and survival appears to be one of the main common functions between the most strongly upregulated genes. The most pronounced upregulation associated with THC was found in *Irs4* (Insulin Receptor Substrate 4), which plays a role in glucose homeostasis as well as in cell growth. Nonetheless, genes encoding proteins that can regulate transcriptional activity were also altered, such as *Hsf4* (heat shock transcription factor 4) and *Zfhx3* (Zinc Finger Homeobox 3).

Among the genes most strongly downregulated in adult females by chronic adolescent THC administration there were also transcripts that potentially regulate cell proliferation, like *MYB* (MYB proto-oncogene, transcription factor) and *CD74* (Cluster of Differentiation 74 Molecule, Major Histocompatibility Complex, Class II Invariant Chain). However, most of the genes were involved in cell motility, vesicle transport and binding (*Dynlrb2, Dnah6, Cfap43, Cfap44* and *Prc1*).

5.4. INTERACTIVE TRANCRIPTOME ALTERATIONS

When males and females administered THC were compared the largest number of DE genes was identified, up to 612. Of these, 436 were exclusively altered in this comparison. A Principal Component Analysis of the DE genes performed with dseq2 showed a strong separation of the THC treated groups from the vehicle treated controls, and especially in the case of the females that received adolescent THC, which accounted for 71% of variance. In terms of the other secondary component extracted, which accounts for 13% of the variance, this seemed to be most strongly influenced by sex (See Figure 20).

The subset of 612 DE genes that were identified by comparing the THC treated animals was enriched in several binding related GO terms at the molecular function level. These were presumably most prominent in the axon and they would preferentially alter biological process like axon guidance and adhesion. Up to 7 Kyoto Encyclopaedia of Genes and Genomes (KEEG) pathways were altered by THC: Oxytocin signalling, Calcium signalling, Retrograde endocannabinoid, Vascular smooth muscle contraction, Glutamatergic synapse, Nicotine addiction and Morphine addiction. The genes with a log2fold change that identify these pathways can be seen in Figure 23.

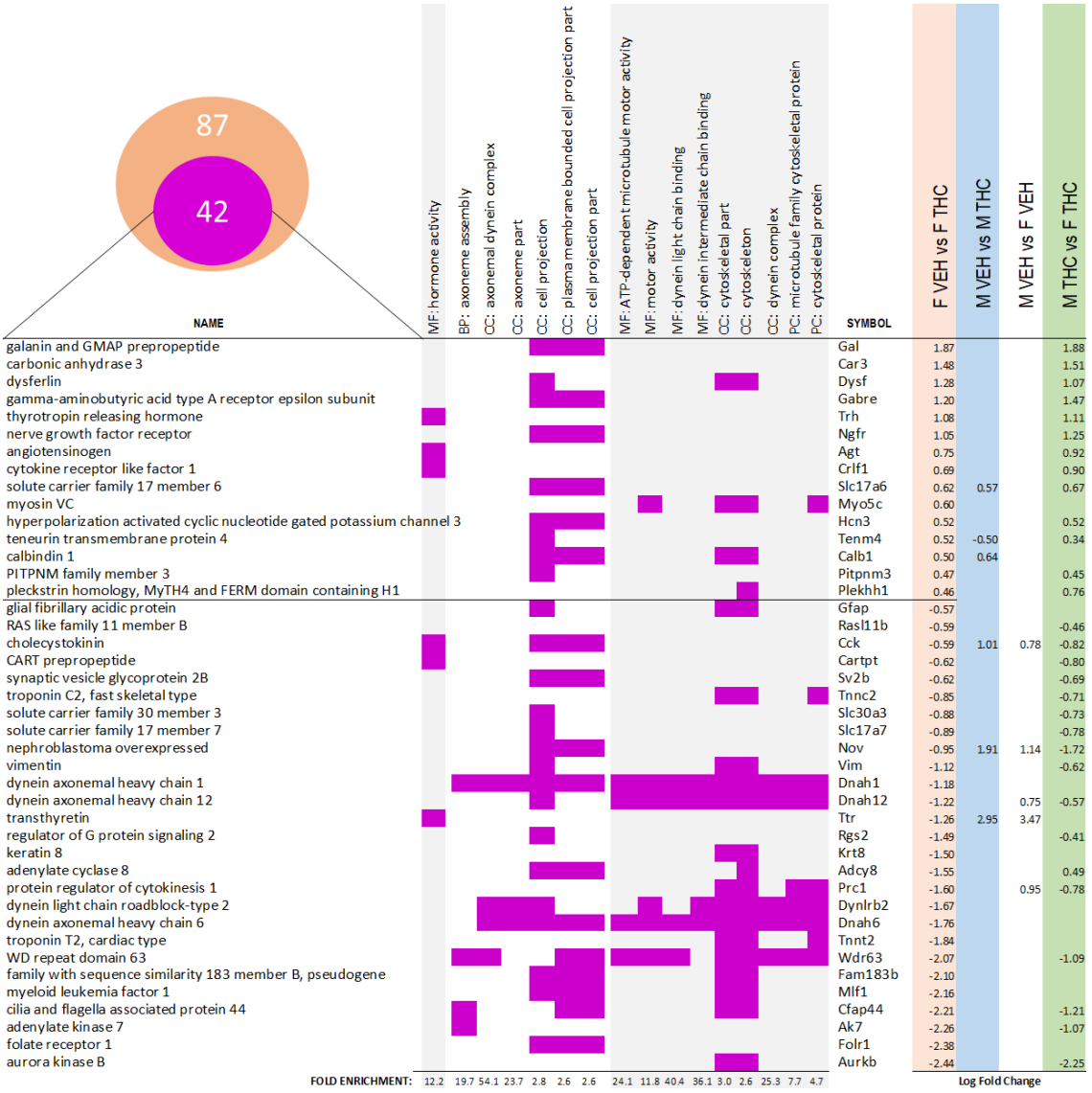


Figure 22. Principal GO terms and DE genes identified when comparing Female rats. DE genes obtained with Cuffdiff in the Female-VEH VS Female-THC comparison, submitted to PANTHER analysis to assess the relevant GO terms. In total there were 87 DE genes in the female-VEH vs female-THC comparison. The Venn diagram represents the 42 DE genes (out of the total of 87) that represent the most representative GO terms depicted in the graph. Each row represents a gene and their associated symbol, an their association with one of the GO terms (MF stands for Molecular Function, BP stands for Biological Process, CC for Cellular Component and PC for Protein class) highlighted with a purple block, as well as the Log Fold Change if DE (Adjusted p-value < 0.05) in any of the Cuffdiff pairwise comparisons. The rows are ordered by the Log Fold Change of the genes in the comparison of Females.

Table 21. Upregulated Female transcripts

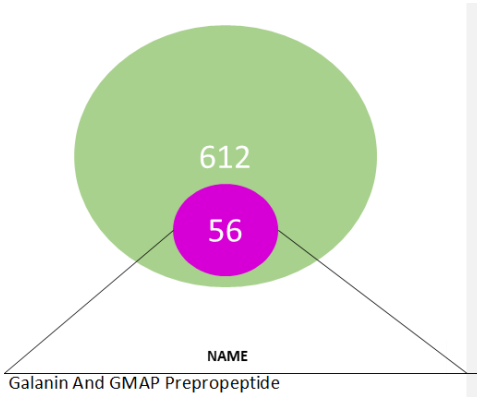
symbol	MALE VEH				MALE THC				FEMALE VEH				FEMALE THC				name	F VEH vs F THC	M VEH vs M THC	M VEH vs F VEH	M THC vs F THC
Irs4	55	128	119	11	12	48	53	85	62	51	98	28	129	166	346	204	insulin receptor substrate 4	1.90			2.19
Gal	16	7	22	38	28	7	8	36	18	17	23	23	18	41	185	24	galanin and GMAP prepropeptide	1.87			1.88
Car3	40	68	112	113	59	90	65	90	41	45	138	107	79	584	121	39	carbonic anhydrase 3	1.48			1.51
Hsf4	45	84	113	89	66	55	58	95	77	71	127	78	110	151	278	93	heat shock transcription factor 4	1.38			1.38
Dgkk	37	50	35	11	13	34	29	25	20	33	38	14	48	59	82	59	diacylglycerol kinase kappa	1.36			1.36
Dysf	26	62	63	39	37	31	42	51	30	25	67	22	76	70	124	45	dysferlin	1.28		-2.26	1.07
Il22ra2	28	99	76	102	87	65	103	93	22	40	80	62	77	110	126	122	interleukin 22 receptor subunit alpha 2	1.22			
Gabre	44	45	58	20	28	25	37	55	48	34	40	46	50	68	164	89	gamma-aminobutyric acid type A receptor epsilon subunit	1.20			1.47
Trh	106	112	132	102	107	130	94	92	135	102	107	95	95	201	435	129	thyrotropin releasing hormone	1.08			1.11
Ngfr	27	30	316	43	61	73	59	411	31	247	383	56	270	411	558	62	nerve growth factor receptor	1.05			1.25
Col2a1	137	191	180	111	143	140	96	103	251	96	117	70	201	143	380	218	collagen type II alpha 1 chain	0.91			1.06
Grin2d	236	362	400	237	190	241	228	380	274	329	321	170	405	471	572	379	glutamate ionotropic receptor NMDA type subunit 2D	0.87			0.88
Col7a1	82	229	84	213	158	86	162	110	117	111	77	103	164	232	103	205	collagen type VII alpha 1 chain	0.85			
Zfhx3	406	557	358	205	223	308	227	168	308	367	242	286	413	420	429	644	zinc finger homeobox 3	0.76			1.13
Magel2	284	343	337	183	218	225	191	233	373	244	245	221	310	254	671	487	MAGE family member L2	0.75			1.08
Agt	2763	4018	4278	2424	2786	2929	2842	5946	2837	3984	6348	3594	4613	7990	8762	4231	angiotensinogen	0.75			0.92
Crlf1	694	913	688	465	678	588	492	684	825	693	785	530	824	1113	1164	1268	cytokine receptor like factor 1	0.69			0.90
Gpx3	234	401	653	341	404	454	277	311	379	405	884	227	546	531	1228	469	glutathione peroxidase 3	0.69			1.06
Baiap3	1627	2284	2473	710	994	1310	1467	1688	2484	1588	1720	992	1960	2360	3672	3109	BAI1 associated protein 3	0.69			0.97
Slc17a6	140	187	271	216	283	207	304	452	167	332	577	259	317	529	743	231	solute carrier family 17 member 6	0.62			0.67
	RAW COUNTS																	FOLD CHANGE			

Associated Fold Change in the expression of genes in Male-THC relative to Males-VEH rats (M VEH vs M THC); Female-THC relative to Female -VEH rats (F VEH vs F THC); Female -VEH relative to Male -VEH rats (M VEH vs F VEH); and Male-THC relative to Female-THC rats (F THC vs M THC).

Table 22. Downregulated Female transcripts

symbol	MALE VEH				MALE THC				FEMALE VEH				FEMALE THC				name	F VEH vs F THC	M VEH vs M THC	M VEH vs F VEH	M THC vs F THC
Adcy8	156	228	279	181	151	170	239	356	171	327	362	209	282	325	317	265	adenylate cyclase 8	-1.55			0.49
Sptlc3	13	58	3	57	18	19	46	12	1	133	55	108	28	21	10	7	serine palmitoyltransferase long chain base subunit 3	-1.58			
Myb	70	24	4	6	95	15	8	14	20	18	11	209	9	8	2	56	MYB proto-oncogene, transcription factor	-1.59			
Prc1	209	76	59	68	225	76	46	113	65	99	46	668	37	64	50	109	protein regulator of cytokinesis 1	-1.60			-0.78
Dynlrb2	106	27	11	3	136	23	17	26	41	45	26	265	6	13	17	79	dynein light chain roadblock-type 2	-1.67		0.95	
Dnah6	259	63	10	13	315	28	9	42	58	56	30	541	9	16	10	152	dynein axonemal heavy chain 6	-1.76			
Cd74	87	134	50	133	96	119	43	47	47	318	110	508	62	59	96	39	CD74 molecule	-1.76			
Armc3	89	26	7	19	103	13	19	25	25	26	28	238	10	16	12	55	armadillo repeat containing 3	-1.78		1.17	
Tnnt2	12	75	16	12	22	70	9	20	15	9	23	231	10	18	25	17	troponin T2, cardiac type	-1.84			
RT1-Da	22	45	19	33	31	26	19	13	16	72	24	149	16	11	22	10		-1.89			
Cfap43	345	68	35	29	352	35	32	68	86	71	48	895	11	23	19	210	cilia and flagella associated protein 43	-1.93			-0.90
Wdr63	158	26	5	6	159	21	17	45	43	35	14	416	15	8	12	83	WD repeat domain 63	-2.07			-1.09
Fam183b	75	14	2	3	64	6	4	22	18	17	6	253	4	4	5	48	family with sequence similarity 183 member B, pseudogene	-2.10			
Slc22a6	4	69	5	19	20	82	10	9	4	9	11	209	5	14	15	7	solute carrier family 22 member 6	-2.12			-1.38
Mlf1	68	21	2	8	116	11	2	26	20	23	17	269	7	4	3	56	myeloid leukemia factor 1	-2.16			
Cfap44	310	54	3	6	251	16	2	27	76	40	13	491	3	8	3	115	cilia and flagella associated protein 44	-2.21			-1.21
Ak7	224	27	6	10	225	12	4	36	43	49	10	596	7	8	4	121	adenylate kinase 7	-2.26			-1.07
Folr1	41	5	1	3	65	1	1	13	12	9	8	137	1	2	2	20	folate receptor 1	-2.38			
Aurkb	205	42	9	10	284	39	26	84	91	34	25	368	18	14	12	44	aurora kinase B	-2.44			-2.25
Tmem212	102	21	1	6	126	14	0	24	34	25	5	328	1	5	1	58	transmembrane protein 212	-2.44			
	RAW COUNTS																	FOLD CHANGE			

Associated Fold Change in the expression of genes in Male-THC relative to Males-VEH rats (M VEH vs M THC); Female-THC relative to Female -VEH rats (F VEH vs F THC); Female -VEH relative to Male -VEH rats (M VEH vs F VEH); and Male-THC relative to Female-THC rats (F THC vs M THC).



NAME	KP: Calcium signallign	KP: Oxytocin signaling pathway	KP: Retrograde endocannabinoid signaling	KP: Nicotine addiction	KP: Morphine addiction	KP: Glutamatergic synapse	BP: Regulation of response to drug	SYMBOL	M THC vs F THC	M VEH vs M THC	F VEH vs F THC	M VEH vs F VEH
Galanin And GMAP Prepropeptide							■	Gal	1.88		1.87	
gamma-aminobutyric acid type A receptor theta subunit			■	■	■			Gabrq	1.73			
gamma-aminobutyric acid type A receptor epsilon subunit			■	■	■			Gabre	1.47		1.20	
purinergic receptor P2X 2	■							P2rx2	1.36			
Angiotensinogen							■	Agt	0.92		0.75	
cholinergic receptor muscarinic 5	■							Chrm5	0.90			
glutamate ionotropic receptor NMDA type subunit 2D	■			■		■		Grin2d	0.88		0.87	
adenylate cyclase 7	■	■	■		■	■		Adcy7	0.86			
solute carrier family 17 member 6			■	■		■		Slc17a6	0.67	0.57	0.62	
potassium voltage-gated channel subfamily J member 5		■	■		■			Kcnj5	0.63			
natriuretic peptide receptor 2								Npr2	0.56			
adenylate cyclase 1	■	■	■		■	■		Adcy1	0.53	-0.68		
calcium voltage-gated channel subunit alpha1 G	■							Cacna1g	0.53			
SH3 and multiple ankyrin repeat domains 1						■		Shank1	0.51			
adenylate cyclase 8	■	■	■		■	■		Adcy8	0.49		-1.55	
glutamate ionotropic receptor kainate type subunit 3						■		Grik3	0.48			
calcium voltage-gated channel auxiliary subunit alpha2del		■						Cacna2d2	0.45			
lysine methyltransferase 2A							■	Kmt2a	0.44			
phospholipase A2 group IVB		■				■		Pla2g4b	0.44			
purinergic receptor P2X 7	■							P2rx7	0.43			
erb-b2 receptor tyrosine kinase 3	■							Erbb3	0.42			
calcium/calmodulin dependent protein kinase IG		■						Camk1g	0.42			
calcium voltage-gated channel subunit alpha1 E	■							Cacna1e	0.42	-0.41		
phospholipase D2						■		Pld2	0.40			
calcium/calmodulin dependent protein kinase II gamma	■	■						Camk2g	0.38			
solute carrier family 1 member 2						■		Slc1a2	0.38	-0.58		
arrestin beta 1					■			Arrb1	0.37			
solute carrier family 8 member A3	■							Slc8a3	0.36			
calcium voltage-gated channel subunit alpha1 B	■		■	■	■			Cacna1b	0.36			
calcium voltage-gated channel subunit alpha1 A	■					■		Cacna1a	0.30			
ryanodine receptor 2	■	■						Ryr2	0.30			
protein phosphatase 1 regulatory subunit 12B		■						Ppp1r12b	0.30			
gamma-aminobutyric acid type A receptor alpha4 subunit			■	■	■		■	Gabra4	-0.30			
potassium voltage-gated channel subfamily J member 4		■						Kcnj4	-0.32			
phospholipase C beta 1	■	■	■			■		Plcb1	-0.33			
protein phosphatase 3 regulatory subunit B, alpha	■	■				■		Ppp3r1	-0.33			
leucine rich repeat kinase 2							■	Lrrk2	-0.34			
RAB3B, member RAS oncogene family							■	Rab3b	-0.35			
protein phosphatase 1 catalytic subunit gamma		■						Ppp1cc	-0.38			
gamma-aminobutyric acid type A receptor delta subunit			■	■	■			Gabrd	-0.40			
synaptotagmin 6							■	Syt6	-0.41			
regulator of G protein signaling 2		■					■	Rgs2	-0.41		-1.49	
prostaglandin-endoperoxide synthase 2		■	■					Ptgs2	-0.41			
myocyte enhancer factor 2C		■						Mef2c	-0.42			
guanylate cyclase 1 soluble subunit beta 1		■						Gucy1b3	-0.44			
Purkinje cell protein 4							■	Pcp4	-0.45			
synaptotagmin 17							■	Syt17	-0.46	0.62		
5-hydroxytryptamine receptor 2C	■							Htr2c	-0.49			
nuclear receptor subfamily 4 group A member 3							■	Nr4a3	-0.51			
synuclein alpha							■	Snca	-0.52			
regulator of G protein signaling 4							■	Rgs4	-0.53			
dopamine receptor D1	■				■			Drd1	-0.54			
guanylate cyclase 1 soluble subunit alpha 1		■						Gucy1a3	-0.59			
troponin C2, fast skeletal type	■							Tnnc2	-0.71		-0.85	
solute carrier family 17 member 7			■	■		■		Slc17a7	-0.78		-0.89	
hepatocyte growth factor							■	Hgf	-0.88			
FOLD ENRICHMENT:	4.0	4.8	5.0	8.2	4.8	4.4	5.4		**Log Fold Change**			

Figure 23. DE genes, and associated GO and KEGG pathways in Male-THC vs Female-THC. The table shows only a subset of the genes implicated, and the relevant GO terms and KEGG pathways (KP) that are more closely related to SUDs.

DISCUSSION

1. MAGNETIC RESONANCE

The time frame of adolescent THC treatment employed here (PND28 to 42) coincides with a developmental window in which we would expect to see an increase in grey and white matter volume in the rat's brain, with a relatively smaller grey matter volume in some areas of the female's brain than in males of the same post-natal age (Sumiyoshi, Nonaka, & Kawashima, 2017). The main differences in the adult rat brain after ACE found in the MRI study are reflected in Table 23 below.

Table 23. MRI Results Summary

	Male VEH vs Male THC	Female VEH vs Female THC
Brain Ventricle Volumetry	↓ Lateral ventricles ↓ Aqueduct	
Grey Matter Volumetry	↓ Right GP	↓dSTR ↓GP (p=0.057)
Grey Matter MD		↓Rostral SNu
Grey Matter FA	↓GP ↓caudal SNu ↓Rostral STR ↑Thalamus	
White Matter FA	↓CC ↓AC	↓HC (p=0.051)
Grey Matter ^{1}H NMR Spectroscopy	↓Cx GPCP+Ch	

1.1. BRAIN VOLUMETRY

1.1.1. DECREASED VENTRICULAR VOLUME IN ADULTS AFTER ADOLESCENT CANNABINOID EXPOSURE

In line with the decrease in ventricular volume found in our animal model, a lower ventricular cerebrospinal fluid (CSF) volume was also reported in young adult humans that frequently used cannabis (Block et al., 2000). Notably, the subjects in this study were current users, such that the stability of this feature in humans remains unclear. The effects of THC on the CSF were also studied elsewhere, this time altering the relative content of eCB ligands, decreasing the AEA in heavy users relative to light users, and increasing 2-Arachidonoylglycerol (2-AG) in heavy users relative to the controls, which may in turn mediate autoinflammatory processes (Morgan et al., 2013).

Human MRI studies into the relationship of cannabis use with psychosis and schizophrenia represent a frequent source of ventricular alterations associated with cannabis (Rapp et al., 2012). Usually, patients with schizophrenia show ventricular enlargement (Welch et al., 2011) and in terms of CSF content, higher levels of AEA have been reported in low-frequency cannabis users with schizophrenia compared to high-frequency users with schizophrenia (Leweke et al., 2007), with a negative correlation between CSF AEA and the persistence of psychotic symptoms also observed following cannabis use (Morgan et al., 2013). In line with a cannabis-induced decrease of ventricular volume, cannabis-naive patients with schizophrenia were seen to have a larger left than right lateral ventricle, although this asymmetry was absent in cannabis-exposed subjects (Cahn et al., 2004). Nonetheless, not all studies have proved to be consistent in this sense and indeed, no differences were evident in patients with schizophrenia irrespective of cannabis use (Ho et al., 2011). Compared to healthy controls, non-cannabis-using schizophrenic patients had a larger third ventricle at the beginning of the study (after the first episode of schizophrenia), although cannabis-using patients had a more pronounced enlargement of the third and lateral ventricles after a 5-year follow-up (Rais et al., 2008). Similarly, frequent cannabis use was seen to be a significant predictor of enlarged third ventricular volume after controlling for other drugs (Welch et al., 2011). In this sense, we did not find a significant change in the volume of the third ventricle here and thus, there is some degree of specificity in both clinical and preclinical findings.

Although a relative loss in the total BVV was evident, analysing the individual ventricles suggests that there is a differential effect over each segment and to some degree, sex differences in the magnitude of these changes. The lateral ventricular volume decreases irrespective of sex, with a similar pattern of evolution during adolescence in both male and female rats, and we would expect an increasing slope with few sex-related differences from at least PND35 to 56 (Piontkewitz et al., 2011). Thus, the decrease in volume in adult animals that received adolescent THC treatment here could be due to the interruption of this normal growth due to THC and eCB signalling. Nonetheless, data from other animal studies indicate that THC has an inhibitory effect on CSF production and flux, presumably affecting ventricle volume. Indeed, this phenomenon was proposed to influence choroidal synaptosomal neurotransmitters (Mancall et al., 1985).

1.1.2. VOLUMETRIC ALTERATIONS TO BASAL GANGLIA AND THEIR RELATIONSHIP TO DA SIGNALLING AND SUDs

There was a reduction in volume evident in two basal ganglia structures. THC decreased the right GP volume although, it only produced a trend towards a smaller total GP volume (p=0.057). By contrast, the STR volume decreased in THC females but not males.

It is well-documented in the literature that human cannabis use reduces the volume of the grey matter in CB_1 rich regions, although this is usually linked to current cannabis use rather than associated with disruptions of normal ontological maturation (Battistella et al., 2014). CB_1 receptors are expressed strongly in the basal ganglia, and especially in the GP of both humans and rodents (Glass et al., 1997; Herkenham et al., 1991). Several studies in human cannabis users have reported alterations within the basal ganglia and to its functional connectivity with other areas (Filbey et al., 2016), although besides morphological alterations, fewer volumetric abnormalities have been reported (Orr et al., 2016; Smith et al., 2015). Most of the studies, including those assessing the STR or other basal ganglia structures, failed to find significant differences even after short periods of abstinence (Chye et al., 2017; Cousijn et al., 2012; Ganzer et al., 2016), although one study found a reduction in the right ventral striatum due to cannabis exposure (Pagliaccio et al., 2015). More recently, increased basal ganglia grey matter volume was found in heavy cannabis users (Moreno-Alcázar et al., 2018). Nonetheless, as this study involved periods of abstinence of 24 h, the results could be influenced by residual cannabis effects and those of cannabis-withdrawal itself, particularly in the light of the absence of studies reporting this same alteration.

The specificity of the change in the STR cannot solely be attributed to a mechanism mediated by CB_1 receptors or a disruption of the developmental trajectory of this nucleus. Other structures have similar developmental trajectories, and female rats also display analogous patterns of CB_1 expression and G-protein activity in other brain areas (Burston et al., 2010; Mengler et al., 2014; Rubino & Parolaro, 2011; Van Waes et al., 2012). It was recently proposed by that dysregulated DAergic activity may modulate volumetric changes in specific areas (Chang et al., 2020). In this regard, conditions associated with dampened DAergic signalling (e.g. depression, anhedonia, SUDs) have been repeatedly associated with a reduction in the volume of basal ganglia structures (Barrós-Loscertales et al., 2011; Belujon & Grace, 2017; Harvey et al., 2007; Kim et al., 2008). Conversely, individuals with hyperdopaminergic pathologies (attention-deficit/hyperactivity disorder, autism spectrum disorder, psychopathy) experience an increase in the volume of basal ganglia structures (Buckholtz et al., 2010; Glenn et al., 2010; O'Dwyer et al., 2016; Onnink et al., 2016), remarkably the increase in DAergic activity through natural means (sports or videogames) can also influence striatal volume (Becker et al., 2016; Erickson et al., 2010). Interestingly, GP is an output region of the dSTR and thus, changes in such structures could affect the other. In this regard, interactive changes in the volume of these two structures were associated with the value of subjects scores in an autism spectrum disorder scale (O'Dwyer et al., 2016). Thus, volumetric changes to the GP and STR might be associated with disrupted DAergic signalling that have already been documented in cannabis users (Bloomfield et al., 2016; Volkow et al., 2014) and in preclinical models (see introduction section 2.2.2). Moreover, hints of dampened DAergic signalling and increased DAT expression, were already found specifically in the adult female STR after ACE (Higuera-Matas et al., 2011).

1.2. GRAY MATTER MEASUREMENTS

1.2.1. EVIDENCE FOR SUBCORTICAL MICROSTRUCTURAL ALTERATIONS AND THEIR RELATIONSHIP TO ADDICTION

From PDN21 to 90 the myelinisation of white matter bundles within the **STR** of Wistar males is still developing and increasing, as inferred by the FA signal. During the same period cell density decreases, as reflected by the slow decline in the MD signal over time (Mengler et al., 2014). In males, adolescent exposure to THC seems to disrupt and delay myelinisation in the STR, as suggested by the lower FA value. The MD values only showed a trend towards an interaction in the STR (male-VEH< male-THC and female-VEH>female-THC: p=0.055 in total STR and p=0.052 in STR Bregma -0.5mm) and thus, this could be a secondary effect and will require further confirmation. Nonetheless, it seems that adolescent THC might have also interacted with some of the sex-specific developmental differences that arise within the striatum and its connections to other areas during adolescence (Lei et al., 2016).

FA has also been associated with changes in myelinisation of DAergic areas and tracts. Severe DAergic alterations as a result of methamphetamine abuse (Volkow et al., 2001) can blunt the FA signal in the STR (Alicata et al., 2009) and increased FA in subcortical DAergic tracts has been found in the circuits underlying symptom generation in schizophrenia (Alba-Ferrara & de Erausquin, 2013). In addition, a relationship between MD and DA synthesis capacity was detected in the posterior caudate and putamen, suggesting that DA

synthesis may be related to the density of DAergic neural fibres (Kawaguchi et al., 2014). In the case of THC females, the lower MD (trend) could parallel the loss of volume observed, further suggesting a DAergic aetiology of volumetric differences and indeed, DAergic alterations after cannabis exposure have already been discussed (Bloomfield et al., 2016; Volkow et al., 2014).

Conversely, the FA signal in THC males was higher in the male rats that received adolescent THC relative to both VEH males and THC females. From a neurodevelopmental perspective, there is evidence of a progressive weakening from adolescence to adulthood of some thalamo-cortical connections (Fair et al., 2010) and thus, the elevation in FA signal in this region could be due to an aberrant axonal pruning provoked by adolescent THC. In this regard, there is evidence of the early influence of eCB signalling in shaping the thalamo-cortical projections (Itami et al., 2016), and that CB_1 agonists may prevent pruning at cortical glutamatergic synapses during adolescence (Rubino et al., 2015). Moreover, an increased FA relative to control or baseline non-pathological conditions has been interpreted as a compensatory mechanism that could be associated or compatible with a loss of FA or other alterations in different regions (Mole et al., 2016), changes that may reflect how aberrant structural connectivity (Hoeft et al., 2007) compromises the diffusion of the signal (Alba-Ferrara & de Erausquin, 2013). Nevertheless, the functional implications of this change in FA remain unknown. The different thalamic nuclei have been associated with a wide variety of functions and remarkably, in the context of SUDs, thalamic activity can modulate drug-related behaviours in both humans and rodents (e.g. drug-seeking and drug-cue reactivity: Huang et al., 2018). However, the resolution in the MRI study did not permit the reliable identification of the specific nuclei altered and thus, a more exhaustive analysis of the changes within each nucleus might be an interesting approach for future studies.

It is noteworthy that a reduced MD was observed in the rostral SNu of THC females. SNu has strong reciprocal connections with the thalamus from the via stria medullaris thalami (Felten, Summo Maida, & O'Banion, 2016), as well as with other common areas altered by cannabis like the hippocampus (via the fornix), the amygdala (via the stria terminalis) and the VTA (via the medial forebrain bundle) among others (Willis & Haines, 2018). All these connections make the SNu an important hub capable of modulating memory formation (Khakpai et al., 2013), reward (and avoidance) related learning and even including drug-related behaviours (Degroot & Parent, 2001; Kirby & Rawlins, 2003; Liu et al., 2012). Moreover, SNu activity might also be relevant for the sex-specific differences in response to cannabis, which also communicates with the hypothalamus and that therefore may regulate neuroendocrine and autonomic responses (Risold, 2004). The SNu is usually divided cytoarchitectonically into the lateral (LS) and medial septal (MS) nucleus, each division having distinct functional implications. The LS is crucial for the appropriate processing of CS-US association, while MS is crucial for appropriate processing of contextual cues (foreground or background information: Calandreau et al., 2007). Again, as in the case of the THA, a more subtle analysis of the SNu is needed to elucidate the potential role of the MS or LS and what changes take place after adolescent cannabinoid exposure, and the probable modulation of the cannabis-induced changes in PIT and PCA. Regarding drug-motivated behaviour, it was shown that the connection of the dorsal hippocampus to the VTA via LS mediates the reinstatement of cocaine-seeking by contextual stimuli (Luo et al., 2011).

1.2.2. THC-TREATMENT DIMINISHED THE CORTICAL GPC+PCh METABOLITE SIGNAL

Choline compounds (downregulated in the cortex of adult THC treated animals) are a marker of cell membrane turnover, cell density and membrane integrity, and they are increased in conditions of membrane breakdown and inflammation, or in associated with an increase in cell density (Dager et al., 2008; Ross & Sachdev, 2004). Difference in basal brain function were also obtained in a parallel PET study with a similar ACE protocol, indicating that adolescent THC administration to males enhanced glucose metabolism in the somatosensory cortex and in the piriform cortex. By contrast, in females similar THC exposure produced hypometabolism in a cluster of voxels corresponding to the inferior colliculus and Cb (See appendix D). Together these functional alterations could be involved in long-term effects on sensorimotor reflexes and coordination, although the true effects of these changes will require further elucidation.

However, an increase in choline compounds has also been associated with myelinisation (Dager et al., 2008; Ross & Sachdev, 2004) and there is evidence for reduced GPC+PCh after exposure to demyelinating agents (Yan et al., 2015), indicating that this parameter may reflect impaired myelinisation. Indeed, the voxel employed for the cortical measurements included a partial segment of the CC that might influence the signal, supporting the association of this reduced GPC+PCh with non-efficient myelination.

1.3. WHITE MATTER: FA TRACTS

White matter alterations associated with axon myelinisation are among the main effects of cannabinoid agonists like THC and they can potentially affect the development of the adolescent brain (Lubman et al., 2015). CB_1 receptors are present in the main white matter structures including the CC, AC, HC, stria terminalis and stria medullaris, presumably modulating their development from the early perinatal stage (Romero et al., 1997). Under normal circumstances, after birth the white matter bundles gradually increase the amount of myelin and myelinated axons they contain as adolescence progresses. White matter maturation may follow slightly different developmental pathways and a certain degree of sexual dimorphism can be observed. For example, the number of myelinated axons in the CC suffer a dramatic increase from PND15 to 25, and while exponential growth continues from PND25 to 60 show a significant male<female sex difference begins to appear (Juraska & Willing, 2017). Thus, adolescent THC treatment coincides with the beginning of one of the most sensitive time windows for white matter development and it seems to coincide with the onset of sex-specific differences.

The analysis of the FA signal in the brain tracts revealed that THC diminished the FA signal in the HC and a section of the rostral CC, AC and IC. Diminished myelinisation and in the integrity of white matter fibres is commonly associated with cannabis consumption in human MRI studies (Becker et al., 2005). Remarkably, an earlier age of onset is associated with the severity of demyelination (Orr et al., 2016) and frontal FA deficits (Gruber et al., 2011). Moreover, impairments in axonal connectivity have been seen in several tracts in long-term cannabis users, including the right fimbria of the hippocampus, the splenium of the CC and AC fibres (Zalesky et al., 2012). An unequivocal causal role in cognitive functions is hard to establish but the loss of white matter integrity is related to a variety of functional implications depending on the location of the axons affected, such that motor, sensory and/or cognitive functions may be altered (Crawford et al., 2009). In this sense, the loss of white matter integrity in frontolimbic areas (uncinate fasciculus and forceps minor) in regular cannabis user's was associated with apathy and depressive-like symptoms (Shollenbarger et al., 2015) that may ultimately affect reward processing (Admon & Pizzagalli, 2015). Thus, it is highly probable that the FA changes detected here may be associated with different behavioural outcomes.

2. BEHAVIOURAL TRAITS

Table 24. Behavioural traits - main results

	Male VEH vs Male THC	Female VEH vs Female THC
Pavlovian-to-instrumental transfer	↑ expression of stronger PIT	
Two-Choice Serial Reaction Time Task	↓ state-dependent impulsivity	↑ reactivity to reward delays
Pavlovian Conditioned Approach	↓ goal tracking	
Habit training	NSD in habit formation	

NSD= No Significant Differences

2.1. PIT

No previous study have analysed the impact of ACE over PIT in animal or human models. Nonetheless, the literature regarding alterations in PIT due to cannabinoid exposure indicated a more than probable interaction. Nonetheless, different models of adolescent cannabinoid exposure have documented alterations in the amygdala, NAc and in DA transmission, key features for the expression of PIT (See Box 19).

It was shown that general transfer (See Box 18) could be eliminated with microinjections of SCH-2339 (a D_1 antagonist) into the NAc (both shell and core subdivisions), while raclopride (a D_2 antagonist) microinjections into the NAc can attenuate but do not eliminate PIT (Lex & Hauber, 2008). These findings support and extend our understanding of DAergic influences on PIT but also, the implications of this for general transfer protocols and outputs that may rely upon areas that are not usually involved in general PIT, like the NAc Shell. Thus, PIT might be potentiated through already known changes in DA signalling, as indicated by PEACE studies that previously reported increased DA turnover and D_1 receptor density in the NAc Shell (Bortolato et al., 2014; Higuera-Matas et al., 2011; Zamberletti et al., 2014). Transcriptome changes in the NAc Shell and its implication for SUDs, reward-related behaviours, and DAergic signalling will be discussed in more detail below.

Beyond the role of the NAc, other structures within the mesolimbic DA pathways that are apparently relevant to PIT expression can be modified by ACE. Interestingly, PIT is differentially modified by dSTR subdivisions and it has led to interpret PIT outcomes within the context of A-O/goal directed behaviour (DMS

dependent) and in S-R learning (DLS dependent: Corbit & Janak, 2007). Importantly this approach has also recently been validated in humans (Seabrooke et al., 2019). In this sense, it was proposed that goal-directed behaviour in the general PIT may arise from the evaluation of the utility of the goal itself (Cartoni et al., 2013) and consequently, CSs evoke future outcome representations and the organism estimates the value of the actions that can be performed. If the outcome is not desirable (e.g. if it has been devaluated by satiation) the associated CS won't enhance instrumental seeking behaviours (Corbit, Janak, & Balleine, 2007), yet with extended instrumental training enhanced transfer and reduced devaluation effects should arise (Holland, 2004). Moreover, goal-directed behaviours happen in PIT procedures as a result of efficacy (the probability of reaching a goal) and context (the availability of certain rewards) evaluation. An action performed in situations with a high reward probability (e.g. in the presence of the appropriate CS^+) can be considered a goal-directed behaviour. Finally, goal-directed actions are actions executed in the right context (e.g. inhibited when a CS^- is present) whereas the opposite would be expected for S-R actions. In the present PIT protocol, an stronger response under CS^+ and a weaker response under CS^- would be consistent with enhanced goal-directed actions, which seems to be the case especially in males exposed to THC. This interpretation also matches the results obtained with the PCA procedure. GT and not ST is subjected to outcome devaluation and thus, ST animals can be seen to be more prone to develop S-R actions (Morrison, Bamkole, & Nicola, 2015). THC animals, especially males, appear to show both higher PIT and increased GT bias, while VEH animals express an intermediate PIT and an intermediate phenotype in the PCA. Remarkably, there were no differences in outcome devaluation and in the contingency degradation tests.

Regarding SUDs, it was recently proven that the strength of PIT correlates with increased CSA behaviour (Takahashi et al., 2019),although we did not see differences in CSA due to THC and neither did we detect a subgroup of males exposed to THC with an enhanced CSA. However, different animals underwent PIT and CSA, and thus there is no way to directly assess this correlation within subjects. Moreover, the present CSA protocol uses a CS linked to drug availability (a discriminative stimulus, DS) instead of a drug-paired CS (the implications for relapse on drug-seeking will be discussed below) and thus, some CS-driven effects that presumably intervene in the results presented elsewhere (Takahashi et al., 2019) may be lost. However, other research groups did find increases in CSA (at low doses) after ACE (Friedman et al., 2019). Remarkably, it was also proven that rats that show a higher PIT did not develop an enhancement of other prototypical SUD criteria (Takahashi et al., 2019). As such, PIT scores do not correlate with enhanced motivation (PR), resistance to punishment (foot-shocks) or persistence of cocaine-seeking (seeking in periods without drug availability), the most common result in PEACE studies of drug SA (Kononoff et al., 2018, Friedman et al., 2019). In the present CSA protocol, there were no differences in the BPs during PR sessions, resistance to punishment or in ALP during time-outs, although we found a difference in mean consumption during PR that will be discussed below.

2.2. 2CSRTT

No differences due to sex were found across baseline sessions, although VEH males showed increased impulsivity in the first test session and a higher overall mean PreR compared to VEH females taking all three sessions together. There is mixed evidence of sex differences in terms of impulsive action in the literature. Sex-differences may arise in response to stress and learning (Papaleo et al., 2012) and are subject to hormonal regulation (Bayless et al., 2012; Jentsch & Taylor, 2003), which is interesting due to the influence of ACE on the HPA axis. Human studies have also shown sex-specific differences, although this may be dependent on the task employed and due to some degree of hormonal influence (Colzato et al., 2010; Weafer & de Wit, 2014). However, in the present experiments the oestrus cycle was not checked (Burton & Fletcher, 2012) after testing, nor during training or in the baseline sessions, so the possibility that the hormonal status interferes with the PreR cannot be ruled out or confirmed. Using a similar 2CSRTT approach to that described in the present experiment, it was found that adult females made more PreR than adult males (Burton & Fletcher, 2012).

In the first test session there was a decrease in waiting-impulsivity (PreR) in ACE males but not females. Nonetheless, THC animals showed the opposite trend relative to the previous baseline session (proportional increase in PreR). An enhanced increase in waiting-impulsivity was evident during the first test session in females treated with THC, and both ACE groups showed a higher proportional increase in PreR during the second test relative to the VEH groups. No differences were evident in the third challenge in any of these measures and thus, the behaviour measured may be reflecting a state-dependent impulsive reaction rather than a stable impulsivity trait.

There are several documented effects of acute and chronic cannabis consumption by humans related to different forms of impulsivity (McDonald et al., 2003; Wrege et al., 2014) and significantly, there is ever more evidence of increased impulsivity even after long periods of abstinence (see introduction, section 2.3). Moreover, it is also common that no clear differences are found in some impulsivity-related tasks in humans, although distinct patterns of brain activity can still be registered in cannabis users during the execution of these tasks

(Eldreth et al., 2004; Jacobus et al., 2017; Tapert et al., 2007). In this sense, white matter alterations in chronic cannabis smokers have been associated with measures of impulsivity, including motor subscale scores correlated with left frontal FA values (Gruber et al., 2011). Loss of frontal white matter tracts, including reduced FA in the anterior CC, are also present in cocaine-dependent subjects and are associated with increased impulsivity.

We did find some alterations to FA that are more pronounced in the rostral sections and indeed, changes in myelinisation associated with PEACE have already been reported (see introduction section 2.2.1). Thus, as expected, there is evidence for increased impulsivity-related traits in different tasks in PEACE animal models (see introduction section 2.3.4), yet there is no information on the effect of adolescent cannabinoid exposure on impulsive action, another form of waiting-impulsivity. After ACE, adult animals show increased preference for large-risky rewards compared to small-certain ones (Jacobs-Brichford et al., 2019) and have a preference bias for small immediate reinforcers compared to large delayed ones (Johnson et al., 2019). These two studies did not explore sex-differences, and the results may appear inconsistent with the % PreR for male THC, or with the transient nature in the proportional increase in PreR. Notably, there is a non-complete overlap of the neural basis within each form of impulsivity (Voon, 2014), and cannabinoids exert a distinct influence of distinct types of impulsivity, and higher "non-planning" scores were recorded in chronic cannabis users and lower "motor impulsivity" scores (Churchwell et al., 2010; Silveri et al., 2011).

Since this impulsivity task was limited to two levers it is less exigent in terms of attentional or of working and short-term memory processes, although other emotional alterations might have a stronger impact (Wrege et al., 2014). Impulsivity shares common substrates with anhedonia and irritability, which may also be altered after adolescent cannabinoid exposure (Kononoff et al., 2018), and which interestingly, implies weaker tonic DA activity and weaker phasic mesolimbic DA responses in associative learning and reward anticipation (Zisner & Beauchaine, 2016). Notably, decreased DA reactivity is a potential effect of cannabis abuse, also linked to amotivational states in cannabis users (Bloomfield et al., 2016; Campbell, 1976; Volkow et al., 2014), and overlapping neurobiological mechanisms within the cortico-striato-thalamo-cortical networks can cause both apathy and impulse control disorders, which is of particular relevance in the light of the effects elicited by the treatment within this circuit seen in the MRI studies (Houeto et al., 2016).

Regarding the behavioural phenotype described through the other tasks (Lovic et al., 2011), ST are more prone to impulsive actions but not impulsive choice. This is consistent with the decrease in impulsive action in males receiving THC, and it should be borne in mind that the shift induced by THC in the PCA was more evident in males. Moreover, the lack of correlation between PIT and impulsivity (Sommer et al., 2017) might further support the interpretation of the PIT results within the prism of A-O/goal-directed behaviours. Using the 5-CSRTT, it was shown that impulsive animals exhibit a decreased DA D_2/D_3 binding in the NAc but not the dSTR (Dalley et al., 2007). Indeed, an imbalance in DA D_2/D_3 receptors is a common feature of different forms of impulsive/compulsive behaviours, including SUDs (Dalley, Everitt, & Robbins, 2011). Disentangling the role of the different NAc structures, it was shown that strong impulsivity (5-CSRTT) is associated with a stronger NAc Shell DA release and weaker release in the NAc Core (Diergaarde et al., 2009). These differences are interesting due to the DA related alterations that will be discussed below RNAseq section. However, even if weak DAergic activity in the vSTR might be associated with a smaller chance of reproducing impulsive actions, the RNAseq study does not provide information about other relevant regions of the circuit, including the NAc core. Moreover, this DAergic configuration might be relevant for the 5-CSRTT but potentially less influential in the 2-CSRTT. The Implications of the impulsivity data for SUDs will be addressed in the corresponding CSA section.

2.3. PCA

The PCA index slowly varies in the control groups across the autoshaping sessions, at the end revealing an intermediate phenotype in most of the VEH treated animals (70% of the males and 70% of the females), a proportion that was slightly higher than expected (Fitzpatrick & Morrow, 2016). The lack of differences in the control groups was expected, as the propensity to attribute incentive salience to food cues previously showed minimal sex differences and does not vary with the oestrous cycle (Pitchers et al., 2015). In THC treated groups, the relative abundance of the GT endophenotype increased, also without significant sex differences (60% in males THC and 50% in THC females).

The direct effects of PEACE in PCA have been evaluated (Schoch et al., 2018: see introduction section 2.3.3), reflecting the emergence of a mixed ST/GT phenotype in ACE animals compared to the controls that were biased towards ST in this PCA protocol. Thus, the increased GT seen in our animals is coherent with the direction of the change in this study. Moreover, the involvement of the eCBS in this task was recently addressed in other studies and using the CB_1 antagonist Rimonabant a dose-dependent decrease in cue-driven lever approaches was seen, preventing the acquisition of the conditioned response to the lever (CS^+) without affecting pellet retrieval or consumption (Bacharach et al., 2018). More recently, and in accordance to the initial

hypothesis, it was found that the CB_1 agonist CP-55,940 decreases ST in a dose-dependent manner and similarly, it appeared to increase GT (Gheidi et al., 2020). These experiments show a clear involvement of the eCBS in the PCA, although as opposite types of CB_1 ligands lead to similar increases in GT behaviour, the involvement of other systems, and the pharmacodynamics and pharmacokinetics of different cannabinoids could be explored in the future to potentially resolve this discrepancy.

The activity of DAergic and cholinergic signalling is well-documented, and it has opposing influences on the PCA (See Box 23). Remarkably CB_1 agonists and antagonists modulate the DA (García, et al., 2016) and cholinergic system (Domino, 1981; Gessa et al., 1998; Gifford et al., 1997; Tripathi et al., 1987), and in this sense they represent a possible source of variation in the present model through direct or indirect alterations of the relative prevalence of DA and cholinergic signalling in critical brain areas as a result of ACE (e.g. in the PFC, ACC, NAc and Amygdala). To enhance GT, cannabis might interfere with the DA signal since ST behaviour seems to be more DA-dependent (Flagel et al., 2011; Saunders & Robinson, 2012), although it may become D_1-independent with more extensive training (15 sessions: Clark et al., 2013).

The idea that STs are more vulnerable to addiction is based on several findings and different lines of evidence. For example, STs were more prone to increase drug-seeking, and they had a higher relapse vulnerability (Saunders & Robinson, 2010) and a biased preference for drugs over food (Tunstall & Kearns, 2015) relative to GTs. Nonetheless, these features may be strongly dependent on the interaction of these different cognitive styles within the particular set-up of the sessions in terms of cues, stimulus, drug access or patterns of self-administration (Pitchers et al., 2017a). In this sense, GTs do not differ from STs when shifted to intermittent drug self-administration regimes, with both endophenotypes expressing similar addiction-like behaviours (Kawa, Bentzley, & Robinson, 2016). More precisely, relapse vulnerability, a core feature originally predicted by this model (Kuhn et al., 2019), seems to be highly dependent on the different relapse "triggers" used (Pitchers, Sarter, & Robinson, 2018). In this regard, contextual cues and discriminative cues are more determinant for GTs (Pitchers et al., 2017b; Saunders et al., 2014). Thus, GT behaviour cannot simply be viewed as a resilient endophenotype towards addiction without considering the context and variables that will take place in the progression towards addiction, and the abstinence period.

2.4. HABIT TRAINING

The protocol employed produced the expected outcomes and after the short non-habit-forming training, all the control animals reduce their responses and subsequent sensory-specific satiety. Moreover, after extended habit-forming training, some animals fail to reduce their response to the same degree. No differences were found due to sex between the control animals and animals that received adolescent THC treatment did not show significant differences in the main indices. Indeed, only the female-THC group showed a higher response rate in the tests, maintaining an equivalent degree of devaluation.

Cannabinoids have a strong influence on S-R learning and memory processes mediated by the dSTR (Goodman & Packard, 2016). PEACE studies did not directly explore a shift towards habitual S-R behaviours in an intact reversal-learning task after ACE but not after adult exposure (Johnson et al., 2019). Contrary to what was expected, adolescents show reduced DA presynaptic activity in the dSTR which may slow down the formation of habits (Matthews et al., 2013), including ethanol-seeking habits (Serlin & Torregrossa, 2015). Moreover, preserved and functional CB_1 signalling, especially in certain areas and pathways, is necessary for the incorporation of S-R driven actions (Gremel et al., 2016; Hilario et al., 2007). Indeed, the ACE-mediated disruption of some maturational processes can impede the proper configuration of midbrain CB_1 signalling (See Introduction section 2.2.2), which means that ACE is not necessarily linked to enhanced S-R. This perspective does not contradict the increased S-R learning associated with adult cannabis exposure (Fernández-Cabrera et al., 2014; Nazzaro et al., 2012). Moreover, STs but not GTs are resistant to outcome devaluation and thus, the increased GT bias of THC rats is also in line with the results obtained here from two independent protocols (Morrison et al., 2015).

Importantly, in the light of recent research into the role of habit in SUDs in both clinical and preclinical settings (Hogarth, 2018; Hogarth et al., 2019), the lack of bias toward S-R seeking is necessarily linked to a SUD-resistant phenotype. Within the CSA results, there was no clear evidence that could account for rigid S-R drug-seeking in THC treated animals, nor a progression to compulsive drug use or increased incentive sensitization. However, other contexts and protocols of drug SA that could exploit goal-directed patterns of drug SA (Hogarth, 2020) might be interesting in the context of PEACE if these animals are truly biased towards A-O/goal-directed learning.

3. COCAINE SELF-ADMINISTRATION

Table 25. Cocaine self-administration main results

	Male VEH vs Male THC	Female VEH vs Female THC
Acquisition	NSD in cocaine acquisition	
Progressive Ratio	Increased infusion intake	NSD
Re-baseline	NSD	Increased reestablishment index
Punished seeking	NSD	
Extended Access	NSD*	
Cue-seeking relapse	NSD*	

NSD= No Significant Differences; * See specific discussion sections.

3.1. ACQUISITION

We found no sex-related differences in the initial acquisition sessions and although there was some evidence of (cannabis-naïve) females being more prone to acquire a CSA behaviour than males, no significant differences were detected in the present set-up and at the doses employed. CSA protocols with lower doses, shorter infusion periods, shorter sessions and with the inclusion of drug-free days seem to be more suitable to produce this effect (Hu et al., 2004; Lynch & Carroll, 1999).

Several lines of evidence suggest that the initial neurological impact of cocaine is indeed modified by ACE (See Introduction, section 2.4), which could also imply alterations to cocaine addiction-like behaviours. We studied cFos immunohistochemistry and we registered a potentiation of the expression of this factor in the motor cortex of ACE animals in response to an acute dose of cocaine, as well as dampened expression in male-THC rats yet enhanced expression in female-THC rats in the dorsomedial hypothalamic nucleus (see appendix E). However, ACE produced no differences in the acquisition of CSA. As reviewed previously, increases in CSA have been reported in females (Higuera-Matas et al., 2008) and adult animals after ACE when a low dose of cocaine (0.1mg/kg) is administered (Friedman et al., 2019). Moreover, dampened acquisition has also been registered when animals started the ACE at PND43 (Kononoff et al., 2018). Thus, differences, in the time of testing and in the dose of cocaine used, and also in the ACE regime used are among the most probable sources of differences between our results and those from previous PEACE studies.

Interestingly, it has been shown that impulsive animals (using the 5-CSRTT) displayed higher rates of intravenous CSA (Dalley et al., 2007). Female-THC rats showed an increase in impulsivity (percentage increase of PreR), although they did not show an enhanced cocaine acquisition or consumption in the CSA regime. The results obtained in the 2CSRTT were limited to the first two tests and thus, they did not reflect a stable trait. However, as noted before, enhanced CSA has been reported previously in ACE females (Higuera-Matas et al., 2008). Moreover, a positive correlation between PIT and increased CSA has also been reported (Takahashi et al., 2019), thus we would have expected to see an increased intake in THC animals and especially in males. In this regard, an important feature is the setting of the drug CS in the CSA sessions, which was used as a DS signalling the availability of the drug instead of pairing it with delivery. Thus, even a DS can be reliably used in self-administration and relapse protocols (Madangopal et al., 2019), in which we might expect a weaker influence of drug-paired cues.

3.2. MOTIVATION FOR CONSUMPTION

PR sessions remained stable across the sessions and thus, no significant session effect was registered in any of the measurements: ALP, Infusions, BP, or motivation index. Repeated testing on a PR schedule remained stable at low cocaine doses (0.38 and 3 mg/kg/inj) and increased with higher doses (0.75 after seven days of testing and 1.5 mg/kg/inj after 5 days) and short infusion times (5s: Liu et al., 2005). Thus, at the infusion rate employed (0.5 mg/kg/inj in 7s) a stable BP was expected, at least in control animals. Females may display higher BPs in PR schedules compared to males (Roberts et al.,1989), yet we did not see any such an effect in BP but a global effect in the infusions, suggesting a higher intake in female-VEH rats compared to male-VEH rats. Remarkably, ACE reversed this trend and male-THC rats exhibited a higher intake compared to male-VEH rats. Regarding PEACE, differences in PR sessions have not been reported (Kononoff et al., 2018; Friedman et al., 2019) and thus, this is probably the first time that ACE produces an increase in cocaine consumption under PR. Although, the lower doses (0.1 and 0.32 mg/kg) employed by Friedman and the long access (6h) phase employed before PR in the study of Kononoff, are two probable sources of this divergence Nonetheless, there was no treatment effect in the first session in any measurement and the absence of a different progression across sessions is in line with the previous studies.

3.3. RE-BOUND INDEX

We observed increased consumption during the re-baseline sessions after the PR sessions, with all groups increasing their mean intake more than 50% compared to last acquisition session. The PR session imposed a period of forced abstinence and/or limited access that seemed to impact on drug-intake. Compared to their male counterparts, female-VEH rats maintained their intake closer to the last acquisition session before the PR phase and notably, THC females showed a higher relative increase in intake than VEH females. This suggests that adolescent THC consumption can have an impact on the underlying process that occur following patterns of discontinued drug use, rendering these subjects more reactive to such drug-administration regimes. Discontinued drug use emulates the human context of drug use and abuse, where access to the drug is frequently discontinuous. As such, different activities and efforts are required to obtain the drug, and abstinence periods are frequent (whether self-imposed or forced). When such conditions are implemented in animal models they have proven to have an impact on cocaine sensitization and they produce changes in DA signalling (Calipari et al., 2013; Calipari et al., 2015). Thus, the results obtained highlight the possibility of exploring the effects of protocols that exploit this phenomenon, such as intermittent access procedures, in the context of the protracted effects of adolescent cannabinoid exposure. Nonetheless, there were no differences between groups in the mean consumption during re-baseline sessions. Thus, the magnitude of this effect does not overcome previous consumption but it can potentially provoke changes that modulate future changes in consumption under different contexts.

3.4. COMPULSIVITY

When tested in punished-seeking sessions, the groups showed no clear differences in compulsivity as all the groups decreased their consumption by 50-25% in terms of the mean consumption when the intake was compared to the FR1 sessions. The lack of sex-related differences has been documented previously, although females are more sensitive to changes in dose and will show more compulsion if instrumental action is rewarded with higher doses. Importantly, female compulsivity in this foot-shock punishment test is not attributable to changes in the oestrus cycle (Dattaet al., 2018).

Compulsivity was not altered by THC consumption, which in the light of the behavioural findings in impulsivity is worth mentioning. Higher levels of waiting-impulsivity in SRTTs should predict higher scores in the compulsivity test (Belin et al., 2008), yet THC groups didn't show a clear change in impulsivity as a trait (defined as a stable increase or decrease across the three test sessions). Hence, the lack of differences is somehow expected as changes exhibited by THC animals should be considered state-dependent impulsivity and they no longer predict concomitant changes in compulsivity. Similarly, the results obtained for the other behavioural measures did not predict changes in compulsivity.

3.5. EXTENDED ACCESS

All groups increased their intake during the extended access sessions compared to the mean number of infusions during the last acquisition and re-baseline sessions. In extended access conditions, while some sex differences may be expected, specifically increased CSA in females (Roth & Carroll, 2004), female and male controls behaved similarly during this phase. Again, differences in the present CSA protocol with others (dose, infusion times, manipulations previous to escalation sessions, and the use of CS and DS) could have obscured this predisposition.

Although no strong statistical effect was present across the sessions, the statistical trend observed for the THC animals in the last sessions pointed to increased overall consumption, visual analysis of the data indicates that this effect may be driven by the female-THC subjects. Extended access sessions are known to produce tolerance, a reduced drug effect that may lead to overconsumption to compensate for this after repeated use (Kawa et al., 2019). Tolerance involves a decreased in the ability of cocaine to increase extracellular DA overflow (Ferris et al., 2012), which may be accompanied by weaker inhibition of the DAT (Siciliano et al., 2018). Notably, this is an effect that has been already documented after ACE in the dSTR of females but not males (Alejandro Higuera-Matas et al., 2011). Moreover, this DA deficiency might be prompted by tolerance and it has also been linked to anhedonia, a commonly reported feature of ACE, and it may motivate drug-seeking and drug-taking behaviours (Koob & Volkow, 2016; Volkow, Koob, & McLellan, 2016). Regarding PEACE and the known changes induced by cannabis in the DA system (Bloomfield et al., 2016; Volkow et al.,

2014: see Introduction, section 2.2) it is not odd that the ACE could have had an impact on the DAergic processes related to cocaine tolerance.

3.6. SEEKING INCUBATION

Female-VEH rats showed a clearly exacerbated incubation of seeking compared to other groups, peaking around withdrawal day 30. The greater vulnerability of females to incubate and reinstate seeking by conditioned cues, and through drug-priming, has been documented previously (Kerstetter et al., 2008; Lynch & Carroll, 2000; Nicolas et al., 2019). Thus, the increased seeking in female-VEH rats is somehow expected, although interestingly, cocaine-seeking in the female-THC group was similar to that in the male groups.

No previous studies have explored cocaine craving incubation after ACE. After ACE, adult male rats did increase heroin seeking in a cue-induced drug-seeking (after forced abstinence) test and also using a stress-induced reinstatement (after extinction training) test (Tomasiewicz et al., 2012; Stopponi et al., 2014). However, we did not detect any significant difference between the male groups in the drug-seeking tests. ACE could have a different impact on different forms of relapse and reinstatement of drug-seeking, although in terms of the protocol and test conditions, the present study did not use a classical cue-induced reinstatement protocol and a drug-DS was employed, in contrast to the drug-CS regime used previously (Tomasiewicz et al., 2012). Nonetheless, another ACE study showed that adult mice with an adolescent exposition to WIN55,212-2 were less susceptible to the anxiogenic effects of cocaine abstinence (Aguilar et al., 2017). Although this latter study did not use self-administration methods, it suggest a potential mechanism for our effects that needs to be further explored, especially concerning its potential sex-specific nature.

4. RNA-seq RESULTS IN THE CONTEXT OF SUBSTANCE USE DISORDERS

The results obtained in the RNA-seq study both corroborate previous findings and expand our understanding of the epigenetic effects cannabinoid (see Introduction, section 2.2.3), this time with an accent on the protracted effects of adolescent THC exposure on the NAc shell transcriptome. Moreover, for the first time this study explores the sex-dependent differential effects of ACE. Although we are aware that changes induced by ACE are not limited to these features, we will centre almost exclusively on reward processes, response to drugs and SUDs, the focus of the present work. We will consider evidence of DAergic alterations due to its intimate relationship with these processes (see boxes 7 and 8). The RNA-seq results, and the DEGs and GO terms identified (see Results, section 5, and Figures 20, 21, and 22) have been scrutinized and regrouped into 3 sections that are each relevant to SUDs: (4.1) Transcription and translation, (4.2) Glutamate, GABA and other ion channels, and (4.3) hormones and neuropeptides. To facilitate a rapid overview of the dimension and direction of the DEG changes considered, the gene names are accompanied by upward or downward-facing triangles, blue triangles in the case of Male-VEH vs Male-THC ▼▲, orange for Female-VEH vs Female-THC ▼▲, and green for Male-THC vs Female-THC ▼▲.

4.1. TRANSCRIPTION & TRANSLATION

4.1.1. CANNABINOID TREATMENT ALTERS D1 AND D2 SIGNALING PATHWAYS IN A SEX DEPENDENT MANNER

The transcriptional factor Zinc Finger Homeobox 3, ***Zfhx3*** (▼▲▲), is one of the DEGs that appeared in several comparisons, and the associated GO terms implicate this gene in neurogenesis and ion binding. Interestingly, *Zfhx3* is a feature of a subtype of D_2 DA neurons of the adult midbrain (Poulin et al., 2014) and thus, the differences observed may be a proxy for the relative amount of a certain type of DAergic neurons in the NAc Shell. Moreover, *Zfhx3* was down-regulated in subjects with alcohol use disorder (Wang et al., 2016), a pathology that for type 1 alcoholics at least entails a loss of D_2/D_3 receptors and DAT in the NAc (Tupala et al., 2001).

Conversely, male-THC rats showed upregulation of the Nuclear receptor subfamily 4 group A member 2, *Nr4a2* (▲), a member of the steroid nuclear hormone receptor superfamily that is associated with relevant GO terms like behaviour and neurogenesis. *Nr4a2* and the Nur subfamily of nuclear receptors has also been related to DA cell proliferation and survival, and differential D_1/D_2 activity (Castillo et al., 1998; Sacchetti et al.,

2006). Interestingly, *Nr4a2* is also modulated by neuronal firing and DA signalling, and a loss of D_2 signalling induces *Nr4a2* upregulation (Tseng et al., 2000). Two other members of the Nurr family were found to be DEGs in the NAc Shell, *Nr4a1* (▼, Nurr77) and *Nr4a3* (▼, Nor-1), although the effect was found exclusively in the comparison between THC treated animals. Changes in this comparison are usually variations in gene expression produced by THC in opposite directions in each sex that are not sufficiently pronounced to result in statically significant differences between the VEH and THC animals of each sex. Both *Nr4a1* and *Nr4a3* are regulated by DA receptor activity, with agonistic D_2 activity inhibiting *Nr4a1* mRNA expression and antagonistic D_2 activity increasing *Nr4a3* mRNA expression (Campos-Melo et al., 2013; Maheux et al., 2005). Thus, a lower D_2 activity in male-THC than in female-THC rats might produce this pattern of expression. Further possible evidence of a weakening of the D_2 signal in male-THC rats is the downregulation of the *Hipk2* enzyme (▼, Homeodomain-interacting protein kinase 2). *Hipk2* interacts with homeodomain transcription factors and is involved in TGFβ-mediated survival of midbrain DA neurons (in mice). Notably, deletion of *Hipk2* produced a series of abnormalities in DA neurons but preserved D_1 functionality (Zhang et al., 2007).

Within the Nac shell the relative weight of D_1/D2 signalling and expression influence motivated and drug related behaviours, and is affected by drug use and abuse. For example, Nac shell loss of D1 signalling delays the induction of sensitized locomotion, while D_2 signalling loss increases the rate of behavioural sensitization evoked by repeated administration of methamphetamine (Kai et al., 2015), which is interesting considering the frequently reported effects of ACE in stimulant sensitization (see introduction section 2.4.2). Noteworthy, several studies forced on the protracted effects of ACE reported changes in DAergic receptor expression, that also showed a sex-dependent nature but with a notable variation across brain areas (Higuera-Matas et al., 2011; Zamberletti et al., 2012).

Remarkable there were no DEG corresponding to D_2 receptors and thus, these effects could be due to a loss of D_2 functionality rather than a downregulation or change in the expression and/or functionality of D_1 receptors. In this regard there was an interactive effect on the *Drd1* (▼) that encodes for the D_1 protein. This effect was restricted to the comparison between THC animals and when the raw expression data was explored, this DE was due to a more marked loss of D_1 receptors in female-THC rats and just a slight gain D_1 in male-THC rats. This could indicate a sex dependent change in the differential predominance of D_1 and D_2 mediated signalling after adolescent THC. However, we don't know if this effect arises in areas other than the NAc Shell. Previous data regarding adolescent cannabinoid exposure showed an enhanced D_1 receptor density in NAc Shell of males but not females (Alejandro Higuera-Matas et al., 2011), whereas when the whole NAc was analysed, D_1 receptor density was enhanced in females but not males after cannabinoid adolescent exposure, and both sexes showed increase in D_2 receptor density (Zamberletti et al., 2012). A more detailed description of this anatomical differences should be addressed in future studies but nonetheless, these differences in DA signalling can potentially modulate reward-related and aversion processes, and ultimately the progression towards SUDs.

4.1.2. CREB-RELATED ALTERATIONS

Significantly, *Hipk2* (▼) can phosphorylate cAMP-response element binding protein (CREB), which plays a key role in transcription related to cell metabolism, proliferation and survival (Mayr & Montminy, 2001). In addition, the CREB-regulated transcription coactivator 1, (▼, *CRTC1*) is decisive for the efficient induction of CREB target genes and it was downregulated by THC in males. CREB alters the transcription of a wide variety of genes, some of them already discussed, like *Nr4a2* (▲) and *Nr4a1* (▼) (Parra-Damas et al., 2017), and others that will be reviewed later in the context of hormone-related alterations, such as: *Gal* (▲▲), *Cck* (▲▼) and *Cartpt* (▼▼) (Picciotto, 2008; Yu et al., 2017); many solute carriers, potassium and calcium channels; and also genes involved in synapse activity like synaptogamin 17 (▲▼, *Syt17*), among others. Within the brain, CREB proteins have been associated with learning processes, such as LTP and SUD (McPherson & Lawrence, 2007). Relevant to the experiments described here, modulation of striatal CREB signalling determines cocaine intake (Hollander et al., 2010) and enhanced CREB activity in the NAc shell can increase motivation for cocaine during self-administration and after cocaine withdrawal (Larson et al., 2011). Notably, activation of D_1 receptors mediate CREB phosphorylation and together with the net result in DA signalling other CREB-related alterations might contribute to shaping the response to different drugs of abuse after ACE.

4.2. GLUTAMATE, GABA & OTHER ION CHANNELS

4.2.1. GLUTAMATERGIC ALTERATIONS

The glutamatergic synapse GO term was significantly over-represented in the DEGs between the male groups, nonetheless these genes and other DEGs related to glutamatergic signalling were also found in the other comparisons. The Vesicular glutamate transporter 2 or VGLUT2 (▲▲▲*Slc17a6*) mediates glutamate uptake

into synaptic vesicles at presynaptic nerve terminals of excitatory neural cells and it was upregulated in both sexes as a result of THC. The effect on this transcript seems to be stronger in female-THC rats than in male-THC rats. In midbrain DA neurons VGLUT2 facilitates the co-release of glutamate in the NAc (Papathanou et al., 2018), a feature shared by the majority of DA neurons projecting to the NAc Shell from the VTA (Mingote et al., 2019). The implications of these neurons in SUDs and DAergic signalling in the NAc was reviewed recently (Buck et al., 2020) and remarkably, VGLUT2 seems to be required in DA neurons for psychostimulant-induced behavioural activation (Birgner et al., 2010), as well as for other basic forms of plasticity and learning. Shell-projecting DA-GLU activity can modulate and enhance extinction learning, both of rewarding and aversive outcomes, as well as behavioural switching, and it may diminish latent inhibition (Mingote et al., 2019), psychological traits that can influence the evolution and expression of SUDs (Buck et al., 2020). From this perspective, VGLUT2 may participate in PEACE related to extinction, behavioural flexibility and the ability to switch tasks (see Introduction, section 2.3), while also introducing potential bias into S-R learning processes and the expression of goal-directed actions. Further confirmation and manipulation of this gene in future PEACE studies could be an interesting pathway to explore.

THC treatment also increases of *Calb1* (▲▲) expression in both sexes. The most well-known role of the CALB1 protein in neurons is to buffer Ca^{+} entry upon stimulation of glutamate receptors, yet it can also affect DA transmission in distinct ways across different brain areas. For example, *Calb1* knock-down in DA neurons elevates DA release in the vSTR but not the dSTR, and it also enhances DA uptake and attenuates cocaine's inhibitory effect on DAT (Brimblecombe et al., 2019). Thus the NAc Shell upregulation of *Calb1* could influence DA release, and it may interact with the acute response to cocaine and modulate cocaine tolerance (Siciliano et al., 2018). Whether this is a global effect or if it is restricted to one type of DA projection to the NAc Shell cannot be clearly inferred from the existing data. Notwithstanding, Calb1 is also a feature of D_2 but not D1 midbrain DA neuron subtypes(Poulin et al., 2014), thus Calb1 upregulation could also be related to the already commented alterations in D_1 and D_2 relative expression and function. However, CALB1 can prevent drug-induced DA neuronal death by lowering intracellular calcium (possibly by inhibiting caspase and calpain activity: Choi et al., 2008) and in this regard, CALB1 elevation may be an adaptation triggered by enhanced GLU signalling as a result of more DA-GLU co-release by neuronal projections.

Among the other glutamatergic alterations, the excitatory amino acid transporter 2 (▼▲, *Slc1a2*) that encodes SLC1A2, the principal glial transporter that clears glutamate from the synaptic cleft, was downregulated exclusively in male-THC rats. Interestingly, many drugs of abuse (cocaine, amphetamines nicotine, opioids, ethanol and cannabinoids) alter the expression of this solute carrier, and pharmacological interventions are being developed to restore *Slc1a2* expression (with N-acetylcysteine or ceftriaxone) in SUDs (Roberts-Wolfe & Kalivas, 2015).

4.2.2. CHANGES IN MALE NAC SHELL ION CHANNEL EXPRESSION PROFILE

Changes in the expression profile of different elements of ion channels found in male-THC rats could indicate particular changes in neural excitability. In this regard, the downregulation of the Voltage-gated potassium channel subunit beta-2 (▼, *KCNAB2*) that resets the resting potential in neurons might delay the counterflow of potassium (K^{+}) ions and may affect the onset of new action potentials. From a behavioural and cognitive perspective, this change may have consequences on motivated behaviours (O'Donovan et al., 2019). Moreover, KCNAB2 depletion was also reported previously in the VTA after chronic morphine exposure (Mazei-Robison et al., 2011). In male-THC rats there was also a downregulation of the ATP-sensitive inward rectifier potassium channel 10 (▼, *Kcnj10*) that participates in inward K^{+} transport from outside the cell, rectifying the potential difference. *Kcnj10* has been implicated in SUDs, and SNPs of this gene are linked to ethanol preference (Zou et al., 2009).

The electrophysiological profile of some accumbens neuronal types in male-THC rats may also be determined by a differential expression of other ion channels. In this regard, the Sodium channel protein type 8 subunit alpha (▼, *Scn8a*) is also downregulated in male-THC rats. Since transport of positively charged sodium ions (Na+) into cells is a key element for the onset of the action potential, a change in potassium outflow may blunt the capacity to initiate an action potential. Additionally, there was evidence of changes in chloride transport in the NAc Shell cell of male-THC rats. The *Slc12a5* (▼) encodes the Potassium-chloride transporter member 5 (KCC2) protein, an extruder of intracellular chloride (Cl^{-}) in mature neurons, and it was downregulated by THC in males. Besides contributing to enhanced excitability, KCC2 maintains low intracellular Cl^{-} concentrations and its downregulation has been associated with altered neuronal migration and formation, and the maturation of glutamatergic and GABAergic synaptic connections (Medina et al., 2014). Electrophysiological approaches and protein expression quantification are needed to confirm these changes and to explore the resulting neuronal response profile after ACE. Remarkably, Slc12a5 has been implicated in MDMA seeking and relapse (Orejarena, 2010).

4.2.3. GLUTAMATERGIC AND GABAERGIC CHANGES IN THE FEMALE NAC SHELL

Other changes in glutamatergic and GABAergic activity exclusively affect females. The Glutamate Receptor Ionotropic NMDA 2D (▲, *Grin2d*) was upregulated by THC and while downregulation of this gene has been observed in human cocaine addicts it is upregulated in alcoholics (Enoch et al., 2014). This gene is associated to the Nicotine addiction KEEG Ontology, as well as alcoholism, amphetamine and cocaine addiction (K05212). The expression of *Grin2d* is positively modulated by oestrogen receptor activity in the hypothalamus (Ikeda et al., 2010) and thus, a hormonal interaction may be behind this effect. *Grin2d* overexpression can enhance Ca^{2+} entry and interestingly, there were many interactive differences in calcium signalling that could be related to differences in glutamatergic and GABAergic activity. The composition of GABA receptors also has a different expression profile after ACE in males and females. Between the GABAergic genes exclusively altered in females, the GABA(A) Subunit Epsilon (▲, *Gabre*) was upregulated by THC. Remarkably, the expression of this gene is also sensitive to exposure to drugs of abuse and *Gabre* was downregulated in the hippocampus three weeks after an a single-dose of MDMA (Petschner et al., 2013). Notably, *Gabre,* and Gabrq were upregulated in the NAc shell of female rats with a genetic predisposition to alcohol consumption (Spence et al., 2018), and like *Grin2d*, *Gabre* expression is also positively regulated by oestrogens (Noriega et al., 2010; Spence et al., 2018). These two examples once again raise the possible interplay of cannabinoids with hormonal activity. Other changes in glutamatergic elements and the linkage of these with oestradiol levels are already known in females after ACE (Rubino et al., 2015). Significantly, the main changes in hormone activity will be addressed in the next section below.

4.3. HORMONES AND NEUROPEPTIDES

4.3.1. SEXUAL DIMORPHISM IN THE CKK EXPRESSION

THC produced a sex-dependent change in the expression of *Cck* ▲▼. Cck signalling pathways have been related to food intake but also with reward and anxiety and even panic (Bradwejn & Vasar, 1995; Rotzinger & Vaccarino, 2003). Moreover, Cck signalling have been studied in the context of drug-related behaviours such as reinstatement and reactivation of morphine induce CPP (Lu et al., 2001; Lu et al., 2002), and to cocaine behavioural sensitization, which is accompanied by increasing levels of Cck in the NAc Shell (Beinfeld, Connolly, & Pierce, 2002). CCK is the most abundant neuropeptide in the central nervous system and influence D_2 medium spiny neurons activity mediating emotional responses like stress susceptibility (Karson et al., 2008; Shen et al., 2019). Moreover, the activity of CCK-8S (the predominant form of CCK in the CNS and NAc) modulates the sensitivity of D_2 type receptors on mesoaccumbens neurons in response to DA inputs (Kelland et al., 1991; Li et al., 1994).

The Cck receptors (CCK1/A and CCK2/B) are differentially expressed in the NAc, being CCK1 more abundant in GABAergic afferent terminals of the shell section, and CCK2 highly expressed in the axons NAc core (Kombianet al., 2004; Mercer & Beart, 1997; Mercer et al., 2000; Noble & Roques, 1999). Shell and core CCK-related activity shows opposing outcomes but in both regions, CCK receptors are thought to modulate interact with the DAergic signaling (Ballaz, 2017). Regarding drug interactions, NAc Shell CCK1 activation seems to lead to DA agonist like activities and facilitates psychostimulant cross-sensitization (Wunderlich et al., 2004). Thus, it could be one of mechanism behind cross-sensitization effects observed in PEACE studies (see introduction 2.4.2).

4.3.2. ALTERED REGULATION OF DOPAMINE IN FEMALES BY HORMONAL NEUROPEPTIDES

GO terms related to hormone activity was enriched in the female subset of DEGs, and together with other neuropeptides they seem to influence DAergic activity in the NAc Shell. For example, Thyrotropin-releasing hormone (▲▲, *Trh*) participates in energy metabolism and hormonal activity but interestingly, its activity can also interfere with DA signalling. TRH directly injected into the NAc can enhance DA release and thus, a heightened level of TRH mRNA in the NAc Shell of THC females could contribute to this effect (Puga et al., 2016). Another peptide that may contributed to a differential DA modulation is angiotensinogen (▲▲, *Agt*), the precursor protein of angiotensin I that can be further converted to the angiotensin II (Agt II) peptide. The main function of Agt II is considered to be in the regulation of blood pressure, which it increases, as well as in the body balance of salts and fluids. Nonetheless, the brain renin-angiotensin system (RAS) participates in other activities like stress response and the processing of sensory information (Raghavendra et al., 1999). Central Agt II manipulation, both genetic and pharmacologically, alters voluntary alcohol consumption and specifically, reduced Agt II and consequently, dampened activation of the Ang II type 1 (AT_1-R) and type 2 (AT_2-R) receptors, are linked to less alcohol consumption and lower levels of DA in the VTA (Maul et al., 2005). Methamphetamine use leads to a profound loss of DAergic tone, which is accompanied by AT_1-R overexpression, and the blockade of this receptor can attenuate methamphetamine-triggered hyperlocomotion in mice (Jiang et al., 2018). Indeed,

increased expression of AT_1-R in the NAc was paralleled by enhanced D_2 expression after a haloperidol treatment, with no effect on D_1 expression (Jenkins et al., 1997). In the same study, regions without DA expression didn't show changes in ATRs. Thus, the elevation in Agt RNA could also indicate enhanced DA release.

We also found a downregulation of the Cocaine- and amphetamine-regulated transcript Prepropeptide (▼▼, *Cartpt*), which is proteolytically processed to generate other peptides, such as CART. Interestingly, CART can act as an endogenous psychostimulant (Kuhar et al., 2002) with known roles in reward and stress responses (Zhang et al., 2012), and in the regulation of appetite and homeostasis (reviewed by Lau & Herzog, 2014). Furthermore, the involvement of CART in SUDs is not only limited to cocaine reward (Yu et al., 2017) but also, it is involved in opioid use (Bakhtazad et al., 2016) and other relevant features for SUDs such as depression (Dandekar et al., 2009). CART injection into the VTA can reduce cocaine-seeking behaviour, while injection into the NAc inhibits the behavioural effects of cocaine (Yu et al., 2017). Mechanistically, CART seems to exert a neuromodulatory role in the NAc, attenuating DA release (Rakovska et al., 2017) possibly by inhibiting Ca^{2+} influx (Yu et al., 2017). Thus, downregulation of this peptide may be associated with increased DA activity in the NAc Shell of female-THC rats.

Lastly, female-THC rats also upregulated the neuropeptide *Galanin* (▲▲) that is involved in pathological food consumption and addiction (Gosnell et al., 1986a; 1986b; Sandi et al., 1988). Galanin receptors activity modulates drug intake, drug-induced place preference and reinstatement of different drugs. The rich and complex interactions of galanin and drug use were recently brought together in a clear review (Genders et al., 2020). In terms of DA activity, a microdialysis study revealed that galanin may have inhibitory effects on DA release in the NAc (Rada et al., 1998). In this sense it was also shown that overabundance of galanin decreased sensitivity to the behavioural effects of amphetamine, in part mediated by NAc Shell DA release (Clarke et al., 1988). Finally, galanin receptor agonists also reduced reinstatement of cocaine-seeking (Ogbonmwan et al., 2015) and cocaine-conditioned place preference (Narasimhaiah et al., 2009). Thus, the modulation of galanin production may also play a relevant role in the response to stimulants after ACE in females.

CONCLUSIONS

1. There is a causal relationship between exposure to THC during adolescence and several alterations in brain structure.

    i. In adult animals exposed to THC during adolescence there are volumetric reductions in subcortical structures, including the lateral ventricles and Dorsal Striatum and Globus Pallidus.
    ii. In adult animals exposed to THC during adolescence there are microstructural alterations to subcortical structures, including the Septal Nuclei, Thalamus, Striatum and Globus Pallidus
    iii. In adult animals exposed to THC during adolescence microstructural alterations are associated with decreased myelinisation in rostral sections of the white matter tracts.
    iv. In adult animals exposed to THC during adolescence the cortical metabolism of choline compounds is altered.

2. There is a causal relationship between exposure to THC during adolescence and a broad set of psychological alterations that affect reward processing and impulsivity

    i. Adult males exposed to THC during adolescence increase instrumental actions in a Pavlovian to instrumental transfer protocol under the influence of a reward-predictive stimulus.
    ii. n adult animals exposed to THC during adolescence, there are sex-specific state-dependent effects on motor impulsivity, with females exposed to THC more reactive to unexpected delays and males exposed to THC less prone to perform premature actions.
    iii. Adolescent THC exposure increases goal-tracking behaviours in a Pavlovian-conditioned approach task.
    iv. Protracted effects of adolescent cannabis exposure do not increase stimulus-response learning in a habit-forming instrumental protocol.

3. There is a causal relationship between exposure to THC during adolescence and sex-specific alterations in cocaine addiction-like behaviours.

4. Adolescent exposure to THC has a protracted effect on the transcriptome of the Nucleus Accumbens Shell in a marked sexually dimorphic fashion.

APPENDICES

APPENDIX A. EVOLUTION OF BODY WEIGHT

The weight of the animals studied in experiments 1 to 5 was recorded and those used for cFos immunohistochemistry. As expected, females had a lower body weight than males at the beginning of the treatment. After the chronic administration, THC-exposed rats (irrespective of the sex) had a marginally lower body weight than the control rats that received the vehicle alone (See Table 26). The increase in body weight was also lower during and after the treatment in females relative to the males, and for animals exposed to THC compared to controls that received the vehicle alone (See Figure 24).

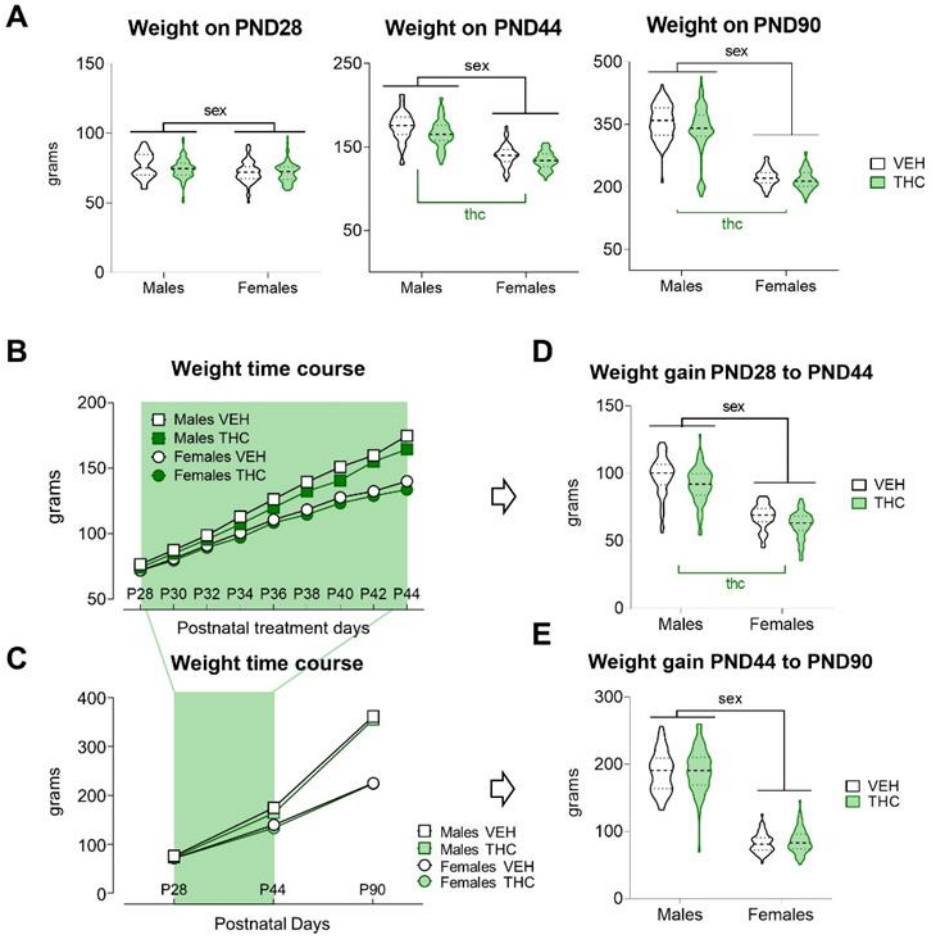


Figure 24. Evolution of body weight. Green lines and "THC" represent a significant difference due to the Adolescent Treatment factor. Black lines and "sex" represent significant difference within the levels of the Sex factor. **A)** Body weight at three different time points. The expected sex differences were only evident on the first treatment day (PND28: $F_{1,297}$=14.686; p<0.000; η_p^2=0.04) indicating no prior weight bias due to the factor Adolescent Treatment. Nonetheless, the mean body weight was lower in the THC groups at the end of the treatment (PND44: $F_{1,299}$=20.162; p<0.000; η_p^2=0.06) and in adulthood (PND90: $F_{1,341}$=14.686; p=0.004; η_p^2=0.02). **B)** Time course evolution of body weight with chronic treatment and **C)**, time course of the evolution of body weight at the three experimental points selected. **D)** The net body weight gain during treatment (Body Weight at PND44 – at Body Weight PND28) and **E)** the net body weight gain during the washout period (Body weight on PND90 – Body weight on PND44). The body weight gain during the THC treatment was slightly lower in the groups of THC-treated animals ($F_{1,296}$=22.585; p<0.000; η_p^2=0.07). However, a significant Sex effect (male>female) was only found in the weight gain during the washout period ($F_{1,294}$=362.205; p<0.000; η_p^2=0.51).

Table 26. Evolution of body weight.

MEASSURE	PHASE		p	Effect	Statistic value	*df*1	*df*e	Effect size	1-β	MALE VEH			MALE THC			FEMALE VEH			FEMALE THC		
										MEAN	SEM	N	MEAN	SEM	N	MEAN	SEM	N	MEAN	SEM	N
Weight	PND28	**	0.000	SEX	F= 14.686	1	297	0.05	0.97	76.69	8.855	76	74.28	7.306	81	72.06	7.744	71	71.96	7.45	73
	PND44	**	0.000	SEX	F= 307.697	1	299	0.51	1	174.9	17.46	79	166.0	16.66	80	140.2	13.20	71	133.8	10.47	73
		**	0.000	TMT	F= 20.162	1	299	0.06	0.99												
	PND90	**	0.000	SEX	F= 767.753	1	341	0.69	1	355.9	41.0	91	336.1	61.2	102	222.7	20.5	70	216.1	23.4	82
		**	0.004	TMT	F= 8.344	1	341	0.02	0.82												
Weight gain	PND28-PND44	**	0.000	SEX	F= 451.228	1	296	0.60	1	98.27	14.52	76	91.39	13.39	80	68.12	9.068	71	61.82	9.89	73
		**	0.000	TMT	F= 22.585	1	296	0.07	1												
	PND44-PND90	**	0.000	SEX	F= 362.205	1	294	0.51	1	186.8	33.58	79	191.0	32.13	78	82.70	14.14	70	85.98	18.25	71

APPENDIX B. COCAINE SELF-ADMINISTRATION SUPPLEMENTARY GRAPHS

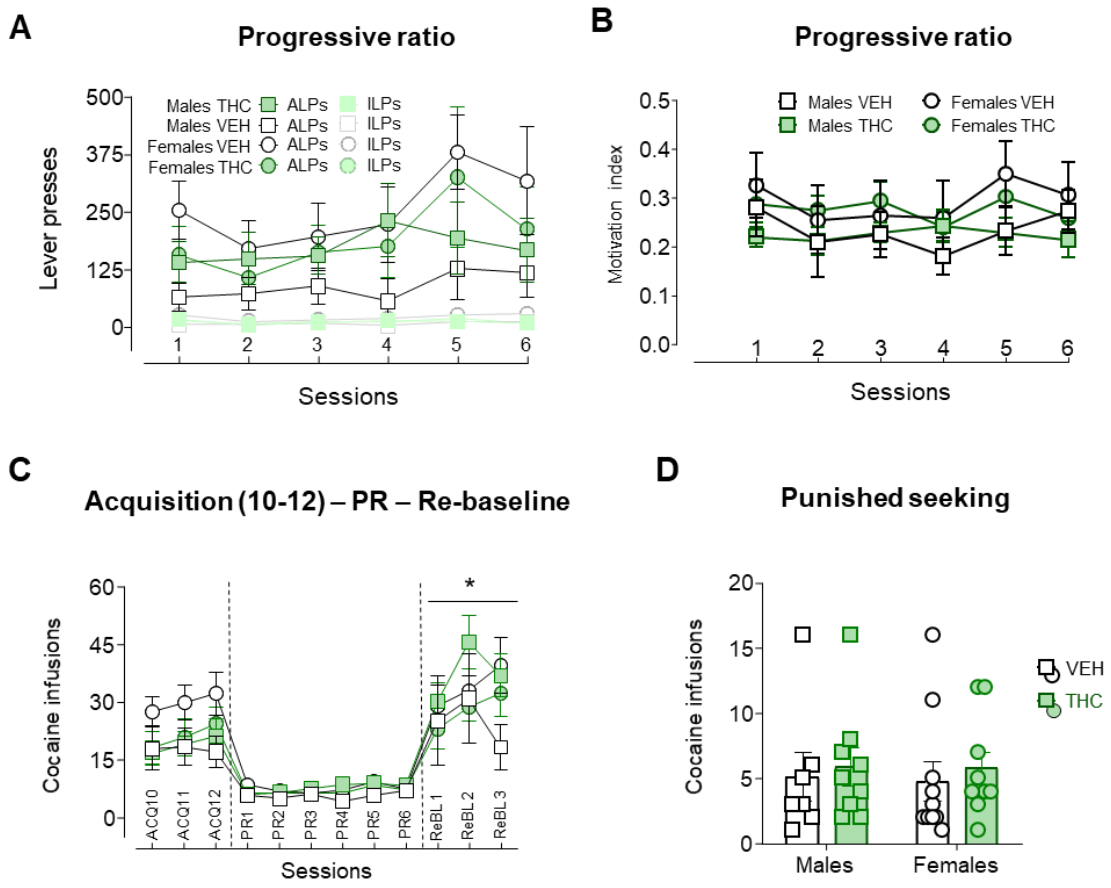


Figure 25: Cocaine self-administration (III). A) Lever presses across the six progressive ratio sessions. There was an effect of the sessions ($F_{5,145}$=3.857; p= 0.003; ηp^2=0.12), but no significant Treatment or Sex effects were detected. B) Motivation index across the progressive ratio sessions remained stable across sessions in all groups without relevant group differences. C) Cocaine infusions form the last three acquisition sessions to re-baseline sessions. The intake incremented during re-baseline sessions ($F_{2,62}$=4.835; p=0.011; ηp^2=0.13) without differences related to Sex or Treatment. D) Cocaine infusions obtained during the punishedseeeking session. No significant Treatment or Sex effects were detected.

APPENDIX C. DIFFERENTIALLY EXPRESSED GENES

MALES

Table 27 Differentially expressed genes in males

Enseml ID	Name	Symbol	log2_FC	q_value
ENSRNOG00000016275	transthyretin	Ttr	2.953	0.014
ENSRNOG00000010188	SATB homeobox 2	Satb2	2.261	0.014
ENSRNOG00000008697	cellular communication network factor 3 - nephroblastoma overexpressed	Nov	1.909	0.014
ENSRNOG00000033984	interferon lambda receptor 1	Ifnlr1	1.834	0.014
ENSRNOG00000037687	R-spondin 2	Rspo2	1.665	0.014
ENSRNOG00000027271	RGD1359290	RGD1359290	1.212	0.014
ENSRNOG00000055608	LOC102548695	LOC102548695	1.161	0.014
ENSRNOG00000029262	RGD1560017	RGD1560017	1.091	0.014
ENSRNOG00000019321	cholecystokinin	Cck	1.014	0.014
ENSRNOG00000002911	albumin	Alb	1.005	0.014
ENSRNOG00000016038	O-6-methylguanine-DNA methyltransferase	Mgmt	0.995	0.024
ENSRNOG00000017459	complement C1q like 3	C1ql3	0.945	0.014
ENSRNOG00000005185	neurexophilin 3	Nxph3	0.935	0.014
ENSRNOG00000005600	nuclear receptor subfamily 4 group A member 2	Nr4a2	0.849	0.014
ENSRNOG00000033517	LOC100360791	LOC100360791	0.791	0.014
ENSRNOG00000021745	basic helix-loop-helix family member e22	Bhlhe22	0.688	0.042
ENSRNOG00000007456	calbindin 1	Calb1	0.639	0.014
ENSRNOG00000032825	ribosomal protein L30	Rpl30	0.638	0.014
ENSRNOG00000017136	synaptotagmin 17	Syt17	0.622	0.024
ENSRNOG00000017328	phosphotriesterase related	Pter	0.592	0.024
ENSRNOG00000016147	solute carrier family 17 member 6	Slc17a6	0.567	0.038
ENSRNOG00000012841	ALG11 alpha-1,2-mannosyltransferase	Alg11	0.506	0.014
ENSRNOG00000007907	transmembrane protein 178A	Tmem178a	0.493	0.038
ENSRNOG00000017843	RNA polymerase III subunit K	Polr3k	0.457	0.014
ENSRNOG00000009968	ERCC excision repair 8, CSA ubiquitin ligase complex subunit	Ercc8	0.430	0.038
ENSRNOG00000016343	dickkopf WNT signaling pathway inhibitor 3	Dkk3	0.417	0.014
ENSRNOG00000012684	biogenesis of lysosomal organelles complex 1 subunit 2	Bloc1s2	0.400	0.042
ENSRNOG00000018111	solute carrier family 12 member 5	Slc12a5	-0.366	0.042
ENSRNOG00000011460	ARFGEF family member 3	Arfgef3	-0.377	0.032
ENSRNOG00000005309	sodium voltage-gated channel alpha subunit 8	Scn8a	-0.379	0.048
ENSRNOG00000019476	ArfGAP with GTPase domain, ankyrin repeat and PH domain 1	Agap1	-0.383	0.042
ENSRNOG00000013140	PDZ domain containing 2	Pdzd2	-0.384	0.048
ENSRNOG00000010302	RAS like family 10 member B	Rasl10b	-0.387	0.042
ENSRNOG00000007957	TSPO associated protein 1	Tspoap1	-0.387	0.048
ENSRNOG00000011550	potassium voltage-gated channel subfamily A regulatory beta subunit 2	Kcnab2	-0.393	0.042
ENSRNOG00000018416	tau tubulin kinase 1	Ttbk1	-0.394	0.014
ENSRNOG00000029510	plexin B1	Plxnb1	-0.398	0.038
ENSRNOG00000013581	exostosin like glycosyltransferase 3	Extl3	-0.411	0.038
ENSRNOG00000002863	calcium voltage-gated channel subunit alpha1 E	Cacna1e	-0.412	0.024
ENSRNOG00000027513	regulator of telomere elongation helicase 1	Rtel1	-0.417	0.038
ENSRNOG00000004310	CASK interacting protein 2	Caskin2	-0.419	0.042
ENSRNOG00000025594	scratch family transcriptional repressor 1	Scrt1	-0.420	0.048
ENSRNOG00000052129	NACHT and WD repeat domain containing 1	Nwd1	-0.421	0.032
ENSRNOG00000018012	TUB like protein 4	Tulp4	-0.422	0.014

ENSRNOG00000004956	jade family PHD finger 2	Jade2	-0.429	0.042
ENSRNOG00000001254	collagen type VI alpha 2 chain	Col6a2	-0.432	0.038
ENSRNOG00000007034	homeodomain interacting protein kinase 2	Hipk2	-0.437	0.014
ENSRNOG00000052687	multiple EGF like domains 8	Megf8	-0.438	0.014
ENSRNOG00000009019	solute carrier family 6 member 6	Slc6a6	-0.438	0.032
ENSRNOG00000022421	CREB regulated transcription coactivator 1	Crtc1	-0.448	0.024
ENSRNOG00000007705	potassium voltage-gated channel subfamily J member 10	Kcnj10	-0.448	0.038
ENSRNOG00000060410	protocadherin 1	Pcdh1	-0.457	0.014
ENSRNOG00000010031	vitronectin	Vtn	-0.466	0.038
ENSRNOG00000014928	amyloid beta precursor protein binding family A member 1	Apba1	-0.468	0.014
ENSRNOG00000056817	mucin 6, oligomeric mucus/gel-forming	Muc6	-0.478	0.014
ENSRNOG00000057569	AHNAK nucleoprotein	Ahnak	-0.480	0.024
ENSRNOG00000006911	spectrin beta, erythrocytic	Sptb	-0.483	0.014
ENSRNOG00000010161	myosin X	Myo10	-0.484	0.032
ENSRNOG00000012830	progestin and adipoQ receptor family member 8	Paqr8	-0.487	0.014
ENSRNOG00000011151	teneurin transmembrane protein 4	Tenm4	-0.495	0.014
ENSRNOG00000019819	dihydrouridine synthase 2	Dus2	-0.497	0.032
ENSRNOG00000008334	ciliary rootlet coiled-coil, rootletin	Crocc	-0.505	0.014
ENSRNOG00000016872	phospholipid phosphatase related 4	Plppr4	-0.515	0.032
ENSRNOG00000032656	protein tyrosine phosphatase receptor type T	Ptprt	-0.516	0.038
ENSRNOG00000019584	delta like non-canonical Notch ligand 1	Dlk1	-0.522	0.032
ENSRNOG00000018366	RGD1310819	RGD1310819	-0.523	0.014
ENSRNOG00000023993	kinesin family member 1A	Kif1a	-0.532	0.014
ENSRNOG00000003098	prominin 1	Prom1	-0.535	0.024
ENSRNOG00000009590	storkhead box 2	Stox2	-0.560	0.014
ENSRNOG00000005479	solute carrier family 1 member 2	Slc1a2	-0.580	0.014
ENSRNOG00000049758	TBC1 domain family member 16	Tbc1d16	-0.581	0.014
ENSRNOG00000010217	proline rich coiled-coil 2B	Prrc2b	-0.602	0.014
ENSRNOG00000014373	tripartite motif containing 66	Trim66	-0.622	0.014
ENSRNOG00000011171	regulating synaptic membrane exocytosis 3	Rims3	-0.627	0.042
ENSRNOG00000047321	hemoglobin alpha, adult chain 2	Hba-a2	-0.630	0.014
ENSRNOG00000001349	minichromosome maintenance complex component 7	Mcm7	-0.634	0.014
ENSRNOG00000036960	ATP binding cassette subfamily C member 9	Abcc9	-0.651	0.014
ENSRNOG00000046602	AABR07006030.1	AABR07006030.1	-0.656	0.024
ENSRNOG00000000940	fms related tyrosine kinase 1	Flt1	-0.666	0.014
ENSRNOG00000029886	hemoglobin alpha, adult chain 2	Hba-a1	-0.673	0.014
ENSRNOG00000059479	adenylate cyclase 1	Adcy1	-0.677	0.014
ENSRNOG00000011154	adhesion G protein-coupled receptor F5	Adgrf5	-0.680	0.014
ENSRNOG00000019689	von Willebrand factor	Vwf	-0.691	0.014
ENSRNOG00000058105	hemoglobin subunit beta	Hbb	-0.714	0.014
ENSRNOG00000053240	suppressor of glucose, autophagy associated 1	Soga1	-0.730	0.014
ENSRNOG00000014182	tensin 1	Tns1	-0.742	0.014
ENSRNOG00000002730	regulator of G protein signaling 5	Rgs5	-0.745	0.014
ENSRNOG00000014452	zinc finger homeobox 3	Zfhx3	-0.774	0.014
ENSRNOG00000014366	SHC adaptor protein 3	Shc3	-0.789	0.014
ENSRNOG00000001375	galactose-3-O-sulfotransferase 4	Gal3st4	-0.809	0.014
ENSRNOG00000055293	protein tyrosine phosphatase receptor type B	Ptprb	-0.857	0.024
ENSRNOG00000004346	notch receptor 3	Notch3	-0.882	0.014
ENSRNOG00000048769	NIMA related kinase 5	Nek5	-0.978	0.014
ENSRNOG00000037206	coiled-coil domain containing 77	Ccdc77	-1.152	0.014
ENSRNOG00000024651	growth regulating estrogen receptor binding 1	Greb1	-1.525	0.014

FEMALES

Table 28. Differentially expressed genes in females

Enseml ID	Name	Symbol	log2_FC	q_value
ENSRNOG00000046790	insulin receptor substrate 4	Irs4	1.902	0.013
ENSRNOG00000015156	galanin and GMAP prepropeptide	Gal	1.873	0.013
ENSRNOG00000010079	carbonic anhydrase 3	Car3	1.481	0.013
ENSRNOG00000015253	heat shock transcription factor 4	Hsf4	1.377	0.038
ENSRNOG00000039323	diacylglycerol kinase kappa	Dgkk	1.360	0.022
ENSRNOG00000054795	AABR07041096.1	AABR07041096.1	1.358	0.013
ENSRNOG00000032788	dysferlin	Dysf	1.277	0.013
ENSRNOG00000012259	interleukin 22 receptor subunit alpha 2	Il22ra2	1.218	0.022
ENSRNOG00000061182	gamma-aminobutyric acid type A receptor epsilon subunit	Gabre	1.201	0.022
ENSRNOG00000011824	thyrotropin releasing hormone	Trh	1.076	0.013
ENSRNOG00000005392	nerve growth factor receptor	Ngfr	1.048	0.013
ENSRNOG00000058560	collagen type II alpha 1 chain	Col2a1	0.907	0.022
ENSRNOG00000021063	glutamate ionotropic receptor NMDA type subunit 2D	Grin2d	0.873	0.013
ENSRNOG00000020579	collagen type VII alpha 1 chain	Col7a1	0.853	0.029
ENSRNOG00000014452	zinc finger homeobox 3	Zfhx3	0.760	0.013
ENSRNOG00000010158	MAGE family member L2	Magel2	0.752	0.013
ENSRNOG00000018445	angiotensinogen	Agt	0.746	0.013
ENSRNOG00000020030	cytokine receptor like factor 1	Crlf1	0.694	0.013
ENSRNOG00000052564	glutathione peroxidase 3	Gpx3	0.690	0.013
ENSRNOG00000017893	BAI1 associated protein 3	Baiap3	0.688	0.013
ENSRNOG00000016147	solute carrier family 17 member 6	Slc17a6	0.618	0.022
ENSRNOG00000008356	myosin VC	Myo5c	0.601	0.045
ENSRNOG00000052022	PNMA family member 3	Pnma3	0.547	0.029
ENSRNOG00000020444	hyperpolarization activated cyclic nucleotide gated potassium channel 3	Hcn3	0.525	0.029
ENSRNOG00000011151	teneurin transmembrane protein 4	Tenm4	0.520	0.045
ENSRNOG00000007456	calbindin 1	Calb1	0.502	0.013
ENSRNOG00000061731	plexin B3	Plxnb3	0.496	0.013
ENSRNOG00000008309	PITPNM family member 3	Pitpnm3	0.470	0.029
ENSRNOG00000020525	collagen type V alpha 3 chain	Col5a3	0.459	0.038
ENSRNOG00000010650	pleckstrin homology, MyTH4 and FERM domain containing H1	Plekhh1	0.457	0.045
ENSRNOG00000021525	neurobeachin like 1	Nbeal1	-0.495	0.013
ENSRNOG00000018366	RGD1310819	RGD1310819	-0.530	0.013
ENSRNOG00000019819	dihydrouridine synthase 2	Dus2	-0.547	0.029
ENSRNOG00000002919	glial fibrillary acidic protein	Gfap	-0.571	0.013
ENSRNOG00000002097	RAS like family 11 member B	Rasl11b	-0.589	0.022
ENSRNOG00000019321	cholecystokinin	Cck	-0.591	0.029
ENSRNOG00000017712	CART prepropeptide	Cartpt	-0.618	0.013
ENSRNOG00000011160	synaptic vesicle glycoprotein 2B	Sv2b	-0.621	0.013
ENSRNOG00000026577	copine 4	Cpne4	-0.675	0.013
ENSRNOG00000009388	serine palmitoyltransferase small subunit B	Sptssb	-0.719	0.038
ENSRNOG00000050767	neuritin 1	Nrn1	-0.744	0.013
ENSRNOG00000015155	troponin C2, fast skeletal type	Tnnc2	-0.854	0.013
ENSRNOG00000012404	thyroid hormone responsive	Thrsp	-0.866	0.013
ENSRNOG00000006204	solute carrier family 30 member 3	Slc30a3	-0.877	0.013
ENSRNOG00000031211	acyl-CoA synthetase medium chain family member 5	Acsm5	-0.892	0.013
ENSRNOG00000020650	solute carrier family 17 member 7	Slc17a7	-0.892	0.013
ENSRNOG00000008697	cellular communication network factor 3 - nephroblastoma overexpressed	Nov	-0.954	0.013

ENSRNOG00000020620	protein phosphatase 1 regulatory subunit 32	Ppp1r32	-1.073	0.045
ENSRNOG00000015550	prostaglandin D2 synthase	Ptgds	-1.083	0.013
ENSRNOG00000018087	vimentin	Vim	-1.124	0.013
ENSRNOG00000003183	fibromodulin	Fmod	-1.134	0.013
ENSRNOG00000026914	dynein axonemal heavy chain 1	Dnah1	-1.180	0.013
ENSRNOG00000016957	insulin like growth factor binding protein 2	Igfbp2	-1.208	0.013
ENSRNOG00000059865	dynein axonemal heavy chain 12	Dnah12	-1.224	0.013
ENSRNOG00000027935	leucine rich repeat containing 34	Lrrc34	-1.231	0.022
ENSRNOG00000005109	reprimo, TP53 dependent G2 arrest mediator homolog	Rprm	-1.235	0.013
ENSRNOG00000039086	coiled-coil domain containing 153	Ccdc153	-1.239	0.013
ENSRNOG00000016275	transthyretin	Ttr	-1.257	0.029
ENSRNOG00000037688	adenylate kinase 9	Ak9	-1.308	0.013
ENSRNOG00000046001	AABR07030823.1	AABR07030823.1	-1.394	0.013
ENSRNOG00000008492	cilia and flagella associated protein 45	Cfap45	-1.473	0.013
ENSRNOG00000003687	regulator of G protein signaling 2	Rgs2	-1.494	0.013
ENSRNOG00000009779	keratin 8	Krt8	-1.502	0.022
ENSRNOG00000025005	coiled-coil domain containing 190	Ccdc190	-1.512	0.013
ENSRNOG00000004890	adenylate cyclase 8	Adcy8	-1.546	0.013
ENSRNOG00000004443	serine palmitoyltransferase long chain base subunit 3	Sptlc3	-1.585	0.013
ENSRNOG00000055858	MYB proto-oncogene, transcription factor	Myb	-1.593	0.029
ENSRNOG00000013057	protein regulator of cytokinesis 1	Prc1	-1.603	0.013
ENSRNOG00000012450	dynein light chain roadblock-type 2	Dynlrb2	-1.668	0.013
ENSRNOG00000015581	dynein axonemal heavy chain 6	Dnah6	-1.759	0.013
ENSRNOG00000018735	CD74 molecule	Cd74	-1.760	0.013
ENSRNOG00000016716	armadillo repeat containing 3	Armc3	-1.777	0.013
ENSRNOG00000033734	troponin T2, cardiac type	Tnnt2	-1.838	0.013
ENSRNOG00000032844	RT1-Da	RT1-Da	-1.894	0.013
ENSRNOG00000036585	cilia and flagella associated protein 43	Cfap43	-1.931	0.013
ENSRNOG00000014893	WD repeat domain 63	Wdr63	-2.066	0.013
ENSRNOG00000002873	family with sequence similarity 183 member B, pseudogene	Fam183b	-2.102	0.013
ENSRNOG00000018215	solute carrier family 22 member 6	Slc22a6	-2.117	0.013
ENSRNOG00000012827	myeloid leukemia factor 1	Mlf1	-2.163	0.013
ENSRNOG00000028077	cilia and flagella associated protein 44	Cfap44	-2.211	0.013
ENSRNOG00000055714	adenylate kinase 7	Ak7	-2.258	0.013
ENSRNOG00000019902	folate receptor 1	Folr1	-2.375	0.045
ENSRNOG00000005659	aurora kinase B	Aurkb	-2.440	0.013
ENSRNOG00000012608	transmembrane protein 212	Tmem212	-2.443	0.022

APPENDIX D. POSITRON EMISSION TOMOGRAPHY

POSITRON EMISSION TOMOGRAPHY - MATERIALS AND METHODS

PET-CT studies were performed on adult rats at PND 32-33 and PND 60 with the collaboration of the Radioisotopes for Biomedicine research group at the Centre for Energy, Environmental and Technological Research (CIEMAT) in Madrid (Spain), using a small-animal PET-CT apparatus (SEDECAL, Madrid, Spain). A total of 20 rats (11 males and 9 females from 5 different litters) underwent two different PET scans at PND28 and again at PND65. From PND38 to PND54, the rats received nine intraperitoneal (i.p.) injections of Δ^9-THC (2 mL/kg) at a dose of 3 mg/kg or vehicle (2 mL/kg). Static PET images were obtained for 45 min 30 min post intravenous administration with 176 ± 37 MBq/kg body weight of 2-deoxy-2-[18F] fluoro-D-glucose ([18F]FDG). Briefly, the rats were anesthetized with 2–3% isoflurane in medical oxygen (1 L/min) and their temperature was maintained at 37 ºC using a heating pad during PET acquisition. The PET data obtained was reconstructed using a 2D-OSEM algorithm (16 subsets and 3 iterations) with random and scatter corrections. The PET images underwent pre-processing using a protocol described previously (Casquero-Veiga et al., 2019). Briefly, each PET image was spatially co-registered to a common reference CT scan for each sex by an automatic method based on mutual information (Pascau et al., 2009), then subjected to 9 point scaling in the three spatial directions. The PET intensity values were then normalized to the mean brain intensity and four brain ROIs were segmented: hippocampus, prefrontal cortex, caudate nucleus, and cortex. Statistical analysis was performed with a three-way mixed analysis of variance (ANOVA), followed by a Bonferroni multiple comparison post-test, with the between-subject factors representing gender and treatment (THC or vehicle), and time as the within-subject factor. Statistical analyses were performed in SSPS 14.0 and a p-value of less than 0.05 was considered statistically significant.

For the PND65 scans, voxel-based 2-sample t-tests ($p<0.05$ uncorrected) were performed for each sex with Statistical Parametric Mapping (SPM) software (http://www.fil.ion.ucl.ac.uk/spm/software/spm12/). The PET images were smoothed with a Gaussian kernel 2.5 times the voxel size at full width at a half maximum (FWHM) and masked in order to exclude extracerebral voxels from the analyses. Only clusters more extensive than 50 adjacent voxels were considered in order to minimize the effect of type I errors.

POSITRON EMISSION TOMOGRAPHY - RESULTS

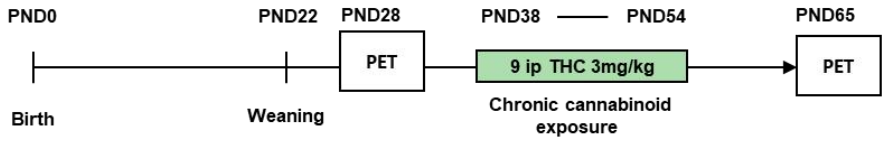
There were no statistically significant differences in the standardized uptake value ratio (SUVR) values between the rats exposed to THC or the vehicle alone in any of the regions analysed (see Table 29). We observed a developmental effect in the SUVRs that increased in all the ROIs analysed at PND65 relative to PND32. We obtained a significant effect of Sex in the hippocampus and a significant Sex x Time interaction, which suggested that males had higher SUVRs than females at both developmental ages (see Table 29). The SPM analysis of adult brain PET scans revealed some additional preliminary effects, whereby THC-males had increased metabolism in the somatosensory (S1) and piriform (Pir) cortex. By contrast, THC-exposed females showed hypometabolism in a cluster of voxels comprising the inferior colliculus and the cerebellum. There was also some marginal evidence for a hypometabolism in a cluster located in the cortex (mostly the motor and sensory cortices: see Figure 26).

Table 29. Positron Emission Tomography

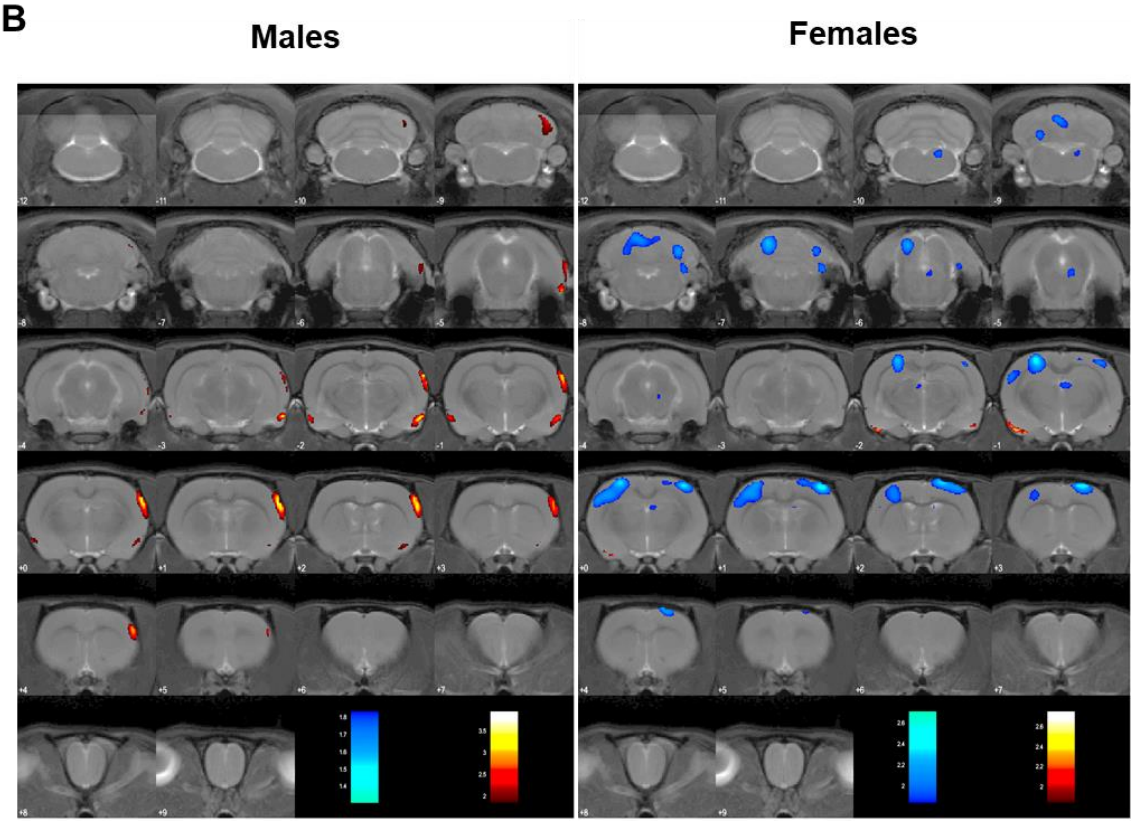
Area	p		Effect	Statistic value	*df* 1	*df* e	Effect size	1-β	MALE VEH MEAN	MALE VEH SEM	MALE VEH N	MALE THC MEAN	MALE THC SEM	MALE THC N	FEMALE VEH MEAN	FEMALE VEH SEM	FEMALE VEH N	FEMALE THC MEAN	FEMALE THC SEM	FEMALE THC N
	**	0.000	TIME	F= 68.96	1	16	0.812	1												
	*	0.022	SEX	F= 6.48	1	16	0.288	0.667												
Hippocampus	ns	0.879	TIME * SEX	F= 0.02	1	16	0.001	0	0.939	0.103	5	0.925	0.148	6	0.958	0.224	5	0.964	0.067	4
	ns	0.561	TIME* TMT	F= 0.35	1	16	0.022	0.52												
	ns	0.901	TIME * SEX*TMT	F= 0.02	1	16	0.001	0.52	1.003	0.085	5	0.996	0.161	6	1.017	0.098	5	1.035	0.049	4
	**	0.000	TIME	F= 81.50	1	16	0.836	1												
	*****	**0.027**	**TIME * SEX**	**F= 5.89**	**1**	**16**	**0.269**	**0.625**												
	*	0.005	TIME in Males	F= 69.02	1	10	0.873	1	1.041	0.094	5	1.059	0.165	6	1.016	0.174	5	1.009	0.027	4
Caudate nucleus	*	0.005	TIME in Females	F= 14.85	1	8	0.650	0.92												
	ns	0.206	TIME* TMT	F= 1.74	1	16	0.098	0.236	1.151	0.134	5	1.162	0.134	6	1.049	0.058	5	1.099	0.165	4
	ns	0.103	TIME * SEX*TMT	F= 3.00	1	16	0.158	0.37												
	**	0.000	TIME	F= 31.32	1	16	0.662	0.999												
Prefrontal cortex	ns	0.309	TIME * SEX	F= 1.11	1	16	0.065	0.168	1.015	0.197	5	1.045	0.358	6	1.044	0.201	5	1.025	0.228	4
	ns	0.784	TIME* TMT	F= 0.08	1	16	0.005	0.58												
	ns	0.132	TIME * SEX*TMT	F= 2.52	1	16	0.136	0.32	1.102	0.085	5	1.082	0.139	6	1.117	0.040	5	1.133	0.080	4
	**	0.000	TIME	F= 22.26	1	16	0.582	0.999												
Cortex	ns	0.588	TIME * SEX	F= 0.31	1	16	0.019	0.082	1.008	0.192	5	1.028	0.313	6	1.040	0.206	5	1.020	0.259	4
	ns	0.970	TIME* TMT	F= 0.00	1	16	0.000	0.05												
	ns	0.218	TIME * SEX*TMT	F= 1.64	1	16	0.093	0.226	1.084	0.089	5	1.068	0.130	6	1.096	0.036	5	1.110	0.049	4

The main test performed are two-ANOVA with Sex (Male/Female) and Treatment (THC/VEH) as within subject factors. Interactions are analysed with a simple effect analysis. Corrected model associated values are reported when factor effects or interactions have associated p values over 0.1.

A



B



THC effects on brain metabolism

ROI	Side	T	k	↓/↑	p_{unc} peak level	p_{unc} cluster level	p_{FWE} cluster level
Males							
S1	L	3.92	293	↑	0.002	0.698	0.804
Pir	L	2.19			0.028		
Females							
M1	L	2.71	185	↓	0.012	0.684	0.896
S1	R	2.46	267	↓	0.018	0.616	0.870
IC	R	2.36	145	↓	0.021	0.724	0.909

Cb: cerebellum, IC: inferior colliculus, M1: primary motor cortex, Pir: piriform cortex, S1: primary somatosensory cortex.
ROI: Region of interest. Side: Right (R) and Left (L). T: t value, k: cluster size. Glucose metabolism: Increase (↑) and Decrease (↓). p_{unc}: p value uncorrected, FWE: Family wise error correction.

Figure 26: Positron emission tomography at PND65: male-VEH n=6; male-THC n=6; female-VEH n=6; female-THC n=5. The effects of THC on brain glucose metabolism in males and females represented as statistical parametric T-maps overlaid on a T2-image as a template. T-maps were obtained as results from 2-sample t-test analyses, applying a cluster size threshold of 50 adjacent voxels and a p-value of 0.05 (uncorrected). The colour bars represent the t-values corresponding to reductions (cold colours) or increases (hot colours) in brain metabolism. The intensity of the colour negatively correlates with the t-value of the difference in the cluster represented. The most solid effects were detected in the females in which THC provoked a hypoactivation in the IC-Cb and the somatosensory Cx. ROIs: Cb, cerebellum; IC, inferior colliculus; M1, primary motor cortex; Pir, piriform cortex; S1: primary somatosensory cortex. Hemispheres: Left (L), Right (R).

POSITRON EMISSION TOMOGRAPHY: DISCUSSION

Our preliminary developmental PET study showed maturational effects in all the ROIs analysed, and interesting sex differences in the hippocampus and the caudate nucleus. Our SPM analysis of the adult brain also suggested hypometabolism in a cluster of voxels comprising the inferior colliculus and the Cb, as well as the motor and sensory cortices of THC-exposed females. To the best of our knowledge, there are no long-term PET studies with [18F]-FDG in humans that have ascertained the functional effects of adolescent cannabis use. However, there are two previous reports suggesting that adolescent exposure to the synthetic cannabinoid CP 55,940 modifies brain metabolism in the frontal and amygdalo-entorhinal cortices in females (Alejandro Higuera-Matas et al., 2008b). Moreover, the brain responses to a cocaine injection were also different in animals exposed to CP 55,940 during adolescence (Alejandro Higuera-Matas et al., 2011). Although these results should be replicated, they represent the first PET evidence indicative of a long-lasting alteration in the Cb. However, prior studies had already documented substantial cellular alterations in the Cb after exposure to THC. The mechanisms underlying these alterations are beginning to be unveiled and they are likely to involve microglial activation. For example, in mice sub-chronic administration of THC activated cerebellar microglia and increased the expression of neuroinflammatory markers, including IL-1β. Moreover, this neuroinflammatory phenotype was correlated with deficits in cerebellar conditioned learning and fine motor coordination (Cutando et al., 2013). The sensorimotor cortex was also affected, specifically in females, as well as the hippocampus and to a lesser extent the inferior colliculus. These functional alterations could also be involved in the long-term cognitive effects of adolescent cannabis use (Higuera-Matas et al., 2015; Meier et al., 2012) either alone as separate structures or in combination as networks. Indeed, the Cb receives indirect connections from the sensory cortices to use sensory information for spatial representation (Rondi-Reig et al., 2014), and there is a hippocampo-cerebellar centred network for the learning and the execution of sequence based navigation (Babayan et al., 2017). There are multiple reports of altered spatial learning and memory deficits in rodents after adolescent exposure to cannabinoids (Higuera-Matas et al., 2015), and the metabolic alterations preliminarily reported here could provide a mechanistic explanation for these.

APPENDIX E. IMMUNOHISTOCHEMISTRY FOR c-FOS

IMMUNOHISTOCHEMISTRY FOR c-FOS - MATERIALS AND METHODS

In this study, 64 rats (32 males and 32 females) from 9 different litters underwent the same chronic adolescent cannabinoid treatment described in section 2 of the Materials and Methods. When the animals reached P90 they were injected either with cocaine (cocaine hydrochloride 20 mg/kg, i.p.: Alcaliber, Spain) or saline (0.9% NaCl sterile solution 1 mL/kg, i.p.: Vitulia, Spain). After 90 minutes they were anesthetized with an injection of a 16% chloral hydrate solution (400 mg/kg, i.p.) and transcardially perfused with PBS 0.1 M followed by 4% paraformaldehyde (PFA). The rat's brain was then extracted and kept in a fixative solution (4% PFA) for 24 h, then transferred to a 30% sucrose solution for another 24 h. They were then kept at -20 ºC in a glycerol/ethylene glycol (30%/30%) and (40%) PB 0.4M solution.

Coronal vibratome brain slices (50 µm) were then obtained, transferred to a 30% glucose solution and kept at -4 ºC for 24 h. They were then transferred to a -20 ºC freezer and kept in a glycerol/ethylene glycol and PB 0.4 M solution until immunohistochemistry was performed. Free-floating tissue was washed in PBS 0.1 M (3 successive, 10 min rinses) and then incubated with 0.3% hydrogen peroxide (v/v) in PBS at room temperature for 30 min. They were then incubated 1 hour in blocking solution (2% v/v: normal goat serum + 0.3% (v/v) Triton-X 100 in PBS) and washed in PBS + Triton-X 100 (PBST, 3x10 min). Subsequently, the sections were incubated at 4 °C for 24 hours with a rabbit c-Fos antibody (1:50,000: Merck ABE457 Lot: 3116957) and after washing in PBST (3x10 min) they were incubated for 1 hour with a biotinylated goat anti-rabbit IgG (1:200: Vectastain, BA-1000-1.5, LOT ZE1218). After washing in PBS (3x10 min), the sections were incubated with ABC reagent (avidin-biotin complex kit, Vector Labs) at room temperature for 1 hour, washed (PBS, 3x10 min), and reacted with diaminobenzidine (DAB) for approximately 5 minutes to visualise the brown precipitate in the neurons

labelled for c-Fos. The sections were then washed (PBS, 3x10 min), allowed to dry, and mounted on microscope slides and cover slipped with DPX.

Tissue images were captured at 10 X optical magnification by bright-field microscopy. The background was subtracted using a rolling ball procedure (radius 12.00) and the c-Fos positive cells per ROI were counted using the particle analysis option in ImageJ (size: 80-200, circularity: 0.50-1.00), carried out by a researcher blind to the experimental conditions.

IMMUNOHISTOCHEMISTRY FOR c-FOS - RESULTS

We first examined how the initial actions of cocaine were modified by adolescent THC exposure. The cell activation induced by cocaine was potentiated in the motor cortex by THC exposure during adolescence (see Table 30 and Figure 27), and we found a significant Sex x Adolescent Treatment x Adult Treatment triple interaction in the dorsomedial nucleus of the hypothalamus. The analysis of this interaction revealed a trend for cocaine to induce significant c-Fos activity relative to the saline-injected animals in females but not males exposed to THC during adolescence. There were also differences in cocaine injected males between the THC and VEH groups. Adolescent exposure to THC also modified the mean c-Fos accumulation (irrespective of cocaine exposure) in the piriform (only in the males), retrosplenial and somatosensory cortices (see Table 31 and Figure 28). There was a significantly higher accumulation of c-Fos after cocaine exposure (Adult Treatment effect) in the lateral orbitofrontal, cingulate, motor, insular and entorhinal cortices (see Table 30), an effect that was also observed in the amygdala. The effects of cocaine in the lateral orbitofrontal cortex were restricted to males. There were some interactive effects in the medial septal nucleus between the sex of the animal and cocaine/saline exposure, although the simple effects did not reach statistical significance, similar to the Islands of Calleja where cocaine induced c-Fos protein expression only in the females (see Table 31 and Figure 29). We also detected THC-related effects in retrosplenial, somatosensory and piriform cortices where THC animals had higher levels of c-Fos protein than animals exposed to the VEH alone. In the latter two cases, this effect was only observed in males (a significant Sex x Adolescent Treatment interaction with significant simple effects in males). We also observed main general effects of the sex of the animals in the medial orbital, prelimbic, cingulate, motor, insular, entorhinal and retrosplenial cortices, and in the Islands of Calleja, with females showing higher values than males (see Table 31, and Figure 28 and 29).

Table 30. Immunohistochemistry for c-Fos - results (I).

							Males									Females								
							VEH						THC			VEH						THC		
			Statistic			Effect	SALINE			COCAINE			SALINE			COCAINE			SALINE			COCAINE			SALINE			COCAINE		
Area	p	Effect	value	*df* 1	*df* e	size	1-β	MEAN	SEM	N	MEAN	SEM	N	MEAN	SEM	N	MEAN	SEM	N	MEAN	SEM	N	MEAN	SEM	N	MEAN	SEM	N	MEAN	SEM	N
Motor cortex	** 0.001	SEX	F= 13.676	1	53	0.21	0.95	42.1	13.3	8	146.3	32.9	7	43.4	11.6	8	250.7	33.4	8	161.9	26.5	7	190.0	21.7	8	105.5	18.3	7	250.5	52.8	8
	** 0.000	ADULT TMT	F= 42.833	1	53	0.45	1.00																							
	*** 0.011**	**SEX * ADULT TMT**	**F= 7.029**	**1**	**53**	**0.12**	**0.74**																							
	** 0.000	COCAINE effects in MALE	F= 43.048	1	57	0.42	1.00																							
	** 0.009	COCAINE effects in FEMALE	F= 7.369	1	57	0.11	0.73																							
	** 0.000	SEX effects in SALINE	F= 18.211	1	57	0.24	0.99																							
	ns 0.548	SEX effects in COCAINE	F= 0.365	1	57	0.01	0.09																							
	*** 0.025**	**ADOL TMT * ADULT TMT**	**F= 5.326**	**1**	**53**	**0.09**	**0.62**																							
	** 0.004	COCAINE effects in VEH	F= 9.204	1	55	0.12	0.79																							
	** 0.000	COCAINE effects in THC	F= 38.931	1	55	0.35	1.00																							
	ns 0.523	SEX effects in SALINE	F= 0.412	1	57	0.01	0.10																							
	* 0.042	SEX effects in COCAINE	F= 4.317	1	57	0.07	0.53																							
Dorsomedial hypothalamic nucleus	*** 0.010**	**SEX * ADOL TMT * ADULT TMT**	**F= 7.148**	**1**	**49**	**0.13**	**0.75**	20.9	3.1	5	30.9	16.9	8	26.6	5.9	7	14.0	13.5	8	16.9	2.9	7	14.7	4.2	7	15.4	3.9	8	29.0	6.8	7
	ns 0.195	COCAINE effects in MALE*VEH	F= 0.531	1	49	0.01	0.11																							
	ns 0.076	COCAINE effects in MALE*THC	F= 6.355	1	49	0.12	0.70																							
	ns 0.766	COCAINE effects in FEMALE*VEH	F= 0.046	1	49	0.00	0.06																							
	t 0.055	COCAINE effects in FEMALE*THC	F= 3.970	1	49	0.08	0.50																							
	ns 0.470	THC effects in MALE*SALINE	F= 0.258	1	49	0.01	0.08																							
	* 0.015	THC effects in MALE*COCAINE	F= 5.441	1	49	0.10	0.63																							
	ns 0.832	THC effects in FEMALE*SALINE	F= 2.601	1	49	0.05	0.35																							
	t 0.052	THC effects in FEMALE*COCAINE	F= 4.669	1	49	0.09	0.56																							
	ns 0.613	SEX effects in VEH *SALINE	F= 1.730	1	49	0.03	0.25																							
	* 0.024	SEX effects in VEH*COCAINE	F= 3.280	1	49	0.06	0.43																							
	ns 0.113	SEX effects in THC*SALINE	F= 0.089	1	49	0.00	0.06																							
	* 0.036	SEX effects in THC*COCAINE	F= 3.852	1	49	0.07	0.49																							
Medial Orbital	** 0.003	SEX	F= 9.602	1	53	0.15	0.86	44.3	5.4	7	70.5	12.6	7	76.8	14.0	8	68.1	10.6	8	105.9	8.5	7	102.3	13.5	8	69.4	18.8	8	97.0	14.0	8
Prelimbic cortex	** 0.000	SEX	F= 20.458	1	53	0.28	0.99	39.6	9.7	7	54.3	9.4	7	51.9	8.9	8	63.1	17.1	8	106.1	10.2	7	121.3	19.2	8	71.8	18.4	8	110.5	17.6	8
Lateral Orbital cortex	** 0.000	ADULT TMT	F= 20.582	1	52	0.28	0.99	18.5	6.2	7	97.6	59.8	7	37.4	12.3	8	72.4	23.0	8	62.5	9.9	6	85.3	14.0	8	52.2	10.7	8	59.8	8.7	8
	**** 0.006**	**SEX * ADULT TMT**	**F= 8.326**	**1**	**52**	**0.14**	**0.81**																							
	** 0.000	COCAINE effects in MALE	F= 26.073	1	56	0.32	1.00																							
	ns 0.223	COCAINE effects FEMALE	F= 1.520	1	56	0.03	0.23																							
	** 0.004	SEX effects in SALINE	F= 9.277	1	56	0.14	0.85																							
	ns 0.437	SEX effects in COCAINE	F= 0.614	1	56	0.01	0.12																							
Cingulate	* 0.042	SEX	F= 4.342	1	0	0.08	0.53	27.0	8.0	7	94.9	31.4	7	25.2	5.5	8	70.4	12.4	8	75.3	11.1	7	88.3	11.5	7	43.1	8.1	8	94.9	13.7	8
	** 0.000	ADULT TMT	F= 19.341	1	0	0.27	0.99																							
Amygdala	* 0.015	ADULT TMT	F= 6.272	1	53	0.11	0.69	18.8	8.2	7	53.7	44.3	7	37.2	6.9	8	57.7	41.3	8	35.2	7.6	7	51.9	7.7	8	42.3	8.7	8	42.3	7.3	8
Insular cortex	** 0.009	SEX	F= 7.292	1	54	0.12	0.76	15.8	3.8	7	26.8	8.5	7	18.1	3.0	8	28.8	10.3	8	29.4	7.0	8	36.3	6.4	8	26.8	7.4	8	37.4	4.9	8
	* 0.011	ADULT TMT	F= 6.851	1	54	0.11	0.73																							

The tests performed were two-ANOVAs with Sex (Male/Female) and Treatment (VEH/THC) as the within subject factors or a Repeated Measures (RM) ANOVA using the between-factor Session. Corrected model values are reported when factor effects and interactions have associated p values above 0.1.

Table 31. Immunohistochemistry for c-Fos - results (II)

								Males												Females									
								VEH						THC						VEH						THC			
			Statistic			Effect		SALINE			COCAINE			SALINE			COCAINE			SALINE			COCAINE			SALINE			COCAINE		
Area	p	Effect	value	*df*1	*df* e	size	1-β	MEAN	SEM	N	MEAN	SEM	N	MEAN	SEM	N	MEAN	SEM	N	MEAN	SEM	N	MEAN	SEM	N	MEAN	SEM	N	MEAN	SEM	N
Entorhinal cortex	* 0.026	SEX	F= 5.204	1	54	0.09	0.61	18.3	2.6	7	31.4	3.3	7	21.8	2.8	8	36.6	5.4	8	30.1	5.2	8	43.2	5.2	8	28.2	4.7	8	36.6	5.8	8
	** 0.000	ADULT TMT	F= 14.179	1	54	0.21	0.96																								
	*** 0.047**	**SEX * ADULT TMT**	**F= 4.159**	**1**	**56**	**0.07**	**0.52**																								
Media Septal nucleus	ns 0.135	COCAINE effects in MALE	F= 2.295	1	56	0.04	0.32	16.5	4.2	7	9.9	7.6	7	16.8	6.0	7	10.6	10.4	7	16.9	5.2	8	15.5	2.9	8	6.8	1.4	8	18.9	4.5	8
	ns 0.181	COCAINE effects FEMALE	F= 1.833	1	56	0.03	0.27																								
	ns 0.245	SEX effects in SALINE	F= 1.380	1	56	0.02	0.21																								
	ns 0.092	SEX effects in COCAINE	F= 2.947	1	56	0.05	0.39																								
Periaqueductal gray	ns 0.978	Corrected model	F= 0.223	7	47	0.03	0.11	10.6	4.5	5	19.2	4.9	7	15.8	9.3	7	15.0	6.3	8	19.2	5.0	6	17.2	7.6	6	14.4	5.8	8	21.4	8.4	8
VTA	ns 0.777	Corrected model	F= 0.568	7	41	0.09	0.22	15.3	3.2	4	16.2	7.5	6	16.0	5.6	7	13.6	11.2	6	12.1	2.9	5	10.6	2.3	7	14.1	2.6	7	17.6	1.8	7
Retrospenial cortex	** 0.003	SEX	F= 9.873	1	53	0.16	0.87	17.6	4.0	7	18.2	1.2	8	40.2	12.4	7	27.9	6.3	8	28.1	4.3	7	33.0	4.2	8	39.7	5.5	8	44.9	6.0	8
	** 0.001	ADOL TMT	F= 11.592	1	53	0.18	0.92																								
Somatosensory cortex	* 0.045	ADOL TMT	F= 4.205	1	51	0.08	0.52	34.8	7.8	7	31.1	5.0	8	69.0	14.5	7	54.3	10.1	8	34.0	6.8	7	45.6	7.4	8	35.0	4.5	7	36.9	4.7	7
	*** 0.015**	**SEX * ADOL TMT**	**F= 6.313**	**1**	**51**	**0.11**	**0.69**																								
	** 0.002	THC effects in MALE	F= 10.585	1	51	0.17	0.89																								
	ns 0.747	THC effects in FEMALE	F= 0.105	1	51	0.00	0.06																								
	ns 0.337	SEX effects in VEH	F= 0.939	1	55	0.02	0.16																								
	* 0.011	SEX effects in THC	F= 7.009	1	55	0.11	0.74																								
Piriform cortex	** 0.003	ADOL TMT	F= 9.466	1	53	0.15	0.86	28.3	4.3	7	25.2	11.5	8	64.6	10.8	7	43.6	21.6	8	45.6	8.4	7	47.6	8.3	8	41.5	6.3	8	65.3	13.0	8
	*** 0.049**	**SEX*ADOL TMT**	**F= 4.056**	**1**	**53**	**0.07**	**0.51**																								
	** 0.001	THC effects in MALE	F= 11.760	1	57	0.17	0.92																								
	ns 0.468	THC effects in FEMALE	F= 0.534	1	57	0.01	0.11																								
	* 0.010	SEX effects in VEH	F= 7.111	1	57	0.11	0.75																								
	ns 0.965	SEX effects in THC	F= 0.002	1	57	0.00	0.05																								
	*** 0.040**	**SEX * ADULT TMT**	**F= 4.447**	**1**	**53**	**0.08**	**0.54**																								
	ns 0.168	COCAINE effects in MALE	F= 1.948	1	57	0.03	0.28																								
	ns 0.185	COCAINE effects FEMALE	F= 1.804	1	57	0.03	0.26																								
	ns 0.853	SEX effects in SALINE	F= 0.035	1	57	0.00	0.05																								
	* 0.011	SEX effects in COCAINE	F= 6.835	1	57	0.11	0.73																								
Islands of Calleja	** 0.000	SEX	F= 73.145	1	52	0.58	1.00	9.4	2.2	7	6.6	1.6	7	10.4	2.1	7	6.7	1.2	8	18.1	3.4	7	28.3	3.6	8	25.6	2.7	8	35.3	5.1	8
	**** 0.004**	**SEX * ADULT TMT**	**F= 9.211**	**1**	**52**	**0.15**	**0.85**																								
	ns 0.316	COCAINE effects in MALE	F= 1.026	1	56	0.02	0.17																								
	** 0.002	COCAINE effects FEMALE	F= 10.048	1	56	0.15	0.88																								
	** 0.000	SEX effects in SALINE	F= 14.890	1	56	0.21	0.97																								
	** 0.000	SEX effects in COCAINE	F= 67.354	1	56	0.55	1.00																								

The tests performed were two-ANOVAs with Sex (Male/Female) and Treatment (VEH/THC) as the within subject factors or a Repeated Measures (RM) ANOVA using the between-factor Session. Corrected model values are reported when factor effects and interactions have associated p values above 0.1.

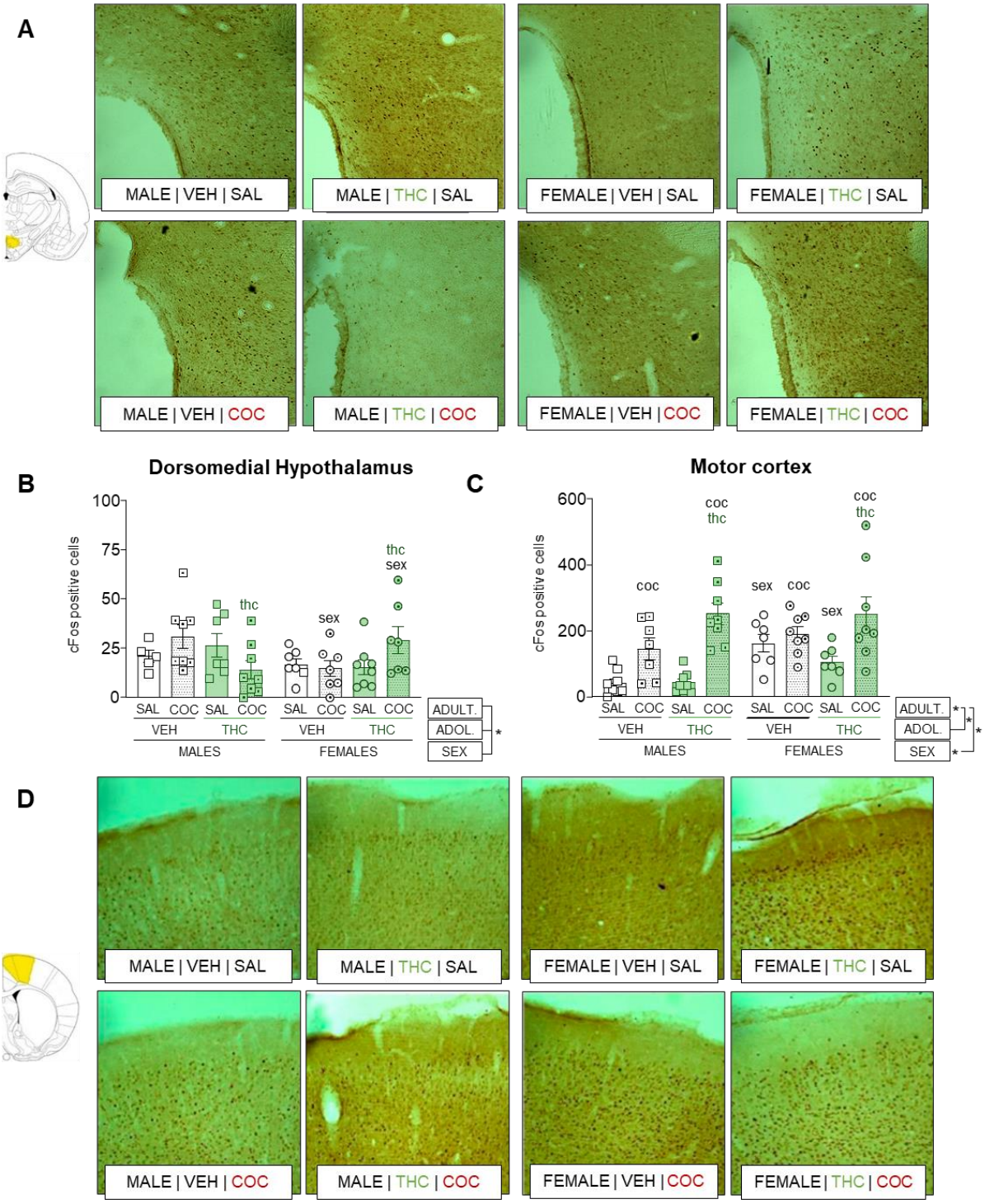


Figure 27: Immunohistochemistry for cFos - the main effects of THC. Fos protein accumulation in response to a single i.p. cocaine (20 mg/kg) or saline injections (n=8 in all the groups). The graphs represent the individual values (dots) and the mean ± SEM (lines). The main effects are indicated with "*" next to the name of the factors (ADULT, ADOL or SEX), the interactions between factors are indicated with lines joining each factor participating in the interaction and the corresponding "*" ($p<0.05$) to the right and significant results of the analysis of the simple effect of the interactions are indicated with "sex", "THC" or "COC" over the experimental group: "sex" stands for differences with the corresponding group of the other sex, and with the same adolescent treatment (THC or VEH) and same adult treatment (cocaine or saline); "THC" stands for differences with the corresponding VEH-adolescent treated group of the same sex and with the same adult exposure to cocaine or saline; "COC" stands for differences with the corresponding saline group of the same sex and same adolescent treatment with THC or VEH. A) Representative pictures of Fos accumulation in response to acute i.p. cocaine in the dorsomedial hypothalamic nuclei. B) Fos expression in the dorsomedial hypothalamic nuclei. C) Fos expression in the motor cortex. D) Representative pictures of Fos expression in response to acute i.p. cocaine in the motor cortex.

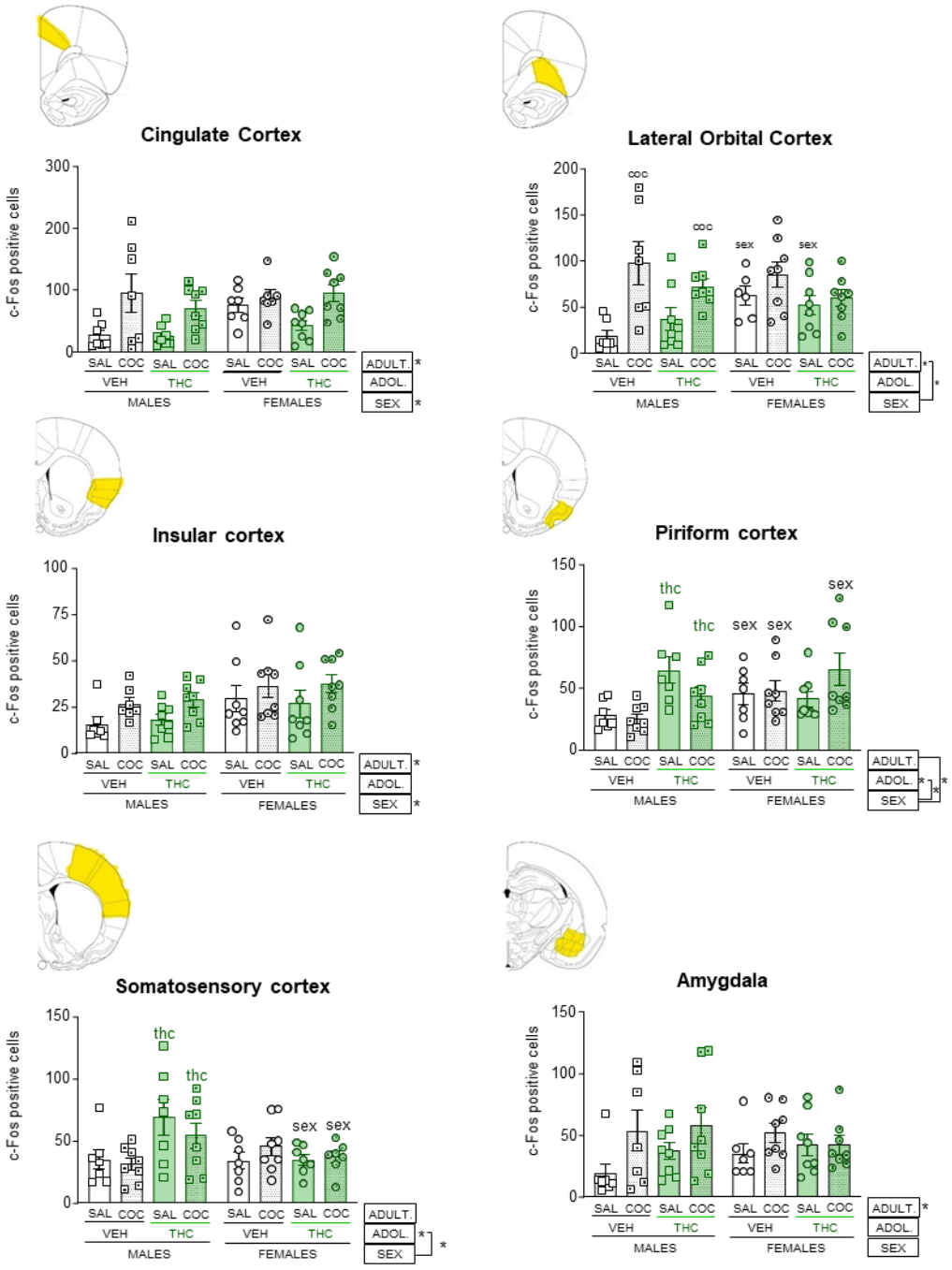


Figure 28. c-Fos immunohistochemistry (I). Brain areas showing significant effects of Sex, Adolescent Treatment or Adult Treatment (cocaine injection) or the interactions among them. Fos protein accumulation in response to a single i.p. cocaine (20 mg/kg) or saline injections (n=8 in all the groups). The graphs represent the individual values (dots) and the mean ± SEM (lines). The main effects are indicated with "*" next to the name of the factors (ADULT, ADOL or SEX). Interactions between the factors are indicated with lines joining each factor participating in the interaction and the corresponding "*" ($p<0.05$) to the right. Significant results of the analysis of the simple effect of the interactions are indicated by "SEX", "THC" or "COCC over the experimental group: "SEX" represents differences with the corresponding group of the other sex with the same adolescent treatment (THC or VEH) and same adult treatment (cocaine or saline); "THC" represents differences with the corresponding VEH-adolescent treated group of the same sex and the same adult exposure to cocaine or saline; "COC" represents differences with the corresponding saline group of the same sex and same adolescent treatment with THC or VEH.

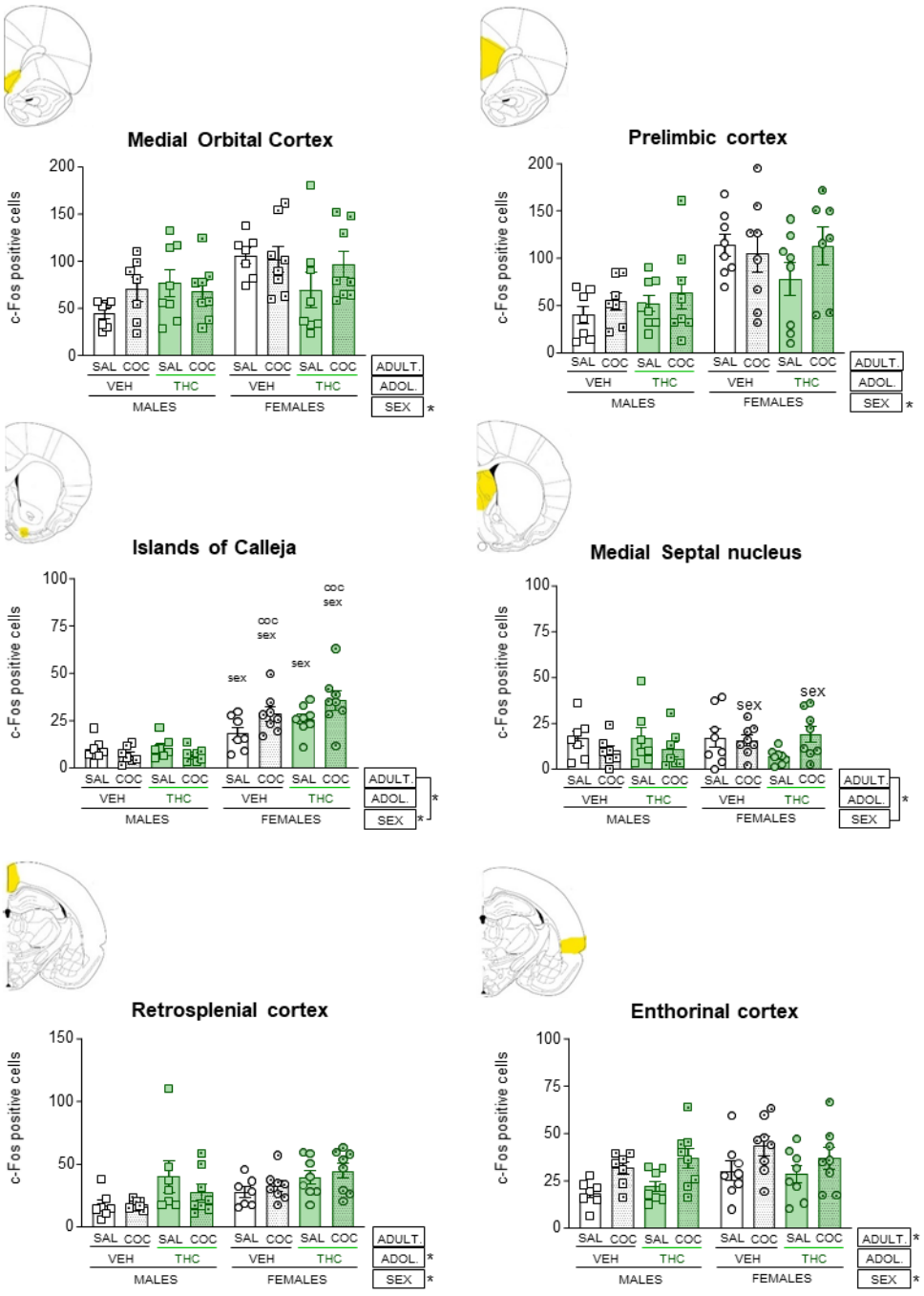


Figure 29. c-Fos immunohistochemistry (II). Brain areas showing significant effects Sex, Adolescent Treatment or Adult Treatment (cocaine injection), or interactions among them. Fos protein accumulation in response to a single i.p. cocaine (20 mg/kg) or saline injections (n=8 in all the groups). The graphs represent individual values (dots) and the mean ± SEM (lines). The main effects are indicated with "*" next to the name of the factors (ADULT, ADOL or SEX), and interactions between the factors are indicated with lines joining each factor participating in the interaction and the corresponding "*" ($p<0.05$) to the right. Significant results of the analysis of the simple effect of the interactions are indicated by "SEX", "THC", or "COC" over the experimental group: "SEX" represents differences with the corresponding group of the other sex, with the same adolescent treatment (THC or VEH) and the same adult treatment (cocaine or saline); "THC" represents differences with the corresponding VEH-adolescent treated group of the same sex and with the same adult exposure to cocaine or saline; "COC" represents differences with the corresponding saline group of the same sex and with the same adolescent treatment with THC or VEH.

IMMUNOHISTOCHEMISTRY FOR C-FOS - DISCUSSION

Cocaine-induced c-Fos accumulation in the motor cortex was potentiated in rats exposed to THC. To the best of our knowledge there is only one report that has studied the interactions between the cannabinoid system and cocaine in the motor cortex. This study suggested that cocaine exerted its effects on the morphology of the neurons in the motor cortex in a CB1-dependent manner, although only morphological parameters were studied in this area and no functional indices were provided (Ballesteros-Yáñez, Valverde, Ledent, Maldonado, & DeFelipe, 2007). The potentiation of cocaine-induced c-Fos accumulation in the motor cortex reported here is intriguing considering that it was previously indicated that adolescent exposure to a cannabinoid does not potentiate the locomotor actions of the drug, at least not in adult male rats (Kononoff et al., 2018; Scherma et al., 2020). The increased cellular activation induced by cocaine in the motor cortex could also be related to the rewarding actions of the drug. Indeed, there are data that suggest that the establishment of cocaine place preference is associated with increased c-Fos levels in the motor cortex, among other regions (Soderman & Unterwald, 2008). In addition, some data also suggest that chronic treatment with the cannabinoid agonist WIN during adolescence augments cocaine-induced conditioned place preference (Rodríguez-Arias et al., 2016), although it remains unclear if this effect persists until adulthood, which is certainly not the case with amphetamine-induced conditioned place preference (Keeley, Bye, Trow, Mcdonald, & Freels, 2018).

Cocaine-induced c-Fos accumulation in the motor cortex was potentiated in rats exposed to THC. To the best of our knowledge there is only one study of the interactions between the cannabinoid system and cocaine in the motor cortex, which suggested that cocaine exerted its effect on the morphology of the neurons in the motor cortex in a CB1-dependent manner. However, in this case only morphological parameters in this area were studied and no functional indices were provided (Ballesteros-Yáñez et al., 2007). The potentiation of cocaine-induced c-Fos accumulation in the motor cortex reported here is intriguing considering that previous reports indicate that adolescent exposure to cannabinoid does not potentiate the locomotor actions of the drug, at least not in adult male rats (Kononoff et al., 2018; Scherma et al., 2020). The increased cellular activation induced by cocaine in the motor cortex could also be related to the rewarding actions of the drug. Indeed, there are data that suggest that the establishment of cocaine place preference is associated with increased Fos levels in the motor cortex, among other regions (Soderman & Unterwald, 2008). In addition, there are data suggesting that chronic treatment with the cannabinoid agonist WIN during adolescence augments cocaine-induced conditioned place preference (Rodríguez-Arias et al., 2016), although it is currently unknown if this effect lasts until adulthood, which is certainly not the case with amphetamine-induced conditioned place preference (Keeley, Bye, Trow, Mcdonald, et al., 2018).

There was a higher accumulation of cFos induced by cocaine in the dorsomedial hypothalamic nucleus of THC females. Interestingly, this effect showed a trend that followed an opposite pattern to males. The sex-specific nature of the phenomenon could be related to the higher levels of CB_1 receptors found in this hypothalamic area of females, although there must be additional mechanisms governing this effect as there are other areas in which sex-differences in the expression of CB1 receptors are evident, such as the orbitofrontal cortex (Liu, Li, Zhao, Wang, & Wang, 2020) but where the results we obtained in the dorsomedial hypothalamic nucleus were not found. The increased reactivity of the dorsomedial hypothalamic neurons after exposure to cocaine may affect the cardiovascular effects of the drug (Stotz-Potter, Willis, & DiMicco, 1996) or its anorexigenic actions (Bellinger & Bernardis, 2002). In terms of this last phenomenon, people with stimulant use disorders are usually underweight, although the causes for this lower body weight are poorly understood (Verdejo-Garcia & Crossin, 2021). The activation of the dorsomedial hypothalamic nucleus by cocaine reported here may play a role in the aforementioned anorexic actions of the drug. For example, there is a recently identified population of TrkB-positive neurons that inhibit food intake upon activation (Houtz, Liao, An, & Xu, 2021). TrkB receptors are activated by BDNF, the levels of which increase after exposure to cocaine (Graham et al., 2007). Moreover, even if we do not have data on the regulation of BDNF levels in the hypothalamus by chronic adolescent THC exposure, it was previously shown that in other areas of the brain like the prefrontal cortex, BDNF levels increase in female rats with a history of THC exposure but decrease in males (Poulia et al., 2019).

If such effects also occur in the dorsomedial hypothalamic nucleus, this may suggest potential synergic effects converging on the BDNF-TrkB axis.

In addition to its role in energy regulation, it should be mentioned that the dorsomedial hypothalamic nucleus projects to brain regions critical for corticosteroid secretion (Thompson et al., 1996, Elmquist et al., 1998), a key element in resilience to stress and addiction (Srinivasan et al., 2013). Therefore, a differential response in the cocaine-induced stress response may occur in this nucleus, leading to the specific pattern of c-Fos levels we observed. However, even if they are mechanistically plausible, whether these aforementioned phenomena (cardiovascular actions, anorexic effects or stress reactivity) are actually potentiated in cannabinoid-exposed female rats remains to be determined.

The precise mechanisms by which our cannabinoid treatment might have provoked the cocaine-induced c-Fos responses in these two areas (motor cortex and dorsal hypothalamus) could be related to the enhanced activity of dopaminergic neurons induced by cocaine after adolescent cannabinoid exposure (Pistis et al., 2004). Alternatively, they may reflect the effects that such cannabinoid exposure during adolescence has on adult dopamine transporter levels, specifically in females (Alejandro Higuera-Matas et al., 2010), or other documented interactions between the endocannabinoid and dopamine systems (Behan et al., 2012; Renard et al., 2017; Zamberletti et al., 2012b). More recently, a series of molecular studies have shown that chronic cannabinoid exposure during adolescence reprograms the molecular and epigenetic responses to cocaine in the cortex, including histone hyperacetylation, chromatin accessibility, nucleosome positioning and ERK signalling (Scherma et al., 2020), all of which might result in the increased c-Fos levels reported here (Monje et al., 2005; Wang & Prywes, 2000).

APPENDIX F. FIRST AUTHOR PUBLICATION

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Cocaine-induced Fos expression in the rat brain: Modulation by prior Δ^9-tetrahydrocannabinol exposure during adolescence and sex-specific effects

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ABSTRACT

It has been suggested that cannabis consumption during adolescence may be an initial step to cocaine use in adulthood. Indeed, previous preclinical data show that adolescent exposure to cannabinoids (both natural and synthetic) potentiates cocaine self-administration in rats. Here we aimed at gaining a deeper understanding of the cellular activation patterns induced by cocaine as revealed by Fos imaging and how these patterns may change due to adolescent exposure to THC. Male and female Wistar rats were administered every other day THC (3 mg/kg i.p.) or vehicle from postnatal day 28–44. At adulthood (PND90) they were given an injection of cocaine (20 mg/kg i.p.) or saline and sacrificed 90 min later. Cocaine-induced Fos activation was measured by immunohistochemistry as an index of cellular activation. We found that cocaine-induced activation in the motor cortex was stronger in THC-exposed rats. Moreover, there was significant sex-dependent interaction between cocaine and adolescent THC exposure in the dorsal hypothalamus, suggesting that cocaine induced a more robust cellular activation in THC-exposed females but not in THC-treated males. Other THC- and cocaine-induced effects were also evident. These results add to the previous literature suggesting that the behavioral, cellular, molecular, and brain-activating actions of cocaine are modulated by early experience with cannabinoids and provide additional knowledge that may explain the enhanced actions of cocaine in rats exposed to cannabinoids during their adolescence.

1. Introduction

Cannabis consumption during adolescence may be associated with the use of other drugs of abuse later in life, including cocaine (Kandel et al., 1992; Lynskey and Agrawal, 2018; Mayet et al., 2016). There is preclinical evidence supporting the relationship between prior exposure to cannabinoids and alterations in the rewarding or activating effects of cocaine. For example, adolescent rats exposed to Δ^9-tetrahydrocannabinol (THC) showed increased locomotor responses to cocaine compared to chronic vehicle-injected controls (however, this was not observed when the THC treatment was administered during adulthood) (Dow-Edwards and Izenwasser, 2012). In addition, adolescent mice exposed to the cannabinoid agonist WIN 55512-2 showed potentiated conditioned place preference (CPP) to cocaine (Rodríguez-Arias et al., 2016). CB_1 and CB_2 receptors may mediate these interactions at the pharmacological level since they play opposite roles in the sensitization and CPP phenomena induced by cocaine (Lopes et al., 2020).

In spite of their interest, the aforementioned studies do not examine the long-term effects of adolescent exposure to cannabinoids on the actions of cocaine. Concerning this, other works have shown that adolescent exposure to cannabinoids increases cocaine conditioned place preference (Aldhafiri et al., 2019) or cocaine self-administration (Friedman et al., 2019; Higuera-Matas et al., 2008) at adulthood in animal models. In order to better understand this phenomenon, it is crucial to gain a more in-depth knowledge of how previous exposure to cannabinoids during adolescence modifies the effects of cocaine on the

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brain. As regards this, a recent study has shown that prior exposure to the cannabinoid agonist WIN 55, 512-2 during adolescence modifies the initial behavioral, molecular and epigenetic responses to cocaine (Scherma et al., 2020). However, even if the in-depth study of specific brain regions is of interest, a brain-wide analysis of cocaine effects in adult animals preexposed to cannabinoid during their adolescence will result in a more complete picture of how the actions of cocaine on the brain differ as a function of prior exposure to THC during adolescence. In this regard, by using positron emission tomography -PET- our group showed that exposure to the synthetic cannabinoid CP 55940 during adolescence modifies the effects of cocaine on adult brain metabolism in a sex-dependent manner (Higuera-Matas et al., 2011). Despite the advantages of the in vivo brain imaging provided by the PET technology, the spatial resolution of the technique is limited. The use of the immediately early gen *cfos* and its protein product Fos as a proxy of cellular activation induced by acute injection of cocaine is an interesting alternative approach (McReynolds et al., 2018). Several studies have examined the effects of exposure to cocaine on Fos levels in the brain. Particularly relevant to the present work are two reports showing that the Fos response to cocaine is antagonistically modulated by CB_1 and CB_2 cannabinoid receptors (Gobira et al., 2019; Lopes et al., 2020). Thus, these studies hint at the possibility that prior exposure to THC during adolescence could indeed modify cocaine-induced Fos levels, the main question that we want to answer with the present experiments.

Most of the previously published papers regarding Fos levels, cocaine and cannabinoids have focused on male rats. However, there are multiple sex-specific effects of cannabinoids (Bara et al., 2018; Blanton et al., 2021; Borsoi et al., 2019; de Salas-Quiroga et al., 2020; Farquhar et al., 2019; Liu et al., 2020; Morena et al., 2021; Rubino and Parolaro, 2015; Sholler et al., 2020; Viveros et al., 2011) and, in addition, we have previously shown a sex-specific potentiating effect of cannabinoid exposure during adolescence on adult cocaine self-administration (it only occurs in the females) (Higuera-Matas et al., 2008). Therefore, a second goal of the present work was to study the potential sex-specific differences in the interactions between cocaine effects and adolescent exposure to THC.

2. Results

2.1. Interactions between adolescent cannabinoid and adult cocaine exposures

The main goal of this work was to explore potential modulations of cocaine effects in Fos levels by previous exposure to THC during adolescence. These modulations are reflected in the statistical interactions between the Adolescent Treatment (THC exposure) and Adult Treatment (cocaine injection) and the interactions between these two factors and the Sex of the animals. As regards this, we found a significant Sex × Adolescent Treatment × Adult Treatment triple interaction ($F_{1,49} = 7.148$; $p = 0.01$; $\eta^2_p = 0.13$) in the dorsomedial nucleus of the hypothalamus. The analysis of this interaction revealed a trend for cocaine to induce a significantly higher Fos accumulation (compared to saline-injected animals) in THC-exposed females, while this effect was absent in the males (see Table 1). Indeed, THC/COC female rats had higher Fos levels in this nucleus than THC/SAL females (effect of Adult Treatment in THC-exposed females: $F_{1,49}$ $p = 0.055$; $\eta^2_p = 0.08$), and this difference was absent in VEH/COC males as compared to VEH/SAL male rats (lack of effect of Adult Treatment in VEH-exposed males $F_{1,49} = 0.046$; $p = 0.766$). In addition, cocaine-injected THC-males showed lower numbers of Fos^+ cells than cocaine-injected VEH-males (reflected in the simple effect of Adult Treatment in THC-exposed males $F_{1,49} = 5.441$; $p = 0.015$; $\eta^2_p = 0.10$, that was absent in VEH-exposed males) (see Table 1 and Fig. 1A, B).

In the motor cortex, the prior exposure to THC during adolescence potentiated the cellular activation induced by cocaine in adult animals. There were no sex-specificity in this interaction. Indeed, there was a significant Adolescent Treatment × Adult Treatment interaction in this region ($F_{1,53} = 5326$; $p = 0025$ $\eta^2_p = 0.09$), and the increase in Fos levels induced by cocaine (Adult Treatment) was higher in THC-exposed rats (regardless of the Sex) than in VEH-treated animals ($\eta^2_p = 0.35$ vs $\eta^2_p = 0.12$) (see Table 1 and Fig. 1C,D).

2.2. Cocaine-related effects

There was a significant effect of the Adult Treatment factor in the cingulate, insular and entorhinal cortices, and in the amygdala, whereby cocaine-injected animals showed higher levels of Fos^+ cells than saline-injected rats, irrespective of the Adolescent Treatment or the Sex (see Tables 1 and 2 and Figs. 1 and 2). In the lateral orbitofrontal cortex, in addition to the Adult Treatment effect, we obtained a significant Adult Treatment × Sex interaction ($F_{1,52} = 8.326$; $p = 0.006$; $\eta^2_p = 0.14$), which showed that the effects of cocaine were significant only in the males (see Table 2 and Fig. 2). There were some interactive effects in the medial septal nucleus between the Sex and cocaine exposure (Adult Treatment), but the analysis of this interaction showed no simple effects of cocaine either in males or females (see Table 2). In the Islands of Calleja, there was a significant Adult Treatment × Sex interaction ($F_{1,52} = 9.211$; $p = 0.004$; $\eta^2_p = 0.15$), showing that cocaine induced a significant elevation of the expression of the Fos protein only in the females (significant effect of the Adult Treatment in the females but not in males) (See Table 2 and Fig. 2). Lastly, there was an Adult Treatment × Sex interaction in the piriform cortex ($F_{1,53} = 4.447$; $p = 0.04$; $\eta^2_p = 0.08$) which only resulted in a significant effect of Sex among cocaine-injected animals (suggesting that Fos levels in this structure were higher in cocaine injected males and females but not among saline-injected males or females) (see Table 2 and Fig. 2).

2.3. Effects of adolescent exposure to THC

Adolescent exposure to THC increased the mean Fos accumulation (irrespective of cocaine exposure -Adult Treatment- or Sex) in the retrosplenial cortex (significant effect of Adolescent Treatment: $F_{1,53} = 11.593$; $p = 0.001$; $\eta^2_p = 0.18$). In the somatosensory and piriform cortices we found significant Sex × Adolescent Treatment interactions ($F_{1,51} = 6.313$; $p = 0.015$; $\eta^2_p = 0.11$ and $F_{1,53} = 4.405$; $p = 0.049$; $\eta^2_p = 0.07$; respectively), which, upon further analysis, revealed higher levels of Fos protein in THC-males than in their VEH-exposed controls (see Table 2 and Fig. 2).

2.4. Main effects of SEX

We have also observed general main effects of the Sex of the animals in the medial orbital, prelimbic, cingulate, motor, insular, entorhinal and retrosplenial cortices and in the Islands of Calleja with females showing higher values than males (see Tables 1 and 2 and Figs. 1 and 2).

3. Discussion

We have provided evidence for a modulation of cocaine-induced activation (as indexed by Fos protein levels) in the motor cortex and dorsal hypothalamus of adult rats with chronic exposure to THC during adolescence. In the case of the dorsal medial nucleus of the hypothalamus, this modulation was evident in female but not male rats while in the motor cortex, cocaine-induced activation was more potent in THC-exposed animals regardless of their sex. We also report differences in cocaine-induced Fos accumulation in different areas of the brain and the cocaine-independent long-term effects of THC on cellular activation.

1. Modulation of cocaine-induced Fos activation by chronic adolescent THC exposure.

Cocaine-induced Fos accumulation in the motor cortex was

Table 1
FOS Expression After Acute Cocaine i.p. Challenge.

			Statistic		Effect		Males								Females							
							VEH				THC				VEH				THC			
							SALINE			COCAINE	SALINE			COCAINE	SALINE			COCAINE	SALINE			COCAINE
Area	p	Effect	value	df1	df e	size	1-β	MEAN	SEM	N	MEAN	SEM	N	MEAN	SEM	N	MEAN	SEM	N	MEAN	SEM	N	MEAN	SEM	N
Motor cortex	** 0.001	SEX	F= 13.676	1	53	0.21	0.95	42.1	13.3	8	146.3	32.9	7	43.4	11.6	8	250.7	33.4	8	161.9	26.5	7	190.0	21.7	8	105.5	18.3	7	250.5	52.8	8
	** 0.000	ADULT TMT	F= 42.833	1	53	0.45	1.00																							
	* 0.011	SEX * ADULT TMT	F= 7.029	1	53	0.12	0.74																							
	** 0.000	COCAINE effects in MALE	F= 43.048	1	57	0.42	1.00																							
	** 0.009	COCAINE effects in FEMALE	F= 7.369	1	57	0.11	0.73																							
	** 0.000	SEX effects in SALINE	F= 18.211	1	57	0.24	0.99																							
	ns 0.548	SEX effects in COCAINE	F= 0.365	1	57	0.01	0.09																							
	* 0.025	ADOL TMT * ADULT TMT	F= 5.326	1	53	0.09	0.62																							
	** 0.004	COCAINE effects in VEH	F= 9.204	1	55	0.12	0.79																							
	** 0.000	COCAINE effects in THC	F= 38.931	1	55	0.35	1.00																							
	ns 0.523	SEX effects in SALINE	F= 0.412	1	57	0.01	0.10																							
	* 0.042	SEX effects in COCAINE	F= 4.317	1	57	0.07	0.53																							
Dorsomedial hypothalamic nucleus	* 0.010	SEX * ADOL TMT * ADULT TMT	F= 7.148	1	49	0.13	0.75	20.9	3.1	5	30.9	16.9	8	26.6	5.9	7	14.0	13.5	8	16.9	2.9	7	14.7	4.2	7	15.4	3.9	8	29.0	6.8	7
	ns 0.195	COCAINE in MALE*VEH	F= 0.531	1	49	0.01	0.11																							
	ns 0.076	COCAINE in MALE*THC	F= 6.355	1	49	0.12	0.70																							
	ns 0.766	COCAINE in FEMALE*VEH	F= 0.046	1	49	0.00	0.06																							
	t 0.055	COCAINE in FEMALE*THC	F= 3.970	1	49	0.08	0.50																							
	ns 0.470	THC effects in MALE*SALINE	F= 0.258	1	49	0.01	0.08																							
	* 0.015	THC effects in MALE*COCA	F= 5.441	1	49	0.10	0.63																							
	ns 0.832	THC effects in FEMALE*SALINE	F= 2.601	1	49	0.05	0.35																							
	t 0.052	THC effects in FEMALE*COCA	F= 4.669	1	49	0.09	0.56																							
	ns 0.613	SEX effects in VEH *SALINE	F= 1.730	1	49	0.03	0.25																							
	* 0.024	SEX effects in VEH*COCAINE	F= 5.280	1	49	0.06	0.63																							
	ns 0.113	SEX effects in THC*SALINE	F= 0.089	1	49	0.00	0.06																							
	* 0.036	SEX effects in THC*COCAINE	F= 3.852	1	49	0.07	0.49																							

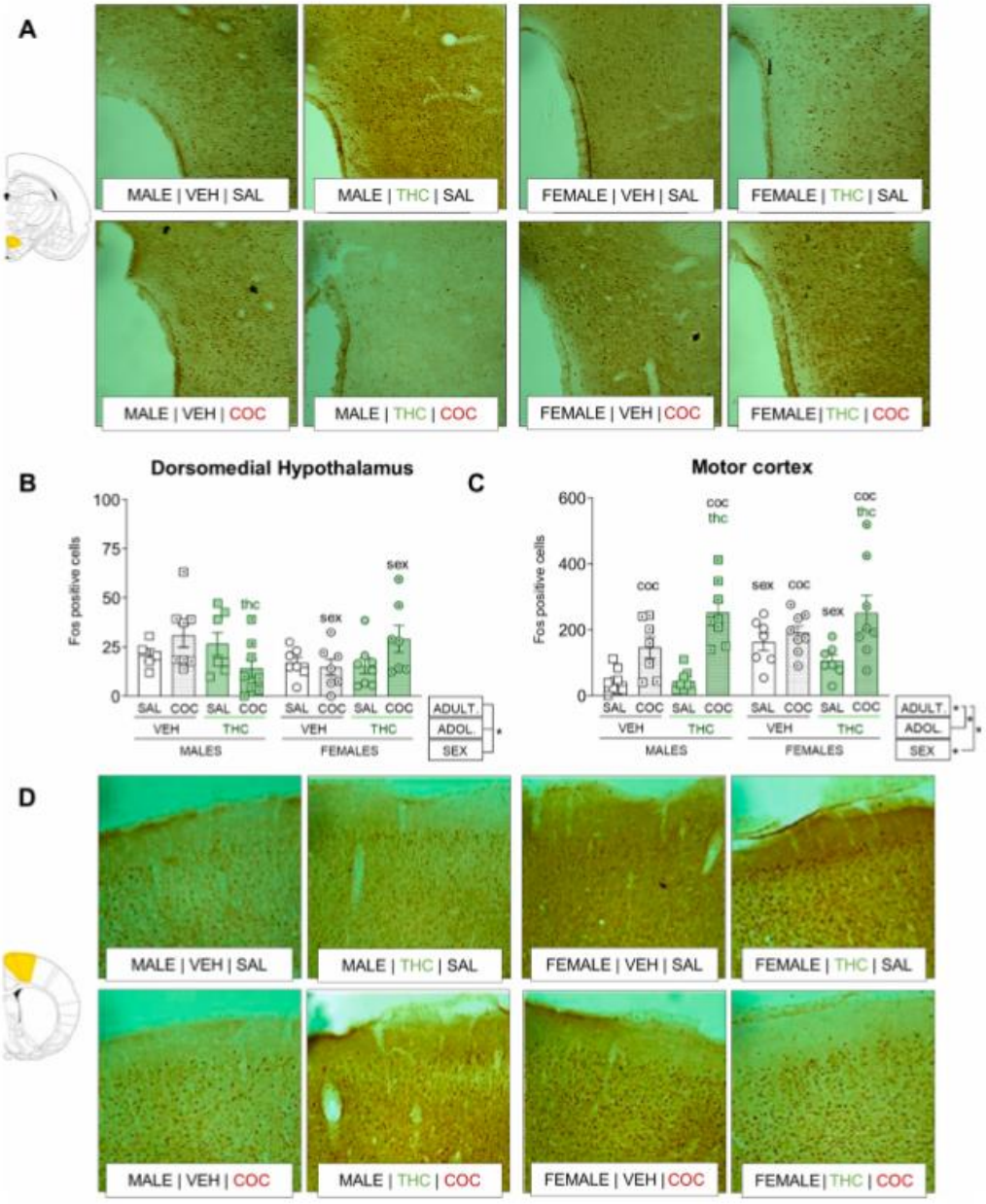

Fig. 1. Fos protein accumulation in response to a single i.p. cocaine (20 mg/kg) or saline injections. n = 8 in all the groups. Graphs represent individual values (dots) and Mean ± SEM (lines). Main effects are indicated with "*" next to the name of the factors (ADULT, ADOL or SEX); interactions between factors are indicated with lines joining each factor participating in the interaction and the corresponding "*" ($p < 0.05$) to their right; significant results of the analysis of the simple effect of the interactions are indicated with "sex", "thc" or "coc" over the experimental group. "sex" stands for differences with the corresponding group of the other sex, of the same adolescent treatment (THC or VEH) and same adult treatment (cocaine or saline); "thc" stands for differences with the corresponding VEH-adolescent treated group of the same sex and same adult exposure to cocaine or saline; "coc" stands for differences with the corresponding saline group of the same sex and same adolescent treatment with THC or VEH. A) Representative pictures of Fos accumulation in response to acute i.p. cocaine in the dorsomedial hypothalamic nuclei. B) Fos expression in the dorsomedial hypothalamic nuclei. C) Fos expression in the motor cortex. D) Representative pictures of Fos expression in response to acute i.p. cocaine in the motor cortex.

Table 2
Fos Expression After Acute Cocaine i.p. Challenge.

									Males												Females									
									VEH						THC						VEH						THC			
				Statistic		Effect			SALINE			COCAINE			SALINE			COCAINE			SALINE			COCAINE			SALINE			COCAINE		
Area		p	Effect	value	df1	df e	size	1-β	MEAN	SEM	N	MEAN	SEM	N	MEAN	SEM	N	MEAN	SEM	N	MEAN	SEM	N	MEAN	SEM	N	MEAN	SEM	N	MEAN	SEM	N
Medial Orbital	**	0.003	SEX	F= 9.602	1	53	0.15	0.86	44.3	5.4	7	70.5	12.6	7	76.8	14.0	8	68.1	10.6	8	105.9	8.5	7	102.3	13.5	8	69.4	18.8	8	97.0	14.0	8
Prelimbic cortex	**	0.000	SEX	F= 20.458	1	53	0.28	0.99	39.6	9.7	7	54.3	9.4	7	51.9	8.9	8	63.1	17.1	8	106.1	10.2	7	121.3	19.2	8	71.8	18.4	8	110.5	17.6	8
Lateral Orbital cortex	**	0.000	ADULT TMT	F= 20.582	1	52	0.28	0.99	18.5	6.2	7	97.6	59.8	7	37.4	12.3	8	72.4	23.0	8	62.5	9.9	6	85.3	14.0	8	52.2	10.7	8	59.8	8.7	8
	**	**0.006**	**SEX * ADULT TMT**	**F= 8.326**	**1**	**52**	**0.14**	**0.81**																								
	**	0.000	COCAINE effects in MALE	F= 26.073	1	56	0.32	1.00																								
	ns	0.223	COCAINE effects FEMALE	F= 1.520	1	56	0.03	0.23																								
	**	0.004	SEX effects in SALINE	F= 9.277	1	56	0.14	0.85																								
	ns	0.437	SEX effects in COCAINE	F= 0.614	1	56	0.01	0.12																								
Cingulate	*	0.042	SEX	F= 4.342	1	0	0.08	0.53	27.0	8.0	7	94.9	31.4	7	25.2	5.5	8	70.4	12.4	8	75.3	11.1	7	88.3	11.5	7	43.1	8.1	8	94.9	13.7	8
	**	0.000	ADULT TMT	F= 19.341	1	0	0.27	0.99																								
Amygdala	*	0.015	ADULT TMT	F= 6.272	1	53	0.11	0.69	18.8	8.2	7	53.7	44.3	7	37.2	6.9	8	57.7	41.3	8	35.2	7.6	7	51.9	7.7	8	42.3	8.7	8	42.3	7.3	8
Insular cortex	**	0.009	SEX	F= 7.292	1	54	0.12	0.76	15.8	3.8	7	26.8	8.5	7	18.1	3.0	8	28.8	10.3	8	29.4	7.0	8	36.3	6.4	8	26.8	7.4	8	37.4	4.9	8
	*	0.011	ADULT TMT	F= 6.851	1	54	0.11	0.73																								
Entorhinal cortex	*	0.026	SEX	F= 5.204	1	54	0.09	0.61	18.3	2.6	7	31.4	3.3	7	21.8	2.8	8	36.6	5.4	8	30.1	5.2	8	43.2	5.2	8	28.2	4.7	8	36.6	5.8	8
	**	0.000	ADULT TMT	F= 14.179	1	54	0.21	0.96																								
Media Septal nucleus	*	**0.047**	**SEX * ADULT TMT**	**F= 4.159**	**1**	**56**	**0.07**	**0.52**	16.5	4.2	#	9.9	7.6	7	16.8	6.0	#	10.6	10.4	7	16.9	5.2	8	15.5	2.9	8	6.8	1.4	#	18.9	4.5	8
	ns	0.135	COCAINE effects in MALE	F= 2.295	1	56	0.04	0.32																								
	ns	0.181	COCAINE effects FEMALE	F= 1.833	1	56	0.03	0.27																								
	ns	0.245	SEX effects in SALINE	F= 1.380	1	56	0.02	0.21																								
	ns	0.092	SEX effects in COCAINE	F= 2.947	1	56	0.05	0.39																								
Periaqueductal gray	ns	0.978	Corrected model	F= 0.223	7	47	0.03	0.11	10.6	4.5	5	19.2	4.9	7	15.8	9.3	7	15.0	6.3	8	19.2	5.0	6	17.2	7.6	6	14.4	5.8	8	21.4	8.4	8
VTA	ns	0.777	Corrected model	F= 0.568	7	41	0.09	0.22	15.3	3.2	4	16.2	7.5	6	16.0	5.6	7	13.6	11.2	6	12.1	2.9	5	10.6	2.3	7	14.1	2.6	7	17.6	1.8	7
Retrospenial cortex	**	0.003	SEX	F= 9.873	1	53	0.16	0.87	17.6	4.0	7	18.2	1.2	8	40.2	12.4	7	27.9	6.3	8	28.1	4.3	7	33.0	4.2	8	39.7	5.5	8	44.9	6.0	8
	**	0.001	ADOL TMT	F= 11.592	1	53	0.18	0.92																								
Somatosensory cortex	*	0.045	ADOL TMT	F= 4.205	1	51	0.08	0.52	34.8	7.8	7	31.1	5.0	8	69.0	14.5	7	54.3	10.1	8	34.0	6.8	7	45.6	7.4	8	35.0	4.5	7	36.9	4.7	7
	*	**0.015**	**SEX * ADOL TMT**	**F= 6.313**	**1**	**51**	**0.11**	**0.69**																								

(continued on next page)

Table 2 (*continued*)

Area		p	Effect	Statistic value	df1	df e	Effect size	1-β	Males VEH SALINE MEAN	SEM	N	Males VEH COCAINE MEAN	SEM	N	Males THC SALINE MEAN	SEM	N	Males THC COCAINE MEAN	SEM	N	Females VEH SALINE MEAN	SEM	N	Females VEH COCAINE MEAN	SEM	N	Females THC SALINE MEAN	SEM	N	Females THC COCAINE MEAN	SEM	N
	**	0.002	THC effects in MALE	F= 10.585	1	51	0.17	0.89																							
	ns	0.747	THC effects in FEMALE	F= 0.105	1	51	0.00	0.06																							
	ns	0.337	SEX effects in VEH	F= 0.939	1	55	0.02	0.16																							
	*	0.011	SEX effects in THC	F= 7.009	1	55	0.11	0.74																							
Piriform cortex	**	0.003	ADOL TMT	F= 9.466	1	53	0.15	0.86	28.3	4.3	7	25.2	11.5	8	64.6	10.8	7	43.6	21.6	8	45.6	8.4	7	47.6	8.3	8	41.5	6.3	8	65.3	13.0	8
	*	**0.049**	**SEX*ADOL TMT**	**F= 4.056**	**1**	**53**	**0.07**	**0.51**																							
	**	0.001	THC effects in MALE	F= 11.760	1	57	0.17	0.92																							
	ns	0.468	THC effects in FEMALE	F= 0.534	1	57	0.01	0.11																							
	*	0.010	SEX effects in VEH	F= 7.111	1	57	0.11	0.75																							
	ns	0.965	SEX effects in THC	F= 0.002	1	57	0.00	0.05																							
	*	**0.040**	**SEX * ADULT TMT**	**F= 4.447**	**1**	**53**	**0.08**	**0.54**																							
	ns	0.168	COCAINE effects in MALE	F= 1.948	1	57	0.03	0.28																							
	ns	0.185	COCAINE effects FEMALE	F= 1.804	1	57	0.03	0.26																							
	ns	0.853	SEX effects in SALINE	F= 0.035	1	57	0.00	0.05																							
	*	0.011	SEX effects in COCAINE	F= 6.835	1	57	0.11	0.73																							
Islands of Calleja	**	0.000	SEX	F= 73.145	1	52	0.58	1.00	9.4	2.2	7	6.6	1.6	7	10.4	2.1	7	6.7	1.2	8	18.1	3.4	7	28.3	3.6	8	25.6	2.7	8	35.3	5.1	8
	**	**0.004**	**SEX * ADULT TMT**	**F= 9.211**	**1**	**52**	**0.15**	**0.85**																							
	ns	0.316	COCAINE effects in MALE	F= 1.026	1	56	0.02	0.17																							
	**	0.002	COCAINE effects FEMALE	F= 10.048	1	56	0.15	0.88																							
	**	0.000	SEX effects in SALINE	F= 14.890	1	56	0.21	0.97																							
	**	0.000	SEX effects in COCAINE	F= 67.354	1	56	0.55	1.00																							

potentiated in THC-exposed rats. There is only one report that has studied the interactions between the cannabinoid system and cocaine in the motor cortex to the best of our knowledge. This study suggested that cocaine exerted its effect on the morphology of the neurons in the motor cortex in a CB_1-dependent manner; however, the authors only studied the morphological parameters in this area, and no functional indices were provided (Ballesteros-Yáñez et al., 2007). The potentiation of cocaine-induced cellular activation in the motor cortex here reported is intriguing considering that previous reports indicate that adolescent exposure to cannabinoid does not potentiate the locomotor actions of the drug, at least not in adult male rats (Kononoff et al., 2018; Scherma et al., 2020). The increased cellular activation induced by cocaine in the motor cortex could also be related to the rewarding actions of the drug. Indeed, some data suggest that the establishment of cocaine place preference is associated with increased Fos levels in the motor cortex, among other regions (Soderman and Unterwald, 2008). Also, previous data suggest that chronic treatment with the cannabinoid agonist WIN during adolescence augments cocaine-induced conditioned place preference (Rodríguez-Arias et al., 2016), however, it is currently unknown if this effect lasts until adulthood (it certainly does not, in the case of amphetamine-induced conditioned place preference (Keeley et al., 2018).

There was a higher cocaine-induced Fos accumulation (compared to the corresponding saline group) in THC females in the dorsomedial hypothalamic nucleus; interestingly, this effect showed a trend that followed an opposite pattern in the males. The sex-specific nature of the phenomenon could be related to the higher levels of CB_1 receptors found in females in this hypothalamic area. However, there must be additional interacting mechanisms governing this effect because there are other areas with sex-differences in the expression of CB_1 receptors, such as the orbitofrontal cortex (Liu et al., 2020) where we have not found the pattern of results obtained in the dorsomedial hypothalamic nucleus. The increased reactivity of the dorsomedial hypothalamic neurons after cocaine may affect the cardiovascular effects of the drug (Stotz-Potter et al., 1996) or its anorexigenic actions (Bellinger and Bernardis, 2002). Indeed, due to reasons that are not entirely clear, people with stimulant use disorders are usually underweight (Verdejo-García and Crossin, 2021). It is tempting to speculate that the activation of the dorsomedial hypothalamic by cocaine reported here may play a role in this last phenomenon. In support of this possibility, a recently identified population of TrkB-positive neurons in the dorsomedial hypothalamic nucleus has been shown to inhibit food intake upon activation (Houtz et al., 2021). TrkB receptors are activated by brain-derived neurotrophic factor (BDNF), which is has been shown to increase its levels after cocaine (Graham et al., 2007). Moreover, even if we do not have data on the regulation of BDNF levels in the hypothalamus by adolescent chronic THC exposure, a previous report has shown that in other areas of the brain, such as the prefrontal cortex, BDNF levels are increased in female rats with a history of THC exposure (while they were decreased in males) (Poulia et al., 2019). If this phenomenon also occurred in the dorsomedial hypothalamic nucleus, it would point to potential synergic effects between THC and cocaine that would converge on the BDNF-TrkB system to regulate food intake.

In addition to its role in energy regulation, it should be mentioned that the dorsomedial hypothalamic nucleus projects to brain regions critical for corticosteroid secretion (Thompson et al., 1996; Elmquist et al., 1998), which is a critical element in stress resilience and addiction (Srinivasan et al., 2013). Therefore, a differential cocaine-induced stress response may occur in this nucleus, leading to the specific pattern of Fos levels that we have obtained.

Of note, even if mechanistically plausible, whether these previously mentioned phenomena (cardiovascular actions, anorectic effects or stress reactivity) are actually potentiated in cannabinoid-exposed female rats remains to be determined.

The precise molecular mechanisms by which the cannabinoid treatment enables the reported modulation of cocaine-induced Fos responses could be related to an increased activity of dopaminergic neurons induced by cocaine after adolescent cannabinoid exposure (Pistis et al., 2004) or the effects that this cannabinoid exposure during adolescence has on adult dopamine transporter levels (specifically in the females) (Higuera-Matas et al., 2010). It could also involve other documented interactions between the endocannabinoid and dopamine systems (Behan et al., 2012; Renard et al., 2017; Zamberletti et al., 2012), or dopamine-independent mechanisms. For example, a series of molecular studies have shown that chronic cannabinoid exposure during adolescence reprograms the molecular and epigenetic responses to cocaine in the cortex, including histone hyperacetylation, chromatin accessibility, nucleosome positioning and ERK signalling (Scherma et al., 2020), which might result in the increased Fos levels reported here (Monje et al., 2005; Wang and Prywes, 2000).

2. Cocaine-induced cellular activation

There were significant increments in Fos accumulation after cocaine (as compared to the saline condition) in several areas of the brain, such as the lateral orbitofrontal, cingulate, motor, insular and entorhinal cortices, the amygdala or the Islands of Calleja. Prior studies have also documented increased Fos levels in cortical areas after cocaine treatments, but they did not differentiate across cortical regions (Zhuang et al., 2000), so it is difficult to compare these results with the ones presented here. Concerning this issue, other studies that differentiated across specific cortical territories did not find changes in Fos levels in the cingulate, medial prefrontal or orbitofrontal cortices in response to cocaine (Zlebnik et al., 2014). The dose of cocaine was slightly lower than the one that we used here (15 mg/kg vs 20 mg/kg), which may explain, to some extent, these divergencies. The reported effects of acute cocaine on the orbitofrontal cortex may be the first steps of a series of cocaine-induced modifications of this structure that run in parallel with the development of cocaine addiction (Lucantonio et al., 2012). The actions of the drug on the cingulate cortex and the amygdala are also coincident with those reported with intravenous cocaine (Zahm et al., 2010) and are consistent with the well-known roles of these structures in different aspects of cocaine reward and addiction (D'Cunha et al., 2017; Ellgren et al., 2004; Lee et al., 2014; Liu et al., 2020; Pelloux et al., 2018, 2013; Puaud et al., 2021; Rich et al., 2020; Rodríguez-Arias et al., 2016; Zhuang et al., 2000; Zlebnik et al., 2014). Acute cocaine also elicited a significant Fos response in the entorhinal cortex, a structure that also exhibits Fos activity following relapse to cocaine by cocaine-associated cues (Kufahl et al., 2009), and also in the insular cortex, which is also involved in different aspects of cocaine-induced reward and relapse (Kufahl et al., 2009; Pelloux et al., 2018; Rotge et al., 2017). The significance of the effects of cocaine on the Islands of Calleja is less clear but this region has been shown to activate during withdrawal from self-administered cocaine in rats (Hammer et al., 1993) and is rich in D3 dopamine receptors (Stößel et al., 2017), suggesting a potential involvement in the actions of cocaine.

3.1. Long-lasting actions of THC on Fos levels

THC exposure during adolescence also had long-lasting effects on Fos accumulation in the piriform and somatosensory cortices in the males and, regardless of the sex, in the retrosplenial cortex. No effects were found in other cortical areas such as the prelimbic cortex, which is in accordance with a previous data (Zamberletti et al., 2014).

In the retrosplenial cortex, the cannabinoid system is involved in fear memory consolidation, reconsolidation, and extinction (Sachser et al., 2015) so the lingering increase in cellular activity in this structure in THC-exposed rats may be related to the changes in fear-related behaviors observed in animals with adolescent cannabinoid exposure, probably in conjunction with stressful experiences (Saravia et al., 2019). The long-lasting effects on the piriform cortex reported here could be related to alterations in olfactory processes (Terral et al., 2020) or other high-

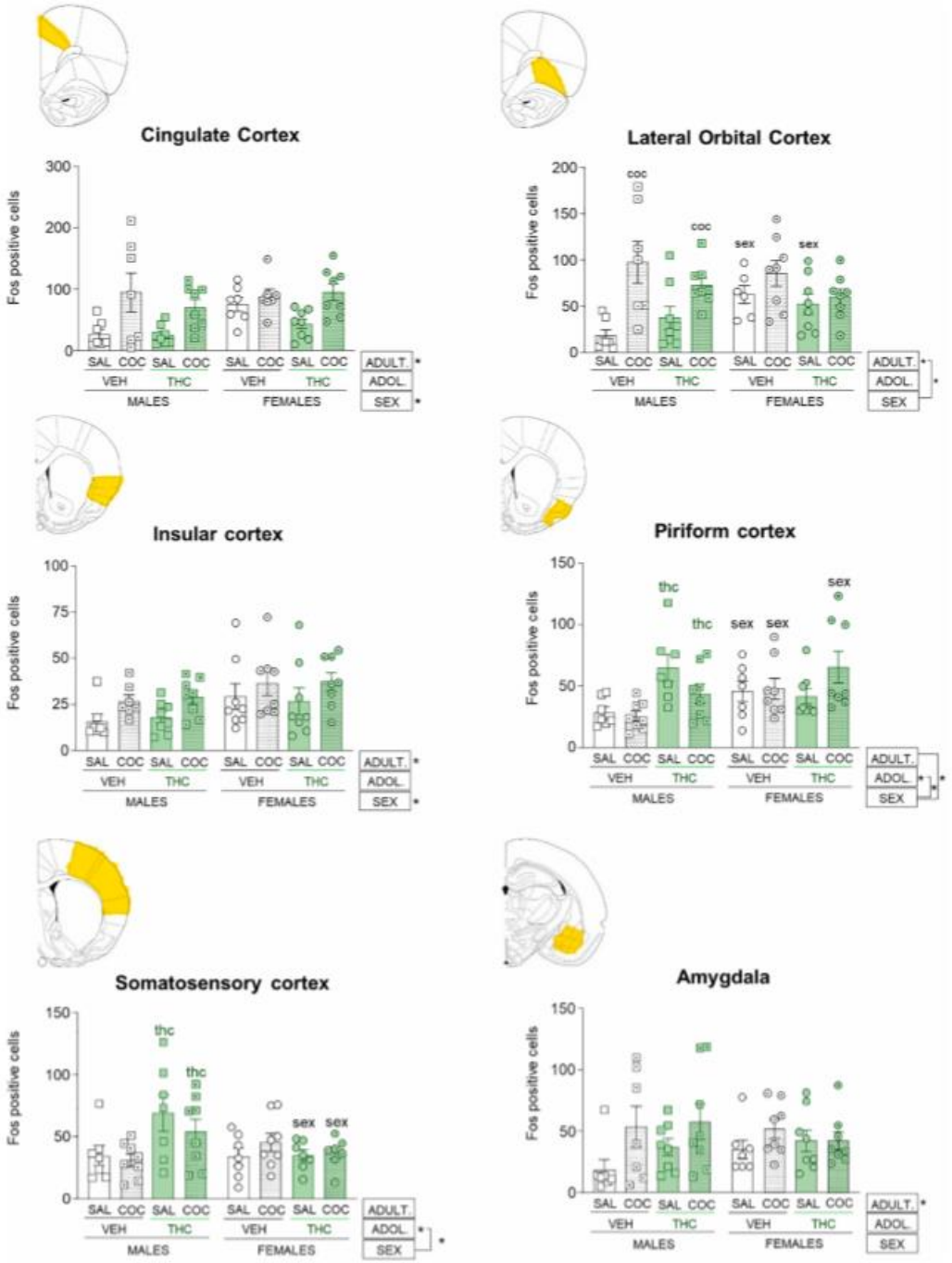

Fig. 2. Brain areas showing significant effects Sex, Adolescent Treatment or Adult Treatment (cocaine injection) or interactions among them. Fos protein accumulation in response to a single i.p. cocaine (20 mg/kg) or saline injections. n = 8 in all the groups. Graphs represent individual values (dots) and Mean ± SEM (lines). Main effects are indicated with "*" next to the name of the factors (ADULT, ADOL or SEX); interactions between factors are indicated with lines joining each factor participating in the interaction and the corresponding "*" ($p < 0.05$) to their right; significant results of the analysis of the simple effect of the interactions are

indicated with "sex", "thc" or "coc" over the experimental group. "sex" stands for differences with the corresponding group of the other sex, of the same adolescent treatment (THC or VEH) and same adult treatment (cocaine or saline); "thc" stands for differences with the corresponding VEH-adolescent treated group of the same sex and same adult exposure to cocaine or saline; "coc" stands for differences with the corresponding saline group of the same sex and same adolescent treatment with THC or VEH.

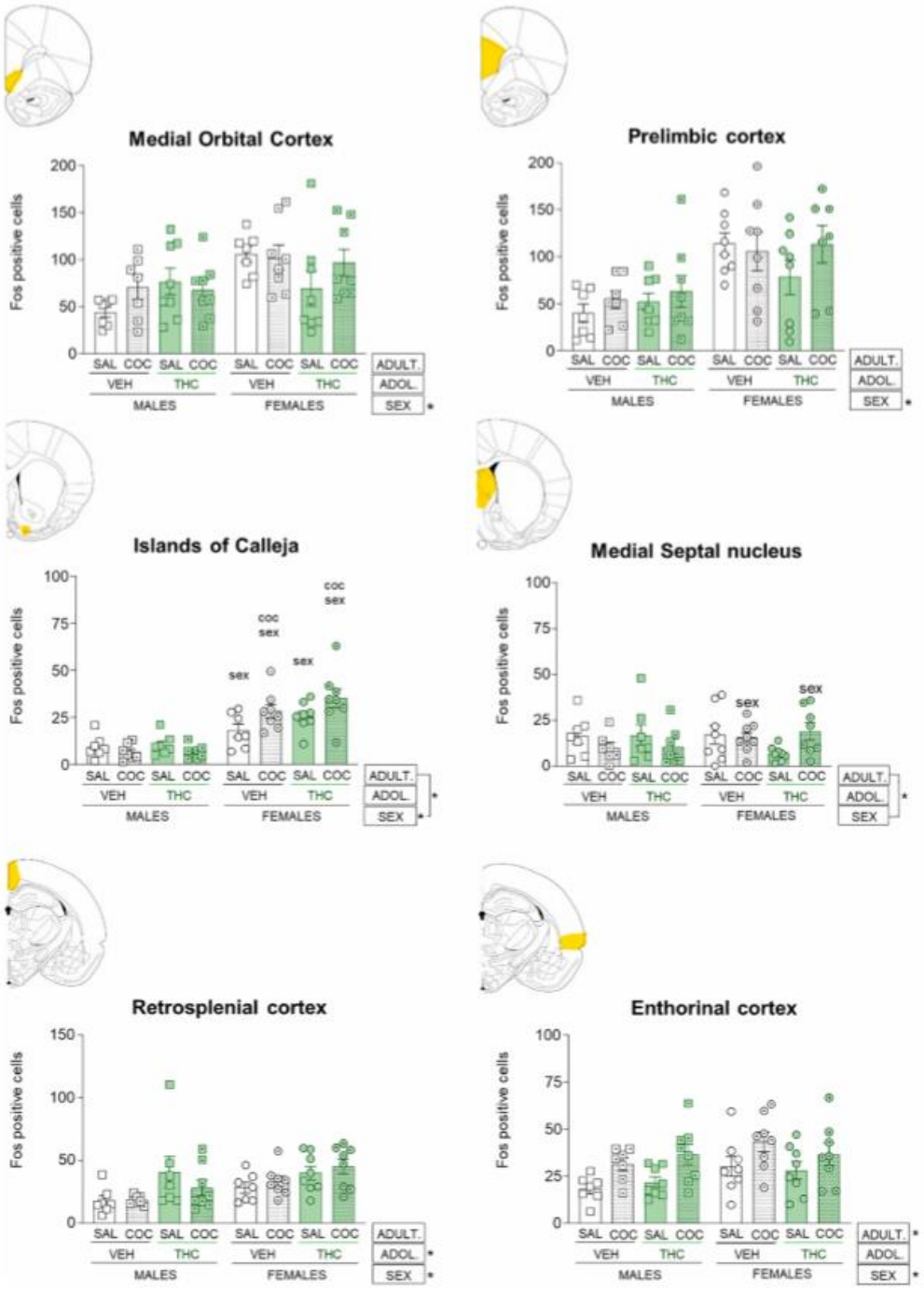

Fig. 2. (*continued*).

order functions such as the retrieval of conditioned odour preference (Terral et al., 2019). It is currently unknown if these processes are altered with adolescent cannabis consumption; however, there is evidence suggesting that cannabis use affects the levels of CB_1-$5HT_{2A}$ receptor heteromers in the olfactory neuroepithelium (Galindo et al., 2018), an effect that negatively correlates with the age of onset of cannabis use, which may point to altered olfactory processing. In spite of these data, it is currently unknown if these cognitive functions involving olfaction are indeed altered in adult individuals with an experience of cannabis consumption during adolescence, which is an attractive new venue for future research.

The increase in Fos+ cells in the somatosensory cortex could be related to altered perceptual functions involving learning or habitual processes. As regards this, it has been shown that the functional connectivity of this cortical area with the striatum is altered in cannabis users (Blanco-Hinojo et al., 2017).

The sex-specific effects of THC exposure on the piriform and somatosensory cortices are interesting and may be due to potential sex differences in the distribution of cannabinoid receptors during adolescence in these areas; however, to the best of our knowledge, no studies have examined these two cortical territories in terms of their sex-specific density of cannabinoids receptors.

4. Conclusion

In conclusion, the main results of this study suggest that a chronic adolescent treatment with THC modulates cocaine-induced cellular activation as indexed by the Fos protein in the adult brain in two specific brain regions, the motor cortex and the dorsomedial hypothalamic nucleus. These results open a venue for further research into the precise behavioral consequences of cocaine in adult animals with prior cannabinoid experience during adolescence, especially regarding the functions governed by these two brain territories.

5. Materials and methods

5.1. Animals

10 male and 10 female Wistar albino rats from Charles-River S.A. (Saint-Germain-sur-l'Arbresle, France) were mated (one male × one female) in our laboratory two weeks after their arrival, and their male and female offspring were used. On the day of birth (postnatal day –PND-0), the litters were sex-balanced and culled to a litter size of 10 ± 2 pups per dam. The animals were weaned at PND 22 and housed in plexiglass cages (2 or 3 sibling animals of the same sex and treatment per cage). All animals were maintained at a constant temperature (20 ± 2 °C) under a reverse 12-h/12-h dark/light cycle (lights on at 20:00 h), with free access to water and food (commercial diet for rodents SAFE. Augy, France), unless otherwise specified. Every attempt was made to minimize the pain and discomfort of the experimental animals, and all procedures were conducted as per the European Union legislation on the protection of animals used for scientific purposes (2010/63/EU Directive) and approved by the Ethics Board of the National University of Distance Learning.

5.2. Δ^9-Tetrahydrocannabinol exposure during adolescence

Δ^9-Tetrahydrocannabinol (THC) was purchased from THCPharm (Frankfurt, Germany) as resin, dissolved in pure ethanol (Merck) and stored at −30 °C in 0.5 mL aliquots in siliconized (Sigmacote; Merck) vials with a nitrogen-saturated atmosphere, protected from the light. Vehicle aliquots were treated in the same way, but no THC was added. Each day the final solution was prepared by adding to each aliquot, kolliphor (PEG-35 castor oil; Merk) and saline (0.9% NaCl solution; Vitulia, Spain) in a 1:1:18 proportion to a final volume of 10 mL. Animals were injected i.p. (2 mL/kg) every other day with THC (3 mg/kg; dose chosen according to the previous literature (Ellgren et al., 2007; Miller et al., 2019; Rubino et al., 2008, 2009; Tomasiewicz et al., 2012; Zamberletti et al., 2014) or vehicle from postnatal day 28–44. The ethanol concentration in both the THC and vehicle solution was 5% v/v, a dose of approximately 0.0789 g/kg that does not induce significant behavioral effects (Frye and Breese, 1981).

5.3. FOS immunohistochemistry after acute cocaine challenge

When animals reached PND 90, they were injected with cocaine (cocaine hydrochloride: 20 mg/kg i.p. Alcaliber, Spain) or saline (1 mL/kg i.p. 0.9% NaCl sterile solution; Vitulia, Spain). Ninety minutes later, they were anaesthetized with an injection of a 16% chloral hydrate solution (400 mg/kg i.p.) and transcardially perfused with PBS 0.1 M followed by 4% paraformaldehyde. Brains were then extracted and kept in fixing solution (4% paraformaldehyde) for 24 h and then transferred to a 30% sucrose solution for another 24 h. They were then kept at −20 °C in a glycerol/ethylene glycol (30%/30%) and (40%) PB 0.4M solution. The dose and timing of the sacrifice were chosen on the basis of previous literature (Young et al., 1991).

Fifty-microns coronal brain slices were obtained in a vibratome, transferred to a 30% glucose solution and kept at −4°C for 24 h. They were then transferred to a −20 °C freezer and kept in a glycerol/ethylene glycol and PB 0.4M solution until immunohistochemistry.

Free-floating tissue samples were washed in PBS 0.1M (3 successive 10 min rinses) and then incubated with 0.3% hydrogen peroxide v/v in PBS at room temperature for 30 min. They were then incubated 1 h in blocking solution (2% (v/v) normal goat serum + 0.3% (v/v) Triton-X 100 in PBST) and washed (PBST, 3 × 10 min). After this, they were incubated at 4 °C for 24 h in a rabbit Fos antibody (1:50000; Merck ABE457 Lot: 3116957). Sections underwent 3 × 10 min PBST rinses followed by a 1-hour incubation in biotinylated goat anti-rabbit IgG (1:200; Vectastain; BA-1000-1.5, LOT: ZE1218). After washing (PBS, 3 × 10 min), sections were incubated in ABC reagent (avidin–biotin complex kit, Vector Labs) at room temperature for 1 h, washed (PBS, 3 × 10 min), and reacted in diaminobenzidine (DAB; approximately 5 min) to reveal neurons labelled for Fos in brown. Sections were then washed (PBS, 3 × 10 min). Sections were allowed to dry and mounted on microscope slides, and coverslipped using DPX.

Tissue images were captured at 10X optical magnification using brightfield microscopy. The background was subtracted using a rolling ball procedure (radius 12.00), and the Fos positive cells per ROI were counted using the particle analysis option in ImageJ (size: 80–200, circularity: 0.50–1.00) by a researcher blind to the experimental conditions. Only those brain areas where consistent and detectable staining against the background was observed were included as ROIs in this study.

5.4. Statistical analysis

We used the IBM Statistics program (v. 25) to perform the statistical analyses. We used a 2 × 2 × 2 ANOVA design with three between-subjects factors: *Sex* (Male|Female), *Adolescent Treatment* (VEH|THC), and *Adult Treatment* (Saline|Cocaine) resulting in eight different groups: Male-VEH-Saline; Male-VEH-Cocaine; Male-THC-Saline; Male-VEH-Cocaine; Female-VEH-Saline; Female -VEH-Cocaine; Female -THC-Saline; Female-VEH-Cocaine. Significant interactions were followed using simple effects analysis. Significance level was set to $\alpha = 0.05$. The partial eta squared (η^2_p) statistic is provided as an index of the effect size, and statistical power is reported as 1-β. We provide all the details regarding F, p, η^2_p and 1-β in Tables 1 and 2 and indicate the statistics of the most critical interactions in the main text.

CRediT authorship contribution statement

Javier Orihuel: Investigation, Methodology, Visualization. **Laura Gómez-Rubio:** Investigation, Data curation. **Claudia Valverde:** Methodology. **Roberto Capellán:** Investigation. **David Roura-Martínez:** Methodology, Data curation, Supervision. **Marcos Ucha:** Methodology, Data curation. **Emilio Ambrosio:** Writing - review & editing, Supervision. **Alejandro Higuera-Matas:** Writing - original draft, Writing - review & editing, Supervision.

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