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Cycle XXXI

INTRAVENOUS SELF-ADMINISTRATION OF THE SYNTHETIC CANNABINOID RECEPTOR AGONIST JWH-018 IN ADOLESCENT MICE: NEUROBIOLOGICAL SEQUELAE IN ADULTHOOD

Scientific Disciplinary Sector

BIO/14 Farmacologia

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List of Abbreviations

2-AG	2-arachidonoylglycerol
2-AGE	2-arachidonyl-glyceryl ether
ABHD6	Alpha/beta domain-containing hydrolase 6
ADP	adenosine diphosphate
AEA	Arachidonoyl ethanol amide
ALDH1L1	Aldehyde dehydrogenase 1 L1
ANT	Adenine nucleotide transporter
AQP	Aquaporin receptor
ATP	Adenosine triphosphate
CA1	Cornu Ammonis1
CB1R	CB1 receptor
CBD	Cannabidiol
Cg	Cingulate cortex
CNS	Central nervous system
COMT	Cateto-O-methyl transferase
CoQ	Coenzyme Q10
COX-2	Cyclooxygenase-2
CPu	Caudate-putamen
CS	Citrate Synthase
EC	Endocannabinoid
eCBs	Endocannabinoids
DA	Dopamine
DAGL-aplha	Diacylglycerol lipase alpha
DAT	Dopamine transporter

DNTB	5,5′- dithiobis-(2-nitrobenzoic) acid
ER	Endoplasmic reticulum
FAAH	Fatty acid amide hydrolase
FADH2	Flavin adenine dinucleotide
FR	Fixed ratio
GABA	Gamma-aminobutyric acid
GASP1	GP-CR-associated sorting protein 1
GFAP	Glial fibrillary acidic protein
Glu	Glutamate
GLAST	Glu aspartate transporter
GLT-1	Glu transporter 1
GLUT	Glucose transport
GnRH	Gonadotropin-releasing hormone
GPCR	G-protein coupled receptors
GS	Glutamine synthetase
GTP	Guanosine-5'-triphosphate
GTPγS	Guanosine-5'-triphosphate-gamma-s
IBA-1	Ionized calcium binding adapter molecule 1
IL	Interleukin
IFN-gamma	Interferon-gamma cytokine
IM	Internal membrane
IVSA	Intravenous self-administration
KA	A-type K^+
Kir	Inwardly rectifying K^+
LTD	Long-Term Depression
LTP	Long-Term Potentiation

M1	Muscarinic receptor
MAGL	Monoacylglycerol lipase
MAO	Monoamine oxidase
MAPKs	Mitogen-activated protein kinases
mGluR5	Metabotropic glutamate receptor 5
mtCB1	Mitochondrial CB1R
NAc	Nucleus accumbens
NADA	N-arachidonoyl-dopamine
NADH	Nicotinamide adenine dinucleotide
NAPE	N-arachidonoyl phosphatydil ethanol
NAPE-PLD	N- arachidonoyl phosphatidyl ethanolamine-preferring phospholipase D
NDUFS4	NADH dehydrogenase iron-sulfur mitochondrial protein 4
NF-kbeta	Nuclear factor kappa-light-chain enhancer of activated B cells
NO	Nitric oxide
OM	Outer membrane
OXPHOS	Oxidative phosphorylation
PAMPs	Pathogen-activated molecular patterns
PEA	Palmitoylethanolamide
PDE	Phosphodiesterase
PFC	Prefrontal cortex
PLC-beta	Phospholipase C beta
PKA	Protein kinase A
PI3K	Phosphatidylinositide 3-kinases
Pi	Inorganic phosphate

PND	Post natal day
PPARs	Peroxysome proliferator activated receptors
PR	Progressive ratio
ROS	Reactive oxygen species
sAC	Soluble form of adenylyl cyclase
SCs	Synthetic cannabinoids
THC	Δ9-tetrahydrocannabinol
TNF-alpha	Tumor necrosis factor
TRL	Toll-like receptor
TRPV1	Transient receptor potential vanilloid receptor
VGCC	Voltage gated Ca^{2+} channels
VTA	Ventral tegmental area

INTRODUCTION

1.1 Cannabis and the endocannabinoid (EC) system

Despite the numerous illicit drugs available nowadays on the street and the Internet, cannabis persists as the most consumed illicit drug in Europe (EMCDDA, 2018) and United States (Zehra et al., 2018) (Fig.1). In Europe, resin (hashish) or herbal (marijuana) cannabis is commonly smoke with tobacco. However, in the last decade, the consume of cannabis has changed and new devices, like e-cigarettes, and new edible products appeared on the scene (EMCDDA, 2018).

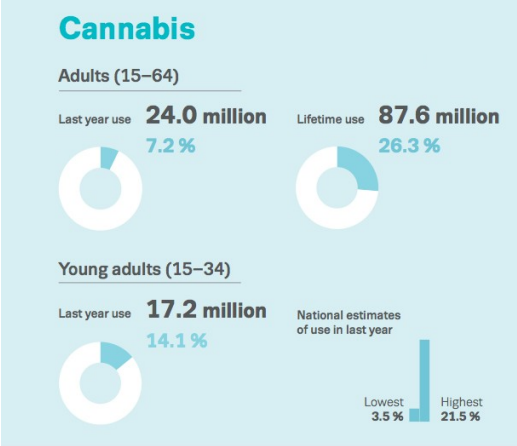

Fig.1 Cannabis use in Europe (Adapted from EMCDDA, 2018)

The active component of cannabis, Δ9-tetrahydrocannabinol (THC), was isolated for the first time in 1964 by Gaoni and Mechoulam (Gaoni and Mechoulam, 1964) and induces pharmacological and psychoactive effects that are mediated by the interaction with the EC system (Chadwick et al., 2013). In humans, THC may induce relaxation and euphoria but other effects such as anxiety and dysphoria are also reported (D'Souza et al., 2004; Wade et al., 2003). Like other cannabinoids, THC typically displays a biphasic activity depending upon the amount of drug consumed, with low and high doses inducing sometimes opposite effects (Mechoulam and Parker, 2013).

The EC system is involved in many functions including neurodevelopment processes, neuroplasticity, reward, learning, stress, emotional responses, memory and cognition (Chadwick et al., 2013; Katona and Freund, 2008; Kirschmann et al., 2017; Parsons and Hurd 2015; Tomas-Roig et al., 2016). The activity of the EC system is modulated by several components including at least two different types of CBRs (CB1Rs, CB2Rs), several endogenous ligands named endocannabinoids (eCBs), and a number of enzymes and metabolic pathways responsible for their biosynthesis, cellular uptake and inactivation (Katona and Freund, 2012) (Fig. 2).

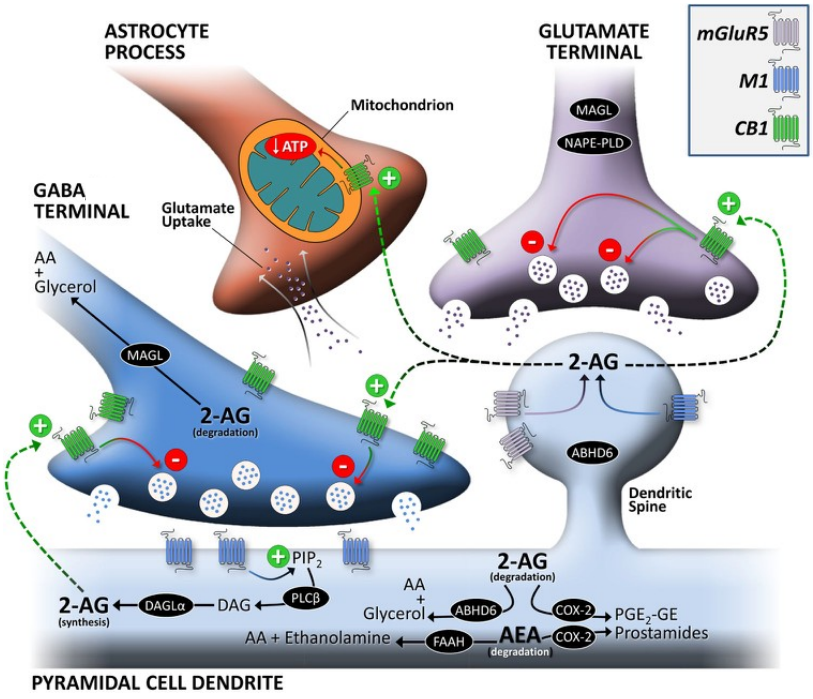

Fig. 2 Overview of the localization of EC system components at the synapse. Schematic of an inhibitory and excitatory terminal synapsing onto the dendritic shaft of a representative cortical principal neuron. Abbreviations: **ABHD6**, alpha/beta domain-containing hydrolase 6; **CB1Rs**; **COX-2**, cyclooxygenase-2; **DAGLα**, diacylglycerol lipase α; **M1**, M1 muscarinic receptor; **MAGL**, monoacylglycerol lipase; **mGluR5**, metabotropic glutamate receptor 5; **NAPE-PLD**, N- arachidonoyl phosphatidyl ethanolamine-preferring phospholipase D; **PLCβ**, phospholipase C β. The increased number of CB1 receptors on the gamma-aminobutyric acid (**GABA**) terminal represents the higher density of CB1Rs found on these axon terminals *(adapted from Lu and Mackie, 2015).*

The most important and better characterized eCBs are the 2-arachidonoylglycerol (2-AG) and the arachidonoyl ethanol amide (anandamide, AEA), followed by 2-

arachidonyl-glyceryl ether (noladin, 2-AGE), O-arachidonoyl-ethanolamine (virhodamine) and N-arachidonoyl-dopamine (NADA), which are endogenous lipids (Pertwee, 2015). The synthesis of AEA occurs in different pathways but the main substrate is N-arachidonoyl phosphatydil ethanol (NAPE), while 2-AG is produced from 2-arachidonoyl-containing phospholipids, in particular from arachidonoyl-containing phosphatidyl inositol biphosphate (Lu and Mackie, 2015). The eCBs are not stored in synaptic vesicles but rather synthesized on demand and act as retrograde synaptic messengers; unlike classic neurotransmitters, their action is mainly postsynaptic. Once released in the extracellular space, they primarily act at CB1Rs and CBR2s, with AEA, NADA and 2-AGE showing more selectivity for CB1Rs and virodhamide exhibiting higher affinity for the CB2R (Mukhopadhyay et al., 2002). Additionally, AEA and some of its analogues do not interact only with CB1Rs and CB2Rs but act also as agonists for the transient receptor potential vanilloid receptor (TRPV1) (Howlett et al., 2002; Pertwee 2004a; Pertwee and Ross, 2002; Ross, 2003; De Petrocellis and Di Marzo, 2010). Once exerted their actions, eCBs are quickly degraded in the cell: AEA is hydrolyzed to arachidonic acid and ethanol amine by fatty acid amide hydrolase (FAAH), while 2-AG can be hydrolyzed by FAAH and MAGL (Gian and Cravatt, 1997; Mechoulam and Parker, 2013).

CB1Rs and CB2Rs belong to the family of G-protein coupled receptors (GPCR), which couple mainly to Gi/o proteins. CB1Rs are located widely throughout the brain while CB2Rs are found primarily in the periphery (Galiegue et al., 1995, Herkemman et al.,1990, Thomas et al., 1992). The distribution of CB1Rs in the central nervous system (CNS) is correlated with its involvement in motor functions, cognition, memory, reward and analgesia. In particular, high density of CB1Rs has been found in the hippocampus,

cortex, basal ganglia, striatum and cerebellum (Marsicano and Lutz, 1999), while other brain regions like the medial hypothalamus, basal amygdala and solitary nucleus present a moderate density of CB1Rs (Herknham et al., 1990). Besides their presence in the CNS, levels of CB1Rs have also been found in peripheral organs/tissues, including fat, skeletal muscle, retina, cardiovascular tissue, reproductive system and gastrointestinal tract (Bouaboula et al., 1993; Croci et al., 1998; Galiegue et al., 1995; Pertwee, 1997; Sazbo et al., 2001). CB1Rs have been detected mainly at the terminals of central and peripheral neurons, where they usually mediate inhibition of the release of a range of excitatory and inhibitory neurotransmitters (Howlett et al., 2002; Pertwee and Ross, 2002; Szabo and Schlicker, 2005). CB1Rs have also been discovered on postsynaptic structures, in astrocytes, microglia and oligodendrocytes (Bosier et al., 2013; Rodriguez et al., 2001; Stella 2010;) which are also able to synthesize and degrade eCBs (Walter et al., 2002; Stella 2009).

CB2Rs are expressed predominantly in peripheral and in immune cells but have also been found in the gastrointestinal system and in numerous other cell types, including pulmonary endothelial cells (Zoratti et al., 2003), adipocytes (Roche et al., 2006), osteocytes, and osteoblasts (Ofek et al., 2006). While at first it was suggested that CB2Rs were absent from the brain (Brown et al., 2002; Griffin et al., 1999), more recent studies have now shown CB2Rs expression in diseased brain cells, including astrocytomas (Ellert-Miklaszewska et al., 2007; Sanchez et al., 2001), microglia and astrocytes in Alzheimer's disease (Benito et al., 2003; Esposito et al., 2007), and T cells, microglia, and astrocytes in multiple sclerosis (Benito et al., 2007).

Notably, several studies indicate that CB2Rs present in microglia are up-regulated in response to immune cell activation and inflammation (Klein, 2005; Stella, 2010).

CB2Rs activation mediates immunosuppressive effects, limits inflammation and is associated with tissue injury under several pathological conditions, including neurodegeneration (Pacher and Mechoulam, 2011). Accordingly, repeated administration of the CB2R agonist JWH-105 reduces the inflammatory response to MDMA and provides partial protection against 5-hydroxytriptamine neurotoxicity (Torres et al., 2010). More recently, expression of CB2Rs has been reported in the healthy CNS (Van Sickle et al., 2005) and in the brain they were detected not only in microglia but also in neurons, i.e., on DAergic neurons in the ventral tegmental area (VTA), suggesting a role of this receptor in pathological conditions, such as neuropsychiatric diseases and drug addiction (Ishiguro et al., 2007; Liu et al., 2017; Morales and Bonci, 2012; Onaivi et al., 2008; Onaivi et al., 2012; Viscomi et al., 2009). Activation of both CB1R and CB2R originates signals through G- protein-dependent and -independent processes (Pertwee 1997; Velasco et al., 2005). Both receptors are preferentially coupled to pertussis toxin-sensitive Gi/o proteins to inhibit adenylate cyclase and cyclic AMP–protein kinase A (PKA) signaling (Howlett et al., 1988). However, under certain condition, coupling to Gs or Gq/11 of CB1Rs has also been reported (Glass and Felder, 1997; Lauckner et al., 2005), while CB2Rs do not transduce signals through stimulatory Gs (Demuth and Molleman, 2006). Moreover, both CB1Rs and CB2Rs regulate the phosphorylation and activation of members of all families of mitogen-activated protein kinases (MAPKs) and carry signal transduction activating phosphatidylinositide 3-kinases (PI3K), but through different mechanisms (Howlett and Shim 2004). Activation of CB1Rs, but not of CB2Rs, inhibits N and P/Q types of voltage gated Ca^{2+} channels (VGCC) while positively regulates A-type K^+ (KA), and inwardly rectifying K^+ (Kir) channels. Beside the G-protein mediated signal transductions,

CB1Rs may also interact with several non-G proteins coupled patterns such as beta−arrestin, adaptor protein AP3, GP-CR-associated sorting protein 1 (GASP1) and the adaptor protein FAN, which activates neutral sphingomyelinase that mediates the generation of ceramide from sphingomyelin (Howlett et al., 2010; Smith et al., 2010). Moreover, although the activity of the eCBs is mainly mediated by CB1Rs and CB2Rs, several other GPCRs have been reported to be activated by cannabinoid drugs or by eCBs and related molecules, including GPR55, GPR18, and GPR119 (Morales et al., 2017). Finally, eCBs and SCs can also explicate their effects also through TRPV channels, or peroxysome proliferator activated receptors (PPARs) (Stella, 2010).

Brain eCBs signaling plays an important role in the motivation for natural rewards (e.g. palatable food, sex, play, social interaction) and modulates the rewarding effects of addictive drugs. Indeed, dysregulated eCB signaling alters synaptic plasticity and stress responsivity and may enhance negative emotional states and craving, all factors that promote addiction. The eCBs produced in the VTA and NAc play an important role in fine-tuning the activity of the VTA-NAc dopamine (DA) projection and its influence on approach and avoidance behaviors that govern reward acquisition (see Parsons and Hurd, 2015 for review).

Moreover, as mentioned above, recent studies have reported the expression of CB2Rs on DA neurons in the VTA, where they are thought to functionally modulate neuronal activity and DA-related behaviors, such as the hedonic effects of drugs of abuse (Zhang et al., 2017). According to this, CB2R antagonists were found to attenuate cocaine-induced reinstatement of drug-seeking behavior (Adamczyk et al., 2012) and cocaine-induced conditioned locomotion in rats (Blanco-Calvo et al., 2014).

1.2 Synthetic cannabinoids (SCs)

The discovery of the primary constituent of cannabis, THC, and of the CBRs were followed by the development of different SCs (such as CP55,940 and WIN 55,212-2) as pharmacological tools to better investigate the EC system and potential therapeutic agents. Indeed, SCs have been used for treatment of different human pathologies and some potent CB1R antagonists are still under clinical evaluation (De Luca and Fattore, 2018). However, while testing SCs improved understanding of the EC system and led to the development of new pharmacotherapeutics, new generation of SCs appeared on the global drug marked and are currently sold as herbal marijuana-legal alternatives. Along with synthetic cathinones, SCs are the most commonly used new psychoactive substances that are posing major medical and psychiatric risk worldwide (Weinstein et al., 2017). Commonly identified in Spice or K2 products (Fig. 3), SCs are typically sprayed on herbal mixtures, enfolded in foil packets with the indication "*not for human consumption*" and marketed via the Internet as "natural legal" highs (Fattore and Fratta, 2011).

Fig. 3 Spice drugs

SCs have been marketed for the first time in 2004, like legal alternative to cannabis (EMCDDA 2009) and became very popular since 2008, causing several cases of accidental or voluntary intoxication (Fisher, 2010; Gay, 2010; Kronstrand et al., 2011;

Simmons et al., 2011; Zimmermann et al., 2009) and being used also to alleviate irritability associated with abstinence or when unable to obtain cannabis (Gunderson et al., 2014). Unlike THC, SCs are extremely potent full agonists of the brain CBRs, show higher affinity for the CB1R and CB2R and induce long-lasting and more adverse effects than cannabis (Cohen and Weinstein, 2018). Use of SCs is increasing worldwide, particularly among adolescents (Ninnemann et al., 2017; Palamar et al., 2017), and represents a serious health problem, both in Europe (EMCDDA 2018) and the USA (https://ndews.umd.edu/resources/synthetic-cannabinoids). Recently, it has become easier to buy SCs through the web, by using the dark net or social networks as sales channels (Miliano et al., 2018; EMCDDA 2018).

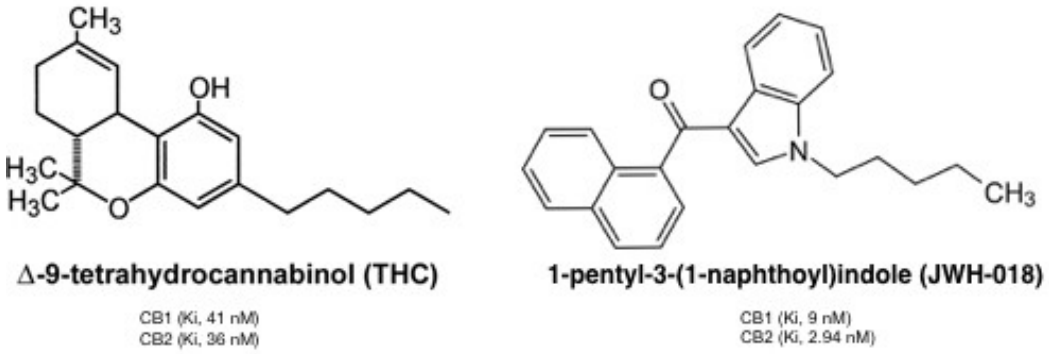

Fig. 4 Chemical structure and constant inhibition (Ki).
Chemical structures of THC and JWH-018 with respective constant inhibition values (Ki reported from Huffman et al., 2005b)

After the identification of the first SCs (e.g., JWH-018, JWH-073, CP-47,497, HU-210, etc) as non-declared active ingredients of a Spice in 2008-2011 (Uchiyama et al., 2011; Vardakou et al., 2010; Auwarter et al., 2009; Uchiyama et al., 2009), many countries have taken measures to control diffusion of these products (DEA 2015, EMCDDA 2018). To bypass regulation, street manufacturers synthesized and marketed new

compounds with minor changes to the chemical composition but with a pharmacological activity very similar to the banned drugs.

To date, it is possible to distinguish three consecutive generations of SCs on the basis of their appearance in Spice products (ACMD, 2009, 2012, 2014). First generation SCs, e.g. JWH-018, CP47,497 and HU-210, are full CB1R agonists with higher affinity than THC. In particular, JWH-018 belongs to the JWH group, a class of molecules synthesized in 80's from Jonh W. Huffman. This compound, which is an aminoalkyl-indol, is present across many different brands and batches of Spice products (Atwood et al., 2010; Moller et al., 2010; Wintermeyer et al., 2010). Previous researches have shown that this compound binds with higher nanomolar affinity to, and very efficaciously activate, CB1Rs *in vitro* (Brents et al., 2011; De Luca et al., 2016; Wiley et al., 2012;); specifically, it has been shown to have binding affinity for CB1Rs in the low nanomolar range (~9 nM vs 41 nM for THC) (Fig. 4) (Aung et al., 2000; Chin et al., 1999; Huffman et al., 1994; Showalter et al., 1996;).

JWH-018 is a potent and effective CB1R agonist that activates multiple signaling pathways, such as inhibition of forskolin-stimulated cAMP production in CHO cells expressing CB1Rs (Chin et al., 1999), inhibition of excitatory postsynaptic currents in a concentration-dependent manner in cultured hippocampal neurons and it potently increased ERK1/2 MAPK phosphorylation (EC50 = 4.4 nM) (Atwood et al., 2010).

Both the MAPK and the G-protein activation induced by JWH-018 were prevented by the CB1R antagonist/inverse agonists rimonabant and AM251, indicating that both are mediated by the GPCR CB1Rs (Atwood et al, 2010; De Luca et al., 2016). In addition, JWH-018 displays *in vivo* cannabimimetic activity, i.e., antinociception, hypothermia, catalepsy and locomotor suppression (Brents et al., 2011; Wiley et al., 2012; Vigolo et

al., 2015), and fully substitutes THC in a drug discrimination procedure (Wiley et al., 2014; 2016).

Additionally, pretreatment with AM251 fully prevented the disruptive effects of JWH-018 and THC on working memory, strengthening the notion that CB1Rs are involved in the effect of cannabinoids on working memory (Barbieri et al., 2016). Importantly, as full potent agonists, SCs activate CBRs in brain areas with high densities of CB1Rs, e.g. the amygdala, cingulate cortex, PFC, ventral pallidum, CPu, NAc and VTA (Glass et al., 1997; Wang et al., 2003). Noteworthy, these brain regions are strongly involved in reward, addiction and cognitive functions (Koob and Volkow, 2010). The last (i.e. 3rd) generation of SCs include compounds, such as 5F-PB22 and BB-22, which display greater CB1R potency and efficacy and higher binding affinity at CB1R compared to 1st SCs generation like JWH-018. Similarly to other SCs, BB-22 produced full substitution for the discriminative stimulus effects of THC, an effect attenuated by pretreatment with rimonabant (Gatch and Forster, 2016). The agonist properties of these compounds are abolished or drastically reduced also by AM251, and no activation of G-protein was observed in mice lacking the CB1R, i.e. CB1R-KO mice (De Luca et al., 2016). Yet, although *in vitro* receptor and guanosine-5'-triphosphate-gamma-s (GTPγS) binding and *in vivo* neurochemical/behavioral findings collected so far suggest that CB1Rs mediate the majority of SC-induced effects, the high affinity and intrinsic activity that these compounds display toward the CB2Rs suggest that they could also be involved in SC-induced effects. Besides CB1Rs and CB2Rs, first generation SCs show only moderate to poor binding to other major non-cannabinoid receptors (Wiley, 2016).

Noteworthy, these compounds act as full agonists in functional tests, in contrast to the partial agonist activity of THC (Atwood et al., 2010, 2011; Huffman et al., 2005a;

Marshell et al., 2014). *In vivo* animal studies showed that all SCs generations produce hypothermia and catalepsy, reduce motor activity and impair sensorimotor responses, effects that are fully prevented by pretreatment with the CB1R antagonist AM251 (Canazza et al., 2016; Ossato et al., 2016; Pintori et al., 2017). The fact that SCs possess higher efficacy and affinity at CB1Rs and CB2Rs than THC might partially explain their high potential to trigger psychotic-like symptoms (Fattore, 2016). Differences in pharmacodynamics between SCs and THC have also been described (Castaneto et al., 2014), which might account for the more intense effects perceived when smoking SCs than cannabis. However, differences in pharmacokinetics might explain the higher severity of toxic side effects reported after use of SC-based products (Brents et al., 2011). In addition, similar to other drugs of abuse (i.e. ethanol, heroin, cocaine), SCs preferentially stimulate DA release in the NAc shell when compared to other DAergic terminal areas arising from VTA (e.g. PFC, NAc core) (Di Chiara et al., 2002, 2004; Imperato and Di Chiara 1988).

1.3 The adolescence period

Adolescence is one of the most important period of brain development (Schneider, 2013) that is involved in many mechanisms such as neuronal plasticity, synaptic pruning, maturation of the corticolimbic circuitries and of neurotransmitters like DA, glutamate (Glu) and GABA (Tomas-Roig et al., 2016) (Fig. 5).

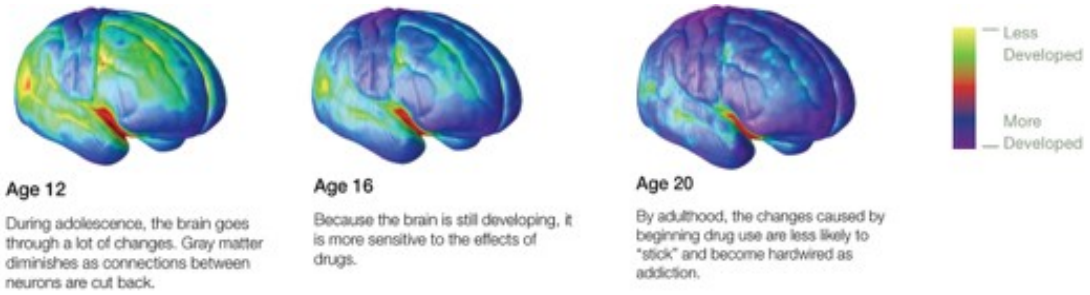

Fig. 5 Brain development in adolescence
Representative schema of brain development during adolescence period (adapted from http:// learn.genetics.utah.edu/content/addiction/adolescent/).

In humans, adolescence is a period of transition between childhood and adulthood and is characterized by many changes in behavior and in the body (Paus et al., 2008). It is an important stage of life involved in the improvement of cognitive and social skills, which brings to the achievement of independence life from the family. However, adolescence is also characterized by impulsivity, higher reward sensitivity and risk-seeking behavior, behaviors evident not only in humans but also in other mammals. During adolescence, the onset of the pubertal period parallels the brain development and the sexual maturity is reached, characterized by a higher secretion of gonadotropin-releasing hormone (GnRH), the main factor of steroid hormone secretion and gonadal maturation (Schneider, 2013). In rodents, the beginning of puberty is identified by different markers: in females it is observed by canalization of the vagina (vaginal opening), which occurs between PND 27-30, while in males the marker is the balanopreputial separation, i.e. the complete separation of the prepuce from the glans penis, observed around PND 40 (Fig. 6).

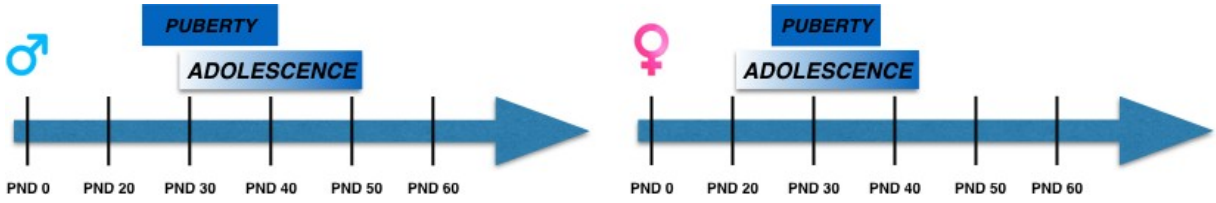

Fig. 6 Timeline of puberty and adolescence in mice
Adolescent and puberty timeline in male and female mice (Adapted from Schneider 2013).

Although puberty is well defined from different markers, is not possible to identify a precise boundary for the adolescence period, which is a transition period. Adolescence is crucial for the changes in the brain's morphology and two different mechanisms occur in parallel in this period:

- the synaptic pruning, which is an essential mechanism that leads to strong neuronal communications;

- the increase of axon caliber/myelination, which allows the neuronal connections to become more efficient and organized.

The neurodevelopment changes have been linked to the onset of puberty, since the activity of steroid hormones (like testicular hormones and testosterone) are implicated in brain development (Schneider, 2013). The maturation of brain areas takes place at different time. The primitive sub-cortical regions (e.g. NAc and amygdala) are the first to develop, followed by the newest regions such as the frontal and temporal lobes. In particular, the PFC, which is the latest region to develop, has a main role in the cognitive functions like abstract thinking, working memory, problem solving and inhibition of impulses (Chambers et al., 2003).

During adolescence the neurotransmitter systems, including the EC system, undergo maturational processes, refinement and crucial changes which peak often with the onset

of puberty (Rodriguez de Fonseca et al., 1993; Andersen et al., 2000; Spear, 2000). The peak of the expression of DA receptors is observed during adolescence, mainly in cortical and subcortical areas. In rats, DA fibers increase until PND 30 in the NAc and striatum, and until PND 60 in the PFC (Kalsbeek et al., 1988; Manitt et al., 2010, Naneix et al., 2012), confirming the difference between brain regions development. DA (D1 and D2) receptors increase during life time, with an inverted U-shape curve, reaching the peak during adolescence followed by a decrease in adulthood (Andersen et al., 2000; Teicher et al., 1995).

The inhibitory neurotransmitter GABA plays an important role during brain development, in particular in the refinement of synapses and in the improvement of efficiency in synaptic GABA uptake systems. The maturation of GABAergic system occurs in hippocampus before the onset of puberty, while in the neocortex is observed not before the end of the adolescence period. The maximum peak of GABA receptors in many brain regions is observed during adolescence (Verdurand et al., 2010).

Also Glu is particularly important for the correct development and remodeling of brain areas (e.g. limbic system). Both Glu NMDA and AMPA receptors achieve the maximum peak around PND 30-35 and then their expression decreases during adulthood. A decrease in the NMDA/AMPA ratio has been reported recently in the cortical interneurons (Parolaro and Rubino, 2015).

Similarly, eCBs and CBRs are present from the early stages of gestation and play an important role for the developing organism during postnatal development, including adolescence and puberty (Chadwick et al., 2013; Fernandez-Ruiz et al., 1999, 2000). Indeed, binding studies have shown that the increase of CB1Rs occurs around PND 30

in female rats and around PND 40 in male rats. Interestingly, the peak of CB1Rs overlaps the onset of puberty (Schneider, 2013).

The expression of CB1R gene has been found to reach the highest values during adolescence while it decreases in adulthood, specifically in cortical regions. In fact, the brain levels of CB1Rs fluctuate during adolescence in a region-dependent manner. In the striatum and NAc shell, CB1Rs have a peak during adolescence and then they are reduced by half in adulthood. On the contrary, a decrease from early to mid adolescence is observed in the NAc core and PFC (Ellgren et al., 2008). In parallel to the changes of CB1Rs levels, a fluctuation of AEA, 2-AG and FAAH levels is also observed (Meyer et al., 2018). Moreover, the EC system is involved in the regulation of the maturation of glutamatergic system in the PFC (Rubino et al., 2015). These changes in the EC system can explain the higher sensitivity in adolescence period to cannabis exposure (Chadwick et al., 2013).

Recent studies demonstrated that administration of the CB1R antagonist AM251 during adolescence prevented the decrease of postsynaptic proteins and glutamatergic receptor subunits. This activity of the EC system could regulate the elimination of excitatory synapses and pruning, supporting the idea that every interference with the physiologic activity of the EC system in adolescence could affect brain development (Rubino and Parolaro, 2016).

Based on all this changes and refinements, the inhibitory and excitatory neurotransmission in adolescence is in a critical balance. Indeed, GABA and Glu functions are essential for Long-Term Potentiation (LTP) and Long-Term Depression (LTD), two mechanisms that are involved in the synaptic plasticity of the adolescent brain (Caballero et al., 2014; Crews et al., 2007). For this reason, different types of stress

acting during brain development could create remarkable damages and trigger the onset of neuropsychiatric disorders (Rubino and Parolaro, 2016). For example, alterations of the hippocampus development increase the possibility to develop depressive or anxiety-like behaviors in adulthood (Schneider, 2013; Tomas-Roig et al., 2016) (Fig. 7).

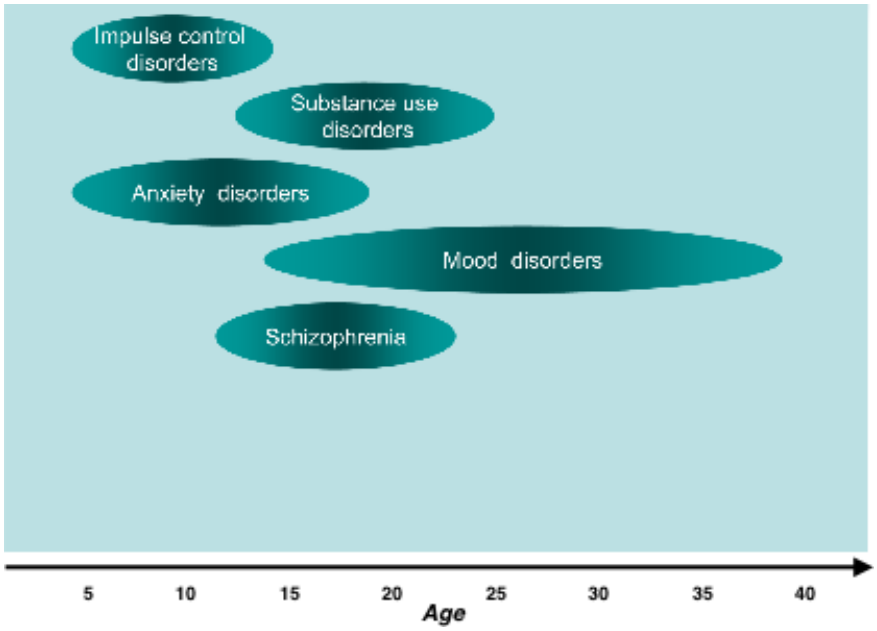

Fig. 7 Timeline onset neuropsychiatric disorder
Onset of neuropsychiatric disorders during life *(adapted from Paus et al., 2008)*

The abnormal neurodevelopment and/or environmental factors are related with different diseases like psychosis, anxiety, eating and mood disorders and substance abuse. Exposure to drugs of abuse during pubertal/adolescence period induces major and permanent adverse effects in adulthood compared to the exposure during juvenile state. The inclination of abusing drugs during adolescence is therefore higher than in adulthood because of the novelty-seeking behavioral trait characteristic of this period (Chambers et al., 2003; Hurd, 2013). In addition, the different time of maturation of brain areas determines an imbalance in the neuronal activity between frontal and

subcortical regions. The activity of the mesolimbic system, in particular of the NAc and amygdala that are involved in the response to rewarding, aversive and emotionally arousing stimuli, is enhanced. On the other hand, the PFC which has the role to control the subcortical areas, develops later than NAc and amygdala, leading to the lack of control of mesolimbic activity.

In general, adolescents have a higher sensitivity to the rewarding effects of drugs of abuse, less sensitivity to their aversive effects and greater metabolic activity (i.e. drugs are metabolized more quickly) than in adulthood, which contribute to the vulnerability of abusing drugs and developing addiction (Spear, 2016).

1.4 The reward system and addiction

Pleasure is a fundamental aspect of motivated behavior in mammals, including humans. The correlation between the sensation of pleasure (edonia) and stimuli of various types, such as moods, emotions, tasty food and substances of abuse, characterizes the system of gratification. Motivated behavior is supported by natural gratifying stimuli through two main properties: the incentive (Baum and Bindra, 1968; Crawford and Masterson 1982; Wilson et al., 1995) and the functional properties (Simpson and Balsam 2016). The incentive properties are essential to activate the subject to search and make a contact with the stimulus. For example, regarding food these properties are: smell, shape and taste (Bassareo et al., 2002). Functional properties are essential for their efficacy in biological and physiological functions, such as the caloric and metabolic properties of food. Motivated behavior is traditionally divided into three main phases: appetitive, consummatory and post-consummatory (Konorski, 1967; Wise et al., 2006). In the appetitive or incentive phase, the organism implements flexible and generic behavior of

research and approach to the most different stimuli (e.g., food, water, sex, mother-child relationship); in the consummatory phase, the behavior is influenced by rigid patterns linked to the nature of the stimulus, like the functional properties. Finally, the post-consummatory phase is characterized by satiety and satisfaction (Konorski, 1967). The motivational properties of pleasure depend on the activity of cerebral areas located in the medial and ventral part of the brain. These areas are the NAc shell and a nucleus complex consisting of the central nucleus of the amygdala, the core of the terminal striae bed and the substantia innominata, traditionally considered part of the limbic system and reclassified to form the so-called "extended amygdala". The activation of the mesolimbic system determines many of the incentive properties of natural gratifying stimuli like stimulated attention and targeted motor behavior (orientation). It also promotes the response by the acquisition of natural gratifying stimuli (primary reinforcements). The ability of no-primary stimuli to acquire motivational properties is related to the stimulation of the same brain areas and same neuronal circuits involved in natural gratification. Drugs of abuse produce a satisfying effect and are typically identified like stimulus with positive valence (positive reinforcement) (Aragona et al., 2008). In experimental animals, positive reinforcements increase the probability of performing any behavior whose goal is the administration of the drug. In this case, the behavior is aimed at searching and approaching the drug, which represents the stimulus (i.e. motivated behavior). By activating the mesolimbic system, drugs of abuse are able to induce motivated behavior and to facilitate learning processes, providing deep motivational meanings. Moreover, they can be considered as surrogates of natural gratifying stimuli, since they cause neurophysiological changes typical of physiological stimuli (e.g. sweet food, sex) and interpretable as gratifying, mimicking the incentive

and functional properties of primary natural stimuli. In addition, drugs of abuse show behavioral characteristics typical of the incentive aspect of natural rewarding stimuli, such as the induction of the alert state, targeted locomotion, incentive learning. In particular, they have the common characteristic of preferentially activating, although with different mechanisms, the DAergic transmission of the mesolimbic system (Di Chiara and Imperato, 1988). Importantly, after consumption of drugs of abuse, the response of DA in the NAc shell is not characterized by rapid habituation which is instead observed in response to gustatory stimuli. This characteristic has a fundamental role in drug addiction, because it increases the consolidation of the associations between the rewarding effects of the drug and the stimuli or contexts, improving the activation of incentive-motivational properties. In this mechanism, the state of appetitive pleasure associated with the stimulating excitement (euphoria) produced by the drug likely plays a reinforcing role.

Craving, i.e. compulsive desire and search for the drug, can be divided into three phases: the drug intake phase (characterized by the gratifying effects caused by the acute administration of the substance), the anxious phase (due to the end of the gratifying effect) and the abstinence phase, with the abstinence symptoms (physical and psychic) typically associated with depression, apathy and irritability.

The DAergic system and the opioid system have been considered for decades the main systems involved in the gratification mechanisms. Recently, it has been shown that also the EC system plays an extremely important role in the processes of gratification. CB1Rs are expressed in many areas of mesolimbic system and their activation by endogenous agonists, natural or synthetic, stimulates the DAergic transmission thus increasing the rewarding effects of the drug stimuli (Solinas et al., 2003; Solinas and

Goldberg, 2005; De Luca et al., 2011). DA, an endogenous neurotransmitter belonging to the catecholamine class, is abundant in the striatum, in the limbic system and in the hypothalamus. DA is synthesized in the cytoplasm and concentrated within the synaptic vesicles until the arrival of a nerve impulse, which allows the release into the synaptic space. After its release from the presynaptic neuron, DA is largely re-exported by the specific transporter (DAT), which belongs to the family of monoamine transporters. Later, DA is metabolized by monoamine oxidase (MAO) associated with mitochondria or cytoplasmic cateto-O-methyl transferase (COMT). DA receptors can be divided into two different types: D1 (subfamily D5) and D2 (subfamily D3 and D4) belonging to the family of trans membrane receptors associated with proteins. D1 receptor agonists induce an increase in adenylate cyclase activity (Dearry et al., 1990, Tiberi and Caron, 1994, Tiberi et al., 1991), through a GTP-dependent Gs (stimulatory) protein, with an increase in intracellular levels of cAMP and an excitatory effect on the postsynaptic membrane. Conversely, stimulation of D2 receptors induces inhibition of adenylate cyclase (Chio et al., 1994a,b) through a GTP-dependent Gi (inhibitory) protein, with consequent reduction of the intracellular concentration of cAMP but also suppression of intracellular Ca^{2+} currents and activation of K^{+} currents leading to neuronal hyperpolarization (Memo et al., 1987). DAergic neurons are connected via axons to large areas of the brain through three main systems: the nigrostriatal and mesocortical mesolimbic pathways, and the tuber-infundibular system. Stimulation of DA neuron in VTA produces a release of DA in the NAc, while stimulation of substantial nigra produces a release of DA in the CPu. DA release has a different role in these two areas. When released into the NAc, DA is associated with reward, motivational stimuli, learning of new behavior and pre-motor cognition, while when released in the CPu, DA

is related to in the initiation of motor activity and habitual behavior. These stimulations are provided from excitatory signals from many areas, specifically from the cortex. The repeated release of DA in the NAc creates neuroplastic changes like cellular morphology alterations, changes in gene expression and alteration of protein implicated in signaling pathways. These changes in the reward system can modify the behavior and affect the learning processes, in particular can impact upon future decisions that are influenced by past experiences (Chambers et al., 2003). Similarly to the other drug of abuse, JWH-018 is able to increase DA levels in the NAc shell. Specifically, this increase has been observed in rats at the dose of 0.25 mg/kg (i.p.) and in mice at dose of 0.3 mg/kg (i.p.) but not at lower or higher doses (De Luca et al., 2015), according to an inverted u-shape which is a characteristic of SCs. The narrow range of doses able to increase DA levels could be influenced by the metabolites of JWH-018, that act as partial agonists or antagonists thus inhibiting the main effects of JWH-018.

A theoretical model of addiction has been proposed by Koob and Volkow (2016) (Fig. 8), who defined drug addiction as a "chronically relapsing disorder" characterized by a lack of control in the seeking and intake of the drug and the development of negative symptoms in the absence of the drug.

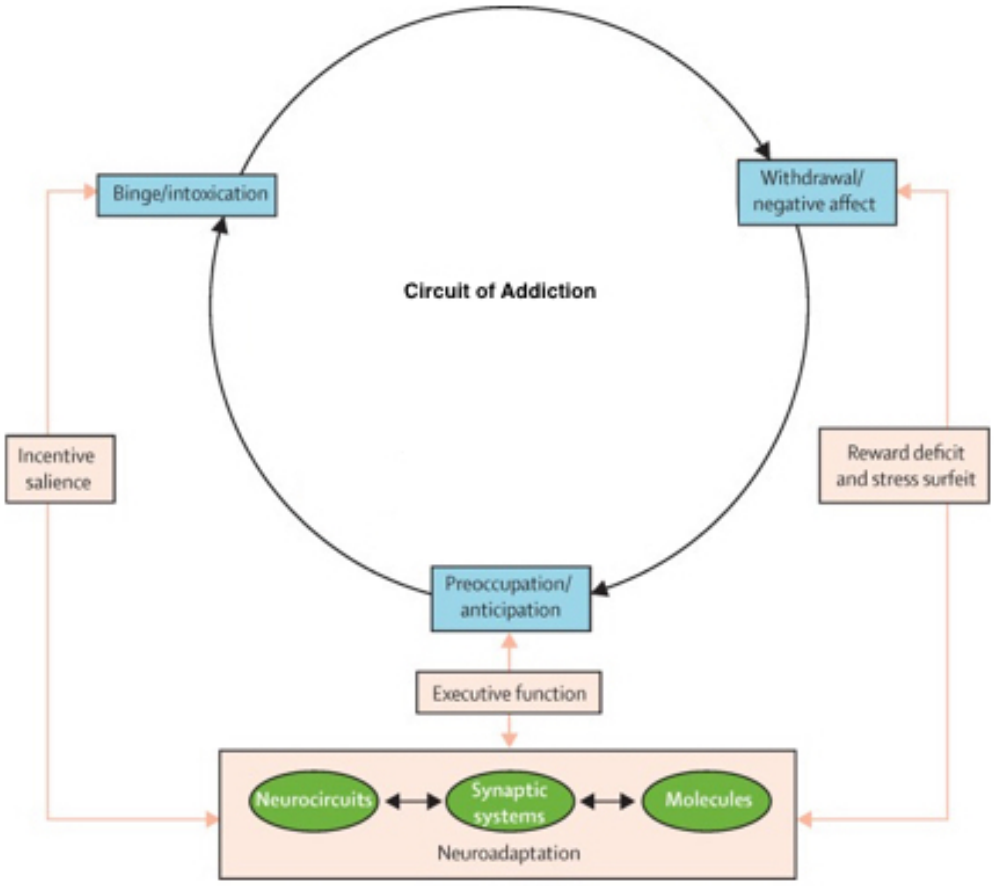

Fig. 8 Circuit of addiction Representation of the different steps in circuit of addiction (adapted from Koob and Volkow 2016)

The model is represented by three stage of addiction, associated to the specific neurocircuits:

- Stage 1: binge/intoxication (determined by changes in the basal ganglia): the rewarding effects are able to produce hyper-activation of the mesocorticolimbic DA transmission.
- Stage 2: withdrawal/negative symptoms (determined by changes in the extended amygdala): the neuroadpatations during the stage of withdrawal/negative symptoms, create a decrease of DAergic signaling in the NAc and dorsal striatum, increasing the rewarding threshold of no-drug reinforcement, which is traduced as amotivation.

- Stage 3: preoccupation/anticipation (determined by changes in the PFC): this stage is characterized by reinstatement of abuse drug after the abstinence period. The abstinence is control by PFC of impulsivity and craving behavior. For this reason, any change in PFC create a higher probability of relapse (Zehra et al., 2018).

1.5 Animal models of drug addiction

The preclinical research of drug addiction provides important tools to the knowledge of brain mechanisms, behavior and genetic profile behind this pathology. Nowadays, animal models are successfully used to study the effects caused by drugs exposure and their consequences. Importantly they allow to study the real pharmacology and the potential abuse of drugs without the complicating socio-cultural aspects that characterize the human beings, including the underlying reasons, the social context and stressful or traumatic situations. Among the strengths of using animal models in addiction research is the fact that the addictive properties of drugs are the same in both the human and the animal brain. Like in humans, in fact, the reinforcing effects of drugs of abuse are able to trigger specific behaviors in animals in order to obtain the drug. Animal models allowed us to understand the whole cycle of drug addiction, which starts with the acquisition of the drug, followed by the maintenance phase and its transition to the compulsive intake of the drug despite the associated negative consequences (Sanchis-Segura and Spanagel, 2006).

Besides the acute drug effects, it is very important to also characterize the chronic neurobiological and behavioral adaptations caused by drug exposure (Belin-Rauscent and Belin, 2012), an aspect particularly crucial when drug exposure occurs during adolescence. Moreover, the use of knockout mice made it possible to identify candidate

genes in drug addiction and provided new approaches for the development of genetic or pharmacological therapies for this pathology (Panlilio and Goldberg, 2007). Animal models of drug addiction are created with the aim to study in animals the same criteria of drug addiction that have analogies with animals' behavior (Belin-Rauscent and Belin, 2012), the main of which are the following:

- the persistence of substance-seeking behaviors even when the animal knows that the substance is unavailable;
- a strong motivation to work for obtaining the substance, which in the animal can be measured as the maximum number of responses it is willing to do to get the substance (breaking point);
- the use of the substance despite its negative effects (animals will continue to self-administer the drug even if the infusion is accompanied by slight electric shock).

The most widely used animal models of drug addiction are the self-administration procedure, the conditioned place preference task, the drug discrimination task, the intracranial self-stimulation (O'Connor et al., 2010).

1.5.1 The intravenous self-administration (IVSA) paradigm

In the IVSA model, animals have to achieve a specific task, such as lever-pressing (O'Connor et al., 2010) or nose-poking (Belin-Rauscent and Belin, 2012), to obtain the drug and is frequently used to assess the risk of abuse liability of new compound or to characterize the reinforcing properties of a substance (O'Connor et al., 2010). This model provides a detailed study of addictive behavior in standard experimental conditions and allows to introduce and/or change pharmacological and environmental components. The paradigm beyond this model is the operant conditioning. In IVSA paradigms, the drug represents the reinforcer and the self-administration is the operant response. The motivation to obtain an infusion of the drug is due to the rewarding and reinforcer effects of the drug. If a drug possesses no effects or induces aversion instead of reward, the animal will refrain to operate to obtain a drug infusion.

The schedule of reinforcement can be easily changed, rendering the experimenter able to identify the elements (e.g. associated cues) that create the strong correlation between the reinforcer (the drug) and the response (lever-pressing or nose-poking). The first self-administration experiments were performed in non human primates. Only after 1962, the scientific community started to use rodents, thankfully to a pioneer work of Weeks, who used rats in a IVSA experiment with an operant procedure (lever-press) to delivery morphine intravenously (Weeks, 1962). In the self-administration models the drugs can be delivered in different ways, among which are the intravenous, oral, intramuscular, and the intranasal routes of administration. In IVSA self-administration protocols, a permanent silastic catheter is implanted in a jugular vein, the drug is delivered through the catheter using an injection pump connected to automatic program that in addition executes recording and scheduling functions. The experiments are performed in sound

and light attenuating isolation chambers called “operant boxes”, where the external influences are reduced.

Self-administration experiment can be performed in operant or no operant model. No operant models are associated to the oral route and are commonly used in rodents for studying alcohol addiction. In the operant model, the drug is delivered only after the pressure on a specific lever located within the operant box. Two different types of levers are in fact available to the animal. Pressure on the lever called the “active lever” triggers the contingent intravenous infusion of the drug, while pressure on the “inactive lever” does not active the infusion pump. The abuse potential of a drug can be therefore determined by the difference between the mean of the active and inactive total lever presses during the test.

A self-administration protocol is typically composed with several phases:

- *Acquisition* phase (variable duration), in which the animal associates’ drug-acting behavior. At this stage, discriminatory stimuli are crucial as they anticipate drug availability;
- *Maintenance* phase, in which the animal regulates the intake of the drug autonomously, maintaining a rather stable of responding session after session;
- *Extinction* phase, where the drug is replaced by a non-responsive solution, for example a physiological or Veh solution. Animals at this stage initially have contingent responses associated with the light stimuli already known, but the number of active lever presses will decrease as the substance they receive has no reinforcing properties;

After the extinction phase, the experimenter can perform reinstatement testing after priming with the previously associated drug, or with associated cue or stressors.

Alternatively, the reacquisition phase can be introduced by replacing the physiological solution with the drug, allowing the animal to resume responding for the drug.

Schedule of reinforcements

In order to study the motivation to obtain the drug, there are many types of schedule of reinforcements to use during the experiment. Usually, when the drug has long lasting rewarding effects, it could be self-administered infrequently during the session. In this case, the FR schedule is often used. With this schedule it is possible to set the number of responses required to obtain a single infusion of the drug. The continuous FR-1 schedule of reinforcement provides a direct link between the response and the delivery of the drug, because the animal has to complete a fixed number of responses to obtain each infusion of the drug. The complexity of the task is predicted to study the reinforcement proprieties of the drug (Belin-Rauscent and Belin, 2012).

- *Progressive-ratio (PR)*

The PR schedule is able to estimate the reinforcer efficacy of the drug. Under this schedule, the number of responses required to obtain each infusion increases exponentially, until the schedule is complete. The main parameter to consider in this schedule is the so-called "breaking point", which is the highest response requirement satisfied in the protocol. The breaking point is defined as the "ascending function of dose per injection" and is a very important parameter that provides a measure of responding without considering the rate of responses (Panlilio and Goldberg, 2007).

Importantly, it is possible to study the relapse in addicted animals, even after a long period of abstinence by using the protocol of extinction-reinstatement and abstinence-relapse.

Mice are able to self-administer intravenously SCs such as WIN 55,212-2, HU-210, CP 55,940 and JWH-018 (Flores et al., 2014; De Luca et al., 2015; Martellotta et al., 1998; Mendizibal et al., 2006; Navarro et al., 2001;). De Luca and colleagues (2015) have shown that both rats and mice self-administer JWH-018, thus confirming the abuse properties of this SC. In particular, adult male mice C57BL6/J acquired operant behavior at dose of 30 μg/kg/inf.

1.6 The neuroimmune system and addiction

In the CNS different types of glia cells are present: microglia, astrocyte, oligodendrocytes and ependymal cells. Oligodendrocytes are involved in the formation of myelin sheath in the neuron while the ependymal cells are present in brain ventricles, where they produce the cerebrospinal fluid (Bachtell et al., 2017).

Microglia and astrocytes are actively involved in many aspects of CNS physiological functions, including synaptic connectivity and dendritic morphology, neurotransmitter release, neuronal development and neurogenesis, programmed cell death and regulation of synapse (Araque et al., 2014; Lacagnina et al., 2017; Salter and Beggs 2014). Recently, increasing evidence suggests that glial cells, including microglia and astrocytes, are also influenced by exposure to drugs of abuse, and that drug-induced alterations might contribute to the behavioral outcomes associated with substance abuse (Coller and Hutchinson, 2012; Miguel-Hidalgo, 2009).

1.6.1 Microglia

Microglia are the resident immune cells in both the brain and spinal cord, display similar characteristics to macrophages, are expressed mostly in the hippocampus, substantia nigra and basal ganglia, and represent 5-20% of glia cells (Lacagnina et al., 2017; Lisboa et al., 2016; Mecha et al., 2016).

The origin of the microglia cells depends on the developmental period: they derive from primitive myeloid progenitors until embryonic day 8, and from infiltrating myeloid cells during the remaining embryonic period and the postnatal period (Alliot et al., 1999; Ginoux et al., 2010). The morphology of these cells is characterized by ramified processes which are extended in the surrounding environment, through which microglia inspects the microenvironment tissue and communicates with adjacent neurons.

On the surface of microglia there are different receptors that are activated by potential threats present in the tissue, such as toxins and bacteria, and by endogenous compounds derived from traumatic brain injuries. As microglia detects these threats, it elaborates different types of responses, e.g. phagocytosis, secretion of cytokines/chemokines and growth factors, lysosomal degradation and enrollment of additional immune cells.

It has been long believed that microglia in the healthy brain exists in a “resting” state (M0) and that a variety of stimuli can activate these cells (state M1-M2). Indeed, in the steady state (M0), microglia cells present a “resting phenotype” with a small soma and ramifications with non-overlapping processes, but they change morphology in response to an insult, and retract the ramifications adopting an ameboid shape characterized by elevate mobility (Fig. 9).

Fig. 9 Representative images of IBA-1 immunostaining. Images are from CPu section of adult mice that underwent to Veh (in the left) or JWH-018 (in the right) IVSA during adolescence. Note the difference between resting (in the left) and activated (in the right) microglia cell. Scale bar = 100μm

However, it has been shown by in vivo two photon laser-scanning microscopy experiments that in the "resting" condition (i.e., intact healthy brains) microglial cells display highly dynamic processes that can elongate or retract more than 1–3 μm/min, excluding the idea that microglia from healthy brain is a resting component (Nimmerjahn et al., 2005). The term "resting" for the microglia in the healthy CNS has been thus updated and modified into "surveillant", as microglial resting state can be considered as an active state of surveillance of the surrounding environment.

Indeed, recent studies have demonstrated that the soma of cortical glia remain stationary while ramifications are highly mobile, and that protrusions retract and extend periodically through the parenchyma. In this way, microglia cells are able to surveil the brain every few hours, acting like active sentinels. Microglia can make direct connections with axon terminals, dendritic spines and astrocytes and is therefore able to monitor synaptic functions and to influence neuronal activity (Lacagnina et al., 2017; Nimmerjahn et al., 2005; Davalos et al., 2005).

It is well established that microglial activation is regulated by alternation of the so-called "On" and "Off" signals that are produced by cells surrounding microglia. The Classical "On" signals, e.g. infections, bacterial wall lipopolysaccharide (LPS), bacterial and viral RNA or DNA, viral structural envelopes, intracellular elements, ATP/UTP released from damaged neurons and astrocytes, heat shock proteins, and pathogen-activated

molecular patterns (PAMPs), activate microglia through microglial toll-like receptor (TRL). Other "On" signalling molecules, including complement proteins, interferon-gamma cytokine (IFN-gamma), or tumor necrosis factor (TNF-alpha) released from lymphocytes, activate innate immune responses producing the transcriptional activation of proinflammatory genes through the nuclear factor kappa-light-chain enhancer of activated B cells (NF-kβ) (Benarroch, 2013; Kierdorf and Prinz, 2013; Kettenmann et al., 2011). Activated microglia cells are sensitive to "Off" signals that include Heat Shock proteins and apoptotic cell membranes, cannabinoids receptors agonists and anti-inflammatory cytokines (Il-4, Il-10, Il-13 and TGF-alpha) (Cutando et al., 2017).

The traditional dichotomic nomenclature of classical activated (M1) and alternatively activated state (M2) disregards the wide spectrum of phenotypes related to (i) different functions of microglia relating M1 with cytotoxic properties and M2 with regeneration and repair (subtype M2a), (ii) immunoregulation (M2b) or (iii) acquired-deactivating phenotype (M2c) (Martínez et al., 2009). As shown in Figure 10, microglia phenotypes, markers and secreted cytokines/chemokines are different during homeostatic conditions (M0), proinflammatory state (M1), and alternative state (M2a, M 2b and 2c).

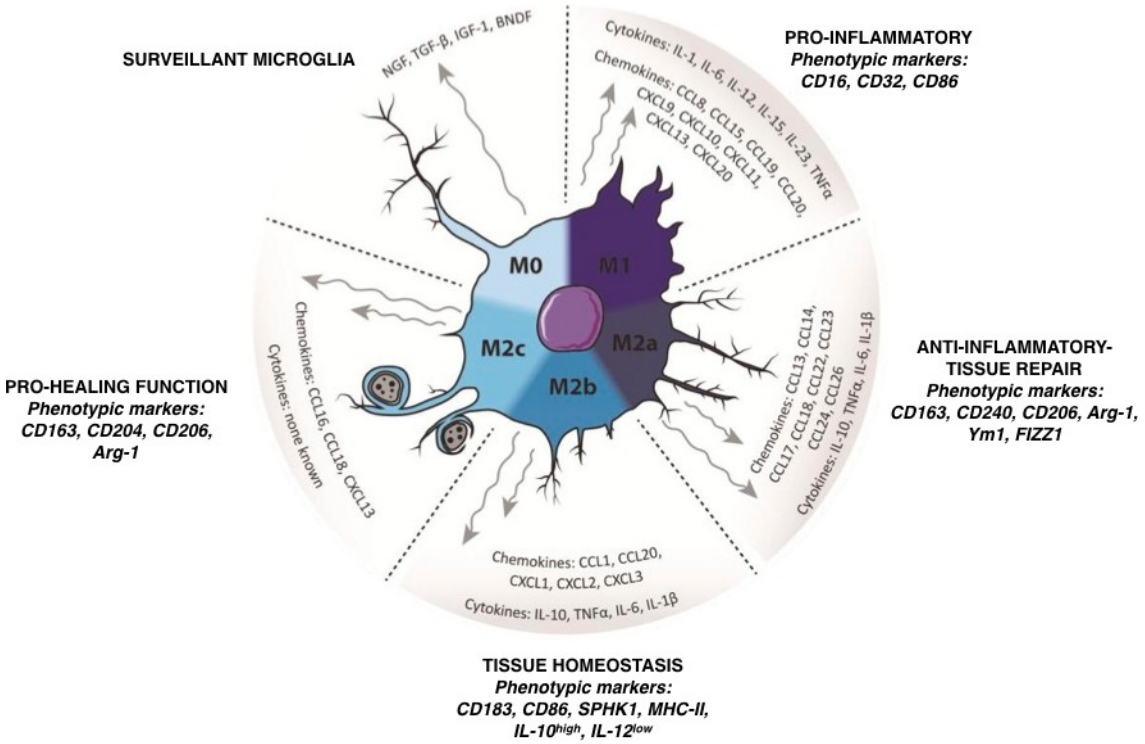

Fig. 10 Microglia phenotypes, markers and secreted cytokines/chemokines.
Different phenotypes and functions of microglia in homeostatic conditions (M0), proinflammatory state (M1), alternative state (M2a, M 2b and 2c) (Adapted from Mecha et al., 2016).

Indeed, several stimuli (stroke, trauma, infectious) can produce a short-lived reaction to injury, called neuro-inflammation, characterized by an increased number of microglia cells showing amoeboid phenotype and by the release of inflammatory mediators (Tansey et al., 2007). This M1 phase can be followed by a shift of microglia towards an M2 phenotype to drive the anti-inflammatory response (e.g., angiogenesis, debris scavenging) or towards a persistent proinflammatory response leading to the production of nitric oxide (NO) and reactive oxygen species (ROS), cytokines and other factors that can damage CNS cells and tissue (Kigerl et al., 2009).

Chronic neuroinflammation, which is characterized by a long-standing and often self-perpetuating inflammatory response, is associated to a long-term activation of microglia with the sustained release of pro-inflammatory mediators and chemokines, as well as

increased oxidative stress (Tansey et al., 2007). Indeed, persistent activated microglia release proinflammatory mediators which could create brain damages and are likely implicated in the development of psychiatric disorders and drug addiction (Lacagnina et al., 2017; Lisboa et al., 2016).

Drugs of abuse could influence glia directly by interacting with the surface receptors or indirectly, through their effects on neurons surveilled by glia or astrocytes, by altering several functions such as neurotransmitters synthesis and/or release, protein trafficking and release of immune signaling molecules and of cytokines/chemokines. Indeed, glia cells stimulated by drugs of abuse may also influence neuronal function either through physical remodeling of structural components (Brown and Neher, 2014; Corty and Freeman, 2013), or through released compounds (Block et al., 2007) that exert neuroprotective (e.g. BDNF, IL-10) or neurotoxic (e.g. IL-1beta, TNF-alpha, and NADPH oxidase) functions.

In particular, reactive persistent microgliosis has been detected in the brain of methamphetamine dependent subjects after many years of abstinence from the drug (Sekine et al., 2008), suggesting that methamphetamine had induced enduring effects on the proliferation of reactive microglia cells. Preclinical research confirms the methamphetamine-induced microglial activation in several brain regions (Thomas et al., 2004; Raineri et al., 2012; Castelli et al., 2014). Along with microglial activation, methamphetamine increases also the striatal mRNA expression levels of IL-6 family pro-inflammatory cytokines, leukemia inhibitory factor, oncostatin m, and IL-6 (Robson et al., 2013). Recent studies have showed alterations of IBA-1, a marker for activated microglia, in different brain areas after exposure to different drugs of abuse (e.g.

cocaine, alcohol, morphine), in particular in regions implicated in drug addiction (Lacagnina et al., 2016).

Altogether these observations are consistent with the idea that the drug of abuse might cause neuronal dysfunction via microglia-secreted pro-inflammatory and toxic factors.

1.6.2 Astrocytes

Astrocytes belong to glia, are considered supportive cells in the brain and are involved in homeostasis and neuronal outliving. Astrocytes are highly heterogeneous in structure and function and play a fundamental role in neurogenesis, CNS development and structural organization (Alvarez-Buylla et al., 2001; Kriegstein and Alvarez-Buylla, 2009; Nedergaard et al., 2003; Pfrieger, 2009). Indeed, astrocytes are essential for synaptogenesis and synaptic maturation and exert an overall control of the synapses by secreting multiple factors such as thrombospondins, cholesterol or neuregulins (Nedergaard and Verkhratsky, 2012). They provide energy and metabolic support to neurons, control ions homeostasis through numerous transporters and, being the major source of glutathione and ascorbic acid, are essential for water transportation and scavenging ROS (Araque et al., 2014; Fields et al., 2015; Haj-Yasein et al., 2011). In addition, astrocytes express Glu transporter 1 (GLT-1) and Glu aspartate transporter (GLAST), which are critical for the clearance of synaptic glutamate, as demonstrated by the intracerebroventricular delivery of antisense oligonucleotides against GLAST or GLT-1, which determine an excessive Glu release, striatal neurotoxicity, and progressive motor deficits (Rothstein et al, 1996). The disruption of astrocyte potassium channels *in vivo* elicits both an impaired synaptic Glu clearance, with devastating effects

to survival (Djukic et al., 2007), and inhibition of astrocyte proliferation, which induces a great sensitivity to Glu toxicity *in vitro* (Rosenberg and Aizenman, 1989).

Astrocytes are commonly identified and classified on the basis of their morphology and expression of the intermediate filament GFAP, along with other astrocyte-specific biomarkers, such as the Ca^{2+} binding protein S100β, the glutamine synthetase (GS), connexin, the aquaporin receptor (AQP4) (Norenberg, 1979; Oliveira and Gonçalves, 2009; Pekny and Pekna, 2004; Rouach et al., 2002) aldehyde dehydrogenase 1 L1 (ALDH1L1) (Li et al., 2013; Yang et al., 2011), GLAST and GLT-1 (for review see Sofroniew and Vinters, 2010). Although GFAP expression is the most common approached used to identify astrocytes, not all astrocytes express GFAP (Kimelberg, 2004; Kettenmann and Verkhratsky, 2011), which expression is also highly variable among brain regions (Sofroniew and Vinters, 2010; Sofroniew, 2009). On the contrary, GFAP is a reliable marker for reactive astrocytes, and increased GFAP levels has been described in the contest of injury and inflammation (Burda and Sofroniew, 2014; Sofroniew, 2009; Verkhratsky and Parpura, 2016). In response to injuries or insults, astrocytes can also secrete a variety of neuroimmune signaling molecules, includings IL-alpha, IL-6, TNF-alpha, and IFN-gamma (Lau and Yu, 2001). Release of immune-related molecules from astrocytes can regulate glial cells function and modulate synaptic function (Lacagnina et al., 2017). Astroglial reactivity is considered initially as a defensive response aimed at limiting the damage and facilitating tissue regeneration; yet, persisting reactive astrogliosis can be maladaptive and dysfunctional, i.e. can increase the release of neurotoxic factors, inhibit neural plasticity and regenerative responses, and exacerbate damage progression. Accordingly, Pekney and colleagues (Pekney et al., 2016) have proposed two different responses of astrocytes, one leading

to astrogliosis, where astrocytes can have neuroprotective or anisomorphic/severe scar forming effects, the other one leading to atrophy/degeneration, with loss of function and pathological remodeling of astrocytes. Atrophic astrocytes are smaller and display a reduced expression of GFAP, AQP4 and GLT-1 (Pekny et al., 2016), resulting in (i) the inability to mediate Glu homeostasis at the synapse, (ii) a reduced tone of signaling molecules and trophic factors, and (iii) in altered glucose metabolism and reduced network astrocytic connectivity (Pekny et al., 2016; Wang et al., 2017a).

To date, astrogliosis has been widely described in neuropsychiatric disorders and there is increasing evidence that astrocytopathy is a characteristic of neuropsychiatric disorders such as schizophrenia, frontotemporal dementia, stress, major depressive disorders and substance use disorders (Bedner et al., 2015; Braun et al., 2009; Czeh et al., 2005; Kim et al., 2017; Niciu et al., 2014; Rajkowska and Stockmeier, 2013; Scofield et al., 2016). Indeed, *post-mortem* human studies have reported numerous changes of astrocytes, including increased or decreased levels of GFAP levels and morphological alterations, such as hypertrophy and atrophy, in addicted patients (Miguel-Hidalgo, 2009; Oehmichen et al., 1996; Suarez et al., 2000; Weber et al., 2013). For example, different *post-mortem* human studies observed hypertrophic astrocytes and areas depleted from GFAP-positive cells in tissue obtained from alcoholics (Cullen and Halliday, 1994; Miguel-Hidalgo, 2005). Similarly, preclinical studies have demonstrated that treatment with several drugs of abuse, such as cocaine (Bowers and Kalivas, 2003; Fattore et al., 2002), methamphetamine (Castelli et al, 2014; Friend and Keefe, 2013; Guilarte et al., 2003;) and morphine (Song and Zhao, 2001) induce GFAP expression and reactive astrogliosis. However, prenatal cocaine exposure in Rhesus monkeys and cocaine self-administration in rats were also found to decrease GFAP

expression in the cortex of monkeys and in the NAc of rats, respectively (Lidow, 1995; Scofield et al., 2016). Moreover, GFAP expression and number of astrocytes were extremely reduced in the prelimbic cortex (Prl) of ethanol-preferring rats (Miguel-Hildago et al., 2005) and following prolonged exposure to alcohol (Franke, 1995; Rintala et al., 2001).

In conclusion, due to their multifaceted physiological functions, astrocytes are essential for the homeostasis of the CNS, and their dysfunction or maladaptive responses contribute to the pathogenesis of most, if not all, neurological diseases, including addictive disorders (Kim et al., 2017).

1.7 EC system and glia cells: role in drug addiction

New roles for glia cells in psychiatric diseases and addiction are emerging, providing new evidence that alterations in these cells, microglia and astrocytes in particular, contribute to the detrimental effects associated with drug abuse (for reviews see Lacagnina et al., 2017; Kim et al., 2017; Melis et al., 2017). Yet, a potential role of glial cells as pharmacotherapeutic targets for addiction has also been proposed (Beardsley and Hauser, 2014; Cooper et al., 2012; Snider et al., 2013;). Exposure to THC and SCs, induce alteration in glia cells suggesting that EC system plays a crucial role in neuroinflammation. In this regard, CB1Rs and CB2Rs are present in both microglia and astrocytes (Stella, 2010; Navarrete and Araque, 2008, 2010) and expression of atrocytes depends on the microglia activation profile. CB2Rs are expressed at low levels in intact healthy brain tissue, while they are rapidly expressed at detectable levels upon microglia activation (Carlisle et al., 2002; Stella, 2010; Walter et al., 2003). These receptors may

be considered as key regulators of the immune system, including microglial activation (Ashton et al., 2007; Correa et al., 2005). Recently, it has been demonstrated that the EC system influences the proliferation, migration and release of pro/anti-inflammatory mediators via CB2Rs and may affect their phagocytic functions by switching the glial cells to the M2 state (Mecha et al., 2015). On the other hand CB1Rs, which is expressed in microglia cells of different species, have been related to the production of NO, the inflammatory mediator released from activated microglia. Indeed, NO production induced by LPS and IFN-alpha was partially inhibited by the cannabinoid receptor agonist CP55,940, and this inhibition was prevented by the selective CB1R antagonist SR41716A (Waksman et al., 1999). Other than the canonic CB1Rs and CB2Rs, when high concentrations of cannabinoid receptor agonists are used, other receptors such as GPR55, PPAR and TRPV may be involved in the microglial immune-related response (Puffenbarger et al., 2000). As previously mentioned, all these receptors are present in microglial cells and respond to eCBs or SCs to modulate microglial activation. For example, PPAR-alpha from microglia cells control ischemia-induced microglia activation and neuronal damage in the hippocampus after transient global cerebral ischemia (Lee and Won, 2014). In addition, TRPV1 and TRPV4 stimulation from microglial cells modulates the abnormal microglial activation processes in an acute model of inflammation (Raboune et al., 2014). Moreover, cannabidiol (CBD) enhances microglial phagocytosis via TPRV channel activation (Hassan et al., 2014) suggesting the pharmacological potential of TRPV channel for treating neuroinflammatory disorders that involve microglia function. Moreover, microglia can produce eCBs at higher levels than neurons *in vitro* (Walter et al., 2003), and AEA, 2-AG and palmitoylethanolamide (PEA) affect immune function by acting mostly through CB2Rs

(Cabral et al., 2015). For instance, 2-AG acting on CB2Rs provokes microglial migration, decreases proinflammatory molecules release, and enhances BDNF release and microglia activation (Carrier et al., 2004).

Taken together, these studies showed that cannabinoid agonists mediate immunosuppressive effects and limit inflammation via microglial cannabinoid-like receptors thus revealing the crucial importance of the EC system in glia physiology.

On these premises, it can be argued that chronic use or abuse of natural cannabinoids (e.g., THC) or of SCs (e.g. Spice drugs) may affect microglia and/or astrocytes. In this respect, Cutando et al. (2013) demonstrated that the repeated administration of THC in healthy naïve mice activated cerebellar microglia reactivity, as revealed by an increase of the CD11 marker, and increased the expression of other neuroinflammatory markers, including Il-1beta. Expression of CB2Rs was increased under neuroinflammatory conditions in activated CD11b-positive microglial cells. Moreover, microglia activation was associated with CB1R down-regulation in the cerebellum while the neuroinflammatory phenotype correlated with deficits in cerebellar conditioned learning and fine motor coordination.

Accordingly, Zamberletti et al. (2015) showed that adolescent THC administration induces long-term microglia activation in the PFC of female (but not male) rats, increases the expression of the pro-inflammatory markers TNF-alpha, iNOS and COX-2, and reduces the expression of the anti-inflammatory cytokine IL-10. This neuroinflammatory phenotype is correlated with a down-regulation of CB1Rs on neuronal cells and with an up-regulation of CB2Rs on microglia cells. All these biochemical data appear to be strongly associated with the cognitive deficits induced by adolescent THC exposure. Interestingly, treatment with ibuldistat, a phosphodiesterase

(PDE) inhibitor that inhibits microglia activation (Ke et al, 2006), attenuates cortical neuroinflammation and cognitive impairments associated with adolescent THC administration. In line with these data, other reports have demonstrated that perinatal exposure to cannabinoids agonist, or THC exposure during adolescence, has long-term effects on peripheral immune function and results in a pro- inflammatory macrophage phenotype in adult mice, respectively (Lombard et al., 2011; Moretti et al., 2014).

Finally, Lopez-Rodriguez (2014), examined the effect of adolescent THC treatment in male and female rats at adulthood, reporting different results with respect to those described by Zamberletti et al. (2015). They found an increased proportion of reactive microglial cells in the male hippocampus, whereas an opposite trend was found in females; THC also reduced CB1Rs levels in the hippocampus of female, but not male, rats. These data suggest a sexual dimorphic effect of adolescent THC exposure on microglia modifications.

Astrocytes express CB1Rs and CB2Rs but can also produce eCBs through Ca^{2+} and ATP-pathway (Stella et al., 2015). To date, there are only a limited number of studies investigating the neurochemical and behavioral consequences of chronic cannabinoids exposure on astrocytes. In particular, the majority has investigated the long-term effects of either prenatal or adolescent THC exposure. Effects of pre- and perinatal exposure to THC vary with sex, showing a reduction of GFAP levels in both the substantia nigra during prepuberty, peri adolescence and adulthood (PND 21, 30 and 70), and in the cerebellar cortex of male rats at prepuberty and adulthood (PND 20 and 70). On the contrary, on PDN21 it was described an increased GFAP expression in the brain of THC-exposed females compared to either their controls and THC-exposed male rats (Suarez, 2000, 2002). When animals were exposed to increasing doses of THC from

PND 28 to 45, a significant increase of GFAP-positive cells was observed in the hilus of hippocampus of both sexes (Lopez-Rodriguez, 2014). Lastly, Zamberletti et al. (2015, 2016), using the same protocol, showed that adolescent treatment with THC results in a persistent neuroinflammatory state, characterized by increased levels of GFAP, TNF and iNOS in the PFC and hippocampus of female rats, but only in the hippocampus of male rats. These alterations are associated with different behavioral phenotype in male and female rats.

Collectively, these data show a clear interplay between the EC system and glia, and suggest that microglia and astrocytes have a role in drug addiction, including cannabinoid addiction.

1.8 Mitochondria and the EC system

Benard and colleagues (Benard et al., 2012) have identified in the mouse brain an intracellular pull of CB1Rs localized in mitochondria (mtCB1). The mtCB1 receptors have been found in outer mitochondrial membranes where they control cellular respiration and energy production. Through activation of mtCB1 receptors, exogenous and endogenous cannabinoids decrease cyclic AMP concentration, PKA activity, complex I enzymatic activity and ATP production. Electron microscopic immunogold experiments have shown that 10-15% of the CB1Rs in the hippocampus Cornu Ammonis 1 (CA1) are localized in neuronal mitochondria. The mtCB1, found in dendrites and axons, are mainly expressed in GABAergic interneurons, in line with the stronger presence of CB1Rs in GABA than in glutamatergic neurons (Marsicano and Lutz, 1999; Marsicano and Kuner, 2008). A recent work has described for the first time

the mitochondrial localization of CB1Rs in hippocampal astrocytes (Gutiérrez-Rodríguez et al., 2018), confirming the complexity of the molecular architecture of the CB1Rs in the brain.

1.8.1 Mitochondria: an overview

Brain consumes 25% of total energy produced by our organism and 20% of total oxygen (Erecinska and Silver, 2001; Kety, 1957; Rolfe and Brown, 1997; Sokoloff, 1960). Margulis proposed that eukaryotic cells have evolved from the acquisition of an aerobic prokaryotic bacterium, that we today call mitochondria (Margulis, 2001). This symbiotic process led to eukaryotic cells able to produce 15-fold more ATP than cytosolic glycolysis and with the possibility to produce ATP from different substrates (Alberts, 2002). Mitochondria have two different types of membrane: an outer membrane (OM) and an internal membrane (IM), separated by an inter membrane space (Alberts, 2002). The OM expresses a transport protein called porine, which creates large channels in the double lipid layer making it permeable to molecules up to 5000 dalton (including ATP) (Shoshan- Barmatz and Gincel, 2003). On the other hand, IM is less permeable and allows the passage of a small number of molecules only. The OM creates an internal lumen called matrix while the IM forms two structurally and functionally different domains: the inner-boundary membrane closely opposed to OM, involved in the control of ions exchange and the import of metabolites between cytosol and matrix (Davies et al., 2011; Vogel et al., 2006; Wurm and Jakobs, 2006), and the invaginations called CRISTAE, where the enzymes that form the respiratory chain are present (Stroud and Ryan, 2013, Herrmann and Riemer, 2010).

Based on cell conditions, mitochondria could appear in different shapes as numerous small organelles or as a single large interconnected membrane-bound tubular network (Picard et al., 2013; Rafelski, 2013). These morphological transitions are determined by two different mechanisms, i.e. fission and fusion, which constantly remold mitochondria connections. The balance between mitochondrial fission and fusion could change among sub cellular compartments (Popov et al., 2005) and dramatically influence the mitochondrial function (Itoh et al., 2013).

The main roles of mitochondria are to:

- transform energetic resources in adenosine triphosphate (ATP), through oxidative phosphorylation (OXPHOS);
- control calcium homeostasis;
- produce ROS (Turrens, 2003).

Moreover, mitochondria are involved in many other functions, including the mediation of the apoptotic processes, the control of the synthesis and metabolism of neurotransmitters, the regulation of neurotransmission, synaptic plasticity and behavior. They have also a relation with the onset and the progression of several neurodegenerative diseases (Attwell and Laughlin, 2001; Benard et al., 2012; Manji et al., 2012; McInnes, 2013).

1.8.1.1 Focus on mitochondrial ATP synthesis

Brain cells need a continuous energy support from different metabolic pathways, from cytosol (glycolysis) and from mitochondria (Alberts, 2002) (Fig. 11).

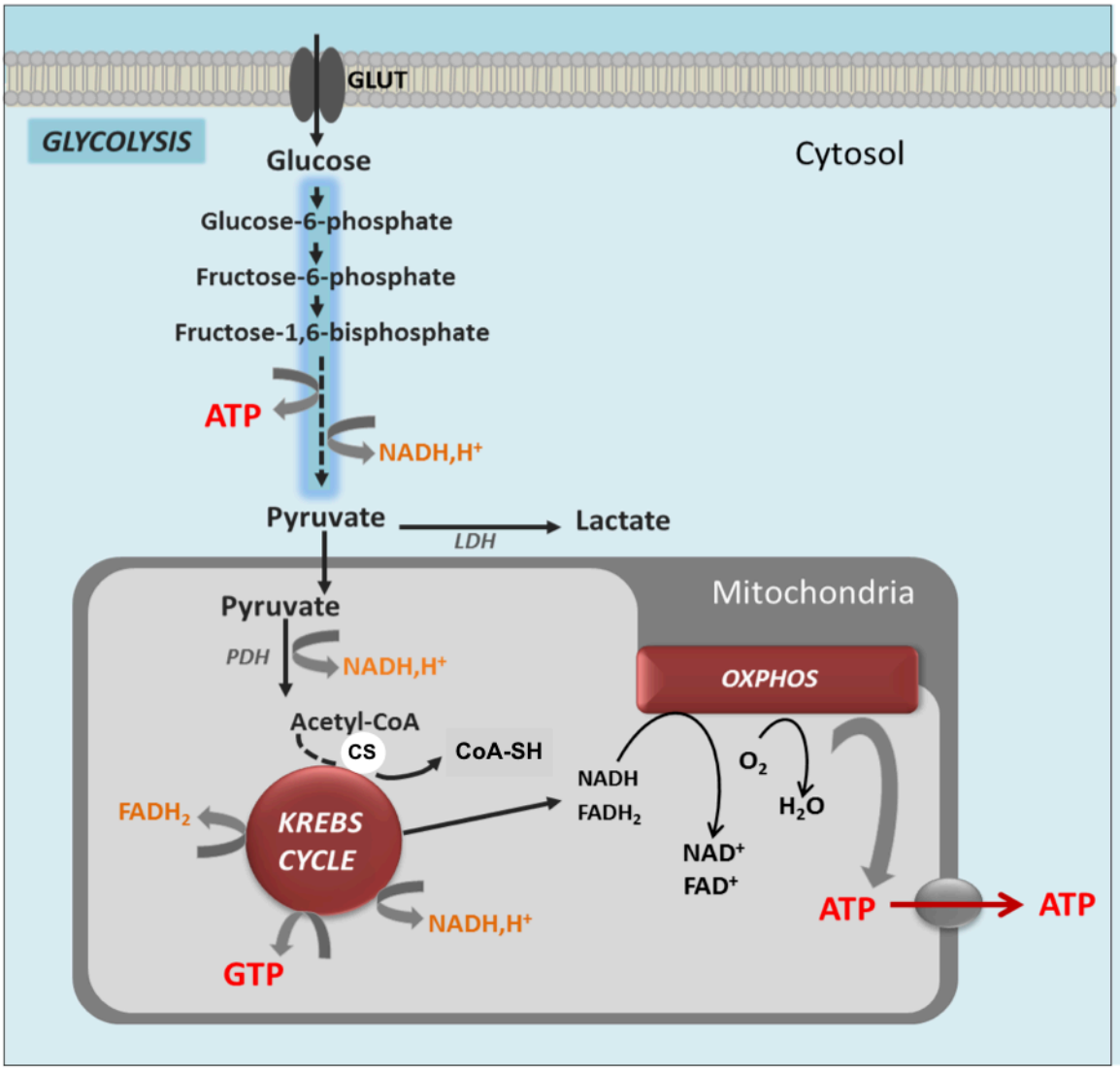

Fig. 11 Main pathways of cellular and mitochondrial energy metabolism. The two main metabolic pathways, i.e. glycolysis and OXPHOS are linked by the enzyme complex pyruvate dehydrogenase (PDH). Briefly, glucose is transported inside the cell and oxidized to pyruvate. Under aerobic conditions, the complete oxidation of pyruvate occurs through the Kreb's cycle to produce NADH and/or FADH2. These reduced equivalents are further oxidized by the mitochondrial respiratory chain. Adapted from Bellance 2008.

The energy utilized in nervous cells mainly derives from degradation of glucose. Once glucose reaches the brain, it is internalized both in neurons and astrocytes through the glucose transport (GLUT) (Leybaert, 2005).

The glycolysis that occurs in the cytosol without oxygen allows to convert one glucose

molecule in two molecules of pyruvate (Alberts, 2002). Then, pyruvate can be converted in lactate by lactate dehydrogenase. Once pyruvate is synthesized in the cytosol, it is moved inside the mitochondria and enzymatically degraded to produce Acetyl-CoA that enters into Kreb's cycle.

CS canalizes the condensation of acetyl-CoA with oxaloacetate to form citrate, which is then transported through the mitochondrial membrane to the cytoplasm. CS is a pacemaker enzyme in the Kreb's cycle and its activity is crucial for the cellular respiration (Ochia et al., 1951). Although CS activity is usually used as marker to quantify the level of mitochondria (Holloszy et al., 1970; Hood et al., 1989) its function is highly regulated through allosteric inhibition.

Nicotinamide adenine dinucleotide (NADH), flavin adenine dinucleotide ($FADH_2$), CO_2 and guanosine-5'-triphosphate (GTP) are the end products of the Kreb's cycle: GTP is involved in the transfer of energy in the cell and can be used to synthesize ATP, while NADH and FADH are used as substrates for the OXPHOS (Nelson and Rajagopalan, 2004).

OXPHOS is an efficient way to produce ATP (30 molecules of ATP/glucose more than glycolysis) through redox reactions and the associated proton pumping, mediated by 5 enzymatic complexes (Garret, 2013) (Fig. 12). Complex I-IV are multi-enzymatic sub-unities which create the electronic transport chain, while complex V is not involved in

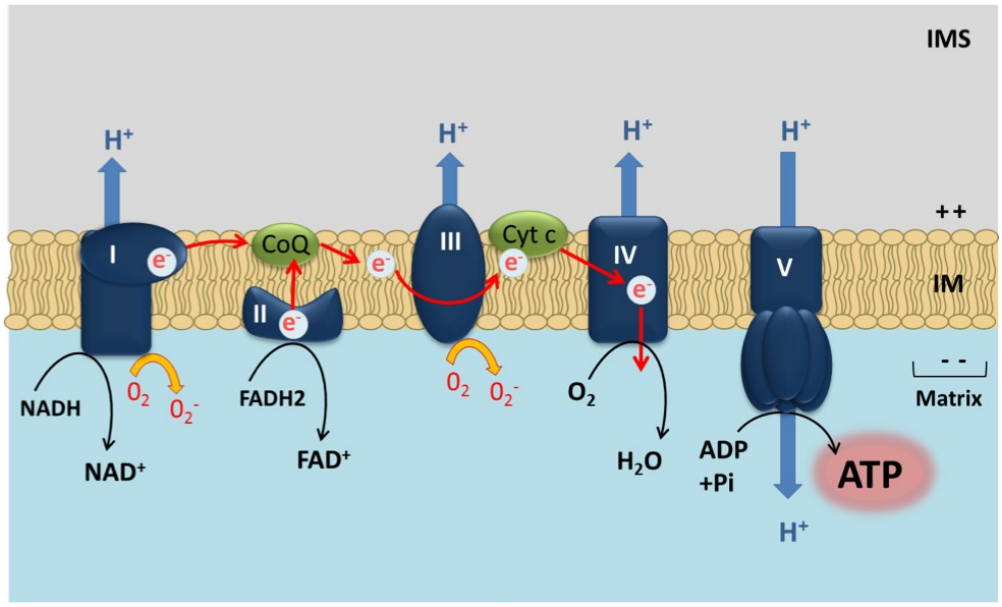

Fig. 12 Mitochondrial respiratory chain.
Mitochondrial respiratory chain. For mammals, the respiratory chain consists of four enzyme complexes (complexes I - IV) and two intermediary substrates (coenzyme Q and cytochrome c). The NADH and FADH2 are oxidized by the mitochondrial respiratory chain to establish an electrochemical gradient of protons, which is finally used by the F1F0- ATP synthase (complex V) to produce ATP, the only form of energy used by the cell (Adapted from Bellance 2008).

electron transfer but in the production of ATP (Garret, 2013). The electrons that are transported in the chain are provided by redox reaction between electrons donor (NADH and FADH) and electrons acceptors (O_2). Complexes have different activity:

- complex I (NADH ubiquinone oxidoreductase) is responsible for the oxidation of NADH;

- complex II (succinate ubiquinone reductase) oxidizes FADH2.

The electrons converge from these two complexes to the coenzyme Q10 (CoQ) (Garret, 2013), a lipid-soluble electron carrier that transfers electrons from complexes I/II to complex III. During this step, CoQ is reduced and is called ubiquinol.

- in complex III (cytochrome bc1 complex), CoQ will be converted back to its oxidized state (ubiquinone) and in this way it catalyses the reduction of Cytochrome C, another electron carrier;

- in complex IV (cytochrome oxidase) (Garret, 2013), the electrons transferred from the Cytochrome C are delivered. This is the last enzyme in the respiratory chain in mitochondria where the electrons are transferred to an O_2 molecule, forming two molecules of water;

- complex V (ATP synthase) transports protons back to the matrix. The energy produced by this transport is used to synthesize ATP from adenosine diphosphate (ADP) and inorganic phosphate (Pi). The ATP is then translocated out of mitochondria, through the adenine nucleotide transporter (ANT) at the IM, and through porins at the OM level.

In the brain, the energy request is very high and it can be modulated in time and space based on activities levels. This regulation can be due to changes in (i) the OXPHOS based on cellular and mitochondrial microenvironment, (ii) the OXPHOS through kinetics parameters (e.g. increase of efficiency), (iii) in the activity of oxidative chain, e.g, post-translational modification, in particular in the complex I (De Rasmo et al., 2010).

Several data show that cAMP-dependent phosphorylation controls directly the cellular respiration through the modulation of complex I activity (Scacco et al., 2000). In addition, the post-transcriptional phosphorylation of NDUFS4, subunit of the complex I, mediates the increase of NADH oxidation (De Rasmo et al., 2010; Scacco et al., 2000).

1.8.1.1.2 MITOCHONDRIA: Beyond the ATP synthesis

Beyond their role in generating ATP, neuronal mitochondria contribute to Ca^{2+} homeostasis. Many characteristics of synaptic transmission and plasticity depend on

changes in presynaptic and post-synaptic Ca^{2+} levels (Zucker, 1999). Under physiological resting conditions, the cytosolic Ca^{2+} concentration is remarkably low (about 50–100 nM), but it can transiently rise during electrical activity to levels that are 10 to 100 times higher (Berridge et al., 2000). Together with the endoplasmic reticulum (ER), mitochondria play a key role in modulating the concentration of cytosolic Ca^{2+} (Grimm, 2012).

In excitable cells, elevation of Ca^{2+} increases OXPHOS indirectly, by increasing ATP consumption via active Ca^{2+} transport, and directly, by stimulating ATP production via activation of complex V and Kreb's cycle enzyme activity (Bender and Kadenbach, 2000; Carafoli and Crompton, 1978; Gunter et al., 2004; Lehninger et al., 1978; Vale et al., 1983). Many data suggested that Ca^{2+} could be one of the positive regulators of mitochondrial energy metabolism at nerve terminals by potentially acting as a feed-forward mechanism to boost ATP synthesis in order to prevent energy drop during synaptic activity (Rangaraju et al., 2014).

During mitochondrial respiration, significant amounts of ROS are formed. Free radicals, such as the hydroxyl radical (OH^-) and the superoxide anion ($O2^-$), are transient and chemically unstable due to unpaired electrons in their atomic orbitals. Hence, they tend to rapidly react with other compounds, in an attempt to capture the electron they need to gain stability (Balaban et al., 2005).

Mitochondria are considered the major sites of ROS production in the cell (Cadenas and Davies, 2000): when electrons are transferred along the electron transport chain, a small quantity of electrons can leak out from the classical redox reactions pathway and be transferred directly to O_2. Complex I and III are thought to be the main ROS generation sites as compared to complex II (Balaban et al., 2005). ROS could have either

deleterious effects, by promoting cellular damages, and physiologic roles, through a signaling activity (Popa-Wagner et al., 2013). ROS have been proposed to act as signal molecules modulating a myriad of cellular processes including apoptosis, inflammation, synaptic plasticity and memory formation (Hu et al., 2006; Massaad and Klann, 2011). The mitochondrial function is essential for neuronal activity. Mutations of mitochondrial genes have been identified as neurological phenotypes in rare diseases. Mitochondria-mediated oxidative stress, alteration of Ca^{2+} homeostasis and apoptosis may also contribute to the pathogenesis of neurological diseases including Alzheimer's, Parkinson's and psychiatric disorders (Mattson et al., 2014).

1.8.2 Link between cannabinoids and mitochondria

It is known that cannabinoids affect mitochondrial morphology and activity (Chari-Bitron and Bino, 1971; Bino et al., 1972; Bartova and Birmingham, 1976; Schurr and Livne, 1975, 1976; Schurr et al., 1978). In neuronal cell cultures, THC could induce the release of mitochondrial Cytochrome C (an intermediary substrate in the mitochondrial respiratory chain) into the cytosol and activate caspases and nuclear DNA fragmentation (Campbell, 2001). Through the release of Cytochrome C in the cytosol, mitochondria are involved in the apoptosis and programmed cell death, mechanisms related also to synaptic plasticity and neurodegenerative disorders (Mattson, 2000). The THC-induced apoptosis can be blocked by the CB1R antagonist AM251, suggesting that the apoptosis is CB1R-dependent and involved in in the activation of G proteins.

Mitochondria control calcium homeostasis, but also eCBs react to and modulate calcium flux in different ways. eCBs modulate the activity of NO, which is able to control the

mitochondrial biogenesis but also the cell fate via modulation of mitochondrial function (Nunn et al., 2012). Many studies (Bartova and Birmingham, 1976; Bino et al., 1972; Chari-Bitron and Bino, 1971) have reported the effects of cannabinoids on respiratory function, including:

- decreased activity of NADH-oxidase (complex I) in isolated mitochondria from different brain regions after acute THC treatment (Bartova and Birmingham, 1976).

- inhibition of the consume of mitochondrial oxygen and disruption of the membrane potential in purified mitochondrial of rat heart after exposure to THC, HU210 and AEA (Athanasiou et al., 2007; Silva et al., 2013; Zaccagnino et al., 2011). These mechanisms are associated with a decline of cellular ATP bioavailability, indicating a potential decrease of mitochondrial respiration and contributing to the depletion of ATP (Sarafian et al., 2003).

In the past, these effects were described as aspecific alterations of mitochondrial membrane. Yet, in 2012 Marsicano's team demonstrated that CB1Rs are expressed on brain mitochondria and that cannabinoids can directly regulate OXPHOS activity (Benard et al., 2012; Hebert-Chatelain et al., 2014; Vallee et al., 2014). mtCB1 receptors have a topological orientation, with a cytoplasmatic N-terminal and an intra-mitochondria C-terminal, and modulate brain functions through different signaling pathways including, among others, inhibition of transmembrane adenylyl cyclase, modulation of ion channels and activation of MAPKs and focal adhesion kinases (Howlett, 2005; Piomelli, 2003). The activation of mtCB1 receptors decreases mitochondrial respiration and complex I activity through inhibition of a soluble form of adenylyl cyclase (sAC).

Inhibition of sAC induced by cannabinoids down-regulates cAMP level and leads to the decrease of PKA activity and the consequent reduction of complex I phosphorylation. Hebert-Chatelain and colleagues observed a specific effect of THC (800nM) on complex-I-dependent respiration in purified brain mitochondria, suggesting that mtCB1 receptor signaling specifically targets this complex (Hebert-Chatelain et al., 2016). Analysis of the PKA-dependent phosphorylation of brain mitochondrial proteins revealed that the subunit NDUFS2 was consistently targeted by PKA in an mtCB1-signaling-dependent manner. Indeed, mitochondrial respiration is blocked by the sAC activator bicarbonate and occluded by the sAC inhibitor KH7. In this line, co-immunoprecipitation of Gα proteins with sAC from purified brain mitochondria revealed a physical interaction between mitochondrial sAC and Gα proteins in the brain, promoted by mtCB1 receptor activation. Additionally, targeting of sAC into mitochondria rescues the decrease of O_2 consumption induced by cannabinoids. Overall, these findings clearly establish a causal role of sAC activity in mediating the effect of mtCB1 receptor signaling on OXPHOS within mitochondria.

This study (Hebert-Chatelain et al., 2016) is the first to report a direct modulation of sAC activity via heterotrimeric G-protein alpha subunits present in the mitochondria. This is actually surprising, because sAC was shown to be independent from G protein signaling in certain tissues, such as testis (Buck et al., 1999). This apparent discrepancy may underlie tissue-specific differential regulatory pathways of sAC activity.

Thus, the mitochondrial cAMP/PKA pathway appears to be involved in the decrease of complex I activity and respiratory activity produced by cannabinoids (Fig. 13).

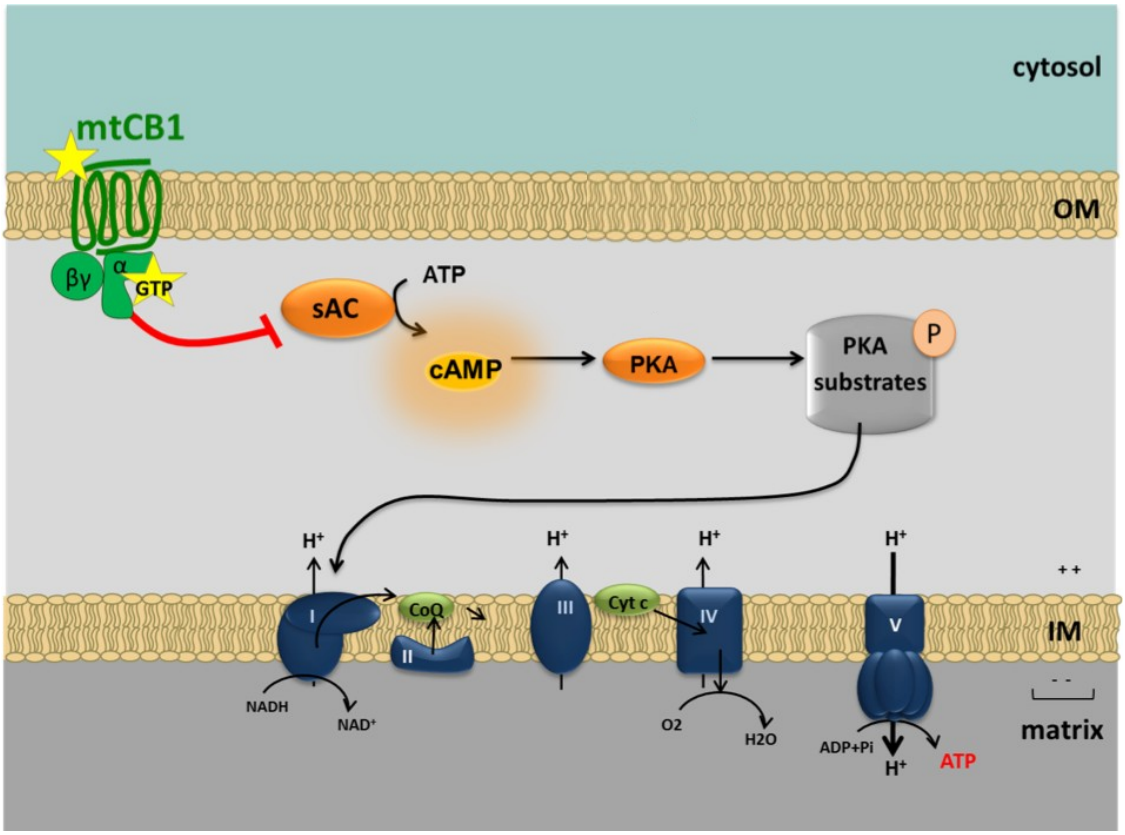

Fig.13 Schematic representation of activation mtCB1 receptors.
Activation of mtCB1 receptors decreases mitochondrial respiration and complex I activity through inhibition of a soluble form of adenylyl cyclase (AC). sAC inhibition induced by cannabinoids down-regulates cAMP level and leads to the decrease of PKA activity and the consequent reduction of Complex I phosphorylation (adapted from Desprez T.)

Exposure to cannabinoids or an increase in the EC tone (for example, by inhibiting MAGL) (Benard et al., 2012) creates remarkable changes in the mitochondrial function. Nowadays, it is well known that mitochondrial dysfunction, such as changes in mitochondrial dynamics and mobility, and perturbation of mitochondrial turnover are involved in the pathology of some neurodegenerative and neurological disorders. In particular, damaged mitochondria fail to produce ATP and appropriate levels of buffer Ca^{2+} and to release apoptotic cell death signals. Moreover, defects in mitochondrial transport may cause local energy depletion and toxic changes in Ca^{2+} buffering that may

trigger synaptic dysfunction and loss, thus contributing to neurodegenerative disorders (Sheng and Cai, 2016).

Mitochondrial deficits could trigger neuropsychiatric disorders. Indeed, disruption of mitochondrial activity or a decreased activity of OXPHOS and complex I create dramatic effects, which consequences are implicated in several neurological diseases (Moran et al., 2012; Papa et al., 2012; Zsurka and Kunz, 2013). For example, complex IV activity in frontal cortex and caudate nucleus is reduced in schizophrenic patients where, in parallel, the mitochondrial respiratory function decreases due to the alteration of complex I activity and a decreased expression of its subunits (Lezi and Swerdlow, 2012; Mattson et al., 2008; Rozenfeld and Devi, 2011). The central role of mitochondria in neuropsychiatric disorders and the effects of (endo)cannabinoids on brain bioenergetics pose new questions about the effect of the exposure to CB1R agonists during the different stages of brain development.

AIM OF THE STUDY

Adolescence is an important period in brain development and maturation, during which a strong and efficient neuronal organization takes place (Chambers et al., 2013). From a neuroanatomical point of view, the development of subcortical regions such as the amygdala, limbic areas and the hippocampus occurs in the first stage of adolescence, while cortical regions reach maturity only later. These neuroanatomical changes are reflected in the maturation of neurotransmitter systems, with DA and GABA being crucial for cortical functions (Bossong and Niesink, 2010; Casey et al., 2008; Spear, 2000).

The EC system plays an important role in both the development and the homeostasis of neurotransmitters, from early life to adulthood. In particular, the activity of Glu and GABA is mediated by the EC system, since the activation of CB1Rs decreases the release of neurotransmitters from the presynaptic terminals (Lu and Mackie et al., 2016; Wilson and Nicoll, 2002). During the onset of adolescence, eCBs and CB1Rs achieve the maximum expression in life (Meyer et al., 2018).

The major expression of CB1Rs has been observed in regions involved in cognitive and rewarding functions such as the cortex, striatum and hippocampus (Parsons and Hurd, 2015; Kawamura et al., 2006; Zamberletti et al., 2015), with AEA playing a dominant role in brain development (Meyer et al., 2018). The EC system is able to modulate DA response to natural reward, as it finely controls the activity of DA projections from the VTA to the NAc, modulating rewarding behaviors and, ultimately, affecting drug use and abuse (Parsons and Hurd, 2015).

Exposure to drugs during adolescence, including cannabinoids, creates aberrations in brain development that lead to important modifications in the EC system and in reward

related circuit in adulthood (Rubino and Parolaro, 2016; Puighermanal et al., 2012; Levine et al., 2017).

Nowadays, cannabis is still the most consumed illicit drug among adolescents in Europe and United States (EMCDDA, 2018; Zehra et al., 2018). During 2017, 14.1% of young adults used cannabis compared to the 7.2% of adults. Notably, the trend to abuse SCs, the main ingredient of Spice drugs, posed adolescents at risk of acute intoxication and deaths (EMCDDA, 2018).

Despite the frequent use of SCs by adolescents (Wagner et al., 2014), no basic research has been conducted to evaluate SCs ability to sustain a reliable IVSA behavior in adolescent animals and their long-term effects. Given the growing popularity of SCs among young adults, lack of knowledge on their long-term effects represents an important unanswered medical and social question.

The first SC identified in Spice drugs was JWH-018, which displays a greater potency and efficacy than THC (Atwood et al., 2010). These pharmacodynamic differences may partially explain reports of toxicity and overdosing by users expecting a cannabis-like potency (EMCDDA 2009; Harris and Brown, 2013). Yet, other mechanisms may be responsible for the different severity of the side effects induced by THC and JWH-018. Indeed, discrepancies in adverse effects frequency and severity might be due to distinct JWH-018 metabolites activity at the CB1R (Brents et al., 2011). The adverse effects reported by acute intoxication of SCs are usually hallucinations, seizure, agitation, tachycardia, psychosis and anxiety (Every-Palmer 2011; Harris and Brown 2013; Papanti et al., 2013).

To date, a few studies have examined the acute effects of SCs in adolescents and the long-term effects caused by adolescent exposure to THC using animal models (see

Rubino and Parolaro, 2015 and Lubman et al., 2015 for review). However, the absence of a reliable adolescent animal model that mimics the voluntarily daily intake of SCs impedes research in the field. Such a model would facilitate the study of the long-term consequences of SCs in adolescent animals at behavioral and neurochemical level. To fill the gap, this study was aimed to develop a reliable adolescent animal model that mimics the voluntarily daily intake of the prototypical SC JWH-018, which would provide a unique tool to evaluate the long-term behavioral and neurochemical consequences of adolescent voluntary consumption of SCs. Thus, the aims of my thesis are the following:

(i) To investigate the reinforcing properties of JWH-018 in adolescent mice.

First of all, we evaluated the ability of this SC to initiate and sustain IVSA in mice and characterized drug-taking behavior in all its phases, including acquisition, maintenance, extinction and reacquisition.

(ii) To evaluate the respective role of the CB1R and the CB2R in modulating JWH-018 IVSA.

In order to understand if operant behavior was effectively mediated by the action of JWH-018 on CB1Rs, we tested the effect of the pretreatment with the CB1R antagonist AM251 on responding for the SC. Moreover, since JWH-018 possesses high affinity not only for the CB1R but also for the CB2R, we also tested the effect of the pretreatment with the CB2R antagonist AM630, to ascertain if also CB2R may be involved in JWH-018 IVSA.

(iii) To investigate the long-term effect of adolescent exposure to JWH-018 on anxiety-like behavior.

Spice drugs users frequently report adverse effects, mostly anxiety and emotional altered states. Since anxiety disorders are often characterized by compulsive and repetitive-like behavior (Angoa-Pérez et al., 2013), in this study we investigated the presence of compulsive behavior as potential consequence of adolescent exposure to JWH-018 by testing adult mice (PND 79) in the nestlet shredding test.

(iv) To evaluate potential alterations of glial cells, CB1R levels and mitochondrial activity during adulthood as long-term consequences of adolescent exposure to JWH-018.

Given the growing evidence that glia cells are influenced by exposure to drugs of abuse (e.g. cocaine, alcohol, morphine) and that drug-induced alterations might contribute to the behavioral outcomes associated with substance abuse (Lacagnina et al., 2017; Linker et al., 2018; Coller and Hutchinson, 2012), we hypothesized that adolescent exposure to JWH-018 might affect microglia and astrocyte activity. For this purpose, we studied the possible long-term effects induced by adolescent exposure to JWH-018 in brain at adulthood, focusing on IBA-1 and GFAP as markers of neuroinflammation for microglia and astrocytes, respectively.

Moreover, since JWH-018 activity is CB1R-mediated, we further evaluated potential receptor alterations in areas involved in drug addiction, such as cortex, hippocampus and striatum. In light of the recent discovery of mtCB1 by Marsicano's group suggesting that these receptors may directly control cellular respiration and energy production (Benard et al., 2012), we investigated the long-term effects induced by JWH-018 on mitochondrial activity through enzymatic and biochemical analysis. The possibility in fact exists that, being JWH-018 a CB1R agonist, it may also act as mtCB1 agonists thus altering mitochondrial activity in adulthood.

MATERIALS AND METHODS

3.1 Animals

Adolescent CD1 male mice (Envigo, Netherlands) were used for all these studies. All animal experiments were carried out in accordance with the Guidelines for the Care and Use of Mammals in Neuroscience and Behavioral Research according to Italian (D.L. 116/92, 152/06, 26/2014) and European Council directives (609/86 and 63/2010) and in compliance with the approved animal policies by the Ethical Committee for Animal Experiments (CESA, University of Cagliari) and the Italian Ministry of Health (6/2016-PR). We made all efforts to minimize pain and suffering, and to reduce the number of animals used. Animals were housed in groups of three to six in standard conditions of temperature (21 ± 1°C) and humidity (60%) under a 12h/12h light/dark cycle (lights on at 7.00 am) with food and water available *ad libitum*.

3.2 Catheter preparation

Starting from previous studies (Rocha et al., 1998, David et al., 2001, De Luca et al., 2015), intravenous catheters have been prepared as follows:

-A silastic tube of 5 cm length (0.30 mm I.D. x 0.64 mm O.D.), was submerged into limonene for dilate it and allow to insert it in a cannula guide, (22GA, 11mm length; PlasticOne, Roanoke, VA, USA) which was previously modeled in the metallic part to obtain a L shape;

-After it was dried overnight, a second silastic tube of 4 mm length (0.64 mm I.D. x 1.19 mm), previously submerged into limonene, was insert between the metallic part of the cannula and the 5cm silastic in order to protect this part;

-Using a silk suture thread, three nodes were made to secure the 5cm silastic with the cannula;

-The skeleton of the catheter was placed in a mould where DuraLay resin (Dental Mfg. Co., Pl. Worth, Illinois, USA) was split to create the final form of the catheter;

-Once the cement was dried, using silicone was made a little sphere at 1.2 cm from the end of 5cm silastic.

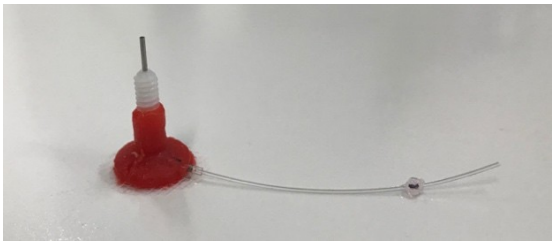

Fig.14 Representative image of intravenous catheter

Catheter for adolescent mice made with Medical-grade tubing Silastic (Dow Corning Corporation, Midland, MI, USA).

3.3 Surgery

On PND 25-26, mice were anesthetized with isoflurane gas, and maintained under anaesthesia using a breathing tube under a scavenging system. Eye lubricant (Tears Naturale P.M) is applied to both eyes to prevent them from drying out during the procedure. A chronic intravenous catheter for mice (Fig.14), prepared as previously described, was implanted with an indwelling intravenous catheter in the right jugular, as described previously.

The catheter, after being inserted and blocked inside the vein, was fixed in the scapular region, from which it comes out with the second end, to allow the administration of drugs. After surgery, mice were housed individually, under the same environmental

conditions, with food and water ad libitum, and underwent post-operative drug treatment for 3 days, before starting the self-administration experiments, to ensure full recovery (Kmiotek et al., 2012).

3.4 Drugs

JWH-018 was purchased from Tocris (Bristol, UK), and solubilized in 2% EtOH, 2% Tween 20 and 96% saline. AM251 and AM630 were purchased from Tocris (Bristol, UK), and solubilized in 9% EtOH, 9% Tween 20 and 82% saline.

3.5 Experimental protocol

After three days of recovery, IVSA experiment under different FR schedule of reinforcement during adolescence (7 days/weeks). At adulthood, mice underwent on drug-free washout period until PND 80 and then they have been sacrificed for the successive neurochemical assays (Fig.15).

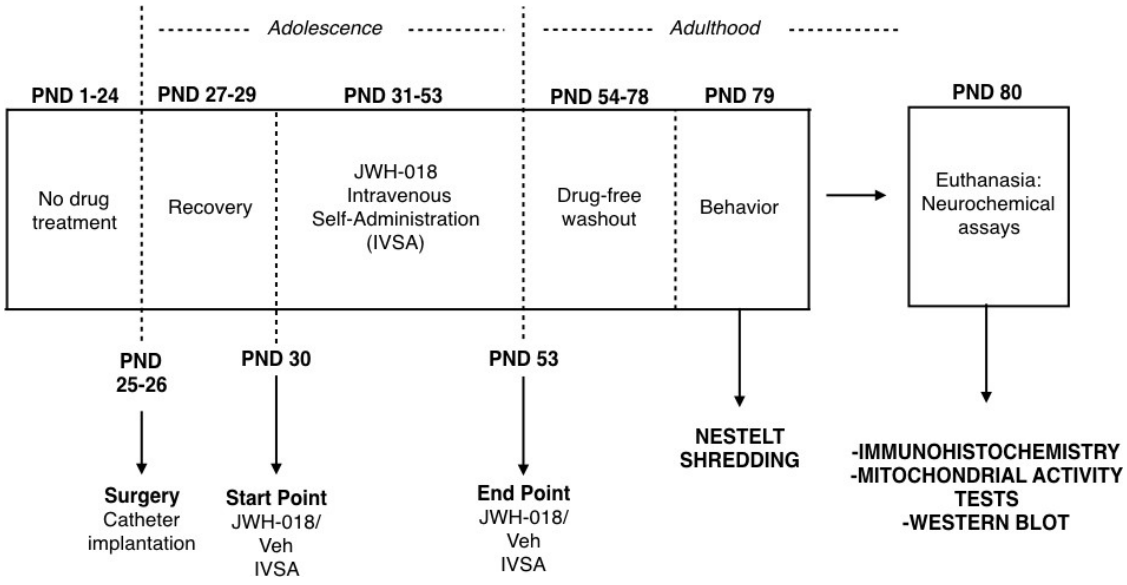

Fig.15 Experimental timeline
Experimental protocol used for the project

3.6 Self-administration experiments

Daily IVSA sessions (2h) were carried out in chambers housed in sound proof boxes (model LE 26M; Panlab Harvard Apparatus, Spain).

Chambers were equipped with two levers, and the responses on one lever (active lever) delivers one reinforcer, whereas responses on the second lever had no programmed consequences (inactive lever). A white light was placed over the active lever as discriminative stimuli. Prior to each daily session, the jugular catheter was flushed with 0.1 ml of sterile saline and the mice were placed in the SA box.

At the end of each SA session, the catheters were flushed with 0.1 ml of heparinized saline and the mice were returned to their home cages where a daily ratio of 4 g of food was made available, which maintained body weights at stable levels throughout these studies.

The responses performed by each mouse on both lever for the entire 2h daily session and the corresponding number of reinforcers received was recorded (Panlab software, Panlab Harvard Apparatus, Spain).

The self-administration session was monitored by a computer system (Panlab Harvard Apparatus, Spain) characterized by the following phases:

1. Ready phase (S1), characterized by the activation of the infusion pump for 5 seconds to fill the chronic catheter with dead volume of 15 µl;

2. Drug available phase (S2), during which the active lever presses behavior of mice allowed the transition to the next phase;

3. Infusion phase (S3), consisting of intravenous injection of 2.5-15 (µg /kg−1/0.025 ml−1) in 25 µl of JWH-018 in 5 seconds;

4. Time-out phase (S4), following infusion of the drug and lasting 20 seconds, during which each lever presser was recorded but did not result in any additional infusion. During this period the cage light was switched off, in a programmed manner. At the end of the time-out the availability of the drug was signaled by the switching on of the cage light on the active lever.

Based on the mouse's behavioral activity, the software cyclically activated the S2-S3-S4 states for the entire programmed duration of the session, ending with the Finish State phase (FIN) characterized by the shutdown of all stimuli.

3.6.1 Exp. I: Characterization of JWH-018 dose-response curve

The first experiment performed, aims to characterizing a dose-response curve of JWH-018 (2.5-15 µg /kg−1/0.025 ml−1) in adolescent mice. Mice IVSA experiment was carry

out under FR1 schedule (1 lever press: 1 injection), from PND 30 until PND 53 (24 sessions).

3.6.2 Exp. II: Acquisition of JWH-018 and Veh over consecutive sessions

Once the dose at which adolescent mice acquired operant behavior is established, (7.5 µg /kg−1/0.025 ml−1) we used different FR protocols to evaluate the abuse properties of JWH-018. After mice acquired operant behavior under FR1 and the number of lever pressing was stable for at least three sessions (6 sessions in total), mice underwent on FR3 (3:1) for 9 sessions. The same protocol was used for Veh group which performed IVSA with Veh solution (2% ethanol, 2% Tween 20 and 96% saline) instead JWH-018 solution.

The final session, the experiment of JWH-018 group was performed under PR schedule under which the number of active lever presses required to obtain each subsequent injection was based on the adapted exponential sequence: 1, 2, 4, 6, 9, 12, 15… (Prieto et al., 2016). PR sessions lasted for 2 h or until mice did not complete the ratio for delivery of one injection within 1 h.

3.6.3 Exp. III: Effect of CB1/CB2Rs blockade on JWH-018 IVSA (7.5 µg /kg−1/0.025 ml−1)

In order to investigate the role of CBRs on operant behavior, we used the CB1R antagonist/inverse agonist AM251 and the CB2R antagonist/inverse agonist AM630. AM251 and AM630 have been administered intraperitoneally (i.p.) 30 minutes before putting the mice in operant box for only one session (during FR3 schedule).

We used a range of doses to well understand the effect on CBRs on operant behavior. For AM251 the range was from 0.3 to 1 mg/kg i.p., whereas for AM630 we administered only the two doses 0.5 and 1 mg/kg i.p..

3.6.4. Exp. IV: Extinction and reacquisition phase

Once mice acquired stable behavior during FR3 schedule, it is possible to use the extinction and reacquisition phase protocols, which allow to study the seeking behavior to JWH-018 and the reacquisition of operant behavior with JWH-018 after the extinction phase, respectively.

In fact, during extinction period, JWH-018 was substituted with the Veh solution and the pump of infusion was turned off in attempt to reduce the cues.

After 6 sessions performed in extinction phase, the protocol switch to reacquisition phase (7 sessions) where Veh was replaced by JWH-018 and the pump was turned on.

In all the self-administration experiments performed, the patency of intravenous catheters was evaluated at the end of the experiments by an infusion of 0.05 ml of thiopental sodium (5 mg/ml) (Braun Medical) through the catheter. If the signs of anesthesia were not apparent within 3 seconds of the infusion, the mouse was removed from the experiment.

3.7 Behavioral experiment

3.7.1 Nestlet shredding

We performed nestlet shredding test to evaluate compulsive and repetitive-like behavior

in mice, since they used to shred material for nest building.

At PND 79, mice were individually placed in habituation for 1 hour in the room of experiment using a light source with an intensity in a range between 20 and 30 lux.

After the habituation time, mice were placed in a clean mouse cage with 2 cm (height) bedding material and 5x5 cm packed cotton nestlet (Ancare Corp; Bellmore, NY) laid on the top of the bedding.

Mice performed test for 75 minutes, after this period the remaining nestlet was collected, dried overnight, and weighed, and the percentage of the nestlet shredded was calculated (Murphy et al., 2017).

3.8 Immunohistochemistry experiment

3.8.1 Brain tissue preparation and GFAP and IBA-1 immunofluorescent staining

At adulthood (PND 80), after a drug free period (~3 week) from the last session of SA, mice were deeply anaesthetized with equithesin (0.97 g pentobarbital, 2.1 g magnesium sulphate, 4.25 g chloral hydrate, 42.8 mL propylene glycol, 11.5 mL ethanol 90%, 5 mL kg 1, intraperitoneal) and then transcardially perfused with 4% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M phosphate-buffered saline (PBS, pH 7.4). Brains were rapidly removed and post-fixed in the same fixative for 6 h. After repeated washing in 0.1 M PBS, brains were cryoprotected in 30% sucrose in PBS for 48 h. Immunostaining was performed on free-floating coronal sections (thickness: 40 mm) which were obtained using a cryostat at levels comprising the brain areas selected for this study. To facilitate the identification of the selected brain areas, adjacent sections were also collected and stained with Neutral Red.

Tissue sections were incubated for 1 hour at 20°C in working solution of mouse immunoglobulin-blocking reagent, prepared as specified by the manufacturer (Vector Laboratories, Burlingame, CA). Following washing, pre-blocking of tissue sections was performed using normal goat serum (NGS, 5% and NDS, 5%), bovine serum albumin (BSA, 1%) and Triton X-100 (0.2%) in PBS for 1 h at room temperature. As concerns GFAP-immunofluorescence, tissue sections were incubated at 4°C for 24 h with a

mouse monoclonal anti-GFAP antibody (1:5000; Millipore Temecula, CA, USA) in PBS containing 0.2% Triton X-100, 0.1% BSA, 0.5% NGS and 0.5% NDS. Then, sections were washed in PBS containing 0.2% Triton X-100 and incubated them with Alexa Fluor 594-labeled donkey anti-mouse IgG (1:500; Molecular Probes, Eugene, OR, USA) for 1 h in the dark at room temperature.

As concerns IBA-1 immunofluorescence, we incubated tissue sections at 4°C for 24 h with a rabbit monoclonal anti-IBA-1 antibody (1:2000; Wako Pure Chemical Industries, Chuo-Ku, Osaka, Japan) in PBS containing 0.2% Triton X-100, 0.1% BSA, 0.5% NGS and 0.5% NDS. Then, sections were washed in PBS containing 0.2% Triton X-100 and incubated them with Alexa Fluor 488-labeled goat anti-rabbit IgG (1:500; Molecular Probes, Eugene, OR, USA) for 1 h in the dark at room temperature.

3.8.2. Imaging and quantitative analysis of GFAP and IBA-1 immunofluorescent staining

An Olympus IX 61 microscope, furnished with 2.5, 4, 10, 20 and 60X planapochromatic oil immersion objectives, was used for observations. An Olympus 12-bit cooled F View II camera (Hamburg, Germany) was used for capturing the images. Excitation light was attenuated with a 6% transmittance neutral density filter.

For each animal, analysis of GFAP and IBA-1 immunoreacitivity (IR) was performed on one tissue section out of every 2 successive sections for a total of 3 sections containing the PFC, 1 tissue section out of every 3 successive sections for a total of 4 and 10 sections containing the NAc shell and core and the CPu, respectively (Fig.16) . In order to include almost the whole area (either PFC, CPu, NAc), according to the extension of the region under analysis, we have chosen the total size of the examined

area in which GFAP-IR and IBA-1-IR cells were counted. The selected coronal levels of these sections corresponded to the levels of plates 12-15 for the PFC (AP: +2.2 to +1.94), 21–31 for the CPu (AP: +1.18 to +0.02), 12-24 for NAc shell and core (AP: +1.18 to +0.86) (Atlas of Mouse Brain, Paxinos and Franklin, 2001).

Semi-quantitative analysis of GFAP was carried out using the 20X objective on 3 non-overlapping regions of interest (ROIs, roughly 140000 mm2) from 1 out of either every 3 slices of NAc and CPu region or every 2 slices of the PFC region. The focus depth was extended by summing the maximum intensity of several images taken at focus steps of 0.25 mm depth intervals to a total of 2 mm thickness using the Z-stack module (Olympus Soft Imaging Solution, GNHB, Munster, Germany). After capture, images were analyzed using the Cell P Analysis software module. Density thresholding to the single channel grey scale images was applied to detect positively stained fibers. Subsequently, for each image we estimated the proportion (%) of area occupied by fibers, and for each animal we calculated average values from images of all tissue sections.

The number of IBA-1 positive cells was counted bilaterally in 3 (PFC), 4 (NAc) and 10 (CPu) sections per animal. In these sections, non-overlapping randomly selected ROIs of 0.15 mm^2 (n 6), were examined with a 20X objective by two trained observers blind to drug treatment. Limits of the ROI were defined based on structural details within the tissue sections to ensure the ROIs did not overlap. The distance among the 6 ROIs was superior to 40 μm to avoid overlapping. IBA-1 positive cells touching the inferior or the

right sides of the ROI were excluded from counting. The number of IBA-1 cells was expressed as mean/mm^2 SEM.

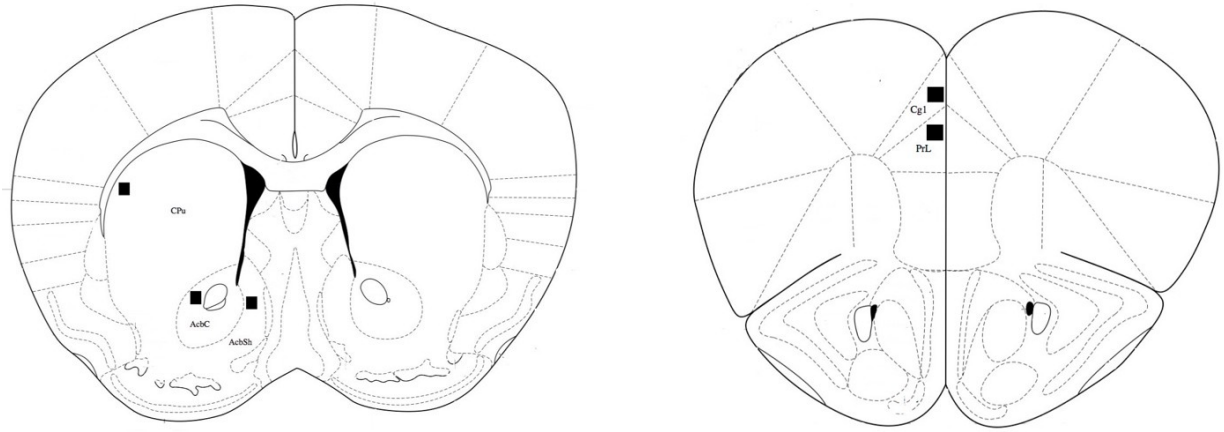

Fig.16 Coronal section of brain areas selected.
Schematic representation of the PFC (Cingulate cortex Cg1 and Prl, CPu, NAc Core and NAc Shell (Coordinate adapted from The mouse brain, Paxinos and Watson 2001).

3.9 Ezymatic assays

3.9.1 Complex I enzyme activity

At PND 80, after a drug free period (~3 week) from the last session of SA, mice were sacrificed by cervical dislocation and hippocampus, striatum and cortex were quickly collected and snap frozen in nitrogen liquid and stored at -80°C.

The day of experiment samples were homogenized in PBS pH 7.4 1X (Gibco by life technologies, Carlsbad, US) supplemented with protease and phosphatase inhibitors purchased from Roche (Basel, Switzerland), using the Tissue Lyser (Quiagen, Hilden, Germany). The protein contents were determined with Roti-Nanoquant protein quantitation assay, following manufacturer's instruction (Carl Roth, Karlsruhe, Germany).

The measurement of complex I enzyme activity was determined with complex I Enzyme Activity Microplate Assay Kit (Colorimetric) following manufacturer's instruction (Abcam), the final samples concentration before the assay procedure was 100µg/200µL. The test has been perform using POLARstar Omega plate reader (BMG Labtech, Offenburg, Germany).

3.9.2 CS enzyme activity

The same samples prepared for the complex I enzyme activity were used for CS activity. CS activity was measured as a color change of 5,5′- dithiobis-(2-nitrobenzoic) acid (DNTB). Incubation buffer was composed of 20 mmol/l Tris/HCl (pH 8), 0.1 % Triton X-100, 0.1 mmol/l DTNB, and 0.42 mmol/l acetyl-CoA. The reaction was initiated by the addition of 0.5 mmol/l oxaloacetate and absorbance was measured at 412 nm for 3 min at 37°C (Singh et al., 2015; Eigentler et al., 2015). The test has been performed in 12 well plate using POLARstar Omega plate reader (BMG Labtech, Offenburg, Germany).

3.10 Western blot

The same samples prepared for the complex I enzyme activity were used for western blot analysis. Protein extracts were mixed with denaturing 4x Laemmli loading buffer and warmed for 30 minutes at 37°C. Samples (18-24 µg per lane) were analyzed on 4-20% precast polyacrylamide gels (Bio-Rad, Hercules, California) and transferred onto PVDF membranes 0.45µm (Merk Millipore, Billerica, MA). Membranes were blocked in a mixture of Tris-buffered saline and polysorbate 20 (20mM Tris-HCl pH 7.6,

150mM NaCl, 0.05% Tween 20) containing 5% of non-fat milk for 1 h at room temperature (RT). Membrane were incubated at RT for 1h using antibodies against rabbit CB1R (CB1, ab23703; 1:200, Abcam, Cambridge, UK), mouse NDUFS4 (ab87399; 1µg/mL, Abcam, Cambridge, UK), mouse alpha-tubulin (used as loading control) (sc69969; 1:5000, Santa Cruz, Dallas, US).

After transfer's step, the membranes were cut in order to perform the incubation for 1 h with the primary antibody anti CB1R or anti NDUFS4 separately.

After stripping with Restore western Blot Stripping Buffer (Thermo Fisher Scientific, Massachusetts, USA), for normalization the membranes were re-blotted with a mouse monoclonal anti alpha-tubulin antibody.

Bound primary antibodies were detected with HRP-linked antibodies (1:2000, Cell Signaling Technology, Danvers, MA) and visualized by enhanced chemiluminescence detection (Clarity western ECL Substrate, Bio-Rad, Hercules, California). The Optical densities of immunoreactive bands were quantified by the Image Lab software (Bio-Rad, Hercules, California) after acquisition on ChemiDoc Touch (Bio-Rad, Hercules, California, US).

3.11 Data analysis

Self-administration

The statistical analysis was performed using the StatSoftware for Windows V.8 (StatSoft, Inc. Tulsa, OK, USA). The results obtained during each SA session were analyzed by means of a two-way variance analysis (ANOVA), using the number of

lever presses (active vs. inactive) as factors and the days as repeated measurements. Two way-ANOVA was followed by LSD post hoc test (Exp I, II, IV).

JWH-018 consume: JWH-018 intake (μg /kg−1/0.025 ml−1) was expressed as average of JWH-018 consumption administered at each dose during 2h of IVSA sessions. One way-ANOVA analysis was followed by LSD post hoc test (Exp I).

CB1/CB2Rs blockade on IVSA: the effects of CBRs blockade on IVSA was expressed as average of the last session under JWH-018 IVSA (7.5 μg/kg/inf) condition, and under CB1Rs or CB2Rs blockade pre-treatment condition for one session. One way ANOVA followed by LSD post-hoc test (Exp III).

Behavior

The percentage of nestlet shredding behavior in the two groups of mice (Veh and JWH-018) was carried out using the GraphPad Prism program version 6.00 for Windows (GrapPad Software San Diego, CA, USA) and unpaired Student's t-test.

Immunohistochemistry

The number of IBA-1 positive cells counted per mm^2 and the percentage of density of GFAP immunoreactivity in the two group of mice (Veh and JWH-018) were carried out using the GraphPad Prism program version 6.00 for Windows (GrapPad Software San Diego, CA, USA) and unpaired Student's t-test.

Western Blot

The expression of CB1Rs and NDUSF4 was expressed as percentage of Veh conditions in every area tested. Relative CB1R and NDUFS4 expression were calculated by

normalizing densitometric values of CB1R and NDUFS4 bands to the corresponding alpha-actin values and the values are expressed as arbitrary units.

The analysis was carried out using the GraphPad Prism program version 6.00 for Windows (GrapPad Software San Diego, CA, USA) and unpaired Student's t-test.

Enzymatic assays

The values of complex I and CS activity were expressed as values of mOD/minute and slope/minute respectively for the two groups (Veh and JWH-018). The analysis was carried out using the GraphPad Prism program version 6.00 for Windows (GrapPad Software San Diego, CA, USA) and unpaired Student's t-test.

RESULTS

ADOLESCENT MICE

4.1 Experiment I: Characterization of JWH-018 dose-response curve in the IVSA experimental paradigm

JWH-018 IVSA by adolescent CD1 mice at different doses under fixed (FR1) reinforcement schedules.

In attempt to characterize the pattern of response of CD1 male adolescent mice (PND 30-53), we firstly investigate on the characterization of JWH-018 dose-response curve in the IVSA experimental paradigm. Based on previous studies performed in adult C57 mice (De Luca et al., 2015), we chose a range of doses from 2.5 to 15 μg/kg−1/0.025 ml−1. This experiment showed that, under appropriate experimental conditions, mice readily acquired operant behavior (lever pressing, FR 1:1) for JWH-018 at the dose of 7.5 μg/kg−1/0.025 ml−1 (Fig.17A). Two way-ANOVA applied to the sessions under the dose of 7.5 μg/kg−1/0.025 ml−1 showed a main effect of session ($F_{(23,322)}$ =7.4, P<0.001). LSD post hoc test showed significant difference between the number of active and inactive lever pressing in the 12° and 13° sessions, performed with 7.5 μg /kg−1/0.025 ml−1.

Moreover, one way-ANOVA shows (Fig.17B) a difference in the total intake during 2h session within the doses tested; LSD post hoc test showed the intake at dose of 7.5 μg /kg−1/0.025 ml−1 was significant higher compare to the other doses tested.

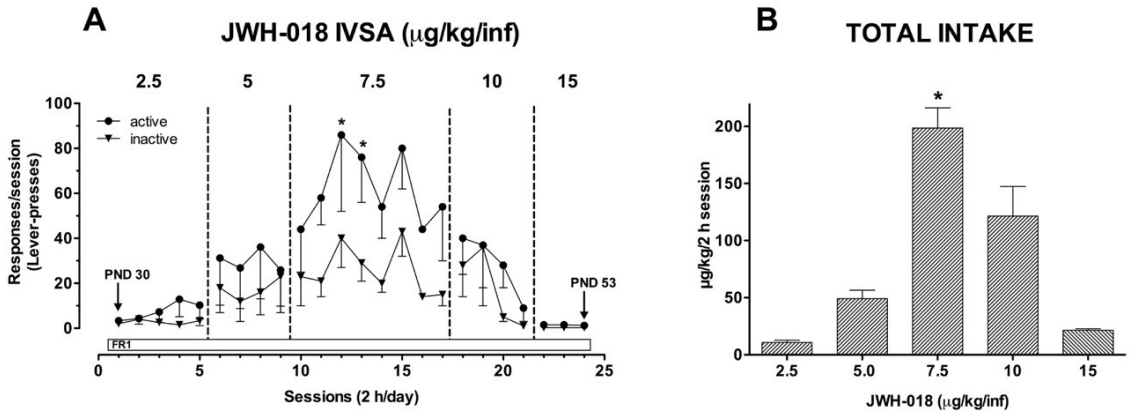

Fig.17 (A) Number of presses on the active lever (*circles*) that resulted in JWH-018 infusion (2.5-15 μg/kg/inf) and on the inactive lever (*triangles*) during each 2h daily session under FR1 reinforcement schedules from PND 30 to 53. Results are expressed as mean ± SEM (n=8-6), *p<0.05 vs inactive lever-presses. (B) Daily intake during JWH-018 IVSA (2.5-15 μg/kg/inf) at FR1. Data are expressed as μg/kg of JWH-018 self-administered during each 2-h daily session. Each bar represents the mean ± SEM of the drug self-administered at each dose during the IVSA sessions as indicated in the panel A, *p<0.05 vs all the other groups.

4.2 Experiment II: Acquisition of JWH-018 and Veh over consecutive sessions

JWH-018 or Veh IVSA by adolescent CD1 mice under different fixed reinforcement schedules (FR1, FR3, PR).

Once the dose at which adolescent mice acquired operant behavior was established, (7.5 μg /kg−1/0.025 ml−1) we used different FR protocols to evaluate the abuse properties of JWH-018. In addition, after mice acquired operant behavior under FR1, the schedule was changed to FR3. The same protocol was used for control group which performed IVSA with Veh instead JWH-018 solution.

In the final session, the experiment of JWH-018 group was performed under PR schedule; under PR, the number of active lever presses required to obtain each subsequent injection was based on the adapted exponential sequence.

Importantly, the operant behavior was specifically directed at obtaining JWH-018 since mice increased responding under a PR schedule of reinforcement (Fig. 18A), where the response requirements increase systematically within the session. Two way-ANOVA showed a main effect of responses ($F_{(1,18)} = 13.27$, $P<0.01$), sessions ($F_{(15,270)} = 10.13$, $P<0.001$) and responses x sessions ($F_{(15,270)} = 2.7$, $P<0.001$). LSD post hoc test showed significant difference between the number of active and inactive lever presses, in the 3°, 6°, 7°, 8°, 9° sessions under FR3 schedule of reinforcement and in the last session performed under PR.

A separate group of animals failed to acquired Veh IVSA (Fig. 18B), two way-ANOVA test show: responses $F_{(1,12)} = 7.8$, $P<0.05$; sessions $F_{(14,168)} = 3.2$, $P<0.01$. The following LSD post hoc shows no significant difference.

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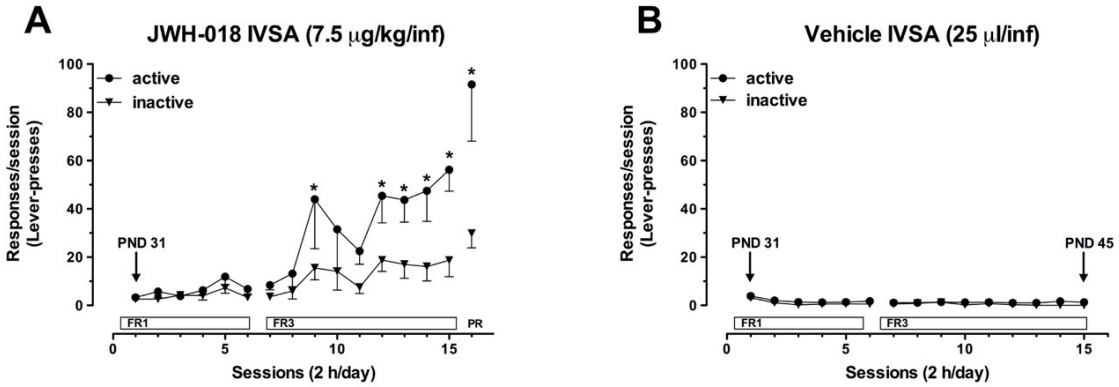

Fig.18
(A) Number of responses (lever presses) on the active lever (*circles*) that resulted in JWH-018 infusion (7.5 μg/kg/inf) and on the inactive lever (*triangles*) during each 2h daily session under FR1, FR3 and PR reinforcement schedules from PND 31 to PND 46. Results are expressed as mean ± SEM (n=10), *p<0.05 vs inactive lever-presses. (B) Number of responses on the active lever (*circles*) that resulted in Veh infusion (25 μl/inf) and on the inactive lever (*triangles*) during each 2h daily session under FR1, FR3 reinforcement schedules from PND 31 to PND 45. Results are expressed as mean ± SEM (n=7).

4.3 Experiment III: Effect of CB1/CB2Rs blockade on JWH-018 IVSA

In order to investigate the involvement of CBRs on operant behavior, we studied the effect of the administration of either CB1R antagonist AM251 or CB2R antagonist AM630 on JWH-018 (7.5 μg /kg−1/0.025 ml−1) IVSA behavior. To better understand the role of CBRs in the maintaining of IVSA behavior, we used a range of doses of AM251 (0.3-1 mg/kg i.p.) and AM630 (0.5-1.0 mg/kg i.p.).

Our results show that IVSA behavior was significantly reduced by the i.p. administration of the selective CB1R antagonist/inverse agonist AM251 (0.7 and 1.0 mg/kg i.p., 30min before the IVSA session) (Fig. 19A). One-way ANOVA showed significant difference of treatment ($F_{(4,24)}$ =16.22, P<0.0001), responses ($F_{(1,24)}$ =54.85, P<0.00001) and treatment x responses ($F_{(4,24)}$ =6.1, P<0.005). LSD post-hoc test showed a significant difference at dose of 0.7 and 1.0 mg/kg i.p. compared to Veh treatment. On the other hand, the administration of the selective CB2R antagonist/inverse agonist AM630 (0.5-

1.0 mg/kg i.p., 30 min before the IVSA session) did not affect IVSA operant behavior (Fig. 19B). One-way ANOVA showed significant differences for responses ($F_{(1,12)}$=26.13, P<0.001). LSD post-hoc test shows no significant difference.

These data show that the rewarding effects of JWH-018 are CB1R-mediated, since the administration of AM251 0.7 mg/kg i.p. significantly reduced operant behavior.

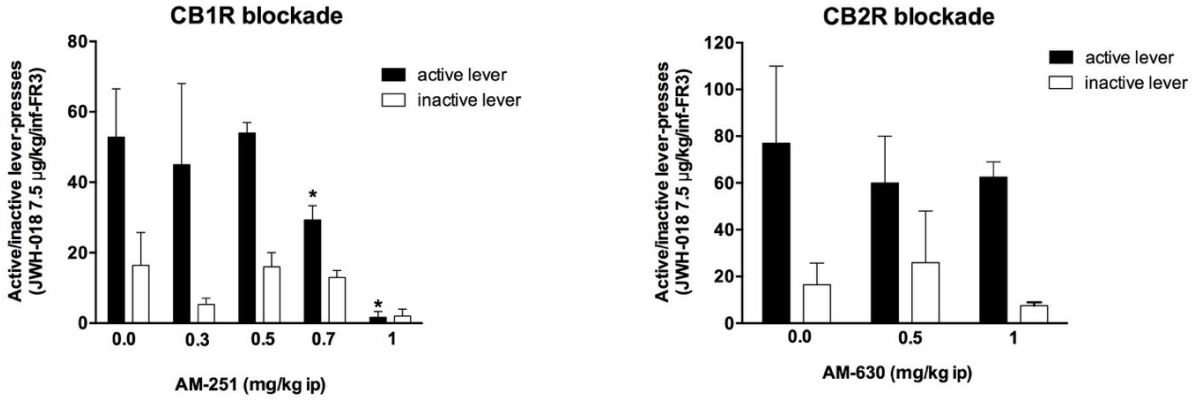

Fig. 19
(A) Number of responses during JWH-018 IVSA and effect of the CB1R antagonist AM251. Bars represent the mean±SEM of the last session under JWH-018 IVSA (7.5 µg/kg/inf) condition (black bar, active lever; white bar, inactive lever), and under AM251 pre- treatment condition for one session (AM251, 0.3, 0.5, 0.7, and 1.0 mg/kg i.p., 30min before each session); *p<0.05 vs Veh. (B) Number of responses during JWH- 018 IVSA and effect of the CB2R antagonist AM630 on IVSA. Bars represent the mean±SEM of the last session under JWH- 018 IVSA (7.5 µg/kg/inf) condition (black bar, active lever; white bar, inactive lever), and under AM630 pre-treatment condition for one session (AM630, 0.5 and 1.0 mg/kg i.p., 30min before each session).

4.4 Experiment IV: Extinction and reacquisition phase

To further investigate on the abuse properties of JWH-018, once mice acquired stable behavior during FR3 schedule, we observed the seeking behavior for JWH-018 through the extinction phase and the following reacquisition of operant behavior. As observed in the previous experiment, during the IVSA sessions under FR3 (Fig. 20), two way ANOVA showed a main effect of sessions ($F_{(5,50)}$=6.03, P<0.001). LSD post-hoc test showed significant difference between active and inactive lever presses in the 1°, 3°, 4°, 5°, 6° sessions.

Two way ANOVA analysis of the extinction phase, showed significant difference in sessions ($F_{(5,50)}$=3.09, P<0.05). LSD post-hoc test shows significant difference between active and inactive lever presses in the 2° and 3° sessions.

During the reacquisition phase, where Veh was replaced by JWH-018, mice were able to recover the operant behavior with values of lever presses similar to the acquisition phase. Two way ANOVA shows significant difference in responses ($F_{(1,6)}$=7.03, P<0.05). LSD post-hoc test shows significant difference between active and inactive lever presses at the 1°, 6° and 7° sessions.

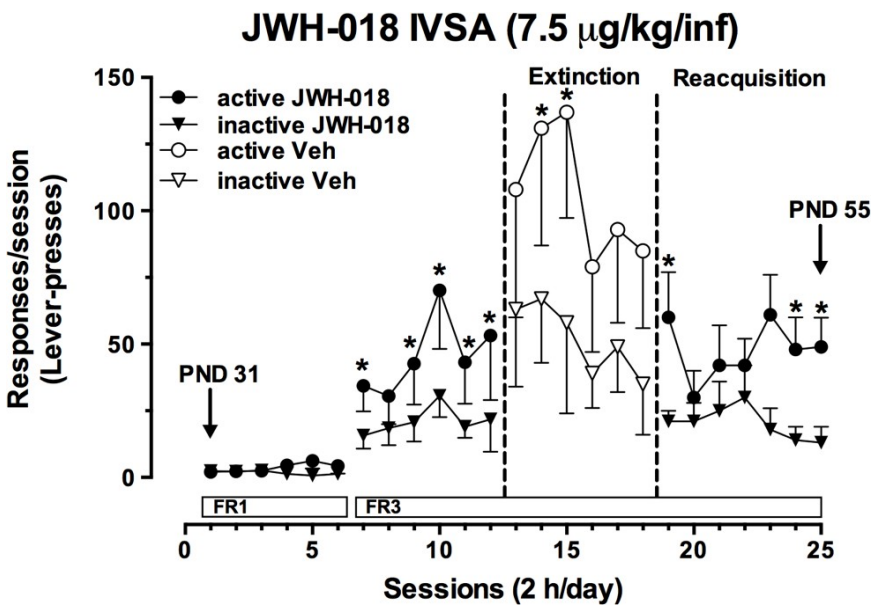

Fig. 20 Number of responses (lever presses) on the active lever (circles) that resulted in JWH-018 infusion (7.5 μg/kg/inf) and on the inactive lever (triangles) during each 2h daily session under FR1 and FR3 during acquisition (sessions 1 to 12), extinction (sessions 13 to 18) and reacquisition (sessions 19 to 25) phases. Results are expressed as mean ± SEM (n=6-4), *$p<0.05$ vs inactive lever-presses.

ADULT MICE

4.5. Behavior

4.5.1. Nestlet shredding test

In order to study a possible compulsive behavior caused by adolescent exposure to JWH-018, we additionally performed nestlet shredding test to evaluate compulsive and repetitive-like behavior in mice, since they used to shred material for nest building. The behavioral test performed on PND 79, showed that adolescent exposure to JWH-018 increases nestlet shredding compared to Veh group (Fig.21) ($t_{(11)}$ =3.14; $p<0.001$; +62%).

These data suggest permanent long-lasting compulsive behavioral effects due to JWH-018 self-administration during adolescence.

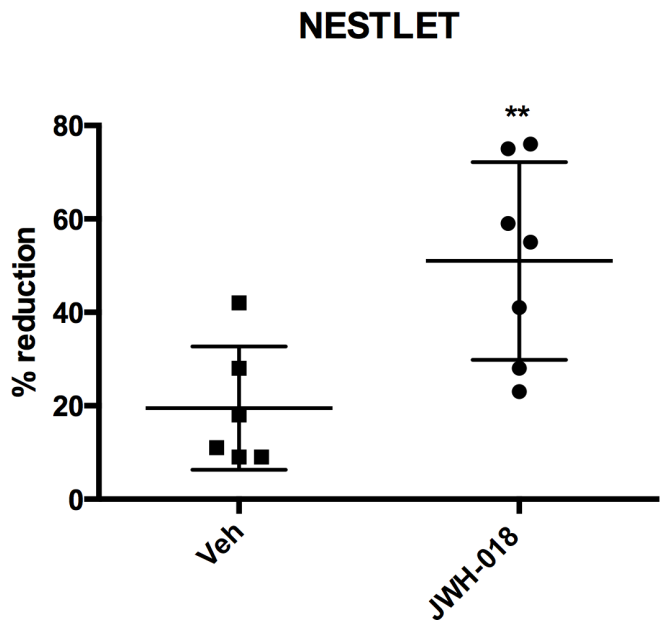

Fig.21 Adolescent exposure to JWH-018 increases nestlet shredding. The amount of Nestlet shred was determined by measuring unshred Nestlet 75 min after placing the nestlet in with the mouse. Values represent mean±SEM (n=7-6) of either percentage of shredded. **p<0.001 compared to Veh group, i.e. mice that did not acquire a stable IVSA behavior (unpaired Student's t- test).

4.6 Effects of JWH-018 IVSA on IBA-1 and GFAP immunoreactivity in selected brain areas

To further elucidate the enduring consequences of adolescent exposure to JWH-018, we measured adult (PND 80) levels of specific markers of neuroinflammation (GFAP and IBA-1) in mPFC, NAc (shell/core) and CPu of adult mice that underwent IVSA during adolescence. In these animals we observed an association between adolescent IVSA of JWH-018 and long-term microglia activation within the NAc core and shell ($t_{(6)}$ =2.47; $p<0.05$; +9%; $t_{(6)}$ =3.02; $p<0.05$; +10% respectively) and CPu ($t_{(6)}$ =3.58; $p<0.05$; +13%), as demonstrated by increased expression of IBA-1, a marker of activated microglia (Fig. 22A), no significant differences are shown in the mPFC. Moreover,

morphological analysis of IBA1-staining revealed that most of microglial cells displayed a rounded ameboid-like aspect, characteristic of activated microglia, while resting microglia in the NAc of control mice (Veh) showed a ramified appearance (Fig.23).

On the contrary, GFAP levels were significantly lower in the CPu of JWH-018 mice compared to Veh group ($t_{(5)}$ =3.63; $p<0.05$; -33%); a trend towards a lower but non-significant expression of GFAP was also observed in all the other analyzed brain areas (Fig. 22B; 24), indicating an astrocytopathy.

Taken together, our data suggest an alarming vulnerability of adolescent mice to JWH-018 as revealed by long-lasting detrimental behavioral and neurochemical effects.

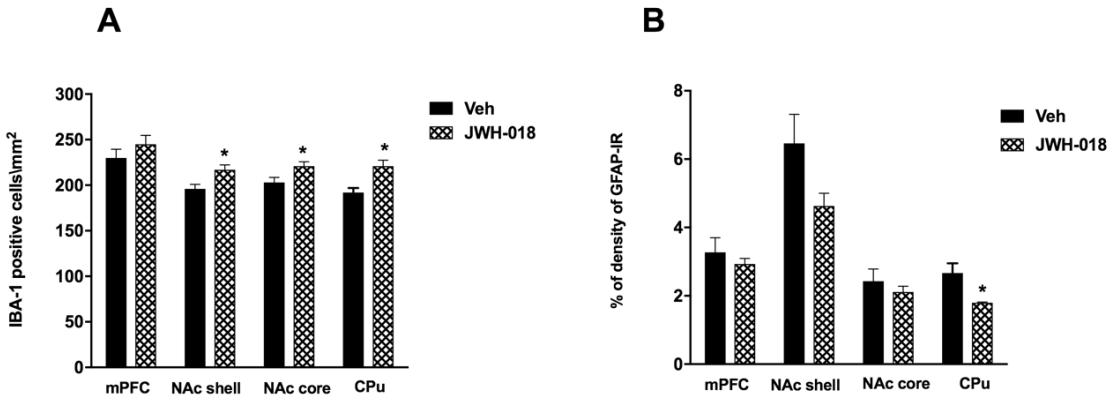

Fig. 22 Long-term effects of JWH-018 IVSA on IBA-1 and GFAP immunoreactivity in selected brain areas. Adolescent IVSA of JWH-018 significantly increased the number of IBA-1positive cells in the dorsal CPu and in the NAc core and shell at adulthood. On the contrary, GFAP- immunoreactivity was significantly decreased in the CPu and a trend of decrease is observed in the other areas. Values represent mean ± SEM (n=5) of either number of IBA-1 positive cells, expressed per mm2 (A) or as percentage of GFAP-IR density (B). *p<0.05 compared to Veh group, i.e. mice that did not acquire a stable IVSA behavior (unpaired Student's t- test).

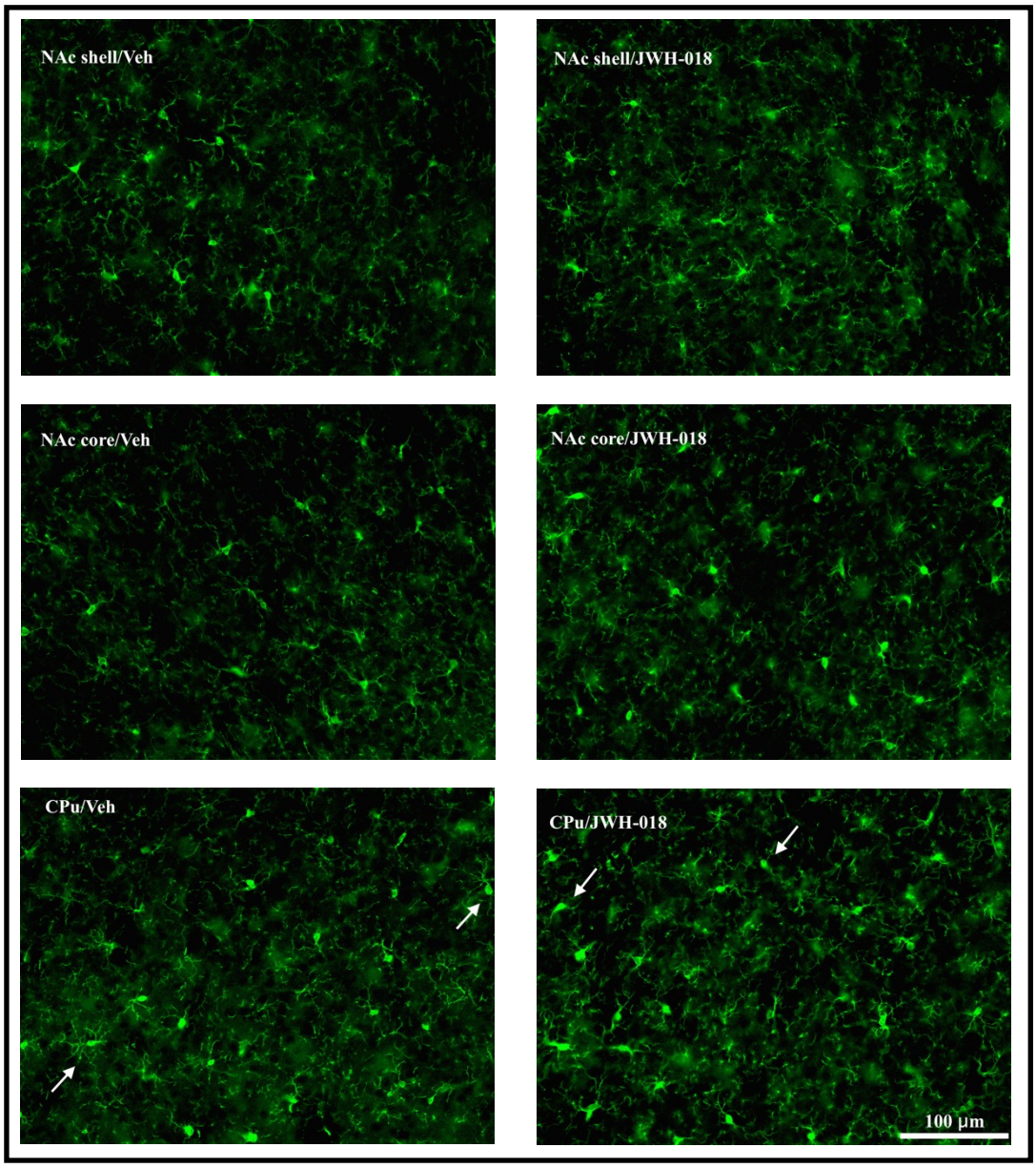

Fig. 23 *Representative images of IBA-1 immunostaining in the CPu, NAc core and shell of adult mice that underwent to JWH-018 or Veh IVSA during adolescence*. Note in the CPu the amoeboid microglia (arrows, activated state in the right panel) and the resting microglia (arrows, resting state in the left panel). Images are shown at 20X magnification. CPu,; NAc; Veh.

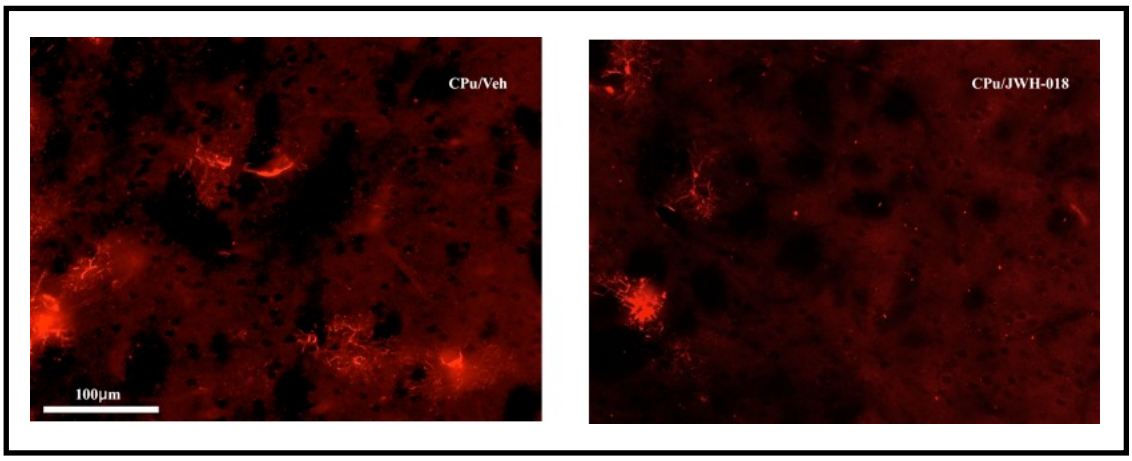

Fig.24 Representative images of GFAP immunohistochemical staining. Representative images of GFAP immunostaining in the CPu of adult mice that underwent to JWH-018 or Veh IVSA during adolescence. Note the decreased density of GFAP-IR in the CPu of JWH-018 mice compared to control mice (Veh). Images are shown at 20X magnification. CPu; IR, immunoreactivity; Veh.

4.7 CB1R expression in the cortex, hippocampus and striatum of adult mice after adolescent JWH-018 IVSA.

Previous experiments (Fig. 19) showed that IVSA of JWH-018 in adolescent mice is mediated by the activation of CB1R. Moreover, CB1R are steadily activated for the whole adolescence period and their expression is crucial in brain areas involved in addiction and motivated behavior (Zamberletti et al., 2015; Parsons and Hurd 2015; Lopez-Gallardo et al., 2012). Therefore, in collaboration with Marsicano's research group (Neurocentre Magendie, INSERM, Bordeaux, France) we investigated by western blot analysis about possible modification in the CB1R expression in cortex, hippocampus and striatum of adult mice pre-exposed to JWH-018 during adolescence. Our results identified a significant decrease of CB1Rs only in the hippocampus of JWH-018 group compared to the Veh ($t_{(11)}$ =2.47; $p<0.01$; -42%). Indeed, no significant differences have been found in the cortex and striatum, although, in this last region, there is a trend of increase of CB1 levels (Fig. 25).

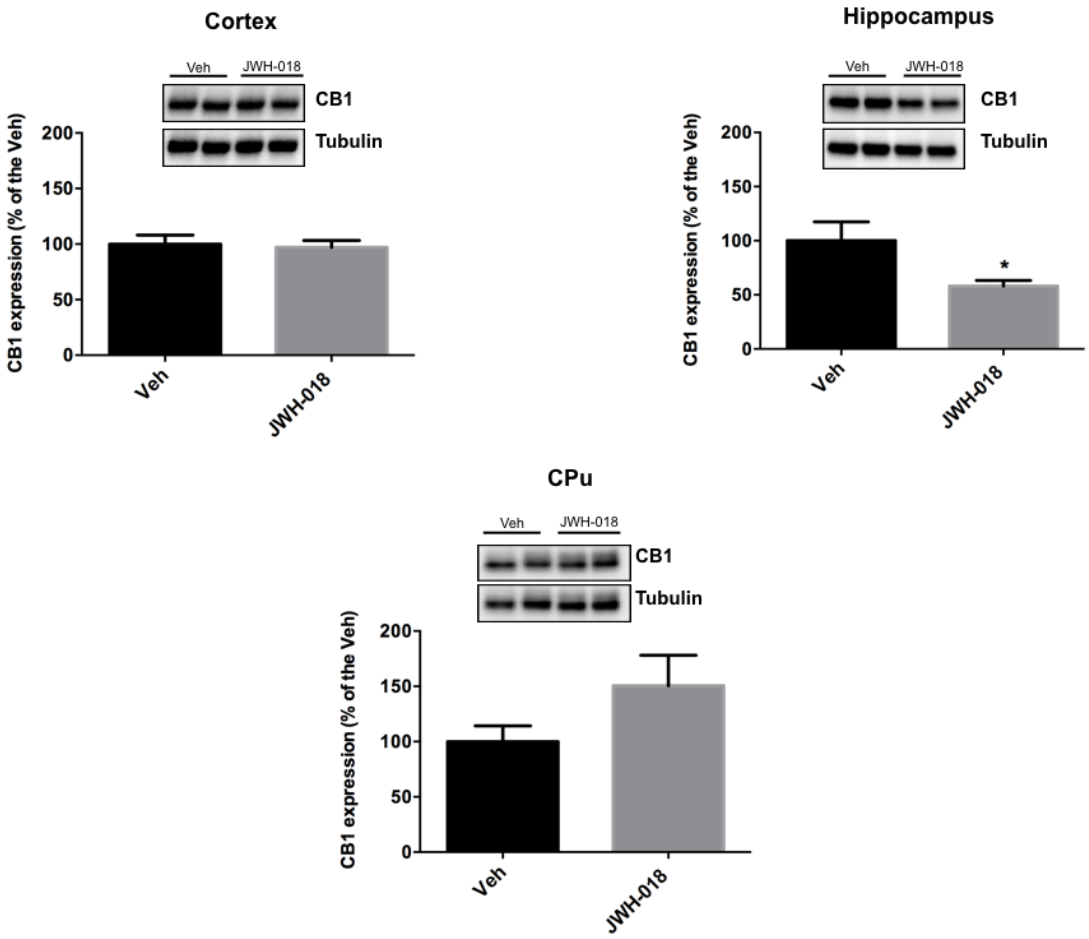

Fig. 25 *CB1R expression in cortex, hippocampus and striatum of adult mice.* Representative immunoblot (top) and optical densitometric quantification (bottom) of cortex (A), hippocampal (B) and striatum (C) samples from adult mice after adolescents IVSA of JWH-018. (two dependent experiments).
Values represent mean±SEM (n=6-7) of CB1 levels expressed as percentage of Veh condition, normalized on tubular levels. *p<0.01 (unpaired Student's t- test).

4.8 Complex I and CS activity in the cortex, hippocampus and striatum of adult mice after adolescent JWH-018 IVSA.

The recent discovery of mtCB1 increases the complexity of eCBs system. We know that cannabinoids modulate OXPHOS, through the inhibition of complex I activity (Benard et al., 2012).

To investigate if repeated exposure to cannabinoids during adolescence creates alterations in the mitochondrial functions, in collaboration with Marsicano's research group (Neurocentre Magendie, INSERM, Bordeaux, France) we studied the effect of IVSA of JWH-018 in adolescent mice (PND 31-53) on the activity of two mitochondrial enzymes during adulthood (PND 80): complex I and CS. As we did for the previous experiments, we performed the assays for cortex, hippocampus and striatum of adult mice.

We didn't find significant differences in complex I activity between Veh and JWH-018 group. The same samples have been processed for the CS activity assay. As shown in Fig. 26, the CS activity of JWH-018 group is significantly lower than the Veh group in all the selected areas, suggesting an alteration on the first step of Kreb's cycle (cortex: $t_{(11)}=2.59$; $p<0.01$; -18%; hippocampus: $t_{(11)}=2.24$; $p<0.05$; -10%; striatum: $t_{(10)}=3.43$; $p<0.001$; -17%).

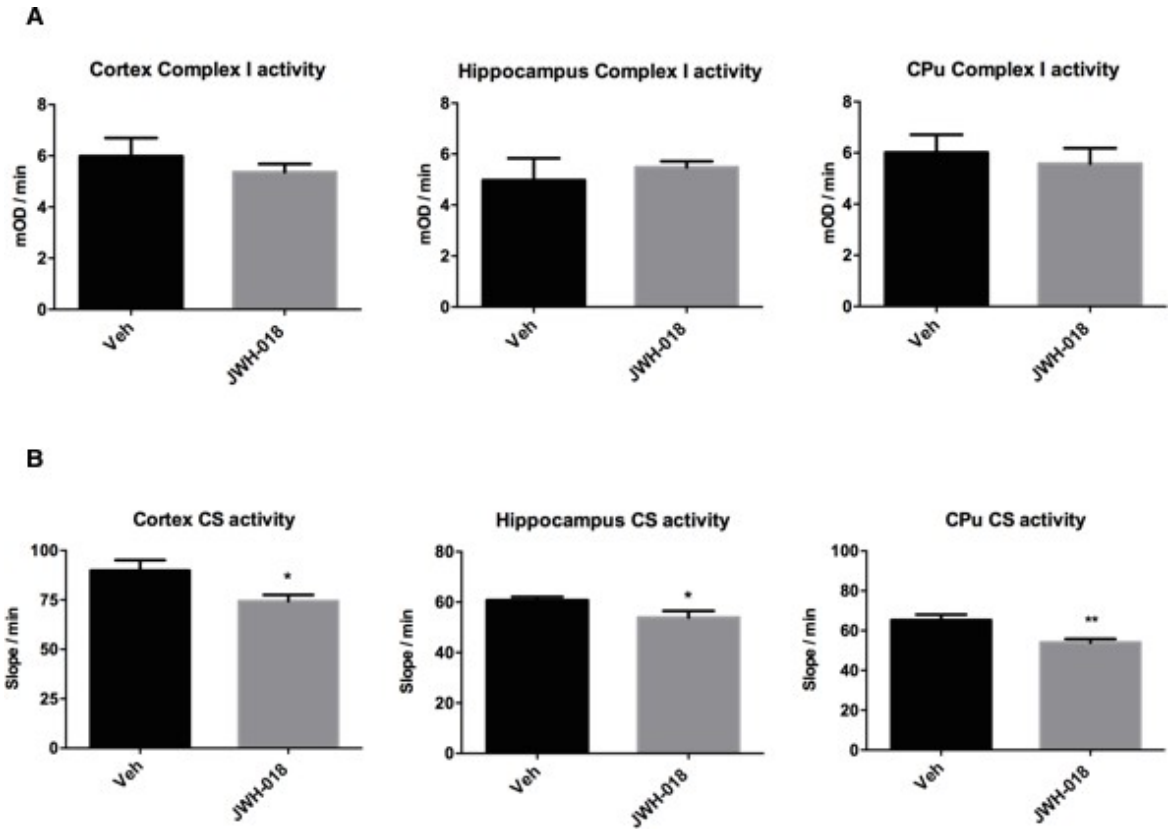

Fig. 26 *Mitochondrial Complex I and CS activity in cortex hippocampus and striatum of adult mice.*
Effects of JWH-018 IVSA during adolescence on complex I (A) and CS (B) activity in selected brain areas of adult mice. Values represent mean±SEM (n=6-7) of mOD/minute for complex I activity and slope/minute for CS activity. *p<0.05, **p<0.001 (unpaired Student's t- test).

4.9 NDUFS4 expression levels in the cortex, hippocampus and striatum of adult mice after adolescent JWH-018 IVSA.

To further investigate the long-term effects of JWH-018 on mitochondrial activity, in collaboration with Marsicano's research group (Neurocentre Magendie, INSERM, Bordeaux, France) we evaluated, by western blot, the expression of NDUFS4, protein involved in complex I assembly and function.

The immunoblots performed in cortex and hippocampus of adult mice show a significant increase of NDUFS4 in JWH-018 group compared to the Veh (cortex: $t_{(11)}$ =1.78; p=0.05; +55%; hippocampus: $t_{(11)}$ =1.93; p<0.05; +55%). On the other hand, no difference is shown of NDUFS4 in the striatum (Fig. 27). This data could suggest an increase of NDUFS4 as compensatory mechanism in the mitochondrial activity, since we didn't find changes in the complex I activity.

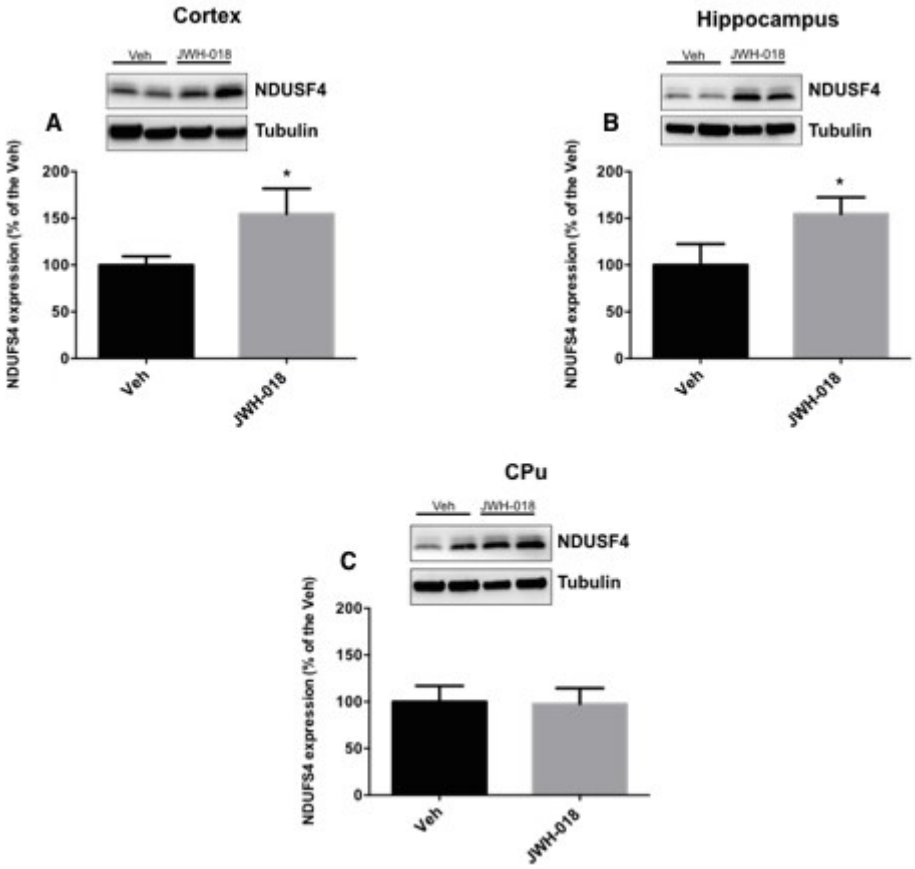

Fig. 27 *NDUFS4 expression in cortex, hippocampus and striatum.*
Representative immunoblot (top) and opitcal densitometric quantification (bottom) of cortex (A), hippocampus (B) and striatum (C) from adult mice after adolescents IVSA of JWH-018 (two dependent experiments).
Values represent mean±SEM (n=6-7) of NDUFS4 levels, normalized on tubulin levels, expressed as percentage of Veh condition. *$p<0.05$ (unpaired Student's t- test).

DISCUSSION

The main objectives of this study are twofold: i) to investigate the reinforcing properties and abuse potential of the synthetic cannabinoid JWH-018 during adolescence; ii) to characterize at different levels (i.e. behavioral, neuroinflammatory, CB1R expression, mitochondrial activity) the effects induced in adult mice by cannabinoid self-administration during adolescence.

Data show that adolescent mice readily acquired IVSA behavior at the dose of 7.5 µg/kg/inf (Fig. 17). A response-study to varying doses (2.5-15 ug/kg/inf) shows an inverted U-shaped trend, a characteristic congruent with previous studies investigating SC-induced effects, with particular reference to those identified in Spice/K2 products (De Luca et al., 2015, 2016). To date, no other studies have evaluated IVSA of synthetic cannabinoids identified in Spice drugs in adolescent mice. Here we provide the first evidence that JWH-018 IVSA is rapidly acquired and sustained by adolescent mice, although within a narrow window of doses. Importantly, based on the number of lever presses, CD1 adolescent mice show higher sensitivity to JWH-018 than C57BL/6 (C57) adult mice undergoing IVSA at the dose of 30 µg/kg/inf(De Luca et al., 2015). It is possible that the different sensitivity to the cannabinoid is due to a strain related effect, since adult C57 mice show better discrimination than CD1 mice when responding for the SC WIN 55,212 IVSA under a nose-poking procedure (Mendizábal et al., 2006), and the number of sessions required to achieve the acquisition criteria are significantly lower in C57 than in CD1 mice. This is not specific to cannabinoids, as other studies have showed that C57 mice acquire operant behavior for cocaine faster than other strains (Grahame and Cunningham, 1995) and perform better in spatial memory tasks than CD1 mice, as shown in the Morris water maze test (Wright et al., 2004).

An important aspect of this study is that we were successful in setting up a protocol of JWH-018 IVSA in the less sensitive strain of mice, which implies greater reinforcing properties of this synthetic cannabinoid with respect to those pf previous generation, such as WIN 55,212-2. Notably, the JWH-018 IVSA paradigm seems to be more feasible than THC IVSA, possibly

due to THC's co-occurring aversive effects, which impact drug reinforcement. The study of THC IVSA behavior is very challenging using both IVSA paradigm and voluntary drug inhalation, likely because of its high lipophilicity, long-half life and distribution in fatty tissue (Huestis, 2005), which are pharmacokinetic characteristics that may strongly influence reinforcement and, hence, the responding rate, animals' behavior under extinction conditions and reinstatement of drug-seeking behavior. Due to its lipophilicity, THC intravenous infusion may create a delay in the onset of the rewarding effects thus impeding the contingent association of its delivery with the operant response (Tanda and Goldberg, 2003). Notably, a recent study has shown that rats exposed to 5 daily treatments with non-contingent THC with cannabidiol (CBD) vapor (10:1) are able to self-administer the same 10:1 ratio of THC+CBD (Melis et al., 2017). According to this, a novel rodent model of intravenous THC+CBD self-administration and reinstatement induced by conditioned context, cues, and stress has been recently been developed (Spencer et al., 2018). Although cannabinoid self-administration studies can be troublesome and involve a number of complicated issues (Tanda and Goldberg 2003; Justinova et al., 2005; Melis et al., 2017), it is evident that the rewarding properties of JWH-018 are stronger than those of THC. In more general terms, all SCs show to possess higher positive reinforcing effect than THC, and different protocol of IVSA of other SCs, such as WIN 55,212, have been developed and characterized so far in adult mice (Martellotta et al., 1998; Navarro et al., 2001; Mendizábal et al., 2006;) and rats (Fattore et al., 2001; Spano et al., 2004; Lecca et al., 2006).

Once identified the dose necessary and sufficient to acquire and sustain operant behavior, we tested adolescent mice under different FR schedules of reinforcement, to better characterize the strength of the rewarding properties of JWH-018 and highlight is abuse potential. We observed that as the FR schedule of reinforcement was increased, the difference between active and inactive lever presses became more significant. Importantly, operant behavior was specifically directed at obtaining JWH-018, since it increased under PR schedule of reinforcement, reaching

the breaking point after 92 active responses, and was absent in the vehicle group. The reinforcing properties of JWH-018 were already shown to be CB1R-mediated (De Luca et al., 2015). Since JWH-018 acts as full agonist at both the CB1R and the CB2R with an *in vitro* difference of three-fold (CB1R Ki ~ 9 nM; CB2R Ki ~ 3 nM), we decided to assess the role of both types of receptors on operant behavior and found that JWH-018 IVSA was reduced by the CB1R antagonist AM251 (0.7 mg/kg ip) while not affected by the CB2R antagonist AM630 (0.5, 1 mg/kg ip). As a matter of fact, the highest dose tested of AM251 (1 mg/kg i.p.) strongly reduced both active and inactive lever-pressing activity most likely due to a possible hypo-locomotor activity. In order to clarify this issue, further experiments of locomotor activity evaluation are in progress. Although JWH-018 is also a CB2R agonist, we did not observe any IVSA differences after the administration of AM630, which suggest that CB2Rs are not involved in the rewarding mechanisms triggered by JWH-018. The results of the antagonistic study are in line with our previous findings that administration of the CB1R antagonist/inverse agonist Rimonabant (1 mg/kg ip, 30 min before the IVSA session) significantly reduced JWH-018 IVSA behavior in mice (De Luca et al., 2015). Intriguingly, the day after pretreatment with Rimonabant, the rate of responding for JWH-018 increased, suggesting a rebound effect in the attempt to counterbalance the reduction of JWH-018-mediated reinforcement. Moreover, when JWH-018 was replaced by vehicle, the number of active and inactive lever presses was not significantly different from those registered during the acquisition phase and the responding rate (and, hence, the number of infusions) did not decrease, in spite of the absence of drug associated cues. Our data show that mice under JWH-018 IVSA are not able to extinguish the operant behavior (see extinction phase, Fig. 20), likely due to a spontaneous withdrawal signs characterized by repetitive/persisting drug-seeking behavior and compulsive searching of the drug. The resistance to extinction of instrumental responding after acquisition of JWH-018 IVSA could be related to the acquisition of a habit modality, consistent with a role of cannabinoids in habit learning formation (Hilario et al., 2007; Goodman and Packard, 2015).

In order to study a possible compulsive behavior caused by adolescent exposure to JWH-018, we additionally performed nestlet shredding test during adulthood (PND 79). This test, based on the spontaneous behavior of mice (i.e. build the nest), represents a valid and simple test for evaluating compulsive- and repetitive-like behavior in animals and possesses predictive validity for the human obsessive-compulsive disorder. These repetitive behaviors are natural in rodents and their frequency and/or intensity can vary after treatment with drugs of abuse (Angoa-Pérez et al., 2013). Compulsive and repetitive-like behaviors represent important and typical features of the anxiety disorders (McGuire et al., 2012), and among different adverse effects induced by acute exposure to SCs, anxiety is frequently reported by drugs users (Every-Palmer, 2011; Papanti et al., 2013). Our data show that adolescent exposure to JWH-018 significantly increases nestlet shredding behavior compared to vehicle group (Fig. 21) and suggest the occurrence of long-lasting compulsive behavioral effects. In line with our results, Murphy et al. (2017) have recently demonstrated that chronic administration of THC during adolescence increases nestlet-shredding behavior, confirming the occurrence of repetitive behavior during adulthood as a consequence of adolescence exposure to cannabinoids.

In the second part of this study, we demonstrated that adolescent mice that readily self-administer JWH-018 develop long-lasting glia alterations. As showed in Fig. 22, we observed that JWH-018-IVSA during adolescence induces at adulthood an increase of IBA-1 positive cells in the NAc shell and core and in the CPu, together with a decrease of GFAP level in the CPu. Despite the consistency of data showing a close correlation between neuroinflammation (i.e., gliosis and microglia activation) and drug addiction (Kim et al., 2017; Lacagnina et al., 2017; Pekny et al., 2016; Melis et al., 2017), no evidence of long-term effect of SCs IVSA on glia has been reported so far. In fact, although lack of information on the risks of new SCs, including long-lasting toxicity, represents a serious health and medical problem, only two studies investigating the relationship between exposure to SCs in adolescence and the

manifestation of significant glia alterations in adulthood are present in the literature. These studies showed that JWH-018 induces apoptosis in the forebrain neuronal cultures (Tomijama et al., 2014) and increases the number of neural cells with distorted and pyknotic nuclei in the NAc and hippocampus, which suggests neurotoxicity (Cha et al., 2015). Our study demonstrates for the first time that adolescent exposure to JWH-018 induces long-lasting alteration of microglia, as supported by increased levels of IBA-1 positive cells, in all brain areas analyzed with the only exception of the PFC. Furthermore, microscopic morphological analysis, through IBA-1 staining, revealed that these cells show a de-ramified morphology and an ameboid-like appearance, which are indicative of an activated state of microglia. Conversely, in adult mice that underwent vehicle IVSA the microglia cells display a small soma and ramifications with non-overlapping processes, features that are characteristic of "resting" or "surveillant" microglia. In line with our data, Zamberletti et al. (2015) reported that a passive administration of THC during adolescence determines, in female rats, a persistent neuroinflammatory profile characterized by altered microglia morphology, increased expression of the pro-inflammatory markers TNF-α, iNOS and COX-2, and a reduction of the anti-inflammatory cytokine, IL-10. The altered levels and morphology of microglia cells were detected only in the PFC, while no difference was observed in the NAc, hippocampus and amygdala. Moreover, this neuroinflammatory profile in the PFC of adult female rats was associated with cognitive impairment and depressive-like behaviors. Conversely, by using a similar protocol in male rats, the same research group did not observe any persistent changes of microglia markers IBA-1 and CD11 in the hippocampus and PFC(Zamberletti et al., 2016). Yet, when examining the effect of adolescent THC treatment in male and female rats at adulthood Lopez-Rodriguez et al. (2014) provided different results. In fact, they reported an increased proportion of reactive microglial cells in the hilus of the dentate gyrus in the hippocampus of males, whereas an opposite trend was found in females. Additionally, they found reported that chronic THC intake in mice during adolescence triggers immune

dysfunctions that persist after the drug intake period, switching the murine immune system to a proinflammatory status in adulthood (Moretti et al., 2014). In support to the important link between microglia and drug abuse, reactive microgliosis has been detected in brain areas of adult methamphetamine addicts who had been abstinent for several years, suggesting that methamphetamine exposure aroused a process that led to persistent effects on proliferation of microglia (Sekine et al., 2008). In parallel, preclinical studies confirm that methamphetamine induces microglial activation in the brain along with increased striatal mRNA expression levels of IL-6 family pro-inflammatory cytokines (Robson et al., 2013). Altogether, present data confirm those available from literature concerning the association between adolescent cannabinoid exposure and altered levels of microglia cells, where the different results obtained among the studies could depend on the type of the cannabinoids (self)administered (i.e., natural or synthetic cannabinoid), the route of treatment (i.e., passive exposure vs active drug self-administration), sex/gender and the specific brain area analyzed as well as the methodologies used to investigate the number of IBA-1 positive cells (i.e., western vs immunohistochemical assay). Despite these discrepancies, collectively these observations point out the important role of microglia cells in the drug addiction, especially during adolescence.

As concerns astrocytes, our study shows a significant decrease of GFAP expression in the CPu and a trend of decrease also in the NAc (core and shell), providing direct evidence that adolescent JWH-018-IVSA can lead to a reduction of the amount of GFAP that persisted later in life. Further, our data also reveals that the JWH-018-induced decrease of GFAP expression is likely region-specific, since no alterations were detected in the PFC. Accordingly, it has been reported that THC inhibits astroglial growth *in vitro* (Tahir et al., 1992) and affects its development *in vivo* (Suarez et al., 2000). In particular, pre-natal and perinatal THC exposure causes opposite effects in the two sexes, since it induces a reduction of the astroglial GFAP expression in the substantia nigra of male rats but a significant increased expression in the substantia nigra of females (Suarez et al., 2000). Moreover, GFAP expression in THC-exposed

males increase with age but it does not reach control levels before PND 70, suggesting a delayed astrocytic maturation in THC-exposed males. The same author (Suarez, 2002) reported that THC administration during development (pre- and perinatal) reduced GFAP content in astrocytes and Bergmann glial cells in the rat cerebellum in a sex-dependent manner. As regards the effect of adolescence treatment, conflicting results have been reported in the levels of GFAP, with studies showing no changes, increased or decreased levels. For example, THC exposure during adolescence at adulthood significantly increased the percentage of GFAP immunoreactive area in the hippocampus of both sexes in one study (Lopez-Rodiguez, 2014) but only in male rats in a different study (Zamberletti et al., 2016). Of note, responsiveness of astrocytes to several insults (e.g., injuries, inflammation, neuropsychiatric disease, drugs of abuse) ranges from astrocytopathy to astrogliosis, characterized by several phenotypes including increased or decreased levels of GFAP and morphological features (i.e., hypertrophy/atrophy) (Kim et al., 2017). Consistent with our data, several studies have demonstrated that prenatal cocaine exposure in rhesus monkeys and cocaine self-administration in rats reduce the content of GFAP in the cortex of monkeys and in the NAc of rats, respectively (Lidow, 1995; Scofield et al., 2016). Further, GFAP expression as well as number of astrocytes were reduced in the Prl cortex of ethanol-preferring rats (Miguel-Hildago et al., 2005) as well as following prolonged exposure to alcohol (Franke, 1995; Rintala et al., 2001). Here we confirm the crucial role of glial cells in drug abuse, and extend such a relationship to SCs. Exposure during adolescence to SCs are particularly worrisome due to the critical involvement of the eCB system in brain development (Galve-Roperh et al., 2009; Downer and Campbell, 2010) and the potentially disruptive impact of these drugs on processes associated to brain development, such as white matter development (Solowij et al., 2011b) and synaptic pruning (Bossong and Niesink, 2010).

In the third part of the study, we verify the possibility that JWH-018 IVSA during adolescence can induce long-term alterations in the expression of the brain CB1Rs, the

expression of which is crucial in brain areas involved in addiction and motivated behavior (Zamberletti et al., 2015; Parsons and Hurd, 2015; Lopez-Gallardo et al., 2012). For these reasons, we selected brain areas involved in addiction and with high expression of CB1Rs, such as the cortex, the hippocampus and the striatum. Our results reveal a significant decrease of CB1Rs in the hippocampus of JWH-018-treated animals compared to vehicle-treated group (Fig. 25). No significant differences were found in the whole cortex, while in the striatum we observed a positive trend. Our data are in line with the study of Silva and colleagues (2015), that shows after adolescent treatment with THC in both female and male rats, a decrease of CB1Rs in the hippocampus not only at the end of treatment with THC, but also after 2 weeks of wash out, identifying these changes as long-term effects. Different studies reported that THC administration induces no changes (Ellgren et al., 2007) or a permanent down-regulation of CB1R binding density within amygdala and hippocampal formation of male rats (Rubino et al., 2008). Recently it was demonstrated that chronic THC treatment during adolescence induces no difference in CB1Rs expression in the PFC of female rats (Zamberletti et al., 2015). Moreover, Cutando and colleagues (Cutando et al., 2013), showed that sub-chronic THC treatment induces adaptive changes in the CNS characterized by a down-regulation of CB1Rs in the cerebellum of male adult mice. A recent study using stochastic optical reconstruction microscopy (STORM) demonstrated that chronic exposure to THC results in loss of CB1Rs on terminals of perisomatically projecting GABA interneurons in the mouse hippocampus, and internalization of the remaining CB1Rs (Dudok et al., 2015). As concern SCs, Lopez-Gallardo and colleagues (2012), after a chronic treatment with CP55,940 during adolescence, found a decrease of CB1Rs expression in the hippocampus of adult male rats. Further, the adolescent subchronic CP55,940 exposure induced an increased CB1R-mediated G protein function in the hippocampal CA1 and CA2 fields of males but not females, indicating both sex- and region-dependent effects of the cannabinoid treatment, whereas no effect on CB1R density was found in any of the regions analyzed (Mateos et al., 2011). In humans, chronic cannabis use disrupts

brain CBRs availability and function. Indeed, Hirvonen et al. (2012), using the *in vivo* technique of positron emission tomography (PET) imaging, showed a down-regulation of brain CB1Rs in subjects with a protracted history of daily cannabis abuse, which was reversed after one month of monitored abstinence. Prolonged exposure to CB1R antagonists Rimonabant (Howlett et al., 2004) and AM251 (Martini et al., 2007) led to drug tolerance. In human and animal models, this mechanism has been attributed to desensitization (with a decrease of biological responses induced by receptor activation) and/or to internalization (with the reduction in the number of cell surface-expressed receptors) (Kendall and Yudowski, 2017). Overall, the differences reported by studies investigating CB1R expression could be ascribed to the different protocols and methods used. In particular, the treatments and the doses of the drug used, animal species (mice/rats), sex, the brain areas studied and the methodology of quantification (autoradiography, western blot, immunohistochemistry) can make the difference. The recent discovery of CB1Rs on mtCB1 membrane increases the complexity of the EC system. Since cannabinoids modulate OXPHOS through the inhibition of complex I activity (Benard et al., 2012), it is possible that repeated exposure to cannabinoids could create remarkable changes in the mitochondrial function. Recent studies suggest the possibility that a mitochondrial deficit could trigger neuropsychiatric disorders. In patients with schizophrenia the activity of complex IV is reduced in frontal cortex and caudate nucleus, in turn the mitochondrial respiratory function decreases due to the alteration of complex I activity and by a decrease of expression of its numerous subunits (Lezi and Swerdlow, 2012; Mattson et al., 2008; Rozenfeld and Devi, 2011). Benard and colleagues (Benard et al., 2012), showed that acute administration of THC in mice reduces mitochondrial respiratory chain complex I activity in purified hippocampal mitochondria. In addition, *in vitro* treatment of brain purified mitochondria with the synthetic CB1R agonist WIN55,212-2 decreases oxygen consumption in a dose-dependent way, indicating that SCs may decrease mitochondrial complex I activity, a notion confirmed in the next years by other experiments (Hebert-Chatelain et al., 2016; Fisar et

al., 2014; Singh et al., 2014). Based on this evidence, we supposed that the activity of complex I could be reduced also by JWH-018. However, we did not find significant differences in the brain areas selected (cortex, hippocampus and striatum) between JWH-018 and vehicle groups of adult mice, suggesting that JWH-018 self-administered during adolescence does not induce long-lasting impairments in OXPHOS activity, a finding is in line with Costa et al. (1996) that showed that chronic treatment with CP55,940 in rats does not lead to significant changes in the mitochondrial respiration. Another enzyme that represents a marker for distinguishing intact mitochondria is CS. Although Costa et al. (1996) did not find difference in CS activity after CP55,940 chronic treatment, we observed a significant decrease of CS activity in all the areas investigated of JWH-018 when adolescent, in line with a previous study showing a decrease of CS activity in a pig mouse brain treated *in vitro* with THC (Singh et al., 2015). To further investigate the long-term effects of JWH-018 on mitochondrial activity, we evaluated the expression of NDUFS4, one of the proteins involved in complex I assembly and function. Indeed, a previous study showed a decreased PKA-dependent phosphorylation of the complex I subunit NDUFS2 in purified brain mitochondria treated with WIN55,212-2 or THC (Hebert-Chatelain et al., 2016). Finding of a significant increase of NDUFS4 in the cortex and hippocampus of adult mice that underwent adolescent JWH-018IVSA as compared to those exposed to vehicle could suggest a possible compensatory mechanism in this subunit synthesis, aimed at providing the correct mitochondria activity.

CONCLUSIONS

Adolescence is a critical stage of brain development that brings adolescents to be more prone to abuse drugs than adults (Tomas-Roig et al., 2016). The ECs system plays a key role in brain maturation as well as in the neurobiology of drug reward, motivation and dependence (Parsons and Hurd, 2015). By managing to model a continuous stimulation of the ECs system during the whole adolescence period, we demonstrated that adolescent mice are more vulnerable to JWH-018 than adults and that JWH-018 IVSA induced remarkable changes in the adult brain. Changes consisted in persistent compulsive behavior, alteration of CB1Rs expression that, in turn, may modify mitochondrial activity, and neuroinflammation. Indeed, we observed an increase of IBA-1 and a decrease in GFAP levels in adult mice that self-administered the SC during adolescence, thus providing the first evidence of glia cell system alterations in the long-term as response to adolescent JWH-018 IVSA. Mitochondria activity was also affected by JWH-018 IVSA, suggesting a complex synergy between brain development, the ECs and the neuroimmune systems, which collaborate together during adolescence originating significant effects persisting in adulthood.

Taken together, findings from these studies revealed severe long-term effects of adolescent exposure to JWH-018, increasing the alarming consequences related to the misuse ofsynthetic cannabinoids by young people.

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