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ADOLESCENT Δ^9-TETRAHYDROCANNABINOL EXPOSURE DIFFERENTLY AFFECTS HISTONE MODIFICATIONS IN THE BRAIN OF FEMALE AND MALE RATS

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ABSTRACT

Despite the increasing evidence of a possible interaction between adolescent Cannabis abuse and the subsequent development of psychiatric disorders, Cannabis remains the illicit drug most abused by adolescents.

We have previously demonstrated that female rats chronically treated during adolescence with increasing doses of delta-9-tetrahydrocannabinol (THC), the main psychoactive ingredient of cannabis, develop a depressive/psychotic-like phenotype in adulthood. Interestingly, only chronic adolescent exposure to THC, but not adult exposure, led to this complex phenotype, suggesting that adolescence may represent a more vulnerable period for the adverse effect of THC. However, the neurobiology of this vulnerability is not still clear.

Considering the important role assumed by epigenetics in the etiopathogenesis of psychiatric disorders, the main goal of this thesis is to extend our knowledge on the impact of adolescent THC exposure on histone modifications occurring in other brain areas involved in the different aspects of the depressive/psychotic-like phenotype described in our animals. Specifically, we considered the Hippocampus for its involvement in cognition, the Nucleus Accumbens for its role in the reward circuit, and the Amygdala for its relevance in the emotional behaviour. To investigate the existence of age-specificity of THC effects, we performed the same analysis also after adult THC treatment. To investigate sex-dependency of THC response, we also checked THC response in adolescent male animals.

First of all, adolescent (PND 35-45) and adult (PND 75-85) female Sprague-Dawley rats were treated twice a day with increasing intraperitoneal (ip) doses of THC: 2.5 mg/kg, 5 mg/kg, and 10 mg/kg or with its vehicle. Two, 24 and 48 hours after the end of the treatment, the brain areas of interest were collected and. Histone modifications associated with both transcriptional repression (H3K9 di- and tri-methylation, H3K27 trimethylation) and activation (H3K9 and H3K14 acetylation) were evaluated.

Chronic THC exposure affected histone modifications in the brain of female rats in a region- and age-specific manner. Indeed, THC acted on different targets depending on

the considered brain areas and, remarkably, the adolescent brain was generally more sensitive to THC exposure compared to the adult one. Specifically, in the Hippocampus of adolescent rats, THC induced a reduction of H3K14ac levels 2 hours after the end of the treatment. This was followed by a significant increase in di- and tri-methylation of H3K9 at 24 hours. Regarding the Nucleus Accumbens, H3K9me3 was significantly increased 2 hours after the end of the treatment. This enhancement was maintained 24 hours later, and it was paralleled by a significant increase in H3K9me2 and H3K14ac levels. On the contrary, at 48h, H3K9me3 levels, as well as H3K9me2 and H3K14ac levels were significantly reduced. In the Amygdala, THC administration induced a significant increase in H3K9me2 levels 2 hours after the end of the treatment. Twenty-four hours later, while this alteration returned to control values, H3K9me3 levels were significantly enhanced.

Adult female rats exposed to chronic THC showed a different pattern of histone alterations. In the Hippocampus and Nucleus Accumbens, H3K14 acetylation levels were significantly increased, respectively, 2 and 24 hours after the end of the treatment. Intriguingly, a more complex picture is present in the adult Amygdala, in which a significant decrease in H3K9me2 and H3K27me3 were induced immediately after the cease of the treatment. Twenty-four hours later H3K9ac was significantly reduced, and at 48 hours, H3K14ac levels were significantly decreased.

As a whole, the investigation performed in female rats suggests that in the adolescent brain THC induced a primary effect represented by changes leading to transcriptional repression, whereas the primary effect induced by adult THC exposure led to transcriptional activation. Interestingly, only in the adolescent brain, the primary effect was followed by a homeostatic response to counterbalance the THC-induced repressive effect, except in the amygdala. The presence of a more complex response in the adolescent brain may be part of the mechanisms that make the adolescent brain vulnerable to THC adverse effects.

The second aim of this thesis was to extend our knowledge on the impact of adolescent THC exposure on histone modifications occurring in different brain areas of male rats. To this aim, adolescent (PND 35-45) male Sprague-Dawley rats were treated with the same

protocol previously described for females and we conducted the same analysis in the Prefrontal Cortex, Hippocampus and Nucleus Accumbens.

Chronic THC exposure affected histone modifications in the brain of male rats in a region-specific manner. Surprisingly, in the Prefrontal cortex and Hippocampus, we did not found any histone alterations at any intervals of time, and only in the Nucleus Accumbens we found significant alterations in H3K9me3 levels. Specifically, H3K9me3 was decreased immediately after the end of the treatment and then increased 24h later.

Further studies are needed to clarify the epigenetic landscape in the brain of male rats and how it could account for the development of the psychotic-like phenotype described in these animals.

However, it is possible to conclude that Cannabis abuse during adolescence could impair the brain network functionality acting through a mechanism involving histone modifications that is characterized by sex-specificity.

INTRODUCTION

THE ENDOCANNABINOID SYSTEM

The discovery in 1990 that a 473-amino-acid G-protein coupled receptor encoded by a rat brain cDNA clone mediated the effects of Δ9-tetrahydrocannabinol (THC), the main psychoactive component present in Cannabis Sativa, represented the starting point for the identification and characterization of the now known as endocannabinoid system (ECS).

The term “endocannabinoid system” refers to a lipid signalling system characterized by specific receptors, their endogenous lipid ligands called endocannabinoids, [the best known are N-arachidonoylethanolamide (anandamide, AEA) and 2-arachidonoylglycerol (2-AG)], and the associated enzymatic machinery (transporters, biosynthetic and degradative enzymes) involved in the synthesis and degradation of endocannabinoids.

Based on the intense research carried out in the last decades, it is known that the ECS is implicated in the modulation of numerous physiological functions and pathological conditions not only in the central nervous system (CNS) but in the entire organism.

CANNABINOID RECEPTORS

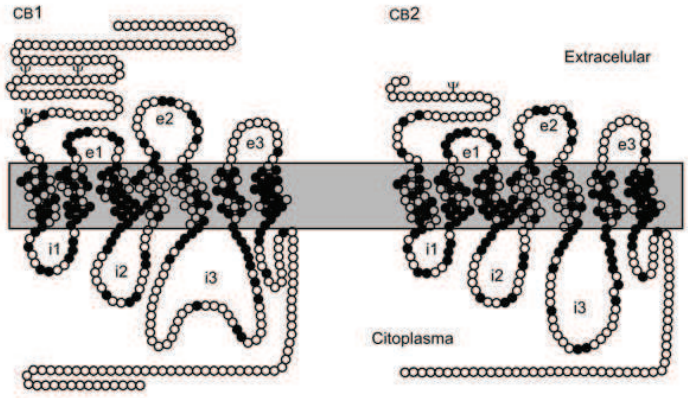

Figure 1.Stucture of cannabinoid receptors (CB1 and CB2)

The first sequenced and cloned cannabinoid receptor, called CB_1R, (Matsuda et al., 1990), is located mainly presynaptically and widely distributed in different regions of the central nervous system (Fig.1).

In particular, it is present in high density in the basal ganglia, cerebellum, hippocampus, and cerebral cortex (Herkenham et al., 1991). Moreover, it is also observed from moderate to low densities in the Amygdala, Nucleus accumbens, Hypothalamus, Midbrain, Medulla oblongata, and Spinal cord. Finally, CB_1 is present at lower levels in peripheral tissues such as the liver, adipose tissue, exocrine pancreas, skeletal muscles, gastrointestinal tract, and immune cells. It belongs to the superfamily of G protein coupled receptors, which have seven transmembrane segments connected by three extracellular and three intracellular loops, an extracellular N-terminal tail, and an intracellular C-terminal tail. CB1 receptor is mostly associated with Gi and Go. Consequently, its activation (i) inhibits adenylyl cyclase activity reducing cAMP levels, (ii) stimulates the mitogen activated protein kinases (MAPK) pathway (iii) modulates the activation of potassium channel of type A and “inward rectifier”, and (iv) inhibits N and P/Q type calcium currents (Iannotti et al., 2016). Some studies have shown that CB1 in certain cell types can regulate adenylyl cyclase (AC) also via Gs or Gq (Turu et al., 2010), or be coupled, via Gi/o or Gq/11, to other types of intracellular signals, such as the protein kinase B (Akt/PKB), phosphoinositide 3-kinase (PI3K) and phospholipase C/inositol 1,4,5-trisphosphate/protein kinase C (PLCβ/IP3/PKC) pathways (Navarrete et al.,2010; Gómez del Pulgar et al., 2000).

Within the brain, CB_1 receptors are expressed on GABAergic, glutamatergic, serotonergic, noradrenergic, and dopaminergic terminals (Azad et al., 2008; Hermann et al., 2002; Kano et al., 2009; Morozov et al., 2009; Oropeza et al., 2007), but given the relative abundance of excitatory and inhibitory neurons in the brain, and the high levels of CB1 receptor expression on these terminals, the predominant effects of eCB signalling occur at GABAergic and glutamatergic synapses (Katona and Freund, 2012). Finally, CB1 receptor is also found in non-neuronal cells of the brain, particularly in astrocytes, where its activation promotes the release of neurotransmitters (Stella, 2010; Oliveira da Cruz et al., 2015). Overall, CB_1 receptor is the most abundant GPCR in the brain.

The function of the CB2 receptor is often related to that of CB1 receptor, even though its protein sequence shows only 44% homology to that of its cognate receptor (Fig1). CB2 receptors were cloned a few years after CB1R, and initially, their localization was thought to be restricted to peripheral tissues and in particular to immune tissues where they modulated cell migration and cytokine release (Klein et al., 1995; Schatz et al., 1997). It is known that CB2Rs are located in the spleen, liver, lungs, gastrointestinal tract, and adipocytes as well as in cardiac and bone tissue. In contrast to CB1, CB2 levels in the brain are very low, and emerging studies have shown that its expression has been found mostly in activated microglia, especially during inflammatory and neurodegenerative processes. The presence of CB2 receptors in neuronal cells is still under debate (Stella, 2010; Pertwee et al., 2010; Di Marzo, 2009). Similar to CB1, CB2 is a GPCR and is coupled to Gi proteins. Therefore, its activation induces adenylyl cyclase inhibition and MAP kinase pathway activation. However, it does not present any effect on voltage-gated ion channels.

OTHER "CANNABINOID" RECEPTORS

Although the International Union of Basic and Clinical Pharmacology recognized CB_1 and CB_2 as the only cannabinoid receptors, recent research shows that the orphan G protein receptor GPR55 could be considered a potential cannabinoid receptor. The endogenous ligand of this receptor is lysophosphatidylinositol (LPI) (Oka et al., 2007; Sharir et al., 2012), but GPR55 seems to be activated by Δ9-THC as well as by some synthetic inverse agonists of CB1 receptors, and antagonized by the other major, nonpsychotomimetic phytocannabinoid, cannabidiol (CBD). Contrasting data exist regarding the possibility that low concentrations of endocannabinoids also activate GPR55 (Sharir et al., 2012; Pertwee, 2007), and such controversies might be due either to biased signalling of these molecules at this receptor, depending on the cell type and conditions used for the assay, or to the recently discovered formation of heteromers between GPR55 and CB1 (Kargl et al., 2012; Martínez-Pinilla et al., 2014).This receptor is couplrd to Gq and G12/13 proteins,

therefore it activates the phospholipase C and small GTPase family proteins, among which RhoA is present.

Another atypical cannabinoid receptor is the transient receptor potential vanilloid type 1 (TRPV1) receptor. The endocannabinoid anandamide, but not 2-arachidonoylglycerol, binds to a cytosolic site of the receptor increasing intracellular calcium concentration, (Di Marzo and De Petrocellis, 2012). However, anandamide affinity is lower than that observed for CB1 and CB2 receptors.

Finally, it has been demonstrated that AEA and its congeners are able to activate peroxisome proliferator-activated receptors (PPAR) alfa and gamma. This action could mediate, at least in part, some of the biological effects of these compounds, such as regulation of glucose and lipid metabolism as well as inflammatory responses. This activity could also explain the antidepressant and antiepileptic effectiveness of AEA and its congeners that has been shown in some preclinical and clinical studies.

ENDOGENOUS LIGANDS

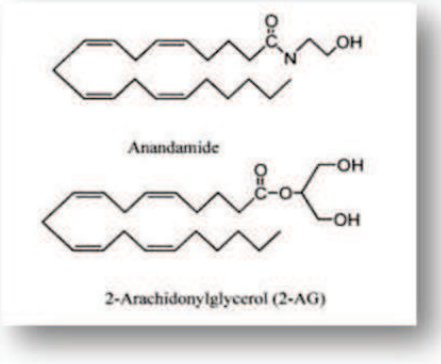

Figure 2. N-arachidonoylethanolamine (AEA), 2-Arachidonoylglycerol (2-AG)

The discovery of these two receptors immediately suggested the hypothesis of the existence of their endogenous ligands, or, as defined later, “endocannabinoids” (Fig2).

Thus, in 1992, the first endogenous agonist of both cannabinoid receptors was isolated from the pig brain, identified as N-arachidonoyl-ethanolamine (AEA) and named anandamide from the Sanskrit word ananda for “bliss” (Devane et al., 1992). Three years

later, a second ligand of both cannabinoid receptors was isolated from the canine gut and turned out to be a common intermediate in phospholipid and triglyceride metabolism, i.e. 2-arachidonoyl-glycerol (2-AG) (Mechoulam et al., 1995).

AEA is a derivative of arachidonic acid (AA) and ethanolamine. This endocannabinoid behaves as an agonist of CB_1 and the affinity of this compound for CB_2 is far less than for CB_1 (Pacher et al., 2006; Lambert and Fowler, 2005). The concentration of AEA in the brain is very low since this endocannabinoid is synthesized on demand from a phospholipidic precursor present in the cell membrane. Higher levels of AEA in the brain are found in areas where there is a high density of CB1 receptors, such as hippocampus and cerebral cortex (Felder et al., 1996).

2-AG is an ester of glycerol. Since it was firstly isolated in the dog intestine and pancreas, it was considered a peripheral ligand (Mechoulam et al., 1995). Later on it was identified in the brain where it was found at a higher concentration than AEA (Sugiura et al., 1995). Recently, other ECs have also been proposed during the last years, including 2-arachidonylglycerol ether (noladin ether), N-arachidonoyl-dopamine (NADA), and virodhamine, but their pharmacological activity and metabolism have not yet been thoroughly investigated. Two AEA-related compounds, i.e. N-oleoylethanolamine (OEA) and N-palmitoylethanolamine (PEA), have been included in an “extended” ECS. Although these two latter molecules lack strong affinity for either CB1 or CB2 receptors, they are biosynthetized by the same class of enzymes for AEA (Iannotti et al., 2016)

ENDOCANNABINOID-BIOSYNTHETIC AND CATABOLIC PATHWAYS

It is generally accepted that endocannabinoids are synthetized mainly “on demand” by stimulus-dependent cleavage of membrane phospholipid precursors following elevation of intracellular calcium (Piomelli, 2003). However, recent data suggest the existence of a storage system also for these mediators, according to which endocannabinoids are secreted from microglial cells through extracellular membrane vesicles (known as exosomes)(Gabrielli et al., 2015). In general, endocannabinoids do not share the same

metabolic or biosynthetic pathways; indeed, distinct regulatory mechanisms for AEA and 2-AG have been demonstrated (Ligresti et al., 2016).

SYNTHESIS

AEA (Fig.3) is produced by the cleavage of a phospholipid precursor, the N-arachidonoyl-phosphatidylethanolamine (NAPE), through at least three different pathways: the most direct one is catalysed by an N-acyl-phosphatidylethanolamine-selective phospholipase D (NAPE-PLD) (Okamoto et al., 2009), localized in the inner layer of cell membrane. Alternative pathways include the formation of phospho-AEA from hydrolysis of NArPE catalyzed by phospholipase C enzyme(s), followed by its conversion into AEA by protein tyrosine phosphatase PTPN22; or the conversion of NArPE into 2-lyso-NArPE by a soluble form of phospholipase A2, followed by the action of a lysophospholipase D.

The biosynthesis of 2-AG (Fig3.) appears to occur almost exclusively via diacylglycerol (DAG) hydrolysis by either of two sn-1-specific diacylglycerol lipases (DAGL) alpha or beta, the alpha-isoform being more important in the adult brain (Ligresti et al., 2016).

RELEASE AND UPTAKE

In the brain, after biosynthesis, endocannabinoids are released from the post-synaptic neuron, cross the synapse as retrograde messengers, and bind to CB1Rs expressed pre-synaptically, thus inhibiting neurotransmitter release. Due to their lipophilic nature, endocannabinoids can diffuse through the plasma membrane, if their levels in the extracellular space are higher than their intracellular concentration. (Ligresti et al., 2016).

The intracellular sequestration of endocannabinoids by intracellular proteins has also been suggested as a mechanism for endocannabinoids transport in the cytoplasm (Hillard and Jarrahian, 2005): Hsp70, FABP5 and FABP7 are reported to be anandamide intracellular binding proteins, being responsible for intracellular sequestration of AEA (Kaczocha et al., 2009; Oddi et al., 2009).

DEGRADATION

After reuptake, endocannabinoids may be degraded by their metabolic enzymes into molecules that can be recycled and used to synthesize new endocannabinoids or other endogenous compounds or may be converted into metabolites that may have biological activity, interfering with cell homeostasis. AEA is primarily degraded by FAAH (Fig 3) through hydrolysis into arachidonic acid and ethanolamine (Cravatt et al., 1996) but since it presents structural similarities with polyunsatured fatty acids, it can also serve as substrate for the inducible cyclooxygenase-2 (COX-2) (Ross, 2003). The molecular mechanism underlying 2-AG hydrolysis appears more complicated than AEA. Although FAAH can also degrade 2-AG into glycerol and arachidonic acid (Goparaju et al., 1998) the main enzyme responsible for its inactivation is monoacylglycerol lipase (MAGL) (Dinh et al., 2002), a cytosolic enzyme belonging to the serine hydrolase superfamily (Lambert and Fowler, 2005). The development of MAGL inhibitors has been reported to raise brain 2-AG levels (Makara et al., 2005). However, the evidence that microglial cells hydrolyze 2-AG even in the absence of MAGL (Muccioli et al., 2007) as well as immunodepletion experiments strongly suggest the existence of additional 2-AG-hydrolyzing enzymes other than MAGL. Indeed, recent data have shown that other enzymes such as ABHD6 (Marrs et al., 2010) and ABHD12, (Blankman et al., 2007), are also involved in 2-AG hydrolysis.

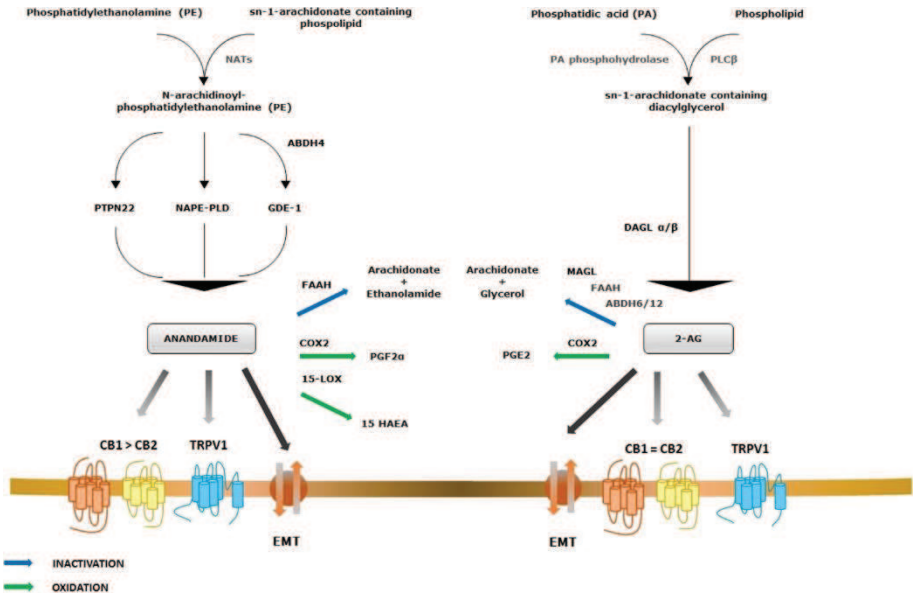

Figure 3. Synthesis, inactivation and oxidation of AEA and 2-AG (Iannotti et al., 2016)

CANNABIS AND ADOLESCENCE

Cannabis continues to dominate the world's illicit drug markets in terms of pervasiveness of cultivation, volume of production and number of consumers. Indeed in 2017, UNODC estimates that 183 million people (3.8% of the global population aged 15-64) used Cannabis (United Nations Office on Drugs and Crime, 2017). The main psychoactive constituent of Cannabis is delta-9-tetrahydrocannabinol (THC), which was isolated by Gaoni and Mechoulam in 1964 (Gaoni and Mechoulam, 1964). In the plant, THC is mainly found at the level of female inflorescences. Over the last two decades, the percentage of this compound in Cannabis has increased from 4% to 12% in the United States (ElSohly et al., 2016). Following Cannabis consumption, the subject may switch between moments of relaxation and euphoria with perceptual alterations including temporal, auditory, and visual distortion, as well as decline in cognitive functions. Furthermore, it is possible to find significant physiological alterations associated with cannabis use, such as heart rate increase, conjunctival hyperaemia, dry mouth, and appetite stimulation. The pharmacological action of THC mainly depends on its binding with CB1 receptor (Howlett, 2002; Matsuda et al., 1990, 1992).

Despite the increasing evidence of a possible relationship between adolescent Cannabis abuse and the later development of psychiatric disorders (Di Forti et al., 2014; Wilkinson et al., 2014), Cannabis remains the most widely used illicit drug among adolescents. According to NIDA report in 2017, 45% of adolescents (aged 12-19) tried Cannabis in their lifetime and 5.90% of them used it daily (National Institute on Drug Abuse, 2017). The high prevalence of its use and the evolving policy surrounding the legalization of cannabis in the western countries predicts an increasing amount of young people suffering from psychiatric disorders related to Cannabis exposure in the next future. Therefore, there is an urgent need to elucidate the molecular underpinnings that link adolescent Cannabis consumption to the development of mental illnesses later in life.

Adolescence is the period between childhood and adulthood, encompassing not only reproductive maturation, but also cognitive, emotional and social maturation. The adolescent brain is a brain in transition that differs anatomically and neurochemically

from that of the adult. During adolescence, the brain undergoes intense structural remodelling (Gogtay and Thompson, 2010; Paus, 2010) in which the endocannabinoid system seems to play a crucial role due to its modulatory activity (Galve-Roperh, 2009; Rubino et al., 2015). Despite the fact that the brain reaches its maximum size around 5 years of age, essential maturation processes that include myelinisation, synaptic refinement and volume reduction of grey matter, continue to occur during adolescence extending well into young adulthood (Blakemore, 2013; Whitford et al., 2007; Cohen-Cory, 2002; Katz and Shatz, 1996; Luna, 2009). These changes lead to functional and structural increases in connectivity and integrative processing, and a modification in the balance between limbic/subcortical and frontal lobe functions (Giedd, 2008). The perfectly orchestrated occurrence of all these dynamic changes is fundamental for attaining a correctly shaped adult brain. Any interference with these developmental processes might represent a risk factor for mental disease (Spear, 2000; Rubino and Parolaro, 2016). Adolescence is also the period in which drug consumption begins, especially Cannabis use, and it is known that chronic consumption of drugs of abuse induces important changes in the brain, mainly in the mesocorticolimbic circuitry (Koob and Volkow, 2016). Moreover, recent studies demonstrated the active and dynamic nature of development of the endocannabinoid system during the adolescent period (Rubino and Parolaro, 2016), together with the suggestion that the endocannabinoid tone could play a role in the elimination of excitatory synapses and thus in pruning processes. Therefore, it is possible to hypothesize that heavy use of Cannabis, during adolescence might interfere with the physiological role played by the endocannabinoid system in adolescent neuronal remodelling thus leading to altered brain maturation.

LONG TERM EFFECTS INDUCED BY ADOLESCENT CANNABIS ABUSE IN HUMANS

Cannabis is the illegal drug most consumed by adolescents and Cannabis use among this population has been accompanied by a decrease in the age of first use in the last years. Even if the majority of abusers does not report adverse reactions, an increasing number of epidemiological studies seems to suggest that sustained Cannabis use during adolescence may increase the risk for developing cognitive deficits, psychosis, depression, and may act as a gateway to other drugs of abuse.

COGNITIVE ASPECTS

Regular use of Cannabis during puberty has a negative impact on memory, learning, and attention (Blakemore, 2013). Several case-control and longitudinal studies have provided evidence of an association between adolescent Cannabis exposure and poorer educational performances (Fergusson et al., 2003; Silins et al., 2014; Townsend et al., 2007). Some data suggest that even adult Cannabis consumers have a negative impact on memory, however they can regain their cognitive performances, reaching almost the control levels, through an appropriate and prolonged period of abstinence. This same improvement does not occur in adolescent abusers who still present cognitive deficits even months after the end of Cannabis use (Medina et al., 2007). The precise mechanism through which Cannabis induces negative effects on cognitive functions has not been entirely clarified yet; nevertheless, recent research has demonstrated a close association between the chronic use of Cannabis during adolescence and anatomic and/or functional brain alterations. These alterations may include hippocampal asymmetry, aberrant activation of some signal transduction pathways in different cerebral regions, and changes in white matter microstructures (Jacobus and Tapert, 2013).

EMOTIONAL ASPECTS

Several studies have revealed a link between adolescent Cannabis exposure and depression, especially in female subjects (Chadwick et al., 2013; Lee et al., 2008). The amount of drug consumed seems to be relevant; indeed, the risk for developing depressive disorders occurs only in habitual Cannabis abusers (Van Laar et al., 2007). Furthermore, if the use begins before the age of 17, the resulting risk is even higher (Hayatbakhsh et al., 2007). It seems therefore clear that a continuative and premature use of Cannabis is associated with an increased risk of developing depression, especially among female abusers.

PSYCHOTIC SYMPTOMS

Epidemiological studies have shown that the use of Cannabis during adolescence increases the risk of later development of psychotic symptoms (Chadwick et al., 2013). The association between Cannabis abuse and the subsequent development of psychosis seems to be related to the age at which drug consumption begins (Arseneault et al., 2002). Moreover, the lower the age of the first exposure to Cannabis, the earlier the onset of psychotic symptoms, especially in frequent smokers of enriched THC strains, such as skunk (Di Forti et al., 2014). The reason why major effects are observed in those who started Cannabis abuse earlier is still unclear. Nevertheless, it is well known that significant cerebral modifications occur during adolescence and the abuse of drugs during this period of life may influence cerebral circuit maturation, affecting brain functionality. These functions include reward and decisional processes, attention, learning and memory, and behavioural control. Since the ECS may play a role in the adolescent brain maturation, the use of Cannabis in this critical period might affect maturation processes, thus increasing the risk of psychosis. However, not all Cannabis abusers will develop psychosis, thus suggesting the importance of factors of vulnerability with which Cannabis consumption can interact. These factors may include genetic susceptibility (such as the

Val108Met polymorphisms of COMT, or Val66Met polymorphisms for BDNF, with the female population carrying Met variant more at risk of early psychosis), along with environmental factors (such as adverse experiences during childhood) (Chadwick et al., 2013; Decoster et al., 2011; Henquet et al., 2008).

LONG TERM EFFECTS INDUCED BY ADOLESCENT CANNABIS ABUSE IN ANIMAL MODELS

Epidemiologic data provide evidence that Cannabis exposure in adolescence is an important contributing factor to psychiatric vulnerability; however, the molecular mechanisms underlying this association are poorly understood. The use of animal models may be considered an alternative strategy in order to successfully evaluate the link between Cannabis abuse and the development of psychiatric disorders. Indeed, animal models of early Cannabis exposure represent a unique tool to characterize the long-lasting behavioural consequences of Cannabis use and to clarify the underlying cellular mechanisms.

The experimental studies that assess long-term effects induced by chronic cannabinoid treatment during adolescence are relatively few and have sometimes shown conflicting results, probably due to differences in the parameters used in the studies (rat strain, cannabinoid agonist, and dose administered). Overall, existing data show the presence of subtle changes in adult cerebral circuits following cannabinoid treatment in adolescence. These changes are known to affect cognitive and emotional processes, thus they may contribute to the development of the alleged psychiatric disorders ensuing from adolescent cannabinoid exposure.

COGNITIVE ASPECTS

In rodents, chronic administration with either synthetic or natural cannabinoids during adolescence induces recognition memory deficits that are evident from 15 days to 30 days after discontinuing the treatment (O'Shea et al., 2004, 2006; Quinn et al., 2008; Realini et

al., 2011; Schneider and Koch, 2003; Zamberletti et al., 2014). This alteration is present in both female and male rats, but it does not appear when the treatment is performed in adult rats (Renard et al., 2013; Realini et al., 2011). Moreover, in the spatial version of the object recognition task, Abush and Akirav (2012) found that chronically treated rats showed impairment in short-term memory evident up to 75 days after the last injection. Similarly, spatial working memory deficits (tested by the eight-arm radial maze) were described after adolescent THC exposure in adult rats (Rubino et al., 2009a, 2009b). A persistent THC effect selective for spatial working memory was also observed under well-controlled experimental conditions in adolescent monkeys (Verrico et al., 2014). Finally, adult animals exposed to WIN 55,212-2 in adolescence showed impairments in the attentional set-shifting task and thus in cognitive flexibility (Gomes et al., 2014). When pure spatial learning was assessed in the Morris Water Maze, researchers found no effect of the adolescent exposure to natural or synthetic cannabinoids, in both male and female rats (Cha et al., 2006, 2007; Higuera-Matas et al., 2009). No effect was also observed when aversive memory was monitored (Rubino et al., 2009a, 2009b). These findings suggest that adolescent cannabinoid exposure may impair specific components of memory, and more likely the forms of memory where PFC plays a role.

EMOTIONAL ASPECTS

There is evidence that adolescent cannabinoid exposure alters emotional regulation in adulthood, although these results vary depending on the particular index or process examined.

Contrasting data were reported regarding anxiety monitored in the elevated plus maze or open field test. Some authors reported no changes in the anxiety profile of animals pre-treated with cannabinoid compounds during adolescence, whereas others described an anxiolytic or even an anxiogenic effect (Rubino and Parolaro, 2016). A stronger agreement exists about social anxiety: all the authors who studied sociability after adolescent exposure to cannabinoids found an impairment in social behaviours, and

reduction in social interaction has been considered as an anxiogenic behaviour in rodents (File and Hyde, 1978). Thus, it appears that depending on the test used, cannabinoids may differently affect the anxiety profile, inducing anxiolytic or anxiogenic effects. However, it is worth noting that often results in the social interaction test do not correlate with performance in other animal tests of anxiety. This suggests that the reduction in social behaviour observed in the social interaction test might reflect other distinct psychological domains that could be relevant to other psychopathological disorders such as depression (Tonissaar et al., 2008). At this regard, besides reduced social behaviour, two other features of the depressive-like phenotype in animals are behavioural despair/passive coping strategy and anhedonia. Both these behaviours were present after adolescent exposure to either synthetic or natural cannabinoids (Bambico et al., 2010; Realini et al., 2011; Rubino et al., 2008), suggesting the presence of a depressive-like phenotype in adult animals after adolescent exposure to cannabinoids. This behavioural picture was associated with biochemical parameters related to depression, such as decreased CREB activation in the prefrontal cortex and hippocampus, increased CREB activation and dynorphin levels in the Nucleus accumbens, decreased neurogenesis in the dentate gyrus of the hippocampus. Interestingly, the depressive-like phenotype did not develop when the chronic cannabinoid administration was performed in adult animals (Bambico et al., 2010; Realini et al., 2011), thus suggesting the existence of an age-dependent susceptibility of the brain to adverse effects of cannabinoids.

PSYCHOTIC SYMPTOMS

Experimental studies regarding long-lasting effects of adolescent cannabinoid exposure on psychosis-related behaviours in adult rodents are still scarce. It is impossible to model schizophrenia in its entirety in animals since this psychiatric disorder represents a complex condition with a very heterogeneous presentation of a variety of symptoms. Patients typically experience a combination of positive (e.g. hallucinations, delusions, thought disorganizations), negative (e.g. loss of motivation, affective blunting, alogia,

social withdrawal, and reduced hedonic capacity) and cognitive symptoms (e.g. deficits in attention, memory and executive functions). Since long-lasting effects of adolescent exposure to cannabinoids on the cognitive dimension and emotional reactivity have already been discussed above, this section will mainly discuss the positive symptoms. Since positive symptoms of schizophrenia such as auditory hallucinations and delusions are uniquely human, animal studies have focused on two main categories of behaviour: locomotor hyperactivity and disruption of prepulse inhibition (PPI). PPI measures sensorimotor gating that is the ability to filter out insignificant sensory information, a cognitive abnormality present in schizophrenia. Accordingly, loss of normal PPI is widely accepted as an endophenotype of schizophrenia with high translational validity, since it can be assessed in both animals and humans. Impairments in PPI in rats and mice were observed long after chronic treatment with the cannabinoid agonist WIN 55,212-2 in adolescence, suggesting the presence of disrupted sensorimotor gating (Wegener et al., 2009; Schneider et al., 2003; Gleason et al., 2012). In contrast, other groups reported no alterations in this behaviour (Llorente-Berzal et al., 2011; Klug et al., 2013). The reason for this discrepancy is unclear: all groups used synthetic cannabinoid agonists, but the last three groups performed a longer treatment (15 or 21 days) with the same dose of agonist (CP55,940 or WIN 55,212-2), triggering a deep state of tolerance in animals. The former groups instead performed a shorter treatment (10 days) or used an irregular protocol of injections (none, one, or two daily injections for 25 days), and this could have led to a less profound tolerance.

The concept of testing locomotor hyperactivity in animal models as a symptom of psychosis is based on the observation that enhanced dopaminergic activity in rodents leads to enhanced motor activity (Geyer, 2008). Accordingly, locomotor hyperactivity may have some face validity for certain components of the positive symptoms of schizophrenia, such as psychotic agitation (Van den Buuse, 2010). Rodent locomotor hyperactivity, either at baseline or induced by the treatment with psychoactive drugs, such as amphetamine or phencyclidine (PCP), has become commonly used as a behavioural tool to study the agitation that is typically present in human psychosis. Nonetheless, few papers have extensively investigated basal locomotor activity in adult

animals with pre-exposure to cannabinoids during adolescence, and they reported discrepant results. Indeed, no significant alterations in the open field recordings after CP-55,940 (Biscaia et al., 2003) and THC (Rubino et al., 2008) were reported, but also the presence of locomotor hyperactivity after WIN 55,212-2 (Wegener et al., 2009), and reduced baseline locomotor activity after CP-55,940 (Klung et al., 2013). More recently, Zamberletti and colleagues (2014) monitored the effect of adolescent THC exposure on PCP-induced locomotor hyperactivity, and they found that THC increases the locomotor activating effect of acute PCP in adulthood. Similarly, PCP-induced stereotyped behavior was significantly enhanced in THC-treated rats.

GATEWAY HYPOTHESIS

An extremely important aspect emerging from the debate on Cannabis abuse among adolescents concerns the possibility that this behaviour could facilitate the subsequent use of other illicit substances. Known as the “gateway” hypothesis, this theory suggests that exposure to Cannabis increases the likelihood that an individual will use illicit drugs other than cannabis at some later point (Ellgren et al., 2008; Eggan et al., 2010; Lee et al., 2013; Leweke et al., 2011; O’Shea et al., 2006, 2004; Quinn et al., 2008). According to this hypothesis, facilitated learning of cocaine self-administration was reported in adult female, but not male, rats pre-treated with cannabinoids in adolescence (Higuera-Matas et al., 2008). This could be associated with an up-regulation of dopamine transporter (DAT) in the caudate putamen (Higuera-Matas et al., 2010). Adolescent exposure to synthetic or natural cannabinoids did not alter dopaminergic or behavioural responses to amphetamine (Ellgren et al., 2004), even though an increased reinforcing effects of MDMA was observed in mice treated with cannabinoids during adolescence (Rodriguez-Arias et al., 2010). This is in contrast with data obtained by Pistis and colleagues (2004); who showed that dopaminergic neurons were significantly less responsive to stimulation by WIN 55-212,2 (WIN) in animals pretreated for 3 days during adolescence or adulthood with WIN and allowed a 2-week washout (Pistis et al., 2004). However, in the group pre-

treated in adolescence but not in adulthood, long-lasting cross-tolerance to morphine, cocaine and amphetamine developed, suggesting an enduring form of neuronal adaptation in dopaminergic neurons after sub-chronic cannabinoid intake at a young age. This, in turn, would have affected the subsequent responses to drugs of abuse. Regarding opioids, male rats showed a significant increase in the acquisition of both morphine and heroin self-administration, and this might be due to cannabinoid-induced alterations in the limbic mu opioid receptor system (Biscaia et al., 2008; Ellgren et al., 2008). Curiously, female rats seem to be unaffected by the same treatment (Biscaia et al., 2008). Even the different rat strain may affect the response: in Lewis rats, THC exposure did not affect heroin conditioned place preference (CPP), but potentiated reinstatement, while in Fisher 344 rats THC exposure increased heroin CPP and made it resistant to extinction (Cadoni et al., 2015). These studies suggest the importance of sex and genetic background in determining the influence of THC exposure on opioid effects related to addiction.

SEX-DIFFERENCES IN RESPONSE TO CANNABIS EXPOSURE

Drug addiction is a widespread phenomenon affecting both men and women in different ways: sex-dependent differences have been frequently observed in the biological and behavioural effects of substances of abuse. Since drug abuse in female is a heavily increasing phenomenon, researchers are giving lot of importance to possible sex differences related to drug consumption, including Cannabis. Sex differences have been observed in the cannabinoid-induce effects related to cannabis abuse and dependence for example, men consume Cannabis in greater amounts and at higher rates than women do (Perez-Reyes et al., 1981) and, consequently, they appear to be more likely than females to become dependent on Cannabis (Wagner and Anthony, 2007; Fermer et al., 2015). Males also exhibit higher circulating levels of THC (Jones et al., 2008), show larger cardiovascular and subjective effects than female smokers (Leatherdale et al., 2007), display more evident withdrawal symptoms (Crowley et al., 1998), are less likely to be Cannabis-only users and have a higher prevalence of panic disorder and personality disorder (Hasin et al., 2008). However, females tend to have shorter intervals between

the onset of use and regular consumption or development of dependence (Ridenour et al., 2006; Schepis et al., 2011). They also seem to experience negative consequences of drug use earlier than males, and to be more likely to suffer from an internalizing disorder, such as depressive and anxiety disorders (Kloos et al., 2009). One of the first study describing this correlation reported that daily Cannabis use was associated with a fivefold increase in anxiety and depression in young females, but not males (Patton et al., 2005). Accordingly, higher rates of comorbid mood and anxiety disorders in women have been recently observed in a large epidemiological study performed in the United States (Khan et al., 2013).

Potential sex-differences have also been reported for Cannabis use and neurocognitive functioning (Crane et al, 2013): in fact, Cannabis use appears to be more consistently associated with poorer episodic memory performance in females and with poorer decision-making performance in males. Females Cannabis users present a larger prefrontal cortex volume compared to controls, whereas male users show a smaller one (Medina et al., 2009). It is worth noting that among users, larger prefrontal cortex total volume was associated with worse executive functioning.

Animal models seem to confirm the existence of some sex-dependent responses to adolescent cannabinoid exposure, with females appearing more sensitive than males in the emotional sphere. On the contrary, literature suggests that adolescent exposure to cannabinoids induces long-term cognitive impairments specifically in recognition and spatial working memory, but these effects do not displays sex differences, since they are present in both male and female animals. Differences in behaviour are substantiated by differences at the cellular/molecular level, as female rats exhibit pronounced metabolism of THC to still active compound 11-OH-THC compared to their male conspecifics, particularly after repeated THC administration (Wiley and Burston, 2014). This fact, together with the observation that adolescent female rats possess more efficient CB1 receptors (Rubino and Parolaro, 2011), suggests that they may be more vulnerable to THC effects.

Accordingly, chronic THC exposure in adolescence induced more intense CB1 receptor desensitization in females, with more brain areas involved, despite similar down-

regulation (Rubino et al., 2008; Burston et al., 2010). If confirmed also in humans, this would explain, at least in part, why females tend to have shorter intervals between the onset of use and the development of dependence (Ridenour et al., 2006; Schepis et al., 2011; Khan et al., 2013; Hernandez-Avila et al., 2004).

POSSIBLE MECHANISMS OF ADOLESCENT VULNERABILITY TO CANNABINOID EXPOSURE

The first target of exogenous cannabinoids is the endocannabinoid system. Therefore, alterations in components of this system are expected after exposure to these compounds. According to this assumption, a profound CB1 receptor downregulation and desensitization has been observed after chronic THC treatment during adolescence in different cerebral areas (Rubino et al., 2015, 2008; Burston et al., 2010). This downregulation is less evident in male rats than in females, probably as a result of the different THC metabolism in the sexes (Wiley and Burston, 2014) and the alleged presence of more efficient receptors in adolescent female rats (Rubino et al., 2011). Moreover, in the PFC of THC-exposed female animals, the significant decrease of CB1 receptor binding described immediately after the last THC injection and still present in adulthood was paralleled by a significant decrease of anandamide levels (Rubino et al., 2015). Thus adolescent cannabinoid exposure alters the dynamic changes present in the endocannabinoid system during adolescence, likely affecting the neurodevelopmental processes in which this system might play a role.

One of the most relevant events induced by chronic adolescent cannabinoid exposure in rodent models is the long-lasting negative impact on working memory and decision-making. These high brain functions are refined during adolescence and are mainly dependent on the functional maturation of the PFC. According to recent literature, the endocannabinoid tone seems to play a fundamental role in some maturational processes within the glutamatergic system**.** Indeed, adolescent THC exposure induces a significant decrease in K+-evoked glutamate release in the adult hippocampus (Higuera-Matas et al., 2012) as well as changes in the maturational fluctuations of NMDA and AMPA subunits in

the PFC of female animals, leading to larger amounts of GluN2B and GluA1 in adulthood (Rubino et al, 2015). Since NMDA receptors play a pivotal role in regulating the peri-adolescent maturation of GABAergic networks in the PFC (Thomases et al., 2013), it might be assumed that even the GABAergic system could be affected by adolescent cannabinoid exposure. In line with this hypothesis, Zamberletti and colleagues (2014) demonstrated that chronic treatment with THC during adolescence in female rats resulted in reduced glutamic acid decarboxylase 67 (GAD67) and basal GABA levels in the PFC. These findings seem to indicate that adolescent cannabinoid exposure affects not only the endocannabinoid system but also the glutamatergic and GABAergic systems. These three systems are important in shaping cortical oscillations, a neural network activity in the neocortex (Uhlhaas et al., 2009) that is implicated in cognitive and sensory processing (Buzsáki et al., 2004; Wang et al., 2010).

Furthermore, the adolescent brain is characterised by a high rate of synaptic pruning, especially in regions that govern higher cognitive function such as the PFC (Selemon, 2013). Recent work suggests an important role of the endocannabinoid system in synaptic pruning. Thus, another event that might play a part in the brain alterations triggered by adolescent cannabinoid exposure should include changes in dendritic spines. Consistent with this assumption, male rats treated with a synthetic cannabinoid showed a significant reduction of dendritic spine density in the nucleus accumbens immediately after the treatment (Carvalho et al., 2016). Similarly, adolescent THC in male rats reduced spine density in the dentate gyrus of the hippocampus in adulthood, as well as dendrite length and number (Rubino et al., 2009). In adult female rats exposed to THC in adolescence, a significant decrease in the number of spines present on PFC pyramidal neurons was observed (Rubino et al., 2015).

Recent evidence supports the involvement of epigenetic mechanisms in the development of psychiatric disorders (Renthal et al., 2009). For example, impairments in chromatin remodelling enzymes are increasingly being recognized as playing a crucial role in the development and maintenance of addictive disorders. It is, therefore, possible that Cannabis abuse during adolescence could impair the brain network functionality acting

through a mechanism involving epigenetic mechanisms, thus leading to long-term behavioral impairments.

EPIGENETICS

The term epigenetics is considered a neologism coined by Conrad Waddington in the early 1940s, reflecting his attempt to integrate genetics with embryology. Indeed, Waddington used this term to explain how identical genotypes could produce different phenotypes during developmental processes through the epigenetic landscape metaphor (Waddington, 1957). From the conceptual point of view, this epigenetic landscape represents a series of choices facing an organism. Genes imposed the initial constraints and starting points, however, environmental and physiologic forces emerge during development. These forces then operate in coordination with genes, pushing the organism into typically deeper canals, the so-called attractors of the landscape, resulting in the organism's phenotype. Through this process of canalization, individual organisms that might have identical genotypes could develop radically different phenotypes (Waddington, 1957). The recent use of this concept focuses on the molecular modifications of DNA and chromatin that are thought to change gene expression allowing transcriptional regulation of genes without altering DNA sequence (Jaenisch and Bird, 2003).

Chromatin is made up by a fundamental unit named nucleosome, which is composed of 147 bp of DNA wrapped around an octamer of core histone proteins (two copies each of H2A, H2B, H3, and H4). Nucleosomes are connected one to the other by linker DNA, with a length ranges between 20–90 bp, and by histone H1 that condenses nucleosomes into higher order structures (Jenuwein and Allis, 2001; Gardner et al., 2011). Since DNA is closely associated with histones and often embedded within chromatin supercoils, there are cellular mechanisms that modify and reshape the chromatin structure to allow the coordinated expression of specific transcriptional programs and the silencing of others.

Among the epigenetic mechanisms, we are going to mention methylation of DNA and histone modifications. (Fig.4)

DNA METHYLATION

DNA methylation (Fig.4) is an enzymatic process by which methyl groups are added to the DNA at the level of cytosine in the 5 position, resulting in the formation of a methyl group protruding into the major groove of DNA (Newell-Price et al., 2000). In mammals, these 5-methylcytosine are mainly present at 5'-CpG-3'palindromic sequences and do not interfere with the normal hydrogen bonds with the complementary base guanine. These CpG dinucleotide sequences are randomly distributed in the genome, presenting the higher concentration at the level of the so-called CpG islands. CpG islands coincide with 50-60% of human promoter regions of genes and are typically methylated to a much lesser extent than the CpG dinucleotides located outside the islands (Wang and Leung, 2004). Methylation of CpG dinucleotides in gene promoter regions is generally associated to transcriptional repression because of its ability to prevent the binding of transcription factors to their target sequences. About 3% of all cytosine in the human genome is methylated (Nafee et al., 2008), and the correct methylation of these bases is essential for normal development. This is associated with a number of key processes including cellular differentiation, genetic imprinting, repeated elements suppression, and X-chromosome inactivation (Bird, 2008). DNA methylation is catalysed by DNA methyl-transferases (DNMTs), which transfer a methyl group from S-adenosyl methionine (SAM) to cytosine residues of CpG dinucleotides. DNMTs family of enzymes include DNMT1, DNMT2, DNMT3a and DNMT3b (Weber and Schubeler, 2007). These enzymes play different roles, equally crucial: DNMT1 maintains the methylation pattern during DNA replication, DNMT3a and DNMT3b seem to catalyse the de novo methylation of the previously unmethylated DNA double filament (Newell-Price et al., 2000; Kim et al., 2009), and DNMT2, on the other hand, is capable of methylating RNA (Goll et al., 2006).

HISTONE MODIFICATIONS

Histone modifications (Fig.4) represent an epigenetic mechanism that acts on the degree of chromatin condensation. Histones are basic proteins (H1, H2A, H2B, H3 and H4) that form the histone octamer core around which DNA is wrapped to form the nucleosome, the fundamental unit of chromatin.

The histone N-terminal tail may undergo a variety of post-translational changes such as acetylation, methylation, phosphorylation, ADP-ribosylation and ubiquitination. In particular, some of these modifications (e.g. H3K9me and H3K27me) make the chromatin more compact (heterochromatin), excluding therefore the transcriptional machinery. Conversely, other modifications (e.g. H3K4me; H3K9ac) relax chromatin structure (euchromatin) so that the nucleosome assumes a transcriptionally active structure exposing promoter regions for transcription factors and allowing gene transcription. This suggests that histone modifications influence gene expression by means of chromatin remodelling and recruitment of transcription factors, which can activate or repress gene transcription (Berger, 2007; Borrelli et al., 2008; Jenuwein and Allis, 2001).

HISTONE ACETYLATION

Histone acetylation is a dynamic process controlled by specific enzymes that add or remove acetyl groups. Indeed, different classes of histone acetyl-transferases (HATs) are able to add acetyl groups to lysine residues of histone tails. This modification reduces electrostatic interaction between DNA and histone proteins, relaxing the chromatin structure and enabling transcription factor to bind promoter regions of specific genes (Kouzarides, 2007). Histone acetylation is therefore associated with transcriptional activation; on the contrary, its lack is associated with gene repression. HATs use acetyl-CoA as a substrate for the reaction and they can add acetyl group even to non-histone proteins such as transcription factors (Doi et al., 2006; Kawasaki et al., 2000). In contrast, histone deacetylases (HDACs) remove acetyl groups from the histones and they are

divided into four classes. Class I HDACs (HDAC 1, 2, 3 and 8) are ubiquitously expressed and probably mediate most of the deacetylase activity in the cells. Class II HDACs (HDACs 4, 5, 7, 9 and 10) are only expressed in specific tissues such as heart and brain (Chawla et al., 2003). Lastly, class III HDACs (sirtuins) are different from other HDACs and they have been implicated in the regulation of life expectancy and metabolism (Haigis and Guarente, 2006).

HISTONE METHYLATION

Histone methylation can be associated with both transcriptional activation and repression. This depends on which amino acidic residue is methylated and on its own degree of methylation. Histone methyl-transferases (HMTs) are enzymes that catalyse the transfer of one, two, or even three methyl groups to lysine and arginine residues of histone H3 and H4, by using S-adenosylmethionine as a substrate. In contrast to acetylation, methylation does not alter the target residue charge. However, methylation can widely modify the steric profile and the potential molecular interactions by adding mono-, di- or tri-methyl groups. This modification makes each site capable of recruiting specific co-regulators and exerting different effects on transcriptional activity. For example, H3K4 trimethylation is associated with gene activation, while H3K9 or H3K27 trimethylation is repressive (Maze et al., 2010).

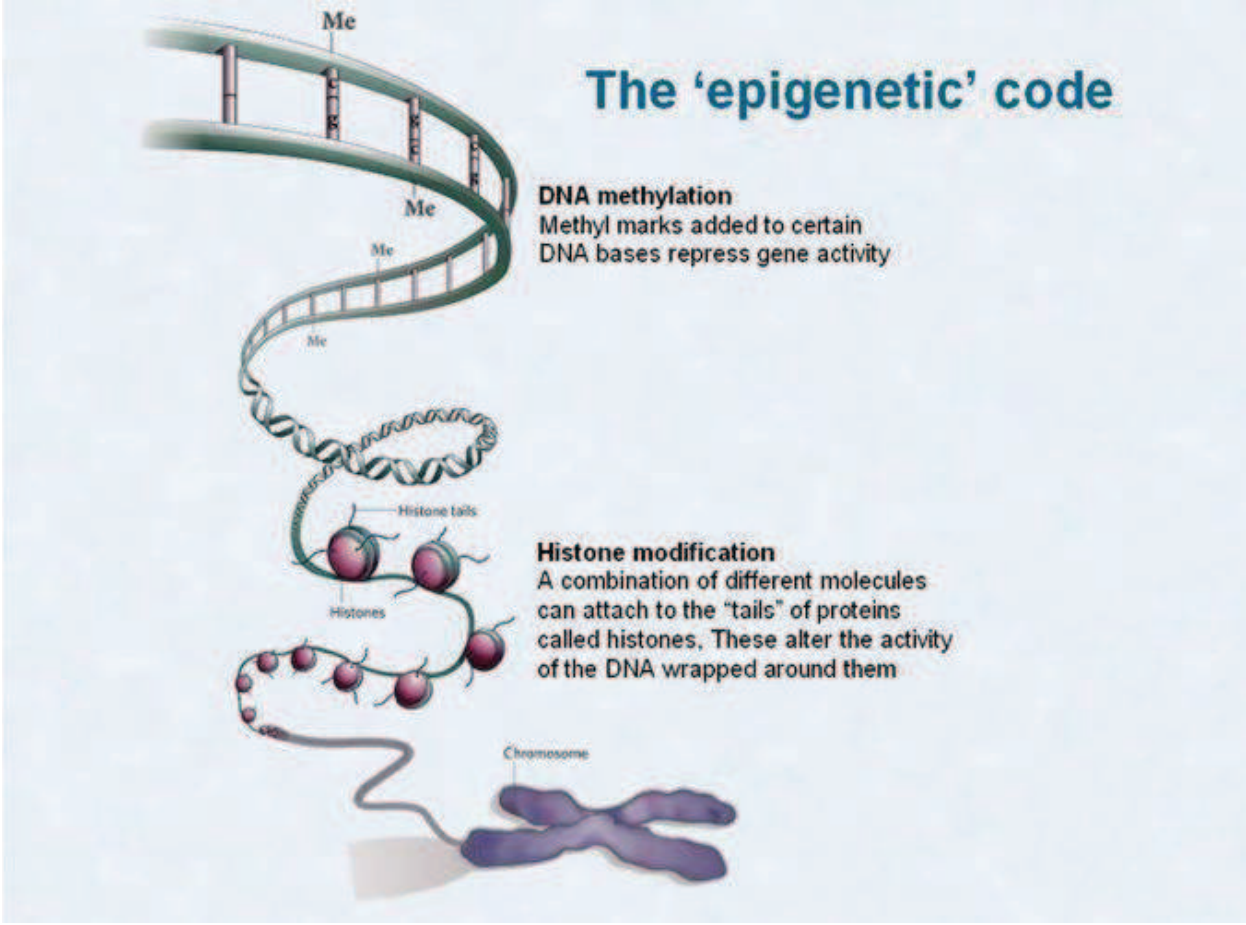

Figure.4 Epigenetic mechanisms: methylation of DNA and histone modifications

EPIGENETIC MECHANISMS IN PSYCHIATRIC DISORDERS

Epigenetic mechanisms control normal brain function, and histone modifications have been related to neural plasticity and multiple forms of behavioural memory (Levenson and Sweatt, 2005; Borrelli et al., 2008; Dulac, 2010; Nestler, 2011). Through the investigation of epigenetic markers, it is possible to understand whether a gene is transcribed or silenced during a particular stage of a brain disease. Indeed, epigenetics can provide bases for determining different transcriptional states, enabling us to identify genes and proteins involved in neuropsychiatric disorders. Moreover, the study of epigenetic mechanisms could provide a valid theory that links genetic factors to different environmental factors, helping us to understand the etiology of the various neuropsychiatric pathologies.

Starting from this assumption, recent studies have reported that alterations in some epigenetic markers are present in suicide victims suffering from depression, suggesting involvement of epigenetic mechanisms in this pathology (Autry and Monteggia, 2009). In particular, histone acetylation seems to play an important role in depression. Indeed, mice subjected to chronic social defeat stress (an animal model for depression) show a transient reduction in acetylated histone H3 at lysine 14 (H3K14Ac) in the Nucleus Accumbens (NAc). The NAc is a brain area that is deeply important for processing stimuli related to reward (Wise, 1987; Koob, 1996; Carelli, 2002). Clinical depression often includes anhedonic, motivational, and arousal deficits, suggesting that the NAc may present an important role in mediating these symptoms (Nestler and Carlezon, 2006). Local infusion of MS275 (selective inhibitor of class I HDAC) has an antidepressant effect and, conversely, over-expression of HDAC2 has a depressive effect, suggesting the importance of these enzymes in the development of depression. Furthermore, the chronic stress paradigm alters acetylation levels even in the Hippocampus, a brain region highly sensitive to the effects of stress and implicated in regulating stress responses, as well as in the Amygdala, an area involved in integrating higher brain functions such as emotions and cognitive memory (Covington HE et al., 2011). Chronic social defeat stress decreases di-methylated histone H3 at lysine 9 levels (H3K9me2) in the Nucleus accumbens, resulting in transcriptional repression. This alteration is related to G9a down-regulation, a histone methyl-transferase responsible for this modification. More studies are necessary to fully understand the role played by these histone modifications in depression.

Considering schizophrenia (SZ), a mental disorder characterized by psychotic symptoms such as delusions and hallucinations, experimental evidence suggests the involvement of epigenetics in its pathogenesis. Indeed, previous research reported the presence of a hyper-methylated state of the promoter of genes encoding for glutamate decarboxylase 67 (GAD67), an enzyme that catalyses the decarboxylation of glutamate to GABA and CO2, and reelin, an extracellular matrix glycoprotein involved in neuronal migration and in schizophrenia (Akbarian et al., 2009; Huang et al., 2007; Chen et al., 2002; Costa et al., 2002). This suggests that hyper-methylated DNA in the promoter region of the coding

gene for reelin may be responsible for the reduced expression of this protein in SZ patients. Furthermore, it has recently been demonstrated that the administration of HDAC inhibitors is able to reactivate reelin and GAD67 transcription to a dose comparable to that of DNMT inhibitors (Kundakovic et al., 2009). These findings therefore suggest the possibility of using epigenetic drugs, either individually or in combination, as new potential therapies to reduce protein deficiencies and the clinical symptoms associated with SZ.

Epigenetic mechanisms have also been described after exposure to abused drugs. Data reported in literature show that after acute cocaine administration a rapid increase in acetylation of histone H4 (a marker associated with transcriptional activation) occurs in specific genes known to be involved in behaviours related to cocaine intake (Kumar et al., 2005). Moreover, a global increase of H3K9me3 (a marker associated with transcriptional repression) is observed (Kumar et al., 2005). This could be a compensatory mechanism carried out by the body to counterbalance the negative effects of histone hyper-acetylation induced by cocaine exposure. According to this data, recent studies performed in our laboratory have shown that adolescent THC exposure induces significant epigenetic alterations in the Prefrontal cortex of female rats. Indeed, two hours after the last THC injection, a significant increase in H3K9me3 levels is observed. This alteration increases its intensity 24 hours later and is paralleled by a significant raise of H3K9me2 and H3K14ac levels. Finally, 48 hours after the end of the treatment, the observed alterations are returned to control levels, meanwhile K9 acetylation levels significantly increase. Such alterations are associated with significant changes in the transcription of genes involved in synaptic plasticity (Prini et al., 2018). A previuos study has found persistent changes in repressive H3K9me2 and H3K9me3 at proenkephalin opioid neuropeptide (Penk) locus in the NAc of adult rats following adolescent THC exposure in line with enduring upregulation of Penk mRNA levels (Tomasiewicz HC et al., 2012).

Overall, these data suggest that epigenetic modifications are strongly related to the etiopathogenesis of psychiatric disorders such as depression, schizophrenia and drug abuse. Consistently with this hypothesis, studies performed over the last years have

demonstrated that histone modifications represent an important epigenetic mechanism for the translation of environmental stimuli -including drug abuse- into changes of gene expression (Feng and Nestler, 2013; Schmidt et al., 2013).

It is therefore possible that Cannabis abuse during adolescence could impair the brain network functionality acting through a mechanism involving histone modifications, thus leading to long-term behavioural impairments. Nevertheless, further studies are needed in order to understand how changes in these mechanisms may contribute to brain and behaviour alteration induced by adolescent THC exposure.

AIM

Despite the accumulation of evidence of a possible relationship between adolescent Cannabis abuse and the later development of psychiatric disorders, Cannabis remains the most common illegal drug used by adolescents. Consistently with the epidemiological data, we have previously demonstrated that THC administration to adolescent female rats induces the onset of a depressive/psychotic-like phenotype. Interestingly, only adolescent THC exposure, but not the adult one, led to this complex phenotype, suggesting that adolescence may represent a vulnerable period for the adverse effect of THC. However, the neurobiology of this vulnerability is not completely understood and thus needs further investigation.

Several papers support the involvement of histone modifications in the pathogenesis of psychiatric illnesses. In line with these data, we demonstrated that THC alters histone modifications in the Prefrontal cortex (PFC) of female rats. Alterations in histone modifications were paralleled by changes in the expression of a subset of plasticity genes relevant for the development of cognitive deficits present at adulthood. Interestingly, alterations in both histone modifications and gene expression were more intense and widespread following adolescent THC treatment compared to adult. This differential effect clearly indicates an age-dependency in THC-induced molecular alterations, at least in the PFC, highlighting new possible molecular mechanisms underlying adolescent susceptibility to develop substance-induced psychopathologies.

On these bases, the first aim of this thesis is to extend our knowledge on the impact of THC exposure on histone modifications occurring in other brain areas that, along with the PFC, are important for the different aspects of the phenotype described previously in female animals.

To this aim, adolescent (PND 35-45) and adult (PND 75-85) female Sprague-Dawley rats will be treated twice a day with increasing intraperitoneal (ip) doses of THC: 2.5 mg/kg, 5 mg/kg, 10 mg/kg or its vehicle. Two, 24 and 48 hours after the end of the treatment, the animals will be sacrificed in order to collect the brain areas of interest: Hippocampus (Hippo), Nucleus Accumbens (NAc), and Amygdala (Amy). These brain areas undergo dramatic changes during adolescence and are mainly affected by Cannabis consumption.

Western blot assays will be performed in order to evaluate some of the histone modifications that are involved in the development of psychiatric disorders. Specifically, we will investigate histone modifications associated with both transcriptional repression (H3K9 di- and tri-methylation, H3K27 trimethylation) and activation (H3K9 and H3K14 acetylation). Moreover, to investigate the existence of age-specificity of THC effects, the study will be performed after both adolescent and adult exposure. By means of this approach, we will be able to complete the picture of the possible histone modifications induced by THC exposure during adolescence and adulthood in female rats.

Recent literature reported that Cannabis consumption is able to impair behaviour in a sex-dependent manner. Indeed, women mainly develop depression/anxiety, whereas men mainly suffer from attention-deficit hyperactivity and anti-social personality disorders. Accordingly, our research group was able to reproduce age- and sex-dependent effect of THC in animal models. Specifically, chronic THC exposure during adolescence causes long-term disturbances of cognitive performances and emotional reactivity in adult female rats. In contrast, adolescent male rats exposed to THC developed a psychotic-like phenotype at adulthood, characterized by anhedonia, cognitive deficits and phencyclidine sensitization.

Based on these results, the second aim of this thesis is to extend our knowledge on the impact of adolescent THC exposure on histone modifications occurring in different brain areas of male rats.

To this aim, adolescent (PND 35-45) male Sprague-Dawley rats will be treated with the same protocol previously described. Two, 24 and 48 hours after the end of the treatment, the animals will be sacrificed in order to collect the brain areas of interest: Prefrontal cortex (PFC), Hippocampus (Hippo), and Nucleus Accumbens (NAc). Western blot assays and histone markers evaluation will be performed with the same protocol used for females.

Data obtained will contribute to better understand part of the molecular events that make the adolescent brain more sensitive to the adverse effects of Cannabis and thus contribute to the development of psychiatric disorders.

MATERIALS AND METHODS

ANIMALS

The experiments have been conducted on adolescent female and male (PND 35-45) and adult female (PND 75-85) Sprague-Dawley rats (Charles River, Calco, Italy) with an average weight of 90-110 grams (PND 28) and 200-220 grams (PND 70) at the beginning of the treatment. The animals were fed with pellet diet and ad libitum water, and they were housed in groups of five animals per cage under standard conditions (22±2°C, 60±5% humidity, twelve hours of artificial daytime lighting). All the experiments were carried out in strict accordance with the guidelines for care and use of experimental animals in the European Communities Council directive (2010/63/UE L 276 20/10/2010) and approved by the Ethical Committee for Animal Research at the University of Insubria and by the Italian Ministry of Health (Aut. N.302/2015-PR). All efforts have been made to reduce the suffering and the number of animals utilized.

DRUGS

The following drug was used to carry out the experiments of this thesis: THC. THC was generously offered by GW Pharmaceuticals (Salisbury, UK). THC was dissolved in ethanol, Kolliphor and saline (1:1:18).

TREATMENTS

After a 7-day relapse period, the rats were randomized into two experimental groups: the first group was treated with the natural cannabinoid agonist Δ9-THC, and the second one with its vehicle. For adolescent animals, chronic treatment began 35 days after birth (PND 35) and ended 10 days after (PND 45). Rats were treated twice a day with increasing intraperitoneal (ip) doses of THC: 2.5 mg/kg (PND 35-37), 5 mg/kg (PND 38-41), 10 mg/kg (PND 42-45) or with its respective vehicle. The same scheme of treatment was performed on adult animals starting 75 days after birth (PND 75). According to the transformation in human equivalent dose proposed by FDA and the average content of THC in a joint, this

protocol mimics a heavy Cannabis abuse. Indeed, the first dose approximately corresponds to one joint containing 7% of THC, the second one to two joints, and the higher one to four joints. Currently, existing strains of Cannabis have reached a content of THC up to 14%. In this case, our treatment would mimic the consumption of half a joint

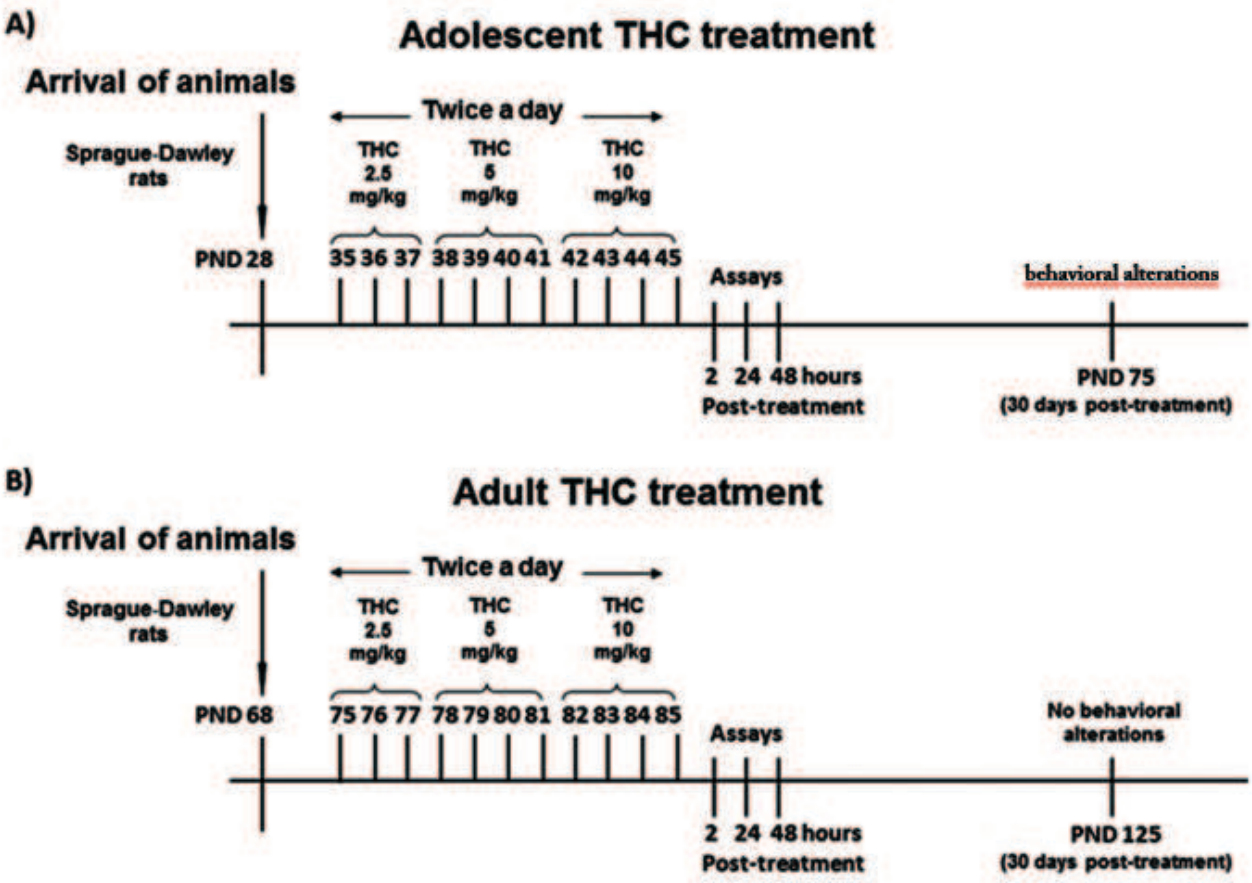

Figure 5. Adolescent (A) and adult (B) THC treatment schedule.

BIOCHEMICAL ASSAYS

Animals were sacrificed and their brains collected at 2, 24 and 48 hours after the last THC administration. For biochemical assays, the brains were quickly removed and brain areas of interest (Hippo, NAc, and Amy) were obtained by regional dissection on ice through an aluminium block by following the Heffner method (Rodent Brain Matrix, ASI instrument, Warren, MI USA) which allows to obtain brain sections of 1 mm thick. In the section of

interest, identified with the aid of Paxinos and Watson (1986) atlas, the area was collected from both the right and the left hemispheres. The obtained cerebral areas were immediately frozen in liquid nitrogen and stored at -80°C until processing.

HISTONE EXTRACTION

The brain areas of interest were homogenized by using a glass/Teflon potter in ice-cold buffer (50 mM Tris-HCl pH 7.5, 1 mM EDTA, 5 mM MgCl2, NaCl 50 mM, 5% Glycerol, 1% Triton, 2 mM DTT, 2 mM PMSF, 2µg/ml Aprotinin; 2µg/ml Leupeptin, 50 mM NaF) and centrifuged at 13.000 rpm at 4 °C for 5 min. The pellet was resuspended in nuclear lysis buffer (Hepes 20 mM pH 8, MgCl2 1.5 mM, NaCl 420 mM, DTT 2 mM, PMSF 2 mM, EDTA 0.2 mM, NaF 50 mM, 25% Glycerol, aprotinin 10 µg/ml, and leupeptin 10 µg/ml) and incubated on ice for 30 min with mild agitation. The samples were centrifuged at 13000 rpm for 10 minutes at 4°C. The pellet was resuspended in 0.2 M HCl, incubated on ice for 30 minutes with mild agitation and then overnight at 4°C to acid extract histones. The samples were then centrifuged at 10000 rpm for 10 minutes at 4°C. The supernatant containing the histone proteins was mixed with 6 volumes of cold acetone and incubated at -20°C overnight to precipitate histones. The following day, the samples were centrifuged at 4000 rpm at 4°C for 10 minutes and the pellets were resuspended in RIPA Buffer (Tris-HCl 50 mM pH 7.5, EDTA 2 mM, NaCl 150 mM, Triton 1%, PMSF 2 mM, Aprotinin 5µg/ml; Leupeptin 5µg/ml). The protein concentrations were determined according to the Micro-BCA assay kit (Pierce, Rockford, IL).

PROTEIN ASSAY THROUGH BCA METHOD

The assay is a detergent-compatible formulation based on bicinchoninic acid (BCA) used for colorimetric determination and quantification of total proteins. This method combines the copper reduction (Cu2+ → Cu1+) mediated by the protein into an alkaline medium (biuret reaction) with highly sensitive and selective colorimetric determination of Cu+1 cation using a single BCA containing reagent. The colorimetric reaction is due to

the chelation of two BCA molecules with a copper ion. This water-soluble complex exhibits a strong absorbance at 570nm, which is linear with the increase in protein concentration within a broad spectrum ranging from 20 μg/ml to 2000 μg/ml. In this assay, a protein set with known concentration was prepared by diluting the stock of standard BSA (Bovine Serum Albumin, 2 mg/ml; Pierce). The concentrations used to dose the samples should fall into the standard protein set, appropriately chosen in relation to the concentrations of the samples themselves. The appropriate concentrations of each standard and samples that need to be dosed were placed in wells of a special plate. The "Working Reagent" solution was prepared by mixing 50 parts of reagent A (Sodium carbonate, Sodium Bicarbonate, reagent for BSA detection and Sodium Tartrate in 0.2 N NaOH) with 1 part of reagent B (25 Copper sulphate at 4%). At this point, 200 μl of the Working Reagent solution was added to each well of the plate, which was finally incubated for 30 minutes at 37 °C. The absorbance of each sample was then measured at 570 nm. The linear equation from which inferring the protein concentrations of the individual samples expressed in micrograms of protein/microliter (μg/μl) of homogenated tissue was calculated by a linear regression analysis performed on the computer (Prism, Graph Pad).

SDS-PAGE

The SDS-PAGE technique is definitely the most commonly used to analyze complex protein mixtures. Proteins react with sodium dodecyl sulphate (SDS), an anionic detergent that coats proteins forming negatively charged complexes. The amount of SDS bound to a protein, and hence the total charge of the complex, is proportional to its molecular mass. SDS binds to polypeptides in a constant weight ratio of 1.4g SDS/g of polypeptide. The proteins are denatured and solubilized by binding to the SDS. At this point, the complex assumes a helicoidal form depending on the molecular weight of the protein. Thus, proteins at the specific isoelectric point, either acidic or basic, form negatively charged complexes that can be separated according to their mass differences by means of electrophoresis and by passing through a sieve composed by a polyacrylamide gel

matrix. The widespread use of this technique is due to the excellent resolution power, linked to the use of a discontinuous system that employs two gels, a "stacking gel" and a "running gel", each one having a different density. This system uses the principle of “isotachophoresis ", which effectively concentrates large volumetric samples into very small bands, thus obtaining a better separation of the different molecular species present in the mixture. The system is set up by superimposing the "stacking gel" to the “running gel”, which present different pH and polyacrylamide concentrations. Samples are loaded into wells that are located at the level of the "stacking gel", and when an electric field is applied, negative charge ions migrate to the anode (positive pole). However, protein-SDS complexes have intermediate mobility between chlorine ions (present throughout the system) and glycinate ions (present in the Running Buffer) at the pH present in the "stacking gel" (pH 6.8), whereas chlorine ions have a greater mobility. Immediately, larger ions concentrate in tight areas in the "stacking gel", but here they are not effectively separated. When complexes reach the "running gel", their respective mobility varies according to the different prevailing pH (pH 8.8) and the glycinate-formed front reaches the bands of protein-SDS complexes. This allows the separation of proteins by their mass in a uniformly buffered electric field. The system used and described by Laemmli uses the following reagents:

1) Acrylamide / bisacrylamide solution (ratio 37:1);

2) Blends of running gel at 14%: Tris buffer 8.8, 1.5 M (mother), consisting of Trizma Base, SDS and H2Od;

3) Blends of stacking gel at 4.5%: Tris Buffer 125mM (Mother 0.5M pH 6.8), Acrylamide 4.5% (Mother 40%), Ammonium Persulfate 10%, Temed;

4) SDS-PAGE 5X buffer: Glycine, 0.96M, Tris base 0.125 M SDS 0.5%.

Both Temed and ammonium persulfate reagents, lastly added to the blends, trigger the polymerization of the polyacrylamide solution. The "stacking gel" is superimposed on the "running gel" when the latter is polymerized. During the polymerization, a suitable comb that allows to obtain wells with a capacity of about 20-25µl is inserted in the stacking gel.

Once polymerized, the gels are placed into the electrophoretic chamber and the combs removed in order to form the wells in which the samples are loaded. Both the samples and the appropriate reference standards (protein markers), consisting of a known molecular weight protein mixture, are loaded into the wells. The samples run into the gel by applying a constant current of 20 mA/gel. A protein amount corresponding to 15 γ for histone samples and 30 γ for nuclear extracts was loaded in each well of the gel. The samples were appropriately prepared in Laemmli buffer 5X (Tris HCl 1M pH 6.8, SDS 10%, Bromophenol blue 0.05%, glycerol 50%, DTT 5%) and diluted with H2Od to obtain a concentration of Laemmli Buffer 1X. The so prepared samples were boiled for 3 minutes (10 minutes in the case of histone proteins), to ensure complete protein denaturation and, eventually, they were placed in ice ready to be loaded.

WESTERN BLOT

Western blot assay is based on the ability to transfer proteins, previously separated by gel electrophoresis, onto a suitable membrane by using a device called "semi-dry cell" (Trans-blot, Biorad) and by applying a constant voltage for about 15 minutes. On the surface of the device representing the anode, four sheets of Whatmann 3MM filter paper, previously soaked into glycine buffer (Trizma base 25mM, glycine 150mM, 10% methanol, pH 8.3), were placed. After being activated into the glycine buffer for at least 30 minutes, PVDF membranes were located onto the four sheets of filter paper along with the gel in which proteins had migrated according their molecular weight. The sandwich was completed by adding four other Whatmann filters. The system was then hermetically closed by placing the lid of the device representing the cathode on this pile. In this way, the “semi-dry” transfer begins. After the transfer, PVDF membranes are incubated for about two hours in a "blocking solution" (lean milk in powder Regilait 5% dissolved into TBS-Tween 20: Trizma Base 20mM, NaCl 150mM, Tween 20 0.1%, pH 7.5), in order to saturate the nonspecific binding sites. After this process, we proceeded with an overnight incubation at 4°C with the primary antibody. The primary antibodies that were used are the following: (I) monoclonal anti-Histone H3 di methyl K9 (1:1000, AbCam), (II) polyclonal

anti-Histone H3 tri methyl K9 (1:1000, AbCam), (III) polyclonal anti-Histone H3 tri methyl K27 (1:1000; Merck Millipore, Darmstadt, Germania), (IV) polyclonal anti-Histone H3 acetyl K9 (1:1000; Merck Millipore), monoclonal anti-Histone H3 acetyl K14 (1:1000; Merck Millipore), (V) polyclonal anti-Histone H3 (1:5000; AbCam). The membranes went through 5 five-minute-lasting washings in TBS-Tween 20, and 1 two-minute-lasting washing in blocking solution. Bound antibodies were detected with horseradish peroxidase linked to anti-rabbit or anti-mouse antibody (1:3000/5000; Chemicon International, Temecula, CA). We then proceeded with 5 other 5-minute washings in TBS-Tween 20 and a 2-minute washing in TBS 1X. Finally, membrane were removed from the last washing and processed for development through electrochemiluminescence (ECL) method.

DEVELOPMENT THROUGH ECL METHOD

Electrochemiluminescence is a method that allows to detect immobilized proteins present on PVDF membranes and conjugated, either directly or indirectly, with HRP antibodies. Such protein detection system consists of a chemo-luminescence reaction: the HRP, linked to the secondary antibody, oxidizes a peracid salt present in the solutions for the ECL, causing HRP oxidation and catalyzing the oxidation of luminol. This, in turn, causes the passage of the electrons to an excited state followed by a decay to baseline levels, which determines the emission of light at a wavelength of 428 nm. G:BOX iChemi-XT16 Imaging System (Syngene, Cambridge, UK) was used for image acquisition. The relevant immunoreactive bands were quantified through scanning densitometry using ImageJ software. Expression of the proteins was normalized to total histone H3 for histone samples. This technique allows to point out whether the protein differences obtained are real or due to experimental errors; indeed, after performing the stripping, the PVDF membranes are incubated with a primary antibody recognizing total histone H3. This protein is encoded by a "housekeeping gene" that allows to quantify the total protein concentration of the loaded sample.

STRIPPING

By means of this method, it is possible to remove the protein-antibody binding and re-use the same membrane with a new antibody. The procedure presents the following steps:

- Washing of the membrane with the stripping solution maintained at 37°C for 15 minutes;
- 3 five-minute-lasting washings in TBS Tween;
- Possibly, repeat the procedure a second time;
- Incubation of the membrane in blocking solution for about 2/3 hours;
- Incubation with primary antibody.

STATISTICAL ANALYSIS

Statistical analysis is performed by using Prism 4.0 version (Graph Pad software, inc., San Diego, CA, USA). Data are presented as mean ± SEM of at least five animals per group. Statistical analyses were performed by using Student's t-test in order to compare two groups of data.

Data on THC- treated animals were further analyzed by two-way MANOVA (using Wilks's Λ statistics). In particular, fold change values were log transformed and then analyzed by two-way MANOVA, one for each time point (2, 24, 48 hours), considering the brain area (hippocampus, nucleus accumbens and amygdala) and the status (adolescent and adult) as independent variables. Results were confirmed by two-way ANOVA (one for each time point/histone modification pair), followed by the Tukey post-hoc test with the Benjamini-Hochberg correction of p values, in order to highlight significant contrasts.

RESULTS

ANALYSIS OF THC-INDUCED HISTONE MODIFICATIONS IN DIFFERENT BRAIN AREAS OF FEMALE RATS

We and others have demonstrated that adolescent exposure to THC, or to synthetic cannabinoids, induces sex-dependent brain and behavioural alterations at adulthood (Rubino et al., 2008, 2009, 2015; Realini et al., 2011; Zamberletti et al., 2014, 2015, 2016; Higuera-Matas et al., 2008, 2012, Biscaia et al., 2003; Schneider and Koch, 2003). In female rats, the phenotype was more complex, as both depressive-like and psychotic-like signs were present, thus, firstly, we decided to further our understanding of adolescent cannabis exposure in females. We hypothesized that cannabis abuse during adolescence could impair the brain network functionality acting through a mechanism involving histone modifications, thus leading to long-term behavioural impairments. Accordingly, we demonstrated in the PFC that THC alters histone modifications, mainly methylation of H3K9, and the expression of a subset of plasticity genes relevant for the development of cognitive deficits present in the adult phenotype (Prini et al., 2018). Interestingly, the alterations induced by THC exposure were age-specific. Indeed, not only the behavioural phenotype developed after adolescent, and not adult, exposure (Realini et al., 2011), but also changes in both histone modifications and gene expression were more widespread and intense after adolescent treatment (Prini et al., 2018].

On these bases, the first aim of this thesis was to extend our knowledge of the impact of THC exposure on histone modifications occurring in other brain areas that, together with the PFC, are important for the different aspects of the depressive/psychotic-like phenotype described in these animals (Rubino et al., 2008, 2009, 2015; Realini et al., 2011; Zamberletti et al., 2015).

To this aim, we investigated histone modifications associated with both transcriptional repression (H3K9 di- and tri-methylation, H3K27 trimethylation) and activation (H3K9 and H3K14 acetylation), since they have already been reported to be modulated by drug treatment (Peña et al., 2014). These analyses were performed 2, 24, and 48 h after the

last THC injection in the Hippocampus (Hippo), Nucleus accumbens (NAc), and Amygdala (Amy), brain areas which undergo dramatic changes during adolescence and which are mainly affected by drug consumption (Koob and Vilkow, 2016; Sharma and Morrow, 2016). To investigate the existence of age-specificity of THC effects, the study was performed after both adolescent and adult exposure, which consisted of increasing doses of THC for 11 days, as reported in Figure 5.

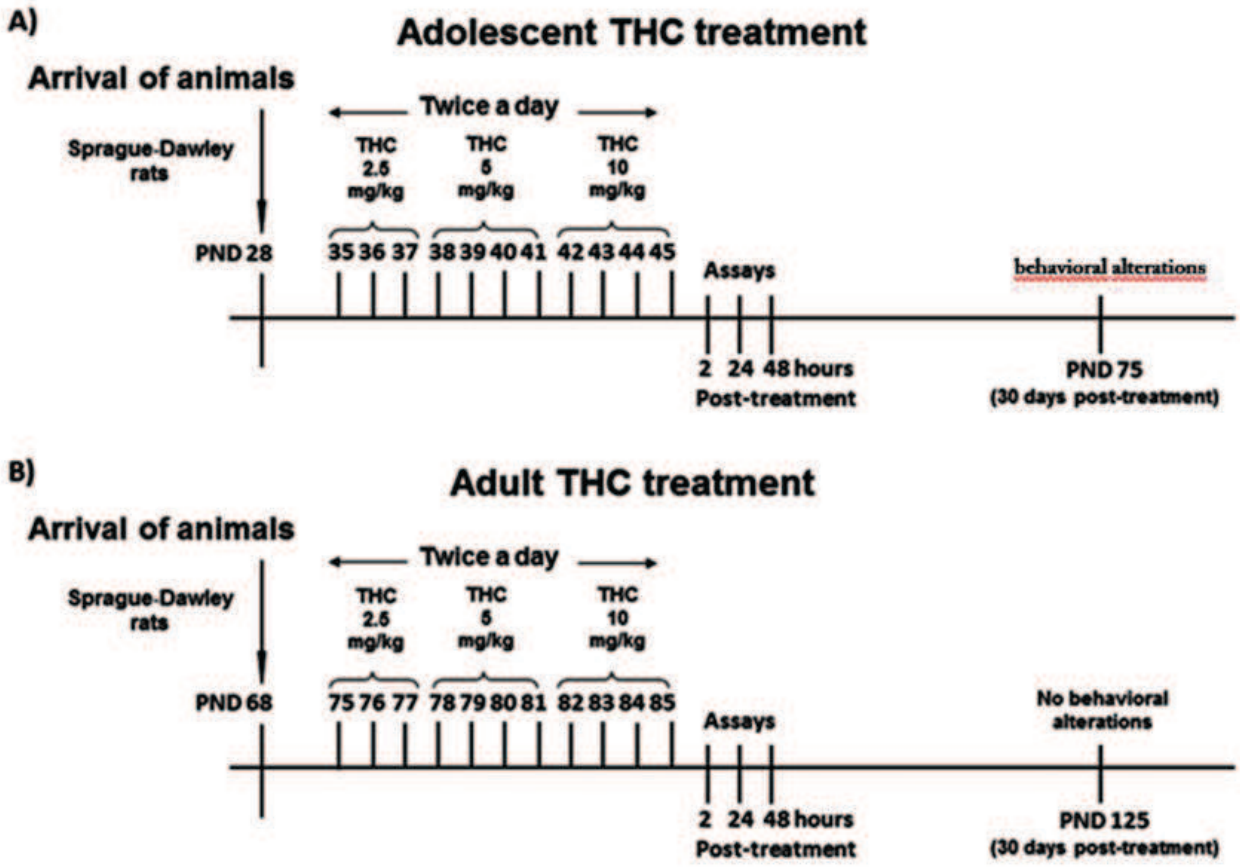

Figure 5. Adolescent (A) and adult (B) THC treatment schedule.

EFFECT OF ADOLESCENT THC EXPOSURE ON HISTONE MODIFICATIONS

In the Hippocampus of female rats (Fig.6), H3K14ac levels were significantly reduced (34%) 2 hours after the end of THC treatment. On the contrary, a significant increase (57%) in H3K14ac levels was observed 24 hours later. At this same interval of time, an enhancement of H3K9me2 (36%) and H3K9me3 (30%) was also observed. Finally, 48 hours after the end of the treatment, all these alterations returned to control values.

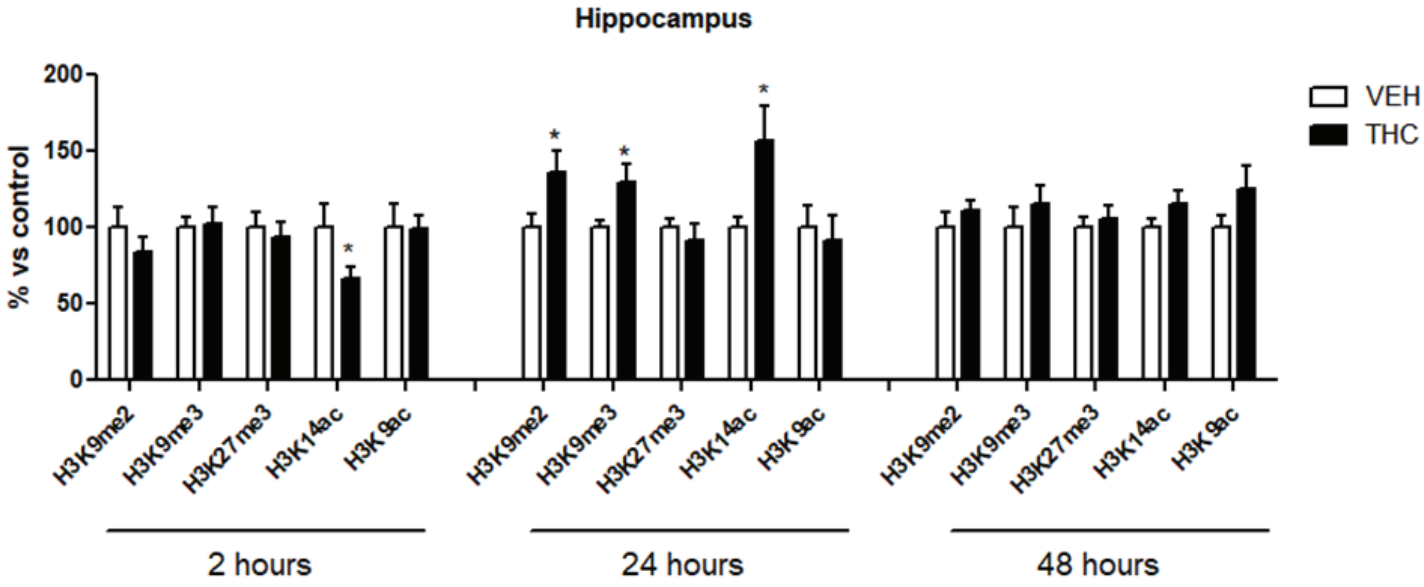

Figure 6. Effect of adolescent THC exposure on histone modifications occurring in the hippocampus of female rats, 2, 24 and 48 hours after the last THC injection. Data are expressed as mean ± SEM of at least eight animals for each experimental group. *p<0.05 versus controls (Student's t-test analysis).

Concerning the Nucleus Accumbens (Fig. 7), H3K9me3 was significantly increased (50%) 2 hours after the end of THC treatment. This enhancement was maintained (43%) 24 hours later, and it was paralleled by a significant increase in H3K9me2 (42%) and H3K14ac (26%) levels. On the contrary, at the later time-point studied (48h), H3K9me3 levels, as well as H3K9me2 and H3K14ac levels were significantly reduced (16%, 23%, and 28%, respectively).

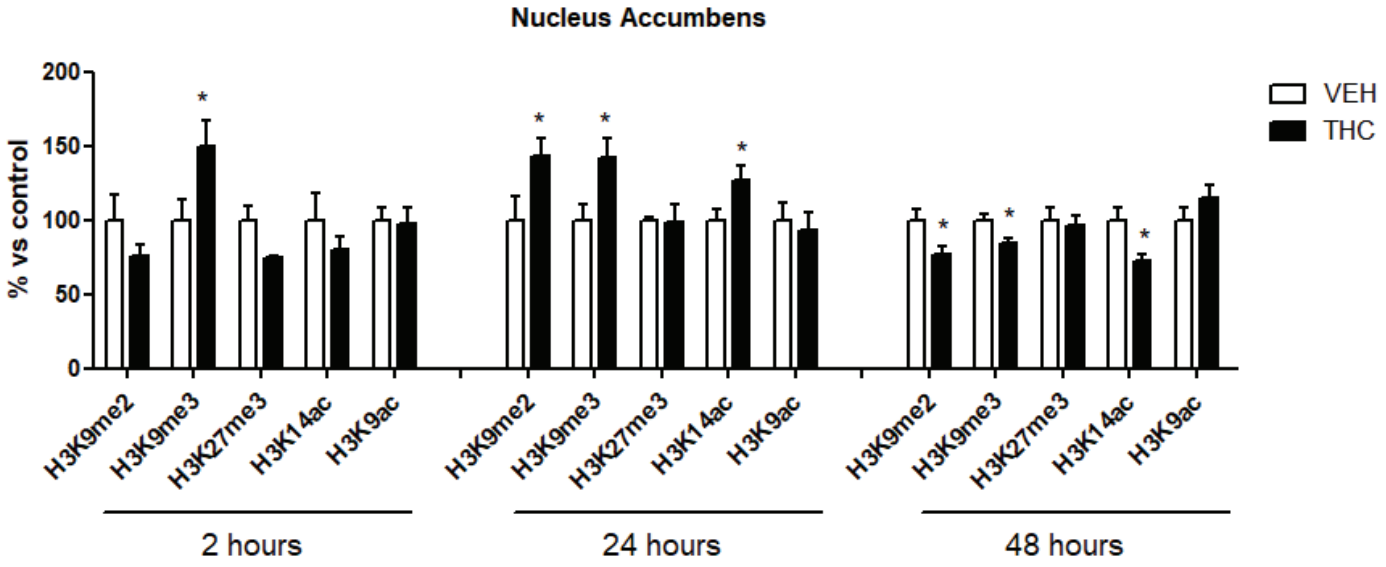

Figure 7. Effect of adolescent THC exposure on histone modifications occurring in the Nucleus Accumbens of female rats, 2, 24 and 48 hours after the last THC injection. Data are expressed as mean ± SEM of at least eight animals for each experimental group. *p<0.05 versus controls (Student's t-test analysis)

Regarding the amygdala (Fig.8), adolescent THC administration induced a significant increase in H3K9me2 (33%) levels 2 hours after the end of the treatment. Twenty-four hours later, while this alteration returned to control values, H3K9me3 levels were significantly enhanced (33%). Finally, 48 hours after the last THC administration, no significant changes were observe

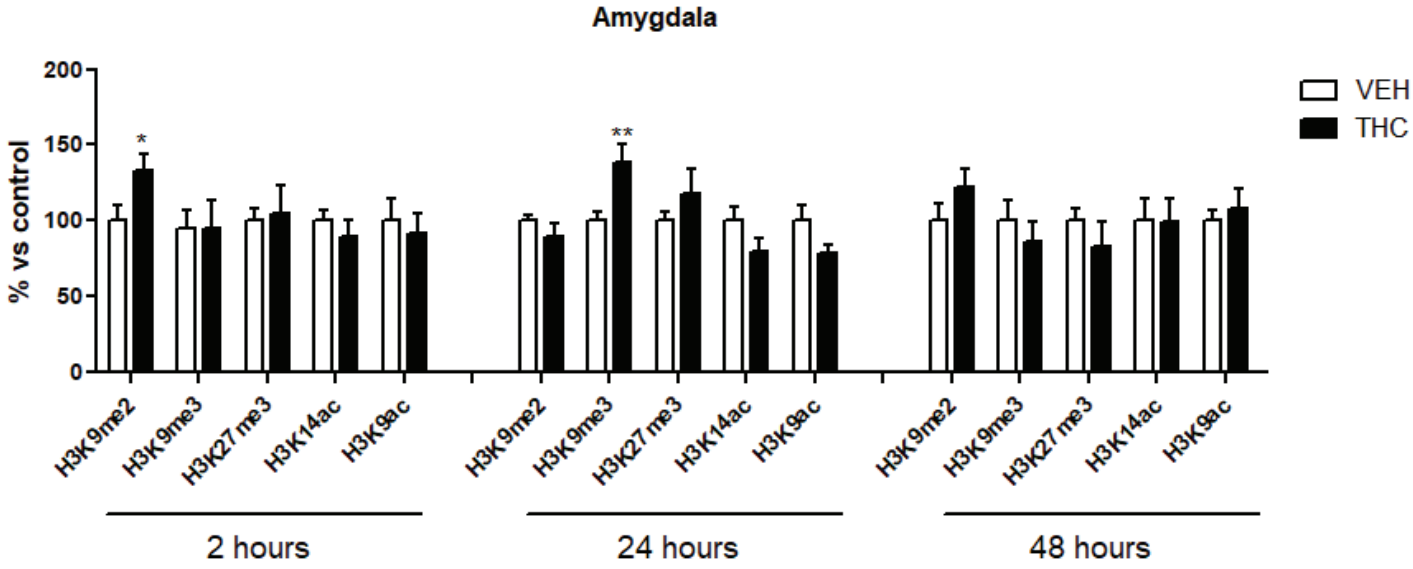

Figure 8. Effect of adolescent THC exposure on histone modifications occurring in the amygdala of female rats, 2, 24 and 48 hours after the last THC injection. Data are expressed as mean ± SEM of at least eight animals for each experimental group. *p<0.05,**p<0.01 versus controls (Student's t-test analysis).

As a whole, these data suggest that THC effect on histone modifications is strictly dependent on the brain region under consideration.

EFFECT OF ADULT THC EXPOSURE ON HISTONE MODIFICATIONS

Since the development of the depressive/psychotic-like phenotype in female rats is restricted to adolescent THC exposure, we decided to investigate whether also THC-induced histone modifications are restricted to the adolescent treatment. This would support their involvement in the development of the altered phenotype. To this aim, we performed the same treatment and the same time-course study of histone modifications in adult female rats exposed to THC. Briefly, female rats were injected with increasing doses of THC from 75 to 85 PND and 2, 24 and 48 hours after the last injection, the chosen histone modifications were analysed.

In the hippocampus of adult animals (Fig.9), H3K14ac was significantly increased (67%) 2 hours after the end of the treatment. This was the only alteration induced by adult THC treatment in this cerebral area, as 24 and 48 hours after the last THC injection, no significant changes were observed.

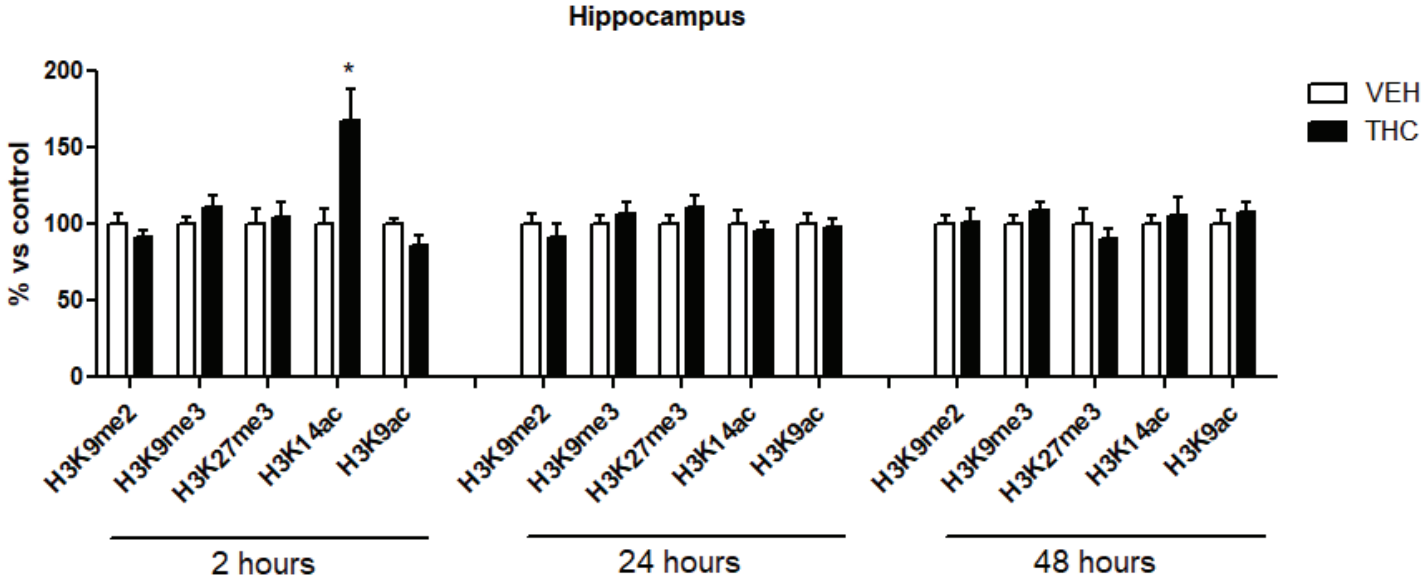

Figure 9. Effect of adult THC exposure on histone modifications occurring in the hippocampus of female rats, 2, 24 and 48 hours after the last THC injection. Data are expressed as mean ± SEM of at least five animals for each experimental group. *p<0.05 versus controls (Student's t-test analysis).

In the Nucleus Accumbens (Fig.10), chronic THC administration did not induce significant alterations of histone markers 2 and 48 hours after the end of the treatment. However, 24 hours after the last THC injection a significant increase in H3K14ac (34%) levels was observed.

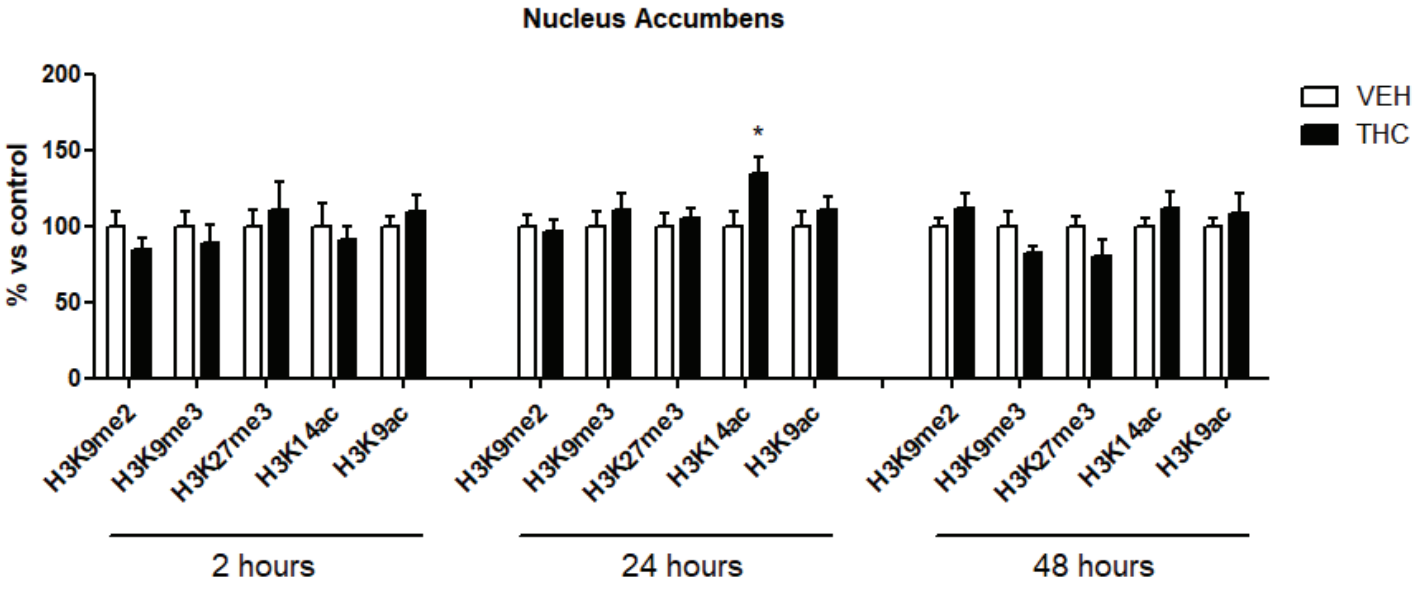

Figure 10. Effect of adult THC exposure on histone modifications occurring in the Nucleus Accumbens of female rats, 2, 24 and 48 hours after the last THC injection. Data are expressed as mean ± SEM of at least five animals for each experimental group. *p<0.05 versus controls (Student's t-test analysis)

Concerning the Amygdala (Fig.11), both H3K9me2 and H3K27me3 levels were significantly decreased 2 hours after the end of THC treatment(31% and 36%, respectively), and then returned to control 24 hours later. At this same interval of time, H3K9ac was significantly reduced (39%). Lastly, 48 hours after the last THC injection, H3K14ac levels were significantly decreased (28%).

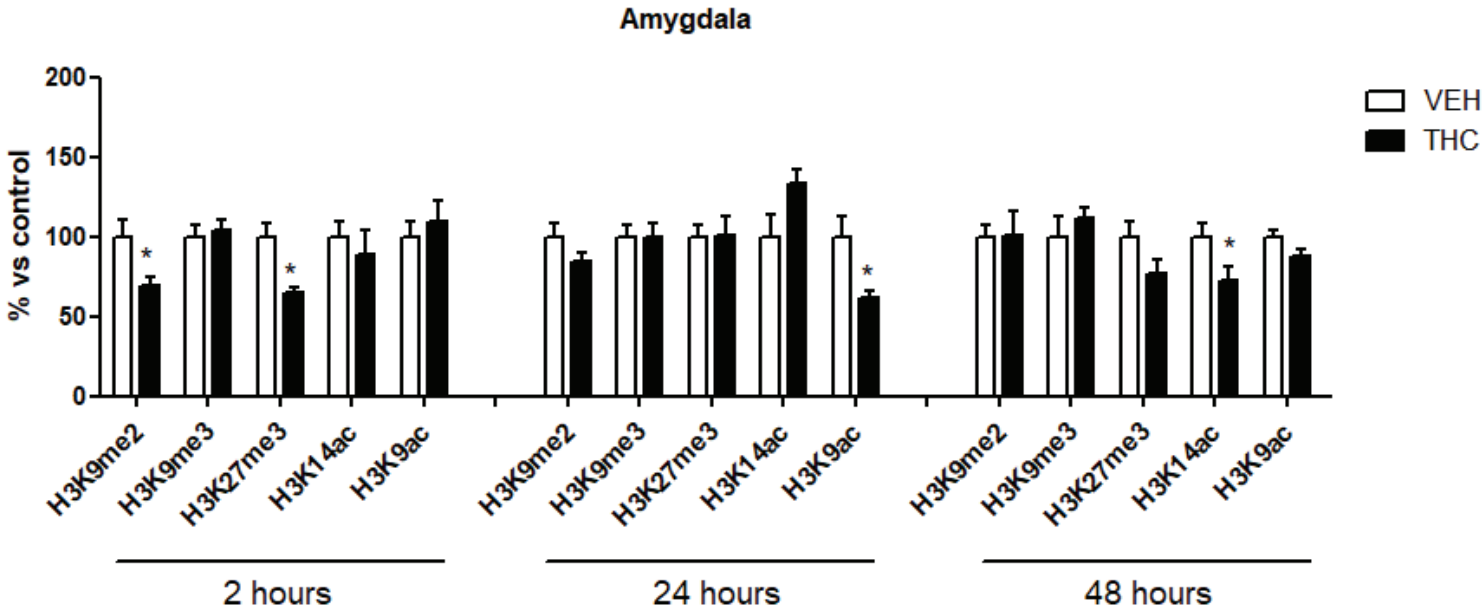

Figure 11. Effect of adult THC exposure on histone modifications occurring in the Amygdala of female rats, 2, 24 and 48 hours after the last THC injection. Data are expressed as mean ± SEM of at least five animals for each experimental group. *p<0.05 versus controls (Student's t-test analysis).

To sum up, these data suggest that adult THC exposure induced changes that are restricted to just one histone modification occurring within 24 h after the cessation of the treatment. The only exception is represented by the amygdala, where changes were present in the entire monitored time window.

A GLOBAL VIEW ON THE EFFECTS OF THC EXPOSURE ON HISTONE MODIFICATIONS

In order to consider as a whole all the data obtained from adolescent and adult rats exposed to THC, we analyzed results in treated animals at each time point (i.e., 2, 24 and 48 hours) by two-way multivariate analysis of variance (MANOVA), considering as independent variables the different brain areas and the age (adolescent/adult). Analysis of variance (ANOVA) and the Tukey post-hoc test were used to confirm the effects obtained via the MANOVAs and highlight significant (and relevant) contrasts. In almost all cases, the ANOVA confirmed the findings of the MANOVA. We decided to evidence

relevant differences shown by the Tukey post-hoc test in Figures 12–16. In particular, they highlight a different response between age groups in the same brain area, or among brain areas in the same age group.

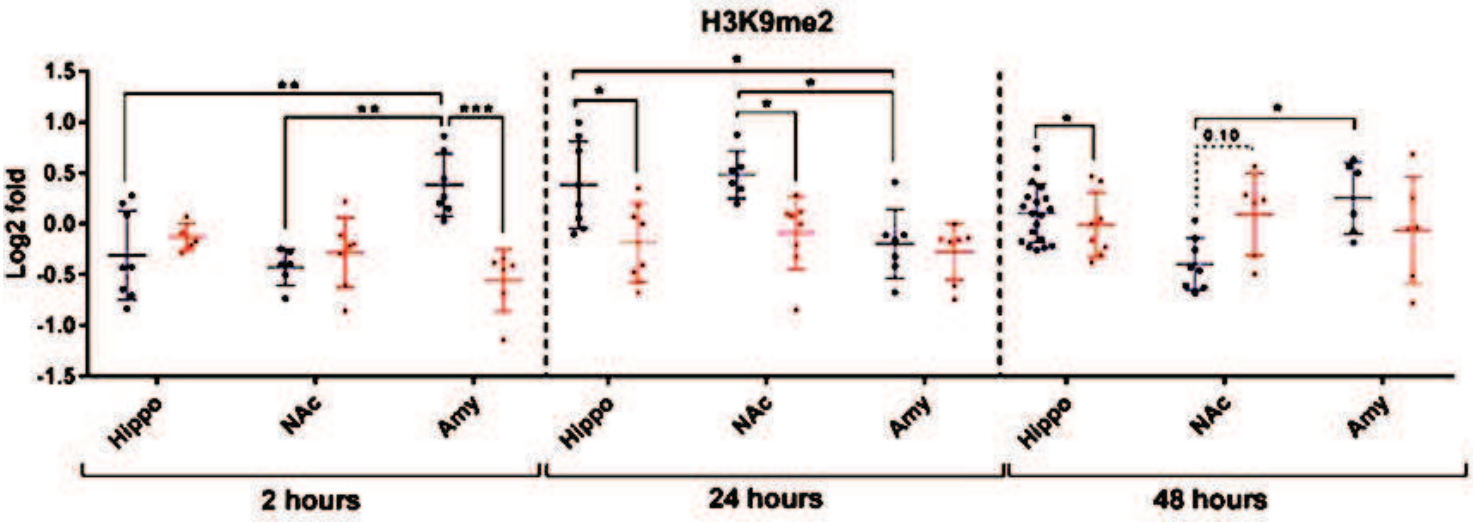

Figure 12. Comparison of THC effect on H3K9me2 in adolescent (THC ado in black) and adult (THC adu in red) animals in the different brain areas. Data are reported as log2 fold. Mean ± SD are shown. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (Tukey post-hoc test)

Regarding H3K9me2 (Figure 12), the multivariate analysis revealed that: (i) age ($f = 5.05$; p = 0.035) and the interaction between the age and the brain area ($f = 7.4$; p = 0.0035) had significant main effects 2 h after the end of the treatment; (ii) age ($f = 8.2$; p = 0.0080) and the brain area ($f = 6.8$; p = 0.0040) had significant effects at 24 h; and (iii) the interaction between age and the brain area ($f = 3.9$; p = 0.031) had significant effects 48 h after the last injection.

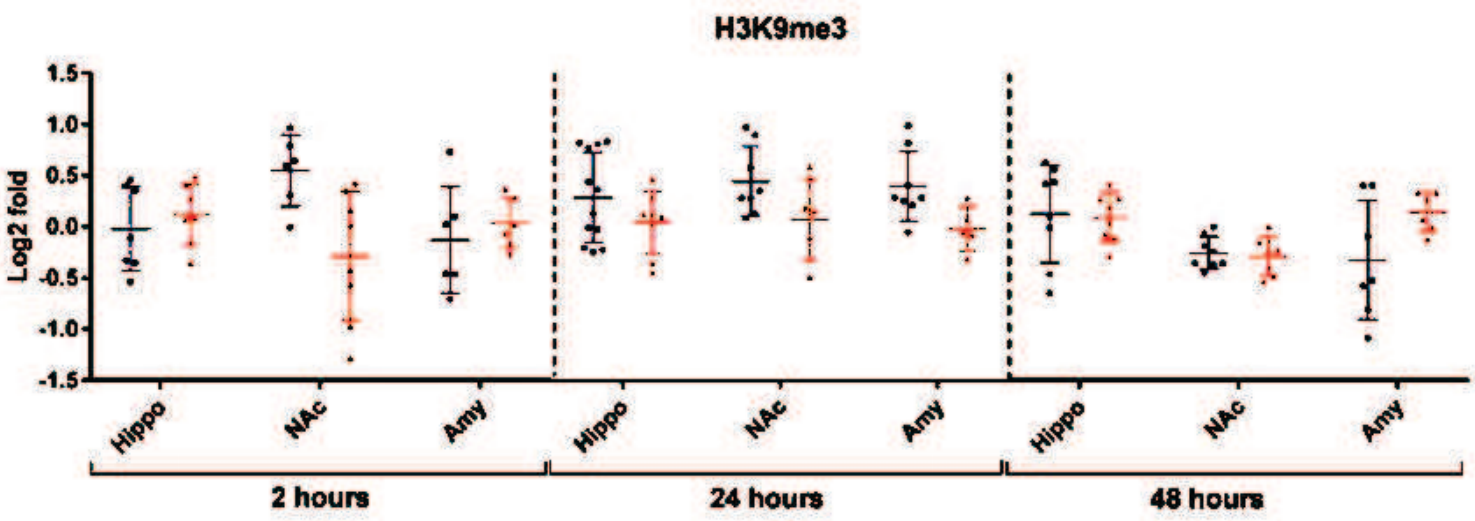

Figure 13. Comparison of THC effect on H3K9me3 in adolescent (THC ado in black) and adult (THC adu in red) animals in the different brain areas. Data are reported as log2 fold. Mean ± SD are shown. Hippo, hippocampus; NAc, nucleus accumbens; Amy, amygdala.

Regarding H3K9me3 (Fig. 13), the multivariate analysis revealed that age (*f*=9.5; p=0.0047) had significant effects at 24 hours after the end of the treatment.

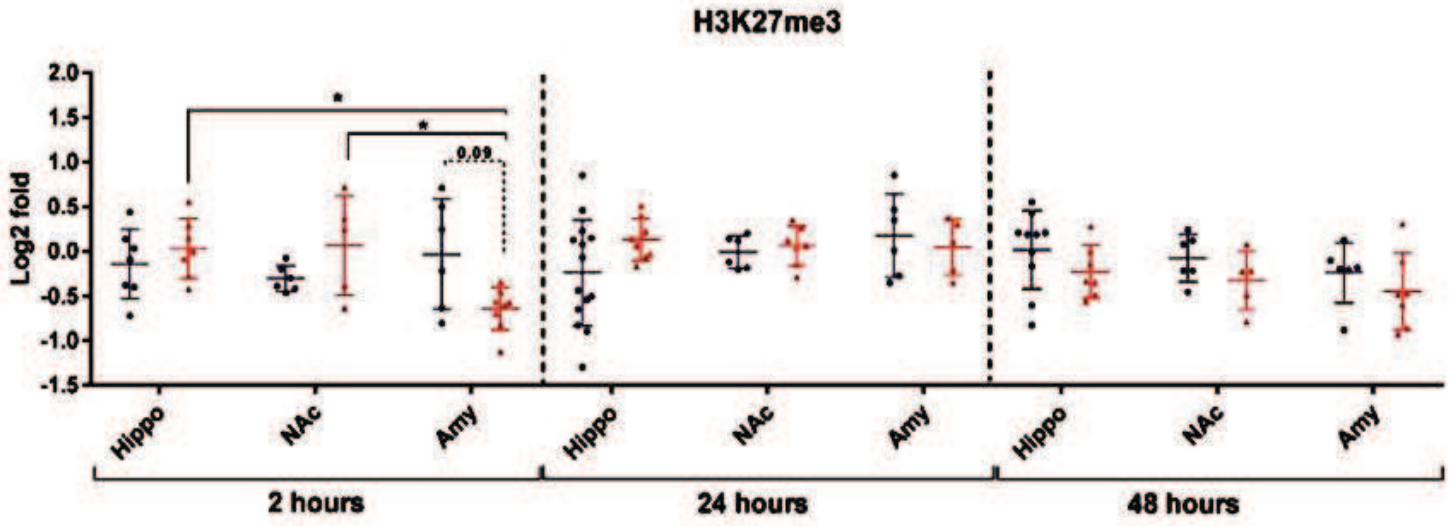

Figure 14. Comparison of THC effect on H3K27me3 in adolescent (THC ado in black) and adult (THC adu in red) animals in the different brain areas. Data are reported as log2 fold. Mean ± SD are shown. * $p < 0.05$ (Tukey post-hoc test)

Regarding H3K27me3 (Fig. 14), the multivariate analysis revealed that brain area (*f*=4.03; p=0.032) had significant main effects 2 hours after the end of the treatment.

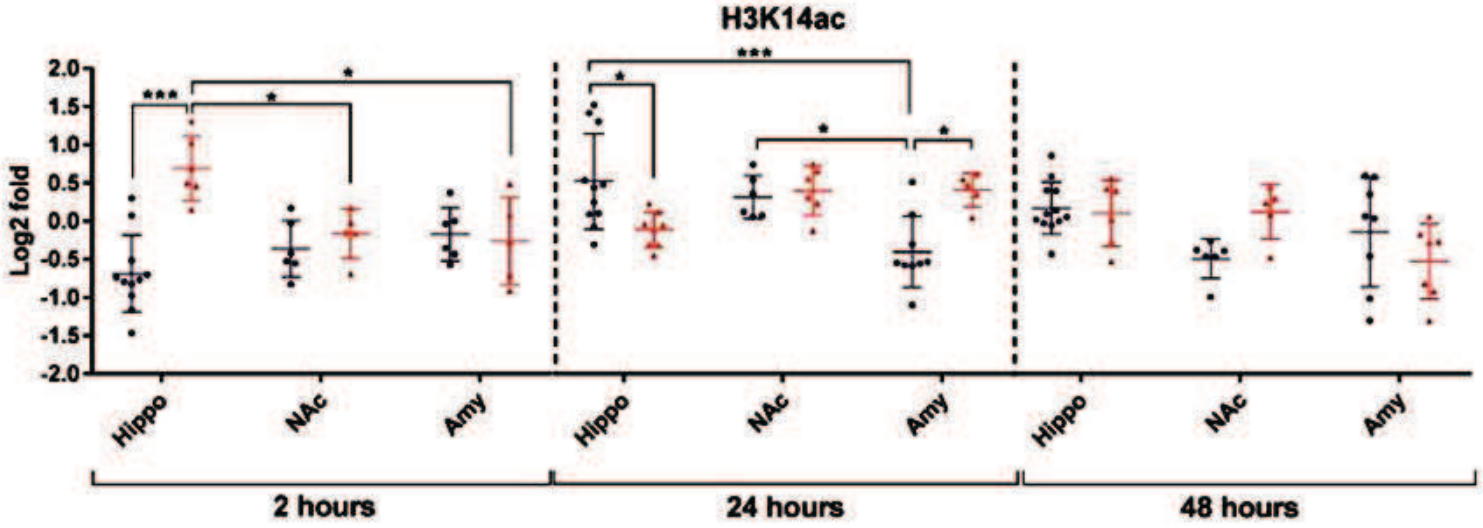

Figure 15. Comparison of THC effect on H3K14ac in adolescent (THC ado in black) and adult (THC adu in red) animals in the different brain areas. Data are reported as log2 fold. Mean ± SD are shown. * $p < 0.05$, *** $p < 0.001$ (Tukey post-hoc test).

Regarding H3K14ac (Fig. 15), the multivariate analysis revealed that: i) age (f= 10.6; p=0.0036) and the interaction between the age and the brain area (f=5.8; p=0.0093) had significant main effects 2 hours after the end of the treatment; ii) the interaction between the age and the brain area (f=6.1; p=0.0063) had significant main effects at 24 hours.

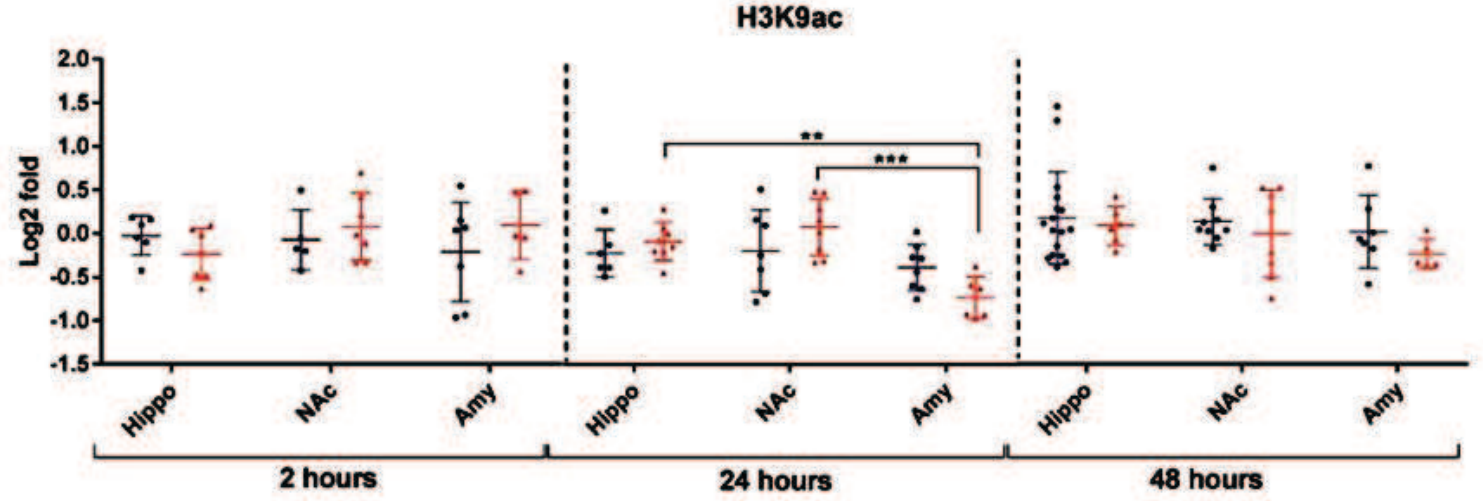

Figure 16. Comparison of THC effect on H3K9ac in adolescent (THC ado in black) and adult (THC adu in red) animals in the different brain areas. Data are reported as log2 fold. Mean ± SD are shown. ** $p < 0.01$, *** $p < 0.001$ (Tukey post-hoc test)

Regarding H3K9ac (Fig. 16), the multivariate analysis revealed that the brain area (f=8.1; p=0.0018) had significant main effects 24 hours after the end of the treatment.

ANALYSIS OF THC-INDUCED HISTONE MODIFICATIONS IN DIFFERENT BRAIN AREAS OF MALE RATS

In the last years, the gender gap in Cannabis use, usually characterized by greater prevalence in men, is narrowing, highlighting the importance of understanding sex and gender differences. Interestingly, abused drugs, Cannabis included, impairs behaviour in a sex-dependent manner: women develop depression/anxiety, whereas men suffer from attention-deficit hyperactivity and anti-social personality disorders. In line with this, we have demonstrated that adolescent THC exposure induces a depressive-psychotic-like phenotype in adult female rats, and a psychotic-like phenotype in males. Thus, the second aim of this thesis is to evaluate whether THC-induced histone modification changes are different in male and female animals. If this is the case, they could be part of the molecular underpinnings leading to the development of the sex-dependent phenotype.

To this aim, we performed the same time-course study of histone modifications in adolescent male rats exposed to THC.

In the Prefrontal Cortex of adolescent male rats (Fig.17), THC exposure did not induce any significant change in the evaluated histone markers 2, 24 and 48 hours after the last THC injection.

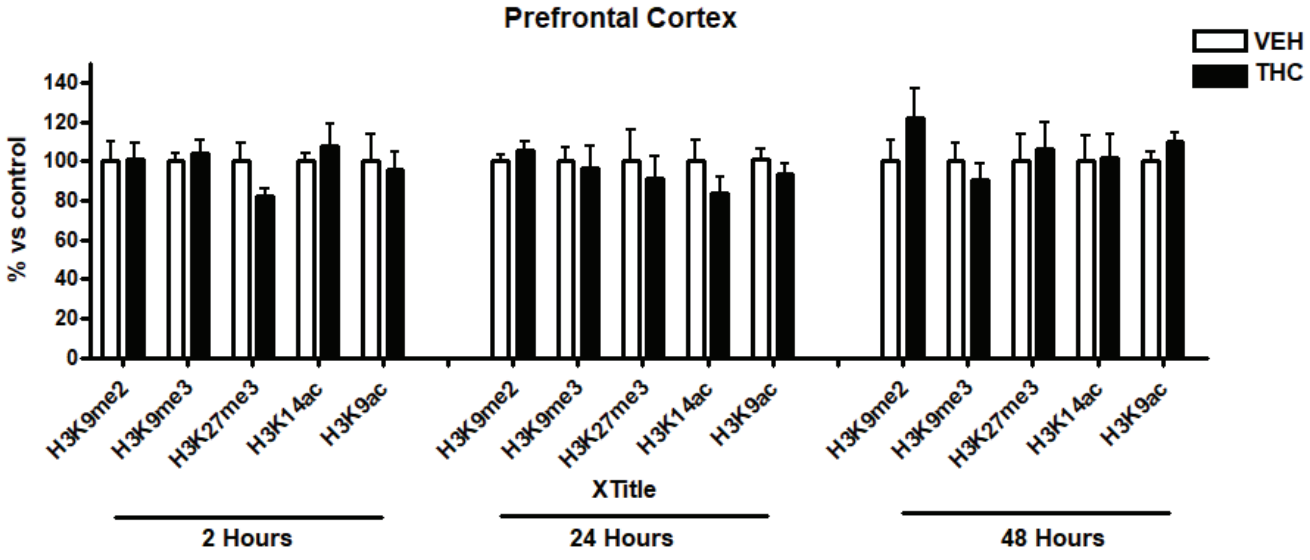

Figure 17. Effect of adolescent THC exposure on histone modifications occurring in the Prefrontal Cortex of male rats, 2, 24 and 48 hours after the last THC injection. Data are expressed as mean ± SEM of at least eight animals for each experimental group.

Similarly, in the Hippocampus (Fig.18), adolescent THC exposure did not alter histone modifications at any interval of time.

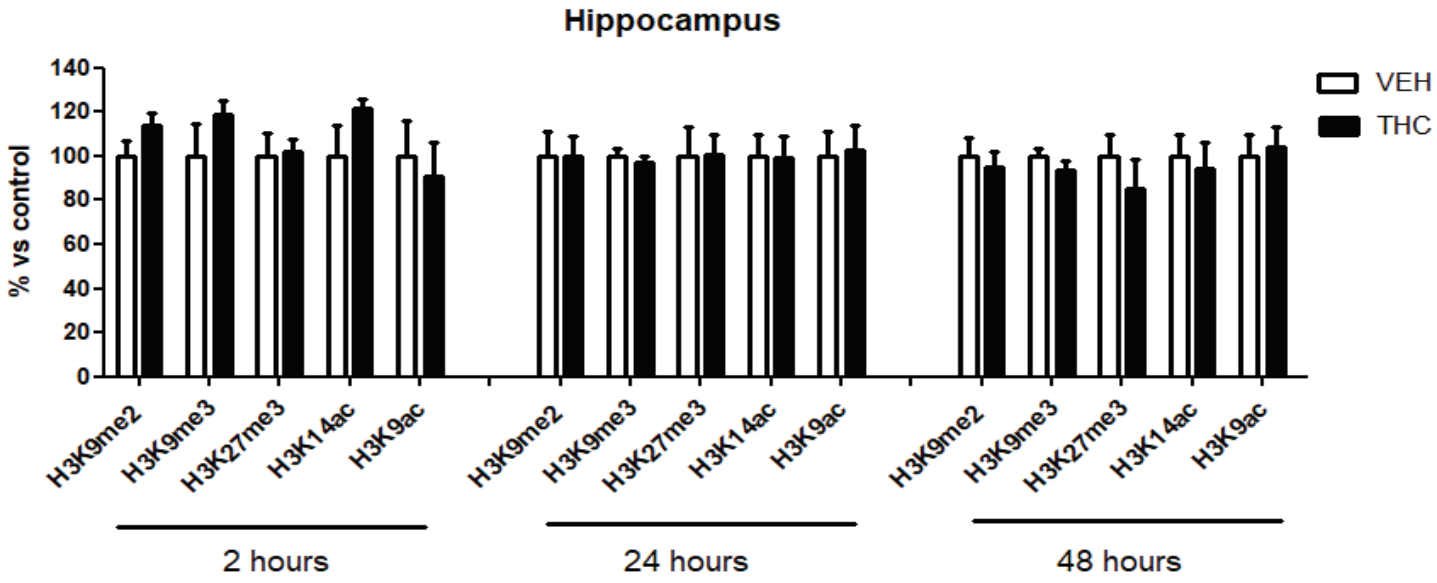

Figure 18. Effect of adolescent THC exposure on histone modifications occurring in the Hippocampus of male rats, 2, 24 and 48 hours after the last THC injection. Data are expressed as mean ± SEM of at least eight animals for each experimental group.

In the Nucleus Accumbens (Fig.19), H3K9me3 was significantly decreased (18%) 2 hours after the end of the treatment. On the contrary, this histone modification significantly increased over control levels (18%) 24 hours later. No changes were observed at the last time point analysed.

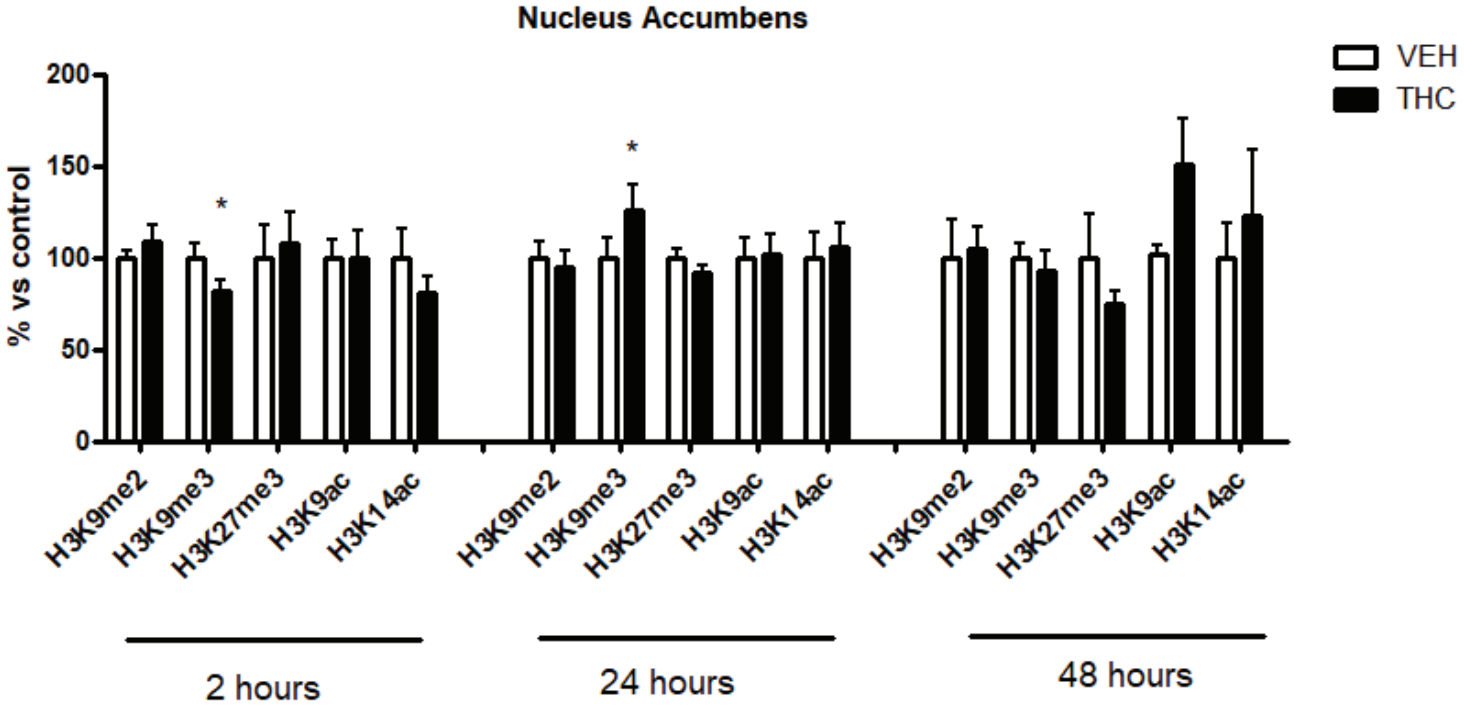

Figure 19. Effect of adolescent THC exposure on histone modifications occurring in the Nucleus Accumbens of male rats, 2, 24 and 48 hours after the last THC injection. Data are expressed as mean ± SEM of at least eight animals for each experimental group. *p<0.05 versus controls (Student's t-test analysis).

To summarize, these results suggest that adolescent THC exposure in male rats induces histone modification changes only in the Nucleus Accumbens, thus suggesting a sex-dependent effect on histone markers.

DISCUSSION

There is an increasingly widespread diffusion of Cannabis abuse among adolescents, and the political debate concerning Cannabis legalisation is emphasizing the need to understand the relationship between Cannabis exposure and the development of psychiatric disorders later in life. Although many epidemiological studies seem to confirm this association, the molecular mechanisms underlying the risk to develop psychiatric illness after adolescent THC exposure are still unclear.

Since epigenetics plays a crucial role in the etiopathogenesis of psychiatric disorders, we wanted to investigate whether chronic THC treatment during adolescence could alter histone modifications.

Our results show that chronic THC exposure affects histone modifications in the brain of female rats in a region- and age-specific manner. Indeed, THC acts on different targets depending on the considered brain area and, remarkably, the adolescent brain generally results more sensitive than the adult brain as evidenced by the multivariate analysis.

From a kinetic point of view, we may describe two different epigenetic effects that influence the adolescent brain. The first effect, mainly repressive, is observed immediately after the end of the treatment (2-24 hours) and likely represents a direct effect of THC exposure. The second one, which is detectable in the next temporal window (i.e. from 24 hours on), may represent a homeostatic response to counterbalance THC-induced repressive effect, since it has an opposite outcome at the transcriptional level. Specifically, the primary effect induced by adolescent THC exposure is the negative modulation of gene transcription in all the analyzed brain areas, although this is obtained through changes in different histone modifications.

In the Hippocampus, the primary effect is the reduction of H3K14ac, a marker known for promoting gene expression. This is followed by a significant increase in di- and tri-methylation of H3K9, markers known to repress gene transcription, at 24 hours. Regarding the Nucleus Accumbens and the Amygdala, the primary effect is represented by the increase of H3K9me, both H3K9me3 and H3K9me2, within 24 hours of the end of the treatment. However, in the Nucleus Accumbens, even the significant increase in

H3K14 acetylation at 24 hours could represent a primary event. Indeed, increased H3 acetylation in this brain area has been observed after chronic administration of different drugs of abuse (Malvaez et al., 2011; Wang et al., 2010; Levine et al., 2011), suggesting that maybe it could be triggered by the activation of the mesolimbic reward circuitry.

These data join the results we have recently described in the Prefrontal Cortex (Prini et al., 2018), where the same adolescent THC exposure in female rats induced an increase in H3K9 methylation. Collectively, these data suggest that H3K9me may represent a common target affected by THC in different brain areas, whereas the increased acetylation described in the Nucleus Accumbens may be due to THC activation of the mesolimbic dopaminergic rewarding circuitry.

This first wave of THC-induced histone changes drives subsequent chromatin rearrangements that are, again, area-specific. Indeed, we observed a switch from down to up-regulation of H3K14ac levels in the Hippocampus at 24 hours. All the observed histone changes were then reported to control 48 hours after the end of the treatment. In agreement with these data, several papers support the hypothesis that the Hippocampus is able to adapt quickly in response to chronic THC exposure (Hoffman et al., 2007; Robert et al., 2003). Indeed, the hippocampus has proved to be the first responding area to chronic treatment with THC through a reduction of both density and functionality of CB1 receptors, even after the first administration. In the Nucleus Accumbens, the enhancements in H3K9 methylation and H3K14 acetylation were completely reversed at 48 hours, when a significant reduction was still present. These further histone changes suggest the presence of a homeostatic response to the primary effect induced by THC in the adolescent brain, as we have previously described in the Prefrontal Cortex (Prini et al., 2018). The only exception is represented by the Amygdala, in which primary histone modifications were reported to control levels without further mechanisms of counterbalance.

An important finding of this study regards the age-dependency of THC-induced histone modifications. Indeed, adult female rats exposed to chronic THC showed a different pattern of histone alterations: whilst the primary effect in the adolescent brain was

represented by changes leading to transcriptional repression, the one observed after adult treatment leads to transcriptional activation. Accordingly, a significant increase in H3K14 acetylation was present in the Hippocampus and Nucleus Accumbens respectively 2 and 24 hours after the end of the adult treatment. Although in very different experimental conditions, Bilkei-Gorzo and colleagues have recently reported similar opposing effects of THC exposure in the hippocampus of young and mature mice (Bilkei-Gorzo et al., 2017). Indeed, they have reported increased H3 acetylation after chronic THC exposure in mature mice and a trend to decrease in the young animals, thus strengthening the hypothesis of an age-dependent effect of THC on histone modifications. Regarding the increase in Nucleus Accumbens H3K14 acetylation, this was also observed after adolescent treatment, supporting the hypothesis that it may be linked to the activation of the mesolimbic rewarding circuit (Malvaez et al., 2011; Wang et al., 2010; Levine et al., 2011). Consistently, experimental alterations in Nucleus Accumbens histone acetylation affect addiction-related behaviours such as place conditioning and locomotor responses to psychostimulants (Robison and Nestler, 2011).

Intriguingly, a more complex picture is present in the adult amygdala. The primary effect of THC exposure in the amygdala is represented by a decrease in H3K9 dimethylation and H3K27 trimethylation, which leads to transcriptional activation. On the other hand, in adolescent animals we have observed an increase in H3K9 methylation that returned to control levels within 48 hours. This difference was further validated by the MANOVA analysis, which highlighted a significant association between H3K9 di-methylation and the adolescent/adult status after 2 hours from the end of the treatment. The univariate ANOVA, followed by the Tukey post-hoc test, confirmed this result and evidenced that the significance was due to opposite variations in the amygdala. Another distinctive feature of the adult amygdala is the presence of a counterbalance event. Indeed, a second wave of changes that leads to transcriptional repression, mediated by a decrease in H3 acetylation, is present 24 and 48 hours after the end of the treatment. This suggests that the amygdala is more responsive in adult than adolescent animals. Again, these differences were evidenced by the MANOVA with a significant effect of the interaction

between the age and the brain area on H3K14ac. The univariate ANOVA confirmed the effect of the brain area and the post-hoc test highlighted the difference between adolescent amygdala and adult amygdala. Since it has been established that the amygdala is activated during exposure to aversive stimuli, functioning as a "behavioural brake" (Zald, 2003), the different response between adult and adolescent animals could represent the biological bases of the adolescent propensity for risk-taking and novelty-seeking behaviours. Remarkably, also in adolescent humans, neuroimaging studies have shown a weaker involvement of the amygdala, and a greater contribution of the Nucleus Accumbens, in response to negative and positive stimuli, compared to adults (Ernst et al., 2005). This fits well with the triadic model of neurodevelopment (Ernst et al., 2006), suggesting that motivated behaviour is mediated by the tension between reward (Nucleus Accumbens) and threat (amygdala) systems. According to this model, adolescence would be characterized by an imbalance in the tension between reward and threat systems in favour of reward. This would explain the increase in reward seeking and lower regard for negative consequences that characterize adolescent behaviour. In line with this model, our results showed a greater responsiveness of the adolescent Nucleus Accumbens and a weaker sensitivity of the amygdala to THC exposure compared to adults.

As a whole, these results suggest that in the adolescent brain THC triggers a more complex response characterized by a primary effect followed by compensatory changes, except for the amygdala. These changes may perturb adolescent brain refinement, thus underlying the behavioural alterations observed after adolescent THC (Rubino and Parolaro, 2016). The presence of compensatory events may be part of the mechanisms that make the female adolescent brain more vulnerable to THC adverse effects.

However, despite women show an increased susceptibility to developing Cannabis use disorder and comorbidities relative to men, we have to consider that men are more likely to endorse past month cannabis use and are more frequently diagnosed with Cannabis use disorder. Thus, it is important to extend our knowledge on THC-induced histone

modifications in males, to better understand whether an epigenetic mechanism may contribute to this sex-dependent THC response.

Our results show that chronic THC exposure affects histone modifications in the brain of adolescent male rats in a region-dependent manner.

Surprisingly, in the Prefrontal Cortex and Hippocampus, adolescent THC exposure did not affect the investigated histone modifications. In contrast, in the Nucleus Accumbens, we observed immediately after the end of the treatment a decrease in H3K9 trimethylation that suggests transcriptional activation. This primary event of transcriptional activation was counterbalanced 24 hours later, by a significant enhancement of H3K9 trimethylation levels. Alterations in methylation of H3K9 in the Nucleus Accumbens has been already described after chronic exposure to different drugs of abuse, such as MDMA (Caputi et al., 2016), morphine (Sun et al., 2012), cocaine (Maze et al., 2010), amphetamine and methamphetamine (Godino et al., 2015). Since all these drugs have in common the ability to activate the mesolimbic dopaminergic system, we may speculate that changes in the methylation status of H3K9 could be related to the activation of this circuit.

In general, the picture observed in male brain is completely different from that observed in females. Indeed, THC-induced changes in histone modifications were more intense and widespread in female brains.

These results are quite surprising and different hypotheses could be suggested.

First of all, we investigated only a subset of histone modifications, thus it could be possible that other histone modifications could be affected by THC exposure in adolescent males. In the same line, other brain areas could play a major role in THC effects in males. Regardless these limitations, our data further highlight the existence of a sex-dependent response to THC in adolescent animals.

Another consideration to take into account is based on results obtained by gene expression studies we performed in the prefrontal cortex of adolescent female rats. We found that alterations in histone modifications in this brain area mainly affected the expression of genes related to neuroplasticity and brain refinement processes.

Remarkably, it has been reported that brain developmental trajectories are significantly different between males and females, and synaptic refinement appears to occur earlier in females (Lenroot et al., 2007; see for review Lenroot and Giedd, 2010). We may speculate that during early-mid adolescence (the developmental window we focused on) maturational processes are mainly present in the female brain. Thus, THC treatment during this specific period might primary impair female brain.

Finally, another explanation regarding the higher intensity of THC effect on histone modifications observed in female rats could be ascribed to different pharmacokinetics in the two sexes. Indeed it has been reported that adolescent female rats exhibit pronounced metabolism of THC to the still active compound 11-OH-THC compared to their male conspecifics, particularly after repeated THC administration (Wiley et al., 2014). Thus, THC effect could be potentiated by its active metabolite in female adolescents. This fact together with the observation that adolescent female rats possess more efficient CB1 receptors (Rubino and Parolaro, 2011) suggests that they may be more vulnerable to THC effects.

These findings join few others present in literature regarding epigenetic mechanism involvement in long-term consequences of cannabinoid exposure during adolescence. As already suggested by Szutorisz and Hurd (2018), we may hypothesize that THC exposure can imprint on the epigenetic landscape of adolescent brain developmental trajectories and thus alter adult brain functionality via the dysregulation of genes that have important neurobiological functions. However, whether adolescent cannabinoid exposure may also affect the functionality of other tissues/organs is still an unexplored question. Thus, gaining knowledge on if and how epigenetic processes are disrupted by cannabinoids, for example in the germline, will be of paramount importance to understand the possibility to transmit THC effects from parent to offspring. At this regard, Szutorisz et al (2014) have demonstrated that exposure of male and female adolescent rats before mating led to behavioural and molecular abnormalities in their unexposed offspring suggesting an important role for epigenetic mechanism in the heritability of altered phenotypes.

In conclusion, our results suggest that Cannabis abuse during adolescence could impair the brain network functionality acting through a mechanism involving histone modifications that is characterized by sex-specificity. Further studies are needed to clarify the epigenetic landscape in male rats and how it impacts on gene expression, and ultimately, on adolescent brain refinement.

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THE RESULTS OF THIS P.h.D THESIS HAVE BEEN THE OBJECT OF THE FOLLOWING SCIENTIFIC PUBBLICATION:

"Chronic Δ^9-THC Exposure Differently Affects Histone Modifications in the Adolescent and Adult Rat Brain"

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