Paolo Masia gratefully acknowledges Sardinian Regional Government for the financial support of his PhD scholarship (P.O.R. Sardegna F.S.E. - Operational Programme of the Autonomous Region of Sardinia, European Social Fund 2014-2020 - Axis III Education and training, Thematic goal 10, Investment Priority 10ii), Specific goal 10.5.

Università degli Studi di Cagliari

Dottorato in Neuroscienze

XXXIII Cycle

BIO/14 FARMACOLOGIA

Adolescent WIN 55,212-2 pre-exposure affects rat behavioral and neurochemical response to cocaine

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Final Exam Academic Year 2019 – 2020
Thesis discussed on January 2021

INDEX

THESIS ABSTRACT

Introduction and objectives: Recently, the effects of cannabis use on the brain received increasing attention in relationship with the implications for public health (Hall and Linskey, 2016). Noteworthy, cannabis consumption is also associated with later use of cocaine. The epidemiological studies describing such progressive pattern of different substance use, refers to the Gateway Hypothesis (Kandel, 1975). In this context, since the endocannabinoid system plays a central role in the development and in the reward circuits of the adolescent brain (Diaz Alonso et al., 2012), it is relevant to understand if and how early exposure to cannabinoids could cause neurobiological changes that increase the risk of vulnerability to abuse other drugs.

For this purpose, we investigated the prospective gateway effect of WIN55,212-2 (WIN), a synthetic cannabinoid and full agonist of the CB1 receptors, evaluating drug's cross-sensitizing behavioral and neurobiological effects to cocaine in both adolescence and adulthood.

Results: Adolescent and adult male rats received administration of increasing doses of WIN, or its vehicle, twice-daily for 11 consecutive days. After 7 days of abstinence, rats were treated with cocaine, and tested with voltammetry in the nucleus accumbens (NAcc), or with locomotor activity 24 hours after the last day of abstinence. Adolescent, but not adult WIN-pre-treated rats later exposed to cocaine, showed an increase in the amplitude of dopamine release in the NAcc, and in the motor-activating effects of cocaine compared to vehicle-pre-treated animals. Furthermore, using a multi-omics approach, we found that the cocaine-induced behavioral cross-sensitization of WIN-pre-treated rats correlates with a variety of molecular and epigenetic modifications at the level of the pre-frontal cortex.

Moreover, since substance use disorders are triggered by repeated exposures that involve drastic epigenetic and synaptic alterations, we also evaluated the long-term persistence of motor cross-sensitization and the possible positive reinforcement after repetitive cocaine administrations. We found a close-to-significant persistence of motor cross-sensitization between WIN and cocaine and a conditioned place preference for cocaine in adolescent WIN pre-treated animals.

Conclusions: The findings of the present thesis aim to provide a contribution to the literature to better understand the effects of cannabis use on the brain, and to provide a significant piece of knowledge for decision makers to address more effectively the subtle issue of cannabis legalization.

PREFACE

According to the United National Office on Drugs and Crime (UNODC), the magnitude of worldwide substance use is estimated at 269 million people aged 15–64 who consumed drugs at least once in 2018. This represents the 5.4% of the global population aged 15-64, and it is 28% higher than the 210 milion (4.8%) past-year users estimated over the period 2009-2018 (UNODC, 2020).

Furthermore, among all 269 milion users, and especially in Western countries, over 35 milion people are estimated to suffer from drug use disorders, such as drug dependence and/or require specific treatment (UNODC, 2020).

Cannabis is the most commonly used illegal substance, accounting for an estimated 80% of illicit drug use worldwide, and that include 192 milion users in 2018, with 3.9% of the global population aged 15-64 having used cannabis in the past year (UNODC, 2020). Prevalence of use as a fraction of the world's population began to increase during the early 1990s and the first decade of the 21st century in most European countries (following different regional patterns), with the percentage of individuals aged 18 to 29 years in the United States who reported using cannabis in the past year almost doubling between 2001 to 2002 and 2012 to 2013, from 10.5% to 21.2% (Hasin et al., 2015). In European countries, the prevalence of past-year cannabis use oscillated over the last decade between 6 and 7% among the population aged 15–64 (UNODC, 2020), while Americas represent the parts of the world with the highest annual prevalence of cannabis use (8.8% among the population aged 15–64). In the United States, cannabis use hugely increased since 2007, and especially among adolescents (18-25 years old) or young adults (from 26 years old) (NSDUH, 2019).

To this regard, it is well known that adolescence and early adulthood are important transition periods, with crucial brain changes and cognitive and emotional development (Fuhrmann et al., 2015). For some, this is also a time of vulnerability to the use of drugs (Taylor et al., 2017). Between 12 and 17 years of age is the range of critical risk period for the first substance use, and within the population aged 15–64, peak levels of drug use are seen among those aged 18–25 (EMCDDA 2017).

Considering all the illegal substances, cannabis is again the most widely used drug among young people. It is estimated that all over the world, 13 million are the past-year users of any drug among students aged 15–16 in 2018, with an estimated 11.6 million past-year users of cannabis (with an annual prevalence of 4.7% among this age group, higher than the rate of prevalence of cannabis use among the general population aged 15–64, which is 3.8%) (UNODC, 2020).

Nevertheless, while overall the use of cannabis increased from 22% in 1992 to 40% in 2011 (Johnston et al., 2012), and even more in the last decade (UNODC, 2020), and despite cannabis exposure during adolescence is well proved to lead to significant health consequences (Sharma et al., 2012; Hall and Degenhardt, 2009), the perceived risk of regular use decreased from 80% to 45% (Johnston et al., 2012).

Furthermore, from a couple of decades on, the cannabis–policy landscape has profoundly changed and it is continuously changing after cannabis legalization. More and more countries or jurisdictions have in fact allowed different forms of legalization or decriminalization in the past years. This, accompanied with the increased acceptance of cannabis use especially among adolescents, heightens the attention of how politics, health care system and society should approach and be informed on the risk of marijuana use.

The effects of legalization of reducing the price of cannabis represent the key mechanism through which legalization is likely to increase cannabis use in the long-term period (Hall and Lynskey, 2016), even if it is hard to predict the degree of this trend (Pacula and Sevigny, 2017). Legalization of tobacco and alcohol followed this rule in the past (Pacula et al., 2014; Pacula et al., 2015), but there is still limited evidence the same consideration can be also made for cannabis (Pacula et al., 2014; Pacula et al., 2015). Despite of still limited epidemiological data, important contributions in the literature have been recently provided. Zvonarev and colleagues offered a thorough analysis, showing pre- and post-legalization rates of marijuana use in different US jurisdictions. They found that, legalization led everywhere to an overall increase in cannabis users, even if the heterogeneity in population sub-groups and policies has to be considered (Zvonarev et al., 2019). A survey of the National Survey on Drug Use and Health (NSDUH) performed from 2008 through 2016, confirms the results concerning the increase of cannabis use after legalization (NSDUH, 2019).

When debating if legalization increased cannabis use among adolescents, some authors claimed that the regulated commercial market for recreational cannabis aims to provide mainly access for adults (Gruzca et al., 2019; Cerdà et al., 2017; Pacula and Sevigny, 2017; Hasin, 2018). Other studies though, showed that adolescents are actually continuously exposed to and engage with marketing for recreational use on social media in states where cannabis has been legalized (Trangenstein et al., 2019), therefore it would not be surprising if an increase also in young users will be recorded in the long-term period (Grucza et al., 2019). Interestingly, a concern from a public health perspective arose from the NSDUH, being that a significant increase (reaching as high as 25% to 35%) in the prevalence of frequent use (i.e. more than 20 days per month) and cannabis-use associated disorders have been reported among adolescents or young adults in the past years (Gruzca et al., 2019).

Furthermore, it is becoming increasingly popular the practice of consuming different substances at the same time, either to experience a drug synergy effect, or to compensate the supply of the primary drug. This widespread phenomenon is known as polydrug use, and it is very frequent among adolescents (EMCDDA, 2009). It is proved not only to worsen dependence, but it is also associated with a higher number of health-related harms and elevated risk of drug overdose (EMCDDA, 2009). In 2003, the European school survey project on alcohol and other drugs (ESPAD) carried out surveys to investigate the features of polydrug use among over 70000 students between 15 and 16-year-old coming from 22 European countries. It came out that almost 30% of the study participants reported having used at least two or more substances one month before the survey was carried out, with cannabis and cocaine representing the most popular combination among illegal drugs (EMCDDA, 2009).

Cocaine represents in fact the second most used illegal substance of abuse. Globally, the data indicate that in the 15 to 64 age group, approximately 19 million people were past-year users of cocaine in 2018 (UNODC, 2020). Europeans have used cocaine at least once in their lifetime (equal to 5.2% of the total population in this age group) and around 2.3 million young people between the ages of 15 and 34 (1.9% in this age group) used cocaine in 2016 (EMCDDA, 2017).

Moreover, in the context of polydrug use fits a broader theory, the so called Gateway Hypothesis, which postulates that an adolescent's early consumption of alcohol or tobacco or cannabis, escalates to more addictive illicit drugs at a later stage in life (Hall and Lynskey, 2005). Thus, according to this hypothesis that was proposed by Denise Kandel for the first time in 1975 (Kandel et al., 1975), a drug has the intrinsic potential to function as a bridge for the use of other substances.

Adolescence constitutes the main substrate on which the whole concept of the gateway hypothesis is based, since in this period the brain development is far to be completed. For instance, myelinogenesis continues and the neurocircuitry is structurally and functionally vulnerable to increase in sex hormones (Arain et al., 2013). In addition, during adolescence, glutamatergic neurotransmission prevails on the gamma-aminobutyric acid neurotransmission, giving rise to immature and impulsive behaviors (Bossong and Niesink, 2010). The endocannabinoid system is also deeply involved in the developmental processes, and undergoes significant transient fluctuations in the levels of the endocannabinoids and in the expression of cannabinoid receptors in some crucial brain areas, some of them involved in the processes of reward and cognition, such as the nucleus accumbens (NAcc) (Porter et al., 2015) and the prefrontal cortex (PFC) (Stevens et al., 2009; Fox et al., 2005). For this reason, early adolescent onset of substance use represents a strong predictor of future substance use disorders (Weissman et al., 2015).

In particular, the Cannabis Gateway Hypothesis hugely influenced drug policy and legislation in many countries or states over the last decades, and represented a popular topic in debates regarding legalization or decriminalization of cannabis. But even if some epidemiological data provided strong evidence that hard drug users began at first with cannabis (Kandel, 2002; Kandel, 1975), the matter remains still controversial (Pudney, 2003, Van Ours, 2001), especially when trying to answer the main crucial question of whether the observed sequential pattern of drug use from cannabis to harder drugs is due to correlation or causality.

However, since causality cannot be proved using epidemiological studies, a translational approach to preclinical research is needed. At this purpose, the use of animal models is fundamental to provide important insights into the molecular aspects that are possibly involved in the transition from cannabis to other drugs. Studies performed in rodents over the past 15 years have shown that the abuse of cannabis or synthetic cannabinoids lead to interference with endocannabinoid signaling (Ellgren et al., 2008; Mechoulam and Parker, 2013), and to molecular and epigenetic changes that remodel the vulnerable adolescent brain in a way that it becomes sensitive to more addictive substances, such as heroin (Solinas et al., 2004; Panlilio et al., 2007; Ellgren et al., 2007; Cadoni et al., 2015); and cocaine (Dow-Edwards et al., 2012; Higuera-Matas et al, 2008; Aguilar et al., 2017; Kononoff et al; 2018; Melas et al., 2018; Friedman et al., 2019).

INTRODUCTION

1. Cannabis and the endocannabinoid system

1.1 Cannabis plant

Cannabis Sativa is an annual dioeciously flowering plant belonging to the Cannabaceae family (Cronquist et al., 1981) and its derivatives, i.e., marijuana, are among the best-known mind-altering substances used by man in ancient times. Its first appearance is believed to be in central Asia around 5000 BC, and for millennia the plant has been also used for fiber, oil production, and traditional uses (Farag and Kayser, 2017). It is characterized by having palmate leaves, each one consisting of 5 to 13 lanceolate leaflets, with serrated margin. Plants generally have their period of germination during spring, while that of flowering occurs more frequently during the summer. Pollination is anemophilous (through the wind) with the appearance of the first fruits in autumn, each of which contains a single endosperm seed (Bonini et al., 2018).

The cannabis plant contains a total of 483 compounds among which more than 120 bioactive constituents, collectively known as phytocannabinoids, responsible for the psychoactive effects of the plant (Brenneisen, 2007; Figueroa-Protti et al., 2019); alkaloids (Turner and Elsohly, 1976); terpenoids (Booth and Bohlmann, 2019); flavonoids (Andre et al., 2016), and many others (Brenneisen, 2007).

Among the phytocannabinoids, the main component is called Δ^9-tetrahydrocannabinol (Δ^9-THC), isolated for the first time in 1964 (Gaoni and Mechoulam, 1964). Other compounds include cannabidiol (CBD) (Adams et al., 1940; Mechoulam et al., 2002); cannabigerol (CBG) (Gaoni and Mechoulam, 1964), cannabichromene (CBC) (Gaoni and Mechoulam, 1966), cannabidivarin (CBDV) (Vollner et al., 1969), and tetrahydrocannabivarin (THCV) (Gill et al., 1970).

Marijuana is the most common term indicating the female inflorescence of cannabis following a drying treatment, while hashish is the most common definition for the cannabis resin preparation. The concentration of THC varies according to the sex of the plant; the geographical source, the plant strain, and the type of preparation intended for human consumption (Huestis, 2007). Although estimates vary for the aforementioned reasons, according to Fairbairn, the average content of THC was up to 8%, in hashish up to 14%, and in hash oil up to 60% (Brown, 1998; UNODC, 2017 Drug Price Report). Consumption of cannabis for inhalation in the form of cigarettes (but also through cigars, pipes, water pipes, or “blunts”, which is marijuana rolled in the tobacco-leaf wrapper from a cigar) is the most common delivery intake among recreational therapeutic users. Such form of consumption is associated with a variety of side effects, including a chronic cough,

bronchitis, and especially inhalation of toxic combustion products (Volkow et al., 2014; Health Canada, 2018). This route of administration make cannabinoids reach their maximum concentration in the blood and brain in a few minutes after smoking. The bioavailability of the major cannabinoids consumed through inhalation is very high (around 25%, with differences among individuals) (Health Canada, 2018).

Some other cannabis products used by patients are consumed orally, oromucosally or sublingually (Sativex®, extracts, oils, foods). In these cases, cannabis peak concentration in the blood takes longer to occur (0.5-6 hours) in comparison to inhalation. Moreover, the effects last longer and, at an equal dose administered, they are generally less intense (since the bioavailability is less than 15%). Nevertheless, for the delayed appearances and intensity of the given effects, these edible products are more difficult to control, and this is why this route of administration is associated with more overdose episodes. Absorption is slower than when resins or pure oils are consumed, and the bioavailability is lower but also more variable between individuals (4-12%) (Health Canada, 2018).

1.2 The endocannabinoid system

The study of the mechanisms that underlie the effects of the action of phytocannabinoids led to the discovery of a complex biological cell communication system known as the endocannabinoid system. It consists of specific receptors called cannabinoid receptors (CBRs), lipid compounds which act as endogenous molecules and a series of protein mechanisms that finely regulate the synthesis, transport and degradation of these ligands. The wide distribution of cannabinoid receptors is indicative of the number of physiological and cognitive processes in which the endocannabinoid system is directly involved, both peripherally and centrally **(Fig. 1)** (Ligresti et al., 2016; Piomelli, 2003).

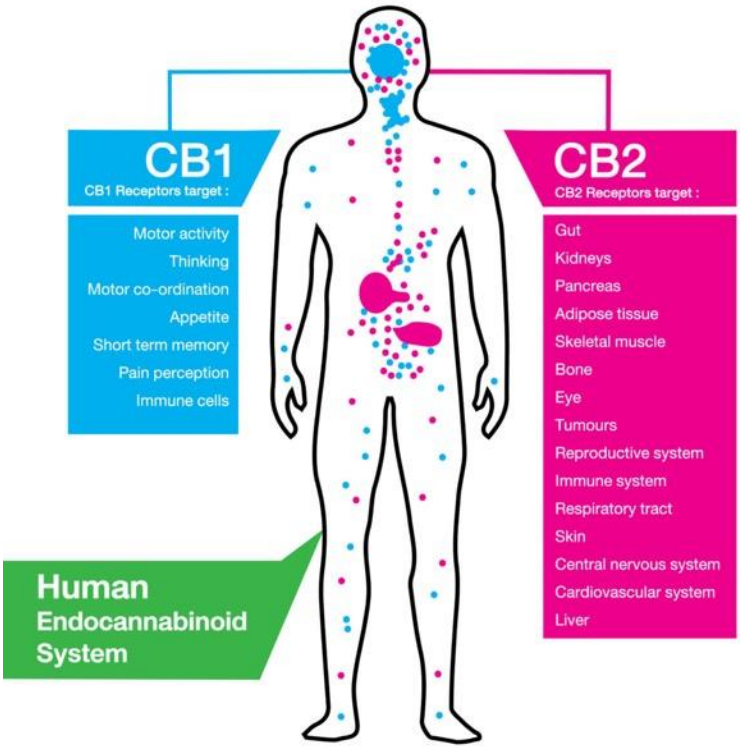

Fig.1 – *Endocannabinoid system functions (©Chris Shade, Natural Partners)*

THC was isolated and its chemical structure clarified by Gaoni and Mechoulam in 1964 (Gaoni, 1964). Since that year, THC has been synthesized, and many studies have been conducted on its activity (Mechoulam, 1988). However, before the discovery of CB1 in 1988 (Devane et al., 1988), the processes underlying the action of THC on the brain were difficult to understand, with only some speculation claiming a generic activity of the molecule on the neuronal cell membrane (Leuschner et al., 1984, Paton, 1975). With the discovery of CB1 receptors (Matsuda et al., 1990), that represent the primary pharmacological target of THC, several studies were conducted in the search of the endogenous ligands for these receptors. Anandamide (AEA) was the first endocannabinoid to be discovered, and it was isolated for the first time in 1992 from the pig brain by William Devane and colleagues (Devane et al., 1992). Anandamide is a word that comes from the Sanskrit “ananda” (internal bliss), which underline its role as an endogenous marijuana-like substance self-delivered by the brain (Scherma et al., 2019). After that, a second endocannabinoid has been identified and named 2-Arachidonoylglycerol (2-AG). The latter was isolated from the rat brain and the canine gut (Mechoulam et al., 1995; Sugiura et al., 1995). The discovery of AEA and 2-AG paved the way to numerous investigation that will show years later that the behavioral and molecular effects exerted by the two endogenous cannabinoids only partially overlap, with other differences observed between them and THC (Luchicchi and Pistis, 2012).

The endocannabinoid system is a lipid signaling system that made its appearance before the evolution of vertebrates and it is widely preserved across organisms (Elphick et al., 2003). As previously mentioned, in mammals it is involved in several physiological processes, that include inflammation and pain (Jhaveri et al., 2007; Woodhams et al., 2017), appetite (Kirkham, 2005), and mood (Ashton and Moore, 2011). Importantly, the endocannabinoid system plays a pivotal role both in developmental mechanisms (Fride, 2004), and in the reinforcement and reward processes of the brain (Gardner, 2005; Parsons and Hurd, 2015). In fact, as will be later discussed in detail, it participates in mediating the rewarding and pharmacological responses induced not only by cannabinoids (Gonzalez et al., 2007), but also by other drugs of abuse (Parsons and Hurd, 2015; Maldonado et al., 2006; Tanda, 2007) with AEA being by far the most studied among endocannabinoids in this context (for a review see Scherma et al., 2019).

AEA and 2-AG are bioactive lipids, and they belong to the N-acylethanolamines (NAEs) and monoacylglycerols (MAGs) subclasses, respectively (De Petrocellis and Di Marzo, 2009). Despite of the fact that they display a similar structure (they are both arachidonic acid derivatives conjugated either with ethanolamine or glycerol, respectively), these compounds exert a large variety and different physiological actions from one another. First of all, both AEA and 2-AG are involved in distinct biosynthetic and metabolitic pathways (Ahn et al., 2008). Furthermore, 2-AG brain tissue levels are ten to a hundred times higher than AEA levels (Shen and Thayer, 1999), with the latter, similarly to THC, acting as a partial agonist and thus activating cannabinoid receptors with low intrinsic efficacy, whereas 2-AG acts as a full agonist (Mackie, 2008).Over the years, also other endocannabinoid-like activity lipid molecules have been discovered and isolated (Di Marzo and De Petrocellis, 2012), including 2-arachidonylglyceryl ether (2-AGE, noladin), O-arachidonylethanolamine (virodhamine), and N-arachidonyldopamine (NADA) (Hanus et al., 2001; Porter et al., 2002). Nevertheless, since their mechanism of action has not been clarified, it still remains to be determined whether they can be classified as endogenous cannabinoids or not.

1.2.1 Endocannabinoid biosynthesis and metabolism

Although endocannabinoids are considered as neurotransmitter, they do not show the usual neurotransmitter properties. First of all, they are hydrophobic molecules, and therefore precluded from typical storage into synaptic vesicles. Furthermore, there are no cannabinoid neurons or cannabinoid neuronal pathways, even if both endocannabinoids have precursor molecules located in all the cells as membrane components, and usually synthesized on demand in a Ca2+-dependent

manner after cellular depolarization or receptor stimulation (De Petrocellis and Di Marzo, 2009; Di Marzo et al., 1994).

The biosynthesis and metabolism of AEA and 2-AG can occur following different pathways, and represent critical regulative mechanisms that determine their tissue levels. The main AEA biosynthetic pathway comes from the cleavage of a phospholipid precursor, N-arachidonoyl phosphatidylethanolamine (NAPE); NAPE, in turn, is derived from the enzymatic transfer, catalyzed by N-acyltransferase (NAT), of an acyl group from the sn-1 position of arachidonic acid to the amino group of a phosphatidylethanolamine (PE). NAPE is hydrolyzed to AEA and phosphatidic acid by a phosphodiesterase, a substrate-specific phospholipase D (NAPE-PLD). In addition to NAPE-PLD, NAPE can also be hydrolyzed by other enzymes such as phospholipase A2 (PA2), phospholipase C (PLC) and α/β-hydrolase 4 (Abh4) (Bisogno et al., 2005).On the other hand, 2-AG mainly arises from inositol phospholipids (PI) via diacylglycerol (DAG), by the phospholipase C (PLC)/DAG lipase pathway (Piomelli et al., 2003; Sugiura and Waku., 2000). Alternatively, 2-AG is also synthesized from PI by a hydrolysis that is catalyzed by PI-specific phospholipase A1 (PLA1) and lyso-PI-specific PLC (Ueda et al., 2011).

Endocannabinoids, as typical bioactive lipids, have a very short half-life, due to the rapid metabolic deactivation they undergo.

Degradation of AEA is mediated by the intracellular enzyme fatty acid amide hydrolase (FAAH), which breaks AEA down into two compounds: free arachidonic acid and ethanolamine (Ahn et al., 2008; Di Marzo and Maccarrone, 2008). However, AEA degradation also occurs through the action of N-acylethanolamine acid amidase (NAAA) (Ueda et al., 1999). Both FAAH e NAAA degradation mechanisms culminate in the same hydrolysis of oleoylethanolamide (OEA) and palmitoylethanolamide (PEA), two endogenous fatty acid amides that bind primarily to the α-type of peroxisome proliferator-activated receptors (PPARα) and are reported to increase the AEA activity through an entourage effect (Ho et al., 2008). Apart from the hydrolytic pathways, AEA can be oxygenated by cyclooxygenase-2 (COX-2), lipoxygenase (LOX) isoenzymes, and by cytochrome P-450 (Maccarrone, 2017). FAAH is not only the preferred way of AEA degradation, but it is also a potential therapeutic target for a huge variety of CNS conditions (for a review see Ulugol, 2014). For instance, experiments involving FAAH knockout mice have shown a 10 times increase in AEA levels within many brain areas (Cravatt et al., 2001) leading to a CBR-mediated analgesic phenotype (Lichtman et al., 2004). The latter findings led to the characterization of important FAAH inhibitors, such as URB597 ((3-(3-carbamoylphenyl) phenyl) N-cyclohexylcarbamate). URB597 is able to prolong AEA half-life and therefore its activity (Piomelli et al., 2006). Moreover, URB597 is also involved in depression (Gobbi et al., 2005) and anxiety

(Kathuria et al., 2003); neuropathic (Russo et al., 2007), inflammatory (Holt et al., 2005) and acute pain (Kathuria et al., 2003). Despite of the knowledge acquired in the last decades about the biosynthetic and metabolic pathways, there is no report regarding the molecular identification and cloning of the AEA transporter (Maccarrone, 2017, Alexander and Cravatt, 2006; Beltramo et al., 1997; Glaser et al., 2005). However to date,one model hypothesizes that AEA uptake is strictly linked to FAAH (Glaser et al., 2003). For instance, some of thediscovered AEA transport inhibitors, such as N-arachidonoyl-aminophenol (AM404) (Costa et al., 2006), N-arachidonyl-2-methyl,4-hydroxyphenylamine (VDM11) (Vandevoorde et al., 2005) and LY218240 (Alexander and Cravatt, 2006) prevent AEA to be recycled and to be reuptaken into the cells from the synaptic cleft, a mechanism that also lead to the inhibition of FAAH. On contrary, other transport inhibitors, such as N-(5Z, 8Z, 11Z, 14Z eicosatetraenyl)-4-hydroxybenzamide (AM1172), N-arachidonyl3-furylmethylamine (UCM707), and (R)-N-(1-(4-hydroxyphenyl)- 2-hydroxyethyl)oleamide (OMDM2), are not able (or only slightly) to inhibit FAAH (Kaczocha et al., 2006).

2-AG as well is also fastly degraded by several enzymes. The main mechanism of 2-AG degradation occurs through monoacylglycerol lipase (Bisogno et al., 2005). Nevertheless, interestingly, some investigation have shown evidence that 2-AG may also be degraded by FAAH (Bisogno et al., 1998; Goparaju et al., 1998). In addition, a small but relevant percentage of 2-AG degradation in the brain can also occur through the action of a series of serine hydrolase α-β-hydrolase domain 6 or 12 (Marrs et al., 2010; Savinainen et al., 2012). Finally, in a similar fashion to FAAH inhibitors, the MAGL inhibitor N-arachidonyl maleimide (NAM) reduces the 2-AG hydrolysis by almost 85% (Saario et al., 2005).

1.2.2 Endocannabinoid signaling

The mechanism of action of endocannabinoids at synaptic level is quite peculiar, since they retrogradely regulate synaptic neurotransmission: after being synthesized and finally released on demand from the postsynaptic neurons, they travel through the synaptic cleft and then bind to and activate the CB1 receptors at the level of the pre-synaptic bouton, momentarily blocking the release of GABA or glutamate, therefore modulating both excitatory and inhibitory inputs (Ohno-Shosaku and Kano, 2014; Tanimura et al., 2010). More precisely, the inhibition of neurotransmission release occurs through the activation of presynaptic K+ channels and the inhibition f N- and P/Q-type Ca2+ channels as schematically shown in **Fig. 2** (Ohno-Shosaku and Kano, 2014; Alger, 2012; Kano et al., 2009). Such endocannabinoid-induced retrograde inhibition of neurotransmitter release give rises to two well-known different types of synaptic plasticity: 1) if the activation of CBRs occurs on

axon terminals of GABAergic neurons, this process mediate the so called depolarization-induced suppression of inhibition (DSI); 2) if the activation of CBRs occurs on axon terminals glutamatergic neurons endocannabinoids mediate the so called depolarization-induced suppression of excitation (DSE) (Wilson and Nicoll, 2002). Furthermore, the suppression of neurotransmitter release can be either transient (endocannabinoid-mediated short-term depression) or persistent (endocannabinoid-mediated long-term depression) (Ohno-Shosaku and Kano, 2014). This retrograde signaling function of endocannabinoids appears to be widely distributed throughout the CNS (Maejima et al., 2001).

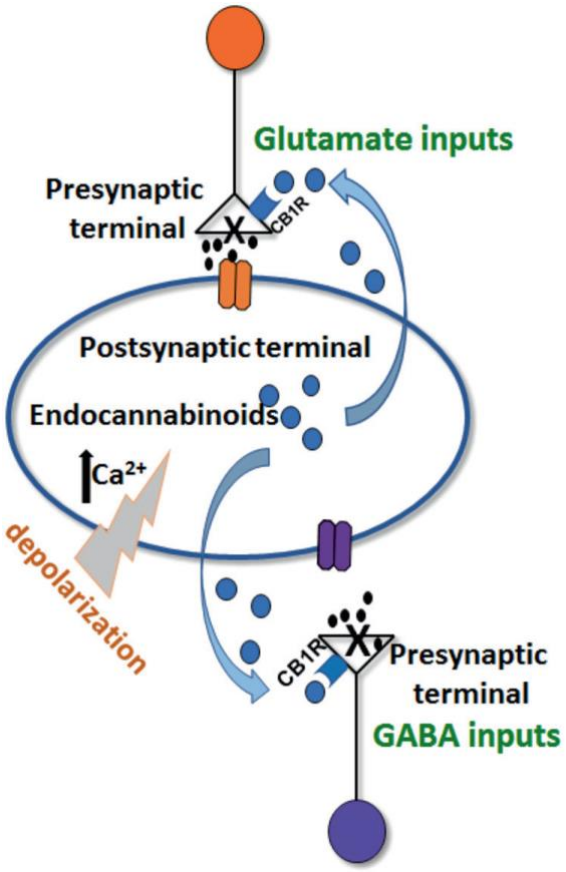

Fig. 2 - *Schematic representation of the "retrograde" regulation of endogenous cannabinoids, culminating in the inhibition of neurotransmission (©Scherma et al., 2019)*

1.2.3 Cannabinoid receptors

Before the 1980s, the pharmacological action of THC was believed to be related to its intrinsic lipophilicity and consequent ability of penetrating cell membranes and to change specific membrane properties (Leuschner et al., 1984; Paton, 1975). The existence of cannabinoid receptors (CBRs) was only proposed for the first time in the last years of 1980s after ligand-binding studies

were performed, but their presence was afterwards confirmed when CBRs were finally cloned, demonstrating unequivocally that they were able to modulate the behavioral-pharmacological effects of marijuana (Devane et al., 1988; Matsuda et al., 1990). Thus, CBRs, discovered years after other neurotransmitter receptors, were found to be the most expressed receptors in the brain. Nevertheless, endogenous ligands are only released on demand by cleavage of lipid precursors, but no cannabinoid neurons actually exist. It is important to underline that while most of the classical neurotransmitter-neuromodulator receptor systems have only one endogenous ligand, CBRs have at least 2, likely more, endogenous ligands (Di Marzo and Petrocellis, 2012). Worth to note is also that the opioid system has more than one endogenous ligand as well, and that the endogenous ligands of both systems (i.e. endocannabinoid and opioid) are known to display reinforcing properties in self-administration investigation in rodents. To date, two CBRs have been discovered: the CB1 receptor (CB1R) identified in 1988 (Devane et al., 1988), and cloned in the early 1990s from rat cerebral cortex (Matsuda et al., 1990), and later from human (Gerard et al., 1991) and mouse brain (Chakrabarti et al., 1995); the second CBR subtype, CB2 receptor (CB2R), was derived from human promyelocytic-leukemia cells (HL-60 cells) (Munro et al., 1993). CBRs belong to the the G-protein-coupled receptor (GPCRs). Endogenous cannabinoids, THC and other synthetic cannabinoids such as CP55940,5 and WIN 55,212-2 bind with a high affinity to CB1Rs. CB1Rs are the most abundant G-protein-coupled receptor in the brain: it has been found at very high density in the cingulate gyrus, frontal cortex, hippocampus, cerebellum and basal ganglia (Mackie et al., 2005; Tsou et al., 1998; Glass et al., 1997); at moderate density in the basal forebrain, amygdala, nucleus accumbens, periaqueductal gray and hypothalamus; and at low density in the midbrain, pons, medulla, primary motor cortex and thalamus (Hu and Mackie, 2015). CB1Rs are mainly expressed on axons and synaptic boutons of neurons but traces can be found on interneurons and astrocytes (Breivogel et al., 1998; Howlett, 1985). When CB1Rs are activated, they mostly couple with G proteins of the αi and αo subtypes. The general mechanism comprises a signaling cascade that leads to inhibition of adenylyl cyclase (Howlett, 1985), inhibition of the opening of voltage gated calcium channels (Mackie and Hille, 1992), an increase in potassium channel conductance (Mackie and Hille, 1992; Deadwyler et al., 1995), activation of the mitogen-activated protein kinases (MAPKs) (Bouaboula et al., 1995), culminating in an overall suppression of neurotransmitter release. CB1Rs are also present at the level of peripheral sympathetic axon terminals, where they exert a suppression action on norepinephrine release (Ishac et al., 1996; Vizi et al., 2001), and at the level of the enteric nervous system, where they inhibit intestinal motility and secretion (Izzo and Sharkey, 2010). The concentration of CB2Rs is strictly linked to the immune system, being that they are located in the marginal zone of the spleen, the thymus, the tonsils, and the surface of immune cells

(Munro et al., 1993), but more recent studies have demonstrated that CB2Rs can be also found in the brain, especially in microglial cells (Nunez et al., 2004). To date, it is still not clear if CBRs are also involved in mechanisms that mediate substance abuse (Ishiguro et al., 2010; Onaivi et al., 2008). For instance, CB2Rs are expressed in dopamine neurons located in the midbrain DA (Liu et al., 2017; Zhang et al., 2017), where they are likely responsible for the modulation of alcohol preference and the reinforcing and neurochemical effects of cocaine (Zhang et al., 2014). In humans, CB2R are encoded by the CNR2 gene that shares 68% identity with human CB1R within the transmembrane regions and only 44% homology throughout the total protein (Munro et al., 1993). Although CB1 and CB2Rs belong to the same family of G proteins and interact with some identical ligands, they display different signaling mechanisms: CB2Rs are able only to poorly modulate calcium channels and inwardly rectifying potassium channels (Felder et al., 1996). In addition, CB2Rs from different species give rise to a large spectrum of pharmacological outcomes in response to activation induced by the same drugs (Bingham et al., 2007; Yao et al., 2006).

Besides the binding to CBRs, AEA and 2-AG also stimulate other receptor types or cannabinoid-like receptors. For example, AEA (but not 2-AG), is able to activate TRPV1 vanilloid receptors, the molecular target of capsaicin (Toth et al., 2009). Indeed, Zygmunt et al. (Zygmunt et al., 1999) described in their work that AEA leads to a vasodilation effect when activates TRPV1 receptors on perivascular sensory nerves. The interaction of AEA with TRPV1 receptors is specific and depends on the ability of AEA to reach the intracellular binding site (Bisogno et al., 2005). The two ligands AEA and capsaicin both display a similar affinity for TRPV1 receptors, although capsaicin has significantly lower potency in comparison to AEA, and higher concentrations of the latter ligand are needed to induce the physiological TRPV1 responses than those required for CB1 activation (Ross, 2003). Moreover, levels of TRPV1 receptor expression are related to the pharmacological action that AEA displays: when the TRPV1 receptor expression is low, AEA acts as a partial agonist; on contrary when the receptor expression is high AEA acts as a full agonist (Toth et al., 2009; Zygmunt et al., 1999). Overall, it has been found that the binding and activation of TRPV1 receptors by AEA might have therapeutic potential for the treatment of inflammatory, respiratory, and cardiovascular disorders (Ross, 2003).

Concerning other receptors at which endocannabinoids bind to, many studies have described the orphan G-protein coupled receptor GPR55 as a cannabinoid-like receptor showing differences in signaling mechanisms in comparison to the CB1 and CB2 receptors (Johns et al., 2007; Ryberg et al., 2007; Sharir et al., 2012). In particular, recent evidence showed that GPR55 might represent a pharmacological target for AEA (Brown and Hiley, 2009). GPR55 is highly expressed in large dorsal root ganglion neurons and its activation is able to increase intracellular calcium in these

neurons. GPR55 exerts its physiological actions through distinct signaling. For instance, signaling pathway in HEK293 cells that transiently express GPR55 indicate that the calcium increase involves G(q), G(12), RhoA, actin, phospholipase C, and calcium release from IP(3)R-gated stores (Brown and Hiley, 2009; Sharir and Abood, 2010). GPR55 activation also inhibits M currents (Lauckner et al., 2008).

Furthermore, it has been speculated by several investigation that endocannabinoids and endogenous cannabinoid-like molecules activate the peroxisome proliferator-activated receptors (PPARs) (O'Sullivan and Kendall, 2010; Sun et al., 2007). PPARs are a family of nuclear receptors that regulate the transcription and expression of different genes (Michalik and Wahli et al., 2006) and modulate pivotal physiological functions such as inflammation, cell differentiation and homeostasis (O'Sullivan and Kendall, 2010; Ferré, 2004). Recently, evidence have been provided suggesting that between endogenous PPAR molecules and AEA there is a structural and functional analogy, such that AEA has the ability to activate some members of the PPAR family (Sun et al., 2007; Bouaboula et al., 2005). In fact, AEA acts as a weak PPARα ligand and also able to activate PPARγ (O'Sullivan and Kendall, 2010; Bouaboula et al., 2005). About the latter receptor, AEA has anti-inflammatory effects inhibiting the release of the pro-inflammatory cytokine IL-2 in a CB1R/CB2R-independent manner (Rockwell and Kaminski, 2004). Furthermore, the possibility that AEA causes PPARγ and CB1 receptor upregulation has been suggested (Karaliota et al., 2009).

1.2.4 Synthetic CB1 agonists and antagonists

The importance of the endogenous cannabinoid system also resides in its therapeutic target potential, as it can be involved in various pathological states. This has led researchers to synthesize a variety of agonists and antagonists of the CB receptors to study the complex mechanisms featured by the endocannabinoid system.

We can distinguish between 4 main classes of cannabinoids (EMCDDA, 2009): (i) the so called classic cannabinoids, which are analogues of THC and they are characterized by a structure containing a dibenzopyrane ring which includes HU-210, nabilone and dronabinol (Ottani and Giuliani, 2001); ii) the non-classic cannabinoids, such as the cyclohexylphenol (CP) series, among which we find the molecules CP 55,940, CP 47,497 and their n-alkyl homologues (Compton et al., 1992); (iii) the class of aminoalkylindoles or JWH compounds (named after the name of their inventor JW Huffman; Wiley et al., 2009), which includes several subclasses such as naphthylmethylindols, naphthylpyrroles, naphthylmethylindanes, phenylacetylindoles and naphthylindols, to which WIN 55,212-2 (WIN) belongs, non-selective full agonist of the CB

receptors (Pertwee et al., 1997; Selley et al., 2001); finally a class of heterogeneous compounds, synthetic derivatives of arachidonic acid structurally related to the AEA. Like the agonist compounds, numerous molecules have been synthesized acting as antagonists towards CB receptors (Pertwee, 2005), or as reverse agonists, such as the SR141716A, known by the name Rimonabant (Rinaldi-Carmona et al., 1995). The latter prevents nicotine from establishing a conditioned place preference in rats (Le Foll and Goldberg, 2004; Forget et al., 2005). Furthermore, rimonabant reduces nicotine self-administration and drug-seeking behavior (Gamaleddin et al., 2012; Cohen et al., 2005). Since endogenous cannabinoids facilitate dopamine release in the nucleus accumbens, by blocking CB1 receptors rimonabant reduces the reinforcing effects of nicotine. However, rimonabant has been taken away from the market worldwide shortly after its introduction, due to the dangerous psychiatric side effects associated with it (Moreira and Crippa, 2009).

1.2.5 Endocannabinoid system and neurodevelopment

The endocannabinoid system and its related lipid mediators play a crucial role in the earliest phases of ontogenetic development, regulating neural progenitor commitment, survival (Aguado et al., 2006; Alonso et al., 2012) and synaptic connectivity in the developing brain (Mulder et al., 2008; Berghuis et al., 2007) of several species, including rodents and humans
(Rodriguez De Fonseca et al., 1993; Mato et al., 2003; Harkany et al., 2008; Mulder et al., 2008; Zurolo et al., 2010; Maccarrone et al., 2014). The endocannabinoid system has also been shown to highly influence the morphological and molecular processes that eventually lead to neuronal differentiation (Berghuis et al., 2005), including growth cone differentiation and axon guidance (Bisogno et al., 2003).

In rodents, the endocannabinoid system regulates the first synaptic transmission since postnatal day (PND) 10 and then it increases throughout development and into adulthood (Rodriguez De Fonseca et al., 1993; Liang et al, 2014). The peak expression of CB1 receptors is observed around PND 30 (earlier in females, later in males, Rodriguez De Fonseca et al., 1993), especially at the level of the PFC and striatum, and then it starts decreasing when approaching adulthood, around PND 70 (Rodriguez De Fonseca et al., 1993; Ellgren et al., 2008; Klugmann et al., 2011). 2-AG is high around birth and may fluctuate throughout adolescence, but with a decisivedecreased expression during mid-adolescence. AEA gradually increases during early life (earlier in females than males, Wenger et al., 2002) showing a strong increase from early adolescence to adulthood, and its levels remain relatively consistent all across development in corticolimbic regions, including PFC,

hippocampus, amygdala, and hypothalamus. While the cellular distribution of MAGL during development is not known (Basavarajappa et al., 2009), FAAH activity fluctuates with an inverted fashion to AEA during adolescence (**Fig.3) (**Ellgren et al, 2008; Heng et al, 2011; Lee et al, 2013; Rubino et al, 2015). In a similar fashion, both CB1 receptors and endocannabinoid ligands can also be detected in the human brain (Mato et al., 2003) during early developmental stages (Mato et al., 2003; Viveros et al., 2005).Interestingly, high densities of CB1 receptors have been observed to be localized in white matter areas during the prenatal stages, while fibre-enriched areas in adults present almost a lack of CB1 clusters. This evidence underline the role of the endocannabinoid system guidance processes eventually culminating in the establishment of cortical–subcortical connections (Mato et al., 2003; Viveros et al., 2005).

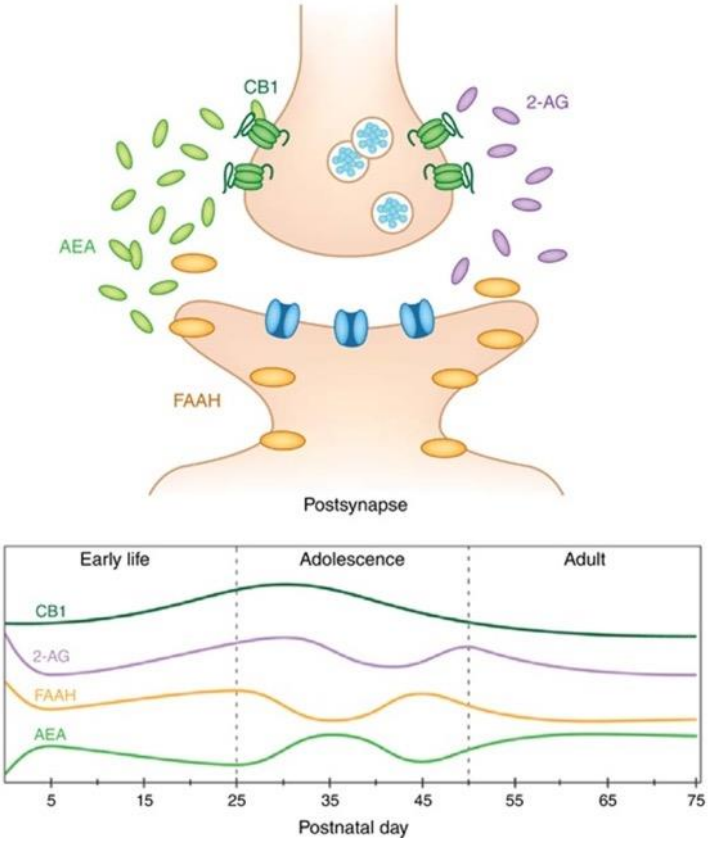

Fig. 3 *– Schematic representation of changes related to the endocannabinoid signaling across rodent development (© Meyer et al., 2018)*

It is likely that level changes in gonadal hormones (androgens, estrogen, and progesterone) and gonadotrophins during puberty are strictly linked to fluctuations in the endocannabinoid signaling later on during adolescence. It has been shown both in rodents (Wenger et al, 2001; Tsutahara et al, 2011) and in humans (Kolodny et al., 1974) that the endocannabinoid activity seems to attenuate the release of gonadal hormones to keep the correct physiological balance (Gorzalka and Dang, 2012).

On the other hand, alterations of gonadal hormone normal levels can influence the endocannabinoid signaling through a feedback loop involving the hypothalamus, pituitary, and limbic regions (Maccarrone et al, 2003; Nguyen and Wagner, 2006; Gorzalka and Dang, 2012).

The delicate balancing role of physiological processes that the endocannabinoid system contribute to maintain across all the developmental stages, can be altered by the perinatal exposure of cannabinoids, which are in fact able to modify the maturation of neurotransmitter systems and their related behaviors (Basavarajappa et al., 2009). These effects are carried out through the activation of CB1 receptors, that, as we previously mentioned, emerge early in the developing brain (Berrendero et al., 1998; Fernández-Ruiz et al., 1999). Psychoactive cannabinoids act as epigenetic factors, anticipating or delaying the expression of crucial genes implicated in the synthesis of receptors at a very specific moment of development. This can alter the activity related to the physiological function of the CB1 receptors, resulting in an increase or a decrease in their concentration or in changes in the activities of the signaling pathways related to the activation of the CB1 receptors. For instance, adult animals perinatally exposed to marijuana, through perturbations of normal neurotransmitter development pattern, have shown a huge variety of long-term neurobehavioral disturbances (for a review see Basavarajappa et al., 2009). Furthermore, different studies conducted by Fried and colleagues on the effects of cannabinoids in humans, have shown that the use of marijuana by pregnant women is able to affect the neurobehavioral development of their children (Fried et al., 1992, 1998, 2003; Fried and Watkinson, 2001)

1.2.6 Endocannabinoid system and reward

Each drug of abuse, including cannabis, gives rise, through their psychoactive effects, to gratification and a condition of well-being that is defined as reinforcement (Edwards, 2016). The compulsive tendency in engaging rewarding stimuli, the preoccupation of the behavior, the loss of control and suffering negative consequences, ultimately defines addiction as a disorder of the brain's reward system (Sussman, 2011).

The meso-cortico-limbic system is the set of brain areas in which the physiological mechanisms underlying gratification, positive reinforcement and motivational processes are integrated (Kelley and Berridge, 2002). This systemtransmits dopamine from the ventral tegmental area (VTA), which is located in the midbrain, to forebrain areas, such as the prefrontal cortex, assigned to control intellectual and conscience processes; to deeper areas such as the amygdala and the hippocampus, the latter a convoluted cortex structure located in the temporal lobe, which play roles in regulating

the mechanisms of learning and memory, and emotional states (Koob and Volkow, 2010; Schoenbaum et al., 2006); and to subcortical areas belonging to the striatum including the nucleus accumbens, located in the ventral area of the striatum, important in controlling involuntary movement and pivotal for the integration of reward and motivational mechanisms (Di Chiara, 2002).

Cannabinoid CB1 receptors are located in the VTA and in several mesocorticolimbic areas projecting from it, therefore acquiring an important role in brain reinforcement and reward processes (Gardner, 2005). As already mentioned, CB1 receptors are mainly located at the presynaptic level, exerting, through the retrograde action of endocannabinoids, inhibition of neurotransmitter release (Ohno-Shosaku and Kano, 2014; Tanimura et al., 2010). Such modulation of either excitatory or inhibitory inputs control the dopaminergic transmission of the whole mesocorticolimbic system (Kreitzer and Regehr, 2001; Wilson, 2001; Ohno-Shosaku and Kano, 2014). More specifically, endocannabinoids can be released in response to depolarization at the level of nucleus accumbens (Robbe et al., 2002) and VTA dopaminergic neurons (Melis et al., 2004; Lupica and Riegel, 2005). The activation of CB1 receptors on pre-synaptic terminals of GABAergic neurons in the VTA and of glutamatergic neurons in both the VTA and nucleus accumbens, leads to the inhibition of both GABAergic- and glutamatergic-mediated neurotransmission (Ohno-Shosaku and Kano, 2014; Melis et al., 2004; Lupica and Riegel, 2005; Robbe et al., 2002). The activity-dependent release of endocannabinoids eventually modulate the dopaminergic activity in the VTA and in the VTA-related dopamine projection areas depending on the relative level of activation of these inputs under distinct behavioral circumstances (Scherma et al., 2008; Melis et al., 2004; Lupica and Riegel, 2005; Robbe et al., 2002).

In general, the endocannabinoid system mediates the reinforcing and rewarding effects of cannabinoids (Maldonado et al., 2006; Tanda and Goldberg, 2003), and of other abused drugs, such as alcohol (Gonzalez et al., 2002) and nicotine (Maldonado and Rodriguez De Fonseca, 2002).

The action of most substances of abuse in fact leads, directly or indirectly, to an increase of extracellular dopamine levels in the nucleus accumbens (Di Chiara and Imperato, 1988).

It is known that THC generates an indirect increase in the dopaminergic tone at the level of the nucleus accumbens, with the activation of the CB1 receptors in the midbrain and at proencephalic level, inducing a regulatory effect that creates a change in the firing pattern of the dopaminergic neurons in those areas (French and Dillon, 1997; Diana et al., 1998; Gessa et al., 1998) which in turn generate another dopaminergic signal in different projection areas, among which the nucleus accumbens (Cheer et al., 2004; Gessa et al., 1998; French and Dillon, 1997). This is established

from in vivo microdialysis studies which confirmed that cannabinoids induce a significant increase in extracellular dopamine levels in the area of the nucleus accumbens (NgCheong Ton et al., 1988; Tanda et al. 1997; Malone and Taylor, 1999). The same effect was also observed with the synthetic cannabinoid WIN 55,212-2 in voltammetry in freely-moving animal (Cheer et al., 2004), and self-administration (Tanda et al., 1997; Fadda et al., 2006) experiments. This increase, both in the case of THC and WIN 55,212-2, occurred selectively at the shell level (Tanda et al., 1997), a sub-region of the nucleus accumbens involved in the mediation of reward induced by the substances of abuse (Johnson et al., 1995; Pontieri et al., 1995; Wise, 1996; Gardner and Vorel, 1998).

The drugs of abuse that are able to activate the dopaminergic system and increase dopamine levels in the nucleus accumbens are also known to facilitate brain stimulation reward, an operant procedure where animals receive a small electrical current by pressing a lever (Olds and Milner, 1954). Also THC for instance, has been shown to facilitate brain stimulation reward (Lepore et al., 1995; Gardner et al., 1988). On the other hand, drugs such as opioid antagonists that block dopamine receptors (Schaefer, 1988) reduce thresholds for self-stimulation (Wise, 2002; Kornetsky, 2004).

Furthermore, studies performed using the self-administration operant conditioning have shown that THC, but also the synthetic cannabinoid WIN 55,212-2 and HU-210, are able to increase the reinforcing effects of heroin (De Vries et al., 2003; Solinas et al., 2005); nicotine (Valjent et al., 2002; Gamaleddin et al., 2011); and alcohol (Colombo et al., 2002). On the other hand, one study showed that WIN 55,212-2 decreases intravenous cocaine self-administration in rats (Fattore et al., 1999), even if HU-210 is able to induce relapse to cocaine seeking in rats, after prolonged withdrawal periods (De Vries, 2001), and thus the mechanism responsible for regulating cocaine's reinforcing effect might be influenced by the endocannabinoid system (Vlachou et al., 2003).

The effects that the endocannabinoid system exerts on the reward processes also directly involve its main molecular characters (Solinas et al., 2008). For example AEA, and its metabolically stable AEA analogue meth-AEA, represent two types of reinforcement of self-administration behavior in non-human primates when injected intravenously (Justinova et al., 2005), and are also able to increase dopamine levels in the accumbens shell (Solinas et al., 2006). Moreover, different drugs of abuse alter AEA levels in the brain. In particular, nicotine and THC are known to decrease AEA levels in the striatum (Gonzalez et al., 2002), alcohol in the midbrain (Gonzalez et al., 2002), while cocaine and heroin do not exert any effects on AEA levels (Gonzalez et al., 2002). THC also decreases tissue levels of 2-AG in the striatum (Di Marzo et al., 2000) ethanol decreases tissue levels of 2-AG only in the midbrain (Gonzalez et al., 2002), while nicotine and cocaine do not seem to alter 2-AG levels in any brain region (Gonzalez et al., 2002).

1.1.8 Endocannabinoid system and food consumption

The appetite-stimulating properties associated with cannabis have been known for centuries, however the effects of THC on food consumption has only recently been demonstrated (Williams et al., 1998). There is a large amount of evidence that supports the involvement of the endocannabinoid system in the regulation of food behavior and energy balance through both central and peripheral mechanisms (Coutts and Izzo, 2004; Di Marzo and Matias, 2005). In fact, it is well known that the pharmacological manipulation of the system, through the action of exogenous cannabinoids such as THC, CP55940 and WIN, determines an increase in food consumption both in humans and in laboratory animals (Cota et al., 2003; Hart et al., 2002; Hollister, 1971; Koch and Matthews, 2001; Williams et al., 1998). The main central areas in which the endocannabinoid system performs its modulatory action for food intake are the hypothalamus and the mesolimbic system (Matias and Di Marzo, 2007). Indeed, it has been shown that both THC and endocannabinoids, when administered locally at the level of the hypothalamus (Anderson-Baker et al., 1979; Jamshidi and Taylor, 2001; Verty et al., 2005) and nucleus accumbens (Jamshidi and Taylor, 2001; Kirkham et al., 2002; Soria-Gòmez et al., 2007), are able to increase food consumption in rats. It has also been found that the generation of these hyperphagia effects are mediated by the activation of CB1 receptors, in fact their pharmacological inactivation induces the disappearance of the aforementioned effects (Kirkham et al., 2002; Soria-Gòmez et al., 2007), and their gene silencing (CB1-KO) leads to conditions of thinness and hypophagy in mice (Wiley et al., 2005).

However, the endocannabioid system seems to be able to mediate diametrically opposite effects compared to those reported so far, in fact there are several preclinical studies that indicate a biphasic dose dependent effect of cannabinioid agonists on eating behavior. The administration of high doses of exogenous cannabinoids, natural or synthetic, can lead to a decrease in the amount of food consumed and a consequent slowdown in body growth (Rubino et al., 2008; Scherma et al., 2016; Radziszewska and Bojanowska, 2013).

At the beginning of the present thesis we briefly mentioned about the tendency of adolescents towards risk-taking behavior, and the vulnerability of young people's brain to drugs of abuse. Here below we will go a little bit deeper inside these pivotal topics.

2. Adolescence

Adolescence is a crucial and transitional phase of physical, psychological growth and social development between puberty and legal adulthood, and it is widely defined as a period of time characterized by vulnerability and adjustment (Steinberg, 2005).

Despite of the World Health Organization (WHO) definition by which the adolescence period occurs between ages 10 and 19, its onset has been fasten in the last decades almost worldwide for the occurrence of an anticipated puberty. At the same time though, completion of education, marriage, parenthood, have been shifted the perception of when adulthood actually begins.

For this reason, some researchers prefer a definition of 10-24 years as a better range to describe this life stage (Susan M Sawyer, 2018). The very same definition is also affected by a certain amount of variability according to environmental (i.e. geographic, social, moral) features. In non-western countries for example, it is usually a social event, like marriage, that delineate the beginning of adulthood, while in western countries, more influenced by individualism and independence, the onset of adulthood is marked individually, with financial independence, behavioral self-control, and so forth (Arnett and Taber, 1994). Despite many similarities, also sex differences have to be underlined: adolescence of boys is usually different and longer than that of girls (Schlegel, A., & Barry, 1991).

While keeping into account all these differences, other than the inter-individual genetic variability, it is possible to recognize some common behavioral features that characterize adolescence. Overall, adolescents seem to have less ability than adults in decision making and fixing goals (Byrnes, 2002). It is well known about the tendencies of adolescents toward risk taking when compared with adults (Balocchini et al., 2013), for example for being particularly prone to binge drink, smoke cigarettes, use illegal drugs, violence, and have unintended pregnancy and sexually transmitted diseases (Eaton et al., 2006; Casey and Jones, 2008). Furthermore, as reported by the National Center for Health Statistics, almost the 70% of deaths among 13000 adolescents in the US every year is the result of motor vehicle crashes, unintentional injuries, homicide and suicide (Eaton et al., 2006).

Another common aspect that characterizes adolescence is an increased emotional sensitivity, mainly related to the social environment shift from childhood, being that the time spent with peers become

dominant, at the expanse of adults, which also cause more conflicts between the adolescent and his/her parents (Csikszentmihalyi et al., 1977;Steinberg, 1989). Such greater emotional sensitivity has been linked with a higher incidence in triggering affective disorders (Pine et al., 2001;Steinberg, 2005).

Broadly speaking, poor judgment and scarce ability in decision making during adolescence are deeply linked to the anatomy of the adolescent brain, which is not fully mature at least until 25 years old, with some of the crucial developmental processes that are still continuing into adulthood (Steinberg, 2008; Casey et al., 2008).

Motor and sensory systems are the first to develop, while the regions of the brain involved in advanced tasks, such as spatial orientation, language, and reasoning, develop further on, during late adolescence (Gogtay et al., 2004; Sowell et al., 2004). In addition, while limbic structures, implicated in behaviors like impulsivity and emotions, develop early during adolescence (Giedd et al., 1999), the frontal and the pre-frontal cortex (PFC), involved in cognition and rationality, and that serve to control and inhibit the functions of the limbic system, develop in late adolescence and early adulthood (Lenrott et al., 2010). The progressive increasing activity of the PFC over the years (Rubia et al., 2006), and at the same time a decreased activity of irrelevant brain regions (Durston et al., 2005), show that a linear increase in the cognitive and neurobiological development can be described, underlying the transition from adolescence to adulthood (Casey et al., 2008).

During adolescence, the neurocircuitry is structurally and functionally vulnerable to a huge variety of factors. First of all, increases in sex hormones (estrogen, progesterone, and testosterone) play a major role during puberty and adolescence, leading also to, along with environmental stimuli, radical changes in sex, eating, and sleeping habits (Vigil et al., 2016). Studies on rodents showed the strong impact that reproductive puberty-related hormones have on brain development (Schulz and Sisk, 2006, Sisk and Foster, 2004); with gonadal steroids controlling neurogenesis and neurite outgrowth (McEwen and Alves, 1999), axonal myelination (Yates and Juraska, 2008), and growth of astrocyte processes in white matter (Chowen et al., 2000). The white matter is typically distributed into bundles called tracts that contain myelin-coated axons. Overall, these tracts create neural networks characterized by cortico-cortical and cortico-subcortical connections, important for cognitive and affective functions (Ladoceur et al., 2012; Sturman and Moghddam, 2011). During adolescence there is an increase in the white matter density, due to the process of myelination of the neural fibers, which acquire the feature to better conduct the neuronal signals (Jernigan and Gamst, 2005; Hüppi and Dubois, 2006).

Volumetric longitudinal studies suggest that sex may affect the timing of white matter development (in females starts before and it is slower than that of males), as well as the myelination and

organization of specific white matter tracts, especially with regards to the corpus callosum, whose white matter volume is higher with age in males than females (Ladoceur et al., 2012). Functional magnetic resonance (fMRI) studies show that there is a differential recruitment of prefrontal regions during different stages in life (i.e. childhood, adolescence, and adulthood) during cognitive control tasks (Velanova et al. 2008; Geier et al. 2009); while structural MRI studies provides histological evidences showing that myelination follows a posterior to anterior gradient (Yakovlev and Lecours1967), and there is a decrease in cortical gray matter in the context of white matter development (Gogtay et al. 2004; Giorgio et al. 2009).

In fact, the remodeling phase of the white matter during adolescence occurs in conjunction with changes at the level of the gray matter. The maturation of the brain is also strictly related to the gray matter density and its continuous functional remodeling throughout life. Since from the very first years, grey matter keeps increasing until reaching a plateau around 12-13 years of age, and then begin to reduce during adolescence, that is instead typically characterized by an increased synaptogenesis. Immediately after that, a process of synaptic pruning takes over, in which an elimination of the poorly used synapses occurs, and thus also a functional reduction well known as *'use it or lose it'* (Edelman, 1987).

A lot of the understanding about the neurobiological and behavioral insights of the adolescent brain comes from studies on animal models. Even though the ontogenetic transitions characteristics of adolescence can be found among mammalian species (Spear, 2000), and both rodents and nonhuman primates show a variety of features in common with humans, it has been often argued that only humans deal with stress in adolescence (Bogin, 1994).

Another point of controversy when studying the features of adolescence in animal models, is related to the characterization of the actual adolescent period, which should reflect as much as possible the time window frame of adolescence in humans. As in humans though, also in other mammals is difficult to define the boundaries between puberty, adolescence and early adulthood (Spear, 2000). The age range of postnatal days (PND) 28-42 is considered by some researchers as the prototypic adolescence in the rat (Spear, 2000), one of the most used animal model in research. Nevertheless, depending on the aim of the study, researchers can extend this period for several weeks (until PND 60), or begin a week earlier if interested in the very first precursors of adolescence (Spear, 2000).

From a behavioral point of view, animal models also show to be more curious about engaging risky behaviors (Spear, 2000), as well as having curiosity toward peers affiliation (Primus and Kellogg, 1989); parental conflict (Csikszentmihalyi, 1977; Steinberg et al., 1989; Primus and Kellogg, 1989);

and sensation seeking (Adriani et al., 2004). From a molecular point of view, it has been shown that rodents undergo specific changes in limbic and prefrontal areas during adolescence, and this period of time it is also characterized by a first massive synaptogenesis activity (puberty) and then by a rapid pruning at the end of the adolescence period (Crews et al., 2007) in different brain areas, among which amygdale (Zher et al., 2006), nucleus accumbens (Teicher et al., 1995) and PFC (Andersen and Teicher, 2004). Furthermore, studies on adolescent animal models revealed that dopamine signaling is crucial among these three regions, and that there is an overall fine balance between excitatory and inhibitory dopamine transmission (Floresco and Tse, 2007; Grace et al., 2007).

3. Adolescence and cannabis use

In general, it has been shown that the adolescent human brain morphology changes after use of cannabis, and that includes a reduction in the cerebral sulci of both hemispheres,and a thinning of the cortical thickness at the level of the right frontal lobe, culminating in a slowed brain gyrifacation with consequent anatomical alterations, hemispherical asymmetries, and aberration of the normal cerebral evolutionary course (Mata et al., 2010).

Epidemiological investigation have provided significant piece of information regarding the effects of cannabis use during adolescence over the course of the last decades. One of the most important contribution in literature about adolescent cannabis use is given by a 38-years follow-up longitudinal study performed by Meier and coworkers in 2012 aiming to test the relationship between adolescent cannabis use and neuropsychological decline. The study began in New Zealand in 1972-1973 and involved 1037 participants followed from birth to adulthood (Meier et al., 2012). They found that cannabis use is related to cognitive decline broadly across domains of functioning, such as the intelligent quotient (IQ) and other neuropsychological performances, and that the impairments were particularly severe among early-onset cannabis users, with chronic use associated with grater decline (Meier et al., 2012). Noteworthy, as also reported by other authors in a similar fashion, only participants who began to use cannabis in adolescence, as opposed to adulthood, were affected by the aforementioned cognitive impairments (Meier et al., 2012; Fontes et al., 2011; Pope et al ., 2003).

Other studies performed on adolescents arrived at partial different conclusions, specifically pertinent with non-acute impact of cannabis use. Pope and coworkers for example reported that only current and heavy cannabis users showed impairments, but exclusively in verbal memory compared to controls, and only 1 and 7 days after abstinence, not after 4 weeks (Pope et al., 2001). Fried and colleagues found that only heavy cannabis users showed declines in IQ, memory and processing speed at ages 17-20 years in comparison to their baseline performance at ages 9-12 years (Fried et al., 2005; Fried et al., 2002), and seemingly Gonzalez et al. found a modest significance concerning only measures of episodic memory (Gonzalez et al., 2012). Adverse effects on learning, visual cognitive processes and attention that can persist for more than a month have also been reported (Harvey et al., 2007; Medina et al., 2007; Tapert et al., 2002; Fried et al., 2005).

All these investigation add important insights to the work published by Meier et al., underlying how adverse cannabis effects can be specific only to some cognitive functions, and not to only general IQ measures; and that the length of the abstinence period might be decisive to observe either persistence or recovering of the cognitive deficits (Gonzalez et al., 2012).

The use of marijuana in adolescence, and especially before the age of 15, has been also associated with the appearance of mood disorders (Fergusson et al., 2003) and in an increased risk of developing depression, anxiety (Hayatbakhsh et al., 2007; Henquet et al., 2006; Rey et al., 2004; Van Laar et al., 2007), and in the tendency of having suicidal thoughts (Moore et al., 2007; Van Ours et al., 2013). It is also known how cannabis exposure during adolescence can generate both acute and long-term psychotic-related disorders (Ferdinand et al., 2005; Henquet et al., 2005; Le Bec et al., 2009) and may induce a worsening of symptoms in patients already affected by these disorders (Levine et al., 2017).

Despite of all this, and as previously mentioned, a survey published by Johnston and colleagues in 2012, among secondary school students in the United States, underlined a very crucial point: the use of cannabis increased from 22% in 1992 to 40% in 2011, concurrently with a decrease in the perceived risk of regular use from 80% to approximately 45% (Johnston et al., 2012), with males significantly more likely to rate some of the harms associated with cannabis use as less risky, and reporting higher rates of cannabis use (Hellemans et al., 2019). If these trends of adolescent onset, as well as the less risk perceived of persistent cannabis use, and public acceptance will continue, a larger segment of the population is likely to be inclined matching these criteria in the future. In response to this, the degree of control for mental health and education is pivotal to address the issue of early onset and regular use of cannabis (Gonzalez and Swanson, 2012). Education is of course necessary but probably not sufficient to stop chronic marijuana users, as suggested by similar legalized drugs with adverse effects (tobacco and alcohol). Thus, a combination between prevention and intervention is needed, with politics that should be aware and strongly informed to deal better with adolescent drug abuse, at least delaying to adulthood the permission of cannabis use (Meier et al., 2012; Gonzalez and Swanson, 2012).

Although epidemiological studies suggest that cannabis use during adolescence might lead to an increased risk for developing psychotic-related symptoms during adulthood, human-based data are not conclusive, basically due to their heterogeneity. Therefore, the use of animal models in their adolescent phase is useful to study the impact of cannabis use on the adolescent brain development and the claimed vulnerability to later develop psychotic disorders (Rubino and Parolaro, 2014).

We described how cannabis exposure in adolescence affects levels and activity of the endocannabinoids and their interactions with the cannabinoid receptors, which will eventually impair the physiological activity and the neuronal refinement in pivotal brain areas, disrupting the delicate balance provided by the endocannabinoid system.

To date, preclinical data on this topic are relatively rare, but some of them seem to support epidemiological evidence, suggesting that adolescent cannabinoid exposure may trigger a complex behavioral phenotype at a later stage in life (Rubino and Parolaro, 2014).

For instance, it has been observed that rats treated with cannabinoids in adolescence show a reduced social interaction, indicative for anxiety, (Leweke and Schneider, 2011; O'Shea et al., 2004 and 2006; Realini et al., 2011) as well as altered levels of anhedonia (Bambico et al., 2010; Rubino and Parolaro, 2008; Realini et al., 2011), which connote a depressive-like behavioral framework. In fact, molecular changes related to depression have been reported, such as the disregulation in the levels of the transcription factor CREB and in the normal activity of the CB1 receptor in areas such as PFC, nucleus accumbens and amygdala (Rubino et al., 2008; Realini et al., 2011).

The exposure to cannabinoids in adolescence but not in adulthood, has also been shown to induce deficits in spatial and working memory (O'Shea et al., 2004 and 2006; Quinn et al., 2008; Realini et al., 2011;). These deficits correlate with intraneuronal alterations such as the decrease of some synaptic plasticity markers; the expression levels of NMDA glutamatergic receptor in the PFC and the hippocampus; and the density of dendritic spines in the dentate gyrus of the hippocampus (Rubino et al., 2009).

4. The Gateway Hypothesis and cannabis as a gateway drug

4.1. Epidemiological evidence

We underlined how the effects of cannabis use on mental health and adjustment have been largely investigated over the past decades (Macleod et al., 2004) also in strict relationship with implications for public policy. One of the most relevant topic about cannabis use is related to the "Gateway Hypothesis", an epidemiological theory introduced for the first time in 1975 by Denise Kandel, postulating that the consumption of a lighter drug (such as cannabis) during adolescence increases the probability to consume illicit and harder drugs (i.e. cocaine, heroin, metamphetamine) at a later point in life (Kandel D., 1975).

In the seminal paper of Denise Kandel dated 1975, she observed that the first way to consumption always concerned the legal substances, such as alcohol and tobacco. Furthermore, cannabis was a crucial intermediate in the transition from legal to illegal substances (**Fig.4**). So the regular sequence of phases described by Kandel provided the knowledge that the use of alcohol and cigarettes very often preceded the use of cannabis which in turn may lead to approach other illegal drugs such as psychedelics, cocaine and heroin (Kandel, 1975). These evidences though, only indicated that people who used one drug might have an increased chance of progressing to the use of another drug, but no causal relationship can be claimed from these findings.

Fig.4 – *Sequential temporal pattern of drug use according to the Gateway Hypothesis(©Noel and Wang, 2018)*

In general, mixed results can be found in literature showing either a link or sequence of licit drug use to illicit drug use (Guxens et al., 2007, Korhonen et al., 2010, Lessem et al., 2006, Mayet et al., 2012) or no association (Mackesy-Amiti et al., 1997, Golub and Johnson, 1994).

Afterwards, Kandel and others expanded the studies on the Gateway Theory first investigating what are the predictors of progression (Yamaguchi and Kandel, 1984; Kandel, 1992) and what are the sex behavioral differences, showing that whereas progression to illicit drugs among men is dependent upon prior use of alcohol, among women either nicotine or alcohol is a sufficient condition for progression to cannabis (Kandel, 1992); and then authors start in particular to concentrate their

efforts on the Cannabis Gateway Hypothesis, considering cannabis as one of the main gateway substances that play a pivotal role in the succession of substances of abuse.

Recent statistics published in 2018 by SAMHSA give us an important estimation that 118.2 million Americans aged 12 and older have used cannabis at least once. Among those who have used cannabis, 32%also used cocaine, 12% have used methamphetamines, and 4% have used heroin, even if only 0.3 % cannabis users have abused heroin,0.2% have abused cocaine, and 0.1% have abused methamphetamines later in life.

Human-based studies showed that the frequency of one's cannabis use during adolescence had a statistically significant association with other illicit drug use and cannabis dependence in adulthood (Silins et al., 2014; Fergusson et al., 2008; Van Gundy et al., 2010; Mayet et al., 2012; Mayet et al., 2016), or the association is present only if childhood adversities are experienced (Melberg et al., 2010) or for particular genetic predispositions (Grant et al., 2010; Harrington et al., 2008).

At the methodological level, when Kandel published the first studies on the Gateway Hypothesis, she identified the sequences and stages of drug use as chain of events that allow to characterize three epidemiological features: 1) the concept of sequence that fixes the relationship between two substances, where the consumption of a substance A always precedes the consumption of another substance B; 2) the concept of association, for which the consumption of a substance A very often increases the probability of starting the consumption of a second substance B (Collins, 2002; Fergusson et al., 2006); 3) the evidence of causation (Kandel, 2006).

Nevertheless, many authors pointed out that some environmental elements may not allow to establish a full causal relationship between cannabis use and the use of other illicit drugs, or in an earlier stage, between nicotine or alcohol and cannabis. These confounding components are related to the genetic predisposition of the individual; to social factors, such as the fact that the affiliation of cannabis users is to the same black market that provides both cannabis and the second drug; and to the environmental aspect in which cannabis consumers are imbedded, characterized by an illicit drug subculture that push toward attitudes that lead to the propensity to experiment illicit drugs (Hall and Lynskey, 2005). Moreover, not only causation, but human-based studies also do not provide any insights regarding what are the molecular mechanisms underlying drug use progression.

For these reasons, over the last years has been necessary to use a translational approach from epidemiology to molecular biology (i.e. from an observational to an experimental approach). Results obtained from animal models are thus pivotal and can then inform back and help epidemiology to better characterize the Gateway Hypothesis.

Although the preclinical findings may not translate to human subjects (Perel et al., 2007), unlike human-based studies, animal-based ones allow researchers to create controlled environments to test the effects of cannabis use on while reducing or eliminating confounding factors. Thus, the importance of these studies resides in the ability to investigate drug use progression independently of any social or legal constraints that regulate and defines drug use. Furthermore, animal models also allow to alternate specifications of the specific sequential order of drug presentation, that becomes the only experimental determinant of outcome, with other factors (such as the relative availability of different substances), loosing relevance (Kandel et al., 2002)

4.2. Preclinical evidence

For instance, preclinical studies investigating a variety of cannabinoid agonists, have shown that they are able to modulate the reinforcement effects (Caillé and Parsons, 2003; Navarro et al., 2001; Norwood et al., 2003; Solinas et al., 2005), and the motor effects (Cadoni et al., 2001 ; Gorriti et al., 1999; Lamarque et al., 2001; Muschamp and Sivy, 2002; Pontieri et al., 2001) of substances such as opioid agonists and amphetamine. Rodríguez-Arias and colleagues (2010) observed how the exposure to WIN in adolescence increases the reinforcing effects of MDMA in the Conditioned Place Preference (CPP) test, and Scherma and colleagues (2016) reported that the exposure in adolescence to THC increases the reinforcing effects of WIN in adults, with the self-administration model.

Insights regarding an interaction between the endocannabinoid system and the effects of cocaine have been also provided. For example, it has been observed that CB1 receptor knockout mice shows a reduction in cocaine self-administration (Soria et al., 2005), and the state of anxiety related to the effects of cocaine is modified by a pre-exposure to THC (Panlilio et al., 2007). Furthermore, adolescent rats treated with the synthetic cannabinoid WIN were observed to amplify cocaine withdrawal symptoms, including anxiety and depressive related behaviors (Aguilar et al., 2017). Pre-treatment in adolescence (but not in adulthood) with THC was also found to generate a cross-sensitization to the cocaine motor-related responses (Dow-Edwards and Izenwasser, 2012), and an exposure to the synthetic cannabinoid CP 55.940 in adolescence results in an increase in cocaine self-administration in female rats (Higuera-Matas et al., 2010).Moreover, WIN exposure in adolescence was found to reduce the levels of eIF2α and other key transcription factors, in the nucleus accumbens, and to induce cocaine motor cross-sensitization in rats (Melas et al., 2018).

Other authors have shown that the use of some substances during adolescence, such as nicotine (Collins and Izenwasser, 2004; McQuown et al., 2007), MDMA (Aberg et al., 2007; Achat-Mendes et al., 2003; Daza-Losada et al., 2008) and alcohol (Ledesma et al., 2017), are all able to modify the response to the effects of cocaine.

Also the relationship between cannabis and subsequent use of opioids is relevant. For instance, it was found that the effects of THC exposure on the rats' heroin use varied with genetic background (Cadoni et al., 2001), and rats exposed to THC during adolescence voluntarily used more heroin doses and greater amounts of heroin during adulthood (Ellgren et al., 2007). The same authors also showed in a more recent investigation, that THC-induced changes in the endogenous cannabinoids at the level of specific brain regions provided additional evidence of increased opioid reward-related behavior after adolescent THC exposure (Ellgren et al., 2008).

Preclinical investigation, even if they are not exhaustive nor sufficient to confirm the presence of a causal link of different drug use, have provided important data showing the ability of some substances of abuse, including cannabinoids, to induce neurobiological alterations, especially in adolescence. Nevertheless, further molecular experiments should be addressed to prove this relationship.

AIMS OF THE STUDY

As previously described, the endocannabinoid system plays a crucial role in developmental processes, such as synaptic plasticity and pruning during adolescence. Nevertheless, the abuse of cannabis or synthetic cannabinoids leads to interference with endocannabinoid signaling, and to molecular and epigenetic changes that remodel the vulnerable adolescent brain in a way that it becomes more sensitive to addictive substances. The endocannabinoid system also plays an overall modulatory role for the brain reward circuitry, and represents the primary site of action for the reinforcing and rewarding effects not only of cannabinoids, but also of psychostimulant drugs, including cocaine (Tanda, 2007).

We also mentioned how the observation of a regular pattern of involvement across different classes of drugs, collocates the Cannabis Gateway Hypothesis as one of the best replicated findings in the epidemiology of drug use. But although epidemiological studies have established the sequence between cannabis and other substances and specified their association, they are not able to establish causal progression nor can they identify the underlying cellular and molecular mechanisms that could contribute to the Gateway sequence of drug use.

For these reasons, in the present study we bridged the epidemiology of drug abuse and molecular biology by developing a rat model of this epidemiological sequence to explore the behavioral, physiological and molecular mechanisms underlying the gateway sequence transition from cannabis to cocaine. Cocaine was chosen for its huge relevance and impact in our society, and for being the second most used illegal drug worldwide especially among adolescents. Importantly, cannabis use in adolescence is known to be an important risk factor for starting cocaine consumption, and to predict cocaine dependence (Wit and Phillips, 2012).
Some preclinical investigation paved the way to our study establishing the features underlying the interplay between cannabis and cocaine use. For instance, it was found that cannabinoids are able to cross-sensitize to cocaine (Melas et al., 2018; Dow-Edwards et al., 2012; Kononoff et al., 2018); induce irritability-like behavior (Kononoff et al., 2018); increase the acquisition of cocaine self-administration (Higuera-Matas et al. 2010; Friedman et al., 2019), and alter cocaine-related withdrawal symptoms (Aguilar et al., 2017) in rats.

Background and aims of the study

The rationale behind this project comes from a prior collaboration between our group and Professor Denise and Erik Kandel's group of the Columbia University (New York).

In this preliminary investigation (Melas et al., 2018), *in vitro* studies were performed in neuronal-like cell cultures, showing how a prolonged exposure to WIN gives rise to a significant increase in the levels of the non-phosphorylated form of eIF2α. The latter is a crucial transcription factor involved in the regulation of protein synthesis, and the decreased levels of its p-form has been found to account for adolescent drug sensitivity (Huang et al., 2016). Furthermore, single-cell RNA sequencing showed that Gadd34 gene (Ppp1r15a) expression was increased, and its protein product, GADD34, acts as a scaffold protein recruiting the protein phosphatase 1 (PP1), which eventually induces a dephosphorylation of p-eIF2α.

According to the overall model the authors proposed (**Fig. 6**), this enhanced GADD34 levels is caused by the acetylation modifications (H3K27ac) along the entire Gadd34 gene (indicating an increased transcription), and this acetylation is caused in turn by the CREB-binding protein (CBP) (an enzyme with histone acetyl transferase activity active on the lysine residues of histone 3) increase at its nuclear location compared to the cytoplasm, and it was linked in general to the ERK/CREB signaling. Histone acetylation modifications are in fact one type of the possible epigenetic changes induced by drugs of abuse. Epigenetic changes are due to alterations in gene expression that are transmitted across generations and that take place without a modification in the DNA sequence, but are due to modulation of chromatin associated factors by environmental effects (Heinbockel and Csoka, 2018). Cannabinoids are well known to induce such epigenetic changes (Watson et al., 2015; Szutorisz et al., 2018; Prini et al., 2018).

To address whether WIN can induce similar effects *in vivo*, adolescent (PND 42) and adult (PND 77) male Sprague Dawley rats were treated sub-chronically with WIN or saline for 11 consecutive days at increasing doses, with intraperitoneal injections. It was found that WIN led to a significant decrease in p-eIF2α levels at the level of the nucleus accumbens (NAcc), and only in adolescent rats. In line with the *in vitro* data, they found a significant upregulation of p-ERK 1 and 2; a trend toward upregulation of p-CREB; and an increase in the nuclear/cytoplasmic localization of CBP. On contrary, GADD34 levels showed a trend toward decrease.

At this point, it was investigated whether the WIN-induced decrease in p-eIF2α levels in the adolescent NAcc could be associated with the gateway drug properties of cannabinoids. For this purpose, they used the same sub-chronic WIN treatment, and afterwards, to assess whether the cannabinoid is able to affect the vulnerability to the use of other drugs of abuse, such as psychostimulants, 24 hours after the last WIN administration animals were challenged with 10 mg/kg cocaine intraperitoneally (i.p) and assessed with locomotor cross-sensitization (**Fig. 5**).

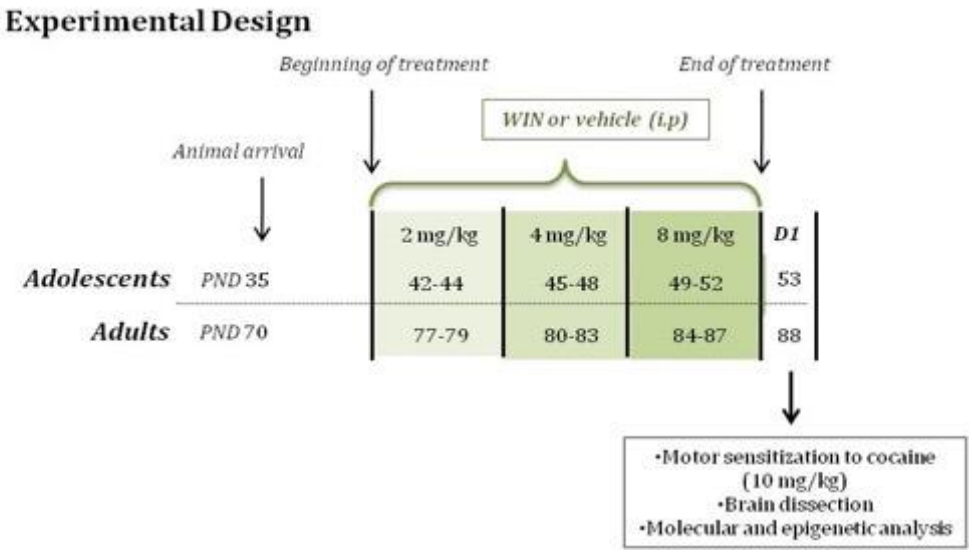

Fig. 5 –*Overview of the experimental protocol used by Melas et al., 2018*

Drug sensitization is defined as the increased effect of drug following repeated doses (the opposite of drug tolerance), and as a relatively simple manifestation of learning and memory that refers to an increase in the strength of a response to a stimulus induced by past experiences with the same or related stimuli (Robinson and Berridge, 2003; Stewart and Badiani, 1993). The cross-sensitization happens when the stimulus (i.e. one particular drug) is generalized to a related stimulus (i.e. another drug), resulting in the amplification of a particular response (Yang et al., 2011).

Behavioral sensitization can be divided into two main classes of effects: incentive-motivational effects and excito-motor effects (Robinson and Berridge, 2003). Cannabioids (both THC and WIN) represent a class of substances of abuse that are widely demonstrate to be able to produce behavioral cross-sensitization to other substances, such as morphine (Cadoni et al., 2001, 2008); heroin (Pontieri et al., 2001); amphetamines (Lamarque et al., 2001) and cocaine (Dow-Edwards and Izenwasser, 2012). Importantly, the sensitization phenomenon is strictly correlated to the increase in the activity of dopaminergic neurons at the level of the VTA and the substantia nigra pars compacta, which in turn leads to an increase in the dopaminergic activity at the level of the NAcc (Robinson and Berridge, 1993; Kalivas and Stewart, 1991). Thus, the mesolimbic dopaminergic system is crucial for the sensitization phenomenon.

Melas et al. (2018), found that adolescent WIN pre-treated rats showed cocaine-induced motor cross-sensitization compared to controls. And this effect was not observed in adult rats.

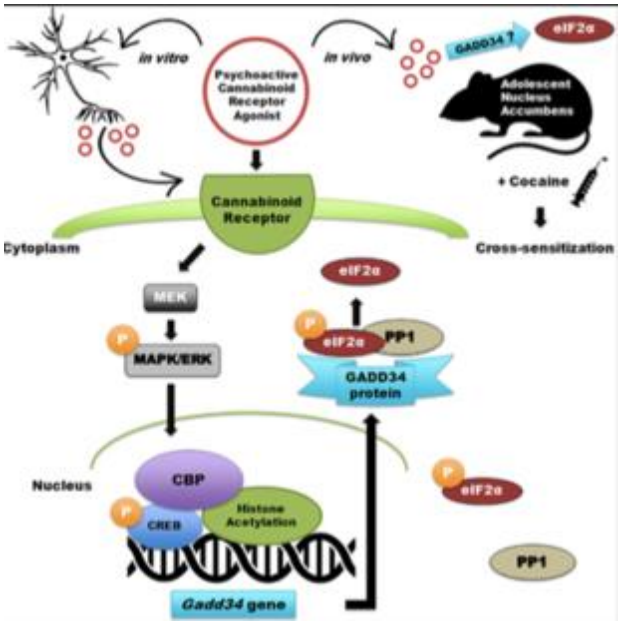

Fig. 6 –*Model proposed by Melas et al., 2018 showing how chronic treatment with WIN is able to cause cross-sensitization to cocaine and decrease of p-eiF2a levels (©Melas et al., 2018)*

Considering the aforementioned studies and findings as a starting point for the advancement of our research, we next investigated the effects of the chronic exposure to WIN in both adolescent and adult rats to evaluate:

1) the long-term persistence of the observed cross-sensitization to cocaine in adolescence, assessed with the *locomotor activity test* after a period of drug abstinence. Since the mechanism that increases the basal response to cocaine is a consequence of changes in the neurophysiological substrate that are determined in the induction phase (exposure to WIN), we asked whether the same cocaine-induced behavioral motor response can still be observed after a prolonged period of suspension of the cannabinoid pre-treatment.

2) whether molecular and epigenetic changes are associated with the behavioral cross-sensitization in adolescence found after a period of drug abstinence. For this purpose, in collaboration with the Columbia University, we performed *western-blot* experiments to assess the levels of the most relevant molecules.

3) since there is a correlation between the sensitization phenomenon and an increase in the activity of dopaminergic neurons of the nucleus accumbens, what are the dopamine dynamics at the level of this brain region. To this purpose, *fast-scan cyclic voltammetry* was used to monitor electrically-

evoked dopamine release in the accumbens shell of anesthetized rats. This part of the study was conducted at the National Institute On Drug Abuse (NIDA, NIH) of Baltimore, thanks to a collaboration that our group held with Dr. Gianluigi Tanda;

4) the long-term persistence of motor cross-sensitization after repetitive cocaine administrations, since substance use disorders are triggered by repeated exposures that involve drastic epigenetic and synaptic modifications. For this purpose, we performed the *locomotor activity test* after 4 days straight of cocaine treatment. Furthermore, we evaluated after the same repetitive cocaine administrations the possible positive reinforcement effects using the *conditioned place preference test.*

5) the bidirectionality of behavioral sensitization, using the *locomotor activity test*. Since the order of consumption between two drugs characterize patterns of drug use progression that lead to different molecular and behavioral outcomes, we asked whether, reversing our drug administration protocol, adolescent rats chronically treated with cocaine can cross-sensitize to WIN after a period of abstinence.

Overall, the present thesis aims to provide new insights on the behavioral and molecular aspects underpinning the gateway theory. Secondly, since cannabis use for both medical and recreational purposes is increasing throughout the world, this investigation aims to give a contribution to the literature of the field to advice policymakers to efficiently address the subtle issue of cannabis legalization.

MATERIAL AND METHODS

Animals

Male Sprague-Dawley rats (pre-natal day (PND)35 [adolescents] and PND70 [adults]; ENVIGO, Italy) were housed 5 per cage in a climate-controlled animal room (21°C ± 2°C, 60% humidity) under a reversed 12-hr light/dark cycle (lights on at 07:00 a.m.), with *ad libitum* access to water and food. All procedures and experiments were carried out in an animal facility according to Italian (D.L. 26/2014) and European Council directives (63/2010) and in compliance with the approved animal policies by the Ethical Committee for Animal Experiments at the University of Cagliari (Sardinia, Italy) and the Italian Department of Health (881/2016-PR). All possible efforts were made to minimize animal pain and discomfort, as well as to reduce the number of experimental subjects.

Drugs

WIN55,212-2 mesylate (Tocris, Tocris Bioscience, UK) was dissolved in 2% Tween-80, 2% ethanol, 96% saline and injected intraperitoneally (i.p.) in a volume of 1 ml/kg of body weight. Cocaine hydrochloride (MacFarlan Smith Ltd., Edinburgh, UK; and Mallinckrodt, Saint Louis, MO) was dissolved in 0.9% sodium chloride solution and was injected i.p. in a volume of 1 ml/kg of body weight.

Treatments

Rats were acclimated to the colony for 1 week before starting treatment with WIN or vehicle. Increasing doses of WIN (2 mg/kg, 3 days; 4 mg/kg, 4 days; 8 mg/kg, 4 days) or vehicle were given twice/day for 11 consecutive days during mid-adolescence (PND 42–52), or adulthood (PND 77–87). As previously described, this WIN dose regimen has been shown to induce psychomotor cross-sensitization to the effects of cocaine in adolescence (Melas et al., 2018; Kononoff et al., 2018). After a week of WIN abstinence, experiments were started (an overview of the protocol is shown in **(Fig. 7)**:

-for voltammetry experiments, rats received an acute injection of saline or cocaine (10mg/kg i.p.; see timeline in **Fig. 8**) ;

-for locomotor sensitization experiments, rats received one saline or cocaine (10mg/kg i.p.) injection daily for 4 days and underwent locomotor testing 30 minutes before and immediately after the last injection;

-for conditioned place preference experiments, rats received one saline or cocaine (10mg/kg i.p.) injection daily during the conditioning session (see below).

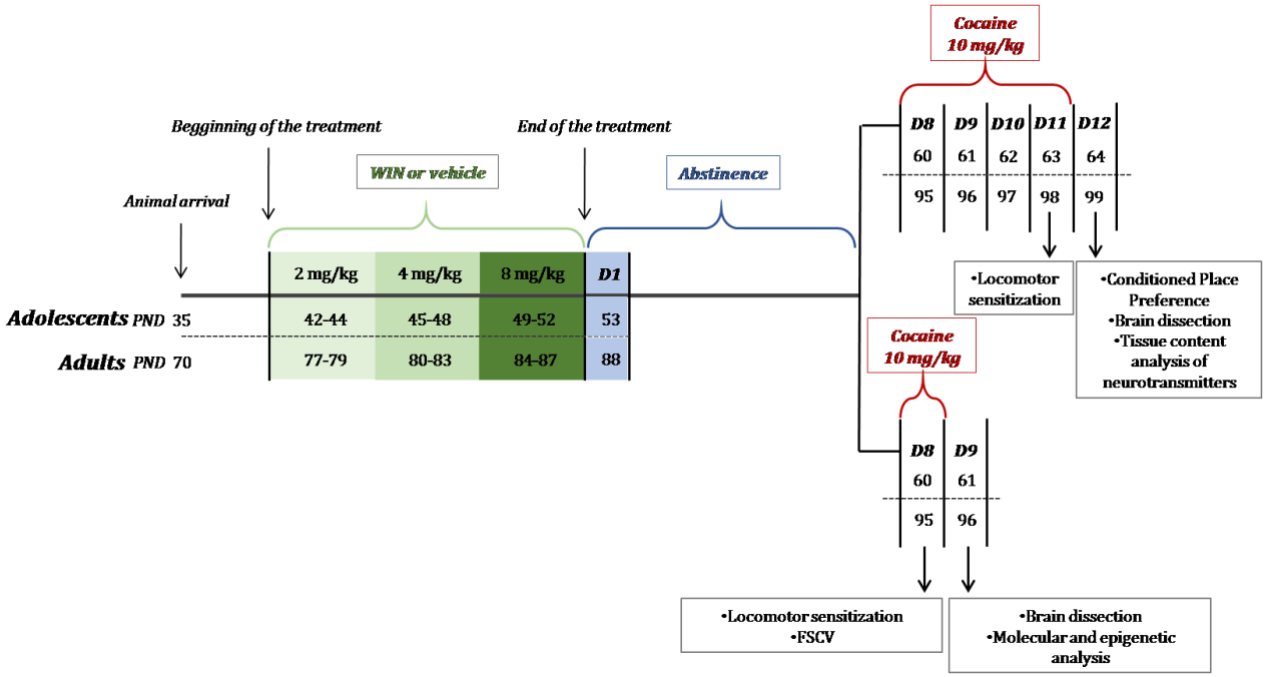

Fig.7- ***Schematic overview of the drug administration protocol and timeline of all the experiments performed.*** *D1 = WIN abstinence day 1. For both FSCV and conditioned place preference experiments only adolescent animals were used. Brain dissection for molecular analysis or tissue content analysis of neurotransmitters have been performed 24 hours after the cocaine challenge and locomotor activity test. No brain dissection was performed after FSCV and conditioned place preference experiments.*

Food Intake and Body Weight

At the beginning of vehicle or WIN treatment, both adolescent and adult rats were divided into two groups matched for body weight and food intake. These two parameters were monitored throughout the entire treatment. As animals were housed five per cage, chow amounts consumed per animal per day were averaged by dividing the amount per cage by five.

Locomotor sensitization

A total number of 40 adolescent and 40 adult rats were utilized for this experiment. Each experimental group was randomly divided into 4 sub-groups (n = 10 per sub-group): vehicle +

saline; vehicle + Cocaine; WIN + saline, WIN + cocaine. Each rat was individually tested for locomotor activity using the Digiscan Animal Activity Analyzer (Omnitech Electronics, USA) in a room dimly illuminated by a neon lamp (50 Lux) as previously described (Melas et al, 2018; Scherma et al, 2020).The boxes were composed of transparent Plexiglas cages (42 cm × 30 cm × 60 cm) fitted with two sets of 16 photocells located at right angles to each other, projecting horizontal infrared beams 2.5 cm apart and 4 cm above the cage floor and a further set of 16 horizontal beams which height could be adapted to the size of the animals. The rats were moved to the experimental room with controlled conditions 1 h previous to the beginning of the experiments. Distance travelled and time in the center were recorded 30 minutes before and 60 minutes after each i.p. challenge with cocaine 10 mg/kg, in 10 min intervals. Between one session and the other, the walls of the apparatus were carefully cleaned with H2O2 in order to leave any possible olfactory trace.

Western blotting

Rats were sacrificed and brains quickly removed 24 hours after the cocaine injection and locomotor sensitization experiments at abstinence day 9. Protein concentrations were measured using the Pierce™ Detergent Compatible Bradford Assay Kit(Thermo Scientific) and equal amounts of sample were run on 4–20% Mini-PROTEAN® TGX™ PrecastProtein Gels (Biorad; Bio-Rad Laboratories Inc., Hercules, CA, USA), and after that transferred to Immobilon-FLPVDF membranes (EMD Millipore; EMD Millipore Corp., Billerica, MA, USA) as standard Western blotting practices require. Following primary and secondary antibody incubations, including appropriate washes, protein bands of interest were visualized using a fluorescent detection system(Odyssey Classic; LI-COR, LI-COR Biotechnology, Lincoln, NE, USA). For blot re-probing, the membrane was stripped using the Restore™ Fluorescent Western Blot Stripping Buffer (Thermo Scientific). The primary antibodies used were the following: acetyl-H3K27 (#39133, Active Motif; Active Motif, Inc.,Carlsbad, CA, USA), acetyl-H4K5-K16 (#06-866, EMD Millipore), alpha α-tubulin (sc-5286, Santa Cruz), beta β-actin (ab6276, Abcam; Abcam PLC, Cambridge, UK), beta III β-tubulin (ab52901, Abcam), eIF2α (#2103, Cell Signaling; Cell Signaling Technology, Inc., Danvers, MA, USA), ERK 1/2 (sc-514302, Santa Cruz; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), HDAC6 (#7612, Cell Signaling), histone H3 antibody (ab10799, Abcam), histone H4 (ab17036, Abcam), phospho eIF2α (Ser51) (#9721, Cell Signaling), phospho H3S10 (#9791, Cell Signaling), trimethyl H3K4 (#9751, Cell Signaling). Images were acquired and targets were quantified using the Image Studio™ Software (LI-COR).

Global quantitative phosphoproteomics by mass-spectrometry

For TMT-based phosphoproteomics, frozen PFC tissues were lysed by bead-beating in 9 M urea and 200mM EPPS (pH 8.5), supplemented with protease and phosphatase inhibitors. Samples were reduced with 5 mM TCEP and alkylated with 10 mM iodoacetamide (IAA) that was quenched with 10 mM DTT. Atotal of 300 μg of protein was chloroform−methanol precipitated. Protein was reconstituted in 200 mMEPPS (pH 8.5) and digested by Lys-C overnight and trypsin for 6 h, both at a 1:50 protease-to-peptide ratio. Digested peptides were quantified using a Nanodrop at 280nm and 200 μg of peptide from each sample were labeled with 800 μg TMT reagent using 10-plex TMT kit (16). TMT labels were checked, 2 μg of each sample was pooled, desalted and analyzed by short SPS-MS3 method, and using normalization factor, samples were bulk mixed at 1:1 across all channels and desalted using a 200 mg Sep-Pak solid-phase extraction column. Desalted peptides were enriched for phosphopeptides using a mixture of MagReSynTi-IMAC and Zr-IMAC resins according to the manufacturer's instructions (ReSyn Biosciences; ReSyn Biosciences Ltd, Edenvale, Gauteng, South Africa). In brief, 2 mg of labeled peptide were dissolved in 1 ml of binding buffer (80% Acetonitrile, 1M glycolic acid and 5% TFA) and incubated with equilibrated 300 μl (150 μl of each Ti-IMAC and Zr-IMAC) resins at room temperature for 30 min, and the resin was washed 3 three times to remove the unbound, non-phosphorylated peptides.

Phosphopeptides were eluted using 1% ammonium hydroxide. The enriched phosphopeptides were further fractionated in nine fractions using Pierce™ High pH Reversed-Phase Peptide Fractionation Kit (Thermo Scientific) and each fraction dried down in a speed-vac. Dried phosphopeptides were dissolved in 10 μl of 3% acetonitrile/ 0.1% formic acid injected using MSA-SPS-MS3 and NL SPS-MS3 methods. The UltiMate™ 3000 RSLCnano system (Thermo Scientific) and EASY Spray™ source (Thermo Scientific) with Acclaim™ PepMap™100 2 cm x 75 μm trap column (Thermo Scientific) and EASY-Spray™ PepMap™ RSLC C18 50 cm x 75 μm ID column (Thermo Scientific) were used to separate fractioned peptides with a 5-30% acetonitrile gradient in 0.1% formic acid over 45 min at a flow rate of 250 nL/min. After each gradient, the column was washed with 90% buffer B for 10 min and re-equilibrated with 98% buffer A(0.1% formic acid, 100% HPLC-grade water) for 40min. For the phosphopeptide analysis, two methods were used for each fraction. For both methods, the full MS spectra were acquired in the Orbitrap at are solution of 120,000. The 10 most intense MS1 ions were selected for MS2 analysis. Following

acquisition of each MS2 spectrum, a synchronous-precursor-selection (SPS)-MS3 scan was collected on the top 10 most intense ions in the MS2 spectrum. The isolation width was set at 0.7 Da and isolated precursors were fragmented using two methods. In the first method, we used collision induced

dissociation (CID) at a normalized collision energy (NCE) of 35% with MultiStage Activation (MSA), and in the second method with NL-triggered MS3 using higher energy collision induced dissociation (HCD) at anormalized collision energy (NCE) of 35%. In both cases, following acquisition of each MS2 spectrum, asynchronous precursor selection (SPS) MS3 scan was collected on the top 10 most intense fragment ions in the MS2 spectrum. SPS-MS3 precursors were fragmented by higher energy collision-induced dissociation (HCD) at a NCE of 65% and analyzed using the Orbitrap. Raw mass spectrometric data were analyzed using Proteome Discoverer 2.2 to perform database search and TMT reporter ions
quantification. TMT tags on lysine residues and peptide N termini (+229.163 Da) and the carbamidomethylation of cysteine residues (+57.021 Da) was set as static modifications, while the oxidation of methionine residues (+15.995 Da), deamidation (+0.984) on asparagine and glutamine and phosphorylation (+79.966) on serine, threonine, and tyrosine were set as a variable modification. Data were searched against a UniProt rat with peptide-spectrum match (PSMs) and protein-level at 1% FDR. The signal-to-noise (S/N) measurement for each protein was normalized so that the sum of the signal for all proteins in each channel was equivalent to account for equal protein loading. The total number of phosphomodifications identified was 8,691 (Dataset S9; fifth worksheet) and the Qlucore Omics Explorerpackage was used to perform multi-group statistical analysis (Qlucore, Lund, Sweden).

Fast Scan Cyclic Voltammetry (FSCV)

A total number of 14 adolescent rats were used for this experiment. All the surgery procedures reprise the protocol used by Tanda and colleagues in previous studies (Keighron et al., 2019). Both vehicles and WIN pre-treated animals were anesthetized with 1.1 g/kg (i.p.) urethane in sterile saline and received boosters of one third the original dose, not before 10 minutes from the first booster, and until completely anesthetized. Rats were delicately placed in a stereotaxic apparatus where the dura was exposed by drilling four small holes. Animals were then implanted with a bipolar tungsten stimulation electrode (SE) [posterior -4.6 mm, and lateral ±1.0 mm from bregma, and ventral -6.5 from dura (Paxinos and Watson, 1998)]; whose proper functioning was tested by delivering a train of 24 pulses of 180 μA, 60 Hz, 4 ms in duration which caused a detectable movement of the whiskers. The SE is placed at the level of the medial forebrain bundle (MFB). A carbon-fiber microelectrode (CFME) working electrode (anterior +1.7, lateral ±0.8), was carefully and slowly lowered (ventral -6.8 to -8.0 from dura) in the ipsilateral hemisphere, while testing the dopamine (DA) response to electrical stimulus, until reaching our ideal final position (i.e. the nucleus accumbens shell, NAcc) where a robust DA signal was found. Furthermore, an Ag/AgCl

reference electrode was implanted in the contralateral hemisphere. At the end of the fast-scan cyclic voltammetry (FSCV) experiment, and with the purpose of making sure that the working electrode was in our desired brain region (NAcc shell), the electrode was marked by applying 10 V cathodically for 30 s, generating a lesion, well recognizable in the histological investigation.

FSCV electrochemistry

DA was detected using a cylindrical glass sealed CFME (Huffman and Venton, 2009). The latter was created by enclosing a carbon fiber (0.007 mm diameter; Goodfellow Cambridge Limited, Huntingdon, England, UK) in a borosilicate glass capillary tube (1.2 mm o.d.; A-M Systems, Sequim, WA, USA) and then pulled to a tapered point with a micropipette puller (Narishige, Tokyo, Japan). The carbon fiber was cut, extending 100 μm past the tapered end, and pre-calibrated in vitro with known concentrations of DA, in the days prior the day of the experiment. DA was identified via FSCV with a triangular waveform scan from -0.4 to 1.3 V at 400 V/s with a holding potential of -0.4 V. During the experiment, a stimulus of 24 pulses of 180 μA, 60 Hz, 4 ms in duration was applied each five minutes throughout the entire period of recording, which was characterized by a 15 minutes period of baseline, a subsequent 15 minutes period of saline, and by at least a 2 hours period (or until the DA signal significantly decaded) of cocaine (10 mg/kg, i.p.), applied to both vehicles or WIN pre-treated rats **(Fig. 8).**Data was collected using a UEI potentiostat and breakout box running Tarheel-CV (University of North Carolina, Chapel Hill electronics shop) using a pair of DigitimerNeurolog NL800A (Digitimer North America LLC, Ft. Lauderdale, Fl. USA) current stimulus isolators to control the stimulation. The presence of DA was confirmed by cyclic voltammogram produced by the peak after stimulation.

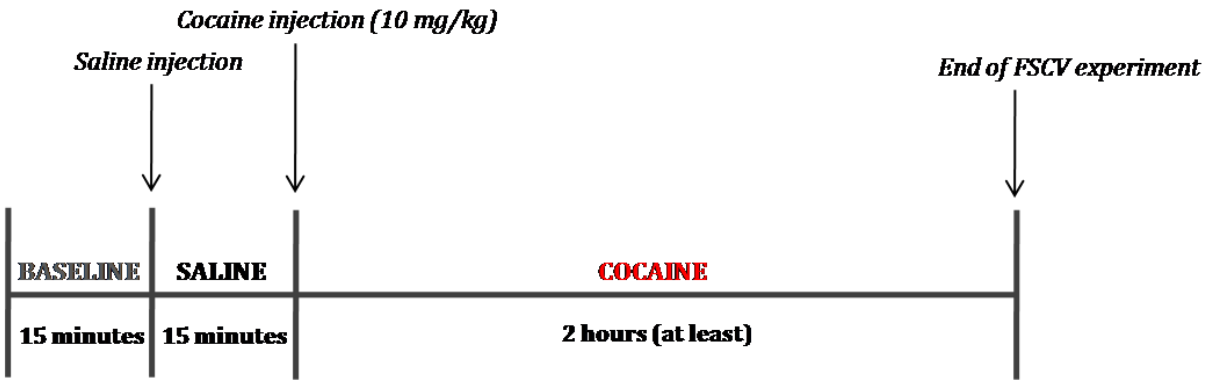

Fig. 8 - *Schematic description of the FSCV experiment*

Conditioned place preference (CPP)

A total number of 32 adolescent rats were utilized for this experiment. Each experimental group was randomly divided into 4 sub-groups (n = 8 per sub-group): vehicle + saline; vehicle + Cocaine; WIN + saline, WIN + cocaine. . Apparatus and procedure were as described previously (Scherma et al., 2008, 2012). The general procedure consisted of three consecutive phases:

Pre-test

Rats were placed at the intersection of two compartments, with the guillotine door separating the two compartments raised to allow exploration of both sides for 15 min. Time spent by the animal in each of the two compartments was recorded to monitor any initial preference for one side versus the other side. Animals showing a pronounced unconditioned preference for one compartment (more than 600 s spent in one compartment) were excluded from the subsequent (conditioning) phase of the experiment.

Conditioning

Conditioning sessions were conducted over 4 consecutive days, with two sessions per day. In the morning, all rats received an injection of saline before being placed in one of the two compartments for 30 min, with the door separating the two compartments closed. Four hours later, the rats received an injection of saline or cocaine (10 mg/kg) and were placed in the opposite compartment for 30 min.

CPP test

On the day after the last conditioning day, a test session was conducted using the same 15 min procedure as the pretest. Time spent by the animal in each of the compartments was recorded.

Measurements of neurotransmitters (DA and Glutamate)

Rats were sacrificed and brains quickly removed 24 hours after the locomotor sensitization experiments at abstinence day 12. Brain areas of interest [i.e., prefrontal cortex, , nucleus accumbens, and hippocampus] were obtained by regional dissection, followed by immediate freezing in liquid nitrogen and storage at -80°C until further processing.

For measurements of dopamine (DA), tissue samples were weighted and homogenized in ice-cold 0.1M $HClO_4$(1:20 weight tissue per solvent volume). After centrifugation (23,000g, 30 min), the supernatants were filtered through a 0.22 µm Spin-X Centrifuge Tube Filter (Costar, Corning Incorporated, Corning, NY) and finally injected into a High Performance Liquid Chromatography (HPLC) system with a C18 column (LC18 DB Supelco, 5 µm, 4.6 X 150 mm) and the Coulochem

III detector (ESA Inc., Chelmsford, MA, USA). The first electrode of the detector analytical cell was set at +20 mV and the second at +320 mV; column temperature was set at 26°C. The mobile phase consisted of 50 mM sodium acetate buffer (pH 4.2), supplemented with 0.07 mM EDTA, 0.35 mM sodium octyl sulfonate, 10 % methanol. Flow rate was maintained constant at 1 mL/min. DA, as well as its metabolites were quantified by peak area comparisons with standards, run on the day of analysis. Data were collected and analyzed using the EZchrom SI 3.2 software. The values obtained were expressed as ng/mg tissue wet weight. Measurements of glutamic acid concentrations in NAcc homogenate supernatants were performed in this way: determination of glutamic acid concentration was carried out in 5 µl aliquots of samples in HClO4 0.1M after pre-column derivatization with orto-phtalaldialdehyde and 2-mercaptoethanol, by HPLC. The chromatograph was equipped with a 15 x 0.4 cm Supelco C18 column, 5 µm particle size, and coupled to fluorescence detection (excitation wavelength: 318 nm; emission wavelength: 452 nm; SFM 25 spectrofluorimeter, Kontron, Milan, Italy), using an automatic injector. The mobile phase was phosphate buffer 0.1 M, pH 6.2 containing methanol 30 % v/v and the flow rate was 1 ml/min. The column temperature was maintained at 35 °C. The sensitivity of the assay was 10 nM. The values obtained were expressed in mM.

Statistical Analysis

All data are expressed as mean values and error bars, which represent standard error of the mean (SEM). The number of biological and/or technical replicates used for statistical analyses is denoted in the legend of the corresponding figure for each experiment.

Locomotor activity data were analyzed using two-way ANOVA for repeated measures, with drug treatment (vehicle and WIN) and time as between-groups factors, and time as a repeated factor. Post hoc multiple comparisons were performed by Bonferroni's test.

CPP data are expressed as CPP score calculated as the time spent in the drug paired compartment during the test session minus the time spent in the drug-paired compartment during the pre-test session and were analyzed by one-way ANOVA. Post hoc comparisons, were performed by Tukey’s multiple comparisons test.

FSCV data were analyzed for significance using a two-way ANOVA with a Bonferroni post hoc test analysis. HDCV software (University of North Carolina, Chapel Hill) was used to collect the amperometric data recorded by each stimulus, and to analyze the concentrations of the chemical components contributing to the stimulated peak (Bucher et al., 2013). The DAmax was determined by using a custom macro written in Igor Pro (Wavemetrics) which identified peaks greater than 3 X

root mean square noise and fit the descending portion of the peak as showed in the following equation (Keighron et al., 2019; Sabeti et al., 2002; Berglund et al., 2013):

$$DA(t) = DA_{Max}e^{-k(t-t_0)}$$

For Western blot analysis, two-group comparisons were performed using two-tailed unpaired Student's t-test, and four-group comparisons were performed using one-way or two-way ANOVA's, followed by correction for multiple comparisons using Tukey's or Holm-Sidak's multiple comparisons tests. Correlation analyses were computed using Pearson correlation coefficients with two-tailed P-values.

DA and glutamate values were analyzed by two way ANOVA, followed by correction for multiple comparisons using Tukey's or Holm-Sidak's multiple comparisons tests.

In all cases, differences with a $P<0.05$ were considered significant. All statistical analyzes were performed with the GraphPad Prism 8 program (GraphPad Software, Inc., La Jolla, CA, USA).

RESULTS

Body weight and food intake in rats chronically exposed to WIN or cocaine

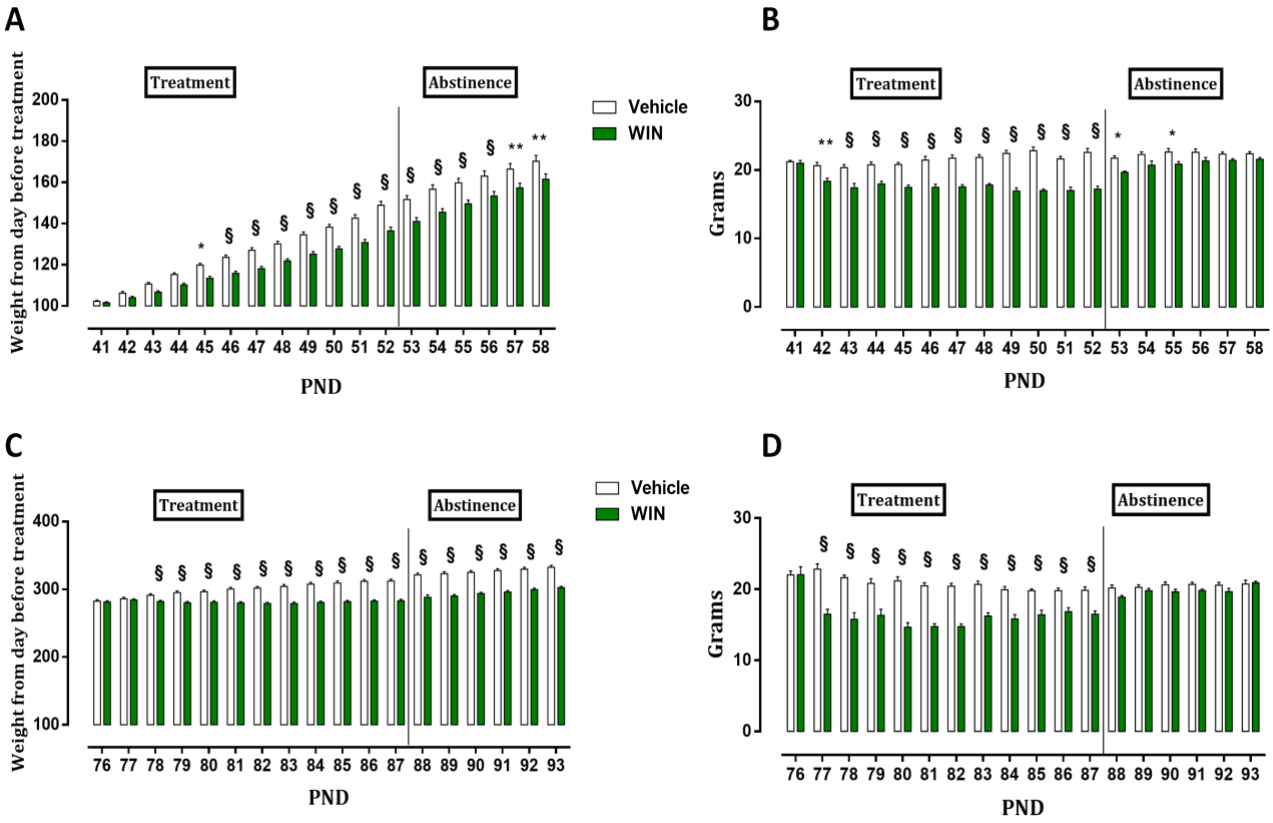

Fig. 9 - Body weight and food intake throughout the WIN treatment *-Body weight and food intake were monitored once daily during the WIN treatment period of both adolescent (**A, B**) and adult (**C, D**) rats. Data are represented as mean ± SEM. Two-way ANOVA's followed by Bonferroni post-hoc analysis; P-values: * P < 0.05, ** P < 0.01, § P < 0.001.*

The temporal curves of animal weight (**Fig. 9 A,C**) are expressed as daily increment from day 0 (day before the WIN treatment started). We found a significant difference in body weight gain between WIN- and vehicle-exposed rats in both adolescent and adult groups. The weight gain of WIN-exposed rats was lower than that of rats exposed to vehicle (two-way ANOVA significant main effect of drug treatment × days interaction for adolescent animals: $F(17, 1084) = 2,794$, $P < 0.001$ followed by Bonferroni post-hoc correction: $P < 0.05$ for day 4; $P < 0.001$ from day 5 to day 15; $P < 0.01$ for days 16 and 17; and two-way ANOVA drug treatment x days interaction for adult animals: $F(17, 1644) = 32,61$, $P < 0.0001$, with Bonferroni post-hoc correction ($P < 0.001$ from day 2 to day 17). Moreover, the effect on weight gain found in WIN-exposed rats was accompanied by a

concomitant reduction in food intake as compared with vehicle-exposed rats (two-way ANOVA significant main effect of drug treatment × days interaction for adolescent animals: F(17, 324) = 8,601, P = 0.0001, followed by Bonferroni post-hoc analysis: P < 0.05 on day 1, P < 0.01 on day 12 and day 14, and P < 0.001 from day 2 to day 12; and two-way ANOVA drug treatment × days interaction for adult animals: (F(16, 282) = 9,094, P < 0.0001 with Bonferroni post-hoc analysis: P < 0.001 from day 1 to 11). The differences in body weight in both groups were not restricted to the period of WIN exposure since they persist even in the post-treatment period.

On contrary, when we reversed the drug administration protocol (see **Fig. 23** for more details) cocaine chronic treatment in adolescent animals resulted in no significant changes in body weight (**Fig. 10A**) or food intake (**Fig. 10B**) compared to vehicles.

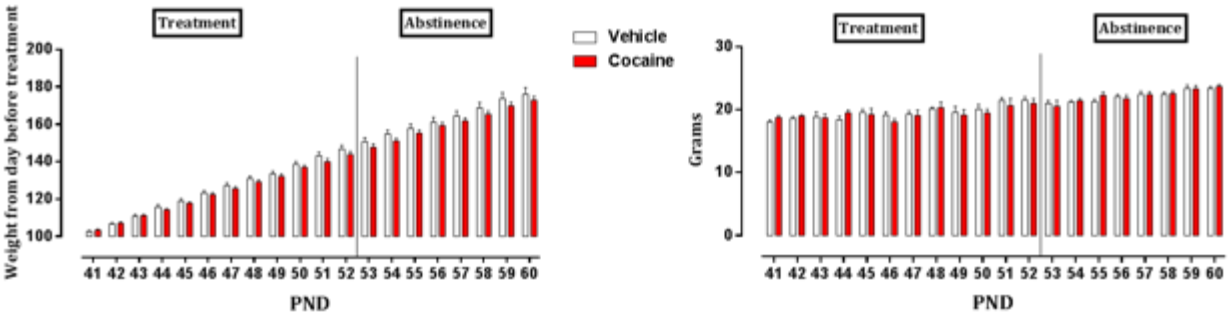

Fig. 10 - Body weight and food intake throughout the cocaine treatment - *Body weight **A)** and food intake **B)** were monitored once daily during the cocaine chronic treatment of adolescent rats. Data are represented as mean ± SEM. Two-way ANOVA's followed by Bonferroni post-hoc analysis. P>0.05.*

Evaluation of persistence of cross-sensitization between cannabinoids and cocaine in adolescent rats

As already reported, chronically WIN pre-treated adolescent rats showed cocaine-induced motor cross-sensitization compared to controls24 hours after the last WIN administration (Melas et al., 2018).

We next asked whether the latter results are able to persist also after 7 days of WIN abstinence(see **Fig. 7** for drug administration protocol), following an intraperitoneal (i.p) challenge of cocaine (10 mg/kg) that was given on abstinence day 8. A significant cross-sensitization between WIN and cocaine in adolescent rats was again observed [(two-way repeated-measures ANOVA: treatment F(3, 75) = 13.31, P < 0.001; time F(8, 600) = 123.0, P < 0.001; subject F(75, 600) = 5.447, P <

0.001; interaction F(24, 600) = 10.26, P < 0.001; Tukey's multiple comparisons test for groups of interest, cocaine vs. WIN–cocaine: 20 min, P = 0.002; 30 min, P = 0.042; n = 18 to 23 animals per group)]**(Fig. 11A)** while no effects was found for adult rats (Tukey's multiple comparisons test for groups of interest, cocaine vs. WIN–cocaine: P > 0.195 for all comparisons from 0 to 60 min; n = 19 to 20 animals per group) **(Figure 11B).**

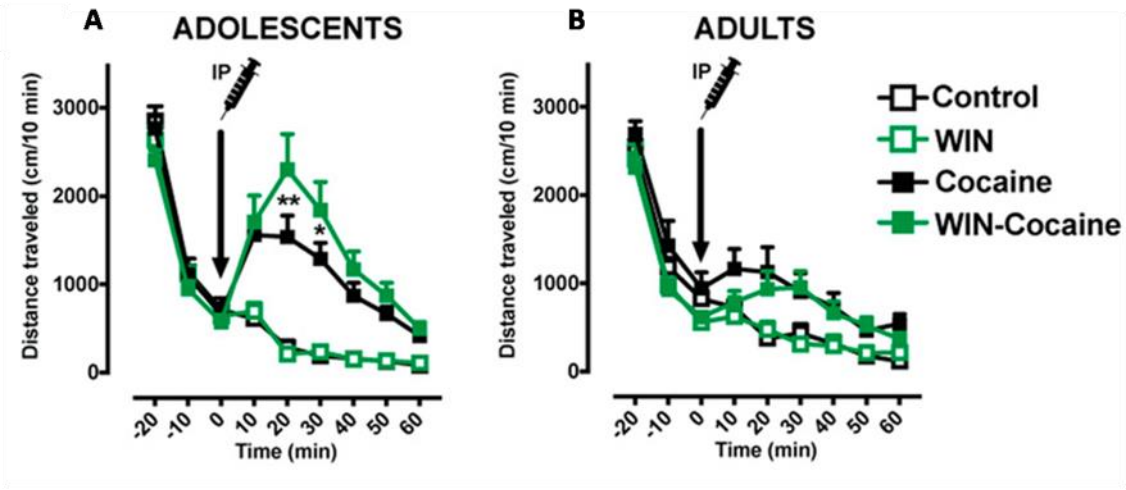

Fig.11 - ***Cross-sensitization between WIN and cocaine*** *only in adolescent* ***A),*** *not adult* ***B)*** *rats. Graph data are presented as mean ± SEM. Two-way ANOVA's followed by Tukey's multiple comparisons test. P values: *P ≤ 0.05, **P < 0.01 (©Scherma et al., 2020).*

Evaluation of cocaine-induced histone modifications and molecular changes

We mentioned that in previous studies (Melas et al., 2018) the pre-exposure to WIN *in vitro* leads to a histone hyperacetylation at H3K27*ac* along the Gadd34 gene, such that its protein product GADD34 is able to recruit the phosphatase 1 (PP1) and to dephosphorylate p-eIF2α. The aforementioned results obtained *in vitro* were then confirmed *in vivo*: histone acetylation at H3K27 and p-eIf2α levels were affected by WIN exposure up to 24 hours following the last WIN administration in adolescent rats (Melas et al, 2018). For these reasons, both H3K27 and eIF2α represent key molecular markers that are altered by WIN exposure.

At this point we asked whether after a period of abstinence (7 days) and a challenge with cocaine (i.p. 10 mg/kg) delivered on WIN abstinence day 8(**Fig.12**), similar or different molecular and epigenetic modifications were related to the behavioral cross-sensitization found in adolescent animals. Thus, 24 hours after the cocaine injection, we performed brain dissections that included the following brain areas: amygdala, dorsal striatum, hippocampus, NAcc and PFC.

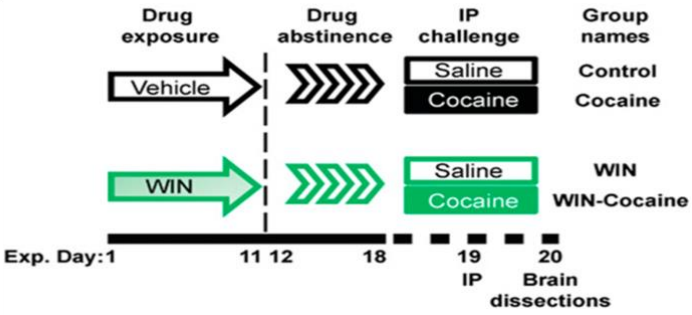

Fig. 12 – *Schematic representation of the experimental design*. *Experimental day 1 corresponds to PND 42 and 77 for adolescent and adult rats, respectively (©Scherma et al., 2020).*

Western-blotting experiments were performed to assess levels of both H3K27 and p-eIf2α. We found a 50% increase in global H3K27 acetylation in the adolescent PFC of the WIN and cocaine groups; and an almost double increase in the WIN-cocaine group [two-way ANOVA: treatment $F(3, 75) = 18.68$, $P < 0.001$; brain region $F(4, 75) = 23.35$, $P < 0.001$; interaction $F(12, 75) = 6.231$, $P < 0.001$; Tukey's multiple comparisons test in PFC: control vs. WIN, $P = 0.01$; control vs. cocaine, $P < 0.001$; control vs. WIN–cocaine, $P < 0.001$; WIN vs. WIN–cocaine, $P < 0.001$; cocaine vs. WIN–cocaine, $P < 0.001$; n = 4 to 6 animals per group(**Fig. 13A**). In adult rats, only the cocaine group showed an increase in H3K27 acetylation (ANOVA $F(3, 12) = 8.424$, $P = 0.002$; Tukey's multiple comparisons test: Control vs. cocaine, $P = 0.001$; WIN vs. cocaine, $P = 0.024$; cocaine vs. WIN–cocaine, $P = 0.05$; n = 4 animals per group) (**Fig. 13B**).

To determine whether the epigenetic changes observed were specific at H3, we then investigated other core histones, and we therefore assessed levels of H4 acetylation (K5-K16). Moreover, we studied other histone modifications such as trimethylation at H3K4 and phosphorylation at H3S10(**Fig. 13C**). The pattern we found was similar and coherent to that of H3 among both adolescent and adult PFC: in adolescents there was a significant increase in global H4 acetylation for the WIN and cocaine groups and an additional increase in the WIN-cocaine group (two-way ANOVA: treatment $F(3, 81) = 19.07$, $P < 0.001$; histone modification $F(5, 81) = 30.45$, $P < 0.001$; interaction $F(15, 81) = 8.327$, $P < 0.001$; Tukey's multiple comparisons test for H4K5-K16ac: control vs. WIN, $P = 0.009$; control vs. cocaine, $P = 0.001$; control vs. WIN–cocaine, $P < 0.001$; WIN vs. WIN–cocaine, $P < 0.001$; cocaine vs. WIN–cocaine, $P < 0.001$; n = 4 to 6 animals per group] . Similarly, in adults the most relevant increase in H4 acetylation was related to the cocaine group (Tukey's multiple comparisons tests; H4K5-K16ac: control vs. WIN, $P = 0.035$; control vs. cocaine, $P < 0.001$; control vs. WIN–cocaine, $P = 0.001$; WIN vs. cocaine, $P < 0.001$; cocaine vs.

WIN–cocaine, P = 0.026; n = 4 animals per group). No changes were found for trimethylation and phosphorylation neither in adolescents nor in adults.

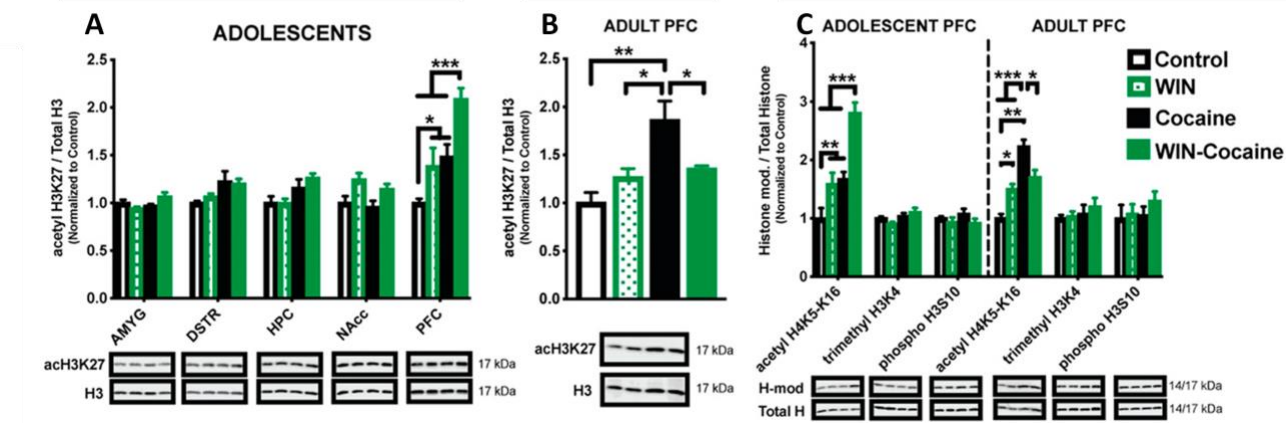

Fig. 13 –*Behavioral cocaine-induced motor cross-sensitization in adolescent rats is linked with histone hyperacetylation in the PFC. A)* *a global H3K27 50% increase for the WIN and the cocaine groups; an almost 100% increase for the WIN-cocaine groups in the PFC of adolescent rats;* ***B)****a global H3K27 increase for the cocaine group in the PFC of adult rats;* ***C)*** *Left part: a global H3K5-K16 increase for the WIN, cocaine and WIN-cocaine groups in the PFC of adolescent rats. No changes in trimethyl H3K4 and in the phosphorylation of H3S10. Right part: a global H3K5-K16 increase for the cocaine group in the PFC of adult rats. No changes in trimethyl H3K4 and in the phosphorylation of H3S10. Legend: AMYG, amygdala; DSTR, dorsal striatum; H-mod, histone modification; HPC, hippocampus; IP, Intraperitoneal; kDa, kilodaltons; NAcc, nucleus accumbens; PFC, prefrontal cortex; Total H, total histone; WIN, WIN 55,212-2 mesylate. Graph data are presented as mean ± SEM. Representative Western blots are shown below the graphs, with the approximate molecular weights of observed band sizes indicated to the right.Two-way ANOVA's withTukey's multiple comparisons test. P Values: *P ≤ 0.05, **P < 0.01, and ***P < 0.001 (©Scherma et al., 2020).*

No relevant changes in levels of p-eIf2α were found in the five brain regions under examination of the adolescent rats (two-way ANOVA: Treatment F (3, 75) = 0.4366, P = 0.727; Brain region F (4, 75) = 1.344, p = 0.262; Interaction F (12, 75) = 1.208, P = 0.294; n = 4-6 animals/group) (**Fig.14**).

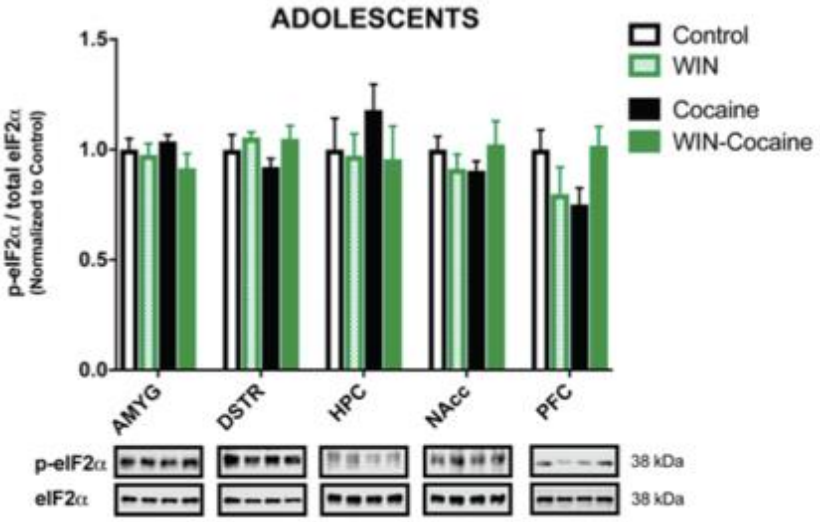

Fig.14 - No changes in phospho-eIF2α levels in any of the adolescent brain regions. *Graph data are presented as mean ± SEM. Two-way ANOVA's. P Values: P>0.05 (©Scherma et al., 2020).*

After we found that the WIN-cocaine group is susceptible to histone hyperacetylation in the PFC of adolescent rats, we tried to understand which epigenetic enzyme may be involved in this process. Histone deacetylases (HDACs) and histone acetyltransferases (HATs) are widely distributed in cells. They play an opposite role, and a subtle equilibrium between them control the state of acetylation of lysine amino acids on histone proteins. They usually work within a multisubunit complex, and the other subunits are crucial for them to modify histone residues around the binding site. We used adolescent PFC samples from the cocaine and WIN-cocaine groups, and we then separated the nuclear fraction from the cytoplasm, subjecting the nuclear extract to TMT-based quantitative proteomic analysis. We found significantly reduced levels of HDAC6 in the WIN-cocaine group (($P \leq 7.6e\text{-}4$; $q \leq 0.01$; n = 5 animals per group and n = 3 technical replicates per animal) (**Fig. 15A**), but since HDAC6 (a type II HDAC) is present both in the cell nucleus and in the cytoplasm, we used Western Blot to investigate the presence of the HDAC6 at the level of both components. We found a significant reduction in HDAC6 nuclear levels in WIN pre-treated rats then challenged with cocaine ([two-way ANOVA: treatment $F(3, 27) = 4.486$, $P = 0.011$; cell fraction $F(1, 27) = 18.36$, $P < 0.001$; interaction $F(3, 27) = 5.086$, $P = 0.006$; Tukey's multiple comparisons test for nuclear fraction: control vs. WIN–cocaine, $P < 0.001$; WIN vs. WIN–cocaine, $P = 0.05$; cocaine vs. WIN–cocaine, $P = 0.09$; n = 4 to 6 animals per group) (**Fig. 15B**), and a significative negative correlation between nuclear HDAC6 levels and levels of H3 acetylation (Pearson's $r = -0.5495$; $P = 0.0275$; n = 16 animals for which both histone acetylation and HDAC6 measurements were available) and H4 acetylation (Pearson's $r = -0.6949$; $P = 0.0028$; n = 16 animals) (**Fig. 15C**). Interestingly, in adult rats we found a cocaine-induced increase in nuclear

HDAC6 levels (two-way ANOVA: treatment F(3, 24) = 4.353, P = 0.014; cell fraction F(1, 24) = 5.939, P = 0.023; interaction F(3, 24) = 4.783, P = 0.009; Tukey's multiple comparisons test for nuclear fraction: control vs. WIN–cocaine, P = 0.001; WIN vs. WIN–cocaine, P < 0.001; cocaine vs. WIN–cocaine, P = 0.029; n = 4 animals per group) (**Fig. 15D**), but no significant negative correlation between nuclear HDAC6 levels and levels of histone acetylation (P > 0.05; n = 16 animals) (**Fig. 15E**).

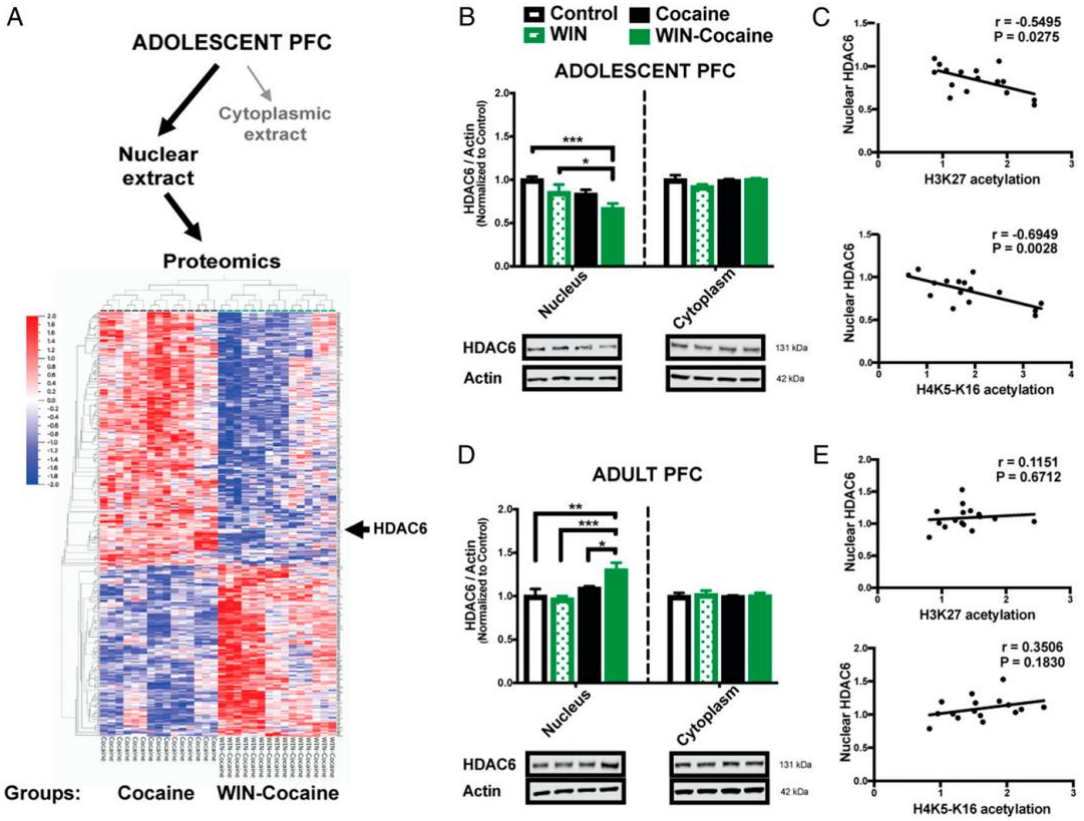

Fig. 15 – ***An association between WIN pre-treatment and cocaine-induced effects in HDAC6 levels. (A)****Adolescent cocaine and WIN–cocaine groups' nuclear PFC extractsunder quantitative proteomic analysis. Hierarchical clustering heatmap is presented for the differentially expressed proteins. The arrow to the right of the heatmap denotes the significant decrease in levels of HDAC6 in the WIN–cocaine group.* ***B)****Reduction in nuclear HDAC6 in the WIN–cocaine group.* ***C Upper)****Regression line of normalized nuclear HDAC6 (HDAC6/Actin) and H3 acetylation (H3K27ac/H3) levels, and corresponding correlation coefficients, using data from all four adolescent treatment groups* ***C Lower)*** *Regression line of normalized nuclear HDAC6 (HDAC6/Actin) and H4 acetylation (acH4K5-K16/H4) levels, and corresponding correlation coefficients, using data from all four adolescent treatment groups* ***D)****A significant increase of HDAC6 levels in the PFC of WIN–cocaine adult group* ***E)****No significant correlations for the adult PFC (P > 0.05; n = 16 animals). Graph data are presented as mean ± SEM. Representative*

*Western blots are shown below the graphs, with the approximate molecular weights of observed band sizes indicated to the right. Two-way ANOVA's with Tukey's multiple comparisons test. P Values: *P ≤ 0.05, **P < 0.01, and ***P < 0.001 (©Scherma et al., 2020).*

This fine modulation of cocaine-induced histone acetylation after WIN pre-exposure by HDAC6, led us to also investigate the remaining majority of differentially expressed proteins at the cytoplasmic level. Using quantitative proteomics, we found a significant increase in a mitogen-activated protein kinase (MAPK3, that is also known as ERK1) in the WIN-cocaine group ($P \leq 7.6e\text{-}4$; $q \leq 0.01$; n = 5 animals per group and n = 3 technical replicates per animal) (**Fig. 16A**). The role of ERK1 and its homolog ERK2 is well known in cocaine-induced behavioral sensitization (Lu et al., 2006). Using Western Blot we found that cytoplasmic ERK1/2 levels were increased in the adolescent WIN-cocaine group [ANOVA: $F(3, 15) = 15.87$, $P < 0.001$; Tukey's multiple comparisons test: control vs. WIN–cocaine, $P = 0.0019$; WIN vs. WIN–cocaine, $P < 0.001$; cocaine vs. WIN–cocaine, $P = 0.0012$; n = 4 to 6 animals per group], but not in adult rats [ANOVA: $F(3, 12) = 0.298$, $P = 0.825$; n = 4 animals per group] (**Fig. 16B**).

Since recent studies also showed that there is a regulatory interaction between ERK1/2 and HDAC6 (Wu et al., 2018; Williams et al., 2013), we performed correlation analysis that showed an almost significant negative correlation between them in adolescents ($P = 0.08$) but not in adults ($P = 0.96$) (figure not shown). This led us to investigate the dynamic and complex relationship between HDAC6 and ERK1/2 by creating CRISPR/Cas9 knockout (KO) and overexpression cell lines, both for HDAC6 and ERK1/2. Western blotting experiments using lysates from HDAC6 KO revealed significantly increased levels of ERK1/2 (t test, $t = 14.18$, $df = 4$; $P < 0.001$; n = 3 technical replicates per group) (**Fig 16C**); and lysates from ERK1/2 KO revealed significantly decreased levels of HDAC6 (t-test, $t = 4.875$, $df = 4$; $P = 0.008$; n = 3 technical replicates per group) (**Fig 16D**). Overexpression of HDAC6 led to increased levels in ERK1, but no changes in ERK2 (ERK1: t-test, $t = 4.732$, $df = 4$; $P = 0.009$; ERK2: t-test, $t = 0.355$, $df = 4$; $P = 0.740$; n = 3 biological replicates per group) (**Fig. 16E**), while overexpression of ERK2 led to increased levels of HDAC6 (t-test, $t = 11.81$, $df = 4$; $P < 0.001$; n = 3 technical replicates per group) (**Fig. 16F**).

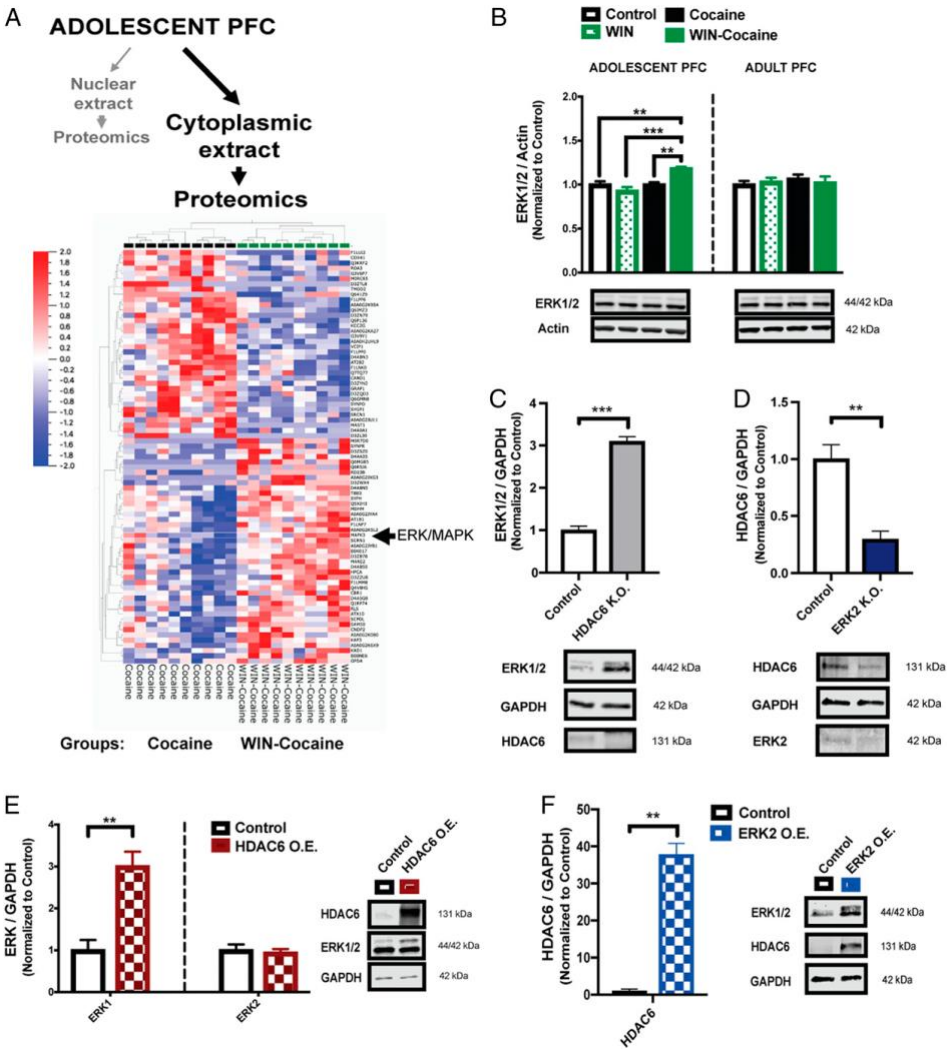

Fig. 16 – ***ERK levels are altered by cocaine and after WIN chronic pre-exposure****. **A)**Quantitative proteomics performed on the cytoplasmic extracts of the adolescent PFC of the cocaine and WIN–cocaine groups. Hierarchical clustering heatmap is presented for the differentially expressed proteins. The arrow shows changes in levels of ERK1/MAPK3 between groups.**B)**Increase in ERK1/2 levels in the adolescent cytoplasmic PFC extracts of the WIN–cocaine group (Left). No changes in ERK1/2 levels were found in the adult PFC cytoplasmic extracts (Right).**C)** CRISPR-Cas9 HDAC6 knockout cell line (HeLa) showed increased ERK1/2 levels. **D)**CRISPR-Cas9 ERK2 knockout cell line (HeLa) showed a reduction in HDAC6 levels **E)**Transient HDAC6 overexpression cell line (HEK293T) showed increased levels of ERK1 but no changes in ERK2 levels **F)** Transient ERK2 overexpression cell line (HEK293T) showed increased HDAC6 levels. Graph data are presented as mean ± SEM. Representative Western blots are shown below the graphs, with the approximate molecular weights of observed band sizes indicated to the right. T-test or Two-way ANOVA's withTukey's multiple comparisons test.P Values: **P < 0.01 and ***P < 0.001 (©Scherma et al., 2020).*

Evaluation of sub-second dopamine dynamics in the NAcc shell of anesthetized rats

We mentioned that the drug sensitization phenomenon is strictly linked to modifications within the mesolimbic dopamine pathway, culminating in changes in dopamine (DA) extracellular concentration at the level of the NAcc (Kalivas and Stewart, 1991). For this reason, FSCV was used to monitor the sub-second dynamics of extracellular DA in the NAcc shell following stimulation. It is well established that electrical stimulation of the MFB evokes dopamine release at the level of the NAcc (Garris et al., 1997), in both anesthetized (Shu et al., 2014) and freely-moving animals (Cheer et al., 2004). These experiments were conducted in urethane anesthetized rats using an electrical stimulation allowed for the detection of changes in elicited DA release with a uniform stimulus across animals. Once both the carbon-fiber working electrode and the stimulating electrode are in the correct position (NAcc and MFB, respectively), the representative recording obtained for optimal response to DA appears as shown in **Fig. 17.** The red spot indicates the moment when the stimulation is delivered. The green spot at +600 mV represents the oxidation current for DA. The extracellular DA concentration rapidly increases after the stimulation begins, and then it is washed out from the extracellular space. The main mechanism known to be involved in the DA clearance is through the dopamine transporter DAT (David et al., 1998). The inset cyclic voltammogram (CV) insert in each color plot validates that the elicited substance is DA, while the amplitude and duration of each DA stimulation characterize the effect of either vehicle or cocaine on the phasic properties of DA in the NAcc shell of both WIN- and vehicle pre-treated rats.

The experimental groups for FSCV are the same used in previous experiments, although it is important to underline what follows: for FSCV at the same animal was given both saline and the cocaine challenge during the electrophysiological recording (see **Fig. 7**and **8** for the drug administration protocol). Representative color plots from rats receiving 1 mL/kg vehicle (i.p.) or 10 mg/kg cocaine (i.p), and both chronically pre-treated with either vehicle (i.p.), or WIN (i.p.) are shown in **Fig. 18 A-D**.

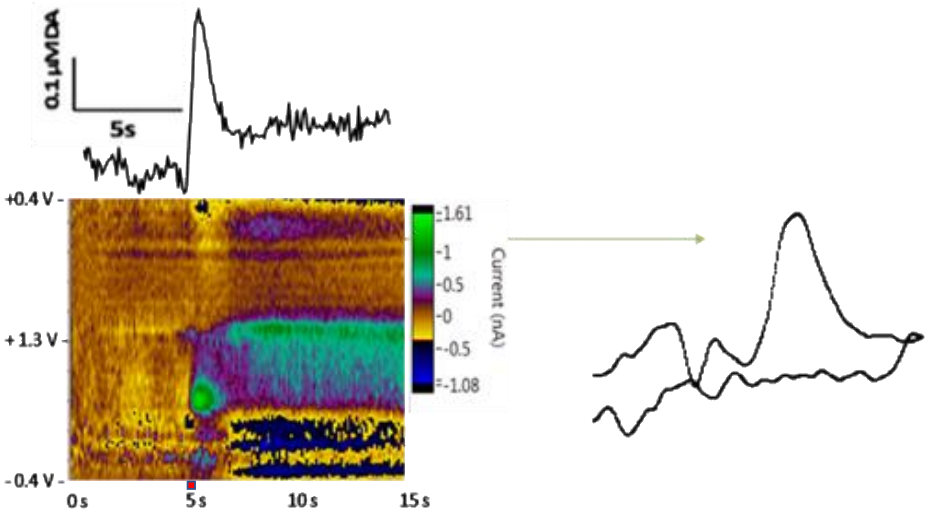

Fig. 17 – ***(Left) Representative recording obtained for optimal response to DA.*** *The voltammetric current (color coded) is plotted against the applied potential (y) and the acquisition time (x). The red spot indicates the moment when the electrical stimulation is delivered at the level of the MFB (biphasic pulses, 24 pulses of 180 µA, 60 Hz, 4 ms in duration). After the electrical stimulation, DA levels rapidly increased (green spot is indicative of the oxidative current). The green spot at +600 mV represents the oxidation current for DA. Dark blue and green spots after the DA peak represent non-dopaminergic changes;* ***(Right)*** *The cyclic voltammogram (CV) indicates that the electrically evoked substance is DA.*

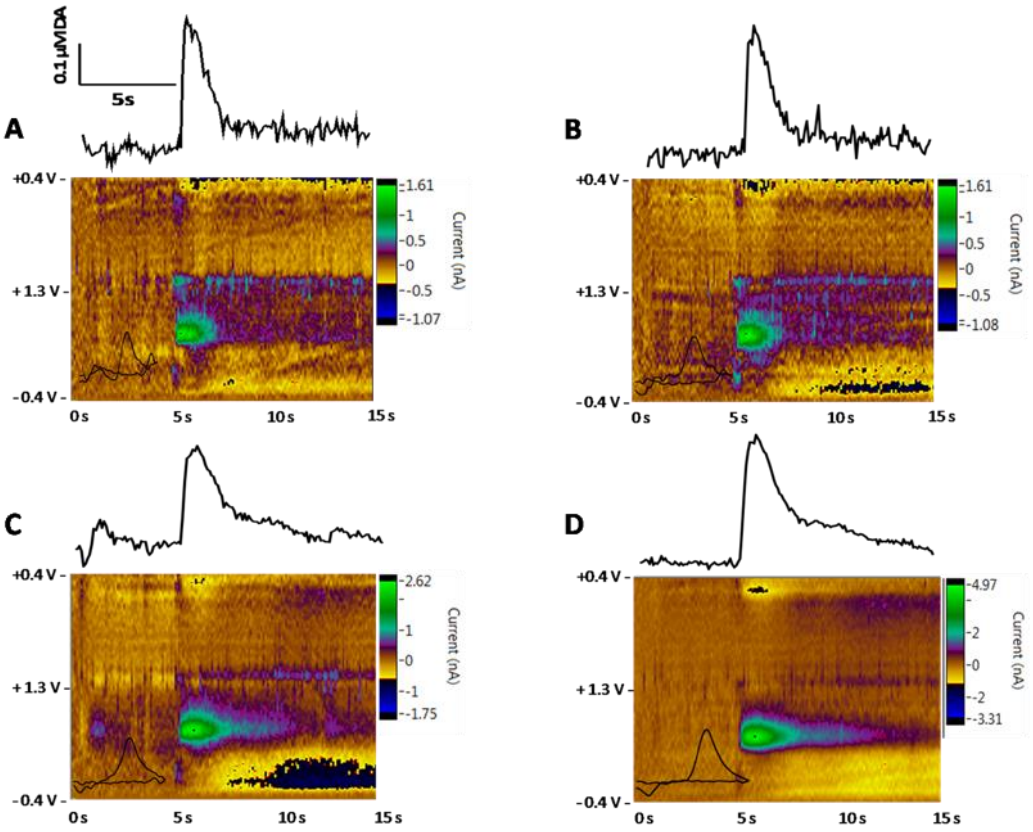

Fig. 18 -*Representative color plots demonstrating the effects of **A)** control; **B)** cocaine; **C)** WIN; **D)** WIN-cocaine groups on the electrically evoked dopamine peak (DAmax) during FSCV experiments.*

As shown in the color plots, the stimulation of the MFB leads to an instantaneous rise of the extracellular DA. Both vehicle- and WIN pre-treated animals were able to increase maximal DA concentration (DAmax) after the cocaine challenge, reaching a maximal effect in the first 35–60 min post injection, with DAmax rising approximately to 60–120% in comparison to baseline values. Importantly, the WIN pre-treated group showed an enhancement in the amplitude of DA peaks in comparison to the vehicle pre-treated, although no significance was found (Two-way ANOVA time x treatment: $F(1,27) = 1.1247$, $P = 0.3102$); interaction $F(1,27) = 1.551$, $P = 0.3077$; number of animals per group = 7).

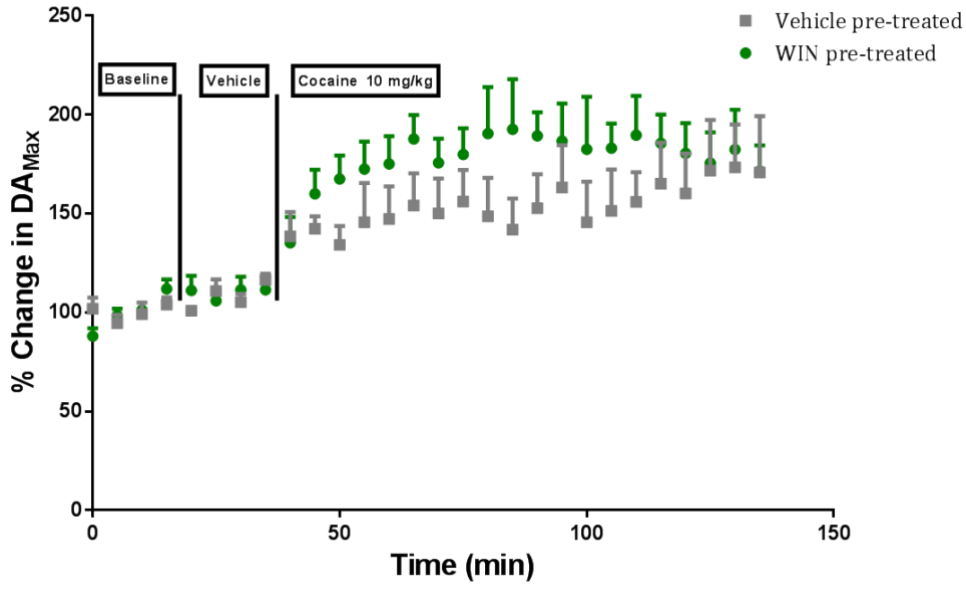

Fig. 19 - *Effect of cocaine administration on the DAMax in vehicle and Win pre-treated animals.* *Urethane anesthetized adolescent rats were stimulated in the MFB each 5 minutes during the FSCV experiment. A baseline period was recorded for 15 minutes. Afterwards, vehicle (i.p. 1ml/kg) was injected. After minute 35, cocaine (i.p 10 mg/kg) was administered and DA peaks values were recorded for at least 2 hours. Cocaine-induced DA increase in the NAcc shell tends to be higher in WIN pre-treated animals in comparison to vehicle pre-treated ones. Graph data are presented as mean ± SEM. Two-way ANOVA's. P Values: P > 0.05.*

Evaluation of the persistence of cross-sensitization between cannabinoids and cocaine after repeated exposure to the drug

After we found that adolescent WIN pre-treated animals cross-sensitize to a single cocaine injection, we next asked whether the same behavioral motor effect persist also after 4 days of cocaine (i.p 10 mg/kg) administration (see **Fig. 7** for drug administration protocol), that was given from WIN abstinence day 8 to day 11. Cocaine treatment resulted in an increased locomotor activity for the cocaine and WIN-cocaine group in comparison to the WIN and control group **(Fig.20A)** (two-way ANOVA: control vs. cocaine group treatment x time: $F(8,144)= 15.05$, $P < 0.0001$; and WIN vs WIN-cocaine group: $F(8,81)= 11.77$, $P < 0.0001$). Although no cross-sensitization between WIN and cocaine in adolescent rats was observed this time, the effect of cocaine on the motor response of WIN pre-treated animal is enhanced and shows a tendency towards significance. In adult rats, cocaine treatment exerted a similar effect when we compared the WIN-cocaine and

cocaine groups with the WIN and control group **(Fig. 20B)** (two-way ANOVA: control vs cocaine group treatment x time: F(8,144)= 16.34, P < 0.0001; and WIN vs WIN-cocaine group: F(8,81)= 10.67, P < 0.0001). Moreover, the cocaine group displayed an increased motor response in comparison to the WIN-cocaine group, although no statistical significance was found.

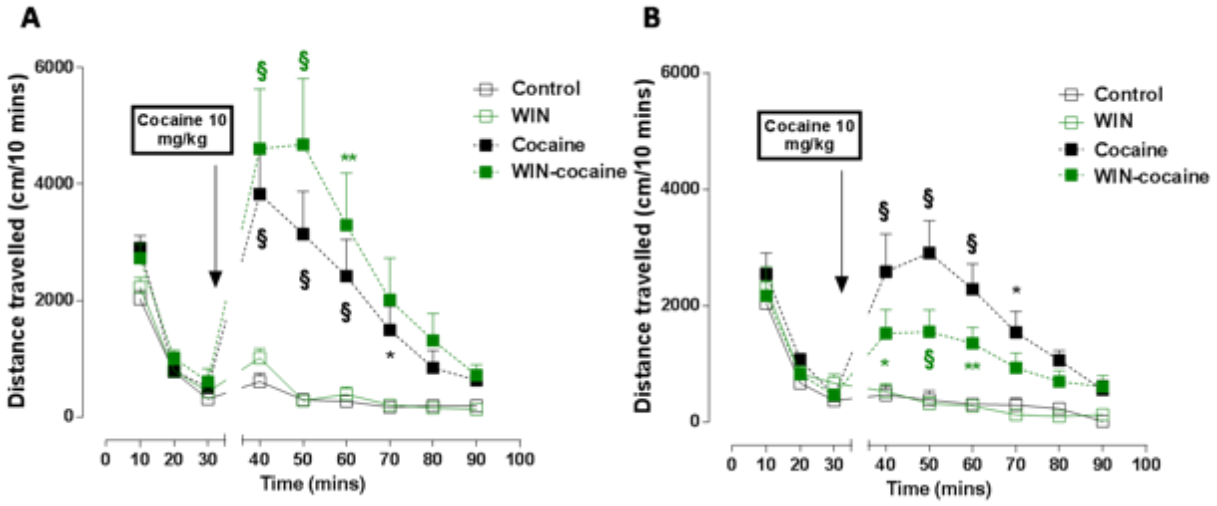

Fig. 20 - ***Cross-sensitization between WIN and cocaine after 4 repeated cocaine injections.*** *Total distance travelled by A) adolescent, and B) adult animals. Data are represented as mean ± SEM. Two-way ANOVA's followed by Bonferroni post-hoc analysis. P Values: (*P <0.05, **P<0.01, §P<0.001vs controls).*

Evaluation of the positive reinforcement in cannabinoid pre-treated adolescent animals after repeated exposure to the drug

We next asked whether prolonged treatment with WIN in adolescence is able to modify the cocaine-induced conditioned place preference (CPP). The latter test was performed on WIN abstinence day 12 after 4 consecutive days of cocaine (i.p. 10 mg/kg) treatment (see Figure 7 for drug administration protocol)and only in adolescent animals.

For both cocaine and WIN-cocaine groups a significant increase was found in the CPP compared to the control [(Two-way ANOVA: (F (3.31) = 8.715, P <0.0003) post-hoc analysis:(P <0.05)] and the WIN (P <0.01) group **(Fig. 21).**

Furthermore, although without reaching any statistical significance, the WIN-cocaine group spent more time in the compartment associated with cocaine administration in comparison to the cocaine

group, suggesting that WIN pre-exposure is able to enhance the reinforcement effect induced by the cocaine treatment.

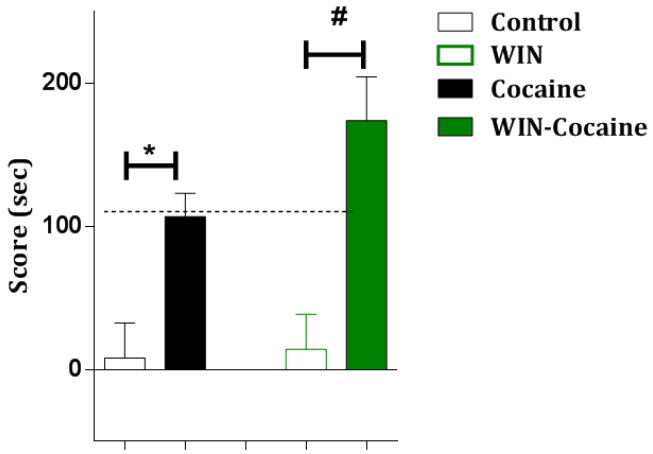

Fig. 21 - *Effects of WIN or vehicle on the cocaine-induced CPP in adolescent animals*. *Data are represented as mean ± SEM. Two-way ANOVA's followed by Bonferroni post-hoc analysis; P-values: * P< 0.05, #P <0.001.*

Evaluation of dopamine and glutamate tissue levels in crucial brain areas after repeated cocaine exposure

We next aimed at investigating the effects of repeated cocaine injections on dopaminergic and glutamatergic transmission in specific brain areas involved in the reward processes, such as the PFC, NAcc and hippocampus (Hipp) in animals chronically pre-treated with WIN.

We found that tissue dopamine levels in the PFC of adolescent animals were significantly decreased in the WIN group in comparison to the vehicle group (4.797 ± 0.2534, Student's t-test, P = 0.0309, n = 5 animals per group) (**Fig.**22**A).** On the other hand, tissue glutamate levels were not affected by WIN treatment (0.3278 ± 0.07099, Student's t-test, p = 0.0585, n = 5 animals per group) but they were affected by cocaine treatment in the PFC of adolescent animals (two-way ANOVA: treatment F(1,13) = 9.818, P = 0.0079; Tukey's multiple comparisons test: cocaine vs. control, P = 0.0046; cocaine vs. WIN, P = 0.0332; WIN-cocaine vs. WIN, P = 0.0012; n = 5 animals per group) **(Fig. 22B).** Furthermore, tissue dopamine levels in the NAcc of adolescent animals were significantly higher for the WIN group in comparison to all the other groups **(Fig. 22C)** (two-way ANOVA: treatment F(1,15) = 11.61, P = 0.039; interaction F(1,15) = 5.436 P = 0.00341; Tukey's multiple comparisons test: WIN vs. control, P = 0.0064; WIN vs. cocaine, P = 0.0115; WIN vs. WIN-

cocaine, P = 0.0481; n = 5 animals per group). On contrary, there was no significance for tissue glutamate levels in the NAcc of adolescent animals **(Fig. 22D)** (two-way ANOVA: treatment F(1,15) = 3.189, P = 0.0944; interaction: F(1,15) = 0.5066, P = 0.04875). Moreover, we found that tissue dopamine levels in the Hipp of adolescent animals were significantly increased for the WIN group in comparison to all the other groups **(Fig. 22E)** (two-way ANOVA: treatment F(1,15) = 0.8975, P = 0.03585; interaction F(1, 15) = 10.73, P = 0.0051; Tukey's multiple comparisons test: WIN vs. control, P = 0.0482; WIN vs. cocaine, P = 0.0332;WIN vs. WIN-cocaine, P = 0.0012; n = 5 animals per group). On the other hand, no significance evidence was found for tissue glutamate levels in the Hipp of adolescent animals **(Fig. 22F)** (two-way ANOVA: treatment F(1,16) = 0.03012, P = 0.8644; interaction: F(1,16) = 0.8798, P = 0.03622).

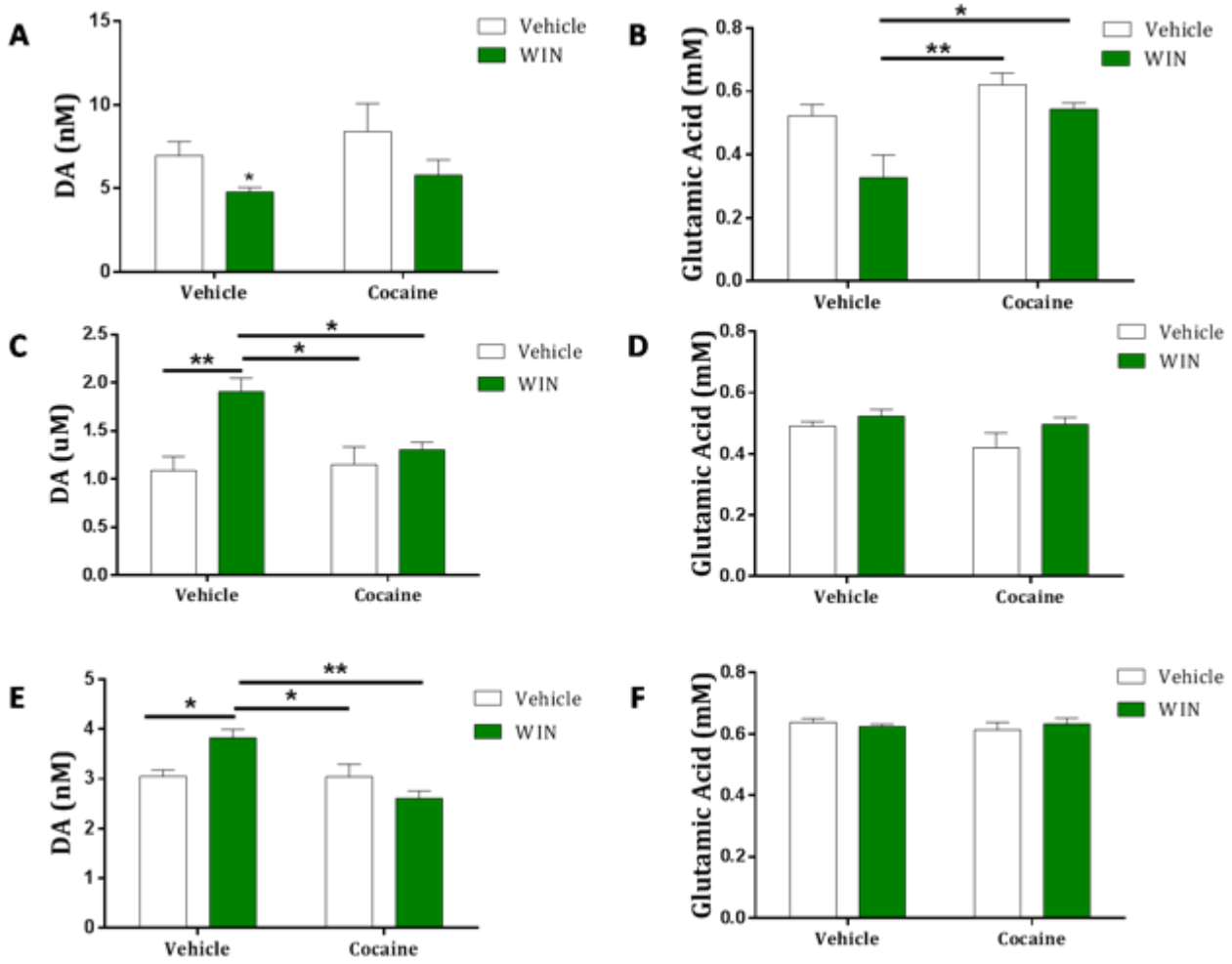

**Fig. 22 - *Tissue dopamine levels* in *A)*PFC, *C)*NAcc and *E)*Hipp of adolescent animals. *Tissue glutamate levels* in *B)* PFC; *D)*NAcc; *F)*Hipp of adolescent animals. *Data are represented as mean ± SEM. Student's t-test for two sample and Two-way ANOVA's with Tukey's multiple comparison test; P-values: *P <0.05, **P<0.01.*

Evaluation of the directionality of behavioral cross-sensitization

We also investigated the directionality of behavioral cross-sensitization by reversing the drug administration paradigm (**Fig. 23A**) and exposing adolescent rats to increasing doses of cocaine (11 days, i.p. 10 mg/kg, 3 days; 15 mg/kg, 4 days; 20 mg/kg, 4 days) and, following a week of cocaine abstinence, challenging them with an i.p. injection of WIN at a dosage of 0.1 mg/kg. We found, however, no evidence for cross-sensitization between cocaine and WIN (Two-way RM ANOVA: Time x Treatment, F (8, 160) = 0.486, p = 0.864; Time, F (3.401, 68.02) = 78.55, p< 0.001; Treatment, F (1, 20) = 0.055, p = 0.816; Subject, F (20, 160) = 6.147, p < 0.001; Sidak's multiple comparisons test: P > 0.985 for all time points; n = 11 animals/group) (**Fig. 23B**). We therefore also tested cross-sensitization following repeated challenges with WIN (once daily for four consecutive days),but again found only modest non-significant effects (data not shown).

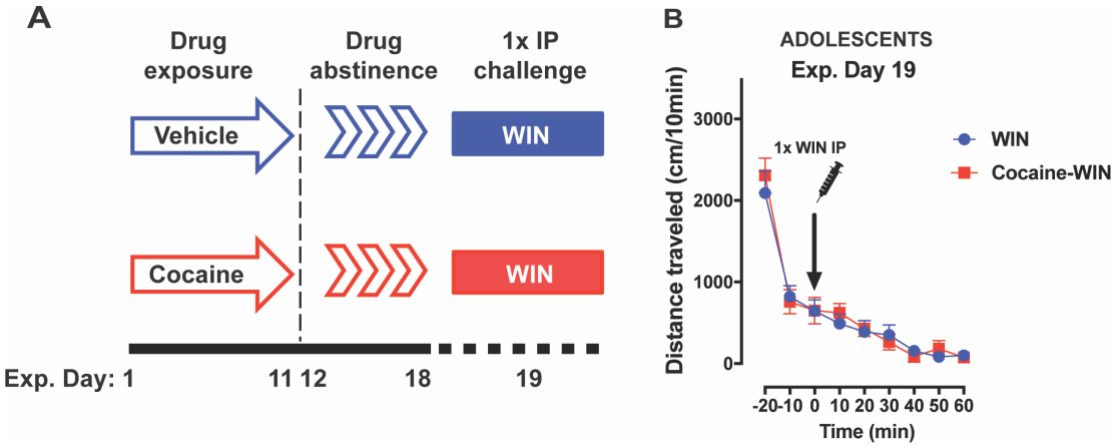

**Fig. 23 - *Lack of cross-sensitization between cocaine and cannabinoids in adolescence*. *A)* Schematic representation of the experimental design. Experimental day 1 corresponds to postnatal day 42. *B)* There was no locomotor sensitization following an i.p. challenge with WIN. Data are represented as mean ± SEM. Two-way ANOVA's. P-values: P >0.05 (©Scherma et al., 2020).*

DISCUSSION

The exposure to certain drugs of abuse can interfere with the delicate balance of the still undeveloped adolescent central nervous system, giving rise to long-term modifications in the reward system, and increasing the vulnerability to the use of other more addictive substances. The existence of a developmental sequence of involvement in drugs of abuse refers to the so called Gateway Hypothesis, a term that was introduced to emphasize that certain drugs can serve as a gateway for the use of other substances in a specific temporal pattern (Kandel et al., 1975).
In the present preclinical study, we bridged the epidemiology of drug abuse and molecular biology by developing a rat model of the epidemiological sequence that characterize the transition from cannabis to cocaine. In particular, we investigated the hypothesis that a chronic pre-exposure to a synthetic cannabinoid (WIN), may amplify the behavioral motor-related response to cocaine, with the attempt to characterize the electrophysiological correlates, and the possible molecular and epigenetic alterations that may have reprogramed pivotal brain regions of the dopamine mesolimbic pathway, to both a first and repetitive encounters with the drug.

A first significant result in our study comes from the effects of the chronic exposure to WIN on rat's body growth and food intake. Both parameters showed a decisive decrease during the whole chronic exposure to the cannabinoid, in comparison to the exposure to vehicle. The endocannabinoid system is known to modulate eating behavior and metabolism of macronutrients (Watkins and Kim, 2015). In fact, the activation of CB1 receptors is linked to an oroxygenic effect caused by both endogenous (Reyes-Cabello et al., 2012; Kola et al., 2008; Mechoulam and Fride, 2001; Di Marzo et al., 2001; Cota et al., 2003; Kirkham et al., 2002; Williams et al., 1998) and non-endogenous cannabinoids (Simiand et al., 1998; Colombo et al., 1998; Freedland et al., 2000; Pacher et al., 2006; Haney et al., 2007; Kirkham, 2009). In humans, cannabinoids induce hyperphagia (Williams et al., 2005; Berry and Mechoulam, 2002; Fride et al., 2005; Cota et al., 2003) and increase the preference for palatable foods (Cristino et al., 2014; Kirkham, 2009). Our observations go in the opposite direction, but they are in line with the preclinical evidence reported by other authors. Reduction in body growth and food intake has been found following treatment with different cannabinoids such as THC (Keeley et al., 2015; Rubino et al., 2008; Stopponi et al., 2013; Scherma et al., 2016), HU210 (Giuliani et al., 2000); CP- 55,940 (Biscaia et al., 2003), and WIN (Abalo et al., 2009; Merroun et al., 2009; Radziszewska and Bojanowska, 2013).

These findings suggest a biphasic activity of cannabinoids that are likely correlated with the dosage: low dosages usually generate hyperphagia, while the anorexigenic effect takes over with higher dosages.

We observed a WIN dose-dependent effect on body weight and food intake reduction hat might be linked to the WIN-induced decrease in locomotion, as it has been reported by some authors for different cannabinoids (Kasten et al., 2019; Bruijnzeel et al., 2016; Merroun et al., 2009) and described as a kind of ataxia through which animals lose their ability to perform even simple movements (Dewey, 1986). This condition, also observed in our experiments, can be explained by the wide distribution of CB1 receptors at the level of the cerebellum and basal ganglia, brain regions involved in motor control (Rodríguez de Fonseca et al., 1998), and that in turn can affect food intake behavior.

On contrary, we observed that adolescent and adult rats chronically treated with cocaine did not show changes in body weight or food intake. In humans, cocaine use is known to inhibit appetite, and therefore to also reduce body weight (Cochrane, Malcolm, and Brewerton, 1998). Other explanations for body weight loss can be linked to cocaine-induced generalized major disturbance in eating behaviors and metabolism (Ersche et al., 2013).However, in line with our findings, other preclinical data reported that cocaine's anorexic effects might not be observable or being only relatively transient (Balopole, Hansult, and Dorph, 1979), with food intake only postponed but not actually reduced (Cooper and Vanderhoek, 1993).

In the preliminary study (Melas et al., 2018) conducted by our group, it was found a cannabinoid-mediated decrease in the phosphorylated form of eIF2a at the level of the NAcc of adolescent rats. This finding provided a molecular basis that aimed at characterizing the gateway drug properties of cannabinoids described both in preclinical studies (Biscaia et al., 2008; Ellgren et al., 2007 Dow-Edwards and Izenwasser, 2012; Cadoni et al., 2001; Solinas et al., 2004; Higuera-Matas et al., 2008; Manzanedo et al., 2004; Manzanedo et al., 2010; Panlilio et al., 2013; Rodrı´guez-Arias et al., 2010) and in human populations (Fergusson et al., 2006; Kandel, 1975; Kandel, 2003; Silins et al., 2014; Ferguson et al., 2008; Van Gundy et al., 2010; Mayet et al., 2012; Mayet et al., 2016). In line with the hypothesis, cross-sensitization between WIN and cocaine was again observed in adolescent animals only, one day after the last WIN administration. In the present study we confirmed the persistence of the same cross-sensitization after a period of abstinence (lasted 7 days): following acute administration of cocaine (i.p. 10 mg/kg) the day after the end of WIN washout, adolescents pre-exposed to WIN (that received increasing doses twice a day, i.p.: 3 days 2 mg/kg; 4 days 4 mg/kg; 4 days 8 mg/kg) showed an increase in the total distance travelled in the locomotor activity

test compared to the control group, while adults, continued to display no differences. This evidence underline a crucial point of our results, being that the adolescent brain is still in its delicate developmental phase, and particularly susceptible to drug exposure (Izenwasser, 2005). Our findings are also in agreement with other investigation that showed the different responses to drugs of abuse according to the age of consumption. For instance, cross-sensitization between THC and cocaine was reported in adolescent rats, not in adults, by Dow-Edwards and Izenwasser (2012). Higuera-Matas and colleagues (2008), also described the susceptibility in the acquisition of cocaine self-administration in animals exposed at an early age to cannabinoids. Furthermore early and prolonged exposure to cannabinoids can generate cross-sensitization to other dopaminergic psychostimulants such as amphetamine (Lamarque et al., 2001; Muschamp and Siviy, 2002). In addition, other authors showed how a prolonged exposure to THC (Panlilio et al., 2007) or to the synthetic cannabinoid CP 55,940 (Arnold et al., 1998) does not induce changes in the cocaine-related motor response in adult animals.

When we reversed the drug administration protocol, and assessed the cross-sensitization between cocaine and WIN, increasing doses of cocaine were given twice a day for 11 consecutive days, followed by a week of cocaine abstinence and an i.p. challenge (on abstinence day 8) with WIN, 0.1 mg/kg, a dosage previously found to induce locomotor sensitization (Polissidis et al., 2013), no changes were observed, therefore suggesting a persistent and unidirectional cross-sensitization between cannabinoids and cocaine in adolescence.

Evidence show that psychostimulants are able to increase extracellular dopamine in terminal dopamine areas by exerting an action on DAT, therefore modulating the motor stimulant and reinforcing properties of the drugs (Cadoni et al., 2000, Robinson and Berridge, 1993; Stewart and Badiani, 1993; Koob et al., 1992; Bozarth, 1986). The mesolimbic dopamine system is therefore hugely involved in the sensitization phenomenon, producing in particular an increase in the activity of dopaminergic neurons at the level of the VTA and substantia nigra pars compacta, which in turn lead to an increase in the dopaminergic activity at the level of the NAcc (Nestler and Aghajanian, 1997; Cadoni et al., 2000; Vanderschuren and Kalivas, 2000; White and Kalivas, 1998). That is why we next asked what are the dopamine dynamics at the level of this brain region. To this aim, FSCV was used to monitor electrically-evoked dopamine release in anesthetized rats, and we particularly aimed our investigation at the level of the NAcc shell. We found that both vehicle and WIN pre-treated rats showed an increase in the DA release, but this effect was significantly enhanced for the WIN-cocaine group. Controversial results are shown in literature about the different functionality and responsiveness to drugs of abuse (or also to natural rewards) in the two

NAcc subdivision: the shell and the core (Bassareo and Di Chiara, 1999). FSCV in awake-behaving rats was performed by Singer and colleagues (2017) showing that a single injection of cocaine produces sensitization-related plasticity in the mesolimbic system with a strong DA release in the NAcc shell. Pierce and Kalivas (1995) also reported a preferential increase of dopamine in the shell compared to the core after amphetamine in rats sensitized to cocaine. Other authors using FSCV in vivo provided demonstration of rapid and similar DA sensitization in the NAcc core and shell after a short abstinence period from chronic cocaine exposure (Addy et al., 2010) On contrary, behavioral sensitization to morphine, cocaine, amphetamine and nicotine was linked with a rise in the DA response in the core and/or a decreased DA response in the shell (Cadoni and Di Chiara, 2000; Cadoni et al., 2000). Our results are in agreement with the authors claiming a sensitization-related increase in the DA transmission at the level of the accumbens shell, but to date we are not able to provide insights into the possible different responsiveness to cocaine present at the level of the accumbens core.

As already mentioned, p-eIF2a was found to be affected by WIN up to 24 h following its last administration (Melas et al., 2018). This time, when we assessed the levels of p-eIF2a after a period of abstinence and the day after the cocaine challenge, no changes were found in any of the adolescent brain regions under investigation. An explanation can be provided considering either (or both): a) the 7 days-suspension of WIN might have had an impact on the molecular pathways involved; b) cocaine-induced cross-sensitization might have altered the WIN-related p-eIF2a decrease levels previously observed. However, interestingly, we found that the same type of epigenetic modification (i.e. increase in H3K27ac), previously reported to be present after a prolonged WIN exposure *in vitro* (Melas et al., 2018), is also induced by cocaine in the PFC of adolescent WIN pre-exposed rats. More precisely, the latter effect is shown in the cocaine and in the WIN group, with an almost 50% increase in H3K27 acetylation, but the increase was significantly enhanced in the WIN–cocaine group (an∼100% increase inH3K27 acetylation), compared to the control group. There was also a significant change in H4acetylation (similar to H3) but no changes in histone phosphorylation or methylation were found in both adolescent and adult rats. These findings suggest that prior exposure to WIN modulates the effect of cocaine on histone acetylation at the level of the PFC.

In line with our data, other gateway drugs, such as nicotine (Kandel and Kandel, 2014; Levine et al., 2011) and alcohol (Griffin et al., 2017) were reported to have priming properties in mediating epigenetic effects, and specifically modifying histone acetylation levels by inhibiting the action of histone deacetylases (HDACs). One type of HDACs, HDAC6, is a class IIb HDAC, known to

deacetylates microtubules in the cytoplasm (Hubbert et al., 2002) but it also exert a nuclear action in histone deacetylation (Wang et al, 2009), and it has been associated with the rewarding effects of cocaine (Taniguchi et al., 2017; Renthal et al., 2007), generating the so called class II HDAC hypothesis of addiction (Griffin et al., 2018). Such downstream epigenetic mechanism characterized by the drug-induced reduction in the activity of HDACs was also confirmed by our results. We found indeed a reduction in HDAC6 levels in the adolescent WIN-cocaine group, and, on contrary, an increase of HDAC6 levels in the adult WIN-cocaine group. These findings suggest a possible role for HDAC6 in modulating cocaine-induced histone acetylation following WIN pre-exposure in both adolescence and adulthood.

The pre-exposure to WIN also led to cocaine-induced molecular changes. Since it was previously found that a significant upregulation of the phosphorylated forms of ERK1/2 was present after chronic WIN exposure (Melas et al., 2018), we evaluated possible cocaine-induced ERK modifications. We found a significant rise in the levels of MAP kinases ERK 1/2 (ERK) in the PFC of adolescent rats. In line with our results, ERK has not only been linked to cocaine-induced psychomotor sensitization as reviewed by Lu and coworkers (2006), but also a regulatory interaction between ERK and HDAC6 has been reported, with ERK that is able to phosphorylate HDAC6, which in turn deacetylates ERK (Wu et al., 2018; Williams et al, 2013). In agreement with these discoveries, using knockout and overexpression cell lines to investigate the relationship between these two enzymes, we found a causal link between ERK and HDAC6.

So far, we emphasized how a single response to cocaine is able to produce changes in the rat brain already pre-treated with cannabinoids, exposing the animals to the risk of future use or dependence (De Wit and Phillips, 2012). However, substance use disorders usually develop only after repeated exposures to a particular drug, leading to enduring epigenetic and synaptic changes (Hamilton and Nestler, 2019; Nestler and Luscher, 2019). For the aforementioned reasons, while maintaining the same drug administration protocol followed by 7 days of WIN abstinence, we consecutively challenged the animals with cocaine (i.p. 10 mg/kg) for 4 days. This time, we did not observed cross-sensitization in adolescent WIN pre-treated animals, even if a tendency towards significance is present. On the other hand, the adult WIN-cocaine group showed a distinctive close-to-significant suppression of the motor response in comparison to the cocaine group. The latter result can be explained considering the different effects that cannabis use exert on adolescent and adult brain (for a review see Dhein, 2020).

In addition, since several studies have shown that repeated exposure to drugs increase their ability to produce rewarding effects evaluated through the conditioned place preference (CPP) model (Lett

et al., 1989; Shippenberg et al., 1996), we studied this behavioral model in adolescent animals challenged for four days in a row with cocaine (i.p. 10 mg/kg) and chronically pre-exposed to WIN. In line with other studies (for an overview see Prus and James, 2009; Nomikos and Spyraki, 1998), we found a significant enhanced CPP for the cocaine and the WIN-cocaine groups in comparison to controls. The animals therefore show a marked preference for the compartment associated with the cocaine exposure rather than the one associated with saline injection. But interestingly, we also observed a close-to-significant enhanced CPP for the WIN-cocaine group in comparison to the cocaine group, suggesting that WIN may be able to increase the magnitude of the positive reinforcing properties associated with cocaine administration.

24 hours after the 4-days period of cocaine treatment, we also evaluated tissue levels of pivotal neurotransmitters, such as dopamine and glutamate, to understand whether a prolonged cocaine exposure may be associated with alterations in the physiological synaptic activity at the level of PFC, NAcc and hippocampus. In the present work we extensively reported how different drugs of abuse are able to increase extracellular dopamine in the NAcc. However, this process only describes the effects of a relatively short-term exposure to drugs, but it lacks of speculating on the fundamental long-term substance abuse-related mechanisms, such as drug craving and relapse. It is known that the positive reinforcing effects of cocaine (and many other drugs) play a crucial role not only in the beginning, but also in the maintenance of the drug-taking habit (D'Souza, 2015). A lot have been discussed in the last decades on the role of the excitatory neurotransmitter glutamate to be involved in many aspects of drug reward (D'Souza, 2015) and in mediating natural reward as well (Bisaga et al., 2008; Pitchers et al., 2012; Mietlicki-Baase et al., 2013). We found a significant increase in glutamate tissue levels in the PFC for the cocaine and WIN-cocaine groups in comparison to the WIN group. In agreement with our results, Màrquez and colleagues found increased glutamate levels in the PFC after cocaine exposure, accompanied with a decrease in FAAH and MAGL levels, and with an increase in CB1 receptor expression, suggesting an interaction between the glutamatergic and endocannabinoidergic systems (Màrquez et al., 2017). Moreover, we also found a significant decrease in the PFC of both glutamate and dopamine tissue levels for the WIN group. A possible explanation for the latter findings may be that the persistent WIN-induced stimulation of CB receptors during the chronic treatment is able to alter the normal receptor distribution, altering the endocannabinoid modulation that is responsible for the future decrease of these neurotransmitters in the PFC (Rodriguèz De Fonseca et al., 1998). This is also in line with other studies showing that the chronic administration of drugs of abuse, including cannabinoids, followed by a period of abstinence, visibly decreases the levels of dopamine and

glutamate in the PFC, and how these tend to increase again after re-exposure to the same stimulus (Kroener and Lavin , 2010). Moreover, we found that WIN chronic treatment enhances dopamine levels in the NAcc in comparison to controls. This is in line with the well known and already discussed features of cannabinoids and other drugs of abuse that lead to an increase of dopamine levels in this brain region. However, for this reason, it may be argued that dopamine levels in the NAcc for the WIN group are also significantly higher in comparison to those of the cocaine and WIN-cocaine group. Our hypothesis, also in agreement with other authors (for a review see Kuhar and Pilotte, 1996) is related to the timing chosen for brain dissections, i.e. 24 hours after the last cocaine injection. This might have been not long enough to restore physiological dopamine levels in the accumbens, therefore characterizing a withdrawal phase such that DAT (and thus dopamine reuptake) levels were altered, eventually decreasing dopamine levels for the cocaine and WIN-cocaine groups.

Glutamate tissue levels in the NAcc of the WIN group is not significantly higher in comparison to controls, although we would aspect to observe a significant difference when increasing the number of animals, since also glutamate is known to increase its levels in the accumbens after drug exposure (Chiu and Jahr, 2017; Schmidt and Pierce, 2010; Schultz, 2011; Britt et al., 2012). Finally, we found that dopamine tissue levels significantly increase in the hippocampus of the WIN group in comparison to all the other groups, according to the role of this area in mediating cannabinoid-related reward Lupica and Hoffman, 2018). Note that the tendency for both neurotransmitter levels are very similar to those found for the NAcc, strengthening the knowledge that reward behavior is finely regulated by hippocampus–nucleus accumbens synapses (LeGates et al., 2018). Nevertheless, it is important to underline that we assessed dopamine tissue levels considering the whole hippocampus, while projections to brain regions regulating reward come specifically from the ventral part of the hippocampus (Eagle, 2020).

Other improvement to our work can derive from the use of self-administration experiments, that better resemble the human approach towards the use of drugs. In particular, it has been shown that adolescent rats exposed to THC i.p. significantly increase WIN self-administration during adulthood (Scherma et al., 2016). Furthermore, the long-access to cocaine self-administration is one of the most validated animal models of cocaine use disorder and drug addiction (Kononoff et al., 2018; Edwards and Koob, 2013; Ahmed and Koob, 1998). About this topic, it is worth to mention the work performed by Kononoff and colleagues (2018), where adolescent rats undergone to the same drug administration protocol we used, but they were further exposed to cocaine self-administration for several weeks during adulthood and then assessed with the locomotor activity

test. They found an induced cross-sensitization to the motor-stimulating effect of cocaine (i.p.) in adolescence, which however did not persist into adulthood after cocaine self administration (Kononoff et al., 2018). These studies also provide insights into two different life stages (adolescent and then adulthood) in which the individuals are exposed to drug consumption, better characterizing the involvement of a determined gateway sequence.

Both Scherma and colleagues (2016) and Kononoff et al, 2018 though, chronically pre-treated the animals with a different cannabinoid i.p., and then assess self-administration i.v. in adulthood. Ideally, self-administration should be assessed in both life stages, however in rats the adolescent time window is too narrow to let the animals both recovering from surgery (necessary for self-administration) and acquiring the drug (Kononoff et al., 2018).

Future investigation may be also directed towards the use of different endpoints, for example establishing another length of the abstinence period or a prolonged duration of cocaine administration (regardless of the administration route), to give us a broader framework of the molecular pathways and epigenetic modifications involved in the behavioral-related responses. In addition, we should further investigate the features of dopamine dynamics related to the cross-sensitization observed also in the core of the nucleus accumbens. This might be done by using a dose-response curve that would allow us to better characterize different pharmacological profiles, since the response to concentration may be complex and it is often nonlinear.

Despite of necessary future progress, our study demonstrates that pre-exposure to cannabinoids modifies the initial behavioral, molecular, and epigenetic response to cocaine in the vulnerable adolescent brain. Furthermore, the overall picture offered by the present thesis provides a contribution that increases the knowledge of the Gateway Hypothesis of substance abuse, informing on the risks that cannabis or synthetic cannabinoids have on the adolescent brain, also in reference with the relatively recent increasing openness towards cannabis legalization policies worldwide.

ACKNOWLEDGEMENTS

Throughout the writing of this thesis, I have received a great deal of support and assistance.

I would first like to thank my tutor, Professor Paola Fadda, whose expertise was invaluable in formulating the research questions and methodology. You always encouraged me not to give up in tough moments, and your wise advices have always been outstanding and really helped me out along the way.

I would like to acknowledge my co-tutor as well, Dr. Maria Scherma, who patiently taught me many things about biomedical research and more. Your guidance throughout my studies has been really unique. You always supported all the group, and this made the work environment positive and productive.

Furthermore, a special thanks goes to all the collaborators of this research project. In particular, Dr. Gianluigi Tanda of the NIDA (NIH, Baltimore, USA), for having me warmly welcomed in the US, and in particular within his amazing working group; and to Dr. Philippe Melas, Dr. Denise and Dr. Erik Kandel of the Columbia University (New York, USA).

Last, but not least, I would like to thank all my colleagues, my friends and family, for their wise counsel and sympathetic ear. Without you this achievement would not have been possible.

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Paolo Masia gratefully acknowledges Sardinian Regional Government for the financial support of his PhD scholarship (P.O.R. Sardegna F.S.E. - Operational Programme of the Autonomous Region of Sardinia, European Social Fund 2014-2020 - Axis III Education and training, Thematic goal 10, Investment Priority 10ii), Specific goal 10.5.

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