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THE ROLE OF THE ENDOCANNABINOID SYSTEM IN PLASTICITY AND MYELINATION IN ADOLESCENT FEMALE RATS

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ABSTRACT

Although scientific evidence suggests that exposure to Cannabis during adolescence increases the risk of mental diseases later in life, this illicit drug remains the most commonly used among adolescents. Adolescence is a very sensitive period, characterized by morphological changes such as a loss of grey matter and an increase in white matter volume. We have previously demonstrated that THC adolescent exposure leads to significant alterations in dynamic changes occurring in the glutamatergic system during the adolescent development of the prefrontal cortex (Rubino et al., 2015). In order to understand if the harmful effect produced by adolescent exposure to THC could be linked to its interference with the physiological role of the endocannabinoid system, the first aim of our work was to investigate the role that the eCB system plays on the processes of neuronal remodelling taking place in the glutamatergic system during adolescence.

To reach our first aim, the adolescent developmental window (from PND 28 to 52) was divided into five sub-periods of five days each. Sprague-Dawley female rats of each subgroups received daily injections with the selective CB1 receptor antagonist AM251 (0.5 mg/kg, i.p.) or its vehicle. At the end of the treatment we studied, through western blot analysis, the effect of this modulation on some markers of plasticity. Specifically, we monitored plasticity relevant proteins present at postsynaptic sites such as PSD95, subunits of NMDA (GluN2A and GluN3A) and A2 AMPA subunits.

The results showed that the blockade of the endocannabinoid system negatively impacts on the dynamic changes typically occurring in the adolescent brain. In particular, adolescent AM251 administration prevented the decrease in PSD95 levels, caused alterations in GluN3A levels and slightly reduced the increase of GluN2A ones. As a whole, these data seem to indicate that the eCB tone could have a role in the adolescent elimination of the excitatory synapses and also that its blockade could delay the maturation of the glutamatergic system in adolescence.

At this point, since we showed that the blockade of the eCB system disrupts some of the physiological developmental changes occurring in the prefrontal cortex during adolescence, our second aim was to identify the most sensitive period of the adolescent THC exposure.

To this purpose, we chose a much milder treatment compared to that used in our previous studies. Specifically, following the same treatment schedule used before, animals of each period (PND 28-32, 33-37, 38-42, 43-47 and 48-52) were injected daily, for 5 days with a THC dose which corresponded to half of a joint (2.5 mg/kg i.p.) or its vehicle. As before, markers of plasticity were monitored at the end of the treatment.

Our results showed that the most delicate periods to this psychoactive component exposure were the early and mid-adolescence.

Interestingly, THC administration impacts on the dynamic changes occurring in the glutamatergic synapses during adolescence in a similar way to that observed after the modulation with the antagonist of CB1 receptor, AM251. These results could suggest a possible down regulation of CB1 receptors following the THC exposure, but they may also indicate that the adolescent synaptic remodelling could be especially promoted by the endocannabinoid 2AG and thereby THC, being a partial agonist of CB1 receptors, may behave as an antagonist towards the full agonist 2-AG when its levels are high.

Another important process occurring during the adolescent brain development is the white matter increase. Since the involvement of the eCB system in adolescent myelination remains completely unknown, our next aim was to investigate the effect of CB1 receptor blockade on myelination process in adolescent female rats. To reach this objective, female rats were injected with AM251 (0.5 mg/kg i.p) according to the same treatment protocol previously mentioned. Then, we monitored through western blot analysis MBP and MOG levels, two of the main white matter markers.

Since the modulation with the antagonist of CB1 receptors prevented the increase of both these markers we could assert that the eCB tone plays a fundamental role also in the adolescent myelination enhancement.

The gain in axonal myelination, that serves to enhance communication efficiency, has previously been associated with the development of language and memory skills in adolescence. Interestingly, recent studies have shown that individual variability in myelin growth trajectories, investigated during the transition from adolescence to adulthood, could be also linked to the expression of impulsivity traits. Specifically, it has been reported that

impulsivity is associated with a reduction in myelin growth in the lateral and medial prefrontal cortex. On these grounds, first we wanted to investigate through the wire-Beam Bridge Test, whether any correlation exists between impulsivity and white matter development in our animal model. Furthermore, since it has been demonstrated that the blockade of eCB system affects the adolescent myelination enhancement, we also wanted to investigate if animals treated with the CB1 receptor antagonist, AM251, were effectively more impulsive then controls. To reach this aim we injected adolescent female rats from PND 28 to PND 45 (period in which the eCB system functionality system seems to be fundamental for the myelination process) with AM251 and then we tested their impulsivity through the Wire-Beam Bridge Test.

As a whole, our results first of all confirm that there is an association between the increase of myelin and the reduction of impulsivity in adolescent naïve rats and thereby that the reduction of impulsivity is age-related. Secondly, they suggest that the eCB system blockade during this specific developmental window, preventing the myelin enhancement, leads concurrently to a higher expression of impulsivity.

Finally, we tried to identify the cellular mechanism which could be involved in the myelination enhancement induced by the activation of CB1 receptors during this specific developmental window.

Our results suggest that CB1 receptor stimulation could induce the activation of the AKT pathway that in turn could inhibit the Hippo signalling pathway, thereby promoting the translocation of YAP into the nucleus, where it could promote myelin gene transcription.

As previously done in the study of the eCB system role in plasticity, we also wanted to evaluate whether the administration of THC would affect the increase of white matter in adolescent rats. Again, our results showed that THC prevented the myelination increase in a similar way to that observed after the blockade of the eCB system.

To sum up, the present work suggests that adolescent cannabis exposure could be detrimental for the physiological brain development during this precise developmental period, affecting the two main physiological processes occurring during this age: synaptic pruning and myelination events. This study also describes for the first time the relationship

between the age-related increase of myelin and the reduction of impulsivity in female rats. Furthermore, it also demonstrates that the blockade of the eCB tone, preventing the increase of myelin, leads to increased levels of impulsivity. Finally, it sheds light on a possible cellular mechanism that, beginning with the stimulation of the CB1 receptor, could favor the enhancement of myelin through both AKT and hippo pathways.

INTRODUCTION

THE ADOLESCENT BRAIN

Adolescence is a very delicate period in which brain has not reached the full maturation yet. During this "work in progress" span of time, the brain undergoes changes in gross morphology characterized by a loss of grey matter (GM) and a rise of white matter (WM) (Giedd et al., 1999; Sowell et al., 1999; Sowell et al., 1999; Sowell et al., 2004). The decrease in GM seems to be mediated by synaptic pruning that serves to create more efficient neuronal pathways and seems to affect predominantly the asymmetric synapses located on dendritic spines, which are primarily excitatory. Thus, pruning seems to mainly involve glutamatergic neurotransmission (Bourgeois and Rakic, 1993). On the other hand, the increase in WM seems to be associated with the myelination processes, which have an essential role in creating more efficient neuronal networks by promoting the smooth flow of information (Paus, 2010). Both these refinements occur mainly in the prefrontal cortex (PFC), the last brain region to reach the maturation.

Furthermore, animal studies suggest that, during this transient period, maturational events also occur in different neurotransmitter systems and in some components of the endocannabinoid system. Specifically, in adolescents, adrenergic, dopaminergic and serotonergic receptors are over-expressed across many brain regions (Lidow and Rakic, 1992; Rodríguez de Fonseca et al., 1993). During adolescence, dopamine neuron activity peaks and then decreases whereas the excitatory/ inhibitory balance is remarkably different in adolescents compared to adults. As a matter of fact, contrary to the linearly increasing of GABA levels, which has been demonstrated to be the main inhibitory neurotransmitter, the expression of different subunits of glutamate N-methyl-D-aspartate (NMDA) and alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptors are characterized by dynamic changes during this sensitive period. Furthermore, the expression of glutamate NMDA receptors on fast-spiking interneurons is highly altered. In the PFC of adolescents, these inhibitory interneurons do not exhibit NMDA receptor-mediated currents (Wang and Gao, 2009). Finally, the whole adolescent period is also characterized by fluctuations in endocannabinoid levels among brain regions involved in cognition, reward and motivation such as nucleus accumbens (NAc), hippocampus, amygdala and PFC. Specifically in PFC anandamide (AEA) levels strongly increase from early to late adolescence whereas 2-

arachidonoylglycerol (2-AG) is lower during the later adolescent phase comparing to the beginning, a finding similar to what has been observed in NAc (Ellgren et al., 2008; Rubino et al., 2015; Rubino, personal communication).

Since all this neuronal signalling is essential in both cognitive and emotional process, its dysfunction or any interference with its physiological development (as THC adolescence consumption) may represent a risk factor for numerous psychiatric illnesses including mood disorders, addiction or schizophrenia (Spear, 2000; Rubino and Parolaro, 2016).

CANNABIS AND THE ENDOCANNABINOID SYSTEM

The last European drug report confirms that Cannabis continues to be the most used illicit drug among adolescents (European Drug Report, 2021). Furthermore, it has been found that the delta9-tetrahydrocannabinol (THC) content in Cannabis resin is highly increased compared to the past years. Since several studies provide evidence that the exposure to Cannabis (or to its most psychoactive component, THC) in adolescence may acts as a risk factor for developing mental disorders later in life (Schneider, 2008; Rubino et al., 2012; Rubino and Parolaro, 2015; Renard et al. 2014), monitoring of the Cannabis market changes and the prevalence of use among young people is relevant

The main psychoactive component of Cannabis Sativa, THC, exerts its role by acting through the endocannabinoid receptors (CB1 and CB2). The endocannabinoid receptors together with the endocannabinoids (the best known are AEA and 2-AG) and all the enzymes responsible for their synthesis and degradation, form the endocannabinoid system (ECS), a neuromodulatory system involved in the CNS development and in neuronal activity modulation.

CANNABINOID RECEPTORS

The two best-known cannabinoid receptors are CB1 and CB2 (Fig.1). Both these receptors belong to the G proteins–coupled receptor family (GPCRs) and mostly couple with inhibitory G proteins. Therefore, their activation lead to (i) inhibition of adenylyl cyclase activity, consequently reducing cellular cAMP levels, (ii) stimulation of MAP (mitogen-activated

protein) kinases, (iii) activation of inwardly rectifying potassium channels (GIRKs), and finally to (iv) inhibition of N- or P/Q-type of Ca2+ channels (Iannotti et al., 2016).

Fig.1 Cannabinoid receptors CB1 and CB2.

CB1 receptors are primarily expressed in the CNS but they are also present in the peripheral nervous system and in organs such as the gastrointestinal tract, liver, adipose tissue, heart, lungs, male and female reproductive systems, bone and skin (Iannotti et al., 2013; Iannotti et al., 2016). In CNS neurons, CB1 receptors are widely found in glutamatergic, cholinergic and serotonergic neurons (Shu-Jung Hu and Mackie, 2015). However, they are most abundant on certain GABAergic interneurons (Bodor et al., 2005). Finally, low expression of CB1 receptors has been also found on oligodendrocytes precursors, oligodendrocytes and microglia (Molina-Holgado et al., 2002).

In contrast to CB1, CB2 receptors are mainly expressed in cells of immune origin including monocytes, macrophages, B- and T-cells and microglia, the immune cells of the central nervous system (Cabral et al., 2015; Stella, 2010). Furthermore, CB2 receptors are also present in peripheral organs important for the immune response such as spleen, thymus gland, tonsils and gastrointestinal system (Galiègue et al., 1995; Ständer et al., 2005; Campora et al., 2012).

OTHER CANNABINOID RECEPTORS

Although CB1 and CB2 are the only two official recognized cannabinoid receptors, evidence suggests that cannabinoids are also able to interact with other receptors (Fig.2). As a matter

of fact, it has been demonstrated that the transient receptor potential vanilloid type-1 (TRPV1) channel can be activated by the endocannabinoid AEA. Its activation leads to the rise of intracellular Ca2+ (Cortright and Szallasi, 2004). However, AEA affinity for TRPV1 is lower comparing to that for CB1 and CB2 receptors (Di Marzo and De Petrocellis, 2012).

The GPR55 receptor, which belongs to the G proteins-coupled receptors family, is considered another 'atypical' cannabinoid receptor. Its endogenous ligand is lysophosphatidylinositol (LPI) (Oka et al., 2007; Henstridge et al., 2009; Sharir et al., 2012) but it has been demonstrated that it can also be activated by Δ9-THC and by some synthetic inverse agonists of CB1 receptors. Moreover, it seems to be antagonized by cannabidiol (CBD), which is the major, non-psychotropic phytocannabinoid. Furthermore, there are contrasting data suggesting that it could be activated by low concentrations of AEA, 2-AG, virodhamine, noladin ether and PEA (Sharir et al., 2012).

Finally, 2-AG and AEA can also bind various Peroxisome proliferator-activated receptors (PPARs) isotypes (Lenman and Fowler, 2007) that are intracellular receptors, able to regulate the gene expression when activated. PPAR-γ can be activated by THC, CBD, WIN and CP as well (O'Sullivan et al., 2006; O'Sullivana and Kendallb, 2010).

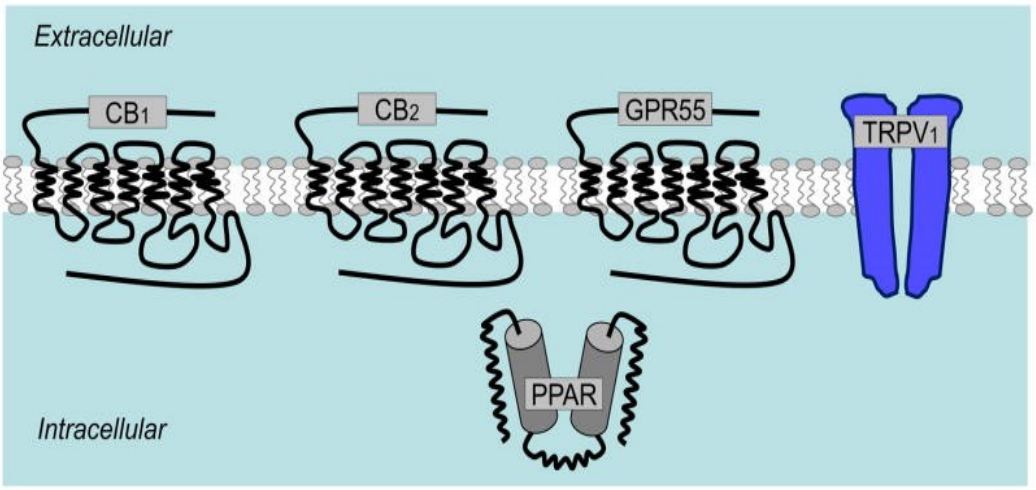

Fig.2 Receptors activated by cannabinoids.

ENDOGENOUS LIGANDS

The discovery of cannabinoids receptors, CB1 and CB2 led to further investigation that ended with the identification of their endogenous ligands or "endocannabinoids". For the first time in 1992, AEA was isolated (Devane et al., 1992) and then three years later 2-AG (Mechoulam et al., 1995). They are able to bind both cannabinoid receptors. Later on, other endogenous molecules able to exert cannabinoid-like effects have been identified. Among them, noteworthy are 2-arachidonyl-glycerol ether (noladin ether), virodhamine, N-arachidonoyl-dopamine (NA-DA), N-homo-γ-linolenoylethanolamine (HEA) and N-docosatetraenoylethanolamine (DEA) (Hanus et al., 2001; Porter et al., 2002; Hanus et al., 1993; Bisogno et al., 2000). However, their pharmacological activity and metabolism have not been fairly investigated yet. Finally, two AEA-related compounds, N-palmitoylethanolamine (PEA) and N-oleoylethanolamine (OEA) have also been identified (Re et al., 2007; Fonseca et al., 2001). Since these molecules lack strong affinity with both CB receptors, AEA and 2-AG are still considered the primary endogenous ligand of cannabinoid signalling.

The chemical structures of AEA and 2-AG (Fig.3) are both characterized by arachidonic acid-containing lipid molecules. Despite this similarity, they present some crucial differences that are responsible for their in vivo roles. First, 2-AG is a full agonist of CB1 and CB2 receptors, whereas AEA (as well THC) acts as a partial agonist of both (Shen and Thayer, 1999; Mackie, 2008). Secondly, in the brain, the concentration of 2-AG is 170 times higher than that of AEA (Stella et al., 1997) and finally, they are regulated by different biosynthetic and catabolic pathways (Ahn et al., 2008).

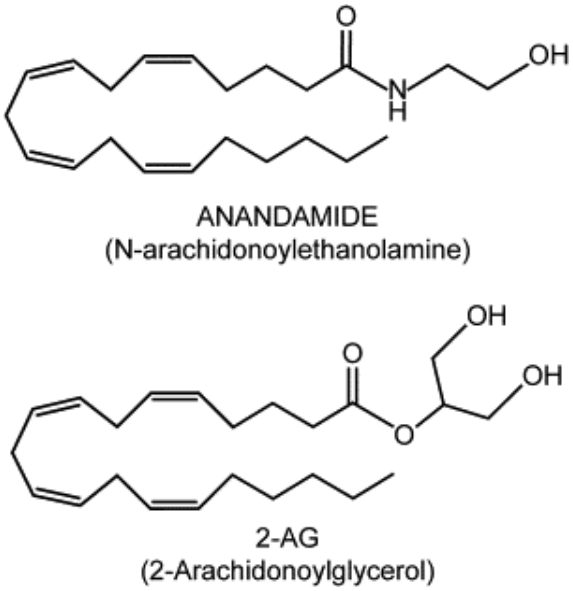

Fig.3 Structures of AEA and 2-AG.

BIOSYNTHETIC AND CATABOLIC PATHWAY OF AEA AND 2-AG

In contrast to classic neurotransmitters, which are synthesized and stored in vesicles, the major form of endocannabinoid (eCB) synthesis occurs "on demand". In particular, endocannabinoids that exist as precursors in form of membrane lipids, are released thanks to the activation of enzymes after specific signals such as, for example, an increasing of intracellular calcium.

There are different pathways for eCB production (Fig.4). The canonical way for producing 2-AG is a 2-steps pathway:

1- removal of the inositol triphosphate from arachidonoyl-containing phosphatidylinositol bisphosphate (PIP2) by a PLCβ;

2- removal of the acyl group in the 1 position;

This last step is made by a diacylglycerol (DAG) lipase. In the brain, two DAG lipase isoforms have been identified: DAG lipase alpha (α) and DAG lipase beta (β) (Bisogno et al., 2003; Jung et al., 2007). The former is more relevant in synaptic production of 2-AG whereas the latter seems to be more important for the production of 2-AG in microglia (Tanimura et al., 2010). Regarding AEA, it is synthesized by the cleavage of an N-arachidonoyl phosphatidyl

ethanolamine (NAPE). The most canonical and direct route is produced by NAPE phospholipase D (NAPE-PLD) (Okamoto et al., 2009). Other alternative pathways include:

- The formation of phospo-AEA catalyzed by NAPE-phospholipase C (PLC) followed by its conversion in AEA by PTPN22 phosphatase

-The hydrolysis of the acyl groups by ABHD4, followed by hydrolysis by GDE1

Once synthesized by the postsynaptic cell, endocannabinoids travel retrogradely in order to activate the presynaptic cannabinoid receptors. Then, they are rapidly deactivated by uptake into cells and subsequent intracellular enzymatic degradation. Generally, both 2-AG and AEA hydrolysis consists of arachidonic group removal. Specifically, in CNS, the 2-AG hydrolysis is mainly performed by monoacylglycerol lipase (MAGL) or alpha/beta-hydrolase domain containing 6 (ABHD6), whereas the AEA action is primarily terminated by fatty acid amino hydrolase (FAAH). Nevertheless, there is evidence suggesting that FAAH can also be activated by 2-AG and thereby, its contribution to 2-AG degradation, under certain conditions or in specific brain areas, cannot be excluded. However, it has been reported that MAGL is the enzyme responsible for at least 85% of 2-AG breakdown (Blankman et al., 2007). Over the years, many specific FAAH and MAGL inhibitors have been developed. Among them, noteworthy are URB597, PF-3845, PF-04457845, OL-135, for FAAH; and JZL184, URB602, OMDM169, CAY10499 and KML29 for MAGL (Mor et al., 2004; Ahn et al., 2009; Ahn et al., 2011; Lichtman et al., 2004; Long et al., 2009; King et al., 2007; Bisogno et al., 2009; Muccioli et al., 2008; Chang et al., 2012).

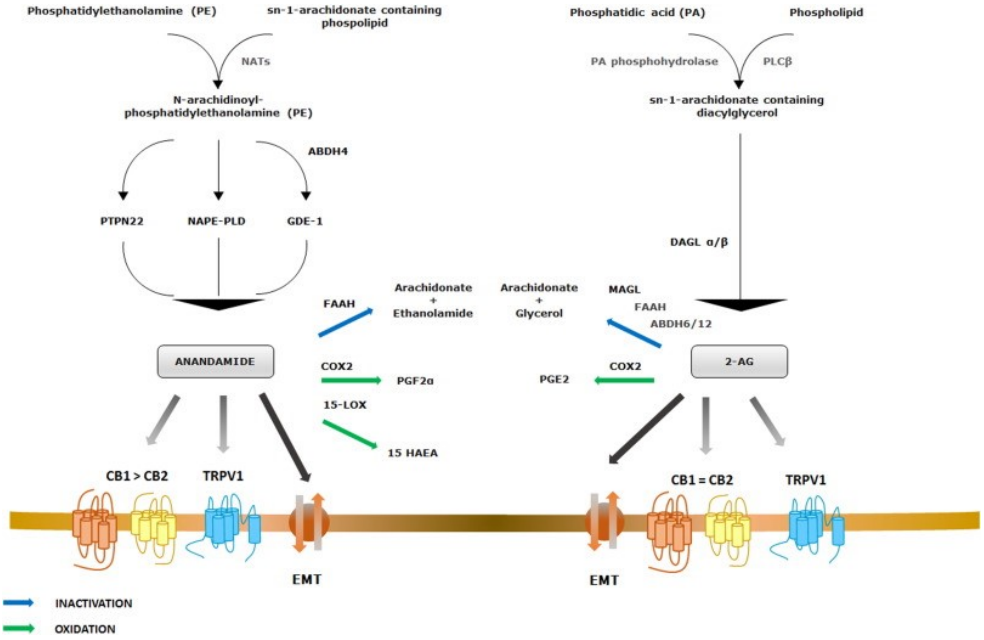

Fig.4 2-AG and AEA synthesis and degradation.

LONG-LASTING EFFECTS OF CANNABINOID EXPOSURE DURING ADOLESCENCE IN ANIMAL MODELS

The use of animal models is a valid and strategic way to evaluate the molecular mechanisms underlying the association between the use of Cannabis during adolescence and the development of psychiatric disorders later in life. However, first of all it is important to reproduce in animal models what has already been observed in epidemiological studies, that is the occurrence of alterations in adult behavior after adolescent cannabinoid exposure.

CANNABINOIDS AND EMOTIONAL BEHAVIOR

The link between cannabis use and the development of anxiety in animal models is not as consistent as in humans. Contrasting data are reported regarding anxiety tested using the elevated plus maze or the open field. For instance, the exposure to cannabinoids during adolescence has been reported to lead to either anxiety, an anxiolytic-like response or even no behavioural changes in adulthood (Renard et al., 2016). Even more recent findings failed to draw a clear conclusion. An anxiolytic effect has been described preferentially in males when the exposure to THC occurs at a very early stage of adolescence. No effects on

measures of anxiety have been reported after cannabis or THC smoke during adolescence (Bruijnzeel et al., 2019). Based on current data is thereby not possible to provide a clear conclusion on the lasting effects of the exposure to cannabinoids in adolescence on measures of anxiety. However, we may speculate that these contrasting data might be influenced by different factors such as the animal strain chosen, the type of cannabinoid used (natural or synthetic), the precise adolescent time of cannabinoid exposure or the behavioural test used to assess anxiety. These different experimental parameters seem to have a minor impact when social anxiety has been investigated. Specifically, studies suggest a decrease in social interaction in adult male and female rats after adolescent exposure to both synthetic and natural cannabinoid agonists (Leweke and Schneider, 2011; O'Shea et al., 2004; 2006; Quinn et al., 2008; Realini et al., 2011; Zamberletti et al., 2014; Renard et al., 2016). The only exception is the Gleason's paper (2012), in which no significant differences in interaction time between the control group and the group administered with WIN 55,212-2 were measured. This could be explained by the fact in Gleason's work the behaviour was checked nearly 3 months later the end of the treatment whereas in other experiments it was monitored within two months. This result suggests that some of the altered behaviours induced by the adolescent cannabinoid exposure might be normalized with time. It is well known that reduction in social interaction can be considered not only an anxiogenic behaviour in rodents (File and Hyde, 1978), but also a sign of major depressive disorder (Hirschfeld et al., 2000). Accordingly, the effect of adolescent exposure to cannabinoids (both natural and synthetic) on measures of depressive-like behaviors in adulthood is much clearer. Adult animals, exposed to cannabinoids in adolescence have shown a depressive-like phenotype, tested in the social interaction test, in the sucrose preference test and in the forced swim test (Amir Levine et al., 2017). Even adolescent exposure to very low doses of THC (1 mg/kg) seems to be related to aspects of depressive reactivity (De Gregorio et al., 2020). It is worth noting that females treated with cannabinoids in adolescence are likely to display more intense depressive-behaviors compared with males (Rubino and Parolaro, 2016). Thereby, these data may suggest that female rats may be more sensitive to the emotional consequences of adolescent cannabis exposure.

CANNABINOIDS AND COGNITION

Many studies have reported that in rodents, chronic exposure to cannabinoids during adolescence, but not adult treatment, leads to long-lasting alterations in cognitive functions. As a matter of fact, it has been observed that adult rats exposed to cannabinoids agonists (both synthetic and natural) in adolescence showed impairments in the performance of the classic novel object recognition (NOR) test and spatial version as well (Zamberletti et al., 2014; O'Shea et al., 2006; O'Shea et al., 2004; Quinn et al., 2007; Realini et al., 2011; Abush and Akirav, 2012; Renard et al., 2012; Schneider and Koch, 2003). Deficits in the spatial working memory assessed by the eight-arm radial maze were described also in adult rats treated with THC in adolescence (Rubino et al., 2009a; Rubino et al., 2009b). Finally, impairments in the attentional set-shifting task, which is a measure of the cognitive flexibility, were found in adult animal exposed to WIN 55,212-2 in adolescence (Gomes et al., 2014). Conversely, data showed no long-lasting effects of adolescent cannabinoids exposure when the pure spatial memory was measured using the Morris water maze and the Barnes maze test (Higuera-Matas et al., 2009; Abush and Akirav, 2012; Cha et al., 2007). Overall, these findings seem to suggest that adolescent cannabinoid exposure is likely to impair the memory form in which PFC plays a role. However, cannabinoid exposure under conditions of intravenous and smoke self-administration seems to have less detrimental effects on cognition. Indeed, working memory performance was unaltered or slightly improved after adolescent self-administration of THC, as also reported after THC smoke exposure (Stringfield et al., 2021; Bruijnzeel et al. 2019).

CANNABINOIDS AND PSYCHOSIS

The pre-pulse inhibition (PPI) paradigm is the most reliable animal assay used to evaluate psychotic-like states in rodents. It has been demonstrated that impairment in the PPI can be linked to a dysfunction in the sensor motor gating mechanism, which is a cognitive alteration typical in schizophrenia. Another way used to investigate psychotic-like signs in animal models is through the study of hyperlocomotion, either at baseline or after the administration of stimulating dopaminergic and glutamatergic agents. The majority of data show that the exposure to WIN55, 212-2 or THC can lead to long-lasting impairment of PPI in

rats only when the administration occurs during adolescence not in adulthood. However, depending on the rodents species used, it is possible to found discrepant findings (De Felice et al., 2021; Rubino and Parolaro, 2016). Regarding spontaneous locomotor activity following adolescent cannabinoid exposure, data have not reveal consistent results. However, investigations of hyperlocomotion induced by dopaminergic and glutamatergic agents seem to be more consistent and suggest that THC treatments in adolescence may increase the locomotor response (Rodríguez et al., 2017; Zamberletti et al., 2014).

CANNABINOIDS AND GATEWAY EFFECT

Epidemiological studies suggest an association between early cannabis use and a higher risk to progress toward addiction to other illicit drugs. This is known as the "gateway hypothesis" which posits that the relationship between early cannabis use and the subsequent abuse of other addictive substances is causal in nature (Kandel et al. 2015). Animal studies examining a causal link between adolescent cannabinoid exposure and subsequent abuse of illicit drugs at adulthood have mainly focused on opioids and cocaine. Adolescent THC exposure increased heroin intake (Ellgren et al. 2007) but did not facilitate the acquisition of heroin self-administration in adult rats (Ellgren et al. 2007; Stopponi et al. 2014). Furthermore, exposure to THC during adolescence was shown to increase the vulnerability to heroin relapse in adulthood (Stopponi et al. 2014), suggesting that a previous history of cannabinoid use during adolescence might be a risk factor for relapse following opioid use later in life. Interestingly, THC pre-exposure during adolescence increased fentanyl self-administration in female rats only (Nguyen et al. 2020). Overall, available data suggest that the association between adolescent THC exposure and adult vulnerability to opioids might be dependent on the sex of the animals and the potency of opioid tested. Recently, the influence of adolescent cannabis exposure on adult heroin reinforcement has been demonstrated in a model of genetic vulnerability to drug addiction. THC pre-exposure increased responding for heroin, heroin intake and reinstatement in vulnerable male rats (Lecca et al. 2020), suggesting that genetic factors might play a pivotal role in determining the outcome of adolescent cannabinoid exposure on opioid vulnerability later in life.

With regard to a possible association between adolescent cannabinoid exposure and subsequent use of legal drugs of abuse, such as nicotine and alcohol, adolescent THC

exposure did not alter the performance of intravenous nicotine self-administration in adulthood (Flores et al. 2020), but increased self-administration of nicotine was observed following adolescent administration of WIN 55,212-2 (Dukes et al. 2020). WIN 55,212-2 during the early adolescent period also enhanced preference for alcohol consumption in adult rodents (Frontera et al. 2018).

SEX-DEPENDENT EFFECT OF CANNABIS EXPOSURE

Drug addiction is a widespread phenomenon affecting both men and women. For many years, preclinical and clinical studies have ignored the potential sex influence, focusing their investigations mainly on males or considering all the subjects as "unisex". Fortunately, the view that biological sex is not relevant has been overcome over the years. Human studies have shown that, as it has been seen for other drugs, also cannabis abuse in humans appears to be associated with different responses in males and females. For examples, generally, female abusers are likely to develop internalizing disorder, such as depressive and anxiety disorders (Kloos A et al., 2009). Conversely, male peers seem to suffer from more externalizing behaviors, such as aggressiveness and impulsivity (Kloos A et al., 2009). Animal models, which provide a valuable strategy in order to evaluate the molecular bases of these sex differences, seem to confirm the existence of sex-dependent responses to adolescent cannabinoid exposure. Specifically, in line with human studies, female animals appear more sensitive than males in the emotional sphere. Pharmacokinetics seems to play an important part in these differences. Specifically, females show a greater metabolism of THC to a still active compound (11-OH-THC) compared with their male peers (Wiley and Burston, 2014), suggesting the fact that in females the effect of THC can be potentiated by its active metabolite. Furthermore, since studies have also revealed that female animals have more efficient CB1 receptors (Rubino and Parolaro, 2011), it is possible to hypothesize that females may be more vulnerable to THC exposure. It is also worthwhile noting that the THC treatment seems to affect different brain regions according to gender. As a matter of fact, among all the cerebral areas investigated, PFC seems to be more sensitive in females, whereas it is the hippocampus in males. In agreement with these findings, our group has

demonstrated that after adolescent THC administration, females were characterized by a significant decrease in pre- and post-synaptic markers in the PFC, whereas the same proteins were found altered in the hippocampus of male peers (Rubino et al., 2009). The differences in the brain regions affected may in part explain why females are more likely to suffer from internalizing disorders, compared to males. This already complicated picture becomes worse if we take into account the fact that some brain developmental characteristics are different in the two sexes. Worth noting are, for examples, the neurodevelopmental trajectories (Lenroot RK et al., 2007). Specifically, the total brain size and regional gray matter that are characterized by an inverted U shaped maturational curve, peak earlier in females, suggesting a different intensity of pruning events in adolescent boys and girls of the same age. Furthermore, since recent studies have demonstrated that the ECS could play a role in pruning (Rubino T et al., 2015), adolescent cannabinoid exposure might interact with this event leading to different impairments in brain and behavior. Finally, interactions of the endocannabinoid system with gonadal hormones may also play a part in the complex pruning event. It has been shown that sex hormones together with the ECS work in symphony for promoting maturational events in adolescent brains (Chadwick B et al., 2011). Overall, further investigations of all these interactions are necessary in order to design proper sex-specific treatments and to prevent long-term side effects caused by heavy Cannabis abuse.

POSSIBLE MECHANISMS OF ADOLESCENT VULNERABILITY TO CANNABINOID EXPOSURE

The endocannabinoid system is the first target of exogenous cannabinoids, thereby it would be consistent expecting alterations in components of this system after exposure to these compounds. In line with this assumption, studies have revealed a marked CB1 receptor downregulation and desensitization after chronic THC treatment during adolescence in different cerebral areas (Rubino et al., 2015; Rubino et al., 2008; Burston et al., 2010). This effect was described to be more evident in female than in male rats (Rubino et al., 2008;

Wiley and Burston, 2014). This difference can be due to the metabolism of THC, which is different in the two sexes and to the presumed greater efficiency of receptors in adolescent female rats (Rubino and Parolaro, 2011). Furthermore, the long-term reduction in CB1 receptor density, described after adolescent THC exposure in different brain areas, was more marked in adult female animals rather than in males, (Zamberletti et al., 2012). Lastly, in the PFC of female rats exposed to THC, this significant reduction of CB1 receptor binding, which was found immediately after the last THC injection and persisted in adulthood, was also accompanied by a decrease of anandamide levels (Rubino et al., 2015). Long-lasting negative impact on working memory and decision-making was the relevant event observed in rats after chronic adolescent cannabinoids exposure. Since these processes could be mainly linked to alterations in the maturation of PFC, studies of developmental impairment of the GABAergic and glutamatergic system, occurring after chronic adolescent cannabis exposure, focused their investigation primarily on the PFC. Evidence suggests that adolescent CB1 receptor overstimulation might disrupt the maturation of the glutamatergic system. Specifically, female animals exposed to CP-55,940 or THC in adolescence were characterized in adulthood by decreased K+-evoked glutamate release in hippocampus (Higuera-Matas et al., 2012) and by altered maturational events of AMPA and NMDA subunits in PFC, which appeared to be enriched in GluN2B and GluA1 (Rubino et al., 2015). Since the well-known key role played by NMDA receptors in the regulation of the peri-adolescent maturation of GABAergic system in the PFC (Thomases et al., 2013), an alteration in GABAergic system can be also hypothesized. In accordance with this assumption a downregulation of GABAergic transmission has been described in PFC of rats exposed to WIN 55,212-2 or THC during early adolescence or mid-adolescence (Cass et al., 2014; Zamberletti et al., 2014). Thereby it can be alleged that the adolescent exposure to cannabinoid can affect the endocannabinoid, glutamatergic and GABAergic systems as well. Another event that strongly characterizes the adolescent brain maturation is synaptic pruning, which occurs especially in PFC, the most important brain region in the control of cognitive function (Selemon, 2013). It has been suggested that the endocannabinoid system could be also involved in the adolescent synaptic pruning regulation and so changes in dendritic spines might be triggered by adolescent cannabinoid exposure. Consistently, in

male animals the exposure to WIN 55,212-2 in adolescence significantly decreased spine density in the Nuceus Accumbens (NAc) (Carvalho et al., 2016). Furthermore, it has been found in adult male rats reduced spine density in the dentate gyrus of the hippocampus and a decrease in dendrite length and number after THC adolescent exposure (Rubino et al., 2009). Research has also shown that adolescent THC exposure in male rats resulted in the premature pruning of spines and protracted atrophy of distal apical trees in the PFC (Miller et al., 2019), suggesting that adolescent THC exposure reduces the complexity of pyramidal neurons, which might enhance vulnerability to psychiatric disorders. Finally, similar findings were observed in adolescent female rats exposed to THC which showed a decrease in the number of spines present on PFC pyramidal neurons (Rubino et al., 2015).

THE ENDOCANNABINOID SYSTEM AND OLIGODENDROCYTES

The Myelin sheath has important functional roles, such as promoting rapid transmission of axons and providing their metabolic support (Simons and Nave, 2015). Short portions of axons are left unwrapped at the nodes of Ranvier where Sodium Channels are located. Myelin works as an electrical insulator and this allows the action potential to “jump” from one node of Ranvier to the next. This process is called saltatory conduction and is a form of transmission much faster than the one in non-myelinated axons. Furthermore, it has been also demonstrated that oligodendrocytes (myelin-forming cells of SNC) metabolically support axons. Specifically Oligodendrocytes (OLs) can generate lactate, which can be relocated to axons in order to produce energy in the form of ATP (Tepavčević, 2021). Curiously, emerging evidence suggests that alterations in myelin growth trajectories can have an impact in the development of individual differences. Specifically in a humans study, using a novel MT imaging protocol, it has been demonstrated that the cingulate cortex expresses the greatest myelin-related growth during late adolescence and early adulthood and a reduction in the myelin growth, during this period, could be associated with higher impulsivity traits (Ziegler et al., 2019).

OLs are generated from oligodendrocyte progenitor cells (OPCs). OPCs arise from neuroectodermal stem cells and rely on processes of survival, proliferation, migration and

differentiation to reach their maturation (Baumann et al., 2001). With exception of migration, these processes seem to be also regulated by the eCB system (Ilyasov et al., 2018). (Fig.5).

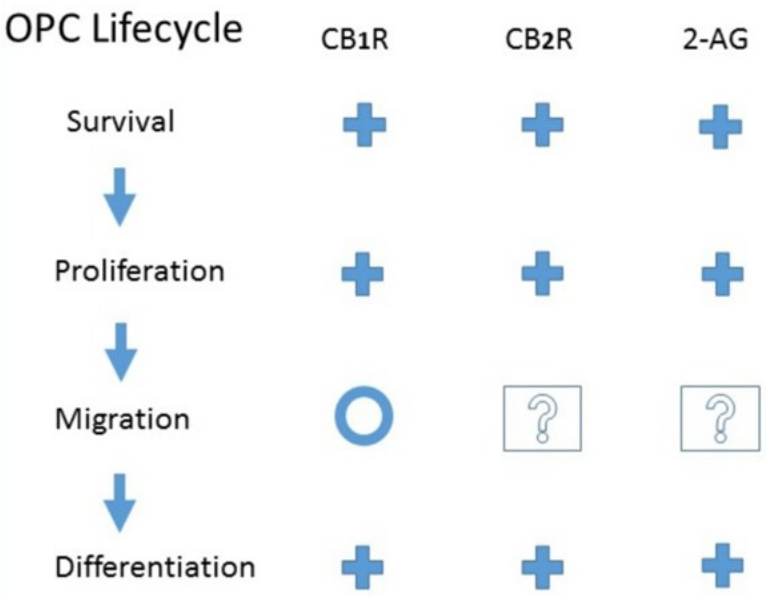

Fig.5 Evidence for CB1 or CB2 receptor activation or endogenous 2-Arachidonoylglycerol modulation of OPCs life Cycle.

THE ENDOCANNABINOID SYSTEM AND OPCs SURVIVAL

In vitro studies showed that OPC survival could be promoted by the activation of either CB1 or CB2 receptors under conditions of trophic factor deprivation. The mechanism important for the reduction of apoptotic processes and for the promotion of OPCs survival seems to involve the (PI3K)/Akt pathway (Molina-Holgado et al., 2002).

THE ENDOCANNABINOID SYSTEM AND OPCs PROLIFERATION

It has been shown that OPCs proliferation can be modulated by eCB. Specifically Molina-Holgado et al. demonstrated in cultured OPCs a reduction in PDGF/FGF-stimulated OPC proliferation after blockade of CB1, CB2 receptors and synthesis of 2-AG by DAGLs, using respectively AM281 (1 µM), AM630 (1 µM) and RHC80267 (5 µM) (Molina-Holgado et al., 2002). These effects are related to the phosphorylation of Akt and mammalian target of

rapamycin (mTOR). Furthermore, cells treated with 2-AG synthesis inhibitor had lower levels of extracellular signal regulated-kinase (ERK) 1/2 phosphorylation. Thus, OPCs proliferation seems to depend on Akt/mTOR and ERK pathways (Gonsalvez et al., 2016).

THE ENDOCANNABINOID SYSTEM AND OPCs DIFFERENTATION TO MATURE OLIGODENDROCYTES

Molina-Holgado et al. using isolated differentiated OPCs, in the absence of PDGF/FGF, have investigated oPCs differentiation (Molina-Holgado et al. 2002). They reported an increasing in OPC arborization and production of MBP (marker of OLs maturity (Fig.6)) when CB receptors were activated with CB1, CB2 and CB1/CB2 receptor agonists (ACEA, JWH133 and HU210, respectively). This result could be reverted in the presence of CB1 and CB2 antagonists such as AM281 and AM630. In addition, they provided evidence that this effect was exerted through a mechanism dependent on the activation of the PI3K/Akt and mTOR signaling pathways, since these effects resulted in being blocked with both LY290042 (PI3K inhibitor) and rapamycin (mTOR inhibitor). Furthermore, evidence suggests that, while AEA levels were low and did not change between the cell stages, the amount of 2-AG was greater in OPCs rather than in mature OLs (Gomez et al., 2010). Differentiation resulted in being increased by MAGL inhibitor, JZL184 and decreased by DAGL inhibitor, RHC80267, effect reverted with exogenous 2-AG. Finally, inhibition of ERK pathway abolished Western blot staining for MBP, suggesting also its involvement in the differentiation mechanism.

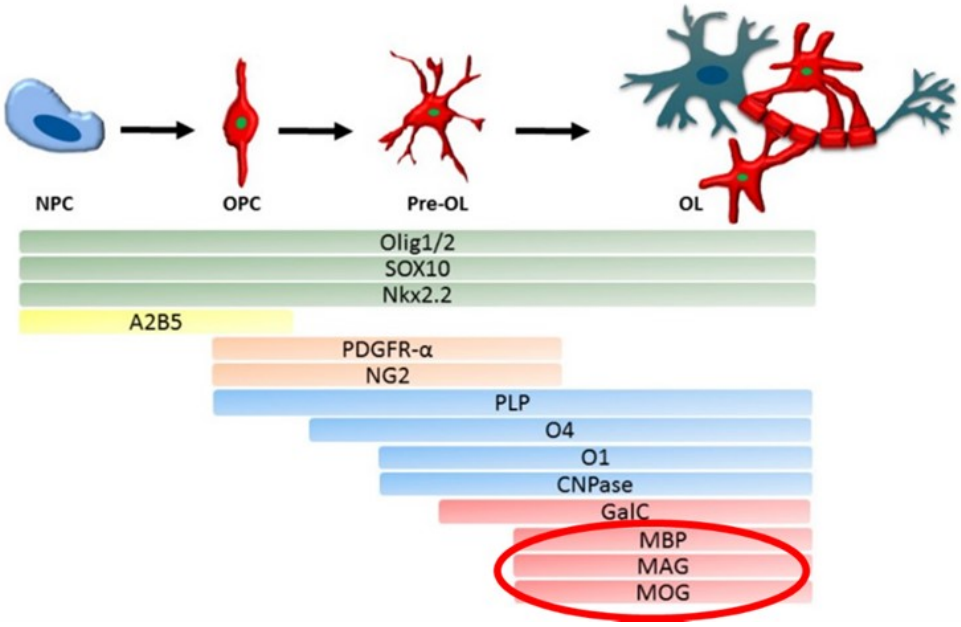

Fig.6 Markers of OLs maturity.

POSSIBLE INVOLVEMENT OF YAP IN CENTRAL MYELINATION

YAP (yes associated protein) and its paralogue, TAZ (Transcriptional coactivator with PDZ-binding motif), are Hippo pathway transcriptional co-activators able to shuttle between cytoplasm and nucleus (Hansen et al., 2015). Specifically, when YAP and TAZ are activated they can translocate into the nucleus and promote cell proliferation. In contrast, following phosphorylation mediated by activated Hippo pathway, they are inactivated, retained and degraded within the cytoplasm. Accumulating evidence suggests the implication of YAP/TAZ in myelination of the developing peripheral nerve. Specifically, studies showed that knockout mice lacking YAP/TAZ in Schwann cells are peripherally unmyelinated. Further investigations assumed that the regulation of myelination could be due to the upregulation of myelin genes, including Krox20 (Lopez-Anido et al., 2016). Interestingly, recent studies have raised the possibility that the activation of YAP may promote the morphogenesis and maturation of white matter in OLs through mechanical stress.

Indeed, it has been shown that mechanical factors can influence cellular properties. For example, when exogenous forces are applied, they are able to stimulate integrins (Tzima et

al., 2001), activate downstream mechanotransducers including YAP (Low et al., 2014). Specifically, external stretching forces change cell morphology and stress fiber quantity. In order to counterbalance the external forces cells produce stress fibers, such as for example F-actin. These fibers are able to upstream modulate the Hippo pathway, which is principally composed by two core kinases, MST and LATS. When cells develop low contractile forces and do not allow the development of acto-myosin contractility, LATS phosphorylates YAP. When phosphorylated, YAP remains localized in the cytoplasm. In contrast, when mechanical stimulation inhibits LATS, YAP can enter the nucleus and start different genes transcription.

Recent in vitro studies reported that nuclear components of OLs are altered by mechanical stresses and also their differentiation can be mechanically modulated (Hernandez et al., 2016; Jagielska et al., 2012; Lourenço et al., 2016).

The timing of myelination is strongly influenced by interactions between OLs and neurons (Ishibashi et al., 2006; Jakovcevski et al., 2007; Wake et al., 2011; Wang et al., 1998) and by some physical property of neurons. It is possible that OLs experience mechanical stress as a result of axonal movements. OLs morphology itself is very dynamic during myelination. First, OLs need to extend their processes in order to form contacts with axons and then myelin sheath needs to be compressed to form compact myelin. Thereby, myelination is thought to occur under mechanical stress loading. Furthermore, myelination is a process characterized by cell-cell contacts between OLs and neurons, and since in vitro studies have reported that YAP is also involved in contact inhibition of the cells through its phosphorylation and nuclear localization (Ota and Sasaki, 2008; Wada et al., 2011), the activation of YAP during the myelination processes by itself can be reasonably hypothesized.

AIM

Although a lot of scientific evidence suggests that exposure to Cannabis (or to its most psychoactive component, THC) in adolescence may act as a risk factor for developing mental disorders later in life, this illicit drug remains the most commonly used among adolescents. Adolescence is a very sensitive period, in which brain has not reached the full maturation yet. It has been extensively described that this "work in progress" span of time is characterized by changes in brain morphology such as a loss of grey and a rise of white matter.

Previous studies performed in our laboratory have demonstrated that adolescent THC exposure leads to significant alterations in the physiological changes occurring in the glutamatergic system of adolescent female rats (Rubino et al., 2015). On these grounds, the first aim of the present work will be to thoroughly investigate if the harmful effect produced by the adolescent exposure to THC could be linked to its interference with the physiological role of the eCB system. Furthermore, we will also evaluate which are the most critical periods to cannabis exposure during this sensitive window. To this aim, the adolescent developmental window (from PND 28 to 52) will be divided into five sub-periods of five days each (PND 28-32, 33-37, 38-42, 43-47 and 48-52). Sprague-Dawley female rats of each subgroups will receive daily injection with the selective CB1 receptor antagonist AM251 (0.5 mg/kg, i.p.), the main psychoactive component of cannabis THC (2.5 mg/kg, i.p) or its vehicle. At the end of the treatment, through western blot analysis, we will study the impact of this modulation on some markers of plasticity. Specifically, we are going to monitor plasticity relevant proteins present at glutamatergic postsynaptic sites such as PSD95, subunits of NMDA receptors (GluN2A and GluN3A) and GluA2 AMPA subunits.

Furthermore, since as mentioned above, another important event characteristic of the adolescent brain development is represented by myelination, the second aim of our work will be to investigate also the role played by the eCB system on this second fundamental process occurring in adolescence. In order to reach our second goal, following the previous treatment schedule, we will treat female rats of each sub-group with the antagonist of CB1 receptors, AM251, and then we will monitor through western blot analysis MBP and MOG, two of the main white matter markers.

A very recent work has shown that individual variability in myelin growth trajectories, investigated during the transition from adolescence to adulthood, could be linked to the expression of impulsivity traits (Ziegler et al., 2019). Specifically, the authors indicated that impulsivity is associated with a reduction in myelin growth in the lateral and medial prefrontal cortex.

On these grounds, our next aim will be to investigate whether any correlation exists between impulsivity and the development of white matter in our animal model. To reach this purpose, adolescent female rats will be treated with AM251, the antagonist of CB1 receptors and then through the Wire-Beam Bridge test their impulsivity will be evaluated.

Furthermore, we will investigate the possible cellular mechanism involved in the myelination enhancement induced by the activation of CB1 receptor during this specific developmental window.

Finally, as previously done in the study of the eCB system role on plasticity, our last goal will be to evaluate whether the administration of THC would affect the physiological increase of white matter in adolescent rats.

Data obtained will contribute to better understand the consequences of adolescent cannabis exposure and to shed light on the molecular mechanisms that make the adolescent brain sensitive to the adverse effects of Cannabis.

MATERIALS AND METHODS

ANIMALS

Female adolescent Sprague Dawley rats, aged between PND 28 and 52, obtained from Charles River laboratories S.R.L (Calco, Lecco, Italy), were housed in groups of five on a 12 h light-dark cycle, under standard conditions of temperature and humidity (22±2°C, 60±5% humidity). All animals had free access to food and water. Experiments took place during the light phase and were carried out in strict accordance with the guidelines released by the Italian Ministry of Health (D.L. 2014/26), and the European Community directives regulating animal research (2010/63/EU). The research project and research procedures were examined and approved by the Body for the Protection of Animals (OPBA) at the University of Insubria, and protocols were then approved by the Italian Minister for Scientific Research. All efforts were made to minimize the number of animals used and their suffering.

DRUGS

The following drugs were used to carry out the experiments of this thesis: THC and AM251. THC, a generous gift from GW Pharmaceuticals (Salisbury, UK) was dissolved in ethanol, cremophor and saline (1:1:18). AM251, the CB1 receptor antagonist, was obtained from Tocris Biosciences (Avonmounth, UK) and dissolved in Tween 80, DMSO, and saline (1:1:8).

PHARMACOLOGICAL TREATMENTS

In order to thoroughly analyse each step of brain maturation and to detect the most critical period to cannabinoid exposure, the entire adolescent developmental window (from PND 28 to 52) was divided into five sub-periods of five days each, as depicted in the below treatment schedule 1. Sprague-Dawley female rats of each subgroup were injected daily, for five days with AM251 (0.5 mg/kg, i.p.) a selective CB1 receptor antagonist or its vehicle. Following the same treatment schedule, another group of animals received THC (2.5 mg/kg, i.p.), the main psychoactive component of cannabis. According to the transformation of human-equivalent

doses proposed by the Food and Drug Administration (FDA), and considering an average THC content of herbal cannabis of about 10.22% in Europe (Freeman et al., 2019), the dose used (2.5 mg/kg) corresponded to half of a joint.

To deepen if in our animal model there was a correlation between the increase in myelination and a reduction in impulsivity and then to investigate the possible mechanism involved, another group of Sprague-Dawley female rats received daily intraperitoneal injections of AM251 (0.5 mg/kg). Since a longer treatment period is required for post-treatment behavioral tests, we chose to inject our animals from PND 28 to 45, which is the period that appeared to be most sensitive to the block of endocannabinoid tone in our experiments (as illustrated in the below treatment schedule 2).

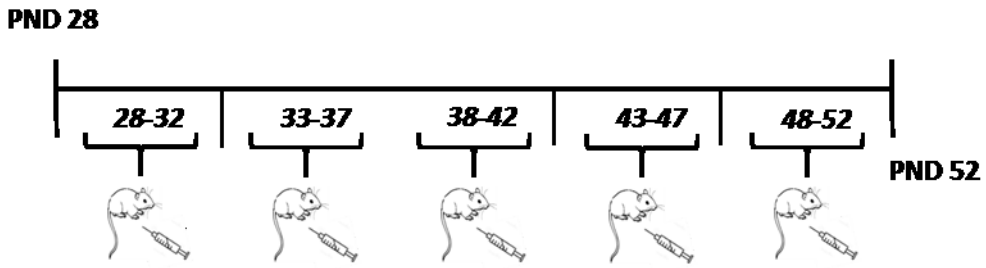

Fig. 7 Treatment schedule 1.

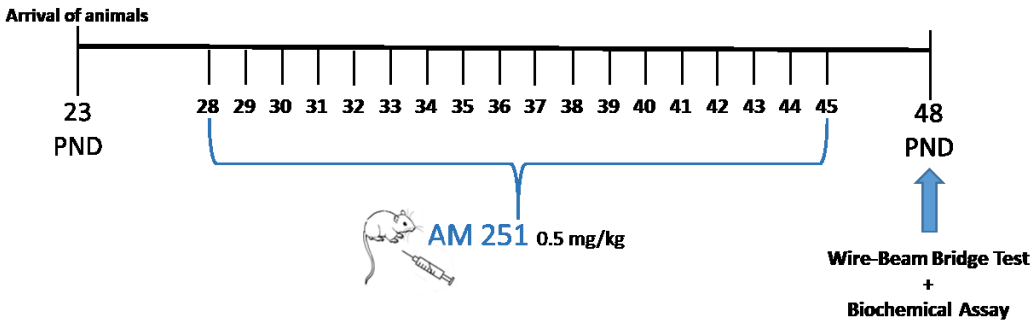

Fig. 8 Treatment schedule 2.

BEHAVIORAL TEST

Wire-Beam Bridge test

The Wire-Beam Bridge test was performed to evaluate impulsivity. Specifically, it has been used a variant of a previously detailed protocol by Frau et al. (2019). The apparatus consisted of two 100-cm high Plexiglas platforms, oppositely placed at 100-cm distance and connected by a horizontal, flexible wire-beam. The bridge consisted of a grid, 10-cm wide, made in black aluminum wire. It was modestly flexible, with a downward deflection of 2 cm per 100-g load at the center point. In order to promote movement, the starting position was made uncomfortable by placing a 50-cm high Plexiglas wall 5 cm from the edge of the platform. Rats were individually placed in the start position, and the time needed to cross the bridge and reach the other platform was recorded. The cut off for the experiment was fixed at 180s.

BIOCHEMICAL ASSAYS

Animals were sacrificed and their brains collected five hours after the last injection, following the treatment schedule 1 or five hours after the Wire-Beam Bridge Test, following the treatment schedule 2. For western blot analyses, PFCs, obtained by regional dissection on ice were immediately frozen in liquid nitrogen and stored at -80°C until processing.

Proteins Extraction

For protein lysate, the PFC was homogenized in an appropriate volume of ice-cold buffer (10 mM Hepes pH 7.5, 1.5 mM $MgCl_2$, 10 mM KCl, 2 mM DTT, 1 mM PMSF, 1 mM EDTA, 1 mM EGTA, 2 mM sodium orthovanadate, 50 mM NaF, 10 mM sodium pyrophosphate, 0.5% Triton, 5 µg/ml aprotinin and 5 µg/ml leupeptin) and centrifuged at 13,000 rpm at 4 °C for 3 min. The supernatant was used as total protein lysate. The pellet was resuspendend in nuclear lysis buffer(20 mM Hepes pH 7.5, 1.5 mM $MgCl_2$, 420mM NaCl, 0.2mM EDTA, Glycerol 25%, 2 mM DTT, 1 mM PMSF, 5 µg/ml aprotinin and 5 µg/ml leupeptin). After 30

minutes of agitation at 4°C, samples were centrifuged at 13,000 rpm at 4 °C for 10 min. The supernatant was used as nuclear extract.

Crude synaptosome fractions from rat PFC were obtained through homogenization in a glass homogenizer in a sucrose solution (20 mM Hepes pH 7.5, 1 mM $MgCl_2$, 0.5mM CaCl2, 1 mM PMSF and protease inhibition cocktail). The homogenized tissue was centrifuged at 500*g* for 2 min. The resulting supernatant (S1) was centrifuged at 13,000 g at 4°C for 20 minutes. The obtained pellet (P2) was resuspended with equal volume of 20 mM Hepes pH 7.5, 1,5 mM MgCl2, 420mM NaCl, 0,2 mM EDTA, Glycerol 25%, 2mM DTT, 0.5mM CaCl2, 2 mM, 2mM PMSF and protease inhibition cocktail).

Protein concentrations were determined according to the Micro-BCA assay kit (Pierce, Rockford, IL).

SDS-Page and Western blot analyses

Equal amount of protein (30 µg) were run on a 6, 8, 10 or 14% SDS-polyacrylamide gel. The proteins were then transferred to polyvinylidene difluoride (PVDF) membranes, blocked for 2 h at room temperature in 5% dry skimmed milk in TBS 1×, 0.1% tween-20 before incubation overnight at 4°C with the primary antibody. The following primary antibodies were used: mouse monoclonal anti-MOG (1:2000; Novus), mouse monoclonal anti-PSD95 (1:2000; Novus), rabbit polyclonal anti-MBP (1:1000; Abcam), rabbit polyclonal anti-NR2A (1:1000; Millipore), rabbit polyclonal anti-NR3A (1:1000; Millipore), rabbit polyclonal anti-Phospho-YAP (1:1000; cell signalling), rabbit polyclonal anti-YAP (1:1000; cell signalling), rabbit monoclonal anti-Phospho-MTOR (1:1000; cell signalling), rabbit monoclonal anti-MTOR (1:1000; cell signalling), rabbit polyclonal anti-Phospho AKT (1:1000; cell signalling), rabbit polyclonal anti-AKT (1:1000; cell signalling), rabbit polyclone anti-Phospho-p44/42 MAP Kinase (1:1000; cell signalling), rabbit polyclone p44/42 MAPK (ERK1/2) (1:1000; cell signalling) and rabbit polyclonal anti-GluA2 (1:2000; Immunological sciences). Bound antibodies were detected with horseradish peroxidase (HRP) conjugated secondary antibody for 1h at room temperature and visualized using WESTAR ECL Substrate for Western Blotting (Cyanagen, Bologna, Italy), and bands were detected with a G-Box XT camera (Syngene, Cambridge, UK). For detection of β-actin or B-lamin, the blots were stripped with Renew

Stripping Buffer (Cyanagen, Italy), re-blotted with mouse monoclonal anti-β-actin (1:2000; Sigma Aldrich) or rabbit anti-lamin (1:2000; Sigma Aldrich) overnight at 4 °C and visualized as described above. For densitometry, the optical density of the bands was quantified using ImageJ software (NIH, Bethesda, MD, USA), normalized to controls and expressed as arbitrary units.

Electron microscopy

PFCs from rat brain were extracted and cut into 1-mm^3 tissue. Samples were fixed for 2 h in 0.1 M cacodylate buffer at pH 7.4 containing 2% glutaraldehyde. After several washes in the same buffer, tissue samples were postfixed for 1 h with 1% osmium tetroxide in cacodylate buffer, pH 7.4. After standard dehydration in ethanol series (70, 90, 100%), samples were embedded in an Epon-Araldite 812 mixture (Sigma-Aldrich, Milan, Italy) and sectioned with a Reichert Ultracut S ultratome (Leica, Wien, Austria). For TEM, thin sections (80 nm) were stained by uranyl acetate and lead citrate and observed with a Jeol 1010 electron microscope (Jeol, Tokyo, Japan).

Statistical analysis

Statistical analysis was performed using Prism 4.0 version (Graph Pad software, San Diego, CA, USA). Data are presented as mean ± SEM and were analyzed with Student's *t* test or one-way ANOVA followed by Tukey's post hoc analysis when appropriate.

RESULTS

EFFECT OF CB1 CANNABINOID RECEPTOR BLOCKADE ON SYNAPTIC PLASTICITY IN ADOLESCENT FEMALE RATS

We have previously demonstrated that THC adolescent exposure leads to significant alterations in dynamic changes occurring in the glutamatergic system during the adolescent development of the prefrontal cortex (Rubino et al., 2015). In order to understand if the harmful effect produced by adolescent exposure to THC could be linked to its interference with the physiological role of the eCB system, we decided to investigate the role of the eCB system on the processes of neuronal remodeling that take place in the glutamatergic system during adolescence. On this basis, to test our hypothesis and to fully evaluate which are the most critical periods to cannabinoid exposure during the brain maturation, the adolescent developmental window (from PND 28 to 52) was divided into five sub-periods of five days each, as depicted in the treatment schedule 1. Sprague-Dawley female rats of each subgroups received daily injection with the selective CB1 receptor antagonist AM251 (0.5 mg/kg, i.p.) or its vehicle.

We next analyzed the effect of CB1 receptor blockade on synaptic and structural markers of the glutamatergic system. First, we monitored alterations in Postsynaptic Density Protein-95 (PSD-95), which is widely considered the main postsynaptic marker of glutamatergic synaptic sites (Okabe et al., 1999, Aoki et al., 2001). Furthermore, since specific changes in the subunit composition of NMDA receptors have been demonstrated to be relevant in the synaptic rearrangements that occur during the postnatal brain development, we also decided to study GluN3A and GluN2A levels. Specifically, it has been suggested that the overexpression of GluN3A increases spine elimination and decreases spine stability (Kehoe et al., 2014), processes that are actively occurring in the adolescent brain. With regard to GluN2A, it is considered a marker of synaptic maturation. As a matter of fact, in the forebrain, immature synapses mainly contain NMDA receptors enriched in N2B subunits that are replaced by N2A subunits when they become mature, an event that take place massively during late postnatal development (reviewed in Dumas, 2005). Finally another major component of excitatory glutamatergic synapse maturation is a switch in the subtype of AMPA glutamate receptor. Specifically, in the developing brain immature synapses contain

calcium-permeable AMPA glutamate receptors that are subsequently replaced with GluA2-containing calcium impermeable AMPARs as synapses stabilize and mature (Brill and Huguenard, 2008; Kumar et al., 2002). Since this switch in AMPA subunits contributes to synapse maturation and stabilization, we then investigated also the levels of GluA2 AMPA subunits.

- **PSD95**

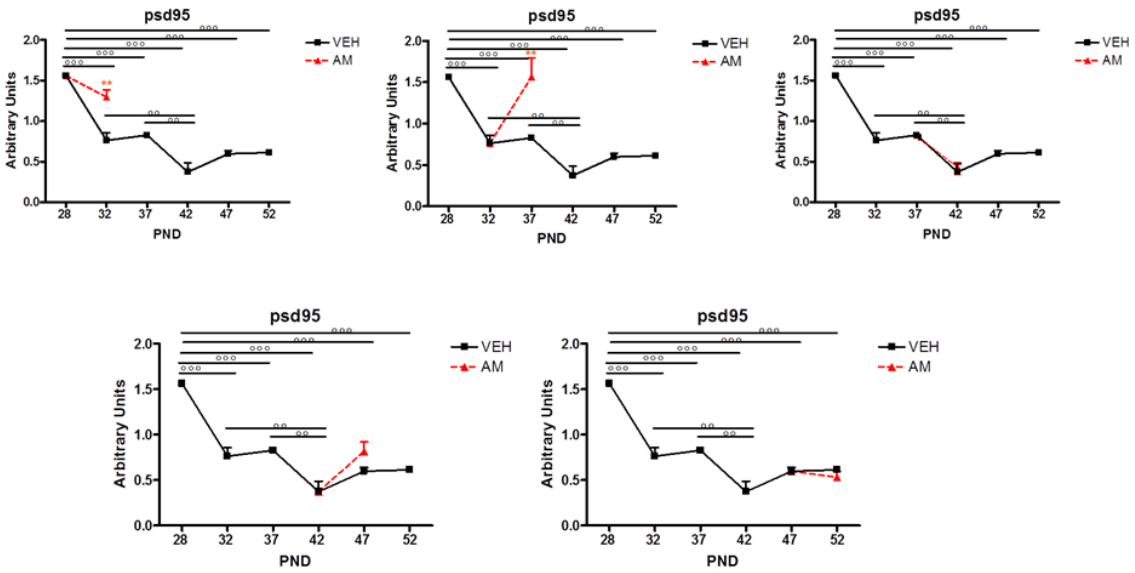

Fig. 9 Effect of AM251 administration during different periods of adolescence on PSD-95. (A) AM251 was administered from PND 28 to 32; (B) from PND 33 to 37; (C) from PND 38 to 42; (D) from PND 43 to 47 and (E) from PND 48 to 52. Data are expressed as mean ± SEM of four animals for each time-point. °°$p < 0.01$, °°°$p < 0.001$ significant difference between age in control group; **$p < 0.01$, significant difference in AM251-treated animals versus controls.

In control animals, PSD95 expression (Fig.9) decreased from PND 28 to 42 and then it seems to stabilize from PND 43 to 52. The administration of the antagonist AM251 significantly prevented PSD95 decrease at PND 32 and 37, whereas it did not alter its physiological trend at PND 42, 47 and 52.

- **GluN3A**

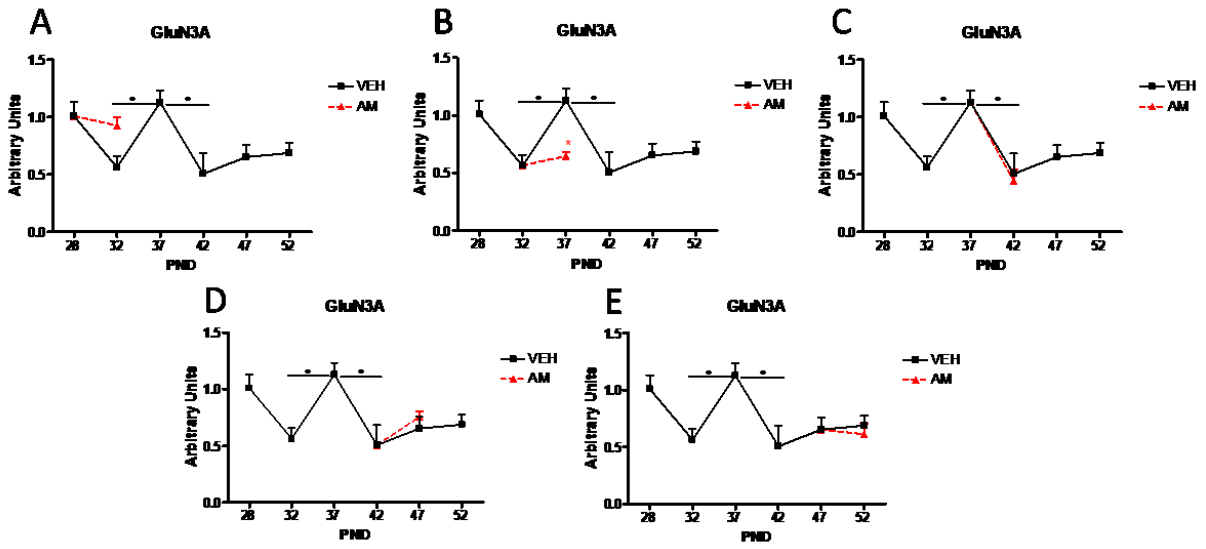

Fig. 10 Effect of AM251 administration during different periods of adolescence on NMDA GluN3A. (A) AM251 was administered from PND 28 to 32; (B) from PND 33 to 37; (C) from PND 38 to 42; (D) from PND 43 to 47 and (E) from PND 48 to 52. Data are expressed as mean ± SEM of four animals for each time-point. °$p < 0.05$ significant difference between age in control group; *$P < 0.05$ significant difference in AM251-treated animals versus controls.

GluN3A physiological levels (Fig.10) decreased from PND 28 to 32, their trend was completely reversed from PND 33 to 37, to eventually decrease again up to PND 42 and stabilize from PND 43 to 52. The modulation with AM251 between PND 33 to 37 significantly prevented the increase of this marker at PND 37. Even though non statistically significant, this modulation seems also to blunt the decrease of GluN3A levels when AM251 is administered from PND 28 to 32. Conversely, this modulation did not alter the physiological trend of GluN3A during the other periods investigated.

- **GluN2A**

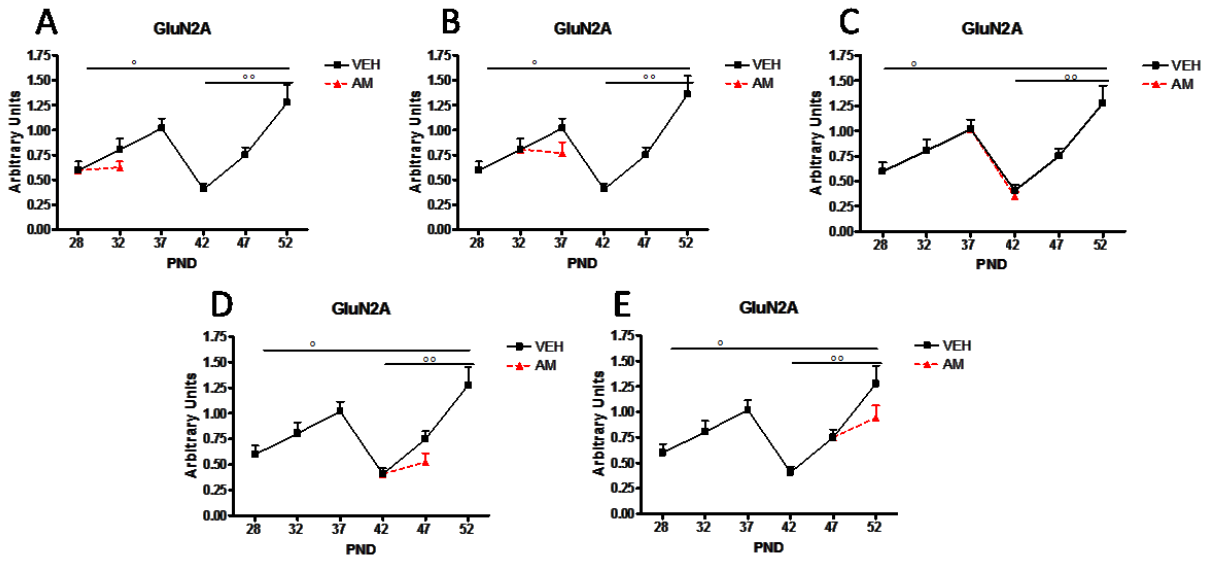

Fig. 11 Effect of AM251 administration during different periods of adolescence on NMDA GluN2A. (A) AM251 was administered from PND 28 to 32; (B) from PND 33 to 37; (C) from PND 38 to 42; (D) from PND 43 to 47 and (E) from PND 48 to 52. Data are expressed as mean ± SEM of four animals for each time-point. °$p < 0.05$, °°$p < 0.01$ significant difference between age in control group.

GluN2A levels (Fig.11), in control animals, increased from PND 28 to 37 and then they decreased until PND 42, to eventually increase again up to PND 52. The administration of the CB1 receptor antagonist, AM251, did not significantly alter the physiological trend of this marker, even though this modulation seems to slightly reduce the increase of GluN2A levels mainly during the last two time periods investigated.

- **GluA2**

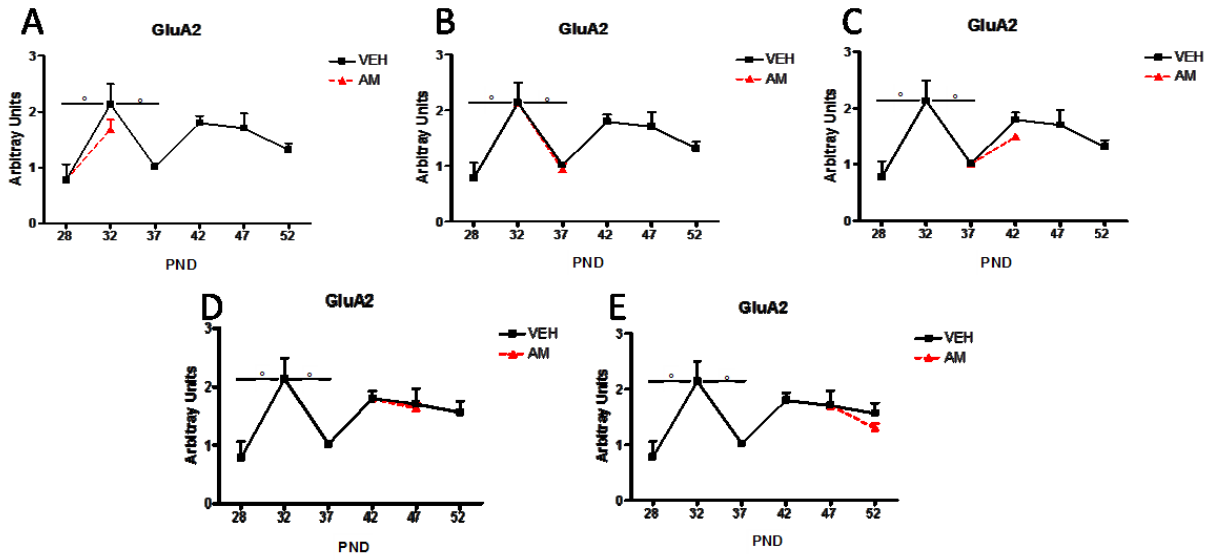

Fig. 12 Effect of AM251 administration during different periods of adolescence on AMPA GluA2. (A) AM251 was administered from PND 28 to 32; (B) from PND 33 to 37; (C) from PND 38 to 42; (D) from PND 43 to 47 and (E) from PND 48 to 52. Data are expressed as mean ± SEM of four animals for each time-point. °$p < 0.05$ significant difference between age in control group.

GluA2 physiological levels (Fig.12) increased from PND 28 to 32, they decreased from PND 33 to 37, to eventually increase again until PND 42. Finally, GluA2 physiological levels seem to stabilize from PND 43 to 52. The administration of AM251 did not alter the physiological trend of this protein.

Overall, these data confirm that PFC glutamatergic synapses of adolescent female rats are characterized by the occurrence of many maturational events. Specifically, data show a reduction in PSD95 levels during the early and mid-adolescence accompanied by fluctuation in GluN2A, GluN3A and GluA2 levels. All these events are thought to be essential in order to determining the final organization of functional network connections within this brain region. Furthermore, our results show that the blockade of the endocannabinoid system negatively impacts on the dynamic changes typically occurring in adolescent brain. In particular AM251 administration, preventing the decrease in PSD95 levels, suggests the role of the eCB tone in the elimination of the excitatory synapses. Moreover, this modulation, slightly reduces the increase of GluN2A levels during the late adolescence, indicating also a

possible delay in the maturation of the glutamatergic system, maybe resulting in a less functional adult PFC. Thereby we can speculate that the physiological adolescent stimulation of the CB1 receptor is fundamental for the normal maturation of the PFC in female rats.

EFFECT OF THC ADMINISTRATION ON SYNAPTIC PLASTICITY IN ADOLESCENT FEMALE RATS

At this point, since we observed that the blockade of the eCB system disrupts the developmental changes occurring in the prefrontal cortex during adolescence, we decided to identify which is the most sensitive period of the adolescent THC exposure. To this aim, we chose a much milder treatment compared to that used in our previous studies. Specifically, following the treatment schedule 1 (as before), animals of each period (PND 28-32, 33-37, 38-42, 43-47 and 48-52) were injected daily, for 5 days with THC (2.5 mg/kg i.p.) or its vehicle.

- **PSD95**

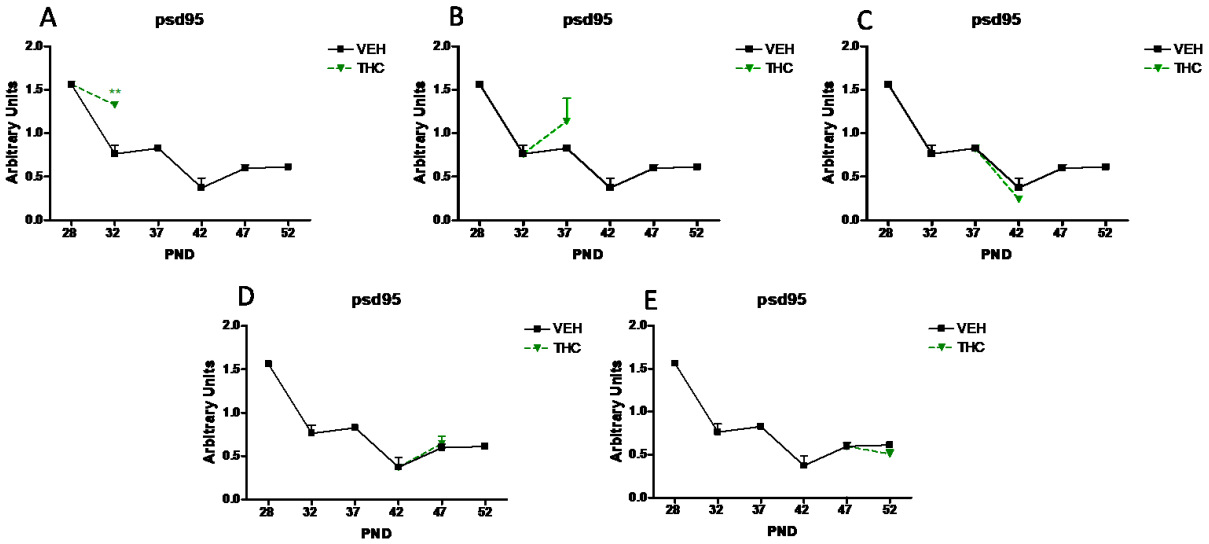

Fig. 13 Effect of THC administration during different periods of adolescence on PSD-95. (A) THC was administered from PND 28 to 32; (B) from PND 33 to 37; (C) from PND 38 to 42; (D) from PND 43 to 47 and (E)

from PND 48 to 52. Data are expressed as mean ± SEM of four animals for each time-point. **p < 0.01, significant difference in THC-treated animals versus controls.

Interestingly, similarly to what observed after the blockade of the eCB system, also the administration of THC during the early adolescence, seems to prevent the PSD95 physiological decrease (Fig. 13). As a matter of fact, compared to controls, animals treated with THC showed significantly higher PSD95 levels at PND 32 and although not statistically significant PSD95 levels were also higher at PND 37. The administration of THC, as well as of AM251, during the mid and late adolescence (PND 42, 47 and 52) did not affect PSD95 physiological trend.

- **GluN3A**

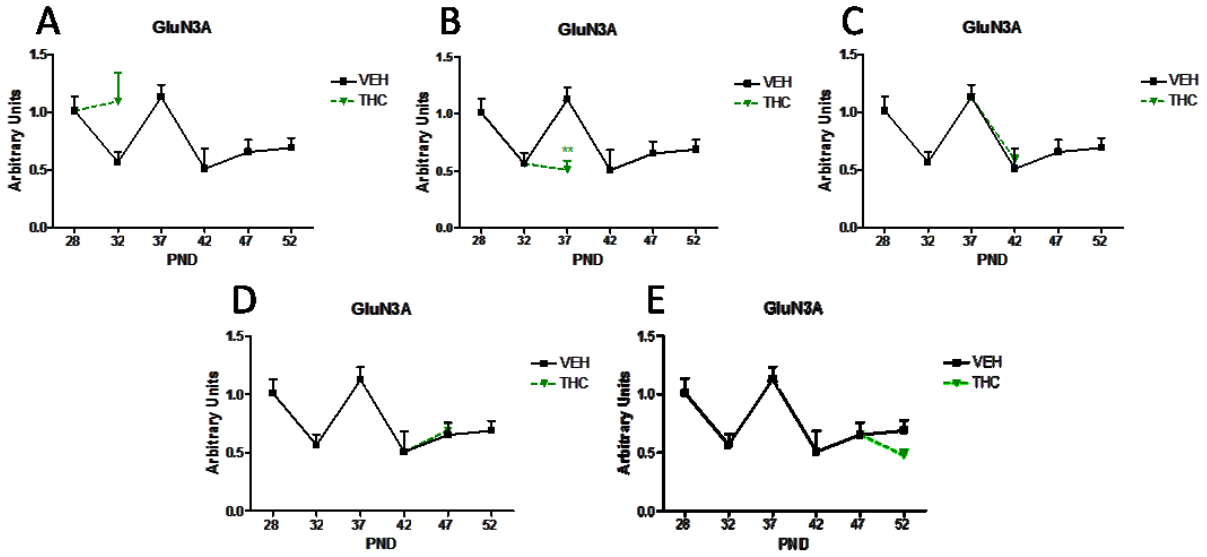

Fig. 14 Effect of THC administration during different periods of adolescence on NMDA GluN3A. (A) THC was administered from PND 28 to 32; (B) from PND 33 to 37; (C) from PND 38 to 42; (D) from PND 43 to 47 and (E) from PND 48 to 52. Data are expressed as mean ± SEM of four animals for each time-point. **p < 0.01 significant difference in THC-treated animals versus controls.

The administration of THC from PND 33 to 37, similarly to what observed following the administration of AM251, significantly prevented the increase of GluN3A at PND 37 (Fig. 14). As in the modulation with the antagonist of CB1 receptor, GluN3A physiological levels were not altered when THC was administrated during the other periods investigated.

- **GluN2A**

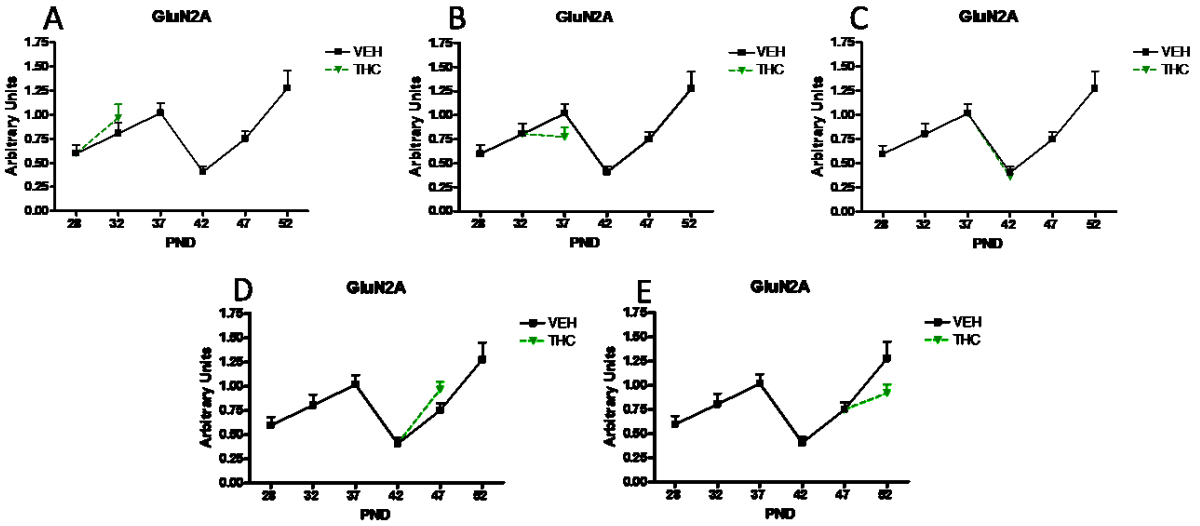

Fig. 15 Effect of THC administration during different periods of adolescence on NMDA GluN2A. (A) THC was administered from PND 28 to 32; (B) from PND 33 to 37; (C) from PND 38 to 42; (D) from PND 43 to 47 and (E) from PND 48 to 52. Data are expressed as mean ± SEM of four animals for each time-point.

- **GluA2**

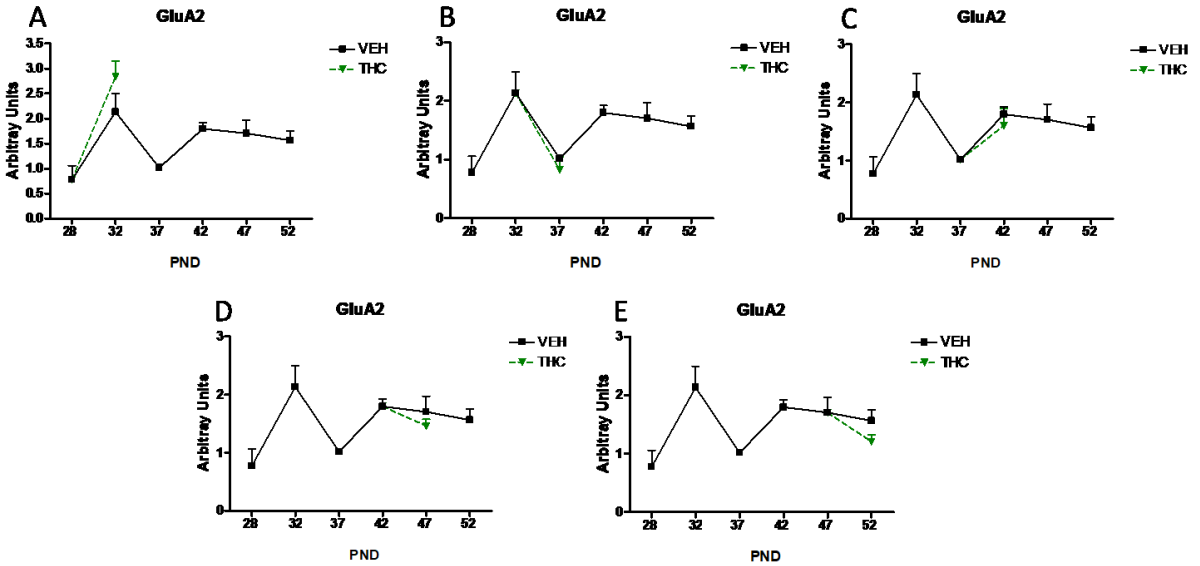

Fig. 16 Effect of THC administration during different periods of adolescence on AMPA GluA2. (A) THC was administered from PND 28 to 32; (B) from PND 33 to 37; (C) from PND 38 to 42; (D) from PND 43 to 47 and (E) from PND 48 to 52. Data are expressed as mean ± SEM of four animals for each time-point.

The administration of THC did not significantly alter the physiological trend of both GluN2A (Fig. 15) and GluA2 (Fig. 16). GluN2A levels appeared to be slightly reduced with respect to controls only when THC was administered during the last time period investigated.

As a whole, these data suggest that the administration of THC impacts on the dynamic changes occurring in the glutamatergic synapses during adolescence in a similarly way to that observed after the modulation with the antagonist of CB1 receptor, AM251.

EFFECT OF CB1 CANNABINOID RECEPTOR BLOCKADE ON MYELINATION IN ADOLESCENT FEMALE RATS

Since another important process occurring during the adolescent brain development is the white matter increase, the second aim of our work was to investigate the effect of CB1 receptor blockade on myelination process in adolescent female rats. In order to reach our second goal, we monitored MBP and MOG, two of the main white matter markers correlated to mature OLs which are the ones active during the myelination process.

- **MBP**

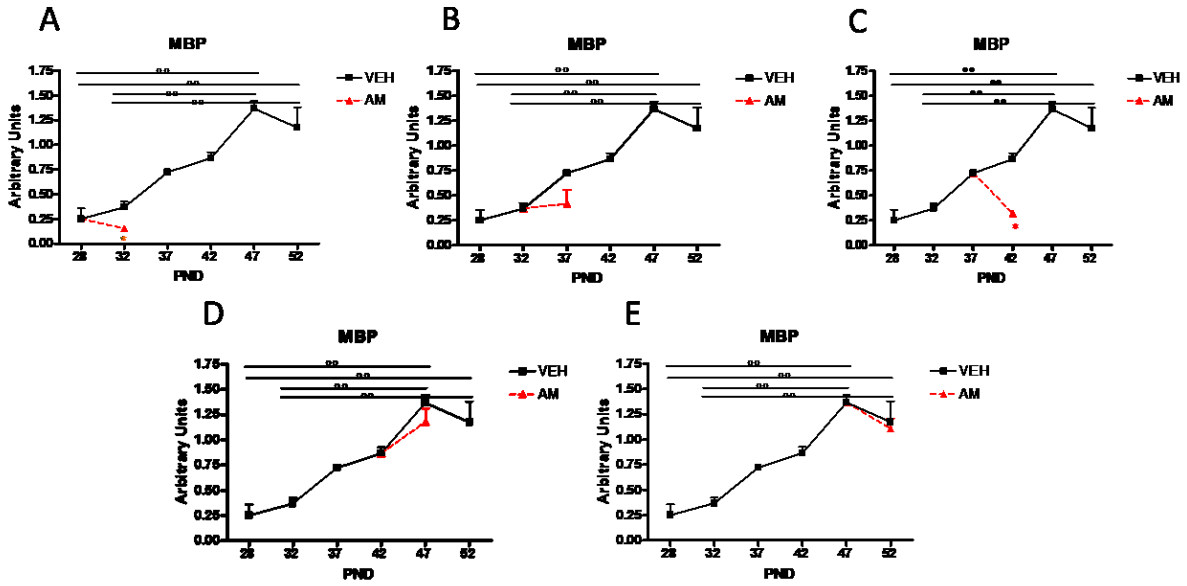

Fig. 17 Effect of AM251 administration during different periods of adolescence on MBP levels. (A) AM251 was administered from PND 28 to 32; (B) from PND 33 to 37; (C) from PND 38 to 42; (D) from PND 43 to 47 and (E) from PND 48 to 52. Data are expressed as mean ± SEM of four animals for each time-point. °°p < 0.01 significant difference between ages in control group; *P < 0.05 significant difference in AM251-treated animals versus controls.

- **MOG**

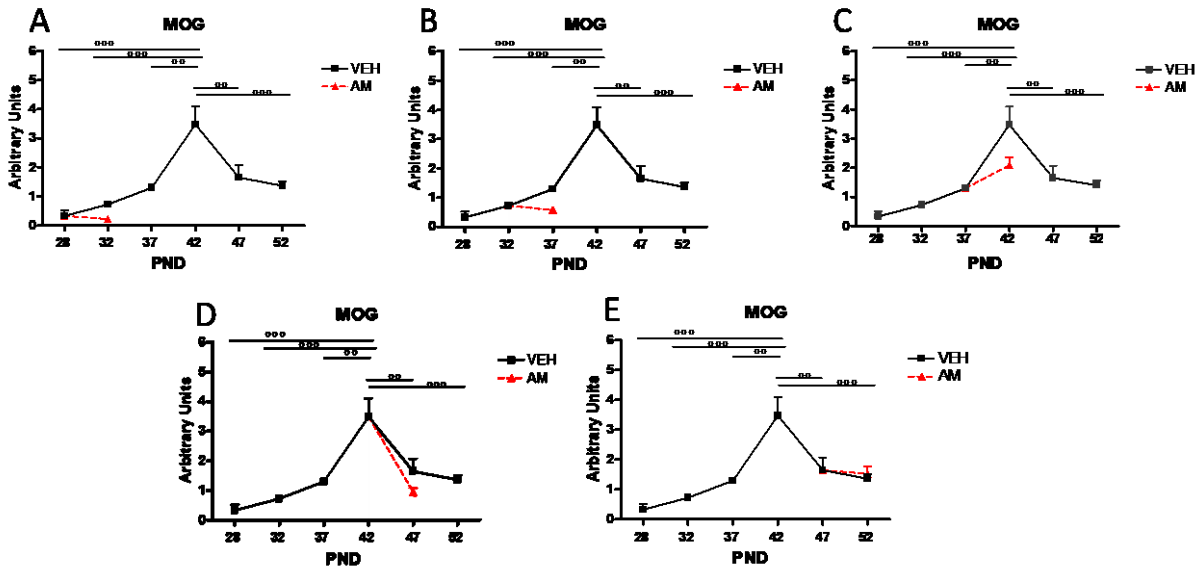

Fig. 18 Effect of AM251 administration during different periods of adolescence on MOG levels. (A) AM251 was administered from PND 28 to 32; (B) from PND 33 to 37; (C) from PND 38 to 42; (D) from PND 43 to 47 and (E) from PND 48 to 52. Data are expressed as mean ± SEM of four animals for each time-point. °°p < 0.01, °°°p < 0.001 difference between ages in control group.

Under physiological conditions, MBP increased from PND 28 to 47 and then it seemed to reach a plateau at PND 52. MOG had a slower increase than MBP during early adolescence (from PND 28 to 37) and then peaked at PND 42. Afterwards MOG levels decreased from PND 42 to 47 and then stabilized at PND 52. The administration of AM251 significantly prevented the increase of MBP expression from PND 28 to 32 and 37 to 42. Even though not statistically significant, MBP levels at PND 37, PND 47 and MOG levels during the early and mid-adolescence (from PND 28 to 47) were always lower in animals exposed to AM251. The administration of AM251 did not alter the physiological trend of both MBP and MOG during the last time window.

Overall, these data suggest that the endocannabinoid tone is fundamental for the occurrence of adolescent myelination enhancement.

EFFECT OF CB1 CANNABINOID RECEPTOR BLOCKADE ON IMPULSIVITY IN ADOLESCENT FEMALE RATS

Since recent studies have associated the attenuated developmental frontostriatal myelination trajectories with the presence of impulsivity traits in adolescents (Ziegler et al., 2019), we decided to investigate if any correlation between impulsivity and the development of white matter in our animal model exists and how the blockade of eCB system functionality can impact on it. To reach this aim we treated adolescent female rats for a longer period, as described in the treatment schedule 2. Specifically, animals were administered from PND 28 to PND 45 (period in which the eCB system functionality system seems to be fundamental for the myelination process) with AM251.

Considering that there are no existing data in literature regarding the correlation between the increase of myelin and the reduction of impulsivity in animals, first, we tested through the Wire-Beam Bridge Test, if younger animals that have a lower amount of myelin, were effectively characterized by higher impulsivity than older animals, whose amount of myelin is greater. Then, we investigated whether preventing the myelin increase by blocking the functionality of the eCB system led to a reduction in the impulsiveness of animals.

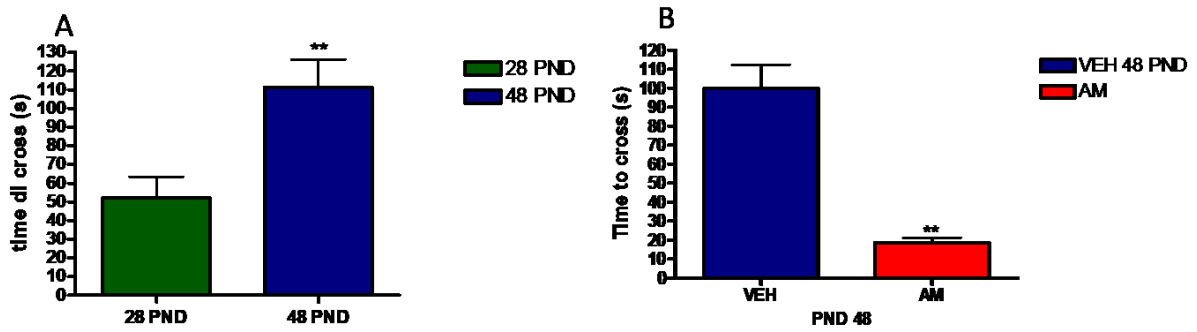

Fig.19 Crossing time of (A) rats at PND 28 vs rats at PND 48 and (B) Veh vs AM251 measured at PND 48. Data are expressed as mean ± SEM of at least eight animals for each group. **p < 0.01 significant difference in animals at PND 28 versus animals at PND 48 and AM251-treated animals versus controls.

In the Wire-Beam Bridge test, Student's t-test showed a significant difference in the time needed to cross the bridge between rats at PND 28 and rats at PND 48. In particular, the time that rats take to cross the bridge was significantly lower (- 53%) in younger animals compared to the older group. Student's t-test highlights a significant difference also between the AM251 and the control group. Specifically, animals exposed to AM251 were faster (-75 %) in crossing the bridge compared with controls.

To confirm that this longer treatment with AM251 had actually reduced the myelin production and that therefore these behavioral results were related to this myelin modification, the same animals previously used for behavioral tests, were then used to investigate the myelin levels through western blot assays and electron microscope as well.

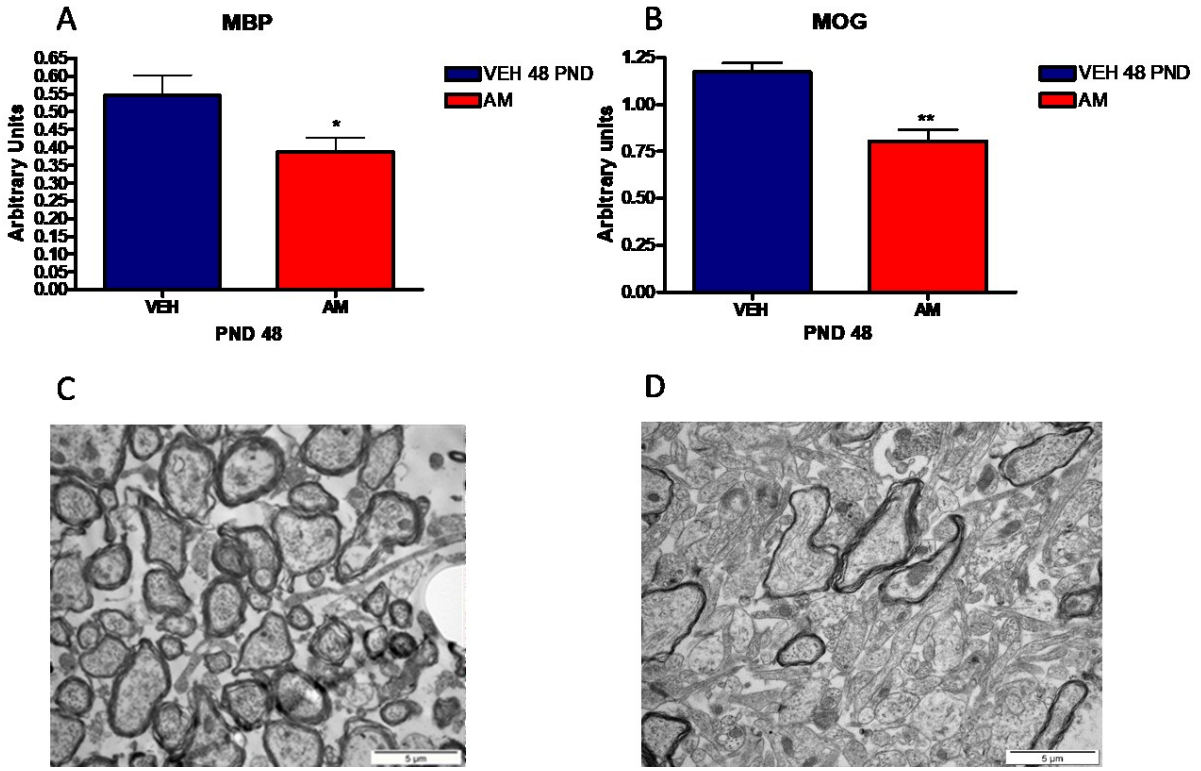

Fig. 20 Effect of AM251 administration from PND 28 to 45 on (A) MBP and (B) MOG levels. Data are expressed as mean ± SEM of eight animals for each experimental group. *P < 0.05, **p < 0.01 significant difference in AM251-treated animals versus controls. Representative Electron micrographs of a cross-section of rat PFC at PND 48. (C) control rat; (D) rat administered with AM251.

The administration of AM251 from PND 28 to 45 significantly prevented the increase of MBP (-28%) and MOG (-31%) levels analysed through western blot assays (Fig. 20 A and B) and also the number of myelinated axons, observed through microscope electron, appeared to be lower in animals treated with AM251 compared to the control group (Fig. 20 C and D).

Collectively these data firstly confirm the association between the increase of myelin and the reduction of impulsivity with increasing age in our animal model. Secondly, they suggest that the eCB system blockade during this specific developmental window (from PND 28 to 45), prevents the myelin enhancement and leads concurrently to a higher expression of impulsivity.

POSSIBLE MECHANISMS IN CB1 RECEPTORS MEDIATED MYELINATION PROCESS

At this point, we tried to identify which could be the cellular mechanism involved in the myelination enhancement through the activation of CB1 receptor during this specific developmental window. Since in the past years different studies have established that Akt/mTOR (mammalian Target Of Rapamycin) and Erk (Extracellular Signal-regulated Kinase) pathways are critical for oligodendrocyte differentiation both in vitro and in vivo (Flores et al. 2000; Flores et al. 2008; Narayanan et al. 2009; Tyler et al. 2009; Fyffe-Maricich et al. 2011; Ishii et al. 2012; Guardiola-Diaz et al. 2012; Ishii et al. 2013), we investigated by western blot the phosphorylation of these kinases with phospho-specific antibodies after the blockade of eCB.

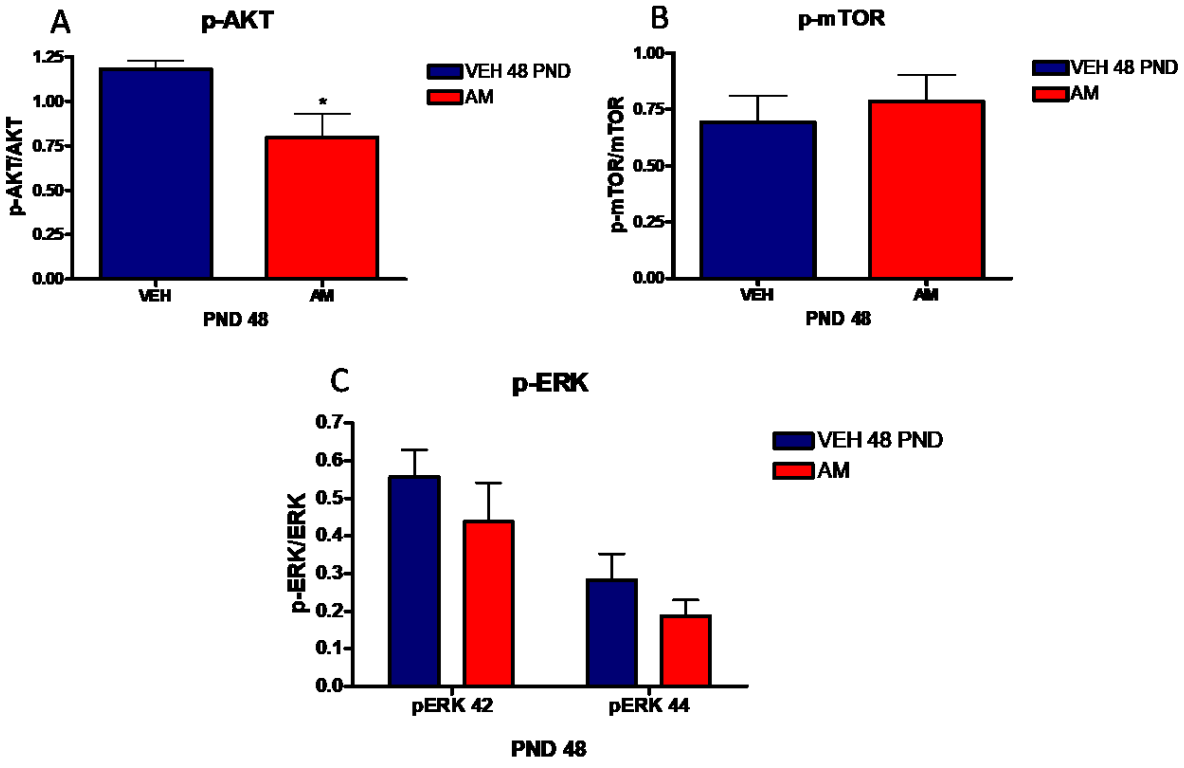

Fig. 21 Effect of AM251 administration from PND 28 to 45 on (A) pAKT (B) p-mTOR and (C) pERK levels. Data are expressed as mean ± SEM of four animals for each experimental group. *P < 0.05 significant difference in AM251-treated animals versus controls.

As expected, AKT phosphorylation levels were significantly lower (-31 %) in animals treated with the antagonist of CB1 receptor, AM251. Being mTOR a direct downstream target of AKT, we hypothesized a similar reduction in its activation status, in animals exposed to AM251. Curiously, we did not found alterations in p-mTOR levels between the control and the treated group. There were not significant differences between the two groups even as regard of p-ERK levels, although the presence of a slight reduced trend.

Taken together, these data clearly suggest that the blockade of eCB system during this specific developmental window (from PND 28 to 45) seems to prevent the myelination enhancement through a pathway involving AKT, but with a different downstream mediator.

YAP INVOLVEMENT

YAP is a key effector downstream of the Hippo signaling pathway and plays an important role in regulating different process such as cell proliferation, differentiation, migration, and apoptosis. It has also been demonstrated that YAP controls peripheral myelination (Poitelon et al., 2016) and as mentioned in the introduction, recent studies have raised the possibility that the activation of YAP could also promote the morphogenesis and maturation of white matter in OLs through mechanical stress (Shimizu et al., 2016).

Since it has been suggested that PI3K/AKT pathway can block the Hippo pathway and induce YAP nuclear localization through inhibiting the phosphorylation of MST1/2 and Lats1/2, we tried to investigate whether the mechanism involved, able to promote the myelination process in our animal model, could be mediated by YAP.

First of all, in order to analyze whether YAP could effectively be involved in the physiological modulation of the myelination process during this specific developmental period, we decided to study through western blot, YAP levels in naïve rats of PND 28 and 48. The former, as already demonstrated, are characterized by still low myelin levels whereas the latter have significant higher myelin levels. Since YAP could migrate into the nucleus and act as a coactivator for gene transcription only when it is not phosphorylated, to investigate its

possible implication in myelination, we studied both the amount of retained into cytosol p-YAP and nuclear YAP.

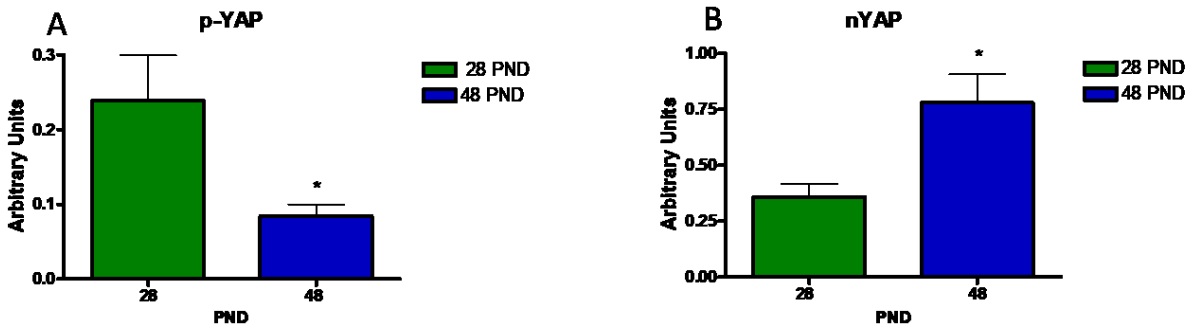

Fig. 22 (A) p-YAP and (B) nuclear YAP levels of rats at PND 28 vs rats at PND 48. Data are expressed as mean ± SEM of four animals for each experimental group. *P < 0.05 significant difference between animals at PND 28 versus animals at PND 48.

Student's t-test showed a significant difference in both cytosolic p-YAP and nuclear YAP levels between rats at PND 28 and rats at PND 48. In particular, older animals were characterized by a reduction in the amount of cytosolic p-YAP of about -64% and by an increase in nuclear YAP levels of about 116% compared to younger ones.

Collectively these data seem to confirm the involvement of YAP in the physiological myelination enhancement during this specific developmental window.

After the evidence that in our animal model YAP appears to have a role in the physiological myelination process, we then investigated whether effectively the blockade of the eCB system could actually affected this pathway leading to an increase in retained p-YAP in the cytosol and a decrease in nuclear YAP.

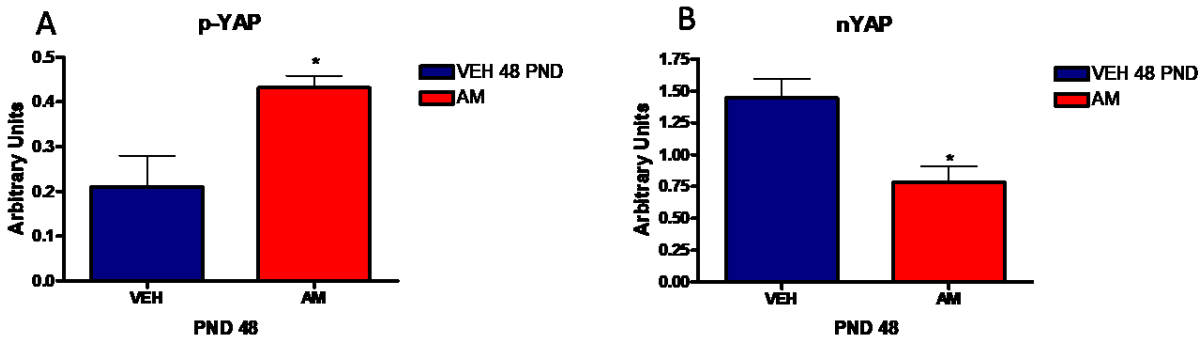

Fig. 23 Effect of AM251 administration from PND 28 to 45 on (A) cytosolic p-YAP and (B) nuclear. Data are expressed as mean ± SEM of four animals for each experimental group. *P < 0.05 significant difference in AM251-treated animals versus controls.

Consistently with our hypothesis, rats treated with CB1 receptor antagonist showed an increase in the amount of phosphorylated yap in the cytoplasm (105%) and a reduction in the amount of nuclear YAP (-45%), thus, confirming the involvement of this pathway during the adolescent myelination enhancement.

EFFECT OF THC ADMINISTRATION ON MYELINATION IN ADOLESCENT FEMALE RATS

Finally, as previously done in the study of the eCB system role on plasticity, we wanted to evaluate whether the administration of THC would affect the increase of white matter in adolescent rats in a similar way to that observed after the exposure to the antagonist of CB1 receptor AM251.

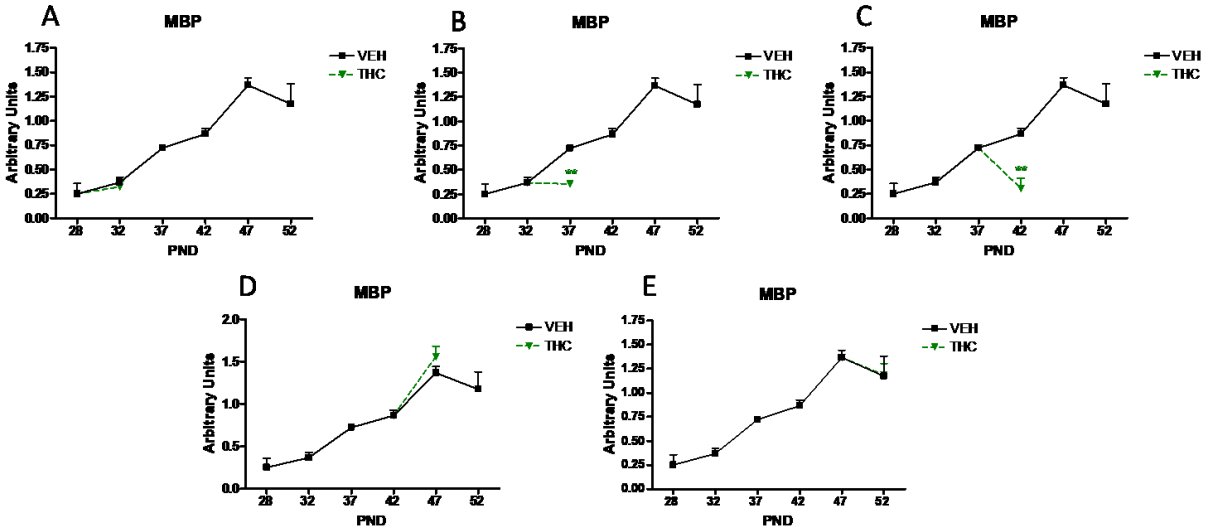

Fig. 24 Effect of THC administration during different periods of adolescence on MBP levels. (A) AM251 was administered from PND 28 to 32; (B) from PND 33 to 37; (C) from PND 38 to 42; (D) from PND 43 to 47 and (E) from PND 48 to 52. Data are expressed as mean ± SEM of four animals for each time-point. **P < 0.01 significant difference in AM251-treated animals versus controls.

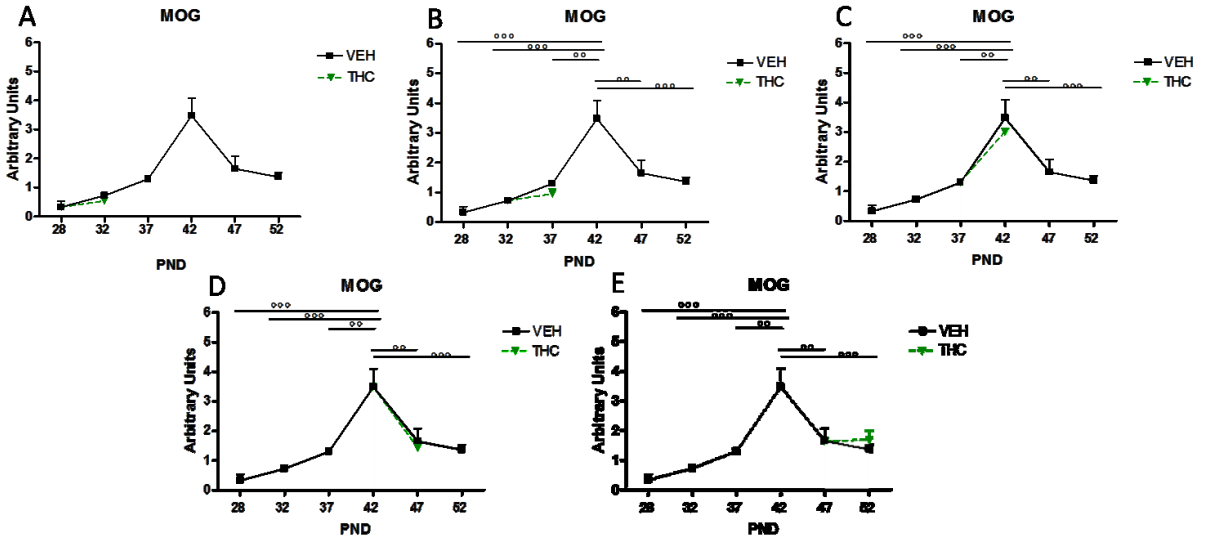

Fig. 25 Effect of THC administration during different periods of adolescence on MOG levels. (A) AM251 was administered from PND 28 to 32; (B) from PND 33 to 37; (C) from PND 38 to 42; (D) from PND 43 to 47 and (E) from PND 48 to 52. Data are expressed as mean ± SEM of four animals for each time-point.

The administration of THC significantly prevented the increase of MBP levels at PND 37 and 42. Even though not statistically significant, at PND 37 and 42, also MOG levels were slightly reduced with respect to controls.

DISCUSSION AND CONCLUSION

THE ENDOCANNABINOID TONE IS FUNDAMENTAL FOR THE MATURATION OF THE GLUTAMATERGIC SYSTEM IN ADOLESCENT FEMALE RATS

The last European drug report shows that the widespread diffusion of Cannabis abuse among adolescents is continuously increasing. Previous studies have reported a relationship between Cannabis exposure and significant alterations in dynamic changes occurring in the glutamatergic system during the adolescent development of the prefrontal cortex (Rubino et al., 2015). On these grounds, we wanted to investigate whether the harmful effect produced by adolescent exposure to THC could be due to its interference with the physiological role of the eCB system. To this aim, we first studied the role of the eCB system on synaptic plasticity in adolescent female rats by blocking the eCB system functionality with AM251, a selective CB1 receptor antagonist.

Our results showed that in control animals PSD-95, a pivotal postsynaptic scaffolding protein in excitatory neurons, decreased from PND 28 to 42 to then stabilize from PND 43 to 52 (Fig.9). The decrease could be due to the elimination of the excitatory synapses (pruning event) that is a critical remodelling process already observed in adolescent brains (Giedd et al., 1999, Paus, 2005).

The spine-mechanism pruning seems to be also regulated by GluN3A (Kehoe et al., 2014). Specifically it has been suggested that the expression of GluN3A-containing NMDARs at synapses interferes with mechanisms for spine stabilization, decreasing the lifetime of spine synapses and promoting their elimination. Consistently, we found a sharp reduction in PSD95 levels following GluN3A peaks. In particular, in control animals GluN3A levels peak at PND 28 and 37 while PSD95 levels undergo a strong reduction in the subsequent sub-periods investigated (from PND 29 to 32 and from 38 to 42), thus suggesting that the increase of GluN3A plays a key role in the pruning occurring in this time-frame (Fig.10).

Maturation and strengthening of glutamatergic synapses are also characterized by a switch from predominance of NMDA receptors rich in gluN2B to those rich in gluN2A subunits (van Zundert et al., 2004). In control animals GluN2A levels increase from PND 28 to 37, they

decrease from PND 38 to 42 to eventually increase again from PND 42 to 52 (Fig.11). The initial fluctuations in GluN2A levels may be due to the pruning events occurring in excitatory synapses during the early and mid-adolescence. Starting from PND 42 when the PSD95 levels stabilize and thereby the pruning events ended, GluN2A levels strongly increase, suggesting a strengthening of the remaining synapses.

The process of maturation and strengthening of glutamatergic synapses is also characterized by the acquisition of AMPA receptors. As a matter of fact, GluA2 subunits similarly to GluN2A ones, promote the development of large spines (Chen et al., 2009). Consistently, it is possible to note that the physiological trend of GluA2, a marker of synaptic maturation, is totally specular to that of GluN3A, a marker of instability. In control animals GluA2 physiological levels increased from PND 28 to 32, they decreased from PND 33 to 37, to eventually increase again until PND 42 (Fig.12). Finally, GluA2 levels seem to stabilize from PND 43 to 52. Likewise to that observed in GluN2A levels, the decrease in GluA2 observed during the early adolescence could be a result of the pruning events that involve the mature excitatory synapses.

Furthermore, it has been suggested that the Ca2+ permeability of AMPARs strongly depend on GluA2. Specifically, AMPA receptors containing GluA2 are Ca2+ impermeable (CI-AMPARs) whereas those lacking GluA2 are Ca2+ permeable (CP-AMPARs) (Hollmann and Heinemann, 1994; Jonas & Burnashev, 1995). Thereby the decrease in GluA2 levels from PND 32 to 37 without changes in GluA1 subunits (data not shown) might suggest the presence of GluA2-lacking and thereby calcium permeable AMPA receptors. In line with this, previous studies reported a greater amount of fast spiking interneurons with Ca2 +-permeable (CP)-AMPA receptors in the prefrontal cortex of adolescent rats (Wang and Gao et al., 2010).

The blockade of the eCB system functionality through the administration of AM251 disrupted some of these maturational events occurring in PFC glutamatergic synapses, leading to a different final organization of functional network connections. Specifically, the administration of AM251 significantly prevented the decrease of PSD95 during the early

adolescence (Fig.9), thus suggesting a possible involvement of the endocannabinoid tone in the pruning of excitatory synapses. Blockade of CB1 receptor also altered the maturational fluctuations of NMDA subunits. In particular, it prevented the decrease of GluN3A levels at PND 32 and their increase at PND 37 (Fig.10). Furthermore, it seems also to slightly modify the physiological fluctuations of GluN2A leading to a reduction in their levels, mostly in the two last periods investigated (Fig.11). This late reduction may suggests that the blockade of the eCB tone leads to a delay in the maturation of the remaining excitatory synapses.

At this point, since we proved that the eCB system is fundamental for the developmental changes occurring in the prefrontal cortex during adolescence, we decided to study with a similar approach which is the most sensitive period for the adolescent THC exposure.

Our results showed that the most delicate periods to this psychoactive component exposure were the early and mid-adolescence. Specifically, this administration significantly prevented the decrease of PSD95 levels and altered the physiological trend of GluN3A levels mainly at PND 32 and 37 with respect to controls.

Curiously we observed that the modulation with THC disrupts the developmental changes occurring in the prefrontal cortex of female rats in a similar way to that observed after the modulation with the antagonist of CB1 receptor, AM251 (Fig. 13,14,15 and 16). We can speculate that this similarity could be due to the fact that the modulation with THC may have produced the down regulation of CB1 receptors leading then to the same effect observed after the administration of the antagonist of CB1 receptor. However, the low THC dose used and the short period of exposure make this assumption very unlikely. Alternatively, since 2-AG, the most abundant eCB in the brain, has previously been demonstrated to control synaptic strength throughout the nervous system, playing important roles in brain development (Oudin end Doherty, 2011), we cannot exclude the possibility that the adolescent synaptic remodelling may be promoted especially by 2AG. Thus, we can also hypothesize that the similar effects induced by these two different modulations could be due to the fact that THC, being a partial agonist of CB1 receptors, may behave as an antagonist towards the full agonist 2-AG when this is acting at high concentrations.

THE ENDOCANNABINOID TONE IS FUNDAMENTAL FOR THE ADOLESCENT MYELINATION PROCESS IN FEMALE RATS

As already reported, adolescence is a very critical period, characterized by substantial changes in brain morphology such as a loss of grey and a rise of white matter. We have previously demonstrated that the eCB tone seems to be relevant in developing a final functional network of connections by promoting the extensive pruning of cortical excitatory synapses and the later maturation and strengthening of the remaining ones. We then wanted to study whether the eCB system could be also involved in the other fundamental event occurring in the adolescent brain: the myelination enhancement.

In literature there are no data regarding the trend of myelin levels in the developing brain of adolescent rats yet. Thereby, with a similar approach to that used before, we tried for the first time to evaluate, in an animal model, how myelin levels vary during the adolescence.

Previously, human studies have reported that myelination begins in in the prenatal developmental phase and culminates during adolescence (Sowell et al., 1999). In line with these data, we found, in our animal model, that MOG and MBP, the two white matter markers, increased from PND 28 to 42/47 respectively (Fig 17 and 18). It is well known that myelination is important to guarantee the rapid and efficient conduction of the electrical impulses along the axons, but also for preserving axonal integrity. Several studies have reported that myelination deficits can contribute to neurological disorders, including mental health disorders, multiple sclerosis, epilepsy and dementias (Gibson et al. 2018). However, the specific importance and role of the adolescent myelination enhancement in the developing PFC is not totally clear yet.

A very recent work in humans has shown that reductions in myelin-related growth mostly in dorsomedial lateral and medial prefrontal regions, during the transition from adolescence to adulthood, can be linked to the development of impulsivity traits (Gabriel Ziegler et al., 2019). Thereby we hypothesized that the increase of white matter, observed in our animals could not only enhance the communication efficiency but also reduce the impulsivity levels.

To test whether in our animal model, younger animals, characterized by a lower amount of myelin, were effectively marked by higher impulsivity compared to older ones, whose amount of myelin is greater, we performed the Wire-Beam Bridge Test. Since the test showed that younger animals were significantly faster in crossing the bridge compared to the older group (Fig 19.A), we confirmed our thesis.

Studies focused on age-related impulsivity differences that span through adolescence and adulthood are not many and the ones present in literature are mostly performed in humans. However, all these clinical reports are in line with our finding, suggesting a significant decline in impulsivity from childhood through adolescence and adulthood (Galvan et al., 2007; Leshem and Glicksohn, 2007).

Our results also showed that the eCB system plays an essential role in the adolescent myelination enhancement. As a matter of fact, the increase of both white markers, MOG and MBP were prevented by the administration of the antagonist of CB1 receptor, AM251 (Fig 17 and 18). This evidence has been also confirmed by the difference in the number of myelinated fibres observed with the electron microscope between control animals and the one treated with AM251. In line with our findings, previous studies reported that the OPCs survival, proliferation and differentiation to mature OLs seem to be promoted by 2-AG (Ilyasov et al., 2018), thus confirming our thesis according to which the eCB system is a relevant player in the myelination process.

As we demonstrated in our animal model a correlation between the reduction of impulsivity and the increase of white matter, we then decided to deeply investigate whether the blockade of the eCB system during this specific developmental window, preventing the increase of myelin, could in parallel lead to a higher behavioural expression of impulsivity.

Through the Wire-Beam Bridge test we found that animals administered with the antagonist of CB1 receptor, during the whole period in which the eCB system functionality system appeared to be fundamental for the myelination process (from PND 28 to 45), were actually characterized by higher levels of impulsivity. As a matter of fact, they were faster in crossing the bridge compared with controls, as showed in Fig 19.B. Thus, confirming the presence of a parallelism between the myelination process and the impulsivity levels.

At this point, we focused our attention on trying to identify which could be the cellular mechanism involved in the myelination enhancement through the activation of CB1 receptor during this specific developmental window. Since the PI3K/Akt/mTOR and ERK have emerged to be the two major pathways involved in the regulation of oligodendrocyte differentiation and myelin growth in the CNS (Flores et al. 2000; Flores et al. 2008; Narayanan et al. 2009; Tyler et al. 2009; Fyffe-Maricich et al. 2011; Ishii et al. 2012; Guardiola-Diaz et al. 2012; Ishii et al. 2013; Fedder-Semmes and Appel, 2021), we started to investigate the effect of AM251 on these kinases.

In line with these finding, our results showed that animals treated with the antagonist of CB1 receptor expressed significant lower levels of phosphorylated AKT (Fig 21.A). In contrast we did not find differences in both p-mTOR and p-ERK levels (Fig 21.B and C). This may suggest that the CB1 cannabinoid receptor regulates the adolescent myelination enhancement through a pathway that involved the AKT activation but without having mTOR as direct downstream target and without leading to the activation of the ERK pathway. These differences could be due to different factors. For example, Tyler et al. (2009) and Gomez et al. (2010) examined the relevance of mTOR and ERK, respectively in the oligodendrocytes differentiation and maturation, studying cultures of oligodendrocyte precursor cells in vitro. It is well know that myelination cell culture systems are useful tools for studying myelin biology however they are systems reproduced under particular controlled conditions, very different from the reality, where oligodendrocytes interact with the surrounding environment including neurons. Whereas, Flores et al. (2008) and Narayanan et al. (2009) demonstrated the involvement of mTOR in regulating CNS Myelination, using Plp-Akt-DD mice at P10, P14, P21, and P30 or between 6-8 weeks, therefore focusing more on the pre-adolescent phase or in any case in a specific period different from ours, maybe indicating that each developmental phase involves different pathways. Furthermore, since they used animal models of demyelination, It is possible speculate that different mechanisms may underlie the regulation of myelin production between health and disease.

In the peripheral myelination process, Schwann cells differentiation as well as the initiation and maintenance of myelin are regulated by YAP and TAZ (Grove et al., 2017; Fernando et al., 2016; Poitelon et al., 2016), which are two transcriptional co-activators, belonging to the Hippo pathway (Dupont et al., 2011). Recent studies have also raised the possibility that the activation of YAP could promote the morphogenesis and maturation of white matter in OLs through mechanical stress (Shimizu et al., 2017). The Hippo pathway is formed by core mediators such as Mst1/2, LATS1/2, Mob and Sav. The activation of the Hippo pathway leads to the direct phosphorylation of YAP that prevents its nuclear localization. In contrast, when the Hippo pathway is inactive, YAP can migrate into the nucleus and activate transcription of genes including myelin genes.

Since previous studies reported a possible cross-talk between the Hippo and AKT pathways, suggesting that the suppression of AKT activity significantly induces phosphorylation of YAP (Xiu et al. 2013), we thereby hypothesized that the adolescent myelination enhancement could be promoted by the inhibition of the hippo pathway through the activation of AKT, following the CB1 receptor activation. To assess if this speculation could be true and thereby if YAP could be the pathway involved in the adolescent myelination process we studied in naïve rats of PND 28 and 48 both the amount of p-YAP retained into cytosol and nuclear YAP. In agreement with our supposition, we found that older animals, characterized by higher myelin levels, were characterized by a significant reduction in the amount of cytosolic p-YAP and by an increase in nuclear YAP levels compared to the younger ones (Fig 22), thus confirming the involvement of the YAP pathway in the adolescent physiological myelination enhancement during this specific developmental window.

At this point, we wanted to confirm whether the blockade of the eCB system could prevent the increase in myelin levels affecting the HiPPO pathway. Consistently with our hypothesis, rats treated with CB1 receptor antagonist showed higher levels of phosphorylated YAP in the cytoplasm and lower levels of nuclear YAP (Fig. 23), thus confirming the involvement of this pathway during the adolescent myelination process.

Finally, as previously done in the study of the eCB system role on plasticity, we evaluated whether the administration of THC would affect the myelination enhancement in adolescent

rats in a similar way to that observed after the exposure to the antagonist of CB1 receptor AM251.

As expected, similarly to what observed following the administration of AM251, THC prevented mainly the increase of MBP at PND 37 and 42 (Fig. 24). As before, we tried to explain why THC, a CB1 partial agonist, could act in the same way to that observed after the administration of the antagonist of CB1 receptor, AM251. Once again, we hypothesized not only that THC may have produced a down regulation of CB1 receptors leading then to the same effect observed after the administration of the antagonist of CB1 receptor, but also that THC, being a CB1 partial agonist, may behave as an antagonist towards the full agonist 2-AG when this is present at high concentrations. As a matter of fact, as previously mentioned, studies highlight that 2-AG is the eCB able to promote the OPCs survival, proliferation and differentiation to mature OLs (Ilyasov et al., 2019).

Aguado et al. (2021), in contrast with our finding, reported a positive effect of THC in stimulating OPCs differentiation and in promoting central nervous system re-myelination and functional recovery. Consistently with them, Huerga-Gómez et al., 2020 identified THC as an effective pharmacological strategy to enhance CNS myelination in vivo. These differences could be attribute to the fact that in the former case, the study was conducted in animal models of demyelination, whereas in the latter, THC was administrated in mice at early postnatal ages, not in the physiological adolescence period as occurred in our experiments. The animal model used and the period of administration as well could thereby led to different results compared to ours. Furthermore, since 2-AG appears to be the key effector of the myelination process, it seems logical to hypothesize that its physiological levels (which are age-related) are essential to determine whether the administration of a partial agonist, such as THC, can act as a CB1 receptor agonist and thus promote the myelination enhancement (when 2-AG is low) or can behave as a CB1 receptor antagonist (when 2-AG levels are high) and thereby prevent its action.

CONCLUSION

As a whole, the present study confirms the presence of maturational events in the prefrontal cortex of female rats during adolescence. The blockade of the eCB system during this period seems to negatively impact on some of these adolescent developmental changes such as synapse refinement and myelination enhancement. Furthermore, the blockade of the eCB system, preventing the increase of myelin, seems to in parallel lead also to a higher expression of impulsivity. This study also suggests a possible molecular mechanism involved in the promotion of the physiological adolescent myelination process through the activation of CB1 receptor. Specifically, we propone that CB1 receptor stimulation can induce the activation of the AKT pathway that in turn can inhibit the Hippo signaling pathway, thereby promoting the translocation of YAP into the nucleus, where it can promote myelin gene transcription. Finally, since we also proved that THC administration produces similar effects to those observed after the administration of the antagonist of the CB1 receptor, AM251, we may speculate that cannabis adolescent exposure could be detrimental for the physiological brain maturation during this specific developmental period.

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