Crosstalk between the transcriptional regulation of dopamine D2 and cannabinoid CB1 receptors in perinatal Δ9-tetrahydrocannabinol occurs in schizophrenia: analyses in patients and in animal model of the disease.

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ABSTRACT

Background

Perinatal exposure to Δ^9-tetrahydrocannabinol (THC) affects brain development and might increase the incidence of psychopathology later in life, which seem to be related to a dysregulation of endocannabinoid (ECS) and/or the dopaminergic (DAergic) systems.

Methods

We here evaluated the transcriptional regulation of the genes coding the cannabinoid CB1 receptor (*CNR1*) and the dopamine D2 receptor (*DRD2*) in perinatally THC exposed male rats (n=7-15), focusing on the role of DNA methylation analyzed by pyrosequencing. Simultaneously, the behavioral abnormalities at two different time points (i.e. neonatal age and adulthood) and the potential preventive effect of peripubertal treatment with the non-intoxicating component of the *Cannabis* cannabidiol (CBD) were assessed. The DRD2 methylation was also evaluated in a cohort of schizophrenic subjects (n=36).

Results

We observed an increase in both *CNR1* and *DRD2* mRNA levels selectively in the prefrontal cortex (PFC) of adult THC exposed rats with a consistent reduction in DNA methylation at *DRD2* promoter, paralleled by social withdrawal and cognitive impairment which were reversed by peripubertal CBD treatment. These adult abnormalities were preceded at neonatal age both by delayed appearance of neonatal reflexes, by higher *DRD2* mRNA and lower 2-arachidonoylglycerol (2-AG) brain level, which persisted until adulthood. Alterations of the epigenetic mark for *DRD2* was also found in subjects with SCZ.

Conclusions

Our data add further evidence to the dopamine-cannabinoid interaction in terms of *DRD2* and *CNR1* dysregulation which could underlie schizoaffective disorders, suggesting that CBD treatment may normalize THC induced psychopathology modulating DAergic activity.

INTRODUCTION

Psychosis is a characteristic trait of schizophrenia (SCZ) spectrum disorders, characterized by a complex etiology, which cannot be explained only by an undeniable genetic component **(1,2)** but also by the exposure to environmental factors **(3,4)**, including recreational drugs **(5–8).** According to the neurodevelopmental hypothesis of SCZ **(9–11)**, the interaction between genetic and environmental factors during early and vulnerable neurodevelopmental phases, such as the peri/prenatal stages **(12)**, can have long-lasting negative consequences for brain function and behavior, increasing the risk to develop psychosis later in life.

Cannabis is the illicit drug most widely consumed during pregnancy in Western societies (13). The prenatal exposure to Δ^9-tetrahydrocannabinol (THC), the main psychoactive constituent of *Cannabis*, could affect the temporal dynamics of cannabinoid CB1 receptors (CNR1), which are present and active from the early embryonic stage **(14)**. Therefore, CNR1 overstimulation could alter the neurodevelopmental process leading to rewire endocannabinoid system (ECS) signaling, which could in part underlie the detrimental effects induced by perinatal *Cannabis* exposure at adulthood **(15,16)**. It is well accepted a functional interaction between the ECS, molecular target of THC and the dopaminergic (DAergic) system both in terms of co-expression of CNR1 and dopamine D2 receptors (DRD2) **(17)** and in signal transduction convergence **(18)** in several brain regions such as prefrontal cortex (PFC), nucleus accumbens (NAc), that have received extensive focus as possible neuroanatomical loci for SCZ related psychopathology **(19)**. Thus, it cannot also be excluded that in the long-lasting detrimental effect induced by perinatal THC exposure the aberrant DAergic neurotransmission could be also play a role.

Antipsychotic treatments have traditionally targeted the DAergic neurotransmitter system. However, they are associated with side effects and a significant proportion of patients do not achieve an adequate remission of symptoms **(20)**. Recently, there has been considerable interest, based on preclinical and human studies in the potential antipsychotic activity and safety profile of

cannabidiol (CBD), a non-intoxicating component of the *Cannabis* **(21)**. In agreement with the hypothesis that preventive treatment with antipsychotics in the early phase of the disease could reduce the risk of progression to first-episode psychosis **(22)**, peripubertal CBD treatment counteracted the development of SCZ-like abnormalities in a neurodevelopmental animal model **(23)**. Although human studies provide relevant information regarding the long-term detrimental effects on neurodevelopment of prenatal *Cannabis* exposure, several confounding factors such as different THC concentration, malnutrition induced by *Cannabis* consumption, could limit the conclusions of such studies. In this regard, animal studies based on perinatal THC exposure, which induces cognitive and behavioral alterations resembling functional and neuropathological deficits at adulthood **(15,25)**, resulted in a very useful experimental tool to reproduce the human condition.

Based on this background, the aim of the present study was to investigate the possible early presence of subtle neurodevelopmental impairments in perinatally THC exposed rats at neonatal age. Moreover, at adult age, we assessed the behavioral phenotype and measured potential ECS and DAergic changes in specific brain regions involved neurodevelopmental disorders such as prefrontal PFC, NAc, hippocampus (HIP) and ventral tegmental area (VTA) **(26,27)**. We also attempted to reverse the behavioral and molecular alterations induced by perinatal THC exposure by peripubertal CBD treatment. Moreover, to further support the translational profile of perinatal THC exposure model, we assessed the epigenetic regulation of DRD2 in peripheral blood mononuclear cells (PBMCs) of schizophrenic subjects.

Methods and Materials

Human Subjects

The recruitment of SCZ subjects which were conducted in accordance with all relevant laws and regulations of …..is described in supplementary materials.

Animals, THC model and experimental design

All experiments were conducted in accordance with all relevant laws and regulations of animal care and welfare. The perinatal THC administration in Sprague-Dawley rats was based on previous study (Drazanova et al., 2019). A detailed description of the experimental procedures and drugs administration can be found in Fig. 1, or as previously described (Uttl et al., 2018; Stark et al., 2019) and detailed in supplementary material.

Behavioral Testing

Evaluation of neonatal reflexes and nest-seeking behavior were assessed as previously described (Lo Pumo et al., 2006; Tamburella et al., 2012; Baharnoori et al., 2012) and detailed in supplementary materials. At adulthood, the spontaneous locomotor activity in the open field test, the social activity in the social interaction (SI) test and the cognitive performance in the novel object recognition (NOR) test were assessed as previously described (Stark et al., 2019) and detailed in supplementary material.

Biochemical methods

The ECs level by liquid chromatography mass spectrometry, the mRNA quantification of ECS and non-ECS elements by quantitative real-time polymerase chain reaction, the protein expression of cannabinoid CNR1 and dopamine DRD2 receptor by western blotting analysis, the DNA methylation status at level of CNR1or DRD2 gene promoter were assessed by Pyrosequencing as previously described (Stark et al., 2019) and detailed in supplementary material.

Statistical Analysis

Data were analyzed using ANOVA, Mann-Whitney, unpaired or Fisher's exact t-test when appropriate (See supplementary material). Statistical significance was set at $p< 0.05$.

RESULTS

THC Rats at Neonatal Age

Behavioral Phenotype. The appearance rate of neonatal reflexes in perinatally THC-exposed rats and respective CNT group are shown in Fig. 2. The percent appearance and the completion of neonatal reflexes had a significant delay in THC rats. Fisher's exact t-test revealed several time points where the percentage of pups exhibiting righting (PND 1-2, $p<0.05$; Fig. 2A), cliff aversion (PND 2-8, $p<0.05$-$p<0.01$; Fig. 2B), forelimb placing (PND 3-9, $p<0.05$-$p<0.001$; Fig. 2C), forelimb grasping (PND 2-4, $p<0.05$; Fig. 2D), bar holding (PND 5, $p<0.05$; Fig. 2E) and negative geotaxis (PND 3-4, $p<0.05$; Fig. 2F) was significantly lower in the THC group as compared to CNT. Perinatal THC-treated pups made significantly fewer entries toward maternal nest as compared to the CNT group ($t=2.095$, $p=0.0491$; Fig. 2G), while no significant difference was found in the next exploration ($t=1.667$, $p=0.1112$; Fig. 2H).

Biochemical Analyses. In the whole brain of independent perinatally THC exposed rats at PND10 there was a significant decrease of 2-AG (MWU-test: $p=0.0286$ vs respective CNT group; Fig. 2J), but not of AEA ($p=0.4857$ vs respective CNT group; Fig. 2I), PEA ($p>0.999$ vs respective CNT group; Fig. 2J) or OEA ($p=0.6286$ vs CNT group; Fig. 2J) content at PND 10. In order to extend these findings, we performed a wide transcriptomic analysis for all the genes known to encode for the large class of enzymes involved in the metabolism of the endocannabinoids (AEA, 2-AG, PEA and OEA). Among the genes involved in the AEA metabolism, we found that protein tyrosine phosphatase non-receptor type 22 (ptn22: for the synthesis of AEA) and fatty acid amide hydrolase (FAAH; for the degradation of AEA) were increased (ptn22: $p=0.057$ vs CNT group; FAAH: $p=0.0286$ vs CNT group). Among the class of genes regulating the metabolism of 2-AG, we found a significant increase in monoacylglycerol lipase (MAGL, involved in the degradation of 2-AG) transcript levels ($p=0.0104$ vs CNT group) (Fig 2K). In the brain of THC animals, with respect to control rats, the expression levels of the two endocannabinoids responsive receptors (CNR1: $p=0.3429$ and TRPV1: $p>0.999$) was not significantly different. In contrast, the DRD2 expression was increased in the brain of perinatally THC exposed rats ($p=0.057$ vs CNT group; Fig 2L).

Perinatally THC exposed Rats and the Response to the CBD Treatment at Adulthood

Behavioural Phenotype

Neither perinatal THC exposure nor peripubertal CBD treatment affected the spontaneous horizontal (number of crossings, factor THC: $F_{1,37}$=2.505, p=0.1220; factor CBD treatment: $F_{1,37}$=0.02824, p=0.1220; THC × CBD treatment interaction: $F_{1,37}$=1.010, p=0.3215; Fig. 3A) or vertical (number of rearings, factor THC: $F_{1,37}$=1.350, p=0.2528; factor CBD treatment: $F_{1,37}$=0.3642, p=0.5499; THC x CBD treatment interaction: $F_{1,37}$=0.06086, p=0.8065; Fig. 3D) locomotor activity in a novel environment at adulthood.

The effects of peripubertal CBD treatment, alone or combined with perinatal THC exposure, on behavioural performance in the social interaction test is depicted in Fig.3B/E. Two-way ANOVA revealed for the time of interaction a main effect of THC ($F_{1,24}$=9.802, p=0.0045), a main effect of CBD treatment ($F_{1,24}$=11.56, p=0.0024) and a significant THC x CBD treatment interaction ($F_{1,24}$=4.937, p=0.0360). *Post-hoc* analysis revealed that THC/VHC group spent less time in social interaction compared to CNT/VHC rats (p<0.001), indicating impaired social behavior. Intraperitoneal treatment with CBD, improved social performance in the THC group as compared to the THC/VHC group (p<0.001). Furthermore, in CNT group CBD did not affect the social activity (Fig. 3B). Neither perinatal THC exposure ($F_{1,24}$=0.08423, p=0.7741) nor CBD treatment ($F_{1,24}$=1.523, p=0.2292) affected the number of interaction (THC × CBD treatment interaction $F_{1,24}$=0.08423, p=0.7741), as index of locomotor activity (Fig. 3E).

In the rats tested in the novel object recognition (NOR) test, two-way ANOVA showed a main effect of THC ($F_{1,38}$=5.387, p=0.0258), a significant THC x CBD treatment interaction ($F_{1,38}$=11.47, p=0.0017) but not a main effect of CBD treatment ($F_{1,38}$=0.03349, p=0.8558) for the discrimination index (DI). *Post-hoc* analysis revealed that perinatally THC exposure affected the recognition memory as demonstrated by a significant reduction (p<0.01) in the DI during the test phase, which

was reversed by CBD ($p<0.01$; Fig. 3C). However, no difference was found in the total exploration time among the groups (two-way ANOVA, factor THC: $F_{1,38}=2.045$, $p=0.1609$; factor CBD treatment: $F_{1,38}=0.1345$, $p=0.7159$; THC× CBD treatment interaction: $F_{1,38}=0.6776$, $p=0.4155$; Fig. 3F), as well as in the time spent to explore the two identical object in the familiarization phase (two-way ANOVA, factor THC: $F_{1,38}=2.045$, $p=0.1609$; factor CBD treatment: $F_{1,38}=0.1345$, $p=0.7159$; THC x CBD treatment interaction: $F_{1,38}=0.6776$, $p=0.4155$; Supplementary Fig. 1).

Regulation of *CNR1* and *DRD2* Gene Transcription.

Among the different cerebral areas (PFC, NAc, VTA, HIP), in our analysis PFC of THC/VHC group was the only one showing significant alterations in CNR1 and DRD2 gene transcription as compared to the CNT/VHC group (**Table 1**).

In the PFC of perinatal THC exposed rats there was a significant CNR1 up-regulation (two-way ANOVA, factor THC: $F_{1,17}=4.635$; $p=0.0460$; MWU test, $p=0.0303$ vs CNT/VHC) which was not modified by CBD treatment (factor CBD treatment: $F_{1,17}=0.3583$, $p=0.5573$; THC x CBD treatment interaction: $F_{1,17}=2.706$, $p=0.1183$; Fig. 4B). However, neither perinatal THC exposure nor CBD treatment modified DNA methylation of *CNR1* promoter (two-way ANOVA, factor THC: $F_{1,18}=3.215$, $p=0.0898$; factor CBD treatment: $F_{1,18}=0.6625$, $p=0.4263$; THC x CBD treatment interaction: $F_{1,18}=2.166$, $p=0.1584$; Fig 4A). No difference was found in the CNR1 protein expression (two-way ANOVA, factor THC: $F_{1,11}=0.1455$, $p=0.7102$; factor CBD treatment $F_{1,11}=4.666$, $p=0.0537$; factor THC x CBD treatment $F_{1,11}=0.2235$, $p=0.6456$; Fig 4C).

Consistent with the increase of DRD2 mRNA expression ($p<0.01$ vs CNT/VHC) (two-way ANOVA, factor THC: $F_{1,18}=16.57$, $p=0.0007$; factor CBD treatment: $F_{1,18}=12.86$, $p=0.0021$; factor THC x CBD treatment interaction: $F_{1,18}=5.283$, $p=0.0337$, Fig. 4E), we observed both a significant reduction in DNA methylation of the DRD2 promoter in the 6 CpGs average of THC/VHC group ($p<0.05$ vs CNT/VHC) (two-way ANOVA, factor THC: $F_{1,16}=0.7572$, $p=0.3971$; factor CBD treatment: $F_{1,16}=2.343$, $p=0.1454$; factor THC x CBD treatment interaction: $F_{1,16}=26.53$, $p<0.0001$,

Fig. 4D) and a significant increase of DRD2 protein expression (Two-way ANOVA, factor THC: $F_{1,11}$=11.78, p=0.0056; MWU-test, p=0.0286 vs CNT/VHC; factor CBD treatment: $F_{1,11}$=0.355, p=0.5634; THC ×CBD treatment interaction: $F_{1,11}$=1.651, p=0.2244; Fig. 4F). Peripubertal CBD treatment reversed THC-induced changes in DNA methylation ($p<0.05$), mRNA ($p<0.01$) but not protein ($p>0.05$) expression at adulthood. Significant inverse correlation between *DRD2* gene expression and DNA methylation was observed for *DRD2* (Spearman r=0.6078, p=0.0111; Fig 5B) but not for CNR1 (Spearman r=0.05965, p=0.8083; Fig 5A).

Moreover, a significant correlation between the *CNR1* gene expression and the DI in the NOR test was highlighted when all the groups under study were considered (Spearman r=0.5714, p=0.0449; **Supplementary Fig. 3A**), while a significant correlation between the *DRD2* gene expression and the DI was shown only when CNT/VHC and THC/VHC were considered (Spearman r=0.8857, p=0.0333; **Supplementary Fig. 3B**).

Finally, a significant correlation between the *CNR1* (Spearman r=0.7169; p=0.0160, **Supplementary Fig. 4A)** and the *DRD2* (Spearman r=0.6697; p=0.0283, **Supplementary Fig. 4B)** gene expression and the time of interaction as index of social behavior in the SI test was highlighted.

DNA Methylation at *DRD2* Promoter Region in Clinical Samples

Patients and controls were age and gender matched to allow consistent comparisons.

DNA methylation was significantly reduced in SCZ patients at CpG site 1 (CNT: 3.57 ± 0.14; SCZ: 3.06 ± 0.11; p=0.0114), CpG 2 (CNT: 3.49 ± 0.14; SCZ: 2.81 ± 0.11; p=0.00079), CpG 3 (CNT: 3.16 ± 0.14; SCZ: 2.62 ± 0.12; p=0.0092) as well as in the average of the 3 CpG sites investigated (CNT: 3.40 ± 0.13; SCZ: 2.82 ± 0.10; p=0.0032, **Fig. 6**). No differences were found when the schizophrenic population was stratified based on gender, age and pharmacotherapy (**see Supplementary Figures 5S, 6S, 7S**). A significant correlation between *CNR1* and *DRD2* % change

of DNA methylation (AVE CpG sites) in schizophrenic subjects was observed (Spearman r=0.4606; p=0.0104, **Fig. 7**).

DISCUSSION

These findings provide evidence that perinatal exposure to THC, at the dose of 5 mg/kg which is not associated with overt signs of toxicity (REF??), produces subtle and enduring neurobehavioral and molecular changes in the rat offspring which appear already at neonatal age and persist through adulthood. Interestingly, the detrimental effects induced by THC are counteracted by peripubertal CBD treatment, further supporting its potential therapeutic for the treatment of neuropsychiatric disorders.

More specifically, we showed that perinatal THC exposure leads to delayed onset of neonatal reflexes, which could represent a predictive factor for psychopathology at adulthood, as previously described (38,40,47,48). We also found that perinatal THC exposed pups made fewer choices toward nest compartment as compared to control animals, indicating decreased preference toward maternal nest. A similar experimental approach, based on the ability of the offspring to use olfactory orientation cues in order to locate maternal nest, has been used in several animal models of prenatal insult such as gestational exposure to drugs of abuse (49) and prenatal malnutrition model for SCZ (50). Although the limits per se of animal models, our findings are in agreement with longitudinal human cohort studies showing behavioral and cognitive alterations in neonates born from Cannabis users (15). At molecular level the impaired neurobehavioral development of THC pups was evident at PND 10 (when the reflexes are fully expressed) with decreased brain 2-AG level due to increased expression of the degradative enzyme MAGL. It has been seen that deficits in 2-AG signaling may have several different deleterious effects on cortical circuitry function in SCZ, such as affecting neurite formation and dendritic maintenance (51,52) and cortical GABA neuron development, which, this latter has been commonly reported to be altered in SCZ (53). Thus, the altered 2-AG content at neonatal age might contribute to explain some of the

behavioral deficits reported at adulthood. Moreover, it should be also considered a long lasting potential detrimental effect of the observed early DRD2 gene over expression, even if marginally significant in the pathophysiology of neurodevelopmental disorders.

At adulthood, perinatal THC treatment elicited cognitive and social deficits in rats, as described by lower discrimination ratio in the NOR (as index of impaired short term recognition memory) and the reduced time of interaction in the SI (as index of social withdrawal), which are often considered the two signs of SCZ-like symptoms (54). In our study, THC offspring, which spent the same time to explore the two identical objects during familiarization phase, showed no preference for the novel object during the testing phase, suggesting an inability to recognize the familiar object. The total object exploration time did not differ among the groups; thus the THC rats are thought to have a deficit in short-term object recognition memory in contrast to a deficit in object exploration, which this latter could be related to impaired locomotor activity. This finding is consistent with observations in preclinical (25) and clinical studies of perinatal THC exposure (15,16), further supporting the face validity of this model. Similarly, the social deficit observed in SI is also not related to changes in motor activity, since no difference was found in the number of interactions. Given that no spontaneous enhanced locomotor activity has been detected in THC animals, partially in line with previous results (25), overall our study reinforces the original findings that cognitive deficit in the NOR and social withdrawal in the SI are a robust phenotype in the THC model. Nevertheless, the behavioral assays (i.e. NOR, SI and spontaneous locomotor activity) used in the present study are not strictly specific for SCZ and could be applicable to assess symptoms domains shared with other neuropsychiatric disorders (i.e. autism, depression, anxiety). Thus, further behavioral tasks to evaluate to assess positive-like deficits and the different cognitive or negative-like symptoms would be useful.

In agreement with preclinical data and human studies suggesting that early adolescence could represent the promising window of opportunity for a course-altering strategy (22,56), we showed

that peripubertal treatment with CBD rescued recognition memory deficit and the decreased sociability in THC offspring. It is noteworthy that the peripubertal age corresponds to mid-to-late adolescence in humans, which is a pivotal period for PFC development; thus, dysregulation in the PFC during this period could play a role in the pathophysiology of SCZ (57). Our results align with the therapeutic benefit of CBD to improve the negative symptoms and cognitive deficits in clinical studies (58) and in most of preclinical models (21). However, further investigations are needed to assess the effects of CBD on additional cognitive and social domains, which are impaired in schizo-affective disorders.

The mechanisms underlying the beneficial effects of CBD on SCZ-like symptoms are still elusive. Aberrant DAergic transmission in the brain is a common target of all current antipsychotics and a well-established neuropathological feature both in SCZ and in THC model (59). Recently, CBD has been found to attenuate DAergic hyperfunction in the mesolimbic pathway (19) and, in agreement with its pharmacological profile as atypical antipsychotic, also showed partial agonistic activity at D2Rs, similarly to aripiprazole (60), which may at least in part account for its antipsychotic effects. Further investigations into the impact of CBD on altered DAergic system may shed light on the mechanisms underlying the improvement of recognition memory and social behavior induced by perinatal THC exposure.

At molecular level, our data showing, at both preclinical and clinical levels, the transcriptional regulation of both CNR1 and DRD2 genes, add new piece of evidence to the dopamine-cannabinoid interactions as molecular substrate of psychosis (61).

Namely, we report the up-regulation of both receptor mRNAs and a consistent reduction in DNA methylation selectively at DRD2 gene promoter in adult PFC of perinatal THC exposed rats. Of note, we also reported a significant correlation between receptor genes expression and cognitive and social impairment in rats. In agreement, DRD2 DNA methylation resulted to be reduced in PBMCs of SCZ subjects as compared to controls. We previously reported the reduction in the epigenetic

mark also for CNR1 in the same human samples (26) and, interestingly, here we highlight the significant correlation between CNR1 and DRD2 methylation levels in our samples. Moreover, the modulation in CNR1 expression is similar to that previously observed in the neurodevelopmental MAM model of SCZ (26). Consistently with our clinical data, CNR1 expression and promoter DNA methylation resulted to be altered and negatively correlated at peripheral level both in subject with THC dependence (62) and in schizophrenic with *Cannabis* abuse (63), whereas no alterations in receptor mRNA levels were observed in different brain regions of prenatally *Cannabis* exposed human fetus (64).

Increased DAergic transmission has also been described in acute SCZ (65,66) and a hyperdopaminergic state evoked by prenatal THC was also recently reported (67). Thus, the increased DAergic neurotransmission, in agreement with the DAergic hypothesis of SCZ (68), could be a potential mechanism by which THC might induces psychosis.

On the other hand, different results were described by DiNieri et al. (69) showing that, both at preclinical and clinical level, that maternal *Cannabis* consumption induce a down-regulation of D2Rs in the NAc of the offspring. The discrepancies in the experimental procedures used by DiNieri and colleagues (*i* THC dose: 0.15 mg/kg, i.v from GD5 to PND2; *ii* rat strain: Long Evans evaluated at two different time points; *iii* human samples: maternal *Cannabis* exposure and fetal brain specimens; *iv* brain region: NAc) in part could explain the divergent results.

Collectively, it appears that changes DRD2 receptor mRNA levels are quite dynamic and it is important to monitor the pattern of alterations at different developmental stages of the psychotic phenotype.

It is important also to highlight the strict correlation we observed between the transcriptional regulation of CNR1 and DRD2, since they are co-localized in the same presynaptic terminals of different brain regions (70,71) and their coactivation is relevant for the modulation of GABAergic neurotransmission in the Globus Pallidus (72,73).

Perinatal THC exposure was also able to affect the AEA and 2-AG content in the PFC of THC rats, suggesting that altered ECs levels could be also involved in the behavioral deficits. To date, we could just speculate if these alterations are signs of THC exposure or compensative response to it. We also observed that peripubertal CBD treatment reverted the molecular modulation of DRD2 but not of CNR1 in rats, with a consistent inverse correlation between DRD2 gene expression and DNA methylation. This is different from previously observation in the MAM model, since CBD reverted CNR1 (23), but not DRD2 molecular changes (data under review). This let us to hypothesize that CBD could acts via different mechanisms (i.e.: CNR1 or DRD2) based on experimental conditions.

Another relevant aspect of our work is the finding on the epigenetic modulation of receptor genes expression. In the last years, already few studies evaluated the effects of THC exposure on epigenetic mechanisms (74) and, when this occurs at prenatal level, the epigenetic modulation of DRD2 (69,75) has been reported. Moreno and coworkers hypothesize that perinatal THC might act as an epigenetic factor which could interfere with central nervous system development. DiNieri et al. focused their attention on histone 3 lysine 9 dymethylation, a repressive mark, and histone 3 lysine 4 trimethylation, an activation mark. Here we shown for the first-time that DNA hypomethylation at receptor gene promoters is significantly correlated with the alteration in mRNA levels. Our results further stress the evidence of the key role of DNA methylation in psychosis etiology, as previously reported (76).

These observations are in agreement with the hypothesis that maternal *Cannabis* consumption could induce neurodevelopmental alterations which could induce the onset of psychosis later in life via epigenetic mechanisms (77). The data on clinical samples confirm the role of the epigenetic regulation of DRD2 and that this is closely linked with CNR1 modulation. Data stratification based on gender, age or pharmacological treatment did not show any difference among subjects suggesting that these do not affect the epigenetic mark, even if it should be considered that sample size for human subjects was relatively limited making the data stratification difficult. In this frame,

another limitation of our study is that we did not have sufficient data to stratify the data on the possible maternal exposure of the subjects, however there are several limits in human studies when considering the vast heterogeneity of *Cannabis* intake (78) that instead can be controlled in laboratory animals. It is important also to point out that the clinical study was carried as a proof of concept of receptors role in psychosis as observed in animal model. Even if the selected cohort of subjects has been selected under rigorous psychiatric criterions not easy to match, further studies are indeed needed to extend these findings to a large cohort, in order to better understand the transcriptional regulation of these key genes and better provide a clear link between peripheral and central changes.

Thus, we here provide further evidence of a close connection between CNR1 and DRD2 gene transcriptional regulation that might be relevant on the development of psychotic outcomes. Of note, it is important to recall that DNA methylation is reversible and this may open new trajectories in the attempt to develop treatments of neurodevelopmental disorders targeting specifically this epigenetic mark.

In conclusion, we shown that peripubertal CBD treatment prevented the THC-induced social and cognitive deficits at adulthood, in line to the hypothesis of preventive antipsychotic treatment in individuals that are at risk of developing SCZ (22). Conveniently, CBD did not negatively affect control offspring, supporting its safety profile (79), which is a pivotal ethical issue when we consider both the preventive treatment as intervention strategy and the rates of individuals (~30%) developing the disease (24,80). We here provide further evidence of a close connection between CNR1 and DRD2 gene transcriptional regulation that might be relevant on the development of psychotic outcomes. Although several possible mechanisms of action have been suggested (81), to the best of our knowledge, none of them have been conclusively identified as the prime mechanism of CBD for the treatment of neuropsychiatric disorders. Based on our results, peripubertal age may

be a promising window for CBD treatment to prevent the emergence of SCZ-like deficits at adulthood, which may in part relate to the reversal of DAergic dysregulation in the PFC.

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CONFLICT OF INTEREST STATEMENT

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FIGURE LEGENDS

Fig. 1.

Experimental design used to investigate the effects of peripubertal cannabidiol (CBD) treatment on perinatally THC exposed rats. Pregnant rats were treated with THC (5 mg/kg; *per os*) or vehicle (CNT; 1 ml/kg; *per os*) from gestational day (GD) 15 to post-natal day (PND) 9. From PND 1 to PND 12 male offspring were subjected to behavioral tests. After completion, the neurochemical analyses were performed. From PND 19 to PND 39 the resulting male offspring were subjected to repeated treatment with vehicle (VHC), or cannabidiol (CBD: 30 mg/kg/day; i.p.). Behavioral tests of the offspring were conducted at adulthood from PND 100. After completion, the neurochemical analyses were performed.

Fig. 2.

Effects of perinatal THC exposure on neonatal behavior, endocannabinoid system (ECS)
elements and dopamine D2 receptor (DRD2) expression in rat pups. Values are mean of percent cumulative appearance of each reflex (A-F) in each day, per group of animals (n=18-20). Data are presented as means±S.E.M. (n=11) of (G) the number of approaches toward maternal nest (nest-seeking behavior) and of (H) exploration number of the nest (nest exploration), of (n=3-5) (I) AEA (J) 2-AG, PEA, OEA levels of (K) gene expression of ECS elements (receptors and metabolic enzymes) and of (L) dopamine D2 receptor (DRD2). *p<0.05, **p<0.01, ***p<0.001 vs CNT.

Fig. 3.

Effects of peripubertal treatment with cannabidiol (CBD: 30 mg/kg/day, i.p.) on the behavioral phenotype of perinatally THC exposed rats in the open field test (A: number of crossings; D: number of rearings), in the social interaction test (B: Time of interaction; E: Number of interactions) and in the novel object recognition test (C: discrimination index; F: Total exploration time) at adulthood. Data are presented as means±S.E.M. (n=7-15). ***P<0.001 vs CNT/VHC; #P<0.05 and ###P<0.001 vs THC/VHC, Bonferroni *post-hoc* test.

Fig. 4. Effects of peripubertal treatment with cannabidiol (CBD) on cannabinoid CB1 (CNR1) and dopamine D2 (DRD2) receptor in the prefrontal cortex of perinatally THC exposed rats at adulthood. Data are presented as means ± S.E.M (n=3–8) of (A) DNA methylation of CNR1 gene (AVE four CpG sites), (B) CNR1 mRNA expression, (C) and CNR1 protein expression; (D) DNA methylation of DRD2 gene (AVE 6 CpG sites), (E) DRD2 mRNA expression and (F) DRD2 protein expression. *p<0.05, **p<0.01 vs CNT/VHC; #p<0.05, ##p<0.01 vs THC/VHC, Bonferroni *post-hoc* test; §p<0.05 MWU test.

Fig. 5. Correlation between (A) CNR1 or (B) DRD2 expression and respective % change of DNA methylation of the average (AVE) in PFC of rats. Data were compared by Spearman's rank correlation coefficient: p<0.05.

Fig. 6. Comparison of DNA methylation status at *DRD2* promoter in the schizophrenic (SCZ) population and (CNT) subjects. DNA methylation data are presented as the mean of the % of methylation values of single C-phosphate-G (CpG) sites under study as well as of the average (AVE) of the three CpG sites ± the SEM. (*n*=36-58) *p<0.05, **p<0.01, ***p<0.001 vs CNT.

Fig. 7.

Correlation between *CNR1* and *DRD2* % change of DNA methylation (AVE CpG sites) in human peripheral blood mononuclear cells (PBMCs) of schizophrenic (SCZ) subjects. Data were compared by Spearman's rank correlation coefficient (p<0.05).

Table 1. *CNR1* and *DRD2* gene expression in perinatally THC rats cerebral areas.

CEREBRAL AREAS	CNR1		DRD2	
	CNT	**THC**	**CNT**	**THC**
PFC	1.12 ± 0.24	*** 2.13 ± 0.23**	1.02 ± 0.09	****2.29 ± 0.23**
NAc	1.02 ± 0.10	1.29 ± 0.12	1.01 ± 0.07	1.00 ± 0.11
VTA	1.04 ± 0.16	0.92 ± 0.06	1.04 ± 0.15	0.73 ± 0.11
HIP	1.05 ± 0.16	0.74 ± 0.13	1.23 ± 0.50	0.56 ± 0.13

CNR1 and *DRD2* gene expression in Prefrontal Cortex (PFC), Nucleus Accumbens (NAc), Ventral Tegmental Area (VTA) and Hippocampus (HIP) of perinatal THC (THC) or vehicle (CNT) exposed rats reported as $2^{-\Delta\Delta Ct}$ values calculated by Delta-Delta Ct (ΔΔCt) method vs controls (CNT) posed equal to 1. Expression was normalized to GAPDH and ß-Actin. Data are reported as means ± SEM (*N* = 5-8).

*p<0.05; **p<0.01.