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TITLE OF THE PhD THESIS

Maternal immune activation impairs endocannabinoid signaling in the mesolimbic system of adolescent offspring

Scientific and Disciplinary Sector

BIO/14 Pharmacology

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Summary

1. INTRODUCTION

1.1 Maternal Immune Activation

The disastrous effects of the ongoing pandemic of Covid-19 highlighted that infective diseases represent a significant concern in our society. Pregnancy is a significantly vulnerable period for viral infections, not only concerning maternal health but also for the unpredictable consequences for the offspring (Allotey et al., 2020; Dashraath et al., 2020; Wastnedge et al., 2021). Mounting evidence from preclinical to clinical studies suggests a relationship between maternal immune activation (MIA), as induced by viral or bacterial infections, and the emergence of neurodevelopmental disorders, such as autism and schizophrenia (Allswede et al., 2020; Miller et al., 2013). Indeed, several epidemiological studies have pointed out a correlation between an imbalance in pro- and anti-inflammatory cytokine levels during pregnancy and an increased risk of developing psychosis later in life (Allswede et al., 2020; Khandaker et al., 2013). Fundamental factors, such as the timing and the intensity of the infection, genetic predisposition, gene-environment interaction that eventually could lead to epigenetic modifications, may influence the neurodevelopmental trajectories in the offspring (Cheslack-Postava & Brown, 2021). About the precise timing of the infection, numerous epidemiological studies revealed an association between maternal infection during the second trimester of a human pregnancy and a higher incidence of schizophrenia in the descendants, underlining the importance of late gestation (Allswede et al., 2016; Limosin et al., 2003). However,

it has been highlighted the importance of an earlier time point in gestation, given that neurodevelopment during early gestation could be particularly sensitive to cytokine imbalance (Allswede et al., 2020; Atladóttir et al., 2010). Nevertheless, it should be stated that the majority offspring exposed to maternal infection during prenatal life do not develop schizophrenia nor related disorders. In fact, the relationship between maternal genetic factors and gene-environment interaction plays a fundamental role in the etiology of neurodevelopmental disorders (Estes & McAllister, 2016; Karmiloff-Smith, 2018) (**Figure 1**).

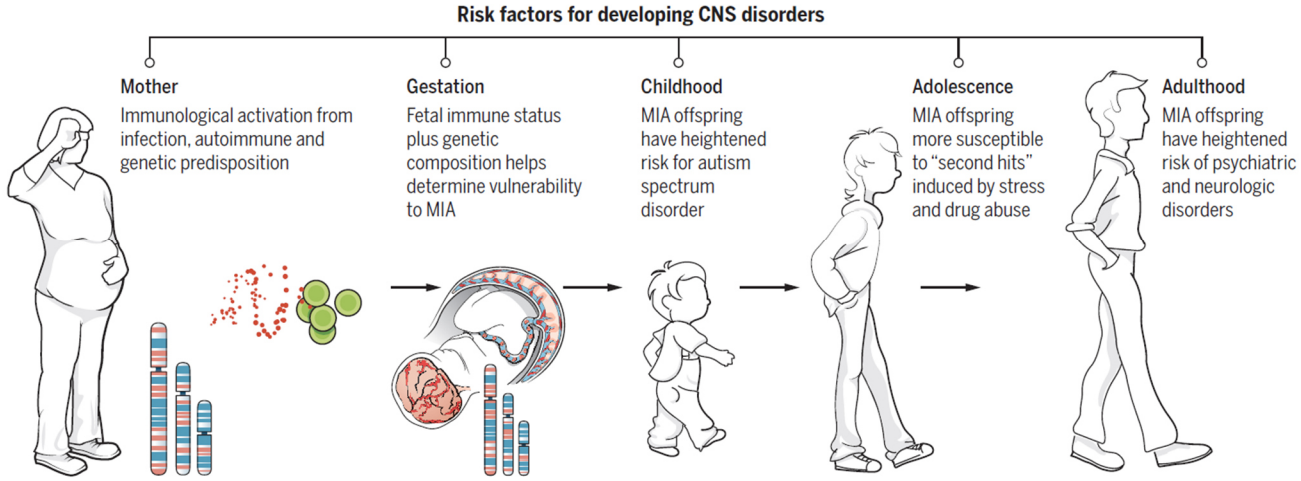


Figure 1. The current model for how MIA leads to neurodevelopmental disorders in offspring. Maternal infections lead to the release of proinflammatory cytokines. A combination of genetic background, autoimmune status and second hit during childhood and adolescence (e.g. stress and drug abuse) increases the risks to develop psychiatric disorders in the adult offspring. (Adapted from Estes & McAllister, 2016).

Thus, investigating the incidence and the prevalence of numerous factors in humans is challenging, but, at the same time, it is imperative to study these aspects in animal models to better clarify the effects of exposure to MIA during pregnancy to assess the neurodevelopmental trajectories in the offspring. Animal models based on MIA have shown that perinatal insults could lead to detrimental effects in the descendants (U. Meyer et al., 2011; U. Meyer & Feldon, 2009a; Zuckerman et al., 2003). These outcomes are similar in diverse mammalian species (e.g. mice, rats, nonhuman primates)

(Bauman et al., 2019; Gumusoglu & Stevens, 2019). Pioneering studies have used prenatal exposure to live pathogens, such as the influenza virus (Cotter et al., 1995; Fatemi et al., 1998; Shi et al., 2003). Models that use live pathogens are helpful for assessing a causal relationship between the pathogen and the outcomes in the offspring but require technical cautions. Another animal model of MIA is based on immune-activating agents that stimulate the innate immune system. One of the most characterized models is based on the exposure during pregnancy to the polyriboinosinic-polyribocytidylic acid (poly (I:C)), a double-stranded synthetic RNA, that triggers an innate immune response by mimicking a viral infection, inducing a schizophrenia-like phenotype in the offspring (U. Meyer et al., 2006; Talukdar et al., 2020; Zuckerman et al., 2003). Poly (I:C) binds to transmembrane protein toll-like receptor 3 (TLR-3) which activation induces a viral-like acute inflammatory state, leading to the synthesis of some proinflammatory cytokines, such as IL-1, IL-6, TNFα and IFNs (Engel et al., 2011). Indeed, MIA offspring showed a plethora of behavioral impairments, such as deficits in sensorimotor gating, social interaction, working memory, behavioral despair (Ding et al., 2019; Kreitz et al., 2020a; Reisinger et al., 2016). Besides, in MIA progeny, key brain regions in the pathophysiology of schizophrenia, such as the ventral tegmental area (VTA), prefrontal cortex (PFC), nucleus accumbens (NAc), and hippocampus showed marked alterations (Ito et al., 2010; U. Meyer & Feldon, 2009b; Vuillermot et al., 2012). Moreover, in utero insults in rodents lead to abnormal neurodevelopmental trajectories interfering with postnatal brain maturation, brain anatomical changes, which might ultimately cause behavioral abnormalities (Kreitz et al., 2020; Piontkewitz et al., 2011). Notably, an impaired dopamine system is considered a hallmark in schizophrenia (Abi-Dargham et al., 2000; Weinstein et al., 2017). Several studies found elevated dopamine levels in some brain regions, but lower or not changed levels were detected in other regions in MIA offspring (Abazyan et al., 2010; Giovanoli & Meyer, 2013; Kirsten et al., 2010). In our previous studies, we reported, in males but not females, behavioral deficits in sensorimotor gating, social interaction, and memory as well as alterations in the mesocorticolimbic pathway. Consistently, we reported a marked change of VTA dopamine neuron activity and a higher release of dopamine in the NAc (Luchicchi et

al., 2016). Moreover, in the two-hit hypothesis of schizophrenia, it has been postulated that MIA could act as a prime and, later on in life, a second hit (e.g. stress or drugs of abuse) could exacerbate latent abnormalities (Giovanoli et al., 2013). Thus, our group previously tested whether a second hit during adolescence could exacerbate the dysfunctions observed in adult offspring. For this purpose, we treated adolescent rats with Δ9-Tetrahydrocannabinol (THC), the main psychoactive compound in cannabis (Lecca et al., 2019). Unexpectedly, THC treatment did not exacerbate the dysfunctions observed in adult rats but attenuated them. These results guided us to focus our attention on the complex interplay between the endocannabinoid system and the dopamine system and their entangled neurodevelopmental trajectories in a MIA model.

1.2 Endocannabinoid system

The endocannabinoid system comprises a family of lipid molecules, receptors, and enzymes whose role in the central and peripheral nervous systems has been studied in the last 30 years. Furthermore, the discovery of the two principal endocannabinoids such as arachidonoylethanolamide (anandamide, AEA (Devane et al., 1992) and 2-arachidonoylglycerol (2-AG; Mechoulam et al., 1995; Sugiura & Waku, 2000) has provided the basis to better elucidate the localization, the structure, and functionality of the endocannabinoid system. Their discovery is associated with the identification and functional expression of cannabinoid receptors such as the cannabinoid receptor 1 (CB1-R) (Matsuda et al., 1990) and the cannabinoid receptor 2 (CB2-R) (Munro et al., 1993). AEA and 2-AG are derived from arachidonic acid (AA)-containing membrane phospholipids and include in their molecule an ethanolamine or a glycerol moiety, respectively (Di Marzo, 2011; Luchicchi & Pistis, 2012; H. C. Meyer et al., 2018). Despite their structural resemblance, they display some distinctions concerning their receptor affinity, biosynthetic and breakdown pathways. Although AEA and 2-AG both bind to two inhibitory G-protein-coupled receptors (GPCRs), such as CB1-R and CB2-R, they do not have

the same affinity. Moreover, AEA can be synthesized via a specific N-acyl phosphatidylethanolamine phospholipase D (NAPE-PLD)-dependent mechanism. On the other hand, 2-AG mainly emerges from inositol phospholipids via a combined interplay between diacylglycerol-lipase (DAGL), and phospholipase C (PLC) (Di Marzo, 2011; Piomelli, 2003). Despite several potential pathways exist for the formation of the direct biosynthetic precursors for 2-AG, they are mainly produced by PLCβ acting on membrane phosphatidylinositols, and then converted to 2-AG by the action of either of two isoforms of the same enzyme, the sn-1-diacylglycerol lipases α and β (DAGLα and DAGLβ). In addition, endocannabinoid signaling is highly regulated by complex machinery that includes metabolic enzymes such as fatty acid amide hydrolase (FAAH) and monoacylglyceride lipase (MAGL) which mainly hydrolyze AEA and 2-AG, respectively. Besides MAGL, 2-AG can be hydrolyzed by two different serine hydrolase α-β-hydrolase domain 6 or 12 (ABHD6, ABHD12). AEA and 2-AG catabolism might take place from the activity of other enzymes (e.g., NAAA, COX-2, and several LOX isoenzymes) (Luchicchi & Pistis, 2012).

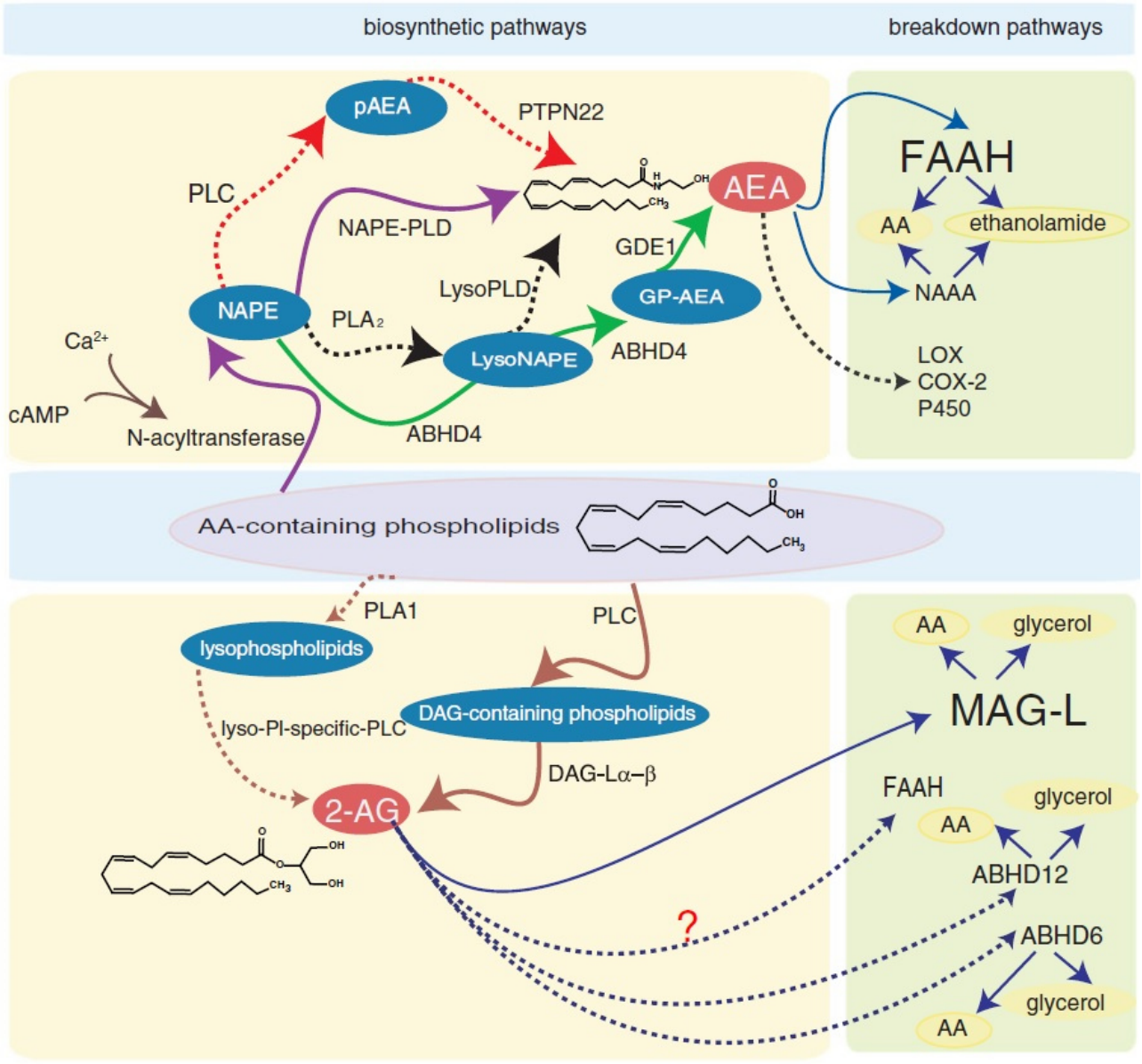


Figure 2. Schematic depiction showing the main pathways involved in AEA and 2-AG formation and catabolism (Adapted from Luchicchi and Pistis et al., 2012).

Thus, AEA and 2-AG are synthesized 'on demand' and travel in a retrograde fashion across the synaptic cleft to bind to endocannabinoid receptors at the presynapse and modulate the release of other neurotransmitters (e.g. glutamate, GABA, dopamine, serotonin, and acetylcholine) (Katona & Freund, 2008; Lovinger, 2008; Piomelli, 2003). "On-demand" synthesis can modulate synaptic transmission in different ways (Castillo et al., 2012; Kano et al., 2009). Among the diverse types of

synaptic plasticity, the first that was demonstrated to be mediated by the endocannabinoid system was depolarization-induced suppression of inhibition (DSI) (Diana & Marty, 2004; Pitler & Alger, 1994). DSI is a short-term form of synaptic plasticity whereby intense depolarization (e.g., repeated action potentials or a 1–5 s step depolarization to 0 mV) of a postsynaptic neuron leads to temporary suppression of inhibitory transmission onto that neuron(Pitler & Alger, 1992, 1994). Postsynaptic depolarization leads to an increase of intracellular calcium ($[Ca^{2+}]_i$) by activating calcium channels, thereby stimulating the release of endocannabinoids. The released endocannabinoids then activate the CB1-Rs at presynaptic terminals and suppress the release of GABA. The equivalent form of short-term endocannabinoid-mediated plasticity involving excitatory transmission is called depolarization-induced suppression of excitation (DSE) (Kreitzer and Regehr 2001). These forms of short-term synaptic plasticity require CB1-Rs involvement, whereas CB2-Rs have not been observed to participate, even if they seem capable of supporting these short-term forms synaptic plasticityif they are appropriately expressed in neurons (Atwood et al., 2012). Both DSI and DSE are concluded when 2-AG is degraded, either by MAGL and/or cyclooxygenase. (Kim & Alger, 2004; Straiker et al., 2009). Our group previously demonstrated a fundamental role of 2-AG in the regulation of both excitatory and inhibitory transmission to dopamine neurons in the VTA (Melis et al., 2004; Miriam Melis et al., 2013, 2014). These results point to both 2-AG and CB1-Rs as one of the main characters in synaptic plasticity in the mesolimbic dopamine system. Indeed, the endocannabinoid system regulates the development of dopamine system, the differentiation of GABA interneurons, the control of short-and long-term plasticity, and the processes that regulate synaptogenesis and neural pruning (Basavarajappa et al., 2009; Kreitzer & Regehr, 2001).

1.3 Role of the endocannabinoid system in neurodevelopment

The endocannabinoid system is central in neurodevelopment, due to its role from the earliest phase of gestation, in the uterus, in the placenta, and the fetal brain (Harkany et al., 2007, 2008). Since postnatal day (PND) 10, the endocannabinoid system modulates synaptic transmission (H. C. Meyer et al., 2018). Endocannabinoids levels, receptors and associated enzyme machinery fluctuate across development. CB1-Rs reach their higher levels around PND 30, at the onset of adolescence, especially in the PFC and striatum. Their levels start declining around PND 70, at the beginning of adulthood (de Fonseca et al., 1994; Klugmann et al., 2011). 2-AG levels are high at birth and may oscillate until adolescence, but with a net decrease during adolescence. AEA gradually increases until adolescence, reaching its peak between adolescence and adulthood, remaining consistent in several corticolimbic regions such as hypothalamus, hippocampus, amygdala and PFC (H. C. Meyer et al., 2018). On the other hand, FAAH levels oscillates with an opposite trend compared to AEA. Similarly, both endocannabinoids' ligands and CB1-R have been observed in white matter areas during the prenatal life. Less is known about the trajectory of MAGL expression, but in humans would seem to decrease from the first year of life with a more pronounced decline at the beginning of adolescence (Long et al., 2012) (**Figure 3**).

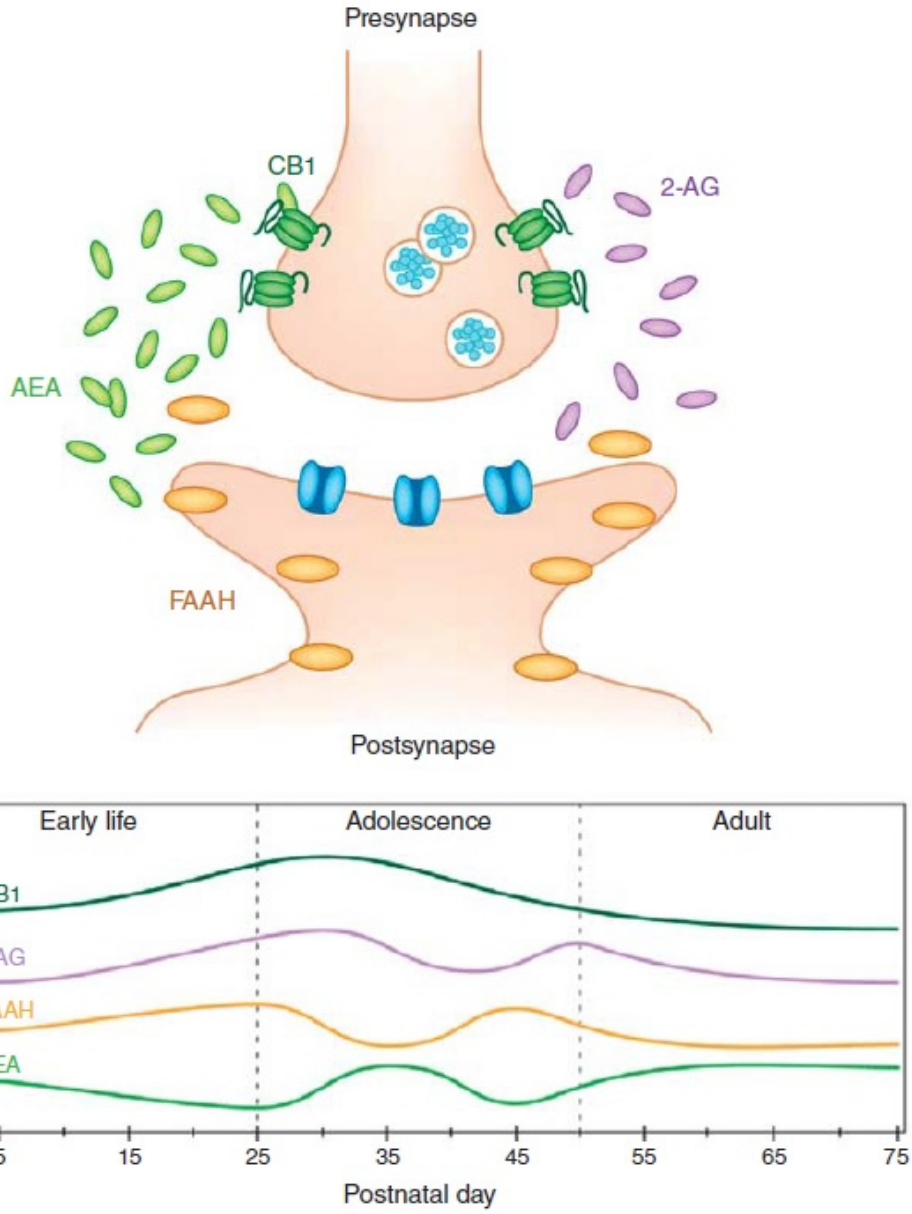


Figure 3. Corticolimbic endocannabinoid signaling changes dynamically across rodent development (Adapted from Meyer et al., 2018).

Thus, a plausible explanation for the developmental changes emerging in the endocannabinoid system during pubertal maturation could be ascribed to the relationship between the endocannabinoid system and gonadal hormones (de Fonseca et al., 1994) that may contribute to the developmental changes emerging during the pubertal period.

Nevertheless, the fragile balancing role of physiological processes that the endocannabinoid system contributes to maintain across all the developmental stages can be altered by several environmental insults (e.g. social stress, maternal infections, drugs of abuse) that differ by their nature and the stage of development at which they occur.

1.4 Role of the endocannabinoid system in neurodevelopmental disorders

Accumulating evidence suggests that disrupting the endocannabinoid system in critical periods of neurodevelopment may lead to aberrant developmental trajectories eventually leading to neurodevelopmental disorders (Galve-Roperh et al., 2009; Viveros et al., 2012; Zamberletti & Rubino, 2021). Indeed, the endocannabinoid system is also crucially involved in fetal neurodevelopment, including synaptic plasticity, neuronal cell proliferation and differentiation (Harkany et al., 2007, 2008). Several meta-analyses and systematic reviews have pointed out that cannabis use during pregnancy leads to postnatal neurodevelopmental disorders in the offspring, however the outcomes are confounded by sociodemographic factors and the fact that users often used other drugs (e.g., tobacco, alcohol) (Connor et al., 2016; Gunn et al., 2016; Metz & Stickrath, 2015). Given that THC crosses the placental barrier from the mother to the fetus, the related fetal developmental processes and their long-term outcomes are potentially vulnerable to impairments in utero Cannabis exposure. Cognitive impairments have also been assessed in animal models of prenatal cannabis exposure. Both adolescent and adult rodents prenatally exposed to either THC or a synthetic CB1-R agonist have been shown to display alterations in learning, long-term memory, sensorimotor gating, attention, and spatial working memory when compared to controls (Beggiato et al., 2020; Campolongo et al., 2007; Frau et al., 2019; Mereu et al., 2003). Thus, exposure to cannabinoids during pregnancy may modify the maturation of neurotransmitter systems and how they govern behavior (Basavarajappa et al., 2009). In fact, adult animals perinatally exposed to THC have shown a plethora of long-term neurobehavioral disturbances induced by perturbations of normal

neurotransmitter development patterns (Basavarajappa et al., 2009). These effects are carried out through the activation of CB1-Rs, which emerge early during neurodevelopment (Berrendero et al., 1999; Fernández-ruiz et al., 2000). The activation of CB1-R interferes with neuronal network oscillations and impairs sensory gating function in the limbic circuitry, further supporting the link between cannabis abuse and increased risks to developing schizophrenia (D'Souza et al., 2005; Semple et al., 2005).

Among the several events in which the endocannabinoid system participates during the prenatal period, the maturation of the population of GABAergic interneurons and the dopaminergic system plays a key role in the emergence of psychosis in adulthood (Zamberletti & Rubino, 2021). Thus, it is plausible that altering the physiological neurodevelopmental trajectories of the endocannabinoid system during the prenatal period could eventually lead to the emergence of neurodevelopmental disorders such as schizophrenia.

1.5 Two-Hit Hypothesis of Schizophrenia: focus on the interplay between MIA, endocannabinoid and dopamine system

In the two-hit hypothesis of schizophrenia, it has been postulated that MIA could act as a prime and, later on in life, a second hit (e.g. stress or drugs of abuse) could exacerbate latent abnormalities (Giovanoli et al., 2013). Clinical and epidemiological studies have identified several risk factors associated with the emergence of schizophrenia. One of the major limitations of clinical and epidemiological studies is that they cannot disentangle the mechanisms involved in the development of schizophrenia. In the case of the two-hit hypothesis of schizophrenia, the vast plethora of multiple factors associated with the emergence of the disorder (e.g., the severity of stress, the timing of exposure, number of stressors) makes the scenario much more complex. Animal studies are fundamental in the investigation of the mechanisms associated with schizophrenia-related disorders

(Brown & Meyer, 2018; U. Meyer et al., 2011). Moreover, experimental variables such as stressful stimuli, their severity and timing, can be more easily handled in rodent models.

Therefore, several two-hit approaches in animal models, using different combinations of insults with different intensities and at different ages (perinatal, adolescence, or adulthood), have been used for research on the etiology of neurodevelopmental disorders (Deslauriers et al., 2013; Gaskin et al., 2014; Giovanoli et al., 2013; Monte et al., 2017). MIA may act as a primer to make the offspring more susceptible to the effect of environmental insults later in life. Thus, given that the endocannabinoid system is involved in fetal neurodevelopment (Harkany et al., 2007, 2008), it is plausible that MIA may impair the correct development of the endocannabinoid system. As described above, one of the fundamental roles of the endocannabinoid system is ascribed to the maturation of the dopamine system. An impaired endocannabinoid system caused by MIA may lead to an aberrant dopamine system maturation that eventually leads to the emergence of schizophrenia-related disorders. Indeed, an impaired dopamine system is considered a hallmark in schizophrenia (Abi-Dargham et al., 2000; Weinstein et al., 2017).

Thus, when we characterized the dopamine system in a MIA model of schizophrenia (Luchicchi et al., 2016), we observed the disruption of the dopamine system in the male offspring, subsequently confirmed in De Felice et al., 2018. The “first hit” due to the poly (I:C) injection that stimulates the Toll Like Receptor (TLR)-pathway modulating the activation of the nuclear factor κB (NFκB) and activator protein 1 (AP-1) through mitogen-activated protein kinases (MAPK) cascade, eventually leading to the synthesis of several proinflammatory cytokines, such as IL-1, IL-6, TNFα, and interferons (Engel et al., 2011), was sufficient to disrupt the dopamine system in the male offspring. Thus, we hypothesized that a “second hit”, a chronic THC treatment during adolescence, could exacerbate the dysfunctions observed in the adult offspring (Lecca et al., 2019). Unexpectedly, THC did not exacerbate the dysfunctions observed in adult animals but attenuated them. A recent study by Stollenwerk & Hillard, 2021 confirmed our previous observations, in which a THC treatment during adolescence failed to potentiate the behavioral effects in adulthood of MIA. These results prompted

us to focus our attention on the complex interplay between the endocannabinoid system and the dopamine system and their entangled neurodevelopmental trajectories in a MIA model. We hypothesize that the "first hit" due to the MIA, may interfere with the correct development of the endocannabinoid system. Thus, impaired developmental trajectories of the endocannabinoid system may eventually influence the physiological maturation of the dopamine system, which is disrupted in adulthood (De Felice et al., 2018; Lecca et al., 2019; Luchicchi et al., 2016).

1.6 AIM

This study aimed to investigate the hypothesis that MIA affects the endocannabinoid system and the endocannabinoid-mediated modulation of dopamine functions. Rats were tested during adolescence to assess (i) the behavioral endophenoype evaluated by locomotor activity in response to THC and prepulse inhibition of startle reflex, (ii) the properties of ventral tegmental area (VTA) dopamine neurons in vivo and their response to cumulative doses of THC, (iii) the endocannabinoid-mediated synaptic plasticity in VTA dopamine neurons, (iiii) the expression of cannabinoid receptors and enzymes involved in endocannabinoid synthesis and catabolism in mesolimbic structures.

2. METHODS

2.1 Animals

Female Sprague Dawley rats (Envigo, Italy) were mated at the age of 3 months. Pregnant dams on gestation day (GD) 15 were randomly assigned to receiving either a single injection of poly(I:C) or an equivalent volume of endotoxin free saline solution in the lateral vein of the tail. Offspring were weaned and sexed on postnatal day (PND) 21. After weaning, offspring were housed with littermates and maintained undisturbed until experiments. Subsequently, male rats were randomly assigned to

the experimental procedures and care was taken to avoid assigning more than three animals from the same litter to the same experimental group. In fact, for the experiments described here, a total of 21 dams were utilized (11 were treated with vehicle and 10 with poly I:C). Each rat underwent only one experimental procedure, with the exception of those tested for spontaneuous locotomotor activity and prepulse inhibition.

Ex vivo electrophysiological experiments were carried out before weaning (PND 12-20) with the aim to detect early abnormalities in dopamine neuron activity and synaptic properties. In vivo electrophysiological recordings were performed at the beginning of adolescence (PND 34-36). Brains and tissues for the analysis of cannabinoid receptors and enzyme levels were collected at PND 34-36.

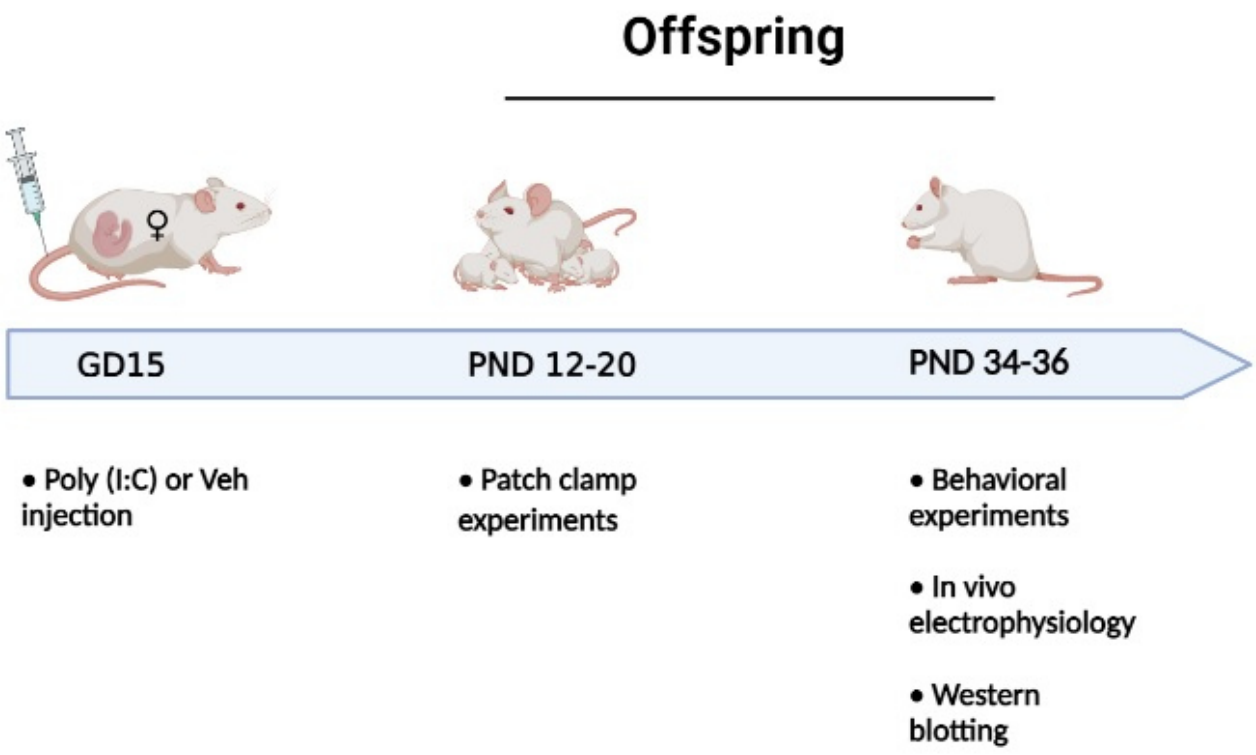


Figure 4. Representation of the experimental protocol. Poly (I:C) treatment during pregnancy consisted of a single i.v. injection of poly (I:C) (4 mg/kg) or vehicle (sterile pyrogen-free saline) at GD15. Ex vivo electrophysiology recordings were performed between PND 12 and 20. Behavioral experiments, in vivo electrophysiology recordings and biochemical studies were performed between PND 34 and 36.

2.2 Drugs and Treatments

Poly (I:C) was purchased from InvivoGen. Poly (I:C) was dissolved in endotoxin-free saline solution, 4.0 mg/kg, i.v poly (I:C) was injected in the lateral vein of the tail of pregnant dams. To assess the efficacy of poly I:C injection, all pregnant rats were weighed for the first 3 days after the administration of either poly I:C or saline to evaluate weight loss as underlined by previous investigations. (Zuckerman et al., 2003). THC (THC-PharmGmbH) or vehicle (1% ethanol, 2% Tween 80 and saline) were injected in control and poly (I:C) for locomotor (1.25 mg/kg ; 2.5 mg/kg, s.c.), and electrophysiological (cumulative 2.4 mg/kg i.v) in vivo experiments. WIN55,212-2 and JZL184 were dissolved in DMSO. The final concentration of DMSO was <0.01 %.

2.3 Behavioral Tests

Testing started on PND 35, with a recovery period of 2 days between each testing condition in the experiment.

Locomotor Activity

The open field test was performed to measure spontaneous locomotor activity. Rats were recorded by a videocamera to track the animal position and movements, and data were analyzed by Anymaze behavioral tracking software. The rats were individually placed in the center of a squared arena (100 × 100 cm), and spontaneous motor activity was monitored for 10 min (test period). Total distance travelled (m), number of entries into the center zone, and time spent in the center zone of the arena were scored. On PND35, the rats were placed in the open field for 30 min of habituation, after which

they were challenged with different doses of THC (1.25 mg/kg ; 2.5 mg/kg, s.c.), and locomotor activity was monitored for 60 min.

Prepulse inhibition (PPI)

On the day of the experiment, rats were placed for a 2 h period of acclimatization into the experimental room. The startle reflex system consisted of four standard cages, each placed inside a sound-attenuated and ventilated chamber (Med Associated, USA). Startle cages were non-restrictive Plexiglas cylinders (diameter 9 cm) mounted on a piezoelectric accelerometer platform connected to an analogue–digital converter. Background noise and acoustic bursts were conveyed through two speakers placed in proximity to the startle cage to produce a variation in sound intensity within 1 dB. On the test day, each rat was placed in the experimental cage for a 5-min acclimation period with a 70-dB white noise background; this was continued for the remainder of the session. Animals were then tested on three consecutive trial blocks. The first and the third blocks consisted of five pulse-alone trials of 40 ms at 115 dB, while the second block (test block) was a pseudorandom sequence of 50 trials including 12 pulse-alone trials, 30 pulse trials preceded by 73, 76, or 82 dB prepulses (10 for each level of prepulse loudness), and eight no-stimulus trials (where the only background noise was delivered). The percentage of PPI was calculated based only on the values relative to the second block and using the following formula: $100 - [(\text{mean startle amplitude for prepulse} + \text{pulse trials/mean startle amplitude for pulse-alone trials}) \times 100]$ (Scherma et al., 2016).

2.4 *In Vivo* Electrophysiology

Rats were anaesthetized with urethane 1.3 g/kg, IP. Rats were placed in a stereotaxic apparatus (Kopf, Tujunga, CA, USA) with their body temperature maintained at 37 ± 1°C by a heating pad. The recording electrode was placed above the VTA (5.6–6.2 posterior to bregma, 0.4–0.8 mm lateral to

midline, 7.0–8.0 mm from cortical surface), according to the stereotaxic rat brain atlas of Paxinos and Watson (Paxinos and Watson, 2007). Single unit activity was recorded extracellularly (bandpass filter 0.1–10,000 Hz) with glass micropipettes filled with 2% Pontamine sky blue dissolved in 0.5 M sodium acetate. Individual action potentials were isolated and amplified through a window discriminator (Neurolog System, Digitimer, Hertfordshire, UK) and displayed on a digital storage oscilloscope (TDS 3012, Tektronics, Marlow, UK). Experiments were sampled online and offline with Spike2 software by a computer connected to CED1401 interface (Cambridge Electronic Design, Cambridge, UK). Dopamine neurons were isolated and identified according to previously described electrophysiological characteristics (Grace & Bunney, 1984; McCutcheon et al., 2012; Ungless & Grace, 2012). VTA dopamine neurons were recorded only when criteria for identification were fulfilled (firing rate 0.5- 10 Hz, duration of action potential ≥ 2.5 ms). Bursts were defined as the occurrence of two spikes at interspike interval < 80 ms and terminated when the interspike interval exceeded 160 ms.

2.5 *Ex Vivo* Electrophysiology

The preparation of VTA slices was as described previously (Melis, 2004; Melis et al., 2013). Briefly, male Sprague Dawley rats (PND 12–30; Envigo), were anesthetized with isoflurane until loss of righting reflex and decapitated. Then, a block of tissue containing the midbrain was rapidly dissected and sliced in the horizontal plane (300 μm) with a vibratome (Leica) in ice-cold (4–6°C) low-Ca^{2+} solution containing the following (in mM): 126 NaCl, 1.6 KCl, 1.2 NaH_2PO_4, 1.2 $MgCl_2$, 0.625 $CaCl_2$, 18 $NaHCO_3$, and 11 glucose (300-306 mOsm). Immediately after cutting, slices were transferred to a holding chamber with artificial cerebrospinal fluid (aCSF) (37°C) and saturated with 95% O_2 and 5% CO_2 containing the following (in mM): 126 NaCl, 1.6 KCl, 1.2 NaH_2PO_4, 1.2 $MgCl_2$, 2.4 $CaCl_2$, 18 $NaHCO_3$, and 11 glucose (300-306 mOsm). Slices were allowed to recover for at least 1 h before being placed, as hemislices, in the recording chamber and superfused with the (aCSF)

(34−36°C) saturated with 95% O_2 and 5% CO_2. Cells were visualized with an upright microscope with infrared illumination (Axioskop FS 2 plus; Zeiss), and whole-cell patch-clamp recordings were made by using an Axopatch 200B amplifier (Molecular Devices). Voltage-clamp recordings were made with electrodes filled with a solution containing the following (in mM): 144 KCl, 10 HEPES, 3.45 BAPTA, 1 $CaCl_2$, 2.5 Mg_2ATP, and 0.25 Mg_2GTP, pH 7.2–7.4, 275–285 mOsm. All GABAA IPSCs were recorded in the presence of, 6-cyano-2,3-dihydroxy-7- nitro-quinoxaline (10 μM) and 2-amino-5-phosphonopentanoic acid (AP5; 100 μM) to block AMPA and NMDA -receptors-mediated synaptic currents, respectively. Experiments were begun only after series resistance had stabilized (typically 10–30 MΩ). Series and input resistance were monitored continuously on-line with a 5 mV depolarizing step (25 ms). Data were filtered at 2 kHz, digitized at 10 kHz, and collected on-line with acquisition software (pClamp 10; Molecular Devices). DA neurons from the lateral portion of the posterior VTA were identified according to the already published criteria (Melis et al., 2013b): cell morphology and anatomical location (i.e., medial to the medial terminal nucleus of the accessory optic tract), slow pacemaker-like firing rate (<5 Hz), long action potential duration (>2 ms), and the presence of a large hyperpolarization activated current (I_h) (>100 pA; Johnson and North, 1992), which was assayed immediately after break-in, using 13 incremental 10 mV hyperpolarizing steps (250 ms) from a holding potential of −70 mV. A bipolar, stainless steel stimulating electrode (FHC) was placed ∼100-200 μm rostral to the recording electrode and was used to stimulate at a frequency of 0.1 Hz. Paired stimuli were given with an interstimulus interval of 50 ms, and the ratio between the second and the first IPSCs (IPSC2/IPSC1) was calculated and averaged for a 5 min baseline (Melis et al., 2002). The depolarizing pulse used to evoke depolarization-induced suppression of inhibition (DSI; Pitler & Alger, 1994) was a 500 ms to 5 s step to +40 mV from holding potential (Melis et al., 2004a). This protocol was chosen on the evidence of an endocannabinoid tone when DA cells are held at +40 mV (M. Melis, 2004). The magnitude of DSI was measured as a percentage of the mean amplitude of consecutive IPSCs after depolarization (acquired between 5 and 15 s after the end of the pulse) relative to that of 5 IPSCs before the depolarization. Bath application of WIN was

performed as follows: WIN was applied for 5 min at the lowest concentration, then, another 5 min with the next increasing WIN concentration. The effect of WIN on GABAA IPSCs was taken at the 5th minute of bath application and normalized to the baseline (5 min before drug application). We chose this protocol because it has been shown that at physiological temperatures WIN-induced effects on GABAA IPSCs recorded from VTA DA neurons reached their peak at this time. The effect of JZL (100 nM) on GABAA IPSCs was taken after 5 min bath application (Lecca et al., 2012). Each hemislice received only a single drug exposure. All drugs were dissolved in DMSO when it was needed. The final concentration of DMSO was <0.01 %.

2.6 Biochemical studies: Western Blotting

For Western blot analysis, nucleus accumbens and midbrain were dissected, frozen in liquid nitrogen and stored at −80◦C; for immunohistochemistry, the brains were quickly removed and post-fixed in 4% paraformaldehyde in 100 mM phosphate buffer pH7.4, stored in fixative for 48 h, kept in 30% sucrose for 24 h. Coronal sections were serially collected using a Leica cryostat CM1510 set to 40 µm thickness and a −20◦C chamber temperature.

Cytosolic fractions from rat nucleus accumbens and midbrain were obtained using a protocol published by (Zamberletti et al., 2019). In brief, animals were sacrificed, and cerebral areas quickly dissected. Samples were homogenized by 25 strokes in a glass-glass homogenizer in 0.32 M sucrose solution containing 20 mM HEPES, 1 mM MgCl2, protease inhibition cocktail, and 0.1 mM phenylmethylsulfonyl fluoride (PMSF) (pH 7.4). The homogenized tissue was centrifuged at 500 × g for 2 min. Resultant pellets (P1) were resuspended in 500 µL of a solution containing HEPES 20 mM, MgCl2 1.5 mM, NaCl 420 mM, EDTA 0.2 mM, glycerol 25%, DTT 2 mM, PMSF 2 mM, protease inhibition cocktail and stored as nuclear fraction. The resulting supernatant (S1) was centrifuged at 10,000 × g for 10 min to obtain a fraction containing mitochondria and synaptosome enriched pellets

(P2) and the supernatant (S2) containing soluble proteins. S2 fraction was conserved as cytosolic fraction while the P2 fraction was resuspended in 0.32 M sucrose, layered onto 0.8 M sucrose and centrifuged at 4100 rpm for 15 min in a swinging bucket rotor to obtain crude synaptosome fractions. The protein concentrations were determined according to the Micro-BCA assay kit (Pierce, Rockford, IL, United States). Equal amount of protein lysates from the cytosolic fractions (30 μg) were run on a 10% 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: rabbit polyclonal anti-CB1 (1:1000; Cayman Chemical, United States), rabbit polyclonal anti-CB2 (1:1000; Cayman Chemical, United States),

rabbit polyclonal anti-FAAH (1:1000; Cayman Chemical, United States), rabbit polyclonal anti-MAGL (1:1000; Cayman Chemical, United States), rabbit polyclonal anti-NAPE-PLD (1:3000; Cayman Chemical, United States), goat polyclonal anti-DAGLα (1:1000; AbCam, United Kingdom), rabbit polyclonal anti-GFAP (1:1000; Sigma Aldrich, United States), rabbit polyclonal anti-CD11b (1:1000; Novus Biologicals, United States), rabbit polyclonal anti-TNF-α (1:2000; Millipore, United States). Bound antibodies were detected with horseradish peroxidase (HRP) conjugated secondary anti-rabbit, anti-mouse or antigoat antibodies (1:1000–10000; Santa Cruz Biotechnology, United States) for 1 h at room temperature and visualized using ECL Western Blotting Detection Reagents (Bio-Rad Laboratories, Hercules, CA, United States). For detection of β-actin, the blots were stripped with Restore Western Blot Stripping Buffer (Thermo Scientific, Rockford, IL, United States) and re-blotted with mouse monoclonal anti-β-actin (1:20000; Sigma Aldrich, United States) overnight at 4◦C and visualized as described above. Bands were detected with G-Box (Syngene) instrument.

For densitometry, images were digitally scanned and optical density of the bands was quantified using ImageJ software (NIH, Bethesda, MD, United States) and normalized to controls. To allow comparison between different blots, the density of the bands was expressed as arbitrary units.

2.7 Statistical Analysis

Data normality was preliminarily verified using the goodness of fit tests Kolmogorov-Smirnov tests. Once assessed these ANOVA assumptions, we analyzed the data by one or multiway ANOVAs followed by Tukey's test for post-hoc comparisons, whenever necessary. Chi-square test and t-test were applied when necessary. Significance threshold was set at 0.05.

3. RESULTS

3.1 Effects of THC on Locomotor Activity in MIA adolescent rats

On PND35, the rats were placed in the open field for 30 min of habituation, after which they were challenged with different doses of THC (1.25 mg/kg; 2.5 mg/kg, s.c.), and locomotor activity was monitored for 60 min (**Figure 5A**) For these experiments we used n= 9 control [5 (1.25 mg/kg) + 4 (2.5 mg/kg)]. and n=11 poly (I:C) male offspring [6 (1.25 mg/kg) + 5 (2.5 mg/kg)]. Two-way ANOVA revealed an interaction between treatment with THC and poly (I:C) pretreatment ($F_{(2,24)}$ = 6.11, P= 0.007) and an effect of treatment with THC ($F_{(2,24)}$ = 4.37, P= 0.024). Post hoc analysis (Sidak test multiple comparisons) revealed a difference between poly (I:C) and control group,

revealing that THC 1.25 mg/kg s.c induced hyperlocomotion in poly (I:C) rats compared to control (**Figure 5E**). With regard to spontaneous locomotor activity, there was no significant difference between poly (I:C) and control rats on distance traveled during the session (unpaired Student's t-test: t(14)=1.186, P=0.256) (**Figure 5B**). Moreover, no significant group differences were found on the number of entries into the center zone (**Figure 5C**) as well as on time spent in the center zone (unpaired Student's t-test: t(14)=1.275, P=0.223 and t(14)=1.634, P=0.125, respectively) (**Figure 5D**).

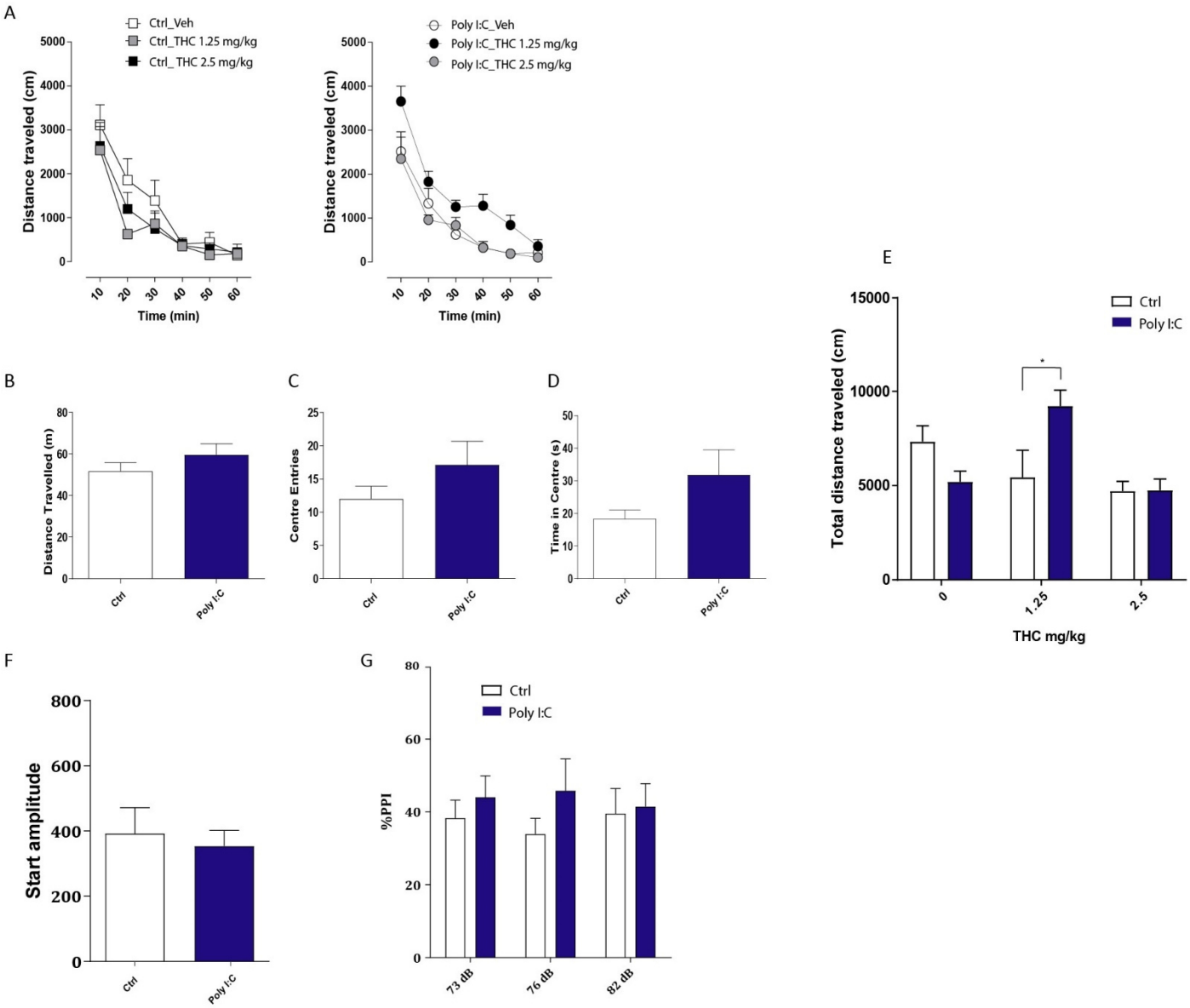


Figure 5. Behavioral phenotypes in adolescent (PND 34-36) male offspring poly (I:C) and control rats. The rats were placed in the open field for 30 min of habituation, after which they were challenged with different doses of vehicle or THC (1.25

mg/kg; 2.5 mg/kg, s.c.), and locomotor activity was monitored for 60 min (A). Bar graphs in (B) shows the spontaneous locomotor activity, described with the distance traveled (m) in the open field . Bar graphs in (C) displays the counts of entries in the center of the open field. Bar graphs in (D) shows the time (seconds) spent in the center of the open field. (D). MIA effects on adolescent male rats in prepulse inhibition (PPI) parameters. Bar graphs in (F) and (G) show the startle amplitude and the % of PPI, respectively. m, metres; s, seconds; dB, decibel. Data are expressed as means ± SEM. *P < .05.

3.2 Effects of MIA on prepulse inhibition of startle reflex in adolescent rats

In our previous studies, we observed that MIA offspring disrupted PPI in adulthood (De Felice et al., 2018; Luchicchi et al., 2016). Given that during adolescence this impairment might be latent (Ding et al., 2019), we investigated whether MIA affected PPI in adolescent male offspring. For this purpose, we utilized n= 8 control and n= 8 poly (I:C) male offspring. Control and poly (I:C) rats did not show differences in acoustic-startle response amplitude (unpaired Student's t-test: $t(14)=0.412$, $P=0.687$) (**Figure 5F**). Moreover, we also found no significant differences between the two groups when analyzing the inhibition (PPI) of startle response at the three different prepulse intensities (two-way ANOVA treatment × prepulse interaction $F_{(2,28)}=1.85$, $P=0.176$) (**Figure 5G**).

3.3 Effects of MIA and THC on dopamine cell activity and functionality *in vivo*

We previously demonstrated that MIA negatively affects electrophysiological properties of VTA dopamine neurons in adult male offspring, but not females (De Felice et al., 2018; Luchicchi et al., 2016). Given that dopamine neurons in the VTA fire faster in adolescent compared to adult rats (McCutcheon et al., 2012), we assessed whether MIA may impact VTA DA neurons activity during adolescence. For this purpose, we performed in-vivo single-unit extracellular recordings of VTA dopamine neurons from adolescent rats under urethane anesthesia. In these experiments, we utilized n= 7 control and n= 8 poly (I:C) male offspring. Analysis of the number of cells/track (**Figure 6D**),

which is an index that represents the spontaneous population activity of dopamine neurons, did not reveal any difference (1.39 ± 0.07 vs 1.61 ± 0.31, control vs poly I:C; P >0.05; Student's t test). Dopamine neurons recorded from control rats fired at 4.55 ± 0.17 Hz (n=78), whereas poly I:C rats showed a firing frequency of 4.52 ± 0.20 Hz (n=93); (P> 0.05. Student's t test) (**Figure 6E**). Moreover, dopamine neurons recorded from poly (I:C) presented a higher percentage of spikes in burst compared to controls (20.81 ± 2.65 %, n=93 vs 13.47 ± 1.91 %, n=78; (t=2.25), P= 0.013. Student's t test, Welch Correction); (**Figure 6F**). Consistently, autocorrelogram analysis corroborated that poly I:C increases the percentage of dopamine cells showing bursting activity (37% poly I:C vs 12% control), while decreasing the percentage of regularly-firing cells (29% poy I:C vs 39% control). (p = 0.001, Chi-Square test; **Figure 6G**). These results suggest that adolescent poly (I:C) rats could present an imbalance between inhibitory and excitatory afferents that fine-tune dopamine neurons' bursting activity. We next determined whether this imbalance could influence the response of VTA dopamine neurons to cumulative doses of THC. For these experiments we utilized n= 6 control and n= 7 poly I:C male offspring. Two-way ANOVA analysis revealed an interaction between factors (poly (I:C)-pretreatment x THC-treatment) and cumulative doses of THC (from 0.3 to 2.4 mg/kg, i.v.) produced a dose-dependent decrease in the firing rate of VTA dopamine neurons in control rats whereas its effects was absent in poly (I:C) rats (Two-way ANOVA, $F_{(4, 54)} = 2.58$, $P < 0.05$) . Post hoc analysis (Sidak test multiple comparisons) revealed a difference in several points of the dose-response curve (**Figure 6H**). (0.6 mg/kg; 1.2 mg/kg; 2.4 mg/kg).

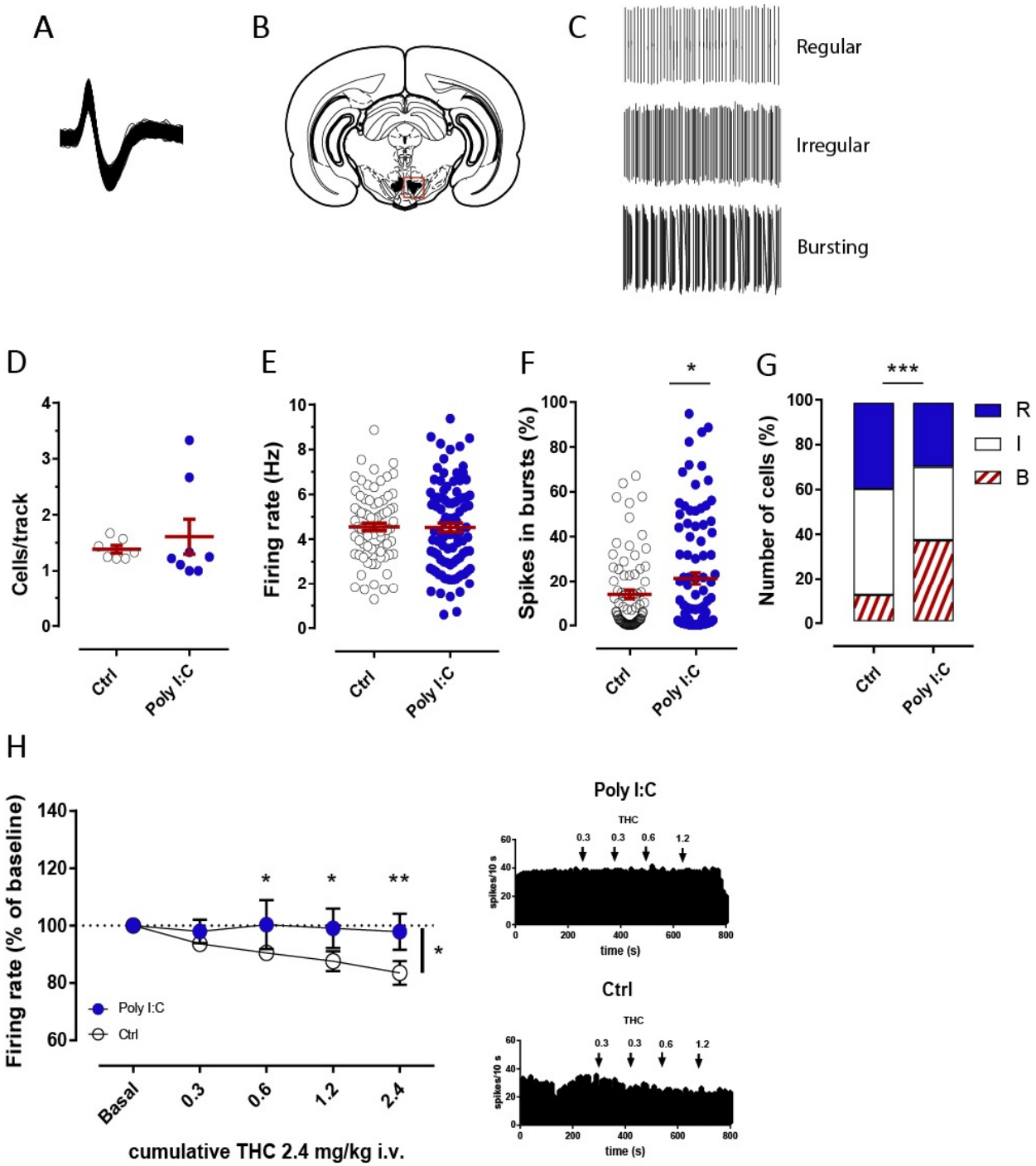


Figure 6. MIA effects on dopamine neuron firing activity in adolescent (PND 34-46) rats. (A) The typical broad spike waveform of a dopamine neuron. (B) Representative localization of recording sites of VTA putative dopamine neurons in red, as verified by histological sections. (C) Autocorrelograms of dopamine neurons were generated from action potential recordings and allowed the classification of their firing pattern into regular (top), irregular (middle), and bursting (bottom) mode. (D) The scatter plot shows the number of spontaneously active dopamine neurons. (E) Scatter plot shows individual dopamine neuron firing rates in Ctrl and Poly (I:C) rats. The scatter plot in (F) displays individual percentages of spikes in bursts between Ctrl and Poly (I:C). Bar graphs in (G) show the percentage of cells that display a different discharge pattern .

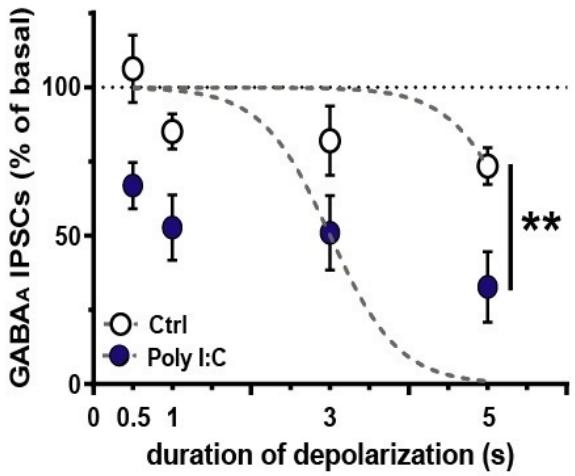
(H) On the left, cumulative THC (2.4 mg/kg i.v.) dose curve response of VTA dopamine neurons in Ctrl and Poly (I:C) adolescent rats., on the right, representative firing rate histograms of VTA dopamine neurons recorded from a Poly (I:C) (top) and Ctrl (bottom) rat. R, regular; I, irregular; B, bursting. Data are expressed as means ± SEM. *P < .05.

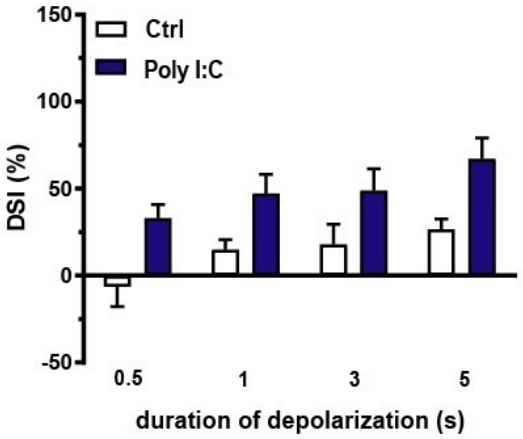
3.4 Effects of MIA on endocannabinoid-mediated transmission *in vitro*

To further investigate the plausible imbalance between excitatory and inhibitory inputs to VTA DA cells observed *in vivo*, we assessed the involvement of eCB signaling in the effects observed in poly (I:C) rats. To this aim, we phasically activated eCB signaling by depolarizing DA cells (from -70 to +40 mV with increasing duration from 0.5 to 5 s) to elicit the endocannabinoid-mediated short-term forms of synaptic plasticity termed depolarization-induced suppression inhibition (DSI) in both poly (I:C) and control groups (Diana & Marty, 2004; Pitler & Alger, 1994). In these experiments we utilized n= 12 control and n= 14 poly (I:C) male offspring. Poly (I:C) offspring displayed a larger DSI than controls (control n = 6 cells; poly I:C n= 8 cells; Two-way ANOVA, $F_{(9, 90)}$=2.17, P=0.03) (**Figure 7A**). The larger DSI expressed by VTA DA cells in poly (I:C) may reflect either (i) higher levels of 2-AG, and or (ii) an increased number or function of CB1-R. MAGL (the principal degrading enzyme for 2-AG) is the rate-limiting step determining the time course of 2-AG- dependent DSI (Blankman et al., 2007; Di Marzo, 2011). Therefore, we next examined the effects of the potent MAGL inhibitor JZL184 on DSI (Long et al., 2009). We found that bath application of JZL184 (100nM) enhanced DSI in controls (**Fig.7B**) (Two-way ANOVA control n= 9 cells; poly (I:C) n= 13 cells) $F_{(3, 60)}$ = 4.65; P< 0.005) (**Figure 7B**). Moreover, after bath application of JZL184, DSI was no longer different among experimental groups (**Figure 7B**). Notably, in the presence of JZL184, DSI was saturated, being its effect independent upon duration of depolarization (**Figure 7B)**. Next, to assess the hypothesis of whether differences in CB1-R number or function might occur at GABAergic synapses arising from rostral afferents onto VTA DA neurons of poly (I:C) rats, we built a dose-

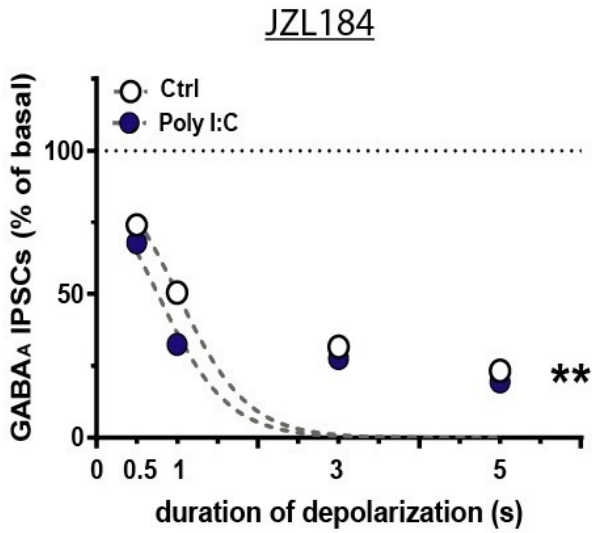
response relationship for CB1-R/CB2-R agonist WIN55,212-2 (WIN;0.01-3 μM, 5 min). No difference were observed among groups (Two-way ANOVA, control n= 7; poly (I:C) n= 8, $F_{(1, 13)}$=0.37, P > 0.05) (**Figure 7C**). Together these findings suggest that the larger DSI observed in poly I:C rats was not due to differences in CB1-R number or function but to a difference in the rate of 2-AG degradation.

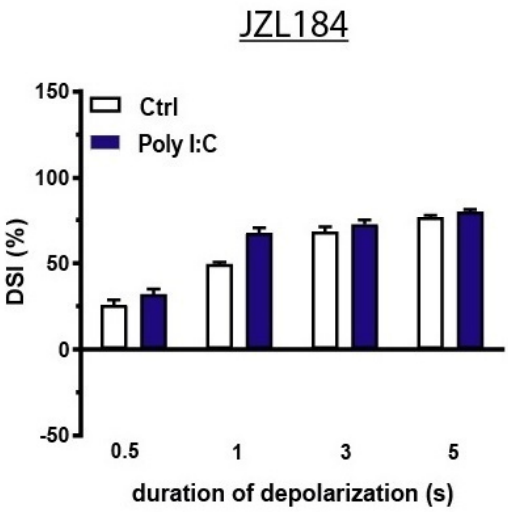
A


GABAA IPSCs (% of basal)
100
50
0
Ctrl
Poly I:C
**
0 0.5 1 3 5
duration of depolarization (s)

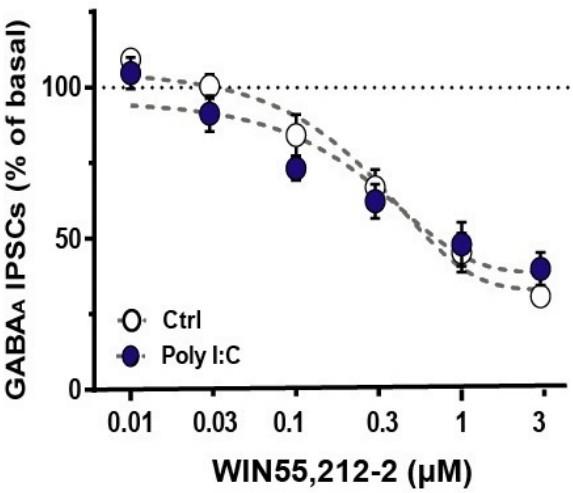

150
100
50
0
-50
DSI (%)
Ctrl
Poly I:C
0.5 1 3 5
duration of depolarization (s)

B


JZL184
GABAA IPSCs (% of basal)
100
50
0
Ctrl
Poly I:C
**
0 0.5 1 3 5
duration of depolarization (s)


JZL184
150
100
50
0
-50
DSI (%)
Ctrl
Poly I:C
0.5 1 3 5
duration of depolarization (s)

C


GABAA IPSCs (% of basal)
100
50
0
Ctrl
Poly I:C
0.01 0.03 0.1 0.3 1 3
WIN55,212-2 (μM)

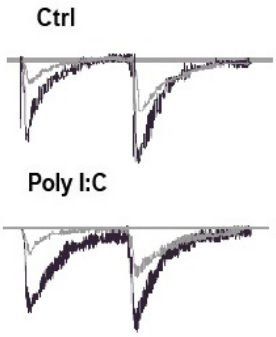

Ctrl
Poly I:C

Figure 7. MIA effects on endocannabinoid-mediated transmission in vitro. (A) On the left, the relationship between the depolarizing pulse duration and the relative amplitude of $GABA_A$ IPSCs obtained after the end of depolarization is plotted. $GABA_A$ IPSC amplitude was normalized to the averaged value (dotted line) before depolarization. Each symbol represents the averaged value obtained from different cells, on the right averaged data for DSI induced by depolarizing pulses with a duration of 0.5, 1, 3, and 5 s are plotted. (B) On the left, the relationship between the depolarizing pulse duration and the relative amplitude of caudal $GABA_A$ IPSCs obtained after 5–15 s after the end of depolarization in the presence of MAGL inhibitor JZL184 in male rats. On the right, bar graphs summarize the enhancing effect of JZL184 on DSI on Ctrl male rats. (C) Dose–response curves for percentage inhibition in amplitude of $GABA_A$ IPSCs by the CB1-R agonist WIN55,212–2 as recorded from VTA DA cells and evoked by stimulating caudal afferents. Data are expressed as means ± SEM. *P < .05.

3.5 Effects of MIA on Components of the Endocannabinoid System in the mesolimbic system

We next assessed the protein levels of components of the endocannabinoid system in key brain regions of the mesolimbic system such as the nucleus accumbens and the midbrain. In these experiments we utilized n= 5 control and n= 5 poly (I:C) male adolescent offspring. With regard to the nucleus accumbens **Figure 8** shows no differences between groups nor in CB1-R (unpaired Student t-test, controls 0.472 ± 0.048, vs poly (I:C) 0.460 ± 0.035; P>0.05) neither in CB2-R expression (controls 0.394 ± 0.045, vs poly (I:C) 0.348 ± 0.068; P>0.05). In accordance with electrophysiological experiments, MIA significantly reduced MAGL expression in poly (I:C) rats compared to controls (controls 0.394 ± 0.037, vs poly (I:C) 0.207 ± 0.038; P=0.004). Interestingly, MIA also reduced NAPE-PLD expression in poly (I:C) rats compared to controls (controls 0.149 ± 0.020, vs poly (I:C) 0.083 ± 0.009; P= 0.009). There was no significant difference between poly (I:C) and control rats nor on FAAH (controls 0.438 ± 0.0749, vs poly (I:C) 0.405 ± 0.060; P= 0.369) neither on DAGLα expression (controls 0.093 ± 0.017, vs poly (I:C) 0.142 ± 0.028, P= 0.090).

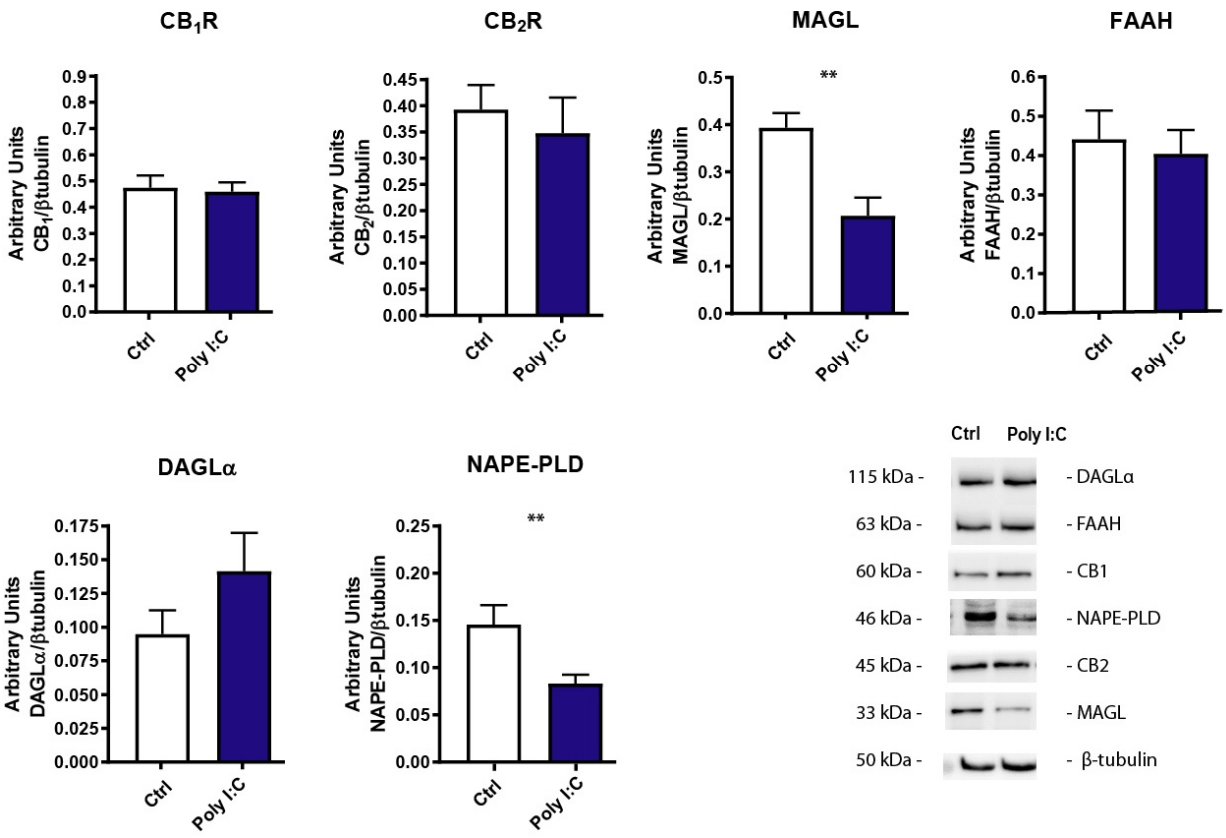


Figure 8. Effect of MIA on components of the endocannabinoid system in the nucleus accumbens of the adolescent (PND 34-36) male offspring rats measured by means of Western blot analysis in cytosolic fractions. Data represent mean ± SEM. *P < .05.

With regard to the midbrain **Figure 9** shows that there was no difference between groups nor in CB1-R (Student t-test, controls 0.349 ± 0.050, vs poly (I:C) 0.328 ± 0.047; P>0.05) receptor neither in CB2-R expression (controls 0.604 ± 0.094, vs poly (I:C); P>0.05). Nevertheless, MIA significantly reduced both MAGL (controls 0.262 ± 0.034, vs poly (I:C) 0.149 ± 0.013; P=0.075) and DAGLα expression (controls 0.212 ± 0.017, vs poly (I:C) 0.169 ± 0.011; P=0.033) in poly (I:C) rats compared to controls. However, MIA did not affect FAAH (controls 0.457 ± 0.051, vs poly (I:C) 0.460 ± 0.012;

P>0.05) neither NAPE-PLD expression (controls 0.740 ± 0.0503, vs poly (I:C) 0.791 ± 0.057: P>0.05) between groups.

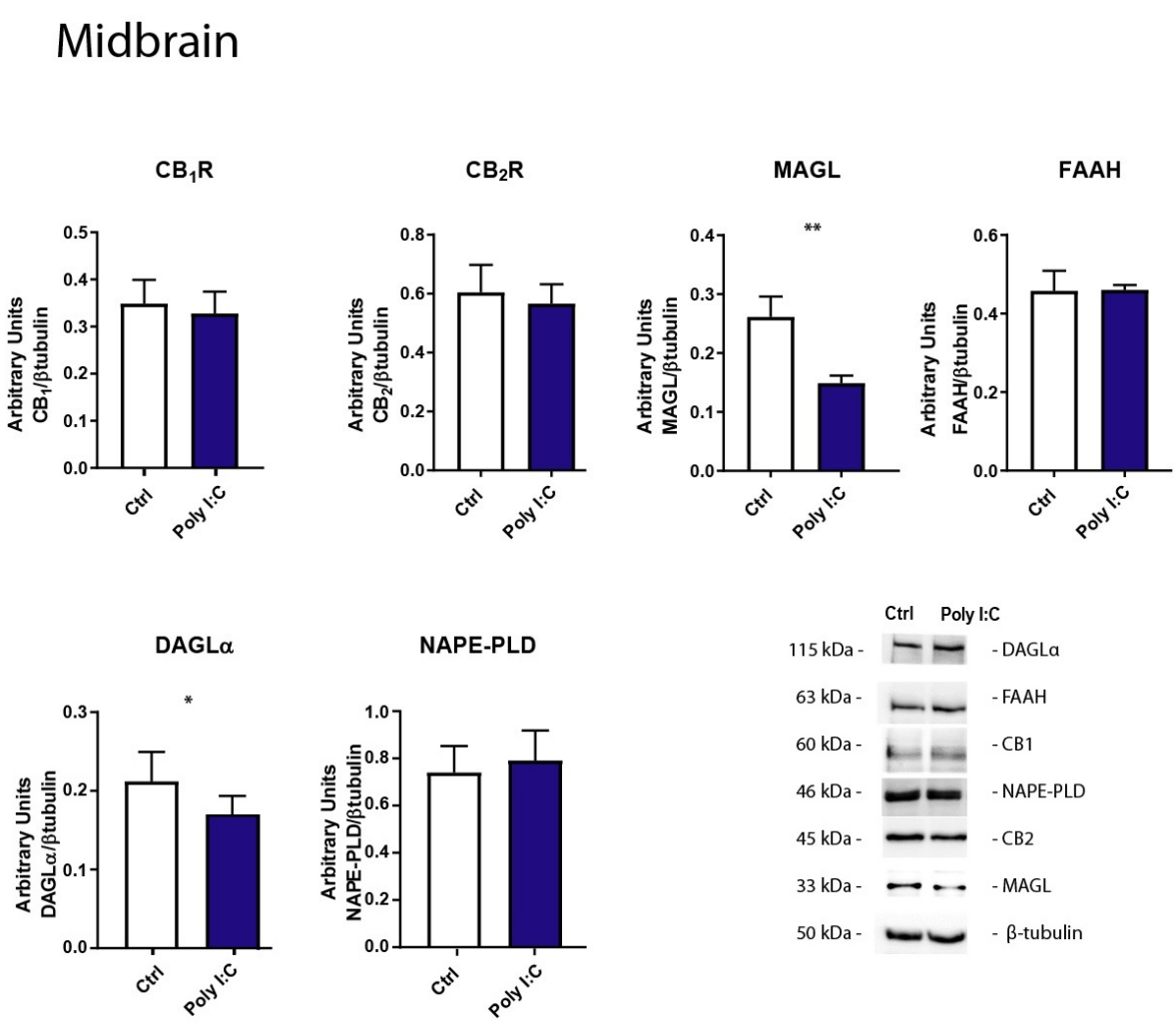


Figure 9. Effect of MIA on components of the endocannabinoid system in the midbrain of the adolescent (PND 34-36) male offspring rats measured by means of Western blot analysis in cytosolic fractions. Data represent mean ± SEM. *P < .05.

4. DISCUSSION

Our results confirm that MIA perturbs the complex interplay between dopamine and endocannabinoid systems during adolescence and might generate a pathological endophenotype that leads to psychotic-like symptoms later in life. When we previously characterized the dopamine system in a MIA model of schizophrenia, we and other groups observed the disruption of the dopamine system in the male adult offspring (De Felice et al., 2018; Luchicchi et al., 2016; Vuillermot et al., 2011; Zuckerman et al., 2003), subsequently maintained across generations (Weber-Stadlbauer et al., 2021; Santoni et al., 2022 in press). Next, we tested whether a THC treatment during adolescence could exacerbate the dysfunctions observed in adult offspring. Unexpectedly, THC did not exacerbate the dysfunctions observed in adult rats but attenuated them (Lecca et al., 2019). A recent study by Stollenwerk & Hillard, 2021 confirmed our previous observations, in which a THC treatment during adolescence failed to potentiate the behavioral effects in adulthood of MIA. Thus, we focused on the complex interplay between the endocannabinoid and the dopamine systems and their entangled neurodevelopmental trajectories in a MIA model. Our hypothesis was that the inflammation caused by MIA might interfere with the proper development of the endocannabinoid system that later leads to an aberrant dopamine system only in adulthood. In line with our hypothesis, the endocannabinoid-mediated control of synaptic transmission is altered by the excess of proinflammatory cytokines (e.g. IL-6, TNFα) with consequent effects in neurodevelopmental disorders (Garay et al., 2013; Rossi et al., 2015). Moreover, a recent systematic review pointed out the critical role of Toll-like receptors (TLR) as a putative pathway linking maternal inflammation during pregnancy and neurodevelopmental disorders in human offspring (Han et al., 2022). Conversely, it has been shown that the synthetic cannabinoid WIN55,212–2 acts on TLR3 and TLR4 signaling by inhibiting the pro-inflammatory signaling axis triggered by TLR3 and TLR4, whereas selectively augmenting

TLR3-induced activation of interferon (IFN) regulatory factor 3 and expression of IFN-β (Downer et al., 2011). These observations strengthen the idea that the poly (I:C) in the MIA model stimulates the TLR-pathway modulating the activation of NFκB and AP-1 through MAPK cascade, eventually leading to the synthesis of several proinflammatory cytokines, such as IL-1, IL-6, TNFα, and interferons (Engel et al., 2011). On the other hand, WIN55,212–2, which acts on TLR3 and TLR4 signaling by inhibiting the pro-inflammatory cascade triggered by the activation of TLR3 (e.g. by poly (I:C)) could partially explain the controversial results obtained by us and Stollenwerk and colleagues. However, the poly (I:C) MIA model does not reproduce the precise immunological scenario that emerges in the human environment following a viral infection. Viral infection models (e.g., influenza virus) would fit a higher level of construct and face validity for the schizophrenia-related phenotype (Shi et al., 2003). To further support the link between inflammation and endocannabinoid system, it should be mentioned the crucial role of CB2-R in neuroinflammation (Rom & Persidsky, 2013). Indeed, it has been shown that CB2-R activation decreased IL-1β, IL-6 levels and inhibited the release of TNFα in microglia (Facchinetti et al., 2003; Ramírez et al., 2005).

In line with our hypothesis, the present findings revealed the persistence of long-term latent modifications on endocannabinoid system in mesolimbic regions of male adolescent rats exposed to MIA. Indeed, in this MIA model, we showed that sensorimotor gating, one of the core behavioral correlates of schizophrenia, is not altered during adolescence (Ding et al., 2019) but only later in life (De Felice et al., 2019; Luchicchi et al., 2016). Consistently, while there was no significant difference between poly (I:C) and control rats on spontaneous locomotor activity, a THC challenge induced hyperlocomotion only in poly (I:C) offspring, suggesting a hidden impairment of the endocannabinoid system that resurfaces only after the THC administration. Accordingly, a similar scenario is shown when considering the VTA dopamine neuron activity. In fact, there are no differences between groups in basal firing frequency or in the cells per track during adolescence, whereas MIA animals displayed a disrupted VTA dopamine neurons activity in adulthood (De Felice et al., 2019; Luchicchi et al., 2016). However, MIA adolescent rats displayed a higher bursting

activity than controls, the opposite of what we observed in adulthood (Luchicchi et al., 2016). Moreover, cumulative doses of THC inhibited VTA dopamine cells in control animals, whereas in MIA offspring, they were unresponsive. This observation is in contrast with the scenario observed in adult control rats in which cannabinoids increase the firing rate of VTA dopamine neurons (French, 1997; Gessa et al., 1998; Wu & French, 2000). There are several possible explanations i) an incomplete maturation of the excitatory and inhibitory inputs to the VTA DA neurons to ii) a different expression of CB1-R between adolescence and adulthood (H. C. Meyer et al., 2018) and to iii) altered 2-AG signaling in the mesolimbic pathway. To explain the lack of response to cumulative doses of THC in MIA rats, we can refer to our previously published data in MIA male rats (De Felice et al., 2019), where we demonstrated that both inhibitory and excitatory afferents to dopamine cells displayed an increased paired-pulse ratio in males (i.e. facilitation) at both types of synapse. This facilitation, together with the reduced frequency of spontaneous miniature of AMPA EPSCs (ie, mEPSCs) and $GABA_A$ IPSCs (ie, mIPSCs), is predictive of a reduced probability of glutamate and GABA release, respectively. Given that the balance between excitatory and inhibitory inputs plays a crucial role in VTA dopamine neuron's activity, any alteration to this balance may affect the response to THC. In line with our hypothesis, MIA offspring displayed a larger 2-AG-mediated DSI than controls. The larger DSI in poly (I:C) rats was not due to differences in CB1-R number or function but to a reduced 2-AG degradation. These results provide further evidence that 2-AG is a key player of DSE/DSI in the VTA (Melis & Pistis, 2007; Melis et al., 2013, 2014). Consistently, poly (I:C) rats showed a decreased level of MAGL, the enzyme that hydrolyzes 2-AG, both in the nucleus accumbens and midbrain. Furthermore, poly (I:C) animals displayed a decreased expression of DAGLα in the midbrain, that could be explained as an adaptation to an excessive 2-AG tone due to reduced MAGL expression both in midbrain and nucleus accumbens. Remarkably, several animal models of psychiatric diseases that use different prenatal insults, showed an altered endocannabinoid-mediated synaptic plasticity, and enhanced 2-AG signaling in mesolimbic structures suggesting a common deranged pathway that eventually leads to a neurodevelopmental impairment (Castelli et al.,

2007; Frau et al., 2019; Melis et al., 2014). Thus, it should be pointed out that 2-AG exerts its anti-inflammatory effects through CB1-R/CB2-R and peroxisome proliferator-activated receptor (PPAR) (Alhouayek et al., 2014). Given that 2-AG has been considered to take the role of 'reservoir' for arachidonic acid production by cells in inflammatory conditions (Alhouayek et al., 2014), we could speculate that enhanced 2-AG levels could be an adaptive process to increased proinflammatory cytokines levels caused by MIA. Consistently, 2-AG inhibits pro-inflammatory cytokine production in lipopolysaccharide-activated cultures of rat microglial cells, murine splenocytes, peritoneal macrophages and exhibits antiproliferative effects toward lymphocytes (Facchinetti et al., 2003; Gallily et al., 2000; Lourbopoulos et al., 2011; Rockwell et al., 2006). However, an imbalance in 2-AG levels leads to an altered endocannabinoid-mediated synaptic plasticity in mesolimbic structures, eventually leading to a disrupted dopamine system in adulthood, resembling a schizophrenia-like endophenotype (**Figure 10**). Furthermore, given that the interplay between mesolimbic structures and the endocannabinoid system is pivotal in addiction, it is surprising that there are very few studies based on maternal infections addressing addiction-like disorders (Aguilar et al., 2018; Gomes et al., 2015; Lecca et al., 2019; Waterhouse et al., 2018). Among the neuropsychiatric disorders linked to MIA, strong epidemiological and experimental evidence indicates that addiction is involved in the etiology of psychiatric diseases with neurodevelopmental components, especially schizophrenia (D'Souza et al., 2005; Van Os et al., 2002). In conclusion the present findings contribute to unveil the neurobiological mechanisms through which inflammation caused by MIA influences the proper development of the endocannabinoid signaling that negatively impacts on dopamine system that eventually leads to psychotic-like symptoms in adulthood.

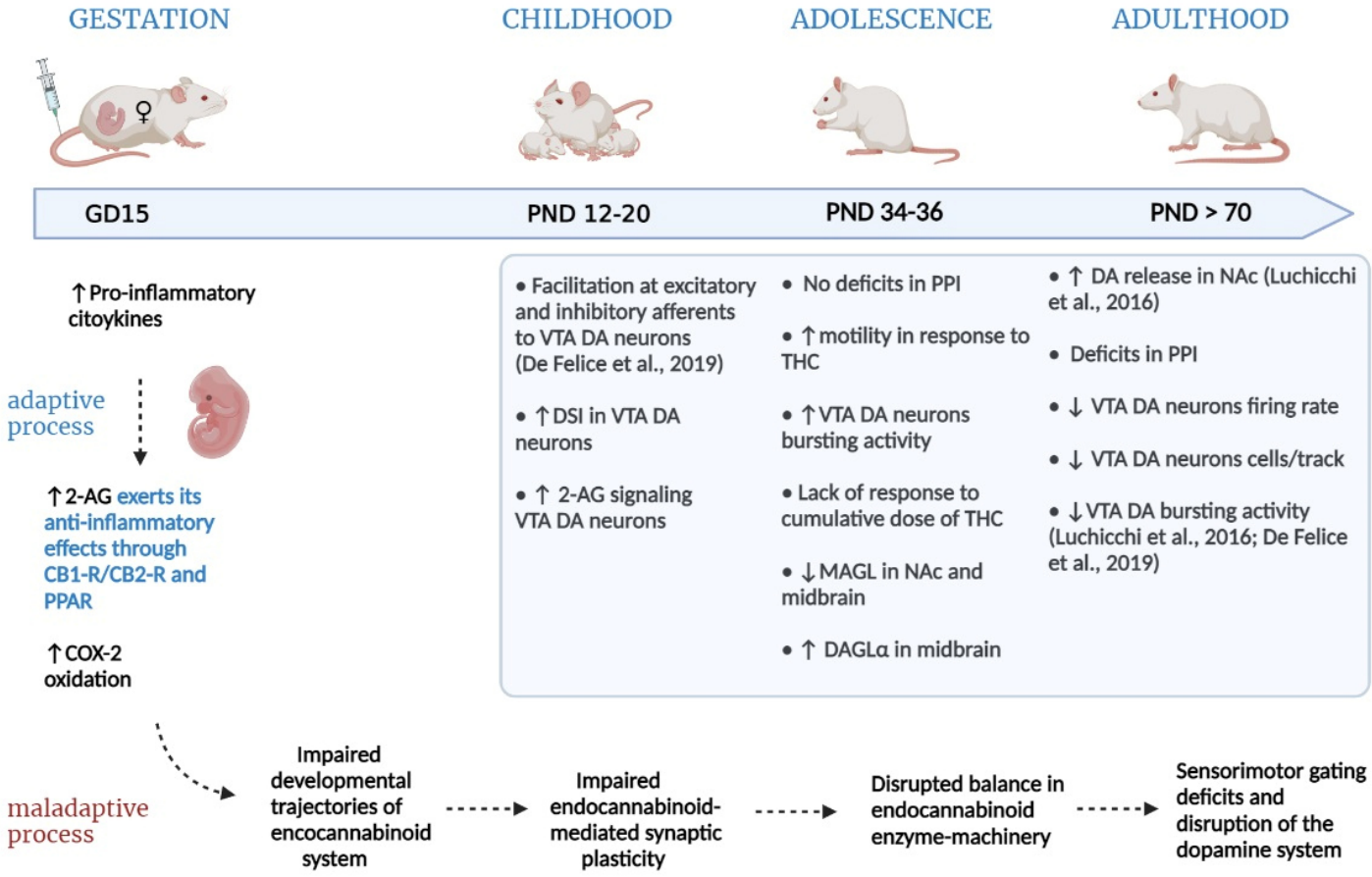


Figure 10. Representation of the hypothesized mechanisms of the neurodevelopmental trajectories in a MIA model of schizophrenia. We hypothesize that enhanced 2-AG signaling could be an adaptive process to increased pro-inflammatory cytokines levels caused by MIA. However, this adaptive process leads to improper developmental trajectories that ultimately lead to an impaired endocannabinoid-mediated synaptic plasticity and a disruption of the dopamine system.

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