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Adolescent cannabinoid exposure increases nigrostriatal dopaminergic transmission

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Por

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RESUMEN (Español e Inglés):

Evidencia sugiere una relación entre el uso de cannabinoides en adolescentes y trastornos psiquiátricos, caracterizados por alteraciones en sistemas dopaminérgicas, como esquizofrenia y adicción a drogas en la adultez. Sin embargo, el impacto de la exposición a cannabinoides en la adolescencia en la vía dopaminérgica nigroestriatal en adultos no está completamente dilucidado. El objetivo es estudiar efecto del agonismo CB1/2 en la adolescencia sobre la dinámica dopaminérgica en el estriadodorsolateral en la adultez. Para esto se administró repetidamente un agonista CB1/2 en ratas adolescentes, se determinó el impacto en la transmisión dopaminérgica nigroestriatal mediante experimentos de microdiálisis y registro electrofisiológico en ratas adultas. Ambas mediciones muestran que la activación repetida de los receptores CB1/2 incrementan el tono dopaminérgico en la vía nigroestriatal. Interesantemente, la activación del pálido ventral (VP) atenuó el dicho incremento en las neuronas dopaminérgicas de la sustancia nigra *par compacta* inducida por la exposición a cannabinoides en adolescentes. Estos resultados indican que la exposición adolescente de cannabinoides produce una desinhibición de la transmisión dopaminérgica nigroestriatal mediada por una disminución del tono gabaérgico del VP. Esta investigación muestra la susceptibilidad de la transmisión dopaminérgica adolescente a estímulos del medio y como pueden moldear los circuitos neuronales en la adultez.

Evidence suggests a relationship between the use of cannabinoids in adolescents and psychiatric disorders, characterized by alterations in dopaminergic systems, such as schizophrenia and drug addiction in adulthood. However, the impact of exposure to cannabinoids in adolescence in the nigrostriatal dopaminergic pathway in adults is not completely elucidated. The objective is to study the effect of CB1/2 agonism in adolescence on dopaminergic dynamics in the dorsolateral striatum in adulthood. For this, a CB1/2 agonist was administered repeatedly in adolescent rats, and the impact on nigrostriatal dopaminergic transmission was determined by microdialysis and electrophysiological recording experiments in adult rats. Both measurements show that repeated activation of CB1/2 receptors increases the dopaminergic tone in the nigrostriatal pathway. Interestingly, the activation of the ventral pallidum (VP) attenuated the increase in the dopaminergic neurons of the substantia nigra *par compacta* induced by exposure to cannabinoids in adolescents. These results indicate that the adolescent exposure of cannabinoids produces a disinhibition of the nigrostriatal dopaminergic transmission mediated by a decrease in the gabaergic tone of the VP. This research shows the susceptibility of adolescent dopaminergic transmission to environmental stimuli and how they can shape neuronal circuits in adulthood.

Dedicatoria

Este trabajo de investigación está dedicado a todas las personas que han estado presente y me han apoyado en el transcurso de estos años.

Particularmente quiero dedicar este trabajo a mi Madre, la cual pudo verme partir en este camino, pero por desgracia del destino tuvo que marchar. Se que ella estaría muy feliz y orgullosa de todo lo logrado. Y quiero agradecerle y que sepa que, sin ella, sin su esfuerzo y dedicación yo no sería el hombre ni científico que soy ahora.

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Quisiera terminar con un poema que ha inspirado a muchas personas, personas que han pasado por muchas adversidades, pero que se levantan y siguen adelante.

Out of the night that covers me,
Black as the pit from pole to pole,
I thank whatever gods may be
For my unconquerable soul.

In the fell clutch of circumstance
I have not winced nor cried aloud.
Under the bludgeoning's of chance
My head is bloody, but unbowed.

Beyond this place of wrath and tears
Looms but the horror of the shade,
And yet the menace of the years
Finds and shall find me unafraid.

It matters not how strait the gate,
How charged with punishments the scroll,
I am the master of my fate:
I am the captain of my soul.

Invictus

William Ernest Henley (1849-1903)

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1. Introduction

1.1. Adolescence and neuroplastic changes in subcortical dopamine transmission

The adolescence is a life period characterized by gradual physiological and behavioral changes during the transition from childhood to adulthood (Schneider 2008; Spear 2000; Sisk and Foster 2004). This period is triggered by the first surge of sex hormones, which are responsible for beginning the reproductive stage, sexual maturity, and the development of the characteristic behavior of adulthood. The limits of adolescence in rodents vary according to the literature. Some researchers suggest that the puberty begins with balano-preputial separation for male rats and with the vaginal opening for female rats at postnatal day (PD) 40 and 35 respectively (Lewis et al. 2002; Engelbregt et al. 2001; Delemarre-van de Waal, van Coeverden, and Engelbregt 2002; Korenbrot, Huhtaniemi, and Weiner 1977; Fernández-Fernández et al. 2005). Other researchers have established different stages for adolescence in rats: early adolescence (PD21 – 34), mid-adolescence (PD34 – 46) and late adolescence (PD46 – 59) (Laviola et al. 2003; Lukkes et al. 2009; Tirelli, Laviola, and Adriani 2003). These stages would correspond to early (10–14 years), middle (15–17 years), and late (18–21 years) human adolescence (Braet et al. 2012; Wills et al. 1996).

During adolescence, transitory behavioral changes have been associated with modifications in neuronal connections (Spear 2000). For instance, adolescents are more sensitive to rewarding stimulus such as social peer interactions, novelty seeking, and palatable food compared to adults (Doremus-Fitzwater and Spear 2016; Friemel, Spanagel, and Schneider 2010; Douglas, Varlinskaya, and Spear 2004; Adriani, Chiarotti,

and Laviola 1998). This “sensitive reward” phenotype in adolescents has been associated with differential changes in dopaminergic pathways (Robinson et al. 2016; Spanagel and Weiss 1999; Doremus-Fitzwater and Spear 2016). The evidence indicates that the maturation of dopaminergic subcortical regions is earlier than the maturation of dopaminergic cortical region (mPFC) (Burke and Miczek 2014). Tarazi et al. observed that dopaminergic synaptic terminals increase in the Striatum and Nucleus Accembens (NAc) of the rats until early adolescence (PD35) while in the mPFC, continue growing up throughout late adolescence (PD60) (Tarazi, Tomasini, and Baldessarini 1998). Another hypothesis proposes that “sensitive reward” phenotype during adolescence is provoked by a transitory increase of dopaminergic activity in this period compared to adulthood. It has been observed in rats that basal extracellular levels of dopamine (DA) in the NAc and firing rate of DA neurons of the ventral tegmental area (VTA) show an age-dependent U-shaped trajectory. Both DA concentration and the firing rate of DA neurons, present a maximum during middle adolescence and then decrease in the adulthood (James Edgar McCutcheon and Marinelli 2009; Badanich, Adler, and Kirstein 2006). Interestingly, the time-window of dopaminergic subcortical transitory changes is different between mesolimbic and nigrostriatal pathway. It has been observed that the tissue content of DA in the NAc reaches the maximum level earlier than DLS (Naneix et al. 2012).

1.2. Mesencephalic Dopaminergic Pathways: Role and modulation

Midbrain DA neurons represent around 0.03% of the total number of cells in the central nervous system in rodents (Steiner and Tseng 2017). Interestingly, this small fraction of cells modulates various essential behaviors to survive, such us reward-based learning, motor control, and cognition. Furthermore, pathologies associated with changes in these behaviors like drug addiction, Parkinson´s disease, and schizophrenia have been associated with modifications of dopaminergic transmission. Anatomical studies have described different subtypes of DA neurons in specific regions (Hillarp, Fuxe, and Dahlström 1966; Fuxe et al. 2010). Using immunocytochemical approaches with antibodies against molecular marker of DA neurons as tyrosine hydroxylase, vesicular monoamine transporter 2, and DA transporter (DAT), it has allowed distinguishing three main groups of DA neuron in the midbrain: substantia nigra *par compacta* (SNc), VTA and retrorubral field (Hillarp, Fuxe, and Dahlström 1966; Fuxe et al. 2010). Interestingly, these groups of DA neurons project their axon to different areas. DA neurons from VTA project to limbic forebrain areas such as the amygdala, ventral pallidum (VP), prefrontal cortex, and NAc (Björklund and Dunnett 2007; Root et al. 2015), while DA neurons from SNc and retrorubral field project send their axons to the striatum (Björklund and Dunnett 2007).

Previous evidence supports that the activity of dopaminergic mesocorticolimbic pathway modulates neural process involved with working memory, decision making, motivation and reward signaling (Floresco and Magyar 2006; Salamone et al. 2015; Baik 2013). On the other hand the activity of the dopaminergic nigrostriatal pathway has been mainly

associated with habit learning (Willuhn et al. 2012; Everitt and Robbins 2013). Both dopaminergic pathways are strongly related to the development of addictive behavior (Everitt and Robbins 2013). An increase of dopaminergic mesolimbic activity accompanies the rewarding effects of natural reinforcers and drugs of abuse (Robinson et al. 2016; Everitt and Robbins 2016). Instead, the transition from an initial intake of drug of abuse to repeated consumption is associated with a progressive increase in the neurotransmission of the dopaminergic nigrostriatal pathway (Willuhn et al. 2012). In this sense, the release of DA in the DLS is crucial for the acquisition and the persistence of drugs habitual seeking. The local perfusion of a non-selective dopaminergic antagonist in the DLS reduces cocaine-seeking behavior in late stages of training and fails to modify cocaine-seeking when it is a goal-directed behavior (Murray, Belin, and Everitt 2012; Vanderschuren, Di Ciano, and Everitt 2005). In addition, it has been observed that the perfusion in the DLS of raclopride, a D2 antagonist, restores the goal-directed behavior in rats with habitual responding to ethanol-seeking (Corbit, Nie, and Janak 2014). Due to the critical role of DA transmission in the DLS in the acquisition and persistence of habitual seeking of abused drugs, it is fundamental to understand the regulation of this neurotransmission in physiological and pathological conditions.

DA transmission is mainly regulated through mechanisms localized in the terminal and somatic region of DA neurons. In the terminal region, DA extracellular concentration is the consequence of the balance between two processes: release and uptake of DA (Sulzer, Cragg, and Rice 2016). DA release is fundamentally mediated by the fusion of DA-containing vesicles in the cell membrane (Sulzer, Cragg, and Rice 2016), by an augmentation of intracellular Ca^{+2} induced by an increase in firing rate (Fulop and Smith

2006). Also, a rise in DA synthesis (Pothos et al. 1998; Pothos, Davila, and Sulzer 1998) and a higher expression of vesicular monoamine transporter 2 (Pothos et al. 2000) increase the content of DA in vesicles associated with an enhancement in quantal DA release. In turn, the evidence proposes that the activation of receptors expressed in dopaminergic terminals modifies the probability of DA release. For instance, the activation of D2 (Pothos et al. 1998; Schmitz et al. 2003), kappa opioid (Di Chiara and Imperato 1988), mGlutR1 (H. Zhang and Sulzer 2003) and $GABA_B$ (Smolders et al. 1995) receptors reduce the release of DA, whereas, the activation of nicotinic receptors enhances the release of DA (Zhou, Liang, and Dani 2001; Rice and Cragg 2004).

DA uptake is the main mechanism controlling the duration of DA transmission and allow the replenishment of DA-containing vesicles in the axon terminal (Uhl 2003). DAT is the primary mechanism responsible for the clearance of DA compared to extracellular degradation, as evidenced in striatal regions of mice lacking DAT (Benoit-Marand, Jaber, and Gonon 2000). On the other hand, in brain regions with lower or null presence of DAT, such as the PFC and hippocampus, DA is cleared by the norepinephrine transporter (Mundorf et al. 2008; Borgkvist et al. 2011). As mentioned before, the activation of receptors in the dopaminergic terminals modulate DA extracellular concentration. In several cases, the mechanism involved is indirectly by modulating the activity of DAT. Strong evidence supports that the activation of D2 presynaptic autoreceptors allows the positive regulation of DAT activity, thus increasing DA clearance and the duration of the transmission (W. A. Cass and Gerhardt 1994; Meiergerd, Patterson, and Schenk 2006; Rothblat and Schneider 1997; Wieczorek and Kruk 1994; Wu et al. 2002). In addition, the activity of kappa opioid receptors has been associated with the control of DAT activity in

the striatum (Azocar et al. 2018; Chefer et al. 2006). DAT activity is also regulated by other factors as nitric oxide (Kiss et al. 1999) and post-translational modifications (Patel et al. 1994; Li et al. 2004; Rastedt, Vaughan, and Foster 2017; Foster and Vaughan 2017).

Regarding the regulation of DA transmission in the cell body, using simultaneously microdialysis and extracellular recording experiments, it was shown that an increase of DA neurons activity in the VTA and SNc, increases DA dialysate in the ventral and dorsal striatum, respectively (Panin et al. 2012). This evidence indicates that somatic activity of DA neurons plays a key role in the dopaminergic transmission in terminals. Midbrain DA neurons may change between three different states: no-firing state, tonic firing state, and phasic firing state (Gomes, Rincón-Cortés, and Grace 2016). Tonic firing is characterized by a slow frequency of spike between 4 and 8 spikes per second (Bunney et al. 1973; Aghajanian and Bunney 1973). Phasic firing state is the transition into a rapid burst-firing mode which can reach an intra-burst frequency of 30 spikes per second (Grace and Bunney 1984; Hyland et al. 2002).

The transition between these states of activity is modulated by inhibitory and excitatory inputs. Local perfusion of $GABA_A$ receptor antagonists significantly increase the activity of DA neurons in the SNc (Paladini and Tepper 1999; J M Tepper, Martin, and Anderson 1995) and VTA (Ikemoto, Kohl, and McBride 2002). In addition, it has been observed that GABAergic input to DA neuron determine the proportion of DA neuron firing spontaneously (Floresco et al. 2003). On the other hand, the glutamatergic input to DA neurons has been associated with changes in the spontaneous burst pattern. A significant reduction of burst firing has been observed in DA neurons using local perfusion of NMDA

receptor antagonist in SNc and specific genetic deletion of this receptor (Chergui et al. 1993; Overton and Clark 1992; Charlety et al. 1991; Zweifel et al. 2009). In addition, the local stimulation of pedunculopontine nucleus, a glutamatergic (as well as cholinergic and GABAergic) nucleus with direct projections to DA neurons (Watabe-Uchida et al. 2012), produces a significant increase of burst firing of DA neurons in the SNc and VTA (Floresco et al. 2003; Steiner and Tseng 2017). Besides, nigrostriatal DA neurons are regulated by various GABAergic and glutamatergic inputs, which modulate the inhibitory-excitatory balance. Some of the GABAergic inputs to nigrostriatal DA neurons come from the striatum, VP, globus pallidus and substantia nigra reticulata (Watabe-Uchida et al. 2012; Steiner and Tseng 2017). On the other hand, described glutamatergic inputs to the nigrostriatal DA neurons come mainly from the Subthalamic and Pedunculopontine nuclei (Watabe-Uchida et al. 2012; Steiner and Tseng 2017). In summary, the activity of DA neurons depends on the balance between glutamatergic and GABAergic inputs. While the activation of NMDA receptors induced by glutamate provokes an increase in burst firing, the hyperpolarization of DA neurons induced by GABA decrease of DA neurons spontaneous activity.

1.3. Cannabinoid Agonist and Dopaminergic transmission.

Cannabis is the third more popular substance of abuse, after nicotine and alcohol, with an estimated of 192.2 million of people that have used cannabis at least once in the last year (UNODC 2018). The annual prevalence of cannabis on the adolescent population is higher than the general population of Europe and the Americas (UNODC 2018). Interestingly, approximately 17% of those who initiate the use of cannabis during adolescence develop disorders due to the use of cannabis, in contrast, only 9% of those who experiment with cannabis in adulthood develop disorders due to the use of cannabis (Volkow et al. 2014; Lopez-Quintero et al. 2011; Anthony 2006). This evidence suggests that the neurobiological substrates associated with the drugs abuse disorder are more susceptible to cannabinoids in adolescence compared to adulthood.

Currently, it has been identified around of 545 natural compounds from *Cannabis Sativa L.* of which, 104 correspond to the chemical group of "phytocannabinoids". One of these phytocannabinoid, the delta-9-tetrahydrocannabinol (Δ^9-THC), is considered the primary psychoactive molecule and responsible for the addictive effects of this plant (Pertwee 2014). Δ^9-THC content in herbal cannabis has increased from 1% in the 70s until around 10 % in 2010 (Fidelia Cascini, Carola Aiello, and GianLuca Di Tanna 2012). The physiological effects of acute exposure of Δ^9-THC have been mainly attributed to the agonism of CB1 and CB2 receptors. Due to the high use of cannabis during adolescence, it is crucial to understand the effects of CB1/2 agonism in the neurobiological substrates associated with drugs of abuse disorder.

CB1 and CB2 receptors are fundamental in different physiological processes, such as appetite, pain-sensation, mood, memory, and motivation (Mechoulam and Parker 2012; Pertwee 2014). The activation of both cannabinoid receptors inhibits adenylyl cyclase via Gi/o protein (Howlett, Qualy, and Khachatrian 1986; Felder et al. 1995) and modifies different functions in mammals. The CB1 receptors (CB1-R) are mainly localized in axon-terminals in the central nervous system, where their activation inhibits the release of different neurotransmitters like, *gamma*-aminobutyric acid (GABA), glutamate, histamine, serotonin, acetylcholine, and DA (Kano et al. 2009; Heifets and Castillo 2009; Castillo et al. 2012). On the other hand, CB2 receptors (CB2-R) expression is higher in cells of the immune system compared than in brain neurons (Grotenhermen 2005). Recent evidence has shown an active role of CB2 agonism in reward/aversion signaling and modulation of DA neuronal activity (Jordan and Xi 2019).

In the case of dopaminergic transmission, it has been observed that an acute systemic administration of WIN55212-2, a CB1/2 agonist, increases DA extracellular levels in the NAc and DLS of adult rats (Tanda, Pontieri, and Di Chiara 1997; A. Polissidis et al. 2014), associated with a higher firing rate of DA neurons in VTA and SNc, respectively (French, Dillon, and Wu 1997). Both effects are attenuated after a dose of CB1 antagonist, indicating that the effects on dopaminergic transmission are depended of CB1-R (Tanda, Pontieri, and Di Chiara 1997; Gessa et al. 1998; Melis, Gessa, and Diana 2000). Since CB1-R are not expressed in DA neurons (García et al. 2015; Herkenham et al. 1991), an indirect mechanism has been proposed to explain the cannabinoid modulation of dopaminergic transmission. The activation of CB1-R on presynaptic GABAergic neurons of the VTA and SNc inhibits the inhibitory GABAergic tone over DA neurons (García et

al. 2015; Beardsley and Thomas 2005; Yanovsky, Mades, and Misgeld 2003). Furthermore using in vitro experiments, the CB2 agonism decreases the spontaneous activity of DA neuron in VTA mediated by an increase the currents of potassium (Ma et al. 2019). A local perfusion of CB2 agonist in the NAc reduces the basal DA release and DA release induced by cocaine in adult rats (H.-Y. Zhang et al. 2017). In addition, it has been proposed that cannabinoids can interact directly with DAT (N Chen et al. 2003). For instance, the acute administration of the synthetic cannabinoid WIN55212-2 decreases the activity of DAT in the DS of adult rodents (Price et al. 2007; Pandolfo et al. 2011).

Despite the vast knowledge about the acute effect of cannabinoids in DA transmission during the adulthood (Price et al. 2007; Tanda, Pontieri, and Di Chiara 1997; A. Polissidis et al. 2014; Alexia Polissidis et al. 2013; Nianhang Chen et al. 2003; French, Dillon, and Wu 1997; Pandolfo et al. 2011; Yanovsky, Mades, and Misgeld 2003; Ma et al. 2019; H.-Y. Zhang et al. 2017; Jordan and Xi 2019), the effects of cannabinoids on DA release and uptake in the DLS during adolescence and the consequences in the adulthood of the repeated uses during adolescence remains to be addressed.

CB1-R expression shows transitory changes through CNS development and, between adolescence and adulthood. In the striatal areas in male rats, the expression of the CB1-R increases gradually until a maximum at PD40, then decreases until it reaches adult values (PD70) (Rodríguez de Fonseca et al. 1993). Conversely, in midbrain regions, it has been shown that the expression of the CB1-R is lower in adolescent rats (PD 37) compared to adult rats (PD72) (Verdurand et al. 2011).

The above evidence supports the idea of an age-dependent effect of CB1-R in the nigrostriatal dopaminergic transmission. Furthermore, since age-dependent changes in CB1-R expression occur in parallel with the development of dopaminergic pathways (J. E. McCutcheon et al. 2012), it is possible to suggest that repeated activation of CB1-R during adolescence could modify the development of the dopaminergic system.

Preclinical evidence indicates that the repeated activation of the CB1-R during adolescence modifies dopaminergic transmission of the mesolimbic pathway. Gomes et al. 2015 showed that the exposure to WIN55212-2 during adolescence increases the spontaneous activity of dopaminergic neurons of the VTA in adult rats and increases hyperlocomotion induced by amphetamine (Gomes, Guimarães, and Grace 2015). Furthermore, Pistis et al. (2004), observed that exposure to WIN55212-2 during adolescence produces long-term cross-tolerance to the effects of other drugs of abuse like morphine, cocaine, and amphetamine, on firing rate of DA neurons of VTA (Pistis et al. 2004). While the adolescent exposure of cannabinoid modifies the dopaminergic mesolimbic pathways during adulthood, the effects in dopaminergic nigrostriatal pathway remain unclear (Pistis et al. 2004; Schneider 2008; Higuera-Matas et al. 2010; Gomes, Guimarães, and Grace 2015).

This research is presented in two chapters:

The first chapter aims to compare the extracellular concentration (Cext) of DA in the DLS between adolescents and adults and to study the age-dependent effect of WIN55212-2 in DA release and DA extraction fraction (Ed), an indirect measure of DA uptake, in the DLS. Our results show increased DA release and DA Ed in the DLS of urethane-

anesthetized adolescent rats. Moreover, decreased DA Ed in DLS of urethane-anesthetized adolescent rats is observed after an acute injection of WIN55212-2, an outcome that was not found in young-adult rats.

The second chapter aims to study the effects of exposure to WIN55212-2 during the adolescence in dopaminergic dynamics of the nigrostriatal pathway in adult rats and to determine the mechanism associated with dopaminergic regulation. Microdialysis associated with No-net flux DA quantification was used to assess long-term changes induced by adolescent exposure to WIN55212-2 in the balance between DA release and uptake in the DLS. Furthermore, single unit recordings were carried out to study the firing pattern of DA neurons of SNc in adult rats, which were treated with cannabinoid agonists during adolescence.

2. Hypothesis

2.1. Chapter 1:

The exposure to an acute dose of WIN55212-2 increased the dopaminergic transmission in the DLS in adolescent rats associated with decreased activity of the dopamine transporter.

2.2. Chapter 2:

The repeated treatment with WIN55212-2 during adolescence increases the activity of the dopaminergic nigrostriatal pathways associated with a reduction of GABAergic input in the SNc as a consequence of lower activity of the ventral pallidum

3. Aims

3.1. Chapter 1:

3.1.1 General Aim:

To assess the age-dependent changes in the dopaminergic transmission in the DLS after an acute exposure of cannabinoid.

3.1.2 Specific Aims:

1. To compare DA extracellular concentration and DA uptake and release in the DLS of adolescent and adult male rats.

2. To determine the effects of an acute systemic dose of WIN55212-2 on DA extracellular concentration and DA uptake and release in the DLS of adolescent and adult male rats.

3.2. Chapter 2:

3.2.1 General Aim

To study the effects and mechanisms of repeated treatment with WIN55212-2 during adolescence on the dynamics of dopamine and the neuronal activity of the nigrostriatal pathway of adult rats

3.2.2 Specific Aims

1. To quantify basal DA release and uptake in the DLS of adult rats that have been repeatedly treated with WIN55212-2 during adolescence.

2. To measure the electrical activity of DA neurons in the Substantia Nigra of adult rats that have been repeatedly treated with WIN55212-2 during adolescence.

3. To determine the extracellular concentration of GABA and Glutamate in the SNc of adult rats that have been repeatedly treated with WIN55212-2 during adolescence.

4. To determine the role of ventral pallidum in the electrical activity of DA neurons in the SNc of adult rats that have been repeatedly treated with WIN55212-2 during adolescence.

4. Chapter 1: Comparing dopaminergic dynamics in the dorsolateral striatum between adolescent and adult rats: Effect of an acute dose of WIN55212-2

Comparing dopaminergic dynamics in the dorsolateral striatum between adolescent and adult rats: Effect of an acute dose of WIN55212-2

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Abbreviations used: DLS: Dorsolateral Striatum; DA: Dopamine; Cext: Extracellular concentration; NAc: Nucleus Accumbens; VTA: Ventral Tegmental Area; DS: Dorsal Striatum; CB1-R: CB1 Receptor; DAT: Dopamine Transporter; KRP: Krebs Ringer Phosphate; AA-KRP: Krebs Ringer Phosphate with 0.2 mM ascorbic acid; PFA: paraformaldehyde; CPu: Caudate putamen; HPLC: High-Performance-Liquid-Chromatography; Ed: Extraction Fraction; SEM: Standard Error of Mean

4.1. Abstract

During adolescence, dopaminergic neurotransmission shows transient changes until reaching adulthood. The administration of CB1 agonists such as WIN55212-2 during adulthood increases dopamine (DA) extracellular levels. However, the effects of acute administration of cannabinoids on nigrostriatal dopamine neurotransmission during adolescence are not fully elucidated. The aim of this research is to compare dorsolateral striatum (DLS) DA dynamics and to study the effect of WIN55212-2 on DLS DA dynamics during adolescence and adulthood. No-net flux microdialysis experiments were carried out in adolescent (post-natal day 35-40) and young-adult (post-natal day 70-75) urethane-anesthetized rats. Basal DA dialysate, DA extraction fraction (Ed) and extracellular concentration of DA (Cext) in DLS were assessed after an acute injection of WIN55212-2 (1.2 mg/kg) or vehicle. An increased basal DA dialysate and DA Ed were observed during adolescence compared to adulthood. Moreover, WIN55212-2 increases DLS DA Cext rising basal DA dialysate in adulthood and decreasing DA Ed in adolescence. Our results suggest that an age-dependent mechanism underlies the effect of WIN 55212-2 on DA balance between release and uptake in DLS.

Keywords: Dopamine, WIN55212-2, Adolescence, no-net flux microdialysis; Dorsolateral Striatum

4.2. Introduction

Adolescence is a period characterized by gradual physiological and behavioral transitions from childhood to adulthood (Schneider, 2008; Sisk and Foster, 2004; Spear, 2000). During adolescence, neuronal connections overproduced in early life are amply pruned to respond to physiological changes and to adapt to environmental factors (Burke and Miczek, 2014; Casey et al., 2008; De Bellis et al., 2001; Gogtay et al., 2004; Powell, 2006; Schneider, 2008; Teicher et al., 1995). It has been observed that adolescents are more sensitive to rewarding stimuli such as social peer interactions, novelty seeking, and palatable food compared to adults (Adriani et al., 1998; Doremus-Fitzwater and Spear, 2016; Douglas et al., 2004; Friemel et al., 2010). In addition, adolescence has been associated with a transitory change in the dopaminergic transmission. It has been observed that dopamine (DA) basal extracellular levels in Nucleus Accumbens (NAc) and the firing rate of dopamine neurons from the ventral tegmental area (VTA) show an age-dependent U-shaped trajectory. Both DA concentration and firing rate of dopamine neurons present a maximum peak during middle adolescence, followed by a decrease during adulthood (Badanich et al., 2006; McCutcheon and Marinelli, 2009). Similarly, age-dependent differences in nigrostriatal dopaminergic transmission have been observed. A transitory increase in the expression of DA receptors, accompanied by a higher turnover of DA, has been observed in the adolescent dorsal striatum (DS) compared to the adult (Naneix et al., 2012). In addition, Nakano and Mizumo (1996) observed a higher DA basal dialysate in adolescent DS compared to adult (Nakano and Mizuno, 1996).

Epidemiological evidence shows that the annual prevalence of cannabis use by the adolescent population is higher compared to the general population of Europe and the Americas (UNODC, 2018). Compared to adulthood, cannabis use during adolescence is associated with a higher risk of cannabis use dependence (Volkow et al., 2014). This evidence suggests that the neurobiological substrates underlying drug abuse are more susceptible to cannabinoid effects in adolescence compared to adulthood. One of the main neuronal pathways associated with the intake and development of drug addiction is the dopaminergic mesolimbic pathway (Everitt and Robbins, 2016; Robinson et al., 2016). Moreover, recent evidence shows that DA release in DLS can encode drugs abuse related information. Using the self-administration paradigm and fast scan voltammetry recording in the freely-moving rat has been observed that habitual cocaine intake is accompanied by a progressive increase in DLS DA release (Willuhn et al., 2012). In addition, repeated exposure to cocaine is associated with an increase in DLS neuronal activity induced by a cue in a go/no-go task, suggesting changes in neural process in the striatum after the repeated exposure to the drug (Takahashi et al., 2007). While the effects of cannabinoids on DA transmission have been extensively studied in mesolimbic and nigrostriatal pathways in adult rats, less explored are the effects of cannabinoids on the adolescent nigrostriatal dopaminergic pathway (Covey et al., 2017; French et al., 1997; Higuera-Matas et al., 2010; Pistis et al., 2004; Schneider, 2008).

Cannabinoids modulate different physiological responses in mammals, such as appetite, pain perception, mood, memory, and motivation (Mechoulam and Parker, 2012; Pertwee, 2014). The CB1 receptors (CB1-R) are mainly localized in the central nervous system, and their activation is key to modulate the function of different neurotransmitters such as

GABA, glutamate, acetylcholine, DA, and serotonin (Castillo et al., 2012; Heifets and Castillo, 2009; Kano et al., 2009). In the case of adult dopaminergic transmission, it has been observed that an acute systemic administration of CB1 agonists such as WIN55212-2 produces an increase in dopamine extracellular levels in the NAc (Tanda et al., 1997) and dorsolateral striatum (DLS) (Polissidis et al., 2014, 2013). Also, the acute administration of WIN55212-2 decreases the activity of the DA transporter (DAT) in the DS of adult rodents (Pandolfo et al., 2011; Price et al., 2007). However, the effects of cannabinoids on DA release and DA uptake in the DS during adolescence remains to be addressed. Interestingly, it has been observed that the expression of CB1-R presents transitory changes through development (Rodríguez de Fonseca et al., 1993; Van Waes et al., 2012; Verdurand et al., 2011). The binding of CB1-R increases in parallel with the development until reaching its maximum in adulthood in several areas such as midbrain, hippocampus, and striatum (Verdurand et al., 2011). Then, it is possible to suggest that CB1-R agonists could have different effects depending on the current development stage. Specifically, there is no evidence regarding the impact of acute administration of CB1-R agonists on dopaminergic transmission in the nigrostriatal pathway of adolescent rats. The main aim of this research is to compare DLS DA extracellular concentration (Cext) during adolescence and adulthood, and to study the age-dependent effect of WIN55212-2 in DLS DA release and DA extraction fraction (Ed), an indirect measure of DA uptake (Chefer et al., 2006; Smith and Justice, 1994). Using no-net flux microdialysis, our results show an increased DA release and DA Ed in the DLS of urethane-anesthetized adolescent rats. Moreover, a decrease in DA Ed in DLS of urethane-anesthetized adolescent rats is

observed after an acute injection of WIN55212-2, an outcome that was not observed in young adult rats.

4.3. Results

4.3.1 Age-dependent differences in dopaminergic neurotransmission in DLS

Basal and stimulated DA dialysate:

Conventional microdialysis experiments in adolescent and adult rats were carried out to compare age-dependent differences in basal and stimulated DA dialysate in DLS (see Fig 1).

Basal DA dialysate was higher in adolescent rats compared to adult rats (Fig 2 Adolescent group: 0.89 ± 0.18 nM; n= 6; vs Adult group: 0.52 ± 0.04 nM; n= 5; $p< 0.05$ according to unpaired t-test). The perfusion of 40 mM K+-KRP increased extracellular concentration of DA in both groups (Fig 2 Basal Adolescent group: 0.89 ± 0.18 nM; n= 6; vs 40 mM K+-KRP adolescent group: 37.16 ± 7.42 nM; n= 6; $p< 0.01$ according to paired t-test; Basal Adult group: 0.52 ± 0.04 nM; n= 5; vs 40 mM K+-KRP adult group: 22.37 ± 8.95 nM; n= 5; $p< 0.05$ according to paired t-test). No significant difference in high potassium-stimulated DA dialysate was observed between adolescent and adult rats (Fig 2 Adolescent group: 37.16 ± 7.42 nM; n= 6; vs Adult group: 22.37 ± 8.95 nM; n= 5; $p> 0.05$ according to unpaired t-test).

DA extraction fraction and extracellular concentration:

No-net flux microdialysis experiments in adolescent and adult rats were carried out to compare age-dependent differences in DA Cext and Ed in DLS.

As previously shown (fig 2), basal DA dialysate in adolescent rats was significantly higher than in adult rats (Fig 3a adolescent group: 0.69 ± 0.05 nM; n= 6; vs adult group: 0.53 ± 0.04 nM; n= 5; $p< 0.05$ according to unpaired t-test). Interestingly, adolescent rats showed a higher DA Ed compared to adult rats (Fig 3b adolescent group: 0.56 ± 0.06 nM; n= 6; vs adult group: 0.41 ± 0.06 nM; n= 5; $p< 0.05$ according to unpaired t-test), suggesting a transitory increase in DAT activity during adolescence. Consequently, DA Cext remained constant in both adolescence and adulthood (Fig 3c adolescent group: 1.23 ± 0.14 nM; n= 6; vs adult group: 1.36 ± 0.14 nM; n= 5; $p> 0.05$ according to unpaired t-test). Neither the perfusion of ascorbic acid nor systemic acute injection of vehicle modified basal DA dialysate (Figure supplementary 1).

4.3.2 Effects of acute exposure of WIN55212-2 in DLS dopamine dynamics

No-net flux microdialysis was carried out in adolescent and adult rats to assess age-dependent consequences of acute systemic injection of WIN55212-2 in DLS DA Cext and Ed.

Adolescent Rats:

The acute intraperitoneal (ip) injection of WIN55212-2 did not modify basal DA dialysate in DLS compared to vehicle group (Fig 4a vehicle group: 0.69 ± 0.05 nM; n= 6; vs WIN group: 0.67 ± 0.04 nM; n= 5; $p> 0.05$ according to unpaired t-test). However, the acute injection of WIN was accompanied by a significant decrease of DA Ed compared to vehicle group (Fig 4b vehicle group: 0.59 ± 0.05; n= 6; vs WIN group: 0.39 ± 0.05; n= 5; $p< 0.05$ according to unpaired t-test). Consequently, an acute injection of WIN55212-2 significantly increased the Cext compared to vehicle injection (Fig 4c vehicle group: 1.23

± 0.14 nM; n= 6; vs WIN group: 1.91 ± 0.37 nM; n= 5; $p< 0.05$ according to unpaired t-test).

Adult Rats:

Contrary to what was observed in adolescent rats, an acute ip injection of WIN increased basal DA dialysate in DLS compared to vehicle group (Fig 5a vehicle group: 0.53 ± 0.04 nM; n= 5; vs WIN group: 0.80 ± 0.06 nM; n= 5; $p< 0.05$ according to unpaired t-test). The acute injection of WIN55212-2 did not modify DA Ed compared to vehicle injection (Fig 5b vehicle group: 0.412 ± 0.056; n= 5; vs WIN group: 0.418 ± 0.065; n= 5; $p> 0.05$ according to unpaired t-test). Consequently, the acute injection of WIN55212-2 was accompanied by a significant increase of Cext compared to an acute injection of vehicle (Fig 5c vehicle group: 1.36 ± 0.14 nM; n= 5; vs WIN group: 2.05 ± 0.26 nM; n= 5; $p< 0.05$ according to unpaired t-test).

4.4. Discussion

Transitory changes in DA neurotransmission have been observed between adolescence and adulthood (Badanich et al., 2006; Matthews et al., 2013; McCutcheon and Marinelli, 2009; Nakano and Mizuno, 1996). Accordingly, our results show age-dependent differences in homeostatic control of DLS DA extracellular levels. Conventional microdialysis experiments show a higher DLS basal DA dialysate in adolescent rats compared to adult rats, without differences in high potassium-stimulated DA dialysate. Moreover, no-net flux microdialysis experiments in DLS indicate that DA Ed during adolescence is higher than in adulthood. Consequently, age-dependent differences in DLS DA Cext were not observed. Interestingly, the results show an age-dependent difference

after an acute administration of WIN55212-2. A significant increase in DLS basal DA dialysate after an acute injection of WIN55212-2 is only observed in adult rats. Adolescence is accompanied by a significant decrease in DLS DA Ed after an acute injection of WIN55212-2, which is not observed in adult rats. Consequently, the increase of DLS DA Cext induced by WIN55212-2 depends on mechanisms that change with age: a decrease in DA Ed in adolescent rats and an increase in DA release in adult rats. In addition, our results suggest that the DLS DAT activity shows an age-dependent vulnerability to the effects of WIN55212-2.

Contributing to evidence showing transitory changes in dopaminergic neurotransmission associated with the transition from adolescence to adulthood (McCutcheon et al., 2012; McCutcheon and Marinelli, 2009; Naneix et al., 2012), a higher basal DLS DA dialysate is observed in adolescent compared to adult rats (Fig. 2a). The high potassium perfusion increases DA dialysate in a similar magnitude in adolescent and adult rats (Fig 2b), suggesting no age-dependent differences in vesicular DA storage (Castañeda et al., 1988; Kantor et al., 1999). Taken together, it is possible to suggest that the increased basal DA release observed in adolescent rats is associated with increased electrical activity of DA neurons from SNc (Tepper et al., 1990).

Using various experimental approaches, it has been demonstrated that there is a more efficient uptake of DA during adolescence (Stamford, 1989; Volz et al., 2009). However, the evidence about DAT expression in the striatal region is not conclusive. Binding essays (Tarazi et al., 1998) have demonstrated that the transition from adolescence to adulthood is accompanied by a systematic increase in DAT expression in DLS. On the other hand,

western blot experiments have shown contradictory results in DAT expression in DS. While Matthews et al. (2013) observed a lower expression of DAT (Matthews et al., 2013), Volz et al. (2009) showed a higher DAT expression in adolescent compared to adult rats (Volz et al., 2009). In this sense, our results using no-net flux microdialysis show an increased DA Ed during adolescence compared to adulthood. This data is in agreement with results showed by Volz et al. (2009), suggesting that higher activity of DAT in adolescent rats is associated with higher DAT expression in adolescent DS (Volz et al., 2009).

The DA Cext is an estimated concentration of DA in the synaptic cleft, which depends on basal DA release and uptake, associated with DA Ed (Chefer et al., 2006; Smith and Justice, 1994). As mentioned before, a higher basal DA dialysate in DLS is observed in adolescent rats compared to adult rats. Moreover, a higher DA Ed in DLS is observed during adolescence. Consequently, no significant differences were observed in Cext of DA in DLS of adolescent rats compared to adult rats. Thus, the higher dopaminergic nigrostriatal release observed during adolescence is tuned by an increase in DAT activity, preserving DA Cext at homeostatic levels similar to adulthood. Interestingly, a transitory increase of DA Cext during adolescence was observed in NAc, without significant changes in DA Ed (Badanich et al., 2006). Thus, the lower DA Cext observed during adulthood in NAc would depend on a late decrease on DA release.

The significant increase in basal dialysate of DA in DLS of adult rats induced by CB1 agonist is in line with previous pre-clinical evidence. An increase in basal DA release in DS (Polissidis et al., 2014, 2013) associated with an increase of neuronal activity of DA

neuron from the substantia nigra pars compacta (French et al., 1997) has been observed after an acute administration of WIN55212-2 in adult rats. The increase in neuronal activity of DA neurons from SNc induced by WIN55212-2 is mediated by a decrease in gabaergic transmission, as tested in ex vivo experiments (Yanovsky et al., 2003). In contrast, an acute administration of WIN55212-2 is not accompanied by significant changes in basal DA dialysate in the DLS of adolescent rats. To our knowledge, this is the first evidence related to the acute effects of WIN55212-2 in DA release in the DLS of adolescent rats. A lower expression of CB1-R in adolescent midbrain (Verdurand et al., 2011) could explain the lack of effect of WIN55212-2 exposure in basal DA dialysate.

The no-net flux microdialysis experiments show age-dependent effects of cannabinoids on DA uptake. The acute exposure to WIN55212-2 is accompanied by a significant decrease in DA Ed in adolescent rats, a result that was not observed in adult rats. While there is no evidence related to the effects of WIN55212-2 on DA uptake in adolescent rats, an inhibitory effect of WIN55212-2 on DA uptake has been observed in adult rats. Interestingly, the inhibitory effect of WIN55212-2 on DA uptake has been shown independent of CB1-R activation (Price et al., 2007). In fact, it has been proposed that WIN55212-2 interacts directly with the DAT protein (Pandolfo et al., 2011; Price et al., 2007; Steffens and Feuerstein, 2004). Whether a similar mechanism than the one observed in adult rats underlies an inhibitory effect of WIN55212-2 in DA uptake in adolescent rats remains to be addressed. The age-dependent effects observed under our experimental conditions could be explained by lower glycosylation of DAT in adolescent rats. (Patel et al., 1994). It has been described that the higher glycosylation of DAT observed in adult rats attenuates the inhibitory effect of drugs on DA uptake (Li et al., 2004). It is tempting

to suggest that the lower dose used in our experiment could explain the lack of inhibitory effects of WIN55212-2 in DA Ed observed in adult rats. Further experiments using higher doses of WIN55212-2 are necessary to address this proposal.

A significant increase in DLS DA Cext is observed after an acute administration of WIN55212-2 in adult and adolescent rats. Interestingly, age-dependent mechanisms underlie the increase in DA Cext. An increase in DA release contributes to the increase in DA Cext induced by WIN55212-2 during adulthood, meanwhile, a decrease in DA uptake is associated with the increase in DA Cext in DLS of adolescent rats.

In summary, our results show that adolescence is accompanied by a higher basal DA release and also a higher DA Ed in the DLS compared to adulthood. The acute exposure to WIN55212-2 increases DA release in adulthood and decreases DA Ed in adolescence, resulting in an increase in DA Cext in both groups. These findings suggest that an increased DAT activity is opposed to a higher DA release in adolescent rats, preserving the DLS DA Cext at homeostatic levels similar to those observed in adult rats. Moreover, an age-dependent mechanism underlies the effect of WIN 55212-2 on DA Cext in DLS, suggesting a high vulnerability to inhibitory effects of cannabinoids on DAT activity during adolescence.

4.5. Experimental Procedure

The study was not pre-registered. All procedures were in strict accordance with the guidelines published in the "NIH Guide for the Care and Use of Laboratory Animals" (8°Edition) and the principles presented in the "Guidelines for the Use of Animals in Neuroscience Research" by the Society for Neuroscience. Also, the protocols were approved by the local bioethics committees, verifying that it complies with the basic principles set forth in Chilean Law 20.380 on Animal Protection 2009 (ID project: 160816013). The rats were identified with markings on the tail and numbered accordingly. Using pseudo randomization (random number generator), the adult and adolescent animals were divided into two experimental groups (vehicle exposure and WIN55212-2 exposure). All control and experimental protocols were performed in parallel. A priori statistical power, effect, and sample size calculations were performed by [G*power (Faul et al., 2007), α=0.05; power >0.8] based on preliminary results of this study. The results obtained suggested a sample size of n=5/group for the experiment. Exact numbers for all experiments are provided in the figure legends and results section.

4.5.1 Animals

Adult (post-natal day from 72 to 78) and adolescent (post-natal day from 35 to 40) male Sprague-Dawley rats were grown from the Animal Care Faculty of the Biological Sciences, Pontificia Universidad Católica de Chile (Charles River; Wilmington, MA, USA; RRID: RGD_728193), under the supervision of a veterinarian. One week before microdialysis experiments, rats were maintained in the Animal Care Faculty of the Department of Pharmacy, Pontificia Universidad Católica de Chile, following the

instruction of a protocol approved by the veterinarian. Rats with similar age were housed in groups of three per cage and kept at room temperature between 22–24°C on a 12 h light/dark cycle (lights on at 7:00 EST) with access to food and water ad libitum. Rats were handled for one week before starting the experiments. A total of 11 adolescent rats and 10 adult rats were divided into two groups; 6 adolescent rats and 5 adult rats for vehicle treatment, and 5 adolescent rats and 5 adult rats for WIN55212-2 treatment. No animals died during the experiments.

4.5.2 Reagents

The CB1/2 agonist, WIN55212-2 mesylate, was purchased from Medchemexpress (Monmouth Junction, NJ, USA). WIN55212-2 was emulsified in 2% Tween 80, then diluted in saline solution (NaCl 0.9%) at concentration of 1.2 mg/mL and sonicated for 5 min. Urethane and Tween 80 were obtained from Sigma Aldrich (St. Louis, MO, USA). The compounds of Krebs-Ringer phosphate buffer (NaCl, KCl, CaCl2, NaH2PO4, Na2HPO4 and ascorbic acid) and the compounds of the mobile phase (octane-1-sulfonic acid sodium salt, acetonitrile, and EDTA) were purchased from Merck (Darmstadt, Germany).

4.5.3 Microdialysis experiments

Adult and adolescent rats were anesthetized with urethane 1.5 g/kg ip and placed in a stereotaxic apparatus. Urethane was chosen due to the extended half-life (Gumbleton and Benet, 1991). In addition, urethane does not modify basal and stimulated DA dialysate (Howard and Feigenbaum, 1997; Tepper et al., 1991). The skull of the rat was exposed, and a hole was drilled targeting the DLS. A concentric microdialysis probe (CMA 11 Microdialysis, Holliston, MA) was lowered into the DLS using the following coordinates:

for adult rats +1.2 AP, -3.6 ML and -4.8 DV relative to bregma and for adolescent rats +1.0 AP, -3.6 ML and -4.6 DV relative to bregma (Paxinos and Whaton, 2009). Body temperature was maintained by a thermostatically controlled electric heating pad. The microdialysis probe was perfused for 40 minutes to allow equilibration with Krebs–Ringer Phosphate (KRP) buffer at a rate of 2 µL/min using a Harvard infusion pump (Harvard Apparatus, Holliston, MA). The composition of the KRP was 120 mM NaCl, 2.4 mM KCl, 1.2 mM CaCl2, 0.9 mM NaH2PO4, 1.4 mM Na2HPO4, and 0.2 mM of ascorbic acid (pH 7.4). Perfusion samples were collected every 5 min in 2 µL of perchloric acid (0.2 N) and maintained on ice (4°C) until DA determination. To determine the DA release induced by depolarization, conventional microdialysis was carried out, while, no-net flux microdialysis was carried out to determine the effect of acute exposure to WIN55212-2 on dopaminergic presynaptic dynamics. Fifteen minutes after conventional microdialysis, adult and adolescent rats were exposed to an acute dose ip of 1.2 mg/kg of WIN55212-2 or vehicle solution (2% tween 80, dissolved in saline solution). Rats were separated in 4 experimental groups: Adult Vehicle (n= 5), Adult WIN (n= 5), Adolescent Vehicle (n= 6) and Adolescent WIN (n= 5). A random number generator method was applied for pharmacological treatment. In the conventional microdialysis, after 40 minutes of stabilization, three consecutive samples were collected every 5 min for determination of an average DA basal level. The DA-evoked release was stimulated for 5 min using a 40 mM K+-KRP. Subsequently, N-KRP with 0.2 mM acid ascorbic (AA-KRP) was perfused during fifteen minutes prior to an acute injection of WIN55212-2 or vehicle. No-net flux microdialysis was carried out twenty minutes after the injection, according to Azocar et al. (2018) (Azocar et al., 2018). The probe was randomly perfused with five different

concentrations of dopamine: 0.0, 5.0, 10.0, 20.0 and 40.0 nM in AA-KRP to determine DA Cext and Ed, an indirect measure of DA uptake (Smith and Justice, 1994). A random number generator method was applied for perfusion of the different concentrations of DA. After a stabilization period of 20 minutes, three consecutive samples were collected every 5 minutes for each concentration of DA. The end of the microdialysis procedure was considered the endpoint of the experiments.

4.5.4 Histology

After microdialysis experiments, rats were decapitated under deep anesthesia (urethane 1.5 g/kg ip), and brains were extracted and cleaned with a saline solution (NaCl 0.9%). Brains were stored in 4% paraformaldehyde (PFA). At least two days before slicing, the brains were cryoprotected using a solution of sucrose 30%. To assess the location of probes, brains were frozen and coronally sliced in sections of 50 mm. Slices were stained with cresyl violet and the probe placement, observed in a light microscope, was localized using the atlas of Paxinos & Watson for rats (2009)(Paxinos and Whaton, 2009). Only data coming from correct probe placements were considered for further analysis (figure 1).

4.5.5 Analysis of dialysate samples

Quantification of DA was carried out as described previously (Escobar et al., 2012). Twelve µL of the collected samples were injected in a Rheodyne injector valve to a High-Performance-Liquid-Chromatography (HPLC) system (BASi America, West Lafayette, IN, USA) with the following configuration: a pump (Jasco LC-Net II/ADC), a UNIJET ™ LC column (part number: MF-8954, BASi) and an amperometric detector (LC4C, BASi America). The mobile phase contained 100 mM NaH_2PO_4, 1.0 mM EDTA, 1.0

mM octane-1-sulfonic acid sodium salt, and 5% acetonitrile (pH 3.0), and it was pumped at a flow rate of 700 µl/min. The potential of the amperometric detector was set at 650 mV. Under these experimental conditions, the retention time for DA was 6 min. The technician was blinded to the experimental group during the measurement of the samples in the HPLC.

4.5.6 Data Analysis

No-net flux microdialysis data were analyzed as described by Chefer et al. 2005, 2006 (Chefer et al., 2006, 2005). The amount of DA gained or lost from the probe during the no-net flux microdialysis (Cin-Cout) was calculated for each animal at each DA perfusion concentration (Cin: 0.0, 5.0, 10.0, 20.0 and 40.0 nM). The net change in DA (Cin–Cout) was plotted against Cin and subjected to linear regression. The point when no DA was gained or lost (Cin-Cout=0) represents an estimate of DA extracellular concentration (Cext). The slope of the linear regression line represents the Ed, an indirect measure of DA transporter (DAT) activity. Basal dialysate DA levels were calculated for each animal as the average of the three basal samples (Cin=0). All statistical analyses were performed using Prism 5.0 GraphPad Software. Data points outside the 95% confidence interval are treated as outliers and could be excluded from the data analysis. Neither point was excluded from the data analysis. Normality was checked with the Kolmogorov–Smirnov test. Resultant data were analyzed by unpaired t-test when appropriate. All data were reported as mean ± SEM.

4.6. Acknowledgments

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The authors have no conflict of interest.

4.7. Figures

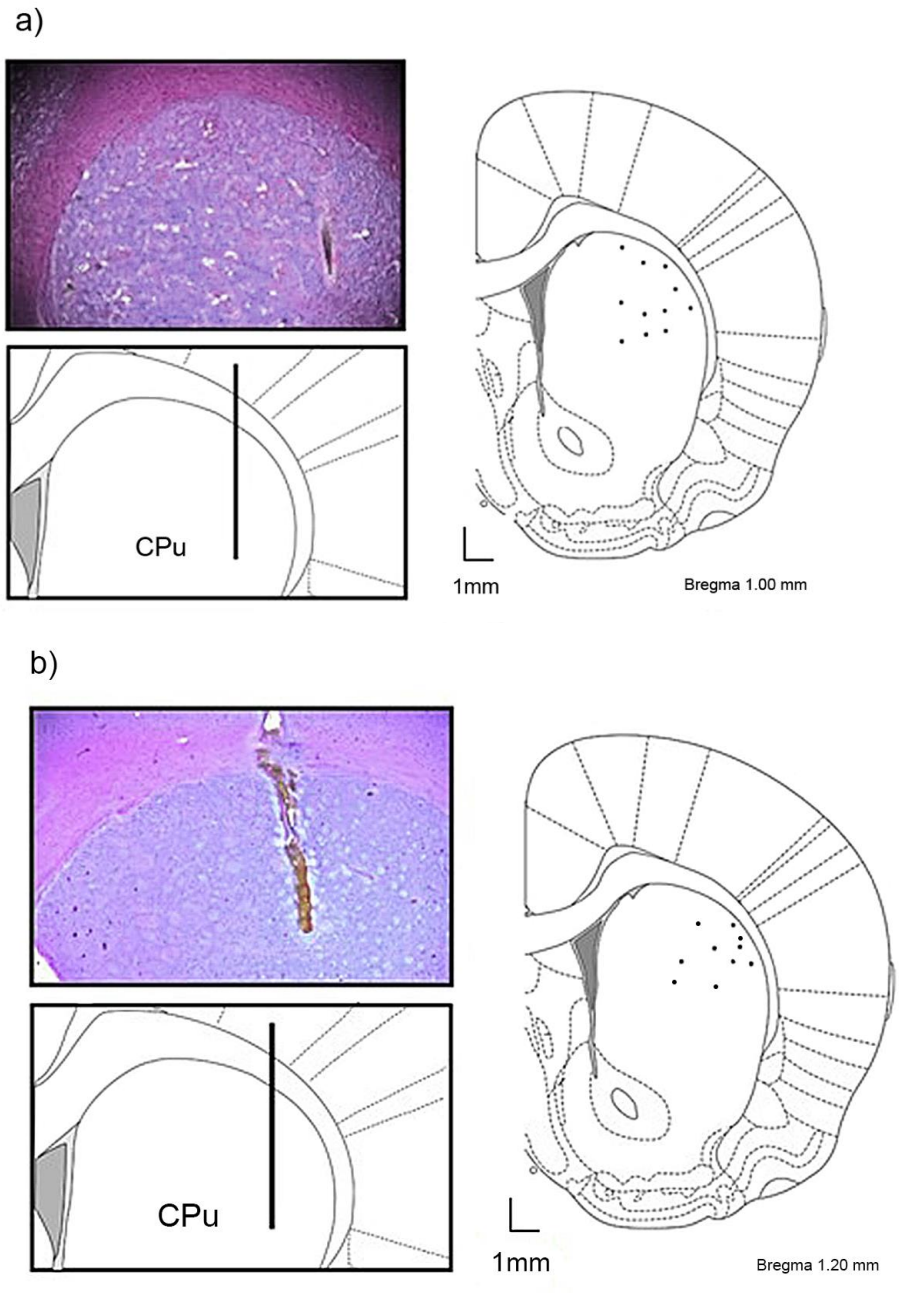

Figure 1: Anatomical placements of the microdialysis probe. The microdialysis probe was implanted in the dorsolateral striatum (DLS) using the coordinates: (a) for adolescent 1.0 mm anterior to bregma, 3.6 mm lateral, and 4.6 mm below dura and (b) for adult 1.2 mm anterior to bregma, 3.6 mm lateral, and 4.8 mm below dura according to the Atlas of Paxinos and Watson (2009). Left. Photo of representative microdialysis probe placement in the DLS of (a) adolescent rats and (b) adult rats (upper panel), and a map showing the

placement of the microdialysis probe (black line) of the representative photo (lower panel). Right. Representative anatomical placements of tips of the microdialysis probe (black dots) of (a) adolescent and (b) adult rats. Diagrams were adapted from Paxinos and Watson (2009). CPu: Caudate putamen

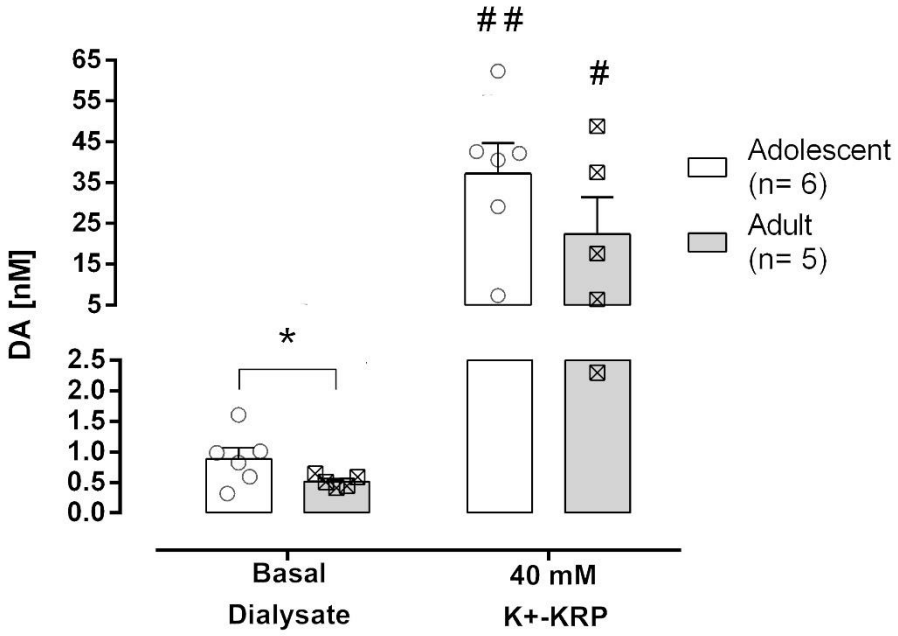

Figure 2: Age-dependent differences in DLS basal and stimulated DA dialysate. In vivo conventional microdialysis in anesthetized rats was carried out in adolescent (n= 6) and adult (n= 5). Data correspond to mean ± SEM. (a) Basal dopamine (DA) dialysate levels. *$p<0.05$ compared with the adolescent group according to paired t-test. (b) stimulated DA dialysate. #$p<0.05$ ## $p<0.01$ compared with respect basal group according to paired t-test.

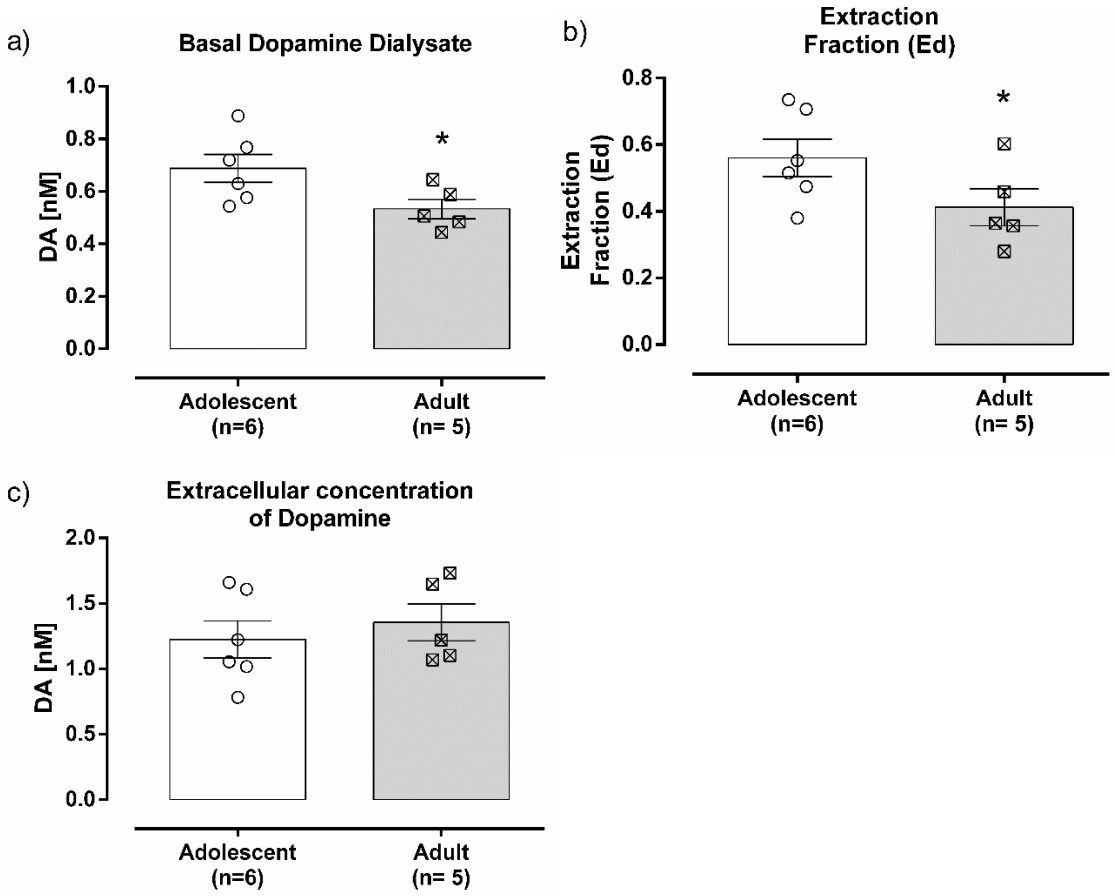

Figure 3: Age-dependent differences in DLS dopaminergic dynamics. In vivo microdialysis no-net flux in anesthetized rats was carried out in adolescent (n= 6) and adult (n= 5). Data correspond to mean ± SEM. (a) Basal dopamine (DA) dialysate levels. *$p<0.05$ compared with the adolescent group according to unpaired t-test. (b) Extraction fraction. *$p<0.05$ compared with the adolescent group according to unpaired t-test. (c) Extracellular dopamine concentration (DA Cext).

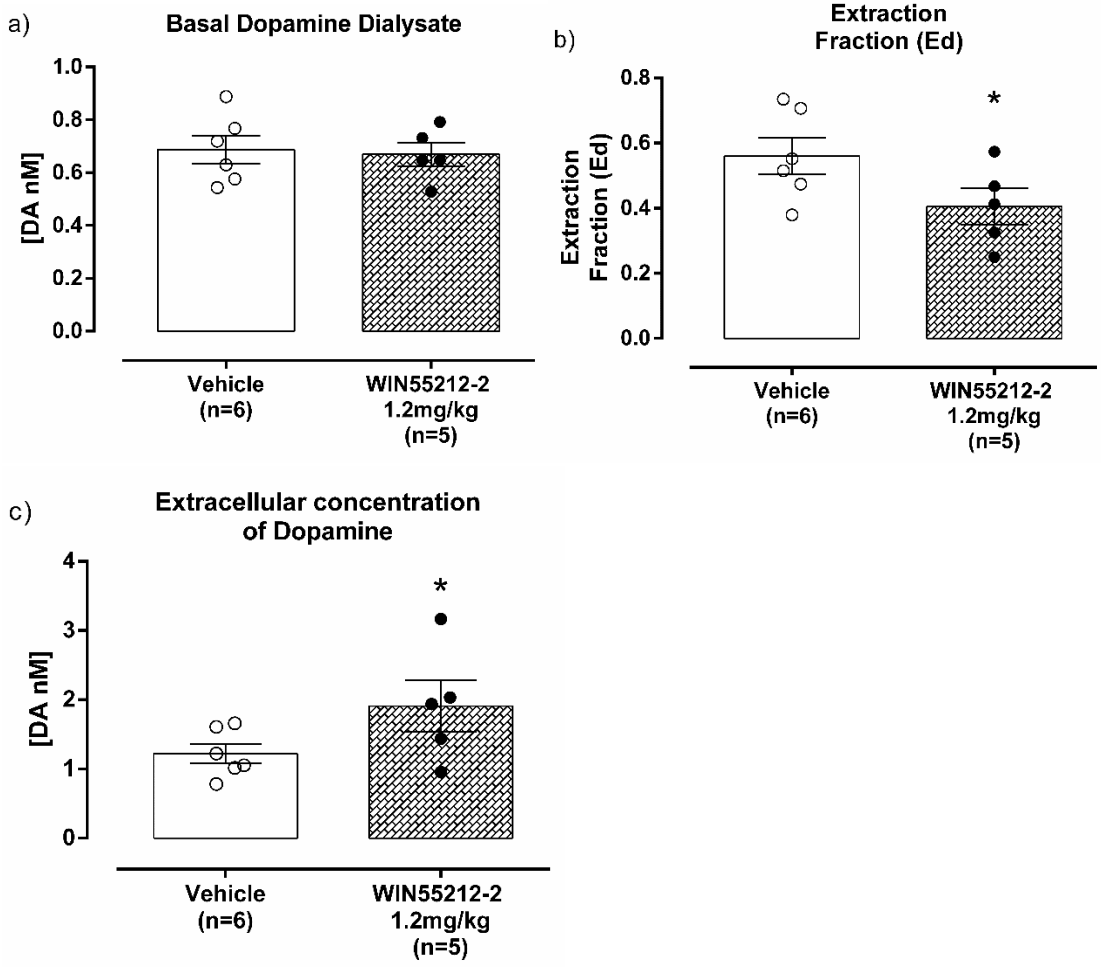

Figure 4: Effect of an acute injection of WIN55212-2 in DLS dopaminergic dynamics in adolescence. In vivo no-net flux microdialysis in anesthetized animals were carried out in vehicle (n= 6) and WIN55212-2 exposed rats (n= 5). Data correspond to mean ± SEM. (a) Basal dopamine (DA) dialysate levels. (b) Extraction fraction. *$p<0.05$ compared with vehicle group according to unpaired t-test. (c) DA Cext. *$p<0.05$ compared with vehicle group according to unpaired t-test.

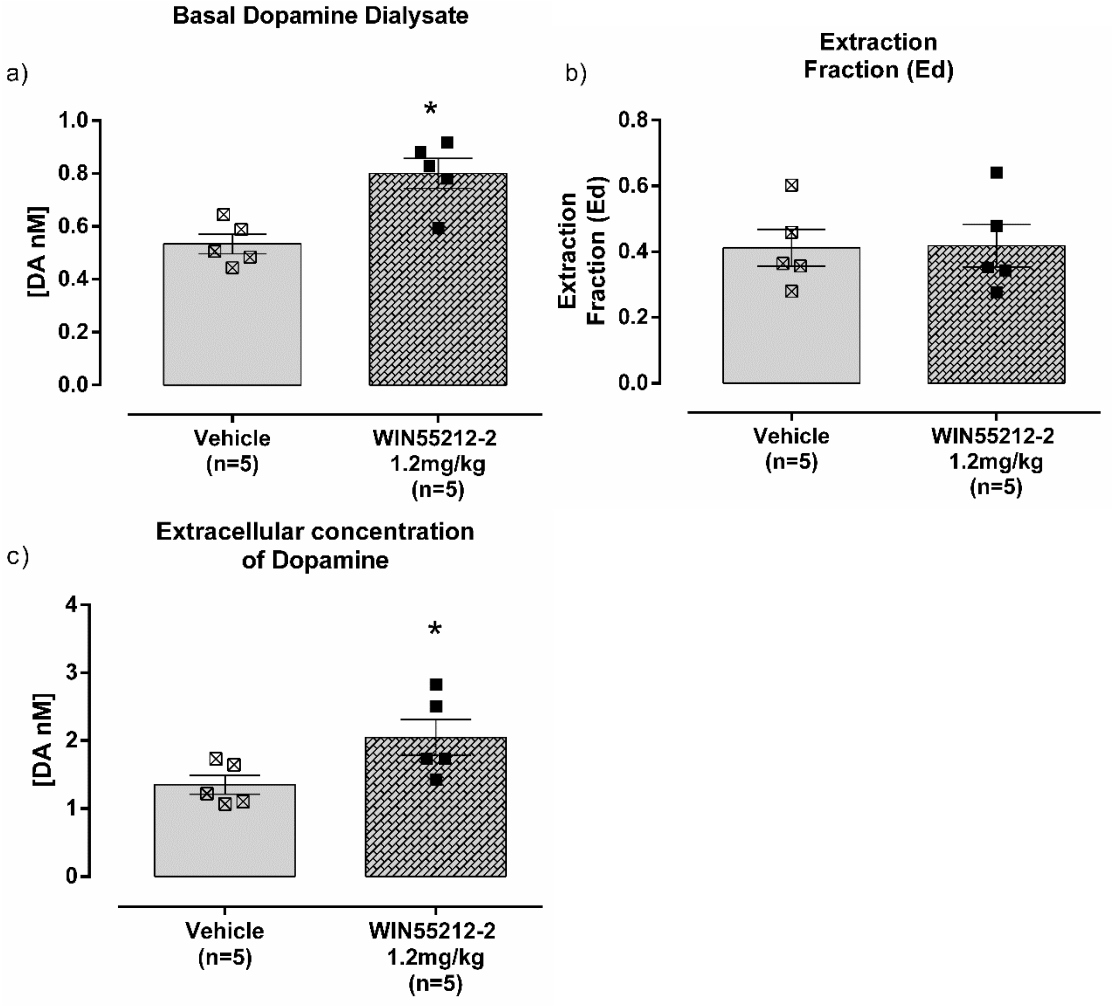

Figure 5: Effect of an acute injection of WIN55212-2 in DLS dopaminergic dynamics in adulthood. In vivo no-net flux microdialysis in adult anesthetized animals were carried out in vehicle (n=5 rats) and WIN55212-2 exposed rats (n=5 rats). Data correspond to mean ± SEM. (a) Basal dopamine (DA) dialysate levels. *$p<0.05$ compared with vehicle group according to unpaired t-test. (b) Extraction fraction. (c) DA Cext. *$p<0.05$ compared with vehicle group according to unpaired t-test.

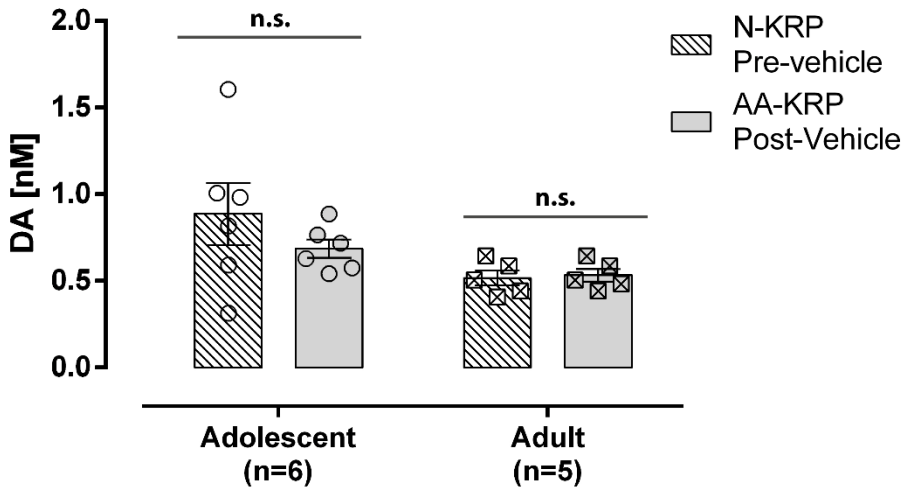

Figure Supplementary 1: DLS basal DA dialysate using KRP and after an acute injection of vehicle using AA-KRP. In vivo conventional microdialysis in anesthetized rats weres carried out in adolescent (n= 6) and adult (n= 5). Data correspond to mean ± SEM. n.s. $p > 0.05$ according to paired t-test.

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5. Chapter 2: Disinhibition of adult nigrostriatal dopaminergic pathway induced by chronic adolescent WIN55212-2 exposure

"Disinhibition of adult nigrostriatal dopaminergic pathway induced by chronic adolescent WIN55212-2 exposure."

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Abbreviations used: DLS: Dorsolateral Striatum; DA: Dopamine; Cext: Extracellular concentration; NAc: Nucleus Accumbens; VTA: Ventral Tegmental Area; CB1-R: CB1 Receptor; DAT: Dopamine Transporter; KRP: Krebs Ringer Phosphate; AA-KRP: Krebs Ringer Phosphate with 0.2 mM ascorbic acid; PFA: paraformaldehyde; CPu: Caudate putamen; HPLC: High-Performance-Liquid-Chromatography; Ed: Extraction Fraction; SEM: Standard Error of Mean.

Keywords: Dopamine, WIN55212-2, Adolescence, no-net flux microdialysis; Ventral pallidum; Dorsolateral Striatum

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5.1. Abstract

Background: During adolescence, critical neuronal circuits exhibit plasticity in response to physiological changes and to adapt to environmental events. In particular, nigrostriatal dopaminergic (DA) pathways are in constant change during development. Evidence suggests a relationship between early use of cannabinoids and psychiatric disorders characterized by altered DA systems, such as schizophrenia and drug addiction. However, it remains unclear what is the impact of adolescent exposure to cannabinoids on nigrostriatal DA pathways in adulthood. The primary aim of this research is to determinate the long-lasting effect of repeated activation of cannabinoid receptors during adolescence on DA activity of nigrostriatal pathways and the mechanisms underlying this impact.

Methods: Male Sprague-Dawley rats were treated with 1.2 mg/kg WIN55212-2, a type 1 (CB1) and 2 (CB2) cannabinoid receptor agonist, daily from postnatal day 40 to 65, then no-net flux microdialysis of DA in the dorsolateral striatum (DLS), DA electrophysiological activity, and microdialysis of GABA and glutamate in substantia nigra par compacta (SNc) were carried out during adulthood (Postnatal day 72 – 78).

Results: Repeated activation of cannabinoid receptors during adolescence increased extracellular levels and release of DA in DLS accompanied by an increase in population activity of DA neurons and a decrease of GABA extracellular levels in SNc in adulthood. Critically, local perfusion of bicuculline, a GABAa antagonist, into the ventral pallidum (VP) reversed the increased DA neuron population activity in SNc induced by adolescent cannabinoid exposure.

Conclusions: These results suggest that adolescent exposure to cannabinoid agonists produces long-lasting disinhibition of nigrostriatal DA transmission mediated by a decrease in GABAergic input from the VP.

5.2. Introduction

Adolescence is characterized by gradual physiological and behavioral changes during the transition from childhood to adulthood [1]–[3]. During the adolescence, neuronal connections overproduced in early life are amply pruned to respond to physiological changes and to adapt to environmental factors [1], [4]–[9].It has been observed that the mesolimbic dopaminergic pathway shows an age-dependent U-shaped trajectory. Both, dopamine (DA) concentration in nucleus accumbens (NAc) and firing rate of dopamine neuron in the ventral tegmental area (VTA), demonstrate a maximum peak during middle adolescence then decrease during adulthood [10], [11]. Therefore, the dynamic state of the dopaminergic circuits during adolescence could produce a time-window of vulnerability to environmental factors that could persist into adulthood.

Epidemiological evidence (based on the general global population) shows that cannabis is the third more popular substance of abuse, after nicotine and alcohol [12]. The annual prevalence of cannabis use by the adolescent population is higher than the general population in Europe and the Americas [12]. Interestingly, approximately a 17% of those who initiate the use of cannabis during adolescence develop cannabis use disorders; in contrast, just 9% who experiment cannabis in adulthood develop cannabis use disorders [13]–[15]. This evidence suggests that the neurobiological substrates associated with drugs abuse are more susceptible to cannabinoids in adolescence compared to adulthood.

Cannabinoids play a role in multiple physiological processes, such as appetite, mood, memory, and motivation [16], [17]. The CB1 receptors (CB1-R) are crucial to synaptic communication due to their ability to modulating the release of different neurotransmitters

such as gamma-aminobutyric acid (GABA), glutamate, and DA [18]–[20]. It has been observed that an acute systemic administration of WIN55212-2, a CB1/2 agonist, increases dopamine extracellular levels in the NAc [21] and dorsolateral striatum (DLS) [22] in conjunction with a higher firing rate of DA neuron in VTA and substantia nigra pars compacta (SNc), respectively [23]. Interestingly, the dopaminergic transmission in the mesolimbic and nigrostriatal pathways are strongly associated with the reinforcing effects of drug of abuses and the development of addictive behavior. The rewarding effects of natural reinforcers and drugs of abuse are accompanied by an increase in dopaminergic mesolimbic activity [24], [25]. The transition from the initial hedonic intake of drug of abuse to repeated and habitual consumption is associated with a progressive increase in DA neurotransmission from the mesoaccumbens to the nigrostriatal DA pathway [26]. Besides the acute effect of CB1 agonists in dopaminergic transmission, preclinical evidence indicates that the repeated activation of CB1-R during adolescence modify dopaminergic transmission in the mesolimbic pathway. Gomes et al. 2015 showed that adolescent exposure of WIN55212-2 increases the spontaneous activity of dopaminergic neurons of the ventral tegmental area (VTA) in adult rats [27]. Furthermore, Pistis et al. 2004 observed that adolescent exposure to WIN55212-2 produced long-term cross-tolerance to the effects of other drugs like morphine, cocaine, and amphetamine on the firing rate of VTA DA neurons [28]. While the long term effects of adolescent exposure to cannabinoid have been studied in the dopaminergic mesolimbic pathways, the enduring effects on the dopaminergic nigrostriatal pathway remain unclear [1], [27]–[29].

The main aim of this research is to study the long-term effects of adolescent exposure of WIN55212-2 on dopaminergic dynamics within the nigrostriatal pathway and to

determine the impact of adolescent exposure of WIN55212-2 on some of the afferent circuits that control the activity of nigral dopaminergic neurons, namely the ventral pallidum (Watabe-Uchida et al. 2012; Ogawa et al. 2014; Root et al. 2015). This research carried out using no-net flux microdialysis to determine the long-term changes induced by adolescent exposure of WIN55212-2 in the balance between release and uptake of DA in DLS. Furthermore, an extracellular single unit recording was carried out to study the firing pattern of DA neuron from SNc in anaesthetized adult rats which were treated with the cannabinoid agonist during adolescence. To establish the effects of adolescent exposure of WIN55212-2 in ventral pallidum-nigro-striatal neurocircuit, basal dialysate of GABA and glutamate was quantified in SNc by conventional microdialysis, and the activity of SNc DA neurons was determined after perfusion of the GABAa antagonist into the ventral pallidum.

5.3. Material and methods

5.3.1 Animals

In experiments of no-net flux and conventional microdialysis adolescent male Sprague-Dawley (post-natal day, PD, 33) rats were obtained from the Animal Care Faculty of the Biological Sciences, Pontificia Universidad Católica de Chile (Charles River; Wilmington, MA, USA; RRID: RGD_728193), under the supervision of a veterinarian. One week before microdialysis experiments, rats were maintained in the Animal Care Faculty of the Department of Pharmacy, Pontificia Universidad Católica de Chile, following the instruction of a protocol approved by the veterinarian. The electrophysiological experiments were carried out in adolescent (post-natal day 33) male Sprague-Dawley rats obtained from Envigo (Indianapolis, IN, USA). All rats were housed in groups of three per cage and kept at room temperature between 22–24°C on a 12 h light/dark cycle (lights on at 7:00 EST) with access to food and water ad libitum. Rats were handled for one week before starting the treatment. A total of 75 adolescent rats were divided into two groups; 38 rats for vehicle treatment, and 37 rats for WIN55212-2 treatment.

5.3.2 Reagents

The CB1/2 agonist, WIN55212-2 mesylate was emulsified in 2% Tween 80 in saline solution 0.9% at concentration of 1.2 mg/mL. GABAa antagonist, bicuculline methylbromide (0.1 µg) was mixed fresh in Dulbecco's buffer before starting recording. Both reagents were obtained from Sigma-Aldrich.

5.3.3 Treatment protocol

Adolescent animals were divided into two treatment groups: vehicle exposure and WIN55212-2 exposure. Adolescent rats were daily injected with the CB1/2 agonist WIN55212-2 (WIN) or vehicle (2% Tween 80 in saline solution 0.9%) since PD40 to PD65 similar as was described by Gomes et al. 2015 [27]. See details in the supplement and figure 1.

5.3.4 No-net flux Microdialysis experiments

To assess the effects of adolescent WIN55212-2 exposure in nigrostriatal dopaminergic transmission, adult rats were anesthetized with urethane 1.5 g/kg i.p. and no-net flux microdialysis experiments in DLS (+1.2 AP, -3.6 ML relative to bregma and -4.8 DV from the dura) were carried out as described in to Azocar et al. (2018) and Perez-Valenzuela et al. (2019). Then, DA basal dialysate, DA extracellular concentration (Cext) and extraction fraction (Ed), an indirect measure of DA uptake [30], were determined. For more details, see supplemental information.

5.3.5 Conventional Microdialysis

To study the mechanism underlying the facilitation of nigrostriatal dopaminergic pathway induced by WIN55212-2, microdialysis experiments in SNc (using a 40° angle from horizontal axis at the following the coordinates: -4.9 AP, -7.6 ML relative to bregma and -8.0 DV from the dura) were performed to quantify basal dialysate of glutamate and GABA. See details in the supplement.

5.3.6 Analysis of dialysate samples

DA was quantified using a High-Performance-Liquid-Chromatography (HPLC) system with an amperometric detector as described previously [31]. Glutamate and GABA were quantified using an HPLC system with a fluorescence detector as described previously[32]. For more details, see supplemental information.

5.3.7 Pharmacological Manipulations

To study the mechanism underlying the facilitation of nigrostriatal dopaminergic pathway induced by WIN55212-2, bicuculline methylbromide (0.1 µg) was mixed fresh in 0.5 µL Dulbecco's buffer and infused in ventral pallidum (VP) through a 30-gauge injection cannula protruding 2.0 mm past the end of the guide, at an injection volume of 0.1 µl every 1 min. The cannula was lowered to the following coordinate: AP -0.7; ML +2.9 from bregma; DV -6.0 from skull [33]. Ten minutes after the perfusion of bicuculline, the single unit recording was carried out. The doses used in this study has shown that it does not change the basal activity of DA neuron [34].

5.3.8 Single unit recording

To assess the effects of adolescent WIN55212-2 exposure in nigrostriatal dopaminergic transmission, and single unit recording in SNc were carried out after at least a week of treatment. Adult rats were anesthetized with chloral hydrate (400 mg/kg; i.p.), and the electrodes were lowered in SNc. The recording procedure was based on Gomes et al. 2015 [27]. Six vertical tracks, separated by 200 µm, were sampled in a predetermined pattern following the coordinate: AP - 4.9 to - 5.1, ML 2.0 to 2.4 from bregma and - 6.5 to - 9.0 DV from brain surface (See figure 2c). The electrode was lowered since 6.5 mm at a rate of 20 µm per each 4 seconds. All of experimental procedures were carried out DA neurons

were be identified according to well-established electrophysiological features [35], [36], which included the following criteria: (1) location; (2) an action potential duration > 2.2 ms; (3) slow firing rate (1–10 Hz); and (4) irregular and burst firing patterns, with the start of burst characterized by interspike interval < 80 ms, and the end of burst characterized by inter-spike interval > 160 ms. Three parameters of the DA neuron activity were analyzed: (1) the number of spontaneously active DA neurons per electrode track; (2) average firing rate; and (3) the percentage of spikes that occurred in bursts. See details in the supplement.

5.3.9 Histology

After experiments, rats were decapitated under deep anesthesia (urethane 1.5 g/kg ip. In the case of microdialysis experiments and chloral hydrate 400 mg/kg; i.p. in the case of single unit recording), and brains were extracted and cleaned with NaCl 0.9%. Brains were stored in 4% paraformaldehyde (PFA). At least two days before slicing, the brains were cryoprotected using a solution of sucrose 30%. To assess the location of probes, brains were frozen and sliced coronally into 50 mm. thick sections. Slices were stained with cresyl violet and the probe and electrodes placement, observed in a light microscope, was localized using the atlas of Paxinos & Watson for rat (2009) [33]. Only data from rats with correct probe placements were considered for further analysis (Figure 2).

5.3.10 Data Analysis

No-net flux microdialysis data were analyzed as described by Chefer et al. 2005, 2006 [37], [38]. The amount of DA gained or lost from the probe during the no-net flux microdialysis (Cin-Cout) was calculated for each animal at each DA perfusion concentration (Cin: 0.0, 5.0, 10.0, 20.0 and 40.0 nM). The net change in DA (Cin–Cout)

was plotted against Cin and subjected to linear regression. The point at which no DA was gained or lost (Cin-Cout = 0) represents an estimate of DA extracellular concentration (Cext). The slope of the linear regression line represents the extraction fraction (Ed), an indirect measure of DA transporter (DAT) activity. Basal dialysate DA levels were calculated for each animal as the average of the three basal samples (Cin=0).

All statistical analyses were performed using Prism 5.0 GraphPad Software. Data points outside of the 95% confidence interval were treated as outliers and could be excluded from the data analysis. No points were excluded from the data analysis. Normality was checked with the Kolmogorov–Smirnov test. Resultant data were analyzed by two-way ANOVA, Sidak post-test, and unpaired t-test when appropriate. All data are reported as mean ± SEM.

5.4. Results

5.4.1 Long-term effects of repeated exposure of WIN55212-2 in adolescence on DLS DA dynamics of adult rats.

Adolescent exposure with WIN55212-2 increased significantly basal DA dialysate in DLS of adult rats (Figure 3a vehicle group: 0.55 ± 0.11 nM; n=6; vs WIN group: 1.00 ± 0.12 nM, n = 6; $p< 0.05$ unpaired t-test). The adolescent exposure of WIN did not modify DA extraction fraction (Ed) compared to vehicle group (Figure 3b vehicle group: 0.38 ± 0.07; n = 6 vs WIN group: 0.42 ± 0.06, n = 6; $p> 0.05$; unpaired t-test). Consequently, DA Cext was higher in adult rats treated with WIN55212-2 during adolescent compared to treated rats with vehicle (Figure 3c vehicle group: 1.51 ± 0.13 nM, n= 6 vs WIN group: 2.42 ± 0.35 nM, n= 6 $p <0.05$; unpaired t-test).

5.4.2 Long-term effects of repeated exposure of WIN55212-2 during adolescence on SNc DA neuron activity

The adolescent administration of WIN55212-2 increases significantly the spontaneous activity of DA neurons of the SNc (Figure 4a vehicle group: 0.86 ± 0.09 cells/track; n= 7 vs WIN group: 1.29 ± 0.17 cells/track; n=7; $p< 0.05$; unpaired t-test). Differences in firing rate are not observed between groups treated with WIN55212-2 and vehicle (Figure 4b vehicle group: 3.10 ± 0.42 Hz; n=36 neurons vs WIN group: 3.54 ± 0.51 Hz; n=54 neurons; $p> 0.05$; unpaired t-test). Adolescent exposure of WIN55212-2 does not modify the percentage of spikes per burst compared to vehicle group (Figure 4c vehicle group: 17.55 ± 4.00 %; n= 36 vs WIN group: 17.29 ± 3.43 %; n=54; $p> 0.05$; unpaired t-test).

5.4.3 The mechanism underlying the long-term disinhibition of the nigrostriatal dopaminergic pathway: dialysate levels of GABA and glutamate in SNc of adult rats

Detection of dendritic basal dialysate of DA was quantified to determinate the correct location of the probe in the substantia nigra (Supplementary Figure 1). Basal DA dialysate in SNc is not modified by WIN treatment WIN adolescence compared to treated rat with vehicle (Figure supplementary 2).

Repeated exposure of WIN55212-2 during adolescence decreased significantly basal GABA dialysate in SNc of adult rats (Figure 5a vehicle group: 0.071 ± 0.006 uM; n=9; vs WIN group: 0.051 ± 0.005 uM; n = 9; $p< 0.05$; unpaired t-test). Adolescent exposure of WIN55212-2 does not modify basal glutamate dialysate levels compared to the vehicle group (Figure 5b vehicle group: 0.65 ± 0.10 uM; n = 9 vs WIN group: 0.57 ± 0.08 uM, n = 9; $p> 0.05$; unpaired t-test).

5.4.4 The mechanism underlying the long-term disinhibition of the nigrostriatal dopaminergic pathway: Role of VP GABAergic transmission

Nigrostriatal Dopaminergic Neuron Activity

There was no significant effect of WIN55212-2 treatment during adolescence ($F_{1.27}$= 1.447; $p > 0.05$) or on the impact of bicuculline perfusion into the VP ($F_{1,27}$= 0.997; $p > 0.05$) on DA neuron activity (two-way ANOVA, with adolescent treatment conditions as the between-subjects variables and perfusion in VP as the within-subjects variable). However, the interaction was significant ($F_{1.27} = 6.535$; $p<0.05$) (Fig. 6a). Reproducing the results observed in Fig. 4a, adolescent exposure of WIN55212-2 increases significantly population activity of DA neurons in adult rats after a buffer perfusion in VP, showing that cannulation did not impact the results (Figure 6a Buffer/Vehicle group: 0.905 ± 0.11 cells/track; n= 7 vs Buffer/WIN group: 1.39 ± 0.15 cells/track; n=7; $p< 0.05$; Sidak post-test).

As observed in VTA [34], changes in DA neuron population activity was not observed in adult rats treated with vehicle after perfusion with bicuculline (Figure 6a Buffer/Vehicle group: 0.905 ± 0.11 cells/track; n= 7 vs Bicuculline/Vehicle group: 1.09 ± 0.16 cells/track; n=9; $p> 0.05$ according to Sidak post-test). Perfusion with bicuculline into the VP reversed the increase in DA neuron population activity induced by adolescent exposure to WIN55212-2 (Figure 6a Bicuculline/Vehicle group: 1.09 ± 0.16 cells/track; n= 9 vs Bicuculline/WIN group: 0.88 ± 0.10 cells/track; n=8; $p> 0.05$; Sidak post-test; Buffer/WIN group: 1.39 ± 0.15 cells/track; n= 7 vs Bicuculline/WIN group: 0.88 ± 0.10 cells/track; n=8; $p< 0.05$; Sidak post-test).

There was no impact of adolescent treatment of WIN55212-2 ($F_{1.187}$= 1.615; $p > 0.05$) or of bicuculline perfusion into the VP ($F_{1.187}$= 3.570; $p > 0.05$) on DA neuron firing rate (two-way ANOVA, with adolescent treatment conditions as the between-subjects variables and perfusion in VP as the within-subjects variable). In addition, the interaction was non-significant ($F_{1.187} = 0.065$; $p>0.05$) (Fig. 6b).

Bicuculline perfusion into the VP did not modify firing rate of dopamine neurons of adult rats treated with vehicle during adolescence (Figure 6b Buffer/Vehicle group: 2.4 ± 0.26 Hz; n= 38 vs. Bicuculline/Vehicle group: 2.99 ± 0.21 Hz; n= 58; $p> 0.05$; Sidak post-test). Furthermore, changes in DA neuron firing rate induced by bicuculline perfusion into the VP were not observed in adult rats treated with WIN55212-2 during adolescence (Figure 6b Buffer/WIN group: 2.01 ± 0.21 Hz; n= 53 vs Bicuculline/WIN group: 2.15 ± 0.35 Hz; n= 42; $p< 0.05$; Sidak post-test).

There was no significant effect of WIN55212-2 treatment during adolescence ($F_{1.187}$= 1.79; $p > 0.05$) or on the impact of bicuculline perfusion into the VP ($F_{1.187}$= 0.21; $p > 0.05$) on DA neuron burst firing pattern (two-way ANOVA, with adolescent treatment conditions as the between-subjects variables and perfusion in VP as the within-subjects variable). In addition, the interaction was ($F_{1.187} = 0.001$; $p>0.05$) (Fig. 6c).

Bicuculline perfusion into the VP did not modify DA neuron burst firing pattern of adult rats treated with vehicle during adolescence (Figure 6c Buffer/Vehicle group: 20.01 ± 4.17 %; n= 38 vs Bicuculline/Vehicle group: 18.52 ± 3.27 %; n= 58; $p> 0.05$; Sidak post-test). Furthermore, changes in DA neuron burst firing pattern induced by bicuculline perfusion in VP were not observed in adult rats treated with WIN55212-2 during adolescence

(Figure 6c Buffer/WIN group: 15.46 ± 3.02 %; n= 53 vs Bicuculline/WIN group: 13.8 ± 3.35 %; n= 42; $p < 0.05$; Sidak post-test).

5.5. Discussion

Cannabinoid use in the adolescent population has increased significantly in the last years in across the world [12]. However, information regards its long-term effects on brain plasticity after adolescent exposure to cannabinoid agonists is still sparse. Our results show that adolescent exposure to the cannabinoid agonist WIN55212-2 renders the nigrostriatal pathway hyper-excitable during adulthood. No-net flux microdialysis experiments show a significant increase in the DA Cext, associated with an increase in DA release, in DLS of adult rats after adolescent exposure to WIN55212-2. Moreover, an increase in population activity of DA neurons in the SNc was accompanied by a decrease in GABA extracellular levels is observed in adult rats after adolescent exposure to WIN 55212-2. Interestingly, the local perfusion of the GABAa antagonist bicuculline into the VP reverses the increases in DA neuron population activity in the SNc. These findings show that the repeated activation of the cannabinoid receptor during adolescence is accompanied by long-lasting disinhibition of nigrostriatal DA transmission mediated by a decrease in GABAergic input from the VP.

The no-net flux experiments indicate that adolescent WIN55212-2 is accompanied by an increase in DLS DA release without significant changes in Ed in DLS of adult rats [30] [38]. An increase in DA release is consistent with previous evidence showing an increase of DA turnover in DLS of adult rats after repeated treatment of 2 mg/kg WIN55212-2 during early adolescent (PD 35 to 48) [39]. Although an inhibitory effect of acute

WIN55212-2 on DA uptake in adult rats has been observed [40], no changes in DA Ed in DLS was evident in adult rats, suggesting that adolescent exposure to WIN55212-2 is not accompanied by enduring modifications in DAT activity. Previous experiments of radioligand binding have shown that treatment with CB1 agonist during early adolescence (PD 28-38) did not modify the binding of DAT in the dorsal striatum of adult male rats [29]. Collectively, the no-net flux experiments support the idea that the increase in DA Cext after adolescent exposure to WIN55212-2 depends on neuronal excitability more than on pre-synaptic control involving DA uptake.

Consistent evidence has reported that an increase in the activity of mesencephalic DA neurons produces an increase in DA efflux in the terminal regions [34] [41]. Simultaneous microdialysis and extracellular recording experiments showed that an increase in firing rate and burst firing of SNc DA neurons increases basal DA dialysate in the DLS [34], [41]. In addition, evidence indicates that an increase in the proportion of spontaneous firing DA neurons produces an increase of DA basal dialysate [34], [41]. Supporting the rise in DA Cext in DLS, in vivo single unit recordings of DA neurons from SNc showed that adolescent exposure of WIN55212-2 increases population activity of DA neurons in the SNc without significant changes in firing rate or burst activity. The similar long-term effect of cannabinoids has been observed in VTA DA neurons [27], [42]. Here, Gomes et al. 2015 showed that the intermittent exposure to WIN552112-2 during adolescence (PD 40 to 65) produces an increase in VTA DA neuron spontaneous activity of [27]. Similarly, Renard et al. 2016 showed that adolescent exposure of Δ9 Tetrahydrocannabinol (Δ^9-THC) increases the firing rate, the bursting activity, and the population activity of VTA DA neurons [42]. Together, no-net flux microdialysis and electrophysiological recording

data indicate that the increase in DA Cext in DLS of adult rats after adolescent exposure to WIN55212-2 is associated with the facilitation of DA release that corresponds to a rise in the number of spontaneously active DA neurons in the SNc.

Mesencephalic DA neurons show three different states of activity: non-firing state, tonic firing state, and phasic firing state [43]. It has been indicated that in VTA DA neurons, these states are modulated by different afferents and neurotransmitters. The population activity, i.e., the number of neurons showing tonic firing, is regulated by GABA input from ventral pallidum (VP) and the phasic or burst pattern of neuronal activity is driven by glutamatergic input from pedunculopontine tegmentum afferents [34]. Due to the fact that the VTA and SNc share some afferent inputs [44], [45], we examined whether a decrease in GABA extracellular levels in SNc driven by VP afferents underlies the increase in population activity in the SNc after adolescent exposure to WIN 55212-2.

To test this hypothesis, conventional microdialysis in SNc and single unit recording experiments in SNc after bicuculline perfusion into VP were carried out in adult rats. In agree with evidence in the VTA [34], adolescent exposure to WIN-55212 is accompanied by a significant decrease in extracellular GABA levels in SNc of adult rats. Moreover, significant changes in extracellular glutamate were not observed after adolescent exposure to WIN55212-2, which is consistent with a lack of impact on SNc DA neuron burst firing pattern. These results are consistent with evidence highlighting the susceptibility of GABAergic neurotransmission to the long-term effects of adolescent cannabinoid exposure. Adults rats exposed to WIN 5212-2 during early and mid-adolescence showed persistent disinhibition of neuronal activity in the medial prefrontal cortex associated with

a decrease in GABA neurotransmission [46]. In addition, adolescent exposure to Δ^9-THC decreased extracellular GABA and reduced the expression of a protein related to biosynthesis of GABA in the PFC [47]. These long-term cortical dysfunctions in GABA neurotransmission observed after early exposure to a cannabinoid has been associated with psychosis risk in adulthood [48]. Furthermore, single unit recording experiments showed that the bicuculline perfusion into the VP reversed the increase in population activity in the SNc observed in adult rats after adolescent WIN 55212-2 exposure. Given that population activity depends on GABA input from the VP [34], these results strongly suggest that inhibition of neuronal activity in the VP may underlie the decrease in extracellular GABA levels that in turn increase SNc DA neuron population activity after adolescent exposure to WIN-55212 (Figure 7).

In summary, our results showed that adolescent exposure of WIN55212-2 decreased GABA release in the SNc that was associated with an increase in DA neuron population activity of presumably driven by a reduction in VP afferent inhibition. Moreover, the adolescent exposure to WIN55212-2 was accompanied by a significant increase in DA release in the DLS, suggesting sustained disinhibition of the nigrostriatal pathway after repeated activation of CB1-R during adolescence. This could underlie a mechanism by which long-term effects of adolescent exposure to cannabinoids on dopaminergic pathways that modulate behavior such us habit learning may contribute to habitual consumption of drugs of abuse.

5.6. Acknowledgments

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5.7. Figures

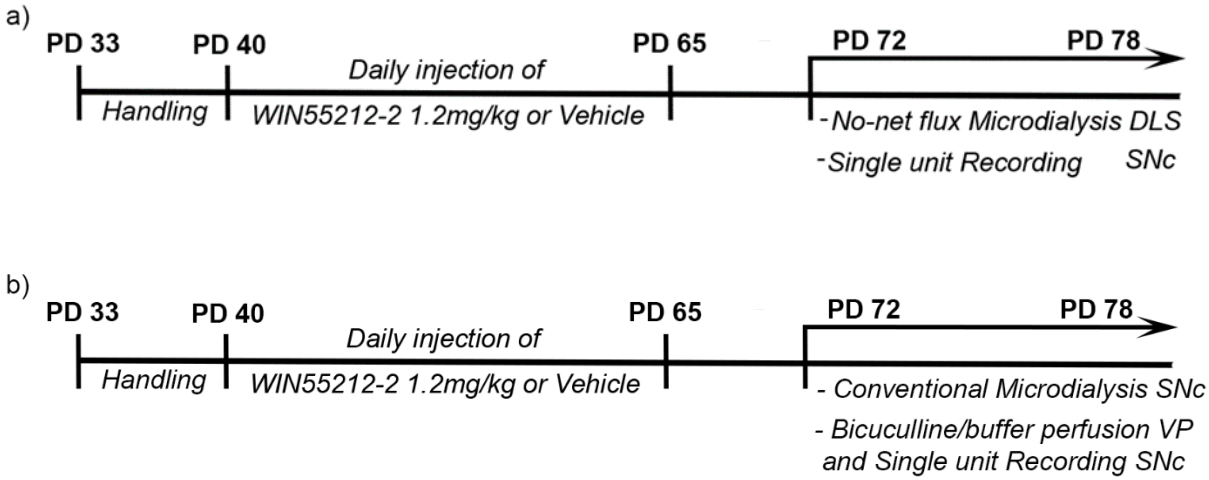

Figure 1: Schematic of the treatment protocol.

All adolescent rats (PD33) were handled for 1 week before injection with WIN55212-2 or vehicle once daily for 25 days (Between PD 40 and PD65). (a) To assess the effects of adolescent WIN exposure on nigrostriatal dopaminergic transmission, no-net flux microdialysis experiments in DLS, and single unit recording in SNc were carried out at least a week after treatment. (b) To study the mechanism underlying the facilitation of nigrostriatal dopaminergic pathway activity induced by WIN55212-2, microdialysis experiments in SNc were performed to quantify glutamate and GABA and single unit activity in SN after local perfusion of bicuculline in after at least a week of treatment.

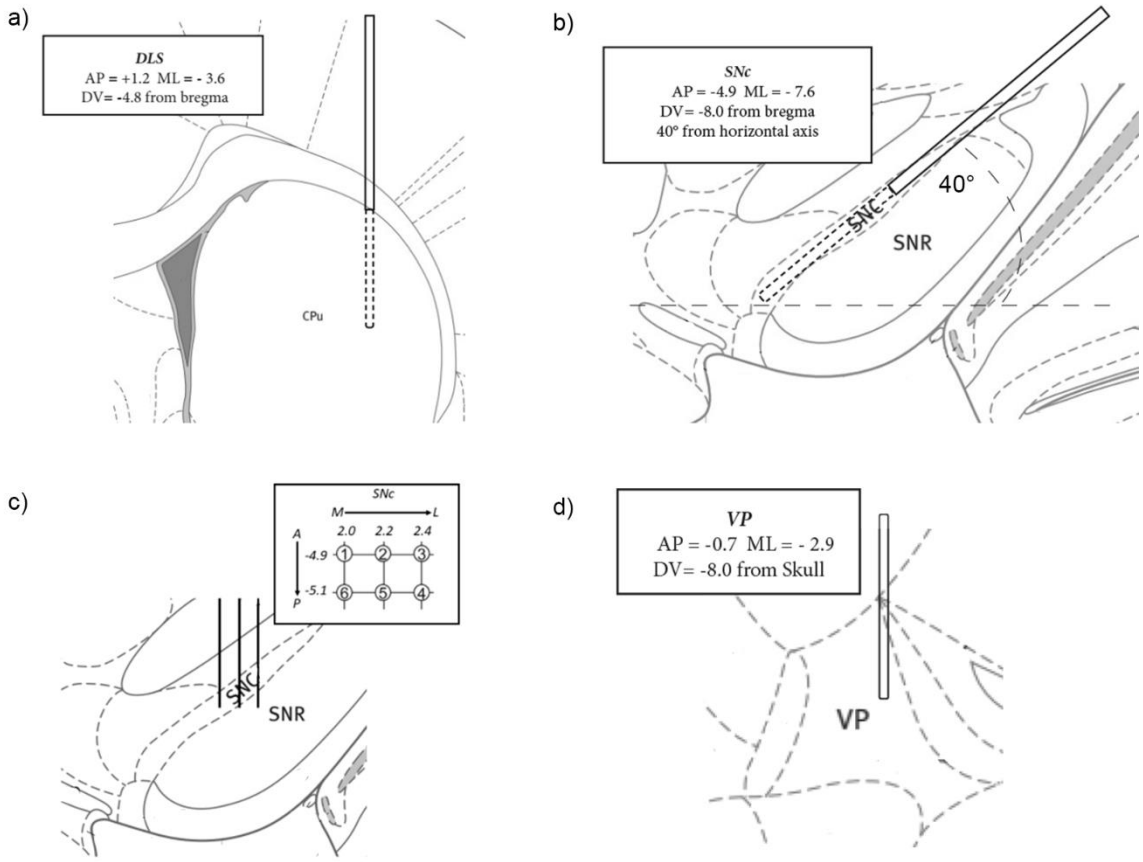

Figure 2: Representative anatomical placements of microdialysis probe, electrode, and cannula.

The microdialysis probe was lowered into the (a) dorsolateral striatum (DLS) and (b) substantia nigra par compacta (SNc) using the coordinates: (a) 1.2 mm anterior to bregma, 3.6 mm lateral, 4.8 mm below dura and (b) 4. mm posterior to bregma, 7.6 mm lateral, 8.0 mm below dura using an angle of 40° from horizontal axis according to Paxinos and Watson (2009). The electrode was lowered in a preset six track pattern with each track separated by 200 µm in the (c) SNc of each rat at the following the coordinates: AP - 4.9 to – 5.1, ML 2.0 to 2.4 from bregma and - 6.5 to - 9.0 DV from the brain surface. A 30-gauge injection cannula protruding 2.0 mm past the end of the guide cannula was used to administer the drug at an injection volume of 0.1 µl per min. A guide cannula was lowered in (d) ventral pallidum using following coordinates: AP -0.7; ML +2.9 from bregma; DV -6.0 from the skull. A 30-gauge injection cannula protruding 2.0 mm past the end of the guide was used to perfuse a solution of bicuculline at a rate of 0.1 µl/min over 5 min.

Diagrams were adapted from Paxinos and Watson (2009). CPu: Caudate putamen; SNC: Substantia nigra pars compacta; SNR: Substantia nigra pars reticulata.

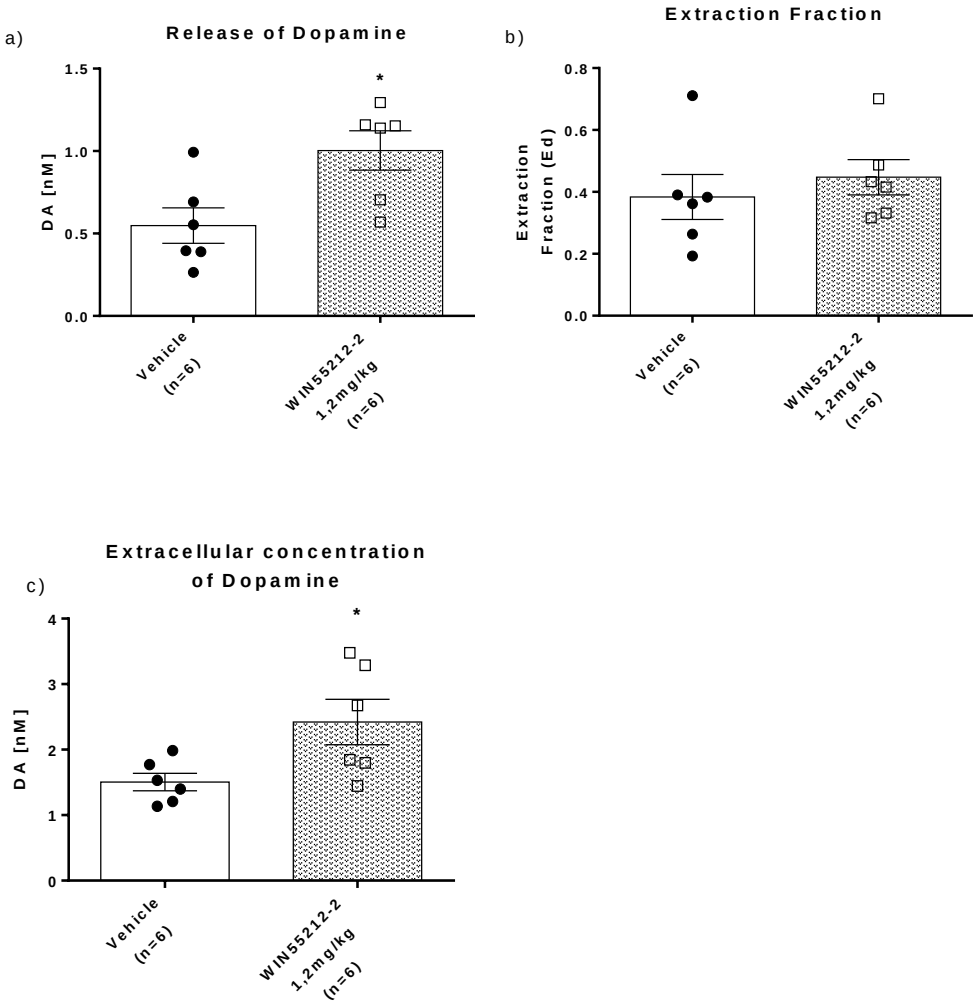

Figure 3: Adolescent exposure to WIN55212-2 increased DA release and DA Cext in DLS in adult rats.

In vivo no-net flux microdialysis in anesthetized adult rats was carried out in vehicle (n= 6) and WIN55212-2 adolescent exposed rats (n= 6). Data correspond to mean ± SEM. (a) Basal DA dialysate levels. *$p<0.05$ compared with vehicle group; unpaired t-test. (b) Extraction fraction. (c) DA Cext. *$p<0.05$ compared with vehicle group; unpaired t-test.

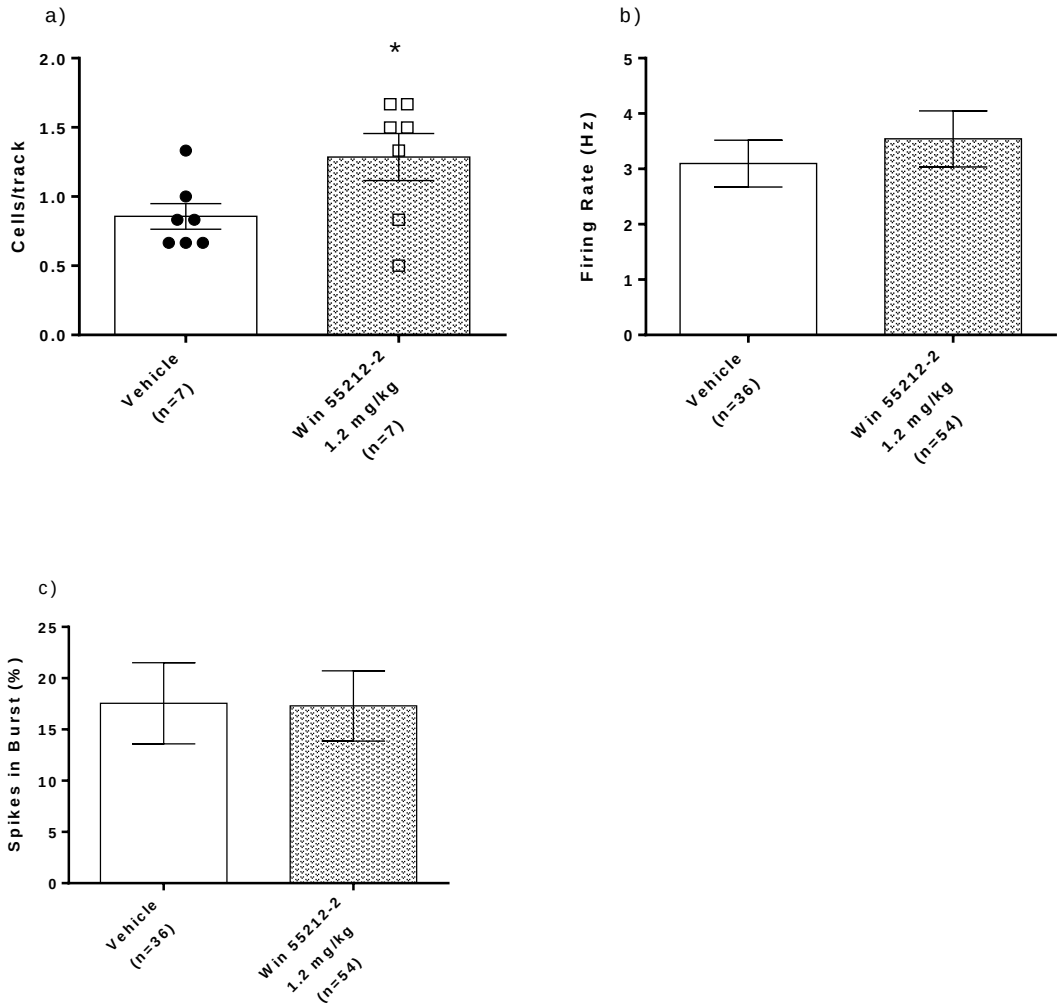

Figure 4: Adolescent exposure to WIN55212-2 increased SNc DA neuron population activity in adult rats.

In vivo single unit recording in anesthetized adult animals was carried out in vehicle (n= 7) and WIN55212-2 adolescent exposed rats (n= 7). Data correspond to mean ± SEM. (a) population activity of DA neuron. *$p<0.05$ compared with vehicle group; unpaired t-test. (b) Average firing rate. (c) Percentage of spike in a burst.

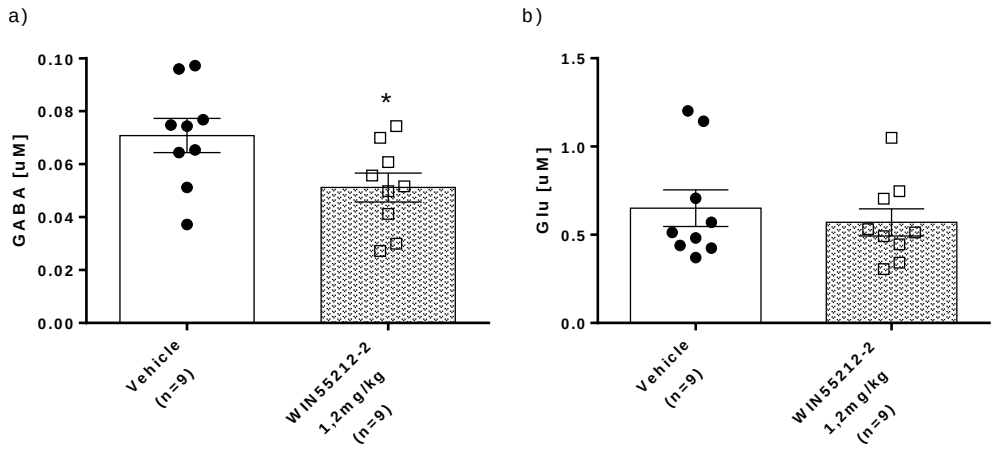

Figure 5: Adolescent exposure to WIN55212-2 decreased basal GABA dialysate in the SNc of adult rats.

In vivo conventional microdialysis in anesthetized adult rats was carried out following vehicle (n= 9) or WIN55212-2 exposure during adolescence (n= 9). Data correspond to mean ± SEM. (a) Basal GABA dialysate. *$p<0.05$ compared with vehicle group; unpaired t-test. (b)Basal Glutamate dialysate.

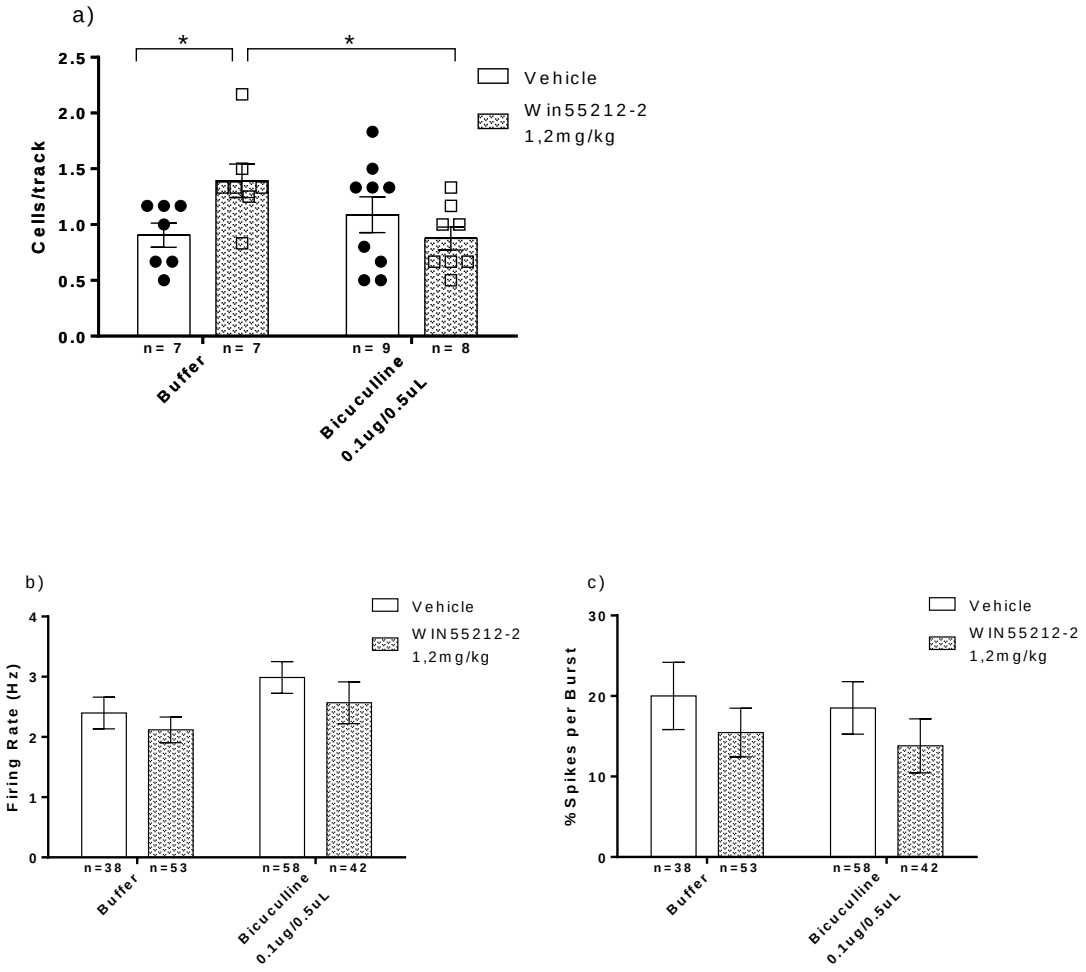

Figure 6: Local perfusion of bicuculline into the VP reversed the increase in DA neuron spontaneous activity induced by adolescent exposure to WIN55212-2.

Local perfusion of bicuculline (0.1 ug/0.5uL) or buffer solution was carried out in VP 10 minutes before in vivo single unit recording. Recordings were performed in anesthetized adult rats following vehicle (buffer/vehicle n= 7 and bicuculline/vehicle n=9) and WIN55212-2 adolescent exposure (buffer/WIN n= 7 and bicuculline/WIN n= 8). Data correspond to mean ± SEM. (a) population activity of DA neurons. *$p<0.05$ compared with buffer/WIN group; Sidak post-test. (b) Average firing rate. (c) Percentage of spike in burst.

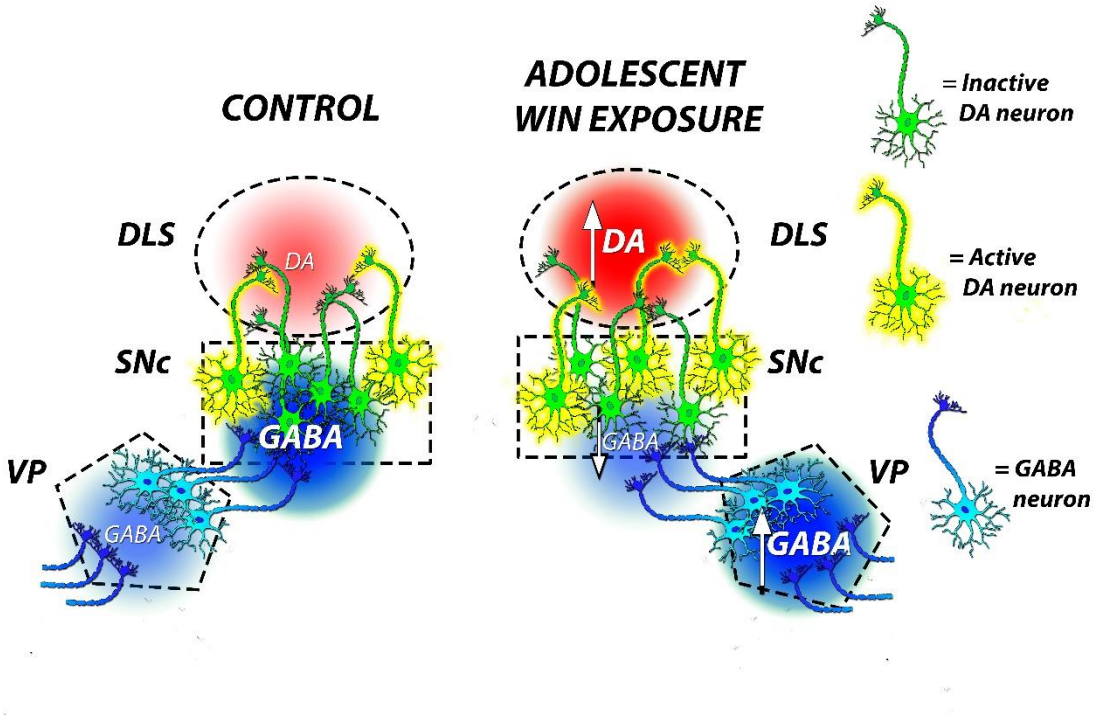

Figure 7: Proposed mechanism of disinhibition of dopaminergic nigrostriatal neurons induced by adolescent exposure to WIN55212-2. In control conditions, the VP is constantly inhibiting DA neuron activity in SNc via GABA release. This basal inhibition maintains a proportion of DA neurons in a hyperpolarized, nonfiring state, causing DA Cext to remain at normal levels. Following adolescent exposure to WIN55212-2, a higher GABAergic tone inhibits GABA neurons in the VP, which in turn produces disinhibition of DA neuron firing and increasing DA Cext in the DLS.

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5.9. Supplemental Information

All procedures were carried out in strict accordance with the guidelines published in the "NIH Guide for the Care and Use of Laboratory Animals" (8th Edition) and the principles presented in the "Guidelines for the Use of Animals in Neuroscience Research" by the Society for Neuroscience. The microdialysis protocols were also approved by the local bioethics committees, verifying that it complies with the basic principles set forth in Chilean Law 20.380 on Animal Protection 2009 (ID project: 160816013). The rats were identified with markings on the tail and numbered accordingly. Single unit recording protocols were approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh.

Treatment protocol

Adolescent animals were divided into two treatment groups: vehicle exposure and WIN55212-2 exposure. Adolescent rats were injected with the CB1/2 agonist WIN55212-2 (WIN) or vehicle since PD40 to PD65 similar as was described by Gomes et al. 2015 [1]. The drug was daily administered intraperitoneally at a dose of 1.2 mg/kg in a volume of 1 mL/kg. A total of 25 injections were carried out per rat.

Microdialysis experiments

Adult rats were anesthetized with urethane 1.5 g/kg i.p. and placed in a stereotaxic apparatus. The urethane was chosen due to the extended half-life. The skull of the rat was exposed, and a hole was drilled.

No-net flux Microdialysis

A concentric microdialysis probe (CMA 11 Microdialysis, Holliston, MA) was lowered vertically into the DLS using the coordinates: +1.2 AP, -3.6 ML relative to bregma and -4.8 DV from the dura [2]. Body temperature was maintained by a thermostatically controlled electric heating pad. The probe was perfused for 40 minutes with Krebs–Ringer Phosphate buffer with 0.2 mM acid ascorbic (AA-KRP) using a Harvard infusion pump (Harvard Apparatus, Holliston, MA) at a rate of 2 µL/min to allow equilibration. The composition of the AA-KRP was 120 mM NaCl, 2.4 mM KCl, 1.2 mM CaCl2, 0.9 mM NaH2PO4, 1.4 mM Na2HPO4, and 0.2 mM of ascorbic acid (pH 7.4). No-net flux microdialysis was carried out after the stabilization period, according to Azocar et al. (2018) and Perez-Valenzuela et al. (2019) [3]. The probe was randomly perfused with five different concentrations of dopamine: 0.0, 5.0, 10.0, 20.0 and 40.0 nM in AA-KRP to determine DA basal dialysate, DA extracellular concentration (Cext) and extraction fraction (Ed), an indirect measure of DA uptake [4]. After a stabilization period of 20 minutes, three consecutive samples were collected every 5 minutes for each concentration of DA. Perfusion samples were collected in 2 µL of perchloric acid (0.2 N) and maintained on ice (4°C) until quantification.

Conventional Microdialysis

A concentric microdialysis probe (CMA 11 Microdialysis, Holliston, MA) was lowered diagonally into the SNc using a 40° angle from the horizontal axis at the following the coordinates: -4.9 AP, -7.6 ML relative to bregma and -8.0 DV from the dura [2]. Body temperature was maintained by a thermostatically controlled electric heating pad. The probe was perfused for 40 minutes with Krebs–Ringer phosphate (KRP) buffer at a rate

of 2 µL/min using a Harvard infusion pump (Harvard Apparatus, Holliston, MA). After a stabilization period using KRP, six consecutive samples were collected every 5 min; three samples for determination of an average of dendritic release of DA and three samples for determination of an average GABA and glutamate basal dialysate. Perfusion samples were collected in 2 µL of perchloric acid (0.2 N) and maintained on ice (4°C) until quantification.

Analysis of dialysate samples

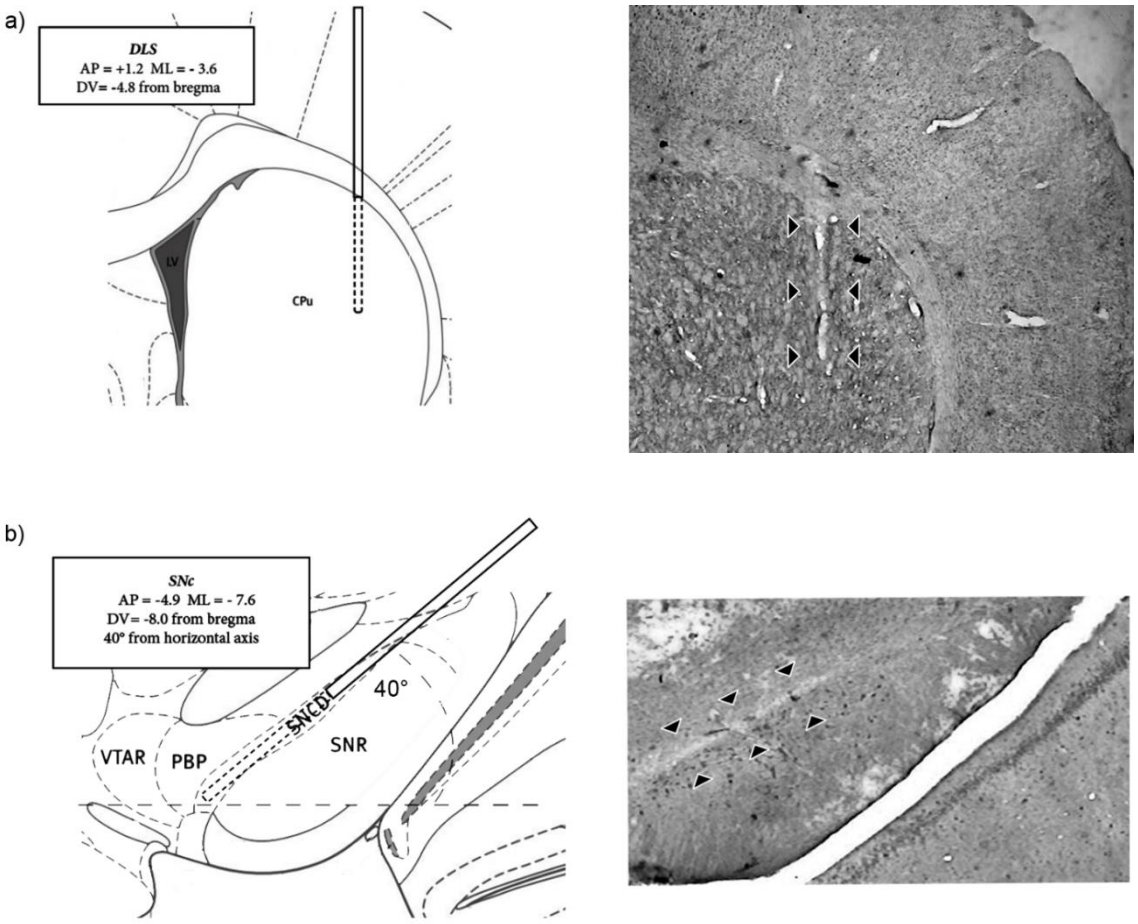
Quantification of DA was carried out as described previously [5]. Twelve (ten µL of sample plus two µL PCA 0.2 N) µL of the collected samples were injected in a Rheodyne injector valve to a High-Performance-Liquid-Chromatography (HPLC) system (BASi America, West Lafayette, IN, USA) with the following configuration: a pump (Jasco LC-Net II/ADC), a UNIJET ™ LC column (part number: MF-8954, BASi) and an amperometric detector (LC4C, BASi America). The mobile phase contained 100 mM NaH2PO4, 1.0 mM EDTA, 1.0 mM octane-1-sulfonic acid sodium salt, and 5% acetonitrile (pH 3.0), and it was pumped at a flow rate of 700 µl/min. The potential of the amperometric detector was set at 650 mV. Under these experimental conditions, the retention time for DA was 6 min. The HPLC-fluorometric determination for GABA and glutamate were similarly performed as described previously [6]. Briefly, 12 µL of the sample of dialysis perfusate and PCA were mixed with 12 µL of KRP, 4 µL of borate buffer (pH 10.8), and then the mixture was derivatized by adding 4µL of fluorogenic reagent (20mg of orthophthaldehyde and 10µL of β-mercaptoethanol in 5mL of ethanol). At 90 seconds after derivatization, samples were injected into an HPLC system with the

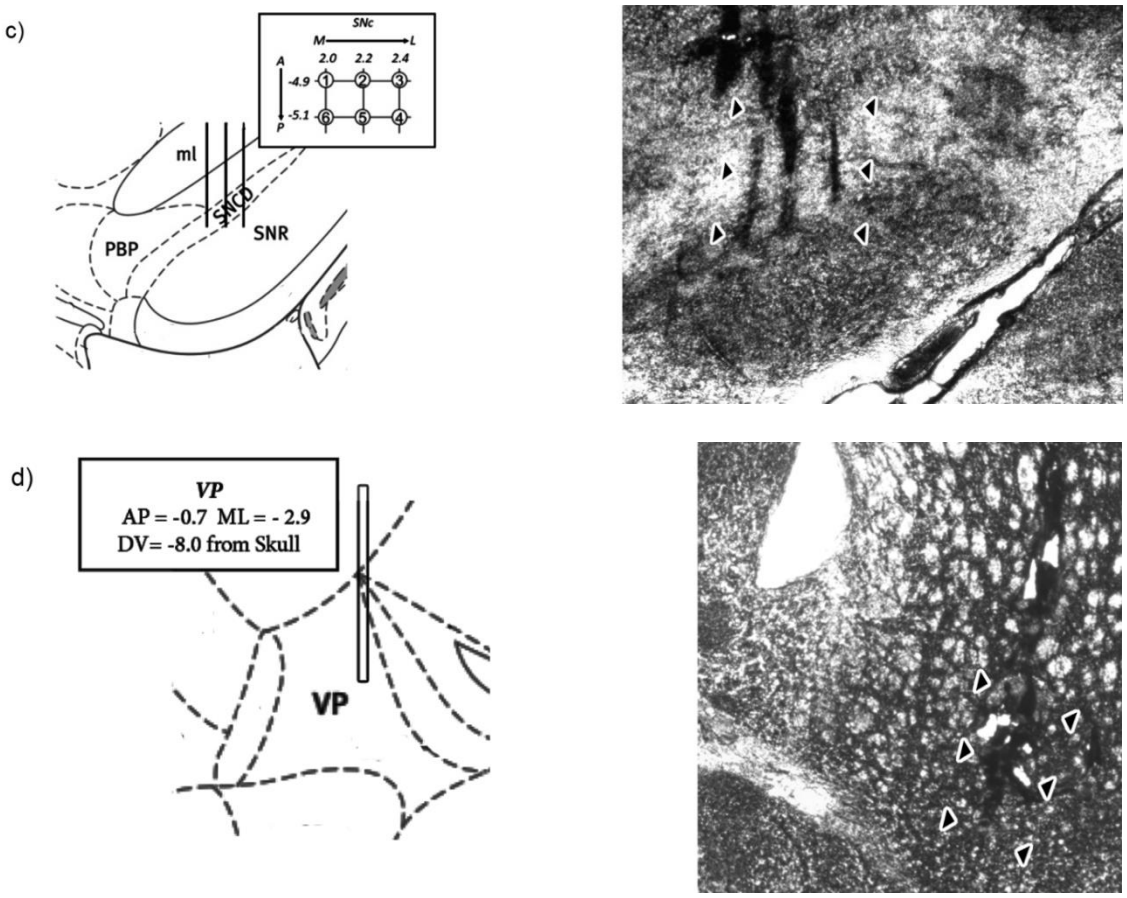
following configuration: quaternary gradient pump (Jasco Co. Ltd., Tokyo, Japan), a C-18 reverse phase column (Kromasil®; Eka Chemicals, Bohus, Sweden), and a fluorescence detector (Jasco Co. Ltd.). A mobile phase containing 0.1M NaH2PO4 and 23% CH3CN (pH 5.7) was pumped for 20 min. The flow rate of the mobile phase was set at 1.2 mL/min and the retention time for glutamate was 3 min and for GABA was 16 min.

Single unit recording

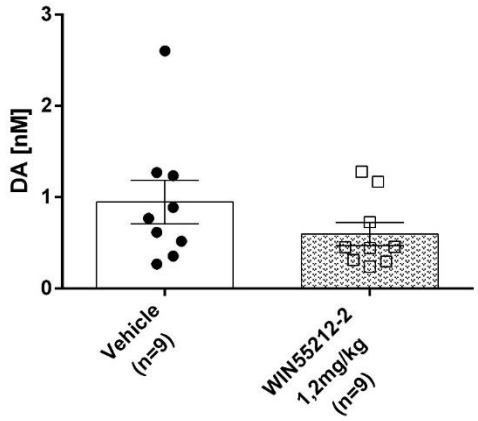
The recording procedure was based on Gomes et al. 2015 [1]. Adult rats were anesthetized with chloral hydrate (400 mg/kg; i.p.) and were placed in a stereotaxic apparatus (Stoelting). A thermostatically will control heating pad sustained core body temperature of 37°C. A burr hole will be drilled in the skull overlying the right SNc (AP – 4.9, ML + 2.2 from bregma) [2]. Extracellular recording microelectrodes were pulled from Omegadot 2.0 mm glass tubing on a Narishige P-5 vertical electrode puller, the tip broken back under microscopic control, and was filled with 2M NaCl containing 2% Pontamine Sky Blue dye. The impedance of the electrodes was tested in situ ranged from 6 to 15 MΩ. Single-unit activity was filtered using a high-pass filter at 30 Hz and low-pass at 10 kHz. All data analysis was performed using custom software (Neuroscope). Only neuronal activity with a signal-to-noise ratio greater than 3:1 and at least 1–3 min of stable spontaneous activity was used. At the end of recordings, the recording sites were marked via electrophoretic ejection of Pontamine Sky Blue dye from the tip of the electrode (20 μA constant negative current, 30 min).

Supplemental Figures:





Supplementary Figure 1: Representative anatomical placements (Left side) and histology example of placements (Right side) of microdialysis probe, electrode, and cannula. Microdialysis probe was lowered in the (a) dorsolateral striatum (DLS) and (b) substantia nigra par compacta (SNc) using the coordinates: (a) 1.2 mm anterior to bregma, 3.6 mm lateral, 4.8 mm below dura and (b) 4. mm posterior to bregma, 7.6 mm lateral, 8.0 mm below dura using an angle of 40° from horizontal axis according to the Atlas of Paxinos and Watson (2009). The electrode was lowered six times, separated by 200 mm, in the (c) SNc of each rat following the coordinates: AP - 4.9 to – 5.1, ML 2.0 to 2.4 from bregma and - 6.5 to - 9.0 DV from the brain surface. A 30-gauge injection cannula protruding 2.0 mm past the end of the guide, at an injection volume of 0.1 µl every 1 min. A guide cannula was lowered in (d) ventral pallidum using following coordinate: AP -0.7; ML +2.9 from bregma; DV -6.0 from the skull. A 30-gauge injection cannula protruding 2.0 mm past the end of the guide was used to perfuse a solution of bicuculline at a rate of 0.1 µl/min during 5 min. Diagrams were adapted from Paxinos and Watson (2009). CPu: Caudate putamen; SNC: Substantia nigra pars compacta; SNR: Substantia nigra pars reticulata.



Supplementary Figure 2: Adolescent exposure to WIN55212-2 did not modify basal DA dialysate in SNc of adult rats. In vivo conventional microdialysis in anesthetized adult animals was carried out in vehicle (n= 9), and WIN55212-2 exposed during adolescence (n= 9). Data correspond to mean ± SEM of basal DA dialysate in SNc.

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6. General Discussion

6.1. Chapter I: Comparing dopaminergic dynamics in the dorsolateral striatum between adolescent and adult rats: Effect of an acute dose of WIN55212-2

6.1.1 Age-dependent differences in dopaminergic neurotransmission in the DLS

Transitory changes in DA neurotransmission have been observed between adolescence and adulthood (James Edgar McCutcheon and Marinelli 2009; Badanich, Adler, and Kirstein 2006; Matthews et al. 2013; Nakano and Mizuno 1996). For example, a transitory increase in the expression of DA receptors, accompanied by a higher turnover of DA, and higher DA basal dialysate was observed in adolescent DS compared to adult (Naneix et al. 2012; Nakano and Mizuno 1996). Accordingly, our results show age-dependent differences in homeostatic control of DA extracellular levels in the DLS. Conventional microdialysis experiments showed higher basal DA dialysate in the DLS of adolescent rats compared to adults. Moreover, no-net flux microdialysis experiments in DLS indicate that DA Ed during adolescence is higher than in adulthood. Thus, similar DA C_{ext} in DLS was observed at both life periods.

A higher basal DLS DA dialysate is observed in adolescent compared to adult rats (Chapter 1, Fig. 2a). The high potassium perfusion increases DA dialysate in a similar magnitude in adolescent and adult rats (Chapter 1, Fig. 2b), suggesting no age-dependent differences in vesicular DA storage (Castañeda, Becker, and Robinson 1988; Kantor, Hewlett, and Gnegy 1999). Taken together, it is possible to suggest that the increased basal DA release observed in adolescent rats is associated to increased electrical activity of DA neurons from SNc (James M. Tepper, Trent, and Nakamura 1990). This increase

in basal dopamine release could be associated with a higher proportion of active neuron in the DS during the anticipation of reward in adolescent rats compared to adult rats (Sturman and Moghaddam 2012).

Using various experimental approaches, it was shown that DA uptake is more efficient during adolescence (Volz et al. 2009; Stamford 1989). However, the evidence about DAT expression in the striatal region is not conclusive. Binding assays (Tarazi, Tomasini, and Baldessarini 1998) showed that the transition from adolescence to adulthood is accompanied by a gradual increase in DAT expression in the DLS. On the other hand, western blot experiments have shown contradictory results regarding DAT protein levels in the DS. While Matthews et al. (2013) observed lower levels of DAT (Matthews et al. 2013), Volz et al. (2009) showed higher levels of DAT in adolescent compared to adult rats (Volz et al. 2009). Our results using no-net flux microdialysis show an increased DA Ed during adolescence compared to adulthood, suggesting that higher activity of DAT could be associated with higher DAT levels in the DS of adolescent rats compared to adults, as shown by Volz et al (Volz et al. 2009).

The DA C_{ext} is an estimated concentration of DA in the synaptic cleft, which depends on basal DA release and uptake, associated with DA Ed (Chefer et al. 2006; Smith and Justice 1994). Our results show no significant differences in the C_{ext} of DA, indicating that the higher dopaminergic nigrostriatal release observed during adolescence is counteracted by an increase in DAT activity, resulting in a DA C_{ext} similar to adulthood. It is possible to suggests that phasic dopaminergic signaling during adolescence would be more intense

and shorter, but due to the time scale and special resolution of microdialysis technique it is observed a DA C_{ext} similar to adult rats.

6.1.2 Effects of acute exposure of WIN55212-2 in DLS dopamine dynamics

WIN55212-2 is a synthetic aminoalkylindole derivate cannabinoid with similar physiological properties that the main psychoactive compound of cannabis, Δ^9-THC. This synthetic cannabinoid presents high affinity to both cannabinoid receptor, while the inhibitory constant (Ki) to the binding of CB1-R is 1.89 nM, the Ki to CB2 receptor is approximately 0.28 nM (Showalter et al. 1996; Pertwee 2014). Related to pharmacokinetic properties, it has been observed in rat pre-clinical model that both Δ^9-THC and WIN55212-2 have similar life-time (Valiveti et al. 2007). However, in functional assays of CB1-R, these molecules have a different pharmacological profile, whereas Δ^9-THC acts as a partial agonist, WIN55212-2 is full agonist (Prather et al. 2000; Pertwee 2014). Despite the different efficacy, WIN55212-2 produce a similar profile of behavioral effects in mice compared to Δ^9-THC such as suppression of motor activity, antinociception, hypothermia, and catalepsy (Martin et al. 1991). According to dopaminergic transmission, it has been observed that both Δ^9-THC and WIN55212-2 increase DA release in DLS in adult rats associated with an increase in the neuronal activity of neuron of SNc (A. Polissidis et al. 2014; Alexia Polissidis et al. 2013; Ton et al. 1988) These effects in dopaminergic transmission are attenuated after a dose of CB1 antagonist, indicating that the potentiation of dopaminergic transmission is dependent of the activation of CB1-R (Tanda, Pontieri, and Di Chiara 1997; Gessa et al. 1998; Melis, Gessa, and Diana 2000). On the other hand, recent evidence shows that the expression of CB2-R in VTA DA neurons in rats and their activation decrease the electrical activity of dopaminergic

neurons (H.-Y. Zhang et al. 2017; Ma et al. 2019). Therefore, effects of WIN55212-2 in the dopaminergic transmission mediated by CB2-R cannot be discarded.

The significant increase in basal dialysate of DA in DLS of adult rats induced by CB1 agonist is in line with previous pre-clinical evidence. An increase in basal DA release in the dorsal striatum (Alexia Polissidis et al. 2013) associated with an increase of neuronal activity of DA neuron from the SNc (French, Dillon, and Wu 1997) has been observed after an acute administration of WIN55212-2 in adult rats. Previous evidence showed that this increase in neuronal activity of DA neurons from SNc induced by WIN55212-2 is mediated by a decrease in GABAergic transmission (Yanovsky, Mades, and Misgeld 2003). Despite the inhibitory role of CB2 agonism in the dopaminergic transmission, previous evidence has shown that the systemic administration of a selective CB2 agonist do not modify the release of DA in striatum (H.-Y. Zhang et al. 2017). Indicating that the effect of CB1 agonism predominate against the CB2 agonism during a systemic dose of WIN55212-2 in adult rats.

In contrast with what is observed in adult rats, an acute administration of WIN55212-2 is not accompanied by significant changes in basal DA dialysate in the DLS of adolescent rats. To our knowledge, this is the first evidence related to the acute effects of WIN55212-2 in dopaminergic dynamics in the DLS of adolescent rats. A lower expression of CB1-R in adolescent midbrain (Verdurand et al. 2011) could explain the lack of effect of WIN55212-2 exposure in basal DA dialysate. It is possible to suggest that the CB1 and CB2 agonism of WIN55212-2 contribute equally in the release of DA in DLS during a systemic administration in adolescent rats. More experiments with selective antagonist are

requested to elucidate the role of CB2-R in the nigrostriatal dopaminergic transmission during adolescence.

The acute exposure to WIN55212-2 induced a significant decrease in DA Ed in adolescent rats, a result that was not observed in adult rats. While there is no evidence relating WIN55212-2 with DAT activity in adolescent rats, an inhibitory effect on DAT activity was observed in adult rats. Interestingly, the inhibitory effect of WIN55212-2 on DAT activity has been shown independent of CB1-R activation (Price et al. 2007). In fact, it has been proposed that WIN55212-2 interacts directly with the DAT protein (Pandolfo et al. 2011; Price et al. 2007; Steffens and Feuerstein 2004). Whether a similar mechanism than the one observed in adult rats underlies an inhibitory effect of WIN55212-2 in DAT activity in adolescent rats remains to be addressed. The age-dependent effects observed under our experimental conditions could be explained by lower glycosylation of DAT in adolescent rats. (Patel et al. 1994). It has been described that the higher N-glycosylation of DAT observed in adult rats attenuates the inhibitory effect of drugs on DA uptake (Li et al. 2004). It is tempting to suggest that the lower dose used in our experiment could explain the lack of inhibitory effects of WIN55212-2 in DA Ed observed in adult rats. Further experiments using higher doses of WIN55212-2 are necessary to address this proposal.

Interestingly, age-dependent mechanisms underlie the increase in DA C_{ext}. An increase in DA release contributes to the increase in DA C_{ext} induced by WIN55212-2 during adulthood, meanwhile, a decrease in DA uptake is associated with the increase in DA C_{ext}

in DLS of adolescent rats. Suggesting a high vulnerability to inhibitory effects of cannabinoids on DAT activity during adolescence.

6.2. Chapter II: Disinhibition of adult nigrostriatal dopaminergic pathway induced by chronic adolescent WIN55212-2 exposure

Cannabinoid use in the adolescent population has increased significantly in the last years across the world (UNODC 2018). However, information regarding its effects on brain plasticity after adolescent exposure is still sparse. Our results show that adolescent exposure to the cannabinoid agonist WIN55212-2 renders the nigrostriatal pathway hyper-excitable during adulthood. Effects of repeated exposure of WIN55212-2 in adolescence on DLS DA dynamics of adult rats.

An increase in DA release can be explained through various mechanisms, for example, an increase in DA synthesis, a decrease in DA metabolism or an increase in the activity of DA neurons. Consistent with previous evidence, repeated treatment with WIN55212-2 during adolescent increased DA turnover in the DLS of adult rats (Bortolato et al. 2014). In this sense, the expression of tyrosine hydroxylase in the DLS and SNc increases after a 7-days withdrawal period, after a chronic treatment with WIN55212-2 during adulthood (Perdikaris et al. 2018). Taken together this evidence, it is possible to suggest that repeated WIN55212-2 exposure increase DA synthesis in the nigrostriatal pathway. On the other hand, an increase in DA release can be attributed to a higher activity of DA neuron (Panin et al. 2012). In this sense, it has been observed that adolescent WIN55212-2 exposure increases the activity of DA neuron in the VTA (Gomes, Guimarães, and Grace 2015),

suggesting that a similar increase in the activity of DA neurons of the SNc contributes to the rise in DA release in the DLS.

Although an inhibitory effect of acute WIN55212-2 on DA uptake has been observed in adult (Pandolfo et al. 2011) and adolescence (Chapter 1, Fig. 4b) rats, no changes in DA Ed in the DLS were evident in adult rats after an adolescent exposure of WIN55212-2. Previous experiments of radioligand binding showed that treatment with a CB1 agonist during early adolescence (PD 28-38) did not modify the binding of DAT in the dorsal striatum of adult male rats (Higuera-Matas et al. 2010). In addition, a similar chronic treatment with WIN55212-2 during adulthood did not modify the binding of DAT protein in the DLS (Fanarioti et al. 2014), suggesting that adolescent exposure to WIN55212-2 is not accompanied by modifications in DAT expression in adult rats.

Collectively, the no-net flux experiments support the idea that the increase in DA Cext after adolescent exposure to WIN55212-2 depends on neuronal excitability more than on pre-synaptic control involving DA uptake.

6.2.1 Long-term effects of repeated exposure of WIN55212-2 during adolescence on SNc DA neuron activity

Consistent evidence indicates that, beside of presynaptic control, changes in the activity of DA neurons can modify the concentration of DA in the terminal regions (Floresco et al. 2003; Panin et al. 2012). Microdialysis and extracellular recording experiments have shown that an increase in the spontaneous activity of DA neurons increases basal DA dialysate in the DLS (Panin et al. 2012; Floresco et al. 2003). Supporting the rise in DA Cext in DLS, our results using in vivo single unit recordings of DA neurons in the SNc

showed that adolescent exposure to WIN55212-2 increases population activity of DA neurons in the SNc, without significant changes in firing rate or burst activity.

A similar effect has been observed after the exposure of CB1/2 agonist to adolescents in VTA DA neurons (Gomes, Guimarães, and Grace 2015; Renard et al. 2016). Here, Gomes et al. 2015 showed that the intermittent exposure to WIN552112-2 during adolescence (PD 40 to 65) produces an increase in the spontaneous activity of DA neurons in the VTA (Gomes, Guimarães, and Grace 2015). Similarly, Renard et al. 2016 showed that adolescent exposure to Δ^{9}-THC increases the firing rate, the bursting activity, and the population activity of DA neurons in the VTA (Renard et al. 2016). Together, no-net flux microdialysis and electrophysiological recording data indicate that the increase in DA Cext in DLS of adult rats after adolescent exposure to WIN55212-2, is associated with the facilitation of DA release induced by a rise in the number of spontaneously active DA neurons in the SNc.

Previous evidence has associated the activity of the DLS with the shift between goal-directed responding to a habitual seeking of rewarding stimuli (Everitt and Robbins 2016; Yin, Knowlton, and Balleine 2004; Corbit, Nie, and Janak 2012). This transition from goal-directed to habitual behavior can be studied using the reward devaluation in the self-administration protocol, in which the devaluation of the reward reduces the goal-directed seeking, but the habitual seeking remains unaltered (Corbit, Nie, and Janak 2014, 2012; Yin, Knowlton, and Balleine 2004). Local excitotoxic lesion of the DLS after a long training of sucrose self-administration reduced the seeking during the extinction test with a devaluated reward, showing that DLS is necessary to maintain the habitual behavior

(Yin, Knowlton, and Balleine 2004). In addition, the pharmacological inhibition of the DLS decreases ethanol seeking in devaluated reward paradigm, in rats with 8 weeks of self-administration training, whereas changes in reward seeking were not observed after the inhibition of DLS in rat with 2 weeks of self-administration training (Corbit, Nie, and Janak 2012). This evidence suggests that the DLS becomes a key in the control of a well-established habitual behavior.

Additionally, the acquisition of habitual seeking depends on an increase in the nigrostriatal dopaminergic transmission (Everitt and Robbins 2016; Nelson and Killcross 2006; Willuhn et al. 2012). An increase in DA levels in DLS has been observed during habitual cocaine-seeking behavior when cocaine cues were presented contingently (Ito et al. 2002). Furthermore, the training of self-administration of cocaine is accompanied by an increase in DA release in the DLS after the second week of the training, suggesting that dopaminergic transmission in this nucleus controls cocaine-seeking when it becomes a habitual behavior (Willuhn et al. 2012). Strengthening this idea, the local perfusion of a non-selective dopaminergic antagonist in the DLS reduced cocaine-seeking behavior in late stages of training but failed to modify cocaine-seeking when it is goal-directed behavior (Murray, Belin, and Everitt 2012; Vanderschuren, Di Ciano, and Everitt 2005). Local perfusion of raclopride, a D2 antagonist, in the DLS restored goal-directed behavior in rats with habitual response to ethanol-seeking (Corbit, Nie, and Janak 2014). These pieces of evidence support the hypothesis that DA release in the DLS is required for maintenance of habitual drug seeking behaviors.

Our results show that adolescent exposure to WIN55212-2 enhances the basal release of DA in the DLS and increase the population activity of DA neurons in the SNc of adult rats. Given that only DA neurons that are already spontaneously firing respond to excitatory signals which trigger the burst firing (Grace 2012), then it is possible to suggest that the exposure to cannabinoids during adolescence amplifies DA signaling in the DLS and facilitates the habitual seeking behavior of drugs of abuse during the adulthood. More experiments are required to assess changes in habitual seeking behavior after an adolescent exposure of cannabinoids.

6.2.2 The mechanism underlying the long-term disinhibition of the nigrostriatal dopaminergic pathway

As previously mentioned, mesencephalic DA neurons show three different states of activity: non-firing state, tonic firing state, and phasic firing state (Gomes, Rincón-Cortés, and Grace 2016). It has been indicated that in control conditions these states of DA neurons in the VTA are modulated by different afferents and neurotransmitters. The population activity, i.e., the number of neurons showing tonic firing, is regulated by GABA input and the phasic or burst pattern of neuronal activity is driven by glutamatergic input (Floresco et al. 2003). Using a conventional microdialysis approach, we observed a significant decrease in extracellular GABA levels but not in glutamate in the SNc of adult rats that were exposed to WIN55212-2 during the adolescence.

The reduction of GABA tone in the somatic region of DA neurons could enhance the dopaminergic nigrostriatal transmission. Dual microdialysis experiments have shown that local perfusion of bicuculine, a GABAa antagonist, in the Substantia Nigra increases DA release in the DLS, indicating that endogenous GABA exerts a tonic inhibitory control on

dopaminergic nigrostriatal activity (Westerink, Santiago, and De Vries 1992). Our results are consistent with evidence highlighting the susceptibility of GABAergic neurotransmission to the long-term effects of adolescent cannabinoid exposure. Adults rats exposed to WIN55212-2 during early and mid-adolescence show persistent disinhibition of neuronal activity in the medial prefrontal cortex associated with a decrease in GABA neurotransmission (D. K. Cass et al. 2014). Furthermore, adolescent exposure to Δ^{9}-THC decreased extracellular GABA and reduced the expression of a protein associated with the biosynthesis of GABA in the PFC of adult rats (Zamberletti et al. 2014). These long-term cortical dysfunctions in GABA neurotransmission observed after early exposure to cannabinoid has been associated with psychosis risk in adulthood (Renard, Rushlow, and Laviolette 2018). Conversely, non-effects on basal GABA and glutamate release in the hippocampus were observed in adult rats after the repeated treatment with CP55940, a synthetic CB1 agonist, during early adolescence (PD 28 to 38) (Higuera-Matas et al. 2012). The effects in the GABA release after a repeated adolescent exposure to cannabinoids could depend on different variables such as the age of exposure, the period of exposure, the length of withdrawal or the brain region.

GABAergic inputs to the SNc come from different regions such as the striatum, VP, external globus pallidus and substantia nigra reticulate (Watabe-Uchida et al. 2012; Steiner and Tseng 2017; Ogawa et al. 2014). A decrease in the activity in any of these GABAergic areas would explain the reduction in GABA levels in the SNc and the disinhibition of nigrostriatal dopaminergic transmission induced by adolescence WIN55212-2 exposure.

Our results and previous evidences show that the population activity of DA neurons in the VTA (Gomes, Guimarães, and Grace 2015) and SNc (Chapter 2, Fig. 3) of adult rats increases after an adolescent repeated exposure of WIN55212-2, suggesting that there is a common area, which mediates the effects of adolescent cannabinoid exposure in both dopaminergic regions. A candidate for mediating the increase of dopaminergic activity in the VTA and the SNc could be the GABAergic neurons of VP, however, changes in others GABA input cannot be discarded. It has been described that GABAergic neurons from VP monosynaptically project to DA neurons in both areas (Watabe-Uchida et al. 2012; Ogawa et al. 2014). Then, it is proposed that adolescent exposure to WIN55212-2 could decrease the neuronal activity of GABAergic neuron from VP, inducing a reduction in GABA extracellular concentration in the SNc accompanied by an increase in population activity of DA neurons in SNc of adult rats. To test this hypothesis, extracellular recording of DA neurons in SNc was carried out after local perfusion of bicuculline in VP.

Our results showed that bicuculline perfusion into the VP attenuated the increase in population activity induced by an adolescent WIN 55212-2 exposure without modifying the dopaminergic activity in basal conditions. Given that the activation of VP induced by bicuculline failed to modify the dopaminergic transmission in control condition, then it is possible to suggest that the increase of GABA in the SNc was not enough to decrease the normal population activity. This result is similar to that observed in the basal condition in the DA population activity of VTA (Floresco et al. 2003). The attenuation of hyperactivity of DA neurons induced by the GABAa antagonism in the VP suggests that adolescence exposure of WIN55212-2 decreases the activity of GABAergic neurons of VP. This

proposal would explain the reduction in GABA extracellular levels and the disinhibition of DA neurons of SNc (Chapter 2, Fig. 7).

A putative mechanism underlying the effect of bicuculline could involve an increase in the inhibitory tone in the VP. Despite the expression of CB1-R in GABAergic projection to VP (Pickel et al. 2012), there is no evidence of changes in the activity of GABA neurons of VP induced by adolescence cannabinoid exposure. However, desensitization of CB1-R in the GABAergic projection could promote a decrease in the activity of VP neurons. Previous evidence indicates that repeated activation of CB1-R in the adolescence induces desensitization of CB1 signaling during adulthood (Tiziana Rubino et al. 2008; T. Rubino and Parolaro 2008). It has been observed that a repeated treatment with Δ^9-THC during adolescence reduces CB1-R expression immediately after the treatment (Burston et al. 2010) and until adulthood (Tiziana Rubino et al. 2008). Taken together with the inhibitory role of CB1-R and their high expression in GABA neurons (Tsou et al. 1998; Pickel et al. 2012; Castillo et al. 2012), a desensitization of CB1-R would increase the inhibitory tone in the VP.

The modulation of VP GABAergic neuron activity by dopaminergic inputs could be an alternative, but it is not the only mechanism that can explain our results. It has been observed that electrical stimulations of VTA and SNc decrease the activity of the sixty percent of VP neurons, whereas thirty percent of the neurons increases the activity. Both responses present a short latency after the stimulation, suggesting a monosynaptic control of VTA and SNc on VP neuron (Maslowski-Cobuzzi and Napier 1994). Furthermore, the local application of D1 and D2 antagonist in VP attenuated this inhibition (Maslowski-

Cobuzzi and Napier 1994). In addition, microiontophoretically application of DA in VP produces a dose-dependent increase in the activity of ten percent of recorded neurons and a dose-dependent decrease in the activity of thirty percent of the neurons (Napier, Simson, and Givens 1991). This evidence indicates that DA release modulates the neuronal activity in VP. Interestingly, D1 and D2 receptor express in the GABAergic projection to VP (Lu, Behnam Ghasemzadeh, and Kalivas 1997), suggesting that the inhibitory effect of DA release in VP can be modulated by presynaptic GABA input. Then, we suggest that the increase of dopaminergic transmission of VTA induced by adolescence cannabinoid exposure (Gomes, Guimarães, and Grace 2015; Renard et al. 2016) could promote the inhibitory response of GABAergic neurons of VP (Napier, Simson, and Givens 1991) and to provoke the disinhibition of DA neuron from SNc.

This research proposes a mechanism based on changes of GABAergic input that could explain the disinhibition of nigrostriatal dopaminergic transmission induced by adolescent cannabinoid exposure. It has been observed that chronic WIN55212-2 exposure during adulthood alters the expression of DAT, D1, D2 and GABAa receptor in dopaminergic neurons of SNc and VTA (Perdikaris et al. 2018; Fanarioti et al. 2014). In addition, the repeated treatment with CB1/2 agonists in adult rats changes the homeostatic levels of neurotransmitter that control the DA release in the terminal region of DA neurons such as GABA, glutamate, and acetylcholine (Covey et al. 2017; Sulzer, Cragg, and Rice 2016). Whether these effects occur after an adolescent exposure to cannabinoid and remains until adulthood is still an open question.

Overall, the results in chapters 1 and 2, allow suggesting that the inhibitory effect of an acute dose of WIN55212-2 on DA uptake observed in adolescent rats is attenuated and the release of DA in basal conditions is promoted in adulthood by the repeated exposure to WIN55212-2 in the adolescence.

In summary, our results showed that adolescent exposure to WIN55212-2 increases the release of DA in the DLS promoted by an increase in DA neuron population activity of the SNc. This disinhibition of nigrostriatal dopaminergic activity is accompanied by a reduction of GABA levels in SNc and driven by a decrease in the VP afferent inhibition. These changes could be the basis of a mechanism by which exposure to cannabinoids during adolescence affect in the long term the dopaminergic pathways that modulate behaviors such as learning and habit, contributing to the habitual consumption of drugs of abuse.

7. References

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