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Behavioral and neurochemical changes in mesostriatal dopaminergic regions of the rat after chronic administration of the cannabinoid receptor agonist WIN55,212-2

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Abstract

Background: The endocannabinoid system interacts extensively with other neurotransmitter systems and has been implicated in a variety of functions, including regulation of basal ganglia circuits and motor behavior. The present study examined the effects of repeated administration of the non-selective cannabinoid receptor 1 (CB1R) agonist WIN55,212-2 on locomotor activity and on binding and mRNA levels of dopamine receptors and transporters (DAT) and GABA receptors in mesostriatal dopaminergic regions of the rat.

Methods: Rats received systemic injections of WIN55,212-2 (0, 0.1, 0.3 or 1 mg/kg, i.p.) for 20 consecutive days. Locomotor activity was measured on days 1, 10 and 20. Following the last measurement, rats were euthanized and prepared for in vitro binding and in situ hybridization experiments.

Results: Acutely, 0.3 and 1 mg/kg of WIN55,212-2 produced hypolocomotion, which was sustained for the next two measurements, compared to vehicle. Repeated administration of WIN55,212-2 decreased the mRNA levels of the D2 autoreceptors in substantia nigra (SN) and ventral tegmental area (VTA) and increased D1 receptor mRNA and binding in nucleus accumbens (NAc). Furthermore, both DAT binding and mRNA levels were decreased in SN. Moreover, repeated administration of WIN55,212-2 decreased GABA receptor binding levels in dorsal striatum and in SN. Conclusions: Our data indicate that chronic WIN55,212-2 administration results in sustained effects on locomotor activity, similar to those observed after acute administration, and modulates the dopaminergic and GABAergic systems in a region-, dose- and neurotransmitter-selective manner.

Key words: WIN55,212-2; locomotor activity; dopamine transporter; dopamine receptors; GABA receptor

Introduction
The rewarding and motor effects produced by Δ⁹-tetrahydrocannabinol (Δ⁹-THC) and synthetic cannabinoid agonists are mediated primarily by cannabinoid 1 receptors (CB1R) (Ameri, 1999). CB1R are abundantly expressed in the basal ganglia (Herkenham et al., 1991a), a brain region involved in motor control (Martín et al., 2008; Morera-Herreras et al., 2008). It has been shown that systemic administration of Δ⁹-THC and other CB1R agonists exert biphasic effects on motor activity, with low doses increasing motor activation and higher doses producing hypolocomotion or even catalepsy (Sañudo-Peña et al., 2000; Drews et al., 2005; Shi et al., 2005; Rodvelt et al., 2007; Polissidis et al., 2010; 2013; Katsidoni et al., 2013).

It has been suggested that cannabinoids elicit their pharmacological effects in part through activation of dopaminergic neurons in the brain and more specifically the mesostriatal dopaminergic system with cell bodies located within the ventral tegmental area (VTA) and substantia nigra pars compacta (SNpc) (French et al., 1997; Rodríguez De Fonseca et al., 2001; Pan et al., 2008; Morera-Herreras et al., 2008), by enhancing dopamine release in their respective dopamine terminal fields, the nucleus accumbens (NAc) (Tanda et al., 1997) and striatum (Taylor et al., 1988).

Interestingly, CB1R do not appear to be expressed at dopaminergic terminals in these main dopaminoceptive regions (Herkenham et al., 1991a; 1991b; Mailleux and Vanderhaeghen, 1992, 1993; Matsuda et al., 1993; Westlake et al., 1994; Julian et al., 2003); but rather on presynaptic GABAergic interneurons (Katona et al., 2000) and glutamatergic projecting neurons (Hermann et al., 2002), indicating that the effects of cannabinoids on dopamine neurotransmission are mainly indirect and modulated via the function of other neurotransmitters, such as GABA and glutamate. Indeed, it is well documented that cannabinoids affect extracellular levels of GABA in hippocampus (Katona et al., 1999), prefrontal cortex (Pistis et al., 2002), amygdala (Katona et al., 2001) and
glutamate in cerebral (Ferraro et al., 2001) and prefrontal cortex (Pistis et al., 2002) and striatum (Polissidis et al., 2013). In the striatum, CB1R are localized presynaptically on GABAergic and glutamatergic terminals (Matsuda et al., 1993) and postsynaptically in the somata, dendrites and axon terminals of striatal medium spiny neurons (Fitzgerald et al., 2012). Activation of CB1R inhibits GABAergic neurotransmission in the globus pallidus (GP) (Pertwee et al., 1988), NAc (Manzoni and Bockaert, 2001), SN (Wallmichrath and Szabo, 2002), and the VTA (Szabo et al., 2002) via inhibition of adenylate cyclase (Pertwee, 2006).

Striatal medium spiny projection neurons (MSN) expressing D1 dopamine receptors (D1DR) form the direct pathway, while neurons expressing D2 receptors (D2DR) form the indirect pathway. Activation of D1DR leads to stimulation of adenylate cyclase (Blandini et al., 2000) and cAMP formation (Gingrich and Caron, 1993) and, in turn, to activation of the direct pathway (van der Stelt and Di Marzo, 2003) while activation of D2DR inhibits adenylate cyclase (Blandini et al., 2000) and cAMP formation, leading to inhibition of striatal medium spiny neurons that project to the nuclei of the indirect pathway.

Several studies suggest an interaction between CB1R and D1/D2 dopamine receptors (D1DR/D2DR) at the cellular level and coupling to the same effector systems (Hermann et al., 2002). Simultaneous activation of both CB1R and D2DR leads to enhanced activation of adenyl cyclase resulting in activation of striatal neurons of the indirect pathway, which in turn activates neurons of the subthalamic nuclei, resulting in hypomotility (Glass and Felder, 1997; Kearn et al., 2005; Martín et al., 2008). Moreover, simultaneous stimulation of CB1R and D1DR leads to a net decrease in adenyl cyclase, which in turn reduces the inhibitory activity of direct striatal projection neurons. This inhibition increases the activity of nigral neurons resulting in a decreased motor response (Martín et al., 2008).
The present study was designed to correlate the behavioral effects of chronic WIN55,212-2 with changes in neurochemical indices. In this context and based on the key role of dopamine and GABA in the mesostriatal dopaminergic system we investigated the effects of systemic acute and chronic administration of low and high doses of WIN55,212-2, a non-selective CB1R agonist, on motor activity patterns, and characterized neurochemical alterations of dopaminergic and GABAergic systems. WIN55,212-2 has higher efficacy than other CB1R agonists (Kearn et al., 1999), and has been well characterized in behavioral and neurochemical studies (Manzoni and Bockaert, 2001; Wallmichrath and Szabo, 2002; Castañé et al., 2004; Vlachou et al., 2008; Moranta et al., 2009; Mavrikaki et al., 2010; Polissidis et al., 2013). We examined the dopamine transporter (DAT), dopamine, and GABA receptors in the striatum, NAc, SN and VTA of WIN-treated and vehicle-treated rats. *In vitro* binding and *in situ* hybridization experiments were used to evaluate binding and mRNA levels of the aforementioned receptors and transporters.
Methods

Animals

Male Sprague-Dawley rats (n=40) weighting 250-300g (PND60 to PND 70) were used. The animals were housed in groups of 2 or 3 under a 12h light/dark cycle with free access to food and water. Experiments were conducted in accordance with the European Communities Council Directive (86/609/EEC) and the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All efforts were made to minimize animal suffering and to reduce the number of animals used.

WIN55,212-2 treatment

WIN55,212-2 (Tocris, Westwoods Bus. Park Ellisville, U.S.A.) was dissolved in a vehicle solution containing 5% dimethylsulfoxide (DMSO), 5% cremophor EL in 0.9% NaCl and injected intraperitoneally (i.p.) at a volume of 3 ml/kg of body weight. Experimental animals were divided into four groups (n=10 per group) receiving a single daily intraperitoneal (i.p.) injection of either vehicle or WIN55,212-2 (vehicle, 0.1, 0.3 and 1 mg/kg) for 20 days. Control animals (n=10) received i.p. the corresponding vehicle solution in the same injection volume. All rats were tested for locomotor activity and used for neurochemical studies (n=10/ group). Animals were euthanized by rapid decapitation 1.5 hr after the last injection.

Locomotor activity

Spontaneous motor activity was measured using an activity recording system (Model 7445, Ugo Basile) consisting of an animal cage and an electronic unit incorporating a counter and a printer. The rectangular animal cage (56x56x30 cm) has transparent sides and lid to allow observation. The cage floor has horizontal and vertical infrared sensors. The
counter sums up the photocell disruptions, and a printer displays the results at preset
intervals. In our studies, a summation of photocell disruptions of ambulatory distance and
rearing, for each 5-min interval period, during the 1h observation period was registered.
Behavioral testing was performed the 1st, the 10th and the 20th day of the drug treatment
between 08:00 and 16:00 hours, 10min after drug administration. The post-injection time
was selected taking into account that the behavioral effects lasted for approximately 1h.

Brain sectioning for neurochemical studies
Rats were euthanized by rapid decapitation. Brains were isolated and quickly frozen in 2-
methyl-butane. Coronal sections, 14-μm-thick, were cut in a cryostat Leica (CM1850), thaw-
mounted on gelatine–chromalum coated glass slides (for autoradiography studies) or poly-L-
lysine- coated slides (for in situ hybridization studies), dried at room temperature and stored
at -75°C until experiments were performed.

Receptor binding autoradiography
DAT binding was assayed according to Dickinson et al. (1999) using 5nM [³H]-WIN35428
(S.A. 87 Ci/mmol; PerkinElmer Life Sciences, Belgium) as radioligand. Sections were
allowed to air-dry at room temperature (RT), preincubated for 30 min in 20 mM sodium
phosphate buffer (PBS) pH 7.4 at 4°C and incubated for 90 min in buffer containing 0.32 M
sucrose, pH 7.4 at 4°C in the presence of radioligand. Sections were washed for 2x1 min in
20 mM PBS pH 7.4 at 4°C, briefly dipped in ice-cold distilled water and airdried.
Nonspecific binding was determined in the presence of 30 μM benztropine (Sigma Aldrich
Greece).

D2-like and D1-like dopamine receptors were assayed as described by Tarazi et al. (1998). Sections were preincubated at RT for 1 h in 50 mM Tris–HCl, 120 mM NaCl, 5 mM KCl, 2
mM CaCl₂, 1 mM MgCl₂, pH 7.4 and incubated for 1 h at RT in the presence of
[^3]H]raclopride (2 nM; S.A. 62.2 Ci/mmol; PerkinElmer Life Sciences, Belgium) and
[^3]H]SCH23390 (2.5 nM; S.A. 85 Ci/mmol; PerkinElmer Life Sciences, Belgium) for D2-like
and D1-like receptors, respectively. For D1-like receptors, the incubation buffer contained
40 nM ketanserin (Tocris, UK) to block the 5-HT2 serotonin binding sites. After incubation,
sections were rinsed 2 x 5 min in ice-cold buffer, briefly dipped in ice-cold distilled water and
air-dried. Nonspecific binding was determined with 1 μM cis-flupenthixol (Sigma–Aldrich,
Greece) for both receptors.

[^3]H]A receptor binding was performed according to Bristow and Martin (1988).
Sections were preincubated at RT for 30 min in buffer containing 50 mM Tris-citrate, 100
mM MgCl₂, pH 7.4, air-dried and incubated with 6.5 nM [^3]H]-SR95531 (S.A. 55.3 Ci/mmol;
PerkinElmer Life Sciences, Belgium) at 4°C for 30 min. Non-specific binding was
determined in the presence of 10 mM GABA (Sigma Aldrich, Greece). Sections were rinsed
3 x 5 min in ice-cold buffer, briefly dipped in ice-cold distilled water and air-dried under a
stream of cold air. The labeled sections were exposed to BioMax MR Film (Kodak) for 4–10
months. Tritium micro scales (Amersham, United Kingdom), calibrated as nCi/mg tissue
equivalent, were exposed along with the tissue samples and used as standards.
In situ hybridization histochemistry

Hybridization was carried out according to Giannakopoulou et al. (2012). Sections were air-dried at RT, fixed for 5 min in 4% paraformaldehyde in DEPC-treated PBS (0.1 M, pH 7.4), rinsed in PBS, dehydrated in graded ethanol and air-dried at RT. The oligonucleotide sequences used are shown in the Supplementary Material.

Each probe was diluted to a concentration of 3pmol/μl and labeled with $^{35}$S-ATP (PerkinElmer Life Sciences, Belgium) at the 3’ end, using the 3’ terminal transferase enzyme (Roche, Germany) to a specific activity of 2 x 10$^5$ cpm/μl. Chromatography Sephadex G-50 columns (BioRad, Greece) were used to remove unincorporated nucleotides. Hybridization was performed in 50% formamide (v/v), 4xSSC (1xSSC: 0.15 M sodium chloride, 0.015 M sodium citrate), 10% dextran sulfate (w/v) and 10 mM dithiothreitol (DTT), with 1:100 labeled probe (0.03 pmol/μl final concentration of labeled probe). Sections were covered with 120 μl of hybridization solution and incubated for 18 h in a humid chamber at 42°C. Nonspecific signal was determined by the addition of 100-fold excess of unlabeled probe to the hybridization solution. After hybridization, sections were washed in 1xSSC for 20 min at 60°C, in 0.1xSSC for 3 min at RT, dehydrated in graded ethanol and air-dried at RT. Sections were exposed to a Kodak BioMax MR film (Kodak) and exposure time ranged from 2-8 weeks depending on probe labeling.

Quantification

 Autoradiographs were scanned, optical densities were measured with MCID 7.0 software (Imaging Research Inc, St. Catharines, ON, Canada). The results are expressed in fmol/mg protein for receptor binding autoradiography and in relative optical density (ROD) values for in situ hybridization. The values are expressed as mean ± standard error of the mean.
The anatomical structures were defined according to the rat brain atlas (Paxinos and Watson, 2007) and analyzed at the same level. The striatal sections were divided in quadrants (dorsolateral, dorsomedial, ventrolateral and ventromedial). Measurements were taken for total as well as nonspecific binding signal from each animal. The specific signal was determined by subtracting nonspecific from total signal.
Statistical Analyses

For the locomotor activity studies, the significance of repeated drug effect was initially evaluated using two-way analysis of variance (ANOVA) with repeated measures followed by one-way ANOVA with (time) or without (drug) repeated measures and the Least Significance Difference (LSD) post hoc test as required. Statistical analysis for autoradiography and \textit{in situ} hybridization assays was performed by one-way ANOVA, followed by the Bonferroni post hoc test to identify differences in receptor binding or mRNA expression levels between WIN-treated and vehicle-treated animals. The level of statistical significance was set at 0.05. Statistical analyses were conducted using the Statistical Package for the Social Sciences v.17.0 (SPSS, Chicago, IL, USA).
Results

Locomotor activity

Overall, both ambulatory activity and rearing decreased after repeated testing from day 1 to days 10 and 20 and in response to administration of vehicle, 0.1 and 0.3mg/kg doses of WIN, and from day 1 to day 10 in response to administration of 1.0mg/kg of WIN (Fig. 1A and B).

Considering the total (60min session) ambulatory activity, two-way ANOVA, with repeated measures indicated a significant drug effect ($F_{3,36}=12.8$, $p<0.001$) as well as a significant time effect ($F_{2,72}=29.08$, $p<0.001$) but not a significant interaction (drug x time, $p>0.05$). One-way ANOVA with repeated measures per group demonstrated a significant time effect in all treatments (WIN 0.1mg/kg: $F_{2,18}=6.26$, $p<0.05$, WIN 0.3mg/kg: $F_{2,18}=20.60$, $p<0.001$ and WIN 1mg/kg: $F_{2,18}=13.63$, $p<0.001$) except vehicle ($F_{2,18}=3.14$, $p=0.067$). One-way ANOVA on each day of measurement indicated significant effects between groups (day 1 $F_{3, 36}=7.54$, $p<0.001$; day 10 $F_{3, 36}=12.49$, $p<0.001$; day 20 $F_{3, 36}=7.5$, $p<0.001$). LSD post hoc test on each day indicated that WIN 1mg/kg and WIN 0.3mg/kg significantly differ from vehicle group (day 1 $p<0.05$ for both doses; day 10 $p<0.01$ and $p<0.001$ for WIN 0.3mg/kg and WIN 1mg/kg, respectively; day 20 $p<0.01$ for both doses), while WIN 0.1mg/kg did not significantly affect total ambulatory activity ($p>0.05$ compared to vehicle).

Considering total (60min session) rearing, two-way ANOVA with repeated measures demonstrated a significant interaction of drug x time ($F_{6,72}=3.27$, $p=0.007$). One-way ANOVA with repeated measures indicated that there was no significant time effect of WIN 1mg/kg ($F_{2,18}=3.45$, $p=0.54$). However, there was a significant time effect on WIN 0.3mg/kg ($F_{2,18}=4.39$, $p<0.050$), WIN 0.1mg/kg ($F_{2,18}=12.6$, $p<0.001$) and vehicle
One-way ANOVA on each measurement (day) demonstrated significant differences between groups (day 1: $F_{3,36} = 7.81$, $p<0.001$, day 10: $F_{3,36} = 6.83$, $p<0.001$, day 20: $F_{3,36} = 5.20$, $p<0.001$). LSD post-hoc test demonstrated that WIN 1mg/kg had a significant effect on rearing on day 1 ($p=0.001$), as well as on days 10 and 20 ($p<0.01$). WIN 0.3mg/kg significantly affected rearing only on day 1 ($p<0.05$) but not on days 10 and 20 ($p>0.05$). WIN 0.1mg/kg did not significantly affect total rearing ($p>0.05$).

Further analyses performed on the 5 min bin (of total 60 min session) on days 1, 10 and 20 of ambulatory activity and rearing are presented in Supplementary Material.

Dopamine Transporter (DAT)

The levels of $[^3H]$-WIN35428 binding in striatum and SN as well as NAc and VTA (Fig. 3A, 3B, 5A,B,C and D) of vehicle and WIN-treated rats are presented in Table 1. $[^3H]$-WIN35428 specific binding decreased significantly in SN ($F_{3,36} = 20.76$, $p<0.001$) at the doses of 0.1, 0.3 and 1mg/kg of WIN55,212-2 ($p<0.001$ at all doses) and in VTA ($F_{3,34} = 6.23$, $p=0.002$) at the same doses (WIN 0.1mg/kg: $p=0.026$, WIN 0.3mg/kg: $p=0.049$ and WIN 1mg/kg: $p=0.001$). DAT binding site levels were also reduced at the dose 0.1mg/kg in NAc core ($F_{3,36} = 7.289$, $p<0.001$) and in the shell subdivision ($F_{3,36} = 8.56$, $p=0.001$). Significant decreases at the tested doses of WIN55,212-2 were also observed in DAT mRNA levels in SNpc ($F_{3,36} = 8.15$, $p<0.001$, Table 2, Fig. 4Aand 5E) at all doses (WIN 0.1mg/kg: $p=0.042$, WIN 0.3mg/kg: $p=0.002$ and WIN 1mg/kg: $p<0.001$) and in VTA ($F_{3,36} = 7.96$, $p<0.001$, Table 2, Fig 4A and 5F) at all doses, as well (WIN 0.1mg/kg: $p=0.002$, WIN 0.3mg/kg: $p=0.001$ and WIN 1mg/kg: $p=0.032$). Furthermore, no significant differences between vehicle and WIN-treated rats were observed in DAT binding levels in the striatum (Table 1).
D1 dopamine receptors

The levels of D1DR binding (Fig. 3C, 6A and B) and mRNA levels (Fig. 4B, 6C and D) in the terminal regions of the mesostriatal dopaminergic system of vehicle and WIN-treated rats are presented in Tables 3 and 4. Specific binding of $[^3]H$-SCH23390 and D1DR mRNA levels increased significantly ($F_{3,34}=3.882$, $p=0.039$, Table 3), ($F_{3,15}=13.176$, $p=0.019$, Table 4), at the dose of 1mg/kg in the core subdivision of NAc. No effects were observed at the other two doses.

D2 Dopamine receptors

The levels of D2 DR binding, as determined by $[^3]H$-raclopride specific binding (Fig. 3D,3E and 7A-D), in the mesostriatal dopaminergic regions of vehicle and WIN-treated rats are presented in Table 5. Significantly decreased levels of D2DR binding levels were observed in the medial quadrants of striatum (DM: $F_{3,35}=4.56$, $p=0.008$; VM: $F_{3,35}=4.64$, $p=0.008$) at the doses of 0.1 (DM: $p=0.011$ and VM: $p=0.013$) and 1 mg/kg (DM: $p=0.030$ and VM: $p=0.019$). However, as seen in Table 6, no significant alterations were observed in D2DR mRNA levels in SN and VTA (Fig. 4C, 7E and F). Furthermore, we examined the mRNA levels of the D2S isoform (Fig. 4D, 7G and H), which corresponds to a splice variant of the D2DR, showing an expression pattern presynaptically on dopaminergic neurons of SNpc and VTA and likely represents the dopamine autoreceptor (Khan et al., 1998). The in situ hybridization histochemistry study for D2S mRNA revealed significant differences in mRNA levels in SNpc ($F_{3,36}=62.74$, $p<0.001$) and VTA ($F_{3,36}=5.93$, $p=0.002$). Particularly, statistically significant decreases were observed at all doses of WIN55,212-2 in SNpc ($p<0.001$ at all doses) as described in Table 7, while in VTA...
significant decreases were observed at the higher doses (WIN 0.3mg/kg: \( p=0.008 \) and WIN 1mg/kg: \( p=0.003 \)).

GABA\textsubscript{A} Receptors

The levels of GABA\textsubscript{A} binding sites were measured in striatum of WIN-treated and vehicle-treated rats using \( [^3H]\)-SR95531 and are presented in Table 8 and fig. 3F, 3G and 3H. Densitometric measurements taken from striatum showed statistically significant decreases in dorsolateral \( (F_{3,36}= 9.95, p<0.001) \) and dorsomedial \( (F_{3,36}= 19.42, p<0.001, ) \) quadrants at all doses tested, as well as at 0.3mg/kg in the ventrolateral subdivision \( (F_{3,36}=5.43, p=0.003) \). More specifically, at the lowest dose of WIN55,212-2 (0.1mg/kg), the observed binding levels of GABA\textsubscript{A} receptor were significantly reduced in the dorsolateral quadrant \( (p=0.001) \) and in the dorsomedial part \( (p<0.001) \). Decreases were also observed with 0.3mg/kg in the dorsolateral and dorsomedial quadrants \( (p<0.001 \text{ in both subdivisions}) \), respectively. The same effect was also observed at the highest dose (1mg/kg) in DL \( (p=0.003) \) and DM \( (p<0.001) \) striatal quadrants. In the ventrolateral quadrant GABA\textsubscript{A} receptor binding levels were significantly reduced at 0.3mg/kg \( (p=0.005) \). Furthermore, a statistically significant decrease in SN \( (F_{3,36}=4.85, p=0.006) \) was observed at 1mg/kg \( (p=0.004) \).
It is generally accepted that cannabinoid agonists induce a dose-dependent inhibition of motor activity in both humans and laboratory animals and even cause catalepsy with high doses (Gerdeman et al., 2008). In line with previous studies (Sañudo-Peña et al., 2000; Drews et al., 2005; Rodvelt et al., 2007; Polissidis et al., 2010; 2013), our results showed that treatment with the non-selective CB1R agonist WIN55,212-2 dose-dependently suppressed ambulatory activity after single and multiple administrations.

At the neurobiological level, increases in mesostriatal dopamine neurotransmission have been associated with increased spontaneous locomotor activity after treatment with a variety of substances of abuse (Nestler, 2005; Lüscher and Malenka, 2011). However, most of the CNS actions of cannabinoids are mediated via CB1R (Gardner, 2005; Hashimotodani et al., 2007; Vlachou et al., 2008) and activation of these receptors in the striatum is associated with inhibition of motor behaviors (Giuliani et al., 2000; Darmani, 2001; Järbe et al., 2002; Schramm-Sapyta et al., 2007). Several studies have implicated that the mechanism of this CB1R-induced hypomotility involves the interaction between CB1R and D1DR/D2DR dopamine receptors at the cellular level and their coupling to the same effector system (Glass and Felder, 1997; Hermann et al., 2002; Kearn et al., 2005; Andersson et al., 2005; Martin et al., 2008).

Importantly, our results indicated that chronic WIN55,212-2 administration did not induce phenomena of tolerance or sensitization of locomotor activity, although the motor-suppressant effects of the highest dose of WIN55,212-2 tested appeared to level off between the day 10 and 20 of testing. Indeed, while the results of the present study demonstrated that chronic WIN55,212-2 can produce acute and sustained motor-suppressant effects, they provide no evidence that repeated administration of WIN55,212-2 leads to a behavioral
sensitization similar to that observed with other drugs of abuse. Similar results have been obtained after chronic treatment with low doses of $\Delta^9$-THC or other CB1R agonists (Arnold et al., 1998; Muschamp and Siviy, 2002; Ellgren et al., 2004; Kolb et al., 2006; Varvel et al., 2007), although considerably higher doses of $\Delta^9$-THC have been reported to produce behavioral sensitization (Cadoni et al., 2001; Rubino et al., 2001). Although our data did not indicate significant tolerance to the motor-suppressant effects of chronic WIN55,212-2, a study by Sim-Selley and Martin (2002) reported that chronic administration of considerably higher doses of WIN55,212-2 in mice produces tolerance to cannabinoid-mediated hypoactivity. Similar results have been also observed with higher doses of $\Delta^9$-THC (Rodríguez de Fonseca et al., 1994; Romero et al., 1997; Sim-Selley and Martin, 2002; Whitlow et al., 2003). Thus, it appears that behavioral tolerance or sensitization after chronic WIN55,212-2 may depend on several aspects of the experimental protocol, such as species used, dose and route of drug administration.

The present study indicated that chronic administration of WIN55,212-2 for 20 consecutive days modulated the dopaminergic and GABAergic systems of adult rat brain. In particular, we observed selective alterations in binding and mRNA levels of the dopamine transporter (DAT) as well as dopamine and GABA$_A$ receptors in somatodendritic and terminal regions of the mesostriatal dopaminergic system (SN and striatum; VTA and NAc). Considering that only high doses of WIN55,212-2 significantly affected motor activity, the extent to which the observed neurochemical alterations are causally related to the locomotor activity findings in response to chronic administration of WIN55,212-2 is not readily apparent. It may be argued that changes in dopaminergic and GABAergic neurotransmission contribute to the sustained suppressant effects measured after high doses of WIN55,212-2.

In the present study, the use of the non-selective CB1R agonist WIN55212-2, could raise the question whether the observed alterations are attributed to CB1R or CB2R activation.
CB2 cannabinoid receptors are localized primarily in immune cells both in the periphery (Griffin et al., 2000) and in brain microglia (Maresz et al., 2007) indicating that they are activated after brain damage or injury. In addition, they are expressed in neurons, including the striatum and midbrain (Gong et al., 2006), but the extent and level of expression remain controversial (Atwood and Mackie, 2010). However, most of the CNS actions of cannabinoids appear to be mediated via CB1R (Gardner, 2005; Hashimotodani et al., 2007; Vlachou et al., 2008). Systemic administration of CB1R agonists, such as $^9\Delta$-tetrahydrocannabinol and WIN55,212-2, suppress motor activity, especially at higher doses (Darmani, 2001; Polissidis et al., 2013) and this effect is reversed by CB1R specific antagonists.

It is well known that DAT is localized to plasma membranes of axon terminals as well as dendrites of SNpc dopaminergic neurons (Nirenberg et al., 1996) and plays a role in reuptake of dopamine into dendrites and axon terminals (Cheramy et al., 1981). It has been reported that endogenous or exogenous (e.g., WIN55,212-2) cannabinoids inhibit DAT activity in vitro (Chen et al., 2003; Steffens and Feuerstein, 2004), while other studies do not show any effect (Cheer et al., 2004; Köfalvi et al., 2005).

In the present study, we observed reduced DAT mRNA and binding levels in VTA and SNpc at all doses of WIN55,212-2, while DAT binding levels were not altered in striatum but reduced in both NAc shell and core only at the lowest dose. These results suggest that the expression of DAT decreased at both the protein and the mRNA level in mesolimbic and nigrostriatal dopaminergic neurons and this effect is not dose-dependent, with the exception of the NAc. It is interesting to point out that the effect of chronic administration of cannabinoids on DAT binding levels is evident only at the somatodendritic level of dopamine neurons and not at their axonal terminals. These brain regional differences may be a consequence of differential effect of CB1R density and function, as it is known that
repeated treatment with cannabinoids (including WIN55,212-2) induces down-regulation of the cannabinoid receptor (Oviedo et al., 1993; Breivogel et al., 1999; Sim-Selley, 2003; Tanda and Goldberg, 2003; Sim-Selley et al., 2006; Wu et al., 2008). In support of this notion, Moranta et al. (2009) reported brain regional differences in the synthesis of brain monoamines after chronic treatment with WIN55,212-2.

In vivo electrophysiological studies have shown that cannabinoid agonists increase cell firing of the dopaminergic neurons located in SNpc and VTA (French et al., 1997; Wu and French, 2000; Melis et al., 2000). The increased activity of SNpc neurons is in agreement with in vivo microdialysis experiments showing enhanced dopamine release in the striatum after cannabinoid agonist treatment (Tanda et al., 1997; Solinas et al., 2008; Moranta et al., 2009; Polissidis et al., 2010; 2013). Taking into account the above findings and our results of decreased binding and mRNA levels of DAT in SN and VTA, we could suggest that chronic activation of cannabinoid receptors may lead to decreased dopamine uptake by the dendrites of mesolimbic and nigrostriatal dopaminergic neurons, but not by the nigrostriatal terminals. Furthermore, the increased extracellular levels of dopamine in the striatum could be attributed to saturation of DAT (Oleson and Cheer, 2012; Tye et al., 2013) and/or to decreased inhibitory control over dopamine release due to stimulation of D2 autoreceptors (see below).

D2DRs exist in two isoforms (D2S, D2L) generated by alternative splicing of the same gene (Giros et al., 1989). D2S are localized presynaptically on both the somatodendritic and terminal regions of midbrain dopaminergic neurons (Khan et al., 1998). Activation of these receptors in mesencephalic dopaminergic neurons elicits hyperpolarization and decreases firing rate (Lacey et al., 1987; Mercuri et al., 1989; 1997; Centonze et al., 2002), while inhibits dopamine release in their somatodendritic (Cragg and Greenfield, 1997) and terminal mesostriatal regions (Starke et al., 1989; Cragg and Greenfield, 1997; Uziel et
Thus, dopamine release is under the inhibitory control of D2DRs (Benoit-Marand et al., 2001). In the present study, the mRNA levels of the short isoform of D2 receptor (D2S), which corresponds to the presynaptic D2DR, were reduced in SNpc and in VTA after chronic administration of the CB1R agonist, WIN55,212-2. Using \[^{3}H]\text{raclopride} which labels D2DR binding sites, but does not distinguish between presynaptic and postsynaptic localization of D2DR, we were unable to detect any changes of the D2DR at the binding site level in SN and VTA. However, decreased D2DR binding levels were found in the striatum of WIN-treated rats. This change could be correlated to the decreased mRNA expression of D2 autoreceptors observed in SNpc and may reflect a reduction in striatal D2 autoreceptors. However, further immunohistochemical studies using an antibody specific for the D2 autoreceptor are required to verify this change. Our finding is in contrast to the results of Ginovart et al. (2012) who showed increased presynaptic D_{2/3} autoreceptor binding levels after chronic Δ^9-THC administration, but in agreement with the results of Bossong et al. (2009), who showed reduced \[^{14}C]\text{raclopride} binding in human striatum after Δ^9-THC inhalation.

Overall, we could suggest that the somatodendritic regions of the mesostriatal dopaminergic system, the SN and VTA, seem to be more affected than the projection regions, the striatum and NAc, by chronic administration of WIN55,212-2. Decreased mRNA levels of D2 autoreceptors (D2S) and DAT may lead to increased activity of SNpc and VTA neurons and enhanced release of dopamine at their somatodendritic and terminal fields. This finding may be attributed to the fact that chronic exposure to WIN55,212-2 can cause neuroadaptive alterations (i.e., down-regulation) of CB1 receptor (density and function) which is region-specific (Sim-Selley and Martin, 2002).

It is generally accepted that regulation of dopamine release in striatum is mainly the
consequence of alterations in dopaminergic cell firing in the SNpc and the VTA. However, several studies have revealed local regulation of DA release by other neurotransmitters and modulators, such as glutamate. Recent studies suggest that the glutamatergic regulation of dopamine release is inhibitory (Rice et al., 2011). Taking into consideration the above and that activation of CB1R receptors on corticostriatal terminals would inhibit glutamate release, this activation may lead to increased DA release.

Our results have also shown that D1DR mRNA and binding were not altered in striatum; however, in NAc core both mRNA and binding levels were increased at the highest dose only. This dose-dependent effect is specific for the mesolimbic pathway, and it may be related to the pronounced motor-suppressant effects of the high dose of WIN22,212-2 that persisted over the course of administration. It has been suggested that simultaneous stimulation of CB1R and D1DR reduces the inhibitory activity of direct striatal projection neurons, resulting in a decreased motor response (Martín et al., 2008).

It is well known that NAc plays a pivotal role in reward and aversive learning and learning flexibility (Graybiel, 2008). In NAc, similar to dorsal striatum, medium spiny neurons (MSNs) express either D1DR or D2DR along with other receptors and neuropeptides and their distinct roles in learning have only recently been explored (Nakanishi et al., 2014). Furthermore, recent investigations suggest a differential involvement of D1-MSNs and D2-MSN cell populations in NAc in drug-related behaviors (Laviolette et al., 2008; Hikida et al., 2010; Smith et al., 2013). In particular, distinct roles of D1DR and D2DR in the core and shell of NAc have been implicated in the modulation of reward by nicotine (Laviolette et al., 2008), and in the acquisition of cocaine-related learning (Smith et al., 2013; España and Jones, 2013).

Seif et al. (2011) has provided evidence that endocannabinoids mediate the ability of DA receptors to enhance action potential firing in NAc core neurons in vitro, requiring co-
activation of D1DR and D2DR. The selective upregulation of D1DRs in NAc core observed in the present study after high doses of chronic cannabinoid administration suggest that the core versus shell and D1DR- versus D2DR MSNs of NAc may respond differently to repeated cannabinoid administration and these cell-type specific alterations in NAc core may contribute to cannabinoid-related behaviors.

It has been also shown that CB1R are localized presynaptically on GABAergic neurons in several brain regions (Matsuda et al., 1993) and their activation inhibits GABA release (French et al., 1997; Pistis et al., 2002; Szabo et al., 2002; Szabo and Schlicker, 2005; Lupica and Riegel, 2005). More precisely, in SN pars reticulata (SNpr), CB1R are located on GABAergic striatonigral terminals. Furthermore, dendrites of dopaminergic neurons in SNpc extend into the SNpr where they form synapses with CB1-containing axon terminals (Fitzgerald et al., 2012). Considering this localization of CB1R, a possible mechanism for the increased dopamine release after cannabinoid treatment may involve an indirect disinhibition of dopamine neurons (French et al., 1997; Szabo et al., 2002; Lupica and Riegel, 2005). It is, thus, suggested that CB1R, due to their localization, can modulate GABA release and in turn control the activity of the dopaminergic cells in the midbrain (Wu and French, 2000; Laviolette and Grace, 2006; Fernández-Ruiz et al., 2010).

Our results have indicated that GABA<sub>A</sub> receptor binding levels were reduced in the dorsal striatum at all doses and SN at the highest dose. Therefore, in addition to the reduction of GABA released from the striatonigral terminals of the direct pathway, the observed reduction of GABA<sub>A</sub> receptors in SN at least at the higher dose would increase the activity of the GABAergic cells of SNr leading to hypomobility. Overall these results indicate that the effects of WIN55,212-2 on motor activity could be mediated at least partially via GABA<sub>A</sub> receptors expressed in the nigrostriatal pathway. In conclusion, our data indicate that chronic administration of the cannabinoid agonist WIN55,212-2 did not induce
phenomena of tolerance or sensitization of locomotor activity. Furthermore, repeated cannabinoid administration induced neuroadaptive alterations of the dopaminergic and GABAergic systems in a region-, dose- and neurotransmitter-dependent manner.
Acknowledgements

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Statement of Interest

None
References


Lacey MG, Mercuri NB, North RA (1987) Dopamine acts on D2 receptors to increase potassium conductance in neurones of the rat substantia nigra zona compacta. J Physiol 392:397-416.


receptor in the rat striatum: localization and effects on D1 and D2 dopamine receptor-mediated motor behaviors. Neuropsychopharmacology 33:1667-1679.


Pertwee RG, Greentree SG, A. SP (1988) Drugs which stimulate or facilitate central GABAergic transmission interact synergistically with delta-9-

34
tetrahydrocannabinol to produce marked catalepsy in mice. Neuropharmacology 27:1265-1270.


Sim-Selley LJ, Martin BR (2002) Effect of chronic administration of R-(+)
Dihydro-5-methyl-3-[(morpholinyl)methyl]pyrrolo[1,2,3-de]-1,4-
benzoaxazinyl]-(1-naphthalenyl)methanone mesylate (WIN55,212-2) or
delta(9)-tetrahydrocannabinol on cannabinoid receptor adaptation in mice. J
Pharmacol Exp Ther 303:36-44.

Sim-Selley LJ, Schechter NS, Rorrer WK, Dalton GD, Hernandez J, Martin BR,
Selley DE (2006) Prolonged recovery rate of CB1 receptor adaptation after
cessation of long-term cannabinoid administration. Mol Pharmacol 70:986-
996.

Smith RJ, Lobo MK, Spencer S, Kalivas PW (2013) Cocaine-induced adaptations in
D1 and D2 accumbens projection neurons (a dichotomy not necessarily
synonymous with direct and indirect pathways). Curr Opin Neurobiol 23:546-
552.


uptake by cannabinoids in rat neocortex--involvement of Na(+)/K(+)ATPase.
Neurochem Int 44:529-538.

Sunahara RK, Niznik HB, Weiner DM, Stormann TM, Brann MR, Kennedy JL,


**Figure Legends**

**Figure 1:** Effects of repeated WIN55,212-2 administration on spontaneous locomotor activity (n=10 per group). Each plot represents the photocell disruptions caused by the animal’s (A) Ambulatory activity, (B) Rearing. The asterisks (*) signify a statistically significant effect compared to the vehicle group: * p<0.05, **p<0.01 and *** p<0.001.

**Figure 2:** Images of the corresponding brain level from the stereotaxic atlas of the rat brain (Paxinos and Watson, 2007) with their subdivisions of A) striatum/NAc and B) SN/VTA. Abbreviations: DL, dorsolateral; DM, dorsomedial; VL, ventrolateral; VM, ventromedial, SN: substantia nigra, VTA: ventral tegmental area.

**Figure 3:** Autoradiographic localization of A) DAT in striatum/NAc and B) SN/ VTA, C) D1DR in striatum/NAc, D) D2DR in striatum/NAc and E) in SN/ VTA and F) GABA receptor in striatum/NAc, and G) SN/ VTA of different doses of WIN55,212-2.

**Figure 4:** Representative *in situ* hybridization images showing the distribution of mRNA levels of A) DAT in SN/ VTA, B) D1DR in striatum/NAc, C) D2DR in SN/VTA and D) D2S autoreceptors in SN/VTA of different doses of WIN55,212-2.

**Figure 5:** DAT binding site levels labeled with [³H]-WIN35428 in A) striatum, B) nucleus accumbens, C) SNpc and D) VTA of chronically WIN55,212-2 (WIN)- and vehicle-treated rats and DAT mRNA levels in E) SNpc and F) VTA. SNpc: substantia nigra pars compacta; VTA: ventral tegmental area. The asterisks (*) signify a statistically significant effect compared to the vehicle group: * p<0.05, **p<0.01 and ***p<0.001, n=9-10.
Figure 6: DRD1 binding site levels labeled with [³H]-SCH23390 in A) striatum and B) nucleus accumbens and D1DR mRNA levels in C) striatum and D) nucleus accumbens of chronically WIN55,212-2 (WIN)- and vehicle-treated rats. The asterisk (*) denotes a statistically significant effect of WIN55,212-2 compared to the vehicle group: *p<0.05; n=9-10 for receptor autoradiography and n=5 per group for in situ hybridization.

Figure 7: DRD2 binding site levels labeled with [³H]-raclopride in A) striatum, B) nucleus accumbens, C) SN and D) VTA, DRD2 receptor mRNA levels in E) SNpc and F) VTA and D2 autoreceptor mRNA levels in G) SNpc and H) VTA of chronically WIN55,212 (WIN)- and vehicle-treated rats. SN: substantia nigra; VTA: ventral tegmental area; The asterisk (*) denotes a statistically significant effect of WIN55,212-2 compared to the vehicle group: *p<0.05, **p<0.01 and ***p<0.001; n=9-10.

Figure 8: GABA<sub>A</sub> receptor binding site levels labeled with [³H]-SR955321 in A) striatum, B) nucleus accumbens, C) SN and D) VTA of chronically WIN55,212-2 (WIN)- and vehicle-treated rats. SN: substantia nigra; VTA: ventral tegmental area. The asterisks (*) signify a statistically significant effect compared to the vehicle group: **p<0.01 and ***p<0.001; n=9-10.
D1DR binding and mRNA levels

A

[Graph showing D1DR binding and mRNA levels in different regions (DL, DM, VL, VM) for VEHICLE, WIN 0.1mg/kg, WIN 0.3mg/kg, WIN 1mg/kg.]

B

[Graph showing D1DR binding and mRNA levels in Shell and Core regions of the Nucleus accumbens for VEHICLE, WIN 0.1mg/kg, WIN 0.3mg/kg, WIN 1mg/kg.]

C

[Graph showing D1DR mRNA levels (RQ) in different regions (DL, DM, VL, VM) for VEHICLE, WIN 0.1mg/kg, WIN 0.3mg/kg, WIN 1mg/kg.]

D

[Graph showing D1DR mRNA levels (RQ) in Shell and Core regions of the Nucleus accumbens for VEHICLE, WIN 0.1mg/kg, WIN 0.3mg/kg, WIN 1mg/kg.]

160x116mm (300 x 300 DPI)
D2DR binding and mRNA levels

A

B

C

D

E

F

G

H

152x205mm (300 x 300 DPI)
Table 1: DAT binding site levels labeled with $[^3]H$-WIN35428 in striatum, nucleus accumbens, SNpc and VTA of chronically WIN55,212-2 (WIN)- and vehicle-treated rats.

<table>
<thead>
<tr>
<th>BRAIN REGION</th>
<th>VEHICLE</th>
<th>WIN 0.1mg/kg</th>
<th>WIN 0.3mg/kg</th>
<th>WIN 1mg/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dorsolateral striatum</td>
<td>46.22±1.08 n=10</td>
<td>46.23±1.93 n=10</td>
<td>48.70±2.18 n=10</td>
<td>46.65±1.09 n=10</td>
</tr>
<tr>
<td>Dorsomedial striatum</td>
<td>40.11±1.01 n=10</td>
<td>39.33±0.96 n=10</td>
<td>38.28±1.04 n=10</td>
<td>43.00±2.09 n=10</td>
</tr>
<tr>
<td>Ventrolateral striatum</td>
<td>41.99±1.56 n=10</td>
<td>42.07±0.98 n=10</td>
<td>42.33±1.11 n=10</td>
<td>45.19±1.68 n=10</td>
</tr>
<tr>
<td>Ventromedial striatum</td>
<td>46.08±1.32 n=10</td>
<td>46.54±1.97 n=10</td>
<td>43.79±1.14 n=10</td>
<td>46.40±1.15 n=10</td>
</tr>
<tr>
<td>Nucleus accumbens shell</td>
<td>38.55±1.27 n=10</td>
<td>32.73±2.14*** n=10</td>
<td>36.51±1.42 n=10</td>
<td>40.43±0.96 n=10</td>
</tr>
<tr>
<td>Nucleus accumbens core</td>
<td>39.92±1.27 n=10</td>
<td>31.59±1.68*** n=10</td>
<td>35.63±1.34 n=10</td>
<td>38.74±1.18 n=10</td>
</tr>
<tr>
<td>SNpc</td>
<td>98.48±3.33 n=10</td>
<td>73.49±1.69*** n=10</td>
<td>83.39±2.33*** n=10</td>
<td>74.79±2.47*** n=10</td>
</tr>
<tr>
<td>VTA</td>
<td>101.47±3.61 n=9</td>
<td>78.10±3.28* n=10</td>
<td>85.70±2.93* n=10</td>
<td>83.85±5.75*** n=9</td>
</tr>
</tbody>
</table>

SNpc: substantia nigra pars compacta; VTA: ventral tegmental area. The asterisks (*) signify a statistically significant effect compared to the vehicle group: * p<0.05, **p<0.01 and *** p<0.001, n=9-10.
Table 2: DAT mRNA levels in SNpc and VTA of chronically WIN55,212-2 (WIN)-
and vehicle-treated rats.

<table>
<thead>
<tr>
<th>BRAIN REGION</th>
<th>VEHICLE</th>
<th>WIN 0.1mg/kg</th>
<th>WIN 0.3mg/kg</th>
<th>WIN 1mg/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>SNpc</td>
<td>0.42±0.008</td>
<td>0.378±0.0122*</td>
<td>0.361±0.010**</td>
<td>0.353±0.011***</td>
</tr>
<tr>
<td></td>
<td>↓10%</td>
<td>↓14.05%</td>
<td>↓15.95%</td>
<td></td>
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<tr>
<td>VTA</td>
<td>0.413±0.011</td>
<td>0.332±0.006**</td>
<td>0.331±0.010***</td>
<td>0.349±0.013*</td>
</tr>
<tr>
<td></td>
<td>↓19.6%</td>
<td>↓19.8%</td>
<td>↓15.5%</td>
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</table>

SNpc: substantia nigra pars compacta; VTA: ventral tegmentall area. The asterisks (*) signify a statistically significant effect compared to the vehicle group: * p<0.05,
**p<0.01 and ***p<0.001; n=10 per group.
Table 3: DRD1 binding site levels labeled with \[^{3}\text{H}]\text{-SCH23390\) in striatum, nucleus accumbens, SNpc and VTA of chronically WIN55,212-2 (WIN)- and vehicle-treated rats.

<table>
<thead>
<tr>
<th>BRAIN REGION</th>
<th>VEHICLE</th>
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<th>WIN0.3mg/kg</th>
<th>WIN1mg/kg</th>
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</thead>
<tbody>
<tr>
<td>Dorsolateral striatum</td>
<td>118.45±3.82</td>
<td>112.27±1.71</td>
<td>121.61±1.22</td>
<td>117.88±4.28</td>
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<tr>
<td>Dorsomedial striatum</td>
<td>118.19±3.31</td>
<td>115.59±2.26</td>
<td>115.92±3.10</td>
<td>117.36±4.76</td>
</tr>
<tr>
<td>Ventrolateral striatum</td>
<td>121.50±1.87</td>
<td>117.91±2.76</td>
<td>120.15±2.82</td>
<td>122.18±3.12</td>
</tr>
<tr>
<td>Ventromedial striatum</td>
<td>119.36±3.10</td>
<td>115.33±1.45</td>
<td>123.24±3.13</td>
<td>123.32±1.83</td>
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<tr>
<td>Nucleus accumbens shell</td>
<td>118.79±3.87</td>
<td>125.80±8.26</td>
<td>116.71±3.56</td>
<td>118.05±5.72</td>
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<tr>
<td>Nucleus accumbens core</td>
<td>102.47±3.47</td>
<td>105.88±4.49</td>
<td>101.72±2.91</td>
<td>116.24±2.15*</td>
</tr>
</tbody>
</table>

The asterisk (*) denotes a statistically significant effect of WIN55,212-2 compared to the vehicle group: p<0.05; n=9-10.
Table 4: DRD1 mRNA levels in striatum and nucleus accumbens of chronically WIN55,212-2 (WIN)- and vehicle-treated rats.

<table>
<thead>
<tr>
<th>BRAIN REGION</th>
<th>VEHICLE</th>
<th>WIN 0.1mg/kg</th>
<th>WIN 0.3mg/kg</th>
<th>WIN 1mg/kg</th>
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<tbody>
<tr>
<td>Dorsolateral striatum</td>
<td>0.20±0.0014</td>
<td>0.202±0.0035</td>
<td>0.202±0.0042</td>
<td>0.208±0.0043</td>
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<tr>
<td>Dorsomedial striatum</td>
<td>0.203±0.0031</td>
<td>0.201±0.0032</td>
<td>0.201±0.0037</td>
<td>0.20872±0.0043</td>
</tr>
<tr>
<td>Ventrolateral striatum</td>
<td>0.21±0.0025</td>
<td>0.209±0.0043</td>
<td>0.209±0.0054</td>
<td>0.209±0.0047</td>
</tr>
<tr>
<td>Ventromedial striatum</td>
<td>0.197±0.0039</td>
<td>0.197±0.0027</td>
<td>0.197±0.0032</td>
<td>0.201±0.0046</td>
</tr>
<tr>
<td>Nucleus accumbens</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Shell</td>
<td>0.22±0.004</td>
<td>0.215±0.007</td>
<td>0.22±0.004</td>
<td>0.24±0.006</td>
</tr>
<tr>
<td>Core</td>
<td>0.18±0.0018</td>
<td>0.18±0.0031</td>
<td>0.172±0.002</td>
<td>0.194±0.003*</td>
</tr>
</tbody>
</table>

The asterisk (*) signify a statistically significant effect compared to the vehicle group:

**p<0.01; n=5 per group.
Table 5: DRD2 binding site levels labeled with $[^3]$H-raclopride in striatum, nucleus accumbens, SN and VTA of chronically WIN55,212 (WIN)- and vehicle-treated rats.

<table>
<thead>
<tr>
<th>BRAIN REGION</th>
<th>VEHICLE</th>
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<th>WIN 0.3mg/kg</th>
<th>WIN1mg/kg</th>
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<tbody>
<tr>
<td>Dorsolateral striatum</td>
<td>221.31±5.89 n=9</td>
<td>207.98±7.35 n=10</td>
<td>214.27±8.47 n=10</td>
<td>204.46±6.88 n=10</td>
</tr>
<tr>
<td>Dorsomedial striatum</td>
<td>210.02±8.87 n=9</td>
<td>175.06±7.29* n=10</td>
<td>191.20±4.75 n=10</td>
<td>178.97±7.68* n=10</td>
</tr>
<tr>
<td>Ventrolateral striatum</td>
<td>221.08±2.87 n=9</td>
<td>191.01±7.40 n=10</td>
<td>191.72±5.34 n=10</td>
<td>205.10±6.84 n=10</td>
</tr>
<tr>
<td>Ventromedial striatum</td>
<td>203.61±5.06 n=9</td>
<td>170.47±5.87* n=10</td>
<td>182.95±5.44 n=10</td>
<td>171.93±6.14* n=10</td>
</tr>
<tr>
<td>Nucleus accumbens shell</td>
<td>54.95±1.87 n=10</td>
<td>53.21±2.95 n=9</td>
<td>62.00±2.43 n=9</td>
<td>58.07±2.88 n=9</td>
</tr>
<tr>
<td>Nucleus accumbens core</td>
<td>56.27±3.26 n=9</td>
<td>59.64±2.68 n=10</td>
<td>51.94±2.17 n=10</td>
<td>48.95±2.68 n=10</td>
</tr>
<tr>
<td>SN</td>
<td>53.53±1.63 n=10</td>
<td>50.42±0.89 n=10</td>
<td>55.05±1.55 n=10</td>
<td>57.09±2.18 n=10</td>
</tr>
<tr>
<td>VTA</td>
<td>41.28±0.83 n=10</td>
<td>45.37±1.61 n=10</td>
<td>44.66±1.74 n=10</td>
<td>46.17±2.07 n=10</td>
</tr>
</tbody>
</table>

SN: substantia nigra; VTA: ventral tegmental area; The asterisk (*) denotes a statistically significant effect of WIN55,212-2 compared to the vehicle group: *$p<0.05$; n=9-10.
Table 6: DRD2 receptor mRNA levels in SNpc and VTA of chronically WIN55,212-2 (WIN)- and vehicle-treated rats.

<table>
<thead>
<tr>
<th>BRAIN REGION</th>
<th>VEHICLE</th>
<th>WIN 0.1mg/kg</th>
<th>WIN0.3mg/kg</th>
<th>WIN1mg/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>SNpc</td>
<td>0.411±0.021 n=9</td>
<td>0.439±0.008 n=10</td>
<td>0.413±0.014 n=10</td>
<td>0.456±0.0052 n=10</td>
</tr>
<tr>
<td>VTA</td>
<td>0.356±0.031 n=9</td>
<td>0.375±0.024 n=10</td>
<td>0.382±0.021 n=10</td>
<td>0.391±0.024 n=10</td>
</tr>
</tbody>
</table>

SNpc: substantia nigra pars compacta; VTA: ventral tegmental area; n=9-10.
Table 7: D2 autoreceptor mRNA levels in SNpc and VTA of chronically WIN55,212-2 (WIN)- and vehicle-treated rats.

<table>
<thead>
<tr>
<th>BRAIN REGION</th>
<th>VEHICLE</th>
<th>WIN 0.1mg/kg</th>
<th>WIN0.3mg/kg</th>
<th>WIN1mg/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>SNpc</td>
<td>0.46±0.007</td>
<td>0.359±0.005***</td>
<td>0.343±0.006**</td>
<td>0.353±0.009***</td>
</tr>
<tr>
<td></td>
<td>↓21.96%</td>
<td>↓25.43%</td>
<td>↓23.26%</td>
<td></td>
</tr>
<tr>
<td>VTA</td>
<td>0.406±0.022</td>
<td>0.36±0.008</td>
<td>0.344±0.005*</td>
<td>0.338±0.005*</td>
</tr>
<tr>
<td></td>
<td>↑15.27%</td>
<td>↑16.75%</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

SNpc: substantia nigra pars compacta; VTA: ventral tegmental area. The asterisks (*) signify a statistically significant effect compared to the vehicle group: * p<0.05, **p<0.01 and ***p<0.001; n=10 per group.
**Table 8**: GABA\_A receptor binding site levels labeled with \([3H]-SR95532\) in striatum, nucleus accumbens, SN and VTA of chronically WIN55,212-2 (WIN)- and vehicle-treated rats.

<table>
<thead>
<tr>
<th>BRAIN REGION</th>
<th>VEHICLE</th>
<th>WIN 0.1mg/kg</th>
<th>WIN0.3mg/kg</th>
<th>WIN1mg/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dorsolateral striatum</td>
<td>82.81±2.08</td>
<td>72.44±0.99***</td>
<td>71.41±1.68***</td>
<td>73.83±1.67**</td>
</tr>
<tr>
<td>n=10</td>
<td></td>
<td>↓12.5% n=10</td>
<td>↓13.8% n=10</td>
<td>↓10.84% n=10</td>
</tr>
<tr>
<td>Dorsomedial striatum</td>
<td>90.81±1.84</td>
<td>79.05±1.29***</td>
<td>78.73±1.23***</td>
<td>77.22±1.25***</td>
</tr>
<tr>
<td>n=10</td>
<td></td>
<td>↓12.9% n=10</td>
<td>↓13.3% n=10</td>
<td>↓14.96% n=10</td>
</tr>
<tr>
<td>Ventrolateral striatum</td>
<td>88.98±1.40</td>
<td>87.16±1.05</td>
<td>80.14±0.96**</td>
<td>83.25±2.75</td>
</tr>
<tr>
<td>n=10</td>
<td></td>
<td></td>
<td>↓9.9% n=10</td>
<td>n=10</td>
</tr>
<tr>
<td>Ventromedial striatum</td>
<td>100.21±1.59</td>
<td>97.21±2.89</td>
<td>95.29±1.62</td>
<td>103.26±1.07</td>
</tr>
<tr>
<td>n=10</td>
<td></td>
<td></td>
<td>n=10</td>
<td>n=10</td>
</tr>
<tr>
<td>Nucleus accumbens shell</td>
<td>111.90±2.49</td>
<td>111.15±1.89</td>
<td>109.23±2.5</td>
<td>121.13±2.44</td>
</tr>
<tr>
<td>n=10</td>
<td></td>
<td></td>
<td>n=10</td>
<td>n=10</td>
</tr>
<tr>
<td>Nucleus accumbens core</td>
<td>116.24±1.72</td>
<td>112.59±3.35</td>
<td>115.06±3.64</td>
<td>124.19±2.91</td>
</tr>
<tr>
<td>n=10</td>
<td></td>
<td></td>
<td>n=10</td>
<td>n=10</td>
</tr>
<tr>
<td>SN</td>
<td>84.13±1.26</td>
<td>77.22±3.13</td>
<td>80.05±3.32</td>
<td>70.39±2.25**</td>
</tr>
<tr>
<td>n=10</td>
<td></td>
<td></td>
<td>n=10</td>
<td>↓16.33% n=10</td>
</tr>
<tr>
<td>VTA</td>
<td>60.95±3.00</td>
<td>55.24±1.66</td>
<td>55.51±1.08</td>
<td>57.62±2.71</td>
</tr>
<tr>
<td>n=9</td>
<td></td>
<td></td>
<td>n=10</td>
<td>n=10</td>
</tr>
</tbody>
</table>

SN: substantia nigra; VTA: ventral tegmental area. The asterisks (*) signify a statistically significant effect compared to the vehicle group: * p<0.05, **p<0.01 and ***p<0.001; n=9-10.
Supplementary Material

Methods

Oligonucleotide probes

The following oligodeoxyribonucleotide probe sequences were used (Institute of Research and Technology, Heraklion, Greece): 1) 5’- ttg atg agg gtg gag ttg gtc agc tgc act ccg ttc tgc tc -3’ corresponding to the DAT mRNA sequence 106-146 (Giros et al., 1991); 2) 5’-att gaa ggg ccg gct cca gtt ctg cct ctc cag atc gtc atc gta cca- 3’ and 5’-ccg cct gtt cac tgg gaa act ccc att aga cat gat aac ggt gca- 3’, corresponding to the D2DR mRNA sequences 22-69 and 699-746, respectively (Bunzow et al., 1988); 3) 5’-ctc ggc ggg cag cat cct tga gtg gtg tct tca ggt- 3’ corresponding to the D2DR mRNA sequence 704–739 (Bunzow et al., 1988) specific for the short isoform of the D2DR (D2S) and corresponding to the dopamine autoreceptor (Khan et al., 1998) and 4) 5’-gga ctg ctg ccc tct cca agg ctg aga tgc gcc gga ttt gct tct ggg-3’ and 5’-tgt cac agt tgt cat cct cgg tgt cct cca ggg agg taa aat tgc cat -3’, corresponding to the D1DR mRNA sequence 519-556 and 665-712 (Sunahara et al., 1990; Zhou et al., 1990). Probe specificity was confirmed using NCBI BLAST.

Results

Analysis of 5-min bins of ambulatory activity

Analysis on the 5min bins (of the 60min session) on day 1 of ambulatory activity using two-way ANOVA with repeated measures demonstrated a significant dose x time interaction ($F_{33, 396}= 2.95, p<0.001$). One-way ANOVA with repeated measures on time (5min bins in total of 60min) indicated a significant time effect in all the doses tested (vehicle: $F_{11, 99}=29.44$, WIN 0.1 mg/kg: $F_{11, 99}=22.06$, WIN 0.3 mg/kg: $F_{11, 99}=21.41$).
and WIN 1 mg/kg: $F_{11,\,99}=3.89$ all for $p<0.001$). One-way ANOVA on each 5min bin (total of 12 5-min bins) demonstrated significant differences on time points 0-5 min ($F_{3,\,36}= 8.14$, $p<0.001$), 10-15min ($F_{3,\,36}= 3.88$, $p<0.05$), 15-20min ($F_{3,\,36}= 3.59$, $p<0.05$) and 40-45min ($F_{3,\,36}= 4.33$, $p<0.01$) while for the rest of the 5min bins differences did not reach statistical significance. LSD post-hoc test indicated that only WIN 1 mg/kg demonstrated a statistically significant effect on time point 0-5min ($p<0.001$) and both WIN 1 mg/kg and WIN 0.3 mg/kg demonstrated significant effect on time point 10-15min ($p<0.05$). On time point 15-20min the effect reached significance only for WIN 0.3 mg/kg ($p<0.05$) while on time point 40-45min WIN 0.1 mg/kg ($p<0.01$) demonstrated a statistically significant difference compared to the control group (Supplementary Figure S1Ai).

On day 10 two-way ANOVA demonstrated a significant dose x time interaction on ambulatory activity ($F_{33,\,396}= 3.56$, $p<0.001$). One-way ANOVA with repeated measures on time (each 5min bin in total of 60min) indicated a significant time effect for vehicle ($F_{11,\,99}=11.57$, $p<0.001$), WIN 0.1 mg/kg ($F_{11,\,99}=9.96$, $p<0.001$) and WIN 0.3 mg/kg ($F_{11,\,99}=6.76$, $p<0.001$) but not WIN 1 mg/kg ($p>0.05$). One-way ANOVA on each 5min bin (total of 12 5-min bins) demonstrated significant differences on time points 0-5 min ($F_{3,\,36}= 9.09$, $p<0.001$), 5-10min ($F_{3,\,36}= 4.66$, $p<0.01$), 15-20min ($F_{3,\,36}= 7.36$, $p<0.001$) and 20-25min ($F_{3,\,36}= 3.87$, $p<0.05$). Furthermore, a strong tendency for statically significant differences was observed for time point 10-15min ($F_{3,\,36}= 2.77$, $p=0.055$) but for the rest of the 5min bins no significant differences were observed. LSD post-hoc test indicated that WIN 0.3 mg/kg and WIN 1 mg/kg significantly affected ambulatory activity on time point 0-5min ($p<0.05$ and $p<0.001$ respectively) while this effect reached significance only for WIN 1 mg/kg on time point 5-10min ($p<0.001$). On time point 15-20min the effect reached significance only for WIN 0.1 mg/kg ($p<0.05$)
although the effect of WIN 1 mg/kg was closed to be significant too (p=0.055). The effect of WIN 0.1 mg/kg reached significant difference also for time point 20-25min (p<0.05) ((Supplementary Figure S1Aii).

On day 20, two-way ANOVA demonstrated a significant dose x time interaction on ambulatory activity (F_{33, 396}= 2.25, p<0.001). One-way ANOVA with repeated measures on time (each 5min bin in total of 60min) indicated a significant time effect for all the doses (vehicle F_{11, 99}=8.1 p<0.001, WIN 0.1 mg/kg F_{11, 99}=8.24 p<0.001, WIN 0.3 mg/kg F_{11, 99}=5.11 p<0.001 and WIN 1 mg/kg F_{11, 99}=2.40 p<0.01). One-way ANOVA on each 5min bin (total of 12 5min bins) demonstrated significant differences on time points 0-5 min (F_{3, 36}= 3.76, p<0.05), 5-10min (F_{3, 36}= 3.00, p<0.05), 10-15min (F_{3, 36}= 3.56, p<0.05) and 15-20min (F_{3, 36}= 3.31, p<0.05) while for the rest of the 5-min bins differences did not reach statistical significance. LSD post-hoc test demonstrated no significant effects of WIN on time points 0-5min and 15-20min but the effect of WIN 0.3 mg/kg and WIN 1 mg/kg reached significance for time points 5-10min (p<0.05 for both doses) and 10-15min (p<0.05 and p<0.01 respectively). (Supplementary Figure S1Aiii).

Analysis of 5-min bins of rearing

Analysis on the 5-min bins on day 1 of rearing using two-way ANOVA demonstrated a significant dose x time interaction (F_{33, 396}= 3.65, p<0.001). One-way ANOVA with repeated measures on time (5min bins in total of 60min) indicated a significant time effect in all the doses tested (vehicle: F_{11, 99}=7.50, WIN 0.1 mg/kg: F_{11, 99}=13.26, WIN 0.3 mg/kg: F_{11, 99}=8.85 and WIN 1 mg/kg: F_{11, 99}=4.28 all for p<0.001). One-way ANOVA on each 5min bin (total of 12 5min bins) demonstrated significant differences only on time points 0-5 min (F_{3, 36}= 16.67, p<0.001) and 40-45min (F_{3, 36}= 4.97, p<0.01).
LSD post-hoc test indicated that WIN 0.1 mg/kg and WIN 1 mg/kg demonstrated a statistically significant effect on time point 0-5min (p<0.001 and p<0.01 respectively) and on time point 40-45min WIN 0.1 mg/kg (p<0.01) demonstrated a statistically significant difference compared to the control group (Supplementary Figure S1Bi).

On day 10, two-way ANOVA demonstrated a significant dose x time interaction on rearing (F_{33, 396} = 2.07, p=0.001). One-way ANOVA with repeated measures on time (each 5min bin in total of 60min) indicated a significant time effect for vehicle (F_{11, 99}=7.23, p<0.001), WIN 0.1 mg/kg (F_{11, 99}=3.33, p<0.001) and WIN 0.3 mg/kg (F_{11, 99}=4.09, p<0.001) but not WIN 1 mg/kg (p>0.05). One-way ANOVA on each 5min bin (total of 12 5min bins) demonstrated significant differences only on time points 0-5 min (F_{3, 36}= 4.99, p<0.01) and 15-20min (F_{3, 36}= 6.50, p=0.001). LSD post-hoc test indicated that only WIN 1 mg/kg significantly affected rearing on time point 0-5min (p<0.01), while the effect reached significance only for WIN 0.1 mg/kg on time point 15-20min (p<0.05) (Supplementary Figure S1Bii).

On day 20, two-way ANOVA demonstrated a significant dose x time interaction on rearing (F_{33, 396} = 1.93, p<0.01). One-way ANOVA with repeated measures on time (each 5min bin in total of 60min) indicated a significant time effect for WIN 0.1 mg/kg (F_{11, 99}=6.05 p<0.001), WIN 0.3 mg/kg (F_{11, 99}=3.90 p<0.001) and WIN 1 mg/kg (F_{11, 99}=2.02 p<0.05) but not vehicle (p>0.05). One-way ANOVA on each 5min bin (total of 12 5min bins) demonstrated significant differences only on time points 0-5 min (F_{3, 36}= 3.51, p<0.05) and 15-20min (F_{3, 36}= 2.95, p<0.05). LSD post-hoc test demonstrated a significant effect of WIN 0.1 mg/kg on time points 0-5min and 15-20min (p<0.05) (Supplementary Figure S1 Biii).
**Supplementary Figure S1:** Effects of repeated WIN55,212-2 administration on spontaneous locomotor activity (n=10 per group). (A) Ambulatory activity (i) on day 1, (ii) on day 10, (iii) on day 20 of treatment. (B) Rearing (i) on day 1, (ii) on day 10, (iii) on day 20 of treatment. The asterisks (*) signify a statistically significant effect compared to the vehicle group: * p<0.05, **p<0.01 and *** p<0.001.