THE POTENT α_2-ADRENOCEPTOR ANTAGONIST RS 79948 ALSO INHIBITS DOPAMINE D_2 -RECEPTORS: COMPARISON WITH ATIPAMEZOLE AND RACLOPRIDE

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ABSTRACT

Neurochemical, electrophysiological and behavioral evidence indicate that the potent α_2-adrenoceptor antagonist RS 79948 is also a dopamine (DA) D_2 receptor antagonist. Thus, results from ligand binding and adenylate cyclase activity indicate that RS 79948 binds to D_2 receptors and antagonized D_2 receptor-mediated inhibition of cAMP synthesis at nanomolar concentrations. Results from microdialysis indicated that RS 79948 shared with the selective α_2-adrenergic antagonist atipamezole the ability to increase the co-release of DA and norepinephrine (NE) from noradrenergic terminals in the medial prefrontal cortex (mPFC), except that RS 79948-induced DA release persisted after noradrenergic denervation, unlike atipamezole effect, indicating that RS 79948 releases DA from dopaminergic terminals as well. Similarly to the D_2 antagonist raclopride, but unlike atipamezole, RS 79948 increased extracellular DA and DOPAC in the caudate nucleus. Electrophysiological results indicate that RS 79948 shared with raclopride the ability to activate the firing of ventral tegmental area (VTA) DA neurons, while atipamezole was ineffective.

Results from behavioral studies indicated that RS 79948 exerted effects mediated by independent, cooperative and contrasting inhibition of α_2- and D_2 receptors. Thus, RS 79948, but not atipamezole, prevented D_2-autoreceptor mediated hypomotility produced by a small dose of quinpirole. RS 79948 potentiated, more effectively than atipamezole, quinpirole-induced motor stimulation. RS 79948 antagonized, less effectively than atipamezole, raclopride-induced catalepsy. Future studies should clarify if the dual α_2-adrenoceptor- and D_2-receptor antagonistic action might endow RS 79948 with potential therapeutic relevance in the treatment of schizophrenia, drug dependence, depression and Parkinson's disease.

KEYWORDS: anti-dopamine beta-hydroxylase saporin, behavior, catecholamines, in vivo electrophysiology, microdialysis

ABBREVIATIONS: dopamine (DA); norepinephrine (NE); norepinephrine transporter (NET); dihydroxyphenylacetic acid (DOPAC); prefrontal cortex (PFC); medial prefrontal cortex (mPFC); anti-dopamine-beta-hydroxylase saporin (aDBH); ventral tegmental area (VTA); cyclic adenosine monophosphate (cAMP).

1. INTRODUCTION

Over the last two decades, α_2-adrenoceptors antagonists have been successfully used as adjunctive therapy to improve pharmacotherapy of antipsychotics (Svensson, 2003; Litman et al, 1993; 1996), antidepressants (Maes et al., 1999), and anti-parkinsonian drugs (Thobois et al., 2013; Perez-Lloret and Rascol, 2016). The beneficial effect has been attributed to their ability to enhance dopamine (DA) output and dopaminergic transmission in the prefrontal cortex (PFC), thereby rescuing a dopaminergic hypoactivity present in schizophrenia (Bird et al., 1979; Tassin, 1992; van Kammen and Kelley, 1991; Slifstein and Abi-Dargham, 2017; Rao et al., 2019), mental depression (Bunney and Davies, 1965; Dunlop and Nemeroff, 2007; El Mansari et al., 2010; Kapur and Mann, 1992),

drug addiction (Koob and Nestler, 1997; Wise, 1998; Weinshenker and Schroeder, 2007; Aston-Jones and Kalivas, 2008; Volkow et al., 2007) and Parkinson's disease (Remy et al., 2005; Starkstein and Brockman, 2011; Passamonti et al., 2018).

However, the mechanism by which α_2-adrenoceptor blockade leads to increased DA output in the PFC is debated. It has been suggested that blockade of α_2-autoreceptors, by increasing norepinephrine (NE) output and extracellular NE concentration in the PFC, reduces DA clearance from extracellular space due to competition of NE and DA for the same NE transporter (NET) (Carboni et al., 1990; Pozzi et al., 1994; Gresch et al., 1995; Moron et al., 2002; Carboni and Silvagni, 2004). Alternatively, it has been postulated that α_2-adrenoceptor blockade increases DA output by removing a tonic α_2-adrenoceptor-mediated inhibitory control by NE on DA release in the medial PFC (mPFC) (Pozzi et al., 1994; Gresch et al., 1995; Hertel et al., 1999).

At variance from these hypotheses, early studies from our laboratory provided evidenced that NE and DA may be co-released from noradrenergic terminals in the PFC (Devoto et al., 2001, Devoto et al., 2002; Devoto et al., 2003; Devoto et al., 2004; Devoto and Flore, 2006). Consistent with this hypothesis, more recently we found that noradrenergic denervation suppressed the increase of extracellular DA induced by the α_2-adrenoceptor antagonist atipamezole in the mPFC, suggesting that noradrenergic terminals are the primary source of α_2-adrenoceptor-mediated DA release in the mPFC (Devoto et al., 2019). Recent data from our laboratory provided more evidence that DA measured by microdialysis in the mPFC originates from noradrenergic terminals, DA release being dissociated from firing of dopaminergic neurons (Devoto et al., 2020). Indeed, D_2-receptor antagonists do not elevate extracellular DA measured by microdialysis in the mPFC, despite that they activate the firing of meso-cortical DA neurons (Gessa et al., 2000) but they disclose their ability to elevate extracellular DA if NET is inactivated or eliminated by noradrenergic denervation (Devoto et al., 2019; 2020).

The compound RS 79948 ((8aR,12aS,13aS)-12-ethylsulfonyl-3-methoxy-5,6,8,8a,9,10,11,12a,13,13a-decahydroisoquinolino[2,1-g][1,6]naphthyridine) is classed among the most potent and selective α_2-adrenoceptor antagonists (Clark et al., 1989). It binds with picomolar affinity to all α_2-adrenoceptor subtypes, while has negligeable affinity for α_1- adrenoceptors, imidazoline-, serotonin- and muscarinic receptors (Clark et al., 1989; Milligan et al., 1997). Due to these properties, RS 799748 has been evaluated in rats as a potential PET ligand for central α_2-adrenoceptors in humans (Hume et al., 1996). Moreover, RS 79948 and the α_2-adrenoceptor antagonist atipamezole, because of their far greater affinity and selectivity for α_2-adrenoceptors with respect to other adrenergic receptor subtypes, are better tools for physiological investigations on α_2-adrenoceptors than other α_2-antagonists commonly used in clinical and experimental research, such as yohimbine or idazoxan.

While trying to extend to RS 79948 the microdialysis results obtained with atipamezole, we found that this compound, expectedly, increased extracellular NE and DA in the mPFC, similarly to atipamezole, but we also found that RS 79948-induced elevation of extracellular DA was not abolished after noradrenergic denervation. This finding led us to investigate whether RS 79948 might induce DA release other than from noradrenergic also from dopaminergic terminals, this property being disclosed after noradrenergic denervation, as in the case of D_2-receptor inhibitors. Thus, this research was aimed at verifying whether RS 79948 display D_2 dopaminergic- other than α_2-adrenergic antagonistic activity. Receptor binding, microdialysis, electrophysiological and behavioral results indicated that RS 79948 inhibits D_2 receptors other that α_2-adrenoceptors. Moreover, we compared the effect of RS 79948 systemic administration with that of atipamezole and raclopride, in tests mediated by α_2-adrenoceptors and D_2 receptors, respectively, to evaluate how its double action would interfere with dopaminergic- or noradrenergic system dependent behaviors.

2. MATERIALS AND METHODS

2.1 Subjects

Male Sprague-Dawley rats (Charles River, Italy) weighing 250–350 g, were group-housed and kept on a regular 12:12 h light/dark cycle, in temperature- and humidity-controlled facilities, with food and water available *ad libitum*. The experimental protocols were conducted to minimize pain and suffering and to reduce the number of animals used. Experiments were authorized by Italian Ministry of Health (aut. N. 611/2017-PR) and were carried out in accordance with the European Directive on the protection of animals used for scientific purposes (2010/63/EU).

2.2 Drugs and treatments

The aDBH immunotoxin was purchased from Advanced Targeting System (San Diego, CA, USA); it was administrated at 5 µg/5 µl ICV. RS 79948 ((8aR,12aS,13aS)-5,8,8a,9,10,11,12,12a,13,13a-dechydro-3-methoxy-12-(ethylsulfonyl)-6H-isoquino[2,1-g][1,6]naphthyridine hydrochloride, CAS 186002-54-0), IP administered at 3 mg/kg, was from Tocris Bio-Techne (Minneapolis, Minnesota, USA); atipamezole hydrochloride (5-(2-Ethyl-2,3-dihydro-1H-indene-2-yl)-1H-imidazole hydrochloride, CAS 104075-48-1), IP administered at 3 mg/kg, was from Orion Pharma (Antisedan™); (-)-quinpirole hydrochloride ((4aR-trans)-4,4a,5,6,7,8,8a,9-Octahydro-5-propyl-1H-pyrazolo[3,4-g]quinoline hydrochloride, CAS 85798-08-9, IP administered at 0.5 or 0.1 mg/kg) and S(−)-Raclopride (+)-tartrate salt (3,5-Dichloro-N-(1-ethylpyrrolidin-2-ylmethyl)-2-hydroxy-6-methoxybenzamide (+)-tartrate salt, CAS n. 98185-20-7, IP administered at 0.5 or 2 mg/kg) were from Sigma-Aldrich. For electrophysiology experiments, atipamezole and RS 79948 were intravenously (IV) administered in cumulative doses from 0.0625 to 0.5 mg/kg, raclopride was from 0.006 to 0.05 mg/kg. Drugs were dissolved in sterile distilled water or saline and administered in a volume of 1 ml/kg body weight. For behavioral experiments, coadministration of quinpirole plus another drug was performed with a single IP injection of the two drugs in 2 ml/kg volume.

2.3 Binding assay

Evaluation of the affinity of RS 79948 for DA D_{2short} and D_{2long} receptors was determined by Eurofins Cerep (Poitiers, France), according to their protocols n. 46 and 1405, respectively. Briefly, radioligand binding assays were performed with 0.3 nM [^{3}H]methyl-spiperone in recombinant HEK-293 cells expressing short- or long isoforms of human DA D_2-receptors (Grandy et al., 1989; Hall and Strange, 1997). Specific binding was determined in the presence of 10 μM butaclamol; [^{3}H]methyl-spiperone K_d was 0.15 and 0.1 nM for short and long isoform, respectively. RS 79948 displacement curves were analyzed by non-linear regression by Prism 9.1.2 for MacOS (GraphPad Software, La Jolla California USA, www.graphpad.com) to calculate its K_i for each DA D_2-receptor isoform.

2.4 Adenyl cyclase assay

Rat insulinoma INS-1 832/13 cells were seeded in white plates and after 24 h were assayed for adenyl cyclase activity. The growing medium was replaced with non-supplemented RPMI-1640 containing the phosphodiesterase inhibitors 1-methyl-3-isobutylxanthine (500 μM) and Ro-20–837 1724 (100 μM), 10 μM forskolin to stimulate adenyl cyclase activity and 10 μM quinpirole, alone or plus increasing concentration of RS 79948. Cell lysates were assayed following manufacture instruction with the cAMP Parameter Assay Kit KGE002B (R&D Systems, Minneapolis, USA). The luminescence was measured using the microplate reader BioTek Synergy H1 Hybrid Multi-Mode Reader. For quantitative analysis, a cAMP standard curve was generated for each set of experiments.

2.5 Microdialysis

Microdialysis experiments were performed as previously described (Devoto et al., 2015). Rats were deeply anaesthetized with Equithesin (0.97 g pentobarbital, 2.1 g $MgSO_4$, 4.25 g chloral hydrate, 42.8 ml propylene glycol, 11.5 ml 90% ethanol in 100 ml; 5 ml/kg, IP) and stereotaxically implanted with vertical microdialysis probes (membrane AN 69-HF, Hospal-Dasco, Bologna, Italy; cut-off 40,000 Daltons), in the mPFC (3 mm active membrane length; AP +3.0, L ± 0.6, V -6.5 from the bregma), or in the caudate nucleus (4 mm active membrane length; AP +0.5, L ± 3.5, V -7

from bregma), according to Paxinos and Watson (2007). A sub-group of rats was implanted with microdialysis probes after noradrenergic denervation (see 2.3 section). The day after probe implantation, artificial cerebrospinal fluid (aCSF: 147 mM NaCl, 4 mM KCl, 1.5 mM $CaCl_2$, 1 mM $MgCl_2$, pH 6-6.5) was pumped by CMA/100 microinjection pump (Carnegie Medicine, Stockholm, Sweden) through the dialysis probes at 1.1 µl/min constant rate in freely moving animals, dialysate samples were collected every 20 min and immediately injected into HPLC. Drugs were administered after stable extracellular levels were obtained, i.e., three consecutive samples with a variance not exceeding 10%. The average of these three values was considered as baseline and used as 100% for the subsequent calculation of the variations induced by drug administration. NE, DOPAC and DA were simultaneously analyzed by HPLC with electrochemical detection, by HPLC systems equipped with 3.0 x 150 mm C18 (3.5 µ) Symmetry columns (Waters, Milan, Italy), maintained at 40°C by Series 1100 thermostats (Agilent Technologies, Waldbronn, Germany), and ESA Coulochem II detectors (Chelmford, MA, USA). The mobile phase was 80 mM Na_2HPO_4, 0.27 mM EDTA, 0.6 mM sodium octyl sulfate, 7% methanol, 4% acetonitrile, pH 2.4 with H_3PO_4, delivered at 0.3 ml/min; the Coulochem analytical cell first electrode was set at +200 mV, the second at -200 mV. Quantification was performed by recording the second electrode signal. Under these conditions, NE and DA detection limit (signal to noise ratio 3:1) was 0.3 pg per injection on column.

On completion of testing, rats were euthanized by Equithesin overdose, the brains removed and sectioned by a cryostat (Leica CM3050 S) in 40 µm thick coronal slices to verify locations of dialysis probes. No animal was found with errant location of the device.

2.6 Noradrenergic denervation

Central noradrenergic system ablation was achieved as previously described (Devoto et al., 2015) by the administration of the selective neurotoxin anti-dopamine-beta-hydroxylase saporin (aDBH). Briefly, rats were anesthetized with Equithesin and placed in a Kopf stereotaxic apparatus. A hole was drilled in the skull, directed to one lateral ventricle (AP -1.0, L ± 1.5 from the bregma, V -4.3

from skull, coordinates according to Paxinos and Watson, 2007) for administration of 5 µg/5 µl immunotoxin or 5 µl vehicle (n=8 rats/group), with a 10 µl syringe operated by a CMA/100 microinjection pump (CMA Microdialysis, Stockholm, Sweden) at 1 µl/min during 5 min, followed by 2 min pause before slowly withdrawing the needle. Injections were randomly distributed into either the left or right lateral ventricle. Rats were given antibiotic therapy (enrofloxacin, Bayer HealthCare, Shawnee Mission, KS) for five days and allowed to recover in their home cages for fifteen to eighteen days before the microdialysis probe insertion.

2.7 In vivo extracellular single unit recordings

Rats were placed in a stereotaxic apparatus (Kopf, Tujunga, CA, USA) under urethane anesthesia (1.3 g/kg, IP), with their body temperature maintained at 37±1°C by a heating pad. The recording electrode was placed above the VTA (5.1-5.7 posterior to bregma, 0.2-0.6 mm lateral to midline, 7.0-8.0 mm from cortical surface), according to the stereotaxic rat brain atlas by Paxinos and Watson (2007). Single unit activity of neurons was recorded extracellularly (bandpass filter 0.1-10,000 Hz) with glass micropipettes filled with 2% Pontamine sky blue dissolved in 0.5 M sodium acetate. Individual action potentials were isolated and amplified by means of a window discriminator (Neurolog System, Digitimer, Hertfordshire, UK) and displayed on a digital storage oscilloscope (TDS 3012, Tektronics, Marlow, UK). Experiments were sampled online and offline with Spike2 software by a computer connected to CED1401 interface (Cambridge Electronic Design, Cambridge, UK). DA neurons were isolated and identified according to previously described electrophysiological characteristics (Grace and Bunney, 1983; Ungless et al, 2004). VTA DA neurons were recorded only when criteria for identification were fulfilled (firing rate $\leq$ 10 Hz, duration of action potential $\geq$ 2.5 ms). Bursts were defined as the occurrence of at least two spikes at interspike interval < 80 ms and terminated when the interspike interval exceeded 160 ms. The drugs were considered as having an effect if they produced a change of at least 15 % of firing frequency and 10 % difference of burst firing from baseline.

2.8 Behavioral tests

2.8.1 Locomotor activity. Locomotor behaviors were assessed in a testing room with a background light and noise maintained at 10 lux and 65 dB, respectively. Testing chambers were placed to avoid any bias due to direct light, dark corners, or shadowed areas. The apparatus consisted of a square arena (40 × 40 cm), surrounded by four 40-cm transparent Plexiglas walls. Each cage had two sets of 16 photocells located at right angles to each other, projecting horizontal infrared beams 2.5 cm apart and 2 cm above the cage floor. To minimize emotional biases, each rat was handled by the same experimenter for 5 min daily for 3 consecutive days prior to the test. At the beginning of the test, rats were placed individually in the center of the arena and horizontal activity counts were collected every 10 min via custom software. To detect the biphasic effects of quinpirole on motor activity (Luque-Rojas et al., 2013), animals were habituated to the apparatus only the day before the test and placed in the activity cages immediately after quinpirole administration.

2.8.2 Stereotyped behaviors. Quantification of stereotyped behaviors was performed by observational time-sampling procedure. Each rat was observed and rated for 60 s, every 10 min, by an experimenter blind to drug treatment. The frequency of different behaviors was measured by scoring the presence or absence of a given behavior, according to the rating scale used by Kelley (2001). Each animal was assigned a final stereotypy score, defined as the sum relative to single or composite stereotypic behaviors over the time bins.

2.8.3 Bar test. Drug-induced catalepsy was assessed via the bar test, as described in Sanberg et al (1988). Rats were placed with forelimbs on a 9-cm high bar and the time during which the animal retained this position was recorded 30 min after drug injection by an observer unaware of the treatment. The average time of three consecutive trials were assessed. Rats were removed from the bar if catalepsy duration exceeded 180 s (cut-off period).

2.9 Statistical Analysis

Statistic was performed using Prism 9.1.2 for MacOS (GraphPad Software, La Jolla California USA, www.graphpad.com). Data, expressed as mean ± standard error (SEM), were analyzed by unpaired Student's t test, with or without Welch's correction, one-way or two-way repeated measures

ANOVA, as appropriate and specified in Results. Post-hoc multiple comparisons were made using Dunnett's or Tukey's multiple comparison tests. In all cases, $P < 0.05$ was considered significant.

3. RESULTS

3.1 Binding and adenyl cyclase assays.

To verify whether RS 79948 binds DA-D_2 receptors, other than α_2-adrenergic receptors, we tested this compound in competition assay against [^{3}H]methyl-spiperone binding on human recombinant HEK-293 cells expressing DA D_{2short}- or DA D_{2long} receptors (fig. 1). Non-linear regression analysis of data evidenced that RS 79948 inhibited [^{3}H]methyl-spiperone specific binding with K_i = 57 and 26 nM for D_{2short}- and D_{2long} receptors, respectively.

To disclose the nature of RS 79948 binding, its action on D_2 receptor-inhibited adenyl cyclase activity was tested in INS-1 832/13 rat insulinoma cells, a cell line that expresses functional dopaminergic receptors (Wu et al., 2008). Results indicated that RS 79948 reverted the cAMP reduction induced by the selective D_2 agonist quinpirole (suppl. fig. 1). The concentration of cAMP in 10 µM quinpirole-treated cells was 70.09 ± 2.11 nM and was significantly increased by co-treatment with RS 79948 (One-way ANOVA: $F_{(5, 12)} = 9.30$, $P = 0.0008$), with IC_{50} = 0.92 nM.

3.2 Microdialysis.

We and others previously demonstrated that α_2-adrenergic antagonists are able to increase extracellular DA levels in the mPFC but not in the striatum, while D_2-receptor antagonists act in the opposite manner, increasing extracellular DA in the striatum but not in the mPFC. Thus, by *in vivo* microdialysis we investigated how the double antagonistic properties of RS 79948 might affect extracellular catecholamine and DOPAC levels in mPFC and caudate nucleus. RS 79948 effects were compared with those of atipamezole and raclopride, selective α_2- and D_2-antagonist, respectively. Baseline levels of NE, DA and DOPAC in the mPFC and caudate nucleus of intact rats and in mPFC of aDBH-lesioned or control rats are shown in the Table.

Table. Extracellular basal values of norepinephrine, dopamine and DOPAC

	NE	DA	DOPAC
mPFC (21)	2.5±0.3	1.5±0.2	188.1±28.9
Caudate nucleus (13)	1.8±0.2	11.9±1.9	3555.3±304.3
mPFC-Veh (8)	2.5±0.5	1.7±0.4	213.6±58.2
mPFC-aDBH (8)	< 0.3 (ND)	2.9±0.7	110.9±34.2

Values are expressed as pg/20 µl sample and are the mean ± SEM of the number of rats in parenthesis. ND: not detectable.

As previously demonstrated (Devoto et al., 2019; 2020), in rats intracerebroventricularly infused with the neurotoxin aDBH, the extracellular NE levels were below HPLC detection limit (0.3 pg), while DA concentration was increased and DOPAC was decreased, even though not significantly (Two-tailed, unpaired T test, DA: t=1.537, df=14, P=0.1466; DOPAC: t=1.522, df=14, P=0.1503). Fig. 2 shows the effect of RS 79948, atipamezole and raclopride on extracellular NE, DA, and DOPAC in the mPFC. RS 79948 and atipamezole (both at the dose of 3 mg/kg, IP) increased extracellular NE by 80 and 140%, respectively, (fig. 2A) and extracellular DA by about 160% (fig. 2B). On the other hand, consistent with previous results (Devoto et al., 2020), raclopride (0.5 mg/kg IP) failed to modify extracellular NE and DA (fig. 2A and B). As shown in fig. 2C, RS 79948 and raclopride increased extracellular DOPAC levels in the mPFC by 190 and 140%, respectively, while atipamezole increased extracellular DOPAC levels by only 60% (fig. 2C). Two-way ANOVA for repeated measures indicated significant effect of treatment for NE ($F_{(2, 18)} = 10.7$, P=0.0009), main effects for treatment, time and their interaction for DA (time: $F_{(3.91, 70.3)} = 3.48$, P=0.0124; treatment: $F_{(2, 18)} = 4.89$, P=0.0201; time x treatment interaction: $F_{(10, 90)} = 2.40$, P=0.0142) and for DOPAC (time: $F_{(2.02, 36.4)} = 18.5$, P<0.0001; treatment: $F_{(2, 18)} = 8.73$, P=0.0022; time x treatment interaction: $F_{(10, 90)} = 2.58$, P=0.0086).

In the caudate nucleus, raclopride and RS 79948 increased extracellular DA by 150 and 80%, respectively, (fig. 3A) and extracellular DOPAC by 150 and 115%, respectively, (fig. 3B), while atipamezole failed to modify extracellular DA (fig. 3A) and reduced extracellular DOPAC by 20%

(fig. 3B). Two-way ANOVA for repeated measures indicated a significant effect of time, treatment and time x treatment interaction for DA (time: $F_{(1.66, 16.6)} = 12.0$, P=0.0009; treatment: $F_{(2, 10)} = 52.8$, P<0.0001; interaction: $F_{(10, 50)} = 4.57$, P=0.0001) and DOPAC (time: $F_{(2.38, 23.8)} = 87.2$, P<0.0001; treatment: $F_{(2, 10)} = 96.5$, P<0.0001; interaction: $F_{(10, 50)} = 40.4$, P<0.0001).

We have previously shown that atipamezole ability to increase extracellular DA level in the mPFC was lost after central noradrenergic denervation (Devoto et al., 2020). Thus, we tested whether RS 79948 effect was affected by noradrenergic system ablation, obtained with ICV administration of the neurotoxin a-DBH. Figure 4 shows that, unlike what was previously observed with atipamezole, noradrenergic denervation did not prevent RS 79948-induced elevation of extracellular DA and DOPAC in the mPFC, actually RS 79948 increased extracellular DA by a maximum of 100% in intact rats and 300% in denervated rats (fig. 4A). Two-way repeated measures ANOVA indicated a significant main effect of time ($F_{(2.14, 30.0)} = 8.47$, P = 0.0010) and time x lesion interaction ($F_{(5, 70)} = 3.03$, P = 0.0157), and a trend of significant lesion effect ($F_{(1, 14)} = 3.97$, P = 0.061). On the other hand, the RS 79948-induced increase of DOPAC levels was modestly reduced after noradrenergic denervation (fig. 4B). ANOVA indicated a significant main effect of time ($F_{(2.39, 33.0)} = 9.14$, P = 0.0004) but no significant lesion effect nor time x lesion interaction for DOPAC.

3.3 Electrophysiology.

Consistent with its D_2 receptor antagonistic activity, RS 79948 (IV injected in cumulative doses up to 0.5 mg/kg) increased firing rate in 7 out of 8 VTA DA neurons examined (fig. 5A), with a mean maximum excitation of 40% (RM one-way ANOVA, $F_{(2.318, 16.23)} = 4.35$; P=0.026). However, RS 79948 did not significantly increase burst firing in these neurons (RM one-way ANOVA, $F_{(2.381, 16.67)} = 3.064$, P>0.05. Fig. 5B).

In contrast, atipamezole, administered in the same route and dosage as RS 79948 modified neither the firing rate nor the bursting activity in 4 cells, increased the firing rate in 2 cells and decreased it in 1 out of the 7 neurons analyzed (fig. 5D and 5E), the mean effect being not significantly different from baseline (RM one-way ANOVA, frequency: $F_{(1.814, 10.88)} = 0.731$, P = 0.49; bursting activity:

$F_{(1.883, 11.30)}$ = 0.6215, P = 0.55). On the other hand, raclopride, administered in cumulative doses up to 0.05 mg/kg IV, increased the firing rate in 6 out of 8 neurons examined to a mean maximum of 55% of baseline at the dose of 0.025 mg/kg (fig. 5G), and significantly increased the difference of burst firing up to 10.44% at the dose of 0.025 mg/kg (fig. 5H). Repeated measures one-way ANOVA indicated that raclopride dose-dependently increased burst activity ($F_{(2.207, 15.45)}$ = 3.78; P = 0.0425), with no significant effect on frequency ($F_{(1.452, 10.17)}$ = 2.303; P = 0.1562). The lack of a significant effect of raclopride on firing rate is mostly due to the relatively high variability in DA neuron response to the drug and to the occurrence of a depolarization block in several cells at the higher doses tested.

3.4 Behavioral experiments.

3.4.1 Effects of RS 79948 *on immobility and hyperactivity induced by low and high dose of quinpirole.*

Low doses of D_2 agonists are known to produce hypomotility in rodents by selectively stimulating D_2 autoreceptors, while high doses to activate motor activity by stimulating postsynaptic D_2 receptors in the striatum. Therefore, we assessed the ability of RS 79948 (3 mg/kg, IP) in comparison with atipamezole (3 mg/kg, IP) to antagonize the hypomotility produced in rats by a low dose of quinpirole (0.1 mg/kg, IP).

As shown in fig 6, RS 79948 significantly reversed the hypomotility induced by the low dose of quinpirole, while atipamezole was ineffective. ANOVA analysis relative to the early phase (20 min) of the low dose of quinpirole detected significant main effects of quinpirole treatment as well as a pretreatment x treatment interaction [Two-way ANOVA, treatment: $F_{(1,36)}$= 49.4, P<0001; interaction: $F_{(2,36)}$= 11.2, P=0.0002]. No main effects of pretreatment (RS 79948 or atipamezole) were detected by ANOVA [$F_{(2,36)}$= 0.194]. Post-hoc analysis also disclosed that the effect of RS 79948 was not paralleled by impairment of motor performance (Fig. 6). Therefore, these data suggest that, differently from atipamezole, RS 79948 is effective in counteracting quinpirole-mediated hypomotility, probably by the blockade of presynaptic DA-D_2 receptors.

Next, we compared the ability of RS 79948, atipamezole and raclopride to modify the hyperactivity induced by a high dose of quinpirole (0.5 mg/kg, IP). To this aim, separate groups of rats were tested in motility cages under the same experimental conditions and design as with the low dose (fig. 7).

As expected, the high dose of quinpirole elicited a significant increase in motor activity [Two-way ANOVA, main effect of treatment: $F_{(1,48)}= 96.7$, $P<0.001$]. Furthermore, ANOVA detected a main effect of pretreatment [$F_{(3,48)}= 35.2$, $P<0.001$] and a significant pretreatment x treatment interaction [$F_{(3,48)}= 21.8$ $P<0.001$]. Multiple comparisons revealed that the selective DA-D_2 antagonist raclopride reversed the hyperactivity induced by the high dose of quinpirole. However, differently from what observed against the low dose of quinpirole, RS 79948 not only failed to counter quinpirole-mediated hyperactivity but, similarly to atipamezole exacerbated this effect. Of note, RS 79948 potentiated quinpirole-induced hyperactivity to a higher degree than atipamezole (Tukey' test, $P < 0.05$).

3.4.2 Effects of RS 79948 on orofacial stereotypies induced by high dose of quinpirole.

Orofacial stereotypies characterized by perseverative sniffing, licking and biting can be produced by high doses of D_2 receptor agonists, being mediated by postsynaptic D_2 receptors in the striatum. To determine if RS 79949, via its D_2 receptor antagonism, antagonized quinpirole-induced stereotypy, the stereotyped behavior induced by quinpirole alone (0.5 mg/kg, IP) was compared with that of quinpirole administered in association with RS 79948 (3 mg/kg, IP), atipamezole (3 mg/kg, IP) or raclopride (0.5 mg/kg, IP). Stereotypy was monitored for two hours at 10 min intervals.

As shown in figure 8, RS 79948 significantly attenuated the stereotyped behaviors elicited by the administration of the high dose of quinpirole (0.5 mg/kg). ANOVA analyses detected significant effects of pretreatment [$F_{(3,52)}= 32.3$, $P<0.001$], treatment [$F_{(1,52)}= 212.0$, $P<0.001$] and their interaction [$F_{(3,52)}= 32.3$ $P<0.001$]. Tukey's multiple comparisons test revealed that raclopride, as

expected, completely reversed the orofacial stereotypes, while RS 79948 significantly attenuated them, but to a lesser degree than raclopride ($P < 0.0001$ versus raclopride effect, fig. 8). Of note, the α_2-adrenergic antagonist atipamezole failed to modify the oral stereotypies induced by quinpirole injection (fig. 8).

3.4.3 Effects of RS 79948 *on raclopride-induced catalepsy.*

Previous findings indicate that α_2-adrenergic antagonists counteracted catalepsy induced by D_2 receptor antagonists in rodent models (Kleven et al., 2005; Invernizzi et al., 2003). Thus, we investigated whether RS 79948 shared these properties with atipamezole, despite of being also a DA-D_2 receptor antagonist. Rats were injected with RS 79948 or atipamezole alone (both at the dose of 3 mg/kg) and in combination with a cataleptogenic dose of raclopride (2 mg/kg); catalepsy was tested 30 and 60 min later by the bar test (Fig. 9). As previously reported (Kleven et al., 2005), atipamezole showed a robust anticataleptic effect, while RS 79948, which given alone was ineffective, counteracted raclopride-induced catalepsy, but to a lesser extent than atipamezole. ANOVA analyses indicated significant main effects of pretreatment [$F_{(2,34)}= 17.1$, $P<0.0001$], treatment [$F_{(1,34)}= 64.5$, $P<0.0001$], and pretreatment x treatment interaction [$F_{(2,34)}= 15.6$, $P<0.0001$].

4. DISCUSSION

The results from binding, microdialysis, electrophysiology and behavioral studies indicate that RS 79948 inhibits other than α_2- also D_2 receptors and modulates cortical and striatal functions in opposite, independent and cooperative manner.

RS 79948 displaced [^{3}H]methyl-spiperone from DA D_2 receptors and reversed D_2 receptor-mediated decrease in cAMP production at nM concentrations, indicating a functional antagonistic action on DA D_2 receptors.

RS 79948 shared with raclopride the ability to stimulate the firing of VTA DA neurons and with atipamezole the ability to increase DA and NE in the mPFC, suggesting that these effects are

mediated by D_2 and α_2- receptors, respectively. The results confirmed previous observations that DA release in the mPFC may be dissociated from the electrical activity of meso-cortical DA neurons (Gessa et al., 2000) and challenge the contention that α_2-adrenoceptor antagonists increase DA output in the mPFC by activating VTA DA neurons (Grenhoff and Svensson, 1993; Hertel et al., 1999).

The effect of noradrenergic denervation on DA release by atipamezole and raclopride provide important information for a better understanding of the mechanism of RS 79948 effect on NE and DA release. As previously shown (Devoto et al., 2019; Devoto et al., 2020), noradrenergic denervation suppressed atipamezole-induced DA release, consistent with the hypothesis that α_2-noradrenergic antagonists release DA from noradrenergic terminals. However, noradrenergic denervation also disclosed the property of raclopride to release DA from dopaminergic terminals. To explain this apparent inconsistency, we postulated that D_2-receptor antagonists normally release DA from dopaminergic terminals in the mPFC, but this DA is not detectable by microdialysis because it is readily recaptured by NET into noradrenergic terminals (Devoto et al., 2020). Therefore, by eliminating NET, consequent to noradrenergic denervation, more DA, which escapes the synaptic cleft, would be detected by microdialysis.

This hypothesis may explain why RS 79948, via α_2-adrenoceptor blockade, increased DA in intact rats and why, by inhibiting D_2 receptors, also increased DA output after noradrenergic denervation. Results from microdialysis analysis of DOPAC concentrations in the mPFC suggest that they are a better indicator of dopaminergic activity and transmission than extracellular DA. Indeed, changes of extracellular DOPAC measured by microdialysis in the mPFC paralleled the effect of RS 79948, atipamezole and raclopride on the firing of DA neurons. Accordingly, both RS 79948 and raclopride, which activated VTA DA neurons, also increased DOPAC levels in the mPFC and in the caudate nucleus, while atipamezole, which failed to stimulate DA neurons, increased DOPAC levels only modestly in the mPFC and even reduced them in the caudate nucleus.

The results from behavioral tests indicated that RS 79948 exhibits effects mediated by the concomitant inhibition of D_2 and α_2-receptors, resulting in changes of DA transmission in opposite, cooperative or independent direction. Thus, RS 79948 antagonized the sedative effect of a low dose of quinpirole, which is thought to be mediated by the stimulation of D_2 autoreceptors and the inhibition of dopaminergic transmission in the striatum (Di Chiara et al., 1976; Frantz and Van Hartesveldt, 1995). This effect was not shared with atipamezole, suggesting that α_2-adrenoceptors are not involved in presynaptic D_2 receptor modulation. RS 79948 contrasted the orofacial stereotypies produced by the high dose of quinpirole, RS 79948 effect being much weaker than that of raclopride, possibly because the blockade of α_2-adrenoceptors modulates DA transmission in opposite direction of D_2 blockade. Accordingly, RS 79948 and atipamezole, at doses that per se had no effect on motor activity, potentiated the motor stimulant effect of quinpirole and contrasted raclopride-induced catalepsy. Facilitation of dopaminergic transmission via α_2-adrenoceptor blockade seems to be the common mechanism by which atipamezole and RS 79948 potentiated quinpirole-induced motor activity and contrasted raclopride-induced catalepsy. Conversely, impaired DA transmission by inhibition of D_2 receptors seems the mechanism by which raclopride and, to a lesser extent, RS 79948 contrasted orofacial stereotypies produced by the high dose of quinpirole. The finding that, in apparent contrast to the DA facilitation hypothesis, atipamezole failed to aggravate orofacial stereotypies may be explained with a supramaximal, ceiling stimulation of D_2 receptors by quinpirole, precluding further stimulation (Dickson et al., 1994), while the lesser efficacy of RS 799948 with respect to raclopride might be the consequence of RS 79948 dual nature, being caused by increased DA transmission due to α_2-adrenoceptor blockade, which opposed to D_2-receptor antagonism.

A reasonable explanation of why RS 79948 efficacy to facilitate DA transmission and hinder D_2 receptor stimulation was, generally, weaker than that of atipamezole and raclopride, respectively,

may be that RS 79948, by inhibiting at the same time α_2- and D_2 receptors, modulated DA transmission in opposite, concurrent directions.

In conclusion, facilitation of DA transmission seems a common mechanism by which α_2-adrenoceptor antagonists potentiated quinpirole-induced motor stimulation: atipamezole and RS 79948 reverted and attenuated, respectively, the cataleptic effect of raclopride. Moreover, the facilitation of dopaminergic transmission in the striatum may also explain why α_2-adrenoceptor antagonists modulate antiparkinsonian-, dyskinetic- and non-motor symptoms produced by L-DOPA (Kleven et al., 2005; Ostock et al., 2015), potentiate DA agonists induced circling behavior in rats with unilateral lesion of the nigro-striatal pathway (Haapalinna et al., 2003), enhance the antipsychotic-like effect of antipsychotic drugs in rats (Hertel et al., 1999; Wadenberg et al., 2006; Masana et al., 2011), potentiate the antidepressant-induced increase of extracellular NE, DA and serotonin in the PFC (Invernizzi and Garattini, 2004; Ortega et al., 2010) and induce reinstatement of cocaine seeking behavior squirrel monkeys (Lee et al., 2004).

However, the mechanism by which α_2-adrenoceptor antagonists facilitate dopaminergic transmission is not clear. Since α_2-adrenoceptor antagonists do not activate the electrical activity of VTA DA neurons, do not elevate DA output in the caudate nucleus, facilitate contra-lateral circling induced by direct D_2 receptor agonists in DA-denervated animals, in the absence of DA, it has been suggested that the facilitatory effect results from an action at post-synaptic sites to the dopaminergic neurons (Chopin et al., 1999; Invernizzi et al., 2003; Lee et al., 2004; Ostock et al., 2015). Future research should investigate whether NE from noradrenergic afferences, particularly to the heavily innervated nucleus accumbens shell (Delfs et al., 1998; McKittrick and Abercrombie, 2007), might surrogate DA in the facilitation process mediated by α_2-adrenoceptor antagonists. It is worth noting that NE may act as a DA D_2-like receptor agonist (Cornil and Ball, 2008; Sanchez-Soto et al., 2016), especially when DA receptor sensitivity is modified, as in the animal models where α_2-adrenoceptor antagonists exhibit the facilitatory effect.

Future studies are warranted to unveil whether the peculiar dual inhibition of α_2- and D_2 receptors of RS 79948 might provide a novel pharmacological tool for the treatment of neuropsychiatric disorders characterized by imbalances of dopamine signaling in the PFC, such as schizophrenia, impulse control disorders, attention deficit with hyperactivity disorder.

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FIGURE LEGENDS

Figure 1. Competition curves of RS 79948 on [^{3}H]methyl-spiperone binding, measured on DA-D_{2short}- and DA-D_{2long} receptors. Data are expressed as % inhibition of specific binding.

Figure 2. Effect of raclopride (0.5 mg/kg IP), atipamezole (3 mg/kg IP) and RS 79948 (3 mg/kg IP) on extracellular NE, DA, and DOPAC levels in the mPFC of rats. Data are expressed as percentage of mean basal value and are the mean ± SEM of the number of rats in parentheses. Drugs were administered at T= 60 min, as indicated by the arrows. * P < 0.05, ** P < 0.01, *** P < 0.001 vs RS 79948; ° P < 0.05, °° P < 0.01, °°° P< 0.001 vs raclopride (Tukey's multiple comparisons test).

Figure 3. Effect of raclopride (0.5 mg/kg IP), atipamezole (3 mg/kg IP) and RS 79948 (3 mg/kg IP) on extracellular DA and DOPAC levels in the caudate nucleus of rats. Data are expressed as percentage of mean basal value and are the mean ± SEM of the number of rats in parenthesis. Drugs were administered at T= 60 min, as indicated by the arrow. Post hoc Tukey's multiple comparisons test indicated that ATIP was significantly different from RS and RACL starting at time point 100 to time point 180 min, for both DA and DOPAC values. * P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001 vs atipamezole (Tukey's multiple comparisons test).

Figure 4. Effect of RS 79948 (3 mg/kg IP) on extracellular DA and DOPAC levels in the mPFC of control- and aDBH-lesioned rats. Data are expressed as percentage of mean basal value and are the mean ± SEM of 8 rats per group. RS 79948 was administered at T= 60 min, as indicated by the arrow.

Figure 5. Effect of cumulative doses of RS 79948 (A, B; n= 8), atipamezole (D, E; n=7) and raclopride (G, H; n=8) on the firing rate of VTA-DA neurons of urethane anesthetized rats. Data are expressed as % of basal frequency and difference of burst firing. Representative histograms of DA neuron activity illustrating the dose-dependent firing rate increase in response to RS 79948 (C) and raclopride (I) and no effect of atipamezole (F). Dosage is expressed in mg/kg, IV administered. * P < 0.05 vs RS 0.025; P < 0.01 vs RS 0.05 (Dunnett's multiple comparisons test).

Fig. 6: Effect of RS 79948 and atipamezole (both at 3 mg/kg IP) on locomotor activity inhibition induced by a low dose (0.1 mg/kg, IP) of quinpirole. Data are expressed as total motility counts in 20 min and are the mean ± SEM of 6 rats/group. * P < 0.05; **** P < 0.0001 (Tukey's multiple comparison test).

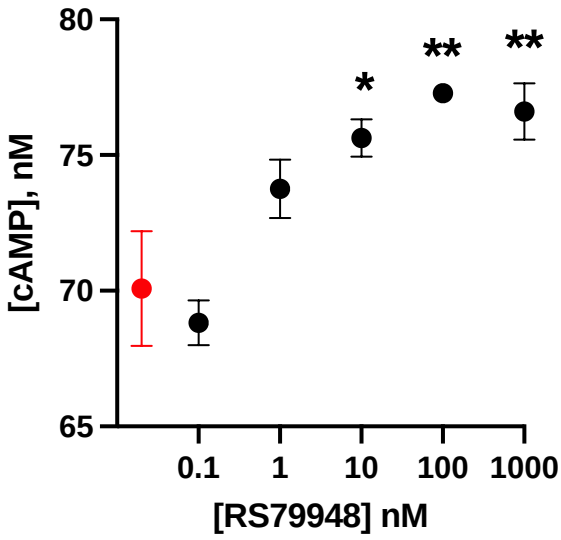
Figure 7: Effect of RS 79948 (3 mg/kg IP), atipamezole (3 mg/kg IP) and raclopride (0.5 mg/kg IP) on quinpirole (0.5 mg/kg)-stimulated locomotor activity. Data are expressed as total motility counts in 120 min and are the mean ± SEM of 6-8 rats. ** P < 0.01; **** P < 0.0001 (Tukey's multiple comparison test).

Figure 8: Effect of RS 79948 (3 mg/kg IP), atipamezole (3 mg/kg IP) and raclopride (0.5 mg/kg IP) on quinpirole (0.5 mg/kg IP)-induced oral stereotypies. Data are expressed as total score in 120 min and are the mean ± SEM of 6-9 rats. *** P < 0.001; **** P < 0.0001 (Tukey's multiple comparison test).

Figure 9: Effect of RS 79948 (3 mg/kg IP) and atipamezole (3 mg/kg IP) on raclopride (2 mg/kg IP)-induced catalepsy. Data represent total time spent on the bar (cut-off 180 sec) and are the mean ± SEM of 6-9 rats. *** P < 0.001; **** P < 0.0001 (Tukey's multiple comparison test).

Supplementary figure 1: Antagonism by RS 79948 on quinpirole (10 µM, red symbol) effect on adenyl cyclase activity. Data are expressed as nM cAMP concentration and are the mean ± SEM of 3 independent experiments. * $P < 0.05$; ** $P < 0.01$ vs quinpirole alone (Dunnett's multiple comparison test).

Supplementary figure 1



Supplementary figure 1: Antagonism by RS 79948 of quinpirole (10 µM, red symbol) effect on adenyl cyclase activity. Data are expressed as nM cAMP concentration and are the mean ± SEM of 3 independent experiments. * P < 0.05; ** P < 0.01 vs quinpirole alone (Dunnett's multiple comparison test).