Dopamine D2 and Adenosine A2A Receptors Interaction on Ca2+ Current Modulation in a Rodent Model of Parkinsonism

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Abstract
Adenosine A1 and A2A receptors are expressed in striatal projection neurons (SPNs). A1 receptors are located in direct (dSPN) and indirect SPNs (iSPN). A2A receptors are only present in iSPNs. Dopamine D2 receptors are also expressed in iSPNs and interactions between D2 and A2A receptors have received attention. iSPNs activity increases during parkinsonism (PD) and A2A receptors may be responsible by enhancing Ca2+ currents (iCa2+). Therefore, A2A receptors blockade is a therapeutic approach. We asked whether A2A receptors need the interaction with D2 receptors (D2R) to exert their actions. By using isolated and identified iSPNs to avoid indirect influences, we show that D2R action habilitates A2A receptors (A2AR) modulation. iCa2+ through voltage gated Ca2+ channels (CaV) was used as a signal to observe this interaction. Voltage-clamp recordings in acutely dissociated iSPNs, current-clamp recordings in slices and calcium imaging in transgenic A2A-Cre mice, showed that D2R reduction in iCa2+ endows A2AR to restore iCa2+ on iSPNs showing an antagonistic interaction between D2 and A2A receptors. A2A receptors were blocked by the antagonist istradefylline, however, this blockade differed in control and dopamine-depleted iSPNs: istradefylline reduced D2R modulation in parkinsonian animals as compared to controls. Calcium imaging recordings show that istradefylline occludes D2R actions in the parkinsonian circuitry and this effect depends on the order of drugs application. Thus, while D2 activation enables A2A receptors action, blockade of A2AR induces a reduction in the action of D2 agonists, confirming a complex interaction.

Summary Statement
A2A receptor required previous D2 receptor activation to modulate Ca2+ currents. Istradefylline decreases pramipexole modulation on Ca2+ currents. Istradefylline reduces A2A neurons activity in striatal microcircuit, but pramipexole failed to further reduce neuronal activity.

Keywords
adenosine A2A receptor, calcium imaging, dopamine D2 receptor, istradefylline, Parkinson’s disease, pramipexole

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Introduction

The main drug for Parkinson’s disease treatment is 3-(3,4-dihydroxyphenyl)-L-alanine (L-DOPA) (e.g.: Mercuri & Bernardi, 2005), although its long-term use entails motor complications such as L-DOPA induced dyskinesias and on/off phenomena (Berthet & Bezard, 2009; LeWitt & Fahn, 2016; Reddy et al., 2014). These are more frequent and appear sooner when using higher doses of L-DOPA (Olanow & Schapira, 2013).

Dopaminergic agonists such as pramipexole may retard these phenomena, although their actions do not completely match those of L-DOPA (Lara-Gonzalez et al., 2019; Millan, 2010). Therefore, non-dopaminergic drugs have been proposed as adjuvants to increase L-DOPA useful time and reduce motor complications.

Parkinsonism exhibits an overactivation of indirect striatal projection neurons (iSNP) and the striatal circuit in general (Jáidar et al., 2010, 2019; Kravitz et al., 2010; Zhai et al.,

Figure 1. Whole-cell Ca$^{2+}$ currents in acutely dissociated $\alpha_2\delta_A$ receptor expressing indirect striatal projection neurons (iSPNs). A) Representative inward Ca$^{2+}$ current (bottom) elicited by rectangular voltage commands from $-80$ to $+50$ mV (top) in 10 mV steps (tail currents are clipped). Empty circles show where Ca$^{2+}$ current amplitude was taken. The cells were in presence of TTX and Ba$^{2+}$ (see Methods), therefore, no Na$^+$ or K$^+$ currents were elicited. B) Ca$^{2+}$ currents (bottom) in the same neuron evoked by a ramp voltage command from $-80$ to $+50$ mV (top) in 0.7 mV/ms (tail currents are not shown). C) Current-voltage relationship (I-V plot) showing superimposed measurements from currents obtained with rectangular voltage commands in A (empty circles) and ramp commands in B (continuous line). Note that current obtained with the ramp command ‘fits’ measurements of currents obtained with rectangular commands, suggesting good voltage control and space clamp. D) Representative time course of Ca$^{2+}$ currents blockade during bath application of 200 μM Cd$^{2+}$ in a different cell.
How much of this overactivation is due to D2-A2A receptors interaction (Ferré & Fuxe 1992; Hillion et al., 2002) is currently debated (Beggiato et al., 2014; Fredholm et al., 2011; Preti et al., 2015). A2A receptors (A2AR) activation activates the Golf/s/cAMP/PKA cascade in iSPNs (Fredholm et al., 2000; Jacobson & Gao, 2006) increasing Ca\(^{2+}\) currents (iCa\(^{2+}\)) and excitability (Hernandez-Gonzalez, et al., 2014; Hernandez-Lopez et al., 1997) and N-methyl-D-Aspartate (NMDA) plateau potentials (Azdad et al., 2009). iSPNs hyperexcitability is reduced by A2AR antagonists (Armentero et al., 2011; Moreau & Huber, 1999; Muller & Ferré, 2007; Preti et al., 2015; Richardson et al., 1997; Tozzi et al., 2007; Yabuuchi, et al., 2006), effective in improving motor deficits in parkinsonian (PD) patients (Hauser et al., 2011; Mizuno et al., 2010; Müller, 2015; LeWitt 2008; Stacy, 2009). How much of this overactivation is due to D2-A2A receptors interaction (Ferré & Fuxe 1992; Hillion et al., 2002) is currently debated (Beggiato et al., 2014; Fredholm et al., 2011; Preti et al., 2015). A2A receptors (A2AR) activation activates the Golf/s/cAMP/PKA cascade in iSPNs (Fredholm et al., 2000; Jacobson & Gao, 2006) increasing Ca\(^{2+}\) currents (iCa\(^{2+}\)) and excitability (Hernandez-Gonzalez, et al., 2014; Hernandez-Lopez et al., 1997) and N-methyl-D-Aspartate (NMDA) plateau potentials (Azdad et al., 2009). iSPNs hyperexcitability is reduced by A2AR antagonists (Armentero et al., 2011; Moreau & Huber, 1999; Muller & Ferré, 2007; Preti et al., 2015; Richardson et al., 1997; Tozzi et al., 2007; Yabuuchi, et al., 2006), effective in improving motor deficits in parkinsonian (PD) patients (Hauser et al., 2011; Mizuno et al., 2010; Müller, 2015; LeWitt 2008; Stacy, 2009). Hence, one aim of the present study was to observe whether A2AR actions depend on a previous habilitation by D2R, thus involving D2-A2A receptors interaction. Indeed, we demonstrate this interaction (Fuxe et al., 2010) by using A2A-Cre mice to identify iSPNs and by recording acutely dissociated neurons to avoid indirect actions. Ca\(^{2+}\) current was seen as a signaling effector in the dorsal striatum, in the nucleus accumbens, globus pallidus and cell lines (Azdad et al., 2009; Floran et al., 2005; Salim et al., 2000). Similarly, activation of A2AR requires the previous activation of adenosine A1 receptors in the dorsal striatum (Hernandez-Gonzalez et al., 2014), and as D2R, A1 receptors probably use the PLC cascade to reduce iCa\(^{2+}\) (Hernandez-Lopez et al., 2000; Jacobson & Gao, 2006; Preti et al., 2015). Here we demonstrate that previous D2R reduction of iCa\(^{2+}\) is necessary for a A2AR agonist to increase and restore iCa\(^{2+}\) on iSPN, thus counteracting D2R modulation and revealing a functional antagonism between both receptors (Ferré et al., 1991, 2008; Stromberg et al., 2000) on iCa\(^{2+}\).

![Figure 2](image-url)

Figure 2. Demonstration of D2-A2A receptors interaction: application of a selective adenosine A2A receptor agonist only modulates Ca\(^{2+}\) current when dopamine D2 receptors are previously activated. A) Left: time course of drugs actions on Ca\(^{2+}\) currents: 1 \(\mu\)M of CGS-21680 (CGS) applied alone has no effect on whole cell Ca\(^{2+}\) currents in isolated indirect SPNs (identified A2A receptor expressing neurons) (1). 10 \(\mu\)M pramipexole, a dopamine D2/3 receptor selective agonist, reduces Ca\(^{2+}\) currents (2). In the presence of pramipexole, administration of CGS increases Ca\(^{2+}\) currents reversing the action of pramipexole (3). Middle: I-V plots taken at different moments during the time course at left. Results suggest that A2AR actions require a previous activation of D2 receptors. Right: box plots summarize these results in a sample of neurons using absolute current amplitudes (n = 6 neurons from different slices and animals; Friedman ANOVA with post hoc Dunnet tests. F(3) = 12.60. Control vs. pramipexole: ** P = .004 and pramipexole vs. pramipexole plus CGS: *P = .04). There were no significant differences between CGS given alone and CGS plus pramipexole. B) Left: representative time course of Ca\(^{2+}\) currents amplitude showing that neither application of 10 \(\mu\)M pramipexole alone nor addition of 1 \(\mu\)M CGS have any effects on Ca\(^{2+}\) currents in A2A negative neurons (putative direct SPNs). Middle: I-V plots taken from the time course at left. Box plots at right summarize these results with absolute current amplitudes (n = 6 from different slices and n = 3 different animals; Friedman ANOVA test. F(2) = 4. P = .184).
Figure 3. Action of A2A receptors on excitability of indirect SPNs after the previous activation of D2 receptors. Evoked firing after 100 pA somatic current injections in all cases. Left column: top: control firing, middle: application of 10 μM quinelorane, a D2 receptors agonist, decreases firing frequency in indirect SPNs (A2A+) from 12.8 ± 0.63 to 7.37 ± 0.8 Hz. CGS application, in the continuous presence of quinelorane, restores firing frequency from 7.37 ± 0.8 to 11 ± 0.6 Hz (bottom). Right column: quinelorane failed to significantly decrease frequency in putative direct SPNs (A2A- neurons). Box plots at the left summarizes the results in a sample of indirect SPNs in all three conditions (n = 8 from 8 different slices and from 8 animals, Friedman ANOVA with post hoc Dunnet tests. F(2) = 13.87, **P = .0014, *P = .0374). Box plot at the right summarizes the action of quinerolane and CGS in putative dSPNs, no differences were found (n = 7 from 7 different slices and from 7 animals, Friedman ANOVA with post hoc Dunnet tests. F(2) = 1, P > .999).
amplitude and excitability, showing a molecular correlate of behavioral and pharmacological findings (Ferré et al., 2008; Prasad et al., 2021). The selective antagonist istradefylline, blocked the action of A2AR. However, istradefylline blockade differed between control and dopamine-depleted iSPN: A2A receptors blockade reduced the modulation of D2R on Ca2+ of dopamine-depleted iSPNs from parkinsonian animals; perhaps, a physiological correlate of D2R affinity decrease by A2AAR ligands (Bonaventura et al., 2015; Preti et al., 2015).

In addition, we show the reduction in hyperexcitability by A2AR blockade on dozens of simultaneously active iSPN from parkinsonian 6-OHDA mice. In particular, we found differences in A2AR blockade when antagonists of these receptors are administered after or before the actions of D2R agonists. In these conditions, with iSPNs interconnected within their network receiving multiple influences, A2AR antagonism virtually occludes D2R actions when applied before the dopamine-agonist.

Materials and Methods

Research Subjects

Protocols were designed and performed in accordance with the international norms for the ethical use of experimental animals established in the National Institutes of Health Guide for Care and Use of Laboratory Animals Eighth Edition (NIH, 2010)*, including minimizing the number of animals to achieve statistical significance and the avoidance of animal suffering. The mouse strain used for this work, Tg(Adora2a-cre)KG139Gsat/MMude, RRID:MMRRC_031168-UCD, was obtained from...
the Mutant Mouse Resource and Research Center at University of California at Davis, an NIH-funded strain repository, and was donated to the Mutant Mouse Resource and Research Center at University of California by Nathaniel Heintz, Ph.D., The Rockefeller University, GENSAT and Charles Gerfen, Ph.D., National Institutes of Health, National Institute of Mental Health. Mating was carried out between homozygous mice to obtain stable transgenic A2A-Cre mice after breeding within a C57BL/6 background. Subjects were housed in acrylic cages (4–5 mice per cage; 19 × 29 × 12 cm), and kept on a 12:12 light/dark period (light beginning at 8:00 am) in a temperature controlled, pathogen-free room allowed food and water ad libitum.

**Experimental Procedures**

To identify isolated iSPNs, A2A-Cre mice at postnatal 25–45 were anesthetized i.p. with ketamine (Bayer 85 mg/kg) and xylazine (Bayer 15 mg/kg). Afterward, they were injected stereotaxically in a laminar flow hood (Telstar Technologies, Model PV-30/60) in a dedicated sterile room with the following viral constructs (University of Pennsylvania Vector Core): AAV2/1.CAG.Flex.tdTomato.WPRE.bGH (Honguki Zeng) for whole-cell recordings in isolated cells. pAAV.Syn.Flex.GCaMP6f.WPRE.SV40 for calcium imaging recordings at the following coordinates relative to bregma (in mm): anteroposterior = 0.9, mediolateral = ±

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**Figure 5.** Istradefylline, a selective A2A receptor antagonist, blocks CGS action in the presence of pramipexole in dopamine-depleted indirect SPNs. A) Left: representative time course of Ca2+ currents reduction by 10 μM pramipexole application. Reduction was 23.7 ± 3.46%. The subsequent action of CGS restored Ca2+ currents in the continuous presence of pramipexole in identified indirect SPNs (A2A+ neurons) to 38 ± 5.26%. Middle: I-V plots taken from the time course at left as indicated by numbers. Right: box plots summarize results from these samples of neurons using absolute Ca2+ currents amplitudes (n = 11 neurons taken from 10 different animals; Friedman ANOVA test with post hoc Dunnet tests F(2) = 16.55. Control vs. pramipexole: ***P = .002; pramipexole vs. pramipexole plus CGS: ***P = .0009), showing restoration of Ca2+ currents by CGS in dopamine-depleted neurons. B) Left: similar experiment as in (A) but in the continuous presence of the A2A receptor antagonist, istradefylline: pramipexole keeps having a reducing effect of 14.9 ± 2.2% on Ca2+ currents but the counteracting action of CGS was blocked. Middle: I-V plots taken from the time course at left as indicated. Box plot summarize the results from these samples of neurons using absolute current amplitudes (n = 10 neurons taken from 9 different animals; Friedman ANOVA test with post hoc Dunnet tests. F(2) = 15.80. istradefylline vs. istradefylline plus pramipexole: *P = .0110 and istradefylline plus pramipexole vs. istradefylline plus pramipexole plus CGS: P > .99), showing that Ca2+ currents restoration caused by A2A receptors activation was blocked.
with 95% O2 and 5% CO2.

1.44 mM NaH2PO4, 0.4 mM CaCl2, 4°C). For dopamine-depleted neurons, there was a 14 days delay between rotational movements (1000 Classic, Warner Instruments, Hamden, USA) and a 125 kHz DMA interface (Axon instruments). The internal solution contained (in mM): 180 N-methyl-d-glucamine (NMDG), 40 HEPES, 10 EGTA, 4 MgCl2, 2 ATP, 0.4 GTP and 0.1 leupeptin (pH 7.2 with H2SO4; 280 ± 5 mOsM/l with glucose). Thereafter, the cells were superfused at 1 ml/min with saline of the same composition at room temperature (approximately 25–30°C). A2A+ neurons were visualized using an UV lamp (X-Cite; EXFO, Ontario, Canada). Dissociated neurons typically lack distal dendrites and prolonged axons (Supplementary Figure 3A).

**Voltage Clamp Recordings of Calcium Currents in Dissociated Neurons**

Voltage-clamp recordings were performed on identified A2A+ neurons (Figure 1 and Supplementary Figure 3) with 8–15 μm diameter (Hernández-Flores et al., 2015; Pérez-Burgos et al., 2010; Rendon-Ochoa et al., 2018). Patch pipettes of borosilicate glass (WPI, Sarasota, FL, USA) were pulled in a Flaming-Brown puller (Sutter instruments Corporation, Novato, CA, USA) and fire polished before use. For whole-cell recordings, electrodes with a D.C. resistance of 4–6 MΩ were used and liquid junction potentials were corrected about 80%. Recordings were obtained with an Axopatch 200B patch-clamp amplifier (Axon instruments, Foster City, CA, USA) and controlled and monitored using Im-Patch© (http://impatch.ifc.unam.mx/) an open access software (Lara-Gonzalez et al., 2019) and a 125 kHz DMA interface (Axon instruments). The internal solution contained (in mM): 180 N-methyl-d-glucamine (NMDG), 40 HEPES, 10 EGTA, 4 MgCl2, 2 ATP, 0.4 GTP and 0.1 leupeptin (pH 7.2 with H2SO4; 280 ± 5 mOsM/l; room temperature around 25–30°C). We record and report Ca2+ currents using Ba2+ as a charge carrier as showed in previous articles (Bargas et al., 1994; Hernández-Flores et al., 2015; Hernández-González et al., 2014; Pérez-Burgos et al., 2010; Rendon-Ochoa et al., 2018). Ba2+ also serves as K+ blocker and Na+ channels were blocked with 1 μM of TTX.

1.2, dorsoventral = −3.2. The total virus volume injected was 0.6 μl over for 10 min to a 0.1 μl/min rate with a dental needle. After surgery, animals were monitored for two weeks to ensure protein expression and complete recovery. A total of 59 mice were used and randomly assigned to different experimental groups.

**Preparation of Acutely Dissociated Neurons and Brain Slices**

Brain slices and acutely dissociated neurons were obtained as described in previous work (Duhne et al., 2021; Hernández-González et al., 2014; Rendon-Ochoa et al., 2018). Briefly, transfected A2A-Cre mice were anesthetized (see above) and perfused intracardially with chilled sucrose solution (234 mM sucrose, 28 mM NaHCO3, 7 mM dextrose, 4.54 mM pyruvate, 0.28 mM ascorbic acid, 2.5 mM KCl, 7 mM MgCl2, 1.44 mM NaH2PO4, 0.4 mM CaCl2, 4°C). For dopamine-depleted mice, there was a 14 days delay between rotational behavioral tests and calcium imaging experiments. Once brains were extracted, 300 μm thick sagittal slices were taken in a vibrotome (1000 Classic, Warner Instruments, Hamden, USA) and kept in saline solution for 1 h at 34°C: 126 mM NaCl, 15 mM glucose, 26 mM NaHCO3, 0.2 mM thiourea, 0.2 mM ascorbic acid, 2.5 mM KCl, 1.3 mM MgCl2, 1.2 mM NaH2PO4 and 2.0 mM CaCl2; pH = 7.4; 300 ± 5 mOsM/L continually perfused with 95% O2 and 5% CO2.

When recordings were done in slices, they were transferred to a submerged chamber and superfused at 5 ml/min with saline solution. For calcium imaging, slices were placed under a 20X immersion objective (Olympus X-LUMPLFLN Objective, 1.00 numerical aperture, 2.0 mm width diameter) while constantly perfused with ACSF and 95% O2 and 5% CO2. When recordings were done in dissociated cells, the dorsal striatum was dissected from the slices and returned into the saline solution containing 10 mM HEPES plus 1 mg/ml of pronase E type XIV (Sigma-Aldrich, Mexico) at 34°C in for 15–20 min. After digestion, dissected striata were transferred to a low calcium saline solution (0.4 mM CaCl2). To obtain acutely dissociated neurons, the tissue was mechanically dissociated with fire-polished Pasteur pipettes with different diameters. The cell suspension (1 ml) was placed into a Petri dish mounted on the stage of an inverted microscope (Nikon Instruments, Melville, NY, 20 × 0.4 numerical aperture). Cells were left for 13 – 15 min to adhere to the bottom of the dish. The dish contained 1 ml of the whole-cell recording saline solution (in mM): 0.001 tetrodotoxin (TTX), 140 NaCl, 3 KCl, 5 BaCl2, 2 MgCl2, 10 HEPES, and 10 glucose (pH: 7.4 with NaOH; 300 ± 5-mOsM/l with glucose). Thereafter, the cells were superfused at 1 ml/min with saline of the same composition at room temperature (approximately 25–30°C). A2A+ neurons were visualized using an UV lamp (X-Cite; EXFO, Ontario, Canada). Dissociated neurons typically lack distal dendrites and prolonged axons (Supplementary Figure. 3A).
Figure 7. Interaction of pramipexole and istradefylline in the striatal parkinsonian circuit. A) Top: Representative raster plot where dots in rows represent the activity of indirect SPNs ($A_{2A}^+$) along time as obtained with calcium imaging (see Methods). Three conditions are shown: first: an initial representative interval showing hyperexcitability of indirect SPNs proper of parkinsonism in a dopamine-depleted striatum (6-OHDA; Jaidar et al., 2019). Second: a reduction of this activity after activation of D2 receptor by the dopamine-agonist pramipexole (blue bar; Lara-Gonzalez et al., 2019). Third: population activity does not change much when istradefylline is applied in the continuous presence of pramipexole (green bar). Histogram at right shows activity per row (active frames / total frames) in the different conditions denoted by colors. Histogram at bottom shows summed neuronal activity column by column (frame by frame). Dashed line is the threshold to detect significant peaks of coactivity. Note their reduction during both drugs. B) Box plots illustrate samples of cumulative activity (taken from cumulative activity -plots, see Methods) from raster plots taken at the three conditions: Activity during pramipexole is significantly reduced when compared to the parkinsonian condition (n = 8 from 8 different slices and 8 different animals, Friedman ANOVA test with post hoc Dunnet test. $F(2) = 9.75$, dopamine-depleted vs. pramipexole: **$P = .0081$). However, activity during pramipexole plus istradefylline was not significantly different to that of the control (dopamine-depleted vs. pramipexole plus istradefylline: $P = .0735$) showing that istradefylline reduced D2 receptors action, in parallel to findings in single cells. Nonetheless, this reduction was not enough to return to the parkinsonian activity (istradefylline vs pramipexole plus istradefylline: $P = .9999$) supporting its therapeutic actions. C) Cumulative distribution functions of cellular activity in the three conditions (dopamine-depleted n1 = 273 neurons, pramipexole n2 = 103 neurons and pramipexole plus istradefylline n3 = 144 neurons; dopamine-depleted vs. pramipexole: $K = 0.67619$, $P = 2.8 \times 10^{-59}$; dopamine-depleted vs. pramipexole plus istradefylline: $K = 0.61264$, $P = 3.68 \times 10^{-52}$ and pramipexole vs. pramipexole plus istradefylline: $K = 0.071795$, $P = .33717$; Kolmogorov–Smirnov tests with the Benjamini–Hochberg method for False Discovery Rate).
In this way, for simplicity, we will refer to Ba\(^{2+}\) currents as Ca\(^{2+}\) currents. Current-voltage relationships (I-V plots) were aquired before, during and after drug applications. Figure 1A shows a representative I-V plot evoked with 20 ms of rectangular voltage commands from −80 to 50 mV in 10 mV steps. In addition, Figure 1B shows the representative current in response to a voltage ramp command (0.7 mV/ms) from −80 to 50 mV. When I-V plots from

![Figure 8](image-url)

**Figure 8.** Istradefylline virtually occludes pramipexole modulation on indirect SPNs (A\(_2A^+\)) activity in the striatal dopamine-depleted microcircuit. A) Raster plot: left, shows spontaneous hyperactivity of indirect SPNs in a dopamine-depleted microcircuit (6-OHDA model). Next, addition of 50 nM istradefylline (green bar) decreases indirect SPNs activity. Further application of 10 μM pramipexole in the continuous presence of istradefylline only shows a tendency for a small additional reduction of activity. Histogram at right shows activity row by row in all three conditions (denoted by colors). Histogram below shows the summed activity of active neurons at each movie frame. Dashed line in the histogram at bottom indicates the presence of statistically significant peaks of coactivity (\(P < .01\)), note their reduction during drugs application. B) Box plots compare slopes distributions of cumulative activity-plots (\(n = 8\) from 8 different slices and animals, Friedman ANOVA with post hoc Dunnet test. \(F(2) = 12.97\). dopamine-depleted vs. istradefylline: *\(P = .022\); dopamine-depleted vs. istradefylline plus pramipexole: **\(P = .0018\); istradefylline vs istradefylline plus pramipexole: \(P = .830\)). C) Cumulative distribution functions of % cellular activity of indirect SPNs in each condition (dopamine-depleted-n\(_1\) = 638 neurons vs. istradefylline-n\(_2\) = 450 neurons: \(K = 0.38\). \(P = 2.4 \times 10^{-16}\); dopamine-depleted vs. istradefylline plus pramipexole-n\(_3\) = 432 neurons: \(K = 0.38\). \(P = 1.5 \times 10^{-31}\); pramipexole vs istradefylline plus pramipexole: \(K = 0.079\). \(P = .032\), from \(n = 8\) from 8 different slices and 8 different animals; Kolmogorov-Smirnov test with the Benjamini–Hochberg method for a desired False Discovery Rate).
both methods coincided, space-clamp was considered acceptable (Figure 1C). For clarity, most figures only show representative responses to voltage ramps. 200 µM Cd²⁺ completely blocked these currents (Figure 1D).

**Current Clamp Recordings**

Neurons were visualized using infrared differential interference contrast microscopy with an upright microscope and a digital camera. SPNs were identified using epifluorescent illumination (Supplementary Figure 3B). The signals were digitized at 10 kHz using an AT-MIO-16E4 board (National Instruments, Austin, TX, USA) and saved in a PC computer using Im-Patch®. Current-clamp recordings were performed with the patch clamp technique in the whole cell configuration in SPNs from the dorsal striatum, the membrane potential was held at −65 mV. Micropipettes were filled with internal saline (3−6 MΩ) containing (in mM): 115 KH₂PO₄, 2M Cl₂, 10 HEPES, 1.1 EGTA, 0.2 ATP, 0.2 GTP, and 5% biocytin (pH 7.2; 285 mOsm/L). Action potential firing was evoked by neuronal depolarizations (100 pA current pulses lasting 1000 ms) in control, in quinelorane (10 µM), and in quinelorane 15 responses for each neuron in every condition. The frequency of every evoked response and averaged the frequency of

**Calcium Imaging Experiments**

Fluorophore stimulation was carried out with a Lambda HPX High power light emitter diode driver coupled to specific excitation emission filters. GCamp6f: excitation BP460–480 nm, emission 495–540 nm (Olympus, U-MGFPHQ). Imaging recordings were obtained with a CoolSnap K4 camera (Photometrics, Tucson, AZ, USA) and a Retiga R1 camera (Teledyne QImagingm, BC, Canada), controlled by Im-Patch®. A₂A + neuron activity was observed using the GCaMP6f signal. Videos were 720–2160 frames long and acquisition rate was 6 frames / second. Noise was subtracted for both cameras since resolution is different (K4 2048 × 2048 resolution and Retiga 1360 × 1025 resolution). To cover long periods, 7–10 videos were taken per experiment, making up to 90 min per experiment. A 15 mM KCl solution was administered at the end of all experiments to test neuronal viability.

Videos were processed with ImPatch®, as previously reported (Aparicio-Juárez et al., 2019). Briefly, probability of firing action potentials during calcium transients was extracted as the first time derivative of the fluorescent signal (Carrillo-Reid et al., 2008). Then we built neuronal activity matrices were the y-axis denote active neurons and x-axis represent the sequence of movie frames as column vectors, transformed to time units. 1 denotes an active frame and 0 an inactive frame for each cell. Whole experiments can be contained in these matrices and expressed as raster plots, where each dot stands for a cell firing in that frame and each row shows the activity of a cell along time. The activity of all cells in all column frames was added to build coactivity histograms graphed below the raster plots. Significant coactivity peaks were determined using several variants of Montecarlo simulations with 10000 iterations each (Pérez-Ortega et al., 2016). Significant coactivity peaks can be observed in the frames in which the threshold was overcome (Supplementary Figure 4).

In this work we were interested in the amount of neuronal activity per experiment and this variable was quantified with cumulative activity plots (CA plots). CA plots were built by adding active raster columns along time (a sumatory of the coactivity histogram along time). To consider samples of experiments in the same condition, we used linear fits to CA plots (Lara-Gonzalez et al., 2019) (Supplementary Figure 1). Slopes of these fits from samples of neurons show whole neuronal activity during each experiment and their distribution was plotted as Tukey box plots (cumulative activity metrics). In this way we compared activity of samples of neurons in both control and during drugs applications. Samples using cumulative activity metrics were contrasted with Friedman ANOVA tests corrected for multiple comparisons using the Dunnet test.

The activity of individual cells through a single experiment was defined as the total number of active frames over the total number of frames, transformed to time units. This value, expressed as percentage of activity, was used to compare neuronal activity between different microcircuit conditions. To do that, cumulative distribution functions were plotted using all neurons from all samples from a given condition. Thus, cumulative distribution functions denote the probability or less that a given cell is active (P has values between 0 and 1). In this way distributions of neuronal populations were compared during different conditions. Cumulative distribution functions were contrasted with Kolmogorov-Smirnov tests corrected for multiple comparisons using the Benjamini-Hochberg procedure with a 0.05 FDR. The dopamine-depleted microcircuit is characterized by enhanced spontaneous activity (Jáidar et al., 2010; Pérez-Ortega et al., 2016). Note that CA plots and cumulative distribution functions are different metrics to measure the activity of the same neurons, in one case, various samples of experiments compared in different conditions are used as it is usually done. In the other case, probability distributions of neuronal activity using the set of all neurons taken from all experiments in the same condition are compared.

**Electrophysiological Analysis**

Digitized data were imported for analysis and plotted using commercial software (Origin 7, Microlab, Northampton, MA, USA). Mean ± SEM of peak Ca²⁺ currents for dissociated neurons and firing rate for current clamp recordings were reported. Free distributions were assumed, therefore, Friedman, Kruskal–Wallis or Wilcoxon test with post hoc Dunn or Dunnet tests for multiple comparisons were used.
(signaled in each result). Friedman and Wilcoxon test were used when comparing the same samples in two or three different conditions (before, during and after application of a drug). P < .05 was used as significance threshold. Analysis was conducted by GraphPad Prism 6.01 (La Joya, CA).

Results

Habituation of A2AR by D2R in Isolated iSPNs from Dorsal striatum

To elucidate whether activation of adenosine A2AR requires a previous activation of D2R in identified iSPNs from dorsal striatum we recorded isolated cells to avoid indirect effects. Figure 2A left shows a temporal course of the action of the A2AR selective agonist, CGS 21680 (CGS), on the maximal iCa2+ amplitude in an identified iSPNs: notably, bath application of 1 μM CGS alone had no significant effects on whole cell iCa2+ from isolated neurons (see also Supplementary Figure 2). iCa2+ amplitude went from 330 ± 56 pA in control (100%) to 315 ± 56 pA during application of the A2 agonist (95.3 ± 2.4%; NS), suggesting that CGS cannot activate A2A receptors by itself. Then, 10 μM pramipexole, a dopamine D2R selective agonist, was applied: it reduced iCa2+ to 256 ± 49(22.83 ± 3.9%), similar to results previously reported (Hernandez-Lopez et al., 2000). Thereafter, in the continuous presence of the D2R agonist, pramipexole, a second application of CGS now increased and restored iCa2+ to nearly previous values: 311 ± 58 pA (increase of 26.7 ± 5.9%), showing that A2AR are present and can be activated in iSPNs if enabled by the previous activation of D2R, suggesting that the interaction between these receptors is necessary for A2AR activity, and also, a negative cooperativity or functional antagonism between both receptors on iCa2+. This result parallels behavioral and biochemical antagonism previously reported (Ferré et al., 1991, 2008; Prasad et al., 2021; Preti et al., 2015; Stromberg et al., 2000; Yang et al., 1995). Figure 2A middle, shows representative I-V plots built from different moments during the time course denoted by numbers. Figure 2A right, summarize these results in samples of neurons using global linear slope measurements of cumulative activity metrics (see above and Supplementary Figure 1) graphed as box plots (n = 6 from different neurons and 6 different animals; Friedman ANOVA with post hoc Dunnet tests. F(3) = 12.60; control versus pramipexole: **P = .004; pramipexole versus pramipexole plus CGS: *P = .04), demonstrating that, after being enabled, A2AR activation counteracts the actions of D2R.

Figure 2B shows that application of pramipexole or CGS produces no change on iCa2+ of a putative dSPN or A2AR neuron (applied in any order; n = 6 from different neurons and 3 animals, Friedman ANOVA with post hoc Dunnet tests. F(2) = 4. P = .1840), suggesting that D2-A2A receptors interaction described above is proper of iSPNs and not present in dSPNs (Beggiato et al., 2014; Ferré et al., 2008).

Action of A2A Receptors on Excitability

Neurons interconnected within their circuit may be the subject of multiple influences. Therefore, we wanted to observe if the activation of A2AR had an impact on neuronal excitability (Pérez-Garcí et al., 2003) by counteracting D2R activation, in non-isolated neurons. Therefore, current-clamp recordings from striatal slices were performed (Figure 3). We observed that, as expected (Hernandez-Lopez et al., 2000; Lara-Gonzalez et al., 2019), application of 10 μM quinolone decreased frequency in iSPNs from 12.9 ± 0.63 Hz in control to 7.4 ± 0.8 Hz during D2R activation (Figure 3 left and bottom, n = 8 neurons taken from 8 different slices and 8 different animals; Friedman ANOVA with post hoc Dunnet test. F(2) = 13.87. **P = .0014). The same effect can be obtained with different D2R agonists (Lara-Gonzalez et al., 2019). Also note that the subsequent application of CGS partially restored firing frequency in iSPNs to 11 ± 0.56 Hz (*P = .0374 as compared with quinolone alone).

We did not observe similar changes in firing frequency in putative dSPNs (A2AR, Figure 3; right, n = 7 neurons from 7 different slices and 7 different animals, P > .99).

Istradefylline Shows Specificity of A2A Receptors

Actions: Differences Between Control and Dopamine-Depleted Mice

Next, we show that A2AR actions are specific, using isolated neurons and compared the D2-A2A receptors interaction without and with the presence of the A2AR antagonist, istradefylline, both in control and dopamine-depleted cells.

First, we show the actions in control (non dopamine-depleted) cells: 10 μM pramipexole decreased iCa2+ from 300 ± 55 pA to 185 ± 24 pA (31.9 ± 6.07% decrease from control current), while subsequent administration of CGS counteracted this action by restoring iCa2+ amplitude to 255 ± 32 pA (an increase of 22.5 ± 4.72%) in control neurons (Figure 4A; n = 8 from different neurons from 8 different animals; Friedman ANOVA with post hoc Dunnet tests. F(2) = 12.25. Control versus pramipexole: **P = .0035 and pramipexole versus pramipexole plus CGS: *P = .0179), confirming a significant recovery of iCa2+ by A2AR activation.

Similar experiments were done in the continuous presence of the A2AR antagonist istradefylline (Figure 4B): pramipexole had its usual reducing action of iCa2+ from 289 ± 69 pA to 234 ± 59 pA or 25.68 ± 2.98% (Figure 4B; n = 8 neurons taken from 8 different animals; Friedman ANOVA with post hoc Dunnet tests F(2) = 13.00. *P = .0374). However, the subsequent addition of CGS (in the continuous presence of pramipexole and istradefylline) had no the counteracting effect (compared with pramipexole alone: P = .9521 and compared to the control: **P = .0014), strongly suggesting that istradefylline was capable to block A2AR actions in spite of D2R enabling effect in control cells (non dopamine-depleted neurons) and that these actions are, therefore, specific.
Next, we wanted to observe if this interaction was preserved in dopamine-depleted iSPNs and in case it was, whether it is found in the same amount. We used neurons taken from the injured side of the 6-OHDA rodent model of parkinsonism to test the habilitation of A2A-R by D2-R. In isolated identified dopamine-depleted iSPNs, application of 10 μM pramipexole decreased iCa²⁺ from 323 ± 42 pA, to 236 ± 30 pA or 23.7 ± 3.46% showing that D2R action on iCa²⁺ is preserved in dopamine-depleted cells (Prieto et al., 2009). During the continuous presence of pramipexole, addition of 1 μM CGS restored iCa²⁺ to 328 ± 37 pA, or 38 ± 5.26% (see box-plots in Figure 5A; n = 11 from different neurons from 10 different animals; Friedman ANOVA test with post hoc Dunnet tests F(2) = 16.55. Control versus pramipexole: **P = .002 and pramipexole versus pramipexole plus CGS: ***P = .0009). These experiments confirmed, first, that in dopamine-depleted neurons D2R activation reduces iCa²⁺ explaining its therapeutic actions (Lara-González et al., 2019), and secondly, they show that the D2-A2A receptors interaction is present since D2R activation habilitates A2AR activation to counteract the same D2R actions that habilitated them on iCa²⁺, in neurons from 6-OHDA injured mice, similarly as in neurons from control mice. Then we wanted to see whether istradefylline blocked these actions in dopamine-depleted iSPNs, an action that would further support its use as a therapeutic tool.

Therefore, we tested the ability of istradefylline to abolish A2AR restoration of iCa²⁺ in dopamine-depleted neurons. Figure 5B shows the effect of 10 μM pramipexole in the continuous presence of 50 nM istradefylline: iCa²⁺ was decreased from 390 ± 100 pA to 333 ± 86 pA or 14.9 ± 2.24% in control neurons. Subsequent application of CGS in the continuous presence of pramipexole and istradefylline did not produce further changes in iCa²⁺ amplitude: 325 ± 82 pA showing that the effects were specific on A2AR, supporting istradefylline role in maintaining a low excitability of iSPNs during parkinsonism and during the action of D2R (n = 10 from different neurons from 9 different animals; Friedman ANOVA test with post hoc Dunnet tests F(2) = 15.80. Istradefylline versus istradefylline plus pramipexole: *P = .0110 and istradefylline plus pramipexole versus istradefylline plus pramipexole plus CGS: P > .99). Istradefylline impeded A2AR to restore iCa²⁺ in dopamine-depleted iSPNs after pramipexole action.

Notably however, when comparing non dopamine-depleted (control) with dopamine-depleted neurons from parkinsonian animals, pramipexole modulation appeared to be reduced in dopamine-depleted neurons when istradefylline was present (Bonaventura et al., 2015; Casadó-Anguera et al., 2016). Modulation was reduced from 25.7 ± 3.0% in control neurons versus 14.9 ± 2.2 in dopamine-depleted neurons, showing that D2-A2A receptors interaction goes both ways: the occupation of A2AR by an antagonist ligand reduced the modulatory action of a D2R agonist during parkinsonism (Bonaventura et al., 2015; Casadó-Anguera, 2016; Prasad et al., 2021), these electrophysiological results support parallel biochemical actions that have been attributed to D2-A2A receptors oligomers and protein-protein interaction, that is, the non neutral action of A2A receptor antagonists. By comparing percentages of iCa²⁺ modulation by pramipexole in control and dopamine-depleted neurons in the presence and absence of istradefylline (Figure 6) we found a decreased modulation by pramipexole in dopamine-depleted neurons when istradefylline was present (n-pramipexole = 11 neurons from different neurons from 10 different animals, n-pramipexole plus istradefylline = 10 neurons from different neurons from 9 different animals; Mann-Whitney U test, F = 7.0, *P = .0127). In control iSPNs, pramipexole modulation showed no significant differences with or without istradefylline. However, istradefylline seemed to decrease the variance in both samples (Figure 6). These results are a molecular correlate of D2-A2A receptors interaction in dopamine-depleted iSPNs (Ferre et al., 2008). Because this change in D2R modulation of iCa²⁺ was present in dopamine-depleted neurons we decided to evaluate the global impact of the D2-A2A receptors interaction in dopamine-depleted neuronal populations in ex-vivo striatal parkinsonian tissue by using Ca²⁺ imaging with single cell resolution.

**Pramipexole and Istradefylline Interaction depends on the Order of Drugs Administration in the Striatal Cirucity**

We focused in the spontaneous hyperexcitability of identified iSPNs due to dopamine-depletion (A2A+ neurons; Figure 7A left; Jáidar et al., 2010, 2019; Plata et al., 2013). Bath application of 10 μM of pramipexole reduced iSPNs activity as reported before in the whole circuit (Lara-González et al., 2019; Figure 7A blue bar). Under these conditions, a subsequent administration of istradefylline in the continuous presence of pramipexole, failed to further decrease neuronal activity but did not restore parkinsonian activity significantly (Figure 7A green bar). Histogram of coactivity (Figure 7A bottom) shows a reduction of significant coactivity peaks and less activity during pramipexole application. The addition of istradefylline caused a small reduction of pramipexole effects, in parallel with single cell experiments. Box plots (Figure 7B) summarize these results (n = 8 experiments from 8 different slices and 8 different animals, Friedman ANOVA test with post hoc Dunnet test. F(2) = 9.75. dopamine-depleted versus pramipexole: **P = .0081; dopamine-depleted versus pramipexole plus istradefylline: P = .0735; pramipexole versus pramipexole plus istradefylline: P = .9999).

Figure 7C shows cumulative distribution functions of cellular activity in the three conditions by taking all the neurons in each condition (dopamine-depleted-n1 = 273 neurons, pramipexole-n2 = 103 neurons and pramipexole plus istradefylline-n3 = 144 neurons. dopamine-depleted vs.
pramipexole: $K = 0.67619$, $P = 2.8091 \times 10^{-59}$; dopamine-depleted vs. pramipexole plus istradefylline: $K = 0.61264$, $P = 3.6841 \times 10^{-52}$. Pramipexole vs. pramipexole plus pramipexole: $K = 0.071795$, $P = .33717$; Kolmogorov–Smirnov test with the Benjamini–Hochberg method for a desired false discovery rate). That is, at the global circuit level, addition of istradefylline did not appear to interfere much with pramipexole anti-parkinsonian action: only a few more neurons were activated after its addition when counting all recorded neurons from all experimental samples (Figure 7A and B); at a larger scale other influences present in the tissue may intervene (cumulative distribution functions in Figure 7C; see Discussion). These results support the use of pramipexole in therapies in spite of the reduction in D2R action.

Nevertheless, the order of drugs administration appears to influence D2-A2AR receptors interaction in DA-depleted ex-vivo tissue: Figure 8A raster plot (left) shows an initial and representative period of the spontaneous hyperactivity of iSPNs present in dopamine-depleted tissue. Unexpectedly, 50 nM istradefylline administered alone decreased parkinsonian striatal activity in a significant amount (Figure 8A green bar) observed as less active neurons and a reduction in significant peaks of coactivity (Figure 7A and B). A subsequent application of pramipexole further tended to decrease the activity by a very small (non-significant) amount (Figure 8A blue bar, see below), showing that istradefylline virtually occluded the dopamine-agonist effect. Figure 8B show a box plot summary of cumulative activity plot samples comparisons in the different conditions ($n$ = 8 experiments from 8 different slices from 8 different animals, Friedman ANOVA with post hoc Dunnet test. $F(2) = 12.97$, dopamine-depleted versus istradefylline: $*P = .022$ was significant, contrary to the lack of significance when istradefylline was applied after pramipexole (Figure 7A and B); dopamine-depleted versus istradefylline plus pramipexole: $**P = .0018$; istradefylline versus istradefylline plus pramipexole: $P = .830)$. Figure 8C shows cumulative distribution functions of cellular activity for all iSPNs taken from all experiments in the different conditions ($n$ = 8 experiments from 8 different slices and 8 different animals: dopamine-depleted-n1 = 638 neurons vs. istradefylline-n2 = 450 neurons: $K = 0.38$, $P = 2.4017 \times 10^{-30}$; dopamine-depleted vs. istradefylline plus pramipexole-n3 = 432 neurons: $K = 0.38$, $P = 1.5061 \times 10^{-51}$ and pramipexole vs. pramipexole plus pramipexole: $K = 0.079$, $P = .032$; Kolmogorov-Smirnov tests with the Benjamini–Hochberg method for a false discovery rate).

**Discussion**

Focusing on iCa$^{2+}$ excitability as indicators of D2-A2AR receptors interactions, future work will investigate the classes of Ca$^{2+}$ channel involved (Bargas et al., 1994; Rendón-Ochoa et al., 2018). The main findings of this work are: 1) A2AR do not modulate iCa$^{2+}$ currents by themselves in iSPNs, they need the previous activation of D2R, confirming an interaction that enables A2AR actions in iSPNs. 2) This interaction resulted to be antagonistic: previous D2R activation as evaluated by a reduction in iCa$^{2+}$, habituates A2AR to counteract D2R actions on iCa$^{2+}$ in dissociated iSPNs, as evaluated by a recovery in the current reduced by D2R action. That is: A2AR were enabled by D2R to oppose their actions. These electrophysiological results parallels previously biochemical antagonistic interactions reported between both receptors. 3) Brain slice experiments were consistent with these findings, as evaluated by neuronal excitability: D2R first decreased firing frequency and then A2AR restored it. 4) The interaction of D2-A2A receptors is preserved in dopamine-depleted iSPNs, using the 6-OHDA PD model. 5) Istradefylline blocked the action of A2AR in both control and dopamine-depleted iSPNs, demonstrating specificity. 6) Istradefylline decreased the modulation on iCa$^{2+}$ by D2R in dopamine-depleted cells, showing that the interaction goes both ways, in parallel with previously reported decrease in D2R actions. 7) Istradefylline prevented further reduction in iSPNs activity while comparing samples of iSPNs previously treated with pramipexole, in fact, the number of active cells slightly increased. However, the tissue did not return to parkinsonian activity, and in a larger sample used to build cumulative distribution functions it is shown that istradefylline does not significantly interfere with the dopamine-agonist action, supporting their adjuvant effect in therapy. 8) When administered before, istradefylline, reduced parkinsonian hyperactivity by a significant amount, virtually occluding the subsequent action of pramipexole, which had only a tendency to a marginal additional activity.

**D2-A2AR Receptors Antagonistic Interaction on Ca$^{2+}$ Channels Modulation in iSPNs**

A D2-A2AR receptors interaction was demonstrated by the fact that in acutely dissociated iSPNs, free from additional interferences, A2AR do not modulate iCa$^{2+}$ by themselves. They need habilitation by a previous activation of D2R. As previously reported with pharmacological and biochemical studies (Fuxe et al., 2010; Hillion et al., 2002; Sheth et al., 2014), the D2-A2AR receptors interaction resulted to be antagonistic: previous D2R activation showed a reduction in iCa$^{2+}$ and the habilitation of A2AR in turn opposed or counteracted D2R action by recovering iCa$^{2+}$ thus paralleling previously reported biochemical interactions (Ferré et al., 1991, 2008; Stromberg et al., 2000; Yang et al., 1995). In addition, brain slice experiments demonstrate that actions on excitability due to activation of A2AR after activation of D2R can be observed in iSPNs recorded in slices: D2R actions decreased firing frequency (Hernandez-Lopez et al., 2000; Lara-Gonzalez et al., 2019) and then A2AR actions restored it reversing the decrease in firing due to D2R, similar to results obtained in nucleus accumbens (Azdad et al., 2009).
where Ca$^{2+}$ channels and membrane to membrane interactions between both receptors are thought to be involved.

Istradefylline blocked the action of A$_{2A}$R in both control and dopamine-depleted iSPNs, demonstrating specificity (Mizuno et al., 2010; Muller, 2015; Preti et al., 2015; Richardson et al., 1997). In its presence, CGS failed to recover or enhance iCa$^{2+}$, proving that A$_{2A}$R was responsible for current enhancement. Furthermore, they confirmed that changes in excitability run in parallel to those of Ca$^{2+}$ fluctuations between both receptors are thought to be involved. The microcircuit behavior of the parkinsonian microcircuit has been reported elsewhere (Perez-Ortega et al., 2016). Here we were mainly interested in whole neuronal activity. We used a genetically coded indicator of Ca$^{2+}$ influx: virally transfected GCaMP6f (see Materials and methods and Supplementary Figure 3), that allows record various identified neurons with single cell resolution (Aparicio-Juarez et al., 2019; Duhne et al., 2021). In these conditions, the parkinsonian circuitry is characterized by hyperactivity followed by the appearance of multiple peaks of coactivity and a highly recurrent neuronal ensemble (Jáidar et al., 2010, 2019; Lara-Gonzalez et al., 2019; Plata et al., 2013). It is also known that adenosine levels in the tissue are correlated with neuronal activity (Prasad et al., 2021).

Istradefylline failed to further significantly reduce iSPNs hyperactivity while comparing samples of iSPNs previously treated with pramipexole, in fact, the number of active cells slightly increased. However, the tissue did not return to parkinsonian activity, and in the larger sample used to build cumulative distribution functions it is shown that istradefylline does not significantly interfere with the dopamine-agonist action, supporting the coadjuvant effect in therapy.

In addition, when administered before, istradefylline, reduced parkinsonian hyperactivity by a significant amount, virtually occluding the subsequent action of pramipexole, which had only a tendency to a marginal additional activity. It is known that istradefylline combined with low doses of L-DOPA, enhances anti-parkinsonian activity in MPTP mar- mosets (Uchida et al., 2015) and in clinical trials (Bara-Jimenez et al., 2003; Hauser et al., 2011; Kondo et al., 2015; LeWitt & Fahn, 2016). The dopamine-agonist had a negligible effect when added after istradefylline, probably due to the reducing action of D$_2$R activation by A$_{2A}$R blockade. But the joint action of these drugs seems to be efficient to diminish microcircuit hyperactivity during parkinsonism, supporting the coadjuvant effects of A$_{2A}$R blockade.

Discrepancies between samples of single cells and measures with larger samples at a greater scale may be explained by additional interferences: for example, a D$_2$-A$_{2A}$ receptors interaction is involved in glutamate release by glial cells (Cervetto et al., 2017), in controlling corticostriatal transmission (Tozzi et al., 2007), the excitability of cholinergic inter-neurons (Tozzi et al., 2011) and A$_{2A}$R are also habituated by A$_1$ receptors (Hernandez-Gonzalez et al., 2014) whose activation depends on adenosine concentration which in turn depends on neuronal activity which is high during parkinsonism.

In this work, we provided evidence that Ca$^{2+}$ channels are molecular effectors of the well known D$_2$-A$_{2A}$ receptors antagonistic interaction reported in several pharmacological tests in the dorsal striatum of mice (Prasad et al., 2021), including the reported antagonist actions of A$_{2A}$R onto D$_2$R function on iSPNs (Ferré et al., 2008; Stromberg et al., 2013).
A2AR agonists counteracting the action of D2R agonists may exhibit negative allosteric protein-protein interactions: the action of A2AR agonists but also reduce the modulatory action of D2R agonists.

However, this effect was of little importance when looking at larger neuronal populations, highlighting the possible differences found between samples of a few neurons and larger samples in parkinsonian tissue subject to several influences. However, to elucidate whether these actions are due to allosteric protein-protein interactions or at the level of signaling-crosstalk is out of the scope of the present work (Casadó-Anguera et al., 2016; Fernandez-Dueñas et al., 2019). Notwithstanding, these experiments open the question about what is the role of Ca\(^{2+}\) channels in the possible heteromers that may be participating. In sum, the present electrophysiological findings support the hypotheses previously found with biochemical work: that A2AR-D2R heteromers may exhibit negative allosteric protein-protein interactions: A2AR agonists counteracting the action of D2R agonists (and vice versa) and that A2AR antagonists not only block the action of A2AR agonists but also reduce the modulatory action of D2R agonists.

**Author Contribution**

EAR-O: Conceptualization, Methodology, Formal analysis, Investigation, Data Curation, Writing - Original Draft, Writing - Review & Editing, Visualization; TH-F: Investigation, Writing - Review & Editing; MP-O: Methodology, Software, Investigation, Resources, Writing - Original Draft, Writing - Review & Editing, Visualization; MBP-R: Methodology, Investigation, Writing - Original Draft, Writing - Review & Editing, Visualization; VHA-R: Methodology, Investigation, Writing - Review & Editing; VMC: Methodology, Software, Investigation, Writing - Original Draft, Writing - Review & Editing, Visualization; OH-G: Investigation; MP-R: Methodology, Resources; EG: Conceptualization, Methodology, Project administration, Funding acquisition, Supervision, Writing - Review & Editing; JB: Conceptualization, Methodology, Project administration, Funding acquisition, Supervision, Writing - Review & Editing

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**Supplemental material**

Supplemental material for this article is available online.

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2000), adding evidence that D2-A2A interaction goes both ways and showing the efficacy of A2AR blockade as an adjuvant in anti-parkinsonian therapy. Habilitation of A2AR to modulate iCa\(^{2+}\) was previously shown with adenosine A1 receptors (Hernandez-Gonzalez et al., 2014), suggesting that other receptors, out of the scope of the present work and in need of further investigation, may be able to habilitate A2AR in iSPNs. When tested in samples of single neurons, A2AR blockade appears to interfere with the action of D2R, however, this effect was of little importance when looking at larger neuronal populations, highlighting the possible differences found between samples of a few neurons and larger samples in parkinsonian tissue subject to several influences. However, to elucidate whether these actions are due to allosteric protein-protein interactions or at the level of signaling-crosstalk is out of the scope of the present work (Casadó-Anguera et al., 2016; Fernandez-Dueñas et al., 2019). Notwithstanding, these experiments open the question about what is the role of Ca\(^{2+}\) channels in the possible heteromers that may be participating. In sum, the present electrophysiological findings support the hypotheses previously found with biochemical work: that A2AR-D2R heteromers may exhibit negative allosteric protein-protein interactions: A2AR agonists counteracting the action of D2R agonists (and vice versa) and that A2AR antagonists not only block the action of A2AR agonists but also reduce the modulatory action of D2R agonists.
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### Abbreviations

- **SPN** Striatal projection neurons
- **iSPN** Indirect striatal projection neurons
- **dSPN** Direct striatal projection neurons
- **PD** Parkinson’s disease
- **CaV** High voltage gated calcium channels
- **D2R** Dopamine D2 receptor
- **A2A-R** Adenosine A2A receptor
- **A2A-Cre** Tg(Adora2a-cre) KG139Gsat
- **6-OHDA** 6-Hydroxydopamine
- **L-DOPA** L-3,4 dihydroxifenilalanine
- **MPTP** 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
- **iCa2** Calcium currents
- **NMDA** N-methyl-D- aspartate
- **NMDG** N-methyl-d glucamine
- **EGTA** Ethylene Glycol Tetraacetic Acid
- **HEPES** 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid
- **TTX** Tetrodotoxin
- **CGS** CGS-21680: 4-[2-[[6-Amino-9-(N-ethyl-β-D-ribofuranuronamidosyl)-9H-purin-2-yl]amino]ethyl]benzenepropanoic acid hydrochloride