ORIGINAL ARTICLE

Adenosine A$_1$ and A$_{2A}$ receptors are involved on guanosine protective effects against oxidative burst and mitochondrial dysfunction induced by 6-OHDA in striatal slices

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Abstract

6-Hydroxydopamine (6-OHDA) is the most used toxin in experimental Parkinson's disease (PD) models. 6-OHDA shows high affinity for the dopamine transporter and once inside the neuron, it accumulates and undergoes non-enzymatic auto-oxidation, promoting reactive oxygen species (ROS) formation and selective damage of catecholaminergic neurons. In this way, our group has established a 6-OHDA in vitro protocol with rat striatal slices as a rapid and effective model for screening of new drugs with protective effects against PD. We have shown that co-incubation with guanosine (GUO, 100 μM) prevented the 6-OHDA-induced damage in striatal slices. As the exact GUO mechanism of action remains unknown, the aim of this study was to investigate if adenosine A$_1$ (A$_1$R) and/or A$_{2A}$ receptors (A$_{2A}$R) are involved on GUO protective effects on striatal slices. Pre-incubation with DPCPX, an A$_1$R antagonist prevented guanosine effects on 6-OHDA-induced ROS formation and mitochondrial membrane potential depolarization, while CCPA, an A$_1$R agonist, did not alter GUO effects. Regarding A$_{2A}$R, the antagonist SCH58261 had similar protective effect as GUO in ROS formation and mitochondrial membrane potential. Additionally, SCH58261 did not affect GUO protective effects. The A$_{2A}$R agonist CGS21680, although, completely blocked GUO effects. Finally, the A$_1$R antagonist DPCPX, and the A$_{2A}$R agonist CGS21680 also abolished the preventive guanosine effect on 6-OHDA-induced ATP levels decrease. These results reinforce previous evidence for a putative interaction of GUO with A$_1$R-A$_{2A}$R heteromer as its molecular target and clearly indicate a dependence on adenosine receptors modulation to GUO protective effect.

Keywords Guanosine · Adenosine A$_1$ and A$_{2A}$ receptors · 6-Hydroxydopamine, Striatal slices

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Introduction

Parkinson's disease (PD) is a neurodegenerative disorder characterized by bradykinesia, tremor, and rigidity, caused by the progressive loss of the dopaminergic neurons in the nigrostriatal pathway [1]. Although the etiology of PD is considered idiopathic, it can be related to other factors as genetics, environmental toxins, oxidative stress, or mitochondrial abnormalities [2]. The main

therapeutic approach is based on dopamine replacement to promote motor symptoms relief; however, it does not stop the progression of the disease [3]. The molecular trigger to neuronal degeneration may involve the oxidative burst, mitochondrial dysfunction, and bioenergetics impairment [4–6]; thus, new strategies of treatment aiming protection of these biochemical unbalance in dopaminergic neurons are necessary.

The experimental study of PD relies, mainly, in the use of animal models administered with toxins to mimic the neurodegeneration in the nigrostriatal pathway. 6-hydroxydopamine (6-OHDA) was the first discovered drug that has specific toxicity for dopaminergic neurons [7], and it is the most used toxin in experimental PD models [8–10]. Because of the similar structure, 6-OHDA also shows affinity for the dopamine transporters [11] and it accumulates inside the neurons, where it undergoes an auto-oxidation, promoting selective damage of catecholaminergic neurons [8, 11].

In this way, our group has established a 6-OHDA in vitro model with rat brain slices, showing that in vitro incubation with 6-OHDA induced a decrease in cellular viability, increase in ROS production, and a disruption in mitochondrial membrane potential in striatal slices [12]. Moreover, we have shown that co-incubation with guanosine (GUO) prevented the 6-OHDA-induced damage in striatal slices [13]. GUO is a purine nucleoside, which has demonstrated neuroprotective effects in several animal and cellular models of neurotoxic conditions and neurodegenerative diseases [14].

Regarding PD, it is already known that GUO exerts protective effects against in vitro 6-OHDA toxicity in two cell lines (C6 glioma and dopaminergic human SH-SY5Y neuroblastoma cells) [15, 16]. Besides that, GUO also have effects on in vivo PD models. In unilaterally 6-OHDA-lesioned rats, GUO acutely administered increased L-DOPA sub-maximal response and decreased L-DOPA-induced dyskinesia, i.e., GUO potentialized the L-DOPA effects diminishing its side effects. In the same way, GUO also reversed reserpine-induced motor disturbance in mice [17]. In search for the molecular target of GUO, our group has already implied adenosine receptors modulation with GUO effects in an ischemic-like damage, in hippocampal slices and cortical astrocytes [18, 19]. In fact, adenosinergic transmission has been pointed out as a promising therapeutic strategy for motor symptoms of PD [20, 21]. This therapeutic potential is mainly due the fact that adenosine A_1 and A_{2A} receptors (A_1R and $A_{2A}R$) are largely expressed in the striatum and have a key role in modulation of dopaminergic transmission [22, 23].

Since the GUO mechanism of action it is still not clearly identified, it is of great interest to understand the signaling behind its effects acting as neuroprotective agent. Therefore, the aim of this study was to investigate if adenosine A_1R and/or $A_{2A}R$ are involved on GUO protective effects on striatal slices against oxidative damage, mitochondrial dysfunction, and ATP depletion due to 6-OHDA-induced toxicity in vitro.

Materials and methods

Animals

Male Wistar rats (3 months old, 350–400 g) were obtained from our local colony, maintained in a 12-h dark/light cycle, at constant room temperature at 23 ± 1 °C and with food and water ad libitum. Experiments followed the ARRIVE Guidelines published in 2010 and were approved by the local Ethical Committee for Animal Research (CEUA/UFSC PP00955).

Brain slices

Animals were euthanized by decapitation and the whole brain were quickly removed and the striatum was rapidly dissected in ice-cold Krebs Ringer buffer (KRB) (122 mM NaCl, 3 mM KCl, 1.2 mM MgSO$_4$, 1.3 mM CaCl$_2$, 0.4 mM KH$_2$PO$_4$, 25 mM NaHCO$_3$, and 10 mM D-glucose, bubbled with 95% O$_2$/ 5% CO$_2$ up to pH 7.4). Striatal slices (0.4 mm) were prepared using a McIlwain Tissue Chopper (The Mickle Laboratory Engineering Co. ltd., England) and separated in KRB at 4 °C. After sectioning, slices were randomly selected (3 slices per group) and incubated in 24-well cell culture plate with KRB (1 mL) for 30 min, at 37 °C, for metabolic recovery from slicing procedure.

Slices treatment

6-OHDA (Sigma, St. Louis, MO, USA) was solubilized in water at 0.1% sodium metabisulfite and stored at − 20 °C. For the experiment, 6-OHDA was diluted to 100 μM in KRB. To investigate 6-OHDA-induced damage, slices from the striatum were exposed to 6-OHDA (100 μM) during 1 h [12]. GUO (Sigma, St. Louis, MO, USA) was freshly prepared and diluted in KRB (100 μM) and co-incubated with 6-OHDA for 1 h to determine its neuroprotective effect. To investigate the role of A_1R or $A_{2A}R$ in the GUO neuroprotective effect, slices were pre-incubated with agonists or antagonists of A_1R (CCPA and DPCPX, respectively) and $A_{2A}R$ (CGS21680 and SCH58261) 15 min prior to the incubation with 6-OHDA and/or GUO (Fig. 1a). Concentrations of adenosine receptors ligands were selected based on previous studies [19, 24]. Slices of control group were incubated in a physiological KRB. All experimental groups were assayed in triplicates. After the 1 h of co-incubation with 6-OHDA and GUO, ROS production, mitochondrial membrane potential, or intracellular ATP levels were evaluated.

ROS levels

ROS production was measured by using the molecular probe 2,7-dichlorofluorescein diacetate (H$_2$DCFDA, Sigma Aldrich, St. Louis, MO, USA). H$_2$DCFDA diffuses through the cell membrane and is hydrolyzed by intracellular esterases to the
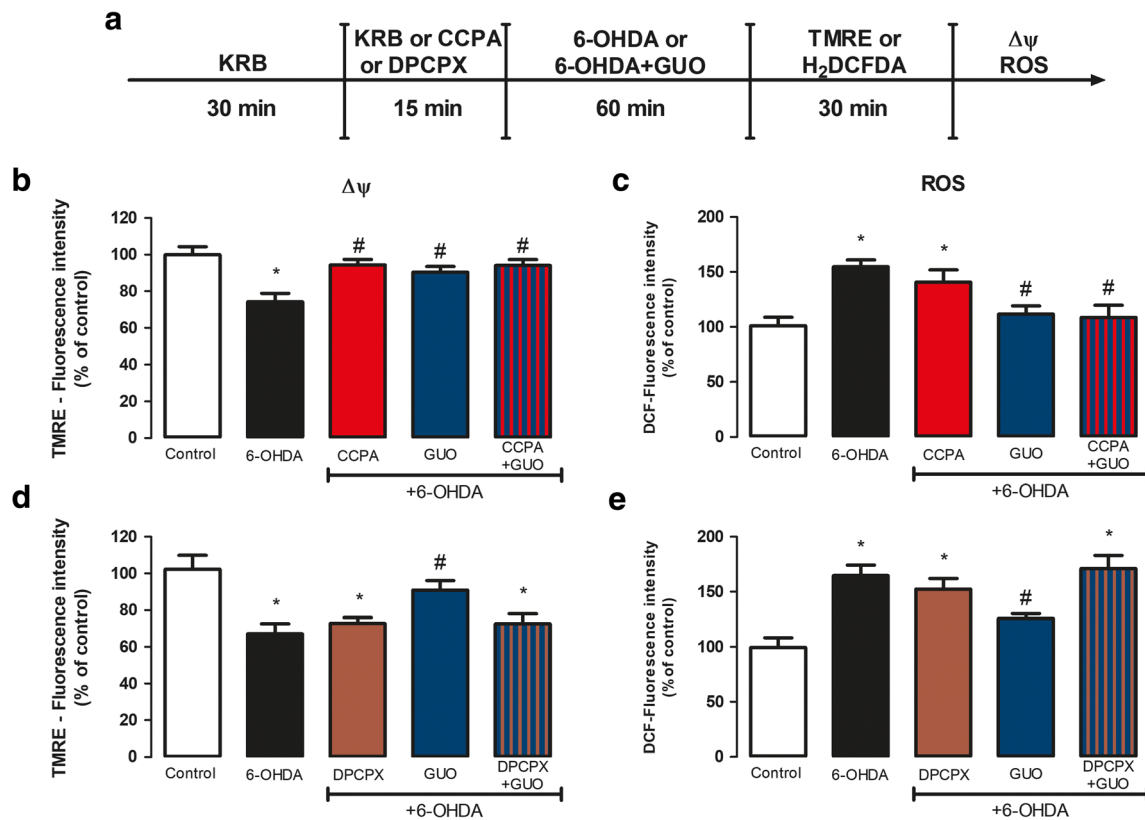
Fig. 1 Effects of A_1R modulation on 6-OHDA-induced toxicity in striatal slices. Experimental design is describe in **a**. Striatal slices were pre-incubated with A_1R agonist CCPA (100 nM; **b, c**) or A_1R antagonist DPCPX (250 nM; **d, e**). Slices were incubated with 6-OHDA (100 μM) and/or co-incubated with GUO (100 μM). 6-OHDA-induced mitochondrial membrane potential ($\Delta\Psi$) (**b, d**) and ROS levels (**c, e**). Data are expressed as percentage of controls normalized among individual experiments and represent means with SEM ($n = 6$). (*) when $p < 0.05$ compared with control or (#) compared to 6-OHDA group (one-way ANOVA followed by Tukey's test)

non-fluorescent form 2′,7′-dichlorofluorescein (DCFH). DCFH reacts with intracellular ROS (such as H_2O_2) to form dichlorofluorescein (DCF), a green fluorescent dye. DCF fluorescence intensity is proportional to the amount of ROS. Striatal slices were incubated with 80 μM of H_2DCFDA diluted in KRB (1 mL) for 30 min at 37 °C. Slices were then washed with and maintained in KRB (1 mL) for fluorescence measurement. Fluorescence was read using excitation and emission wavelengths of 480 and 525 nm, respectively in a fluorescence microplate reader (TECAN®). Results were obtained as arbitrary unit of fluorescence and were expressed in percentage related to control levels.

Mitochondrial membrane potential

Mitochondrial membrane potential ($\Delta\Psi$) was measured by using the molecular probe tetramethylrhodamine ethyl ester (TMRE, Sigma Aldrich, St. Louis, MO, USA). TMRE is a cell-permeant, cationic, red-orange fluorescent dye that is readily sequestered by active mitochondria. Slices were incubated with 10 nM TMRE diluted in KRB (1 mL) for 30 min at 37 °C. Slices were then washed with and maintained in KRB (1 mL) for fluorescence measurement. Fluorescence was

measured using wavelengths of excitation and emission of 550 and 590 nm, respectively. The results are expressed and normalized as percentages relative to the control conditions. Results were obtained as arbitrary unit of fluorescence and were expressed in percentage related to control levels.

ATP levels

After GUO and 6-OHDA treatment, brain slices from striatum were homogenized in trichloroacetic acid (TCA) 2% aqueous solution (350 μL). The homogenates were centrifuged at 14000 rpm at 4 °C for 3 min. The supernatants (100 μL) were used for determination of ATP levels, using bioluminescent assay kit according to the manufacturer's recommendations (#FLAA, Sigma Aldrich, St. Louis, MO, USA). The amount of protein in each sample was measured using the method of [25] and the results are expressed in μmol ATP/μg of protein in each sample (3 slices for group).

Statistical analysis

Results are expressed as means ± standard error (SEM). Comparisons among experimental and control groups were

performed by one-way ANOVA followed by the Tukey post hoc test. Statistical difference was accepted when $p < 0.05$.

Results

A$_1$R modulation

As we previously shown, GUO (100 μM) protects striatal slices against in vitro 6-OHDA-induced mitochondrial membrane depolarization and increased ROS generation [13]. So, we aimed to investigate whether these effects were related to A$_1$R modulation. Slices incubated with 6-OHDA (100 μM) showed a decrease in the florescence of the TMRE dye, that is related to a mitochondrial membrane depolarization, as the same effect was observed when slices were incubated with carbonyl-cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP, 10 μM), a mitochondrial oxidative phosphorylation uncoupler [12]. Interestingly, when slices were pre-incubated with the A$_1$R agonist, CCPA (100 nM), it prevented the 6-OHDA-induced mitochondrial membrane depolarization (Fig. 1a). However, the pre-incubation with CCPA did not affect the GUO protective effect on mitochondrial membrane potential ($\Delta\Psi$). Regarding ROS levels, CCPA pre-incubation had no effect on 6-OHDA-induced ROS increase and did not alter the protective effect of GUO. These results suggest that activation of A$_1$R does not counteract the GUO effects.

On the other hand, pre-incubation with DPCPX (250 nM), an A$_1$R antagonist, showed to be ineffective in preventing the mitochondrial membrane depolarization and ROS increase caused by 6-OHDA (Fig. 2c, d). Besides that, DPCPX pre-incubation totally abolished GUO effects on 6-OHDA-induced mitochondrial membrane depolarization and ROS increase. These experiments indicate that blocking A$_1$R also blocks the GUO effects, showing an A$_1$R dependence on GUO protective effects.

A$_{2A}$R modulation

A putative A$_{2A}$R dependence on the GUO protective effects in the 6-OHDA in vitro protocol was also assessed in striatal slices (Fig. 2a). The pre-incubation with the A$_{2A}$R agonist CGS 21680 (30 nM) exerted no effect on 6-OHDA-induced mitochondrial membrane potential ($\Delta\Psi$) depolarization and ROS levels increase; however, it completely blocked the GUO protective effect in both $\Delta\Psi$ and ROS levels (Fig. 2b, c). These results indicate that A$_{2A}$R activation can affect GUO effects. The blockade of A$_{2A}$R is already seen as an anti-parkinsonian strategy, as it has protective effects on many PD models. In this way, pre-incubation of SCH58261 (50 nM), an A$_{2A}$R antagonist, presented a protective effect upon OHDA-induced $\Delta\Psi$ depolarization and ROS levels increase (Fig. 2c, d). Moreover, this protective effect of SCH58261 does not affect GUO effects.

ATP levels

As mitochondrial depolarization might cause ATP depletion and 6-OHDA also causes changes in the cellular energetic balance [13], we evaluate the effects of adenosine receptors ligands on 6-OHDA and/or GUO incubation, by measuring ATP levels in striatal slices. Since the blockade of A$_1$R or activation of A$_{2A}$R interfered on the GUO neuroprotective effect, we performed ATP measurements with DPCPX and CGS21680. As expected, 6-OHDA incubation decreased the ATP levels and GUO co-incubation prevented this decrease (Fig. 3). Although neither DPCPX nor CGS21680 presented effect on ATP levels decrease by 6-OHDA, both ligands impaired the capability of GUO to prevent it. These results are in line with the observed for $\Delta\Psi$ and ROS levels, indicating that GUO effects are related to both A$_1$R and A$_{2A}$R.

Discussion

In this study, we investigated the modulation of A$_1$R and A$_{2A}$R upon GUO effects against the cellular damage caused by in vitro incubation with 6-OHDA in ex vivo slices obtained from rat striatum. This in vitro protocol of 6-OHDA-induced toxicity is a simple and sensitive protocol that completely suits our goal of evaluating the mechanism of neuroprotection afforded by GUO. We have already shown that co-incubation with GUO (100 μM) prevented the striatal slices against oxidative damage, mitochondrial dysfunction, and ATP depletion caused by 6-OHDA [13]. GUO is a naturally occurring guanine-based purine that has been pointed out to act as a neuromodulator and a neuroprotective agent [14, 26]. GUO have already shown to exert protective effect in different models of PD. In vitro 6-OHDA toxicity in two cell lines (C6 glioma and dopaminergic human SH-SY5Y neuroblastoma cells) was prevented by GUO incubation [15, 16]. In the same way, the toxicity induced by 1-methyl-4-phenylpyridinium (MPP+) in different cultured cells (SH-SY5Y and PC12 cells) was prevented by GUO [27, 28]. Protective effects of GUO were also showed in rodent models of PD [17, 29], and recently, we showed that GUO effects on reducing oral tremor in reserpinized mice were sensitive to adenosine receptors modulation [30].

Among the new classes of drugs developed to improve the clinical features of PD, A$_{2A}$R antagonists appear to be the most promising. A$_{2A}$R blockade has been demonstrated to be effective in both preclinical and clinical PD studies [31–33]. Indeed, istradefylline (KW6002), an A$_{2A}$R antagonist, was already approved for clinical use in Japan, and recently, in the USA [34–36]. Interestingly, the mechanism behind A$_{2A}$R antagonists in PD may rely in part to the existing functional and molecular interaction (i.e., heteromerization) of A$_{2A}$R and D$_2$R within postsynaptic striatal neurons [37, 38]. Moreover, a mutual trans-inhibition between these two
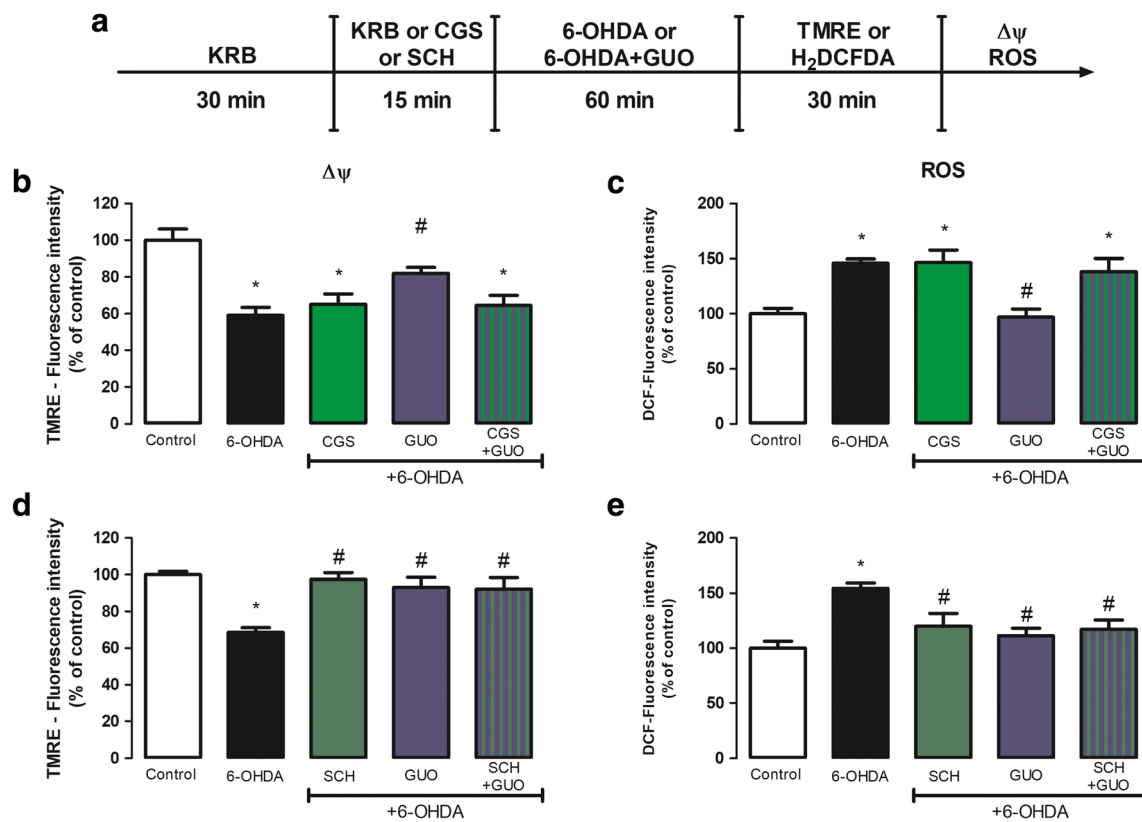
Fig. 2 Effects of $A_{2A}R$ modulation on 6-OHDA-induced toxicity in striatal slices. Experimental design is describe in **a**. Striatal slices were pre-incubated with of $A_{2A}R$ agonist CGS 21680 (CGS, 30 nM; **b**, **c**) or $A_{2A}R$ antagonist SCH 58261 (SCH, 50 nM; **d**, **e**). Slices were incubated with 6-OHDA (100 μM) and/or co-incubated with GUO (100 μM). 6-OHDA-induced mitochondrial membrane depolarization (**b**, **d**) and ROS levels increase (**c**, **e**). Data are expressed as percentage of controls normalized among individual experiments and represent means with SEM ($n = 6$). (*) when $p < 0.05$ compared with control or (#) compared to 6-OHDA group (one-way ANOVA followed by Tukey's test)

receptors has been described [39]. Corroborating with this, in our results, the $A_{2A}R$ antagonist SCH58261 was effective in both parameters analyzed. More important, the protective effect of SCH58261 does not affect GUO effects, suggesting that they do not interfere in each other mechanism. Interestingly, we previously showed that submaximal doses of SCH58261 and GUO can potentiated each other effect in a PD-related behavioral test but not in ROS levels [30]. Taken

Fig. 3 A_1R and $A_{2A}R$ modulation on ATP levels in striatal slices. Experimental design is describe in **a**. Effect of pre-incubation of A_1R antagonist, DPCPX (250 nM) (**b**) or $A_{2A}R$ agonist, CGS21680 (CGS, 30 nM) (**c**) on 6-OHDA-induced ATP depletion. Slices were incubated with 6-OHDA (100 μM) and/or co-incubated with GUO (100 μM). Data are expressed as μmol ATP/μg of protein of each sample and represent means with SEM ($n = 3$). (*) when $p < 0.05$ compared with control or (#) compared to 6-OHDA group (one-way ANOVA followed by Tukey's test)
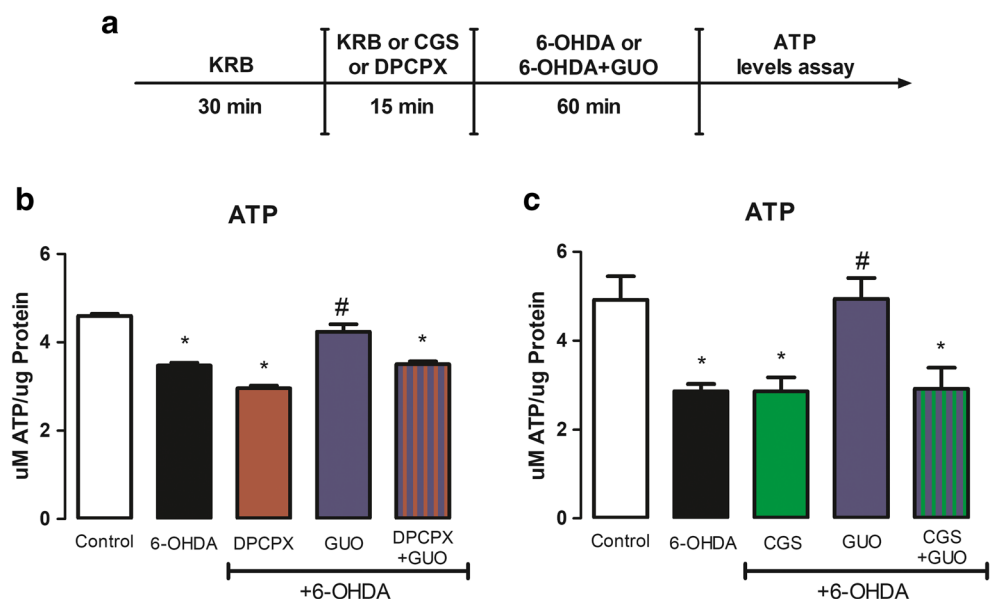
together, these data suggest that GUO might be acting through a negative $A_{2A}R$ modulation.

In addition to this postsynaptic site of action, $A_{2A}R$ can also form heteromeric complexes with A_1R in presynaptic neurons of the basal ganglia, where they can control glutamate release [40] and striatal circuits independently of dopaminergic signaling [41]. Regarding A_1R, some rare mutations on this receptor gene could lead to PD [42]. Also, some studies show that A_1R modulation could control and improve motor function associated with PD [43–45]. In fact, a lot of data show that A_1R stimulation is neuroprotective [46–49]. Surprisingly, CCPA was unable to prevent the ROS increase induced by 6-OHDA, but it did protected mitochondrial function. However, CCPA did not alter the GUO protective effect on mitochondrial $\Delta\Psi$, and this result could be interpreted as a non-additive synergistic effect of CCPA and GUO. Nevertheless, the potential use of A_1R-based therapies, by using A_1R agonists, could lead to deleterious peripheral side-effects, once A_1R is also expressed in the vascular system.

Evidences from other disease models also have pointed to GUO effects via adenosine receptors modulation. In an in vitro brain ischemia model, hippocampal slices subjected to oxygen/glucose deprivation presented increased ROS production prevented by GUO, but this effect is abolished by pre-incubation with DPCPX [19]. Notwithstanding, in the same protocol, not only DPCPX but also CGS21680 blunted the protective effect of GUO in hippocampal slices [19] and in cortical astrocytes [18]. The same pattern of results was seen in our study that used an in vitro PD model and evaluated other brain area. Despite the similarity of these results, one study, where staurosporine-induced apoptosis in astrocytes cultures were evaluated, showed that the neuroprotective effect of GUO was not affected by the selective A_1R or $A_{2A}R$ antagonists [50]. On the other hand, still in the ischemia model, in hippocampal slices of $A_{2A}R$-knockout animals subjected to oxygen/glucose privation, the GUO-protective effects is abolished, evidencing the importance of this receptor for GUO effects. Taken together, these observations strengths the possible mechanism of GUO-effects through the dependence of adenosine A_1 and A_{2A} receptors modulation.

Regarding GUO interaction with a putative specific receptor, it was showed that GUO presented selective binding site in cellular membrane that is not related to adenosine receptors [51, 52]; however, this possible GUO-specific receptor has not been fully described or characterized. Beside this and the controversy data regarding GUO effects via A_1R or $A_{2A}R$ interaction, the possibility of GUO interaction with adenosine receptor heteromers appears to be the most likely scenario to explain this debate [53]. In fact, we recently showed that GUO-induced effects require both A_1R and $A_{2A}R$ co-expression in transfected HEK293 cells [54]. Only in cells expressing both A_1R and $A_{2A}R$, GUO was able to decrease $A_{2A}R$ binding affinity and cAMP response evoked by a selective $A_{2A}R$ ligand. Also, GUO had no effect on A_1R signaling through intracellular calcium increase, even in the presence or absence of $A_{2A}R$ co-expression [54]. Considering all these evidences, our working hypothesis is that we need to interpret GUO interaction with adenosine receptors results not as separated receptors but as a heteromeric entity. GUO could be acting as a negative modulator of $A_{2A}R$, but only in the presence of A_1R. It is feasible to speculate that the physical interaction between A_1R and $A_{2A}R$ could lead to an increase of $A_{2A}R$ affinity for GUO. This could explain why GUO effects are blocked by CGS21680 and DPCPX (by causing allosteric modulation of GUO $A_{2A}R$ affinity) and not by SCH58261 and CCPA. Indeed, GUO modulation over A_1R-$A_{2A}R$ heteromer or A_1R or $A_{2A}R$ individual entities could vary among brain structures, once that it may depend on receptors expression. Therefore, we also cannot exclude the possibility of GUO acting through other heteromer, that could modulate or be associated with A_1R or $A_{2A}R$, and further investigations are necessary to detail GUO mechanism of action.

In conclusion, we demonstrated that GUO protective effects on oxidative damage, mitochondrial dysfunction and ATP depletion caused by 6-OHDA in rat striatal slices are sensitive of both A_1R and $A_{2A}R$ modulation. These results could provide another possibility for GUO action through the A_1R-$A_{2A}R$ heteromer and highlight its importance as a neuroprotective agent in PD.

Compliance with ethical standards The procedures used in this study complied with the guidelines on animal care of the UFSC Ethics Committee on the Use of Animals (CEUA), which follow the principles of laboratory animal care from NIH (2011).

Conflict of interest The authors declare no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

Abbreviations A_1R, Adenosine A_1 receptors; $A_{2A}R$, Adenosine A_{2A} receptors; *CCPA*, 4-[2-[[6-Amino-9-(*N*-ethyl-β-D-ribofuranuronamidosyl)-9H-purin-2-yl]amino]ethyl] benzene propanoic acid hydrochloride; *CGS21680*, 4-(2-[7-Amino-2-(2-furyl)[1, 2, 4] triazolo[2,3-*a*][1,3,5]triazin-5-ylamino]ethyl) phenol; *DPCPX*, 1,3-

Dipropyl-8-cyclopentylxanthine; *GUO,* Guanosine; *6-OHDA,* 6-Hydroxydopamine; *H₂DCFDA,* 2,7-Dichlorofluorescein diacetate; *KRB,* Krebs Ringer buffer; *ΔΨ,* Mitochondrial membrane potential; *MTT,* (4,5 Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; *PD,* Parkinson's disease; *ROS,* Reactive oxygen species; *SCH58261,* 5-Amino-7-(2-phenylethyl)-2-(2-furyl)-pyrazolo(4,3-e)-1,2,4-triazolo(1,5-c)pyrimidine; *TMRE,* Tetramethylrhodamine ethyl ester

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