Journal of Neurochemistry

JOURNAL OF NEUROCHEMISTRY | 2015 | 134 | 740–747 doi: 10.1111/jnc.13162

ORIGINAL ARTICLE

Dual target strategy: combining distinct non-dopaminergic treatments reduces neuronal cell loss and synergistically modulates L-DOPA-induced rotational behavior in a rodent model of Parkinson's disease

Marie-Therese Fuzzati-Armentero,* Silvia Cerri,* Giovanna Levandis,* Giulia Ambrosi,* Elena Montepeloso,* Gianfilippo Antoninetti,* Fabio Blandini,* Younis Baqi,†,‡ Christa E. Müller,† Rosaria Volpini,§ Giulia Costa,¶ Nicola Simola¶ and Annalisa Pinna**

*Laboratory of Functional Neurochemistry, Center for Research In Neurodegenerative Diseases C. Mondino National Neurological Institute, Pavia, Italy

†Pharmaceutical Institute, Pharmaceutical Chemistry I, Pharma Center Bonn, University of Bonn, Bonn, Germany

‡Department of Chemistry, Faculty of Science, Sultan Qaboos University, Muscat, Oman

§School of Pharmacy, Medicinal Chemistry Unit, University of Camerino, Camerino, Italy

¶Department of Biomedical Sciences, University of Cagliari, Cagliari, Italy

**National Research Council of Italy, Neuroscience Institute, Cagliari, Italy

Abstract

The glutamate metabotropic receptor 5 (mGluR5) and the adenosine A_{2A} receptor (A_{2A}R) represent major non-dopaminergic therapeutic targets in Parkinson's disease (PD) to improve motor symptoms and slow down/revert disease progression. The 6-hydroxydopamine rat model of PD was used to determine/compare the neuroprotective and behavioral impacts of single and combined administration of one mGluR5 antagonist, 2-methyl-6-(phenylethynyl)pyridine (MPEP), and two A_{2A}R antagonists, (*E*)-phosphoric acid mono-[3-[8-[2-(3-methoxyphenyl) vinyl]-7-methyl-2,6-dioxo-1-prop-2-ynyl-1,2,6,7-tetrahydropurin-3-yl]propyl] (MSX-3) and 8-ethoxy-9-ethyladenine (ANR 94). Chronic treatment with MPEP or MSX-3 alone, but not with ANR 94, reduced the toxin-induced loss of dopaminergic neurons in the substantia nigra pars compacta. Combining MSX-3 and MPEP further improved the neuroprotective effect of either antagonists. At the behavioral level, ANR 94 and MSX-3 given alone significantly potentiated L-DOPA-induced turning behavior. Combination of either A_{2A}R antagonists with MPEP synergistically increased L-DOPA-induced turning. This effect was dose-dependent and required subthreshold drug concentration, which *per se* had no motor stimulating effect. Our findings suggest that co-treatment with A_{2A}R and mGluR5 antagonists provides better therapeutic benefits than those produced by either drug alone. Our study sheds some light on the efficacy and advantages of combined non-dopaminergic PD treatment using low drug concentration and establishes the basis for in-depth studies to identify optimal doses at which these drugs reach highest efficacy.

Keywords: 6-OHDA, adenosine A_{2A} receptor, antagonist, metabotropic glutamate receptor, neurodegeneration, neuroprotection.

J. Neurochem. (2015) **134**, 740–747.

Received January 14, 2015; revised manuscript received March 31, 2015; accepted May 4, 2015.

Address correspondence and reprint requests to Marie-Therese Fuzzati-Armentero, C. Mondino National Neurological Institute, via Mondino 2, 27100 Pavia, Italy. E-mail: marie.armentero@mondino.it

Abbreviations used: 6-OHDA, 6-hydroxydopamine; A_{2A}R, adenosine A_{2A} receptor; ANR 94, 8-ethoxy-9-ethyladenine; DA, dopamine; DMSO, dimethylsulfoxide; IS, intrastriatal; L-DOPA, L-3,4-dihydroxyphenylalanine; MFB, medial forebrain bundle; mGluR, metabotropic glutamate receptor; MPEP, 2-methyl-6-(phenylethynyl)pyridine; MSX-3, (*E*)-phosphoric acid mono-[3-[8-[2-(3-methoxyphenyl)vinyl]-7-methyl-2,6-dioxo-1-prop-2-ynyl-1,2,6,7-tetrahydropurin-3-yl]propyl]; PD, Parkinson's disease; PEG, polyethylene glycol; SNc, substantia nigra pars compacta; TH, tyrosine hydroxylase.

The increase in age-related neurodegenerative diseases including Parkinson's disease (PD), the most common movement disorder, is imposing a growing economic burden linked to the costs needed for effective treatment. Approved PD therapies, including L-3,4-dihydroxyphenylalanine (L-DOPA) and dopamine (DA) agonists, are mainly symptomatic and principally directed at replacing/restoring the loss of DA that characterizes the motor disorder. As disease progresses, these treatments inevitably lose efficacy, requiring dose escalation regimens that lead to debilitating side effects, including dyskinesia and motor fluctuations. After 5–10 years of treatment, disease management is considerably complicated by the combination of undesired treatment-linked side effects and unyielding neuronal loss. Commonly used adjunct therapies, such as inhibitors of monoamine oxidase B that prevent the breakdown of DA, may effectively reduce motor fluctuations but often worsen dyskinesia and have no effect on 'off' time.

In the past decades, many efforts and resources have been invested in developing innovative therapeutic approaches for PD that may improve motor symptoms with reduced long-term side effects and may slow down or revert disease progression. Two important targets that have emerged and attracted considerable attention for alternative non-dopaminergic therapeutic strategies are the metabotropic glutamate receptors (mGluRs), including mGluR5, and the adenosine 2A receptor ($A_{2A}R$). These receptors are strategically expressed in the basal ganglia nuclei where they may intervene on important motor-loop mechanisms. Both mGluR5 and $A_{2A}R$ are also detected on glial cells, where they may modulate neuroinflammatory processes associated with PD.

Chronic administration of the negative allosteric mGluR5 modulator 2-methyl-6-(phenylethynyl)pyridine (MPEP) has been shown to significantly improve akinesia and L-DOPA-induced abnormal involuntary movements in both rodent and primate models of PD (Levandis *et al.* 2008; Ambrosi *et al*. 2010; Morin *et al*. 2013). Moreover, chronic treatment with MPEP reduced toxin-induced PD-like neurodegenerative processes in rats (Armentero *et al.* 2006; Ambrosi *et al.* 2010), suggesting that antagonizing mGluR5 may also have a disease modifying potential.

Numerous $A_{2A}R$ antagonists have been evaluated in experimental models of PD and some of them have progressed into human clinical trials (Armentero *et al.* 2011; Pinna 2014). The first $A_{2A}R$ antagonist, istradefylline (Nouriast®, Kyowa Hakko Kinn Co, Ltd, Japan), has been approved in 2013 in Japan for the treatment of PD in combination with L-DOPA (Jenner 2014). While there is little evidence that $A_{2A}R$ antagonists may be effective as monotherapy at early stages of PD, these drugs can, however, improve motor symptoms and reduce 'off' time without increasing dyskinesia in advanced PD patients under L-DOPA treatment (Hauser *et al.* 2014).

In this study, we used a well-characterized rodent model of PD, induced by the intracerebral injection of the neurotoxin 6-hydroxydopamine (6-OHDA), to assess and compare the efficacy of the treatment with mGluR5 and $A_{2A}R$ antagonists given alone or combined. Output measures included the evaluation of the 6-OHDA-induced neuronal loss as well as L-DOPA-induced turning behavior, as an index of anti-parkinsonian activity.

Materials and methods

Drugs – reagents

6-Hydroxydopamine (dissolved in saline/0.05% ascorbic acid), L-DOPA, desipramine hydrochloride, benserazide (all dissolved in distilled water), MPEP (mGluR5 antagonist; dissolved in dimethylsulfoxide (DMSO)/polyethylene glycol (PEG 400) solution 50/50 for neuroprotection studies, and in distilled water for behavioral studies) were purchased from Sigma-Aldrich (Milan, Italy). (*E*)-phosphoric acid mono-[3-[8-[2-(3-methoxyphenyl)vinyl]-7-methyl-2,6-dioxo-1-prop-2-ynyl-1,2,6,7-tetrahydropurin-3-yl]propyl] ester disodium salt (MSX-3, pro-drug of MSX-2; $A_{2A}R$ antagonist) was synthesized as previously described (Hockemeyer *et al.*, 2004). Purity above 95% was confirmed by high-performance liquid chromatography coupled to UV and electrospray ionization mass spectroscopic analysis. MSX-3 (free acid) was re-suspended in 0.9% saline and the pH of the MSX-3 solution was adjusted by adding 1.0 M NaOH until the drug was completely dissolved following conversion to its disodium salt (pH 7.1–7.4). The $A_{2A}R$ antagonist 8-ethoxy-9-ethyladenine (ANR 94) was synthesized as previously described (Volpini *et al.* 2009). ANR 94 was dissolved in DMSO/PEG 400/water (ratio: 50/350/600).

Animals

Male Sprague–Dawley rats (Charles River, Calco, Milan, Italy) weighing 275–300 g were maintained under standard conditions of temperature and humidity with free access to food and water. Behavioral tests were performed during the light cycle between 10:00 and 16:00 h. All experiments were performed in accordance with the European Communities Council Directives (2010/63/EEC; D.L., 27.01.1992, number 116) and the guidelines for animal experimentation approved by the Animal Care Committees of the University of Pavia and of the University of Cagliari.

Neuroprotection studies

Rats ($n = 49$) received a single stereotaxic 6-OHDA injection (20 μg/3 μL) into the right striatum as described before (Ambrosi *et al.* 2010), after which they were immediately randomly assigned to six treatment subgroups:

(i) Vehicle (DMSO/PEG)
(ii) MSX-3 (1 mg/kg/day)
(iii) ANR 94 (2 mg/kg/day)
(iv) MPEP (1.5 mg/kg/day)
(v) MPEP (1.5 mg/kg/day) + MSX-3 (1 mg/kg/day)
(vi) MPEP (1.5 mg/kg/day) + ANR 94 (2 mg/kg/day).

All active compounds were delivered by means of osmotic minipumps programmed to deliver 2.5 μL/h/day for 28 days

(Alzet osmotic pump, model 2ML4; Alzet, Palo Alto, CA, USA) that were implanted subcutaneously on the back of each animal immediately after the stereotaxic surgery (Cerri *et al.* 2014). One or two pumps were implanted depending on the treatment subgroup (single or combined, respectively). Drug infusion started within 10 min from intrastriatal (IS) injection of 6-OHDA and lasted 28 days. Three animals developed subcutaneous infection on the site where the osmotic pumps had been implanted and were killed 1 week after the beginning of the experiments. The doses of MSX-3, MPEP and ANR 94 were chosen according to previously published data (Armentero *et al.* 2006; Pinna *et al.* 2010; Pinna 2014).

Twenty-eight days after surgery, animals were killed by decapitation. Brains were rapidly removed and divided in two parts, respectively, containing the striatum and the ventral midbrain and were treated and stored as described elsewhere (Ambrosi *et al.* 2010). Striatal homogenates were obtained and quantified for protein content as described (Ambrosi *et al.* 2010). Survival of tyrosine hydroxylase (TH)-positive striatal terminals was performed by western blot analysis using specific primary and secondary antibodies and data were quantified using an Odyssey® Infrared Imaging System (Li-Cor BioSciences, Lincoln, NE, USA) after normalization on actin levels as described before (Ambrosi *et al.* 2010). Survival of dopaminergic TH-positive neurons in the substantia nigra pars compacta (SNc) was evaluated using unbiased optical fractionator method (Stereo investigator; Microbrightfield Inc., Williston, VT, USA).

Results represent the percentage of TH-positive terminals or neurons in lesioned hemispheres compared to the intact, non-lesioned hemispheres.

Behavioral studies

Rats (n = 80) were pre-treated with desipramine (10 mg/kg i.p.) and received a 6-OHDA injection (8 μg/4 μL) in the left medial forebrain bundle (MFB) as described before (Pinna *et al.* 2014).

Two weeks after unilateral 6-OHDA-infusion, rats were acutely primed with L-DOPA (50 mg/kg i.p.) plus benserazide (30 mg/kg i.p.); only rats displaying at least 300 contralateral turns during the 2-h testing period were included in the study. Three days after priming rats were randomly assigned to different groups and treated acutely (one time) with the following drugs:

(i) L-DOPA (3 mg/kg i.p) + benserazide (3 mg/kg i.p.)
(ii) MPEP (3 mg/kg i.p.)
(iii) MPEP (3 mg/kg) + L-DOPA (3 mg/kg) + benserazide (3 mg/kg)
(iv) ANR 94 (2 or 3 mg/kg i.p)
(v) ANR 94 (2 or 3 mg/kg) + L-DOPA (3 mg/kg) + benserazide (3 mg/kg)
(vi) MPEP (3 mg/kg) + ANR 94 (2 or 3 mg/kg)
(vii) MPEP (3 mg/kg) + ANR 94 (2 or 3 mg/kg) + L-DOPA (3 mg/kg) + benserazide (3 mg/kg)
(viii) MSX-3 (2 mg/kg i.p)
(ix) MSX-3 (2 mg/kg) + L-DOPA (3 mg/kg) + benserazide (3 mg/kg)
(x) MPEP (3 mg/kg) + MSX-3 (2 mg/kg)
(xi) MPEP (3 mg/kg) + MSX-3 (2 mg/kg) + L-DOPA (3 mg/kg) + benserazide (3 mg/kg).

When a drug was not included in a treatment regimen, its vehicle was injected to rats. The subthreshold dose of L-DOPA (3 mg/kg), only induced very low intensity, non-significant of contralateral rotations and dyskinetic movements that did not affect the behavioral evaluation (Morelli and Pinna 2001; Putterman *et al.* 2007). Sub-threshold doses of the A_{2A}R and mGluR5 antagonists, that had themselves no effect on turning behavior, were chosen based on a pilot study performed in our laboratory and according to previous studies (Domenici *et al.* 2005; Pinna *et al.* 2010, 2014).

In all experiments, hemiparkinsonian rats administered with A_{2A}R antagonists plus L-DOPA or with the combination of mGluR5 and A_{2A}R antagonists, showed a very low intensity of dyskinetic movements that had no effect on the rotational behavior. Moreover, the combined administration of antagonists was carried out acutely in no-dyskinetic rats.

Benserazide was always injected 30 min before L-DOPA. The antagonists MPEP, MSX-3, and ANR 94 were administered, respectively, 20, 15, and 10 min, before L-DOPA (Pinna *et al.* 2010, 2014).

The number of contralateral turns was measured for 2 h starting immediately after L-DOPA injection as described before (Pinna *et al.* 2014). To exclude any bias that might result from improper 6-OHDA lesion, the correct nigrostriatal lesion was checked by immunohistochemical staining for the TH in the SNc. Briefly, the mesencephalic part of the brain of all unilateral 6-OHDA-lesioned rats was post-fixed in 4% paraformaldehyde for 3 weeks. Coronal sections containing the SNc were stained for the enzyme TH (T1299; Sigma, St Louis, MO, USA) using a standard protocol and dopaminergic neurons were counted (Pinna *et al.* 2014). All rats used for behavioral studies showed an almost complete lesion with less than 15% neurons surviving in the lesioned side.

Statistics

All values are expressed as mean ± SEM. In all experiments and for all parameters, differences between groups were evaluated by one-way ANOVA followed by Newman–Keuls *post hoc* test or by ANOVA followed by Tukey's *post hoc* test, using a dedicated software (Prism 3 software; GraphPad Software, San Diego, CA, USA). Significance was set a $p < 0.05$.

Results

Co-administration of adenosine A_{2A}R and glutamatergic mGluR5 antagonists reduces the toxin-induced neuronal cell loss

Four weeks after the IS injection of 6-OHDA, only 51.6% survival of striatal TH-positive dopaminergic terminals was observed in the injected hemisphere compared to the intact hemisphere in vehicle-treated animals (Fig. 1a). The treatment with the mGluR5 antagonist (MPEP) or with either A_{2A}R antagonists (MSX-3 or ANR 94), given alone or in A_{2A}R/mGluR5 combination did not modify the survival of dopaminergic terminals in the striatum.

A marked loss of dopaminergic neurons was observed in the SNc 4 weeks after 6-OHDA injection, with only 45.9% ± 4.2 of TH-positive cells surviving the toxic insult

in vehicle-treated animals (Fig. 1a and b). Mono-treatment with ANR 94 did not reduce toxin-induced cell loss, whereas a significant increase in the number of TH-positive neurons in the SNc was observed after single treatment with MPEP (66.9% ± 11.2; $p < 0.05$) or MSX-3 (65.8% ± 6.0; $p < 0.01$). Combination of MPEP with ANR 94 increased cell survival but the level of neuroprotection was similar to that observed with MPEP alone (66.5% ± 6.5). Combination of MSX-3 with MPEP significantly improved neuronal survival in SNc (75.6% ± 8.0; $p < 0.001$), compared with the single administration of either drug.

Co-administration of adenosine A_{2A}R and glutamatergic mGluR5 antagonists potentiates L-DOPA-induced turning behavior

The effects of the combined administration of the A_{2A}R antagonist ANR 94 (2 or 3 mg/kg i.p.) and the mGluR5 antagonist MPEP (3 mg/kg i.p.) on contralateral turning behavior induced by a low dose of L-DOPA (3 mg/kg i.p.) are shown in Fig. 1. Both doses of ANR 94 (2 or 3 mg/kg i.p.), in absence of L-DOPA, and MPEP (3 mg/kg i.p.), singularly and in combination with L-DOPA (3 mg/kg), induced a slight contralateral turning behavior, indicative of motor stimulation but not anti-parkinsonian effect (Fig. 1c and d). In contrast, ANR 94 (2 or 3 mg/kg) in combination with L-DOPA (3 mg/kg) induced a significant and dose-dependent potentiation in contralateral turns with respect to L-DOPA alone (*$p < 0.05$; Fig. 1c and d). Combined administration of ANR 94 (2 or 3 mg/kg) and MPEP (3 mg/kg), in absence of L-DOPA, induced a moderate, non-significant, contralateral turning indicative of motor stimulating effects. Conversely, combination of the low dose of ANR 94 (2 mg/kg) and MPEP (3 mg/kg) plus L-DOPA (3 mg/kg) induced a significant potentiation of contralateral turns with respect to that induced by L-DOPA alone (**$p < 0.005$). Remarkably, this potentiation was significantly higher compared to that induced when the same dose of ANR 94 was combined with L-DOPA in absence of MPEP ($^{\#}p < 0.05$; Fig. 1c). On the other hand, combined administration of the high dose of ANR 94 (3 mg/kg) and MPEP (3 mg/kg) plus L-DOPA (3 mg/kg) induced a significant potentiation of contralateral turns induced by L-DOPA alone (**$p < 0.005$, Fig. 1d), that was, however, not different from that observed when the same dose of ANR 94 was combined to L-DOPA in absence of MPEP (Fig. 1d).

To corroborate these results, we replicated the experiments with the A_{2A}R antagonist MSX-3 (Fig. 1e). Administration of MSX-3 (2 mg/kg i.p.) or MPEP (3 mg/kg), alone or in combination, induced a slight contralateral turning behavior, indicative of motor stimulation but not anti-parkinsonian effects (Fig. 1e). Similar to experiments with ANR 94, when combined with L-DOPA (3 mg/kg) MSX-3 induced a significant potentiation of contralateral turns with respect to L-DOPA alone (*$p < 0.05$; Fig. 1e). Moreover, combination of MSX-3 (2 mg/kg) and MPEP (3 mg/kg) significantly potentiated contralateral turns induced by L-DOPA (**$p < 0.005$; Fig. 1e). Notably, this effect was significantly higher than that observed in rats treated with MSX-3 plus L-DOPA in absence of MPEP (^$p < 0.05$; Fig. 1e).

In all experiments, hemiparkinsonian rats administered with A_{2A}R antagonists plus L-DOPA or with the combination of mGluR5 and A_{2A}R antagonists, showed a very low intensity of dyskinetic movements; the rotational behavior was not hampered by any abnormal involuntary movements (AIMs). Moreover, the combined administration of antagonists was done acutely in no-dyskinetic rats. All animals used in the behavioral study had a complete lesion at the level of the SNc (Fig. 1f).

Discussion

Non-dopaminergic targets have become fundamental in the development of alternative therapeutic strategies for PD. To date, only limited information is available on the efficacy and advantages of combinatorial non-dopaminergic PD treatment. To the best of our knowledge, our study is the first to assess and compare both the neuroprotective potential and the beneficial effect on PD-like motor symptoms of mGluR5 and A_{2A}R antagonists when given as monotherapy and combined. We used two variants of the well-characterized hemiparkinsonian rat model induced by the IS or intra MFB injection of 6-OHDA (Blandini & Armentero 2012). IS infusion of the neurotoxin causes slow and progressive retrograde loss of dopaminergic neurons in the SNc, and is commonly used to evaluate neuroprotective/neuroregenerative treatments. Conversely, MFB injection of 6-OHDA induces rapid and almost complete nigrostriatal neurodegeneration. In this variant, the turning behavior contralateral to the lesioned hemisphere of the brain is typically induced by acute treatments that improve motor deficits or increase the efficacy of L-DOPA (Pinna and Morelli 2014). Either 6-OHDA models mimic pathological and behavioral features observed in PD patients. They provide good model systems to perform rapid screening of pharmacological treatments that may reduce neurodegenerative processes or improve parkinsonian symptoms and offer important clues for potential candidate drugs for therapeutics trials in humans.

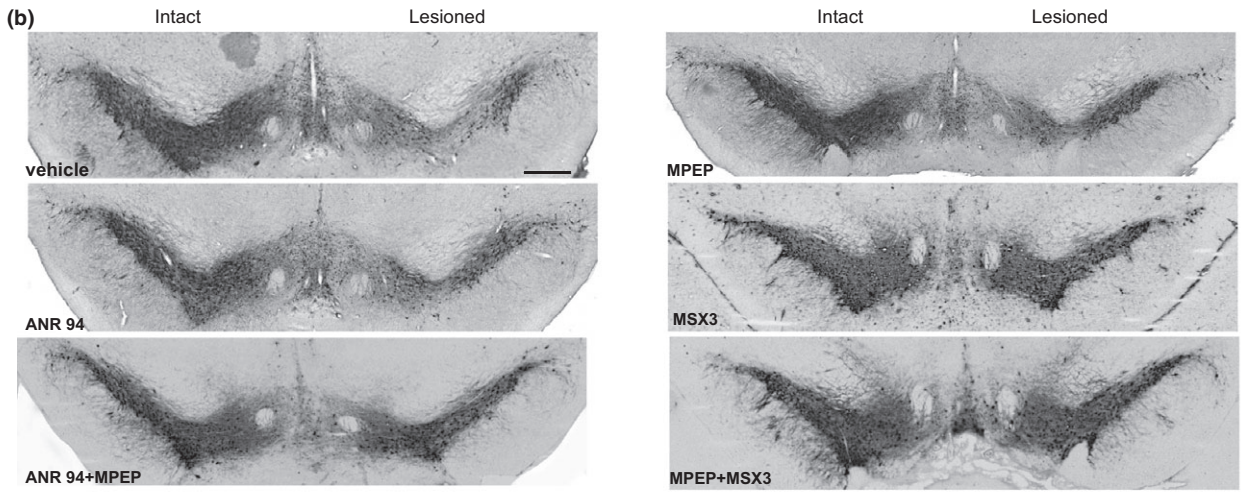
PD is associated with life-long chronic therapies that lose efficacy over time and commonly cause debilitating unwanted side effects. The main objective of this study was to use and combine low concentration ranges of the various antagonists that would conceptually induce little/no side effects, and that when combined would be effective in reducing the neuronal cell loss and would demonstrate improved antiparkinsonian activity.

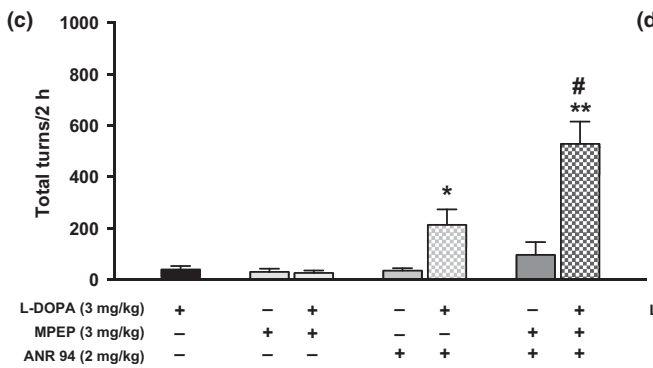
In our behavioral study, we used antagonists and L-DOPA sub-threshold concentrations, which *per se* prompted only a mild turning behavior. Similarly, combining mGluR5 and

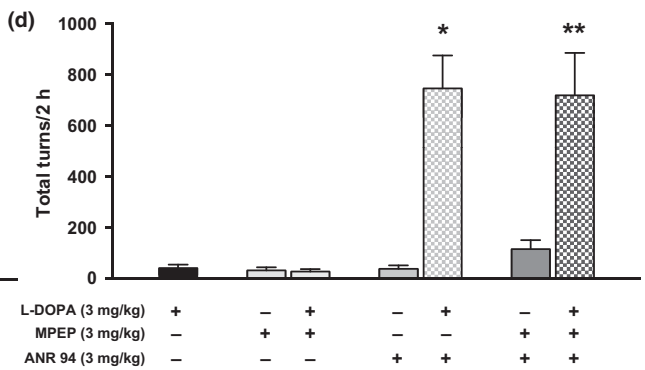
(a)

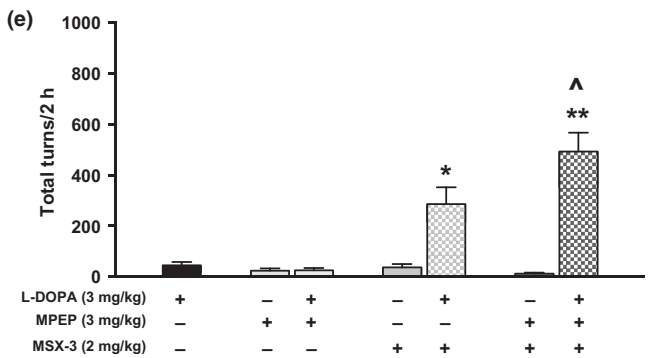
Effect of treatment with mGluR5 and A2AR antagonists on 6-OHDA-induced nigrostriatal lesion

	vehicle	MPEP	ANR 94	MSX-3	MPEP+ANR 94	MPEP + MSX-3
DA terminals (% R/L striatum)[a]	51.6 ± 3.7	45.8 ± 11.1	44.9 ± 8.6	58.1 ± 4.9	55.6 ± 4.0	55.0 ± 4.0
DA Neurons (% R/L SNc)[b]	45.9 ± 4.2	66.9 ± 11.2[c]	55.4 ± 8.4	65.8 ± 6.0[d]	66.5 ± 6.5	75.6 ± 8.0[d]
Intact hemisphere	10172 ± 1061	10334 ± 1164	10131 ± 1555	10671 ± 999	10681 ± 990	10276 ± 1542
Lesioned hemisphere	4511 ± 1041	6316 ± 2658	5963 ± 2195	7045 ± 1120	65588 ± 1677	7707 ± 1431









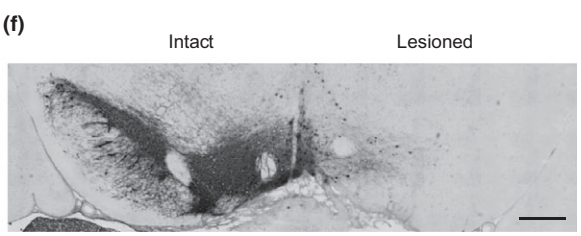


Fig. 1 Neuroprotective and behavioral impact of combined treatment. (a) Partial nigrostriatal neurodegeneration of dopaminergic terminals and cell bodies. Results (mean ± SEM) indicate the survival of striatal tyrosine hydroxylase (TH)-positive terminal (%R/L striatum); the number of dopaminergic neurons in the SNc is indicated as the percentage of surviving neurons in the lesioned hemisphere (%R/L SNc) or as the absolute number of neurons in the intact and lesioned hemisphere. [a]one-way ANOVA $F(5,43) = 0.943$, $p = 0.67$; [b]one-way ANOVA $F(5,43) = 2.857$, $p = 0.025$; [c]$p < 0.05$, [d]$p < 0.001$ (b) Representative photomicrographs of brain coronal sections showing TH-positive cells in the SNc of both the intact and the lesioned hemispheres of animals bearing an intrastriatal (IS) 6-hydroxydopamine (6-OHDA)-induced lesion of the nigrostriatal pathway and that received a 4-week treatment with vehicle, 8-ethoxy-9-ethyladenine (ANR) 94, 2-methyl-6-(phenylethynyl)pyridine (MPEP) or MPEP+MSX-3, as indicated in each panel (scale bar = 500 μm). (c–e) Effect of the administration of the $A_{2A}R$ antagonists, ANR 94 (2 or 3 mg/kg i.p.) (b and c) or MSX-3 (2 mg/kg i.p.) (d), and the metabotropic glutamate receptor (mGluR)5 receptor antagonist MPEP, (3 mg/kg i.p.) on turning behavior induced by a sub-threshold dose of L-DOPA (3 mg/kg i.p.). ANR 94, MSX-3, and MPEP were administered alone or in combination. Ordinates indicate the total contralateral numbers of turns measured in 2 h. Results are reported as mean ± SEM of total turns. Statistical significance was determined by one-way ANOVA followed by Newman–Keuls *post hoc* test. *$p < 0.05$, **$p < 0.005$, versus L-DOPA alone. #$p < 0.05$ versus ANR 94 + L-DOPA; ^$p < 0.05$ versus MSX-3 + L-DOPA. (f) Representative photomicrograph of a brain coronal section showing complete loss TH-positive cells in the SNc following injection of 6-OHDA in the medial forebrain bundle (MFB) (scale bar = 500 μm).

$A_{2A}R$ antagonists in the absence of L-DOPA had little behavioral effect. Conversely, single administration of the $A_{2A}R$ antagonists, MSX-3 or ANR 94 alone, with a sub-threshold dose of L-DOPA induced a significant turning behavior, confirming the known anti-parkinsonian effect of these drugs. Remarkably, the combination of mGluR5 and $A_{2A}R$ antagonists with a low L-DOPA dose resulted in a synergistic potentiation of the anti-parkinsonian effect of L-DOPA. This finding was observed only after the administration of the lower doses of the $A_{2A}R$ antagonists. Indeed, the higher dose of ANR 94 induced a strong behavioral effect that was not further enhanced by addition of MPEP.

The analysis of the 6-OHDA-induced cell loss in the SNc in the presence of low concentration of the same antagonists also highlights that a stronger neuroprotection can be achieved by targeting both mGluR5 and $A_{2A}R$ at the same time, rather than either receptor alone. Globally, the results obtained on the neuroprotective and behavioral effects by combining these pharmacological treatments have a high translational valence. While the therapeutic efficacy of the individual manipulation of these receptors singularly is well documented, our data strongly indicate that the combination sub-threshold doses of mGluR5 and $A_{2A}R$ antagonists, which elicit little beneficial effects on their own, provides significant benefits. Conceptually, the combined use of low drug concentration that reduces neuronal loss and beneficially affects motor symptoms may in the long term also translate into a considerable reduction of unwanted side effects linked to their chronic administration. The effect of the combination of $A_{2A}R$ and mGluR5 blockade on the emergence L-DOPA-induced dyskinesia is under investigation in our laboratory.

Several mechanisms may be involved in the neuroprotective or anti-parkinsonian effects of the combined treatment observed in this study. Glutamatergic transmission is increased in the basal ganglia during PD, and it is likely involved in motor symptoms, L-DOPA -induced dyskinesia, and excitotoxic mechanisms that sustain neurodegenerative processes. Because of their specific localization in the brain, both mGluR5 and $A_{2A}R$ can modulate the excessive glutamatergic transmission caused by the loss of dopaminergic innervation. Both receptors are also found on glial cells, astrocytes, and microglia, where they may modulate the inflammatory response, which parallels and likely sustains neurodegeneration. Our neuroprotective study indicates that MSX-3 singularly is more neuroprotective than ANR 94 when either drug is given alone, and that MSX-3 seems to preferentially benefit from the combination with MPEP. Several explanations may justify these differences: (i) both compounds readily cross the blood brain barrier, but injection of the pro-drug MSX-3 may result in higher brain concentration of MSX-2 compared to that reached by ANR 94, (ii) the two compounds have different inhibitory potency (Ki: 8.0 and 46.0 nm for MSX-2 and ANR 94, respectively) possibly leading to different physiological response when combined with the mGluR5 antagonist, and (iii) differences may arise from the distinct pre- or post-synaptic pharmacological profiles that exist among various $A_{2A}R$ antagonists (Orru *et al.* 2011) and that may lead to different influences at the physiological level.

Dopamine receptors, $A_{2A}R$ and mGluR5 all belong to the G protein-coupled receptor super family. It has been demonstrated that $A_{2A}R$, mGluR5 and D_2R co-localize on dendritic spines. Importantly, evidence indicates that such receptors may form larger heteromeric complexes that have different functional and biochemical properties compared to the single receptor structures (Fuxe *et al.* 2003). In addition, the existence of heteromeric receptors complexes containing $A_{2A}R$-mGluR5-D_2R, in which $A_{2A}R$ and mGluR5 may modulate D_2R activity, has been demonstrated both *in vitro* and *in vivo* (Ferre *et al.* 2002). The localization of these heteromers in specific PD-affected brain areas, such as the basal ganglia, has strengthened the idea that combined treatments capable of targeting these complex structures may produce higher therapeutic efficacy at doses lower than those of drugs that individually target the receptors involved in

these heteromers. On this basis, the combined activation/inhibition of receptors within putative multimeric complexes may be involved in the synergistic behavioral interaction between $A_{2A}R$ and mGluR5 antagonists in hemiparkinsonian rats that received these drugs in combination with L-DOPA.

Our data are of particular importance because the receptors assessed in this study have already been considered in various clinical studies using different compounds approved for clinical testing in humans, and combined treatment might be immediately translated into the clinic. Although several $A_{2A}R$ antagonists have been tested in clinical trials, none of them has yet received approval from the FDA for use in PD patients, probably because of lack of superiority of these drugs over approved treatments. Istradefylline, however, one of the first $A_{2A}R$ antagonists tested in clinical trials, has recently been approved as adjunctive therapy for use in PD patients in Japan (Dungo and Deeks 2013). Importantly, two newly developed mGluR5 modulators – AFQ056 and AX48621 – have recently demonstrated significant anti-dyskinetic activity in advanced PD patients with moderate-to-severe L-DOPA-induced dyskinesia (Bezard *et al.* 2014; Petrov *et al.* 2014; Rascol *et al.* 2014).

Collectively, our findings provides an *in vivo* proof-of-concept that co-treatment with known $A_{2A}R$ and mGluR5 antagonists results in a therapeutic benefit and provides better results than those produced by either drug given alone, both in terms of motor performance and neuroprotection. Considering that the needs of PD patients change together with disease progression, it is essential to identify and optimize novel therapeutic strategies according to the disease stage and make them rapidly available in the clinical settings. Therefore, more pre-clinical studies should be carried out to investigate further the use of mGluR5 and $A_{2A}R$ antagonists in combination treatment regimens to identify the window of opportunity and the optimal doses at which these drugs can effectively improve PD symptoms and at the same time reduce nigrostriatal cell loss.

Acknowledgments and conflict of interest disclosure

This study was funded by in part within the frame of the Era-NET Neuron program by grant RC2008MinSal/Era-NET from the Italian Ministry of Health (MTA, AP) and by a grant from the Italian Ministry of Health (Ricerca Corrente RC2013). YB and CEM are grateful for support by the BMBF, Germany (01EW0911) in the frame of ERA-NET NEURON. Dr Nicola Simola gratefully acknowledges the Sardinian Regional Government for financial support (P.O.R. Sardegna F.S.E. Operational Programme of the Autonomous Region of Sardinia, European Social Fund 2007–2013–Axis IV Human Resources, Objective l.3, Line of Activity l.3.1 'Avviso di chiamata per il finanziamento di Assegni di Ricerca'). The authors have no conflicts of interest to declare.

All experiments were conducted in compliance with the ARRIVE guidelines.

References

Ambrosi G., Armentero M. T., Levandis G., Bramanti P., Nappi G. and Blandini F. (2010) Effects of early and delayed treatment with an mGluR5 antagonist on motor impairment, nigrostriatal damage and neuroinflammation in a rodent model of Parkinson's disease. *Brain Res. Bull.* **82**, 29–38.

Armentero M. T., Fancellu R., Nappi G., Bramanti P. and Blandini F. (2006) Prolonged blockade of NMDA or mGluR5 glutamate receptors reduces nigrostriatal degeneration while inducing selective metabolic changes in the basal ganglia circuitry in a rodent model of Parkinson's disease. *Neurobiol. Dis.* **22**, 1–9.

Armentero M. T., Pinna A., Ferre S., Lanciego J. L., Muller C. E. and Franco R. (2011) Past, present and future of A(2A) adenosine receptor antagonists in the therapy of Parkinson's disease. *Pharmacol. Ther.* **132**, 280–299.

Bezard E., Pioli E. Y., Li Q., Girard F., Mutel V., Keywood C., Tison F., Rascol O. and Poli S. M. (2014) The mGluR5 negative allosteric modulator dipraglurant reduces dyskinesia in the MPTP macaque model. *Mov. Disord.* **29**, 1074–1079.

Blandini F. and Armentero M. T. (2012) Animal models of Parkinson's disease. *FEBS J.* **279**, 1156–1166.

Cerri S., Levandis G., Ambrosi G. *et al.* (2014) Neuroprotective potential of adenosine A2A and cannabinoid CB1 receptor antagonists in an animal model of Parkinson disease. *J. Neuropathol. Exp. Neurol.* **73**, 414–424.

Domenici M. R., Potenza R. L., Martire A., Coccurello R., Pezzola A., Reggio R., Tebano M. T. and Popoli P. (2005) Chronic treatment with the mGlu5R antagonist MPEP reduces the functional effects of the mGlu5R agonist CHPG in the striatum of 6-hydroxydopamine-lesioned rats: possible relevance to the effects of mGlu5R blockade in Parkinson's disease. *J. Neurosci. Res.* **80**, 646–654.

Dungo R. and Deeks E. D. (2013) Istradefylline: first global approval. *Drugs* **73**, 875–882.

Ferre S., Karcz-Kubicha M., Hope B. T. *et al.* (2002) Synergistic interaction between adenosine A2A and glutamate mGlu5 receptors: implications for striatal neuronal function. *Proc. Natl Acad. Sci. USA* **99**, 11940–11945.

Fuxe K., Agnati L. F., Jacobsen K. *et al.* (2003) Receptor heteromerization in adenosine A2A receptor signaling: relevance for striatal function and Parkinson's disease. *Neurology* **61**, S19–S23.

Hauser R. A., Olanow C. W., Kieburtz K. D. *et al.* (2014) Tozadenant (SYN115) in patients with Parkinson's disease who have motor fluctuations on levodopa: a phase 2b, double-blind, randomised trial. *Lancet Neurol.* **13**, 767–776.

Hockemeyer J., Burbiel J. C. and Muller C. E. (2004) Multigram-scale syntheses, stability, and photoreactions of A_{2A} adenosine receptor antagonists with 8-styrylxanthine structure: potential drugs for Parkinson's disease. *J. Org. Chem.* **69**, 3308–3318.

Jenner P. (2014) An overview of adenosine A2A receptor antagonists in Parkinson's disease. *Int. Rev. Neurobiol.* **119**, 71–86.

Levandis G., Bazzini E., Armentero M. T., Nappi G. and Blandini F. (2008) Systemic administration of an mGluR5 antagonist, but not unilateral subthalamic lesion, counteracts l-DOPA-induced dyskinesias in a rodent model of Parkinson's disease. *Neurobiol. Dis.* **29**, 161–168.

Morelli M. and Pinna A. (2001) Interaction between dopamine and adenosine A2A receptors as a basis for the treatment of Parkinson's disease. *Neurol. Sci.* **22**, 71–72.

Morin N., Morissette M., Gregoire L., Gomez-Mancilla B., Gasparini F. and Di Paolo T. (2013) Chronic treatment with MPEP, an mGlu5 receptor antagonist, normalizes basal ganglia glutamate

neurotransmission in L-DOPA-treated parkinsonian monkeys. *Neuropharmacology* **73**, 216–231.

Orru M., Quiroz C., Guitart X. and Ferre S. (2011) Pharmacological evidence for different populations of postsynaptic adenosine A2A receptors in the rat striatum. *Neuropharmacology* **61**, 967–974.

Petrov D., Pedros I., de Lemos M. L., Pallas M., Canudas A. M., Lazarowski A., Beas-Zarate C., Auladell C., Folch J. and Camins A. (2014) Mavoglurant as a treatment for Parkinson's disease. *Expert Opin. Investig. Drugs* **23**, 1165–1179.

Pinna A. (2014) Adenosine A2A receptor antagonists in Parkinson's disease: progress in clinical trials from the newly approved istradefylline to drugs in early development and those already discontinued. *CNS Drugs* **28**, 455–474.

Pinna A. and Morelli M. (2014) A critical evaluation of behavioral rodent models of motor impairment used for screening of antiparkinsonian activity: the case of adenosine A(2A) receptor antagonists. *Neurotox. Res.* **25**, 392–401.

Pinna A., Tronci E., Schintu N., Simola N., Volpini R., Pontis S., Cristalli G. and Morelli M. (2010) A new ethyladenine antagonist of adenosine A(2A) receptors: behavioral and biochemical characterization as an antiparkinsonian drug. *Neuropharmacology* **58**, 613–623.

Pinna A., Bonaventura J., Farre D. *et al.* (2014) L-DOPA disrupts adenosine A(2A)-cannabinoid CB(1)-dopamine D(2) receptor heteromer cross-talk in the striatum of hemiparkinsonian rats: biochemical and behavioral studies. *Exp. Neurol.* **253**, 180–191.

Putterman D. B., Munhall A. C., Kozell L. B., Belknap J. K. and Johnson S. W. (2007) Evaluation of levodopa dose and magnitude of dopamine depletion as risk factors for levodopa-induced dyskinesia in a rat model of Parkinson's disease. *J. Pharmacol. Exp. Ther.* **323**, 277–284.

Rascol O., Fox S., Gasparini F., Kenney C., Di Paolo T. and Gomez-Mancilla B. (2014) Use of metabotropic glutamate 5-receptor antagonists for treatment of levodopa-induced dyskinesias. *Parkinsonism Relat. Disord.* **20**, 947–956.

Volpini R., Dal Ben D., Lambertucci C., Marucci G., Mishra R. C., Ramadori A. T., Klotz K. N., Trincavelli M. L., Martini C. and Cristalli G. (2009) Adenosine A2A receptor antagonists: new 8-substituted 9-ethyladenines as tools for in vivo rat models of Parkinson's disease. *ChemMedChem* **4**, 1010–1019.