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ORIGINAL ARTICLE

Functional role of striatal A2A, D2, and mGlu5 receptor interactions in regulating striatopallidal GABA neuronal transmission

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Abstract

In this study, the functional role of individual striatal receptors for adenosine (A2AR), dopamine (D2R), and the metabotropic glutamate receptor mGlu5R in regulating rat basal ganglia activity was characterized *in vivo* using dual-probe microdialysis in freely moving rats. In particular, intrastriatal perfusion with the D2R agonist quinpirole (10 μM, 60 min) decreased ipsilateral pallidal GABA and glutamate levels, whereas intrastriatal CGS21680 (A2AR agonist; 1 μM, 60 min) was ineffective on either pallidal GABA and glutamate levels or the quinpirole-induced effects. Intrastriatal perfusion with the mGlu5R agonist (RS)-2-chloro-5-hydroxyphenylglycine (600 μM, 60 min), by itself ineffective on pallidal GABA and glutamate levels, partially counteracted the effects of quinpirole. When combined with CGS21680 (1 μM, 60 min), (RS)-2-chloro-5-hydroxyphenylglycine (CHPG; 600 μM, 60 min) fully counteracted the quinpirole (10 μM, 60 min)-induced reduction in ipsilateral pallidal GABA and glutamate levels. These effects were fully counteracted by local perfusion with the mGlu5R antagonist MPEP (300 μM) or the A2AR antagonist ZM 241385 (100 nM). These results suggest that A2ARs and mGlu5Rs interact synergistically in modulating the D2R-mediated control of striatopallidal GABA neurons.

Keywords: CGS21680, CHPG, extracellular GABA and glutamate levels, Parkinson's disease, quinpirole, receptor heteromers.

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It has been suggested that striatal adenosine regulates the striatopallidal GABA neuron excitability possibly through its capability to counteract dopamine D2 receptor (D2R) signaling to multiple effectors. Therefore, adenosine A2A receptor (A2AR)-induced reduction in D2R signaling leads to increased activity of striatopallidal GABA neurons and, thus, to motor inhibition. Although the interactions between the two receptors could occur at several levels, it has been reported that striatal adenosine might exert its effects also by acting on an A2AR-D2R heteromer. In fact, G-protein-coupled receptors can function not only as monomers but also as oligomers transducing different integrated intracellular signals (Agnati *et al.* 2003; Fuxe *et al.* 2010, 2014; Borroto-Escuela *et al.* 2011). Heterodimers composed by A2AR and D2R are present on GABAergic striatopallidal neurons, and activation of A2AR leads to a reduced D2R recognition, G-protein coupling, and signaling and motor inhibition (Fuxe *et al.* 2003; Tanganelli *et al.* 2004; Schiffmann *et al.* 2007). In line with this view, results obtained in animal models of Parkinson's disease (PD) and some clinical data provide considerable evidence supporting the anti-parkinsonian activity of A2AR antagonists (Schwarzschild

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Abbreviations used: A2AR, adenosine A2 receptor; D2R, dopamine D2 receptor; mGlu5R, metabotropic glutamate receptor 5.

et al. 2006; Simola *et al.* 2008; Armentero *et al.* 2011; Ferraro *et al.* 2012a).

In rat striatopallidal GABA neurons, the metabotropic glutamate receptor 5 subtype receptor (mGlu5R) showed a similar localization of A2AR and D2R (Fuxe *et al.* 2003, 2007; Cabello *et al.* 2009; Morin and Di Paolo 2014). Particularly, mGlu5R stimulation decreases D2R agonist affinity and the co-stimulation of both A2AR and mGlu5R produces a modulation of D2R agonist binding that is stronger than the reduction induced by stimulation of each receptor alone (Popoli *et al.* 2001). *In vivo* microdialysis results demonstrated a synergistic interaction between A2AR and mGlu5R on ventral striopallidal GABAergic neurons (Díaz-Cabiale *et al.* 2002). However, there is a lack of data on the possible role of A2AR, D2R and mGlu5R interactions in regulating striatal neurotransmission. In this study, the *in vivo* characterization of the functional role of individual A2AR, D2R and mGlu5R in regulating rat basal ganglia activity has been performed using dual-probe microdialysis in freely moving rats.

Methods

Animals

Adult male Sprague–Dawley rats (225–250 g, Harlan Italy S.r.l., Udine, Italy) were used. The animals, housed in a temperature- and relative humidity-controlled environment with a regular 12 h light/dark cycle, had free access to food and water.

Experiments were carried out in accordance with the European Communities Council Directive (86/609/EEC) and the Guidelines released by the Italian Ministry of Health (D.L. 116/92 and D.L. 111/94-B). An approval to conduct the experiments was obtained by the local Ethics Committee. Efforts were made to minimize the number of animals used and to reduce pain and discomfort.

Chemicals

The A2AR agonist 4-[2-[[6-Amino-9-(*N*-ethyl-β-D-ribofuranuronamidosyl)-9*H*-purin-2-yl]amino]ethyl]-benzenepropanoic acid (CGS21680), the selective A2AR antagonist 4-(2-[7-amino-2-(2-furyl)[1,2,4]triazolo[2,3-a][1,3,5] triazin-5-ylamino]ethyl)phenol (ZM 241385), the mGlu5R agonist (RS)-2-chloro-5-hydroxyphenylglycine (CHPG) and the mGlu5R antagonist 2-methyl-6-(phenylethynyl)-pyridine (MPEP) were purchased from Tocris Cookson (Bristol, UK). For administration by reverse microdialysis, they were dissolved in dimethyl sulfoxide and then included in Ringer solution. The final concentration of dimethyl sulfoxide in the perfusion medium was < 0.001% and this did not influence basal dialysate GABA and glutamate levels. The D2R agonist quinpirole and the D2R antagonist eticlopride were purchased from Sigma-Aldrich (Milan, Italy) and dissolved in Ringer solution. All other chemicals were of the highest commercially available purity.

Surgery

Following induction of anesthesia (1.5% mixture of isoflurane/air) animals were mounted in a David Kopf stereotaxic frame with the upper incisor bar set at −2.5 mm below the interaural line. Two microdialysis probes of concentric design (molecular weight cut-off, 20 kDa; CMA 12; Carnegie Medicine, Stockholm, Sweden) were implanted: one probe was implanted into the right striatum (outer diameter, 0.5 mm; length of dialyzing membrane, 2 mm), and the other (outer diameter, 0.5 mm; length of dialyzing membrane, 1 mm) was implanted into the ipsilateral globus pallidus (GP). The coordinates relative to the bregma were as follows: striatum, anterior (A): +0.3 mm; lateral (L): +3.1 mm; ventral (V): −7.5 mm; GP, A: −1.3 mm; L: +3.3 mm; V: −7.0 mm (Paxinos and Watson 1982). After the implantation, the probes were permanently secured to the skull with methacrylic cement and 36 h later microdialysis was performed in the freely moving rats.

Dual-probe microdialysis experiments

On the day of microdialysis, the probes were perfused with Ringer's solution (in millimolar: Na^+ 147, K^+ 4, Ca^{2+} 1.4, Cl^- 156, and glucose 2.7) at a constant flow rate (2 μL/min) using a microinfusion pump. Dialysate sample collection commenced 300 min after the onset of perfusion to achieve stable dialysis glutamate levels; perfusates were collected every 20 min thereafter (Ferraro *et al.* 2012a,b). Following the collection of three stable basal values, the D2R agonist quinpirole (10 μM), the A2AR agonist CGS21680 (1 μM) and the selective mGlu5R agonist CHPG (600 and 1000 μM) were included for 60 min, alone and in combination, in the Ringer solution perfusing the striatum. This medium was then replaced with the original perfusate until the end of the collection period (total sample collection time = 180 min). The concentrations of quinpirole, CGS21680 and CHPG have been selected on the basis of previous studies suggesting that, in this concentration range, the compounds selectively act on D2R, A2AR, and mGlu5R, respectively (Andersen and Gazzara 1994; Pintor *et al.* 2000; Fazal *et al.* 2003; Coleman *et al.* 2006). When required, the selective A2AR antagonist ZM 241385, the mGlu5R antagonist MPEP and the D2R antagonist eticlopride were added to the perfusion medium 10 min before the agonists.

At the end of each experiment the brain was removed from the skull, and the position of the probes in the striatum and globus pallidus was carefully verified in 30-μm-thick coronal cryostat sections. Only those animals in which both probes were correctly located were included in this study.

The glutamate and GABA levels in each sample were measured by HPLC with fluorimetric detection as previously described (Beggiato *et al.* 2013a,b).

Data evaluation

Data from individual time points are reported as percentages of the mean ± SEM of the three basal samples collected prior to treatment. For clarity, only the statistical significance of the peak effects (maximal responses) is shown in the Figures. In addition, the area created by the curve, reflecting the duration of the effect, was determined for each animal. Area values (overall effects) were calculated as percentages of changes in baseline value over time using the trapezoidal rule (Beggiato *et al.* 2013a,b). Statistical analysis was carried out by the analysis of variance (ANOVA) followed by the Newman–Keuls test for multiple comparisons. Statistical significance was assumed for $p < 0.05$.

Results

Effects of quinpirole, CGS21680, or CHPG on striatal extracellular glutamate levels

Striatal extracellular glutamate levels were 123 ± 9 nM (n = 24). These levels were stable over the time course of the experiments. Intrastriatal perfusion with quinpirole (10 μM) or CGS21680 (1 μM) did not affect rat striatal glutamate levels (Fig. 1). On the contrary, intrastriatal perfusion with CHPG (1000 μM) significantly increased extracellular glutamate levels in rat striatum (main peak effect = 143 ± 7% of basal values; $p < 0.01$ Fig. 2). At a lower concentration (600 μM), the mGlu5R agonist was ineffective. The effect of CHPG (1000 μM) was fully counteracted by the mGlu5R antagonist MPEP (300 μM), added to the intrastriatal perfusion medium 10 min before the agonist (Fig. 2). By itself, intrastriatal perfusion with MPEP (300 μM) did not affect local extracellular glutamate levels (*data not shown*).

Effects of intrastriatal perfusion with quinpirole and CGS21680, alone or in combination, on pallidal GABA and glutamate levels

Basal dialysate GABA and glutamate levels in the GP of control rats were 11.08 ± 1.12 nM and 177 ± 2.16 μM (n = 24), respectively, and remained constant over the duration of the experiment.

Intrastriatal quinpirole (10 μM, 60 min) perfusion was associated with a long-lasting decrease in ipsilateral pallidal GABA and glutamate levels, and these effects were completely counteracted by intrastriatal perfusion with the D2R antagonist eticlopride (10 nM), by itself ineffective (Fig. 3). The intrastriatal CGS21680 (1 μM, 60 min) perfusion did not affect the ipsilateral pallidal GABA and glutamate levels (Fig. 4). Intrastriatal CGS21680 (1 μM, 60 min) perfusion did not significantly affect the quinpirole (10 μM, 60 min)-induced reduction in ipsilateral pallidal GABA and glutamate levels, although a trend to a reduction in quinpirole-induced effects was observed by analyzing the area created by the curve data (Fig. 4).

Effects of intrastriatal perfusion with CHPG, alone or in combination with quinpirole, on pallidal GABA, and glutamate levels

Intrastriatal CHPG (1000 μM, 60 min) perfusion significantly increased ipsilateral pallidal GABA and glutamate levels and these effects were completely counteracted by intrastriatal perfusion with the mGlu5R antagonist MPEP (300 μM; Fig. 5), by itself ineffective on pallidal GABA and glutamate levels (*data not shown*). At a lower concentration (600 μM), CHPG was ineffective.

Intrastriatal CHPG (600 μM, 60 min) perfusion partially decreased the quinpirole (10 μM, 60 min)-induced reduction in ipsilateral pallidal GABA and glutamate levels (Fig. 6).

Effects of intrastriatal perfusion with CHPG in combination with CGS21680 on intrastriatal quinpirole-induced reduction in pallidal GABA and glutamate levels

Intrastriatal CHPG (600 μM, 60 min) in combination with CGS21680 (1 μM, 60 min) perfusion fully counteracted the quinpirole (10 μM, 60 min)-induced reduction in ipsilateral pallidal GABA and glutamate levels (Fig. 7). Interestingly,

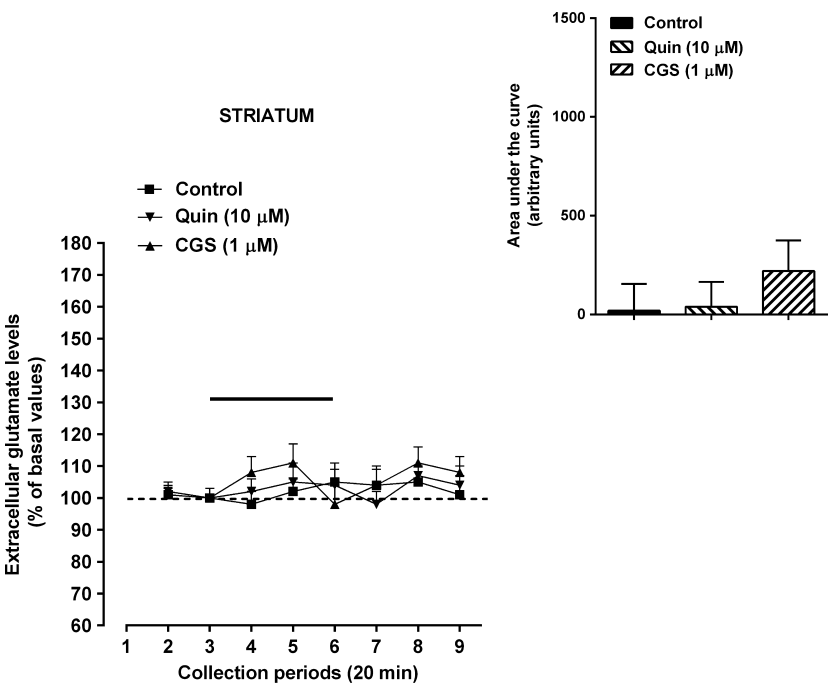

Fig. 1 Effects of intrastriatal perfusion with quinpirole (Quin) or CGS21680 (CGS) on local glutamate levels in the awake rats. The solid bar indicates the period of intrastriatal perfusion with the compounds (60 min). The results are expressed as percentage of the mean of the three basal values before treatment. Each point represents the mean ± SEM of 5–6 animals. Control rats were perfused with normal Ringer perfusion medium throughout the experiment. The areas under the curves, which mainly represent the integrated time-response curve of the overall effects, calculated as % changes in basal value over time (Δ basal % × time) using the trapezoidal rule, are reported on the right side of the figure.

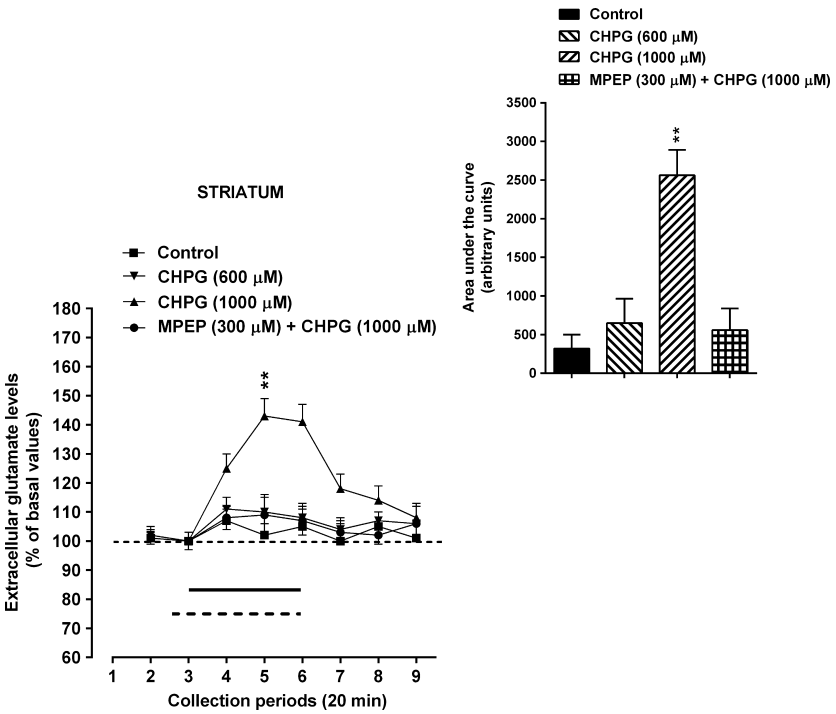

Fig. 2 Effects of intrastriatal perfusion with CHPG, alone or in combination with 2-methyl-6-(phenylethynyl)-pyridine (MPEP), on local glutamate levels in the awake rats. The solid and the dotted bars indicate the period of intrastriatal perfusion with the mGlu5R agonist (60 min) and antagonist (70 min), respectively. The results are expressed as percentage of the mean of the three basal values before treatment. Each point represents the mean ± SEM of 5–6 animals. Control rats were perfused with normal Ringer perfusion medium throughout the experiment. The areas under the curves, which mainly represent the integrated time-response curve of the overall effects, calculated as % changes in basal value over time (Δ basal % × time) using the trapezoidal rule, are reported on the right side of the figure. $^{**}p < 0.01$ significantly different from all the other groups (ANOVA followed by the Student–Newman–Keuls test for multiple comparisons).

these effects were fully counteracted by the local perfusion with either MPEP (300 μM) or the A2AR antagonist ZM 241385 (100 nM).

Discussion

A2ARs are co-localized with D2Rs on the dendritic spines of the striatopallidal GABA neurons, where they form functional A2AR-D2R heteromers (Fuxe *et al.* 2003, 2005; Ciruela *et al.* 2012). In addition, mGlu5Rs displayed a similar localization to that described for A2AR and D2R in rats (Yung *et al.* 1995; Hettinger *et al.* 2001) and in the striatopallidal complex in non-human primates (Paquet and Smith 2013). A2ARs and group I mGluRs synergistically increased the K_d value of the high affinity D2R agonist binding sites, which was associated with an ability of these two receptors when activated to synergistically counteract D2R agonist induced contralateral turning behavior in a rat model of PD (Ferre′ *et al.* 1999; Popoli *et al.* 2001). In addition, a synergism between A2AR agonists and mGlu5R agonists in increasing GABA release in the ventral striatopallidal GABA pathway, has been reported (Díaz-Cabiale *et al.* 2002).

The present results indicate the existence of a synergistic interaction between A2AR and mGlu5R on striatopallidal GABA neurons, whereby together they exert a concerted inhibition of D2R-mediated control of striatopallidal GABA along with pallidal glutamate levels.

Previous microdialysis studies demonstrated that rat striatopallidal GABA release is inhibited by DA, an effect mediated by the D2R activation (Reid *et al.* 1990; Ferré *et al.* 1993). In line with this, in this study intrastriatal quinpirole perfusion reduced pallidal GABA levels. The evidence that the D2R antagonist eticlopride fully counteracted the quinpirole-induced reduction in GABA (and glutamate) levels, further confirm the role of D2R in these effects. The quinpirole-induced decrease in striatopallidal GABA release probably leads via a disinhibition of the GP-subthalamic nucleus (STN) GABA neurons to an inhibition of the STN/SNr glutamate neurons, which send axon collaterals to the GP, explaining the observed intrastriatal DA D2R agonist induced reduction in basal pallidal glutamate levels (Ferraro *et al.* 2012b). This is in line with previous data demonstrating that the intrapallidal perfusion with the GABA-A receptor antagonist (−)-bicuculline antagonized the intrastriatal neurotensin-induced effects on

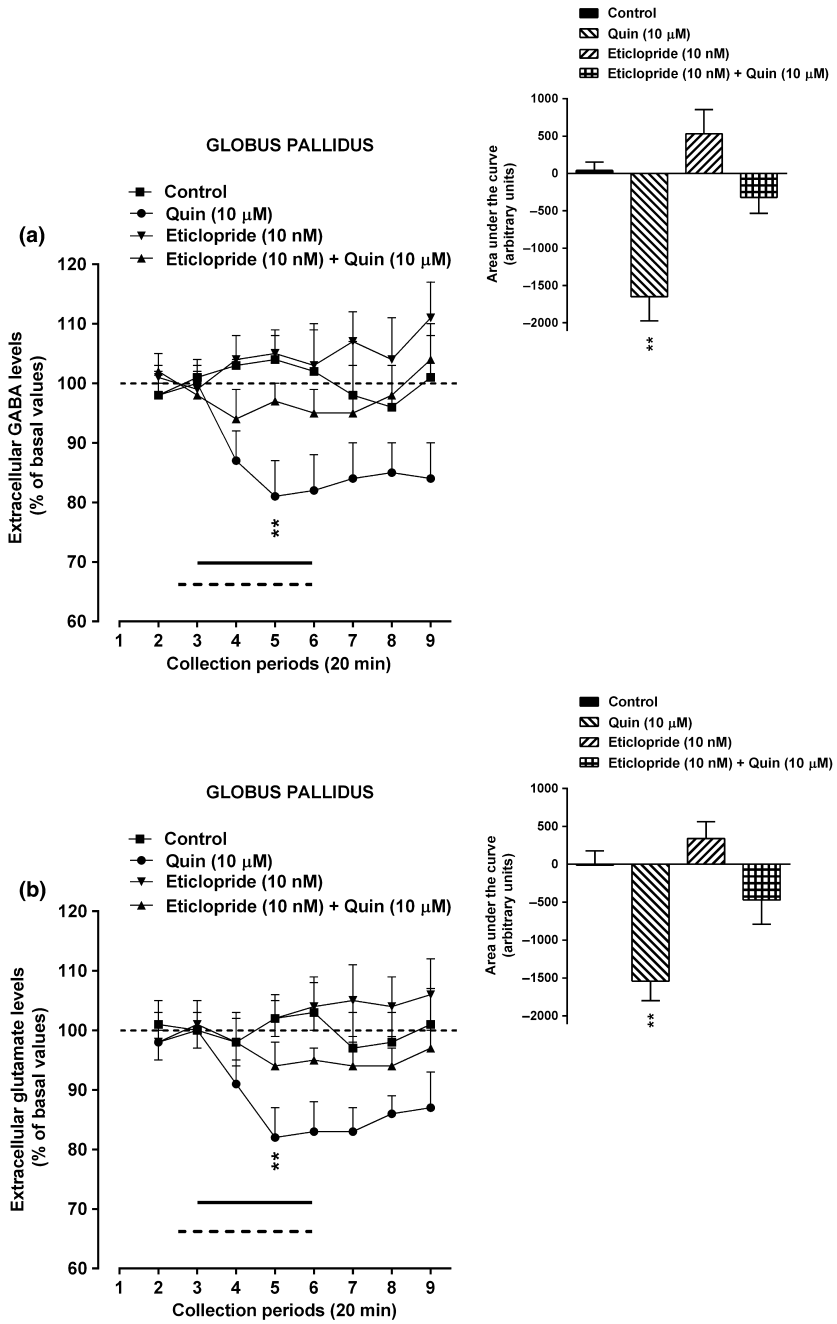

Fig. 3 Effects of intrastriatal perfusion with quinpirole (Quin), alone or in combination with eticlopride, on GABA (a) and glutamate (b) levels from the ipsilateral external globus pallidus of awake rats. The solid and dotted bars indicate the period of intrastriatal perfusion with the dopamine D2 receptor agonist (60 min) and antagonist (70 min), respectively. The results are expressed as percentage of the mean of the three basal values before treatment. Each point represents the mean ± SEM of 5–6 animals. Control rats were perfused with normal Ringer perfusion medium throughout the experiment. The significance with regard to the peak effects (maximal responses) is shown. The areas under the curves, which mainly represent the integrated time-response curve of the overall effects, calculated as % changes in basal value over time (Δ basal % × time) using the trapezoidal rule, are reported on the right side of each panel. $^{**}p < 0.01$ significantly different from all the respective other groups (ANOVA followed by the Student–Newman–Keuls test for multiple comparisons).

pallidal glutamate release (Ferraro *et al.* 1998). Furthermore, previous electrophysiological study (Soltis *et al.* 1994), demonstrated that the infusion of bicuculline into the STN induced an increase in the firing rate of pallidal neurons.

In this study, we found that intrastriatal perfusion with the A2AR agonist CGS21680 (1 μM) failed to significantly affect pallidal GABA and glutamate levels. Furthermore, the compound did not modify the reduction in the two neurotransmitters induced by the intrastriatal perfusion with quinpirole. These findings are in line with previous results showing that in sham-operated rats the intrastriatal quinpirole (10 μM)-induced reduction in pallidal glutamate levels was counteracted by the co-perfusion of CGS21680 at 10 μM, but not 1 μM, concentration (Tanganelli *et al.* 2004).

Intrastriatal CHPG (1000 μM) perfusion induced a significant increase in pallidal GABA levels, which was associated to an increase in extracellular glutamate (see above). The fact that the mGlu5R was only effective at 1000 μM is consistent with the low potency of the drug (Doherty *et al.* 1997; Pintor *et al.* 2000). The role of mGluR5 in these effects is confirmed by the evidence that they are fully counteracted by intrastriatal perfusion with the mGluR5 antagonist MPEP

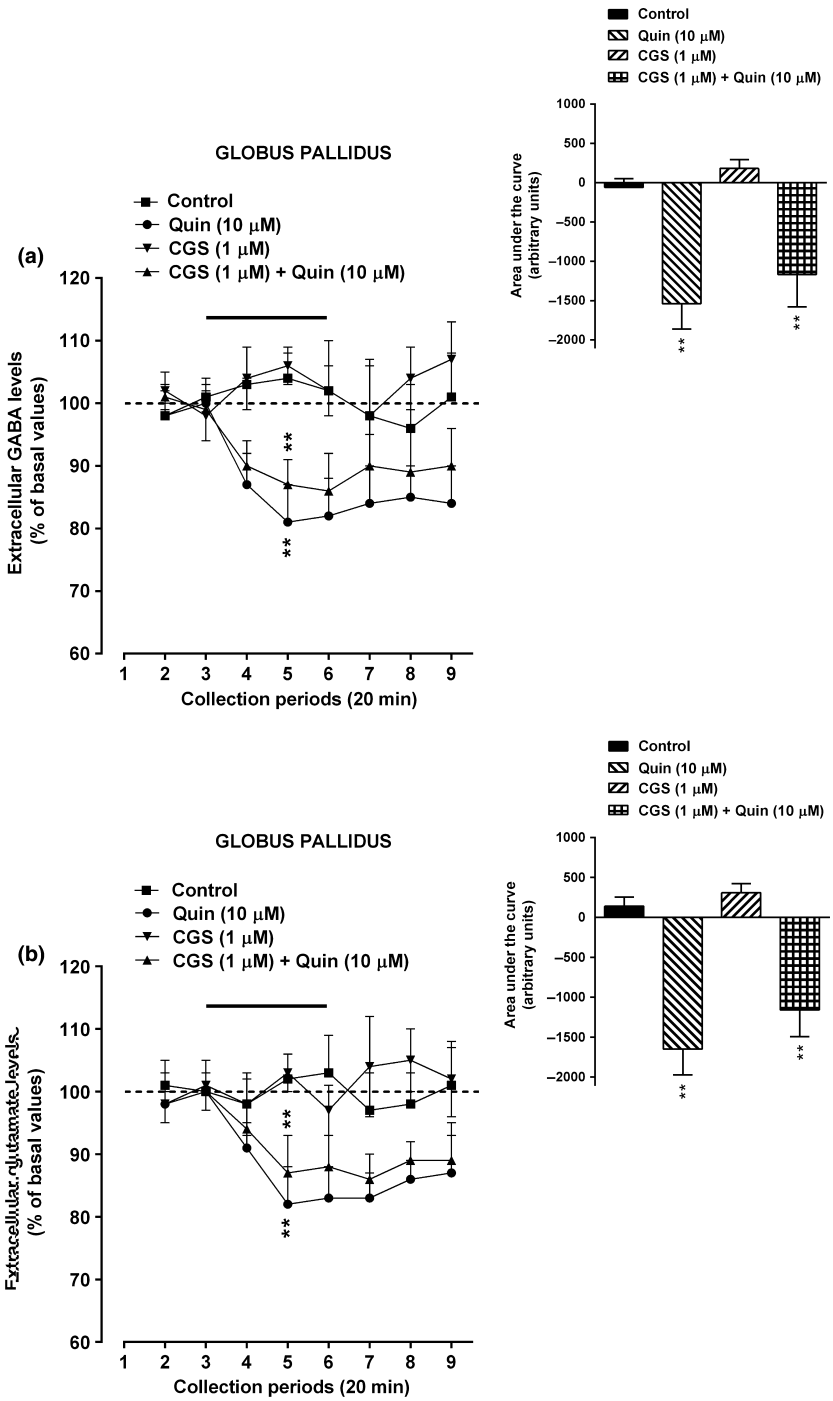

Fig. 4 Effects of intrastriatal perfusion with quinpirole (Quin) and CGS21680 (CGS), alone and in combination, on GABA (a) and glutamate (b) levels from the ipsilateral external globus pallidus of awake rats. The solid bars indicate the period of intrastriatal perfusion with the compounds (60 min). The results are expressed as percentage of the mean of the three basal values before treatment. Each point represents the mean ± SEM of 5–6 animals. Control rats were perfused with normal Ringer perfusion medium throughout the experiment. The significance with regard to the peak effects (maximal responses) is shown. The areas under the curves, which mainly represent the integrated time-response curve of the overall effects, calculated as % changes in basal value over time (Δ basal % × time) using the trapezoidal rule, are reported on the right side of each panel. $^{**}p < 0.01$ significantly differ from the respective control and CGS alone groups (ANOVA followed by the Student–Newman–Keuls test for multiple comparisons).

(Beggiato *et al.* 2013a,b). These findings suggest that the CHPG-induced inhibition of over-all motor activity (Popoli *et al.* 2001) may be explained by its ability to increase neurotransmission of the striatopallidal GABA neurons in the indirect pathway. This leads to increases in the excitatory STN-SNr and STN-GPi drives on the inhibitory nigrothalamic GABA pathway, which results in a reduction in the excitatory thalamocortical glutamate drive to the motor cortex and associated areas. The ability of CHPG to also increase striatal extracellular glutamate levels may represent an important integrative mechanism in the regulation of basal ganglia functioning, and may be one of the factors underlying the critical role played by mGlu5Rs in synaptic plasticity. Interestingly, the intrastriatal perfusion with CHPG at a lower concentration (600 μM) did not affect pallidal GABA and glutamate levels but partially

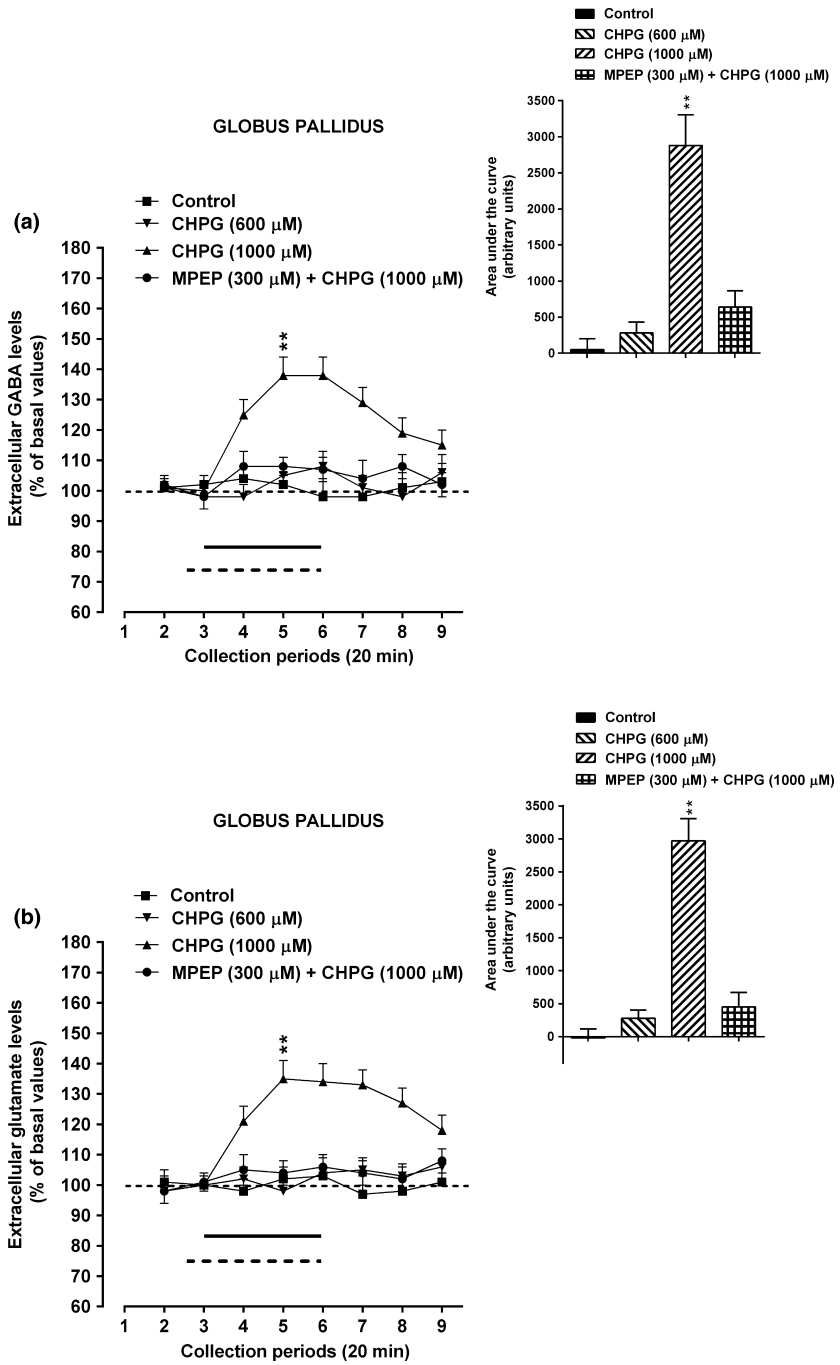

Fig. 5 Effects of intrastriatal perfusion with CHPG, alone or in combination with 2-methyl-6-(phenylethynyl)-pyridine (MPEP), on GABA (a) and glutamate (b) levels from the ipsilateral external globus pallidus of awake rats. The solid and dotted bars indicate the period of intrastriatal perfusion with the mGlu5R agonist (60 min) and antagonist (70 min), respectively. The results are expressed as percentage of the mean of the three basal values before treatment. Each point represents the mean ± SEM of 5–6 animals. Control rats were perfused with normal Ringer perfusion medium throughout the experiment. The significance with regard to the peak effects (maximal responses) is shown. The areas under the curves, which mainly represent the integrated time-response curve of the overall effects, calculated as % changes in basal value over time (Δ basal % × time) using the trapezoidal rule, are reported on the right side of each panel. $^{**}p < 0.01$ significantly differ from all other respective groups (ANOVA followed by the Student–Newman–Keuls test for multiple comparisons).

counteracted the quinpirole-induced reduction in the two neurotransmitter levels. These effects were counteracted by intrastriatal perfusion with MPEP, thus suggesting the involvement of mGluR5. These data validate the existence of a functional interaction between mGlu5Rs and D2Rs. Although other mechanisms are possible, it seems likely that the effect of CHPG could be because of its ability to reduce the affinity of the high-affinity state of striatal D2R receptor for the agonists (Ferre′ *et al.* 1999; Rimondini *et al.* 1999; Popoli *et al.* 2001; Kachroo *et al.* 2005). In addition, the effect of CHPG on quinpirole-induced reduction in striatopallidal GABA neuron activity could underlie the ability of the compound to antagonize quinpirole-induced contralateral rotations in 6-OHDA lesioned rat (Popoli *et al.* 2001).

The main finding of this study is the demonstration of a synergistic functional interaction between A2AR and mGlu5R on striatopallidal GABA neurons, whereby they exert a concerted inhibition of D2R-mediated control of

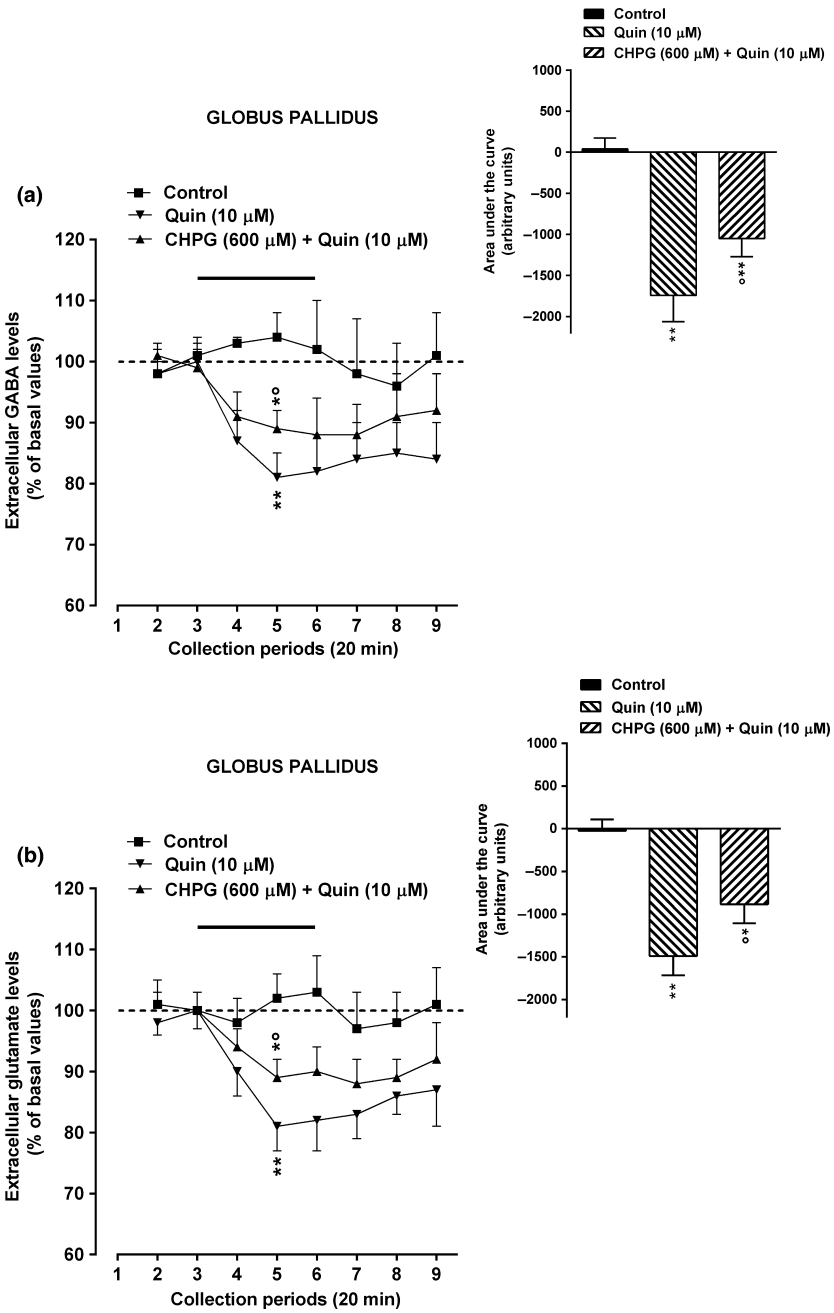

Fig. 6 Effects of intrastriatal perfusion with quinpirole (Quin) alone and in combination with CHPG on GABA (a) and glutamate (b) levels from the ipsilateral external globus pallidus of awake rats. The solid bars indicate the period of intrastriatal perfusion with the compounds (60 min). The results are expressed as percentage of the mean of the three basal values before treatment. Each point represents the mean ± SEM of 5–7 animals. Control rats were perfused with normal Ringer perfusion medium throughout the experiment. The significance with regard to the peak effects (maximal responses) is shown. The areas under the curves, which mainly represent the integrated time-response curve of the overall effects and are calculated as % changes in basal value over time (Δ basal % × time) using the trapezoidal rule, are reported on the right side of each panel. $*p < 0.05$; $**p < 0.01$ significantly differ from the respective control group; $°p < 0.05$ significantly differ from the respective Quin group (ANOVA followed by the Student–Newman–Keuls test for multiple comparisons).

striatopallidal GABA release and pallidal glutamate levels. In fact, in the presence of CGS21680 (1 μM), by itself ineffective, CHPG (600 μM) fully counteracted the quinpirole-induced reduction in pallidal GABA and glutamate levels. These effects were fully counteracted by the local perfusion with MPEP (300 μM) or the A2AR antagonist ZM241385 (100 nM). The evidence that ZM241385 completely blocks the effect of CHPG suggests an involvement of A2AR in some mGlu5R-mediated responses in the striatum. An interaction between A2AR, D2R, and mGlu5R was also demonstrated in a previous study by investigating the effects of CHPG on D2R-dependent contralateral rotations in 6-OHDA lesioned rats (Popoli *et al.* 2001). Thus, it was reported that these effects were significantly potentiated by CGS21680 and attenuated by the adenosine A2A receptor antagonist SCH58261, thus suggesting an involvement of A2A receptors in CHPG-dependent inhibition of D2R-mediated locomotor effects in this animal model of PD. It has been proposed that these interactions between A2AR, D2R, and

mGlu5R ligands could be realized, at least partially, within a heterotrimer where A2ARs and mGlu5Rs, when stimulated, synergize to inhibit D2Rs signaling (Cabello *et al.* 2009; Canela *et al.* 2012). However, the present data do not provide any direct evidence of this heterotrimer involvement in the observed effects, and the synergistic

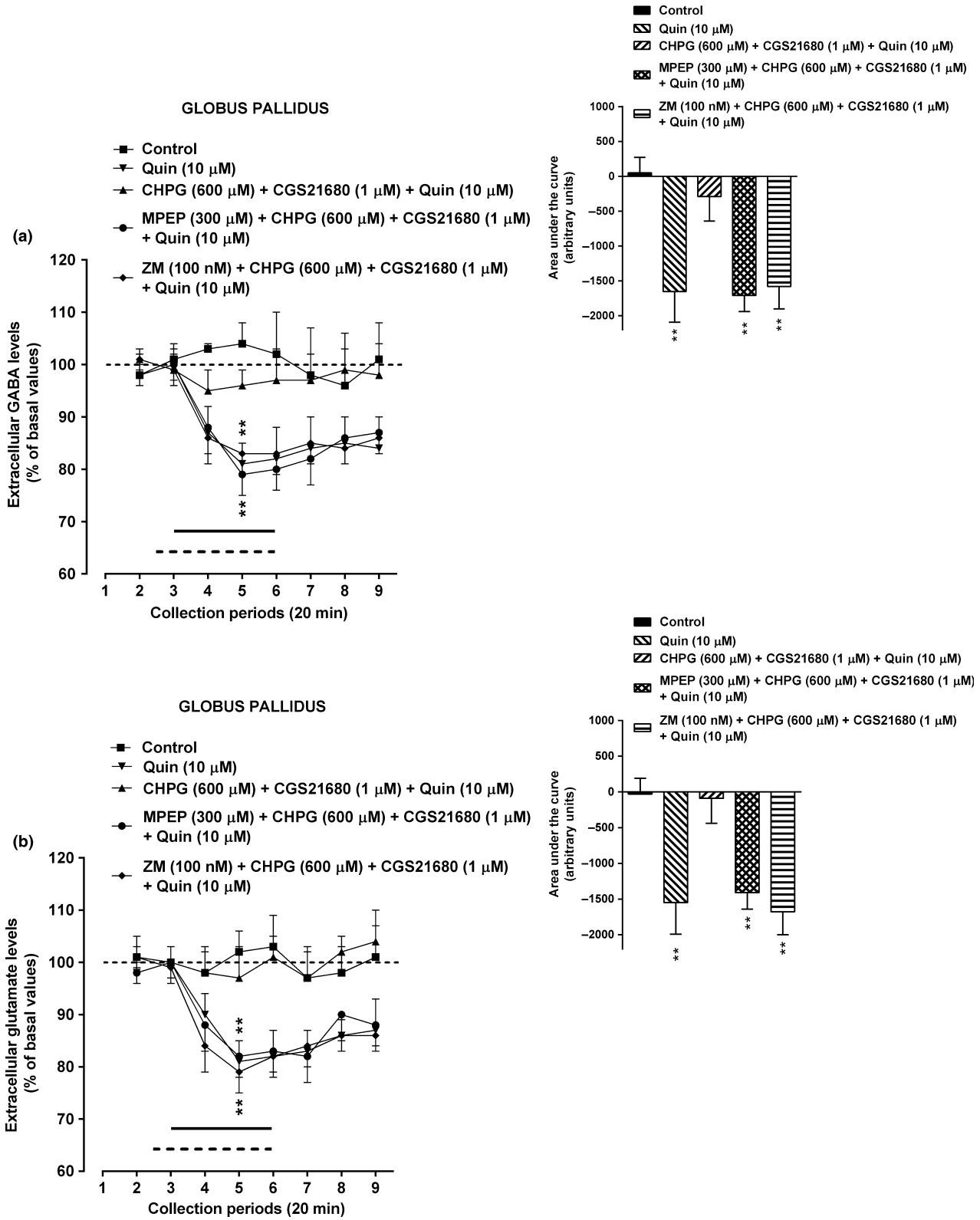

Fig. 7 Effects of intrastriatal perfusion with quinpirole (Quin), alone and in combination with CGS21680 (CGS) + (RS)-2-chloro-5-hydroxyphenylglycine (CHPG), 2-methyl-6-(phenylethynyl)-pyridine (MPEP) + CGS21680 (CGS) + CHPG or CHPG, ZM241385 (ZM) + CGS21680 (CGS) + CHPG on extracellular GABA (a) and glutamate (b) levels from the ipsilateral external globus pallidus of awake rats. The solid and the dotted bars indicate the period of intrastriatal perfusion with the agonists (60 min) or the antagonists (70 min). The results are expressed as percentage of the mean of the three basal values before treatment. Each point represents the mean ± SEM of 6–7 animals. Control rats were perfused with normal Ringer perfusion medium throughout the experiment. The significance with regard to the peak effects (maximal responses) is shown. The areas under the curves, which mainly represent the integrated time-response curve of the overall effects, calculated as % changes in basal value over time (Δ basal % × time) using the trapezoidal rule, are reported on the right side of each panel. $^{**}p < 0.01$ significantly differ from the respective control and Quin + CGS + CHPG groups (ANOVA followed by the Student–Newman–Keuls test for multiple comparisons).

functional interaction between A2AR and mGlu5R in controlling D2-mediated responses could occur at numerous levels between receptor stimulation and neurotransmitter release processes.

Hyperactivity of the GABAergic striatopallidal neuron is a main pathophysiological mechanism responsible for hypokinesia in PD (Obeso *et al.* 2000). Based on this evidence, the current findings suggest the possible usefulness of using not only A2A antagonists but also mGlu5R antagonists and their combinations in the treatment of PD to increase inhibitory D2 signaling on striatopallidal GABA neurons (Fuxe *et al.* 2015). This hypothesis is supported by the reported synergistic anti-parkinsonian effect of A2AR and mGlu5R antagonists in animal models (Kachroo *et al.* 2005), which also suggest that DA depletion could lead to striatopallidal GABA neuron hyperactivity as a consequence of a synergistic stimulatory endogenous tone of adenosine and glutamate on A2AR and mGlu5R, upon interruption of D2R signaling. Furthermore, this approach could be useful in the treatment of L-DOPA-Induced dyskinesias (Morin *et al.* 2015). This exciting hypothesis will be evaluated in a further study, where the A2AR, D2R, and mGlu5R interactions on striatopallidal GABA transmission will be evaluated in an animal model of PD.

Acknowledgments and conflict of interest disclosure

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All experiments were conducted in compliance with the ARRIVE guidelines.

References

Agnati L. F., Ferre S., Lluis C., Franco R. and Fuxe K. (2003) Molecular mechanisms and therapeutical implications of intramembrane receptor/receptor interactions among heptahelical receptors with examples from the striatopallidal GABA neurons. *Pharmacol. Rev.* **55**, 509–550.

Andersen S. L. and Gazzara R. A. (1994) The development of D2 autoreceptor-mediated modulation of K(+)-evoked dopamine release in the neostriatum. *Brain Res. Dev. Brain Res.* **78**, 123–130.

Armentero M. T., Pinna A., Ferré S., Lanciego J. L., Müller 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.

Beggiato S., O'Connor W. T., Tomasini M. C., Antonelli T., Loche A., Tanganelli S., Cacciaglia R. and Ferraro L. (2013a) GET73 increases rat extracellular hippocampal CA1 GABA levels through a possible involvement of local mGlu5 receptor. *Synapse* **67**, 678–691.

Beggiato S., Antonelli T., Tomasini M. C., Tanganelli S., Fuxe K., Schwarcz R. and Ferraro L. (2013b) Kynurenic acid, by targeting α7 nicotinic acetylcholine receptors, modulates extracellular GABA levels in the rat striatum in vivo. *Eur. J. Neurosci.* **37**, 1470–1477.

Borroto-Escuela D. O., Tarakanov A. O., Guidolin D., Ciruela F., Agnati L. F. and Fuxe K. (2011) Moonlighting characteristics of G protein-coupled receptors: focus on receptor heteromers and relevance for neurodegeneration. *IUBMB Life* **63**, 463–472.

Cabello N., Gandía J., Bertarelli D. C., Watanabe M., Lluís C., Franco R., Ferré S., Luján R. and Ciruela F. (2009) Metabotropic glutamate type 5, dopamine D2 and adenosine A2a receptors form higher-order oligomers in living cells. *J. Neurochem.* **109**, 1497–1507.

Canela L., Selga E., García-Martínez J. M. *et al.* (2012) Transcriptional profiling of striatal neurons in response to single or concurrent activation of dopamine D2, adenosine A(2A) and metabotropic glutamate type 5 receptors: focus on beta-synuclein expression. *Gene* **508**, 199–205.

Ciruela F., Fernández-Dueñas V., Llorente J., Borroto-Escuela D. O., Cuffí M. L., Carbonell L., Sánchez S., Agnati L. F., Fuxe K. and Tasca C. I. (2012) G protein-coupled receptor oligomerization and brain integration: focus on adenosinergic transmission. *Brain Res.* **1476**, 86–95.

Coleman C. G., Baghdoyan H. A. and Lydic R. (2006) Dialysis delivery of an adenosine A2A agonist into the pontine reticular formation of C57BL/6J mouse increases pontine acetylcholine release and sleep. *J. Neurochem.* **96**, 1750–1759.

Díaz-Cabiale Z., Vivó M., Del Arco A., O'Connor W. T., Harte M. K., Müller C. E., Martínez E., Popoli P., Fuxe K. and Ferré S. (2002) Metabotropic glutamate mGlu5 receptor-mediated modulation of the ventral striopallidal GABA pathway in rats. Interactions with adenosine A(2A) and dopamine D(2) receptors. *Neurosci. Lett.* **324**, 154–158.

Doherty A. J., Palmer M. J., Henley J. M., Collingridge G. L. and Jane D. E. (1997) (RS)-2-chloro-5-hydroxyphenylglycine (CHPG) activates mGlu5, but no mGlu1, receptors expressed in CHO

cells and potentiates NMDA responses in the hippocampus. *Neuropharmacology* **36**, 265–267.

Fazal A., Parker F., Palmer A. M. and Croucher M. J. (2003) Characterisation of the actions of group I metabotropic glutamate receptor subtype selective ligands on excitatory amino acid release and sodium-dependent re-uptake in rat cerebrocortical minislices. *J. Neurochem.* **86**, 1346–1358.

Ferraro L., Antonelli T., O'Connor W. T., Fuxe K., Soubrié P. and Tanganelli S. (1998) The striatal neurotensin receptor modulates striatal and pallidal glutamate and GABA release: functional evidence for a pallidal glutamate-GABA interaction via the pallidal-subthalamic nucleus loop. *J. Neurosci.* **18**, 6977–6989.

Ferraro L., Beggiato S., Tomasini M. C., Fuxe K., Antonelli T. and Tanganelli S. (2012a) A(2A)/D(2) receptor heteromerization in a model of Parkinson's disease. Focus on striatal aminoacidergic signaling. *Brain Res.* **1476**, 96–107.

Ferraro L., O'Connor W. T., Beggiato S., Tomasini M. C., Fuxe K., Tanganelli S. and Antonelli T. (2012b) Striatal NTS1, dopamine D2 and NMDA receptor regulation of pallidal GABA and glutamate release–a dual-probe microdialysis study in the intranigral 6-hydroxydopamine unilaterally lesioned rat. *Eur. J. Neurosci.* **35**, 207–220.

Ferré S., O'Connor W. T., Fuxe K. and Ungerstedt U. (1993) The striopallidal neuron: a main locus for adenosine-dopamine interactions in the brain. *J. Neurosci.* **13**, 5402–5406.

Ferre′ S., Popoli P., Rimondini R., Reggio R., Kehr J. and Fuxe K. (1999) Adenosine A2A and group I metabotropic glutamate receptors synergistically modulate the binding characteristics of dopamine D2 receptors in the rat striatum. *Neuropharmacology* **38**, 129–140.

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.

Fuxe K., Ferré S., Canals M. *et al.* (2005) Adenosine A2A and dopamine D2 heteromeric receptor complexes and their function. *J. Mol. Neurosci.* **26**, 209–220.

Fuxe K., Canals M., Torvinen M. *et al.* (2007) Intramembrane receptor-receptor interactions: a novel principle in molecular medicine. *J. Neural. Transm.* **114**, 49–75.

Fuxe K., Marcellino D., Leo G. and Agnati L. F. (2010) Molecular integration via allosteric interactions in receptor heteromers. A working hypothesis. *Curr. Opin. Pharmacol.* **10**, 14–22.

Fuxe K., Borroto-Escuela D. O., Romero-Fernandez W., Palkovits M., Tarakanov A. O., Ciruela F. and Agnati L. F. (2014) Moonlighting proteins and protein-protein interactions as neurotherapeutic targets in the G protein-coupled receptor field. *Neuropsychopharmacology* **39**, 131–155.

Fuxe K., Guidolin D., Agnati L. F. and Borroto-Escuela D. O. (2015) Dopamine heteroreceptor complexes as therapeutic targets in Parkinson's disease. *Expert Opin. Ther. Targets* **19**, 377–398.

Hettinger B. D., Lee A., Linden J. and Rosin D. L. (2001) Ultrastructural localization of adenosine A2A receptors suggests multiple cellular sites for modulation of GABAergic neurons in rat striatum. *J. Comp. Neurol.* **431**, 331–346.

Kachroo A., Orlando L. R., Grandy D. K., Chen J. F., Young A. B. and Schwarzschild M. A. (2005) Interactions between metabotropic glutamate 5 and adenosine A2A receptors in normal and parkinsonian mice. *J. Neurosci.* **25**, 10414–10419.

Morin N. and Di Paolo T. (2014) Interaction of adenosine receptors with other receptors from therapeutic perspective in Parkinson's disease. *Int. Rev. Neurobiol.* **119**, 151–167.

Morin N., Morissette M., Grégoire L. and Di Paolo T. (2015) mGlu5, Dopamine D2 and Adenosine A2A Receptors in L-DOPA-Induced Dyskinesias. *Curr. Neuropharmacol.* DOI 10.2174/1570159X 14666151201185652

Obeso J. A., Rodríguez-Oroz M. C., Rodríguez M., Lanciego J. L., Artieda J., Gonzalo N. and Olanow C. W. (2000) Pathophysiology of the basal ganglia in Parkinson's disease. *Trends Neurosci.* **23**, S8–S19.

Paquet M. and Smith Y. (2013) Group I metabotropic glutamate receptors in the monkey striatum: subsynaptic association with glutamatergic and dopaminergic afferents. *J. Neurosci.* **23**, 7659–7669.

Paxinos G. and Watson C. (1982) *The rat brain in stereotaxic coordinates*. Academic, New York.

Pintor A., Pèzzola A., Reggio R., Quarta D. and Popoli P. (2000) The mGlu5 receptor agonist CHPG stimulates striatal glutamate release: possible involvement of A2A receptors. *NeuroReport* **11**, 3611–3614.

Popoli P., Pèzzola A., Torvinen M., Reggio R., Pintor A., Scarchilli L., Fuxe K. and Ferré S. (2001) The selective mGlu(5) receptor agonist CHPG inhibits quinpirole-induced turning in 6-hydroxydopamine-lesioned rats and modulates the binding characteristics of dopamine D(2) receptors in the rat striatum: interactions with adenosine A(2a) receptors. *Neuropsychopharmacology* **25**, 505–513.

Reid M. S., O'Connor W. T., Herrera-Marschitz M. and Ungerstedt U. (1990) The effects of intranigral GABA and dynorphin A injections on striatal dopamine and GABA release: evidence that dopamine provides inhibitory regulation of striatal GABA neurons via D2 receptors. *Brain Res.* **519**, 255–260.

Rimondini R., Fuxe K. and Ferré S. (1999) Multiple intramembrane receptor-receptor interactions in the regulation of striatal dopamine D2 receptors. *NeuroReport* **10**, 2051–2054.

Schiffmann S. N., Fisone G., Moresco R., Cunha R. A. and Ferré S. (2007) Adenosine A2A receptors and basal ganglia physiology. *Prog. Neurobiol.* **83**, 277–292.

Schwarzschild M. A., Agnati L., Fuxe K., Chen J. F. and Morelli M. (2006) Targeting adenosine A2A receptors in Parkinson's disease. *Trends Neurosci.* **29**, 647–654.

Simola N., Morelli M. and Pinna A. (2008) Adenosine A2A receptor antagonists and Parkinson's disease: state of the art and future directions. *Curr. Pharm. Des.* **14**, 1475–1489.

Soltis R. P., Anderson L. A., Walters J. R. and Kelland M. D. (1994) A role for non-NMDA excitatory amino acid receptors in regulating the basal activity of rat globus pallidus neurons and their activation by the subthalamic nucleus. *Brain Res.* **666**, 21–30.

Tanganelli S., Sandager Nielsen K., Ferraro L., Antonelli T., Kehr J., Franco R., Ferré S., Agnati L. F., Fuxe K. and Scheel-Krüger J. (2004) Striatal plasticity at the network level. Focus on adenosine A2A and D2 interactions in models of Parkinson's disease. *Parkinsonism Relat. Disord.* **10**, 273–280.

Yung K. K., Bolam J. P., Smith A. D., Hersch S. M., Ciliax B. J. and Levey A. I. (1995) Immunocytochemical localization of D1 and D2 dopamine receptors in the basal ganglia of the rat: light and electron microscopy. *Neuroscience* **65**, 709–730.