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GluN2A and GluN2B NMDA Receptor Subunits Differentially Modulate Striatal Output Pathways and Contribute to Levodopa-Induced Abnormal Involuntary Movements in Dyskinetic Rats

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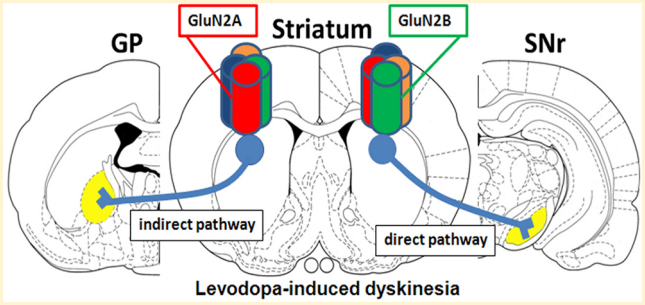
ABSTRACT: Dual probe microdialysis was used to investigate whether GluN2A and GluN2B NMDA receptor subunits regulate striatal output pathways under dyskinetic conditions. The preferential GluN2A antagonist NVP-AAM077 perfused in the dopamine-depleted striatum of 6-hydroxydopamine hemilesioned dyskinetic rats reduced GABA and glutamate levels in globus pallidus whereas the selective GluN2B antagonist Ro 25-6981 elevated glutamate without affecting pallidal GABA. Moreover, intrastriatal NVP-AAM077 did not affect GABA but elevated glutamate levels in substantia nigra reticulata whereas Ro 25-6981 elevated GABA and reduced nigral glutamate. To investigate whether GluN2A and GluN2B NMDA receptor subunits are involved in motor pathways underlying dyskinesia expression, systemic NVP-AAM077 and Ro 25-6981 were tested for their ability to attenuate levodopa-induced abnormal involuntary movements. NVP-AAM077 failed to prevent dyskinesia while Ro 25-6981 mildly attenuated it. We conclude that in the dyskinetic striatum, striatal GluN2A subunits tonically stimulate the striato-pallidal pathway whereas striatal GluN2B subunits tonically inhibit striato-nigral projections. Moreover, GluN2A subunits are not involved in dyskinesia expression whereas GluN2B subunits minimally contribute to it.



KEYWORDS: *GABA, microdialysis, NMDA receptor subunits, NVP-AAM077, 6-OHDA, Ro 25-6981*

Alterations in glutamate (GLU) neurotransmission and, in particular, abnormal function of striatal NMDA receptors, play a key role in driving symptoms of Parkinson's disease (PD) and long-term motor complications (e.g., dyskinesia) associated with L-DOPA therapy.[1−3] The focus on the pathogenic role of NMDA receptors (and the therapeutic potential of NMDA receptor antagonists) has further grown after the discovery of their structural and functional heterogeneity. NMDA receptors are heteromers usually containing two NR1 and two NR2 subunits. NR2 subunits, classified into four (A-D) types,[4−6] govern NMDA channel gating and confer different physiopharmacological properties to the receptor-channel complex.[7,8] NMDA receptors containing the GluN2A and GluN2B subunits are highly expressed in the basal ganglia,[9,10] and several studies have reported plastic changes in striatal NR2 subunit expression levels, phosphorylation state and trafficking in response to dopamine (DA) depletion and chronic L-DOPA therapy (reviewed in ref 3).

These changes have been pathogenically linked to PD since ifenprodil, the first identified GluN2B selective antagonist, and its analogues such as (*R*)-(*R**,*S**)-α-(4-hydroxyphenyl)-β-methyl-4-(phenylmethyl)-1-piperidinepropanol (Ro 25-6981) attenuated motor impairment in different models of parkinsonism[11−15]

By using dual or triple probe microdialysis in awake rats,[16] we have attempted to investigate a possible correlation between the antiparkinsonian/antidyskinetic profiles of GluN2A and GluN2B selective antagonists with their ability to modulate GABA release from globus pallidus (GP) or substantia nigra reticulata (SNr), taken as an index of activity of the striatal output pathways.[17] Two different populations of striatal medium-sized

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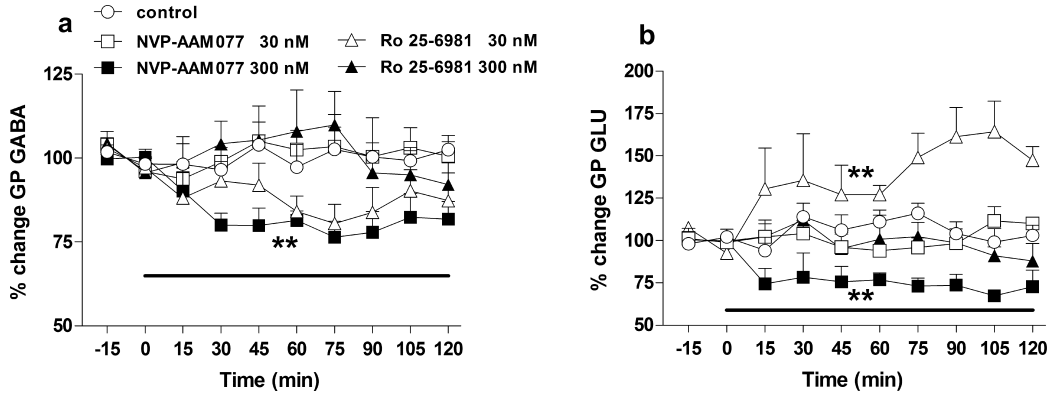

Figure 1. Intrastriatal GluN2A or GluN2B subunit blockade differentially modulated amino acid release in the ipsilateral globus pallidus (GP) of dyskinetic rats. Effect of reverse dialysis of the GluN2A subunit preferential antagonist NVP-AAM077 (30 and 300 nM) and the GluN2B subunit selective antagonist Ro 25-6981 (30 and 300 nM) in the DA-depleted dorsolateral striatum of dyskinetic rats on GABA (panel a) and GLU (panel b) extracellular levels in ipsilateral GP. Data are expressed as percentages ± SEM of basal pretreatment levels (calculated as mean of the two samples before the treatment). Extracellular GABA and GLU levels in GP were 2.5 ± 0.3 nM and 91 ± 20 nM, respectively ($n = 29$). $^{**}p < 0.01$ different from control (ANOVA followed by the Newman-Keuls test for multiple comparisons on AUC values).

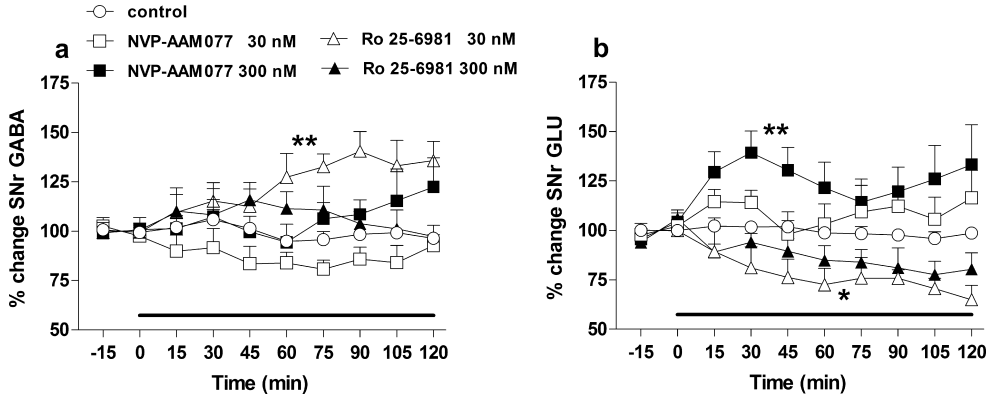

Figure 2. Intrastriatal GluN2A or GluN2B subunit blockade differentially modulates amino acid release in the substantia nigra reticulata (SNr) of dyskinetic rats. Effect of reverse dialysis of the GluN2A subunit preferential antagonist NVP-AAM077 (30 and 300 nM) and the GluN2B subunit selective antagonist Ro 25-6981 (30 and 300 nM) in the DA-depleted dorsolateral striatum of dyskinetic rats on GABA (panel a) and GLU (panel b) extracellular levels in ipsilateral SNr. Data are expressed as percentages ± SEM of basal pretreatment levels (calculated as mean of the two samples before the treatment). Extracellular GABA and GLU levels in SNr were 3.1 ± 0.5 nM and 126 ± 30 nM ($n = 24$), respectively. $^{*}p < 0.05$, $^{**}p < 0.01$ different from control (ANOVA followed by the Newman-Keuls test for multiple comparisons on AUC values).

GABAergic spiny neurons project to GP and SNr, giving rise to the so-called "indirect" and "direct" pathways, respectively.[18,19] According to the classical scheme of basal ganglia functioning, these pathways exert an opposite, facilitatory and inhibitory, respectively, control over nigro-thalamic GABAergic neurons, leading to motor impairment or motor activation, respectively.[20] In situ hybridization studies have shown that striatal GABAergic projection neurons express equal proportions of GluN2A and GluN2B subunits.[9,10] However, we recently provided evidence of GABA release in GP and SNr in response to intrastriatal perfusion of the preferential GluN2A antagonist (*R*)-[(*S*)-1-(4-bromo-phenyl)-ethylamino]-(2,3-dioxo-1,2,3,4-tetrahydroquinoxalin-5-yl)-methyl]-phosphonic acid (NVP-AAM077) or the selective GluN2B selective antagonist Ro 25-6981, respectively.[44] This suggests a "functional" segregation of GluN2A and GluN2B subunits along the striato-pallidal and striato-nigral pathway, respectively.[17]

We therefore followed up these studies and set to investigate whether the control exerted by striatal GluN2A and GluN2B subunits over striatal output pathways is dysregulated in LID. Moreover, we tested whether GluN2A and GluN2B subunits play a different role in the expression of abnormal involuntary movements (AIMs), the rodent correlate of dyskinesia.[21]

RESULTS AND DISCUSSION

Effect of Intrastriatal Perfusion of NVP-AAM077 and Ro 25-6981 on Amino Acid Release in GP and SNr. To unravel the contribution of GluN2A and GluN2B subunits to the regulation of striatal output pathways in LID, reverse dialysis of NVP-AAM077 and Ro 25-6981 through a microdialysis probe implanted in the DA-depleted dorsolateral striatum (DLS) of dyskinetic rats was performed in the absence of (OFF) L-DOPA. GABA and GLU release was monitored in ipsilateral GP or SNr.

Intrastriatal perfusion with NVP-AAM077 (300 nM) caused ∼24% reduction in GABA levels in GP whereas intrastriatal perfusion with Ro 25-6981 was ineffective (Figure 1a). Intrastriatal NVP-AAM077 (300 nM) also caused a reduction (∼33%) of pallidal GLU levels whereas intrastriatal Ro 25-6981 (30 nM) increased them (∼64%) (Figure 1b).

A different pattern of amino acid responses was observed in SNr during intrastriatal perfusion with GluN2 antagonists. Thus, intrastriatal NVP-AAM077 did not affect nigral GABA levels while Ro 25-6981 (30 nM) increased them (∼40%; Figure 2a).

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Moreover, NVP-AAM077 (300 nM) increased (~39%) while Ro 25-6981 (30 nM) decreased (~35%) GLU levels in SNr (Figure 2b).

TH immunohistochemistry revealed a similar degree of denervation in NVP-AAM077 and Ro 25-6981 treated rats. Indeed, the amount of TH-immunoreactive fibers in the lesioned side was 3.7 ± 2.2% (n = 8) and 2.7 ± 1.9% (n = 8) of the unlesioned side in the group of rats treated with NVP-AAM07 and Ro 25-6981, respectively.

Consistent with previous microdialysis studies[17] (Table 1), NVP-AAM077 and Ro 25-6981 preferentially regulated pallidal and nigral GABA, respectively. This is suggestive of a preferential control of GluN2A and GluN2B subunits over the striato-pallidal and striato-nigral pathways, respectively (Figure 3). Nonetheless, while NVPAAM077 consistently inhibited pallidal GABA across different conditions (naïve, 6-OHDA hemilesioned L-DOPA-unprimed, and 6-OHDA hemilesioned L-DOPA-primed dyskinetic rats) (Table 1), Ro 25-6981 inhibited nigral GABA in naïve and L-DOPA-unprimed rats but elevated it in dyskinetic rats.

Table 1. Selective Blockade on Intrastriatal GluN2A and GluN2B Subunits Differentially Modulate Striatal Output Pathways[a]

		globus pallidus		substantia nigra reticulata	
		GABA	GLU	GABA	GLU
NVP-AAM077 intrastriatal	naive	↓	0	0	↑
	6-OHDA	↓[b]	0	0	0
	dyskinetic	↓	↓	0	↑
Ro 25-6981 intrastriatal	naive	0	↑	↓[b]	0
	6-OHDA	0	0	0	0
	dyskinetic[c]	0	↑	↑	↓

[a]Table summary reporting changes of GABA and GLU levels in globus pallidus (GP) and substantia nigra reticulata (SNr) evoked by reverse dialysis of GluN2A and GluN2B subunit antagonists (NVP-AAM077 and Ro 25-6981, respectively) in the dorsolateral striatum of naïve rats, DA-depleted dorsolateral striatum of 6-OHDA hemilesioned L-DOPA-unprimed rats or DA-depleted dorsolateral striatum of 6-OHDA hemilesioned L-DOPA-primed (dyskinetic) rats. Unless otherwise stated the data were obtained by perfusing 300 nM antagonist concentrations. Data obtained in naive or 6-OHDA rats were taken from refs 17 and 16. ↑ denotes an increase; ↓ a decrease; 0 no effect. [b]The effect was observed only at 30 nM. [c]The effects were detected at 30 nM but not 300 nM.

The parkinsonian condition is associated with a reduction in the abundance of GluN2B relative to GluN2A subunits at the postsynaptic level.[22,23] However, chronic L-DOPA treatment restores physiological GluN2B levels.[22−24] These changes may occur at any of the different populations of GluN2B-expressing striatal interneurons and projection neurons,[10] the impact on the activity of striato-nigral neurons being dependent on their activity state. At rest (i.e., OFF L-DOPA), when GLU inputs from the cortex are reduced,[25] indirect modulation by tonically active striatal interneurons may prevail. This indirect GluN2B-driven facilitatory control may be overcome in the presence of (ON) L-DOPA, i.e. when striato-nigral neurons are activated by overactive cortical inputs [26] and phasic D1 receptor stimulation.[41] In this case, GluN2B subunits expressed on striato-nigral neurons may be recruited, leading to the potentiation of the D1 response.[27] GluN2B receptors located on striato-nigral neurons

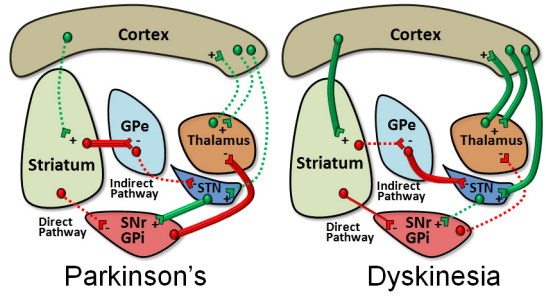

Figure 3. Basal ganglia-thalamo-cortical loop in Parkinson's disease (left) and levodopa-induced dyskinesia (right). Green lines indicate glutamatergic excitatory (+) while red lines are GABAergic inhibitory (−) projection neurons. Dotted lines indicate underactivity of a given pathway while wide lines indicate overactivity. In Parkinson's disease, a loss of dopamine neurons causes an opposite dysregulation of the direct and indirect pathways eventually leading to a loss of thalamo-cortical glutamate signaling (left). In L-DOPA induced dyskenesia, excessive dopamine stimulation results in hyperactivation of thalamo-cortical and cortico-striatal glutamatergic inputs (right).

may thus contribute to LID, as demonstrated by the finding that blockade of GluN2B subunits located in striatum attenuated AIMs expression in dyskinetic rats.[28]

Different from the GluN2B subunit, the inhibitory control operated by the GluN2A subunit over the striato-pallidal pathway seems to undergo only quantitative changes passing from the naïve through the parkinsonian and the dyskinetic states. Based on the comparison of the concentration response-curves of NVP-AAM077 in these conditions[16,17] (Table 1), and the finding that in the dyskinetic state GABA release inhibition appears at higher NVP-AAM077 concentrations (300 nM) than in the parkinsonian state (30 nM), we can conclude that chronic L-DOPA normalizes GluN2A signaling in striato-pallidal neurons and/or the hyperactivity along the indirect pathway. Indeed, L-DOPA inhibits the firing discharge of striato-pallidal neurons in vivo,[29] and, when chronically administered, normalizes *zif-268* expression in striato-pallidal neurons[30] and D2-receptor mediated inhibition of striatal neurons.[31]

Changes in GLU levels induced by intrastriatal perfusion with GluN2 antagonists are more difficult to interpret, since it is believed that striatal projection neurons are GABAergic in nature and there is no direct GLU projection from the striatum to GP or SNr. In some cases, changes in GLU levels may be secondary to changes in local GABA levels [46] while in others, activation of GLU inputs originating from STN, thalamus, or cortex may be involved.

Effect of Systemic Administration of NVP-AAM077 and Ro 25-6981 on AIMs Expression. We next investigated whether NVP-AAM077 or Ro 25-6981 were able to prevent AIMs expression in dyskinetic rats. Preliminary dose-finding studies were conducted in naïve rats to investigate the motor profile of these ligands. NVP-AAM077 did not affect immobility time or stepping activity, although it caused a dose-dependent inhibition of rotarod performance (Figure 4) which was significant at 10 and 20 mg/kg. Conversely, Ro 25-6981 did not alter motor performance up to 10 mg/kg (Figure 5). These data confirm that GluN2A receptor blockade disrupts motor performance whereas GluN2B receptor blockade does not.[32,33] However, since immobility time and stepping activity in the bar (akinesia) and drag (bradykinesia) tests were not affected, we can rule out that GluN2A subunits contribute to motor initiation and execution. The inhibition of rotarod performance may thus be

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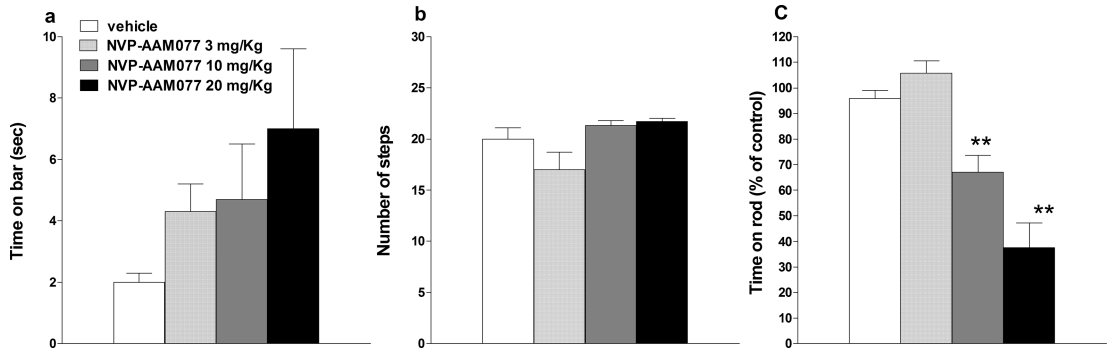

Figure 4. GluN2A subunit blockade inhibited motor performance in naïve rats. Systemic (i.p.) administration of the GluN2A receptor preferential antagonist NVP-AAM077 (3−20 mg/kg) did not affect the time spent on the blocks in the bar test (panel a) and the number of steps in the drag test (panel b) but dose-dependently inhibited motor performance on the rotarod (panel c). Motor tests were performed in a sequence starting at 30 min after drug injection. Motor activity in the bar and drag test was calculated as the average between motor activity at the ipsilateral and contralateral forepaws. Data are expressed as immobility time (in s, panel a), number of steps (panel b), and percentage of the control session (panel c), and are mean ± SEM of seven rats per group. $^{**}p < 0.01$ different from vehicle (ANOVA followed by the Newman-Keuls test for multiple comparisons).

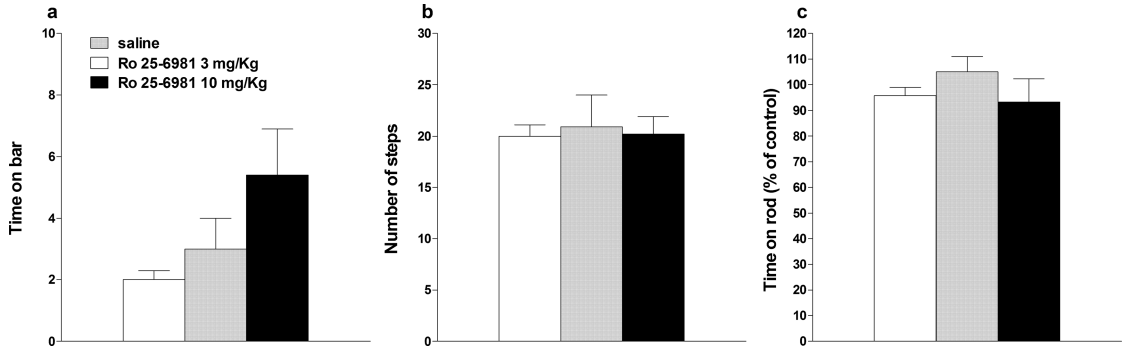

Figure 5. GluN2B receptor blockade did not affect motor performance in naïve rats. Systemic (i.p.) administration of the GluN2B receptor selective antagonist Ro 25-6981 (3−10 mg/kg) did not affect the time spent on the blocks in the bar test (panel a), the number of steps in the drag test (panel b), and the rotarod performance (panel c). Motor tests were performed in a sequence starting at 30 min after drug injection. Motor activity in the bar and drag test was calculated as the average between motor activity at the ipsilateral and contralateral forepaws. Data are expressed as immobility time (in s, panel a), number of steps (panel b), and percentage of the control session (panel c), and are mean ± SEM of seven rats per group.

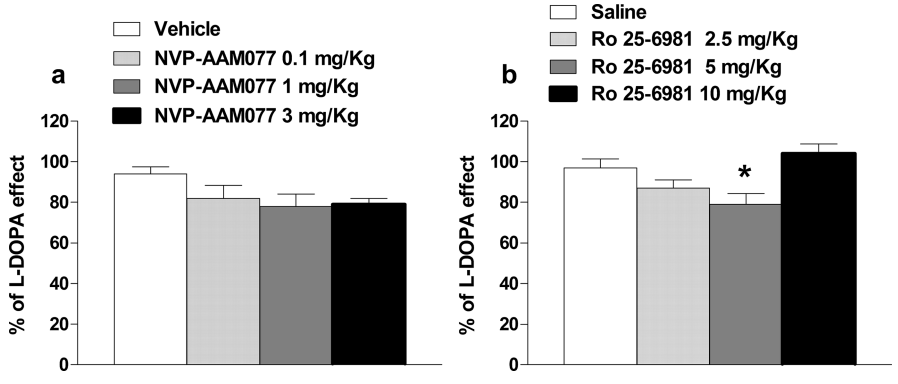

Figure 6. GluN2B but not GluN2A subunit blockade attenuated L-DOPA-induced dyskinesia expression in rats. Systemic (i.p.) administration of the GluN2A receptor preferential antagonist NVP-AAM077 (0.1−3 mg/kg; panel a) failed to affect the expression of axial, limb and orolingual (ALO) abnormal involuntary movements (AIMs) in dyskinetic rats challenged with L-DOPA (6 mg/kg plus benserazide 12 mg/kg; i.p.) whereas the GluN2B receptor selective antagonist Ro 25-6981 (2.5−10 mg/kg; panel b) attenuated it. AIMs were scored every 10 min up to 3 h after L-DOPA administration. Data are expressed as percentage ± SEM of L-DOPA effect (i.e., the effect of L-DOPA alone in the same animal taken as an internal control) and are means of 11 rats per group. $^{*}p < 0.05$ different from saline (ANOVA followed by the Newman-Keuls test for multiple comparisons).

ascribed to changes in motor coordination, stamina or even motivation to run, possibly occurring outside the basal ganglia.

For the study in dyskinetic rats, we selected doses of NVP-AAM077 which did not cause primary hypolocomotive effects. NVP-AAM077 (0.01−3 mg/kg) failed to affect AIMs expression (Figure 6a), whereas Ro 25-6981 mildly attenuated it (22%), but only at 5 mg/kg (Figure 6b).

NMDA receptors containing the GluN2B subunit play a significant role in mediating the changes in glutamatergic function occurring after DA loss, although pharmacological studies aimed at testing the antidyskinetic potential of GluN2B

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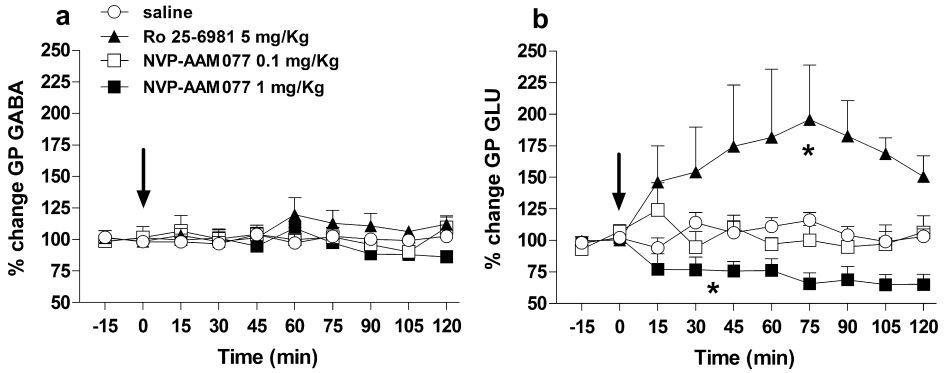

Figure 7. GluN2A and GluN2B subunit blockade oppositely modulated pallidal glutamate (GLU) release in dyskinetic rats. Systemic administration (i.p.; arrow) of the GluN2A subunit preferential antagonist NVP-AAM077 (0.1−1 mg/kg) and the GluN2B subunit selective antagonist Ro 25-6981 (5 mg/kg) did not affect GABA (panel a) but oppositely modulated GLU (panel b) in the globus pallidus (GP) of dyskinetic rats. Extracellular GABA and GLU levels in GP were 4.5 ± 0.7 nM and 174 ± 51 nM, respectively (n = 18). Data are expressed as percentages ± SEM of basal pretreatment levels (calculated as mean of the two samples before the treatment). *p < 0.05 different from saline (ANOVA followed by the Newman-Keuls test for multiple comparisons).

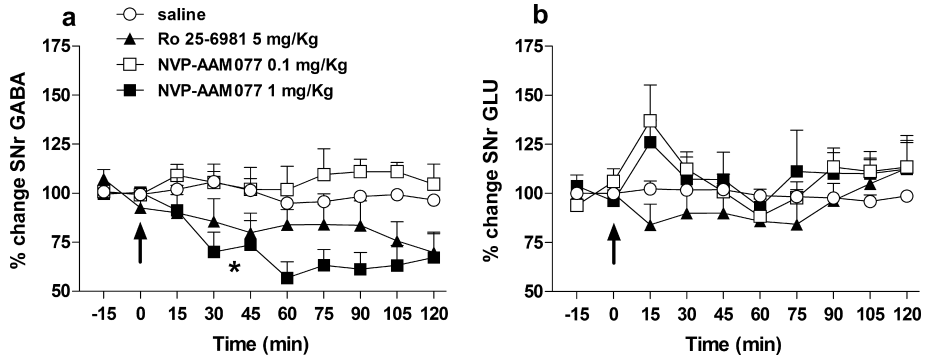

Figure 8. GluN2A but not GluN2B subunit blockade reduced nigral GABA release in dyskinetic rats. Systemic administration (i.p.; arrow) of the GluN2A subunit preferential antagonist NVP-AAM077 (0.1−1 mg/kg) but not the GluN2B subunit selective antagonist Ro 25-6981 (5 mg/kg) reduced GABA (panel a) in the substantia nigra reticulata (SNr) of dyskinetic rats. Neither antagonist affected nigral GLU release (panel b). Data are expressed as percentages ± SEM of basal pretreatment levels (calculated as mean of the two samples before the treatment). Extracellular GABA and GLU levels in SNr were 4.8 ± 0.9 nM and 170 ± 24 nM (n = 18). *p < 0.05 different from saline (ANOVA followed by the Newman-Keuls test for multiple comparisons).

antagonists have generated inconsistent results possibly due to different drug properties and dosages.[3] Thus, chronic administration of CP-101,606 prevented changes of rotational response (sensitization) following L-DOPA treatment (reminiscent of wearing-off phenomena) in 6-OHDA rats.[34] This effect was also observed when this compound was administered acutely after the sensitization phase. Consistently, acute administration of Co 101244 prevented the expression of dyskinesia induced by a L-DOPA challenge in MPTP-treated dyskinetic macaques,[35] while CP-101,606 reduced dyskinesia in PD patients.[36] Nonetheless, another study in dyskinetic rats[37] failed to prove an antidyskinetic effect of GluN2B-selective antagonists given systemically under acute (Ro 63-1908) or chronic (Ro 25-6981) administration protocols. Our data confirm the mild effectiveness of systemic Ro 25-6981 in attenuating AIMs expression, and show its narrow therapeutic dose-range. The reason for the loss of Ro 25-6981 antidyskinetic effect at 10 mg/kg, giving a classical bell-shaped profile to the dose−response curve, remains a matter for speculation. Since local intrastriatal infusion of Ro 25-6981 has been reported to attenuate AIMs expression in dyskinetic rats,[28] it might be conceivable that blockade of extrastriatal GluN2B subunits during systemic administration of Ro 25-6981 opposes the antidyskinetic effect.

The finding that pharmacological blockade of GluN2A receptors does not attenuate LID is consistent with the failure of a selective GluN2A subunit peptide (TATA) to prevent AIMs expression in dyskinetic rats.[38] If we postulate that GluN2A subunit preferentially regulates the striato-pallidal pathway, this finding is in line with the view that the indirect pathway does not contribute to AIMs expression in rodent models of LID.[41,48,39,40] Nonetheless, chronic TATA administration attenuated the development of LID when coadministered with L-DOPA in unprimed 6-OHDA rats, suggesting that the GluN2A subunit contributes to the process of brain sensitization to L-DOPA.[38] Indeed, in vivo electrophysiological evidence has shown that striato-pallidal neurons play a role in the induction phase.[29]

Effect of Systemic Administration of NVP-AAM077 and Ro 25-6981 on Amino Acid Release in GP and SNr. We finally compared the neurochemical patterns of an antidyskinetic dose of Ro 25-6981 (5 mg/kg, i.p.) with that of ineffective doses of NVP-AAM077 (0.1 and 1 mg/kg). Neither antagonist given systemically affected GABA levels in GP (Figure 7a), whereas they both affected GLU levels in this brain area (Figure 7b). NVP-AAM077 reduced (∼35% at 1 mg/kg) pallidal GLU, whereas Ro 25-6981 elevated it to a maximum of ∼80%. NVP-AAM077 reduced GABA levels in SNr at 1 mg/kg (∼43%)

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whereas Ro 25-6981 was ineffective (Figure 8a). Neither antagonist significantly affected GLU levels in SNr (Figure 8b).

Since both systemic administration and intrastriatal perfusion of Ro 25-6981 elevated pallidal GLU without affecting local GABA levels, it is possible that this increase reflects an activation of GLU inputs from STN, thalamus or cortex.

CONCLUDING REMARKS

Dual probe microdialysis revealed that LID was associated with plastic changes in the tonic control operated by GluN2A and GluN2B subunits over striatal output pathways. The data confirm previous studies in naïve and hemiparkinsonian rats showing a preferential control of GluN2A and GluN2B subunits along the striato-pallidal and striato-nigral pathways, respectively. The present study also demonstrates the ineffectiveness of GluN2A subunit selective NMDA receptor antagonists in attenuating LID expression, and confirms that GluN2B selective NMDA receptor antagonists have antidyskinetic potential. However, the poor efficacy of systemic Ro 25-6981 calls for a thorough analysis of the role of striatal and extrastriatal GluN2B subunits as well as the pharmacological profile of different classes of GluN2B subunit selective NMDA receptor antagonists.

METHODS

Experimental Procedures. Male Sprague–Dawley rats (150 g; Harlan Italy; S. Pietro al Natisone, Italy) were housed under regular lighting conditions (12 h light/dark cycle) and given food and water ad libitum. The experimental protocols performed in the present study were in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and were approved by Italian Ministry of Health (license no. 194/2008-B) and Ethical Committee of the University of Ferrara. Adequate measures were taken to minimize the number of animals used and animal pain and discomfort.

Drugs. 6-OHDA hydrobromide, *d*-amphetamine sulfate, L-DOPA methylester hydrochloride, benserazide hydrochloride, and Ro 25-6981 were purchased from Tocris (Bristol, U.K.). NVP-AAM077 was provided by Novartis Institutes for BioMedical Research (Basel, Switzerland). *d*-Amphetamine, L-DOPA, and benserazide were dissolved in saline (doses corrected for salt) immediately prior to use. Ro 25-6981 required heating to dissolve, whereas NVP-AAM077 was dissolved in a small quantity of vehicle containing 0.1 mol/L NaOH, and final pH (7.4) was adjusted with HCl 1 mol/L.

Experimental Design. Fifty-five 6-OHDA hemilesioned rats developed moderate to severe AIMs (global ALO AIMs score >100) after chronic treatment with L-DOPA (6 mg/kg plus 12 mg/kg benserazide, once a day for 21 days) and were enrolled in the study (see below for details). One group of 8 dyskinetic rats underwent microdialysis for 3 consecutive days, and was randomly perfused with intrastriatal vehicle or NVP-AAM077 at two different concentrations (30 and 300 nM). The same protocol was repeated using Ro 25-6981 (30 and 300 nM) or saline on another group of 8 rats. A third group of 11 dyskinetic rats was then challenged with L-DOPA alone or in combination with 3 doses of NVP-AAM077 (0.01, 0.1, and 1 mg/kg) or vehicle. After each pharmacological testing session, animals were allowed 1 day washout and 2 days later challenged again with a standard dose of L-DOPA that worked as an internal control. The same protocol was applied to another 11 animals treated with saline or Ro 25-6981 (2.5, 5, and 10 mg/kg). In the last experiment, 8 dyskinetic animals underwent microdialysis for 3 days,[41] and were treated i.p. with vehicle, NVP-AAM077 (0.1 and 1 mg/kg), or Ro 25-6981 (5 mg/kg). For the dose-finding study, 8 naïve rats were treated i.p. with vehicle or NVP-AAM077 (3–20 mg/kg) and another 8 with i.p. saline or Ro 25-6981 (3 and 10 mg/kg).

Unilateral Lesion with 6-Hydroxydopamine. Unilateral lesion of dopaminergic neurons was induced in isoflurane-anesthetized Sprague–Dawley male rats according to standard procedures.[42,43] Eight micrograms of 6-OHDA free-base (dissolved in 4 μL) were stereotaxically injected into the medial forebrain bundle according to the following coordinates from bregma: antero-posterior (AP) −4.4 mm, medio-lateral (ML) −1.2 mm, dorso-ventral (DV) −7.8 mm below dura.[44] Two weeks after 6-OHDA injection, rats underwent an amphetamine challenge (5 mg/kg i.p.), and rats showing >7 turns/min in the direction ipsilateral to the lesion were enrolled in the study.[45,46]

Histological Evaluation of DA Denervation. Tyrosine hydroxylase (TH) immunohistochemistry was carried out as previously described.[46] Rats were deeply anaesthetized with Zoletil 100 (10 mg/kg, i.m.; Virbac Laboratories, Carros, France), transcardially perfused with 20 mM potassium phosphate-buffered saline (KPBS), and fixed with 4% paraformaldehyde in KPBS at pH 7.4. The brains were removed, fixed in the fixative overnight and transferred to 25% sucrose solution in KPBS for cryoprotection until they sunk. Serial coronal sections of 40 μm thickness were made using a freezing microtome. For each animal, five sections of the striatum were selected from the region spanning bregma −0.5 to +1.5, and processed for TH immunohistochemistry. Sections were rinsed in KPBS and incubated for 15 min in 3% H_2O_2 and 10% methanol in KPBS to block the endogenous peroxidase activity. After washing in KPBS, the sections were preincubated in blocking serum (5% normal horse serum and 0.3% Triton X100 in KPBS) for 60 min, followed by incubation in anti-TH mouse monoclonal antibody solution (1:2000, Chemicon, Temecula, CA) for 16 h at room temperature. The sections were then rinsed in KPBS and incubated for 1 h in biotinylated horse anti-mouse IgG secondary antibody (1:200; Vector Laboratories, Burlingame, CA). After rinsing, sections were incubated with avidin–biotin–peroxidase complex (Vector Laboratories) for 30 min at room temperature. After rinsing with KPBS, immunoreactivity was visualized by incubating the sections in a solution containing 0.05% 3,3-diaminobenzidine (DAB) in 0.013% H_2O_2 in KPBS for about 1 min. The sections were rinsed in KPBS, mounted on gelatin-coated slides, dried with ethanol and xylene, and coverslipped with mounting medium. Every section was viewed with a computer-interfaced light microscopy workstation (Axioskop, Zeiss), acquired with a high-resolution digital camera (AxioCam ICc3, Zeiss), and TH-immunoreactive fiber density analyzed using ImageJ software (Wayne Rasband; NIH). To estimate the TH-density staining, the optical densities were corrected for nonspecific background density which was measured in the corpus callosum. TH-positive fiber density was calculated as ratio between optical density in the lesioned and unlesioned side.

L-DOPA Treatment and AIMs Rating. Two weeks after amphetamine testing, DA-depleted rats underwent a 21 day course of L-DOPA treatment (6 mg/kg + benserazide 12 mg/kg, i.p., once daily) for induction of AIMs.[21,41,47,48] Quantification of L-DOPA-induced AIMs was carried out according to the scale proposed by Cenci and collaborators.[47] Briefly, rats were observed individually for 1 min every 20 min during the 3 h that followed L-DOPA injection. Dyskinetic movements were classified based on their topographic distribution into three subtypes: (i) axial AIMs, that is, twisted posture or choreiform twisting of the neck and upper body toward the side contralateral to the lesion; (ii) forelimb AIMs, that is, jerky or dystonic movements of the contralateral forelimb and/or purposeless grabbing movement of the contralateral paw; (iii) orolingual AIMs, that is, orofacial muscle twitching, empty masticatory movements and contralateral tongue protrusion. Each AIM subtype was rated on a severity scale from 0 to 4 (1 = occasional; 2 = frequent; 3 = continuous but interrupted by sensory distraction; 4 = continuous, severe and not interrupted by sensory distraction) at each monitoring period. In addition, the amplitude of these AIMs was scored based on a scale from 0 to 4 as described previously.[47] Axial, forelimb, and orolingual (ALO) AIMs were calculated as the sum of the products of amplitude and frequency scores from all monitoring periods. In order to select rats exhibiting stable and reproducible dyskinesias, AIM scoring was performed 5 times during the L-DOPA treatment period. All rats included in the microdialysis experiment had developed moderate-severe AIMs (severity grade ≥ 2 on each of the 3 AIM subtypes). GluN2B subunit selective antagonists were administered 15 min before L-DOPA.

Microdialysis Experiments. Dual probe microdialysis was performed as previously described.[41,45,48,49] Two probes of concentric design were

stereotaxically implanted under isoflurane anesthesia in the DA-depleted dorsolateral striatum (DLS; 3 mm dialysing membrane, AN69, Hospal, Bologna, Italy) and ipsilateral SNr (1 mm) or GP (1 mm) of dyskinetic rats according to the following coordinates from bregma and the dural surface:[44] DLS; AP +1.0, ML −3.5, DV −6; SNr, AP −5.5, ML −2.2, VD −8.3; GP, AP −1.3, ML −3.3, VD −6.5. Twenty-four hours after surgery, probes were perfused with a modified Ringer solution ($CaCl_2$ 1.2 mM; KCl 2.7 mM; NaCl 148 mM; $MgCl_2$ 0.85 mM; pH 6.5) at a 3 μL/min flow rate, and, after 6 h rinsing, samples were collected every 15 min. At least three baseline samples were collected before drug treatment. At the end of the experiments, animals were sacrificed and the correct placement of the probes was verified histologically.

NVP-AAM077 and Ro 25-6981 concentrations (30 and 300 nM) were the same as in our previous studies and selected on the basis of affinity and selectivity values.[16,17] NVP-AAM077 is a potent (affinity for recombinant GluN2A receptors of 8 nM)[50] and competitive NMDA antagonist with >100-fold selectivity for human recombinant GluN2A over GluN2B receptors,[50,51] although GluN2A/GluN2B selectivity ratio at rat recombinant receptors is much lower (∼7- to 13-fold);[52−54] suggesting that NVP-AAM077 should be considered a preferential GluN2A antagonist.[55]

Since in vitro recovery rate for GLU under our conditions is ∼18% (Marti et al.[45]), we set maximal NVPAAM077 concentration in the dialysate to 300 nM, in order to provide ∼50 nM in the extracellular space surrounding the probe. In vitro, this concentration has been reported to block 80% of NR2A responses and minimally affect NR2B responses (25%).[55] Different from NVP-AAM077, Ro 25-6981 has high affinity for native NR2B receptors (6 nM)[56] and much greater selectivity (∼5000-fold) for NR2B over NR2A subunits at recombinant rat NMDA receptors.[57] Therefore, 300 nM in the probe is likely to selectively block GluN2B subunits.

Endogenous GLU and GABA Analysis. GLU and GABA were measured by HPLC coupled with fluorometric detection as previously described.[46] Thirty microliters of *o*-phthaldialdehyde/mercaptoethanol reagent were added to 30 μL aliquots of sample, and 50 μL of the mixture was automatically injected (Triathlon autosampler; Spark Holland, Emmen, Netherlands) onto a 5-C18 Chromsep analytical column (3 mm inner diameter, 10 cm length; Chrompack, Middelburg, Netherlands) perfused at a flow rate of 0.48 mL/min (Beckman 125 pump; Beckman Instruments, Fullerton, CA) with a mobile phase containing 0.1 M sodium acetate, 10% methanol, and 2.2% tetrahydrofuran (pH 6.5). GLU and GABA were detected by means of a fluorescence spectrophotometer FP-2020 Plus (Jasco, Tokyo, Japan) with the excitation and the emission wavelengths set at 370 and 450 nm, respectively. The limits of detection for GLU and GABA were ∼1 and ∼0.5 nM, respectively.

Data Presentation and Statistical Analysis. Treatment effects on GABA and GLU levels have been expressed as percentage ± SEM of basal values (calculated as mean of the two samples before treatment). Absolute basal values are given in text and figure legends. Treatment effects on AIMs expression were expressed as percentage ± SEM of L-DOPA effect. Statistical analysis was performed on percentage data or area-under-the-curve (AUC) data by one-way analysis of variance (ANOVA) followed by the Newman-Keuls test for multiple comparisons. Relevant statistical results have been given in text. *P* values <0.05 were considered to be statistically significant.

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Author Contributions

O.S.M., F.M., M.C., M.B., and R.V. carried out the experimental work. A.D., C.G.P., and Y.P.A. provided essential tools (GluN2a and GluN2B antagonists) and were involved in experimental planning and data discussion. M.M. wrote the manuscript and supervised the experiments

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Notes

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ABBREVIATIONS

DA, dopamine; DLS, dorsolateral striatum; GLU, glutamate; GP, globus pallidus; 6-OHDA, 6-hydroxydopamine; NVP-AAM077, (*R*)-[(*S*)-1-(4-bromo-phenyl)-ethylamino]-(2,3-dioxo-1,2,3,4-tetrahydroquinoxalin-5-yl)-methyl]-phosphonic acid; Ro 25-6981, (*R*)-(*R**,*S**)-α-(4-hydroxyphenyl)-β-methyl-4-(phenyl-methyl)-1-piperidinepropanol; SNr, substantia nigra reticulata

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