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THE ROLE OF "NMDA" RECEPTOR SUBUNITS IN MOVEMENT DISORDERS INVOLVING BASAL GANGLIA: A BEHAVIOURAL AND NEUROCHEMICAL STUDY

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I. ABBREVIATIONS

3-NP	3-nitropropionic acid
6-OHDA	6-hydroxydopamine
BG	basal Ganglia
CaMKII	calcium/calmodulin-dependent protein kinase II
CO-I	cythocrome oxidase subunit I
DA	dopamine
DBS	deep-brain stimulation
DLS	dorsolateral striatum
ENK	enkephalin
ERK	extracellular signal-regulated kinases
GAD	glutamic acid decarboxylase
GLU	glutamate
GP	globus pallidus
GPe	external globus pallidus
GPi	internal globus pallidus
HD	Huntington's disease
Htt	huntingtin
L- DOPA	3,4-dihydroxyphenylalanine
LIVBP-like	leucin/isoleucin/valin-binding protein-like
MFB	medial forebrain bundle
m-Htt	mutant huntingtin
MSNs.	medium-sized spiny neurons
NO	nitric oxide
NVP-AAM077	(R)-[(S)-1-(4-bromo-phenyl)-ethylamino]-(2, 3-dioxo-1, 2, 3, 4 tetrahydroquinoxalin-5-yl)-methyl]-phosphonic acid
PD	Parkinson's disease
Ro 25-6981	(R-(R*,S*)-α-(4-hydroxyphenyl)-β-methyl-4-(phenylmethyl)-1-piperidinepropanol
SDH	succinate dehydrogenase
SN	substantia nigra
SNc	substantia nigra compacta
SNr	substantia nigra reticulata
STN	subthalamic nucleus
VTA	ventral tegmental area

1. INTRODUCTION

1.1 Basal Ganglia: circuitry and functions

The Basal Ganglia (BG) are a group of subcortical nuclei which controls the execution of motor programs and represses interfering signals from cortical motor and cognitive areas.

The BG include the striatum, globus pallidus (GP), subthalamic nucleus (STN) and substantia nigra (SN). The striatum is a single structure in rodents whereas is constituted by two distinct nuclei in humans: the caudate and the putamen. On the basis of its anatomical organization and connections, the dorsolateral part of the striatum (DLS) is placed in a crucial position along motor circuits. It integrates the inputs received from the cortex and thalamus and modulates the activity of the output structures of the BG. In humans, the GP is divided into two sub-regions, the external (GPe) and internal (GPi) segments. The rat homologous of these structures is the GP and entopeduncular nucleus, respectively. Finally, the SN is organized in substantia nigra compacta (SNc) and reticulata (SNr).

The current (simplified) model of the BG was first described at the end of the 1980s (Alexander *et al.*, 1986; Albin *et al.*, 1989; Delong, 1990).Briefly, normal motor behaviour depends on the balanced activity of two pathways that originate in the striatum: the so-called “direct” and “indirect” pathways. These two pathways modulate the activity of the BG output structures, GPi and SNr, in opposite way. Both GPi and SNr send inhibitory projections to the thalamus which dishinibits relevant and inhibits irrelevant excitatory thalamo-cortical projections, thus allowing appropriate motor programs to be executed and inappropriate programs to be blocked. The striatum is the major target of inputs arriving to the BG and receives extensive glutamatergic and dopaminergic afferents (Calon *et al.*, 2000; Gerfen, 1992). About 95% of striatal neurons are medium-sized spiny neurons (MSNs). The GABAergic neurons that give rise to the “direct” and the “indirect” pathways are different in their opioid and DA receptor subtypes expression. The neurons of the “direct” pathway express mainly excitatory D1 receptors and the opioids dynorphin and Substance P, while the neurons of the “indirect” pathway mainly express inhibitory D2 receptors and enkephalins (ENK) (Gerfen *et al.*, 1990; Gerfen, 1992). Neurons referred to as the “direct” pathway project monosinaptically to GPi and SNr. Conversely, the “indirect” pathway projects to the GPi/SNr via polysynaptic connections through the relay nuclei GPe and STN. Activation of the “direct” and “indirect” pathways affects the GPi/SNr neurons in

opposing manner, the “direct” pathway being inhibitory and the “indirect” one excitatory. The projection neurons in the GPi/SNr are tonically active and GABAergic in nature. Pausing their activity has been correlated with disinhibition of the thalamo-cortical system and movement initiation (Boraud *et al.*, 2000; Mink *et al.*, 1991).

According to this model, the essential pathophysiological characteristic of the parkinsonian state is the increased neuronal activity of the GPi/SNr, which leads to excessive inhibition of thalamo-cortical and brainstem motor systems (Albin *et al.*, 1989; Crossman, 1989; Obeso *et al.*, 2000; Obeso *et al.*, 1997). The model predicts that reduced activation of striatal DA receptors, caused by DA deficiency, results in disinhibition of neurons of the “indirect” pathway and decreased excitation of the neurons of the “direct” one (Obeso *et al.*, 2000; Penney *et al.*, 1986). Disinhibition of the “indirect” pathway leads to overinhibition of GPe, disinhibition of STN and increased excitation of GPi/SNr, whereas decreased activation of the “direct” pathway causes a reduction in its inhibitory influence on the GPi/SNr. The net result is an excessive activation of the BG output neurons accompanied by excessive inhibition of the motor system, leading to parkinsonian motor features. Following dopaminergic loss, the expression of D2 receptors and preproenkephalin increases in striatal neurons of the indirect pathway, whereas the expression of the D1 receptor, substance P and dynorphin decreases in neurons of the direct pathway (Gerfen *et al.*, 1991; Herrero *et al.*, 1995). Different experiments provide convincing evidence that neuronal activity is increased in STN and GPi. Indeed, an increase in STN and GPi/SNr activity has been demonstrated in MPTP-treated monkeys by 2-deoxyglucose uptake as a marker of synaptic afferent activity (Mitchell *et al.*, 1989), by in situ hybridization for cythocrome oxidase subunit I (CO-I) mRNA as a measure of mitochondrial activity (Vila *et al.*, 1997) and glutamic acid decarboxylase (GAD) mRNA as a measure of GABA activity (Herrero et al., 1996). Furthermore, lesions of STN and GPi induced marked motor improvement in MPTP-treated monkeys. Moreover, lesions or high frequency deep-brain stimulation (DBS) of these regions provide dramatic benefit to PD patients and restore thalamo-cortical activity.

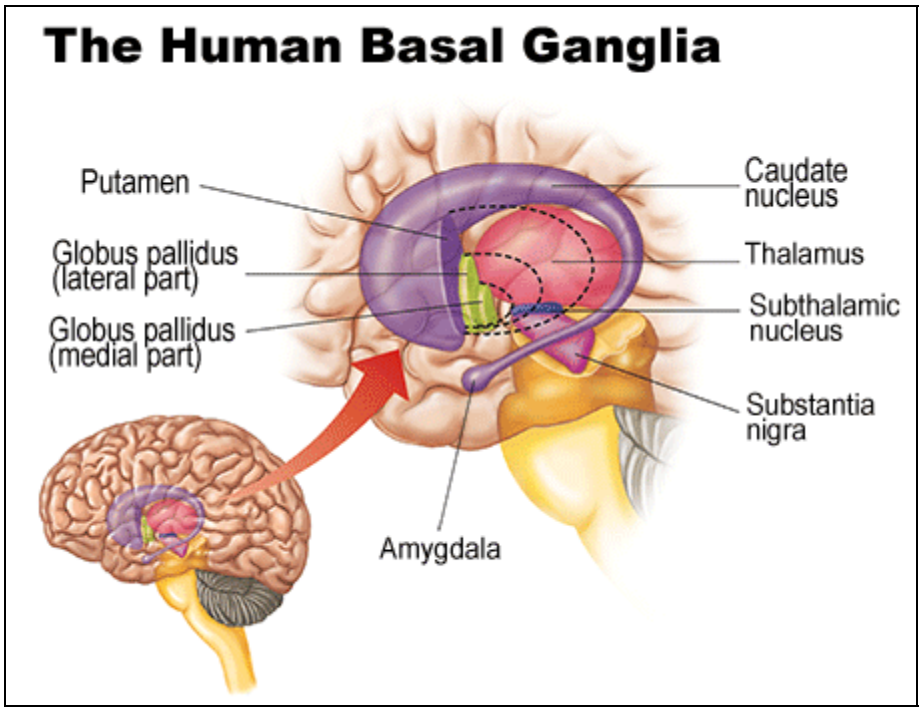


Fig. 1. The basal ganglia structures in humans.

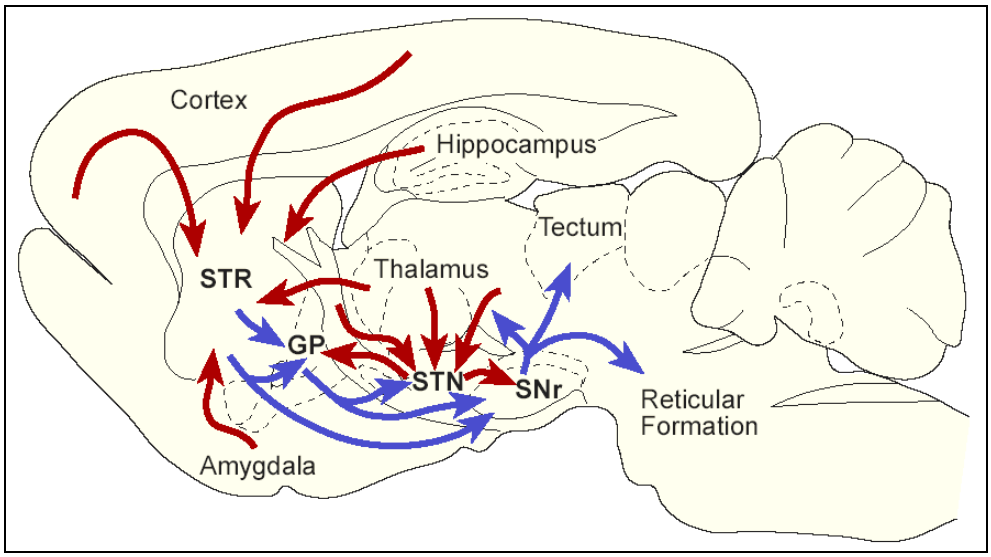


Fig. 2. Schematic diagram depicting the longitudinal organization of rat BG. Striatum, globus pallidus (GP), subthalamic nucleus (STN) and substantia nigra reticulata (SNr). Blue and red arrows indicate inhibitory and excitatory projections, respectively.

1.2 Neurodegenerative disorders affecting the BG

Several neurodegenerative pathologies, targeting specific neuronal types and BG structures, produce severe movement disorders that can give rise to akinetic or hyperkinetic syndromes.

Pathophysiologically, these disorders are all characterized by increased or reduced BG output, i.e. GPi/SNr activity, resulting in excessive inhibition/disinhibition of the thalamocortical system(s), respectively.

In this thesis I focused on Parkinson's disease (PD) and Huntington's disease (HD). The former is a hypokinetic disorder due to progressive degeneration of groups of brain neurons, particularly the dopaminergic neurons of SNc resulting in rigidity, tremor and akinesia. The latter is an inherited, autosomal dominant, neurodegenerative disorder characterized by involuntary choreiform movements and a progressive neuronal degeneration primarily affecting the striatal MSNs, leaving the other subpopulations of striatal cells largely unaffected at least in the early course of the disease.

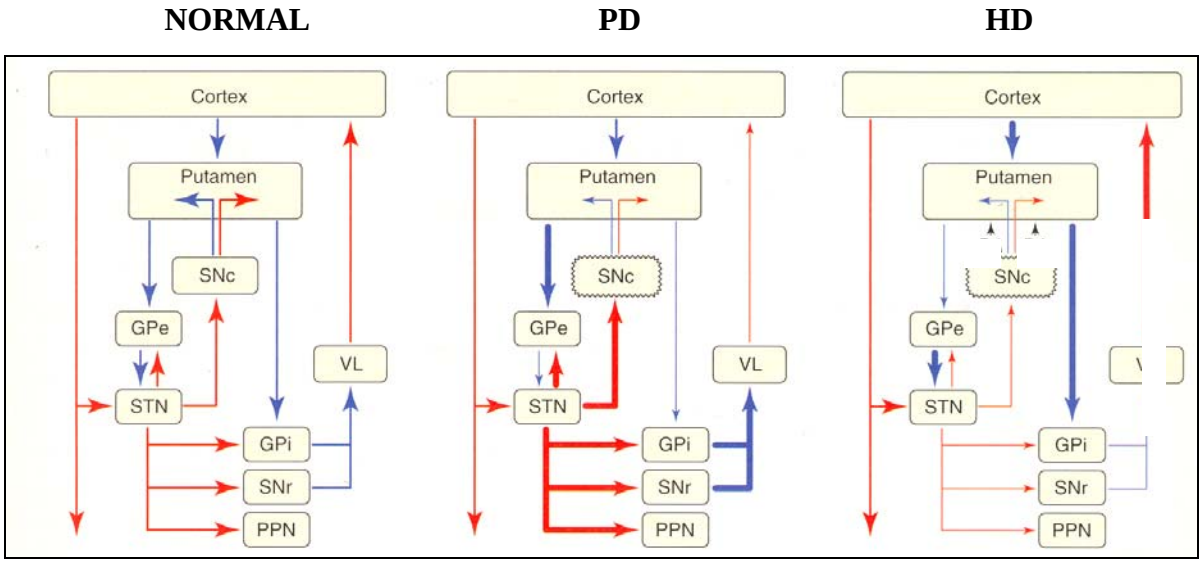


Fig. 3. The normal (left panel), parkinsonian (middle) and huntingtonian (right) states. Blue and red arrows indicate inhibitory and excitatory projections, respectively. Arrow thickness indicates the degree of activation of each projection. Note that the striatum communicates with output neurons in GPi and SNr through a "direct" and "indirect" pathway, the latter synapting in GPe and STN.

1.3 Parkinson's Disease

1.3.1 Clinical features

PD is the second most common neurodegenerative disorder in general population, after Alzheimer 's disease. PD was formally described for the first time in 1817 by James Parkinson in his monograph "Essay on the shaking palsy". Today it affects approximately 0.1% of the world population, with a prevalence of 1% in the population over 60 years of age. The average onset of the disease is between 50 and 60 years (Tanner and Goldman, 1996). The cardinal motor symptoms of PD include akinesia (absence of movements), bradykinesia (slowness in initiation and execution of voluntary movements), hypokinesia (reduced movements), rigidity, postural imbalance and resting tremors. The severity of these symptoms is generally mild in early disease but worsens as the disease progresses with a large variability among patients. In addition to motor symptoms, many patients also develop cognitive impairment, depression, anxiety and autonomic dysfunctions (Owen *et al*., 1992; Martignoni *et al.*, 1995; Aarsland *et al.*, 1999; Owen *et al.*, 2004). PD patients display two-fold to five-fold higher mortality rate than control subjects. Louis and colleagues (1997) showed that patients with PD and coexisting dementia had the highest mortality rate of all subgroups. Causes of death that were overrepresented in these patients (compared with the general population) included pneumonia, urinary tract infections, peptic ulcer disease, accidental injury, and vascular lesions of the central nervous system (Hoehn and Yahr, 1967).

1.3.2 Neuropathology

The main neurophatological feature of PD is the progressive degeneration of dopamine (DA) releasing neurons of the SNc. The first symptoms usually appear when 50-60% of these neurons have degenerated and 70-80% of striatal DA levels is lost (Marsden 1990). The extent of DA cell death well correlates with the severity of the disease-associated motor symptoms (Foley and Riederer, 1999). However, neurodegeneration in PD extends beyond DA neurons: it also affects the noradrenergic locus coeruleus, the serotonergic raphe nuclei, the cholinergic nucleus basalis of Meynert, the cerebral cortex, the olfactory bulb and the autonomic nervous system.

Another classical pathological finding in PD is the presence of Lewy bodies, which are small eosinophilic inclusions found in neurons of SN, locus coeruleus, and even cerebral cortex and brain stem. Although not entirely specific for PD, Lewy bodies have been

demonstrated in the brain of individuals without clinical evidence of the disease and thus may represent marker of the presymptomatic stage.

The aetiology of the disease is poorly understood. Familial PD has been identified in a subset of patients and is associated to mutations in several different genes including alpha-synuclein, parkin/PARK2, UCHL1, PINK1, DJ1/PARK7 and LRRK2 (reviewed by Tan and Skipper, 2007). These patients, however, only account for about 5-10% of all PD cases. The cause of most PD cases, idiopathic or sporadic PD, is largely unknown. Environmental factors may play an important role. This hypothesis is strengthened by the fact that herbicides/pesticides like Paraquat and Rotenone can give rise to parkinsonian symptoms in mammals (Betarbet *et al.*, 2000).

Several pathways are likely to participate in the neurodegenerative process of DA cell death, such as mitochondrial dysfunction, oxidative stress and inflammation (reviewed by Jenner and Olanow, 1998).

1.3.3 Treatment options for Parkinson's Disease

At the moment, there is no cure for PD. Conversely, several treatment options are available to alleviate symptoms and improve quality of life of patients. From a clinical point of view, symptomatic therapy has been the most successful. In particular, 3,4-dihydroxyphenylalanine (L-DOPA) is administered, sooner or later, to all PD patients in order to improve their mobility (Deleu *et al.*, 2002). Indeed L-DOPA, has been the gold standard treatment for PD patients since Cotzias and colleagues described its therapeutic and clinical value in the late 1960s (Cotzias *et al*., 1967). L-DOPA can cross the blood brain barrier and be converted to DA by the enzyme L-aromatic aminoacid decarboxylase. In order to avoid peripheral decarboxylation, L-DOPA is combined with a decarboxylase inhibitor that does not cross the blood brain barrier (Carbidopa or Benserazide). L-DOPA therapy revolutioned the treatment of this disorder, providing dramatic benefit to virtually all PD patients, prolonging the ability of the patients to remain independent and increasing their survival. The standard oral L-DOPA therapy is well established in the clinic today and provides generally satisfactory symptomatic relief for the first 5-10 years of treatment. However, after approximately 5 years of treatment, a series of complications develops in more than 50% of PD patients. These side-effects encompass motor fluctuations and abnormal involuntary movements or dyskinesias (Marsden *et al.*, 1977; Obeso *et al.*, 1989; Obeso *et al.*, 2000). The prevalence of motor fluctuations and dyskinesias increases with the duration and severity of the disease as well as with the duration of treatment (Colosimo *et al.*, 1999;

Grandas *et al.*, 1999). Thus, after 10 years of L-DOPA therapy, motor complications have developed in 70-80% of patients, and in almost 100% of those patients with disease onset below the age of 45-50 years (Quinn *et al.*, 1987). L-DOPA also causes non-motor side effects such as hallucinations and psychosis (Kuzuhara *et al.*, 2001; Nausieda *et al*., 1984).

A number of other medications can be used alone or in combination with L-DOPA to improve movement and reduce L-DOPA dosage, in an attempt to reduce L-DOPA side-effects . The most commonly used medications are DA receptor agonists, amantadine, anticholinergic drugs, MAO-B inhibitors (protecting DA from intraneuronal degradation), COMT inhibitors (reducing catabolism of L-DOPA in the periphery and of DA in the CNS).

Another therapeutic approach to PD is represented by surgical interventions, either via implantation of stimulating electrodes (DBS) or surgical ablation of parts of the BG network.

Dopaminergic dysfunction that cannot be controlled with medications can now be effectively treated with STN DBS (or sometimes with pallidal DBS). Appropriate candidates are not those failing L-DOPA, but rather those with fluctuating L-DOPA responses or dyskinesias. STN is usually the preferred target for DBS surgery in such PD patients (Ashkan *et al.*, 2004).

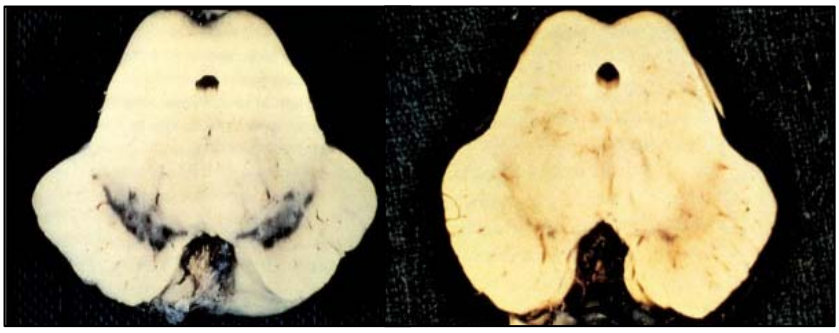


Fig. 4. On the left, the intact SNc, on the right, the SNc of a patient affected by PD

1.4 Huntington's Disease

1.4.1 Clinical features

Huntington's disease (HD) took his eponymous designation from George Huntington, who first described this hereditary form of chorea in 1872. HD is an autosomal, dominantly inherited neurodegenerative disorder characterized by extrapyramidal motor symptoms, cognitive function and behavioural changes (Kremer *et al.*, 1992). The prevalence is 5 to 8 per 100,000, varying geographically: approximately 1 person per 10,000 people of Western European descent and 1 per 1,000,000 of Asian and African descent.

The symptoms of HD can surge at any time between the ages of 1 and 80 years. In the presymptomatic phase, individuals might be characterized by changes of personality, irritability, anxiety, forgetfulness.

The diagnosis is mainly based on family history (although about 8% of patients do not have a known affected family member; Almqvist *et al.* 2001) and typical symptoms are distinct chorea, incoordination, motor impersistence, slowed saccadic eye movements and cognitive decline.

The cognitive dysfunction impairs executive abilities as organising, planning, checking or adapting alternatives, and delays the acquisition of new motor skills. Psychiatric disorders can develop; a typical one is depression and it has been estimated that suicide is about 5 to 10 times higher than in the general population (5-10%; Wahlin *et al.*, 2000). Chorea, useful for diagnosis, is not a good marker of disease severity: in fact it becomes less prominent with the later onset of dystonia and rigidity (Mahant *et al.*, 2003). Motor impersistence, i.e. the inability to maintain a voluntary muscle contraction at a constant level, is linearly progressive and can be considered a marker of disease severity. Fine motor skills are useful for establishing an early diagnosis of HD, while gait and postural maintenance deteriorate later in the disorder course. About 10% of HD cases occurs in people under the age of 20. This is referred to as Juvenile HD, as early as 2 years, and is characterized by prominent slowed saccadic eye movement and rigidity rather than chorea. HD is distinguished from PD by the different time course, the inheritance, the motor impersistence and impaired saccades. There is no cure for HD. Treatment focuses on reducing symptoms, preventing complications, and providing support and assistance to the patient and caregivers. Physicians often prescribe various medications to help control emotional and movement problems: antipsychotics,

antidepressants, tranquilizers, mood-stabilizers and botulinum toxin for dystonia and jaw clenching.

Patients usually die from complications of falls, inanition, dysphagia or aspiration within 20 years from symptom onset.

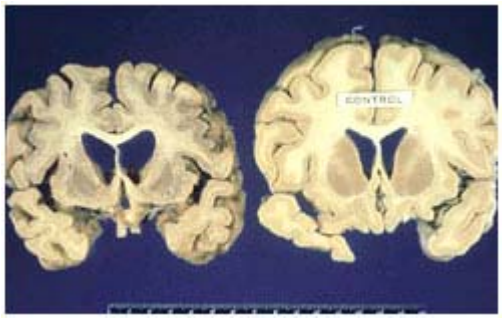


Fig. 5. On the left, a frontal cross-section of the brain of a patient who died of Huntington's disease. On the right a frontal cross-section of a person who died of a non-neurological disorder. In the case of Huntington's disease, neurons are lost in different areas of the brain.

1.4.2 Neuropathology

The most striking neuropathological hallmark of this disorder is atrophy of the striatum (Ferrante *et al.*, 1997). The neurodegeneration preferentially affects the ENK-positive MSNs that project to GPe (Mitchell *et al.*, 1999; Rubunisztein *et al.*, 2003) This is in line with the finding that that chorea dominates early in the course of HD because of a preferential involvement of the “indirect” pathway of BG-thalamocortical circuitry. In physiological conditions, the indirect pathway is involved in inhibition of voluntary movements. Death of the ENK-positive MSNs causes activation of the premotor and supplementary motor cortices and produces the typical hyperkinetic and choreiform movements seen in HD. In later stages of the disease, death also occurs in the neurons of the “direct” pathway, which is normally involved in the initiation of voluntary movements. Death of these neurons blocks the activation of the premotor and supplementary motor cortices, producing hypokinetic symptoms.

The cholinergic and medium-sized aspiny interneurones of the striatum are preserved from the disease. The neurodegeneration affects also other brain areas: the SNr, the cerebral cortex (III, V and VI layers), the CA1 region of the hippocampus, the angular gyrus in the parietal lobe, the Purkinje cell of the cerebellum, the lateral tubercular nuclei of the hypothalamus and the centromedial parafascicular complex of the thalamus.

One of the pathological characteristics of HD is the presence of nuclear and cytoplasmic inclusions containing mutant huntingtin (m-Htt) and polyglutamine in the cerebral cortex and striatum (Di Figlia *et al.* 1997). It has been proposed that soluble or

oligomerized forms of m-Htt are associated with toxicity, whereas aggregates correspond to a protective compensatory event. In fact, it seems that these inclusions are not predictors of cellular dysfunction or disease activity. On the contrary, the neurons containing inclusions survive longer than those that do not develop them, suggesting that inclusion formation can protect neurons by reducing the levels of toxic forms of m-Htt (Arrasate et al, 2004).

1.4.3 Molecular basis of Huntington's disease and possible pathogenic mechanisms

The human HD gene is located on the short arm of chromosome 4 (4p16.3) and encodes the protein Htt, which contains more than 3000 residues. The physiological role of the protein is still not clear, but there is compelling evidence that wild type Htt, particularly expressed in neurones, is a ubiquitous protein that plays major role in regulating cell survival.

HD is related to a CAG trinucleotide repeat expansion in the HD gene that is translated into an abnormally long polyglutamine tract close to the N-terminus of Htt. Asymptomatic individuals have 35 or fewer CAG repeats, whereas HD is caused by expansion to 36 or more repeats (Rubinsztein *et al.*,1996).

Different approaches demonstrate that physiological Htt is involved in several key cellular functions by interacting with various proteins: transcription (CBP, REST, sp1/TAFII130, CA150), intracellular trafficking through microtubule proteins (p150glued, HAP1), vesicle secretion (BDNF vesicles), survival/death signalling (Akt, EGF receptor, HIP1, p53), proteolysis (caspases, calpain), protein degradation (ubiquitin) and neurotransmitter receptor regulation (PSD-95).

The loss-of-function of physiological Htt contributes to degeneration in HD (Cattaneo, 2003) In line with this, Htt inhibits the so called apoptotic “mitochondrial pathway” involving caspase-9 and caspase-3 (Rigamonti *et al.*, 2000,2001). Several early alterations are correlated to the presence of m-Htt: alteration of different transcription factors (Sugars and Rubinsztein, 2003; Luthi–Carter and Cha, 2003), defects in axonal transport, proteasome dysfunction, increased transglutaminase activity, perturbation of calcium homeostasis and synaptic function (Gunawardena *et al.*, 2003; Li *et al.*, 2003; Szebenyi *et al.*, 2003; Tang *et al.*, 2003; Bezprozvanny and Hayden 2004; Gauthier *et al.*, 2004).

Among these mechanisms, early changes in mitochondrial homeostasis may be a key feature of HD. Biochemical studies show consistent defects in mitochondrial complex

II-III in the caudate nucleus of HD patients (Stahl and Swanson, 1974; Browne *et al.*, 1997), with a decrease in succinate oxidation ranging from 39 to 59%. Also in the putamen a 67% decrease in succinate oxidation has been found (Browne *et al.*, 1997). Although also complex IV (cytochrome oxidase) is significantly, but to a lesser extent, affected in these brain areas, the defects appear rather selective, because complex I activity (NADH dehydrogenase) is spared in HD brain samples. In line with these findings, Choo *et al.* (2004) demonstrated that Htt is associated with outer mitochondrial membranes and that m-Htt is able to promote mitochondrial permeability transition as well as decrease in Ca^{2+} threshold necessary to induce it. Moreover, cells expressing a full-length mutant form of Htt displayed increased sensitivity to complex II inhibition, whereas complex I inhibition did not potentiate cell death (Ruan *et al.*, 2004). The m-Htt induced alteration of mitochondrial homeostasis and cell death are promoted through p53-dependent mechanisms (Bae *et al.*, 2005). Altogether, this evidence suggested that defects in mitochondrial function and, more specifically, in complex II/ succinate dehydrogenase (SDH) activity are involved in HD.

1.5 NMDA receptors

1.5.1 General features

Glutamate (GLU) is the main excitatory neurotransmitter in the brain and glutamatergic innervation of BG structures is massive. The cerebral cortex sends abundant glutamatergic projections to the striatum, STN and SNc. The striatum receives glutamatergic projections also from the thalamus (from the midline and intralaminar nuclei). The STN is an excitatory output that innervates the GP and SNr.

The actions of GLU on target neurons are mediated by 3 classes of ionotropic receptors, named (according to the selective agonists) NMDA, AMPA, kainate, and 3 classes of metabotropic receptors classified in 3 groups (I-III) on the basis of their pharmacological properties (Hollmann and Heimann, 1994; Conn and Pin, 1997; Dingledine *et al.*, 1999).

The role of striatal GLU receptors in motor control has been investigated intensively mainly focusing on the NMDA subtype (Schmidt *et al.*, 1992). Native NMDA receptors are believed to be hetero-oligomers, most likely tetramers or pentamers (Laube *et al.*, 1998), of structurally related proteins distinguished in 3 families: NR1, NR2 and NR3 (Monyer *et al.*, 1994; Dingledine *et al.*, 1999).

Functional NMDA receptors are thought to contain at least 1 NR1 subunit, required for channel activity, and 1 NR2 subunit (Hollmann and Heinemann, 1994; Dingledine *et al.*, 1999), whereas the recently discovered NR3 subunit, appear to be scarcely expressed in the adult brain, except for the motor neurons of the spinal cord (Nishi *et al.*, 2001).

Both NR1 and NR2 proteins are transmembrane proteins with three complete transmembrane regions and one intramembrane loop between the first and the second complete transmembrane regions (Dingledine *et al.*, 1999). The aminoterminal region of each protein is extracellular and contains ligand binding regions and multiple glycosylation sites. The carboxyterminal region is intracellular and may control regulation of the receptor by second messenger systems, perhaps through multiple tyrosine and serine phosphorylation sites. This intracellular domain anchors NMDA receptors to specific cellular locations and attaches distinct messenger systems to the receptors (Niethammer *et al.*, 1996). NR2A and NR2B subunits differ from other subunits (NR1, NR2C and NR2D) for being uniquely endowed with COOH terminal extensions of greater than 600 residues that contain scattered regions of conserved sequence between the two forms. The extra peptide sequence in NR2A and NR2B subunits is located intracellularly in the C-terminus, a region of low homology between different NR2 subunits. Since the function of this domain is likely to modulate receptor localization and anchoring, this suggests that different subunits may be associated with different responses to messenger systems. This COOH terminal sequence might provide additional target sites for cellular constituents regulating channel function, location, and assembly.

Eight splice variants of the NR1 subunit have been described (Hollmann *et al.*, 1993), while four different genes encode for the NR2 subunit (NR2A- D). Different combinations of NR1 splice variants and NR2 subunits lead to the formation of NMDA receptors with peculiar functional characteristics (Monyer *et al.*, 1994; Chen *et al.* 1999)

1.5.2 Heterogeneity and modulators

The NMDA receptors are activated by GLU, in the presence of the co-agonist glycine. The binding sites for GLU and glycine have been localized onto the NR2 and NR1 subunits, respectively (Kuryatov *et al.*, 1994 ; Laube *et al.*, 1997). Variations in the proportion of NR2 subunits alter both the affinity of the channel for glutamatergic ligands (Christie *et al.*, 2000) and the interaction between NR1 and glycine (Lynch *et al.*, 1994; Honer *et al.*, 1998).

A feature of the channel coupled to NMDA receptor is the voltage-dependent blockade operated by magnesium ions (Mg^{2+}) at normal resting membrane potentials; thus, opening of the channel requires both agonist binding and membrane depolarization. The Mg^{2+} block is removed once the neuron has been depolarized by another excitatory input, for example by activation of AMPA/kainate receptors. The NR2 subunit composition influences the sensitivity of the channels to Mg^{2+} blockade (Kuner and Schoepfer, 1996). Another divalent cation, zinc (Zn^{2+}), is also able to block the NMDA channel, although this blockade is much weaker than that of Mg^{2+} and voltage-independent.

The NMDA receptors are also sensitive to other modulators. Extracellular polyamines (i.e. putrescine, spermidine, spermine) can interact with the receptor by at least 3 mechanisms, resulting in either potentiation or inhibition of the NMDA receptor function. Firstly, high concentrations of polyamines can cause voltage-dependent inhibition by blocking the channel pore (Williams, 1994). Secondly, low concentrations of polyamines enhance the affinity of the receptor for glycine, thus potentiating the NMDA currents (Dingledine *et al.*, 1999). Thirdly, in the presence of saturating concentrations of glycine, polyamines can also cause a glycine-independent increase in NMDA receptor channel activation (Williams, 1994; Dingledine *et al.*, 1999).

Redox reactions (reduction and oxidation) by covalent modification of sulfhydryl (thiol) groups on cysteine residues can also regulate the NMDA receptor function. Studies on the redox site(s) in recombinant NMDA receptors has lead to identification of two cysteine residues on the NR1 subunit (Sullivan *et al.*, 1994). Oxidation of these cysteine residues by experimental agents can attenuate NMDA receptor responses while their reduction can enhance NMDA receptor responses (Dingledine *et al.*, 1999).

Ion channels phosphorylation of is an important regulatory mechanism in synaptic plasticity (Kohr and Seeburg, 1996). NMDA receptors can be phosphorylated by PKA, PKC, calcium/calmodulin-dependent protein kinase II (CaMKII) and tyrosine kinases (e.g., Src and Fyn) (Dingledine *et al.*, 1999). Phosphorylation serves to increase molecular and functional heterogeneity of NMDA receptors within the NMDA receptor family. The phosphorylation state of NMDA receptors can alter channel opening probability, receptor activation, subcellular distribution, and synaptic targeting of receptors. Several phosphorylation substrate sites have been identified on NR1, NR2A and NR2B subunits (Tingley *et al.*, 1993; Leonard and Hell, 1997; Colbran *et al.*, 2004; Chen and Leonard, 1996).

The predominant NR2 subunits expressed in the adult forebrain are NR2A and NR2B. A variety of studies demonstrate differences in subcellular distributions of NR2A and NR2B- containing NMDA receptors, the former being mainly associated with synapses and the latter predominating at extra-synaptic sites (Li *et al.*, 1998; Barria et Malinow, 2002).

Although recombinant receptors of NR1/NR2A, NR1/NR2B and NR1/NR2A/NR2B types show similarities in single channel conductance, calcium permeability and sensitivity to Mg^{2+} block, they differ in their channel gating properties and sensitivity to agonists and antagonists. (Dingledine *et al.*, 1999; Kew and Kemp, 2005). Indeed, the electrical properties of the channels are modulated by the NR2 subunit, the activation and deactivation kinetics of the NR1/NR2A type in expression systems being faster than those of the NR1/NR2B type (Monyer *et al.*, 1992).

Moreover, the potency of competitive and allosteric antagonists relies on the NR2 subunit and not the NR1 isoform (Lynch and Gallagher, 1996; Fischer *et al.*, 1997). NR1/NR2A heteromers have high affinity for competitive antagonists and thus are defined "antagonist preferring" while NR1/NR2B have high affinity for GLU and are "agonist preferring".

These differences seem to reside in a different geometry and electrostatic potential distribution in the binding site, although the difference in amino acidic sequence in the two subunits reach only the 20%. GLU binds to NR2A and NR2B subunits in correspondence of the site formed by the globular region S1 and S2. However, several studies have demonstrated the existence of another important domain in the region that precedes the binding site domain for GLU. This domain, defined LIVBP-like (leucin/isoleucin/valin-binding protein-like), is important for allosteric modulation of the receptor. This region, constituted by the first ~ 415 N-terminal residues, is homologous to the bacterial leucine, isoleucine and valine binding protein.

The LIVBP-like domain consists of two distinct globular subdomains (or lobes) separated by a deep groove or cleft. Each lobe shows alternation of β strands and α helices arranged as a buried central β sheet flanked on both sides of the plane by α helices. The two lobes are interconnected by a hinge made of three short linkers. The lobe containing the most N-terminal amino acid is referred to as lobe I and the other as lobe II.

Sequence differences in this region play crucial roles in subtype-specific receptor modulation by allosteric modulators such as zinc and ifenprodil. Indeed, the region

containing the LIVBP-like domain constitutes part of a NR2A-specific Zn^{2+} binding site, to which Zn^{2+} binds to promote the channel closure (Fayyazuddin ey al., 2000; Paoletti *et al.*, 2000), whereas in the NR2B subunit is the binding site for selective antagonists like ifenprodil and its derivatives.

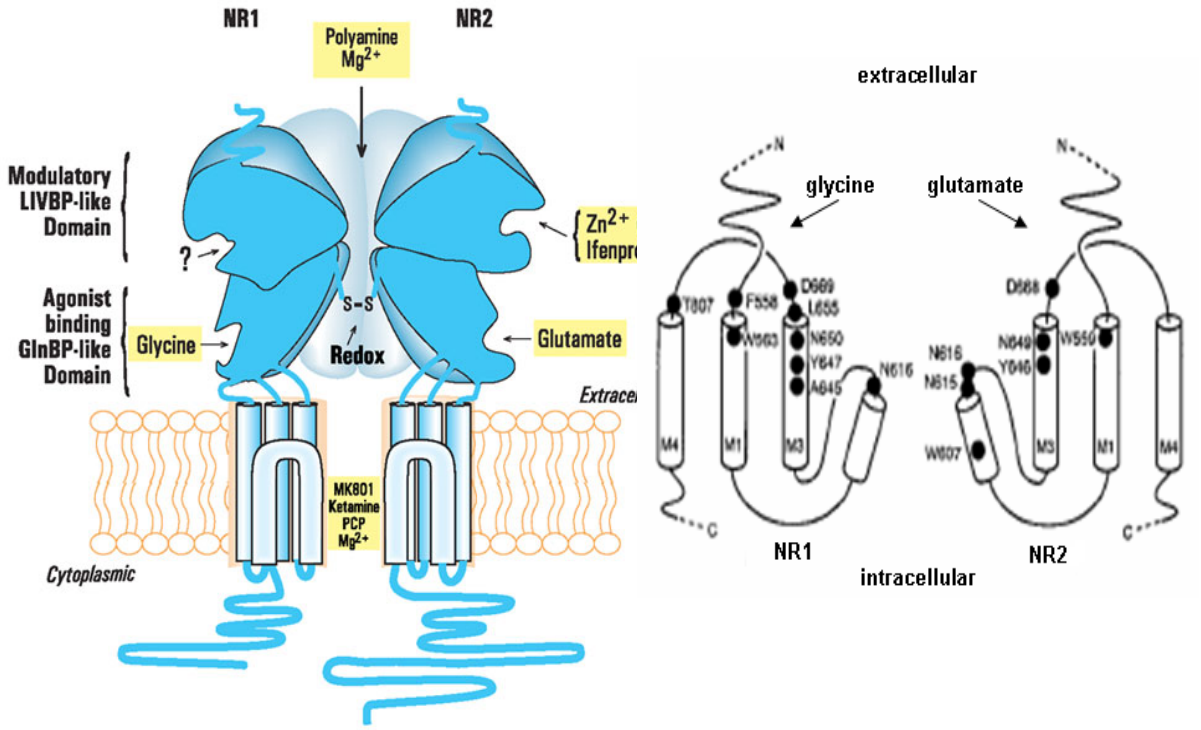


Fig. 6. Schematic representation of NMDA receptor and its modulatory sites (left) Representation of the domains of NR1 and NR2 subunits, with the binding sites for glycine and glutamate on the NR1 and NR2 subunit, respectively (right).

1.6 Localization and function of NMDA receptors in the basal ganglia

NMDA receptors play a role in the regulation of BG function at several levels. The distribution of receptor subunits has been studied in rodent, nonhuman primate and human brains by using both methods to localize mRNA transcripts as well as direct examinations of the encoded protein subunits (Ravenscroft and Brotchie, 2000; Smith *et al.*, 2001). In general, the distribution and relative abundance of NMDA receptors in the various species are remarkably similar. It has been seen that NMDA receptors undergo developmental changes in the brain: the NR2B subunit is expressed throughout the embryonic brain but is essentially restricted to forebrain regions postnatally, whereas the NR2A subunit is progressively added during development and is ubiquitously expressed in the adult brain (Watanabe *et al.*, 1992; Monyer *et al.*, 1994).

1.6.2 Striatum

NMDA receptor binding sites are very abundant (Tallaksen Greene *et al.*, 1992) and the majority of NMDA receptors are located postsynaptically on striatal neurons rather than presynaptically on striatal nerve terminals (Samuel *et al.*, 1990; Wüllner *et al.*, 1994).

In the rat, projection neurons express NR1, NR2A, and NR2B subunits, with little difference in subunit expression between striato pallidal and striato-nigral neurons (Standaert *et al.*, 1994, 1999). Biochemical studies have demonstrated that NR1 and NR2 receptor subunits may be assembled as both heterodimeric receptor complexes (containing NR1 and NR2A, or NR1 and NR2B) and heterotrimeric complexes (containing NR1, NR2A, and NR2B). When striatal tissue is fractionated, the heterotrimeric receptors are found concentrated in synaptic fractions, while the heterodimeric receptors may be found in both synaptic and nonsynaptic pools (Dunah and Standaert, 2003). Cholinergic interneurons can be distinguished from other striatal neurons by the absence of detectable NR2A subunit mRNA. Other types of interneurons express NR1, NR2A, NR2B, and NR2D subunits. NR2C subunits are expressed at very low level in striatal neurons (Standaert *et al.*, 1996, 1999). The cellular distribution of NMDA receptor subunits in human striatum is similar but not identical to that observed in rat striatum (Kuppenbender *et al.*, 2000). A relatively stronger NR2A signal in striato-nigral neurons compared to the striato-pallidal ones has been found. In human cholinergic interneurons, a similar pattern of NMDA receptor subunit distribution is detected, with the presence of NR1, NR2B, and NR2D subunits and the absence of NR2A subunits. NR2A subunits are absent in somatostatin-expressing interneurons of the human but not rat striatum.

1.6.2 Subthalamic nucleus

Binding studies demonstrated that NMDA receptors are present at a relatively lower density than AMPA, kainate and metabotropic receptors (Tallaksen Greene *et al.*, 1992). The NMDA receptors present in the STN are mainly of the NR1/NR2D form. There is little or no expression of NR2A or NR2B subunit in the normal rat STN (Standaert *et al.*, 1994). NMDA-mediated afferent inputs have an important role in controlling the activity of the STN (Nakanishi *et al.*, 1988; Gotz *et al.*, 1997). Blockade of STN NMDA receptors interrupts GP excitation after cortical stimulation, suggesting that these receptors are important in physiological modulation of this outflow pathway (Nambu *et al.*, 2000). STN NMDA receptors also play a role in sustaining pathological hyperactivity of the STN observed in models of parkinsonism, as demonstrated by the

normalization of motor behaviour produced by chronic infusion of an NMDA antagonist into the STN of 6-hydroxydopamine (6-OHDA) lesioned rats (Blandini *et al.*, 2001). Blockade of STN NMDA receptors also blunts the haloperidol-induced akinesia in the rat (Miwa *et al.*, 1998). These observations suggest that inhibition of STN NMDA receptors might provide therapeutic benefit in PD.

1.6.3 Globus pallidus pars interna (GPi) and Substantia nigra reticulate (SNr)

NMDA receptors are present in BG output structures, i.e. GPi and SNr. Both nuclei largely express NR1 and NR2D subunits, which can be detected both at the mRNA and protein level (Standaert *et al.*, 1994; Wenzel *et al.*, 1996). Studies on the role of these receptors in regulating the activity of GPi and SNr neurons have been relatively few. The primary glutamatergic input to GPi/SNr neurons arises from the STN (Parent and Hazrati, 1995b). NMDA antagonists administered into the GPi or SNr are expected to offset the STN hyperactivity seen in PD. Indeed, in a small primate study improvement of parkinsonism after local injection of non selective NMDA antagonists was observed (Graham *et al.*, 1990).

1.6.4 Dopaminergic neurons

NMDA receptors are found on dopaminergic neurons. There is a high density of NMDA receptor ligand binding sites in SNc (Albin *et al.*, 1992). In rat as well as human brain, in situ hybridization studies have demonstrated that the most abundant subunits are NR1 and NR2D (Standaert *et al.*, 1994; Counihan *et al.*, 1998). Only very low levels of NR2A, NR2B and NR2C mRNA are present.

Excitatory inputs to dopaminergic neurons in the SNc arise primarily from the prefrontal cortex, with additional input from the STN and the pedunculopontine tegmental nucleus (Smith *et al.*, 1996, 1998). NMDA receptors present on dopaminergic neurons may play a role in the neurodegenerative process that occurs in PD, but evidence of this is largely circumstantial.

1.6.5 Rat globus pallidus

The rat globus pallidus, the homologous of GPe in primates, has a lower density of NMDA specific binding sites with respect to other BG nuclei (Monhagan and Cotman, 1985). Several studies, by means of in situ hybridization or $[H]^3$ Ro 25-6981 labeling, revealed no detectable NR2A subunits and medium levels of NR2B labelling. The GP shares with entopeduncular nucleus, SNc and the majority of SNr neurons, a similar NMDA receptor phenotype characterized mainly by NR1C and NR2D subunits (Standaert *et al.* 1994; Mutel *et al.* 1998: Monyer *et al.*, 1992; Wenzel *et al.*, 1995).

1.7 NMDA receptors and Parkinson's disease

Several studies have reported an increase in NMDA receptor binding sites (Samuel *et al.*, 1990) and/or change of functional NMDA receptor properties (Schwarting and Huston, 1996; Chase *et al.*, 1998) in DA-depleted rats.

More recently, it has been demonstrated that NR1, NR2A and NR2B subunit expression is differently affected by 6-OHDA lesioning. Thus, NR2A mRNA levels were increased, NR1 mRNA levels were decreased and NR2B mRNA levels were not affected (Kayadjanian *et al.*, 1996; Ulas and Cotman, 1996; Dunah *et al.*, 2000; Ganguly and Keefe, 2001; Betarbet *et al.*, 2004).

A number of reports have demonstrated that increased corticostriatal glutamatergic transmission plays a crucial role in motor symptoms of PD (Mitchell and Carrol, 1997). Accordingly, considerable efforts have been made over the last decade in an attempt to elucidate the molecular mechanisms of dopamine/NMDA receptor interaction, which eventually becomes pathologically abnormal in the DA-depleted striatum (Chase and Oh, 2000; Hallett *et al.*, 2006).

Several kinase pathways are responsible for receptor-mediated gene regulation in striatal neurons. D1 receptor stimulation is physiologically linked to PKA activation, whereas the NMDA receptor, through Ca2+-mediated phosphorylation, is responsible for CaMKII, and extracellular signal-regulated kinases (ERK) activation. In the DA-depleted striatum, up-regulation of these kinases has been detected and proposed as the event underlying the pathological DA-GLU interactions (Chase and Oh, 2000; Gerfen *et al.*, 2002; Picconi *et al.*, 2004). This up-regulation produces abnormal phosporylation of NMDA receptors: increased tyrosine phosphorylation of NR2B subunit as well as CaMKII or PKA-dependent serine phosphorylation of NR2A and NR1 subunits have been reported in the DA-depleted striatum (Menegoz *et al.*, 1995; Oh *et al.*, 1999; Betarbet *et al.*, 2004). In turn, NMDA receptor subunit phosphorylation is an important regulatory mechanism of receptor function, affecting the probability of receptor channel opening, $Ca2^{+}$ influx and subcellular distribution of channel complexes and their anchoring to the membrane (Wang and Salter, 1994; Hisatsune *et al.*, 1997).

1.8 NMDA receptors and Huntington's disease

NMDA receptor-mediated excitotoxicity has been suggested to be involved in the pathogenesis of HD.

The earliest changes associated with neuronal expression of m-Htt are increased NMDA receptor-mediated currents, disrupted calcium homeostasis and enhanced vulnerability to NMDA-mediated excitotoxicity (Li *et al.*, 2004). These changes occur primarily in the striatal projection neurons despite the fact that Htt and NMDA receptors are localized together in most brain areas. Excitotoxic neuronal cell damage is mediated in part by overactivation of NMDA receptors, which results in excessive Ca^{2+} influx through the receptor-associated ion channel and subsequent free radical formation. Riluzole, a neuroproctetive agent used in the therapy of amyotrophic lateral sclerosis, a broad spectrum GLU receptor antagonist and also suppressor of GLU release (Starr *et al.*,1997) reduces the ubiquitin aggregations in the R6/2 mice (Shiefer *et al.*, 2002).

Physiological NMDA receptor activity, however, is also essential for normal neuronal function. This means that potential neuroprotective agents that block virtually all NMDA receptor activity will very likely have unacceptable clinical side-effects. For this reason many NMDA receptor antagonists have disappointingly failed advanced clinical trials for a number of neurodegenerative disorders.

Several lines of evidence suggest that NR2B-containing NMDA receptors are involved in the early neurodegeneration seen in HD. Adult striatal projection neurons are enriched in NR2B-containing NMDA receptors and NR2B expression has been shown to be high in the subpopulation of striatal projection neurons that degenerate first in HD (Li et all., 2003; Kuppenbender *et al.*, 2000). Furthermore, expression of m-Htt enhances the excitotoxicity predominantly mediated by NR2B receptors (Chen *et al.*, 1999; Zeron *et al.*, 2001). Indeed, Li *et al.* (2004) showed that the enhanced striatal NMDA-mediated synaptic currents in a mouse model of HD are caused by potentiation of the postsynaptic NR2B-containing NMDA receptor current. Finally, NR2B containing NMDA receptors are preferentially involved in apoptotic cell death (Chenard and Menniti, 1999; Hardingham et al , 2002) and genotype variation of NR2B subunits has been shown to significantly influence the age of onset of HD in humans (Arning *et al.*, 2005).

In genetic animal models of HD, it has been demonstrated that there is a age-related decrease in striatal expression of NMDA receptors containing the NR2A but not NR2B

subunit (in R6/2 mice; Ali *et al.*, 2004) and that there is an enhanced activity of NR2B containing receptors (YAC72 mice; Li *et al.*, 2004).

Taken together, these data suggest an interaction between Htt and NR2B-containing NMDA receptors in the neurodegeneration associated with early HD, and support the use of NR2B-selective antagonists in the presymptomatic or early stages of the disease.

1.9 NR2A and NR2B subunit selective antagonists employed in the study

In the present study, we employed the most selective and potent NR2A and NR2B subunit prsently available: the NR2A selective antagonist (R)-[(S)-1-(4-bromo-phenyl)-ethylamino]-(2,3-dioxo-1,2,3,4-tetrahydroquinoxalin-5-yl)-methyl]-phosphonic acid (NVP-AAM077) (Auberson *et al.*, 2002) and the NR2B selective antagonist (R-(R*,S*)-α-(4-hydroxyphenyl)-β-methyl-4-(phenylmethyl)-1-piperidinepropanol (Ro 25-6981) (Fischer *et al.*, 1997). In previous studies, NVPAAM077 and Ro 25-6981 have been tested in parallel to investigate the contribution of NR2A and NR2B subunits to locomotion and phencyclidine discrimination (Chaperon et al. 2003), hippocampal plasticity (Massey *et al.* 2004; Mallon *et al.* 2005; Fox *et al.* 2006; Bartlett *et al.* 2007), NMDA neurotoxicity (Zhou and Baudry 2006) and epilepsy (Yang *et al.* 2006). NVP-AAM077 is a potent (affinity for recombinant NR2A receptors of 8 nM, Auberson *et al.* 2002) and competitive NMDA antagonist with >100-fold selectivity for human recombinant NR1/NR2A over NR1/ NR2B receptors (Auberson *et al.* 2002; Liu *et al.* 2004). However, NR2A/NR2B selectivity ratio at rat recombinant receptors is much lower (7- to 13-fold; Feng *et al.* 2004; Neyton and Paoletti 2006; Frizelle *et al.* 2006) and recently NVP-AAM077 has been claimed to be a preferential rather than selective NR2A antagonist (Berberich *et al.* 2005). Therefore, taking into account the in vitro recovery rate for GLU under our conditions (~18% for a 3 mm probe; Marti *et al.* 2002), we set maximal dialysate NVPAAM077 concentration in microdialysis experiments 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%; Berberich *et al.* 2005).

Ro 25-6981 is a 'second generation' ifenprodil analogue (Hoffman La Roche) that exhibits greater selectivity for NR2B over other receptor subtypes and ion channels, suggesting a reduced probability of cardiovascular side effects compared to ifenprodil

(Chenard *et al.*, 1999; Mutel *et al.*, 1998). Like ifenprodil, Ro 25-6981 binds with high affinity to the allosteric modulatory site on the NR2B LIVBP domain, irrespective on whether another NR2 subunit type is present (Chazot *et al.*, 2004).

Ro 25-6981 has been shown to potently bind to rat brain membranes (affinity for native NR2B receptors of ~6 nM; Mutel *et al.* 1998) and have much greater selectivity (~5000-fold) for NR2B over NR2A subunits at recombinant rat NMDA receptors (Fischer *et al.* 1997). It has to be considered that not all brain NMDA receptors are simple binary combinations of NR1 with only one type of NR2 (or NR3) subunit. Triheteromeric NR1/2A/2B are mainly present in the cortex and hippocampus (Cull-Candy and Leszkiewicz, 2004). The pharmacology of triheteromeric NMDA receptors has been characterized recently. It seems that a single copy of NR2A (or NR2B) is sufficient to confer high affinity to zinc (or ifenprodil), but the maximal level of inhibition is greatly reduced compared with diheteromeric receptor containing two copies of the same NR2 subunit (Hatton and Paoletti, 2005).

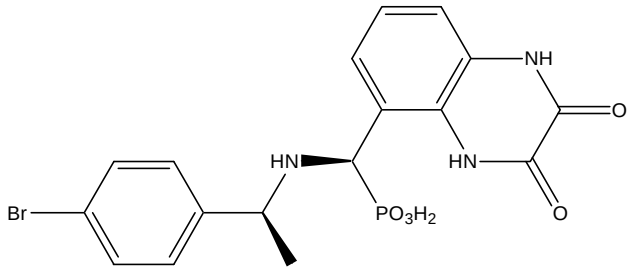


Fig. 7. Chemical structure of the NR1/NR2A selective antagonist NVP-AAM077, employed in the present study

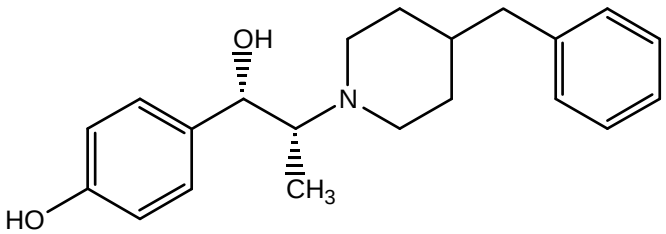


Fig. 8. Chemical structure of the NR1/NR2B selective antagonist Ro 25-6981, employed in the present study

1.10 EXPERIMENTAL ANIMAL MODELS

1.10.1 The 6-OHDA hemilesioned rat model of PD

The most extensively used animal model of PD is the 6-hydroxydopamine (6-OHDA) hemilesioned rat, first described by Urban Ungerstedt (Ungerstedt, 1968). This neurotoxin is still a valuable tool used chiefly in modelling PD in rat and mouse, in order to investigate motor and biochemical dysfunctions associated with the disease. 6-OHDA is a toxin that enters DA neurons through the high affinity DA transporter (DAT) and accumulates in the cytoplasm. It simultaneously inhibits the mitochondrial complexes I and IV (Glinka and Youdim, 1995; Glinka *et al.*, 1996) and forms free radicals, promoting oxidative stress (Olanow, 1993), which synergistically leads to degeneration of the DA neuron (Glinka *et al.*, 1997). The toxin does not cross the blood brain barrier and needs to be injected stereotaxically into the SN, the nigrostriatal tract or the striatum to specifically target the nigrostriatal DA system (Perese *et al.*, 1989; Przedborski *et al.*, 1995). Following this 6-OHDA induced DA neurodegeneration, the rat exhibits many of the disease-related motor symptoms observed in PD, including motor deficit and L-DOPA-induced dyskinesias (reviewed Cenci *et al.*, 2002). The magnitude of the lesion is dependent on the amount of 6-OHDA injected, the site of the injection and species-related sensitivity to the toxin.

The large majority of experimental models available to date are based on the unilateral infusion of 6-OHDA into the medial forebrain bundle (MFB) which contains dopaminergic projections from SNc neurons to striatum. Direct infusion of 6-OHDA in the SNc is rarely performed, in view of its ability to produce non specific and undesired damages to the SNr and mesencephalic ventral tegmental area (VTA).

The injection of 6-OHDA into the MFB, in close vicinity to the DA cell bodies of the SNc, provides an almost complete destruction of the nigrostriatal DA pathway in the rat. Following 6-OHDA injection, dopaminergic neurons start degenerating within the first day (Faull *et al.*, 1969), leading to a severe depletion of DA content in the striatum (80-90%) and an almost complete loss of DA neurons in SNc. This lesion is most commonly performed unilaterally, since animals receiving bilateral lesion develop aphagia and adipsia and require extensive monitoring and care to keep them alive (Ungerstedt, 1971; Zigmond and Stricker, 1972). The unilateral model has the advantage that the behavioural impairment mostly affects the side contralateral to the lesion, and the intact

side can therefore be used as an internal control. The complete lesion of the nigrostriatal DA pathway is created to mimic a mid-late stage of the human disease.

As a general feature, the unilateral 6-OHDA lesioned rat shows spontaneous rotations ipsilateral to the lesioned hemisphere. This behaviour can be increased by administering the indirect DA agonist amphetamine, which increases DA levels in the intact side by stimulating DA release from the pre-synaptic terminals and by reversing DA transport trough the DAT (Sulzer and Rayport, 1990; Jones *et al.*, 1999).

1.10.2 The 3-nitropropionic acid mouse model of HD

3-NP is a suicide inhibitor of the mitochondrial enzyme SDH. The toxin was originally identified after incidental intoxication of animals with Astragalus indigofera, which contains high amounts of the 3-NP precursor, 3-nitropropanol, and humans, after consumption of mildewed sugar cane contaminated by the fungus Arthrinium (He *et al.*, 1995).

Experimental administration of 3-NP produces preferential degeneration of the putamen and caudate nucleus (in rodents, the single anatomical structure named striatum), although SDH inhibition produced by 3-NP is similar in the striatum and other brain regions (Alexi *et al.*, 1998; Brouillet *et al.*, 1998). The neurodegenerative changes produced by 3-NP are similar to those reported in a number of clinical studies caused by primary genetic mitochondrial defects. The primary mechanism underlying striatal 3-NP toxicity is the selective inhibition of respiratory chain complex II, which is consistently found to be reduced in the striatum of patients with HD (Gu *et al.*, 1996; Browne *et al.*, 1997; Tabrizi *et al.*, 1999). The neurodegenerative hallmark of HD, i.e. the striatal degeneration with cognitive and motor dysfunctions, can be to a certain extent replicated by chronic administration of 3-NP, which produces the mitochondrial impairment suspected to occur quite early in the course of HD. 3-NP acts through separate pathways of cell death: rapid NMDA-dependent excitotoxic necrosis and delayed NMDA-independent apoptosis (Pang and Geddes, 1997). Initially, striatal lesions were obtained using various regimens of intoxication. These studies revealed that, in vivo, 3-NP intoxication can vary depending on multiple factors including animal species, strain, sex, age or protocols of toxin administration. 3-NP is more toxic in rats than mice, and the strain influences a lot the degree of toxicity probably due to differences in elimination/detoxification of the compound. Female rats are less sensitive to 3-NP than male rats (Nishino *et al.*, 1998), and toxicity is markedly increased in adult animals (3-4

month old) compared with young ones (Brouillet *et al.*, 1993). For a good experimental reproducibility, it must be taken into account that 3-NP toxicity is not only dose-dependent.but also "concentration-dependent". Indeed, a given dose results less toxic if administered in a more diluited solution.

The irreversible inhibition of SDH produced by 3-NP is similar in the striatum and other brain areas. The 3-NP-induced preferential degeneration of the striatum is not related to a particular distribution of the neurotoxin towards this cerebral structure. Striatal degeneration occurs when steady inhibition of SDH reaches 50-60%. 3-NP toxicity develops through several biochemical pathways. One of the earliest changes is the relocalization of cytochrome c, and subsequent loss of mitochondrial membrane potential (Bizat *et al.*, 2003). 3-NP also induces cytosolic release of apoptogenic mitochondrial factors (Galas *et al.*, 2004) and abnormal production of reactive oxygen species (ROS) as well as highly reactive molecules derived from the formation of nitric oxide (NO). Increased production of ROS and NO probably plays a causal role in 3-NP-induced striatal degeneration because free radical scavenger and/or antioxidants, as well as NO synthase inhibitors, are neuroprotective against 3-NP toxicity (Schulz *et al.*, 1995; Fontaine *et al.*, 2000). Recent studies have shown that an important factor in 3-NP-induced striatal degeneration is calpain activation, which has been found following either acute or chronic 3-NP treatments (Bizat *et al.*, 2005; Galas *et al.*, 2004).

The corticostriatal glutamatergic innervation plays a key role in striatal degeneration produced by systemic administration of 3-NP. Whether 3-NP affects GLU release is still a matter of debate, results produced in our laboratories demonstrated that profound SDH inhibition increased spontaneous GLU efflux from rat striatal synaptosomes and potentiated the K^+-evoked GLU release from striatal slices (Marti *et al.*, 2003). However there is no direct evidence that 3-NP toxicity is mediated by elevation in extracellular GLU concentrations, and microdialysis experiments failed to demonstrate increased extracellular GLU levels in rats treated with the toxin (Beal *et al.*, 1993; Erecinska and Nelson, 1994; Fu *et al.*, 1995). Other studies suggest that 3-NP selectively prevents vesicular GLU storage (Tavares *et al.*, 2001) and that inhibition of GLU reuptake increases 3-NP toxicity (Storgaard *et al.*, 2000) making striatal neurones susceptible to sublethal doses of the toxin (Massieu *et al.*, 2001). It is therefore likely that "physiological" levels of GLU potentiate or play a permissive role in 3-NP neurotoxicity *in vivo*. In fact, inhibition of GLU release in the striatum, either by surgical decortication or by drugs acting at corticostriatal terminals (i.e. riluzole),

provides striatal protection (Beal *et al.*, 1993; Jenkins *et al.*, 1996; Guyot *et al.*, 1997; Kim *et al.*, 2000).

Several studies pointed to a preferential involvement of NMDA receptors in the mechanisms underlying the potentiating effect of GLU on 3-NP toxicity. Blockade of NMDA but not non-NMDA GLU receptors with MK-801 pre-treatment greatly attenuated the 3-NP-induced striatal damage in mice (Kim *et al.*, 2000).

Patch-clamp experiments on cortico-striatal slices revealed that severe SDH inhibition by 3-NP results in a selective long-term enhancement of NMDA-mediated synaptic transmission in striatal MSNs but not in cholinergic interneurones, or in frontal and prefrontal cortical neurones, which appear to be resistant to 3-NP (Calabresi *et al.*, 2001), and promotes striatal expression of genes encoding for proteins linked to NMDA-receptor mediated transmission (Napolitano *et al.*, 2004).

Selective blockade of NR2B subunit containing NMDA receptors with ifenprodil protected the striatum from the injurious effect of 3-NP (Centonze *et al.*, 2006) and abolished the excitotoxic cell death of MSNs induced by quinolinate in the YAC transgenic mice (Zeron *et al.*, 2002). These data suggest the involvement of NR2B-containing NMDA receptors in the 3-NP induced neurodegeneration.

2. AIMS OF THE STUDY

The main aim of this thesis was to investigate the involvement of NMDA receptors in physiological and pathological conditions affecting the BG, and particularly in PD and in HD. The study was performed in the 6-OHDA hemilesioned rat, the most widely used rodent model of PD, and in the 3-NP intoxicated mouse model of HD. The study has been conducted in vivo by using a neurochemical (microdialysis in freely moving rats) and a behavioural approach.

Section I. The first set of experiments was performed in order to prove the concept that NMDA receptors regulate the activity of the MSNs originating the striato-nigral (direct) and the striato-pallidal (indirect) pathways, and that this modulation is altered in parkinsonian condition. We used triple probe microdialysis in awake freely moving rats. We implanted three microdialysis probes simultaneously in striatum, GP and SNr. NMDA was administered by reverse dialysis in the dorsolateral striatum (DLS) and GABA measured in target areas. The study was performed first in naïve rats, then in DA-depleted animals (6-OHDA hemilesioned rats)

Section II *(Paper I).* In the context of a microdialysis study aimed to elucidate the origin of the local increase of GLU levels after intrastriatal NMDA perfusion, we investigated the interaction between endogenous DA and NMDA by employing selective DA receptor antagonists in naïve rats and a model of DA-depletion (the 6-OHDA hemilesioned rat).

Section III *(Paper II).* In this microdialysis study we investigated whether different subsets of NMDA receptors are involved in the modulation of the striato-nigral and striato-pallidal neurons.

NVP-AAM077, a NR2A selective antagonist (Auberson *et al.* 2002), and Ro 25-6981, a NR2B selective antagonist (Fischer *et al.* 1997), were perfused in the DLS, and GABA release was simultaneously monitored in GP and SNr. Each antagonist was tested alone or in combination with NMDA to investigate the contribution of NR2A and NR2B subunits to tonic or phasic regulation of the striatofugal pathways.

Section IV *(Paper III).* We then tested the hypothesis that DA depletion affects the modulation exerted by NR2A and NR2B subunits on the striato-pallidal pathway and striato-nigral pathways by perfusing subunit selective NR2A and NR2B antagonists in the DA-depleted striatum of 6-OHDA hemilesioned rats. In these animals, we also analysed the motor responses induced by acute systemic administration of the two selective antagonists. Finally, to investigate the circuitry underlying motor responses we measured neurotransmitter release in DLS, GP and SNr.

Section V. In a separated set of experiments, we also explored the possibility that acute blockade of the NR2B subunit containing NMDA receptors could affect HD-like motor symptoms in the 3-NP mouse model of HD.

3. METHODS

3.1 Subjects

The experiments were carried out on male Sprague-Dawley rats (purchased from Harlan, S.Pietro al Natisone, Italy, or Stefano Morini, Reggio Emilia, Italy) and C57Bl/6 adult male mice aged 16 weeks (Iffa Credo, France) according to protocols approved by the Ethic Committee of the University of Ferrara, the Italian Guidelines for Animal Care (D.L. 116/92) and European Community Council Directives (86/609/EEC). Animals were raised and housed under a 12-h light cycle with food and water available *ad libitum*. Animals were handled and habituated to the experimenter for 1 week before any behavioural assessment. All efforts were made to minimise the number of animals used and their suffering.

3.2 6-Hydroxydopamine lesion

Unilateral lesion of dopaminergic neurons was induced in isoflurane-anesthetized male Sprague–Dawley rats (150 g; Harlan Italy). Eight micrograms of 6-OHDA (dissolved in 4 μL of saline containing 0.2% ascorbic acid) were stereotaxically injected according to the following coordinates from bregma: anteroposterior (AP) -4.4 mm, mediolateral (ML) -1.2 mm, ventrodorsal (VD) -7.8 mm below dura (Paxinos and Watson 1982). In order to select the rats which had been successfully lesioned the rotational model was employed (Ungerstedt and Arbuthnott 1970). Two weeks after surgery, rats were injected with amphetamine (5 mg/kg, i.p., dissolved in saline) and only those rats performing more than seven ipsilateral turns per minute were enrolled in the study.

This behaviour has been associated with 95% loss of striatal extracellular DA levels (Marti *et al.*, 2002) and TH positive terminals (Marti *et al.*, 2007). Experiments were performed 6-8 weeks after lesion.

3.3 Microdialysis experiments

Three probes of concentric design were stereotaxically implanted under isoflurane anesthesia in the right DLS (3 mm dialysing membrane, AN69; Hospal, Bologna, Italy), ipsilateral SNr (1 mm) and GP (1.5 mm) according to the following coordinates from bregma and the dural surface (Paxinos and Watson 1982): DLS, AP +1.0, ML -3.5, VD -6; SNr, AP -5.5, ML -2.2, VD -8.3; and GP, AP -1.3, ML -3.3, VD -6.5. Forty-eight hours after surgery, probes were perfused with a modified Ringer solution (CaCl2 1.2 mM, KCl 2.7 mM, NaCl 148 mM, and MgCl2 0.85 mM) at a 3 μL/min flow rate. After 6 h rinsing, samples were collected every 10 min. At least three baseline samples were collected before drug perfusion through the probe. Antagonists were perfused alone (3 and 300 nM) for 90 min through the probe implanted in the DLS to unravel tonic influence of NR2A and NR2B subunits on GABA and GLU release in GP and SNr. In separate experiments, to investigate the role of NR2 subunits in phasic

activation of the striatofugal pathways, antagonists were challenged (at 300 nM) against a single concentration of NMDA (10 μmol/L) able to activate both pathways. In this case, antagonists were perfused 60 min before NMDA and maintained until the end of experiment. To investigate circuitry underlying motor effects, NVP-AAM077 and Ro 25-6198 were given systemically (i.p.) at behaviourally relevant doses. These doses were selected on the basis of previous behavioural analysis. In these experiments, sample collection was performed every 15 min for 90 min after drug administration.

At the end of the experiments, animals were sacrificed and the correct placement of the probes was verified histologically.

3.4 Synaptosome preparation

Striatal synaptosomes were prepared as previously described (Marti *et al.*, 2003). Briefly, striata were homogenized in ice-cold 0.32 M sucrose buffer at pH 7.4 and then centrifuged for 10 min at 1,000 g_{max} (4°C). The supernatant was then centrifuged for 20 min at 12,000 g_{max} (4°C) with the synaptosomal pellet being resuspended in oxygenated (95% O_2, 5% CO_2) Krebs solution (mM: NaCl 118.5, KCl 4.7, $CaCl_2$ 1.2, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, glucose 10). One millilitre aliquots of the suspension (~0.35 mg protein) were slowly injected into nylon syringe filters (outer diameter 13 mm, internal volume of about 100 μl; MSI, Westporo, MA, USA). Filters were maintained at 36.5 °C in a thermostatic bath and superfused at a flow rate of 0.4 ml/min with a pre-oxygenated Krebs solution containing glycine (1 μM). Sample collection (every 3 min) was initiated after a 20 min equilibration period. NMDA was added to the perfusion medium 3 min before a KCl pulse (10 mM; 60 sec) and maintained until the end of the experiment. We previously showed that the 10 mM K^+-evoked striatal overflow of GLU and GABA from synaptosomes is largely dependent on exocytotic release mechanisms (i.e. TTX and Ca^{++}-dependent; Marti *et al.*, 2003). In a separate set of experiments, Mg^{++} was omitted from the medium and NMDA was also tested under nominally Mg^{++}-free conditions.

3.5 Endogenous Glutamate and GABA analysis

Glu and GABA were measured by precolumn derivatization with o-phthaldialdehyde and separation by reverse phase HPLC coupled to fluorimetric detection (Lindroth and Mopper 1979). 40 μL of o-phthaldialdehyde/mercaptoethanol reagent (0.4 M borate, 0.04 M o-phthaldialdehyde, 0.4 M 2-mercaptoethanol, pH 10.4) were added to 30 μl aliquots of sample. After a 60-s reaction period at 4°C in a Triathlon autosampler (Spark Holland, Emmen, Netherlands), 50 μl of the mixture were automatically injected onto a 5-C18 Chromsep

analitycal column (3-mm inner diameter, 10 cm length; Chrompack, Middelburg, The Netherlands). The column was eluted at a flow rate of 0.48 ml/min (Beckman 125 pump; Beckman Instruments, Fullerton, CA, USA) with a mobile phase containing 0.1 M sodium acetate, 10% methanol and 2.2% tetrahydrofuran (pH 6.5). GLU and GABA (retention time ~ 4 and 17 minutes, respectively) were detected by means of a fluorescence spectrophotometer RF-551 (Shimadzu, Kyoto, Japan) with the excitation and the emission wavelengths set at 350-450 nm, respectively. Acquisition and analysis of chromatograms was performed by means of a computer controlled system (Beckman System Gold). GLU and GABA retention time were about 3.5 and 17.5, and the sensitivity of the method was 150 fmol/sample.

3.6 Behavioural study in 6-OHDA hemilesioned rats (*Section IV*)

Motor activity in rats was evaluated by means of three behavioural tests specific for different motor abilities. The different tests are useful to evaluate akinesia and motor asymmetry under static conditions (bar test), akinesia, bradykinesia, and asymmetry under dynamic conditions (drag test), and overall motor performance (rotarod test) as an integration of coordination, gait, balance and muscle tone. The tests were performed first in a control session and then 20 and 80 min after drug administration.

1.Bar test. The bar test (Sanberg *et al.*, 1988) measures rat ability to respond to an externally imposed static posture Each rat was placed gently on a table, and the contralateral and ipsilateral forepaws were placed alternatively on blocks of increasing heights (3, 6, and 9 cm). Total time (in seconds) spent by each paw on the blocks was recorded (cutoff time, 20 s).

2.Drag test. The drag test [modification of the "wheelbarrow test" (Schallert *et al.*, 1979)] measures rat ability to balance body posture using forelimbs in response to an externally imposed dynamic stimulus. Each rat was gently lifted from the tail (allowing forepaws on the table) and dragged backwards at a constant speed (20 cm/s) for a fixed distance (100 cm). The number of steps made by each paw was counted by two separate observers.

3.Rotarod test. The fixed-speed rotarod test measures rat ability to run on a rotating cylinder (Rozas *et al.*, 1997). Briefly, rats were trained for 10 days to a specific motor task on the rotarod until their motor performance became reproducible. Rats were tested in a control session at four increasing speeds (10, 15, 20, 25 rpm for hemiparkinsonian rats and 30, 35, 40, and 45 rpm for naive rats; 180 s each), causing a progressive decrement of performance to about 40% of the maximal response (i.e., the experimental cutoff time). Such a protocol was set to detect both facilitatory and inhibitory drug effects. Two other sessions were repeated 20 and 80 min after drug injection, and drug effect was expressed as total time spent on the rod.

Fig. 9 The bar test

Fig. 10 The drag test

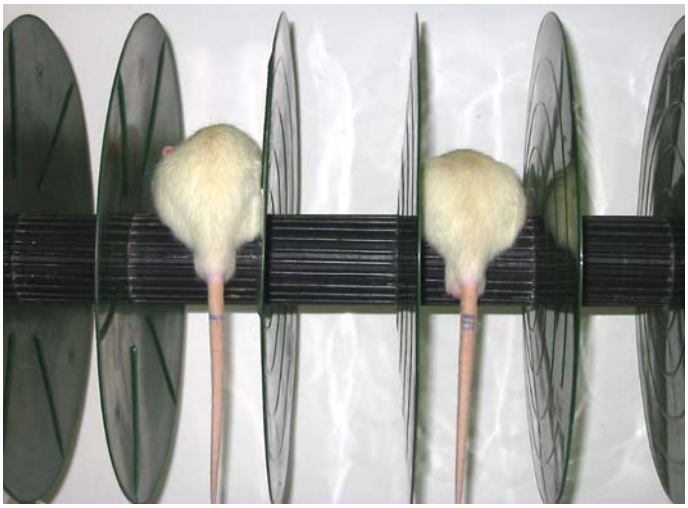


Fig. 11 The rotarod test

3.7 Behavioural study in 3-NP intoxicated mice (*Section V)*

3.7.1 Intoxication paradigm

Mice received twice daily i.p. 3-NP injections 12 h apart according to the following regime: 4 x 10 mg/Kg, 4 x 20 mg/Kg, 4 x 30 mg/Kg, 4 x 40 mg/Kg, 4 x 50 mg/Kg (cumulated dose = 600 mg/Kg in 10 days). The concentration was calculated to keep the injected volume (about 200 µl) stable as doses increased. A 500-µl Hamilton micro-syringe was used for the i.p. injections.

The day of the experiment (d11), 12 h after the last 3-NP injection, mice were singularly videotaped for subsequent blind score. Mice where divided into 4 groups composed of randomly allocated littermates that received a singular injection of saline or Ro 25-6981 (1-3-6 mg/Kg)

3.7.2 Clinical semiquantitative assessment of motor disorders induced by 3-NP (Table 1)

A semi-quantitative scale previously validated (Fernagut *et al.*, 2002b) was used for motor disorder severity rating after preliminary direct observations of 3-NP intoxicated mice and recorded videotapes (Table 1). The three-level scale assessed the severity of the following five items (maximal score=10), which constitute the main motor symptoms observed: hindlimb clasping, reduced global activity in a freely moving environment, hindlimb dystonia, truncal dystonia (kyphosis) and balance adjustments to a postural challenge. The items and guidelines of the rating procedure are detailed in Table 1. Mice were rated after direct observation before each injection (every 12 h) during the intoxication procedure, then on recorded videotapes during experiment with acute Ro 25-6981 administration at baseline and 40 and 90 minutes after injection.

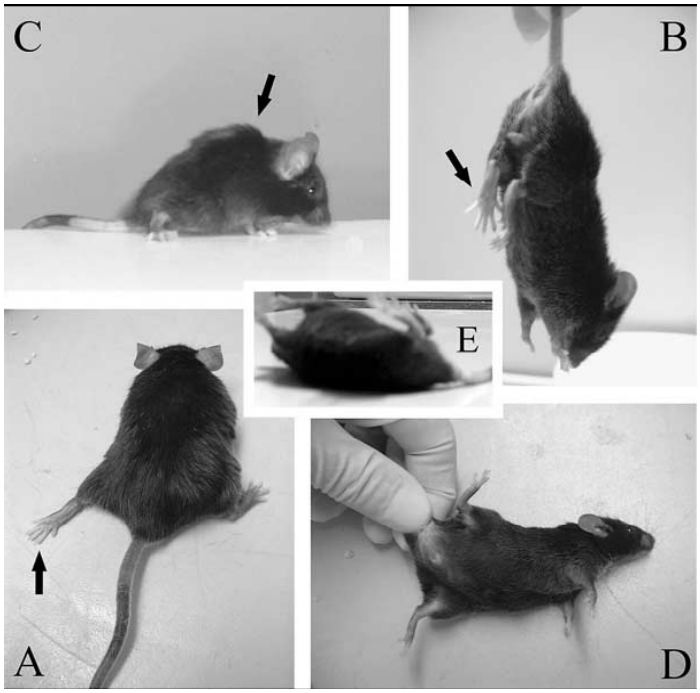


Fig. 12. 3-NP-induced motor and postural abnormalities at day 1 post-intoxication.
(A) Hindlimb dystonia (arrow).
(B) Hindlimb clasping (arrow).
(C)Truncal dystonia (kyphosis) (arrow).
(D,E) Impaired postural adjustments following postural challenge; the mouse is tipped up on its side
(D) and cannot get back on its feet (E).

3-NP mouse motor disorders behavioural scale

General procedure
Gently grasp the mouse by the mid-tail and suspend it for 5-10 s, rate item (A) hindlimb clasping, then place the mouse in the centre of an empty open space under gentle illumination (40 W) for 5 min: rate item (B) general locomotor activity, (C) hindlimb dystonia and (D) truncal dystonia], then challenge its postural adjustment reflexes by grasping the proximal part of its tail and trying to tip the mouse on its side by rotating movements
(at least three trials.

(A) Hindlimb clasping: note the hindlimb movements for postural adjustment and attempt to straighten up while the mouse is suspended by themid-tail (during 5-10 s)
0, normal hindlimb movements; hindlimbs are fully spread and moving about
1, intermittent and/or intermediate clasping of hindlimbs
2, hindlimbs are fully drawn up to the abdomen

(B) General locomotor activity: note the displacement velocity, exploratory behaviour, rearing and grooming
0, normal
1, slight reduction of general activity (<50%) ; displacement velocity, rearings and grooming are reduced
2, marked reduction of general activity (50-100%); the mouse stays in place or moves very slowly with no rearing or grooming

(C) Hindlimb dystonia: note the increased space between hindlimbs, poor hindlimb coordination, abnormal hindlimbs, crouching posture and
impairment of gait
0, absent
1, intermittent or permanent but slight-to-moderate increased hindlimb space and abnormal crouching posture without impaired gait
2, marked hindlimb dystonia with wide interlimb space, poor hindlimb movements and co-ordination, and crouching posture interfering with gait

(D) Truncal dystonia: note the flexed kyphotic posture
0, absent
1, slightly flexed with visible kyphosis
2, markedly abnormal flexed posture with severe kyphosis

(E) Postural challenge: the mice is grasped by the proximal part of its tail and the experimenter tries to tip the mouse on its side by rotating
movements (at least three trials)
0, with normal postural adjustments the mouse cannot usually be tipped on its side
1, moderately impaired postural adjustments; the mouse falls on its side and slowly gets back on its feet.
2, markedly impaired postural adjustments; the mouse falls on its side and cannot get back on its feet on its own

Table 1. Motor behavioural scale for the assessment of the severity of 3-NP-induced motor disorders in the mouse

3.7.3 Standardized motor and sensorimotor integration tests.

The following motor and sensorimotor integration tests were performed the week before intoxication (baseline), then the day of the experiment, 40 minutes (open field) and 60 minutes (traversing a beam) after saline or Ro 25-6981 injection.

Open field spontaneous activity. Mice were placed in the centre of an open field (44x44x32 cm) under red-light (40 W) illumination at the same time of the day. On each side of the open field, two frames at 1 and 5 cm height with 16 photocell beams per side ensured movement detection. The computer defined grid lines that divided the open field into four 'side and corner regions', each line being 10 cm from the wall and the central region measuring 576 cm2 (Actitrack, Panlab, S.L, Barcelona, Spain; (Dulawa *et al.*, 1999)). The tracking, travelling distance, mean velocity, maximal velocity, number of rearings and time spent in the central compartment were computerised during a single 5-min session before and after acute administration of Ro 25-6981.

Traversing a beam. Motor coordination and balance were assessed with the method adapted from Carter *et al.*(1999) by measuring the ability of the mice to traverse a narrow beam to reach a dark goal box. The beams consisted of two different strips of wood (each measuring 50 cm long, one was 1.6 cm and the other 0.9 cm square cross-section) placed horizontally 50 cm above the floor. During training, three daily sessions of three trials (nine crossings) were performed using the 1.6 cm square large beam. Mice were then tested at baseline, then 60 minutes after saline or Ro 25-6981 administration using the 0.9 cm square beam. Mice were allowed to perform three consecutive trials. The number of sideslips was recorded on each trial and the mean number of sideslips during a three-trial session was kept as the variable.

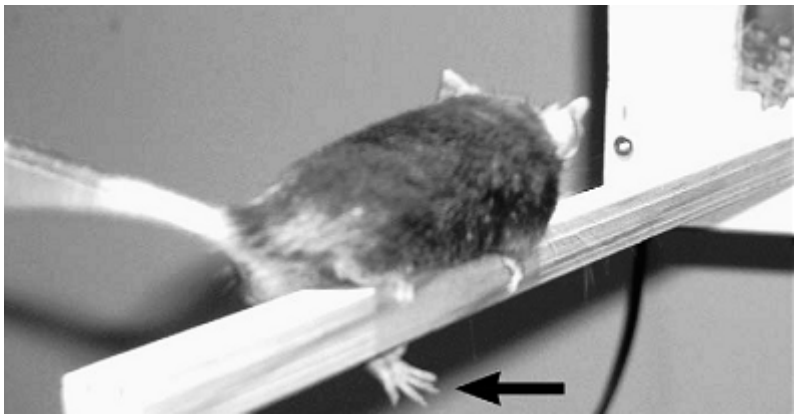


Fig. 13. The Traversing a beam test the day 1 post-intoxication. The arrow indicate the sideslip.

3.8 Data presentation and statistical analysis

Data (mean ± SEM) from microdialysis experiments are reported as percentage of basal value (calculated as the mean of two samples before treatment). Statistical analysis was performed by one-way repeated measure (RM) ANOVA followed by contrast analysis and the sequentially rejective Bonferroni post hoc test for multiple comparisons. The interaction between the antagonists and NMDA was analyzed by two-way RM ANOVA followed by contrast analysis and the sequentially rejective Bonferroni post hoc test for multiple comparisons. Results reported in *Part II* were analyzed on the stimulated area-under-the-curve (AUC) values (calculated on the 0-60 min interval and expressed in arbitrary units; microdialysis) or on absolute neurotransmitter release (in pmol/mg prot/min; synaptosomes) by ANOVA followed by the *post hoc* Newman-Keuls test for multiple comparisons(GraphPad Prism software, San Diego, CA, USA).

In the study conducted on 3-NP intoxicated mice all data are expressed as mean values ± SEM. Comparisons with baseline (pre-intoxication) and between groups were performed using a paired t test.. P values <0.05 were considered to be statistically significant.

3.9 Drugs

6-OHDA, amphetamine, Ro 25-6981 and 3-NP were purchased from Sigma Chemical Company (St Louis, MO, USA); SCH23390 and NMDA and were purchased from Tocris Neuramin (Bristol, UK). Tetrodotoxin (TTX) from Alomone Labs Ltd. (Jerusalem, Israel); raclopride from Research Biochemicals Incorporated (Natick, MA, USA). NVP-AAM077 was produced and kindly supplied by Novartis Institutes for BioMedical Research (Basel, Switzerland). Drugs were dissolved just before use in ringer solution for the intrastriatal perfusion and isoosmotic saline solution for intraperitoneal injection.

3-NP was dissolved in a few drops of distilled water and the pH was adjusted to 7.4 with 1 M NaOH and the final volume adjusted with 0.1 M phosphate-buffered saline solution (PBS), pH 7.4. The solution was filtered (0.22 µm Millipore) and kept at +4°C until use.

Ro 25-6981 required heating to dissolve, whereas NVP-AAM077 was dissolved in small quantity of vehicle containing NaOH 0.1 mol/L and final pH adjusted with HCl 1mol/L.

4. RESULTS

Section I

Striatal NMDA activation modulates striato-pallidal and striato-nigral pathways in physiological and parkinsonian conditions

4.1.1 Activation of striatal NMDA receptors modulates GABA release in GP and SNr in naïve rats.

To prove the concept that NMDA receptors regulate the activity of the medium spiny GABAergic projection neurons originating the striato-nigral (direct) and the striato-pallidal (indirect) pathways, we used triple probe microdialysis in awake rats. We implanted one microdyalisis probe in striatum, one in GP and another in SNr. NMDA was administrated by reverse dialysis in the DLS and GABA measured in the ipsilateral GP and SNr.

Basal GABA extracellular levels in GP (14.1 ± 1.3 nM; n=47) were elevated by intrastriatal perfusion with NMDA. RM ANOVA showed a significant effect of treatment ($F_{4,28}$=6.5, p=0.0008), but not time ($F_{7,28}$=2.0, p=0.056), and a significant time x treatment interaction ($F_{28,261}$=1.81, p=0.0091). Contrast analysis revealed that intrastriatal perfusion with NMDA 10 and 100 µM increases GABA release in GP (p=0.0005 and p=0.0022, respectively) with a maximal increase of about 60% for the 10 µM concentration (**fig. 14**).

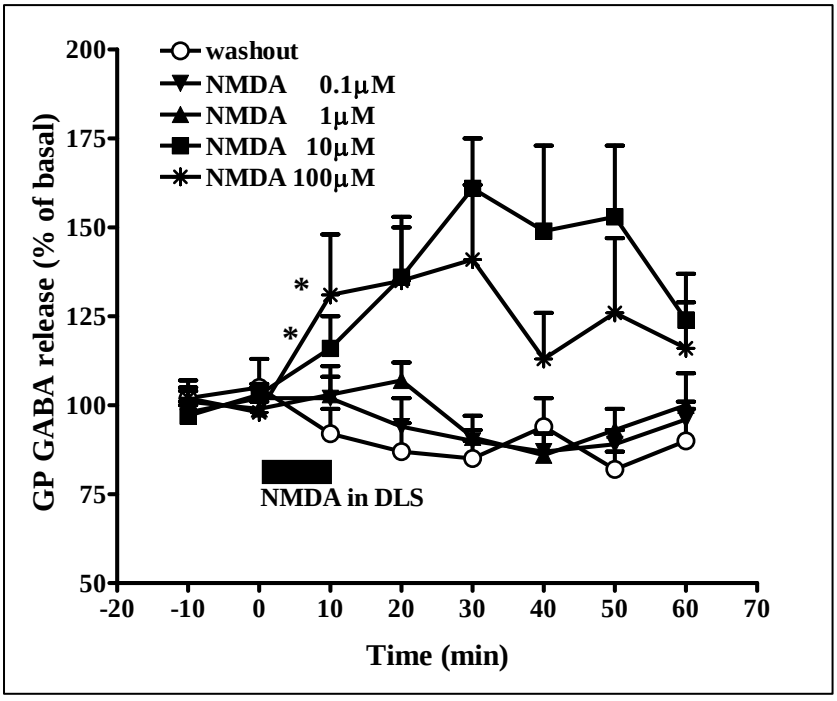


Fig. 14. Striatal NMDA perfusion (10 min) elevated GABA levels in the GP of naive rats. Data are means ± SEM of at least 7 experiments. A significant effect was observed at NMDA 10 and 100µM. *p<0.05 *vs* washout; RM ANOVA followed by contrast analysis and sequentially rejective Bonferroni's test

Basal GABA extracellular levels in SNr (14.0 ± 1.5 nM; n=38) were also elevated by intrastriatal perfusion with NMDA. RM ANOVA showed a significant effect of treatment ($F_{4,28}$=9.6, p<0.0001), time ($F_{7,28}$=6,55, p<0.0001), and a significant time x treatment interaction ($F_{28,261}$=1.88, p=0.0058). Contrast analysis revealed that, as in GP, intrastriatal perfusion with NMDA 10 and 100 μM increased GABA release in SNr (p<0.0001 and p<0.0001, respectively). At variance with GP, also NMDA 1 μM significantly elevated GABA levels (p=0.0029, **fig. 15**).

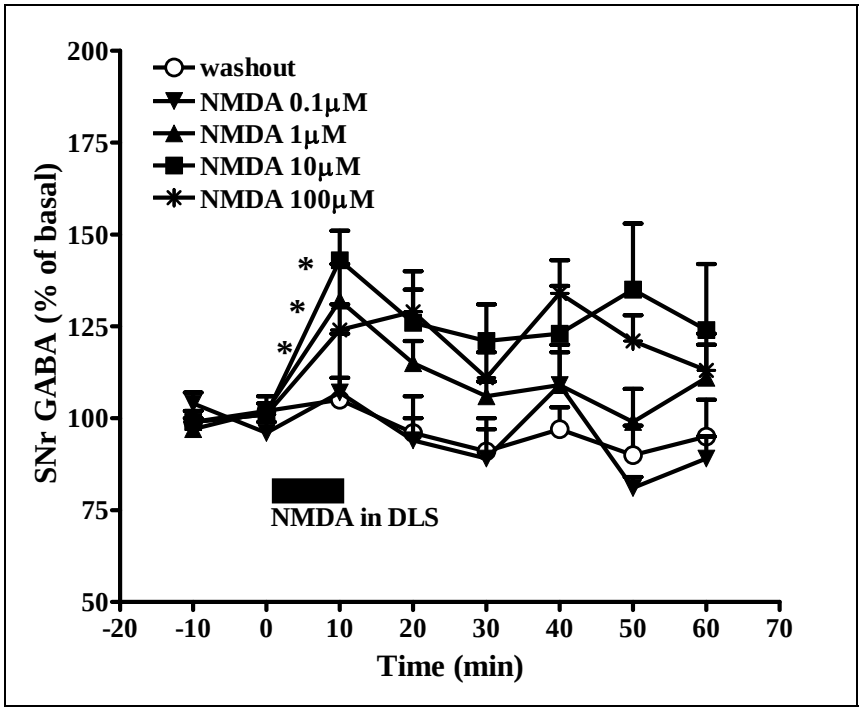


Fig. 15 Striatal NMDA perfusion (10 min) elevated GABA levels in the SNr of naive rats. Data are means ± SEM of at least 7 experiments. A significant effect was observed at NMDA 1-100 μM. *p<0.05 different from washout (RM ANOVA followed by contrast analysis and the sequentially rejective Bonferroni's test).

4.1.2. DA depletion caused an overall increase in sensitivity towards NMDA in hemiparkinsonian rats.

A prediction of the current model of BG function is that the akinesia associated with Parkinson's disease (PD) is due to an imbalance in the activity of the "direct" and "indirect" pathways, the "indirect" pathway becoming hyperactive and the "direct" one hypoactive. To prove that DA depletion produces dysregulation in the control exerted by striatal NMDA receptors over the two pathways, NMDA was perfused by reverse dialysis in the DA-depleted DLS of hemiparkinsonian rats, and GABA measured in the ipsilateral GP and SNr. Under these conditions, we previously demonstrated that striatal DA extracellular levels were reduced by more than 90% compared to naïve rats (Marti *et al.*,2002).

Basal extracellular GABA levels in the GP of hemiparkinsonian rats (9.2 ± 0.9 nM; n=18) were elevated by intrastriatal NMDA perfusion.

RM ANOVA showed a significant effect of treatment ($F_{3,12}$=7.41, p=0.0045), time ($F_{7,21}$=4.26, p=0.0002), and a significant time x treatment interaction ($F_{21,152}$=2.50, p=0.0007). Contrast analysis revealed that intrastriatal perfusion with NMDA 0.1 and 1 µM increases pallidal GABA release being the higher concentration ineffective (p=0.0006 and p=0.0280, respectively; F**ig. 16**). Maximal increase of about 50% was detected for the 0.1 µM NMDA concentration

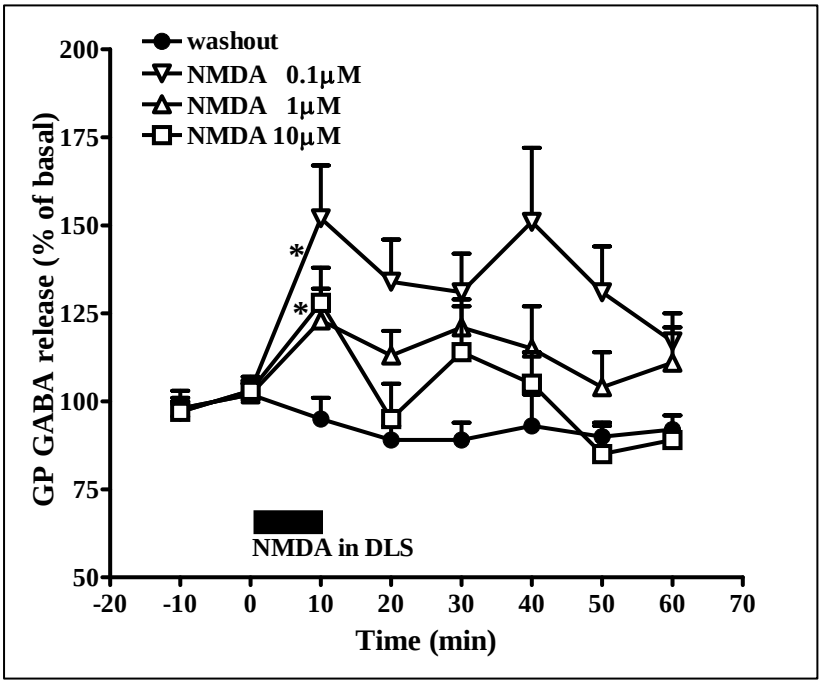


Fig. 16 Striatal NMDA perfusion (10 min) elevated GABA levels in the GP of hemiparkinsonian rats. Data are means ± SEM of at least 7 experiments. A significant effect was observed already at NMDA 0.1 µM. *p<0.05 different from washout (RM ANOVA followed by contrast analysis and the sequentially rejective Bonferroni's test).

Extracellular GABA levels in the SNr of hemiparkinsonian rats (8.8 ± 0.9 nM; n=18) were also elevated by intrastriatal NMDA. RM ANOVA showed a significant effect of treatment ($F_{3,12}$=5.76, p=0.0111), time ($F_{7,21}$=2.91, p=0.0069), and a significant time x treatment interaction ($F_{21,152}$=3.00, p<0.0001). Contrast analysis revealed that only intrastriatal perfusion with NMDA 0.1 µM increased nigral GABA release (p=0.0029) with a maximal increase of about 50% (**Fig. 17**), the other concentrations being ineffective.

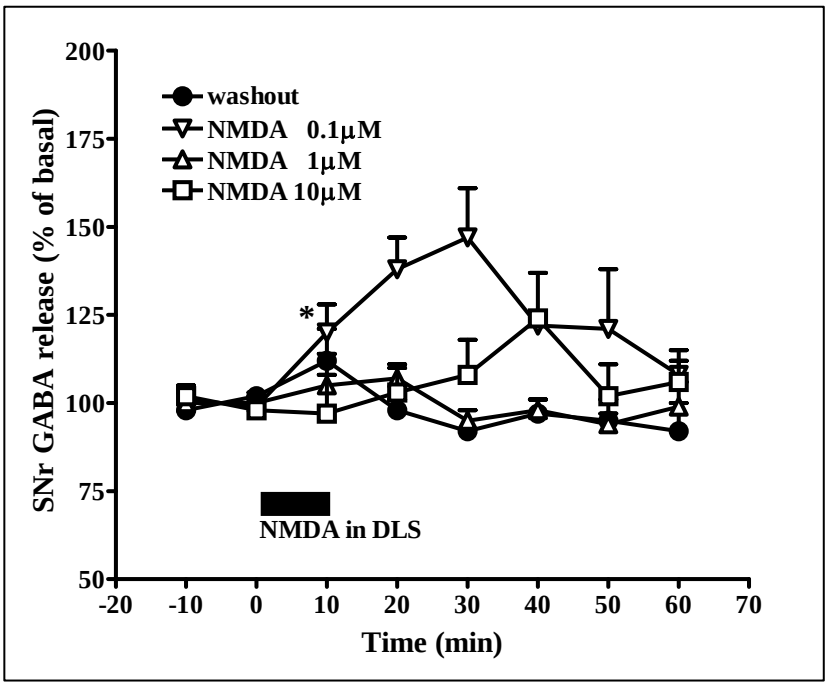


Fig. 17 Striatal NMDA perfusion (10 min) elevated GABA levels in the SNr of hemiparkinsonian rats. Data are means ± SEM of at least 7 experiments. A significant effect was observed at NMDA 0.1 µM, other concentrations being ineffective. *p<0.05 different from washout (RM ANOVA followed by contrast analysis and the sequentially rejective Bonferroni's test).

Section II

Striatal glutamate release evoked in vivo by NMDA is dependent upon ongoing neuronal activity in the substantia nigra and endogenous striatal dopamine

(Paper I)

4.2. Striatal glutamate release evoked by NMDA is dependent upon ongoing neuronal activity in the substantia nigra (*Paper I*)

This study was undertaken to prove the concept that the rise in striatal GLU levels induced by NMDA perfusion is due to striatofugal pathways activation rather than presynaptic modulation of GLU release.

4.2.1 Effect of NMDA on striatal GLU release

Basal GLU extracellular levels in the striatum averaged 199.3 ± 18.8 nM (n=81) and were stable over the time course of the experiment. Intrastriatal perfusion with NMDA dose-dependently elevated GLU dialysate content ($F_{3,50}$=10.64; p<0.0001; Fig. 1). The effect was significant (p<0.01) at 1 and 10 μM NMDA, while the lower (0.1 μM) concentration was ineffective (**fig. 18**)

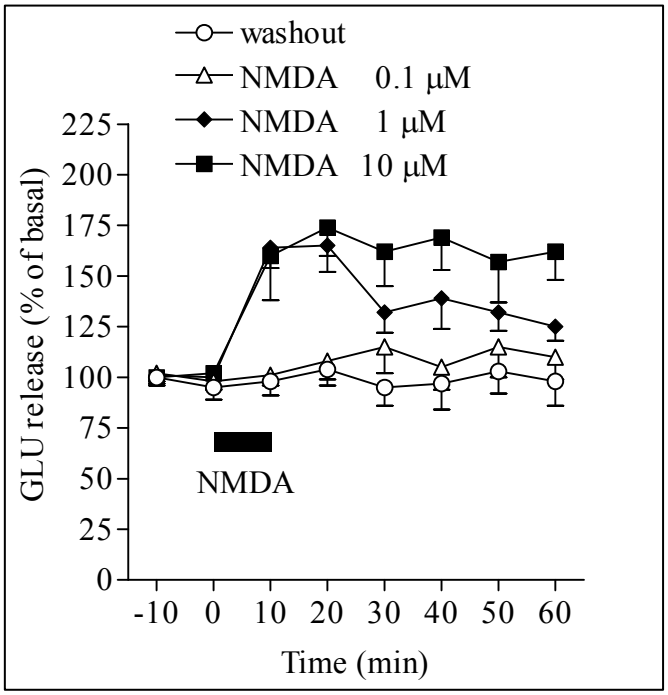


Fig. 18. Effect of local perfusion with NMDA (0.1-10 μM, 10 min; black bar) in the rat striatum on local glutamate (GLU) release. Data are expressed as percentages ± SEM of basal pre-treatment levels (calculated as the mean of the two samples before the treatment). A significant effect (p<0.05 *vs* washout) was observed at NMDA 1 and 10 μM (solid symbols).

4.2.2 Effect of NMDA on striatal synaptosomes

To investigate whether the effect of NMDA could be due to stimulation of NMDA autoreceptors, the ability of NMDA to release GLU release from 10 mM K^+-depolarised striatal synaptosomes in the presence of glycine (1 μM) was tested (Fig. 3). For a comparison, GABA release was simultaneously monitored. Basal GLU and GABA outflows from striatal synaptosomes were 89.1 ± 2.1 (n=41) and 26.9 ± 2.0 (n=43) pmol/mg prot/min, respectively. Ten mM KCl increased by about 75 and 40 % GLU and GABA outflows (net overflow of 66.7 ± 7.2 and 10.3 ± 2.4 pmol/mg prot/min, respectively). NMDA (0.1-100 μM; 6 min) did

not modify either spontaneous or K^+-evoked GLU release. NMDA also failed to alter spontaneous GABA efflux but increased, in a concentration-dependent manner, the K^+-evoked GABA release ($F_{3,35}$=6.84; p=0.001; **Fig 19**). NMDA evoked a significant GABA overflow at 10 μM ($p<0.05$) and almost doubled ($p<0.01$) the effect of 10 mM KCl at 100 μM. To test whether omission of Mg^{++} could disclose an effect of NMDA, NMDA was also applied in the absence of Mg^{++}. NMDA failed to affect both spontaneous and 10 mM K^+-evoked GLU release under nominally Mg^{++}-free conditions (**Fig. 19**). NMDA was also ineffective when challenged against the higher (20 mM) K^+ concentration in the absence of Mg^{++} (103.3 ± 3.3 % and 91.4 ± 6 % of the K^+ stimulation for NMDA 10 and 100 μM, respectively).

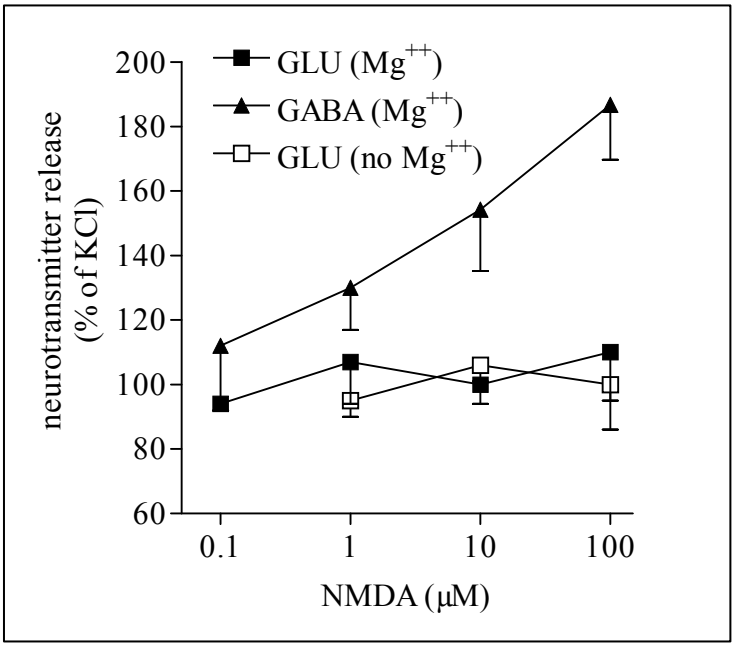


Fig. 19. Effect of superfusion with NMDA (1-100 μM; 6 min) on glutamate (GLU) and GABA release from striatal synaptosomes depolarised with KCl (10 mM, 60 sec) in the presence of Mg^{++} and glycine (1 μM). The effect of NMDA on GLU release in the absence of Mg^{++} is also shown. Data (means of 9 determinations) are expressed as percentages ± SEM of the KCl stimulation.

4.2.3 Blockade of neuronal transmission and $GABA_A$ receptors in the SNr prevents striatal NMDA-evoked GLU release.

To investigate the involvement of the SNr in the striatal GLU-stimulating effects of NMDA, NMDA was applied in the striatum concurrently with TTX in the SNr (**Fig. 20**). Nigral perfusion with TTX (1 μM) alone did not alter striatal spontaneous GLU levels but was able to prevent the stimulation of GLU release induced by 1 μM NMDA. Similarly, to investigate the involvement of nigral GABAergic transmission, the $GABA_A$ receptor antagonist bicuculline was perfused in the SNr (**Fig 20**) at a concentration (10 μM), which is 10 fold higher than bicuculline pA_2 value. This concentration was selected on the basis our previous

work (Marti et al, 2002). Bicuculline, ineffective alone, prevented the stimulation induced by 1 μM NMDA.

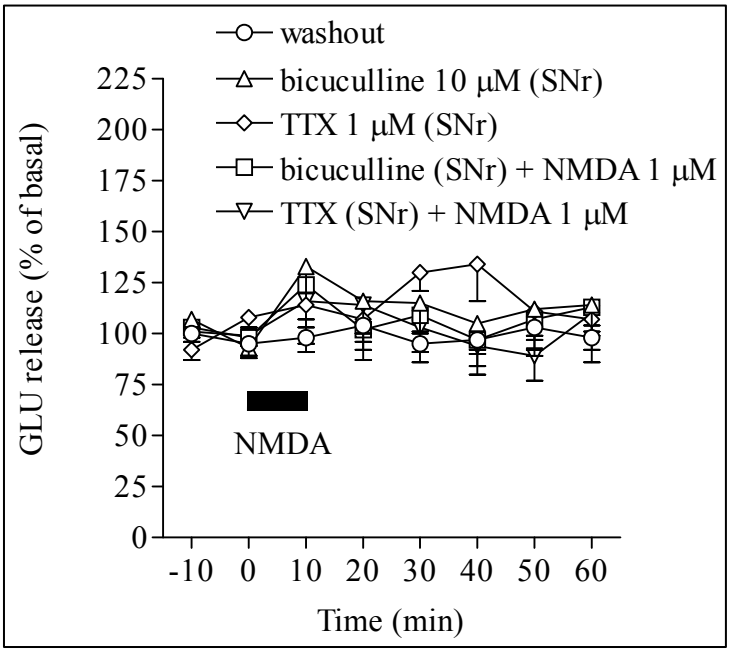
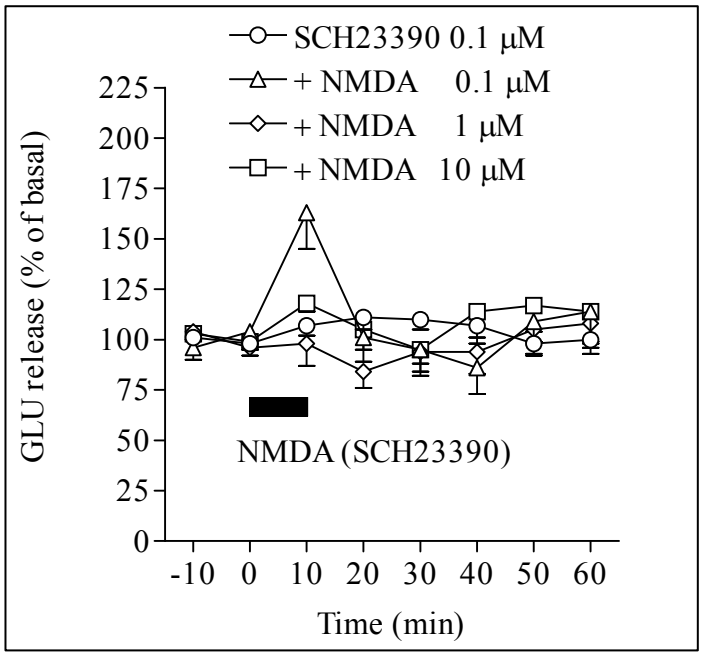


Fig. 20. Effect of perfusion with bicuculline (10 μM) and tetrodotoxin (TTX; 1 μM) in the SNr on the NMDA-stimulated (1 μM, 10 min; black bar) glutamate (GLU) release in the ipsilateral striatum of rats. Perfusion with bicuculline and TTX started 60 min before NMDA and continued until the end of experiment. Data are expressed as percentages ± SEM of basal pre-treatment levels (calculated as the mean of the two samples before the treatment).

4.2.4 Modulation of striatal NMDA-evoked GLU release by endogenous DA.

To investigate the possible role of endogenous striatal DA and DA receptors in mediating the spontaneous or the NMDA-evoked GLU release in the striatum, NMDA was perfused in the presence of selective DA receptor antagonists. According to the classification of DA receptors in D_1-like (D_1 and D_5) and D_2-like (D_2, D_3 and D_4) families (henceforth D_1 and D_2; Sibley and Monsma, 1992), the D_1 and D_2 preferential antagonist, SCH23390 and raclopride, respectively, were used. They were perfused at concentrations effective to achieve selective blockade of D_1/D_5 (0.1 μM SCH23390) or D_2/D_3 receptors (1 μM raclopride; see Marti *et al.*, 2002). Intrastriatal perfusion with raclopride or SCH23390 alone did not modify striatal GLU levels (**Fig. 21-22**). The presence of SCH23390 blocked the GLU output evoked by NMDA up to 10 μM (**Fig. 21**).. Similarly, the presence of raclopride prevented the GLU output evoked by 1 and 10 μM NMDA, whereas allowed the lower 0.1 μM NMDA concentration to elicit a significant ($p<0.01$) stimulation of GLU release ($F_{4,40}=3.64$; $p=0.012$; **Fig 22**). Nevertheless, analysis of the time-course of the response revealed a transient (10 min time-point) increase in GLU levels ($F_{3,30}=3.24$; $p=0.036$; **Fig. 21**) induced by 0.1 μM NMDA in presence of SCH23390 ($p<0.05$).

Fig. 21. Effect of intrastriatal perfusion with SCH23390 (0.1 μM) on striatal glutamate (GLU) release stimulated by NMDA (0.1-10 μM, 10 min; black bar) in rats. Perfusion with SCH23390 started 60 min before NMDA and continued until the end of experiment. Data are expressed as percentages ± SEM of basal pre-treatment levels (calculated as the mean of the two samples before treatment).



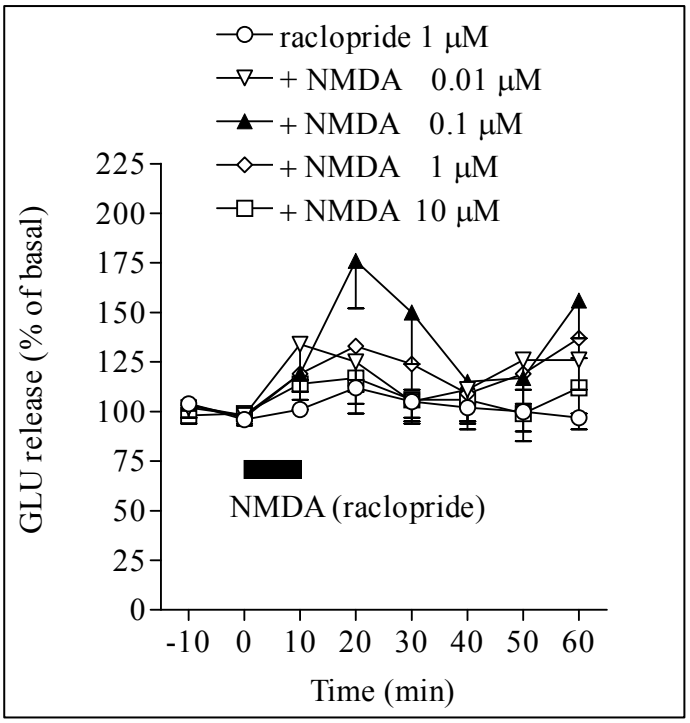


Fig. 22. Effect of intrastriatal perfusion with raclopride (1 μM) on striatal glutamate (GLU) release stimulated by NMDA (0.01-10 μM, 10 min; black bar) in rats. Perfusion with raclopride started 60 min before NMDA and continued until the end of experiment. Data are expressed as percentages ± SEM of basal pre-treatment levels (calculated as the mean of the two samples before treatment). A significant effect ($p<0.05$ *vs* raclopride) was observed at NMDA 0.1 μM (solid symbols).

To confirm the modulatory role of endogenous DA in the NMDA-induced GLU release, NMDA was perfused in the DA-denervated striatum of 6-OHDA hemilesioned rats (a model of Parkinson's disease). Under these conditions, extracellular DA levels were reduced by more than 90% compared with naıve rats, while extracellular GLU levels remained unchanged (Marti *et al.* 2002; Galeffi *et al.* 2003). NMDA perfusion in the DA-denervated striatum (**Fig. 23**) elevated local GLU levels ($F_{4,33}$ = 3.29; p = 0.022). However, NMDA elicited a significant stimulation only at a concentration of 0.01 μM ($p < 0.05$). Overall, the maximal stimulation elicited by NMDA in hemiparkinsonian rats (NMDA 0.01 μM) was significantly reduced as compared with that observed in naive (NMDA 10 μM; p = 0.033) rats.

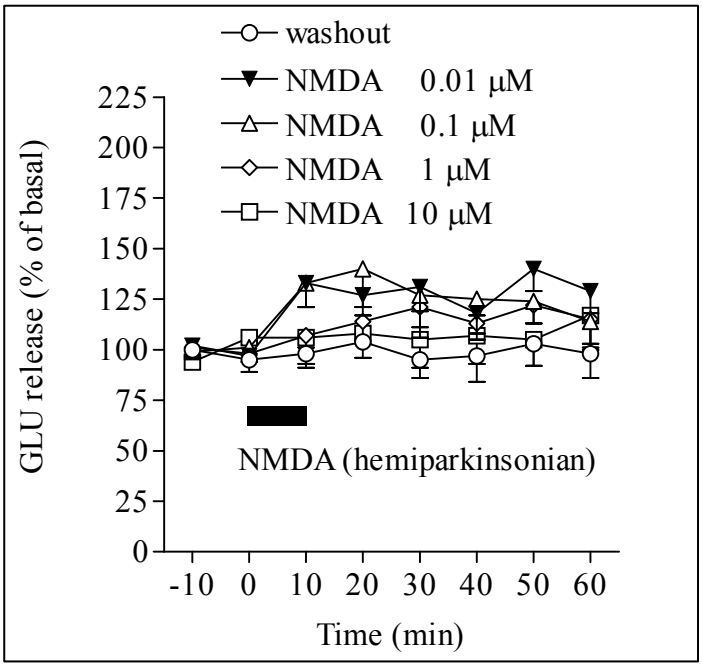


Fig. 23. Effect of intrastriatal perfusion with NMDA (0.01-10 μM, 10 min; black bar) on striatal GLU release in DA-depleted hemiparkinsonian rats. Data are expressed as percentages ± SEM of basal pre-treatment levels (calculated as the mean of the two samples before the treatment). A significant effect ($p<0.05$ *vs* washout) was observed at NMDA 0.01 μM (solid symbols).

Section III

NMDA receptors containing the NR2A and NR2B subunits differentially regulate striatal output pathways

(Paper II)

4.3 NR2A and NR2B subunit containing NMDA receptors differentially regulate striatal output pathways *(Paper II)*

We demonstrated that striatal NMDA receptor activation stimulated GABA release both in GP and SNr, endorsing the view that NMDA receptors in the striatum modulate both the "direct" and "indirect" pathways *(Section I)*. We also previously reported that intrastriatal perfusion with the NMDA channel blocker MK-801 evoked SNr GLU release (Morari *et al.*, 1998b), suggesting that striatal NMDA receptors mediate not only phasic and but also tonic regulation of the striatofugal pathways. In the present study we sought to investigate whether different subsets of NMDA receptors are involved in these modulations.

Triple probe microdialysis was employed in awake rats: probes were implanted in the DLS, ipsilateral SNr and GP. (R)-[(S)-1-(4-bromo-phenyl)-ethylamino]-(2,3-dioxo-1,2,3,4-tetrahydroquinoxalin-5-yl)-methyl]-phosphonic acid (NVP-AAM077) a NR2A selective antagonist (Auberson *et al.*, 2002), and (R-(R*,S*)-α-(4-hydroxyphenyl)-β-methyl-4-(phenylmethyl)-1-piperidinepropanol (Ro 25-6981), a NR2B selective antagonist (Fischer *et al.*, 1997), were perfused in the DLS and GABA release simultaneously monitored in the target areas, GP and SNr.

4.3.1 Effect of blockade of neuronal transmission in the DLS: validation of the method

To confirm the view that changes in GABA release could be related to blockade of tonic activity of striatofugal neurons, the effect of intrastriatal perfusion with the Na^+-channel blocker tetrodotoxin (TTX) was investigated. Also GLU levels were monitored.

TTX did not affect DLS amino acid levels (**Fig 24A**). RM ANOVA on the effect of TTX on pallidal amino acid levels (**Fig. 24B**) showed a significant effect of treatment ($F_{3,24}$=7.10, p=0.0014), time ($F_{7,21}$=7.21, p<0.0001) and a non significant time x treatment interaction ($F_{21,224}$=1.14, p=0.076). Contrast analysis revealed that TTX reduced GABA release (~35 %; p=0.0010) but did not affect GLU levels (p=0.326) compared to control groups. RM ANOVA was also performed on the effect of TTX on nigral amino acid levels (**Fig. 24C**). Significant effects of treatment ($F_{3,21}$=3.55, p=0.031) and time ($F_{7,21}$=5.80, p<0.0001) were found together with a significant time x treatment interaction ($F_{21,204}$=1.73, p=0.028). Contrast analysis revealed that TTX reduced GABA release (~33 %, p=0.0010) but did not affect GLU levels (p=0.210) compared to control groups.

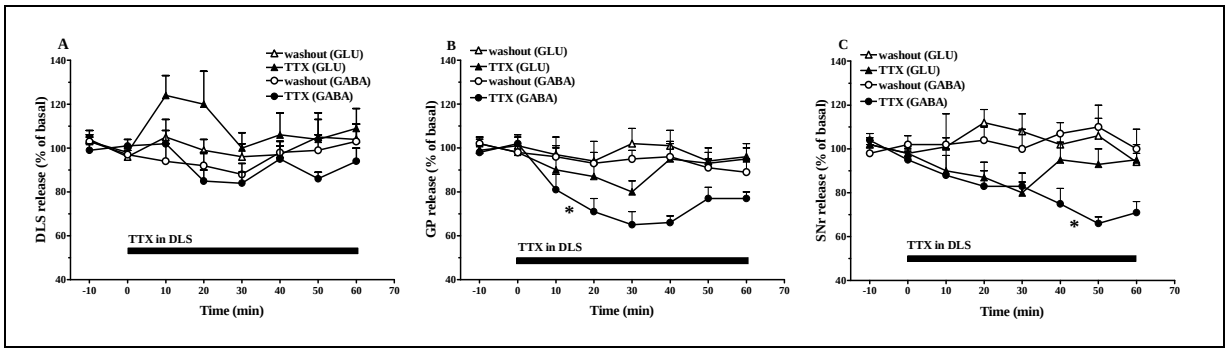


Fig. 24. Effect of tetrodotoxin (TTX) on GABA and GLU release in striatum and its target areas. Effect of reverse dialysis of TTX (1 μM) in the dorsolateral striatum (DLS) of awake rats on GLU and GABA release in DLS (panel A), ipsilateral globus pallidus (GP; panel B) and ipsilateral substantia nigra reticulata (SNr, panel C).
*p<0.05 different from washout (RM ANOVA followed by contrast analysis and the sequentially rejective Bonferroni's test).

4.3.2 Primary effects of antagonists

To investigate whether striatal NR2A and NR2B subunit containing NMDA receptors mediate tonic regulation of striato-pallidal and striato-nigral neurons, reverse dialysis of NR2A and NR2B subunit selective antagonists was performed in DLS, and GABA release monitored in DLS and its projection areas, namely GP and SNr.

Intrastriatal NVP-AAM077 reduced GABA in GP (**Fig 25A**) without affecting GABA release in SNr (not shown). Basal GABA levels in GP were 20.9 ± 2.3 nM. RM ANOVA on the effect of NVP-AAM077 on GABA levels in GP showed a significant effect of treatment ($F_{2,12}$=8.15, p=0.0058) and time ($F_{13,26}$=7.689, p<0.0001) but not a significant time x treatment interaction ($F_{26,220}$=1.04, p=0.41). Contrast analysis revealed that NVP-AAM077 (300 nM) reduced pallidal GABA release (maximal inhibition ~40%) compared to control group (p=0.0023). On the other hand, intrastriatal Ro 25-6981 did not change pallidal GABA release (not shown) but reduced nigral GABA release (**Fig 25B**). RM ANOVA on GABA levels in SNr showed a significant effect of treatment ($F_{2,12}$=4.85, p=0.028), time ($F_{13,26}$=5.15, p<0.0001) and a non significant time x treatment interaction ($F_{26,234}$=1.23, p=0.214). Contrast analysis revealed that both Ro 25-6981 30 nM and 300 nM reduced to the same extent GABA release (~40%) compared to control group (P=0.022 and P=0.017, respectively). Lower concentrations (3 nM) were also tested and found ineffective (data not shown).

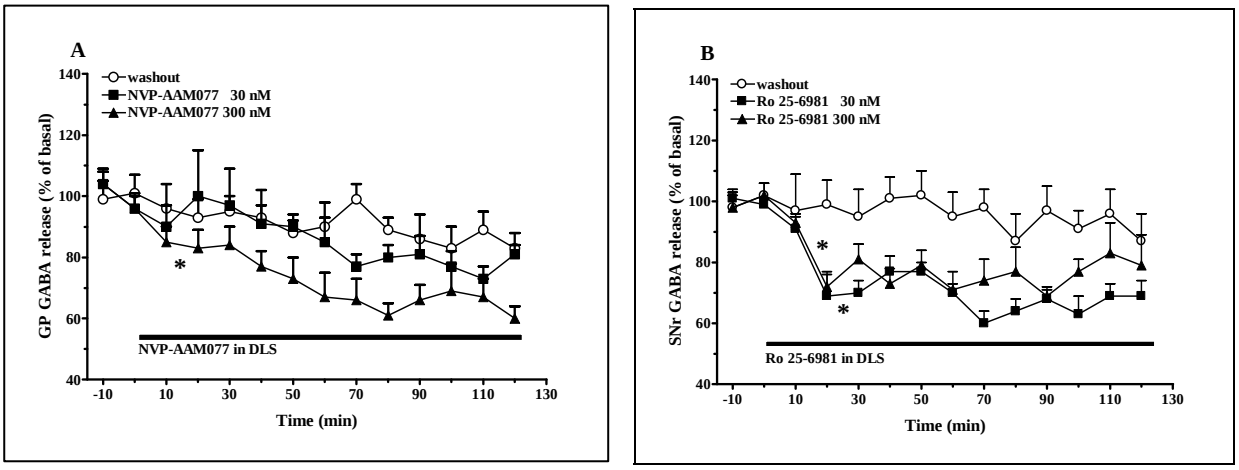


Fig 25. Effect of subunit selective NMDA receptor antagonists on pallidal and nigral GABA release. Effect of reverse dialysis (30 and 300 nM; panel A) of NVP-AAM077 in the dorsolateral striatum (DLS) of awake rats on GABA extracellular levels in ipsilateral globus pallidus (GP). Effect of reverse dialysis of Ro 25-6981 (30 and 300 nM; panel B) in the dorsolateral striatum (DLS) of awake rats on GABA extracellular levels in ipsilateral substantia nigra reticulata (SNr). Data are expressed as percentages ± SEM of basal pre-treatment levels (calculated as mean of the two samples before the treatment).
*p<0.05 different from washout (one-way repeated measure ANOVA followed by contrast analysis and the sequentially rejective Bonferroni's test).

4.3.3 Pharmacological antagonism between NMDA and subunit selective NMDA antagonists

To investigate whether striatal NR2A and NR2B subunit containing NMDA receptors mediate phasic regulation of striato-pallidal and striato-nigral neurons, NVP-AAM077 and Ro 25-6981 were challenged at the higher concentration (i.e. 300 nM) against NMDA (10 μM). This concentration was chosen since in previous experiments (*Section I)* it was shown that reverse dialysis of NMDA 10 μM in the striatum was able to evoke GABA release both in the GP and SNr.

GP. **(Fig. 26)** Only NVP-AAM077 was able to counteract the NMDA-evoked GABA release. RM ANOVA on the effect of NVP-AAM077 showed a significant effect of treatment ($F_{3,21}$=7.65, p=0.0012) but not time ($F_{7,21}$=1.65, p=0.123), and a significant time x treatment interaction ($F_{21,212}$=2.80, p<0.0001). Contrast analysis revealed that intrastriatal NMDA elevated GABA release compared to control group (P<0.0001) and NVP-AAM077 prevented it (P=0.0013 vs NMDA alone). Co-application of NMDA and NVP-AAM077 did not produce a significant stimulation (P=0.95; **fig 26A**). As far as the effect of Ro 25-6981 is concerned, a significant effect of treatment ($F_{3,21}$=7.71, p=0.0011), time ($F_{7,21}$=3.38, p=0.0019) and a significant time x treatment interaction ($F_{21,212}$=2.26, p=0.0018) were found. Contrast analysis revealed that co-application of Ro 25-6981 and NMDA significantly evoked GABA release

(p=0.043 vs washout) and that this stimulation was not different from that evoked by NMDA alone (P=0.077; **fig 26B**).

SNr. Contrary to that observed in the GP, NVP-AAM077 was not able to counteract the NMDA-induced GABA release. RM ANOVA showed a significant effect of treatment ($F_{3,18}$=11.59, p<0.001), time ($F_{7,21}$=5.27, p<0.001), and a significant time x treatment interaction ($F_{21,168}$=3.30, p=0.0075). Contrast analysis revealed that intrastriatal NMDA, alone or in combination with NVP-AAM077, elevated GABA release compared to saline (P=0.0009) and NVP-AAM077 alone was similarly effective (P=0.38 vs NMDA; **fig 27A**). Conversely, Ro 25-6981 attenuated the NMDA response. A significant effect of treatment ($F_{3,21}$=5.27, p=0.0041), but not time ($F_{7,21}$=1.46, p=0.18), and a significant time x treatment interaction ($F_{21,196}$=1.96, p=0.0095) were found. Contrast analysis revealed that NMDA in the presence of Ro 25-6981 produced a significant stimulation (P=0.034 vs Ro 25-6981 alone). However, post hoc analysis on the time-course of the response indicated that Ro 25-6981 prevented the peak at 30 min (p=0.0025 vs NMDA alone; **fig 27B**).

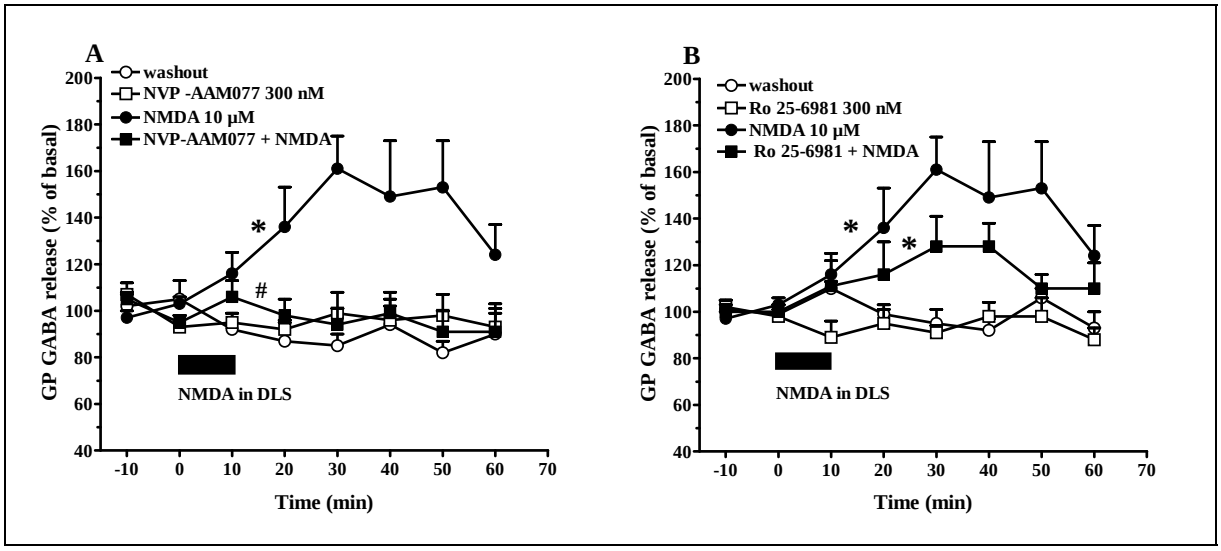


Fig 26. Effect of subunit selective NMDA receptor antagonists on NMDA-evoked pallidal GABA release. Effect of reverse dialysis of NVP-AAM077 (300 nM; panel A) and Ro 25-6981 (300 nM; panel B) in the dorsolateral striatum (DLS) of awake rats on GABA extracellular levels in the ipsilateral globus pallidus (GP) evoked by intrastriatal NMDA (10 µM, 10 min, black bar). Perfusion with antagonists started 90 min before NMDA and continued until the end of experiment. Data are expressed as percentages ± SEM of basal pre-treatment levels (calculated as mean of the two samples before the treatment). Data referring to the NMDA alone groups are the same in panel A and B.
*p<0.05 different from washout; $^{\#}$p<0.05 different from NMDA (two-way repeated measure ANOVA followed by contrast analysis and the sequentially rejective Bonferroni's test).

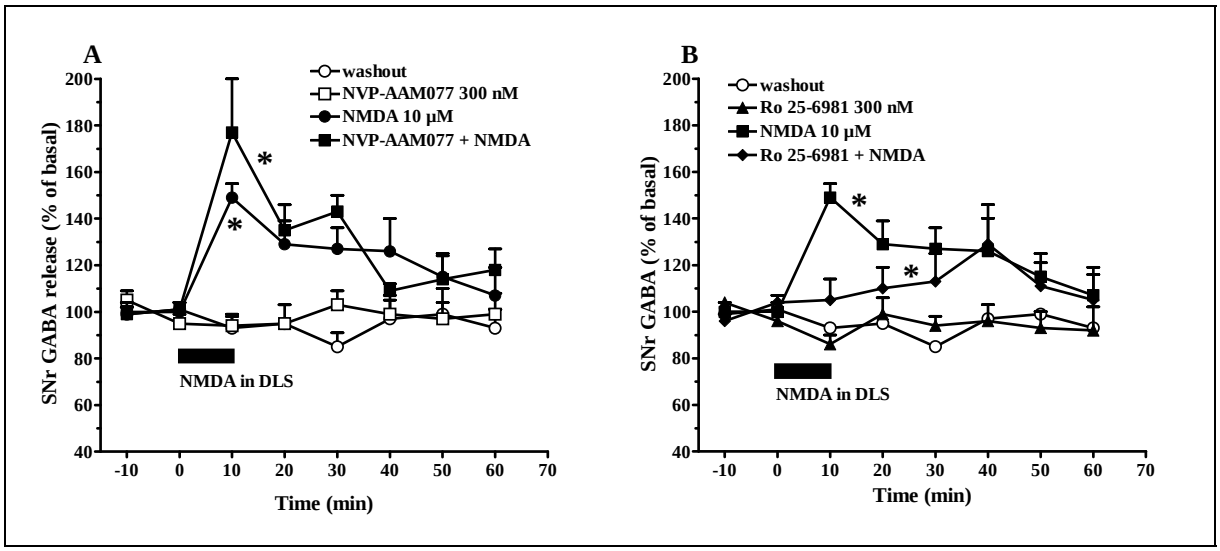


Fig 27. Effect of subunit selective NMDA receptor antagonists on NMDA-evoked nigral GABA release. Effect of reverse dialysis of NVP-AAM077 (300 nM; panel A) and Ro 25-6981 (300 nM; panel B) in the dorsolateral striatum (DLS) of awake rats on GABA extracellular levels in the ipsilateral substantia nigra reticulata (SNr) evoked by intrastriatal NMDA (10 µM, 10 min, black bar). Data are expressed as percentages ± SEM of basal pre-treatment levels (calculated as mean of the two samples before the treatment). Data referring to the NMDA alone groups are the same in panel A and B.
*$p<0.05$ different from washout (two-way repeated measure ANOVA followed by contrast analysis and the sequentially rejective Bonferroni's test).

Section IV

Differential effect of NR2A and NR2B subunit selective NMDA receptor antagonists on striato-pallidal neurons: relationship to motor response in the 6-hydroxydopamine model of parkinsonism

(Paper III)

4.4 Dopamine depletion alters the modulation of striatal output pathways operated by NR2A and NR2B subunit containing NMDA receptors

In the present study, triple probe microdialysis was used to investigate whether the modulation of striatal output pathways operated by NR2A and NR2B subunits is changed under parkinsonian conditions. Three microdialysis probes were implanted in DA depleted DLS, ipsilateral SNr and GP. NVP-AAM077 and Ro 25-6981 were perfused in DLS, and GABA and GLU release simultaneously monitored in the three areas.

4.4.1 Primary effects of subunit selective NMDA antagonists perfused in the DA-depleted striatum

Reverse dialysis of NVP-AAM077 in the DA-depleted striatum of hemiparkinsonian rats reduced GABA levels in GP but not SNr while reverse dialysis of Ro 25-6981 failed to change GABA release in the areas of interest.

RM ANOVA on the effect of NVP-AAM077 on GABA levels (**Fig. 28A**) showed a significant effect of treatment ($F_{3,15}$=8.88, p=0.0013), time ($F_{13,39}$=7.58, p<0.0001) and a significant time x treatment interaction ($F_{39,232}$=1.65, p=0.0136). Contrast analysis revealed that both NVP-AAM077 30 and 300 nM reduced GABA release (maximal inhibition ~40%) compared to control group (p=0.0316 and p=0.0007, respectively). Conversely, intrastriatal Ro 25-6981 did not change GABA (**Fig. 28B**) release in GP.

Basal GABA levels in SNr were 8.0 ± 0.5. Intrastriatal perfusion with NVP-AAM077 (**Fig 29A**) or Ro 25-6981 (**Fig 29B**) did not change nigral GABA and GLU outflow.

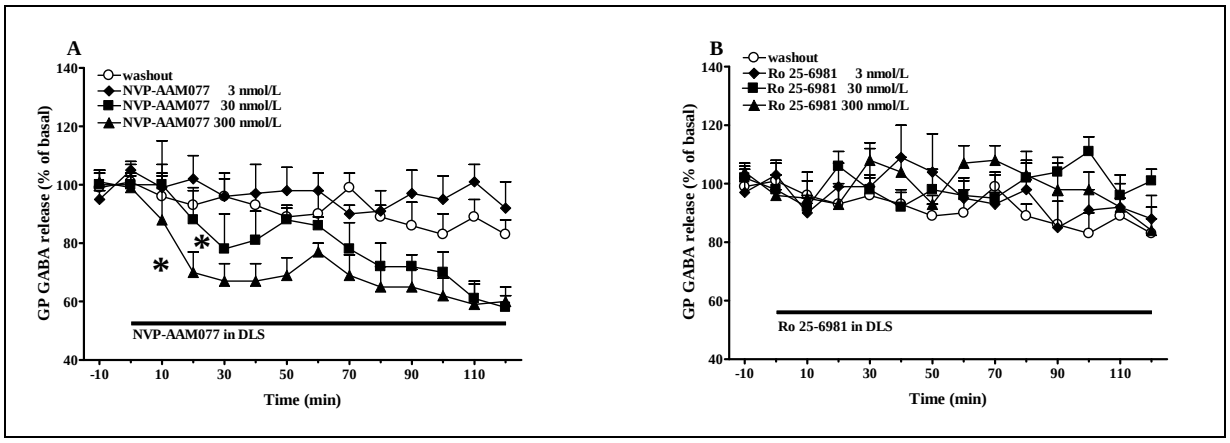


Fig 28. Effect of subunit selective NMDA receptor antagonists on pallidal GABA release in hemiparkinsonian rats. Effect of reverse dialysis of NVP-AAM077 (3, 30 and 300 nM; panel A) and Ro 25-6981 (3, 30 and 300 nM; panel B) in the DA-depleted dorsolateral striatum (DLS) on GABA extracellular levels in ipsilateral globus pallidus (GP). Data are expressed as percentages ± SEM of basal pre-treatment levels (calculated as mean of the two samples before the treatment).
Basal GABA levels (nM) were 10.2 ± 0.6 (n= 34) and no differences between treatemnt groups were detected.
*p<0.05 different from washout, RM ANOVA followed by contrast analysis and the sequentially rejective Bonferroni's test.

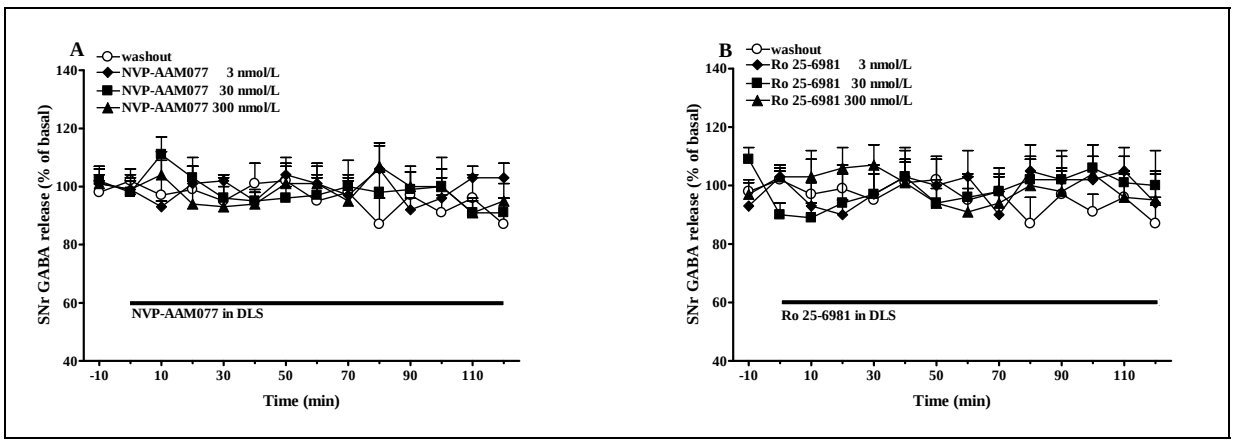


Fig 29. Effect of subunit selective NMDA receptor antagonists on nigral GABA release in hemiparkinsonian rats. Effect of reverse dialysis of NVP-AAM077 (3, 30 and 300 nM; panel A) and Ro 25-6981 (3, 30 and 300 nM; panel B) in the DA-depleted dorsolateral striatum (DLS) on GABA extracellular levels in ipsilateral substantia nigra reticulata (SNr). Data are expressed as percentages ± SEM of basal pre-treatment levels (calculated as mean of the two samples before the treatment).
Basal GABA (nM) levels were 8 ± 0.5 (n= 35) and no differences between treatment groups were detected.
*p<0.05 different from washout, RM ANOVA followed by contrast analysis and the sequentially rejective Bonferroni's test.

4.4.2 Motor behaviour in 6-OHDA hemilesioned rats

To evaluate whether the different neurochemical responses to NR2A and NR2B subunit selective antagonists were predictive of a different capabilities to attenuate the 6-OHDA induced motor deficit, we employed a battery of behavioural tests specific for different motor abilities (Marti *et al.*, 2005, 2007).

Hemiparkinsonian rats displayed overall marked akinesia/hypokinesia compared with vehicle-injected rats (**Fig. 30**), as shown by multiple behavioral tests: increase in the total time spent on the blocks in the bar test (96.8± 1.6 and 2.0 ± 0.2 s, respectively; p <0.001), reduction in the total number of steps in the drag test (14.2 ± 0.4 and 23.5±0.4, respectively; p<0.001), and reduced rotarod performance (406 ± 21 and 1044 ± 50 s in the 5–60 rpm speed range, respectively; p<0.01). Hemiparkinsonian rats also displayed significant motor asymmetry because the contralateral forepaw spent greater time on the blocks (**Fig. 30*A***) and performed lesser number of steps than the ipsilateral one (**Fig. 30*B***). Vehicle-injected rats performed comparably to naïve animals (**Fig. 30*C***).

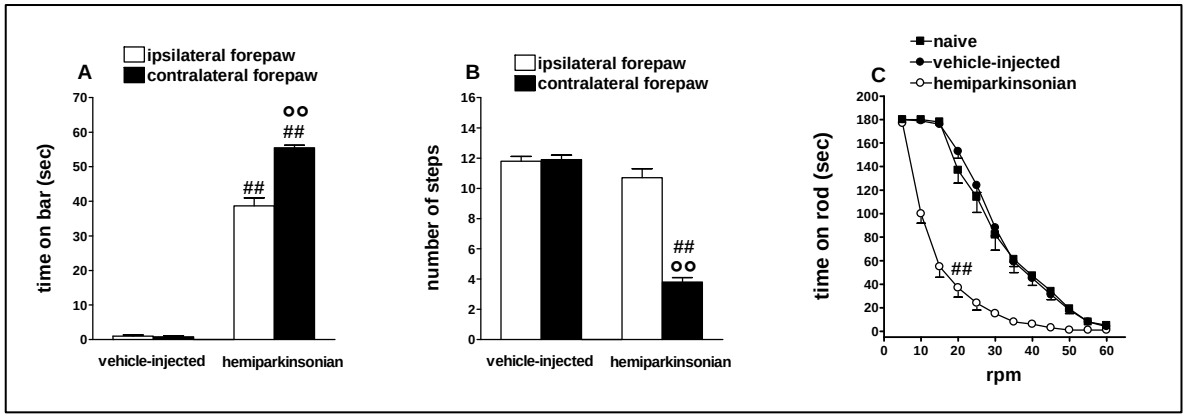


Figure 30. Characterization of motor activity in vehicle-injected and 6-OHDA-injected (hemiparkinsonian) rats. ***A–C***,

Hemiparkinsonian rats displayed overall reduction in motor activity and motor asymmetry compared with vehicle-injected rats, as shown by an increase in the total time spent on the blocks in the bar test (the contralateral forepaw being more severely affected; ***A***), reduction in the number of steps made by the contralateral forepaw in the drag test (***B***), and reduced rotarod performance (***C***). Data are mean ± SEM of 10 –33 determinations obtained from 26 vehicle-injected, 33 hemiparkinsonian, and 18 naive rats. °°p<0.01 versus the ipsilateral forepaw. ##p<0.01 versus vehicle-injected and naive rats (ANOVA followed by Newman–Keuls *post hoc* test).

4.4.3 Systemic administration of subunit selective NMDA antagonists improved motor performance in hemiparkinsonian rats

Motor effects of NVP-AAM077.

The NR2A preferential antagonist NVP-AAM077 (0.001- 1 mg/Kg) did not reduce overall akinesia/bradykinesia in bar test (**fig.31A**) neither altered motor asymmetry in the drag test (**fig. 31B**). NVP-AAM077 affected motor performance in the rotarod test depending on the dose injected (**fig. 31C**). In this respect, RM ANOVA revealed a significant effect of treatment ($F_{4,6}$=13.01,), time ($F_{1,4}$=5.50, p=0.026) and a significant time x treatment interaction ($F_{4,30}$=3.13, p=0.029). *Post hoc* analysis revealed that, 20 min after injection, NVP-AAM077 stimulated motor performance at 0.001 and a.01 mg/Kg (p=0.0125 and p<0.0001, respectively) and inhibited it at 1 mg/Kg (p=0.010) being the intermediate dose 0.1 mg/Kg ineffective. Time spent on the rotarod returned to values not different from vehicle treated 80 min after injection.

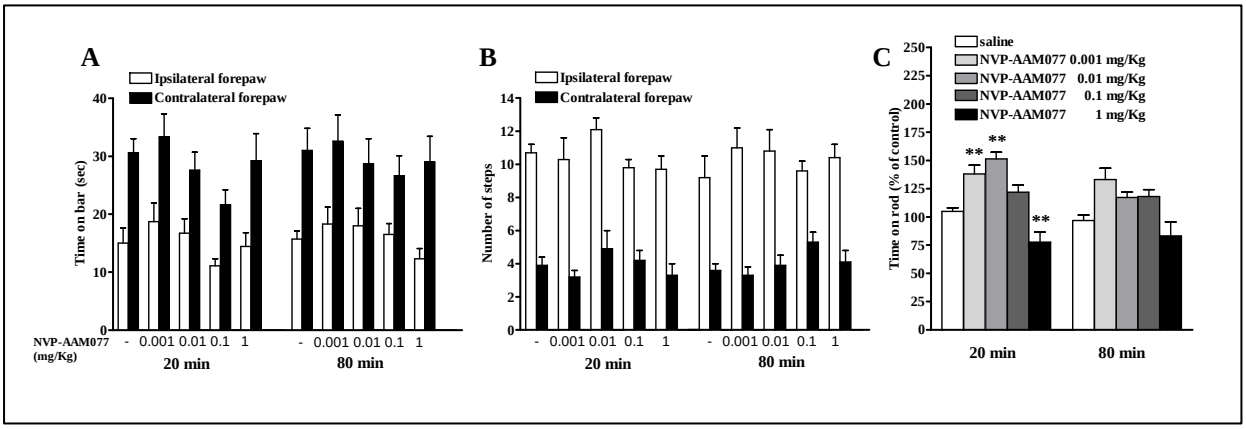


Fig. 31. Motor effects of NVP-AAM077 in hemiparkinsonian rats. Systemic (i.p.) administration of NVP-AAM077 (0.001-1 mg/kg) did not affect the time spent on the blocks in the bar test (***A***) and the number of steps in the drag test (***B***) but affected motor performance on the rotarod (***C***). Motor tests were performed 20 and 80 min after drug injection. Motor activity was evaluated separately at the ipsilateral and contralateral (parkinsonian) forepaws. Data are mean ± SEM of 7 rats per group.
*p<0.05 different from saline (RM ANOVA followed by contrast analysis and the sequentially
rejective Bonferroni test).

Motor effects of Ro 25-6981. The NR2B selective antagonist Ro 25-6981 (0.1-3 mg/Kg) did not reduce overall akinesia/bradykinesia in bar test (**fig.32A**) neither altered motor asymmetry in the drag test (**fig. 32B**) compared to vehicle injected rats. Ro 25-6981 dose-dependently increased motor performance in the rotarod test (**fig. 32C**). In this respect, RM ANOVA revealed a significant effect of treatment ($F_{3,6}$=25.45,) and time ($F_{1,3}$=5.43, p=0.030), but not significant time x treatment interaction ($F_{3,20}$=1.19, p=0.3391). *Post hoc* analysis revealed that, at 20 min after injection, Ro 25-6981 1 and 3 mg/Kg stimulated motor performance (p=0.0167 and p<0.0001, respectively) being the lower dose 0.1 mg/Kg ineffective. Eighty min after injection only Ro 25-6981 3 mg/Kg still significantly improve rotarod performance (p=0.001).

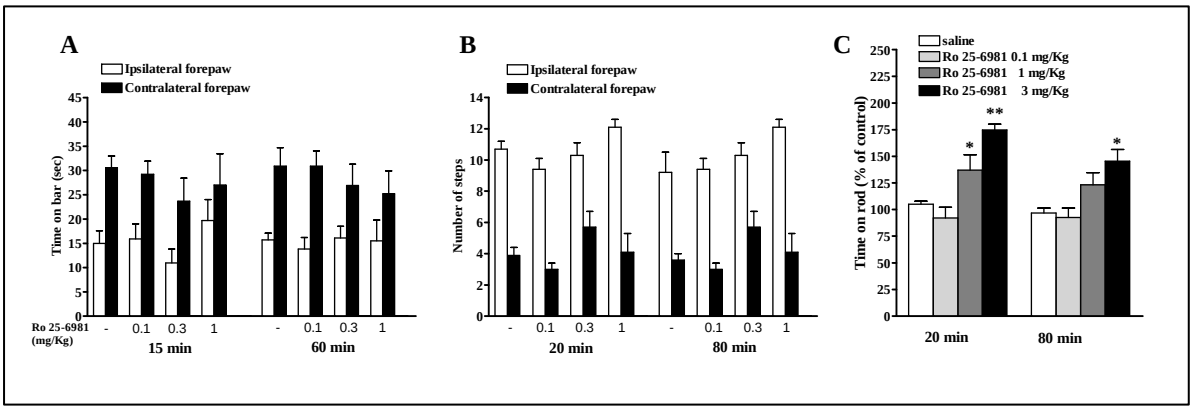


Fig. 32. Motor effects of Ro 25-6981 in hemiparkinsonian rats. Systemic (i.p.) administration of Ro 25-6981 (0.1-3 mg/kg) did not affect the time spent on the blocks in the bar test (**A**) and the number of steps in the drag test (**B**) but affected motor performance on the rotarod (**C**). Motor tests were performed 20 and 80 min after drug injection. Motor activity was evaluated separately at the ipsilateral and contralateral (parkinsonian) forepaws. Data are mean ± SEM of 7 rats per group.
*p<0.05 different from saline (RM ANOVA followed by contrast analysis and the sequentially rejective Bonferroni test).

4.4.4 Motor facilitating doses of subunit selective antagonists decreased GABA levels in GP whereas motor inhibiting doses increased them.

To investigate the circuitry involved in the motor responses to subunit selective antagonists, we employed triple probe microdialysis. GABA release was monitored simultaneously in GP and SNr (ipsilateral to the lesion side) following i.p. administration of behaviourally relevant doses of NVP-AAM077 or Ro 25-6981.

In GP, RM ANOVA on GABA levels showed a significant effect of treatment ($F_{3,18}$=23.83, p<0.0001), time ($F_{7,21}$=3.29, p=0.0028) and a significant time x treatment interaction

($F_{21,152}$=3.88, p<0.0001). Contrast analysis showed that doses improving motor performance (NVP-AAM077 0.01 mg/Kg and Ro 25-6981 1 mg/Kg) decreased pallidal GABA levels (~40 %) while doses inhibiting motor performance (NVP AAM077 1 mg/Kg) elevated them (~45%) (**Fig. 33A**).

In SNr, contrary to that observed in GP, RM ANOVA showed that NVP AAM077 and Ro 25-6981

did not modify GABA levels (**Fig. 33B**).

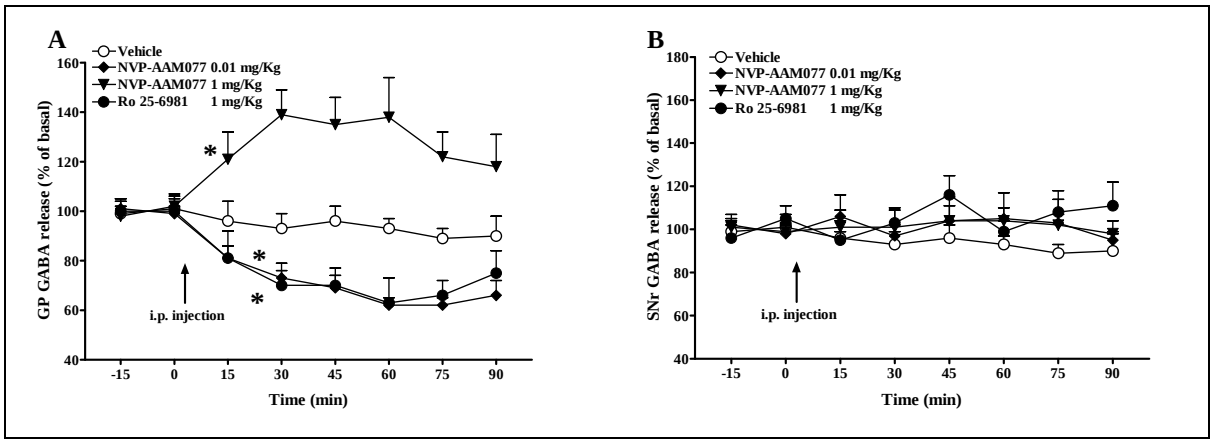


Fig 33. Effect of subunit selective NMDA receptor antagonists on pallidal and nigral GABA release in DA-depleted hemiparkinsonian rats. Effect of intraperitoneal injection (arrow) of NVP-AAM077 (0.01 and 1 mg/Kg) and Ro 25-6981 (1 mg/Kg) on GABA extracellular levels in GP (**A**) and SNr (**B**) ipsilateral to the 6-OHDA lesion. Data are expressed as percentages ± SEM of basal pre-treatment levels (calculated as mean of the two samples before the treatment). Basal GABA (nM) levels in GP and SNr were, respectively: 14.1 ± 1.6 and 9.9 ± 0.8 (n=20). No differences between treatment groups were detected.
*p<0.05 different from vehicle injected, one-way repeated measure ANOVA followed by contrast analysis and the sequentially rejective Bonferroni's test.

Section V

Effect of selective NR2B blockade on motor symptoms in the 3-NP mouse model of Huntington Disease

To investigate the therapeutic potential of NR2B selective blockade on HD-like motor symptoms , we employed a well characterized 3-NP intoxication protocol in mice (Fernagut *et al.*, 2002). At the end of the intoxication protocol, the mice display a moderate to severe motor impairment. We therefore employed a validated set of motor tests to evaluate the effect of acute systemic (i.p.) administration of the NR2B subunit selective antagonist Ro 25-6981 (1-3-6 mg/Kg).

4.5.1 Progressive motor impairment produced by 3-NP intoxication in mice

Lethality

Three out of 32 (10%) 3-NP-intoxicated mice died during the intoxication; in all cases death occurred after the first 50 mg/kg injection.

Motor symptoms monitoring

Monitoring the time course of the motor symptoms during the 3-NP intoxication demonstrated that no significant motor or behavioural signs appeared until the dose of 40 mg/kg of 3-NP was reached (Day 7, $P < 0.05$ from baseline; **Fig. 34**). The earliest motor sign observed was motor slowness, followed by permanent hindlimb dystonia, then hindlimb clasping, followed by truncal dystonia and finally global postural abnormalities with balance impairment. Motor impairment worsened during the incremental increase in dosage from 40 to 50 mg/kg and was maximal on the day following the end of intoxication.

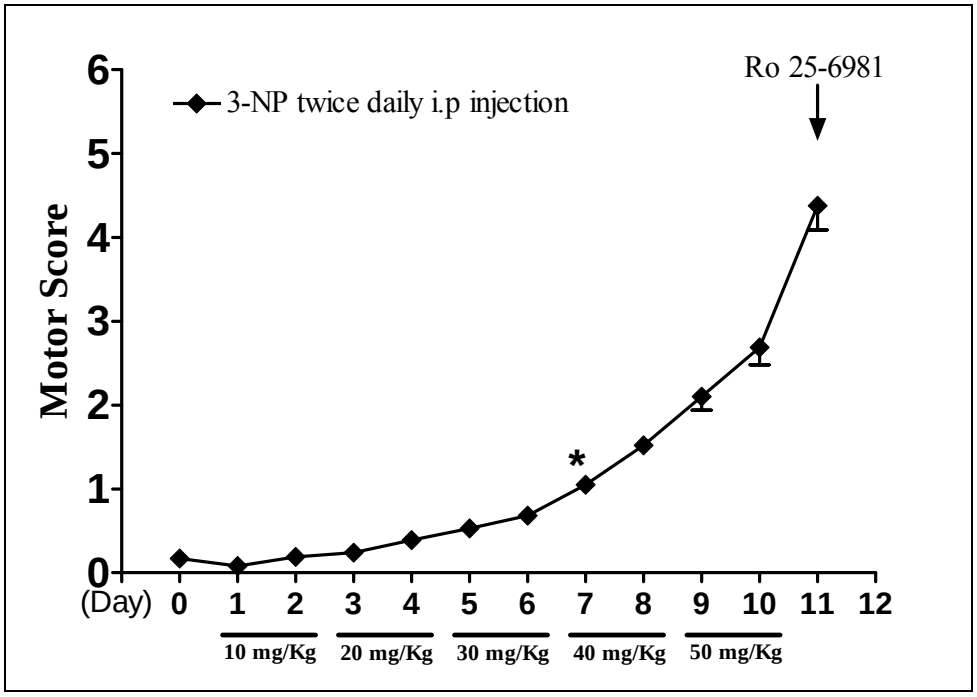


Fig. 34. Evolution of motor behavioural score during 3-NP intoxication. During the intoxication phase, the motor score became significantly different from baseline at day 7 (t test). Mice were scored before each injection during the intoxication phase.

4.5.2 Effect of acute selective NR2B blockade: motor and sensorimotor tests

On the day of the experiment, 12 hours after the last 3-NP injection, mice were singularly videotaped for 1 minute at baseline and then 40 and 90 minutes after injection.

The scores assigned by a blind experimenter on the basis of the videotape are illustrated un **fig. 35**. No significant differences have been detected between saline and Ro 25-6981 (1-3-6 mg/Kg) injected groups.

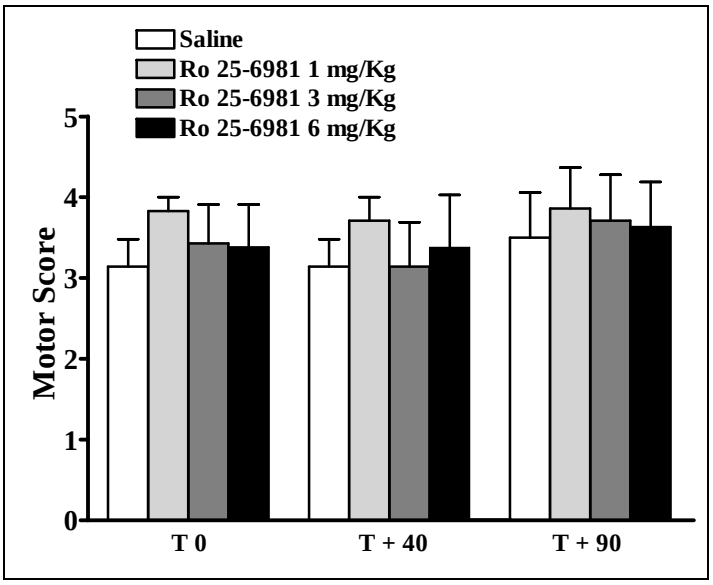


Fig. 35. Motor score immediately before, and 40 or 90 min after intraperitoneal injections of saline and Ro 25-6981 (1, 3, 6 mg/Kg). No significant differences between groups were detected at any time point.

Traversing a beam

Before intoxication, baseline performance levels were not different among the four groups. Sixty minutes after injection, there was a significant increase in the number of sideslips in saline-injected control mice (12.86 ± 2.74 vs. 0.6 ± 0.09, $P < 0.01$) and no significant changes between Ro 25-6981 and saline injected groups (1 mg/Kg: 18.29 ± 1.03; 3 mg/kg: 11.2 ± 2.73; 6 mg/Kg: 14.67 ± 2.02; **fig. 36A**). Similarly, the number of falls from the beam (equal to 0 before the intoxication) did not significantly change between groups (saline: 1.29 ± 0.47; 1 mg/Kg: 2.43 ± 0.3; 3 mg/kg: 1.2 ± 0.58; 6 mg/Kg:1.63±0.52; **fig.36B**)

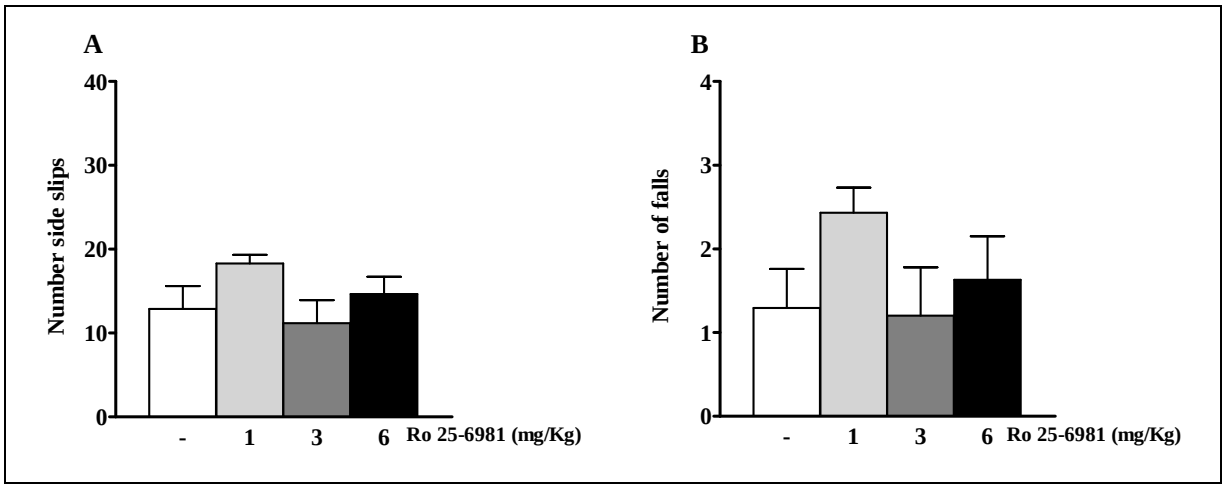


Fig. 36. Mean number of sideslips (**A**) and falls (**B**) during the three trials on beam traversing task 60 minutes after intraperitoneal injection of saline or Ro 25-6981 (1, 3, 6 mg/Kg). No significant differences between groups were detected.

Open field activity

To avoid habituation phenomenon that constitutes the main limit of this test, we measured the spontaneus motor activity in the open field only at baseline (before intoxication) and 40 minutes after drug injection on the day of the experiment, comparing the results obtained with the saline injected group.

Before intoxication, maximal velocity, mean velocity (Vmean), distance travelled, and number of rearings were not different among the four groups

Open field activity demonstrated a significant reduction in all motor parameters considered in all four groups, with no differences between them .

Maximal velocity was: saline, 18,06 ± 1.37; 1 mg/Kg, 15 ± 1.29; 3 mg/kg, 17.2 ± 1.79; 6 mg/Kg, 16.94 ± 1.12 cm/sec (**Fig. 37A**).

Mean velocity (Vmean) was: saline, 3.5 ± 0.63; 1 mg/Kg, 2.29 ± 0.67; 3 mg/kg, 3.04 ± 0.8; 6 mg/Kg, 3.08 ± 0.64 cm/sec (**Fig. 37B**).

The distance travelled was: saline, 1053 ± 190; 1 mg/Kg, 685 ± 203; 3 mg/kg, 918 ± 240; 6 mg/Kg, 923 ± 191 cm (**Fig. 37C**).

The number of rearings was: saline, 2.7 ± 0.95; 1 mg/Kg, 1.14 ± 0.14; 3 mg/kg, 3.57 ± 1.77; 6 mg/Kg, 2.38 ± 1.84 (**Fig. 37D**).

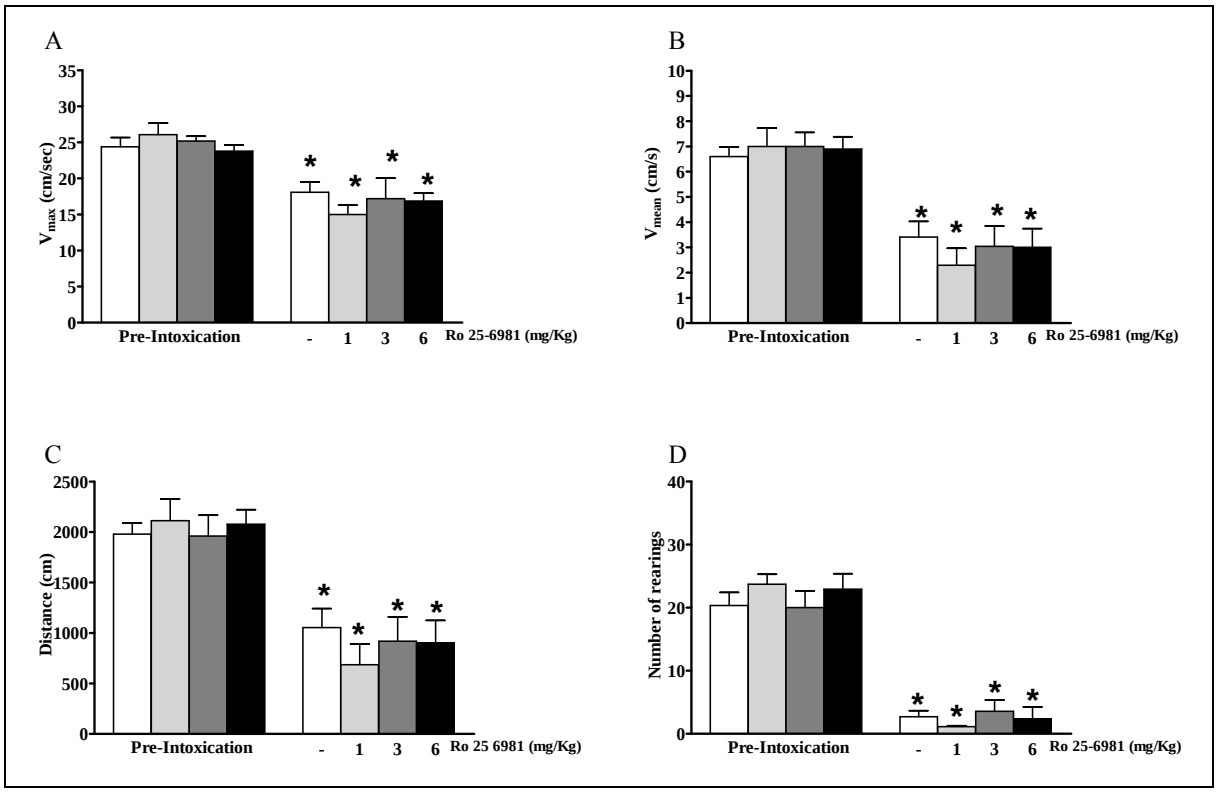


Fig. 37. Open field parameters: (**A**) maximal velocity, (**B**) mean velocity, (**C**) distance travelled, (**D**) number of rearings. Values were taken before intoxication (left) and 40 min after intraperitoneal injection of saline or Ro 25-6981 (1, 3, 6 mg/Kg). No significant differences between groups were detected at pre-intoxication or after drug treatment, while 3-NP treatment produced a significant reduction of all parameters considered compared to baseline (pre-treatment) performance ($p<0.05$).

5. DISCUSSION

Section I: Striatal NMDA activation modulates striatopallidal and striatonigral pathways in physiological and pathological (parkinsonian) conditions

We showed that activation of striatal NMDA receptors increased GABA release both in GP and SNr and that this modulation was altered by DA depletion as in the hemiparkinsonian rat.

Both subpopulation of MSNs, projecting either to GP or SNr, express NMDA receptors (Landwehrmeyer *et al.*, 1995; Tallaksen-Greene *et al.*, 1992). Behavioural evidence that intrastriatal NMDA induced both motor impairment (Schmidt and Bury, 1988; Yoshida *et al.*, 1991; Klockgether and Turski, 1993) and activation (Thanos et al, 1992; Ossowska and Wolfarth, 1995) has been presented, suggesting that striatal NMDA receptors regulate both the striato-nigral and the striato-pallidal pathways. In previous studies, we also provided indirect evidence of this dual modulation, by demonstrating that reverse dialysis of NMDA in the striatum evoked GABA and GLU release in the SNr (Morari *et al.,* 1996; Marti *et al.*, 2005). The present study, provides for the first time, more direct support to this view. Indeed, the triple probe approach allowed us the monitor GABA levels simultaneously in GP and SNr in response to intrastriatal NMDA perfusion. Dynamics of GABA release in striatal projection areas can be considered a reliable marker of the activation of the indirect and direct pathways, respectively (as further demonstrated by the TTX sensitivity of the GABA levels, presented in section III). We showed that intrastriatal perfusion with NMDA 1 μM selectively evoked GABA release in SNr while higher concentrations (10 and 100 μM) were able to stimulate GABA release in both GP and SNr. This led us to speculate that, in physiological conditions, NMDA receptors exert preferential facilitatory control over the direct pathway (activated by a lower NMDA concentration) and are expected to promote movement.

DA loss caused an overall increase in sensitivity towards NMDA (leftward shift of NMDA concentration-response-curve), since pallidal and nigral GABA release became sensitive to lower NMDA concentrations. This indicates that endogenous DA regulates the control phasically operated by NMDA over MSNs. In physiological conditions, the tonic dopaminergic input provided by the nigrostriatal dopaminergic neurons to the striatum may modulate the NMDA transmission at the MSNs through changes in membrane excitability, protein phosphorylation and receptor trafficking (for review see Cepeda and Levine 2006). In this respect, we previously showed that selective blockade of D1 receptors attenuated NMDA effects on striatal GABA and nigral GLU extracellular levels (Morari *et al.*, 1994; Marti *et al.*, 2002), supporting the view of a positive D1-NMDA cooperation in the striatum. Conversely, selective blockade of D2 receptors produced an enhancement of the striatal GABA (Morari *et*

al., 1994) and nigral GLU (Marti *et al.*, 2002) response to NMDA, suggesting the existence of a D2-NMDA negative interaction.

Moreover, chronic DA depletion has been associated with profound adaptative changes of striatal NMDA receptors function, such as increased binding (Samuel *et al.*, 1990; Wullner *et al.*, 1994), expression (Tremblay *et al.*, 1995; Ulas and Cotman, 1996) and phosphorylation (Menegoz *et al.*, 1995; Chase and Oh, 2000) of NMDA receptor subunits, particularly on striato-pallidal neurons (Ganguly and Keefe, 2001), that lead to up-regulation of NMDA transmission. In line with these findings, we demonstrated that DA-denervation is associated with increased responsiveness of striatal NMDA receptors on cholinergic interneurons (Marti *et al.*, 1999).

The fact that DA loss caused a greater increase in sensitivity to NMDA of striato-pallidal vs striato-nigral neurons, compared to naïve rats, supports the view of an imbalance in favour of the indirect pathway during parkinsonism (Alexander and Crutcher, 1990; Chevalier and Deniau, 1990).

In summary, the present study confirms that striatal NMDA receptors activate both the direct and indirect pathways and suggests that under parkinsonian conditions phasic control operated by NMDA receptors over striatofugal pathways is shifted in favor of the "indirect" one.

Section II. Striatal glutamate release evoked in vivo by NMDA is dependent upon ongoing neuronal activity in the substantia nigra and endogenous striatal dopamine

The main finding of this study was that the rise in striatal GLU levels induced by local perfusion with NMDA depends on the activation of extrastriatal loops and endogenous DA.

On the basis of previous morphological (Tarazi *et al.* 1998; Wang and Pickel 2000; but see Greenamyre and Young 1989) and electrophysiological (Garcia-Munoz *et al.* 1991; Berretta and Jones 1996) studies it could be suggested that stimulation of NMDA autoreceptors located on corticostriatal glutamatergic terminals is responsible for the rise in extracellular striatal GLU levels induced by local perfusion with NMDA. The finding that NMDA failed to alter synaptosomal GLU release argues against this hypothesis. As a matter of fact, we cannot completely rule out the possibility that activation of NMDA autoreceptors requires tissue modulators (e.g. nitric oxide, retrograde messengers) and/or the morphological integrity of glutamatergic synapse, which are lost during the procedure of synaptosome preparation and superfusion. However, the NMDA-induced striatal GLU release was abolished by intranigral

perfusion with TTX or bicuculline in the SNr, suggesting that the ability of NMDA to evoke GLU release in the striatum is dependent on ongoing neuronal activity and activation of $GABA_A$ receptors in the SNr. It is therefore possible to speculate that the NMDA effect is mediated by activation of the direct striato-nigral pathway.

We also demonstrated that endogenous striatal DA tonically modulates the NMDA action, depending on the strength of the excitatory input (i.e the NMDA concentrations) and independently of the subtype of DA receptor involved.

The present finding that both SCH23390 and raclopride facilitated the effect of lower NMDA concentrations is consistent with previous observation that both D1 and D2 receptor activation reduces the activity of MSNs (Cepeda and Levine, 1998; Nicola *et al.*, 2000; Onn *et al.*, 2000), possibly depressing their responsiveness to weak (e.g. NMDA 0.1 μM) excitatory inputs. On the other side, the observed inhibition of NMDA effects at micromolar concentration by both DA receptor antagonists, indicates that endogenous DA facilitates stronger excitatory inputs.

The experiments in hemiparkinsonian rats further confirmed the biphasic role of endogenous DA, indicating that D1 or D2 receptor blockade, as well as DA depletion (striatal extracellular levels were reduced by more than 90% compared to naïve rats, Marti *et al.*, 2002), had similar effects on the NMDA-stimulated GLU release. The changes in responsiveness to NMDA observed in hemiparkinsonian rats possibly suggested that not only DA depletion per se but also changes at NMDA receptor level may contribute to enhance striatal sensitivity to NMDA.

Section III. NMDA receptors containing NR2A and NR2B subunits differentially regulate striatal output pathways

Main finding of this study is that blockade of NR2B and NR2A subunit containing NMDA receptors in the striatum differentially modulated GABA release in its projection areas, i.e. GP and SNr. In particular, we showed that Ro 25-6981 and NVP-AAM077 reduced spontaneous and NMDA-induced GABA release in SNr and GP, respectively, suggesting "functional" segregation of NR2B and NR2A subunits in striato-nigral and striato-pallidal neurons.

The finding that reverse dialysis of TTX (1 μM) in the striatum decreased nigral and pallidal GABA levels is consistent with the view that a certain amount (~30 %) of extracellular GABA levels in the SNr and GP derives from neuronal activity of medium size striato-nigral

and striato-pallidal GABAergic neurons. Indeed, about one third of striatal projection neurons tonically discharge at low frequencies (West *et al.*, 2002) and intrastriatal TTX should inhibit axon potential propagation along the two pathways. These results endorse the view that the reduction of GP and SNr GABA levels induced by intrastriatal perfusion with subunit selective NMDA receptor antagonists is due to striatal output pathway inhibition.

In situ hybridization studies have shown that striatal GABAergic projection neurons express NR1 and an equal proportion of NR2A and NR2B subunits (with a tendency for NR2B subunits to predominate in striato-nigral neurons, Landwehrmeyer *et al.*, 1995; Standaert *et al.*, 1999). Thus, the finding that subunit-selective NR2A and NR2B antagonists selectively modulate GABA release in GP and SNr is difficult to reconcile with the lack of segregation of NR2A and NR2B mRNAs in projection neurons. It is possible that mRNA expression does not match the presence of an active protein at the membrane level, due to post-transcriptional or post-translational modifications (e.g. receptor trafficking, phosphorylation). For instance, Dunah *et al.* (2000) were able to detect a reduction in the abundance of NR2B subunit in the DA-depleted striatum only in the membrane fraction (and not the overall receptor population). Alternatively, we could relate inhibition of GP and SNr GABA release to blockade of NR2A or NR2B subunits on striatal interneurons facilitating the activity of projection neurons. Despite the neurobiological substrates remain unknown, the present study indicates that NR2A and NR2B subunit containing NMDA receptors preferentially facilitate the activity of striato-pallidal and striato-nigral neurons, respectively.

In view of the classical scheme of basal ganglia functional organization (Albin *et al.*, 1989; DeLong, 1990), it can be predicted that, in naïve animals, striatal NR2A subunit blockade improves motor activity while striatal NR2B subunit blockade impairs it. The hypothesis that the selective NR2A subunit blockade lead to depression of motor activity is supported by behavioural findings (Standaert *et al.*, 1996; Miyamoto *et al.*, 2001). Contrary to that found for NVP-AAM077, inhibition of SNr GABA release by Ro 25-6491 is not a predictor for a motor depressant action. Indeed, NR2B subunits do not participate in motor control since intrastriatal injections of NR2B antisense nucleotides (Standaert *et al.*, 1996) or NR2B selective antagonists (Nash and Brotchie, 2002) do not affect motor activity in naïve animals.

These data add to previous studies suggesting a differential role for striatal NR2A and NR2B subunits in motor control (Standaert *et al.*, 1996). Moreover, they may provide a neurobiological basis to the finding that increased expression/prevalence of striatal NR2A subunits is associated with parkinsonian hypokinesia in 6-OHDA hemilesioned rats (Dunah *et al.*, 2000; Ganguly and Keefe, 2001, Fiorentini *et al.*, 2006).

Section IV. Differential effect of NR2A and NR2B subunit selective NMDA receptor antagonists on striato-pallidal neurons: relationship to motor response in the 6-hydroxydopamine model of parkinsonism

Main finding of this study is that reverse dialysis of NVP-AAM077 in the DA-depleted striatum of hemiparkinsonian rats reduced GABA levels in GP but not in SNr while reverse dialysis of Ro 25-6981 failed to change amino acid release in these areas. The present results suggest that, under parkinsonian conditions, NR2A subunits facilitate striato-pallidal neurons while NR2B subunits do not modulate striatal output pathways. Systemic administration of low doses of NVP-AAM077 or Ro 25-6981 produced mild improvement of motor performance and reduced pallidal GABA release while systemic administration of high doses of NVP-AAM077 worsened parkinsonism and increased GABA levels. These data are consistent with the view that motor improvement induced by NVP-AAM077 is accomplished by blockade of NR2A subunits on striato-pallidal neurons. Modulation of pallidal activity may also underlie motor improvement induced by Ro 25-6981, although, in this case, blockade of extrastriatal NR2B subunits may be involved.

The pattern of modulation exerted by striatal NR2A and NR2B subunits seen in physiological conditions (see *Sectiont III*) appeared to be dramatically affected by DA depletion. In fact, DA depletion caused a functional imbalance in the control tonically operated by endogenous GLU over striatal output pathways, as suggested by the loss of Ro 25-6981 inhibition of nigral GABA release and the enhancement (when threshold doses are compared) of NVP-AAM077 inhibition of pallidal GABA release.

Several studies demonstrated dysregulation of NMDA subunit expression and composition in the DA-depleted striatum of 6-OHDA hemilesioned rats, such as increased expression of NR2A, but not NR2B, subunits (Ulas and Cotman 1996; Ganguly and Keefe 2001) and subcellular redistribution of NMDA subunits, leading to reduced abundance of NR2B and relative enrichment of NR2A subunits in postsynaptic densities (Dunah *et al.* 2000; Fiorentini *et al.* 2006; Gardoni *et al.* 2006). The present study for the first time provides functional data in support of the view that DA depletion causes an imbalance towards NR2A transmission in the striatum, as indicated by down-regulation of the NR2B facilitation on striato-nigral neurons and up-regulation of the NR2A drive on striato-pallidal neurons.

Therefore, we tested the therapeutical potential of the NR2A and NR2B selective blockade by means of systemic injections of NVP-AAM077 and Ro 25-6981 in the 6-OHDA hemilesioned

rat model of PD, well accepted as valuable tool for investigation on parkinsonian motor deficits.

Indeed, consistently with reports that unilateral DA depletion also affected posture (Whishaw *et al.*, 2003), stepping time, and stepping length (Olsson *et al.*, 1995) at the ipsilateral paw, unilateral lesion of SNc DA neurons caused dramatic bilateral increase in the immobility time on the bar test, with the contralateral ("parkinsonian") forepaw being more severely affected than the ipsilateral one. However, when the animal was forced to move (drag test), motor activity at the ipsilateral paw was only slightly impaired, suggesting that the bar and drag test provide information on different aspects of motor program. Indeed, the bar test essentially measures the time to initiate a movement (akinesia), whereas the drag test measures both the time to initiate and to execute it (bradykinesia). Powerful, dose-dependent attenuation of parkinsonism was produced by increasing L-DOPA doses: reduction of akinesia at both the ipsilateral and the contralateral forepaw (0.1 mg/kg), improvement of exercise-induced motor performance (0.3 mg/kg), and reversal of motor asymmetry both under resting (bar test) and dynamic (drag test) conditions (6 mg/kg).. It is noteworthy that L-DOPA exerted an antiparkinsonian action at doses (0.1–6 mg/kg) lower than those eliciting contralateral rotations (25 mg/ kg), strengthening the view that the development of more subtle test allow a more sensitive evaluation of the effects of a drug on parkinsonian motor symptomps than analysis of pharmacologically induced (e.g., by dopamine agonists) turning behaviour in screening for antiparkinsonian drugs.

The evidence that systemic administration of NVP-AAM077 in hemiparkinsonian rats produced motor improvement (i.e on gait and overall coordination), although mild if compared with the reference antiparkinsonian drug L-DOPA, is consistent with previous data obtained in parkinsonian primates treated with a relatively selective NR2A antagonist (MDL 100,453; Blanchet *et al.*, 1999) and endorses the view that up-regulation of striatal NR2A transmission contributes to sustain motor deficit under parkinsonian conditions.

The reduction of pallidal GABA levels produced by NVP-AAM077, either perfused intrastriatally or administered systemically at motor facilitating doses, confirms the hypothesis that blockade of striatal NR2A subunits reduces the activity of the striato-pallidal pathway.

It is worthy of mention that we observed a dual behavioural response to NVP-AAM077, low doses causing motor improvement and higher ones motor impairment. Due to the high affinity of NVP-AAM077 for NR2C subunits, motor impairment could be related to blockade of cerebellar NMDA receptors (which predominantly express the NR2C subunit), leading to

ataxia. However, this possibility seems unlikely, since motor inhibition produced by NVP-AAM077 was associated with GABA increase in GP. Moreover motor activity was not dramatically reduced by high doses of NVP-AAM077, as expected if ataxia were induced.
This favours the view that motor inhibition was due to striatal NR2A subunit blockade and activation of the striato-pallidal pathway, possibly via inhibition of striatal GABAergic interneurons.
In line with a previous study in primates (Blanchet *et al.*, 1999), Ro 25-6981 produce an overall antiparkinsonian activity similar to NVP-AAM077, suggesting that blockade of either the NR2B or NR2A subunit may lead to attenuation of parkinsonism. The reduction in GABA levels in GP produced by motor facilitating dose of Ro 25-6981 would indicate that motor improvement is accomplished through inhibition of the indirect pathway, as for NVP-AAM077. However, the failure of intrastiatal perfusion of Ro 25-6981 in modulating pallidal GABA release suggests the involvement of extrastriatal NR2B subunits, possibly localized at the presynaptic level on striato-pallidal afferents.

Section V. Effect of selective NR2B blockade on motor symptoms in the 3-NP mouse model of Huntington Disease

The results of the present study failed to demonstrate any beneficial effect of the selective blockade of the NR2B subunit NMDA receptors on motor impairment produced by 3-NP intoxication in the C57BL/6 mice, suggesting that NR2B subunits do not sustain the alteration of motor activity produced by striatal degeneration.
We chose the C57BL/6J mice on the basis of its large use for the development of neurodegenerative animal models and for the elaboration of transgenic lines.
According to a previous study (Fernagut *et al.*, 2002), 3-NP-induced motor symptoms developed once the dose of 40 mg/Kg was reached, with a characteristic pattern including bradykinesia, hindlimb clasping, hindlimb dystonia, truncal dystonia and loss of balance control.That study showed a significant correlation between the motor score of 3-NP intoxicated mice and striatal damage (Fernagut *et al.*, 2002).
A similar but not identical 3-NP intoxication protocol was employed (total cumulative dose 560 mg/Kg in 7 days, against the 600 mg/Kg in 10 days of the present experiment) in the C57BL/6J mouse. The regimen of intoxication has been demonstrated to greatly influence the severity of the lesion, as also outlined by the acute lethality: 25% in the previous study, 10 % in the present one. Mice displayed a significant reduction in striatal volume and/or lateral

circumscribed lesions of the striatum. That study provided indirect evidence that the 3-NP induced degeneration affects striatal output neurons, as shown by reduced calbindin-D-28K immunoreactivity, reduced met-enkephalin immunoreactive projections to GP and substance P immunoreactive projections to SNr, according to features displayed by late-stage HD patients (Goto *et al.*, 1989; Fernagut *et al.*, 2004a).

In another study (Fernagut *et al.*, 2003), the protocol of intoxication was of 450 mg/Kg administered in 9 days. These conditions were more similar for doses and progression to the present one's although milder because the intoxication stopped after the first 50 mg/Kg injection and the overall dose administered was 150 mg/kg lower. Albeit milder, the intoxication produced a significant neuronal loss at the anterior and mid-striatal level which affected calbindin-positive striatofugal cells and was accompanied by astroglial reactivity, considered as a reliable marker for the presence of irreversible neuronal damage.

The histopathological correlates provided by these previous studies proved the usefulness of these protocols of 3-NP intoxication in the C57BL/6 mice to reproduce the main features of HD and confirmed the reliability of this model to test mechanistic and therapeutic hypotheses.

The excitotoxic hypothesis in HD stipulates that selective vulnerability of MSNs results from excessive activation of GLU receptors. In particular, experimental evidence suggests that NMDA receptors are more involved than other Glu receptors in mediating excitotoxic damage (for review, see DiFiglia, 1990).

Notably, a recent study in acutely dissociated MSNs from pre-symptomatic R6/2 mice has shown a decrease in NR2A and no changes in NR2B mRNA levels (Ali and Levine, 2006). These data, together with the disproportionate loss of NMDA receptor enriched striatal neurons observed in patients with HD (Young *et al.*, 1988; Albin *et al.*, 1990) and the preponderance of NR2B expression in the striatum (Landwehrmeyer *et al.*, 1995; Standaert *et al.*, 1999; Christie *et al.*, 2000) have brought the attention on the NR2B subunit as a key mediator of the excitotoxic damage elicited by mutant huntingtin expression.

For this reason, several studies focused on the neuroprotective action of NR2B blockade in asymptomatic or early stages of the disease to delay the onset rather than the progression of the disease, while the clinical practice suggests to test potential drugs in symptomatic conditions.

Therefore, to mimic more closely a human clinical trial setting, we choose to apply the pharmacological intervention and test the potentiality of selective NR2B blockade at the end of the 3-NP intoxication protocol (i.e. when pathological process was ongoing). The literature confirms that the doses of Ro 25-6981 used in the present study are highly selective for the

NR2B subunit and devoid of acute or adverse motor effects in rodents (Loschmann *et a*l., 2004; Kosowsky and Lljequist, 2004; Boyce *et al.*, 1999).

The sensorimotor integration test of traversing the beam has been shown to be sensitive to striatal dysfunction, both in 3-NP intoxicated mice and R6/2 transgenic mice (Carter *et al.*, 1999; Fernagut *et al.*, 2002 a, b). Such impaired motor performance is related to motor slowness, hindlimb incoordinaton with dystonia and impaired balance control.

However, this test disclosed no significant effect of systemic Ro 25-6981, confirming the results obtained by monitoring spontaneous locomotor activity or attributing motor scores.

Overall, these data suggest that NR2B selective antagonists may not be useful for symptomatic therapy of HD.

6. CONCLUDING REMARKS

The results presented in this thesis offer important information about the complex modulation exerted by NMDA receptors over BG circuitry in physiological conditions and in the course of neurodegenerative diseases such as PD and HD. We provided for the first time functional evidence that striatal NR2A and NR2B subunits play a different role in modulating the activity of the striatofugal pathway in physio-pathological conditions. Nevertheless, both NR2A and NR2B antagonists exert motor promoting action in hemiparkinsonian rats. The relevance of this finding may be emphasized in light of the fact that broad-spectrum, non selective NMDA antagonists, although proposed in therapies for several pathologies such as Alzheimer disease, hypoxia/ischemia, seizures disorders or even diabetes, produce dramatic side-effects. Thus, the therapeutic potential of subunit selective NMDA receptor antagonists, which are endowed with better tolerability profile, warrants investigation.

In this respect, the dual effect of NVP-AAM077 on motor behaviour (facilitation/inhibition) and the narrow dose window at which motor improvement was observed makes the possibility to target the NR2A subunit in the symptomatic therapy of PD unlikely. These data, however, have to be confirmed with another and more selective NR2A antagonist. Conversely, the NR2B subunit seems more likely to be a useful target for development of well tolerated therapies of PD, although if compared with L-DOPA, the antiparkinsonan action of Ro 25-6981 was much milder. Nevertheless, the NR2B subunit appears to be a particularly attractive neurobiological target in light of the recently reported neuroprotective and antidiskinetic properties of NR2B antagonists.

Finally, although disappointing, the failure to attenuate HD-like symptoms by NR2B subunit blockade is in line with the ineffectiveness of most clinical trials using NMDA antagonists in the symptomatic therapy of HD (i.e. when the disease is manifest), and can be explained on the basis of the decreased levels of NMDA receptors or reduction in downstream signalling activity at symptom onset. As previously outlined (Diguet et al., 2004), is somewhat discouraging to present negative results in a such pivotal study. However, we think that is useful and necessary to communicate to the scientific community also negative or inconclusive results in order to accelerate, without dispersion of time and resources, the process to the common aim: find a therapy for HD.

As a future outlook, it would be interesting to investigate the circuits underlying the antiparkinsonian action produced by NR2B subunit blockade, as the selective effect on the GP activity suggests a more complex role of this nucleus that may go beyond the simplified concept of only being a relay station along the “indirect” pathway.

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9. ORIGINAL PAPERS

I. Marti M, Manzalini M, **Fantin M**, Bianchi C, Della Corte L and Morari M. (2005) Striatal glutamate release evoked in vivo by NMDA is dependent upon ongoing neuronal activity in the substantia nigra, endogenous striatal substance P and dopamine. *J. Neurochem*. 93 (1): 195-205.

II. Fantin M, Marti M, Auberson Y and Morari M. (2007) NR2A and NR2B subunit containing NMDA receptors differentially regulate striatal output pathways. *J. Neurochem*. 103 (6): 2200-11.

III. Fantin M, Auberson Y and Morari M. (2008) Differential effect of NR2A and NR2B subunit selective NMDA receptor antagonists on striato-pallidal neurons: relationship to motor response in the 6-hydroxydopamine model of parkinsonism *J. Neurochem*. Submitted.

PAPER I

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Striatal glutamate release evoked *in vivo* by NMDA is dependent upon ongoing neuronal activity in the substantia nigra, endogenous striatal substance P and dopamine

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Abstract

The aim of the present microdialysis study was to investigate whether the increase in striatal glutamate levels induced by intrastriatal perfusion with NMDA was dependent on the activation of extrastriatal loops and/or endogenous striatal substance P and dopamine. The NMDA-evoked striatal glutamate release was mediated by selective activation of the NMDA receptor-channel complex and action potential propagation, as it was prevented by local perfusion with dizocilpine and tetrodotoxin, respectively. Tetrodotoxin and bicuculline, perfused distally in the substantia nigra reticulata, prevented the NMDA-evoked striatal glutamate release, suggesting its dependence on ongoing neuronal activity and $GABA_A$ receptor activation, respectively, in the substantia nigra. The NMDA-evoked glutamate release was also dependent on striatal substance P and dopamine, as it was antagonized by intrastriatal perfusion with selective NK_1 (SR140333), D_1-like (SCH23390) and D_2-like (raclopride) receptor antagonists, as well as by striatal dopamine depletion. Furthermore, impairment of dopaminergic transmission unmasked a glutamatergic stimulation by submicromolar NMDA concentrations. We conclude that *in vivo* the NMDA-evoked striatal glutamate release is mediated by activation of striatofugal GABAergic neurons and requires activation of striatal NK_1 and dopamine receptors. Endogenous striatal dopamine inhibits or potentiates the NMDA action depending on the strength of the excitatory stimulus (i.e. the NMDA concentration).

Keywords: dopamine, glutamate, 6-hydroxydopamine, microdialysis, NMDA, substance P.

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The most consistent finding across microdialysis studies focused on NMDA actions in the basal ganglia is that striatal perfusion with NMDA elevates local glutamate (GLU) extracellular levels. This increase has been detected both in awake (Young and Bradford 1991; Carboni *et al*. 1993; Liu and Moghaddam 1995; Morari *et al*. 1996; Bogdanov and Wurtman 1997; Rossetti *et al*. 1999; Yamamoto *et al*. 1999; Hashimoto *et al*. 2000) and anaesthetised (Bustos *et al*. 1992; Morari *et al*. 1993; Kendrick *et al*. 1996; Abarca and Bustos 1999; Bert *et al*. 2002) rats. In spite of such a large number of reports, the nature of glutamate release evoked by NMDA in the striatum remains controversial. The mechanisms proposed point to either stimulation of NMDA autoreceptors, which would directly enhance neurosecretion from striatal afferent GLUergic terminals, or stimulation of postsynaptic NMDA receptors, which would indirectly increase GLU levels by releasing intrastriatal modulators (e.g. nitric oxide) or activation of extrastriatal loops. Indeed, our previous observations (Morari *et al*. 1996, 1998b; Marti *et al*. 2002) that striatal NMDA perfusion elevates, not only GLU release locally in the striatum, but also GLU and GABA release distally in the substantia nigra reticulata

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Abbreviations used: AUC, area under the curve; DA, dopamine; DLS, dorsolateral striatum; GLU, glutamate; GPe, pars externa of the globus pallidus; MK-801, dizocilpine; 6-OHDA, 6-hydroxydopamine; SNr, substantia nigra pars reticulata; SP, substance P; TTX, tetrodotoxin.

(SNr), suggest that the enhanced striatal GLU release may result from the activation of striatofugal GABAergic neurons. Striato-nigral and striato-pallidal neurons originating from the matrix (the so-called direct and indirect pathways) oppositely modulate nigro-thalamic GABAergic neurons, and, consequently, thalamo-striatal and cortico-striatal GLUergic pathways (the cortico-basal ganglia-thalamo-cortical loop; for reviews see Alexander and Crutcher 1990; Smith and Bolam 1990). Moreover, a subset of striato-nigral neurons originating from the striosomes (the so-called striosomal pathway; Graybiel 1990) directly inhibits the dopaminergic neurons of the substantia nigra compacta (SNc), which project back to the striatum (the striato-nigro-striatal loop) and regulate striatal GLU release (for a review see Morari *et al*. 1998a).

The present study was therefore undertaken, firstly, to investigate whether the NMDA-induced GLU release is mediated by activation of extrastriatal loops. Using the dual probe microdialysis approach, the Na^+-channel blocker tetrodotoxin (TTX) and the $GABA_A$ receptor blocker bicuculline were perfused into the SNr to test whether striatal GLU release induced by local application of NMDA was dependent on the propagation of action potentials and on $GABA_A$ receptor stimulation in the SNr. Secondly, to clarify the possible involvement of NMDA autoreceptors in the NMDA-evoked glutamate stimulation, striatal synaptosomes were employed. Finally, the possibility that endogenous striatal substance P (SP) and/or dopamine (DA) could play a role in the effect of NMDA was investigated. Indeed, striato-nigral GABAergic neurons, which innervate striatal cholinergic interneurones through their recurrent collaterals, express and release substance P (Graybiel 1990), which has been shown to regulate the activity of striatofugal pathways (Galarraga *et al*. 1999b; Saka *et al*. 2002) via NK_1 receptors. Moreover, by employing selective DA receptor antagonists and DA-depleted animals (Marti *et al*. 2002), endogenous DA was shown to modulate the NMDA-evoked GLU release from the SNr, a marker of activity of striatofugal pathways. Therefore, intrastriatal NMDA stimulation was also performed in the presence of selective NK_1 (SR140333), D_1-like (SCH23390) and D_2-like (raclopride) receptor antagonists. Furthermore, the effect of NMDA on striatal glutamate release was tested in the DA-depleted striatum of 6-hydroxydopamine (6-OHDA) hemilesioned rats.

Materials and methods

Male Sprague-Dawley rats (300–350 g; Stefano Morini, Reggio Emilia, Italy) were kept under controlled lighting conditions (12 h light/dark cycle) and given food and water *ad libitum*. The experimental protocols involving laboratory animals, approved by the Ethical Committee of the University of Ferrara, were performed according to the Italian Guidelines for Animal Care (D.L. 116/92), which were also in accordance with the European Communities Council Directives (86/609/ECC), taking adequate measures to minimize animal pain and discomfort and to limit the number of animals used.

Microdialysis experiments

One probe of concentric design (3 mm dialysing membrane, AN69, Hospal, Bologna, Italy) was stereotaxically implanted under isoflurane anaesthesia in the right dorsolateral striatum (DLS; AP +0.7, ML −3.5, VD −6 below dura; Paxinos and Watson 1982), as previously described (Marti *et al*. 2002). Forty-eight hours after implantation, the microdialysis probe was perfused at a flow rate of 3 μL/min with a modified Ringer solution (composition in mM: $CaCl_2$ 1.2; KCl 2.7, NaCl 148 and $MgCl_2$ 0.85). Samples were collected every 10 min, starting 6 h after the onset of probe perfusion. Treatments were administered locally via the probe. To test the involvement of SNr in the effect of NMDA, a second probe (1 mm dialysing membrane) was implanted in the ipsilateral SNr (AP −5.5, ML −2.2, VD −8.3) and perfused with bicuculline or TTX, starting from 60 min before the intrastriatal application of NMDA. At the end of each microdialysis experiment, the placement of the probe was verified by microscopic examination.

6-hydroxydopamine lesion

Unilateral lesion of DAergic neurons was induced in isoflurane-anaesthetised male Sprague-Dawley rats (150 g; Harlan Italy; S. Pietro al Natisone, Italy) as previously described (Marti *et al*. 2002). Eight micrograms of 6-OHDA (dissolved in 4 μL of saline containing 0.2% ascorbic acid) were stereotaxically injected into the right SN (AP −4.4, ML −1.2, VD −7.8). Sham-operated animals were unilaterally injected with the same saline/ascorbic acid solution. Two weeks after 6-OHDA or saline injection, rats were tested with a dose of amphetamine (5 mg/kg s.c.) and ipsilateral rotations measured. Rats performing > 70 ipsilateral rotations/10 min were enrolled in this study. This behaviour has been shown to be correlated with > 95% striatal DA depletion (Schmidt *et al*. 1982).

Endogenous GLU analysis

GLU was measured by HPLC coupled to fluorimetric detection, as previously described (Marti *et al*. 2002). Fifteen microlitres of *o*-phthaldialdehyde/mercaptoethanol reagent were added to 15 μL aliquots of sample. Twenty microlitres of the mixture were automatically injected (Triathlon autosampler; Spark Holland, Emmen, the Netherlands) onto a 5-C18 Chromsep analytical column (3 mm inner diameter, 10 cm length; Chrompack, Middelburg, the Netherlands) perfused at a flow rate of 0.75 mL/min (Beckman 125 pump; Beckman Instruments, Fullerton, CA, USA) with a mobile phase containing 0.1 M sodium acetate, 10% methanol and 2.2% tetrahydrofuran (pH 6.5). GLU was detected by means of a fluorescence spectrophotometer RF-551 (Shimadzu, Kyoto, Japan). The limit of detection for GLU was about 150 fmol/sample.

Synaptosome preparation

Striatal synaptosomes were prepared as previously described (Marti *et al*. 2003). Briefly, striata were homogenized in ice-cold

0.32 M sucrose buffer at pH 7.4 and then centrifuged for 10 min at 1000 g_{max} (4°C). The supernatant was then centrifuged for 20 min at 12 000 g_{max} (4°C) with the synaptosomal pellet being re-suspended in oxygenated (95% O_2, 5% CO_2) Krebs solution (mM: NaCl 118.5, KCl 4.7, $CaCl_2$ 1.2, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, glucose 10). One-millilitre aliquots of the suspension (~0.35 mg protein) were slowly injected into nylon syringe filters (outer diameter 13 mm, internal volume of about 100 μL; MSI, Westporo, MA, USA). Filters were maintained at 36.5°C in a thermostatic bath and superfused at a flow rate of 0.4 mL/min with a pre-oxygenated Krebs solution containing glycine (1 μM). Sample collection (every 3 min) was initiated after a 20-min equilibration period. NMDA was added to the perfusion medium 3 min before a KCl pulse (10 mM; 60 s) and maintained until the end of the experiment. We previously showed that the 10 mM K^+-evoked striatal overflow of GLU and GABA from synaptosomes is largely dependent on exocytotic release mechanisms (i.e. TTX and Ca^{2+}-dependent; Marti *et al.* 2003). In a separate set of experiments, Mg^{2+} was omitted from the medium and NMDA was also tested under nominally Mg^{2+}-free conditions.

GLU and GABA analysis

Endogenous GLU and GABA levels in the synaptosome perfusate were simultaneously measured by HPLC coupled to fluorimetric detection, as previously described (Marti *et al*. 2003). One hundred microlitres of each sample was automatically added with 30 μL of *o*-phthaldialdehyde/mercaptoethanol solution, and 50 μL of the mixture was injected onto a 5-C18 Chromsep analytical column. The column was perfused (0.48 mL/min) with a mobile phase containing 0.1 M sodium acetate, 10% methanol and 2.5% tetrahydrofuran (pH 6.5). To achieve a good separation, a two-step linear gradient of methanol in aqueous sodium acetate buffer was provided by a Beckman 125 pump. The limit of detection for both GLU and GABA was about 150 fmol/sample.

Data presentation and statistical analysis

Data (mean ± SEM) from microdialysis experiments are reported as percentage of basal value (calculated as the mean of two samples before treatment), while data from synaptosome experiments are reported as percentage of KCl stimulation. Statistical analysis was performed (GraphPad Prism software, San Diego, CA, USA) on the stimulated area-under-the-curve (AUC) values (calculated on the 0–60 min interval and expressed in arbitrary units; microdialysis) or on absolute neurotransmitter release (in pmol/mg prot/min; synaptosomes) by ANOVA followed by the post-hoc Newman-Keuls test for multiple comparisons. p-values < 0.05 were considered to be statistically significant. The absolute extracellular GLU levels (nM) observed under basal conditions and AUC values are reported in the figure legends.

Materials

Dizocilpine (MK-801), NMDA and SCH23390 were purchased from Tocris Neuramin (Bristol, UK), amphetamine and 6-OHDA bromide from Sigma Chemical Company (St Louis, MO, USA), raclopride from Research Biochemicals Incorporated (Natick, MA, USA) and TTX from Alamone Laboratories Ltd. (Jerusalem, Israel). SR140333 was a generous gift from Sanofi-Synthelabo.

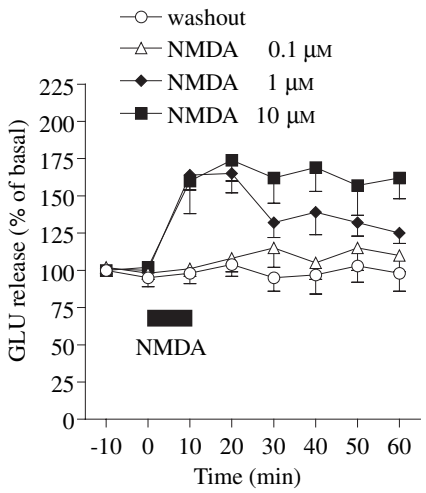

Fig. 1 Effect of local perfusion with NMDA (0.1–10 μM, 10 min; black bar) in the striatum of awake, freely moving rats on striatal glutamate (GLU) release. Data are expressed as percentages ± SEM of basal pre-treatment levels (calculated as the mean of the two samples before the treatment). Basal GLU (nM) and stimulated AUC (0–60 min; arbitrary units) values were, respectively: 151 ± 25, 5591 ± 217 (washout; $n = 13$); 177 ± 52, 5352 ± 361 (NMDA 0.1 μM; $n = 15$); 210 ± 43, 7189 ± 377 (NMDA 1 μM; $n = 16$); 191 ± 51, 7897 ± 481 (NMDA 10 μM; $n = 10$). A significant effect ($p < 0.05$ vs. washout) was observed at NMDA 1 and 10 μM (solid symbols).

Results

Effect of NMDA on striatal GLU release

Basal GLU extracellular levels in the striatum averaged 199.3 ± 18.8 nM ($n = 81$) and were stable over the time course of the experiment. Intrastriatal perfusion with NMDA dose-dependently elevated GLU dialysate content ($F_{3,50} = 10.64$; $p < 0.0001$; Fig. 1). The effect was significant ($p < 0.01$) at 1 and 10 μM NMDA, while the lower (0.1 μM) concentration was ineffective. In a previous study (Morari *et al*. 1998b), MK-801 (1 μM) had been shown to prevent the effect of intrastriatal application of 1 μM NMDA on nigral GLU release. Thus, to analyse the specificity of the effect of striatal NMDA on local GLU release, MK-801 (1 μM) was perfused 60 min before and during the application of 10 μM NMDA. MK-801, while ineffective when applied alone (data not shown), prevented the effect of NMDA (Fig. 2). To investigate whether the effect of intrastriatal NMDA application on local GLU release was dependent on action potential propagation, NMDA (1 and 10 μM) was perfused in the presence of the voltage-dependent Na^+ channel blocker TTX (1 μM, starting 60 min before NMDA) (Fig. 2). TTX alone failed to alter the striatal spontaneous GLU outflow (data not shown) but was effective in preventing the NMDA-evoked GLU release.

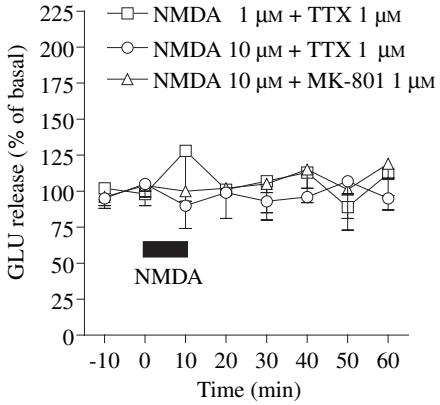

Fig. 2 Effect of striatal perfusion with MK-801 (1 μM) or tetrodotoxin (TTX; 1 μM), in awake, freely moving rats, on striatal glutamate (GLU) release evoked by NMDA (1–10 μM, 10 min; black bar). Perfusion with MK-801 and TTX started 60 min before NMDA and continued until the end of experiment. Data are expressed as percentages ± SEM of basal pre-treatment levels (calculated as the mean of the two samples before treatment). Basal GLU (nM) and stimulated AUC (0–60 min; arbitrary units) values were, respectively: 301 ± 103, 5401 ± 446 (NMDA 1 μM + TTX; $n = 6$); 195 ± 52, 5177 ± 398 (NMDA 10 μM + TTX, $n = 5$); 97 ± 14, 4367 ± 426 (NMDA 10 μM + MK-801; $n = 5$).

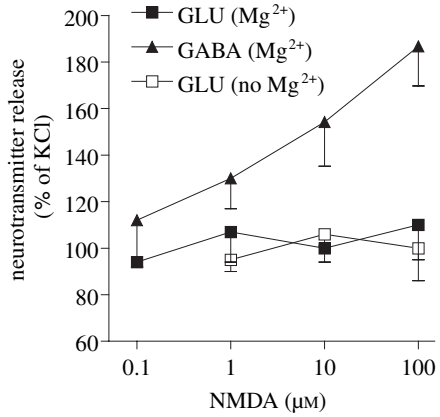

Fig. 3 Effect of superfusion with NMDA (1–100 μM; 6 min) on glutamate (GLU) and GABA release from striatal synaptosomes depolarised with KCl (10 mM, 60 s) in the presence of Mg^{2+} and glycine (1 μM). The effect of NMDA on GLU release in the absence of Mg^{2+} is also shown. Data (means of nine determinations) are expressed as percentages ± SEM of the KCl stimulation.

Effect of NMDA on striatal synaptosomes

To investigate whether the effect of NMDA could be because of stimulation of NMDA autoreceptors, the ability of NMDA to release GLU from 10 mM K^+-depolarised striatal synaptosomes in the presence of glycine (1 μM) was tested (Fig. 3). For a comparison, GABA release was simultaneously monitored. Basal GLU and GABA outflows from striatal synaptosomes were 89.1 ± 2.1 ($n = 41$) and 26.9 ± 2.0 ($n = 43$) pmol/mg prot/min, respectively. KCl (10 mM) increased by about 75 and 40% GLU and GABA outflows (net overflow of 66.7 ± 7.2 and 10.3 ± 2.4 pmol/mg prot/min, respectively). NMDA (0.1–100 μM; 6 min) did not modify either spontaneous (Table 1) or K^+-evoked GLU release. NMDA also failed to alter spontaneous GABA efflux but increased, in a concentration-dependent manner, the K^+-evoked GABA release ($F_{3,35} = 6.84$; $p = 0.001$; Fig. 3). NMDA evoked a significant GABA overflow at 10 μM ($p < 0.05$) and almost doubled ($p < 0.01$) the effect of 10 mM KCl at 100 μM. To test whether omission of Mg^{2+} could disclose an effect of NMDA, NMDA was also applied in the absence of Mg^{2+}. NMDA failed to affect both spontaneous (Table 1) and 10 mM K^+-evoked GLU release under nominally Mg^{2+}-free conditions (Fig. 3). NMDA was also ineffective when challenged against the higher (20 mM) K^+ concentration in the absence of Mg^{2+} (103.3 ± 3.3 and 91.4 ± 6% of the K^+ stimulation for NMDA 10 and 100 μM, respectively).

Table 1 Effect of perfusion with NMDA (1–100 μM) on spontaneous glutamate release from striatal synaptosomes

	Time (min)				
Treatment	−3	0	3	6	9
			■	■	
Washout (Mg^{2+})	109 ± 2	91 ± 2	96 ± 7	74 ± 1	67 ± 3
NMDA 1 μM (Mg2+)	107 ± 2	93 ± 2	92 ± 8	79 ± 4	65 ± 3
NMDA 10 μM (Mg2+)	109 ± 2	91 ± 2	103 ± 11	85 ± 8	74 ± 5
NMDA 100 μM (Mg2+)	108 ± 3	92 ± 3	96 ± 5	82 ± 5	67 ± 2
Washout (no Mg2+)	108 ± 3	92 ± 3	89 ± 5	81 ± 4	76 ± 5
NMDA 1 μM (no Mg2+)	106 ± 3	94 ± 3	86 ± 4	77 ± 7	73 ± 6
NMDA 10 μM (no Mg2+)	108 ± 3	92 ± 3	89 ± 5	72 ± 5	70 ± 5
NMDA 100 μM (no Mg^{2+})	103 ± 3	97 ± 3	98 ± 9	86 ± 8	81 ± 8

NMDA was administered for 6 min (black bar) in the presence and absence of Mg^{2+}. Glycine (1 μM) was co-administered with NMDA. Data (means of at least seven determinations) are expressed as percentages ± SEM of basal values.

Effect of blocking neuronal transmission and $GABA_A$ receptors in the SNr on striatal NMDA-evoked GLU release

To investigate the involvement of the SNr in the striatal GLU-stimulating effects of NMDA, NMDA was applied in the striatum concurrently with TTX in the SNr (Fig. 4). Nigral perfusion with TTX (1 μM) alone did not alter striatal spontaneous GLU levels but was able to prevent the stimulation of GLU release induced by 1 μM NMDA. Similarly, to investigate the involvement of nigral GABAergic transmission, the $GABA_A$ receptor antagonist bicuculline was perfused in the SNr (Fig. 4) at a concentration (10 μM) 10-fold higher than bicuculline pA_2 value, shown to

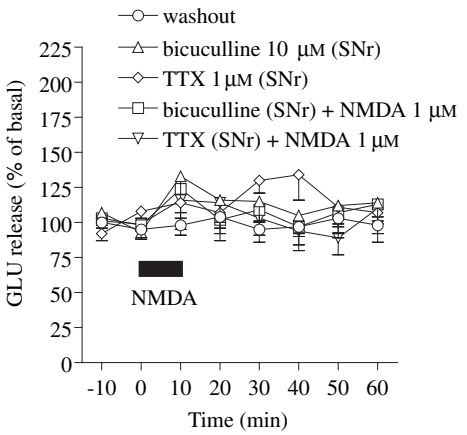

Fig. 4 Effect of perfusion with bicuculline (10 μM) and tetrodotoxin (TTX; 1 μM) in the substantia nigra reticulata (SNr) on the NMDA-stimulated 1 μM, 10 min; black bar glutamate (GLU) release in the ipsilateral striatum of awake, freely moving rats. Perfusion with bicuculline and TTX started 60 min before NMDA and continued until the end of experiment. Data are expressed as percentages ± SEM of basal pre-treatment levels (calculated as the mean of the two samples before the treatment). Basal GLU (nM) and stimulated AUC (0–60 min; arbitrary units) values were, respectively: 181 ± 57, 4938 ± 203 (washout; $n = 5$); 132 ± 32, 5183 ± 403 (bicuculline; $n = 6$); 115 ± 25, 5689 ± 427 (bicuculline + NMDA; $n = 7$); 102 ± 17, 5926 ± 232 (TTX; $n = 5$); 92 ± 2, 5130 ± 483 (TTX + NMDA; $n = 6$).

be effective in our previous work (Marti *et al*. 2002). Bicuculline, ineffective alone, prevented the stimulation induced by 1 μM NMDA.

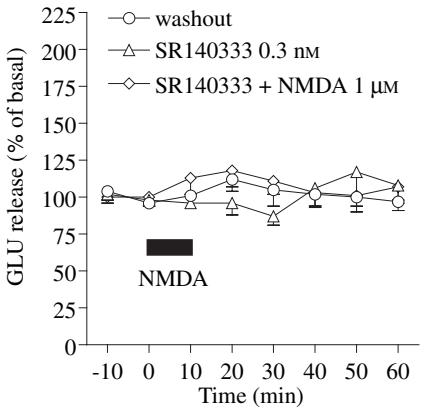

Fig. 5 Effect of intrastriatal perfusion with SR140333 (0.3 nM) on striatal glutamate (GLU) release evoked by 1 μM NMDA (10 min, black bar) in awake, freely moving rats. Perfusion with SR140333 started 60 min before NMDA and continued until the end of experiment. Basal GLU (nM) and stimulated AUC (0–60 min; arbitrary units) values were, respectively: 150 ± 36, 5841 ± 334 (washout; $n = 11$); 312 ± 80, 5153 ± 486 (SR140333; $n = 12$); 285 ± 50, 5505 ± 332 (NMDA + SR140333; $n = 15$). Data are expressed as percentages ± SEM of basal pre-treatment levels (calculated as the mean of the two samples before treatment).

Modulation of striatal NMDA-evoked GLU release by endogenous SP and DA

To test whether striatal spontaneous or NMDA-evoked GLU release is dependent on endogenous striatal SP, the NK_1 receptor antagonist SR140333 was used at a concentration (0.3 nM) selective for NK_1 receptor blockade (Emonds-Alt *et al*. 1993). The intrastriatal perfusion of SR140333 did not alter the spontaneous GLU output, but was able to prevent the stimulation induced by 1 μM NMDA (Fig. 5). To investigate the possible role of endogenous striatal DA and DA receptors in mediating the spontaneous or the NMDA-evoked GLU release in the striatum, NMDA was perfused in the presence of selective DA receptor antagonists. According to the classification of DA receptors in D_1-like (D_1 and D_5) and D_2-like (D_2, D_3 and D_4) families (henceforth D_1 and D_2; Sibley and Monsma 1992), the D_1 and D_2 preferential antagonist, SCH23390 and raclopride, respectively, were used. They were perfused at concentrations effective to achieve selective blockade of D_1/D_5 (0.1 μM SCH23390) or D_2/D_3 receptors (1 μM raclopride; see Marti *et al*. 2002). Intrastriatal perfusion with raclopride or SCH23390 alone did not modify striatal GLU levels (Figs 6 and 7). The presence of raclopride prevented the GLU output evoked by

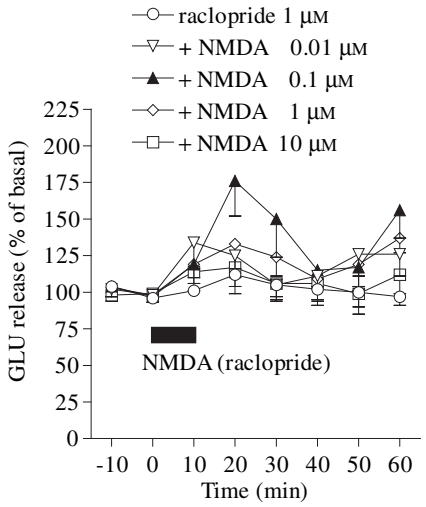

Fig. 6 Effect of intrastriatal perfusion with raclopride (1 μM) on striatal glutamate (GLU) release stimulated by NMDA (0.01–10 μM, 10 min; black bar) in awake, freely moving rats. Perfusion with raclopride started 60 min before NMDA and continued until the end of experiment. Basal GLU (nM) and stimulated AUC (0–60 min; arbitrary units) values were, respectively: 125 ± 41, 5406 ± 222 (raclopride; $n = 16$); 162 ± 67, 6349 ± 333 (raclopride + NMDA 0.01 μM; $n = 5$); 170 ± 63, 7325 ± 663 (raclopride + NMDA 0.1 μM; $n = 9$); 117 ± 51, 6226 ± 529 (raclopride + NMDA 1 μM; $n = 8$); 122 ± 25, 5449 ± 370 (raclopride + NMDA 10 μM; $n = 7$). Data are expressed as percentages ± SEM of basal pre-treatment levels (calculated as the mean of the two samples before treatment). A significant effect ($p < 0.05$ vs. raclopride) was observed at NMDA 0.1 μM (solid symbols).

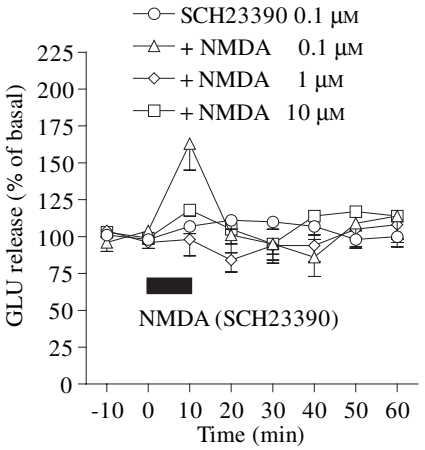

Fig. 7 Effect of intrastriatal perfusion with SCH23390 (0.1 μM) on striatal glutamate (GLU) release stimulated by NMDA (0.1–10 μM, 10 min; black bar) in awake, freely moving rats. Perfusion with SCH23390 started 60 min before NMDA and continued until the end of experiment. Basal GLU (nM) and stimulated AUC (0–60 min; arbitrary units) values were, respectively: 142 ± 27, 5333 ± 279 (SCH23390; $n = 9$); 192 ± 29, 5698 ± 527 (SCH23390 + NMDA 0.1 μM; $n = 6$); 208 ± 56, 5520 ± 568 (SCH23390 + NMDA 1 μM; $n = 9$); 155 ± 29, 5402 ± 297 (SCH23390 + NMDA 10 μM; $n = 8$). Data are expressed as percentages ± SEM of basal pre-treatment levels (calculated as the mean of the two samples before treatment).

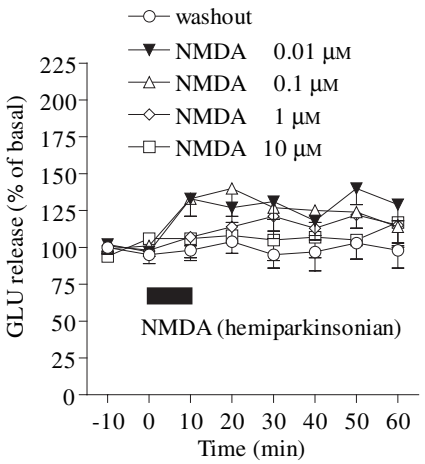

Fig. 8 Effect of intrastriatal perfusion with NMDA (0.01–10 μM, 10 min; black bar) on striatal GLU release in DA-depleted awake, freely moving hemiparkinsonian rats. Basal GLU (nM) and stimulated AUC (0–60 min; arbitrary units) values were, respectively: 120 ± 36, 5097 ± 263 (washout; $n = 9$); 186 ± 58, 6717 ± 277 (NMDA 0.01 μM; $n = 9$); 132 ± 29, 6328 ± 471 (NMDA 0.1 μM; $n = 9$); 148 ± 49, 5911 ± 310 (NMDA 1 μM; $n = 6$); 173 ± 54, 5619 ± 491 (NMDA 10 μM; $n = 5$). Data are expressed as percentages ± SEM of basal pre-treatment levels (calculated as the mean of the two samples before the treatment). A significant effect ($p < 0.05$ vs. washout) was observed at NMDA 0.01 μM (solid symbols).

1 and 10 μM NMDA, whereas allowed the lower 0.1 μM NMDA concentration to elicit a significant ($p < 0.01$) stimulation of GLU release ($F_{4,40} = 3.64$; $p = 0.012$; Fig. 6). Similarly, the presence of SCH23390 blocked the GLU output evoked by NMDA up to 10 μM (Fig. 7). Nevertheless, analysis of the time course of the response revealed a transient (10-min time point) increase in GLU levels ($F_{3,30} = 3.24$; $p = 0.036$; Fig. 7) induced by 0.1 μM NMDA ($p < 0.05$).

To confirm the modulatory role of endogenous DA in the NMDA-induced GLU release, NMDA was perfused in the DA-denervated striatum of 6-OHDA hemilesioned rats (a model of Parkinson's disease; Fig. 8). Under these conditions, extracellular DA levels were reduced by more than 90% compared with naïve rats, while extracellular GLU levels remained unchanged (Marti *et al.* 2002; Galeffi *et al.* 2003). NMDA perfusion in the DA-denervated striatum (Fig. 8) elevated local GLU levels ($F_{4,33} = 3.29$; $p = 0.022$). However, NMDA elicited a significant stimulation only at a concentration of 0.01 μM ($p < 0.05$). In contrast, in sham-operated rats, as in naïve rats, NMDA perfused intrastriatally at micromolar concentrations (1 and 10 μM) induced a significant stimulation ($p < 0.01$; Fig. 9) of GLU release ($F_{3,22} = 6.49$; $p = 0.0026$). Overall, the maximal stimulation elicited by NMDA in hemiparkinsonian rats (NMDA 0.01 μM) was significantly reduced as compared with that observed in sham-operated (NMDA 10 μM; $p = 0.024$) or naïve (NMDA 10 μM; $p = 0.033$) rats.

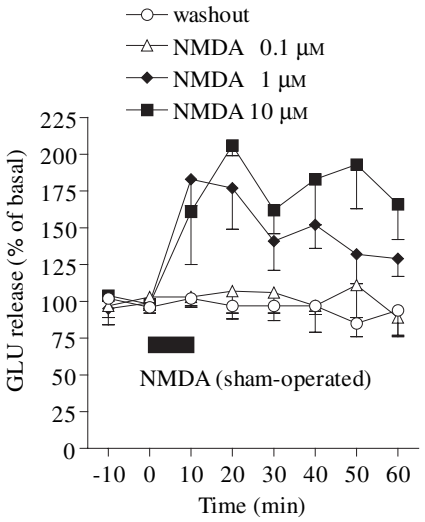

Fig. 9 Effect of intrastriatal perfusion with NMDA (0.01–10 μM, 10 min; black bar) on striatal glutamate (GLU) release in awake, freely moving sham-operated rats. Basal GLU (nM) and stimulated AUC (0–60 min; arbitrary units) values were, respectively: 173 ± 33, 4716 ± 183 (washout; $n = 8$); 139 ± 31, 5529 ± 1004 (NMDA 0.1 μM; $n = 5$); 145 ± 33, 8681 ± 1210 (NMDA 1 μM; $n = 6$); 215 ± 53, 8851 ± 967 (NMDA 10 μM; $n = 7$). Data are expressed as percentages ± SEM of basal pre-treatment levels (calculated as the mean of the two samples before the treatment). A significant effect ($p < 0.05$ vs. washout) was observed at NMDA 1 and 10 μM (solid symbols).

Discussion

The main finding of the present study is that, *in vivo*, the NMDA-evoked striatal GLU release depends on the activation of extrastriatal loops and of striatal NK_1 and DA receptors.

Previous microdialysis studies have demonstrated that extracellular GLU levels under resting conditions are maintained by non-vesicular (Timmerman and Westerink 1997; Baker *et al*. 2002) and, to a lesser extent, vesicular (Morari *et al*. 1993; Herrera-Marschitz *et al*. 1996; Baker *et al*. 2002) release mechanisms. Axon potential propagation does not contribute to extracellular GLU levels, as shown by their insensitivity to TTX (for a review see Timmerman and Westerink 1997). However, under appropriate pharmacological and/or behavioural stimulations, GLU detected in dialysate may reflect neuronal activity. Indeed, the NMDA-evoked striatal GLU release was dependent on activation of the NMDA receptor-channel complex (MK-801 sensitivity: Bustos *et al*. 1992; Morari *et al*. 1993; Kendrick *et al*. 1996; Morari *et al*. 1996) and axon potential propagation (TTX-sensitivity: Bogdanov and Wurtman 1997; Yamamoto *et al*. 1999). The TTX-sensitivity indicates that the NMDA-induced GLU release was neuronal in nature and was not mediated by NMDA autoreceptors. Actually, as NMDA failed to alter synaptosomal GLU release, the present data argue against the presence of functional NMDA autoreceptors on striatal GLUergic terminals. This is consistent with previous studies on K^+-depolarized hippocampal (Barrie and Nicholls 1988; McMahon *et al*. 1989; Zhou *et al*. 1995; Breukel *et al*. 1998), cortical (Barrie and Nicholls 1988), forebrain (Tavares *et al*. 2002) or whole-brain (Sherman *et al*. 1992) synaptosomes. In some reports, NMDA was shown to facilitate spontaneous GLU release (Sherman *et al*. 1992; Breukel *et al*. 1998; Tavares *et al*. 2002), although the exocitotic nature of the phenomenon was questioned (Breukel *et al*. 1998). The existence of NMDA autoreceptors on cortico-striatal terminals has been postulated on the basis of morphological (Tarazi *et al*. 1998; Wang and Pickel 2000; but see Greenamyre and Young 1989) and electrophysiological (Garcia-Munoz *et al*. 1991; Berretta and Jones 1996) studies. Thus, the failure to demonstrate a presynaptic NMDA facilitatory control on GLU release in synaptosomes is puzzling. It is possible that activation of NMDA autoreceptors requires tissue modulators (e.g. nitric oxide, retrograde messengers) and/or the morphological integrity of GLUergic synapse, which are lost during the procedure of synaptosome preparation and superfusion. Alternatively, NMDA autoreceptors may physiologically control aspartate rather than glutamate release (Zhou *et al*. 1995). Further support to the view that the NMDA-induced striatal GLU release was not mediated by striatal NMDA autoreceptors comes from the finding that blockade of impulse propagation or $GABA_A$ receptors in the SNr prevented the striatal effect of NMDA. This indicates that the ability of NMDA to evoke GLU release in the striatum is dependent on ongoing neuronal activity and activation of $GABA_A$ receptors in the SNr. As TTX and bicuculline were perfused in the SNr before the intrastriatal application of NMDA, the inhibition of the NMDA-induced striatal GLU release could be related to a conditioning effect of such compounds on the striatal complex. However, the observation that bicuculline and TTX given into the SNr did not change the basal striatal output of GLU argues against it. Moreover, previous observations that bicuculline increased the activity of nigro-striatal DAergic and nigro-thalamic GABAergic neurons (Santiago and Westerink 1992; Rick and Lacey 1994; Tepper *et al*. 1995), while TTX decreased it (Morari *et al*. 1996), makes it difficult to reconcile such an opposite modulation of nigrofugal outputs with a common inhibitory effect on striatal NMDA-evoked GLU release. Therefore, the simplest explanation is that NMDA elevated striatal GLU release via a phasic activation of striatofugal pathways. NMDA receptors are expressed (Tallaksen-Greene *et al*. 1992; Landwehrmeyer *et al*. 1995; Chen *et al*. 1996) on striatal GABAergic neurons projecting to the SNr (the direct pathway), the SNc (the striosomal pathway) and the GPe (the indirect pathway). The direct and striosomal pathways monosynaptically inhibit SNr GABAergic neurons projecting to the thalamus (Deniau and Chevalier 1985) and SNc DAergic neurons projecting back to the striatum (Grace and Bunney 1985), respectively, while the indirect pathway provides, through the subthalamic nucleus (Deniau *et al*. 1978), excitatory glutamatergic inputs to both SNr GABAergic and SNc DAergic neurons (Robledo and Feger 1990). Which one of the output pathways is specifically involved in the GLU facilitating effect of NMDA remains a matter of conjecture. The finding that the NMDA stimulation of GLU release was antagonized by an NK_1 receptor antagonist, and thus likely mediated by endogenous SP, may suggest that it is because of activation of striato-nigral GABAergic neurons, which release SP (see Introduction). Activation of these neurons would lead to $GABA_A$ receptor-mediated (bicuculline-sensitive) inhibition of nigro-striatal DAergic and/or nigro-thalamic GABAergic neurons. As striatal GLU release evoked by NMDA (1–10 μM) was not associated with changes in striatal DA levels (Morari *et al*. 1996), it would be tempting to speculate that the NMDA-induced GLU release is as a result of disinhibition of thalamo-striatal and cortico-striatal GLUergic projections (i.e. activation of the cortico-basal ganglia-thalamo-cortical loop). In keeping with this view, GABA injection in the thalamus (Barbeito *et al*. 1989) and perfusion with TTX (10 μM) in the SNr (which causes nigral output inhibition and motor disinhibition; Morari *et al*. 1996), elevated basal striatal GLU release.

The finding that NK_1 receptor blockade prevented the effect of NMDA also suggests that striatal interneurones play a relevant role in modulating the NMDA action. Indeed,

although direct depolarising effects of exogenous SP have been reported (Galarraga *et al*. 1999b), the effects exerted by SP on GABAergic projection neurons appear to be mainly mediated by cholinergic interneurones and by interneurones co-expressing neuropeptide Y, somatostatin and nitric oxide, which abundantly express NK_1 receptors. These neurons modulate the activity of striatofugal pathways (Di Chiara *et al*. 1994; Calabresi *et al*. 2000; Saka *et al*. 2002) and, in particular, both nitric oxide (West and Grace 2004) and ACh (via muscarinic M_1 receptors; Galarraga *et al*. 1999a) increase the activity of GABAergic striatal projection neurons. Thus, SP, phasically released by striato-nigral neurons under NMDA activation (Blanchet *et al*. 2000), would directly (Rusin *et al*. 1992; Chizh *et al*. 1995) and indirectly (e.g. via ACh and/or nitric oxide release; Arenas *et al*. 1991; Steinberg *et al*. 1995, 1998; Kemel *et al*. 2002) cooperate with NMDA to activate the striatal output and, as a consequence, to elevate striatal GLU release. In addition, nitric oxide could also act presynaptically to facilitate GLU release from corticostriatal terminals (Meffert *et al*. 1994; West and Grace 2004). This also holds true for ACh, although the net effect may depend on the receptor subtype involved, presynaptic nicotinic receptors being facilitatory (Marchi *et al*. 2002) and muscarinic M_2/M_3 receptors inhibitory (Raiteri *et al*. 1990; Sugita *et al*. 1991; Smolders *et al*. 1997; Niittykoski *et al*. 1999).

Although phasic changes in nigrostriatal DAergic transmission may not underlie the NMDA-evoked striatal GLU release, endogenous striatal DA appeared to tonically modulate the NMDA action. The nature of this modulation was dependent on the strength of the excitatory input (i.e the NMDA concentration) and independent from the subtype of DA receptor involved. The present data may thus be interpreted in terms of NMDA–DA interactions at the membranes of striatal neurons, although this view may be biased by not taking into account the role of endogenous GLU acting on non-NMDA receptors. The present finding that one or the other DA receptor antagonist facilitated the effect of lower NMDA concentrations is consistent with previous observations that both D_1 and D_2 receptor activation reduces the activity of medium spiny neurons (for reviews see Cepeda and Levine 1998; Nicola *et al*. 2000; Onn *et al*. 2000), possibly depressing their responsiveness to weak (e.g. NMDA 0.1 μM) excitatory inputs. Alternatively, the observed inhibition of NMDA effects at micromolar concentrations by both DA receptor antagonists, indicates that endogenous DA facilitates stronger excitatory inputs. A facilitation of the NMDA response by D_1 receptor activation has been clearly demonstrated in striatal neurons (Cepeda *et al*. 1993; Levine *et al*. 1996; Cepeda *et al*. 1998), particularly when membrane potential was decreased above −50 mV and L-type Ca^{2+} currents were activated (Hernandez-Lopez *et al*. 1997). D_2 receptors have also been found to facilitate neuronal activity (Surmeier *et al*. 1992) and NMDA responses (Cepeda *et al*. 1998), although the type of response varies from cell to cell (Nicola *et al*. 2000), negative modulation also being observed. This may reflect involvement of pre- or postsynaptic D_2 receptors (Cepeda and Levine 1998), D_2 receptors located on different neuronal types (Cepeda and Levine 1998; LaHoste *et al*. 2000), D_2 receptor subtypes (Surmeier *et al*. 1992; LaHoste *et al*. 2000) or D_2-receptor dependent transduction mechanisms (Hernandez-Lopez *et al*. 2000). The present experiments in hemiparkinsonian rats confirmed the biphasic role of endogenous DA, indicating that D_1 or D_2 receptor blockade, as well as DA loss, had similar effects on the NMDA-stimulated GLU release. This is consistent with a possible D_1–D_2 cooperative interaction model in which both receptor subclasses need to be simultaneously activated to allow DA to exert its action. In keeping with this model, one or the other DA antagonist (D_1 or D_2) has been shown to prevent DA inhibition of striatal Na^+/K^+ ATPase activity (Bertorello *et al*. 1990), LTD (Calabresi *et al*. 1992) or amphetamine-induced c-*fos* expression in striato-nigral neurons (Ruskin and Marshall 1994), whereas, simultaneous D_1 and D_2 receptor activation was required to stimulate c-*fos* expression (Paul *et al*. 1992; LaHoste *et al.* 1993) or reinstate LTD (Calabresi *et al*. 1992) and DA-GLU cooperative phenomena (Hu and White 1997) in the DA-denervated striatum.

The changes in responsiveness to NMDA observed in hemiparkinsonian rats were not associated with changes in spontaneous GLU output, as DA-denervation did not alter striatal GLU levels (for discussion see Marti *et al*. 2002). This is in keeping with some of the previous studies (Abarca and Bustos 1999; Marti *et al*. 2002; Galeffi *et al*. 2003), although in contrast with others (Tossman *et al*. 1986; Meshul *et al*. 1999; Jonkers *et al*. 2002). Nevertheless, NMDA facilitation was observed at surprisingly low NMDA concentrations, possibly suggesting that not only DA loss per se but also changes at NMDA receptor level, such as increased binding (Samuel *et al*. 1990; Wullner *et al*. 1994), expression (Tremblay *et al*. 1995; Ulas and Cotman 1996; Ganguly and Keefe 2001) and phosphorylation (Menegoz *et al*. 1995; Chase and Oh 2000) of NMDA receptor subunits, and/or network level, such as increased responsiveness of cholinergic interneurones to NMDA (Marti *et al*. 1999), may contribute to enhance striatal sensitivity to NMDA.

Concluding remarks

Evidence has been presented that the NMDA-evoked striatal GLU release *in vivo* does not involve NMDA autoreceptors but is mediated by activation of extrastriatal, possibly the cortico-basal ganglia-thalamo-cortical, loops. Endogenous SP (via NK_1 receptors) and DA (via both D_1 and D_2 receptors) interact with NMDA to stimulate the activity of striatofugal neurons and, as a consequence, striatal GLU release. Loss of NMDA effectiveness under parkinsonian-

like conditions may reflect loss of DA-NMDA positive cooperation in regulating the activity of striato-nigral neurons (Marti *et al*. 2002). As non-NMDA receptor agonists also failed to stimulate SNr GABA release in hemiparkinsonian rats (Galeffi *et al*. 2003), the present data may reinforce the view that DA modulation is crucial to set responsiveness of the direct pathway to GLUergic inputs.

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PAPER II

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NR2A and NR2B subunit containing NMDA receptors differentially regulate striatal output pathways

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Abstract

Triple probe microdialysis was employed to investigate whether striatal NR2A and NR2B subunit containing NMDA receptors regulate the activity of striato-pallidal and striato-nigral projection neurons. Probes were implanted in the striatum, ipsilateral globus pallidus and substantia nigra reticulata. Intrastriatal perfusion with the NR2A subunit selective antagonist (*R*)-[(*S*)-1-(4-bromo-phenyl)-ethylamino]-(2,3-dioxo-1,2,3,4-tetrahydroquinoxalin-5-yl)-methyl]-phosphonic acid (NVP-AAM077) reduced pallidal GABA and increased nigral glutamate (GLU) release whereas perfusion with the NR2B subunit selective antagonist (*R*-(*R**,*S**)-α-(4-hydroxyphenyl)-β-methyl-4-(phenylmethyl)-1-piperidinepropanol (Ro 25-6981) reduced nigral GABA and elevated striatal and pallidal GLU release. To confirm that changes in GABA levels were because of blockade of (GLUergic-driven) tonic activity of striatofugal neurons, tetrodotoxin was perfused in the striatum. Tetrodotoxin reduced both pallidal and nigral GABA release without changing GLU levels. To investigate whether striatal NR2A and NR2B subunits were also involved in phasic activation of striatofugal neurons, NVP-AAM077 and Ro 25-6981 were challenged against a NMDA concentration able to evoke GABA release in the three areas. Both antagonists prevented the NMDA-induced striatal GABA release. NVP-AAM077 also prevented the NMDA-induced surge in GABA release in the globus pallidus, whereas Ro 25-6981 attenuated it in the substantia nigra. We conclude that striatal NMDA receptors containing NR2A and NR2B subunits preferentially regulate the striato-pallidal and striato-nigral projection neurons, respectively.

Keywords: GABA, glutamate, microdialysis, NMDA, NVP-AAM077, Ro 25-6981.

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The striatum represents the main afferent structure of the basal ganglia, receiving massive glutamatergic projections from the cerebral cortex and the thalamus. Striatal NMDA receptors, a subtype of glutamate (GLU) receptors, are key modulators of striatal functions and have been targeted in neurological disorders characterized by GLU receptor over activation (e.g. Parkinson's disease and L-DOPA-induced dyskinesia; Chase and Oh 2000; Hallett and Standaert 2004). NMDA receptors form a heterogeneous family of ligand-gated ion channels assembled in a tetra/pentameric form by different combinations of eight splice variants of NR1 subunits and four isoforms (A–D) of NR2 subunits (Dingledine *et al.* 1999). In the striatum, projection neurons and interneurons express NMDA receptors, although the phenotype varies between cells (Landwehrmeyer *et al.* 1995; Standaert *et al.* 1999). This raises the possibility of a differential pharmacological modulation of striatal functions by subunit selective NMDA receptors ligands. The striatum modulates the activity of basal ganglia output nuclei via two pathways. Medium size GABAergic neurons originating from the matrix and projecting to the substantia nigra reticulata (SNr)/entopeduncular nucleus (the 'direct' pathway) or the globus pallidus (GP; the 'indirect' pathway) oppositely modulate nigro-thalamic GABAergic neurons, and, consequently, motor activity, via the thalamo-cortical GLUergic projections (the cortico-basal ganglia-thalamo-cortical loop; Alexander and Crutcher 1990; Chevalier and Deniau 1990). Behavioral evidence that intrastriatal NMDA induced both motor impairment (Schmidt and Bury 1988; Yoshida *et al.* 1991; Klockgether and Turski 1993) and activation (Thanos *et al.* 1992; Ossowska and Wolfarth 1995) has been presented, suggesting that striatal NMDA receptor regulate both the striato-nigral and striato-pallidal pathways. Consistent with this view, we showed that reverse dialysis (perfusion) of NMDA in the striatum evoked GABA (and GLU) release from SNr (Morari *et al.* 1996; Marti *et al.*

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Abbreviations used: DA, dopamine; DLS, dorsolateral striatum; GLU, glutamate; GP, globus pallidus; MK-801, dizocilpine; 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-(phenylmethyl)-1-piperidinepropanol; SNr, substantia nigra reticulata; TTX, tetrodotoxin.

2005) and GP (Fantin *et al.* 2005). However, intrastriatal perfusion with the NMDA channel blocker dizocilpine (MK-801) evoked SNr GLU release (Morari *et al.* 1998b), overall suggesting that striatal NMDA receptors mediate both phasic and tonic regulation of the striatofugal pathways. In the present study, we sought to investigate whether different subsets of NMDA receptors are involved in these modulations. Triple probe microdialysis was employed in awake rats: probes were implanted in the dorsolateral striatum (DLS), ipsilateral SNr, and GP. (*R*)-[(*S*)-1-(4-bromo-phenyl)-ethylamino]-(2,3-dioxo-1,2,3,4-tetrahydroquinoxalin-5-yl)-methyl]-phosphonic acid (NVP-AAM077) a NR2A selective antagonist (Auberson *et al.* 2002), and (*R*-(*R**,*S**)-α-(4-hydroxyphenyl)-β-methyl-4-(phenylmethyl)-1-piperidinepropanol (Ro 25-6981), a NR2B selective antagonist (Fischer *et al.* 1997), were perfused in the DLS, and GABA (and GLU) release was simultaneously monitored in the three areas. Each antagonist was tested alone or in combination with NMDA to investigate the contribution of NR2A and NR2B subunits to tonic or phasic regulation of the striatofugal pathways. To confirm the view that changes in GABA release produced by NMDA antagonists could be related to blockade of tonic activity of striatofugal neurons, the effect of intrastriatal perfusion with the Na^+-channel blocker tetrodotoxin (TTX) was also investigated.

Materials and methods

Male Sprague–Dawley rats (300–350 g; Stefano Morini, Reggio Emilia, Italy) were kept under controlled lighting conditions (12 h light/dark cycle) and given food and water *ad libitum*. The experimental protocols were approved by the Ethical Committee of the University of Ferrara and were performed according to the Italian Guidelines for Animal Care (D.L. 116/92) and the European Communities Council Directives (86/609/ECC). Adequate measures were taken to minimize animal pain and discomfort and to limit the number of animals used.

Microdialysis experiments

Three probes of concentric design were stereotaxically implanted under isoflurane anesthesia in the right DLS (3 mm dialysing membrane, AN69; Hospal, Bologna, Italy), ipsilateral SNr (1 mm) and GP (1.5 mm) according to the following coordinates from bregma and the dural surface (Paxinos and Watson 1982): DLS, AP +1.0, ML −3.5, VD −6; SNr, AP −5.5, ML −2.2, VD −8.3; and GP, AP −1.3, ML −3.3, VD −6.5. Forty-eight hours after surgery, probes were perfused with a modified Ringer solution ($CaCl_2$ 1.2 mmol/L, KCl 2.7 mmol/L, NaCl 148 mmol/L, and $MgCl_2$ 0.85 mmol/L) at a 3 μL/min flow rate. After 6 h rinsing, samples were collected every 10 min. At least three baseline samples were collected before drug perfusion through the probe. Antagonists were perfused alone (30 and 300 nmol/L) for 90 min through the probe implanted in the DLS to unravel tonic influence of NR2A and NR2B subunits on GABA and GLU release in GP and SNr. In separate experiments, to investigate the role of NR2 subunits in phasic activation of the striatofugal pathways, antagonists were challenged (at 300 nmol/L) against a single concentration of NMDA (10 μmol/L) able to activate both pathways (Fantin *et al.* 2005). In this case, antagonists were perfused 60 min before NMDA and maintained until the end of experiment.

At the end of the experiments, animals were killed and the correct placement of the probes was verified histologically. Endogenous GLU and GABA levels were measured by HPLC coupled to fluorimetric detection according to Marti *et al.* (2003). Briefly, 30 μL sample were pipetted into glass microvials and placed in a thermostated (4°C) Triathlon autosampler (Spark Holland, Emmen, The Netherlands). Forty microliters of *o*-phthaldialdehyde/boric acid solution were added to each sample, and 60 μL of the solution injected onto an analytical column (3 mm inner diameter, 10 cm length; Chrompack, Middelburg, The Netherlands). The column was eluted at a flow rate of 0.48 mL/min with a mobile phase containing 0.1 mol/L sodium acetate, 10% ethanol, and 2.5% tetrahydrofuran (pH 6.5). GLU and GABA retention time were about 3.5 and 17.5 min, and the sensitivity of the method was 150 fmol/sample.

Data presentation and statistical analysis

Data (mean ± SEM) from microdialysis experiments are reported as percentage of basal value (calculated as the mean of two samples before treatment). Statistical analysis was performed by one-way repeated measure (RM) ANOVA followed by contrast analysis and the sequentially rejective Bonferroni *post hoc* test for multiple comparisons. The interaction between the antagonists and NMDA was analyzed by two-way RM ANOVA followed by contrast analysis and the sequentially rejective Bonferroni *post hoc* test for multiple comparisons; p-values < 0.05 were considered to be statistically significant. Mean neurotransmitter levels in the dialysate (in nmol/L) have been reported in text and Figure legends.

Materials

Ro 25-6981 and NMDA were purchased from Sigma Chemical Company (St Louis, MO, USA), TTX from Alomone Labs Ltd. (Jerusalem, Israel) while NVP-AAM077 was obtained by Novartis Institutes for BioMedical Research (Basel, Switzerland).

Results

Primary effects of antagonists

To investigate whether striatal NR2A and NR2B subunit containing NMDA receptors mediate tonic regulation of striato-pallidal and striato-nigral neurons, reverse dialysis of NR2A and NR2B subunit selective antagonists was performed in the DLS, and GABA (and GLU) release monitored in the DLS and its projection areas, namely GP and SNr.

Dorsolateral striatum

Basal GABA and GLU levels in DLS were 15.4 ± 1.7 and 218.6 ± 19.5 nmol/L, respectively. Local perfusion with the NR2A preferential antagonists NVP-AAM077 did not change amino acid levels (Fig. 1a and b). Likewise, local perfusion with the NR2B selective antagonists Ro 25-6981 left striatal GABA levels unaffected (Fig. 1c). In contrast,

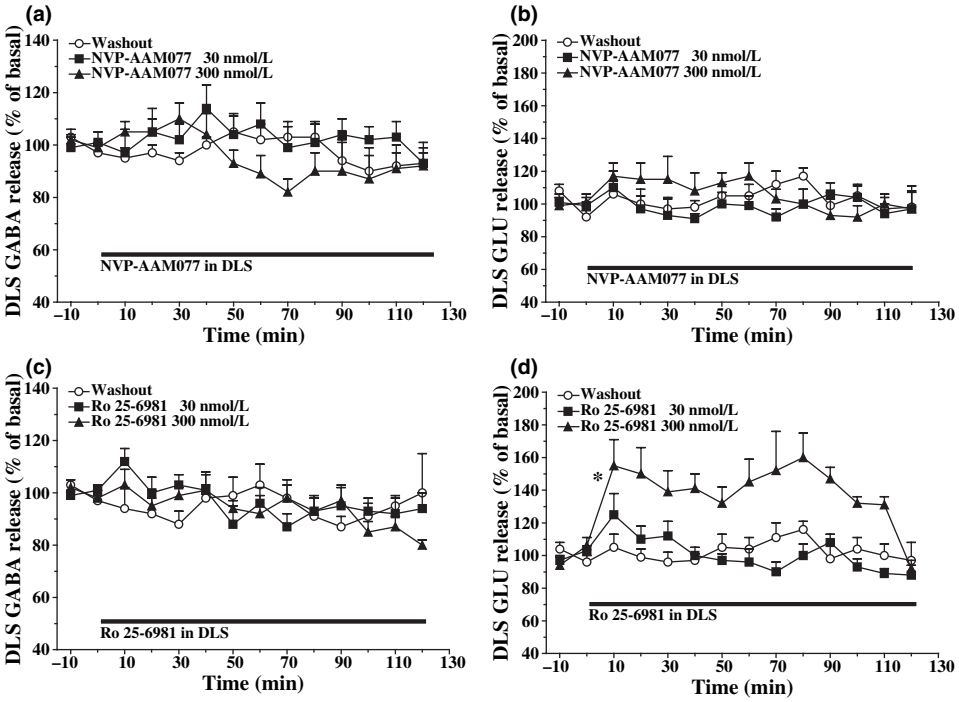

Fig. 1 Effect of subunit selective NMDA receptor antagonists on striatal GABA and GLU release. Effect of reverse dialysis of NVP-AAM077 (30 and 300 nmol/L; panels a and b) and Ro 25-6981 (30 and 300 nmol/L; panels c and d) in the dorsolateral striatum (DLS) of awake rats on local GABA and GLU extracellular levels. Data are expressed as percentages ± SEM of basal pre-treatment levels (calculated as mean of the two samples before the treatment). Panels (a and b): Basal GABA and GLU (nmol/L) levels were, respectively: 21.1 ± 3.8 and 176.8 ± 29.0 (washout, $n = 8$), 12.7 ± 0.6 and 135.6 ± 21.1 (30 nmol/L NVP-AAM077; $n = 8$), 25.2 ± 2.5 and 207 ± 22.2 (300 nmol/L NVP-AAM077; $n = 7$). Panels (c and d): Basal GABA and GLU (nmol/L) levels were, respectively: 19.7 ± 4.0 and 132.5 ± 11.3 (washout, $n = 9$), 17.1 ± 4.5 and 129.4 ± 37.9 (30 nmol/L Ro 25-6981; $n = 6$–9), 22.6 ± 2.9 and 183 ± 32.6 (300 nmol/L Ro 25-6981; $n = 6$). *$p < 0.05$ different from washout (one-way repeated measure ANOVA followed by contrast analysis and the sequentially rejective Bonferroni's test).

RM ANOVA on GLU levels (Fig. 1d) revealed a significant effect of treatment ($F_{2,10} = 31.78$, $p < 0.001$), time ($F_{13,26} = 3.78$, $p < 0.0001$), and a significant time × treatment interaction ($F_{26,195} = 2.65$, $p = 0.0001$). Contrast analysis showed that Ro 25-6981 (300 nmol/L) elevated GLU levels (maximal increase ∼60%) compared with control group (washout; $p < 0.0001$).

Globus pallidus

Basal GABA and GLU levels in GP were 20.9 ± 2.3 nmol/L and 171.4 ± 20.4 nmol/L, respectively. Intrastriatal NVP-AAM077 reduced GABA (Fig. 2a) but did not affect pallidal GLU outflow (Fig. 2b). RM ANOVA on the effect of NVP-AAM077 on GABA levels (Fig. 2a) showed a significant effect of treatment ($F_{2,12} = 8.15$, $p = 0.0058$), time ($F_{13,26} = 7.689$, $p < 0.0001$) but not a significant time × treatment interaction ($F_{26,220} = 1.04$, $p = 0.41$). Contrast analysis revealed that NVP-AAM077 (300 nmol/L) reduced GABA release (maximal inhibition ∼40%) compared with control group ($p = 0.0023$). Intrastriatal Ro 25-6981 did not change pallidal GABA release (Fig. 2c) but evoked GLU release (Fig. 2d). RM ANOVA showed a significant effect of treatment ($F_{2,12} = 8.56$, $p = 0.0049$), but not time ($F_{13,26} = 1.19$, $p = 0.28$) and a significant time × treatment interaction ($F_{26,206} = 2.69$, $p = 0.0001$). Contrast analysis revealed that Ro 25-6981 (300 nmol/L) elevated GLU release (∼50%) compared with control group ($p = 0.0071$).

Substantia nigra reticulata

Basal GABA and GLU levels in SNr were 17.6 ± 1.5 and 181.3 ± 9.6 nmol/L, respectively. Intrastriatal NVP-AAM077 did not change GABA outflow (Fig. 3a) but increased nigral GLU release (Fig. 3b). RM ANOVA showed a significant effect of treatment ($F_{2,12} = 6.36$, $p = 0.0131$), time ($F_{13,26} = 2.12$, $p = 0.014$) but not a significant time × treatment interaction ($F_{26,220} = 1.46$, $p = 0.074$). Contrast analysis revealed that NVP-AAM077 (300 nmol/L) elevated GLU release (maximal increase ∼40%) compared with control group ($p = 0.0064$). Intrastriatal Ro 25-6981 reduced GABA release (Fig. 3c) but did not change nigral GLU levels (Fig. 3d). RM ANOVA on GABA levels showed a significant effect of treatment ($F_{2,12} = 4.85$,

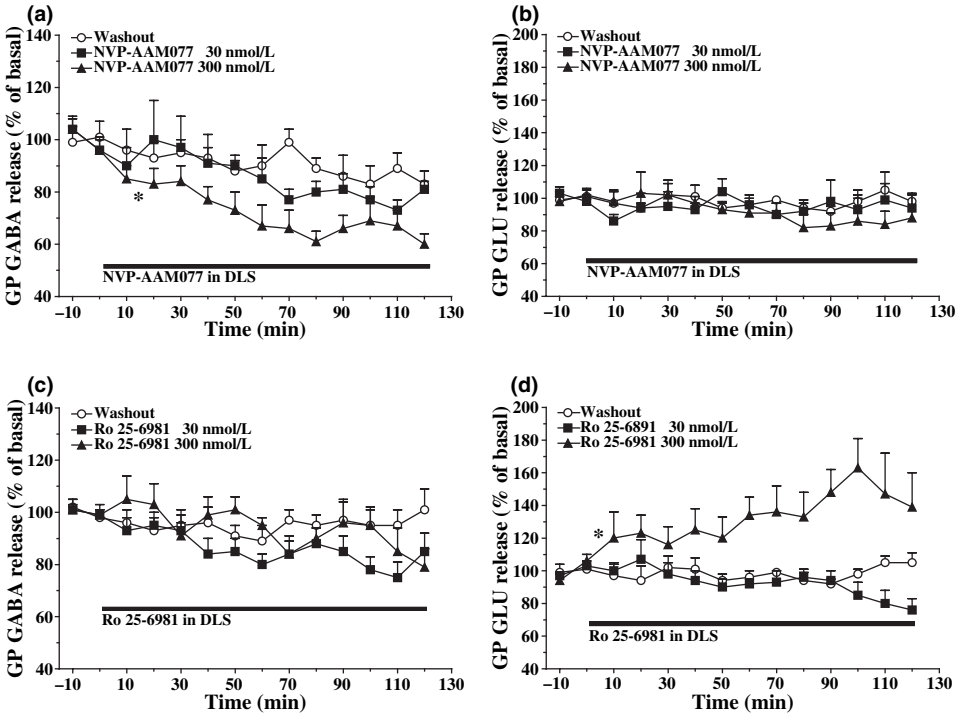

Fig. 2 Effect of subunit selective NMDA receptor antagonists on pallidal GABA and GLU release. Effect of reverse dialysis of NVP-AAM077 (30 and 300 nmol/L; panels a and b) and Ro 25-6981 (30 and 300 nmol/L; panels c and d) in the dorsolateral striatum (DLS) of awake rats on GABA and GLU extracellular levels in ipsilateral globus pallidus (GP). Data are expressed as percentages ± SEM of basal pre-treatment levels (calculated as mean of the two samples before the treatment). Panels (a and b): Basal GABA and GLU (nmol/L) levels were, respectively: 22.1 ± 4.3 and 165.2 ± 34.0 (washout, $n = 7$), 14.1 ± 2.4 and 172.1 ± 39.2 (30 nmol/L NVP-AAM077; $n = 7$–8), 21.0 ± 2.1 and 165.4 ± 33.2 (300 nmol/L NVP-AAM077; $n = 8$). Panels (c and d): Basal GABA and GLU (nmol/L) levels were, respectively: 17.1 ± 2.6 and 210.3 ± 71.2 (washout, $n = 6$), 18.0 ± 2.0 and 225.5 ± 41.9 (30 nmol/L Ro 25-6981; $n = 6$), 21.7 ± 4.1 and 154.3 ± 22.7 (300 nmol/L Ro 25-6981; $n = 7$). *$p < 0.05$ different from washout (one-way repeated measure ANOVA followed by contrast analysis and the sequentially rejective Bonferroni's test).

$p = 0.028$), time ($F_{13,26} = 5.15$, $p < 0.0001$), and a non-significant time × treatment interaction ($F_{26,234} = 1.23$, $p = 0.214$). Contrast analysis revealed that both Ro 25-6981, 30 and 300 nmol/L reduced to the same extent GABA release (~40%) compared with control group ($p = 0.022$ and 0.017, respectively). Lower concentrations (3 nmol/L) were also tested and found ineffective (data not shown).

Effect of tetrodotoxin

To confirm that GABA release in the GP and SNr partly reflected tonic activity of the striato-pallidal and striato-nigral pathways, respectively, reverse dialysis of TTX (1 μmol/L) in DLS was performed, and GABA (and GLU) release monitored in DLS, GP and SNr. TTX did not affect DLS amino acid levels (Fig. 4a). RM ANOVA on the effect of TTX on pallidal amino acid levels (Fig. 4b) showed a significant effect of treatment ($F_{3,24} = 7.10$, $p = 0.0014$), time ($F_{7,21} = 7.21$, $p < 0.0001$), and a non-significant time × treatment interaction ($F_{21,224} = 1.14$, $p = 0.076$). Contrast analysis revealed that TTX reduced GABA release (~35%; $p = 0.0010$) but did not affect GLU levels ($p = 0.326$) compared with control groups. RM ANOVA was also performed on the effect of TTX on nigral amino acid levels (Fig. 4c). Significant effects of treatment ($F_{3,21} = 3.55$, $p = 0.031$) and time ($F_{7,21} = 5.80$, $p < 0.0001$) were found together with a significant time × treatment interaction ($F_{21,204} = 1.73$, $p = 0.028$). Contrast analysis revealed that TTX reduced GABA release (~33%, $p = 0.0010$) but did not affect GLU levels ($p = 0.210$) compared with control groups.

Pharmacological antagonism between NMDA and subunit selective NMDA antagonists

To investigate whether striatal NR2A and NR2B subunit containing NMDA receptors mediate phasic regulation of striato-pallidal and striato-nigral neurons, NVP-AAM077 and Ro 25-6981 were challenged at the higher concentration (i.e. 300 nmol/L) against NMDA (10 μmol/L). This concentration was chosen since in previous experiments (Fantin *et al.* 2005) it was shown that reverse dialysis of NMDA 10 μmol/L in the striatum was able to evoke GABA release both in GP and SNr.

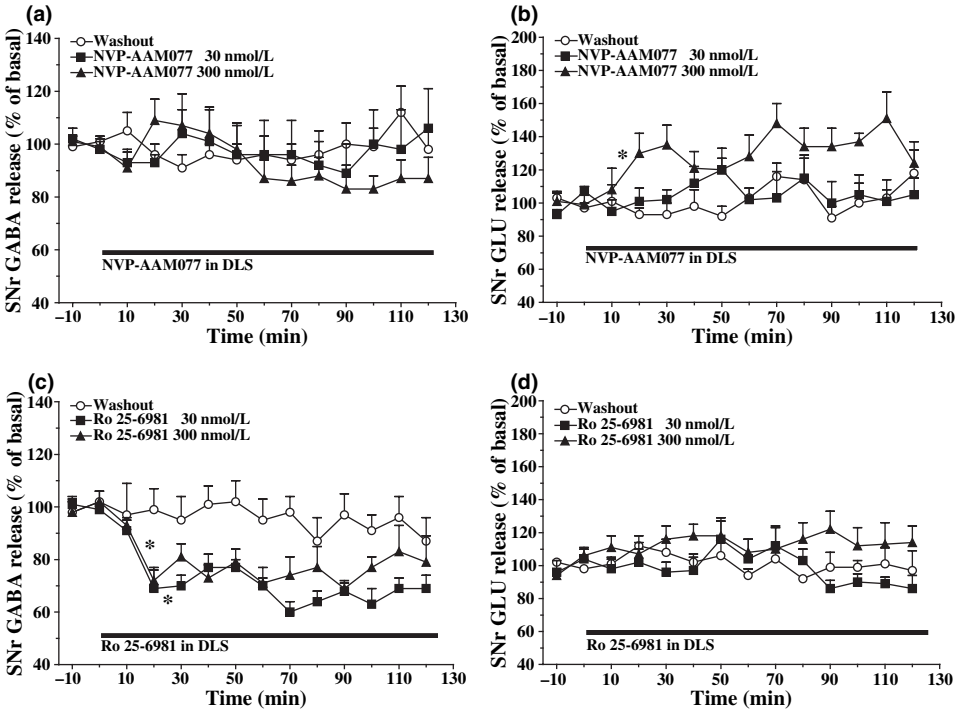

Fig. 3 Effect of subunit selective NMDA receptor antagonists on nigral GABA and GLU release. Effect of reverse dialysis of NVP-AAM077 (30 and 300 nmol/L; panels a and b) and Ro 25-6981 (30 and 300 nmol/L; panels c and d) in the dorsolateral striatum (DLS) of awake rats on GABA and GLU extracellular levels in ipsilateral substantia nigra reticulata (SNr). Data are expressed as percentages ± SEM of basal pre-treatment levels (calculated as mean of the two samples before the treatment). Panels (a and b): Basal GABA and GLU (nmol/L) levels were, respectively: 18.2 ± 2.4 and 138.1 ± 20.2 (washout, $n = 7$), 11.3 ± 1.4 and 70.0 ± 8.5 (30 nmol/L NVP-AAM077; $n = 5$–7), 16.5 ± 2.1 and 152.4 ± 22.9 (300 nmol/L NVP-AAM077; $n = 5$–7). Panels (c and d): Basal GABA and GLU (nmol/L) levels were, respectively: 18.8 ± 3.3 and 147.4 ± 21.6 (washout, $n = 7$), 12.6 ± 1.6 and 152.5 ± 34.0 (30 nmol/L Ro 25-6981; $n = 6$–7), 16.8 ± 2.0 and 156.6 ± 21.1 (300 nmol/L Ro 25-6981; $n = 7$). *$p < 0.05$ different from washout (one-way repeated measure ANOVA followed by contrast analysis and the sequentially rejective Bonferroni's test).

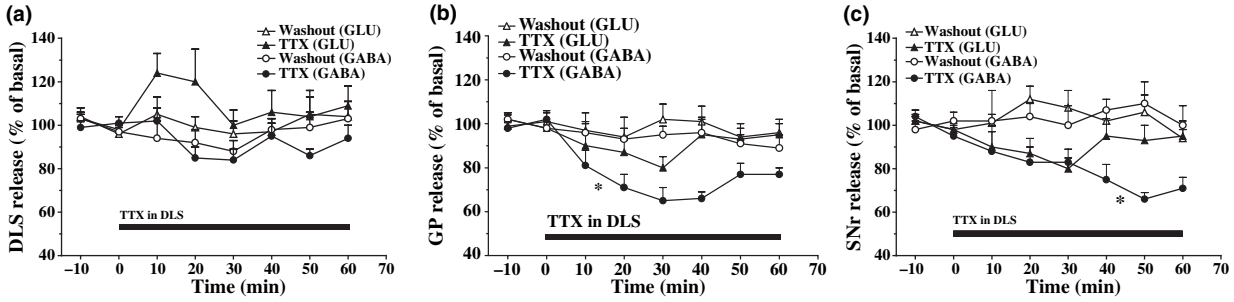

Fig. 4 Effect of tetrodotoxin (TTX) on GABA and GLU release in striatum and its target areas. Effect of reverse dialysis of TTX (1 μmol/L) in the dorsolateral striatum (DLS) of awake rats on GLU and GABA release in DLS (panel a), ipsilateral globus pallidus (GP; panel b) and ipsilateral substantia nigra reticulata (SNr, panel c). Data are expressed as percentages ± SEM of basal pre-treatment levels (calculated as mean of the two samples before the treatment). Data are expressed as percentages ± SEM of basal pre-treatment levels (calculated as mean of the two samples before the treatment). Panel a: Basal GABA and GLU (nmol/L) levels were, respectively: 14.7 ± 3.6 and 143.6 ± 6.3 (washout, $n = 10$), 13.9 ± 1.5 and 73.3 ± 11.5 (TTX; $n = 11$). Panel b: Basal GABA and GLU (nmol/L) levels were, respectively: 15.0 ± 1.9 and 60.1 ± 8.7 (washout, $n = 9$), 8.2 ± 1.1 and 76.2 ± 11.7 (TTX; $n = 9$). Panel c: Basal GABA and GLU (nmol/L) levels were, respectively: 15.3 ± 3.6 and 111.2 ± 26.1 (washout, $n = 9$), 10.1 ± 1.0 and 57.0 ± 13.9 (TTX; $n = 8$). *$p < 0.05$ different from washout (one-way repeated measure ANOVA followed by contrast analysis and the sequentially rejective Bonferroni's test).

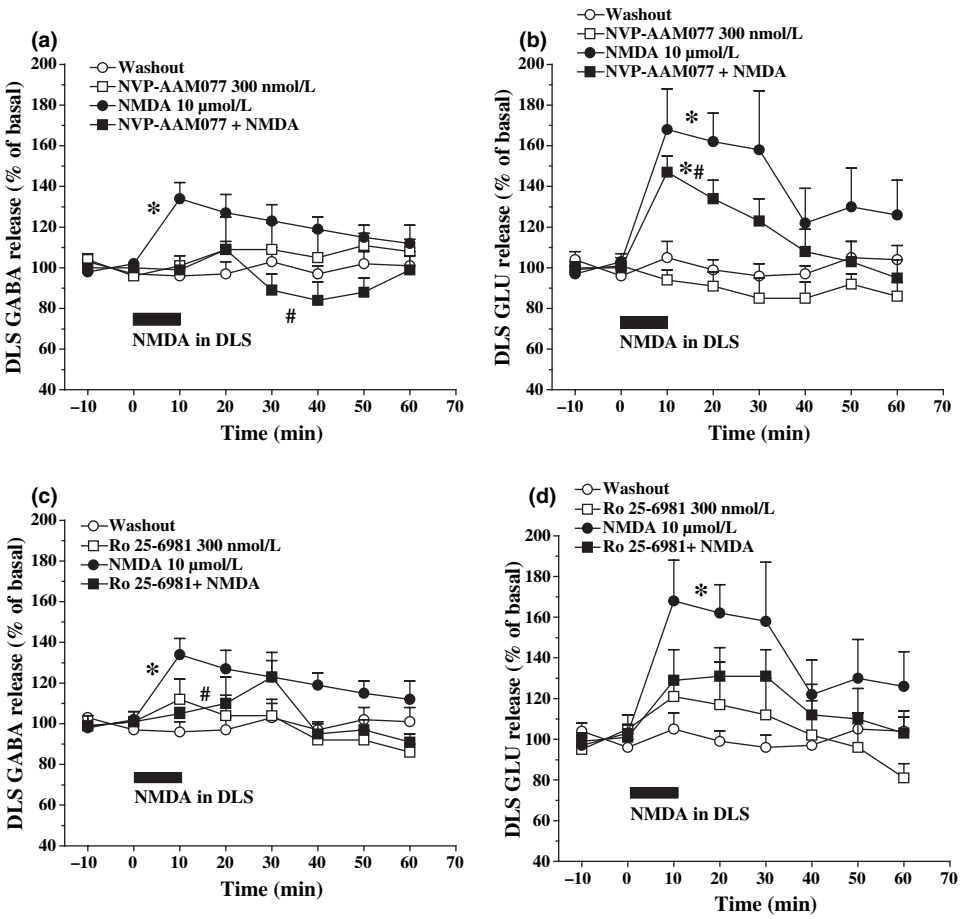

Fig. 5 Effect of subunit selective NMDA receptor antagonists on NMDA-evoked striatal GABA and GLU release. Effect of reverse dialysis of NVP-AAM077 (300 nmol/L; panels a and b) and Ro 25-6981 (300 nmol/L; panels c and d) in the dorsolateral striatum (DLS) of awake rats on local GABA and GLU extracellular levels evoked by intrastriatal NMDA (10 μmol/L, 10 min; black bar). Perfusion with antagonists started 60 min before NMDA and continued until the end of experiment. Data are expressed as percentages ± SEM of basal pre-treatment levels (calculated as mean of the two samples before the treatment). Data referring to NMDA alone groups are the same in panels (a–c) and (b–d). Panels (a and b). Basal GABA and GLU (nmol/L) levels were, respectively: 15.6 ± 3.1 and 199.8 ± 14.7 (washout, n = 7–9), 11.0 ± 2.2 and 221.1 ± 44.9 (10 μmol/L NMDA; n = 9), 15.8 ± 2.3 and 183.9 ± 27.0 (300 nmol/L NVP-AAM077; n = 6–9), 18.6 ± 2.5 and 201.3 ± 36.9 (NVP-AAM077 + NMDA; n = 9). Panels (c and d): Basal GABA and GLU (nmol/L) levels were, respectively: 23.0 ± 2.6 and 144.6 ± 3.5 (washout, n = 7–9), 11.0 ± 2.2, 221.1 ± 44.9 (10 μmol/L NMDA; n = 9), 21.3 ± 3.1 and 183.3 ± 32.7 (300 nmol/L Ro 25-6981; n = 6–8), 23.7 ± 3.1 and 188.6 ± 44.7 (Ro 25-6981 + NMDA; n = 9). $^{*}p < 0.05$ different from washout and $^{\#}p < 0.05$ different from NMDA (two-way repeated measure ANOVA followed by contrast analysis and the sequentially rejective Bonferroni's test).

Dorsolateral striatum

Repeated measure ANOVA on the effect of NVP-AAM077 on GABA values (Fig. 5a) showed a significant effect of treatment ($F_{3,24} = 5.45$, $p = 0.0053$) but not time ($F_{7,21} = 1.55$, $p = 0.152$), and a significant time × treatment interaction ($F_{21,224} = 1.99$, $p = 0.0075$). Contrast analysis revealed that intrastriatal NMDA elevated GABA release compared with controls ($p = 0.0026$) and NVP-AAM077 prevented this effect ($p = 0.0014$ vs. NMDA alone). RM ANOVA on GLU values (Fig. 5b) showed a significant effect of treatment ($F_{3,21} = 9.93$, $p = 0.0003$), time ($F_{7,21} = 1.55$, $p < 0.0001$), and a non-significant time × treatment interaction ($F_{21,180} = 1.55$, $p = 0.063$). Contrast analysis revealed that NMDA elevated GLU release both in the absence and in the presence of NVP-AAM077, although in this case the effect was attenuated ($p < 0.012$ vs. NMDA alone).

As far as the effect of Ro 25-6981 on the NMDA-induced GABA release is concerned (Fig. 5c), a significant effect of treatment ($F_{3,24} = 6.64$, $p = 0.0020$), time ($F_{7,21} = 3.11$, $p = 0.038$), and a significant time × treatment interaction ($F_{21,224} = 1.98$, $p = 0.0079$) were found. Contrast analysis revealed that Ro 25-6981 prevented the NMDA-induced stimulation ($p = 0.0062$ vs. NMDA alone). RM ANOVA on GLU data (Fig. 5d) revealed a significant effect of treatment

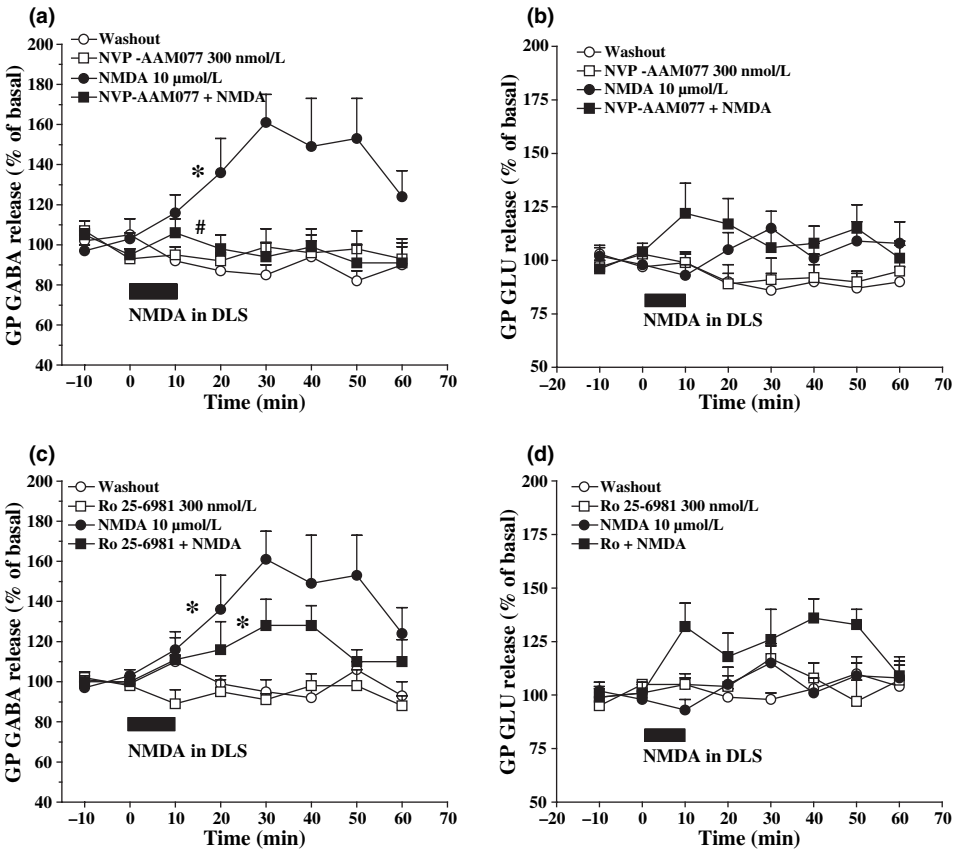

Fig. 6 Effect of subunit selective NMDA receptor antagonists on NMDA-evoked pallidal GABA and GLU release. Effect of reverse dialysis of NVP-AAM077 (300 nmol/L; panels a and b) and Ro 25-6981 (300 nmol/L; panel c and d) in the dorsolateral striatum (DLS) of awake rats on GABA and GLU extracellular levels in ipsilateral globus pallidus (GP) evoked by intrastriatal NMDA (10 μmol/L, 10 min; black bar). Perfusion with antagonists started 60 min before NMDA and continued until the end of experiment. Data are expressed as percentages ± SEM of basal pre-treatment levels (calculated as mean of the two samples before the treatment). Data referring to NMDA alone groups are the same in panels (a–c and b–d). Panels (a and b): Basal GABA and GLU (nmol/L) levels were, respectively: 19.4 ± 3.0 and 165.2 ± 34 (washout, $n = 7$–9), 14.7 ± 3.6 and 154.0 ± 32.5 (10 μmol/L NMDA; $n = 9$), 13.8 ± 1.9 and 165.4 ± 33.2 (300 nmol/L NVP-AAM077; $n = 6$–7), 18.2 ± 2.8 and 120.4 ± 28.5 (NVP-AAM077 + NMDA; $n = 9$). Panels (c and d): Basal GABA and GLU (nmol/L) levels were, respectively: 17.5 ± 1.9 and 98.7 ± 42.1 (washout, $n = 7$–9), 14.7 ± 3.6 and 154.0 ± 32.5 (10 μmol/L NMDA; $n = 9$), 17.3 ± 4.2 and 158.0 ± 12.3 (300 nmol/L Ro 25-6981; $n = 6$–7), 17.8 ± 3.2 and 184.6 ± 61.1 (Ro 25-6981 + NMDA; $n = 9$). $^{*}p < 0.05$ different from washout and $^{\#}p < 0.05$ different from NMDA (two-way repeated measure ANOVA followed by contrast analysis and the sequentially rejective Bonferroni's test).

($F_{3,21} = 3.87$, $p = 0.023$), time ($F_{7,21} = 4.72$, $p = 0.0001$), and a non-significant time × treatment interaction ($F_{21,180} = 0.99$, $p = 0.48$). Contrast analysis revealed that NMDA in the presence of Ro 25-6981 did not evoke GLU levels ($p = 0.35$ vs. Ro 25-6981 alone).

Globus pallidus

Repeated measure ANOVA on the effect of NVP-AAM077 on NMDA-induced GABA release (Fig. 6a) showed a significant effect of treatment ($F_{3,21} = 7.65$, $p = 0.0012$) but not time ($F_{7,21} = 1.65$, $p = 0.123$), and a significant time × treatment interaction ($F_{21,212} = 2.80$, $p < 0.0001$). Contrast analysis revealed that intrastriatal NMDA elevated GABA release compared with control group ($p < 0.0001$) and NVP-AAM077 prevented this increase ($p = 0.0013$ vs. NMDA alone). Intrastriatal NMDA did not evoke GLU release in GP both in the presence and in the absence of NVP-AAM077 (Fig. 6b).

As far as the effect of Ro 25-6981 is concerned (Fig. 6c), a significant effect of treatment ($F_{3,21} = 7.71$, $p = 0.0011$), time ($F_{7,21} = 3.38$, $p = 0.0019$), and a significant time × treatment interaction ($F_{21,212} = 2.26$, $p = 0.0018$) were found. Contrast analysis revealed that co-application of Ro 25-6981 and NMDA significantly evoked GABA release ($p = 0.043$ vs. washout) and that this stimulation was not different from that evoked by NMDA alone ($p = 0.077$). Intrastriatal NMDA did not evoke GLU release in GP both in the presence and in the absence of Ro 25-6981 (Fig. 6d).

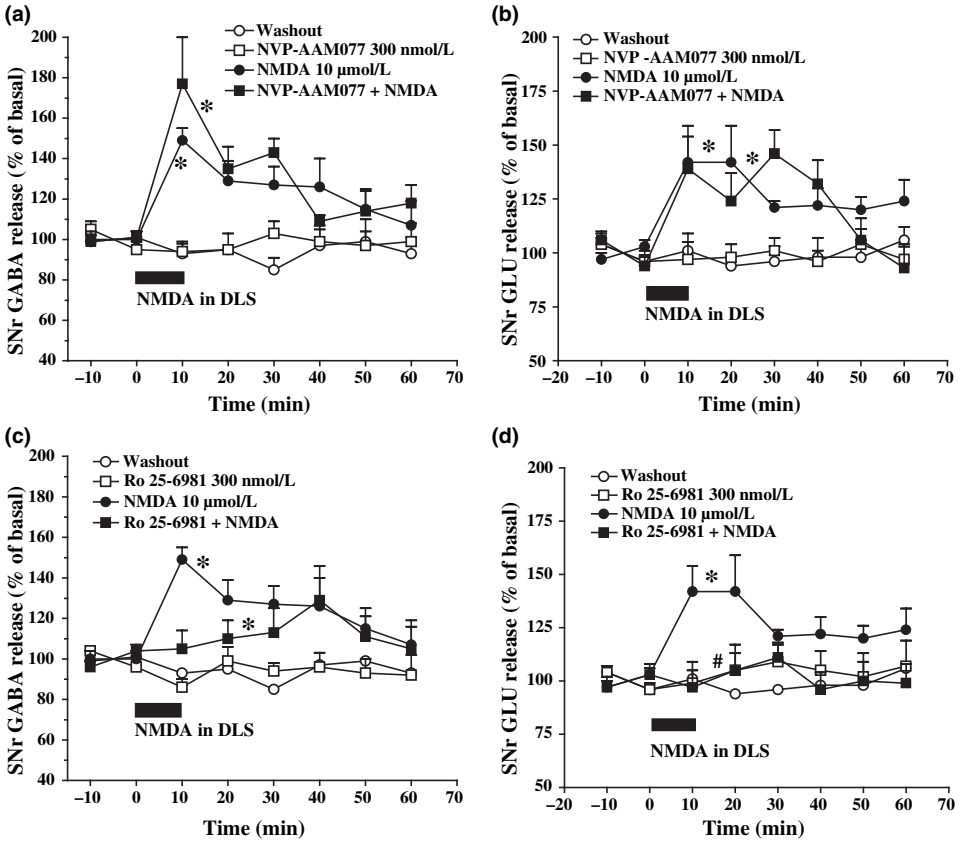

Fig. 7 Effect of subunit selective NMDA receptor antagonists on NMDA-evoked nigral GABA and GLU release. Effect of reverse dialysis of NVP-AAM077 (300 nmol/L; panels a and b) and Ro 25-6981 (300 nmol/L; panels c and d) in the dorsolateral striatum (DLS) of awake rats on GABA and GLU extracellular levels in ipsilateral substantia nigra reticulata (SNr) evoked by intrastriatal NMDA (10 μmol/L, 10 min; black bar). Perfusion with antagonists started 60 min before NMDA and continued until the end of experiment. Data are expressed as percentages ± SEM of basal pre-treatment levels (calculated as mean of the two samples before the treatment). Data referring to NMDA alone groups are the same in panels (a–c and b–d). Panels (a and b): Basal GABA and GLU (nmol/L) levels were, respectively: 15.8 ± 2.1 and 118.4 ± 33 (washout, n = 7–9), 17.3 ± 3.0 and 109.0 ± 21.5 (10 μmol/L NMDA; n = 7–9), 15.4 ± 2.7 and 213.7 ± 29.7 (300 nmol/L NVP-AAM077; n = 7–9), 16.3 ± 2.2 and 129.1 ± 15.8 (NVP-AAM077 + NMDA; n = 7–8). Panels (c and d): Basal GABA and GLU (nmol/L) levels were, respectively: 15.7 ± 13.0 and 147.4 ± 21.6 (washout, n = 7–9), 17.3 ± 3.0 and 109.0 ± 21.5 (10 μmol/L NMDA; n = 7), 12.6 ± 1.9 and 199.3 ± 26.8 (300 nmol/L Ro 25-6981; n = 8), 15.1 ± 2.2 and 189.2 ± 20.0 (Ro 25-6981 + NMDA; n = 7–9). $^{*}p < 0.05$ different from washout and $^{\#}p < 0.05$ different from NMDA (two-way repeated measure ANOVA followed by contrast analysis and the sequentially rejective Bonferroni's test).

Substantia nigra reticulata

Repeated measure ANOVA on the effect of NVP-AAM077 on the NMDA-induced GABA release (Fig. 7a) showed a significant effect of treatment ($F_{3,18} = 11.59$, $p < 0.0001$), time ($F_{7,21} = 5.27$, $p < 0.0001$), and a significant time × treatment interaction ($F_{21,168} = 3.30$, $p = 0.0075$). Contrast analysis revealed that intrastriatal NMDA alone or in combination with NVP-AAM077 elevated GABA release compared with control ($p = 0.0009$) or NVP-AAM077 alone ($p = 0.0004$) groups. Analysis of GLU data (Fig. 7b) yielded to similar results. RM ANOVA showed a significant effect of treatment ($F_{3,21} = 9.19$, $p = 0.0004$), time ($F_{7,21} = 3.30$, $p = 0.0024$), and a significant time × treatment interaction ($F_{21,212} = 3.56$, $p = 0.0004$). Contrast analysis revealed that NVP-AAM077 did not change the NMDA-induced GLU release in SNr.

Conversely, Ro 25-6981 attenuated the responses to NMDA. RM ANOVA on GABA values (Fig. 7c) disclosed a significant effect of treatment ($F_{3,21} = 5.27$, $p = 0.0041$), but not time ($F_{7,21} = 1.46$, $p = 0.18$), and a significant time × treatment interaction ($F_{21,196} = 1.96$, $p = 0.0095$). Contrast analysis revealed that NMDA in the presence of Ro 25-6981 produced a significant stimulation ($p = 0.034$ vs. Ro 25-6981 alone). However, *post hoc* analysis on the time-course of the response indicated that Ro 25-6981 prevented the peak at 10 min ($p = 0.0025$ vs. NMDA alone). Likewise, RM ANOVA on GLU values (Fig. 7d) showed a significant effect of treatment ($F_{3,24} = 5.14$, $p = 0.0069$), but not time

($F_{7,21} = 1.66$, $p = 0.12$), and a significant time × treatment interaction ($F_{21,216} = 1.62$, $p = 0.046$). Contrast analysis showed that Ro 25-6981 counteracted the NMDA-induced GLU release in SNr.

Discussion

The main finding of the present study is that blockade of NR2A and NR2B subunit containing NMDA receptors in the striatum differentially modulated GABA and GLU release in the striatum and target areas. In particular, the finding that NVP-AAM077 and Ro 25-6981 reduced spontaneous and NMDA-induced GABA release in GP and SNr, suggests that NR2A and NR2B subunits preferentially modulate striato-pallidal and striato-nigral neurons, respectively.

The evidence for the heterogeneity of the NMDA receptor family (Meguro *et al.* 1992; Monyer *et al.* 1992; Nakanishi 1992) and the differential distribution of NMDA receptor subtypes in the CNS (Nakanishi 1992; Ishii *et al.* 1993) have fueled considerable interest in dissecting out the role of the different NMDA receptor subtypes in physio-pathological conditions. As the NR2 subunit composition confers pharmacological specificity to the NMDA receptor (Laurie and Seeburg 1994), a number of subunit selective/preferential NMDA receptor antagonists have been developed to target NR2A and NR2B subunits. In previous studies, NVP-AAM077 and Ro 25-6981 have been tested in parallel to investigate the contribution of NR2A and NR2B subunits to locomotion and phencyclidine discrimination (Chaperon *et al.* 2003), hippocampal plasticity (Massey *et al.* 2004; Mallon *et al.* 2005; Fox *et al.* 2006; Bartlett *et al.* 2007), NMDA neurotoxicity (Zhou and Baudry 2006) and epilepsy (Yang *et al.* 2006). NVP-AAM077 is a potent (affinity for recombinant NR2A receptors of 8 nmol/L, Auberson *et al.* 2002) and competitive NMDA antagonist with > 100-fold selectivity for human recombinant NR1/NR2A over NR1/NR2B receptors (Auberson *et al.* 2002; Liu *et al.* 2004). However, NR2A/NR2B selectivity ratio at rat recombinant receptors is much lower (~7- to 13-fold; Feng *et al.* 2004; Neyton and Paoletti 2006; Frizelle *et al.* 2006) and recently NVP-AAM077 has been claimed to be a preferential rather than selective NR2A antagonist (Berberich *et al.* 2005). Based on these reports and taking into account the *in vitro* recovery rate for GLU under our conditions (~18% for a 3 mm probe; Marti *et al.* 2002), we set maximal NVP-AAM077 concentration in the dialysate to 300 nmol/L, in order to provide ~50 nmol/L 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%; Berberich *et al.* 2005). Given the poor NR2A/NR2C selectivity ratio of NVP-AAM077 at rat recombinant receptors (~2, Feng *et al.* 2004), we also cannot rule out the possibility that 300 nmol/L NVP-AAM077 interferes with NR2C responses. Nevertheless, this possibility appears remote as NR2C subunits are expressed at a very low level in the striatum (Standaert *et al.* 1999). Selection of the Ro 25-6981 concentrations (30 and 300 nmol/L) has been less problematic, as this compound has been shown to potently bind to rat brain membranes (affinity for native NR2B receptors of ~6 nmol/L; Mutel *et al.* 1998) and have much greater selectivity (~5000-fold) for NR2B over NR2A subunits at recombinant rat NMDA receptors (Fischer *et al.* 1997).

The finding that reverse dialysis of TTX (1 μmol/L) in the striatum decreased nigral and pallidal GABA levels is consistent with the view that a certain amount (~30%) of extracellular GABA levels in SNr and GP derives from neuronal activity of medium size striato-nigral and striato-pallidal GABAergic neurons. Indeed, about one third of striatal projection neurons tonically discharge at low frequencies (West *et al.* 2002) and intrastriatal TTX should inhibit axon potential propagation along the two pathways. This finding endorses the view that the reduction of GP and SNr GABA release induced by intrastriatal perfusion with subunit selective NMDA receptor antagonists is because of striatal output pathway inhibition. NMDA receptors are abundantly expressed in the striatum (Monaghan and Cotman 1985; Standaert *et al.* 1994) although the subunit composition varies between neurons (Landwehrmeyer *et al.* 1995; Standaert *et al.* 1999), conferring them different functional properties. Indeed, striatal NMDA receptors modulating acetylcholine, dopamine (DA), and GABA release bear different pharmacological profiles (Cai *et al.* 1991; Nankai *et al.* 1996). *In situ* hybridization studies have shown that striatal GABAergic projection neurons express NR1 and an equal proportion of NR2A and NR2B subunits (Landwehrmeyer *et al.* 1995; Standaert *et al.* 1999), with a tendency for NR2B subunits to predominate in striato-nigral neurons (Standaert *et al.* 1999). Thus, the finding that subunit-selective NR2A and NR2B antagonists selectively modulate GABA release in GP and SNr is difficult to reconcile with the lack of segregation of NR2A and NR2B mRNAs in projection neurons. However, such selectivity was displayed against both endogenous GLU and exogenous NMDA, suggesting it relied on NMDA receptor blockade. It is possible that mRNA expression does not match the presence of an active protein at the membrane level because of post-transcriptional or post-translational modifications (e.g. receptor trafficking and phosphorylation). For instance, a number of NR2 subunits could be segregated in submembrane compartments or transported at the terminal levels. In this respect, Dunah *et al.* (2000) were able to detect a reduction in the abundance of NR2B subunit in the DA-depleted striatum only in the membrane fraction (and not in the overall extract). Thus, despite the fact that both populations of GABAergic neurons express NR2A and NR2B subunits, the membranes of striato-pallidal and striato-nigral neurons would be preferentially enriched in NMDA

receptors containing NR2A and NR2B subunits, respectively. Alternatively, we could relate selective inhibition of GP and SNr GABA release by NMDA antagonists to blockade of NR2A or NR2B subunits on subpopulations of striatal interneurons which facilitate the activity of projection neurons. In this respect, cholinergic interneurons may be involved as they express NR2B (although no NR2A) subunits (Standaert *et al.* 1999) and can activate GABAergic projection neurons (Di Chiara *et al.* 1994). Conversely, GABAergic interneurons express both NR2A and NR2B subunits (Landwehrmeyer *et al.* 1995; Standaert *et al.* 1999) but exert predominant inhibition on projection neurons (Koós and Tepper 1999). Despite the fact that the neurobiological substrates remain unknown, the present study indicates that NR2A and NR2B subunit containing NMDA receptors preferentially facilitate the activity of striato-pallidal and striato-nigral neurons, respectively. Therefore, in view of the classical scheme of basal ganglia functional organization (Albin *et al.* 1989; DeLong 1990), it can be predicted that, in naïve animals, striatal NR2A subunit blockade improves motor activity while striatal NR2B subunit blockade impairs it. To support this view, intrastriatal injections of NR2A antisense oligonucleotides induced ipsilateral circling under amphetamine challenge (Standaert *et al.* 1996), whereas NR2A (ε1) subunit knockouts displayed increased locomotion compared with wild-type mice (Miyamoto *et al.* 2001). It is worth mentioning that genetic deletion of the NR2A subunit resulted in potentiation of the NMDA-evoked DA release and attenuation of the NMDA-evoked GABA release in the striatum, suggesting that striatal plasticity underlies the motor phenotype of NR2A knockout mice. The finding that pharmacological blockade of NR2A subunits by NVP-AAM077 abolished the NMDA-induced striatal GABA release *in vivo* is consistent with that *in vitro* study. Moreover, Ro 25-6981 replicated this effect suggesting that simultaneous activation of NR2A and NR2B subunits underlies the NMDA-evoked stimulation of GABA release. Although the majority of striatal NMDA receptors exist in binary NR1/NR2A or NR1/NR2B complexes, ∼30% of them are in NR1/NR2A/NR2B ternary form and are expressed selectively in the synaptosomal fraction (Dunah and Standaert 2003). The involvement of different assemblies of NMDA receptors may explain why some of the NMDA responses (e.g. increase of GP and SNr GABA release) are selectively modulated by either the NR2A or NR2B subunit while others (e.g. increase of striatal GABA release) require activation of both of them. Contrary to that found for NVP-AAM077, inhibition of SNr GABA release by Ro 25-6981 is not a predictor for a motor depressant action. Indeed, NR2B subunits do not participate in motor control as intrastriatal injections of NR2B antisense nucleotides (Standaert *et al.* 1996) or NR2B selective antagonists (Nash and Brotchie 2002) do not affect motor activity in naïve animals. It is possible that other pathways also contribute to motor control. In this respect, inhibition of GABA release induced by NVP-AAM077 in GP and Ro 25-6981 in SNr were mirrored by changes in GLU release: NVP-AAM077 elevated GLU release in SNr while Ro 25-6981 elevated GLU release in GP (and DLS). This is quite puzzling as no direct GLUergic projections from the striatum to SNr or GP have been identified, and TTX perfusion in DLS did not affect GLU levels in projection areas. Thus, GLU cannot be considered a marker of striatal output pathway activity as faithful as GABA. The fact that each antagonist affected neurotransmitter release both in GP and SNr may argue against the view of a 'functional segregation' of NR2A and NR2B responses along the striato-pallidal and striato-nigral pathways. However, the increases of GLU release in GP and SNr were not associated with changes in local GABA levels indicating that these increases are not because of reduction of striatal GABAergic input *per se*. Unless we postulate that GLU, released in GP and SNr, can release GABA from nerve terminals thereby counter-balancing (and masking) inhibition of pathway activity. Conversely, it is more likely that these increases of GLU release are because of activation of polysynaptic pathways. In particular, DAergic pathways may be involved as endogenous DA modulates SNr GLU release induced by intrastriatal MK-801 (Marti *et al.* 2000). For instance, impairment of striato-nigral GABAergic neurons by Ro 25-6981 might disinhibit nigro-striatal and nigro-pallidal DA projections (Smith and Kieval 2000), leading to increased striatal and pallidal GLU release (possibly via D_1 receptor activation; see for a review Morari *et al.* 1998a). On the other hand, impairment of striato-pallidal neurons by NVP-AAM077 would disinhibit the pallido-nigral GABAergic projection, leading to inhibition of nigral DA release and increase of nigral GLU levels (via relief from a D_2 receptor mediated inhibition; Morari *et al.* 1998a).

Concluding remarks

NR2A and NR2B subunit selective antagonists differentially modulated GABA and GLU release in the striatum and target areas (SNr and GP). The patterns of GABA (and GLU) responses (both at rest and under NMDA challenge) are suggestive of a preferential facilitatory control of NR2A subunits over striato-pallidal neurons and NR2B subunits over striato-nigral neurons. These data add to previous studies suggesting a differential role for striatal NR2A and NR2B subunits in motor control (Standaert *et al.* 1996), learning (Schenberg *et al.* 2006), and morphine dependence (Murray *et al.* 2007). Moreover, they may provide a neurobiological basis to the finding that increased expression/prevalence of striatal NR2A subunits is associated with parkinsonian hypokinesia in 6-hydroxydopamine hemilesioned rats (Dunah *et al.* 2000; Ganguly and Keefe 2001; Fiorentini *et al.* 2006).

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PAPER III

Differential effect of NR2A and NR2B subunit selective NMDA receptor antagonists on striato-pallidal neurons: relationship to motor response in the 6-hydroxydopamine model of parkinsonism

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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-(phenylmethyl)-1-piperidinepropanol
SNr	substantia nigra reticulata

Abstract

We previously demonstrated that NMDA receptors containing the NR2A or NR2B subunits differentially regulate striatal output pathways. We now investigate whether such a differential control is altered under parkinsonian conditions and whether subunit selective antagonists have different abilities to attenuate parkinsonian-like motor deficits. Three microdialysis probes were simultaneously implanted in the dopamine-depleted striatum, globus pallidus and substantia nigra reticulata of 6-hydroxydopamine hemilesioned rats. The NR2A antagonist NVP-AAM077 perfused in the striatum reduced pallidal GABA, but not glutamate, levels whereas the NR2B antagonist Ro 25-6981 was ineffective. Neither antagonist affected striatal or nigral amino acid levels. To investigate whether these neurochemical responses were predictive of different antiparkinsonian activities, antagonists were administered systemically and motor activity evaluated in different motor tasks. Neither antagonist attenuated akinesia/bradykinesia in the bar and drag test. However, NVP-AAM077 dually modulated rotarod performance (low doses being facilitatory and higher ones inhibitory) while Ro 25-6981 monotonically improved it. Microdialysis revealed that motor facilitating doses reduced pallidal GABA levels while motor inhibiting doses increased them. We conclude that, under parkinsonian conditions, the striato-pallidal pathway is driven by striatal NR2A subunits. Motor improvement induced by NVP-AAM077 and Ro 25-6981 is accomplished by blockade of striatal NR2A and extrastriatal NR2B subunits, respectively.

Key words: GABA, microdialysis, NMDA receptor subunits, NVP-AAM077, 6-OHDA, Ro 25-6981

Running title: NMDA subunits in parkinsonism

NMDA receptors form a heterogeneous family of ligand-gated ion channels assembled in a tetra/pentameric form by different combinations of 8 splice variants of NR1 subunits and 4 isoforms (A-D) of NR2 subunits (Monyer et al., 1992; Meguro et al., 1992; Dingledine et al., 1999). The assembly of different NR2 subunits confers distinct pharmacological properties to NMDA receptor subtypes (Laurie and Seeburg, 1994; Monaghan and Larsen, 1997). Development of subunit selective ligands has thus raised the possibility to pharmacologically target specific NMDA receptor subtypes, with potentially relevant clinical consequences in the field of Parkinson's disease (PD). Indeed, abnormal function of striatal NMDA receptors plays a key role in driving parkinsonian symptoms and long-term motor complications of L-DOPA therapy (for reviews see Chase and Oh, 2000; Hallet and Standaert, 2004). Non-selective, broad-spectrum NMDA antagonists exert antiparkinsonian and/or antidyskinetic actions in parkinsonian rats (Engber et al., 1994; Papa et al., 1995; Marin et al., 1996), monkeys (Blanchet et al., 1997, 1999; Konitsiotis et al., 2000; Papa and Chase, 1996) and human subjects (Rajput et al., 1998; Verhagen Metman et al., 1998). However, these compounds are characterized by undesired side-effects, mainly ataxia and psychosis, that make clinical use unlikely. Thus, NMDA subunit selective antagonists, which have a better tolerability profile, may represent an alternative therapeutic approach to PD. In particular, NR2B subunit selective antagonists, alone and in combination with L-DOPA, attenuated parkinsonian motor symptoms (Steece-Collier 2000; Löschmann 2004; Wessell et al., 2004) and reduced the development of L-DOPA-induced dyskinesia (Blanchet et al., 1999; Morisette et al., 2006). Likewise, MDL 100,453, an antagonist with 5-10 fold in vitro selectivity for the NR2A over NR2B subunit, improved global motor activity and potentiated the effect of L-DOPA in MPTP-treated marmosets, at the cost, however, of inducing dyskinesia (Blanchet et al., 1999). These findings suggests tha NR2A and NR2B antagonists may have different antiparkinsonian and/or antidyskinetic profiles, although more selective NR2A subunits antagonists need to be used (possibly in comparative studies) to prove this concept. The mechanisms underlying the motor responses to NR2 selective antagonists also remain unknown. Most studies pointed to the striatal

medium-sized GABA spiny neurons as the neurobiological effectors, since they largely express NR2A and NR2B subunits (Standaert et al., 1999; Landwehrmeyer et al., 1995), and subunit expression (Ulas and Cotman, 1996; Ganguly and Keefe, 2001) or subcellular distribution (Dunah et al., 2000; Fiorentini et al., 2006; Gardoni et al., 2006) change following dopamine (DA) depletion. It is worth mentioning that NR2A and NR2B subunits are not segregated in striato-pallidal and striato-nigral projection neurons (Standaert et al., 1999). Nevertheless, in a recent microdialysis study (Fantin et al., 2007), we proposed that NR2A and NR2B subunits differentially regulate striatal output pathways. Indeed, intrastriatal perfusion with the NR2A subunit selective antagonist (R)-[(S)-1-(4-bromo-phenyl)-ethylamino]-(2,3-dioxo-1,2,3,4-tetrahydroquinoxalin-5-yl)-methyl]-phosphonic acid (NVP-AAM077; Auberson et al., 2002) or the NR2B selective antagonist (R-(R*,S*)-α-(4-hydroxyphenyl)-β-methyl-4-(phenylmethyl)-1-piperidinepropanol (Ro 25-6981, Fischer et al., 1997) reduced spontaneous and NMDA-stimulated GABA levels in GP or SNr, respectively. Since these antagonists are the most selective and well characterized NR2A and NR2B antagonists presently available, these data are suggestive of a sort of "functional segregation" of NR2A and NR2B responses along the striato-pallidal and striato-nigral neurons, respectively.

In the present follow-up study, triple probe microdialysis was used to investigate whether NR2A and NR2B modulation of striatal output pathway is changed under parkinsonian conditions. Three microdialysis probes were implanted in dorsolateral striatum (DLS), ipsilateral GP and SNr. NVP-AAM077 and Ro 25-6981 were perfused in DLS, and GABA and GLU release simultaneously monitored in these areas. We next used a battery of previously validated behavioural tests (Marti et al., 2005, 2007) to examine whether NR2 subunit selective antagonists (systemically injected) had different abilities to attenuate parkinsonian-like motor deficits. Finally, microdialysis was used to investigate whether motor changes were associated with different neurochemical responses.

Materials and Methods

Male Sprague-Dawley rats (150 g; Harlan Italy, S. Pietro al Natisone, Italy) were kept under controlled lighting conditions (12 hr light/dark cycle) and given food and water *ad libitum*. The experimental protocols were approved by the Ethical Committee of the University of Ferrara and were performed according to the Italian Guidelines for Animal Care (D.L. 116/92) and the European Communities Council Directives (86/609/ECC). Adequate measures were taken to minimize animal pain and discomfort and to limit the number of animals used.

6-OHDA lesion

Unilateral lesion of DA neurons was induced in isoflurane-anesthetized rats as previously described (Marti et al., 2007). Eight micrograms of 6-OHDA (in 4 µl of saline containing 0.02% ascorbic acid) were stereotaxically injected according to the following coordinates from bregma: anteroposterior (AP) -4.4 mm; mediolateral (ML) -1.2 mm; ventrodorsal (VD) -7.8 mm below dura (Paxinos and Watson, 1982). The rotational model (Ungerstedt and Arbuthnott, 1970) was used to select the rats that had been successfully lesioned. Two weeks after surgery, rats were injected with amphetamine (5 mg/kg, i.p., dissolved in saline) and only those rats performing more than seven ipsilateral turns per minute were enrolled in the study. This behavior has been associated with >95 % loss of striatal DA terminals (Marti et al., 2007) and extracellular DA levels (Marti et al., 2002). Experiments were performed approximately 2 months after lesion.

Microdialysis experiments

Triple probe microdialysis was performed as previously described (Fantin et al., 2007). Three probes of concentric design were stereotaxically implanted under isoflurane anaesthesia in the right DLS (3 mm dialysing membrane, AN69, Hospal, Bologna, Italy), ipsilateral SNr (1 mm) and GP (1.5 mm) according to the following coordinates from bregma and the dural surface (Paxinos and Watson, 1982): DLS; AP +1.0, ML -3.5, VD –6; GP, AP –1.3, ML -3.3, VD –6.5; SNr, AP -5.5, ML -2.2, VD –8.3. Forty-eight 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) at a 3 µl/min flow rate.

After 6 h rinsing, samples were collected every 10 min. At least three baseline samples were collected before drug perfusion through the probe. NVP-AAM077 and Ro 25-6198 were perfused (3-300 nM) for 120 min through the probe implanted in DLS to unravel tonic influence of NR2A and NR2B subunits over striatal output pathways. Drug concentrations were chosen on the basis of affinity and selectivity values and were the same of our previous microdialysis study in naïve rats (see Fantin et al., 2007, for a discussion). In separate experiments, to investigate circuitry underlying motor effects, NVP-AAM077 and Ro 25-6198 were given systemically (i.p.) at behaviourally relevant doses. These doses were selected on the basis of previous behavioural analysis. In these experiments, sample collection was performed every 15 min for 90 min after drug administration. At the end of the experiments, animals were sacrificed and the correct placement of the probes was verified histologically.

Aminoacid analysis

Endogenous GLU and GABA levels were measured by high-performance liquid chromatography (HPLC) coupled to fluorimetric detection according to Marti et al. (2007). Briefly, 30 µl sample were pipetted into glass microvials and placed in a thermostated (4°C) Triathlon autosampler (Spark Holland, Emmen, The Netherlands). Forty microliters of o-phthaldialdehyde/boric acid solution were added to each sample, and 60 µl of the solution injected onto an analytical column (3 mm inner diameter, 10 cm length; Chrompack, Middelburg, The Netherlands). The column was eluted at a flow rate of 0.48 ml/min with a mobile phase containing 0.1 M sodium acetate, 10 % ethanol and 2.5 % tetrahydrofuran (pH 6.5). GLU and GABA retention time were about 3.5 and 17.5 min, and the sensitivity of the method was 150 fmol/sample.

Behavioral analysis

Motor activity in rats was evaluated by means of three behavioural tests specific for different motor abilities, as previously described (Marti et al., 2005, 2007): the bar test (Sanberg et al., 1988), useful to evaluate akinesia and motor asymmetry under static conditions; the drag test (Wessell et al., 2004) which measures akinesia, bradykinesia, and asymmetry under dynamic conditions (backward

dragging); and the fixed-speed rotarod test, which measures rat ability to run on a rotating cylinder and is an indicator of coordination, gait, balance, muscle tone, and motivation to run (Rozas et al., 1997). The three tests were repeated in a fixed sequence (bar, drag and rotarod) before and after drug injection (starting at 20 and at 80 min after treatment). Rats were trained for approximately 10 days to the specific motor tasks until their motor performance became reproducible.

Bar test. Each rat was placed gently on a table, and the contralateral and ipsilateral forepaws were placed alternatively on blocks of increasing heights (3, 6, and 9 cm). Total time spent by each paw on the blocks was recorded (cut-off time, 20 s).

Drag test. Each rat was gently lifted from the tail (allowing forepaws on the table) and dragged backwards at a constant speed (~20 cm/s) for a fixed distance (100 cm). The number of steps made by each paw was counted alternatively by two separate observers.

Rotarod test. Briefly, rats were tested in a control session at four increasing speeds (10, 15, 20, 25 rpm, 180 s each), causing a progressive decrement of performance to ~40% of the maximal response (i.e., the experimental cut-off time). Such a protocol was set to detect both facilitatory and inhibitory drug effects.

Data presentation and statistical analysis

Data (mean ± SEM) from microdialysis experiments are reported as percentage of basal value (calculated as the mean of two samples before treatment). Mean neurotransmitter levels in the dialysate (in nM) have been reported in text and Figure legends. Motor performance has been expressed as time on bar or rod (in seconds; bar and rotarod test) and number of steps (drag test). Statistical analysis was performed by one-way repeated measure (RM) ANOVA followed by contrast analysis and the sequentially rejective Bonferroni *post hoc* test for multiple comparisons. P values <0.05 were considered to be statistically significant.

Materials

Ro 25-6981 was purchased from Sigma Chemical Company (St. Louis, MO, USA) while NVP-AAM077 was provided by Novartis Institutes for BioMedical Research (Basel, Switzerland). Drugs

were dissolved just before use in Ringer solution for the intrastriatal perfusion and isosmotic saline solution for intraperitoneal (i.p.) injection. Ro 25-6981 required heating to dissolve whereas NVP-AAM077 was dissolved in small quantity of vehicle containing NaOH 0.1 mol/L and final pH adjusted with HCl 1mol/L.

Results

Primary effects of subunit selective NMDA antagonists perfused in the DA-depleted striatum

To investigate whether striatal NR2A and NR2B subunit containing NMDA receptors mediate tonic regulation of striato-pallidal and striato-nigral neurons under parkinsonian conditions, reverse dialysis of NR2A and NR2B subunit selective antagonists was performed in the DA-depleted DLS of 6-OHDA hemilesioned rats, and GABA and GLU release monitored in DLS and its projection areas, namely the ipsilateral GP and SNr.

DLS. Basal GABA and GLU levels in DLS were 10.3 ± 0.8 and 277.0 ± 16.3 nmol/L, respectively. Intrastriatal perfusion with the NR2A preferential antagonists NVP-AAM077 (Fig. 1A-B) or the NR2B selective antagonists Ro 25-6981 (Fig 1C-D) did not change local aminoacid levels. Although Ro 25-6981 elevated GLU levels in the first sample after perfusion onset this increase did not reach the level of significance.

GP. Basal GABA and GLU levels in GP were 10.2 ± 0.6 and 226.0 ± 17.3 nmol/L, respectively. RM ANOVA on the effect of NVP-AAM077 on GABA levels (Fig. 2A) showed a significant effect of treatment ($F_{3,15}$=8.88, p=0.0013), time ($F_{13,39}$=7.58, p<0.0001) and a significant time x treatment interaction ($F_{39,232}$=1.65, p=0.0136). Contrast analysis revealed that both NVP-AAM077 30 and 300 nmol/L reduced GABA release (maximal inhibition ~40%) compared to control group (p=0.0316 and p=0.0007, respectively). Conversely, intrastriatal Ro 25-6981 did not change either GABA (Fig. 2C) or GLU (Fig. 2D) release in GP.

SNr. Basal GABA and GLU levels in SNr were 8.0 ± 0.5 and 256.0 ± 19.0 nmol/L, respectively. Intrastriatal perfusion with NVP-AAM077 (Fig 3A-B) or Ro 25-6981 (Fig 3C-D) did not change nigral GABA and GLU outflow.

Systemic administration of subunit selective NMDA antagonists improved motor performance in hemiparkinsonian rats

To evaluate whether the different neurochemical responses to NR2A and NR2B subunit selective antagonists were predictive of a different capabilities to attenuate the 6-OHDA induced motor deficit, we employed a battery of behavioural tests specific for different motor abilities (Marti et al., 2005, 2007).

Motor effects of NVP-AAM077. The NR2A preferential antagonist (0.001- 1 mg/Kg) did not reduce overall akinesia in bar test (Fig. 4A) neither attenuated motor asymmetry in the drag test (Fig. 4B). However, NVP-AAM077 affected rotarod performance depending on the dose injected (Fig. 4C). RM ANOVA revealed a significant effect of treatment ($F_{4,6}$=13.01), time ($F_{1,4}$=5.50, p=0.026) and a significant time x treatment interaction ($F_{4,30}$=3.13, p=0.029). *Post hoc* analysis at 20 min after injection revealed that NVP-AAM077 stimulated motor performance at 0.001 and 0.01 mg/Kg (p=0.0125 and p<0.0001, respectively) and inhibited it at 1 mg/Kg (p=0.010). No effect was detected 80 min after injection.

Motor effects of Ro 25-6981. The NR2B selective antagonist (0.1-3 mg/Kg) did not reduce overall akinesia in bar test (Fig. 5A) neither attenuated motor asymmetry in the drag test (Fig. 5B). Conversely, it dose-dependently increased rotarod performance (Fig. 5C). RM ANOVA revealed a significant effect of treatment ($F_{3,6}$=25.45, p<0.0001) and time ($F_{1,3}$=5.43, p=0.030), but not a significant time x treatment interaction ($F_{3,20}$=1.19, p=0.3391). *Post hoc* analysis at 20 min after injection revealed that Ro 25-6981 stimulated motor performance at 1 and 3 mg/Kg (p=0.0167 and p<0.0001, respectively), the lower dose (0.1 mg/Kg) being ineffective. The stimulation produced by 3 mg/Kg Ro 25-6981 was still evident 80 min after injection (p=0.001).

Subunit selective NMDA antagonists affected GABA levels selectively in GP.

To investigate the circuitry underlying the motor responses to subunit selective NMDA receptor antagonists, GABA and GLU release was monitored simultaneously in DLS, GP and SNr (ipsilateral to the lesion side) following i.p. administration of behaviourally relevant doses of NVP AAM077 or Ro 25-6981.

DLS. RM ANOVA revealed that NVP AAM077 (0.01 and 1 mg/Kg) and Ro 25-6981 (1 mg/Kg) did not alter GABA (Fig. 6A) and GLU levels (Fig. 6B) in DLS.

GP. RM ANOVA on GABA levels (Fig.7A) showed a significant effect of treatment ($F_{3,18}$=23.83, p<0.0001), time ($F_{7,21}$=3.29, p=0.0028) and a significant time x treatment interaction ($F_{21,152}$=3.88, p<0.0001). Contrast analysis showed that doses improving motor performance (NVP-AAM077 0.01 mg/Kg and Ro 25-6981 1 mg/Kg) decreased pallidal GABA levels (~40 %) while doses inhibiting motor performance (NVP AAM077 1 mg/Kg) elevated them (~45%). GLU levels were not affected by drug administration (Fig. 7B).

SNr. Contrary to that observed in GP, RM ANOVA showed that NVP AAM077 and Ro 25-6981 did not modify GABA (Fig. 8A) and GLU levels (Fig. 8B) in SNr.

Discussion

Main finding of the present study is that reverse dialysis of NVP-AAM077 in the DA-depleted striatum of hemiparkinsonian rats reduced GABA levels in GP but not SNr while reverse dialysis of Ro 25-6981 failed to change GABA release in these areas. This suggests that, under parkinsonian conditions, NR2A subunits facilitate striato-pallidal neurons while NR2B subunits do not participate in modulation of striatal output pathways. Systemic administration of low doses of NVP-AAM077 or Ro 25-6981 produced mild improvement of motor performance and reduced pallidal GABA release. These data are consistent with the view that motor improvement induced by NVP-AAM077 is accomplished by blockade of NR2A subunits on striato-pallidal neurons. Modulation of pallidal activity may also underlie motor improvement induced by Ro 25-6981, although, in this case, blockade of extrastriatal NR2B subunits may be involved.

We previously showed (Fantin et al., 2007) that reverse dialysis of NVP-AAM077 and Ro 25-6981 in the striatum of naïve rats reduced spontaneous and NMDA-stimulated GABA release in GP and SNr, respectively. On this basis we proposed the existence of a preferential control of NR2A subunits over striato-pallidal neurons and NR2B subunits over striato-nigral neurons. This view was not contradicted by the finding that NVP-AAM077 and Ro 25-6981 produced specular increases in GLU levels in SNr and GP, respectively (Fantin et al., 2007), since these changes were likely dependent on the activation of polysynaptic pathways. These neurochemical patterns appeared to be dramatically changed following DA denervation. The most striking change was the loss of Ro 25-6981 inhibition of nigral GABA release. Moreover, by comparing threshold doses, we found NVP-AAM077 to be more potent in inhibiting pallidal GABA release in hemiparkinsonian (30 nmol/L) than naïve (300 nmol/L) rats. Finally, both antagonists were unable to evoke GLU release in GP or SNr in the absence of endogenous DA. Several biochemical studies have demonstrated dysregulation of NMDA receptor subunit turnover in the DA-depleted striatum of 6-OHDA hemilesioned rats, such as increased expression of NR2A, but not NR2B, subunits (Ulas and Cotman, 1996; Ganguly and Keefe, 2001) and subcellular redistribution of NMDA subunits,

leading to reduced abundance of NR2B and relative enrichment of NR2A subunits in postsynaptic densities (Dunah et al., 2000; Fiorentini et al., 2006; Gardoni et al., 2006). This latter finding has been confirmed in MPTP-treated macaques (Hallett et al., 2005), overall suggesting that DA depletion causes an imbalance towards NR2A-mediated transmission in the striatum. The present study provides for the first time functional data in support of this view. Indeed, hypersensitivity of the pallidal GABA response to NVP-AAM077 is consistent with an up-regulation of NR2A transmission on striato-pallidal neurons while loss of the nigral GABA response to Ro 25-6981 is consistent with down-regulation of NR2B transmission on striato-nigral neurons. Parkinsonism is accompanied by a functional imbalance between the striato-pallidal "indirect" pathway, which becomes hyperactive due to the loss of a D2 receptor mediated inhibition, and the striato-nigral "direct" pathway which becomes hypoactive due to the loss of a D1 receptor mediated facilitation (Albin et al., 1989; Alexander and Crutcher, 1990). Thus, due to the excitatory nature and voltage-dependence of the NMDA receptor, hypersensitivity of NR2A and loss of NR2B response may be either the cause or the consequence of those global adaptive changes. In this respect, the present data endorse the view that up-regulation of striatal NR2A transmission is pathogenic. Indeed, motor improvement induced by NVP-AAM077 in rats was consistent with the increase in global motor scores observed in parkinsonian primates treated with MDL 100,453 (which is relatively selective for the NR2A subunit; Blanchet et al., 1999), suggesting the NR2A subunits contribute to motor impairment in parkinsonian animals. Moreover, NVP-AAM077 either perfused intrastriatally or given systemically at motor facilitating doses, reduced pallidal GABA levels. Thus, similar to A_{2A} antagonists (Ochi et al., 2000), NVP-AAM077 may attenuate parkinsonism by blocking striatal NR2A subunits and reducing the activity of the striato-pallidal pathway. We also observed that the overall antiparkinsonian effect of NVP-AAM077 in rats, as emerged from the complete behavioural analysis, was mild and limited only to some motor parameters. Indeed, NVP-AAM077 improved rotarod performance (which is a gross measure of coordination and gait ability) but did not ameliorate akinesia/bradykinesia or motor asymmetry in the bar and drag tests. Conversely, the

reference antiparkinsonian drug (L-DOPA) produced consistent motor improvements in all three tests (Marti et al., 2005; 2007). This suggests that NR2A subunit are not involved in movement initiation and execution. An important aspect of NVP-AAM077 behavioural profile was its dual nature, low doses causing motor improvement and higher ones motor impairment. We cannot rule out the possibility that motor inhibition reflects an action beyond NR2A subunits. Indeed, NVP-AAM077 is only ~2-fold selective for NR2A over NR2C subunits, respectively (Feng et al., 2004), and blockade of NR2C subunits in cerebellum causes ataxia. Ataxia was observed at higher doses in naïve rats (20 mg/Kg, i.p.; Y.P. Auberson, personal communication). In our test, however, 1 mg/Kg NVP-AAM077 did not overall inhibit motor behaviour, e.g. did not increase immobility time or reduced number of steps, as expected if ataxia was induced. Moreover, motor inhibition was associated with limited changes in neurotransmitter release, namely increased pallidal GABA levels. This favours the view that motor inhibition was due to striatal NR2A subunit blockade and activation of the striato-pallidal pathway. Indeed, cataleptogenic doses of haloperidol also elevated pallidal GABA levels (Chapman and See, 1996) likely via blockade, among others, of striatal D2 receptors (Sanberg et al., 1988). Moreover, parkinsonian akinesia was associated with increased GABA levels in GP (Robertson et al., 1991; Ochi et al., 2000). Finally, to further confirm the view that sustained GABA inhibition within GP induced hypolocomotion, GABA receptor agonists injected in GP induced catalepsy (Pycock et al., 1976; Vrijmoed-de Vries et al., 1987) while GABA receptors antagonists reversed parkinsonian symptoms (Maneuf et al, 1994). How high doses of NVP-AAM077 activate the indirect pathway remains matter of a conjecture. A possible explanation is that NVP-AAM077 depressed the activity of striatal GABAergic interneurons (which express NR2A, NR2B, NR2D but not NR2C receptors; Standaert et al., 1999), leading to disinhibition of striato-pallidal projection neurons.

In contrast with naïve rats (Fantin et al., 2007), intrastriatal (and systemic) NVP-AAM077 reduced pallidal GABA but did not change nigral GLU release. This indicates that the rise in nigral GLU levels induced by NVP-AAM077 is mediated by (or requires) endogenous DA. DA could act in

SNr to modulate GLU release from nerve terminals (Morari et al., 1998) or in GP to modulate the activity of pallido-nigral or pallido-subthalamic neurons (Hauber and Lutz, 1999). DA denervation also prevented the rise in pallidal GLU levels induced by intrastriatal Ro 25-6981 (Fantin et al., 2007). This phenomenon may be related to the loss of the ability of Ro 25-6981 to inhibit the striato-nigral pathway or, alternatively, to the loss of a D1 facilitating input on pallidal GLU afferents (Hernandez et al., 2007). In this context, the finding that, opposite of subunit selective antagonists, intrastriatal dizocilpine still maintained its ability to evoke nigral GLU release in hemiparkinsonian animals (Marti et al., 1999) may rely on its different affinity profile for NR2 subunits (Laurie and Seeburg, 1994; Monaghan and Larsen, 1997). Indeed, dizocilpine binds to NR2A, NR2B and, with lower affinity, NR2C and NR2D subunits. NR2C (although expressed at very low levels in the striatum) and NR2D subunits may also play a role in modulation of striatal output pathways.

NR2B selective antagonists have already been proven effective in alleviating experimental parkinsonism in rats (Nash et al., 1999; Steece-Collier et al., 2000; Wessel et al., 2004) and nonhuman primates (Mitchell et al., 1995; Steece-Collier et al., 2000; Nash et al., 2000, 2004). Consistently, Ro 25-6981 elicited contralateral turning in 6-OHDA hemilesioned rats (from the dose of 3.13 mg/Kg) and improved motor scores in MPTP-treated marmoset (Löschmann et al., 2004). At variance with that study, however, we did not observe turning at 3 mg/Kg. This discrepancy may be related to the different time after lesion at which experiments were performed in our (~2 months) compared to Löschmann's (9-12 months) study. The overall antiparkinsonian effect of Ro 25-6981 was similar to that of NVP-AAM077 (i.e. no effect in the bar and drag tests and an improvement in the rotarod performance), except that biphasicity was not observed (in the range of doses tested). The lack of acute effect in the drag test was also reported for the NR2B antagonist CP 101,606 (Wessell et al., 2004), confirming that NR2B antagonists given acutely do not affect movement initiation or execution. The finding that blockade of either the NR2A or NR2B subunit produced similar attenuation of parkinsonism is consistent with a previous study in primates

(Blanchet et al., 1999). Similar to NVP-AAM077, motor facilitating doses of Ro 25-6981 reduced pallidal GABA release indicating that motor facilitation was accomplished through inhibition of the indirect pathway. However, at variance with NVP-AAM077, intrastriatal Ro 25-6981 failed to modulate pallidal GABA levels, suggesting that extrastriatal NR2B subunits are involved. The possibility of a local effect of Ro 25-6981 in GP is plausible, since Ro 25-6981 binding sites have been found in this area (Mutel et al., 1998). In this case, we should speculate that a blockade of presynaptic facilitatory NR2B subunits leads to decreased GABA release from striato-pallidal afferents.

Conclusions

Triple probe microdialysis revealed that DA depletion evoked plastic changes in the tonic control operated by endogenous GLU via NMDA receptors over striatal output pathways: up-regulation of the NR2A facilitation of striato-pallidal neurons and down-regulation of the NR2B facilitation of striato-nigral neurons. Despite opposite adaptive changes in NR2A and NR2B control of striatal output pathways, systemic NR2A or NR2B subunit antagonists similarly facilitated motor activity, this action being associated with reductions in pallidal GABA levels. While the effect of NVP-AAM077 is consistent with blockade of striatal NR2A subunits and inhibition of striato-pallidal neurons, that of Ro 25-6981 likely takes place outside the striatum. Overall, the present study confirms the view of a "functional segregation" of NR2A and NR2B responses along the indirect and direct striatofugal pathways, respectively, and suggests the pathogenic role of striatal NR2A and extrastriatal NR2B subunits in sustaining parkinsonian-like motor impairment.

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Figure legends

Fig. 1. Effect of subunit selective NMDA receptor antagonists on striatal GABA and GLU release in hemiparkinsonian rats. Effect of reverse dialysis of NVP-AAM077 (30 and 300 nmol/L; panels A-B) and Ro 25-6981 (30 and 300 nmol/L; panels C-D) in the DA-depleted dorsolateral striatum (DLS) on local GABA and GLU extracellular levels. Data are expressed as percentages ± SEM of basal pre-treatment levels (calculated as mean of the two samples before the treatment). Basal GABA and GLU levels (nmol/L) were 10.3 ± 0.8 (n= 21) and 277.0 ± 16.3 (n=22), respectively.

Fig 2. Effect of subunit selective NMDA receptor antagonists on pallidal GABA and GLU release in hemiparkinsonian rats. Effect of reverse dialysis of NVP-AAM077 (3, 30 and 300 nmol/L; panels A-B) and Ro 25-6981 (3, 30 and 300 nmol/L; panels C-D) in the DA-depleted dorsolateral striatum (DLS) on GABA and GLU extracellular levels in ipsilateral globus pallidus (GP). Data are expressed as percentages ± SEM of basal pre-treatment levels (calculated as mean of the two samples before the treatment). Basal GABA and GLU levels (nmol/L) were 10.2 ± 0.6 (n=34) and 226.3 ± 17.3 (n=33), respectively.

*$p<0.05$ different from washout (RM ANOVA followed by contrast analysis and the sequentially rejective Bonferroni's test).

Fig 3. Effect of subunit selective NMDA receptor antagonists on nigral GABA and GLU release in hemiparkinsonian rats. Effect of reverse dialysis of NVP-AAM077 (3, 30 and 300 nmol/L; panels A-B) and Ro 25-6981 (3, 30 and 300 nmol/L; panels C-D) in the DA-depleted dorsolateral striatum (DLS) on GABA and GLU extracellular levels in ipsilateral substantia nigra reticulata (SNr). Data are expressed as percentages ± SEM of basal pre-treatment levels (calculated as mean of the two samples before the treatment). Basal GABA and GLU levels (nmol/L) were 8.0 ± 0.5 (n=35) and 256.4 ± 19.0 (n=38), respectively.

Fig. 4. Motor effects of NVP-AAM077 in hemiparkinsonian rats. Systemic (i.p.) administration of NVP-AAM077 (0.001-1 mg/kg) did not affect the time spent on the blocks in the bar test (***A***) and the number of steps in the drag test (***B***) but affected motor performance on the rotarod (***C***). Motor tests were performed 20 and 80 min after drug injection. Motor activity was evaluated separately at the ipsilateral and contralateral (parkinsonian) forepaws. Data are mean ± SEM of 7 rats per group. *p<0.05 different from saline (RM ANOVA followed by contrast analysis and the sequentially rejective Bonferroni test).

Fig. 5. Motor effects of Ro 25-6981 in hemiparkinsonian rats. Systemic (i.p.) administration of Ro 25-6981 (0.1-3 mg/kg) did not affect the time spent on the blocks in the bar test (***A***) and the number of steps in the drag test (***B***) but affected motor performance on the rotarod (***C***). Motor tests were performed 20 and 80 min after drug injection. Motor activity was evaluated separately at the ipsilateral and contralateral (parkinsonian) forepaws. Data are mean ± SEM of 7 rats per group. *p<0.05 different from saline (RM ANOVA followed by contrast analysis and the sequentially rejective Bonferroni test).

Fig 6. Effect of subunit selective NMDA receptor antagonists on striatal GABA and GLU release in hemiparkinsonian rats. Systemic (i.p.) administration (arrow) of NVP-AAM077 (0.01 and 1 mg/Kg) and Ro 25-6981 (1 mg/Kg) did not affect GABA (**A**) and GLU (**B**) extracellular levels in the DA-depleted dorsolateral striatum (DLS). Data are expressed as percentages ± SEM of basal pre-treatment levels (calculated as mean of the two samples before the treatment). Basal GABA and GLU levels (nmol/L) were 11.6 ± 1.1 and 223.6 ± 24 (n=26 each), respectively.

Fig 7. Effect of subunit selective NMDA receptor antagonists on pallidal GABA and GLU release in hemiparkinsonian rats. Systemic (i.p.) administration (arrow) of NVP-AAM077 (0.01 and 1 mg/Kg) and Ro 25-6981 (1 mg/Kg) affected GABA (**A**) but not GLU (**B**) extracellular levels in the

globus pallidus (GP) ipsilateral to the lesion side. Data are expressed as percentages ± SEM of basal pre-treatment levels (calculated as mean of the two samples before the treatment). Basal GABA and GLU levels (nmol/L) were 14.1 ± 1.6 and 246.0 ± 29.1 (n=26 each), respectively.

*p<0.05 different from saline (RM ANOVA followed by contrast analysis and the sequentially rejective Bonferroni test).

Fig 8. Effect of subunit selective NMDA receptor antagonists on nigral GABA and GLU release in hemiparkinsonian rats. Systemic (i.p.) administration (arrow) of NVP-AAM077 (0.01 and 1 mg/Kg) and Ro 25-6981 (1 mg/Kg) did not affect GABA (**A**) and GLU (**B**) extracellular levels in the substantia nigra reticulata (SNr) ipsilateral to the lesion side. Data are expressed as percentages ± SEM of basal pre-treatment levels (calculated as mean of the two samples before the treatment). Basal GABA and GLU levels (nmol/L) were 9.9 ± 0.8 and 212.0 ± 31 (n=26 each), respectively.

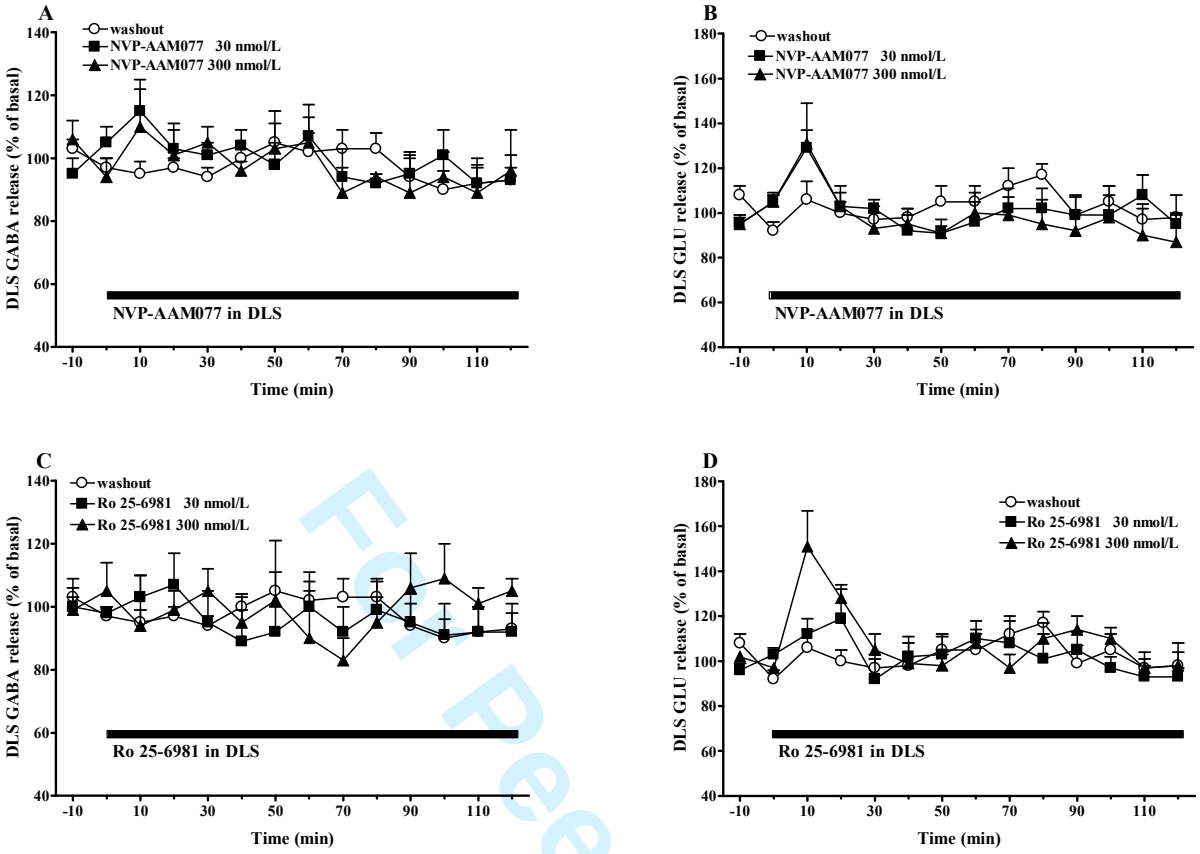
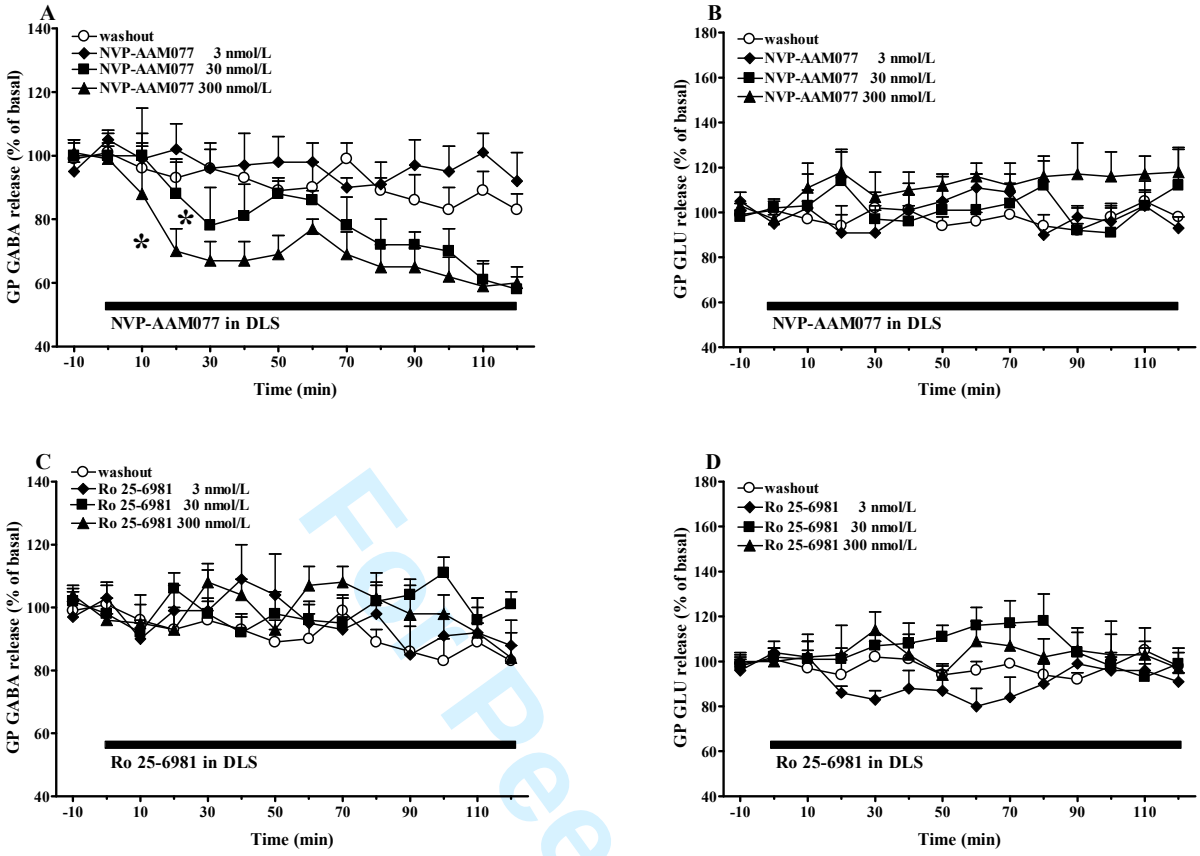


Fig.1 Fantin et al.



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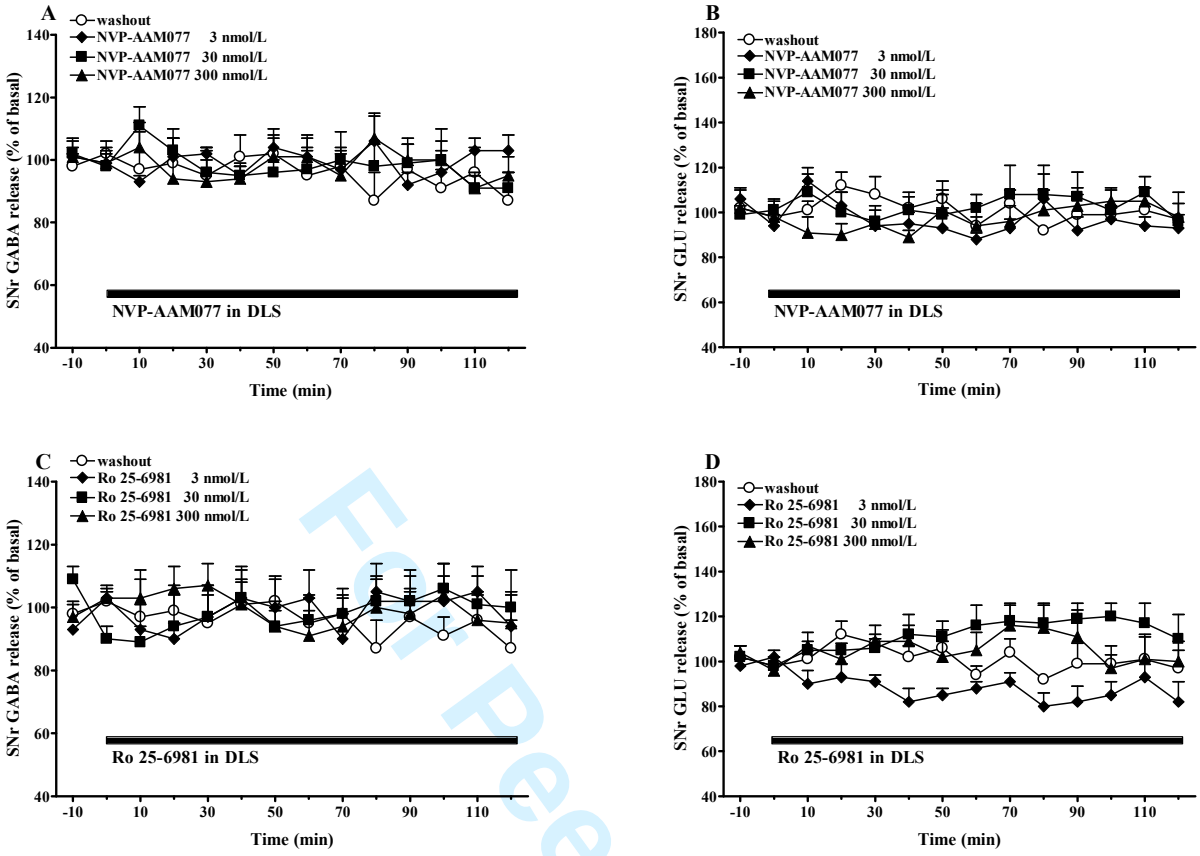

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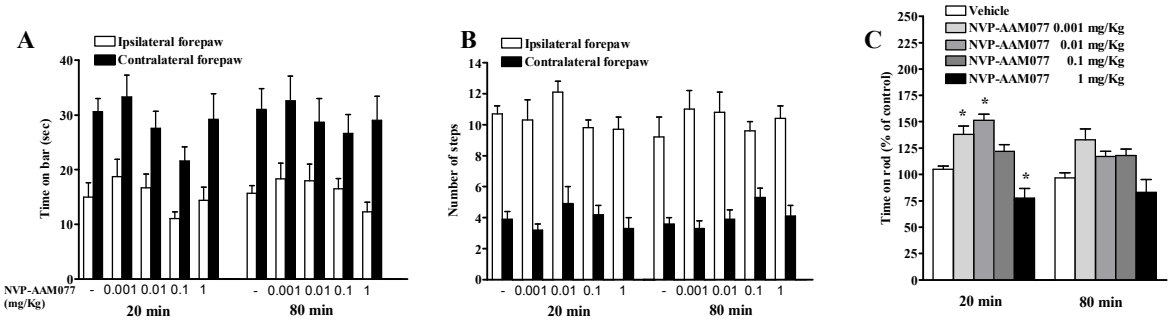

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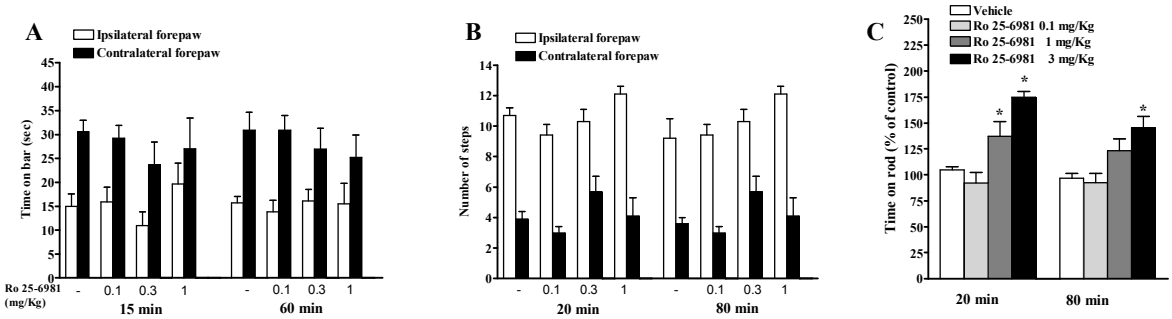

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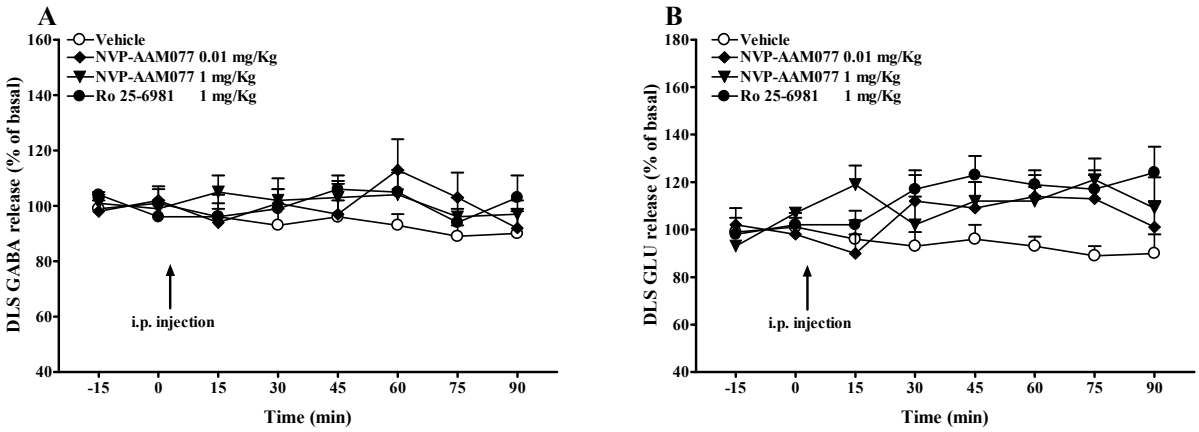

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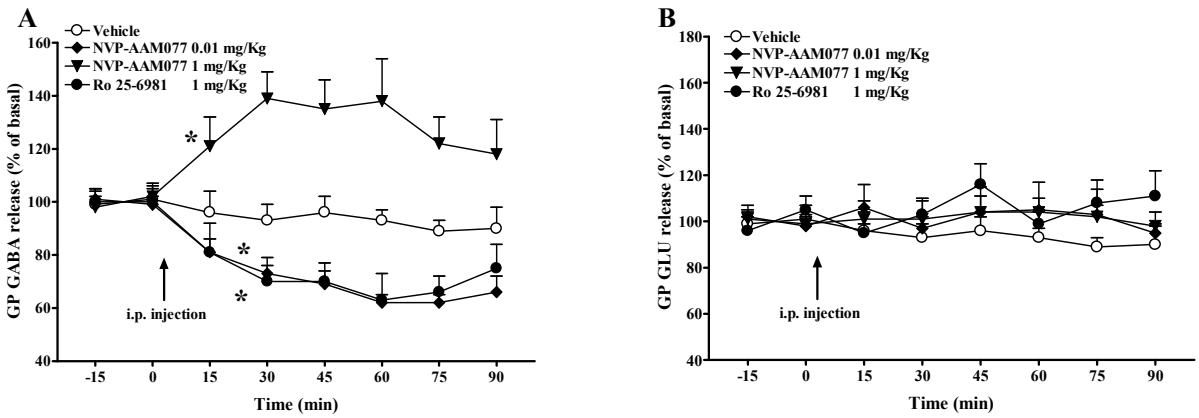

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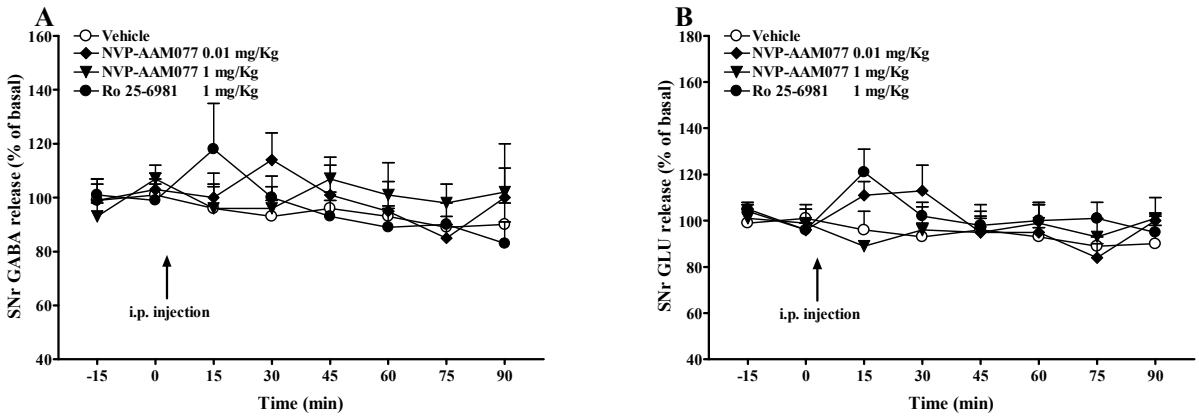

Fig. 8 Fantin et al.