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*The Role of Opioids on Motor Activity in Physio-pathological
Conditions*

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Abbreviations

6-OHDA	6-hydroxydopamine
ACh	acetylcholine
ANOVA	analysis of variance
BBB	blood brain barrier
CNS	central nervous system
CTOP	D-Pen-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH ₂
DA	dopamine
DAMGO	[D-Ala ² , N-MePhe ⁴ , Gly-ol]-enkephalin
DBS	deep-brain stimulation
DLS	dorsolateral striatum
DMSO	dimethyl sulfoxide
DOP	δ-opioid peptide
DPDPE	[D-Pen ² , D-Pen ⁵]-enkephalin
EM-1	endomorphin-1
EN	entopeduncular nucleus
ENK	enkephalins
GABA	γ-amino butyric acid
GLU	glutamate
GP	globus pallidus
GPe	globus pallidus external
GPI	globus pallidus internal
HPLC	high pressure liquid chromatography
i.c.v.	intracerebroventricular
i.p.	intraperitoneal
J-113397	1-[(3R,4R)-1-cyclooctylmethyl-3-hydroxymethyl-4-piperidyl]-3-ethyl-1,3-dihydro-2H-benzimidazol-2-one
KOP	κ-opioid peptide
L-DOPA	3,4-dihydroxyphenylalanine
MOP	μ-opioid peptide
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
N/OFQ	nociceptin/orphanin FQ
NOP	nociceptin/orphanin FQ opioid peptide
NTD	naltrindole
PKC	protein kinase C
PPE-A	preproenkephalin-A
PPE-B	preproenkephalin-B
RM	repeated measures
SN	substantia nigra
SNc	substantia nigra pars compacta
SNr	substantia nigra pars reticulata
SNC-80	(+)-4-[(αR)-α-(2 <i>S</i> ,5 <i>R</i>)-allyl-2,5-dimethyl-1-piperazinyl]-3-methoxy-benzyl]- <i>N-N</i> -diethylbenzamide
STN	subthalamic nucleus
TTX	tetrodotoxin
UFP-512	H-Dmt-Tic-NH-CH(CH ₂ -COOH)-Bid
VMTh	ventromedial thalamus
VTA	ventral tegmental area

Summary

To date, four principal classes of opioid peptide receptors, μ (MOP), κ (KOP), δ (DOP) and ORL-1 (NOP) have been identified and cloned. The experimental and clinical importance of this receptor family has been amply demonstrated in many diseases, though historically they have mainly been associated with antinociception (pain relief). The current work described herein discusses the impact of opioid systems on movement disorders, namely Parkinson's disease (PD). PD is a relatively common neurological disease in which the motor symptoms result from the death of a particular dopamine (DA) containing cell population in the substantia nigra pars compacta (SNc). This inevitably results in a dysregulation of the activity of basal ganglia (BG); a set of midbrain structures responsible for normal movement programming and execution. The BG structures highly express opioid receptors and their endogenous peptides and, throughout the course of PD, opioidergic transmission appears dysregulated. The view that opioids are involved in compensatory or pathogenic mechanisms following loss of DA neurons lends support to the notion that they may represent a suitable target for the treatment of PD.

The work contained herein describes the benefits of opioid receptor ligand administration under physiological or pathological conditions (e.g. in experimental models of PD). For instance, we discovered that the endogenous MOP receptor peptide endomorphin-1 (EM-1), when injected or perfused into the substantia nigra pars reticulata (SNr) of naïve rats facilitates and inhibits spontaneous locomotion depending on dose.

Additionally, we have tested DOP receptor agonists in an experimental model of PD (i.e. 6-hydroxydopamine hemilesioned rats). These studies demonstrated the ability of the selective non-peptide DOP receptor agonist SNC-80 to alleviate symptoms in experimental PD, such as akinesia and poor coordinated movement. We have also showed that when administered systemically, the novel peptide agonist UFP-512 causes both facilitation and inhibition of movement depending on the dose used. Lastly, we demonstrated a functional relationship between NOP and DOP receptors in SNr. We showed that in SNr, blockade of NOP receptors with the selective antagonist J-113397 and activation of DOP receptors with SNC-80 synergistically enhances motor performance in experimental PD. In addition to demonstrating the behavioral outcome of opioid receptor stimulation or blockade on a variety of movement-related parameters, we have correlated these behaviors with novel neurochemical findings in each of the presented studies. The overall findings

of the work presented shed additional light on the subject of opioidergic systems and movement disorders and may contribute to future therapeutics for diseases such as PD.

1. INTRODUCTION

1.1 Opioids and their receptors

The word *opium* is derived from the Greek name for 'juice'; the drug being obtained from the secretions of the poppy plant, *Papaver somniferum*. Cultivation of opium poppies for food, anaesthesia, and ritual purposes dates back to at least the Neolithic Age. The Sumerian, Assyrian, Egyptian, Greek, Roman, Persian and Arab Empires each made widespread use of opium, which was the most potent form of pain relief then available, allowing ancient surgeons to perform prolonged surgical procedures. Opium has been mentioned in the most important medical texts of the ancient world. By the middle of the 16th century, opium's uses were fairly well understood and in the 18th century opium smoking became popular while its overuse by some individuals became more evident.

Opium contains two main groups of alkaloids. Phenanthrenes, including morphine, codeine, and thebaine, are the main narcotic constituents of the plant. Isoquinolines such as papaverine on the other hand have no significant effect on the central nervous system (CNS). In 1805, the German pharmacist Friedrich Wilhem Adam Sertürner reported the isolation of a pure substance in opium that he named *morphium*, after Morpheus, the god of dreams. Incidentally, this was the first ever extraction of a pure medicinal substance from plant material. Morphine is by far the most prevalent and important alkaloid in opium, constituting 10-16% of the total alkaloid amount, and is mainly responsible for its effects such as pain relief, euphoria, addiction, lung edema and respiratory difficulties.

The existence of receptors for opiate drugs was proposed by Beckett and Casy in 1954 based on their studies on structure-activity relationships for antinociceptive (e.g. the ability to relieve pain) activity in a series of synthetic opiates (Beckett and Casy, 1954). These receptors were called 'opioids' since it was understood that their endogenous ligands were peptides with effects resembling those of opiate drugs. As early as 1965, based on structure-activity analysis studies, Portoghese proposed the existence of more than one opioid receptor type or the possibility of multiple modes of interaction between ligands and opioid receptors (Portoghese, 1965). The first clear evidence that opioid receptors were in fact a diverse population was presented by Martin and colleagues in 1976 (Martin et al., 1976). Extensive pharmacological studies since that time have led to the identification of four opioid receptor types based on their diverse pharmacological actions. The earliest proposed receptor types were named after drugs used during the identification process such as the μ (mu for morphine) and κ (kappa for

ketocyclazocine) receptors (Martin et al., 1976). Those findings clearly linked both analgesia and addictive properties with functions of the μ receptor. Pharmacological analysis of opioid peptide effects in guinea pig ileum and mouse vas deferens led to the proposal of a third opioid receptor named the δ (delta for vas deferens) receptor (Lord et al., 1977). Since their discovery, the nomenclature used to refer to these receptors has changed multiple times. According to the International Union of Basic and Clinical Pharmacology (IUPHAR), μ is currently referred to as mu opioid peptide (MOP) receptor, κ is kappa opioid peptide (KOP) receptor and δ is referred to as delta opioid peptide (DOP) receptor. The most recent addition to the opioid peptide family is the nociceptin/orphanin FQ peptide (NOP) receptor which has high sequence homology compared to classical opioid receptors, particularly the KOP receptor (Bunzow et al., 1994). The endogenous ligand of this receptor was first described by Meunier at the International Narcotics Research Conference in 1995 who subsequently reported the ability of this peptide, which he named *nociceptin*, to decrease forskolin-stimulated cAMP production, in vitro (Meunier et al., 1995). A peptide of identical sequence isolated in pig brain was reported simultaneously by Reinscheid and colleagues (Reinscheid et al., 1995), who named it orphanin F/Q (OFQ). For the purposes of this text, the nomenclature used to describe the peptide and its receptor will be *nociceptin/orphanin FQ (N/OFQ)* and the *nociceptin/orphanin FQ peptide (NOP) receptor*, respectively.

Opioid receptors are part of the G protein-coupled receptor superfamily and are composed of 7 hydrophobic transmembrane domains which couple to specific GTP binding proteins (G proteins). These G proteins are heterodimeric proteins consisting of three distinct subunits, α , β and γ . Opioid receptors are known to couple to G_i (Hawes et al., 2000). Through their intracellular actions, opioids are also known to inhibit the formation of cAMP, close voltage sensitive Ca^{2+} channels and enhance outward K^+ conductance (Hawes, et al., 2000). The sum of these actions and their ability to reduce neurotransmitter release and neuronal excitability make the opioids primarily *inhibitory* molecules. Since their discovery, opioid peptide systems have been implicated in variety of diseases and treatments ranging from pain and addiction to mood and movement disorders based on their locations and actions in the CNS.

1.2 Basal Ganglia

The basal ganglia (BG) are a group of subcortical brain structures which control the planning and execution of motor programs. The BG include the striatum, globus pallidus (GP), subthalamic nucleus (STN) and substantia nigra (SN). The dorsolateral part of the striatum (DLS) is positioned along motor circuits where it integrates inputs arriving from thalamus and somatomotor areas of the cortex. The DLS then, in turn, modulates the output structures of BG. In primates (human and non human), the GP is divided into external (GPe) and internal (GPi) segments. In rats, these structures are represented by GP and entopeduncular nucleus (EN), respectively. For the purposes of this text, GPe will be referred to as *GP*. The SN is also organized into two closely linked segments, the substantia nigra pars compacta (SNc) and the substantia nigra pars reticulata (SNr). The disruption of coherent information flow between these structures results in altered output and disturbed motor activity. The primary neurotransmitters of the BG are γ -amino butyric acid (GABA), glutamate (GLU) and dopamine (DA). GABA being the main inhibitory amino acid neurotransmitter in the CNS, GLU being the main excitatory one and DA, depending on which receptor type it activates, may cause inhibitory (D₂-like) or excitatory responses (D₁-like). Two primary pathways (direct and indirect) are described in the current simplified model of BG function (Albin et al., 1989, DeLong, 1990) and these two pathways oppositely modulate the activity of BG output structure (GPi/SNr). These output structures send inhibitory GABAergic projections to the thalamus in effect controlling inhibition and disinhibition of thalamo-cortical projections; the end result being the coordinated execution of motor programs. The striatum is the major input target of projections arriving to the BG. For instance, extensive GLUergic inputs arrive from the cortex as well as the thalamus (Smeal et al., 2008, for review see Smith et al., 2004) while DA arrives via SNc DAergic projections. About 95% of striatal neurons are GABAergic medium-sized spiny neurons (Chang et al., 1982). The remaining neurons found in the striatum are interneurons containing either GABA or acetylcholine (ACh), which act to modulate the activity of the striatofugal pathways (direct and indirect pathways; for review see Pisani et al., 2007).

1.21 Direct Pathway

The direct pathway originates from one population of medium-sized spiny striatal GABAergic neurons which project directly to the GPi/SNr. This cell population mainly express DA D₁ receptors where DA stimulation is responsible for facilitating their activity and causing the

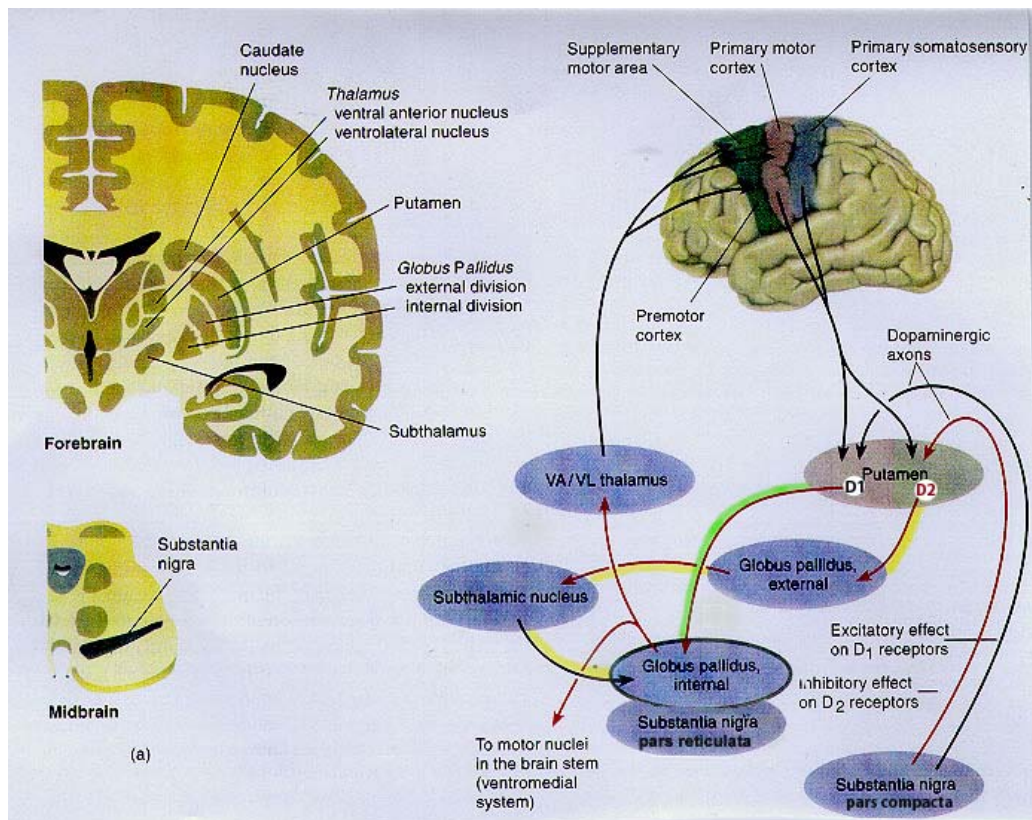
release of GABA and its co-transmitters, dynorphin and substance P, in SNr/GPi (for review see Albin et al., 1989). GPi/SNr then sends GABAergic projections to the ventromedial and ventrolateral thalamus (Kha et al., 2001) and excitatory (GLUergic) projections from the thalamus arrive in the cortex. The cortex excites the striatum which then inhibits the GPi/SNr through this pathway. The GPi/SNr is normally tonically active and inhibitory to the thalamus. When the GPi/SNr is inhibited, the thalamus is relieved from inhibition (disinhibition) and excites the cortex, thereby reinforcing the desired movement (Deniau and Chevalier, 1985).

1.22 Indirect Pathway

In the indirect pathway, a separate group of medium-sized spiny striatal GABAergic neurons project to GP. These striato-pallidal projections use ENK as a co-transmitter. DA D₂ receptors are mainly expressed on this population and DA stimulation is responsible for suppressing their activity (Albin et al., 1989). GP then sends GABAergic projections to STN, while the STN projects to GPi/SNr using the excitatory neurotransmitter, GLU. The GPi/SNr projects to thalamus using GABA and the thalamus projects back to the cortex also using GLU. Therefore, the cortex excites the striatum which then inhibits GP. Since GP is inhibitory to the STN, the STN then becomes more active and excites the GPi/SNr. The GPi/SNr being more active then inhibits the thalamus thereby dampening excitation to cortex. In this way, activation of the indirect pathway through striatum causes a relative inhibition of movement.

1.23 Summation of pathways

The opponent parallel pathway hypothesis emphasizes two major paths of information flow from the striatum to the basal ganglia output nuclei: GPi/SNr (in primates) or EN/SNr (in rodents). One is an inhibitory direct pathway from the striatum to the GPi/SNr. The other is an effectively excitatory multisynaptic pathway from the striatum to GPi/SNr. This hypothesis suggests that the two pathways are in balance such that increased activity in the direct pathway causes decreased GPi/SNr output, and increased activity in the indirect pathway causes increased GPi/SNr output. These two pathways converge on the same cells in the GPi/SNr which ultimately allows for the precise control over BG output needed under normal conditions. By adjusting the balance, the cortical targets of the basal ganglia can be facilitated or inhibited. The hypothesis predicts that abnormally decreased output results in excessive movement (chorea) and abnormally increased output results in decreased movement (Parkinson's disease).



(adapted from Carlson 1998, pg 244)

Fig. 1 The anatomical organization of the basal ganglia in the human brain (left). Shows BG circuitry in the human brain where the red lines indicate inhibitory input and the blue lines indicate excitatory input (right). The yellow accompanying lines refer to the indirect pathway (striato-pallidal) and the green one refers to the direct pathway (striato-nigral).

1.3 Opioids in the Basal Ganglia

Opioid ligands regulate the release of various neurotransmitters in the CNS. The predominant inhibitory effects of opioids on neurotransmitter release was discovered in the 1950's by studies demonstrating that opiates inhibit ACh release from electrically stimulated guinea pig ileum (Paton, 1957, Schaumann, 1957), followed by brain in vivo (Beleslin and Polak, 1965) and then brain slices (Sharkawi and Schulman, 1969). Since that time, opioids have also been shown to inhibit the release of other neurotransmitters such as noradrenaline (Montel et al., 1974), DA (Loh et al., 1976) and GABA (Kondo and Iwatsubo, 1978). The BG have one of the highest levels of endogenous opioids and opioid receptors in the brain (Peckys and Landwehrmeyer, 1999). All opioids have been shown to regulate DA functions in different ways and their net effect on transmitter stimulation or inhibition depends on the anatomical localization of their

receptors. An additional layer of complexity to their modulatory effects is based on the fact that opioid receptors may be located presynaptically, which is the typical scenario, or postsynaptically. In the BG, opioid ligands are found in cells containing other classical neurotransmitters such as GABA where they act to modulate transmitter release through their inhibitory actions. Striatal GABAergic neurons comprising the indirect pathway (projecting to GP) express preproenkephalin-A (PPE-A) whereas striatal neurons of the direct pathway (projecting to SNr) express preproenkephalin-B (PPE-B). Each PPE-A molecule gives rise to 6 copies of methionine-enkephalin (met-ENK) and one copy of leucine-enkephalin (leu-ENK). These ENK are considered the endogenous ligands for the DOP receptor. For the purposes of this text, both leu-ENK and met-ENK will be referred to collectively as ENK. In the BG, ENK are almost exclusively found in striato-pallidal projections (indirect pathway) along with GABA. The direct pathway, on the other hand, is known to express and release substance P and dynorphin, the endogenous ligand of the KOP receptor.

Opioid Receptor Type	Localization
MOP	Striatum, GPe, GPi, STN, SNr and SNe
DOP	Striatum, GPe, GPi, STN and SNr
KOP	Striatum, GPi, SNr and SNe
NOP	GPe, STN, SNe and SNr

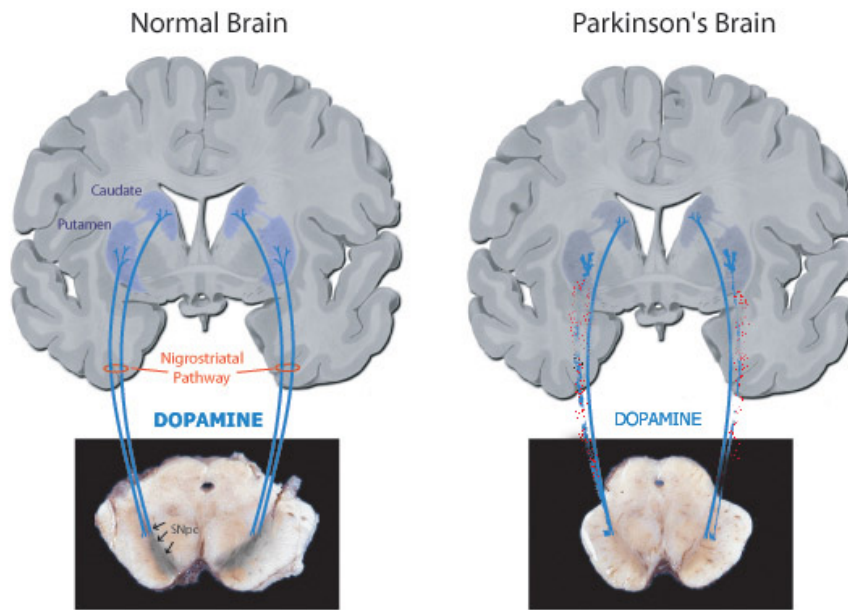
Fig 2. Localization of opioid receptors in BG based on evidence from binding and mRNA expression studies (Mansour et al., 1995, Neal et al., 1999).

1.4 Parkinson's Disease

In 1817, James Parkinson described in his monograph 'An essay on the shaking palsy' a severe movement disorder which he called *paralysis agitans*, later to be named Parkinson's disease (PD). PD is also called "primary parkinsonism" or "idiopathic PD" (classically meaning having no known cause). PD is a relatively common disease affecting 0.1% of the world's population with a prevalence of 1% of the population above 60 years old. Its prevalence increases exponentially from 1% to 5% of the population between 65 and 90 years of age. The cause of the disease is largely unknown and it appears to be without genetic linkage in 90-95% of cases. In those cases, it may be caused in part by the combination of genetic susceptibilities and environmental factors (Lim et al., 2002). While most forms of parkinsonism are idiopathic, secondary cases may result from toxicity most notably from drugs, head trauma, or other medical

disorders. Carlsson and colleagues (1957) experimentally showed that DA loss was a crucial factor underlying the pathological behaviors of PD by depleting catecholamines in mice using reserpine. Following this milestone discovery, DA deficiency was identified in parkinsonian patients (Hornykiewicz, 1966). It is now understood that PD is a progressive neurodegenerative disease which occurs from the loss of DA and non DA neurons in the brain. Nevertheless, PD motor symptoms are caused by the death of SNc DA neurons resulting in a severe reduction in DA at nerve terminals in the caudate nucleus and putamen (e.g. striatum level) as well as in other areas such as GP and STN. However, motor symptoms appear only when the level of degeneration exceeds the critical threshold of 70-80% of nerve terminals and around 50% of midbrain DA containing neurons. This disturbance in BG function results in the dysregulation of movement control leading to tremors, postural instability, rigidity and slowness to initiate (akinesia) and execute (bradykinesia) movements.

Typically, a patient is diagnosed based on medical history and neurological examination conducted by interviewing and observing the patient and applying the Unified Parkinson's Disease Rating Scale which assesses a patient's behaviors, mood, activity and motor behaviors. Early signs and symptoms of PD may sometimes be overlooked as the effects of normal aging. Due to symptom overlap with other diseases, only 75% of clinical diagnoses of PD are confirmed to be idiopathic PD at autopsy (Gelb et al., 1999). The physician may need to observe the person for some time until it is apparent that the symptoms are consistently present. Usually doctors look for shuffling of feet and lack of swing in the arms since these are hallmark symptoms of the disease.



(adapted from Brain-Mind Institute LEN)

Fig 3. A normal (left) and a DA denervated (right) nigro-striatal tract as seen in PD. Note the pigmentation due to a build up of melanin in SNc is markedly darker in the normal brain compared to the parkinsonian brain. The loss of DA neurons in SNc results in altered BG signaling and motor complications.

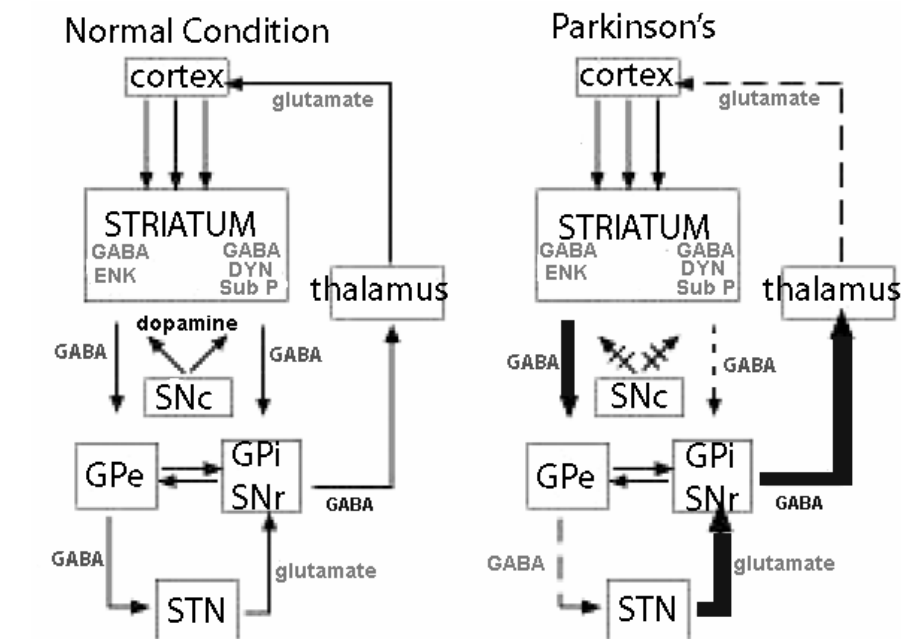


Fig 4. Normal BG inputs and outputs (left) and dyregulated BG inputs and outputs as a result of DA denervation in SNc (right). Lack of DA stimulation in striatal areas causes an increase in GABA release (via lack of inhibitory D₂ tone) to GPe and a decrease (via lack of excitatory D₁ tone) in striato-nigral GABA release. Ultimately the disinhibition of nigro-thalamic output is followed by reduced GLUergic thalamo-cortical drive.

1.5 Parkinson's Disease Treatment Options

Currently, there is no cure for PD, though there are several treatment options that can alleviate symptoms of the disease and improve quality of life of patients. The 'gold standard' symptomatic treatment is 3,4-dihydroxyphenylalanine (L-DOPA) which was discovered in the late 1960's (Cotzias et al., 1967). L-DOPA crosses the blood brain barrier (BBB) when administered orally then is converted to DA by the enzyme L-aromatic amino acid decarboxylase. In order to avoid peripheral decarboxylation, L-DOPA is combined with a decarboxylase inhibitor (Carbidopa or Benserazide) which cannot cross the BBB, limiting its effects to the brain. The net effect of L-DOPA is the replacement of the absent DA in the nigral projections. L-DOPA therapy revolutionized the treatment of PD, providing dramatic benefits to nearly every patient who received it. This treatment is still currently the most widely used and provides satisfactory relief of parkinsonian symptoms for 5-10 years. However, within 5 years, a series of complications begin to develop in more than 40% of PD patients who receive L-DOPA (Ahlskog and Muenter, 2001). These side effects are the "on-off" phenomenon and abnormal involuntary movements (known collectively as dyskinesias). The "on-off" phenomenon refers to the response to L-DOPA either being good (on) or poor (off) and the overall reduction in the duration L-DOPA efficacy. The prevalence and severity of dyskinesias increases with the duration and severity of the disease (Colosimo and De Michele, 1999, Grandas et al., 1999). After 10 years of L-DOPA treatment, dyskinesias have developed in 70-80% of patients and in nearly 100% of patients whose disease onset was below the age of 45-50 years old (Quinn et al., 1987). L-DOPA therapy can also cause non-motor complications such as hallucinations and psychosis (Nausieda et al., 1984, Kuzuhara, 2001) as well as loss of impulse control (Stamey and Jankovic, 2008).

In addition to L-DOPA, DA D₂/D₃ receptor agonists (typically ropinirole or pramipexole), which stimulate DA receptors directly without the need for metabolic conversion, are also regularly used to treat PD, either alone or in conjunction with L-DOPA therapy. These agonists are not as effective as L-DOPA in relieving PD motor symptoms, however, their addition to L-DOPA therapy can significantly reduce dyskinesias (Calne, 1993). Alternative treatments have been used with varying degrees of success such as GLU antagonists (Kornhuber et al., 1991), monoamine oxidase inhibitors (Birkmayer et al., 1975), catechol-O-methyltransferase inhibitors (Ruottinen and Rinne, 1998) and cholinergic antagonists (Duvoisin, 1967). Beside

pharmacological treatment, some surgical procedures have been performed on PD patients. For example ablative surgeries (i.e. pallidotomy or thalamotomy) or deep brain stimulation (DBS) of the thalamus, GPi or STN. The aim of pallidotomy and DBS is to reduce the excessive inhibitory output from the GPi and SNr (Hill et al., 2000). Taken together, it is clear that the currently available treatments for PD are lacking in some key areas such as therapeutic longevity and side effect profiles. For these reasons, investigators continue to search for alternative therapies with an emphasis on neuroprotective agents to slow or halt the progression of the disease.

1.6 Opioids and Parkinson's Disease

PD is classically associated with degeneration of nigro-striatal DA cells although the involvement of other factors such as opioid systems have received more attention in the past two decades in an attempt to elucidate the pathophysiology of PD and L-DOPA induced dyskinesias. Interestingly, opioid receptors and their endogenous ligands are present in high concentration in the BG (Gramsch et al., 1979, Emson et al., 1980, Zamir et al., 1984, Mansour et al., 1995) and alterations in endogenous opioids have been reported in postmortem brain from PD patients (Taquet et al., 1983, 1985, Sivam, 1991). Opioid receptor agonists and antagonists have also been shown to affect the behavior of parkinsonian patients (Trabucchi et al., 1982, Sandyk and Snider, 1986, Groppetti et al., 1990). Most of these investigations have emphasized striatal opioid pathways in the pathological process of PD although several more recent observations suggest the likely importance of opioids in other areas of BG such as SNr, SNc and STN (Shen and Johnson, 2002, Marti et al., 2004, Aubert et al., 2007, Mabrouk et al., 2008). Antagonizing excitatory amino acid neurotransmission in the output structures of the BG can alleviate symptoms of experimental parkinsonism (Klogether and Turski, 1990, Brotchie et al., 1991). Since opioids are generally inhibitory in nature and localized in output structures of the BG, this has led investigators to consider the endogenous opioid system a viable target for the treatment of PD.

1.61 MOP Receptors and Parkinson's Disease

Preclinical and clinical research into the relationship between MOP receptors and PD has been scarce, probably due to the evident unwanted side effects associated with MOP receptor stimulation (e.g. addiction, euphoria and respiratory impairments). Despite this, it is well known

that MOP receptors are highly expressed in several BG structures and the nigro-striatal DA system (Mansour et al., 1995). In non parkinsonian animals, MOP receptor agonists are known to stimulate locomotion when given at lower doses while higher ones cause sedation (Iwamoto, 1981). In fact selective MOP receptor stimulation in SNr (Bontempi and Sharp, 1997) facilitates spontaneous locomotion and turning behavior in rats. This effect on locomotion has been correlated with the effect of MOP receptor stimulation on DA release in striatal areas (Di Chiara and Imperato, 1988) and control of GABAergic nigro-thalamic output neurons (Waszczak et al., 1984). However, studies using the non selective MOP receptor agonist morphine in experimental PD has been shown to be ineffective in enhancing the antiparkinsonian effects of DA agonists (Samadi et al., 2004). Interestingly though, morphine has been shown to reduce the dyskinetic response elicited by L-DOPA in animal models of PD (Samadi et al., 2004) as well as in PD patients (Berg et al., 1999).

1.62 KOP Receptors and Parkinson's Disease

The endogenous opioid peptide ligand for the KOP receptor is dynorphin. Studies examining the result of DA depletion on dynorphin precursor PPE-B mRNA expression have shown that it is decreased (Gerfen et al., 1990) in striato-nigral neurons. Dynorphin serves as a co-transmitter in DA D₁ expressing striatal GABAergic projection neurons extending to the SNr (direct pathway). Additionally, the KOP receptor is found in abundance in both GP and SNr (Haber and Watson, 1983, Mansour et al., 1993). Indeed, prodynorphin peptide immunoreactivity is dense in the SNr but is virtually absent in the SNc (Vincet et al., 1982). Likewise, KOP receptors are localized in the rat SNr but are not detectable in SNc (Mansour et al., 1987), placing this peptide receptor system in a strategic location to modulate the output of the BG and motor function. Despite this, KOP receptor agonists have been shown to produce contrasting effects compared to MOP and DOP receptor agonists in a number of behavioral paradigms. For instance, KOP receptor stimulation was shown to reduce locomotor activity in naïve mice while DOP (Ito et al., 2008) and MOP (Iwamoto, 1981) stimulation enhance it. Additionally, KOP receptor agonists have been shown to produce dysphoria in contrast to the euphoria brought about by MOP agonists (Mucha and Herz, 1985). Additionally, in experimental PD (6-OHDA hemilesioned rats), nigro-striatal denervation is associated with a decrease in PPE-B mRNA expression, the precursor of dynorphin, in striato-nigral neurons (Gerfen et al., 1990). Since the precursor of dynorphin may

be decreased in PD, investigators sought to restore dynorphin signaling using selective agonists with the hopes of reversing movement abnormalities caused by DA depletion. However, results thus far have been conflicting in terms of the potential benefit of KOP receptor agonists in the treatment of PD. Hughes and colleagues (1998) reported that KOP receptor agonists enadoline and U69,593 increased locomotion in monoamine-depleted rats. This group also demonstrated a synergistic effect between enadoline and L-DOPA and has suggested that KOP receptor agonists may have the potential as an adjunct to L-DOPA therapy in PD. On the other hand, clinical studies using spiradoline in PD patients by Giuffra and colleagues (1993) concluded that selective KOP receptor stimulation would not prove useful alone or in combination with L-DOPA in the treatment of PD. Taken together, role of KOP receptors in PD is not clearly understood, yet further studies are warranted due to its localization profile and its ability to elicit behavioral responses in parkinsonian mice.

1.63 DOP Receptors and Parkinson's Disease

The DOP receptor and its endogenous ligands, ENK are widely expressed in the basal ganglia, particularly in STN, striatum and, to a lesser extent, GP and SNr (Abou-Khalil et al., 1984, Mansour et al., 1993, Aubert et al. 2007, Hallett and Brotchie, 2007). ENK act as co-transmitters in striatal GABAergic neurons projecting to GP (Haber and Elde, 1981, Gerfen and Young, 1988) where they inhibit GABA release from striato-pallidal terminals (Maneuf et al., 1994) thereby opposing the inhibitory postsynaptic influence produced by striato-pallidal neurons (Stanford and Cooper, 1999). This modulation may be relevant during PD. Indeed, striato-pallidal neurons become pathogenically overactive following DA depletion, leading to overinhibition of pallido-subthalamic GABAergic and disinhibition of subthalamo-nigral GLUergic projections (DeLong, 1990). Consequently, PPE-A expression in striato-pallidal neurons increases (Young et al., 1986, Gerfen et al., 1990) possibly to attenuate exaggerated GABA release and compensate for motor deficits (Maneuf et al., 1994). Consistently, the DOP receptor agonist SNC-80 (Bilsky et al., 1995) promoted spontaneous or L-DOPA induced contralateral turning in 6-OHDA hemilesioned rats (Pinna and Di Chiara, 1998, Hudzik et al., 2000) and reversed akinesia in reserpinized or haloperidol-treated rats as well as MPTP-treated marmosets (Maneuf et al., 1994, Hill et al., 2000, Hille et al., 2001) Based on the evidence that endogenous ENK inhibited K^+ -evoked GABA release in pallidal slices (Maneuf et al., 1994), it

was proposed that DOP receptor agonists attenuate parkinsonism by inhibiting GABA release from striato-pallidal GABAergic terminals. Recently we showed that SNC-80 attenuated akinesia/bradykinesia and improved overall gait ability in 6-OHDA hemilesioned rats while the selective DOP receptor antagonist naltrindole (NTD; Portoghese et al., 1988) exerted opposite effects (Mabrouk et al., 2008). SNC-80 decreased pallidal GABA as well as nigral GLU and GABA release, suggesting an action at the striatal, pallidal or nigral level. Moreover, only NTD perfusion in SNr (and not GP or DLS) prevented the antiakinetik effect of SNC-80 and its neurochemical correlates. Consistently, microinjections of SNC-80 in SNr (but not GP or DLS) replicated the antiparkinsonian effects of systemic SNC-80. These findings confirm that DOP receptor stimulation, either by synthetic agonists or endogenous ENK, promotes motor function under parkinsonian conditions but challenged the common view that DOP receptor agonists attenuate parkinsonism via intrapallidal mechanisms, suggesting, instead, that nigral DOP receptors are involved.

1.64 NOP Receptors and Parkinson's Disease

N/OFQ is a heptadecapeptide that structurally resembles dynorphin A. Its receptors are expressed in both cortical and subcortical motor areas, particularly in the DA containing neurons of SNc. Since the SNc degenerates during the course of PD, investigators sought to explore the relationship between the NOP system and motor functions. Since its discovery, the N/OFQ-NOP receptor system has been termed 'anti-opioid' since it has been shown to inhibit the effects of classical opioids. For instance, morphine mediated analgesia in the mouse tail flick test was attenuated by NOP receptor stimulation (Mogil et al., 1996, Calò et al., 1998). Additionally, N/OFQ was shown to suppress striatal DA release from ventral tegmental area (VTA; Murphy and Maidment, 1999) and SNc (Marti et al., 2004) as well as locomotor activity (Meunier et al., 1995, Reinscheid et al., 1995) whereas MOP receptor agonists stimulate DA release by inhibiting intranigral (Lacey et al., 1989) or intra-VTA (Johnson and North, 1992) GABAergic interneurons. A link between N/OFQ and PD has been uncovered by our group. Thus N/OFQergic transmission was found to be upregulated in the DA-depleted SNr of 6-OHDA lesioned rats (Marti et al., 2005). Moreover, systemic or intranigral injections of NOP receptor antagonists such as J-113397 can attenuate experimental parkinsonism in 6-OHDA and haloperidol treated rats (Marti et al., 2004b, 2005) as well as MPTP-treated mice and nonhuman

primates (Viaro et al., 2008). Recent studies have also demonstrated that the deletion of the NOP precursor (preproN/OFQ) or NOP receptor genes gives mice partial resistance to MPTP toxicity and haloperidol-induced catalepsy, respectively (Marti et al., 2005). These findings have prompted investigators to identify novel selective NOP receptor antagonists for PD therapy. Indeed, a novel NOP receptor antagonist TRAP-101 was recently shown to reverse experimental PD in accordance with earlier findings using J-113397 (Marti et al., 2008).

Precursor	Peptide	Amino acid sequence	Main Receptor
Pro-enkephalin	[met]-enkephalin	YGGFM	DOP
(PPE-A)	[Leu]-enkephalin	YGGFL	DOP
		YGGFMRF	
		YGGFMRGL	
	Peptide E	YGGFMRRVGRPEWWMQDYQKRYGGFM	
	BAM 22P	YGGFMRRVGRPEWWMQDYQKRYG	
	Metorphamide	YGGFMRRV-NH₂	
Pro-opiomelanocortin	β-endorphin	YGGFMTSEKSQTPLVTLFKNAIHKNAKKGE	MOP/DOP
Pro-dynorphin	Dynorphin A	YGGFLRRIRPKLKWQDNQ	KOP
(PPE-B)	Dynorphin A (1-8)	YGGFLRRI	KOP
	Dynorphin B	YGGFLRRQFKVVT	KOP
	α-Neoendorphin	YGGFLRKYPK	MOP/KOP
	β-Neoendorphin	YGGFLRKYP	KOP
??	Endomorphin-1	YPWF-NH₂	MOP
	Endomorphin-2	YPFF-NH₂	MOP
Pro-nociceptin (PPN/OFQ)	Nociceptin/orphanin FQ	FGGFTGARKSARKLANQ	NOP

Fig 5. Most known endogenous opioids, their precursors, amino acid sequences and known receptors.

2. AIMS OF STUDIES

The main aim of the work presented in this thesis was to continue the investigations into the involvement of opioid receptors in physiological and pathological conditions affecting the BG, particularly as it relates to PD. These studies were performed in both naïve rats (Section I) as well as in 6-OHDA hemilesioned rats (Section II, III, IV), which is widely regarded as a reliable model of PD. We analyzed motor activity of animals which received various opioid ligands either systemically (i.p.) or directly into brain areas of interest (via perfusion or microinjections). In addition we correlated locomotor activity with neurochemical changes using *in vivo* microdialysis to further characterize the mechanism of action of opioid ligands on locomotor behavior.

Section I. The main aim of this study was to investigate whether EM-1, the endogenous ligand of the MOP administered exogenously into the brain of naïve rats could control locomotor activity and through which mechanism this effect occurred. We then implemented the novel grid test to measure subtle animal behaviors in response to drug treatment such as reaction time and speed of movements. These findings were collected in parallel with *in vivo* microdialysis to collect neurochemical evidence which could demonstrate the brain area and neurotransmitters involved in the motor actions of EM-1.

Section II. The main aim of this study was to investigate the role of DOP receptors in parkinsonism and, in particular, to investigate the circuitry underlying the antiparkinsonian actions of the DOP receptor agonist SNC-80. First, we wanted to demonstrate that this drug improved locomotor activity in the 6-OHDA hemilesioned rat model of PD. Following behavioral studies, we sought to determine the precise mechanism of action of DOP receptor agonist induced stimulation of locomotor behavior. We also wanted to understand how endogenous enkephalinergic tone might influence rat behavior. To this end we used the selective DOP receptor antagonist naltrindole systemically and injected into particular brain areas including dorsolateral striatum, globus pallidus (GP) and SNr.

Section III. The main aim of this study was to characterize the ability of the novel DOP receptor agonist H-Dmt-Tic-NH-CH(CH₂-COOH)-Bid (UFP-512) to attenuate motor deficits in 6-OHDA hemilesioned rats. Since UFP-512 caused different behavioral effects compared to typical nonpeptide agonists such as SNC-80, we set out to determine the circuitry involved in motor actions of UFP-512 by using microdialysis coupled to behavioral testing. By coupling *in vivo* microdialysis with the akinesia test, we correlated motor activity with changes in GABA and

GLU release in GP and SNr. Moreover, to test the hypothesis that changes in locomotor behavior were associated with changes in nigro-thalamic transmission, amino acid release in ventromedial thalamus (a target of nigro-thalamic GABAergic projections) was also measured.

Section IV. The main aim of this study was to describe potential interactions between the DOP and NOP receptor using behavioral techniques in 6-OHDA hemilesioned rats. Since we recently demonstrated that DOP receptor agonists and NOP receptor antagonists improved locomotor activity in experimental models of PD via nigral mechanisms, the behavioral and neurochemical profile of this combination was investigated following both systemic and intranigral administration. In order to characterize whether the observed effects were synergistic or additive, we tested these compounds at subthreshold and submaximal doses or concentrations.

3. MATERIALS AND METHODS

3.1 Animal Subjects

Male Sprague-Dawley rats (150 g; Harlan Italy; S. Pietro al Natisone, Italy) were kept under regular lighting conditions (12 hr light/dark cycle) and given food and water *ad libitum*. The experimental protocols performed in the present study were approved by Ethical Committee of the University of Ferrara and adequate measures were taken to minimize animal pain and discomfort.

3.2 6-OHDA lesioning

Unilateral lesion of DAergic neurons was induced in isoflurane-anesthetized male rats as previously described (Marti et al., 2005). Eight micrograms of 6-OHDA (dissolved in 4 μ l of saline containing 0.02% ascorbic acid) were stereotaxically injected according to the following coordinates from bregma: AP= -4.4 mm, ML= -1.2 mm, DV= -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 6-OHDA injection, denervation was evaluated with a test dose of amphetamine (5 mg/kg i.p., dissolved in saline just before use). Rats showing a turning behavior >7 turns/min in the direction ipsilateral to the lesion 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 6-8 weeks after lesion.

3.3 Behavioral experiments

Three behavioral tests were used to evaluate different motor functions as previously described (Marti et al., 2005): 1) the bar test (Kuschinski and Hornykiewicz, 1972; for a review see Sanberg et al., 1988) measures the rat ability to respond to an imposed static posture; 2) the drag test (modification of the “wheelbarrow test”; Schallert et al., 1979) measures rat ability to balance body posture using the forelimbs in response to an externally imposed dynamic stimulus (i.e. backward dragging); 3) the fixed-speed rotarod test (Rozas et al., 1997) measures overall motor performance as an integration of coordination, gait, balance, muscle tone and motivation to run. The three tests were repeated in a fixed sequence (bar, drag and rotarod) before (control) then 20 and 70 min after drug injection. Motor activity was then expressed as percent of performance in

the control session. Rats were trained for approximately 10 days to the specific motor tasks until their motor performance became reproducible.

3.31 Bar Test. Rats were placed on a table and each forepaw was 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 (cut-off time 20 sec).

3.32 Drag test. Rats were lifted from the tail (allowing the forepaws to rest on the table) and dragged backwards at a constant speed (~20 cm/sec) for a fixed distance (100 cm). The number of steps made by each forepaw was counted by two separate observers.

3.33 Rotarod test. The fixed-speed rotarod test was employed using an established protocol (Marti et al., 2004). Briefly, rats were trained for 10 days to a complete motor task on the rotarod (i.e. from 5 to 55 rpm; 180 sec each) until their motor performance became reproducible in three consecutive sessions. Rats were then tested at 4 increasing speeds (usually 10, 15, 20, 25 rpm), causing a progressive decrement of performance to about 40% of the maximal response (i.e. the experimental cut-off time).



Fig 6. The bar test. This test measures akinesia or time to initiate movement after an imposed static posture. Animal is placed on 3 separate block heights, 3, 6 and 9 cm and timed.



Fig 7. The drag test. This test measures rat ability to balance body posture using the forelimbs in response to an externally imposed dynamic stimulus (i.e. backward dragging).

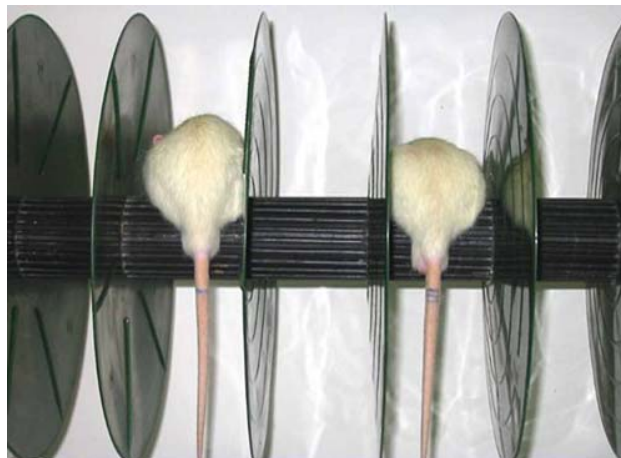


Fig 8. The rotarod test. This test measures overall motor performance as an integration of coordination, gait, balance, muscle tone and motivation to run.

3.34 Inclined grid test. A modified version of the inclined grid test originally developed to evaluate catalepsy in rodents (Ahlenius and Hillegaard, 1986) was employed. A metallic grid (30 x 45 cm) was positioned vertically in the microdialysis cage with a of 60° inclination (Fig 9). Rats undergoing microdialysis were placed in the upper half of the grid and left free to move back to the cage. Each trial started when the rat firmly gripped the grid and terminated when the rat touched the bottom of the cage with both forepaws and hindpaws. Rat motor behavior was videotaped and analyzed posthoc by an experimentally blind investigator using a VCR with slow motion and frame by frame (25 Hz) capabilities. Several motor parameters were recorded: i) latencies of head and forepaw movement (in sec) as a measure of akinesia (Reaction Time; RT);

ii) speed of forepaw movement (in cm/sec) and step length (cm), as a measure of forepaw bradykinesia and gait abnormalities. Each experimental session consisted of 8-10 trials (usually 90-120 sec).



Fig 9. The inclined grid test coupled to microdialysis. This test measures subtle changes in locomotor behavior including reaction time, speed of movements and length of forepaw steps.

3.4 Microinjection technique

Microinjections were performed as previously described (Marti et al., 2004). Microinjection guide cannulas (outer diameter 0.55 mm) were stereotaxically implanted under isoflurane anaesthesia 0.50 mm above the lesioned SNr (AP -5.5, ML -2.2, DV -7.8 from bregma, Paxinos and Watson 1982), GP (AP -1.3, ML -3.3, DV -6.0) and DLS (AP +1.0, ML -3.5, DV -5.5), and secured to the skull by acrylic dental cement and metallic screws. Seven days after surgery, saline or SNC-80 (0.5 μ l volume) were injected through a stainless-steel injector (outer diameter 0.30 mm) protruding 1 mm beyond the cannula tip. In a separate set of experiments, bicuculline (1.5 nmol) was injected in GP. At the end of each experiment the placement of the guide cannula was verified by microscopic examination.

3.5 Microdialysis experiments

3.51 Probe construction

Probes were produced using a concentric design with silica tubing (Polymicro technologies), polyethylene tubing (Smiths Company), stainless steel tubing (outer diameter 0.55 mm) and

dialysis membrane (Hospal Bologna). Inlet portion of probes consisted of 8 cm polyethylene tubing combined with a 3 cm silica tube glued in place with cyanoacrylate (CA) adhesive (Loctite, Attack). The outlet portion of the probe consisted of a 10 cm section of polyethylene tube combined with a 1.5 cm section of silica tubing also glued in place. Silica tubing extended 5 mm into polyethylene tubing when was secured with CA adhesive.

Inlet and outlet silica/polyethylene tubing structures were left to dry and then gently inserted into stainless steel tubing. For SNr, GP and VMTh, the stainless steel used was 20 mm while for studies employee DLS probes, stainless steel was 15 mm. CA adhesive was then used to secure the 3 pieces together and left to dry overnight. Larger tubing was then placed around the point where the 3 pieces converged to further strengthen the structure and more adhesive is added at this site and left to dry overnight.

The inlet portion of the probe with silica extending beyond the stainless steel was then cut to the desired length. For SNr, GP, and VMTh, 1 mm was used while for DLS, 3 mm was used. Membrane is cut and gently inserted over the silica and then cut just beyond where silica ends. Using epoxy adhesive, the membrane is secured to the rest of the structure and the tip is capped leaving a very small space between the end of the silica tubing and the tip of the membrane. The structure is then left to dry for 3 days before experiment. Probes were tested the day of use by pushing purified water through them while checking for any leakage. In vitro recovery for probes were tested periodically and consistently offered ~10-15% recovery.

3.52 Surgery and microdialysis procedure

Microdialysis probes were stereotaxically implanted, under isoflurane anaesthesia, in the DLS (section I), SNr (section I, II, III, IV), GP (Section II, III, IV), and VMTh (Section III and IV) according to the following coordinates from bregma: DLS; AP +1.0, ML -3.5, DV -6.0, SNr; AP -5.5, ML -2.2, DV -8.3, GP; AP -1.3, ML -3.3, DV -6.5, VMTh; AP -2.3, ML -1.4, DV -7.4. Probes were secured to the skull by acrylic dental cement and metallic screws. After surgery, rats were allowed to recover and experiments were run 24 hr after probe implantation. Microdialysis probes were perfused at a flow rate of 3.0 μ l/min with a modified Ringer solution (composition in mM: CaCl₂ 1.2; KCl 2.7, NaCl 148 and MgCl₂ 0.85). Samples were collected every 15 min, starting 6 hr after the onset of probe perfusion. At least 4 stable values were collected before beginning of experiment.

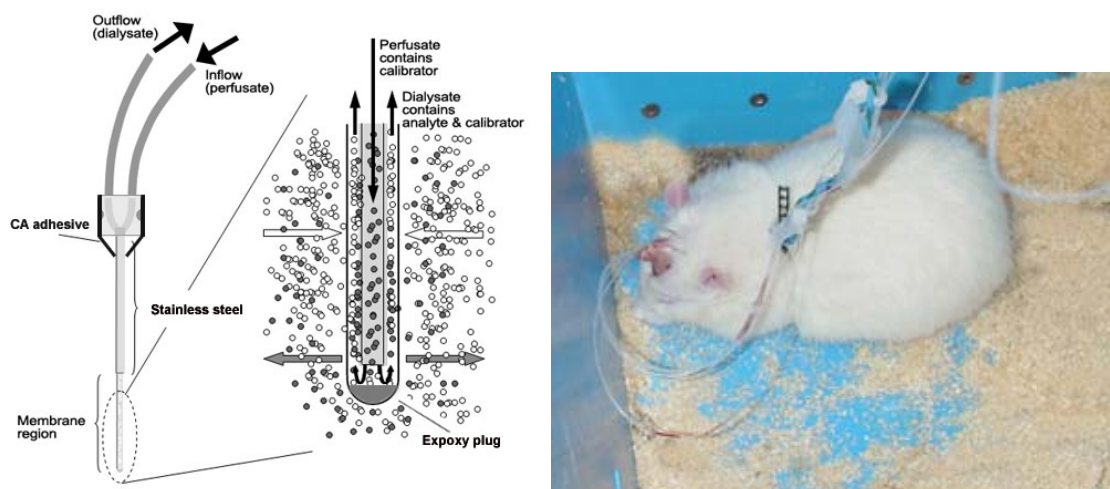


Fig 10. A close up schematic of a microdialysis probe during perfusion (left) and a typical dual probe microdialysis experiment with a rat in home cage (right).

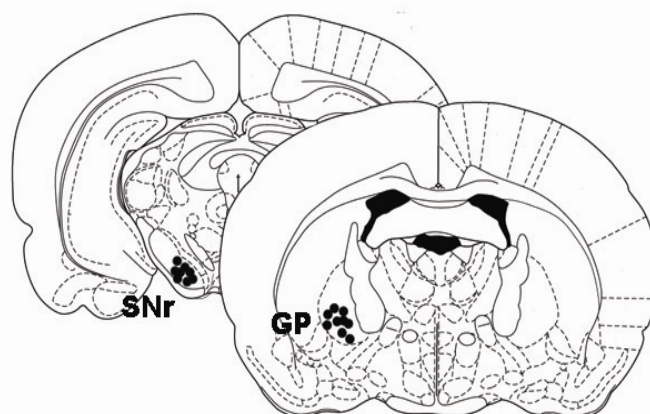


Fig. 11. Example of histological verification of probe placements in SNr and GP. Black points refer to probe tip locations after reviewing brain slices in cryostat.

3.6 Microdialysis + Bar test coupling

The bar test was coupled to microdialysis in order to more accurately characterize the time course of drug effect with respect to neurochemical changes. Bar test, as described above, was simply performed inside microdialysis cages. Animals were handled in their microdialysis cages for 1 hr prior to starting the experiments. Amount of time spent on the bar was measured every 15 minutes (duration of one sample collection) for 45 minutes prior to drug and 90-150 minutes after drug administration. Total time (in sec) spent by each paw on the blocks was recorded (cut-off time 20 sec for each block height).

3.7 Endogenous GLU and GABA analysis

GLU and GABA were measured by HPLC coupled with fluorometric detection as previously described (Marti et al., 2007). Thirty microliters of o-phthaldialdehyde/mercaptoethanol reagent was added to 40 μ l aliquots of sample, and 60 μ l of the mixture was automatically injected (Triathlon autosampler; Spark Holland, Emmen, Netherlands) onto a 5-C18 Chromsep analytical column (3 mm inner diameter, 10 cm length; Chrompack, Middelburg, Netherlands) run 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 were detected by means of a fluorescence spectrophotometer FP-2020 Plus (Jasco, Tokyo Japan) with the excitation and the emission wavelengths set at 370 and 450 nm respectively. The limits of detection for GLU and GABA were about 150 fmol/sample. Retention times for GLU and GABA were 3.5 ± 0.2 min and 18.0 ± 0.5 min respectively.

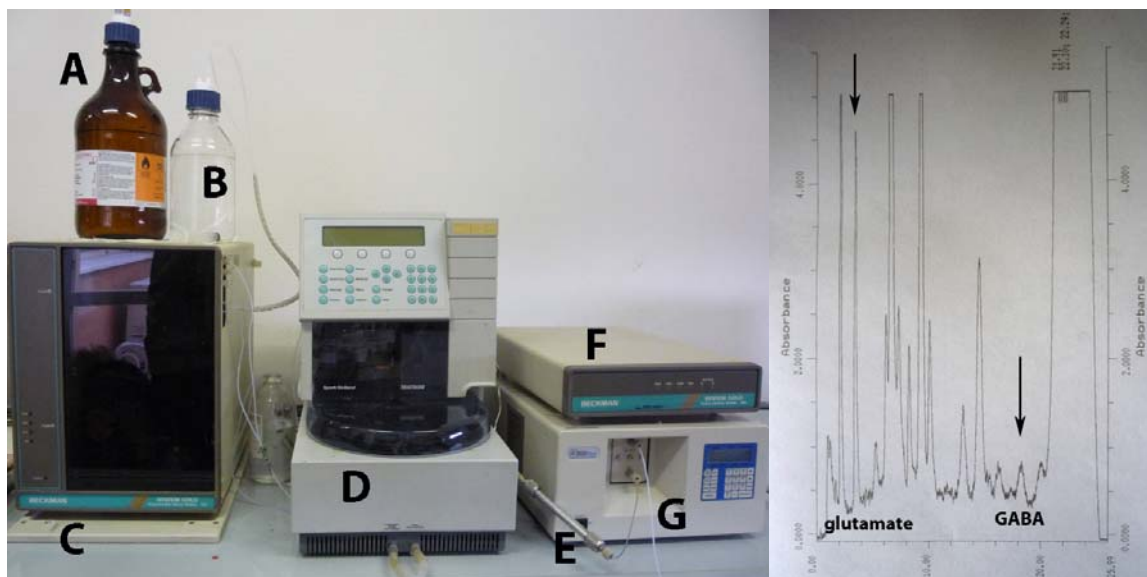


Fig 12. (Left) HPLC coupled to spectrofluorescence detector configured specifically for the detection of GABA and GLU. **A)** Mobile phase **B)** Methanol **C)** Pumps 1 and 2 (Beckman 125) **D)** Autosampler (Triathlon, Spark Holland) **E)** 5C-18 chromsep column **F)** HPLC system interface **G)** Spectrofluorescence detector (Jasco 2020 plus).

(Right) A typical chromatogram showing the retention times for GLU and GABA. Concentration of amino acids were calculated automatically by peak height analysis.

3.8 Endogenous DA analysis

DA was measured by means of reversed phase HPLC coupled with electrochemical detection. Briefly, 27 µl samples were injected onto a 5-C18 Chromsep analytical column run at a flow rate of 0.4 ml/min (Beckman 118 pump, Beckman Instruments, Fullerton, CA, USA) with a mobile phase containing 75 mM NaH₂PO₄, 20 µM EDTA, 0.01% triethylamine, 1.5 mM sodium dodecyl sulphate, 10% methanol and 16% acetonitrile adjusted to pH 5.6 with NaOH. DA was separated under isocratic conditions (~6.5 min retention time) and detected by means of an electrochemical detector (Coulchem II Model 5200; ESA, Inc, Chelmsford, MA, USA) set at +175 mV. The limit of detection for DA was 10 fmol/sample.

3.9 Data presentation and statistical analysis

Motor performance has been calculated as time on bar or on rod (in sec) and number of steps (drag test) and expressed as percent of the control session. In microdialysis studies, GLU and GABA release has been expressed as percentage ± SEM of basal values (calculated as mean of the two samples before the treatment). In text and Figure legends, amino acid dialysate levels were also given in absolute values (in nM).

Motor activity in the inclined grid test has been calculated as time of latency to initiate movements of the head and left and right forepaw (in sec), velocity (in cm/sec) and step length (cm) of left and right forepaw and expressed as percent of the control session. Statistical analysis has been performed on percent data by one-way repeated measure (RM) analysis of variance (ANOVA). In case ANOVA yielded a significant F score, post-hoc analysis was performed by contrast analysis to determine group differences. In case a significant time X treatment interaction was found, the sequentially rejective Bonferroni test was used (implemented on excel spreadsheet) to determine specific differences (i.e. at the single time-point level) between groups. P values <0.05 were considered to be statistically significant.

3.10 Materials

EM-1, UFP-512 and J-113397 were synthesized in the laboratories of the Department of Pharmaceutical Sciences (University of Ferrara). CTOP, bicuculline, raclopride SCH-23390, NTD and SNC-80 were purchased from Tocris Neuramin (Bristol, UK). 6-OHDA hydrobromide and *d*-amphetamine sulphate were purchased from Sigma (St. Louis, MO, USA). All drugs were

freshly dissolved in Ringer or isosmotic saline just before use with the exception of SNC-80. SNC-80 (10 mg/kg) was dissolved in saline containing 5% DMSO while other doses given systemically (0.1-3 mg/kg) or microinjected (0.1 and 1 nmol) only needed 0.5% DMSO. In both cases, vehicle alone did not affect behavioral or neurochemical responses. SNC-80 was vortexed and sonicated for 15 min or until fully dissolved in solution.

4. RESULTS

Section I

The endogenous MOP receptor agonist EM-1 dually affects motor activity and differentially modulates nigro-striatal and nigro-thalamic pathways.

Behavioral Studies I

4.1.1 Effect of i.c.v. injection of EM-1 and CTOP on motor behavior

The effect intracerebroventricular (i.c.v.) administration of EM-1 on physiologically-stimulated motor activity was studied by means of the rotarod test.

In this test, RM ANOVA showed a significant effect of treatment ($F_{4,16}=342.7$, $p<0.0001$), but not time ($F_{1,20}=4.3$, $p=0.051$) and a significant time X treatment interaction ($F_{4,20}=10.5$, $p<0.0001$). EM-1 (0.01-10 nmol) biphasically regulated the rotarod performance (Fig. 13A), causing motor facilitation at 0.1 and 1 nmol (~35% and ~55%, respectively) and motor impairment at 10 nmol (~90%). At this dose, EM-1 increased muscle rigidity which probably impaired the execution of the test.

To unravel tonic regulation by endogenous EM-1 and also investigate the selectivity of exogenous EM-1, the selective MOP receptor antagonist CTOP was employed. RM ANOVA on the effect of CTOP alone showed a significant effect of treatment ($F_{2,8}=14.9$, $p=0.0019$) but not time ($F_{1,12}=0.4$, $p=0.5504$) and a non significant time X treatment interaction ($F_{2,12}=0.4$, $p=0.6722$). CTOP was ineffective at 1 nmol and produced a long-lasting reduction in rotarod performance at 10 nmol (~30%) which persisted up to 60 min (Fig. 13B). An ineffective dose of CTOP (1 nmol) prevented the facilitation caused by EM-1 (1 nmol; Fig. 13 C).

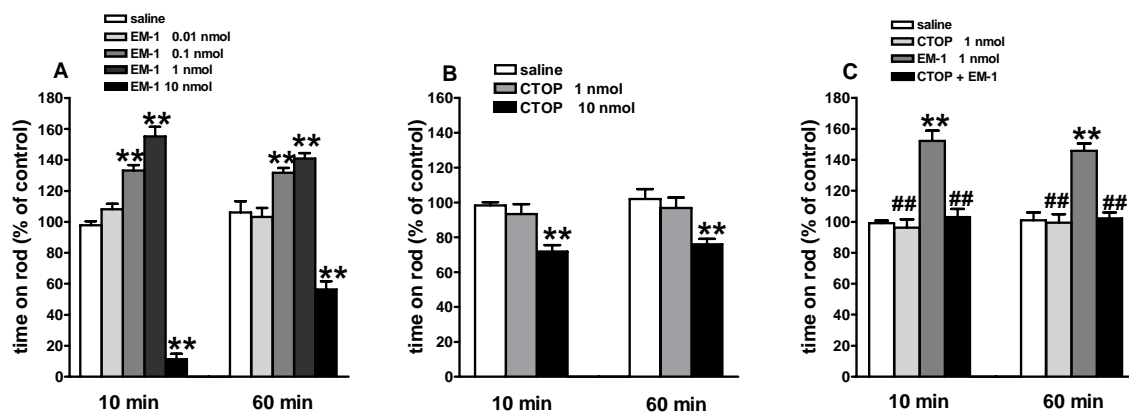


Fig 13. Effect of i.c.v. injection of EM-1 (0.01-10 nmol; A), CTOP (1 and 10 nmol; B) and their co-administration (EM-1 1 nmol and CTOP 1 nmol; C) on rat motor performance on the rotarod. Each experiment consisted of three different sessions: a control session followed (40 min) by other two sessions performed 10 and 60 min after saline, or drug injection (see Methods). Data are expressed as percentages of basal motor activity in the control session and are means \pm SEM of 6-8 determinations per group. Basal motor activity in the rotarod performance was 1044 ± 40 sec (0-55 rpm range).

** $p<0.01$ significantly different from saline.

$p<0.01$ significantly different from EM-1 (RM ANOVA followed by contrast analysis and the sequentially rejective Bonferroni's test).

4.1.2 Effect of intranigral injection of EM-1 on motor behavior

To investigate the involvement of SNr in central motor effects of EM-1, intranigral microinjections of EM-1 and CTOP were performed and rotarod activity evaluated.

EM-1 (0.01-10 nmol) biphasically regulated rotarod performance (Fig. 14A). RM ANOVA showed a significant effect of treatment ($F_{4,24}=402.6$, $p<0.0001$), but not time ($F_{1,26}=0.1$, $p=0.71$) and a non significant time X treatment interaction ($F_{4,26}=1.4$, $p=0.26$). Significant increases were observed at 0.1 and 1 nmol EM-1 (~30% and ~40%, respectively) while marked impairment was observed at 10 nmol (~80%) at 10 min post-injection time and persisted up to 60 min (Fig. 14A).

When administered in SNr, EM-1 evoked spontaneous contralateral turning (Fig. 14B). RM ANOVA showed a significant effect of treatment ($F_{2,12}=35.81$, $p<0.0001$), time ($F_{20,360}=18.06$, $p<0.0001$) and a significant time X treatment interaction ($F_{40,360}=16.98$, $p<0.0001$). EM-1 induced turning at 10 nmol, but not at 1 nmol (Fig. 14B).

The MOP receptor antagonist CTOP modulated rotarod performance (Fig. 14C). RM ANOVA showed a significant effect of treatment ($F_{2,8}=14.9$, $p=0.0019$) but not time ($F_{1,12}=0.024$, $p=0.87$) and a non significant time X treatment interaction ($F_{2,12}=0.026$, $p=0.97$). CTOP was ineffective at 1 nmol yet caused a prolonged reduction of rotarod performance at 10 nmol (~30%; Fig. 14C). An ineffective dose of CTOP (1 nmol) prevented the facilitation of rotarod performance caused by EM-1 (1 nmol; Fig. 14D).

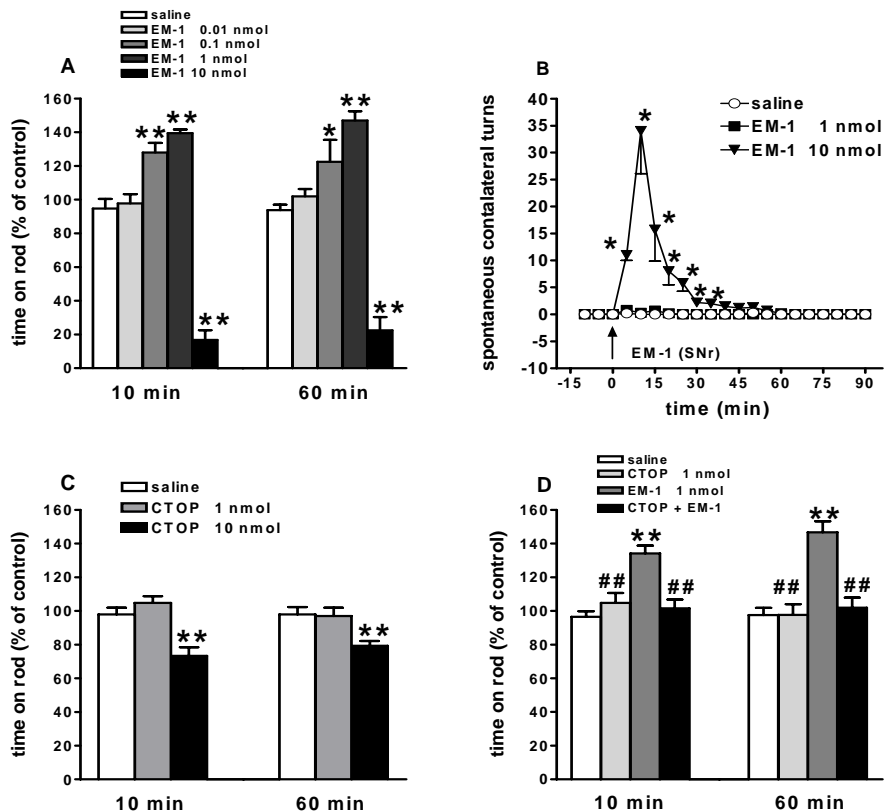


Fig 14. Effect of intranigral injection of EM-1 (0.01-10 nmol; A-B), CTOP (1 and 10 nmol; C) and their co-administration (EM-1 1 nmol and CTOP 1 nmol; D) on rat motor behavior. EM-1 was injected i.c.v. and motor activity evaluated in the rotarod (A) and spontaneous rotational (B) tests. Each experiment consisted of three different sessions: a control session followed (40 min) by other two sessions performed 10 and 60 min after saline, EM-1, CTOP or their co-injection (see Methods). Data are expressed as percentages of basal motor activity in the control session and are means \pm SEM of 6-8 determinations per group. Basal motor activity in the rotarod performance was 1020 ± 29 sec (0-55 rpm range). In spontaneous rotational test data are expressed as absolute values (number of turning executed in 5 min). ** $p < 0.01$ significantly different from saline, ## $p < 0.01$ significantly different from EM-1 (RM ANOVA followed by contrast analysis and the sequentially rejective Bonferroni's test).

Neurochemical studies

4.1.3 Effect of perfusion with EM-1 in SNr on local GABA and GLU extracellular levels

Since the behavioral data pointed to the involvement of nigral MOP receptors in the effects of EM-1, we investigated how local EM-1 perfusion in the SNr affected local GABA and GLU release. RM ANOVA on GABA levels revealed a significant effect of treatment ($F_{4,20}=9.09$, $p < 0.001$), time ($F_{7,167}=2.33$, $p < 0.05$), and a significant time X treatment interaction ($F_{28,167}=2.11$, $p = 0.026$). Post hoc analysis showed that EM-1, ineffective at $0.001 \mu\text{M}$, produced a prompt and transient increase at $0.01 \mu\text{M}$ ($\sim 200\%$ at peak) and a prompt and long lasting decrease at $1 \mu\text{M}$

(~45% at the end of perfusion) while the stimulation induced by an intermediate concentration (i.e. 0.1 μM) was not significant (Fig. 15A).

To test the specificity of EM-1, high EM-1 concentrations (i.e 1 μM) were challenged with CTOP (3 μM ; Fig. 15B). For these data, RM ANOVA on nigral GABA levels revealed a significant effect of treatment ($F_{3,53}=9.66$, $p=0.0002$), time ($F_{7,23}=21.05$, $p<0.0001$) and time X treatment interaction ($F_{21,429} = 12.22$, $p <0.0001$). Post hoc analysis showed that intranigral perfusion with CTOP alone did not modify local GABA levels yet prevented the inhibition induced by EM-1 (Fig. 15B).

Intranigral perfusion with EM-1 (0.01-1 μM ; 90 min) was also associated with changes in nigral GLU levels (Fig. 15C). RM ANOVA on GLU levels revealed a significant effect of treatment ($F_{3,17}=21.42$, $p<0.001$), time ($F_{7,468}=13.45$, $p<0.0001$), and a significant time X treatment interaction ($F_{21,468}=10.68$, $p<0.0001$). Post hoc analysis showed that EM-1, ineffective at 0.01 μM , evoked a prompt and long lasting facilitation (~153% by end of perfusion) at 0.1 μM and a prompt but short-lived increase (~189%) at 1 μM (Fig. 15C).

Again, the specificity of EM-1 was investigated by using CTOP (Fig. 15D). RM ANOVA on nigral GLU levels revealed a non significant effect of treatment ($F_{3,17}=1.55$, $p=0.2130$) but a significant effect of time ($F_{7,436}=14.05$, $p<0.0001$) and time X treatment interaction ($F_{21,436}=9.82$, $p<0.0001$). Intranigral perfusion with CTOP (3 μM), ineffective alone, prevented the increase in GLU induced by EM-1 (1 μM ; Fig. 15D).

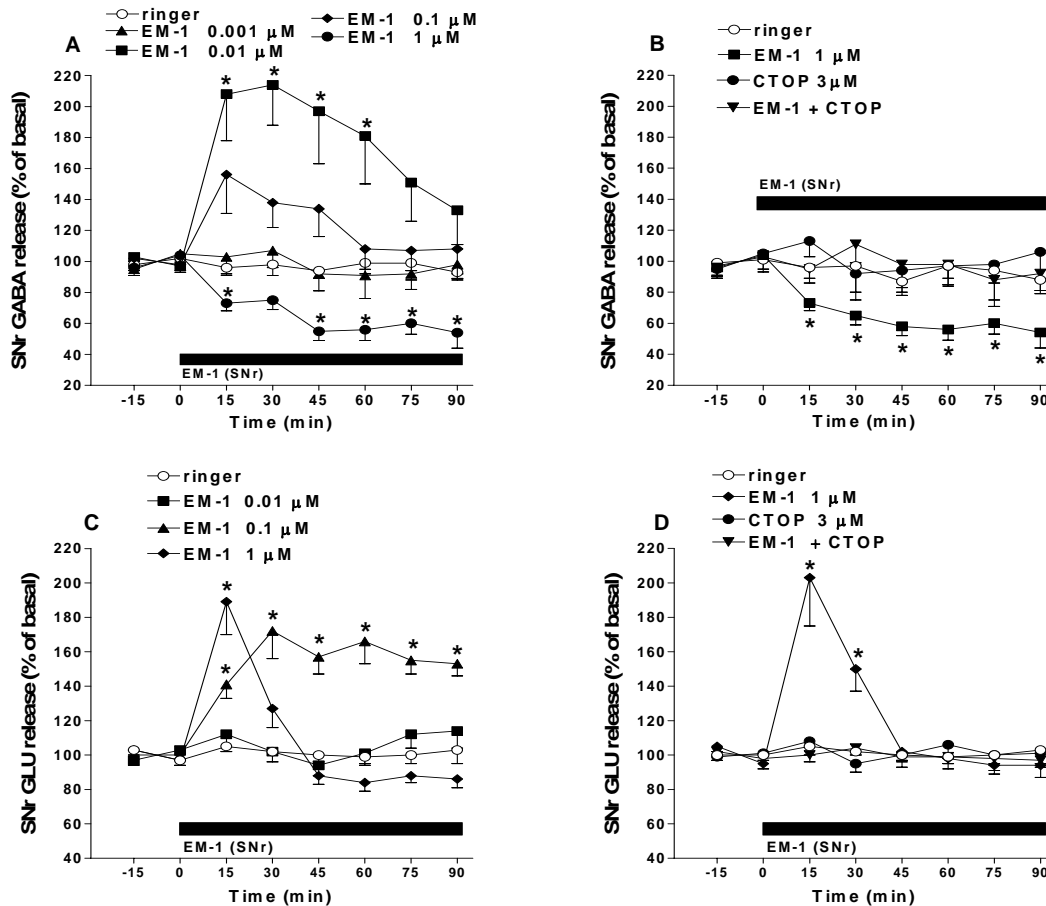


Fig 15. Effect of reverse dialysis of EM-1 (0.01-1 μ M; black bar) and CTOP (3 μ M) in the substantia nigra pars reticulata (SNr) of awake rats on local extracellular GABA (A-B) and GLU (C-D) levels. Perfusion with CTOP started 90 min before EM-1 and continued until the end of experiment. Data are expressed as percentages \pm SEM of basal pre-treatment levels (calculated as mean of the two samples before the treatment). Basal GABA levels in the SNr were 6.0 ± 0.6 nM while GLU levels were 132.9 ± 13.5 nM.

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

Behavioral studies II

4.1.4 Effect of intranigral perfusion of EM-1 on grid test and spontaneous motor activity

In view of the ability of EM-1 to oppositely modulate rotarod performance and SNr GABA release, in a separate series of microdialysis experiments we investigated whether intranigral perfusion with low (0.01 μ M) and high (1 μ M) EM-1 concentrations was accompanied by opposite changes in motor activity. Rat activity was recorded by videotape at rest and during performance of the grid test. Different motor parameters were analyzed post hoc using video recordings.

4.1.41 Effect on neck/trunk and forepaw reaction time

RM ANOVA on neck/trunk reaction time (RT; Fig. 16A), showed a significant effect of treatment ($F_{2,12}=1.75$, $p<0.0001$), time ($F_{7,126}=15.65$, $p<0.0001$) and a significant time X treatment interaction ($F_{14,126}=9.39$, $p<0.0001$). Perfusion with the lower EM-1 concentration (0.01 μM) caused a prompt and long lasting reduction ($\sim 57\%$ at 15 min) while EM-1 (1 μM) caused a biphasic response characterized by a transient reduction ($\sim 46\%$ at 15 min) followed by a slight increase ($\sim 12\%$ at 90 min; Fig. 16A).

RM ANOVA on RT at the ipsilateral forepaw (Fig. 16B) also showed a significant effect of treatment ($F_{2,12}=12.75$, $p=0.0011$), time ($F_{7,126}=21.59$, $p<0.0001$) and a significant time X treatment interaction ($F_{14,126}=9.12$, $p<0.0001$). EM-1 (0.01 μM) caused a prompt and transient reduction in RT at the ipsilateral forepaw ($\sim 59\%$ at 15 min) while EM-1 (1 μM) caused a prompt decrease ($\sim 36\%$ at 15 min) followed by a long lasting increase ($\sim 45\%$ at 75 min; Fig. 16B).

Finally, RM ANOVA on RT at the contralateral paw (Fig. 16C) showed a significant effect of treatment ($F_{2,12}=40.23$, $p<0.0001$), time ($F_{7,126}=20.59$, $p<0.0001$) and a significant time X treatment interaction ($F_{14,126}=24.35$, $p<0.0001$). EM-1 (0.01 μM) caused a prompt and long lasting reduction in RT at the contralateral forepaw ($\sim 72\%$ at 15 min) while EM-1 (1 μM) caused a prompt decrease ($\sim 43\%$ at 15 min) followed by a robust and long lasting increase ($\sim 106\%$ at 60 min; Fig. 16C).

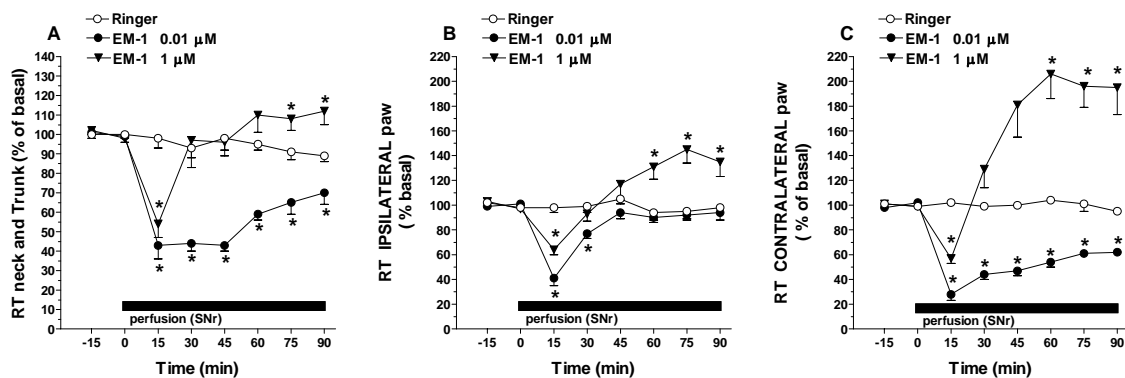


Fig 16. Effect of intranigral EM-1 (0.01-1 μM) perfusion on reaction time (RT) at the neck/trunk and ipsilateral and contralateral forepaws in the grid test. Data are expressed as percentages \pm SEM (7 determinations per group) of basal pre-treatment levels (calculated as mean of the two samples before the treatment). The latency to initiate movements (reaction time, RT) after being positioned on the grid were (in sec): 1.1 ± 0.2 (neck and trunk torsion; A), 2.6 ± 0.4 (ipsilateral paw; B) and 2.8 ± 0.5 (contralateral paw; C).

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

4.1.42 Effect of EM-1 on forepaw speed and gait control

On the speed of the ipsilateral forepaw (Fig. 17A), RM ANOVA showed a significant effect of treatment ($F_{2,12}=151.59$, $p<0.0001$), time ($F_{7,126}=17.30$, $p<0.0001$) and a significant time X treatment interaction ($F_{14,126}=15.77$, $p<0.0001$). Intranigral perfusion with EM-1 (0.01 μM) promptly increased ipsilateral forepaw speed ($\sim 69\%$ at 15 min) while EM-1 (1 μM) was without effect (Fig. 17A).

On the speed of the contralateral forepaw (Fig. 17B), RM ANOVA also showed a significant effect of treatment ($F_{2,12}=151.77$, $p<0.0001$), time ($F_{7,126}=31.53$, $p<0.0001$) and a significant time X treatment interaction ($F_{14,126}=33.62$, $p<0.0001$). EM-1 (0.01 μM) caused a prompt and robust increase in speed of the contralateral paw ($\sim 127\%$ at 15 min) while EM-1 (1 μM) caused a biphasic effect characterized by a prompt transient increase ($\sim 58\%$ at 15 min) then a long lasting decrease ($\sim 46\%$) in contralateral forepaw speed (Fig. 17B).

On step length at the ipsilateral forepaw (Fig. 17C), RM ANOVA showed a significant effect of treatment ($F_{2,12}=19.08$, $p=0.0002$), but not time ($F_{7,126}=1.40$, $p=0.2108$) and a significant time X treatment interaction ($F_{14,126}=33.62$, $p=0.0002$). EM-1 (0.01 μM) caused a mild and transient increase in step length ($\sim 15\%$ at 15 min) at the ipsilateral forepaw while EM-1 (1 μM) caused a mild, yet long lasting decrease (Fig. 17C).

On step length at the contralateral forepaw (Fig. 17D), RM ANOVA also showed a significant effect of treatment ($F_{2,12}=17.29$, $p=0.0003$), time ($F_{7,126}=10.99$, $p<0.0001$) and a significant time X treatment interaction ($F_{14,126}=6.02$, $p<0.0001$). Here, EM-1 (0.01 μM) caused a short-lived increase in step length ($\sim 28\%$ at 15 min) while EM-1 (1 μM) caused a delayed long-lasting reduction (Fig. 17D).

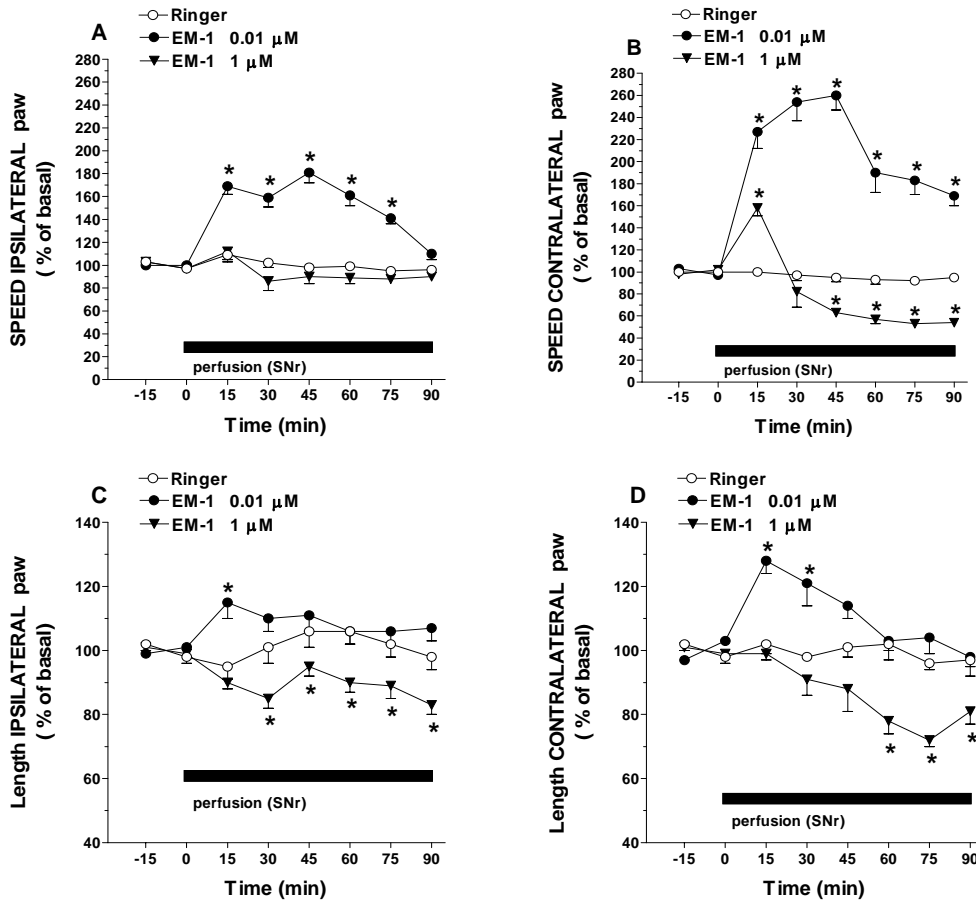


Fig 17. Effect of intranigral EM-1 (0.01-1μM) perfusion on speed and step length of contralateral and ipsilateral forepaws in the grid test. Data are expressed as percentages ± SEM (7 determinations per group) of basal pre-treatment levels (calculated as mean of the two samples before the treatment). Before testing, forepaw speed was 0.43 ± 0.03 and 0.47 ± 0.03 cm/sec for the ipsilateral and contralateral paw, respectively. Step length during movement on the grid was 6.7 ± 0.1 and 6.6 ± 0.2 cm at the ipsilateral and contralateral paw, respectively.

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

Neurochemical studies II

4.1.5 Effect of EM-1 on neurotransmitter release in rats undergoing the grid test

To correlate the behavioral effects of EM-1 to nigral release of GABA and GLU, we performed microdialysis in animals undergoing the grid test.

RM ANOVA on GABA release (Fig. 18A) showed a significant effect of treatment ($F_{2,12}=154.03$, $p < 0.0001$), time ($F_{7,126}=2.10$, $p=0.048$), and a significant time X treatment interaction ($F_{14,126}=7.81$, $p < 0.0001$). Post hoc analysis on nigral GABA release revealed dual

effects depending on concentration used where EM-1 (0.01 μM) caused an increase ($\sim 102\%$ at 45 min) and EM-1 (1 μM) caused a long lasting decrease ($\sim 46\%$ at 90 min; Fig. 18A).

RM ANOVA on nigral GLU levels (Fig. 18B) showed a non significant effect of treatment ($F_{2,12}=2.39$, $p=0.134$), but a significant time ($F_{7,126}=15.87$, $p<0.0001$), and a time X treatment interaction ($F_{14,126}=7.54$, $p<0.0001$). Post hoc analysis showed that EM-1 (0.01 μM) had no effect while EM-1 (1 μM) transiently increased ($\sim 87\%$ at 15 min) nigral GLU levels (Fig. 18B).

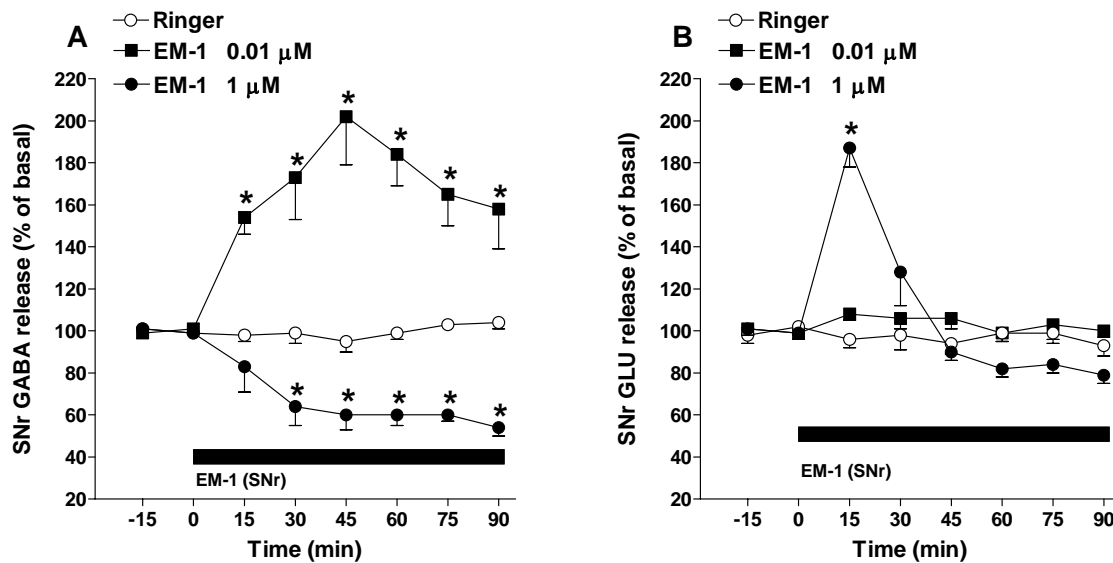


Fig 18. Effect of EM-1 perfusions on nigral GABA (A) and GLU (B) release of rats undergoing the grid test. Data are expressed as percentages \pm SEM of basal pre-treatment levels (calculated as mean of the two samples before the treatment). Basal GABA levels in the SNr were 9.0 ± 0.8 nM while GLU levels were 98.1 ± 13.2 nM.

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

4.1.6 Effect of perfusion with EM-1 in SNr on striatal DA and thalamic GABA release

To test whether EM-1 differentially modulates nigro-striatal DA and nigro-thalamic GABA transmission, low and high EM-1 concentrations were perfused in SNr, and DA release was monitored in DLS together with GABA release in VMTh.

RM ANOVA on DA release (Fig. 19A) showed a significant effect of treatment ($F_{3,24}=149.10$, $p<0.0001$), time ($F_{7,153}=5.41$, $p=0.0011$), and a significant time X treatment interaction ($F_{21,153}=5.95$, $p=0.0001$). Post hoc analysis revealed that EM-1 was ineffective at 0.01 μM but caused a prolonged increase in striatal DA release at 1 μM ($\sim 33\%$ at 75 min) which was blocked by the co-perfusion of CTOP (3 μM ; Fig. 19A).

RM ANOVA on thalamic GABA levels (Fig. 19B) revealed a significant effect of treatment ($F_{2,12}=17.46$, $p<0.0001$), time ($F_{7,126}=7.51$, $p<0.0001$), and a significant time X treatment interaction ($F_{14,126}=15.82$, $p<0.0001$). Post hoc analysis revealed that intranigral EM-1 (0.01 μM) caused a long-lasting reduction in thalamic GABA levels ($\sim 39\%$ at 45 min) while EM-1 μM induced a biphasic response characterized by a mild and transient reduction ($\sim 24\%$ at 15 min) followed by a robust and prolonged stimulation ($\sim 99\%$ at 90 min; Fig. 19B).

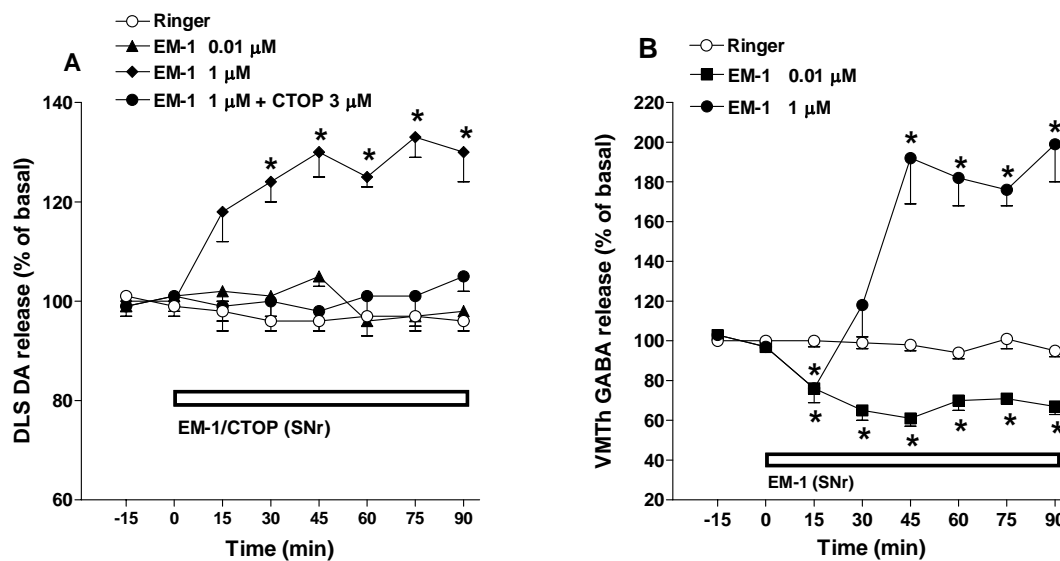


Fig 19. Effect of intranigral EM-1 (0.01-1 μM) perfusion on DA release in DLS (A) and GABA release in VMTh (B). Data are expressed as percentages \pm SEM of basal pre-treatment levels (calculated as mean of the two samples before the treatment). Basal extracellular DA levels in DLS were 1.81 ± 0.23 nM and basal GABA levels in VMTh were 8.0 ± 0.6 nM. Determinations for DLS DA were made from 5-8 per group whereas they were 7 for thalamic groups.

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

4.1.7 TTX sensitivity to modulation of EM-1 effects on nigral GABA

We then investigated the nature of EM-1 modulation of GABA release in SNr. To determine whether EM-1 modulated GABA release through direct or indirect mechanisms, intranigral EM-1 was paired with intrastriatal and/or intranigral perfusion with the Na^+ -dependent channel blocker TTX (1 μM).

RM ANOVA on TTX perfusions against EM-1 0.01 μM (Fig. 20A) showed a significant effect of treatment ($F_{5,39}=22.36$, $p<0.0001$), time ($F_{7,23}=23.234$, $p<0.0001$), and a significant time X treatment interaction ($F_{23,237}=3.55$, $p<0.0001$). Post hoc analysis revealed that perfusion with TTX in DLS or in DLS and SNr reduced nigral GABA release ($\sim 40\%$; Fig. 20A). Moreover, the

increase evoked by EM-1 (0.01 μM) release was attenuated by perfusion of TTX in DLS and abolished in the presence of a combination of intrastriatal and intranigral TTX (Fig. 20A).

RM ANOVA on TTX perfusion against EM-1 1 μM (Fig. 20B) showed a significant effect of treatment ($F_{3,27}=20.326$, $p<0.0001$), time ($F_{7,31}=6.32$, $p<0.0001$) and time X treatment interaction ($F_{31,123}=2.22$, $p<0.001$). TTX did not reduce GABA release in SNr nor did it prevent the reduction induced by EM-1 1 μM .

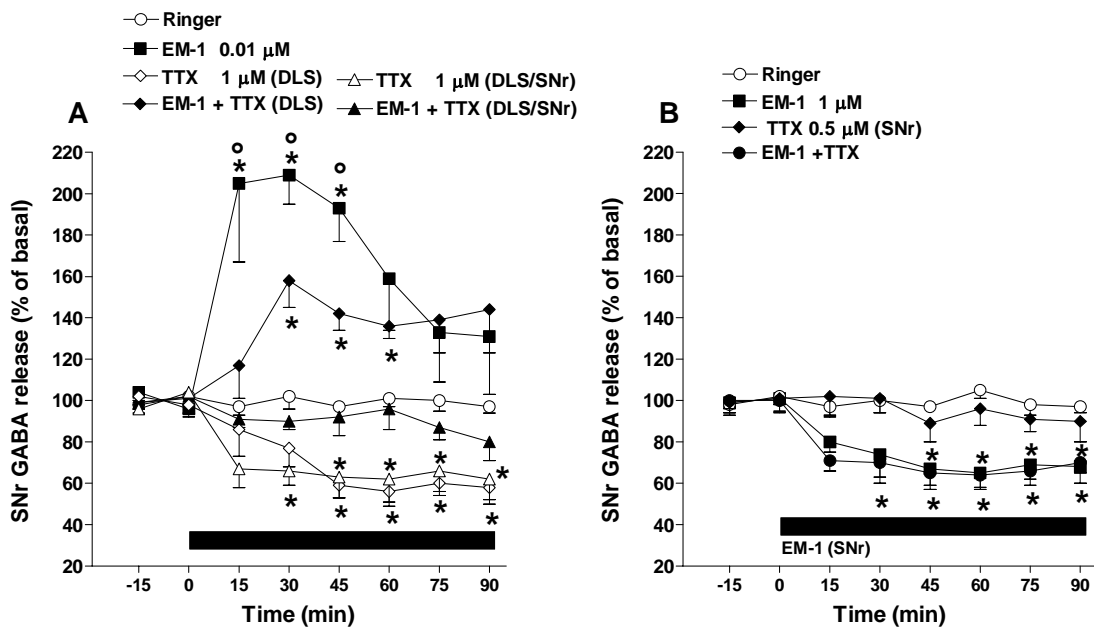


Fig 20. Effect of the voltage operated Na^+ channel blocker TTX on EM-1-evoked nigral GABA release. Effect of reverse dialysis of TTX (1 μM ; 90 min) singularly in the DLS, SNr or simultaneously in both areas, alone or in combination with EM-1 (0.01 and 1 μM , 90 min; black bar) in SNr of awake rats. Perfusion with TTX started 90 min before EM-1 and continued until the end of experiment. Data are expressed as percentages \pm SEM of basal pre-treatment levels (calculated as mean of the two samples before the treatment). Basal GABA levels were 7.1 ± 1.3 nM

* $p < 0.05$ different from Ringer

$^{\circ}p < 0.05$ different from EM-1 + TTX in DLS (RM ANOVA followed by contrast analysis and the sequentially rejective Bonferroni's test).

4.1.8 Modulation of EM-1 effects on nigral GABA by DAergic and GABAergic antagonists

To investigate the involvement of GABAergic and DAergic mechanisms on extracellular GABA release in SNr, EM-1 (0.01 and 1 μM) was perfused in combination with the GABA_A receptor antagonist bicuculline (3 μM), the D₁-like receptor antagonist SCH23390 (0.1 μM) and the D₂-

like receptor antagonist raclopride (1 μM). None of these compounds alone affected local GABA levels (Fig. 21A).

RM ANOVA on the effect of EM-1 (0.01 μM) on nigral GABA levels revealed a significant effect of treatment ($F_{4,35}=50.6$, $p<0.0001$), time ($F_{7,198}=9.78$, $p<0.0001$) and time X treatment interaction ($F_{28,198}=3.75$, $p<0.001$). Post hoc analysis showed that EM-1 (0.01 μM) increased GABA release ($\sim 93\%$), bicuculline and raclopride prevented this effect and SCH23390 only reduced its duration (Fig 21B).

RM ANOVA on EM-1 (1 μM) treatment on nigral GABA levels revealed a significant effect of treatment ($F_{4,24}=10.69$, $p<0.0001$), time ($F_{7,186}=9.43$, $p<0.0001$) and time X treatment interaction ($F_{28,186}=2.27$, $p=0.017$). EM-1 (1 μM) caused a long lasting decrease ($\sim 36\%$ at 90 min; Fig. 21C). This effect being completely prevented by bicuculline and left unchanged by raclopride or SCH23390 (Fig. 21C).

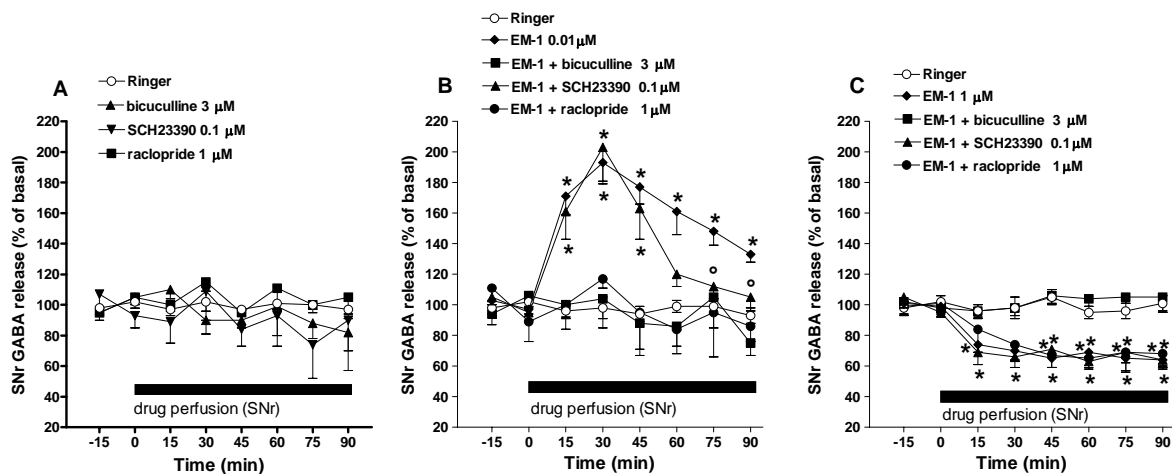


Fig 21. Effect of perfusion of selective antagonists at GABA_A, D₁ and D₂ receptors in SNr on EM-1-evoked nigral GABA release. Effect of reverse dialysis of bicuculline (3 μM), SCH23390 (0.1 μM) and raclopride (1 μM) alone (A) or in combination with EM-1 0.01 μM (90 min; black bar; B) or EM-1 1 μM (90 min; black bar; C) in SNr of awake rats. Perfusion with antagonists started 90 min before EM-1 and continued until the end of experiment. Data are expressed as percentages \pm SEM of basal pre-treatment levels (calculated as mean of the two samples before the treatment). Basal GABA levels were 8.1 ± 1.6 nM and made from 5-8 determinations per group.

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

Section II

Stimulation of delta opioid receptors located in substantia nigra reticulata but not globus pallidus or striatum restores motor activity in 6-hydroxydopamine lesioned rats.

Behavioral Studies

4.2.1 Effect SNC-80 on motor behavior

To investigate whether DOP receptor agonists could attenuate parkinsonian-like symptoms, SNC-80 was administered systemically (i.p.) and motor activity evaluated by the bar, drag and rotarod tests. SNC-80 (0.1-10 mg/kg) dose-dependently improved rat motor performance in all tests performed (Fig. 22).

In the bar test (Fig. 22A), RM ANOVA showed a significant effect of treatment ($F_{4,28}=42.52$, $p<0.0001$), but not time ($F_{1,37}=0.05$, $p=0.82$), and a significant time X treatment interaction ($F_{4,37}=5.23$, $p=0.0019$) at the contralateral paw. RM ANOVA at the ipsilateral paw led to similar results, namely a significant effect of treatment ($F_{4,28}=16.43$, $p<0.0001$), but not time ($F_{1,37}=1.32$, $p=0.26$) or time X treatment interaction ($F_{4,37}=2.08$, $p=0.10$). Post hoc analysis at 20 min after injection revealed that SNC-80 dose-dependently reduced the immobility time at both the contralateral and ipsilateral paws (~89% and ~85%, respectively with highest dose tested; Fig. 22A). Threshold dose was 1 mg/kg and maximal effect was observed with 10 mg/kg. SNC-80 effect persisted up to 70 min but was larger at the contralateral paw.

RM ANOVA showed an overall effect of SNC-80 on the contralateral paw (Fig. 22B) when performed in the drag test ($F_{4,32}=26.45$, $p<0.0001$). However no effect of time ($F_{1,44}=1.09$, $p=0.30$) or time X treatment interaction ($F_{1,44}=1.02$, $p=0.40$) were seen. The number of steps made with the contralateral paw was increased in a prolonged fashion by SNC-80, the 1 mg/kg dose being the threshold. Maximal increase was observed at 10 mg/Kg SNC-80 (~154%). No change was observed at the ipsilateral paw (Fig. 22B).

RM ANOVA on time spent on the rotarod (Fig. 22C) showed an overall effect of SNC-80 ($F_{4,28}=29.91$, $p<0.0001$) but no effect of time ($F_{1,33}=0.90$, $p=0.35$) or a time X treatment interaction ($F_{4,33}=0.64$, $p=0.63$). Post hoc analysis at 20 min revealed an increase in rotarod performance with at 3 mg/kg (~62%) and 10 mg/kg (~101%) SNC-80 (Fig. 22C). This effect persisted at 70 min following treatment.

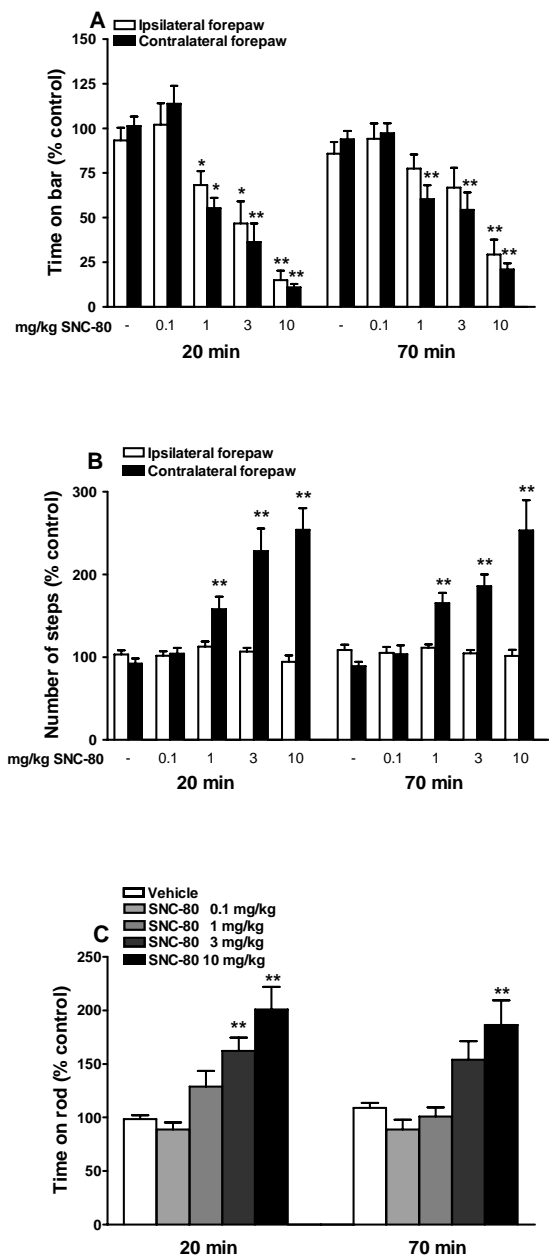


Fig 22. Effect of systemic administration of the nonpeptide DOP receptor agonist SNC-80 (0.1-10 mg/kg, i.p.) in the bar (A) drag (B) and rotarod (C) tests. Each experiment consisted of three different sessions: a control session followed by other two sessions performed 20 and 70 min after vehicle or SNC-80 administration (see Methods). Data are expressed as percentages of basal motor activity in the control session and are means \pm SEM of 8-10 determinations per group. Basal motor activity in the bar test was (sec) 19.4 ± 1.8 (ipsilateral paw) and 35.0 ± 2.2 (contralateral paw). Basal motor activity in the drag test was (number of steps) 10.7 ± 0.3 (ipsilateral paw) and 2.1 ± 0.1 (contralateral paw). Rotarod performance was 540 ± 40 sec (0-55 rpm range).

* $p < 0.05$, ** $p < 0.01$, significantly different from vehicle (RM ANOVA followed by contrast analysis and the sequentially rejective Bonferroni's test).

4.2.2 Effects of the DOP receptor antagonist NTD on motor behavior

To unravel tonic influence of DOP receptors on motor activity the DOP receptor antagonist NTD was given systemically. NTD (0.1-5 mg/kg) dose-dependently impaired motor behavior in all tests performed.

In the bar test (Fig. 23A), RM ANOVA showed a main effect of treatment ($F_{3,15}=5.55$, $p=0.0091$) but not time ($F_{1,18}=0.62$, $p=0.43$), and a significant time X treatment interaction ($F_{3,18}=3.22$, $p=0.047$) at the contralateral paw. Post hoc analysis revealed that NTD increased immobility time at 5 mg/kg (~78%), although a delayed effect was observed also with 1 mg/kg (Fig. 23A). RM ANOVA at the ipsilateral paw showed a significant effect of treatment ($F_{3,15}=15.15$, $p<0.0001$) but not time ($F_{1,18}=4.37$, $p=0.051$) or a time X treatment interaction ($F_{3,18}=1.61$, $p=0.22$). NTD produced a robust elevation in immobility time at 5 mg/kg both at 20 and 70 min after injection (Fig. 23A).

RM ANOVA on the number of steps at the contralateral paw (Fig. 23B) showed an overall effect of NTD ($F_{3,15}=22.11$, $p<0.0001$) but not time ($F_{1,18}=1.13$, $p=0.30$), and a significant time X treatment interaction ($F_{3,18}=3.16$, $p=0.049$). NTD 1 and 5 mg/kg produced significant decreases in stepping activity both at 20 min (~37% and ~45%, respectively) and 70 min (~24% and ~52%, respectively) after administration (Fig. 23B). A delayed decrease in stepping was also detected with the lowest 0.1 mg/kg dose (~28%). No effect was seen in the ipsilateral paw in the drag test (Fig. 23B).

RM ANOVA showed an overall effect of treatment in the rotarod test ($F_{3,15}=27.47$, $p<0.0001$) though no effect of time ($F_{1,18}=0.29$, $p=0.59$) or a time X treatment interaction ($F_{3,18}=1.41$, $p=0.27$) were detected (Fig. 23C). Post hoc analysis revealed that NTD decreased rotarod performance at 5 mg/kg both at 20 min (~40%) and 70 min (~55%) after treatment (Fig. 23C). A delayed reduction in rotarod performance was also observed at 0.1 mg/kg (~25%) and 1 mg/kg (~34%) doses 70 min after treatment (Fig. 23C).

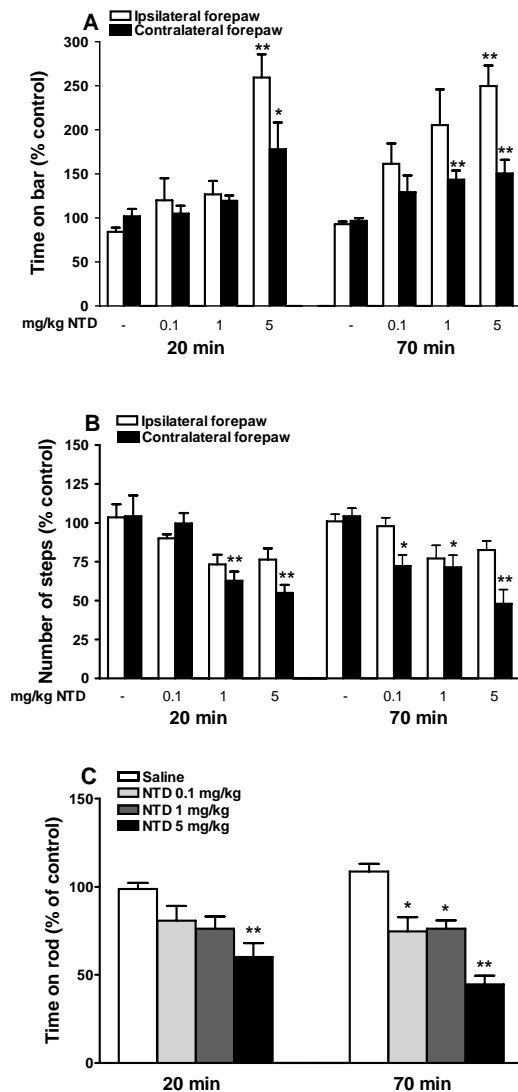


Fig 23. Effect of systemic administration of the DOP receptor antagonist naltrindole (NTD; 0.1-5 mg/kg, i.p.) in the bar (A) drag (B) and rotarod (C) tests. Each experiment consisted of three different sessions: a control session followed by other two sessions performed 20 and 70 min after vehicle or NTD administration (see Materials and Methods). Data are expressed as percentages of basal motor activity in the control session and are means \pm SEM of 6 determinations per group. Basal motor activity in the bar test was (sec) 19.8 ± 2.5 (ipsilateral paw) and 35.2 ± 2.9 (contralateral paw). Basal motor activity in the drag test was (number of steps) 10.2 ± 0.7 (ipsilateral paw) and 2.4 ± 0.3 (contralateral paw). Rotarod performance was 600 ± 32 sec (0-55 rpm range).

* $p < 0.05$, ** $p < 0.01$, significantly different from saline (RM ANOVA followed by contrast analysis and the sequentially rejective Bonferroni's test).

Neurochemical Studies

4.2.3 Effect of systemic administration of DOP receptor ligands on GABA and GLU release in GP and SNr

On the basis of an in vitro study (Maneuf et al., 1994), reduction in GABA release in GP has been proposed as the mechanism underlying the antiparkinsonian action of DOP receptor agonists. We therefore used microdialysis to monitor amino acid levels following behaviorally effective doses of SNC-80 (3 mg/kg). Moreover, NTD (1 and 5 mg/kg) was administered to investigate whether endogenous ENK were involved in tonic regulation of GP amino acid levels. RM ANOVA revealed a significant effect of treatment ($F_{3,15}=16.19$, $p<0.0001$) and time ($F_{5,100}=4.15$, $p=0.0018$), and a time X treatment interaction ($F_{15,100}=3.15$ $p=0.0003$). Post hoc analysis revealed that SNC-80 decreased GABA levels compared to vehicle treated animal (maximal of ~57%; Fig. 24A). Conversely, NTD (5 mg/kg) evoked a robust increase in extracellular GABA levels (~154% at the 30 min time-point) while the lower dose (1 mg/kg) was ineffective (Fig. 24A). Additionally, extracellular GLU levels in the GP were not affected by SNC-80 or NTD (Fig. 24B).

Since microdialysis data lend support to the notion that DOP receptor agonists modulate the indirect pathway, amino acid levels were also monitored in the ipsilateral SNr, which is a target of subthalamonigral GLUergic projections. RM ANOVA on GABA levels revealed a significant effect of treatment ($F_{3,12}=7.48$, $p=0.004$) but not time ($F_{5,80}=1.01$, $p=0.419$) or a time X treatment interaction ($F_{15,80}=1.36$, $p=0.187$). SNC-80 (3 mg/kg) induced a long-lasting decrease in extracellular GABA levels in the SNr with maximal inhibition of ~69% as did NTD (5 mg/kg; maximal inhibition of ~54%; Fig. 24C) Conversely, NTD (1 mg/kg) had no effect. RM ANOVA on GLU levels revealed a significant effect of treatment ($F_{3,12}=6.41$, $p=0.0077$) but not time ($F_{5,80}=0.8$, $p=0.55$) or a time X treatment interaction ($F_{15,80}=0.67$, $p=0.80$). SNC-80 was effective in evoking a long lasting decrease in extracellular GLU levels (~54%) while NTD had no effect at either dose tested (Fig. 24D).

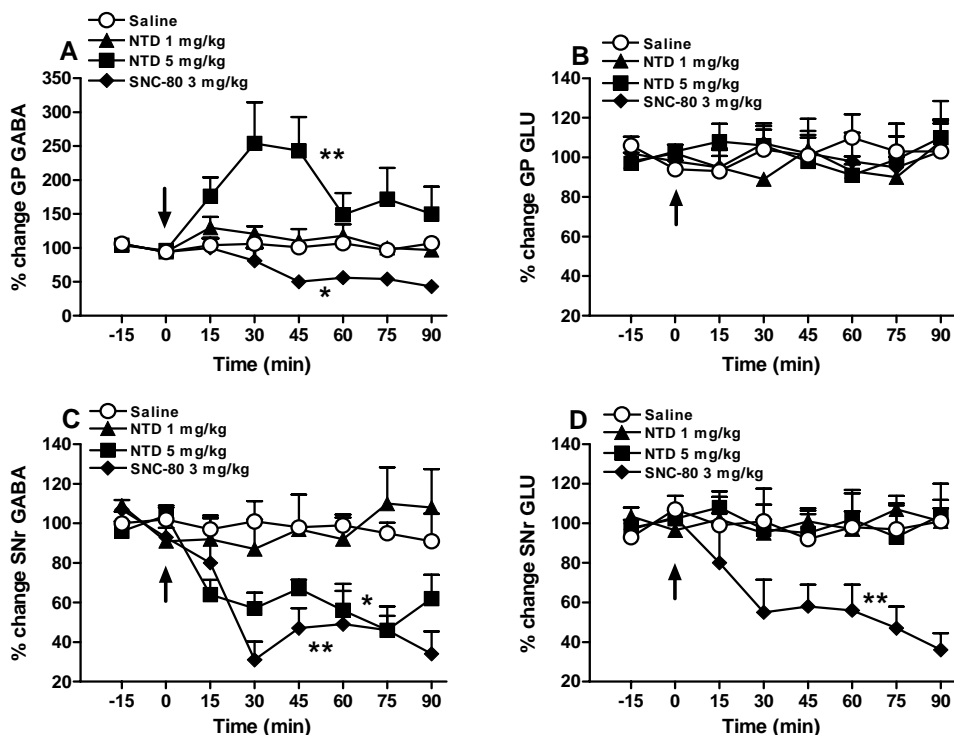


Fig 24. Effect of systemic (i.p.) administration (arrow) of behaviorally relevant doses of SNC-80 (3 mg/kg) and NTD (1 and 5 mg/kg) on extracellular GABA (A-C) and GLU (B-D) levels in SNr. Data are means \pm SEM of 7 (A-B) or 5 (C-D) experiments per group and are expressed as percent baseline (calculated as the mean of the two samples before the treatment). Basal GABA levels in the dialysate were 5.0 ± 1.2 (A) and 6.2 ± 1.4 nM (C) while basal GLU levels were 43 ± 7.3 nM (B) and 62.0 ± 8.1 nM (D).

* $p < 0.05$, ** $p < 0.01$, significantly different from saline (RM ANOVA followed by contrast analysis)

4.2.4 Effect of regional NTD perfusion on the SNC-80 induced antiakinetetic effect

Although microdialysis data were consistent with an action of DOP receptor agonists at the GP level, SNC-80 actions at the nigral or even striatal levels could not be ruled out. Therefore to more specifically investigate the regional specificity of the antiakinetetic effect of SNC-80, a combined microdialysis and behavioral approach was undertaken. Systemic SNC-80 was combined with perfusion of NTD, alternatively in SNr, GP or DLS, in order to obtain a region-selective blockade of DOP receptors. A NTD concentration of 50 nM was chosen since this concentration is expected to provide ~ 5 nM NTD in the surrounding area of the probe (based on a $\sim 10\%$ in vitro recovery). Considering the subnanomolar binding affinity of NTD at DOP receptors (~ 0.16 - 0.25 nM; Rogers et al., 1990, Spetea et al., 1998) and the ~ 80 -fold DOP/KOP

and ~330-fold DOP/MOP selectivity (Spetea et al., 1998), 5 nM appears to be a concentration selective for DOP receptor blockade.

RM ANOVA on immobility time at the contralateral paw (Fig. 25A) showed a significant effect of treatment ($F_{4,24}=27.06$, $p<0.0001$), time ($F_{5,128}=3.47$, $p=0.0056$) but not a significant time X treatment interaction ($F_{30,128}=0.43$, $p=0.99$). Post hoc analysis revealed that 3 mg/kg SNC-80 evoked a rapid onset and prolonged attenuation of akinesia (~57% reduction with respect to basal values; Fig. 25A). NTD perfusion in SNr but not GP or DLS prevented it. Similar data were found at the ipsilateral paw (Fig. 25B). RM ANOVA showed a significant effect of treatment ($F_{4,24}=11.31$, $p<0.0001$), but not time ($F_{5,116}=1.37$, $p=0.24$) or a significant time X treatment interaction ($F_{30,116}=0.42$, $p=0.99$). As for the ipsilateral paw, SNC-80 reduced immobility time, this effect again being prevented by SNr but not GP or DLS perfusion of NTD (Fig. 25B). NTD perfusion alone in each nucleus did not affect the immobility time at the contralateral or ipsilateral paw.

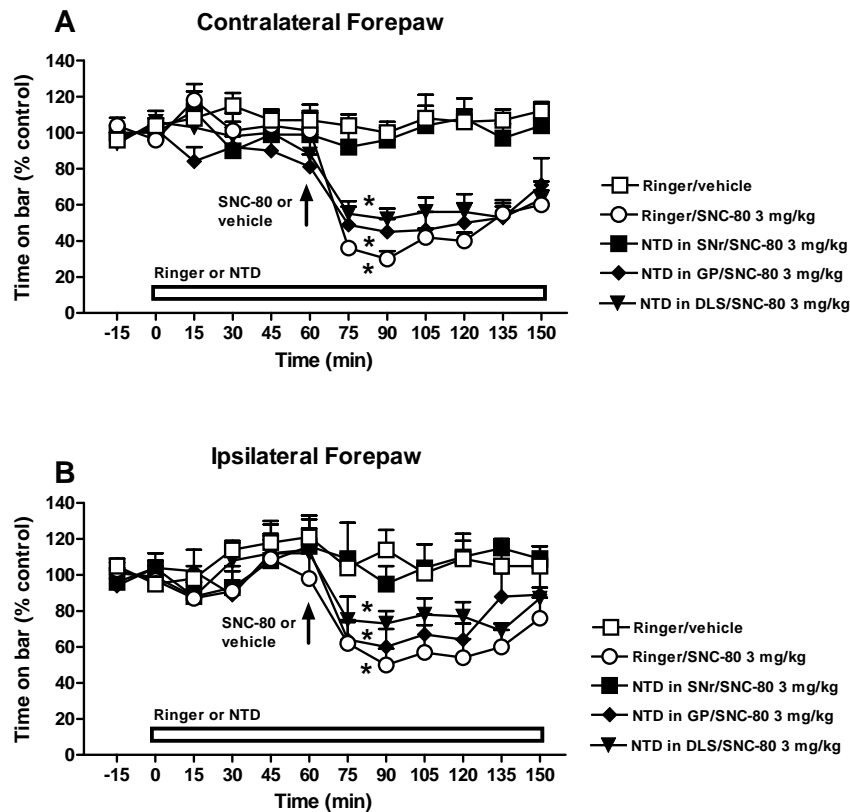


Fig 25. Effect of systemic (i.p.) administration (arrow) of a behaviorally effective dose of SNC-80 (3 mg/kg) while simultaneously perfusing NTD (50 nM, open bar) in SNr, GP or DLS. NTD perfusion started 60 min prior to SNC-80 and continued until the end of experiment. Rats were challenged in the bar test (described in the Methods) while undergoing microdialysis. Akinesia was evaluated (every 15 min) separately at the contralateral (A, n=7) and ipsilateral (B, n=6) forepaws. Data are expressed as mean percentages \pm SEM of motor activity at basal levels. Basal motor activity in the bar test (sec) was 34.5 ± 1.4 and 13.1 ± 1.2 at the contralateral and ipsilateral paw, respectively. * $p < 0.05$, significantly different from vehicle (RM ANOVA followed by contrast analysis)

4.2.5 Effect of regional NTD perfusion on SNC-80 induced neurotransmitter release

Since the behavioral data pointed to the involvement of nigral DOP receptors in the antiakinetik action of SNC-80, we investigated whether NTD perfusion in SNr also prevented the neurotransmitter changes induced by SNC-80.

RM ANOVA on GABA release in SNr (Fig. 26A) showed a significant effect of treatment ($F_{3,18}=12.55$, $p < 0.0001$), time ($F_{5,132}=2.83$, $p=0.0184$) and a significant time X treatment interaction ($F_{15,132}=1.92$, $p=0.0259$). Post hoc analysis showed that SNC-80 reduced nigral GABA release (maximal $\sim 46\%$ reduction) and NTD perfusion in SNr prevented it while NTD perfusion in GP was without effect (Fig. 26A). Similar results were found for GLU levels (Fig.

26B). RM ANOVA showed a significant effect of treatment ($F_{3,18}=5.71$, $p=0.0062$), time ($F_{5,132}=2.83$, $p=0.0184$) and a significant time X treatment interaction ($F_{15,132}=1.92$, $p=0.0259$). SNC-80 reduced GLU levels (maximal ~45% reduction) and intranigral perfusion with NTD prevented it. Conversely, NTD perfusion in GP was without effect. NTD perfusion in the SNr or GP did not affect amino acid levels in SNr (Fig. 26A-B).

RM ANOVA on GABA release in GP showed a significant effect of treatment ($F_{3,15}=6.37$, $p=0.0053$), time ($F_{5,106}=8.13$, $p<0.0001$) but not a significant time X treatment interaction ($F_{15,106}=1.70$, $p=0.062$). Post hoc analysis showed that SNC-80 reduced pallidal GABA release (maximal ~48% reduction; Fig. 26C). NTD perfusion in SNr prevented it while NTD perfusion in GP attenuated it. No changes in GLU levels were observed after systemic administration of SNC-80 alone or simultaneously with NTD perfusion in SNr or GP (Fig. 26D).

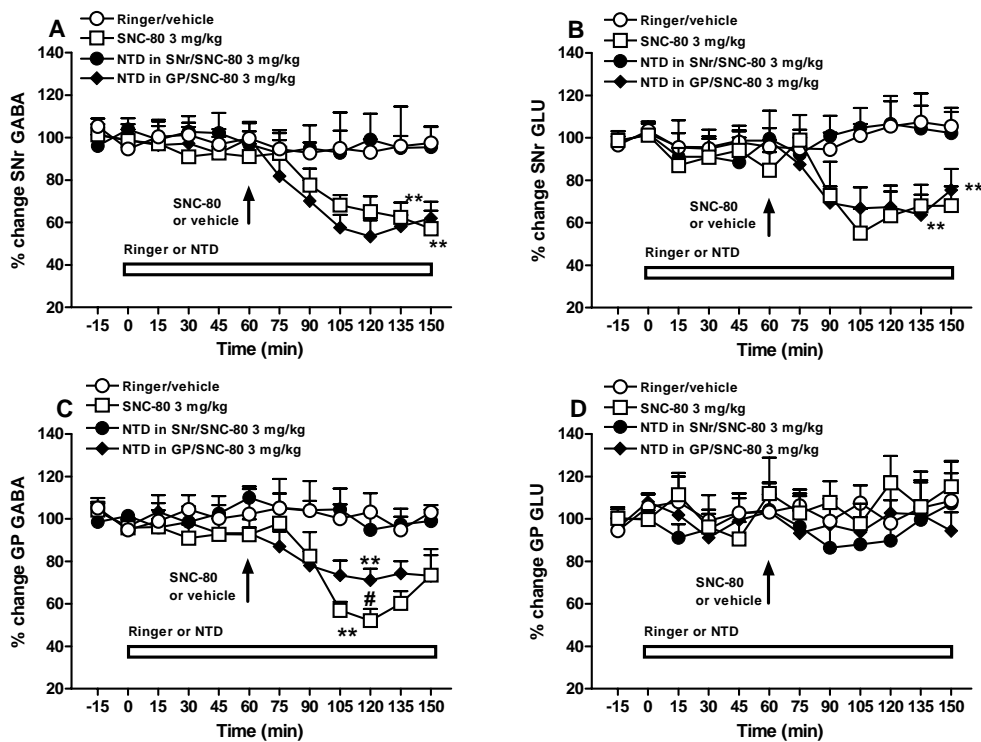


Fig 26. Effect of perfusion of naltrindole (NTD) in SNr or GP on nigral and pallidal GABA and GLU changes induced by SNC-80. Rats treated (arrow) with vehicle or SNC-80 (3 mg/kg) were simultaneously perfused in SNr or GP with NTD (50 nM, open bar). Extracellular GABA and GLU levels in SNr (A-B; $n=7$) and GP (C-D; $n=6$) were monitored. Data are expressed as means \pm SEM of pre-treatment (basal) values. Basal GABA and GLU levels (nM) in the dialysate from SNr were 6.5 ± 1.9 and 33.2 ± 3.7 , respectively, while basal GABA and GLU levels in the dialysate from GP were 4.8 ± 0.8 and 33.8 ± 5.5 , respectively.

* $p<0.05$, ** $p<0.01$, significantly different from vehicle

$p<0.05$, significant difference between drug treated groups (RM ANOVA followed by contrast analysis)

Behavioral Studies II

4.2.6 Effect of SNC-80 microinjections into SNr, GP and DLS on motor behavior

Microdialysis coupled to the bar test clearly indicated that DOP receptors in SNr were involved in the antiakinetetic action of SNC-80. To further confirm this view and to also rule out the possibility that DOP receptors in GP or DLS modulate motor parameters other than akinesia (e.g. bradykinesia), SNC-80 was microinjected (0.1 and 1 nmol) into SNr, GP or DLS and motor activity evaluated in the three tests.

RM ANOVA on immobility time at the contralateral paw following intranigral injection of SNC-80 (Fig. 27A) showed a significant effect of treatment ($F_{2,12}=18.56$, $p<0.0001$), but not time ($F_{1,16}=0.53$, $p=0.43$) or time X treatment interaction ($F_{2,16}=2.47$, $p=0.11$). Post hoc analysis revealed that SNC-80 evoked a long-lasting reduction in immobility time at both doses (maximal 49% at 70 min with 1 nmol; Fig. 27A). RM ANOVA on immobility time at the ipsilateral paw also showed a significant effect of treatment ($F_{2,12}=8.54$, $p=0.0049$), but not time ($F_{1,16}=0.64$, $p=0.53$) or time X treatment interaction ($F_{2,16}=0.58$, $p=0.56$). SNC-80 evoked a transient inhibition (maximal ~36%) of akinesia at the lower 0.1 nmol dose (Fig. 27A).

RM ANOVA on number of steps at the contralateral paw (Fig. 27B) showed a significant effect of treatment ($F_{2,12}=10.25$, $p=0.0025$), but not time ($F_{1,16}=0.59$, $p=0.45$) or time X treatment interaction ($F_{2,16}=0.34$, $p=0.71$). SNC-80 increased stepping activity at 0.1 and 1 nmol (to a maximal ~58% and ~122%, respectively; Fig. 27B). This effect was also observed after 70 min from injection. No change in stepping activity was observed at the ipsilateral paw.

RM ANOVA on time on the rotarod showed a significant effect of treatment ($F_{2,12}=36.46$, $p<0.0001$), but not time ($F_{1,16}=0.15$, $p=0.69$) or time X treatment interaction ($F_{2,16}=0.25$, $p=0.77$). SNC-80 dose-dependently improved rotarod performance (~75% and ~147% improvement at 0.1 and 1 nmol, respectively) when injected in SNr (Fig. 27C).

Microinjection of SNC-80 in GP or DLS failed to alter motor activity in any of the tests performed (Fig. 28).

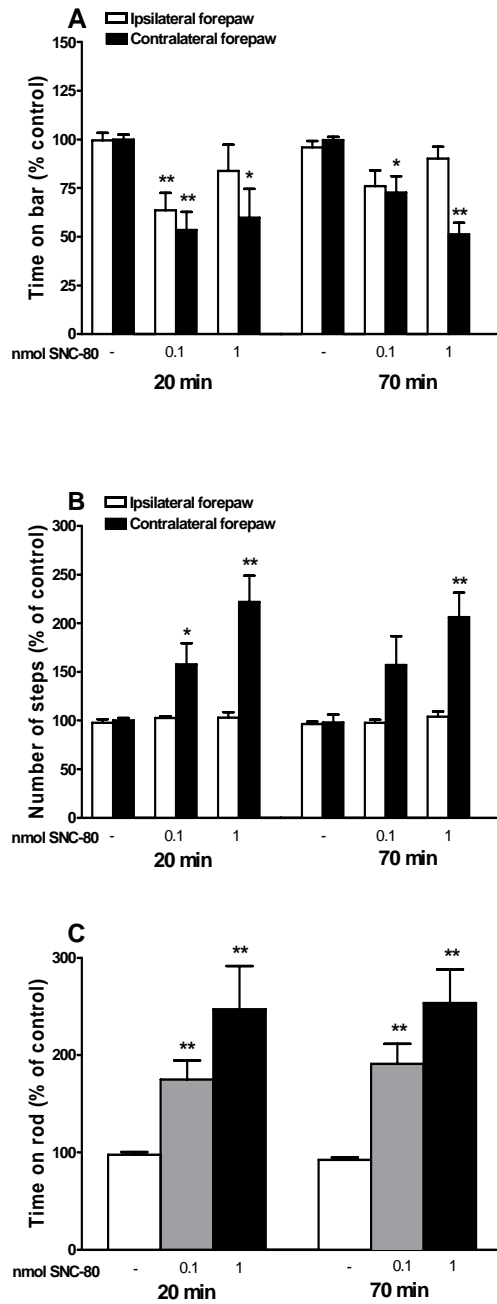


Fig 27. Effect of microinjection of SNC-80 in SNr (0.1 and 1 nmol) in the bar (A) drag (B) and rotarod (C) tests. Each experiment consisted of three different sessions: a control session followed by other two sessions performed 20 and 70 min after vehicle or SNC-80 microinjection. Data are expressed as percentages of basal motor activity in the control session and are means \pm SEM of 7 determinations per group. Basal motor activity in the bar test was (sec) 25.6 ± 4.6 (ipsilateral paw) and 38.5 ± 4.9 (contralateral paw). Basal motor activity in the drag test was (number of steps) 10.8 ± 0.4 (ipsilateral paw) and 3.2 ± 0.6 (contralateral paw). Rotarod performance was 345 ± 55 sec (0-55 rpm range).

* $p < 0.05$, ** $p < 0.01$, significantly different from saline (RM ANOVA followed by contrast analysis and the sequentially rejective Bonferroni's test).

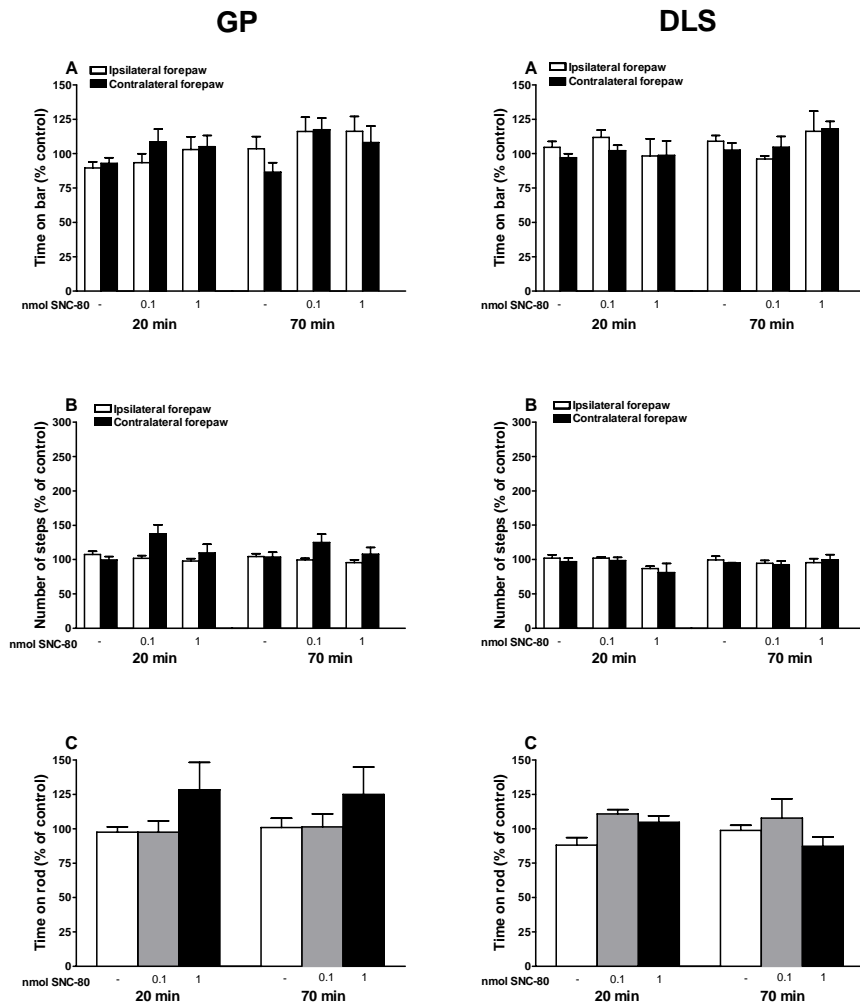


Fig 28. Effects of microinjection of SNC-80 in GP and DLS (0.1 and 1 nmol in 0.5 μ l saline) in the bar (A) drag (B) and rotarod (C) tests. Each experiment consisted of three different sessions: a control session followed by other two sessions performed 20 and 70 min after vehicle or SNC-80 microinjection (see Materials and Methods). Data are expressed as percentages of basal motor activity in the control session and are means \pm SEM of 7 determinations per group. Basal motor activity in the bar test was (sec) 25.6 ± 4.6 (ipsilateral paw) and 38.5 ± 4.9 (contralateral paw). Basal motor activity in the drag test was (number of steps) 10.8 ± 0.4 (ipsilateral paw) and 3.2 ± 0.6 (contralateral paw). Rotarod performance was 345 ± 55 sec (0-55 rpm range).

4.2.7 Effect of bicuculline microinjections in GP on motor behavior

Finally, to confirm that blockade of the striato-pallidal pathway under our experimental conditions leads to attenuation of parkinsonism, the GABA_A receptor antagonist bicuculline was injected in GP at a dose effective in reversing catalepsy in reserpinized rats (1.5 nmol; Maneuf et al., 1994).

RM ANOVA on immobility time at the contralateral paw following pallidal injection of bicuculline (Fig. 29A) showed a significant effect of treatment ($F_{1,4}=37.51$, $p=0.004$), time ($F_{1,8}=8.03$, $p=0.022$) but not a time X treatment interaction ($F_{1,8}=4.89$, $p=0.06$). Bicuculline evoked a short-lasting reduction in immobility time (~30%) since no effect was seen at the 70 min time point (Fig. 29A). There was no effect at the ipsilateral paw in this test.

RM ANOVA on number of steps at the contralateral paw (Fig. 29B) showed a significant effect of treatment ($F_{1,4}=70.58$, $p=0.001$), but not time ($F_{1,8}=3.88$, $p=0.08$), and a significant time X treatment interaction ($F_{1,8}=5.55$, $p=0.046$). Bicuculline transiently increased stepping activity (~54%; Fig. 29B). No change in stepping activity was observed at the ipsilateral paw.

RM ANOVA on time on rotarod showed a significant effect of treatment ($F_{1,4}=15.27$, $p=0.017$), time ($F_{1,8}=25.68$, $p=0.001$) and a time X treatment interaction ($F_{1,8}=22.55$, $p=0.001$). Pallidal injections of bicuculline transiently improved rotarod performance (~73%; Fig. 29C).

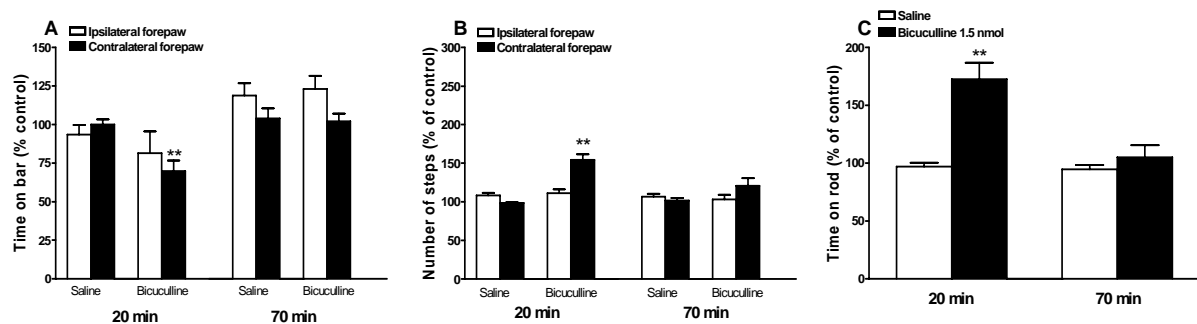


Fig 29. Effects of microinjection of bicuculline in GP (1.5 nmol) in the bar (A) drag (B) and rotarod (C) tests. Each experiment consisted of three different sessions: a control session followed by other two sessions performed 20 and 70 min after saline or bicuculline microinjection. Data are expressed as percentages of basal motor activity in the control session and are means \pm SEM of 5 determinations per group. Basal motor activity in the bar test was (sec) 19.5 ± 4.7 (ipsilateral paw) and 33.8 ± 7.3 (contralateral paw). Basal motor activity in the drag test was (number of steps) 11.2 ± 0.5 (ipsilateral paw) and 2.6 ± 0.6 (contralateral paw). Rotarod performance was 393 ± 39 sec (0-55 rpm range).

** $p < 0.01$, significantly different from saline (RM ANOVA followed by contrast analysis and the sequentially rejective Bonferroni's test)

Section III

The novel delta opioid receptor agonist UFP-512 dually modulates motor activity in experimental parkinsonism via control of the nigro-thalamic pathway

Behavioral Studies

4.3.1 Effect of UFP-512 on motor behavior

To investigate whether UFP-512 could attenuate parkinsonian-like symptoms, it was administered systemically over a wide range of doses (0.1-1000 $\mu\text{g}/\text{kg}$ i.p.) and motor activity evaluated by the drag and rotarod tests.

RM ANOVA on the number of steps at the contralateral paw in the drag test (Fig. 30A) showed an overall effect of treatment ($F_{5,35}=12.28$, $p<0.0001$) and time ($F_{1,32}=5.03$, $p=0.031$) but not a significant time X treatment interaction ($F_{5,32}=1.37$, $p=0.26$). Post hoc analysis at 20 min revealed that UFP-512 increased the number of steps at 1 and 10 $\mu\text{g}/\text{kg}$ (~81% and ~131%, respectively; Fig. 30A). This effect was not detected 70 min later and no effect was observed with higher doses (i.e. 100 or 1000 $\mu\text{g}/\text{kg}$) at either time point (Fig. 30A). No effect was seen at the ipsilateral paw (Fig. 30A).

RM ANOVA on rotarod time spent on the rotarod (Fig. 30B) showed a significant effect of treatment ($F_{5,30}=10.81$, $p<0.0001$), but not time ($F_{1,36}=1.62$, $p=0.21$), and a significant time X treatment interaction ($F_{5,36}=3.30$, $p=0.014$). Post hoc analysis at 20 min revealed that UFP-512 increased motor performance at 1 $\mu\text{g}/\text{kg}$ (~63%) and inhibited it at 100 $\mu\text{g}/\text{kg}$ (~36%) with no effect observed at 70 min following treatment (Fig. 30B).

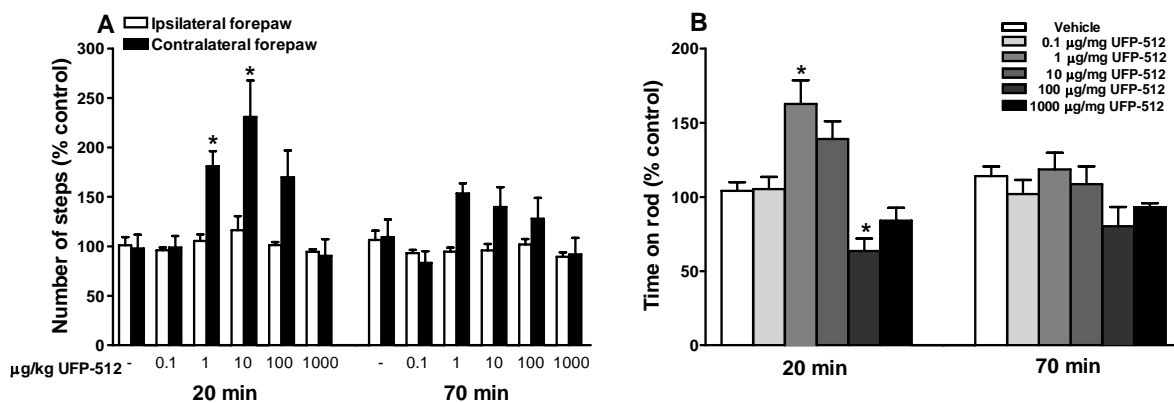


Fig 30. Effect of systemic administration of the novel DOP receptor agonist UFP-512 (0.1-1000 $\mu\text{g}/\text{kg}$ i.p.) in the drag (A) and rotarod (B) tests. Each experiment consisted of three different sessions: a control session followed by other two sessions performed 20 and 70 min after vehicle or UFP-512 administration. Data are expressed as percentages of basal motor activity in the control session and are means \pm SEM of 7 determinations per group. Basal motor activity values in the drag test were (number of steps): 10.0 ± 0.4 (ipsilateral paw) and 2.0 ± 0.1 (contralateral paw). The time spent on the rod (rotarod test) was 499 ± 25 sec (0-55 rpm range).

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

Neurochemical Studies

4.3.2 Effect of UFP-512 on GABA and GLU release in GP

Motor facilitating and inhibiting doses of UFP-512 were administered and in vivo microdialysis was performed to determine how the observed behavioural effects correlated with changes in amino acid neurotransmitters (GABA and GLU).

RM ANOVA on GABA release in GP (Fig. 31A) showed a significant effect of treatment ($F_{2,10}=61.84$, $p<0.0001$) but not time ($F_{7,105}=0.82$, $p=0.56$), and a significant time X treatment interaction ($F_{14,105}=4.85$, $p<0.0001$). Post hoc analysis revealed that UFP-512 10 $\mu\text{g}/\text{kg}$ caused a long-lasting reduction of GABA levels which was significant from 45 min after injection onward (maximal $\sim 35\%$ reduction; Fig. 31A). Conversely, UFP-512 1000 $\mu\text{g}/\text{kg}$ transiently increased GABA levels (maximal $\sim 45\%$), the effect being significant starting at 30 min after injection (Fig. 31A).

Extracellular GLU release was not affected by UFP-512 at either dose tested (Fig. 31B).

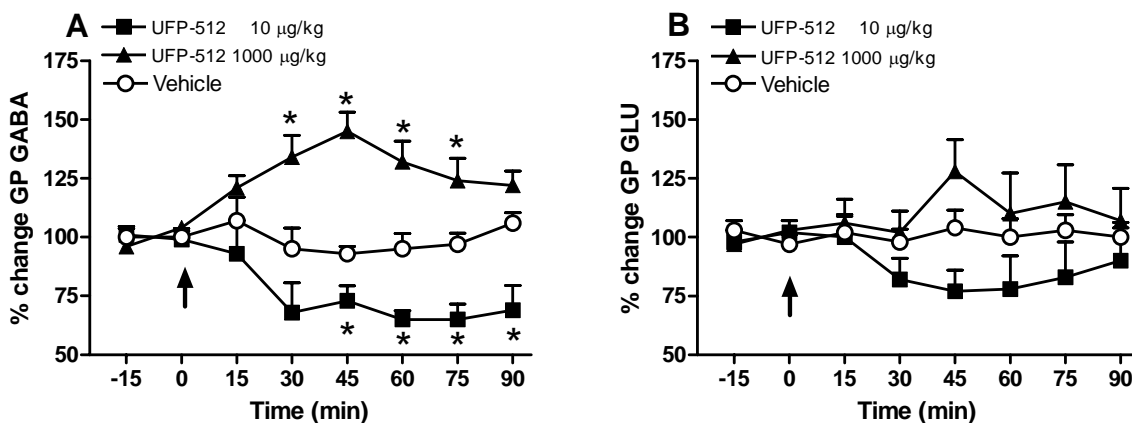


Fig 31. Effect of systemic (i.p.) administration (arrow) of facilitatory (10 $\mu\text{g}/\text{kg}$) and inhibitory (1000 $\mu\text{g}/\text{kg}$) doses of UFP-512 on extracellular GABA (A) and GLU (B) levels in GP. Data are means \pm SEM of 6 (A) or 7 (B) experiments per group and are expressed as percent baseline (calculated as the mean of the two samples before the treatment). Basal GABA levels in the dialysate were 3.4 ± 0.4 nM (A) while basal GLU levels were 62 ± 9.4 nM (B).

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

4.3.3 Effect of UFP-512 on GABA and GLU release in SNr

RM ANOVA on GABA release in SNr (Fig. 32A) showed a significant effect of treatment ($F_{2,10}=7.17$, $p=0.012$), time ($F_{7,105}=4.88$, $p<0.0001$), and a significant time X treatment interaction ($F_{14,105}=2.43$, $p=0.005$). Post hoc analysis revealed that UFP-512 10 $\mu\text{g}/\text{kg}$ caused a

rapid and prolonged decrease in GABA levels (maximal of ~34%) starting 15 min after drug injection (Fig. 32A). Also, UFP-512 1000 µg/kg caused a long lasting decrease (maximal ~30% reduction) in GABA levels which was somewhat delayed (significant from 45 min; Fig. 32A). RM ANOVA on extracellular GLU release in SNr (Fig. 32B) showed a significant effect of treatment ($F_{2,10}=15.69$, $p=0.0008$), but not time ($F_{7,105}=0.35$, $p=0.93$), and a significant time X treatment interaction ($F_{14,105}=2.76$, $p=0.0016$). Post hoc analysis revealed that UFP-512 10 µg/kg caused a transient decrease in GLU levels (maximal ~41%) at 30 and 45 min whereas UFP-512 1000 µg/kg caused a transient increase (maximal ~43%) at 45 after drug injection (Fig. 32B).

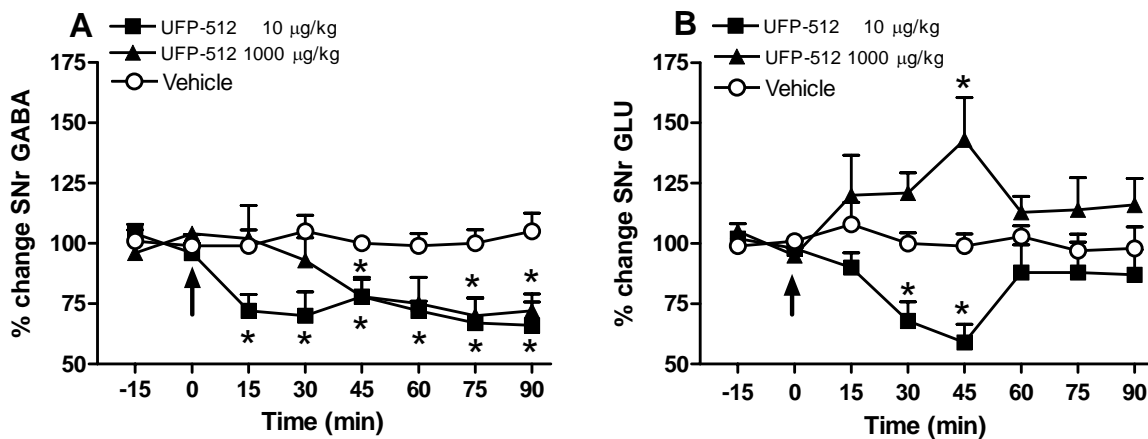


Fig 32. Effect of systemic (i.p.) administration (arrow) of facilitatory (10 µg/kg) and inhibitory (1000 µg/kg) doses of UFP-512 on extracellular GABA (A) and GLU (B) levels in SNr. Data are means \pm SEM of 6 experiments per group (A and B) and are expressed as percent baseline (calculated as the mean of the two samples before the treatment). Basal GABA levels in the dialysate were 4.7 ± 0.5 nM (A) while basal GLU levels were 70 ± 6.2 nM (B).

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

4.3.4 Effect of UFP-512 on GABA and GLU release in VMTh

Since the primary output of SNr projects to the thalamus, and this projection is related to normal movement activity, we used microdialysis to determine how the observed effect on behavior correlated with thalamic amino acid release (GABA and GLU).

RM ANOVA on GABA release in VMTh (Fig. 33A) showed a significant effect of treatment ($F_{2,8}=76.42$, $p < 0.0001$) but not time ($F_{7,100}=0.64$, $p=0.721$), and a significant time X treatment interaction ($F_{14,100}=3.8$, $p < 0.0001$). Post hoc analysis revealed that UFP-512 10 µg/kg caused a decrease in GABA levels (maximal ~19% reduction) at 30 and 45 min whereas UFP 1000 µg/kg

increased GABA release (maximal ~27% increase) at 30 and 45 minutes after drug injection (Fig. 33A).

RM ANOVA on extracellular GLU release (Fig. 33B) in VMTh showed a significant effect of treatment ($F_{2,8}=8.54$, $p=0.01$) but not time ($F_{7,92}=1.94$, $p=0.07$) and a significant time X treatment interaction ($F_{14,92}=2.83$, $p=0.001$). Post hoc analysis revealed that UFP-512 10 $\mu\text{g}/\text{kg}$ increased GLU levels (maximal ~49%) at 30 min whereas UFP 1000 $\mu\text{g}/\text{kg}$ was ineffective, although a trend to decrease was observed (Fig. 33B).

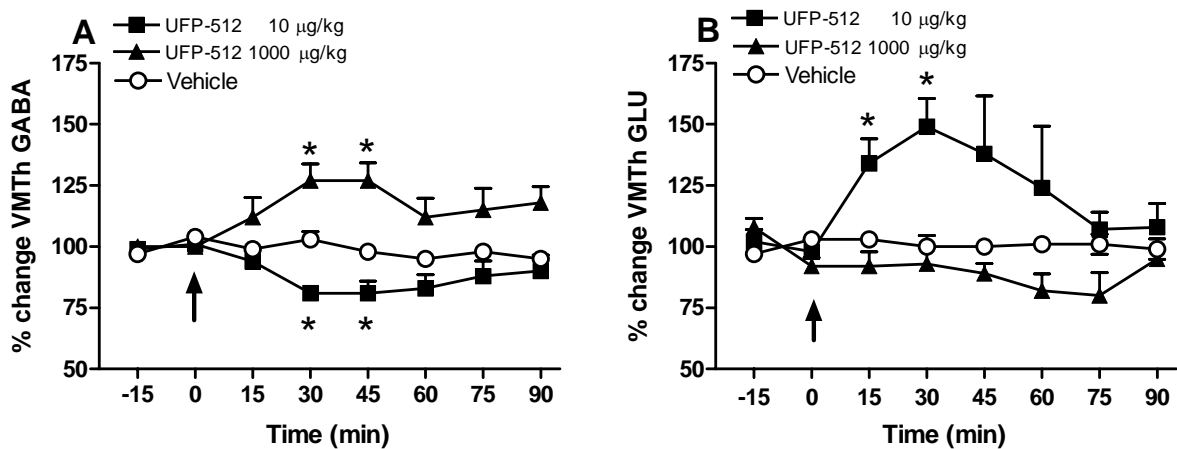


Fig 33. Effect of systemic (i.p.) administration (arrow) of facilitatory (10 $\mu\text{g}/\text{kg}$) and inhibitory (1000 $\mu\text{g}/\text{kg}$) doses of UFP-512 on extracellular GABA (A) and GLU (B) levels in VMTh. Data are means \pm SEM of 5-6 (A, B) experiments per group and are expressed as percent baseline (calculated as the mean of the two samples before the treatment). Basal GABA levels in the dialysate were 3.6 ± 0.14 nM (A) while basal GLU levels were 54 ± 4.4 nM (B).

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

4.3.5 Effect of UFP-512 on akinesia (bar test) while performing microdialysis

Finally, we correlated the changes in amino acid neurotransmission and akinesia by coupling microdialysis to the bar test.

RM ANOVA on immobility time at the contralateral paw (Fig. 34A) showed a significant effect of treatment ($F_{2,8}=27.52$, $p=0.0003$) but not time ($F_{7,92}=1.25$, $p=0.283$), and a significant time X treatment interaction ($F_{14,92}=5.03$, $p < 0.0001$). Post hoc analysis revealed that UFP-512 10 $\mu\text{g}/\text{kg}$ caused a transient attenuation of akinesia (~39% reduction with respect to basal values) 30 and 45 min after injection while UFP-512 1000 $\mu\text{g}/\text{kg}$ inversely caused an increase (~39%) in immobility time also at 30 and 45 min post injection (Fig. 34A).

Similar to the contralateral paw, RM ANOVA on immobility time at the ipsilateral paw (Fig 34B) showed a significant effect of treatment ($F_{2,8}=7.46$, $p=0.015$) but not time ($F_{7,92}=1.15$, $p=0.34$), and a significant time X treatment interaction ($F_{14,92}=2.00$, $p=0.026$). Post hoc analysis revealed that UFP-512 10 $\mu\text{g}/\text{kg}$ caused a mild and transient attenuation of akinesia ($\sim 26\%$) 45 min after injection while UFP-512 1000 $\mu\text{g}/\text{kg}$ inversely caused an increase ($\sim 71\%$) which was significant 15 min after injection (Fig. 34B).

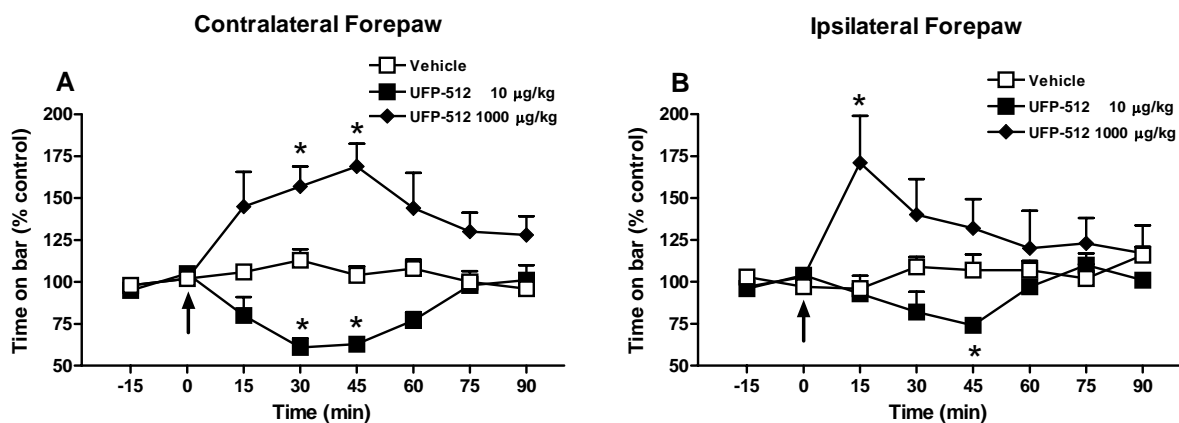


Fig 34. Effect of systemic (i.p.) administration (arrow) of a facilitatory (10 $\mu\text{g}/\text{kg}$) or inhibitory (1000 $\mu\text{g}/\text{kg}$) dose of UFP-512 on the bar test while simultaneously performing microdialysis. Akinesia was evaluated (every 15 min) separately at the contralateral (A) and ipsilateral (B) forepaws. Data are expressed as percent basal motor activity (calculated as the mean of immobility time in the two sessions before treatment) and are means \pm SEM of 5-6 determinations per group. Basal motor activity values in the bar test were (sec): 11.4 ± 1.4 (ipsilateral paw) and 27.5 ± 1.8 (contralateral paw).

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

Section IV

Combined DOP receptor stimulation and NOP receptor blockade synergistically improves motor performance and impairs nigro-thalamic output in experimental parkinsonism

Behavioral Studies

4.4.1 The effect of SNC-80 and J-113397 on motor activity

To investigate whether combined DOP receptor stimulation and NOP receptor blockade caused an additive or synergistic effect on the reduction of parkinsonian-like symptoms, SNC-80 and J-113397 were administered systemically (i.p.) alone or in combination and motor activity evaluated in the bar, drag and rotarod tests. The effect of the subthreshold combination doses (SNC 0.1 mg/kg and J-113397 0.1 mg/kg) were investigated first (Fig. 35A-C).

RM ANOVA showed a significant effect of treatment on the bar test ($F_{3,21}=14.0$, $p<0.0001$), but not time ($F_{1,22}=0.21$, $p=0.65$), or a time X treatment interaction ($F_{3,22}=1.32$, $p=0.29$) at the contralateral paw. Post hoc analysis revealed that each compound was ineffective alone and that only the combination of SNC-80 and J-113397 reduced immobility at the contralateral paw both 20 min (~37%) and 70 min (~34%) after injection (Fig. 35A). Conversely, no effect was observed at the ipsilateral paw.

Likewise, in the drag test, RM ANOVA at the contralateral paw showed an overall effect of treatment at the contralateral paw ($F_{3,27}=44.69$, $p<0.0001$) but not time ($F_{1,20}=0.02$, $p=0.89$) or time X treatment interaction ($F_{3,20}=0.34$, $p=0.79$). Post hoc analysis revealed that only the combination of subthreshold doses of SNC-80 and J-113397 caused an improvement in the drag test at 20 min (~96%) and 70 min (~98%) after treatment, while each compound alone was ineffective (Fig. 35B). No effect was observed at the ipsilateral paw.

RM ANOVA performed on time spent on the rotarod (Fig. 35C) showed an overall effect of treatment ($F_{3,18}=16.37$, $p<0.0001$) but not time ($F_{1,16}=0.001$, $p=0.98$) or a time X treatment interaction ($F_{3,16}=0.36$, $p=0.94$). As with the other tests, an increase in rotarod performance was only seen with the combination of SNC-80 and J-113397 20 min (~65%) and 70 min (~68%) after injection (Fig. 35C).

Submaximal antiparkinsonian doses of J-113397 (1 mg/kg) and SNC-80 (3 mg/kg) were then tested to determine if their motor enhancing effects could be additive.

In the bar test (Fig. 35D), RM ANOVA showed a significant effect of treatment ($F_{3,18}=14.4$, $p<0.0001$), but not time ($F_{1,24}=0.06$, $p=0.81$), or a time X treatment interaction ($F_{3,24}=1.48$, $p=0.24$) at the contralateral paw. At the ipsilateral paw (Fig. 35D), RM ANOVA showed a significant effect of treatment ($F_{3,18}=4.6$, $p=0.015$), but not time ($F_{1,24}=1.81$, $p=0.19$), and a significant X treatment interaction ($F_{3,24}=4.05$, $p=0.018$). Post hoc analysis revealed that SNC-80

reduced immobility at the contralateral paw both 20 (~44%) and 70 min (~32%) after injection while it only reduced immobility time (~39%) after 20 min at the ipsilateral paw (Fig. 35D). On the other hand, J-113397 did not effect the ipsilateral paw and reduced contralateral paw immobility time at 20 min (~23%; Fig. 35D). The combination of SNC-80 and J-113397 however reduced immobility time at the contralateral paw at 20 min (~38%) and 70 min (~47%) as well as at the ipsilateral paw at both 20 (~39%) and 70 min (~41%) following administration (Fig. 35D).

In the drag test (Fig. 35E), RM ANOVA showed an overall effect of treatment at the contralateral paw ($F_{3,18}=15.47$, $p<0.0001$) but no effect of time ($F_{1,26}=3.55$, $p=0.07$) or time X treatment interaction ($F_{3,26}=2.13$, $p=0.12$). Post hoc analysis revealed that the number of steps was increased in a prolonged fashion by both SNC-80 and J-113397 causing a ~28% and ~96% increase after 20 min and ~34% and ~55% after 70 min, respectively. The combination of submaximal SNC-80 and J-113397 also caused a prolonged increase in the number of steps at the contralateral paw at which was not different from that induced by SNC-80 alone (Fig. 35E). No effect was seen at the ipsilateral paw in the drag test with SNC-80, J-113397 or their combination (Fig. 35E).

RM ANOVA performed on time spent on the rotarod using submaximal doses (Fig. 35F) showed an overall effect of treatment ($F_{3,18}=9.36$, $p=0.0006$), but not time ($F_{1,24}=1.15$, $p=0.29$) or a time X treatment interaction ($F_{3,24}=0.66$, $p=0.59$). Post hoc analysis revealed an increase in rotarod performance with SNC-80 (~48% and ~36%) and J-113397 (~47% and ~41%) 20 and 70 min following treatment, respectively. The combination however caused ~82% and ~105% increases 20 and 70 minutes after administration, respectively (Fig. 35F). This increase was greater than either drug given alone 70 min after administration, but not 20 min.

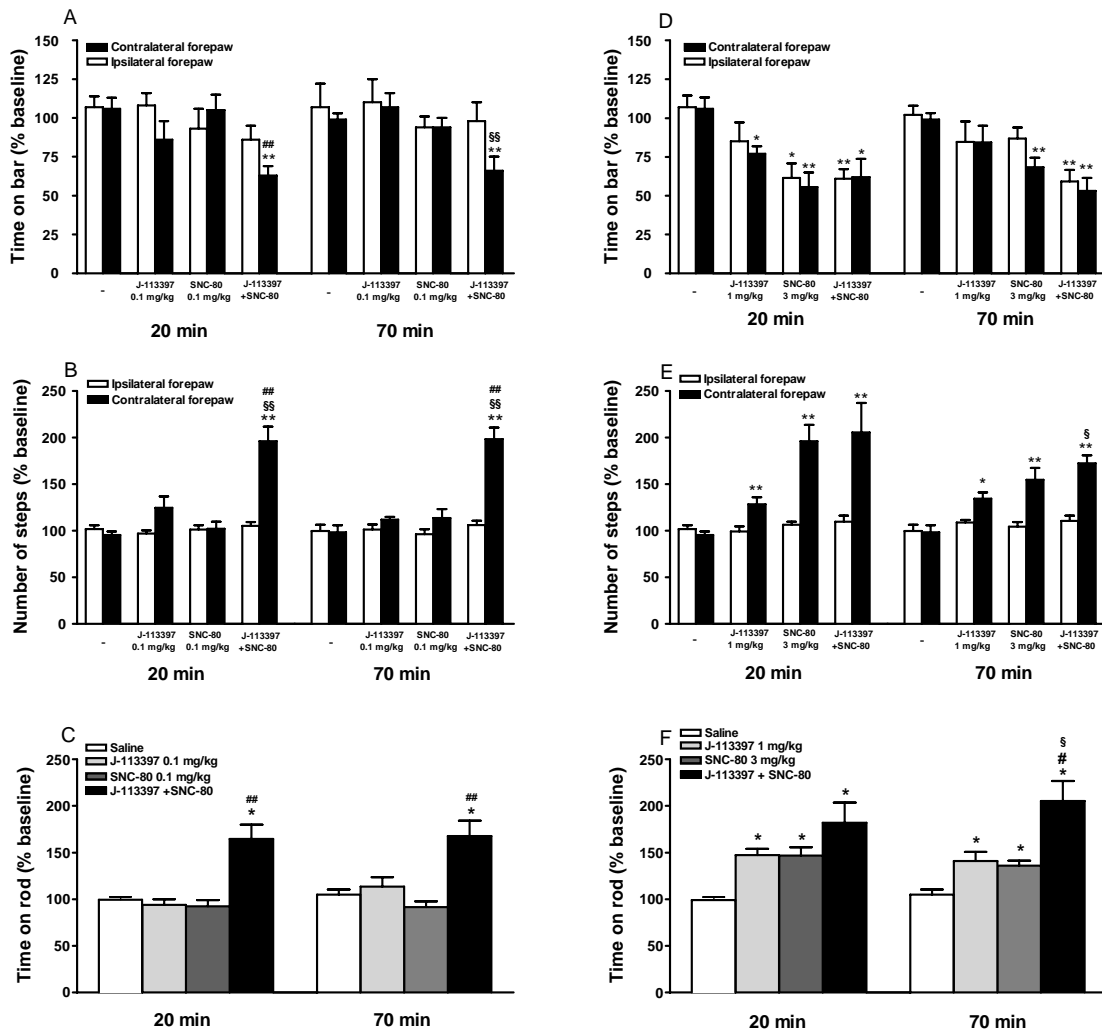


Fig 35. Effect of systemic (i.p.) administration of SNC-80 and J-113397 alone or in combination on bar (A-D), drag (B-E) or rotarod (C-F) tests. Each experiment consisted of three different sessions: a control session followed by other two sessions performed 20 and 70 min after vehicle or drug administration (see Methods). Data are expressed as percentages of basal motor activity in the control session and are means \pm SEM of 6-10 determinations per group. Basal motor activity values in the bar test were (time spent on the bar): 15.9 ± 1.1 (ipsilateral paw) and 27.3 ± 1.6 (contralateral paw). Basal motor activity in the drag test was (number of steps) 11.2 ± 0.2 (ipsilateral paw) and 2.1 ± 0.1 (contralateral paw). Rotarod performance was 480 ± 23 sec (0-55 rpm range).

* $p < 0.05$, ** $p < 0.01$, significantly different from vehicle

$p < 0.01$, significantly different from SNC-80

§ $p < 0.05$, §§ $p < 0.01$, significantly different from J-113397 (RM ANOVA followed by contrast analysis and the sequentially rejective Bonferroni's test).

Neurochemical Studies

4.4.2 The effect of SNC-80 and J-113397 on GABA and GLU release in GP

Since behavioral data supported an interaction between DOP receptor stimulation and NOP receptor blockade, we sought to determine if neurotransmitter changes in the indirect pathway may be responsible. Since the indirect pathway of the BG begins with striatal neurons projecting to GP, and excessive pallidal GABA release has been associated with the consequences of DA depletion (Gerfen et al., 1991, Maneuf et al., 1994), we performed microdialysis in GP using SNC-80 (0.1 mg/kg) and J-113397 (0.1 mg/kg).

RM ANOVA on GABA release in GP (Fig. 36A) revealed a significant effect of treatment ($F_{3,18}=4.27$, $p=0.019$), time ($F_{7,176}=6.86$, $p<0.0001$), and a significant time X treatment interaction ($F_{21,176}=1.86$, $p=0.016$). Post hoc analysis revealed that SNC-80 was ineffective whereas both J-113397 and the combination of SNC-80 and J-113397 decreased GABA levels compared to vehicle treated animal, (maximal of ~37% and ~21%, respectively; Fig 36A). There was no effect of treatment on GLU release (Fig. 36B).

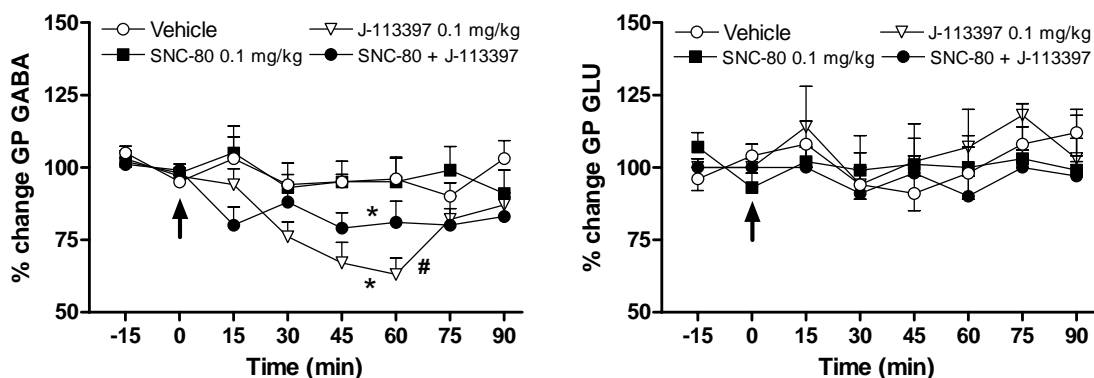


Fig 36. Effect of systemic (i.p.) administration of SNC-80 (0.1 mg/kg) and J-113397 (0.1 mg/kg) alone or in combination on extracellular GABA (A) and GLU (B) levels in GP. Data are means \pm SEM of 6-9 experiments per group and are expressed as percent baseline (calculated as the mean of the two samples before the treatment). Basal GABA levels in the dialysate were 2.9 ± 0.3 nM (A) while basal GLU levels were 40 ± 6.0 nM (B).

* $p<0.05$, significantly different from vehicle

$p<0.05$, significantly different from SNC-80 (RM ANOVA followed by contrast analysis)

4.4.3 The effect of SNC-80 and J-113397 on GABA and GLU release in SNr

Since the direct pathway projects from the striatum to SNr and this area is crucial for normal motor output, we performed microdialysis there to observe how the combination of DOP receptor stimulation and NOP receptor blockade affects nigral neurotransmitter levels.

RM ANOVA on SNr GABA levels (Fig. 37A) showed a significant effect of treatment ($F_{3,18}=16.78$, $p<0.0001$), time ($F_{7,176}=5.06$, $p<0.0001$) and a time X treatment interaction ($F_{21,176}=2.32$, $p=0.002$). Post hoc analysis revealed that when either SNC-80 (0.1 mg/kg) or J-113397 (0.1 mg/kg) were given alone there was no effect while the combination of J-113397 and SNC-80 evoked a robust increase in extracellular GABA levels (~107% at the 30 min time-point; Fig. 37A)

RM ANOVA on SNr GLU levels (Fig. 37B) showed a significant effect of treatment ($F_{3,18}=6.28$, $p=0.004$), but not time ($F_{7,160}=1.22$, $p=0.296$) and a significant time X treatment interaction ($F_{21,160}=2.05$, $p=0.007$). Post hoc analysis revealed that SNC-80 (0.1 mg/kg) given alone had no effect, J-113397 (0.1 mg/kg) caused a transient reduction (maximal ~19% at 45 min) and the combination of J-113397 and SNC-80 evoked a sustained decrease (maximal ~30% at 75 min) in extracellular GLU levels (Fig. 37B).

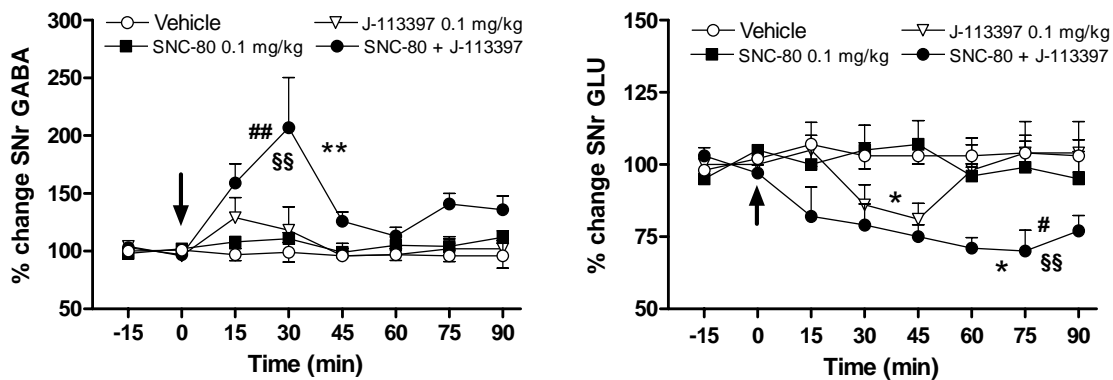


Fig 37. Effect of systemic (i.p.) administration of SNC-80 (0.1 mg/kg) and J-113397 (0.1 mg/kg) alone or in combination on extracellular GABA (A) and GLU (B) levels in SNr. Data are means \pm SEM of 6-9 experiments per group and are expressed as percent baseline (calculated as the mean of the two samples before the treatment). Basal GABA levels in the dialysate were 3.0 ± 0.4 nM (A) while basal GLU levels were 73 ± 7.5 nM (B).

* $p<0.05$, ** $p<0.01$, significantly different from vehicle

$p<0.01$, significantly different from SNC-80

§§ $p<0.01$, significantly different from J-113397 (RM ANOVA followed by contrast analysis).

4.4.4 The effect of SNC-80 and J-113397 on GABA and GLU release in VMTh

Since the thalamus is a major target of the SNr, we performed microdialysis there to observe how thalamic neurotransmitter release reflect changes in SNr and ultimately motor output.

RM ANOVA on GABA release in VMTh (Fig. 38A) showed a significant effect of treatment ($F_{3,15}=9.53$, $p<0.001$), time ($F_{7,108}=2.91$, $p=0.008$) and a time X treatment interaction

($F_{21,108}=1.81$, $p=0.026$). SNC-80 (0.1 mg/kg) or J-113397 (0.1 mg/kg) given alone had no effect while the combination of SNC-80 and J-113397 caused a long lasting decrease (maximal ~25% at 75 min) in VMTh GABA release (Fig. 38A).

RM ANOVA on GLU release in VMTh (Fig. 38B) revealed a significant effect of treatment ($F_{3,12}=5.86$, $p=0.01$) but not time ($F_{7,112}=1.56$, $p=0.15$) and a time X treatment interaction ($F_{21,112}=2.03$, $p=0.009$). Post hoc analysis revealed that either SNC-80 or J-113397 given alone had no effect whereas the combination of SNC-80 and J-113397 caused an increase in VMTh GLU release (maximal ~51% at 45 min; Fig. 38B).

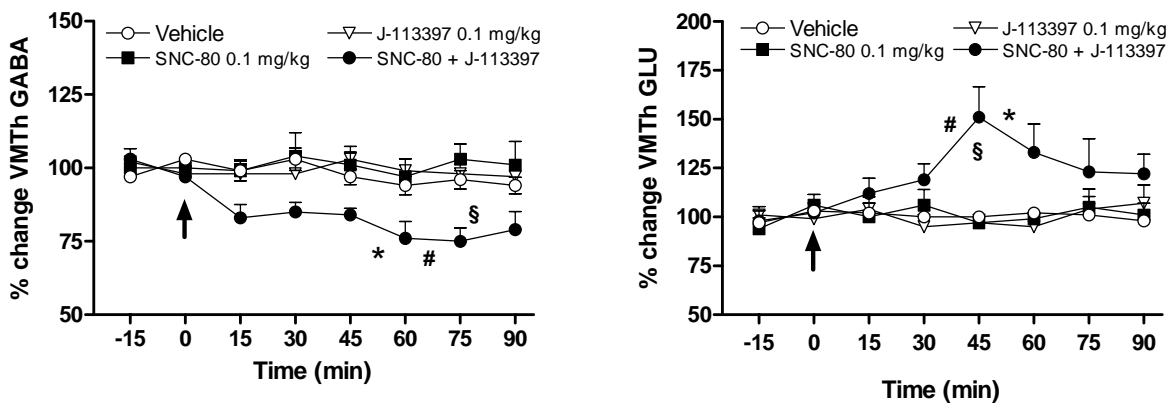


Fig 38. Effect of systemic (i.p.) administration of SNC-80 (0.1 mg/kg) and J-113397 (0.1 mg/kg) alone or in combination on extracellular GABA (A) and GLU (B) levels in VMTh. Data are means \pm SEM of 5 experiments per group and are expressed as percent baseline (calculated as the mean of the two samples before the treatment). Basal GABA levels in the dialysate were 4.1 ± 0.4 nM (A) while basal GLU levels were 90 ± 6.7 nM (B).

* $p < 0.05$, significantly different from vehicle

$p < 0.05$, significantly different from SNC-80

§ $p < 0.05$, significantly different from J-113397 (RM ANOVA followed by contrast analysis).

4.4.5 Effect of systemic SNC-80 and J-113397 on akinesia (bar) test coupled to microdialysis

The bar test was performed simultaneously along with microdialysis to investigate the physiological meaning of the neurochemical changes described here. This method also allows for a more detailed description of the time course of treatment.

RM ANOVA on time spent on the bar with the contralateral paw (Fig. 39A) showed a significant effect of treatment ($F_{3,12}=35.01$, $p < 0.0001$), time ($F_{7,112}=4.99$, $p < 0.0001$) and a time X treatment interaction ($F_{21,112}=6.35$, $p < 0.0001$). Post hoc analysis revealed that each drug given individually had no effect while the combination of SNC-80 (0.1 mg/kg) and J-113397 (0.1 mg/kg) caused

long lasting relief from akinesia with a maximal reduction of ~54% 15 min after administration while (Fig. 39A). No effect was seen at the ipsilateral paw with any treatment (Fig. 39B).

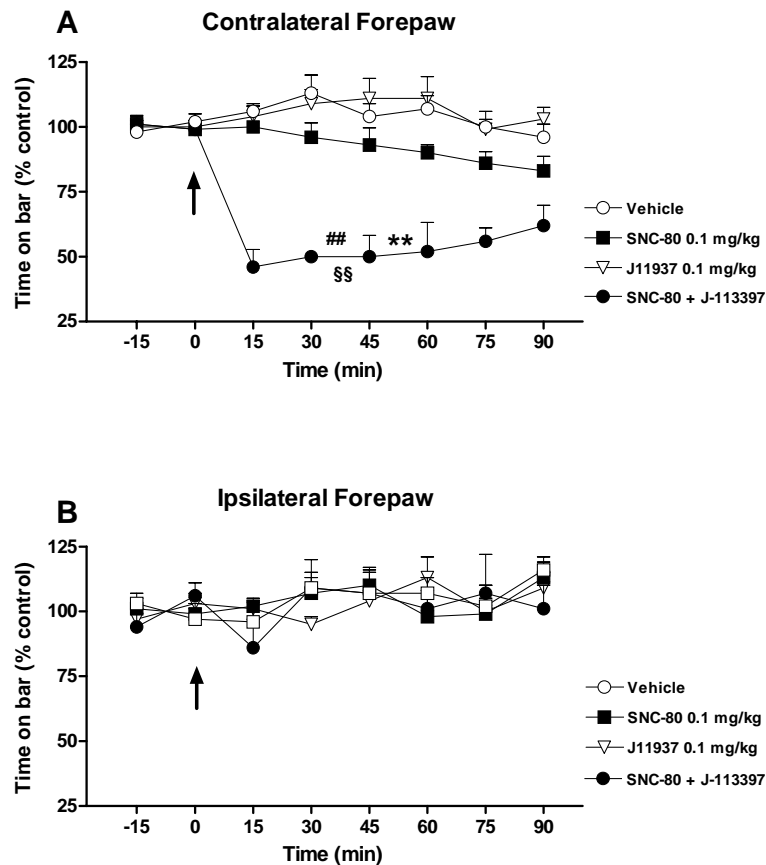


Fig 39. Effect of systemic (i.p.) administration (arrow) of SNC-80 (0.1 mg/kg), J-113397 (0.1 mg/kg) or their combination on the bar test while simultaneously performing microdialysis. Akinesia was evaluated (every 15 min) separately at the contralateral (A) and ipsilateral (B) forepaws. Data are means \pm SEM of 5 experiments per group and are expressed as percent baseline (calculated as the mean of the two samples before the treatment). Basal motor activity values in the bar test were (time spent on the bar): 19.3 ± 2.1 (ipsilateral paw) and 30.3 ± 1.6 (contralateral paw).

** $p < 0.01$, significantly different from vehicle

$p < 0.01$, significantly different from SNC-80

§§ $p < 0.01$, significantly different from J-113397 (RM ANOVA followed by contrast analysis).

4.4.6 Effect of nigral perfusion of SNC-80 and J-113397 on local GABA and GLU release

We recently demonstrated that both DOP receptor agonists (Mabrouk et al., 2008) and NOP receptor antagonists (Marti et al., 2007) promote locomotion via nigral mechanisms, namely through inhibition of nigral-thalamic GABAergic projections. Based on those findings, SNC-80 (0.01 or 0.1 μ M) and J-113397 (0.1 or 1 μ M) were perfused alone to determine the local effects on SNr GABA and GLU release as well as output in the VMTh. The ineffective concentrations of SNC-80 and J-113397 were then perfused together to test whether they could induce synergistic effects. These experiments were performed in parallel with the bar test to correlate neurochemical changes with the alleviation of akinesia.

RM ANOVA on SNr GABA levels (Fig. 40A) revealed a significant effect of treatment ($F_{5,25}=7.20$, $p=0.0003$), time ($F_{7,194}=8.76$, $p<0.0001$) but not a time X treatment interaction ($F_{35,194}=1.34$, $p=0.109$). Post hoc analysis revealed that low concentration nigral perfusions of SNC-80 (0.01 μ M) or J-113397 (0.1 μ M) alone had no effect on GABA levels whereas higher concentrations (0.1 μ M and 1 μ M, respectively) increased nigral GABA release by 32% and 25%, respectively (Fig. 40A). The combination of ineffective concentrations of J-113397 (0.1 μ M) and SNC-80 (0.01 μ M) evoked an increase in extracellular GABA levels (maximal ~40%) which was similar to that induced by the higher concentrations alone (Fig. 40A).

RM ANOVA on SNr GLU levels (Fig. 40B) revealed a significant effect of treatment ($F_{5,25}=14.41$, $p<0.0001$), time ($F_{7,194}=8.30$, $p<0.0001$) and a time X treatment interaction ($F_{35,194}=1.68$, $p=0.014$). Post hoc analysis revealed that low concentrations of SNC-80 or J-113397 alone had no effect whereas high concentrations of SNC-80 and J-113397 caused reductions (maximal ~35% at 75 and ~43% at 45 min, respectively) in GLU release (Fig. 40B). The combination of ineffective concentrations of SNC-80 and J-113397 however induced a long lasting reduction (maximal ~50% at 90 min) in nigral GLU release which was similar to higher concentrations alone (Fig. 40B).

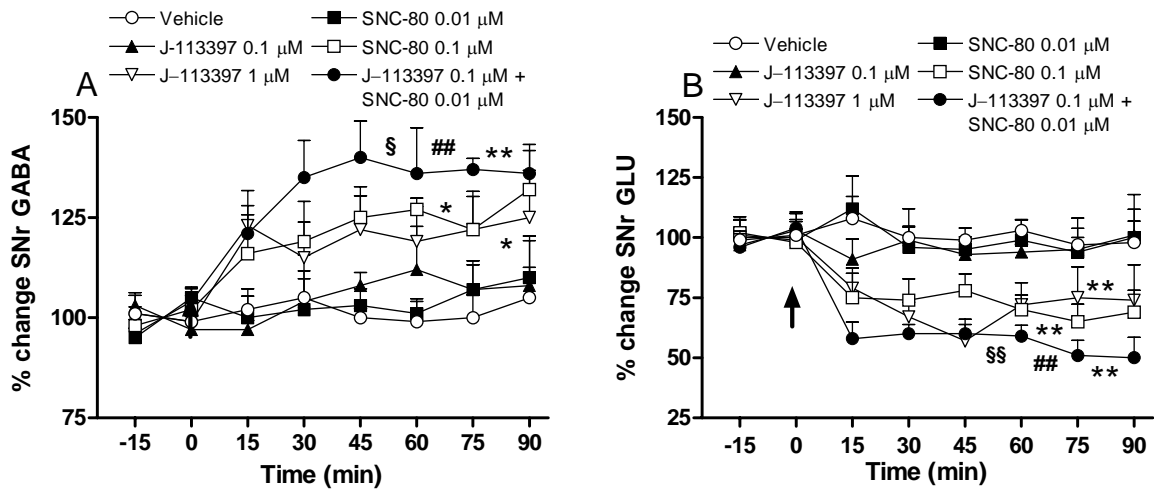


Fig 40. Effect of nigral perfusion of SNC-80 (0.01 or 0.1 μM), J-113397 (0.1 or 1 μM) and their combinations (SNC-80 0.01 μM with J-113397 0.1 μM or SNC-80 0.1 μM with J-113397 1 μM) on local GABA and GLU release. Data are means ± SEM of 5-7 experiments per group and are expressed as percent baseline (calculated as the mean of the two samples before the treatment). Basal GABA levels in the dialysate were 4.2 ± 0.3 nM (A) while basal GLU levels were 140 ± 14.3 nM (B).

* $p < 0.05$, ** $p < 0.01$, significantly different from vehicle

$p < 0.01$, significantly different from SNC-80

§ $p < 0.05$, §§ $p < 0.01$, significantly different from J-113397 (RM ANOVA followed by contrast analysis).

4.4.7 Effect of nigral perfusion of SNC-80 and J-113397 on thalamic GABA and GLU release

To appreciate how the effects observed in the SNr impact nigro-thalamic output neurons, we performed microdialysis in VMTh and measured GABA and GLU release.

RM ANOVA on GABA levels in VMTh showed a significant effect of treatment ($F_{5,25}=20.53$, $p < 0.0001$), time ($F_{7,210}=19.60$, $p < 0.0001$) and a time X treatment interaction ($F_{35,210}=3.92$, $p < 0.0001$). Post hoc analysis revealed that low concentrations of SNC-80 (0.01 μM) or J-113397 (0.1 μM) had no effect on thalamic GABA release while higher concentrations of SNC-80 (0.1 μM) or J-113397 (1.0 μM) reduced it (maximal ~38%, ~26%, respectively; Fig 41A). The combination of the ineffective concentrations also produced a reduction in thalamic GABA release (~39%) which was similar in magnitude to each of the high concentrations alone (Fig. 41A).

RM ANOVA on VMTh GLU levels (Fig. 41B) revealed a significant effect of treatment ($F_{5,25}=6.30$, $p=0.0006$), time ($F_{7,194}=3.98$, $p=0.0004$) and a time X treatment interaction ($F_{35,194}=2.20$, $p=0.0004$). Post hoc analysis revealed that low concentration nigral perfusions of SNC-80 (0.01 μM) and J-113397 (0.1 μM) had no effect on thalamic GLU levels whereas the

higher concentrations (0.1 μM and 1 μM , respectively) increased them by $\sim 41\%$ (at 60 min) and $\sim 53\%$ (at 75 min), respectively (Fig. 41B). The combination of J-113397 (0.1 μM) and SNC-80 (0.01 μM) evoked an increase in extracellular thalamic GLU levels $\sim 52\%$ at 90 min time point which was similar in magnitude as the higher concentrations alone (Fig. 41B).

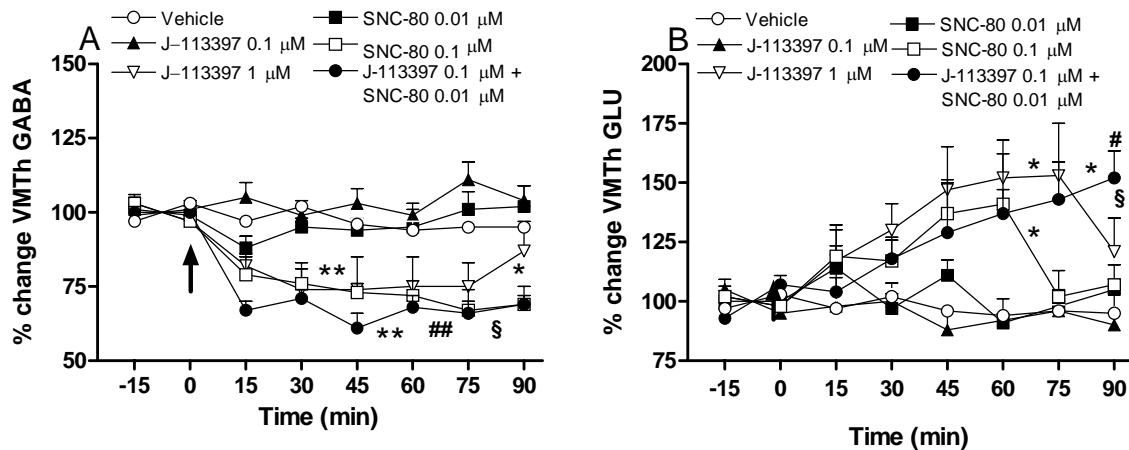


Fig 41. Effect of nigral perfusion of SNC-80 (0.01 or 0.1 μM), J-113397 (0.1 or 1 μM) and their combinations (SNC-80 0.01 μM with J-113397 0.1 μM or SNC-80 0.1 μM with J-113397 1 μM) on thalamic GABA and GLU release. Data are means \pm SEM of 5-7 experiments per group and are expressed as percent baseline (calculated as the mean of the two samples before the treatment). Basal GABA levels in the dialysate were 5.6 ± 0.3 nM (A) while basal GLU levels were 77 ± 6.4 (B).

* $p < 0.05$, ** $p < 0.01$, significantly different from vehicle

$p < 0.05$, ## $p < 0.01$, significantly different from SNC-80

§ $p < 0.05$, §§ $p < 0.01$, significantly different from J-113397 (RM ANOVA followed by contrast analysis).

4.4.8 Effect of nigral perfusion of SNC-80 and J-113397 on akinesia (bar) test coupled to microdialysis

The bar test was performed simultaneously along with these local perfusion microdialysis studies to correlate the alleviation of akinesia with the neurochemical changes described here.

RM ANOVA on time spent on the bar at the contralateral paw (Fig. 42A) showed a significant effect of treatment ($F_{5,20}=30.74$, $p < 0.0001$), time ($F_{7,168}=9.37$, $p < 0.0001$) and a time X treatment interaction ($F_{35,168}=3.11$, $p < 0.0001$). Post hoc analysis revealed that at the contralateral paw, low concentrations of SNC-80 (0.01 μM) or J-113397 (0.1 μM) did not modify immobility time whereas SNC-80 (0.1 μM) and J-113397 (1 μM) reduced time spent on the bar by $\sim 49\%$ and $\sim 42\%$, respectively (Fig. 42A). The combination of low concentrations of SNC-80 (0.01 μM) and J-113397 (0.1 μM) resulted in long lasting relief from akinesia with a maximal immobility time reduction of $\sim 58\%$, 75 min after administration (Fig. 43A).

RM ANOVA on time spent on the bar at the ipsilateral paw (Fig. 42B) showed a significant effect of treatment ($F_{5,20}=8.32$, $p=0.0002$), time ($F_{7,168}=3.78$, $p=0.0008$) and a time X treatment interaction ($F_{35,168}=1.63$, $p=0.02$). Post hoc analysis revealed that low concentrations of SNC-80 or J-113397 alone did not have an effect on immobility time whereas high SNC-80 (0.1 μM) and J-113397 (1 μM) reduced time spent on the bar by $\sim 31\%$ and $\sim 34\%$, respectively (Fig. 42B). The combination of low concentrations of SNC-80 (0.01 μM) and J-113397 (0.1 μM) also caused a reduction (maximal $\sim 48\%$ at 75 min) in immobility time similar to the effect seen with either drug given alone at high concentrations (Fig. 43B).

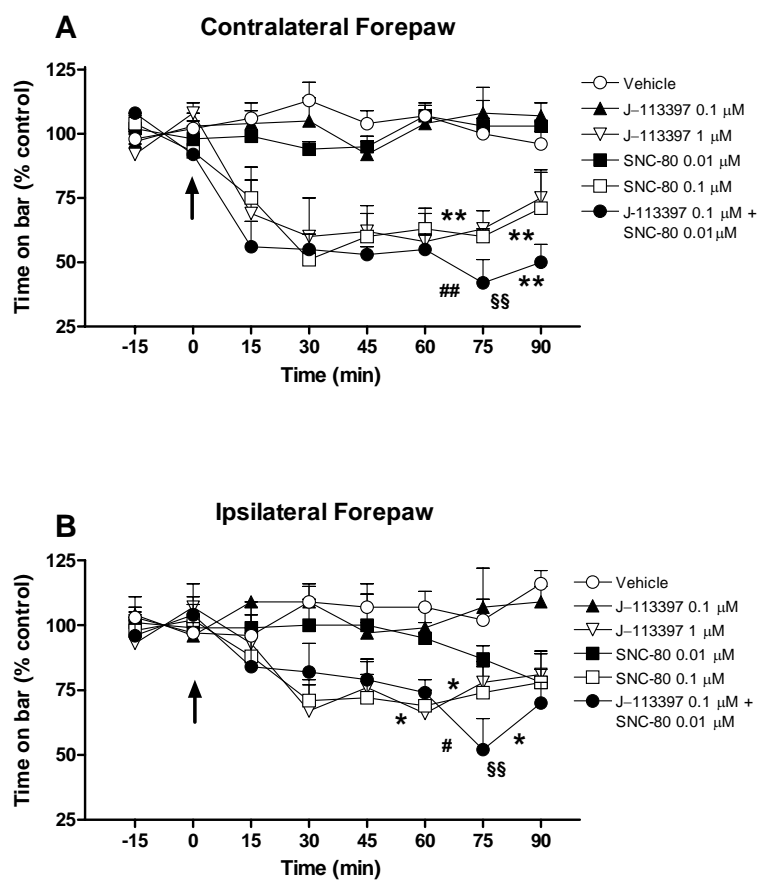


Fig 42. Effect of nigral perfusion of SNC-80 (0.01 μM or 0.1 μM), J-113397 (0.1 μM or 1 μM) or of combination of SNC-80 (0.01 μM) and J-113397 (0.1 μM) on the bar test while undergoing microdialysis. Akinesia was evaluated (every 15 min) separately at the contralateral (A) and ipsilateral (B) forepaws. Data are means \pm SEM of 5 experiments per group and are expressed as percent baseline (calculated as the mean of the two samples before the treatment). Basal motor activity values in the bar test were (time spent on the bar): 11.1 ± 0.7 (ipsilateral paw) and 26.6 ± 1.0 (contralateral paw).

* $p < 0.05$, ** $p < 0.01$, significantly different from vehicle

$p < 0.05$, ## $p < 0.01$, significantly different from SNC-80

§ $p < 0.05$, §§ $p < 0.01$, significantly different from J-113397 (RM ANOVA followed by contrast analysis).

5. DISCUSSION

Section I. *The endogenous MOP receptor agonist Endomorphin-1 dually affects motor activity and differentially modulates nigro-striatal and nigro-thalamic pathways.*

This study showed that exogenous EM-1, produced a dose-dependent biphasic regulation of spontaneous motor activity and physiologically-stimulated locomotion in rats. Facilitation was observed at lower doses (0.1-1 nmol) and inhibition at the higher ones (10 nmol). This effect was prevented by the selective MOP receptor antagonist CTOP, thus likely mediated by MOP receptors. Moreover, CTOP alone inhibited motor performance, suggesting that tonic activation of MOP receptors facilitates motor function. EM-1 and CTOP singularly injected in SNr replicated these effects, suggesting that this brain area is primarily involved in the motor response.

EM-1 plays an important role in the control of motor function in rodents not only at rest (Zangen et al., 2002, Metha et al., 2001, Bujdoso et al., 2001, 2003) but also during continuous movement. The dual control of motor activity induced by administration of EM-1 qualitatively reflects the typical action that MOP (but not DOP and KOP) receptor stimulation produces on spontaneous locomotion in rats. Facilitation, however, seems to be the predominant since it appears at lower doses. Moreover, in keeping with previous finding that naloxone administered at MOP-selective doses reduced locomotion in rats (Timar et al., 2005), CTOP also reduced motor performance.

Consistent with an involvement of nigral MOP receptors in motor effects of EM-1, intranigral microinjections of morphine (Iwamoto and Way, 1977, 1981, Bontempi and Sharp 1997) or the selective MOP receptor agonist DAMGO (Bontempi and Sharp, 1997) induced contralateral turning in rats. It is interesting to point out that while unilateral microinjection of 10 nmol i.c.v. of the peptide reduced rotarod performance possibly due to an increase in muscular rigidity, 10 nmol injection in SNr produced initial hyperlocomotion with contralateral bias overcome by muscular rigidity. Motor facilitation has been attributed to an increase in nigro-striatal DAergic transmission (Iwamoto and Way, 1977, Matsumoto et al., 1988, Bontempi and Sharp, 1997), although an alternative non-DAergic mechanism has been suggested (Morelli and Di Chiara, 1985). Therefore, EM-1 may induce contralateral turning via inhibition of GABAergic interneurons in the SNr and subsequent facilitation of the nigro-striatal DAergic transmission (Lacey et al., 1989). Excessive striatal DA release may increase muscle tone and rigidity, as shown for NOP receptor antagonists (Marti et al., 2004), and impair motor performance.

Alternatively, motor inhibiting actions of EM-1 may be due to modulation of nigral GABAergic output neurons. Indeed, injection of morphine in SNr produce increased rigidity that did not depend on striatal mechanisms but was blocked by intranigral injections of muscimol and enhanced by intranigral injections of bicuculline (Turski et al., 1982). These data suggest that disinhibition of nigro-thalamic neurons may mediate motor inhibition induced by intranigral EM-1.

It is therefore possible to hypothesize that the biphasic modulation of motor functions by EM-1 in SNr results from activation of a facilitatory pathway that prevails during a moderate MOP receptor activation, followed by recruitment of an inhibitory one that appears during a higher degree of receptor activation.

Important clues on the mechanisms underlying the motor effect of EM-1 came from combined microdialysis and behavioral studies. Nigral EM-1 perfusions produced differing effects on neurotransmitter release in SNr and target areas (DLS and VMTh) which were dependent on concentration and correlated with opposite effects on locomotor activity. In particular, perfusion with low EM-1 concentrations reduced thalamic GABA release and facilitated motor activity, while perfusion with higher concentrations elevated striatal DA and thalamic GABA release but inhibited locomotion.

Nigral perfusion with EM-1 (0.01 μ M) caused a facilitation of local GABA release likely via indirect mechanisms. A major source of GABA in SNr is represented by striato-nigral and striato-pallidal GABAergic afferents. TTX perfusion in DLS not only reduced spontaneous but also attenuated the EM-1-evoked GABA release. This suggests that EM-1 stimulates GABA release from striato-nigral terminals. However, simultaneous perfusion of TTX in DLS and SNr blocked the facilitation of GABA release. This may be due to the fact that dual TTX perfusion in SNr induces a deeper blockade on impulse trafficking along striato-nigral fibers or that other intranigral mechanisms contribute to the facilitation of GABA release. In particular, the involvement of GABAergic interneurons should be considered in view of the fact that bicuculline prevented the effect of low concentrations of EM-1. In fact, raclopride also prevented the effect of EM-1. However, since low concentrations of EM-1 did not affect striatal DA release, we suggest that DA released from dendrites of SNc DA cells and acting through D₂ receptors plays a permissive role on the effect of EM-1. Whatever the mechanisms, EM-1 facilitation of nigral GABA release was associated with reduced thalamic GABA release and

facilitation of motor activity. Therefore, stimulation of MOP receptors on local interneurons or local collaterals extending from nigro-thalamic projections could cause disinhibition of GABA release from striato-nigral terminals leading to inhibition of nigro-thalamic projection neurons. This event produces thalamic disinhibition and motor activation (Chevalier and Deniau, 1985), since it is associated with stimulation of thalamo-cortical glutamergic pathways and motor output from cortical areas. The finding that low EM-1 concentrations elevated thalamic glutamate release (data not shown) is consistent with this view.

Differently from EM-1 (0.01 μM), EM-1 (1 μM) reduced nigral GABA and elevated thalamic GABA release. The inhibitory effect of EM-1 was TTX-insensitive, suggesting a presynaptic mechanism of action. However, the reduction in nigral GABA was prevented by co-perfusion with bicuculline. Although quite unexpected, this finding may suggest that GABA_A receptors need to be operative, e.g. play a permissive role, to allow EM-1 inhibition. Interestingly, EM-1 inhibition of GABA release was independent of DA receptor activation in SNr (raclopride and SCH23390-insensitive) but was associated with elevation of striatal DA release, consistent with the view that inhibition of GABA release from nigral interneurons disinhibits striatal DA transmission (Di Chiara and North, 1982). Interestingly, motor stimulatory actions of opioids are typically associated with increased striatal (accumbal) DA release. This work has demonstrated that even with increased striatal DA release, motor inhibition can occur via direct modulation of nigro-thalamic output.

Concluding remarks

In the present study, we showed that EM-1 produced dual effects on motor activity which were dependent on MOP receptor stimulation in SNr. By using microdialysis combined with simultaneous motor testing we found that facilitation and inhibition of motor activity were associated with inhibition and facilitation of GABA release in the thalamus, respectively. Moreover, only motor inhibiting EM-1 concentrations produced increases in striatal DA release. These data suggest that EM-1 affects motor activity through modulation of nigro-thalamic pathways and challenges the common view that motor stimulation following MOP receptor stimulation is mediated only through nigro-striatal DA transmission.

Section II. *Stimulation of delta opioid receptors located in substantia nigra reticulata but not globus pallidus or striatum restores motor activity in 6-hydroxydopamine lesioned rats.*

SNC-80 attenuated akinesia/bradykinesia and improved overall gait ability in 6-OHDA hemilesioned rats while NTD exerted opposite effects. These findings confirm that DOP receptor stimulation, either by synthetic agonists or endogenous ENK, promotes motor function under parkinsonian conditions. SNC-80 decreased pallidal GABA as well as nigral GLU and GABA release, suggesting an action at the striatal, pallidal or nigral level. However, only NTD perfusion in SNr (and not GP or DLS) prevented the antiakinetic effect of SNC-80 and its neurochemical correlates. Consistently, microinjections of SNC-80 in SNr (but not GP or DLS) or bicuculline in GP replicated the antiparkinsonian effects of systemic SNC-80. These findings challenge the common view that DOP receptor agonists attenuate parkinsonism via intrapallidal mechanisms, suggesting, instead, that nigral DOP receptors are involved.

Up-regulation of ENK transmission along the striato-pallidal pathway is thought to play a crucial role in maintaining motor function under parkinsonian conditions. To support the view that such up-regulation is compensatory in nature (Maneuf et al., 1994), DOP receptor agonists promoted movement and attenuated parkinsonian-like motor deficits in rodent and nonhuman primate models of PD (Maneuf et al., 1994, Pinna and Di Chiara, 1998, Hudzik et al., 2000, Hill et al., 2000, Hille et al., 2001). We confirmed the antiparkinsonian potential of DOP receptor agonists by using a battery of behavioral tasks providing information on different motor parameters. Doses of SNC-80 which would not elicit turning were selected since the sensitivity of the tests employed would be disrupted by turning behavior. SNC-80 attenuated akinesia/bradykinesia in the bar and drag test comparable to L-DOPA (Marti et al., 2007) although it was more effective in improving motor performance and gait ability on the rotarod.

Previous studies provided the proof-of-concept that DOP receptor agonists have antiparkinsonian potential, yet these studies did not parallel behavioral with neurochemical data leaving to speculation the circuitry involved and their site of action. We found that systemic administration of motor facilitating doses of SNC-80 decreased pallidal GABA release while systemic administration of motor inhibiting dose of NTD elevated it. This is in accordance with findings that pallidal DOP receptor stimulation inhibits local GABA release in vitro (Dewar et al., 1987; Maneuf et al., 1994, Stanford and Cooper 1999) and in vivo (Schroeder and Schneider, 2002), and corroborates the notion that activation of presynaptic (Stanford and Cooper, 1999) and/or

postsynaptic (Olive et al., 1997) DOP receptors in GP leads to reduced pallidal GABA release and motor improvement. However, the combined behavioral and neurochemical analysis clearly demonstrated that NTD perfusion in GP attenuated SNC-80 induced inhibition of GABA release without having appreciable behavioral consequences. Activation of DOP receptors expressed by striato-pallidal neurons (Mansour et al., 1993) or striatal cholinergic interneurons (Le Moine et al., 1994) may also directly or indirectly (i.e. by inhibition of tonic cholinergic facilitation; Pisani et al., 2003) lead to reduced pallidal GABA release and motor activation. However, DLS perfusion with NTD did not change SNC-80 effect, overall suggesting that neither pallidal nor striatal DOP receptors contributed to SNC-80 motor actions. Conversely, SNr perfusion with NTD prevented both the antiakinetik effect of SNC-80 and its neurochemical correlates, indicating that they were mediated by SNr DOP receptors. One could argue that the bar test only measures akinesia and that DOP receptors located in GP or DLS may regulate other motor parameters. However, microinjections of SNC-80 in GP or DLS also failed to affect motor activity in the drag and rotarod test, which are a (gross) measure of bradykinesia and overall gait ability. In keeping with this view, intranigral injections of DOP receptors agonists induced turning behavior (Jacquet, 1983, Matsumoto et al., 1988) and hyperlocomotion (Morelli et al., 1989) in naïve rats while pallidal injections were ineffective (Dewar et al., 1985). The failure of intrapallidal SNC-80 cannot be attributed to the tests used since blockade of striato-pallidal GABAergic transmission via bicuculline injection in GP attenuated parkinsonism, as shown for systemic or intranigral SNC-80. The efficacy of intrapallidal bicuculline extends to the 6-OHDA hemilesioned rat previous findings in reserpinized rats or MPTP-treated nonhuman primates (Maneuf et al., 1994), confirming the pathogenic contribution of striato-pallidal overactivation to PD symptoms in this model.

The ineffectiveness of intrapallidal SNC-80 as well as intrapallidal NTD to block systemic SNC-80 questions whether pallidal DOP receptors contribute to motor activity under parkinsonian conditions. It is possible that, due to up-regulation of striato-pallidal ENK transmission, intrapallidal DOP receptors are saturated by endogenous ENK making the contribution of exogenous stimulation by SNC-80 irrelevant. Greater DOP receptor saturation by endogenous opioids should result in a greater sensitivity to DOP receptor antagonism. Indeed, systemic administration of NTD doses selective for DOP receptor blockade (Baker and Meert, 2002, Longoni et al., 1991) worsened parkinsonism and increased pallidal GABA release. This

endorses the view that ENK tonically sustain motor function under parkinsonian conditions and that DOP receptor blockade exacerbates pathogenic GABA release in GP and its inhibitory influence on locomotion. However, microdialysis did not reveal neurotransmitter changes in any of the areas in which NTD was perfused alone at doses effective in blocking the exogenous stimulation. Whether a higher NTD concentration is required to unravel tonic DOP receptor modulation in microdialysis studies needs to be investigated. Nevertheless, the finding that DOP receptor agonists and antagonists did not produce overall symmetric neurochemical responses indicates that they did not act strictly through the same circuits.

In this respect, it has previously shown that DOP agonists may activate intranigral DA-dependent (Morelli et al., 1989) and DA-independent (Matsumoto et al., 1988) mechanisms. SNC-80 could activate presynaptic DOP receptors located on GLU nerve terminals (DOP receptor mRNA has been detected in STN; Aubert et al., 2007) resulting in reduced excitatory input onto nigrothalamic neurons and thalamic disinhibition (Deniau and Chevalier, 1985). Such a mechanism underlies the antiakinetik effect of DOP receptor antagonists (Marti et al., 2007). On the other hand, SNC-80 could directly reduce firing activity of nigrothalamic neurons, through an action at DOP receptors expressed on recurrent collaterals (Rick and Lacey, 1994). Both actions would result in reduced nigral GABA release. How stimulation of nigral DOP receptors could cause reduction in GABA release in GP appears puzzling, since no nigro-pallidal GABAergic projection has been identified thus far. A nigro-pallidal DAergic pathway exists (Fallon and Moore, 1978) and pallidal DA inhibits local GABA release via D₂ receptors (Floran et al., 1997, Raevskii et al., 2003). However, this pathway is impaired to some extent by 6-OHDA lesioning (Fuchs and Hauber, 2004, Debeir et al., 2005). Inhibition of GABA release may also reverberate changes occurring at the circuitry level. For instance, in keeping with the view that SNC-80 produces thalamic disinhibition, systemic SNC-80 elevated GLU levels in striatum (data not shown), which may result in activation of GABA or cholinergic interneurons (Zackheim and Abercrombie, 2005) and inhibition of striato-pallidal neurons.

Concluding remarks

The antiparkinsonian potential of DOP receptor agonists has been demonstrated in a number of PD models, although lack of simultaneous *in vivo* neurochemical analysis has left to speculation the brain area targeted and circuitry involved. In this study, SNC-80 promoted movement through activation of DOP receptors in SNr, challenging the common view that pallidal DOP

receptors contribute to antiparkinsonian action of DOP receptor agonists (Maneuf et al., 1994, Hill et al., 2000). However, blockade of DOP receptors resulted in worsening of parkinsonism and increased pallidal GABA release. Whether endogenous ENK sustain motor function and compensate for motor deficits under parkinsonian conditions via activation of pallidal DOP receptors requires further investigation.

Section III. The novel delta opioid receptor agonist UFP-512 dually modulates motor activity in experimental parkinsonism via control of the nigro-thalamic pathway

The main finding of this study is that in hemiparkinsonian rats, the novel DOP receptor agonist UFP-512 caused opposite changes in motor activity which were dose-related and mirrored by changes in GABA release in GP and VMTh as well as GLU release in SNr. In particular, motor facilitation was observed at low doses and was associated with a reduction of thalamic GABA release while motor inhibition appeared at higher doses and was accompanied by increases in thalamic GABA release. These data are consistent with a dual action of UFP-512 on nigro-thalamic output neurons, possibly leading to opposite changes in thalamo-cortical transmission and motor output.

DOP receptor stimulation has been shown to facilitate locomotion and antiparkinsonian behaviors in a number of experimental models of PD (Maneuf et al., 1994, Pinna and Di Chiara, 1998, Hill et al., 2000, Hille et al., 2001, Mabrouk et al., 2008). In these studies, SNC-80 was preferentially used. However, since DOP receptor agonists can lead to different degrees of receptor desensitization and signaling pathway recruitment (for a review see Varga et al., 2004), it is important to examine the behavioral and neurochemical profiles of different classes of DOP receptor agonists in order to develop molecules with antiparkinsonian activity with fewer adverse effects (such as tolerance). UFP-512 is a novel pseudopeptide showing high affinity ($pK_i=10.2$) for the human recombinant DOP receptor as well as high selectivity over MOP (160-fold) and KOP (3500-fold) receptors (Vergura et al., 2007). Compared to SNC-80 in the same tests (Mabrouk et al., 2008), UFP-512 appears 1000-fold more potent in stimulating stepping activity and rotarod performance (effective doses 1 $\mu\text{g}/\text{kg}$) and as effective as (drag test) or slightly less effective (rotarod test) than SNC-80. However, differently from SNC-80, UFP-512 antiparkinsonian effects appear shorter lasting (no significant change observed at 70 min after injection) and lost (or even reversed) at high doses. In past studies, UFP-512 displayed

antidepressant and anxiolytic properties in the forced swimming test (Vergura et al., 2008) as SNC-80 did (Broom et al., 2002; Saitoh et al., 2004), although it was effective at much lower doses both in mice (0.1 mg/kg vs 1 mg/kg, Saitoh et al., 2004) and rats (0.3 mg/kg vs 32 mg/kg, Broom et al., 2002). Moreover, UFP-512 failed to affect spontaneous locomotion in mice (Vergura et al., 2008) while inconsistent effects have been reported for SNC-80 (Saitoh et al., 2004; Ito et al., 2008). Interestingly however, UFP-512 reduced immobility time in the mouse forced swimming test at 0.1 and 0.3 mg/kg while higher doses (1 mg/kg) were ineffective. Analogously in the current study, UFP-512 improved motor behavior at low doses (1-10 μ g/kg) and depressed it at higher ones (100-1000 μ g/kg). The bell-shaped curve cannot be easily explained as simple receptor desensitization phenomenon since *in vitro* studies have shown that UFP-512 causes limited desensitization (as measured by the cAMP pathway) of DOP receptors as well as significant receptor trafficking (recycling back to cell surface) with respect to other DOP agonists such as SNC-80 (Aguila et al., 2007). SNC-80 markedly desensitizes DOP receptors with no significant recycling of the receptor to the cell surface but to lysosomes following internalization (LeCoq et al., 2004). However, SNC-80 has been tested up to 60 mg/kg without causing inhibition of movement in the reserpine-treated rat model of PD (Hill et al., 2000). In line with this study, SNC-80 dose-dependently facilitated locomotor activity in hemiparkinsonian rats with no signs of inhibition even at the highest dose tested (10 mg/kg *i.p.*; Mabrouk et al., 2008). It has been reported that DOP receptor agonists bearing the Dmt-tic pharmacophore, such as UFP-512, undergo metabolism to form diketopiperazine derivatives (Marsden et al., 1993) which are endowed with DOP receptor antagonist characteristics (Balboni et al., 1997). In fact, extensive structure-activity studies with this family of pseudopeptides have shown that slight chemical modifications may cause agonists to become antagonists (Balboni et al., 2008). Since we previously showed that systemic administration of the DOP receptor antagonist NTD caused inhibition of motor activity and a worsening of parkinsonism (Mabrouk et al., 2008), it is possible that the inverse correlation between dose and effect in the 100-1000 μ g/kg range is due to the progressive build-up of extracellular concentrations of a UFP-512 metabolite with antagonist properties at the DOP receptor. The recent finding that UFP-512 behaves as partial agonist at recombinant DOP receptors (T. Costa personal communication) lends further support to the hypothesis that the descending portion of the UFP-512 bell-shaped curve is due to progressive blockade of DOP receptors. Combined neurochemical and behavioral

analysis supported this view and shed light on the circuits involved. A low dose of UFP-512 reduced GABA release in VMTh and attenuated akinesia while a high dose of UFP-512 caused opposite effects. VMTh is one of the main targets of SNr GABA projection neurons, i.e. the basal ganglia output, and it has been demonstrated that changes in VMTh GABA release reflect changes in nigro-thalamic transmission (Timmerman and Westerink, 1997, Mark et al., 2004, Marti et al., 2007). According to the tonic inhibitory role of nigro-thalamic projections on the thalamic filter (Deniau and Chevalier, 1985), it is likely that inhibition and facilitation of thalamic GABA release results in facilitation and inhibition of thalamo-cortical circuits and motor behavior, respectively. It is interesting to note that changes in GABA release in VMTh were not mirrored by changes in GLU release. Thus, low UFP-512 doses inhibited GABA and facilitated GLU release while high UFP-512 elevated GABA without affecting GLU release. The main source of GLU release in VMTh is represented by cortico-thalamic projections (Chevalier and Deniau, 1982, McFarland and Haber, 2002) and an increase in thalamic GLU release likely reflects activation of cortico-thalamic pathways. Indeed, increased activation of thalamic NMDA receptors has been shown to increase locomotion in rats (Klockgether et al., 1986). Alternatively, increased GLU release may arise from disinhibition of GLU nerve terminals from an inhibitory intrathalamic GABAergic tone (possibly mediated by GABA_B receptors; Nyitrai et al., 1999). The lack of changes in GLU release following an increase in GABA release may be related to the saturation of inhibitory presynaptic GABA receptors caused by tonic activity of nigro-thalamic neurons. Thus, further increases in nigro-thalamic activity and GABA release would not cause GLU inhibition.

Consistent with that reported for SNC-80 (Mabrouk et al., 2008), motor facilitatory doses of UFP-512 also reduced GABA release in GP and SNr. Since perfusion with naltrindole in SNr but not GP was able to prevent these changes and associated behaviors, we propose that the antiakinetik action of UFP-512, like SNC-80, originates from DOP receptor stimulation in SNr. However, this cannot be the case of UFP-512 induced motor inhibition. Indeed, we found that both motor inhibiting doses of UFP-512 and naltrindole (Mabrouk et al., 2008) elevated GABA release in GP. Since antagonist action can take place only at those receptors activated by endogenous agonists, and up-regulation of enkephalin release at striato-pallidal synapses occurs as a consequence of DA depletion, it can be speculated that differently from low doses of UFP-512, high UFP-512 doses impair motor behavior by inhibiting (directly or indirectly) DOP

receptors at the pallidal level. Consistent with facilitation of the indirect pathway, high doses of UFP-512 increased nigral GLU release. It must be noted, however, that such an effect was not observed following naltrindole administration (Mabrouk et al., 2008).

The finding that both low and high doses of UFP-512 reduced, although with a different time-course, SNr GABA release is unexpected and quite puzzling. However, it is consistent with that previously reported for SNC-80 and naltrindole (Mabrouk et al., 2008) and possibly related to dynamic changes in different pools of extracellular GABA. Thus, the effect of low doses may reflect a reduction in the activity of nigro-thalamic neurons through an action at DOP receptors expressed on recurrent collaterals (Rick and Lacey, 1994). Conversely, the effect of high doses may reflect a reduction in the activity in pallido-nigral projections due to marked increase in pallidal GABA release. We speculate that the decrease in GABA originating from this specific pool together with the increase in nigral GLU would result in increased nigro-thalamic transmission and, ultimately, a reduction of motor activity observed in behavioral assays.

Concluding remarks

UFP-512 produced opposing motor effects in 6-OHDA hemilesioned rats depending on dose, low ones causing facilitation and higher ones causing inhibition. These changes were accompanied by inhibition and facilitation of GABA release, respectively, in VMTh suggesting that motor changes were due to opposite regulation of nigro-thalamic GABAergic pathway and, ultimately, thalamo-cortical transmission and motor output. Although the precise nature of the UFP-512 bell-shaped curve remains to be elucidated, the present study suggests that high doses of UFP-512 may, directly or indirectly, antagonize DOP receptors. Future studies need to be performed to elucidate the pharmacodynamic profile of UFP-512 as well as to isolate, identify and pharmacologically characterize UFP-512 degradation products.

Section IV. Combined DOP receptor stimulation and NOP receptor blockade synergistically improves motor performance and impairs nigro-thalamic output in experimental parkinsonism

Past studies from our group have demonstrated the importance of opioidergic regulation of locomotor activity occurring via nigral mechanisms under parkinsonian conditions. For instance, NOP receptor antagonists are capable of reversing motor deficits in 6-OHDA hemilesioned (Marti et al., 2005, 2007, 2008) and haloperidol treated (Marti et al., 2004, 2005) rats or MPTP-

treated mice and nonhuman primates (Viario et al., 2008). In 6-OHDA lesioned rats, this action was associated with decreased nigral GLU and increased nigral GABA release, leading to overinhibition of nigro-thalamic projections (Marti et al., 2007, 2008). Recently, we also demonstrated that DOP receptor stimulation in SNr enhanced motor activity in hemiparkinsonian rats (see section II, Mabrouk et al., 2008). This effect was correlated with a reduction in GLU and GABA release in SNr which resulted in reductions of thalamic GABA release (e.g. overinhibition of nigro-thalamic input; see section III). Based on our findings and the possibly similar mechanisms by which NOP receptor blockade and DOP receptor stimulation influence neurotransmission and behavior, we sought to investigate a possible interaction between these two receptor types in vivo. The main finding of this study was that systemic administration of behaviorally ineffective doses of the selective DOP receptor agonist SNC-80 and the selective NOP receptor antagonist J-113397, evoked synergistic attenuation of parkinsonism and improvement in motor performance in hemiparkinsonian rats. These effects were mirrored by neurochemical changes, since the same doses, ineffective alone, caused a synergistic increase in GABA and decrease in GLU release in SNr which was accompanied by a decrease in GABA release in VMTh, suggesting that the nigro-thalamic neurons were the neurobiological substrate of this interaction. Indeed, the effects on behavior (bar test) and neurochemistry were replicated by the co-perfusion of these two compounds in the lesioned SNr, confirming that this brain area is where this interaction occurs. These data are consistent with an opposing regulation of nigro-thalamic output by NOP and DOP receptors and point towards a potential antiparkinsonian therapy based on combined administration of NOP receptor antagonists and DOP receptor agonists.

There is solid evidence suggesting that opioid receptors interact both locally (where receptors are located in the same tissue on the same or adjacent cells) or nonlocally (in different nuclei; for review see Smith and Lee 2003). These interactions may be potentiating or inhibitory and thus result in the enhancement or inhibition of signal transduction pathways normally activated by either of the receptors alone (Fig. 43, 44). These interactions may also be symmetric or asymmetric. Symmetric interactions occur when both receptors affect each other, while asymmetric ones occur when only one receptor alters activity of the other. Moreover, when receptors are located on the same cell, it is possible for them to physically associate and form

dimers which may endow the complex with unique characteristics compared to the single receptors alone.

The NOP receptor has high overlapping mRNA distribution compared to the DOP receptor in the CNS (Bunzow et al., 1994). In particular, DOP and NOP receptor binding and mRNA expression has been found in SNr (Neal et al., 1999, Cahill et al., 2001), although the anatomical localization of these receptors in SNr is mostly unknown leaving to speculation the structures on which these receptors act to produce their effects. Despite this, it was reported that SNc DA cells express NOP receptors (Norton et al., 2002) and that N/OFQ inhibits the nigro-striatal DAergic pathway (Marti et al., 2004). Indeed, stimulation and blockade of NOP receptors in SNr caused a decrease and increase of DA release in striatum, respectively (Marti et al., 2004). On the other hand, DOP receptors have only been demonstrated on striato-nigral terminals (Abou Khalil, 1984) while their presence on nigral GABAergic interneurons has been postulated based on the finding DA antagonists blocked hyperlocomotion induced by DOP receptor stimulation in SNr (Morelli et al., 1989). Indeed, inhibition of GABAergic interneurons would lead to increased striatal DA. However, it was never demonstrated that DOP receptor activation in SNr increases striatal DA release. Moreover, we have to consider that most nigral DA neurons degenerate after 6-OHDA lesioning. This makes the possibility that the synergism between DOP receptor stimulation and NOP receptor blockade relies on DA mechanisms (i.e. stimulation of residual DA neurons) unlikely. The SNr receives a dense innervation of GLU projections from the subthalamic nucleus which becomes pathogenically overactive under parkinsonian conditions (DeLong, 1990). It was suggested that the antiparkinsonian effect of NOP receptor antagonists, such as J-113397, spans from their ability to reduce exaggerated subthalamo-nigral GLUergic transmission (Marti et al., 2004, 2005). Indeed in the current study, systemic J-113397 administration (1 mg/kg) or perfusion in SNr (1 μ M) decreased local GLU and increased GABA release. One possibility is that J-113397 stimulated a moderate release of GABA by blocking endogenous inhibitory N/OFQergic tone on GABAergic interneurons. Blocking this inhibitory tone would result in increased nigral GABA levels which may overinhibit subthalamo-nigral GLU release. DOP receptor stimulation could target a separate mechanism. Indeed, SNC-80 could reduce GLU release via stimulation of DOP receptors located on subthalamo-nigral terminals. This would lead to reduced excitatory drive onto nigro-thalamic neurons. It is interesting to note that we recently showed that systemic administration of SNC-80 at

behaviorally effective doses (3 mg/kg) decreased nigral GABA yet still maintaining its inhibitory effects on GLU release (section II, Mabrouk et al., 2008). This suggests that GLUergic mechanisms are primarily responsible for the motor enhancing effects of DOP receptor stimulation and that influence over GABA release may be a secondary effect which can be altered by indirect non local mechanisms. The discrepancy between the effect of local perfusion (increase; present study) and systemic administration (decrease; Mabrouk et al., 2008) of SNC-80 on nigral GABA release may be explained by the recruitment of inhibitory (possibly extra-nigral) mechanisms by high dose systemic SNC-80. The effect of lower systemic SNC-80 doses should also be investigated. Though the increase in GABA release caused by local perfusion of SNC-80 and the enhancement of this effect caused by the combination seems puzzling, it is important to understand the complex environment of SNr where GABA pools are derived from multiple sources. Local GABAergic interneurons play a crucial part in regulating the output of SNr, yet GABAergic projections arrive from striatum, GP and additionally, collaterals on nigro-thalamic neurons also release GABA. Therefore the increase in nigral GABA release following local SNC-80 perfusion cannot be due to monosynaptic DOP receptor stimulation of GABAergic structures but likely derives from polysynaptic interactions. The possibility that the synergism between SNC-80 and J-113397 occurs via intracellular interaction is also being explored. N/OFQ stimulation of NOP receptors co-expressed with MOP receptors on cell lines was shown to blunt signaling in response to DAMGO. Accordingly it was demonstrated that the NOP receptor antagonist III-BTD blocked heterologous N/OFQ induced phosphorylation of MOP receptors in vitro (Ozsoy et al., 2005). Phosphorylation of GPCRs via G-protein kinases is known to be a main initiator of desensitization phenomenon since it leads to the uncoupling of G-protein effectors from their respective receptors, rendering them inactive. It is also well known that selective DOP receptor agonists such as SNC-80 cause significant receptor desensitization (Lecoq et al., 2004) which may limit their effectiveness as therapeutic agents. Thus we can speculate that the synergism between NOP receptor antagonism and DOP receptor stimulation occurs via modulation of DOP receptor desensitization. Supporting this hypothesis is the fact that at 70 min following treatment with high doses of SNC-80, motor stimulating effects begins to wear off. Moreover, combination of J-113397 with SNC-80 results in a more robust and prolonged motor activation. In addition, mRNA of NOP and DOP receptors has been found in STN (Neal et al., 1999, Cahill et al., 2001) suggesting the possibility that DOP and NOP

receptors are co-expressed on subthalamo-nigral GLUergic projections (see Fig. 44). Therefore, NOP receptor blockade may inhibit desensitization pathways which normally occur after DOP receptor stimulation leading to a synergistic reduction in GLU release in SNr. Future studies focusing on longer time courses of treatment or chronic administration of SNC-80 in the presence of J-113397 would be of interest. Whichever the mechanism, ultimately this enhanced inhibitory tone of SNr (decreased GLU, and increased GABA) inhibits nigro-thalamic output projections, in turn disinhibiting thalamo-cortical projections and thus improving motor performance.

Concluding remarks

Opioid receptor interactions have been investigated in a variety of disease models in order to obtain greater responses or prevent side-effects related to opioid receptor administration. Recent in vitro studies by Ozsoy et al (2005) showed that N/OFQ enhanced MOP receptor agonist induced phosphorylation via PKC and GRK2 dependent pathways which contribute to the rapid desensitization often seen with MOP receptor stimulation. Thus NOP receptor blockade has been viewed as an approach to prevent development of tolerance during chronic pain therapy with morphine. Also, Mogil and colleagues (1996) demonstrated that NOP receptor stimulation via i.c.v. injection of N/OFQ was able to antagonize the antinociceptive effects of the DOP receptor agonist DPDPE (i.c.v.), while NOP receptor blockade would potentiate antinociceptive effects of morphine. The present study proposes for the first time that combined DOP receptor stimulation and NOP receptor blockade may be beneficial in treating PD symptoms. Targeting these two opioid receptor subtypes may have additional benefits with respects to each alone in the treatment of PD. Indeed, DOP receptor stimulation attenuates 6-OHDA induced cell loss in vivo (Borlongan et al., 2000) while indirect evidence that NOP receptor blockade may prevent DA cell loss in the MPTP mouse model of parkinsonism has been presented (Marti et al., 2005). Thus, a strategy aimed to simultaneously stimulate DOP receptors and inhibit NOP receptors may results in additive or synergistic effects on DA cell viability in PD leading to a marked neuroprotection.

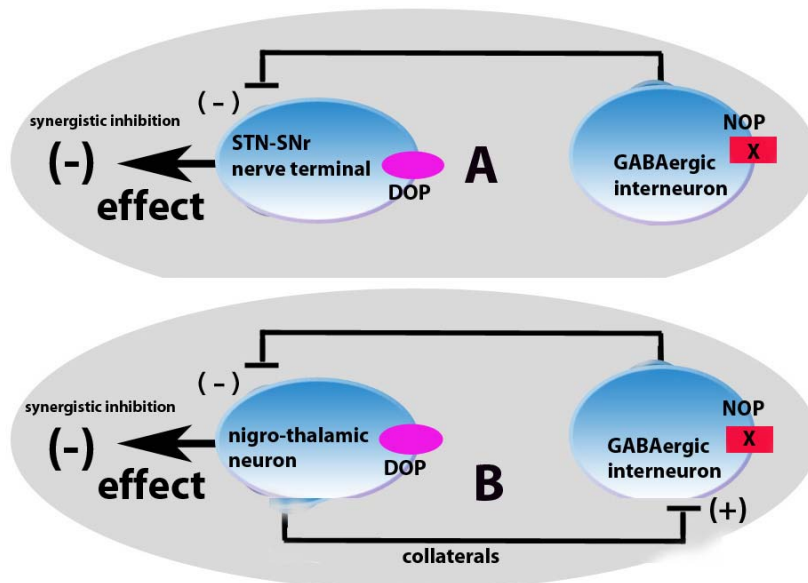


Fig. 43 Possible intercellular interactions between NOP and DOP receptors in SNr. (A) An inhibiting asymmetric relationship between NOP receptor blockade on inhibitory GABAergic interneurons and stimulation of DOP receptors on subthalamo-nigral GLUergic terminals leading to a synergistic reduction of GLU release. (B) Symmetric interaction between NOP receptor blockade on inhibitory GABAergic interneurons and stimulation of DOP receptors on GABAergic nigro-thalamic axon collaterals, leading to synergistic increases in local GABA release followed by further inhibition of nigro-thalamic GLUergic projections. Note that (-) refers to inhibition and (+) refers to disinhibition.

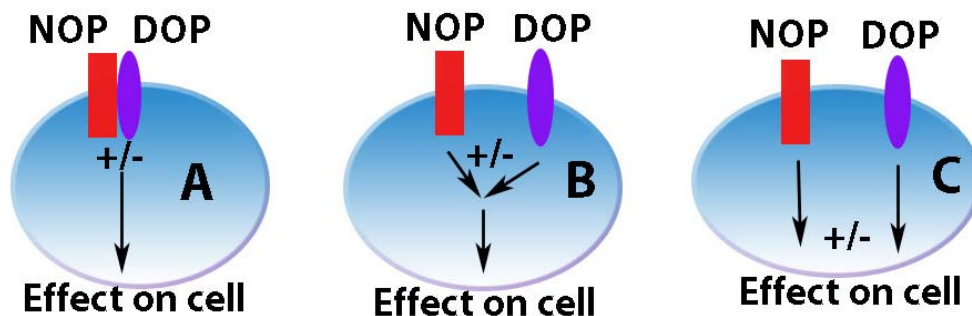


Fig. 44 Possible intracellular interactions between opioid receptors in SNr. Blockade of NOP and stimulation of DOP receptors on the same cell may potentiate or attenuate signal transduction pathways (arrows) leading to synergistic stimulation (+) or inhibition (-) of the cell. The receptors may physically interact (A) or may interact at some point along with signal transduction pathways (B) or they may independently act on the cell via different transduction pathways (C).

6. CONCLUDING REMARKS

The main findings from this work are that, firstly, the endogenous MOP receptor ligand EM-1 administered in SNr biphasically controls motor behavior in rats by modulating nigro-thalamic neurotransmission. We also showed that the systemic administration of the selective DOP receptor agonist, SNC-80, dose dependently improved locomotor activity in hemiparkinsonian rats while the DOP receptor antagonist naltrindole worsened it, suggesting that endogenous ENK tonically sustain movement under parkinsonian conditions. These effects were found to primarily act through nigral mechanisms. We extended our findings with DOP receptors by testing a novel DOP receptor agonist UFP-512. Interestingly, depending on dose, we observed both agonistic and antagonistic properties leading to motor facilitation and inhibition, respectively. Those studies demonstrated that nigro-thalamic output was strictly correlated with motor behavior suggesting that this neuronal population mediates DOP receptor induced changes in motor activity. Finally, we discovered an intriguing interaction between the DOP receptor and the NOP receptor, namely that stimulation of DOP receptors and blockade in NOP receptors causes a synergistic enhancement in locomotor activity and neurotransmitter release. We also demonstrated that this effect was mediated in SNr and that nigro-thalamic output was involved. The cause of the degeneration of nigral DAergic cells that occurs in PD is unknown. Unfortunately, the current treatments available have significant limitations (see section 1.5) and do not protect against cell death and therefore new treatments are needed. Interestingly, DOP receptor agonists have been shown to promote neurogenesis and have neuroprotective properties. This may indicate a potential for DOP receptor agonists in the prevention of neural cell death which leads to parkinsonian motor complications. Our studies here concluded that DOP receptor agonists influence motor behavior, though probably through mechanisms involving their effects on neurotransmission (i.e. reduction of nigral GLU) and not on neural cell proliferation. However, the relationship between these 2 phenomena must be further explored. Indeed, an ideal therapeutic candidate would, in the short term, improve motor function in PD patients while in the long term act to restore degenerated neuronal cells. Additionally, NOP receptor antagonists have been shown to restore motor function in a number of animal models of PD and there is indirect evidence that they can attenuate MPTP induced neurotoxicity. We demonstrated synergistic interactions of these two receptors on their influence over neurotransmitter release and locomotor activity, but studies focusing on their interaction relating to their precise localization and on neuronal viability must be further explored with molecular approaches. These

insights contribute to an overall better understanding of the role of opioids in PD and may lead to the development or improvement of pharmacological strategies to prevent (with neuroprotective opioids) or reduce the severity of the motor complications of the disease alone or in combination with more typical DAergic treatments.

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8. ORIGINAL ARTICLES

Mabrouk OS, Marti M, Salvadori S, Morari M.

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